REGULATION OF ENDOPLASMIC RETICULUM CALCIUM HOMEOSTASIS IN PANCREATIC β CELLS

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REGULATION OF ENDOPLASMIC RETICULUM CALCIUM HOMEOSTASIS IN PANCREATIC β CELLS

Diabetes mellitus is a group of metabolic diseases characterized by disordered insulin secretion from the pancreatic β cell and chronic hyperglycemia. In order to maintain adequate levels of insulin secretion, the β cell relies on a highly developed and active endoplasmic reticulum (ER). Calcium localized in this compartment serves as a cofactor for key proteins and enzymes involved in insulin production and maturation and is critical for ER health and function. The ER Ca^{2+} pool is maintained largely through activity of the sarco-endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) pump, which pumps two Ca^{2+} ions into the ER during each catalytic cycle. The goal of our research is to understand the molecular mechanisms through which SERCA2 maintains β cell function and whole body glucose metabolism.

Our previous work has revealed marked dysregulation of β cell SERCA2 expression and activity under diabetic conditions. Using a mixture of pro-inflammatory cytokines to model the diabetic milieu, we found that SERCA2 activity and protein stability were decreased through nitric oxide and AMP-activated protein kinase (AMPK)-mediated signaling pathways. Moreover, SERCA2 expression, intracellular Ca^{2+} storage, and β cell death under diabetic conditions were rescued by pharmacologic or genetic inhibition of AMPK. These findings provided novel insight into pathways leading to altered β cell Ca^{2+} homeostasis and reduced β cell survival in diabetes.

To next define the role of SERCA2 in the regulation of whole body glucose homeostasis, SERCA2 heterozygous mice (S2HET) were challenged with high fat diet (HFD). Compare to wild-type controls, S2HET mice had lower serum insulin and significantly reduced glucose tolerance with similar adiposity and systemic and tissue-

specific insulin sensitivity, suggesting an impairment in insulin secretion rather than insulin action. Consistent with this, S2HET mice exhibited reduced β cell mass, decreased β cell proliferation, increased ER stress, and impaired insulin production and processing. Furthermore, S2HET islets displayed impaired cytosolic Ca^{2+} oscillations and reduced glucose-stimulated insulin secretion, while a small molecule SERCA2 activator was able to rescue these defects. In aggregate, these data suggest a critical role for SERCA2 and the maintenance of ER Ca^{2+} stores in the β cell compensatory response to diet induced obesity.

Carmella Evans-Molina, MD, PhD, Chair

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ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	AMP-activated protein kinase
ATF6	Activating transcription factor 6
ATM	Atipose tissue macrophage
AUC	Area under the curve
BAT	Brown adipose tissue
BAX	Bcl2-associated X protein
BCL-2	B-cell lymphoma 2
BIM	Bcl2L11
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
CAMKK	Ca^{2+} /calmodulin-dependent protein kinase kinase
cAMP	Cyclic adenosine monophosphate
CC	Compound C
CDK	Cyclin-dependent kinase
CEBP	CCAAT/enhancer-binding protein
CHOP	C/EBP homology protein
cIAP1	Cellular inhibitor of apoptosis protein-1
CICR	Ca^{2+} induced Ca^{2+} release
CPE	Carboxypeptidase E
C-PTIO	Carboxy-PTIO potassium salt
CRISPR	Clustered regularly-interspaced short palindromic repeats
DAG	Diacylglycerol

DEXA	Dual X-ray absorptiometry
DM	Diabetes melitus
DMEM	Dulbecco’s modified essential medium
DTT	Dithiothreitol
EGF	Epidermal growth factor
eIF2a	Eukaryotic initiation factor 2
ELISA	Enzyme-linked immunosorbent assay
Epac2	Exchange protein directly activated by cAMP 2
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FBS	Fetal bovine serum
FDA	Food and drug administration
FFA	Free fatty acid
FoxM1	Forkhead box protein M1
Gck	Glucokinase
GDM	Gestational diabetes melitus
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLT	Glucolipotoxicity
GLUT	Glucose transporter
GPCR	G-protein coupled receptors
GSIS	Glucose stimulated insulin secretion
GWAS	Genome-wide association study
HbA1C	Glycated haemoglobin A1C
HBSS	Hank’s balanced salt solution

HEK293	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HIF-1a	Hypoxia-inducible factor 1-α
IAPP	Islet amyloid polypeptide
IFN-γ	Interferon γ
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IKK	IκB kinase
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
INS-1	Rat insulinoma cell line INS-1 832/13
IP_3R	Inositol 1,4,5-trisphosphate receptor
IPGTT	Intraperitoneal glucose tolerance test
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinases
K_{ATP}	Potassium-linked ATP channels
LKB1	Liver kinase B1
L-NMMA	NG-monomethyl L-arginine
MafA	Avian musculoaponeurotic fibrosarcoma oncogene homolog A
MAPK/ERK	Mitogen-activated protein kinases
MICU1	Mitochondrial Ca^{2+} uptake 1
mTOR	Mechanistic target of rapamycin
NC	Normal chow

NCX	Na^{+} Ca^{2+} exchanger
NEFA	Non-esterified fatty acids
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
nPOD	Pancreatic organ donors database
OGTT	Oral glucose tolerance test
PBA	4-phenylbutyric acid
PC1/3 and PC2	Protein convertase 1/3 and 2
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pdx-1	Pancreatic and duodenal homeobox protein 1
PERK	Protein kinase-R/PKR-like ER kinase
PFK	Phosphofructokinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB/AKT	Protein kinase B
PKC	Protein kinase C
PLC-ε	Phospholipase C ε
PMCA	Plasma membrane Ca^{2+} ATPase
PP cell	Pancreatic polypeptide
PPARγ	Peroxisome proliferator-activated receptor γ
PUMA	P53 upregulated modulator of apoptosis
RNAi	RNA interference
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RRP	Readily releasable pool
RyR	Ryanodine receptor
S2HET	SERCA2 heterozygous
S2KO	SERCA2 knock out
SAB	Secretion buffer
SERCA	Sarco-endoplasmic reticulum calcium ATPase
SG	Secretory granule
siRNA	Small Interfering RNA
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
T1D	Type 1 diabetes melitus
T2D	Type 2 diabetes melitus
TAK1	Transforming growth factor beta-activated kinase 1
TCA	Tricarboxylic acid
TM	Tunicamycin
TNF-α	Tumor necrosis factor α
TUDCA	Tauroursodeoxycholic acid
TZD	Thiazolidinedione
UCP	Uncoupling protein
VOC	Voltage gated Ca^{2+} channel

WAT	White adipose tissue
WFS	Wolfram syndrome gene
WT	Wild-type
Xbp1	X-box Binding Protein 1
ZIP	Zinc influx transporters
ZnT-8	Zinc transporter 8

CHAPTER ONE

Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronically elevated blood glucose that typically results from either a complete or relative lack of the anabolic hormone insulin (1). In certain types of diabetes, the response to insulin may also be blunted, a condition known as insulin resistance. Diabetes is a widespread disease currently affecting more than 415 million individuals worldwide, and this number is estimated to reach 642 million by 2040. Even more striking is the similarly drastic number of individuals (318 million) with pre-diabetes or impaired glucose tolerance. Importantly, various studies have estimated that 70%-90% of this group will develop frank diabetes later in their lives (2). The long-term effects of diabetes mellitus include a variety of microvascular and macrovascular complications including cardiovascular disease, retinopathy, neuropathy, and nephropathy (3). DM is also a major cause of mortality. Nearly 1.5 million people die annually as a result of the disease, and diabetes is also a significant contributor to death from cardiovascular causes (4).

Individuals with DM must carefully manage their blood glucose levels to reduce the risk of complications through rigorous life style interventions (5), medications, and in certain cases weight reduction surgery (6). While there is currently no cure for DM, fundamental basic, translational, and clinical research is critical in providing experimental and intellectual insight into both the pathophysiology of this disorder and novel prevention or treatment approaches.

1.1.1 Different forms of diabetes

The diagnosis of diabetes has historically been made on the basis of fasting blood glucose levels higher than 7 mM (126 mg/dL) or a blood glucose level higher than 11.1 mM (200 mg/dl) 2 hours (h) after a 75 gram oral glucose tolerance test (7). In 2009, a glycated haemoglobin (HbA1C) of 6.5% or higher was also added to the diagnostic criteria (8). There are 3 main types of diabetes: Type 1 diabetes (T1D), Type 2 diabetes (T2D), and gestational diabetes mellitus (GDM).

Type 1 diabetes comprises around 5% of all diabetes cases, and is generally thought to arise from autoimmune-mediated destruction of pancreatic β cells. The main cause of T1D is infiltration of immune cells into the islet, leading to inflammation, and β cell death and dysfunction (9). Thus, the existence of a single or combined circulating autoantibodies against β cell antigens is commonly observed in T1D, and this often serves as the distinguishing feature between T1D and T2D (10). T1D was previously considered to be a condition of children and adolescents, although this notion of an age limitation has changed over the past decade with larger numbers of adults being recognized as having T1D. Data suggest that 70-95% of the insulin producing β cells have been destroyed at the time of T1D diagnosis (9). In a recent analysis based on the Network for Pancreatic Organ Donors Database (nPOD), single antibody positive non-diabetic subjects were found to have equivalent β cell mass with antibody negative control subjects. It was only when the disease progressed that the majority of β cell loss was observed. This suggests that additional research is needed to fully define the dynamics of β cell destruction in the preclinical phase of T1D (11).

T2D accounts for about 90-95% of diabetes cases and is a metabolic disorder that is highly associated with obesity. This form of diabetes is characterized by insulin

resistance coupled with inadequate insulin secretion from the pancreatic β cells (2). During the progression to T2D and in response to insulin resistance and chronic fuel surfeit, the β cells undergo an adaptive phase characterized by compensatory hyperplasia and insulin hypersecretion in order to meet increasing metabolic demands (12). Clinically apparent diabetes develops only in subjects who fail to maintain this compensatory response. In susceptible individuals, this transition is marked by a significant loss of β cell mass, function, and identity (13-15). T2D is also a disease with high prevalence of co-morbidities. In a cohort of over 1,389,016 individuals with T2D, over 97.5% were found to have at least one comorbid condition, and 88.5% had at least two. The most common co-morbid conditions included hypertension, overweight/obesity, and hyperlipidemia (16).

Gestational diabetes mellitus (GDM) is defined as hyperglycemia that is first detected at any time during pregnancy. This form of diabetes is seen in about 7% of all pregnancies. Women with obesity, glucose intolerance, a first-degree relative with T2D, or a history of gestational diabetes are at increased risk of developing GDM (17). GDM tends to occur after the 24th week of pregnancy due to increased insulin resistance and inadequate β cell compensation. However, symptoms normally disappear after birth. Yet women who have been previously diagnosed with GDM have an increased risk of diabetes in subsequent pregnancies. Moreover, 70% of all women with gestational diabetes will develop T2D later in their lives (18). Babies born to mothers with gestational diabetes also have a higher risk of developing T2D in their teens or early adulthood (19).

1.1.2 Mechanisms of glucose homeostasis

Glucose is the main fuel type for most biological processes. This carbohydrate provides energy in the form of ATP through metabolism by glycolysis and the citric acid or tricarboxylic acid (TCA) cycle. Under most physiological conditions, blood glucose levels are controlled by a balance between glucose appearance in the circulation and glucose uptake/metabolism. Glucose appearance is mediated by meal-derived sources and endogenous glucose production mainly from the liver. Glucose disappearance occurs mainly in response to insulin stimulated uptake in peripheral tissues (20). Under normal conditions, blood glucose levels oscillate within a very controlled range. In the fasting state, glucose levels are usually below 99 mg/dL, and blood glucose levels are kept below 139 mg/dL in the post-meal state (7). Depending on the specific physiological cues during post-meal and fasting states, distinctive profiles of hormones are secreted from the pancreas, gut, and brain to regulate the uptake and metabolism of glucose in peripheral tissues including the liver, muscle, and adipose tissue.

Several hormones secreted from pancreas, brain and gut including glucagon, glucocorticoids and epinephrine work on peripheral tissues to increase blood glucose levels through processes including gluconeogenesis and glycogenolysis (20). On the contrary, insulin secreted from pancreatic islets is the only hormone that acts to decrease blood glucose and promote glucose utilization and/or storage. Specifically, insulin binds to the insulin receptor on the surface of a target cell, triggering a series of phosphorylation events starting with a tyrosine kinase and insulin responsive substrates (IRSs) (21). These IRSs serve as docking proteins that further activate downstream signaling pathways, initiating mostly anabolic actions. These actions include glucose transport, glycogen synthesis, lipogenesis, protein synthesis and cellular growth.

Meanwhile, insulin signaling also suppresses gluconeogenesis in the liver, protein hydrolysis in skeletal muscle and lipolysis in adipose tissue (22).

1.2. Pancreatic Islets and the β cell under normal conditions

1.2.1 Pancreatic islet composition and distribution

Pancreas is an endocrine organ that lies in the upper left part of the abdomen. Anatomically, the pancreas is divided into a head, which rests within the concavity of the duodenum, a body lying behind the base of the stomach, and a tail, which is near the hilum of the spleen. Approximately 3 million cell clusters called pancreatic islets are present in the pancreas (23). They are groups of endocrine cells that secrete a multitude of hormones including insulin (β cells), glucagon (α cells), somatostatin (δ cells), pancreatic polypeptide (PP cells) and ghrelin (ε cells). The composition, size and structure of islets shows a wide range of variability at both the individual and species level (24). For example, in rodents, a central core of β cells makes up 60–80% of the islet, while a layer of α cells (15–20%), δ cells (<10%) and PP cells (<1%) surround the core. The composition of human islets, on the other hand, includes a lower percentage of β cells (~50%), α cells (~40%), δ cells (10%) and few PP cells (25). Human islets also have a structure where different cell types tend to be randomly distributed (26). Furthermore, pancreas comprise head which is the , studies of different regions of the human pancreas have revealed: 1) that the density of islets is similar between the head and body regions, but is 2-fold higher in the tail region, 2) no differences exist in glucose-stimulated insulin secretion patterns in islets isolated from different regions, and 3) persons with T2D exhibit a preferential loss of large islets in the head region (27).

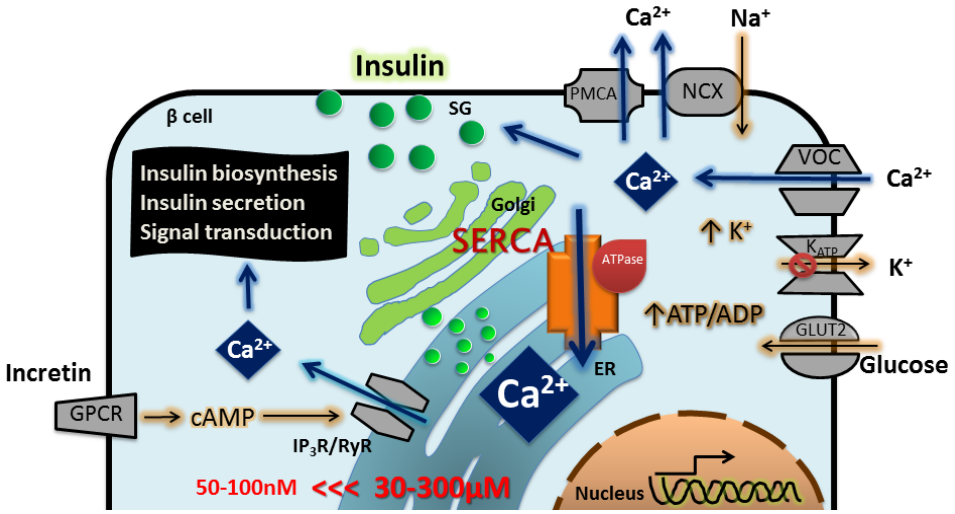

Figure 1. Ca^{2+} regulators and the involvement of Ca^{2+} in β cell homeostasis.

To meet the continuous needs of finely-tuned insulin secretion, the β cell possesses a highly developed ER that plays a central role in protein synthesis, signal transduction, and Ca^{2+} storage. Under normal conditions, the precise regulation of the steep Ca^{2+} gradient between the cytosol and the ER lumen is maintained by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump together with Ryanodine (RyR) and IP_3 receptors (IP_3R). This Ca^{2+} gradient is critical for many β cell functions including insulin biosynthesis, insulin secretion, and cellular signal transduction. Mainly two aspects contribute to the transient elevation of cytosolic Ca^{2+} concentration that ultimately induces insulin secretory granule (SG) exocytosis. In the first, glucose is transported through a glucose transporter (GLUT2). The glucose metabolism leads to an increase in the ATP/ADP ratio. This change triggers closure of K_{ATP} channels and the subsequent opening of voltage gated Ca^{2+} channels (VOC). In the second mechanism, incretins, which are hormones that released from the gut into the blood stream after ingestion of a meal, activate G-protein coupled receptors (GPCR) to induce cAMP elevation which activates IP_3R/RyR to release Ca^{2+} from ER. The clearance of excess cytosolic Ca^{2+} is dependent on SERCA, the plasma membrane Ca^{2+} ATPase (PMCA) and the sodium calcium exchanger (NCX).

1.2.2 Ca^{2+} involvement in the function of pancreatic β cell

Similar to other peptide hormone secreting cells, pancreatic β cells have specialized machinery to meet the needs of continuous protein synthesis and secretion. At peak rates, it is estimated that β cells are capable of producing up to 1 million molecules of proinsulin per minute (28). To meet this high demand of insulin biosynthesis, the β cell has a highly developed endoplasmic reticulum (ER) that plays a central role in protein synthesis and Ca^{2+} storage. Ca^{2+} within the ER and secretory granules play a critical role in insulin production, processing, and maturation (29-31). Ultimately, insulin-containing secretory granules are released through exocytosis in response to physiological cues. This event, termed stimulus-secretion coupling, is primarily a Ca^{2+} dependent event. Because of the central role of Ca^{2+} in insulin biosynthesis and stimulus-secretion coupling, understanding the pathways regulating β cell Ca^{2+} homeostasis under normal and diabetic conditions has been the focus of my dissertation work. A schematic graph summarizing key molecular regulators of Ca^{2+} in the β cell is shown in Figure 1 and is discussed in further detail in the following sections.

a. Mechanisms of insulin biosynthesis

Mice have two insulin genes located on chromosome 6 and 7, *Ins1* and *Ins2* (32, 33) whereas humans have a single insulin gene located on chromosome 11 (34). Insulin mRNA is estimated to occupy ~20% of total mRNA in the β cell under physiological conditions (33).

The insulin genes encode a 110-amino acid precursor known as preproinsulin. After translation, preproinsulin is recognized by the rough ER through its signal recognition particle (SRP). The SRP is subsequently cleaved by signal peptidase to form proinsulin, which contains 86 amino acids. After folding and formation of three

critical disulfide bonds in the ER, proinsulin is transported into the Trans-Golgi network (TGN), where it is packaged and sorted into immature secretory granules. Several key maturation steps occur within immature granules: 1) the granules become acidified via ATP-dependent proton pump activity; 2) proinsulin undergoes proteolytic cleavage by calcium-dependent protein convertase 1/3 (PC1/3) and PC2 and carboxypeptidase E (CPE), resulting in equal molar amounts of insulin and C-peptide; 3) zinc and Ca^{2+} facilitate the crystallization of insulin. The final products of these steps are the dense-core granules that enter the constitutive trafficking pathway for secretion (35). In a mouse β cell, there are roughly 13,000 insulin granules and each contains approximately 200,000 insulin molecules (36, 37).

A number of factors are required to support the high biosynthetic burden of insulin production. Chief among them is a robust ER Ca^{2+} pool. Under normal conditions, Ca^{2+} is distributed unevenly within the cell. A steep gradient exists between the cytosol, where the Ca^{2+} concentration is estimated to be 50-100 nM, and the ER lumen, where the Ca^{2+} concentration is measured to be 30-300 μM (38-40). The high concentration of Ca^{2+} within the ER lumen serves as a required cofactor for a number of steps involved insulin production (41). Most notably, the ER lumen contains several Ca^{2+} related chaperones including calreticulin, heat shock protein 90 kDa β member 1 (HSP90B1 or Grp94), binding immunoglobulin protein (BiP) and protein disulfide-isomerase (PDI) that facilitate protein and lipid synthesis (42). Furthermore, previous work has shown that the ER Ca^{2+} pool serves as the main source of Ca^{2+} within the secretory granules where the Ca^{2+}-dependent convertase enzymes complete the final steps of insulin protein maturation (31).

Nutrient metabolism, especially that of glucose, is one of the dominant driving factors of insulin transcription and translation. The glucose effect on insulin transcription

is to enhance the production of insulin mRNA and to increase its stability. Notably, compared to mature insulin mRNA, unprocessed intron-containing pre-mRNA has a significantly shorter half-life, suggesting that insulin pre-mRNA might serve as a better candidate in measuring the acute response to glucose at the transcriptional level (43). Meanwhile, the translational regulation of insulin in response to glucose is also very rapid, with a 20-fold increase in insulin protein occurring within minutes of glucose stimulation (36).

b. Ca^{2+} channels and pumps in β cells.

ER Ca^{2+} regulators

Three Ca^{2+} pumps or channels are located on the ER membrane and maintain the dynamic Ca^{2+} concentration within this organelle. These include the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R). The SERCA protein is a P-type ATPase that serves as the primary regulator of ER Ca^{2+} homeostasis (discussed in detail in Section 1.4). The SERCA pump hydrolyses 1 ATP molecule in order to move 2 Ca^{2+} molecules from the cytosol into the ER lumen, thereby actively maintaining the steep Ca^{2+} gradient that exists between the ER and cytosol (44). In contrast, the IP_3Rs and RyRs are Ca^{2+} release channels that empty ER Ca^{2+} in a ligand-gated and passive manner. Notably, expression levels and activity of IP_3Rs and RyRs have been found to be significantly lower than the expression of SERCAs in β cells (45). However, interestingly, all isoforms of IP_3Rs were found to localize to insulin secretory granule membranes, with a 2-fold abundance compared to the ER membrane, suggesting a role for IP_3R in granule function (46).

Plasma Ca^{2+} transporters

ER Ca^{2+} storage is also indirectly maintained by two Ca^{2+} transporters on the plasma membrane: the Na^{+}/Ca^{2+}-exchanger (NCX) and the plasma membrane Ca^{2+} ATPase (PMCA). These transporters actively move Ca^{2+} against a tremendous concentration gradient from the inside to the outside of the cell to maintain relatively low cytosolic Ca^{2+} concentrations. PMCA was found to have at least 6 variants with tissue specific functions, while NCX contributes to both Ca^{2+} outflow and influx and has been shown to regulate insulin release (47, 48).

c. Mechanisms of insulin secretion

Insulin secretion is mediated by exocytosis of insulin granules in response to a variety of secretagogues including glucose, amino acids, free fatty acids (FFA) and incretin hormones (49-51). There are mainly 2 routes that will induce the exocytosis of insulin granules. The first depends on β cell depolarization-induced Ca^{2+} mobilization. Glucose is the most important stimuli for this canonical pathway of insulin secretion. In the post-prandial state, when blood glucose rises, glucose molecules are transported into the β cell through the glucose transporter (GLUT2 in rodents or GLUT1 in humans) (52). Glucose is metabolized by glycolysis and the TCA cycle, leading to generation of ATP and an increase in the ATP/ADP ratio. This elevation triggers closure of K_{ATP} channels, resulting in β cell depolarization and Ca^{2+} influx through opening of voltage-gated Ca^{2+} channels (VOC) on the plasma membrane (53). Increased cytosolic Ca^{2+} will induce immediate fusion of insulin granules with the plasma membrane to release insulin. Also, treatments that increase cytosolic Ca^{2+}, such as the application of thapsigargin to inhibit SERCA activity, can acutely induce insulin secretion (29, 54).

Insulin secretion can be amplified by the second route which involves cyclic adenosine monophosphate (cAMP) upregulation, which activates Ca^{2+} release channels located on the ER. This route of release is the main mechanism by which the incretin hormones, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide, induce insulin secretion (GIP) (51). The binding to specific G-protein coupled receptors (GPCR) located on the β cell plasma membrane, activates adenylate cyclase causing an increase in cytosolic cAMP, which in turn activates protein kinase A (PKA). PKA then phosphorylates RyRs and IP_3Rs to increase their activity. In the meantime, cAMP can also directly bind to Epac2 which is a guanine nucleotide exchange factor for the Ras-like small GTPase Rap. Rap subsequently acts on phospholipase C ε (PLC-ε) to generate IP_3 which induces Ca^{2+} efflux from the ER and results in insulin granule exocytosis (55, 56). Increased cytosolic Ca^{2+} then potentiates a sustained release of Ca^{2+} from the ER, which will further facilitate insulin secretion (57). The insulinotropic effects of incretins were further confirmed by inactivation of both GLP-1 and GIP receptors in β cells. This inactivation of the incretin receptors resulted in defect in stimulated insulin secretion (58, 59). Despite having similar molecular mechanisms, GLP-1 and GIP have distinct roles in T2D. Specifically, GIP action is lost in β cells during T2D due to a desensitized GIP receptor (GIPR) or decreased expression of the GIPR whereas GLP-1 is still fully functional. However, due to low circulating GLP-1 levels in T2D, potentiated insulin secretion by oral glucose administration is blunted (60).

Over 50 years ago, it was discovered that insulin secretion is characterized by a biphasic pattern (61-63). Mature secretory granules can be divided into a readily releasable pool (RRP) and a reserve pool, depending on their proximity to the plasma membrane. The RRP is proximal to the plasma membrane and comprises about 5% of

insulin granules, while the vast majority (~95%) of granules are found in the reserve pool located deeper in the cytosol (30).

Ca^{2+} has long been considered the direct driving factor of insulin granule exocytosis. Thus, insulin secretion in β cells and a Ca^{2+} wave almost always parallel with the amount of insulin secreted with the exception of *ob/ob* islets under certain conditions (64). Following glucose or incretin hormone stimulation, acute elevations of cytosolic Ca^{2+} drive the exocytotic fusion of secretory granules in the RRP with the plasma membrane. This forms the typical 1st phase of insulin secretion. Following the acute phase, a sustained release of ER Ca^{2+} through Ca^{2+} induced Ca^{2+} release (CICR) occurs and mediates the formation of Ca^{2+} oscillations. These regulated oscillations drive the movement of reserve pool granules towards the plasma membrane, initiating pulsatile insulin secretion. This slower yet relatively sustained event forms the 2nd phase of insulin secretion (30). Indeed, pulsatile insulin secretion has been observed under basal conditions or during the 2nd phase with a parallel cytosolic Ca^{2+} oscillation (65-68).

Several factors regulate Ca^{2+} oscillation and pulsatile insulin secretion. First, ER Ca^{2+} has been shown to play a critical role in CICR and in the regulation of Ca^{2+} oscillations (68), as inhibition of RyR/IP_3R and ER Ca^{2+} depletion attenuate both processes (69, 70). Metabolic signals are also involved and their influence has been proposed in the glycolytic oscillation model (71). In support of this model, cAMP levels and ATP production from glycolysis have been shown to have a similar oscillatory pattern. This is mainly based on oscillatory changes in the activity of the glycolytic enzyme phosphofructokinase (PFK) (72, 73). In a recent series of studies, a refined dual oscillation model was proposed that has taken into consideration both metabolic and Ca^{2+} feedback effects on PFK activity (74, 75). This is a more comprehensive model that mathematically represents all of the observations above, as well as the distinct types of

oscillatory patterns. In this regard, there are two types of Ca^{2+} oscillations observed in β cells: slow oscillations (with frequency of ~0.2-0.5/min) and fast oscillations (2–3/min). It is believed that fast oscillations are more regulated by changes in membrane potential, while glycolytic oscillations are more greatly influenced by slow Ca^{2+} oscillations (76).

Finally, a key component of healthy oscillations depends upon efficient mechanisms to clear the elevations in cytosolic Ca^{2+} (77). Excess Ca^{2+} can be either extruded to the extracellular space by NCX or PMCA or taken up into the ER lumen by SERCA. A number of studies suggest that the ER serves as the most critical buffering system in these processes and that SERCA activity is responsible for the majority of Ca^{2+} clearance from the cytosol. Specifically, mathematical modelling suggests that SERCA activity is responsible for clearing upwards of 60% of cytosolic Ca^{2+} following glucose stimulation (78).

As individuals progress from normal glucose tolerance to impaired glucose tolerance, changes in the β cell secretory pattern occur (79). Both impaired pulsatility (80) and abnormal biphasic secretion patterns (81) are found in T2D. Specifically, during the early stages of glucose intolerance, 1^{st} phase insulin secretion is decreased (82), while later stages of glucose intolerance result in lower 2^{nd} phase insulin secretion (82-86). Persons with T2D also fail to respond adequately with regular oscillatory insulin secretion in response to glucose excursions (87). A dysregulated oscillatory pattern is also found in rodent diabetes models (88, 89). Taken together, these observations suggest a critical role of cellular Ca^{2+} homeostasis in the regulation of Ca^{2+} oscillations and insulin secretion (90)

d. Other factors regulating insulin secretion

Another important factor regulating insulin secretion is cell-cell contact within the islet and between individual β cells. For example, dispersion of islets into a single cell suspension dramatically reduces glucose stimulated insulin secretion (91-93). A recent study using a mouse knock-out model of connexin 36, a gap junction protein, demonstrated a significant impairment in both 1st and 2nd phase insulin secretion. Connexin 36 knock-out was sufficient to cause whole body glucose intolerance, thus indicating the importance of cell attachment in the dynamics of insulin secretion and coordinated pulsatility of individual islets (94). This inter-cell communication likely involves Ca^{2+} since dispersed β cells are more depolarized at a resting state, yet failed to mount additional Ca^{2+} responses when exposed to glucose (91). These gap junctions within intact islets also allow for the synchronous pattern of cytosolic Ca^{2+} waves that support healthy oscillation patterns (67).

e. The role of Zinc in insulin production and secretion.

In addition to Ca^{2+} ions, zinc (Zn^{2+}) also plays an important role in insulin granulogenesis. Early on, it had been suggested that Zn^{2+} deficiency was linked to both T1D and T2D in terms of impairments in insulin production (95). However, it wasn't until 2004 that Fabrice Chimienti (96) and colleagues first identified and cloned the Zn^{2+} transporter 8 gene (*ZnT-8*) from β cells. It was found that the ZnT-8 protein co-localizes with insulin in the secretory granule, which suggests a critical role for Zn^{2+} in insulin maturation and storage (96). Indeed, *in vivo* studies using ZnT-8 whole body knock out or β cell specific knockout mice demonstrated that although whole body glucose homeostasis was not dramatically compromised, there were consistent β cell insulin packaging defects in ZnT-8 deficient conditions (97, 98). This finding was further

confirmed by an epidemiological study analyzing the interaction of plasma Zn^{2+} and a loss-of-function single nucleotide polymorphism (SNP) of ZnT-8 gene (SLC30A8 rs13266634) in persons with T2D. The results indicated that this SNP was associate with lower plasma Zn^{2+} levels, which increased the odds ratio for T2D and impaired glucose tolerance (IGT) (99). Interestingly, a newly characterized Zn^{2+} transporter (Zn^{2+} influx transporters or ZIP) was recently found to facilitate insulin exocytosis, thus increasing glucose-stimulated insulin secretion without altering insulin production or β cell identity (100).

In summary, insulin biosynthesis and secretion are regulated at multiple levels and each step is critical to ensure optimal glucose stimulated insulin secretion (101, 102). Many of these steps involve Ca^{2+} dependent mechanisms, which represent potential therapeutic targets. Therefore, a more complete understanding of how Ca^{2+} homeostasis is coordinated and maintained in β cells is necessary for future therapeutic strategies to prevent the development of frank T2D

1.3 Natural History of T2D and Deterioration of β Cell Mass and Function

1.3.1 Insulin resistance and compensation in β cells

Clinical studies have provided insight into the natural history of T2D. In a longitudinal study, researchers measured plasma glucose levels 2 h after a glucose tolerance tests in Pima Indians, a group known to have a high genetic risk of T2D. Results indicated that there were two distinct stages in most individuals during their progression to T2D. Blood glucose levels usually became slightly elevated 20-30 years before diabetes diagnosis. Then, an exponential stage of dsyglycemia appeared. During this phase, blood glucose levels quickly increased over 4-5 years prior to clinical

diagnosis. This observation indicates a key transition stage with a rapid glucose rise that differentiates individuals who develop frank diabetes from those who do not (103).

Increasing insulin resistance in peripheral tissues and β cell failure have both been shown to contribute to progression of T2D (104). Insulin resistance is observed in the majority of people with T2D, especially those who are overweight or obese (105). Insulin resistance is defined as the inability of insulin to produce its usual biological effects at physiological concentrations. This defect leads to impaired inhibition of hepatic glucose output, glucose uptake into skeletal muscle, and suppression of lipolysis in adipose tissue (106). Whereas it is difficult to find the true initiating factor in the development of insulin resistance in a pathological setting, several factors including chronic inflammation, lipid accumulation, and changes in gut microbiota have been found to contribute to insulin resistance (107). Notably, both high fat diet (HFD)-fed animals and individuals with T2D exhibit an accumulation of diacylglycerol (DAG) in skeletal muscle and liver. The DAG leads to defects in insulin-stimulated glucose transport activity in skeletal muscle and induces steatotosis in the liver, leading to inhibition of hepatic glucose production and stimulation of glycogen synthesis (108, 109).

Mechanistically, three main aspects contribute to lipid-induced insulin resistance: ectopic lipid accumulation, the development of ‘‘endoplasmic reticulum stress’’ and the contribution of systemic inflammation (110). In regard to systemic inflammation, adipokines secreted by white adipose tissue (WAT) were found to be highly involved, especially in the visceral fat depots. Unlike subcutaneous fat depots, visceral fat has higher adipose tissue macrophage (ATMs) accumulation (111). In the obese state, adipocytes together with pro-inflammatory ATMs preferentially secrete pro-inflammatory factors such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6). These factors directly or indirectly activate c-Jun N-terminal kinases (JNK) and IκB kinase (IKKβ)

signaling pathways that phosphorylate the inhibitory serine residues of IRS-1 (Serine307 in rat and Serine312 in human) to blunt insulin-mediated signal transduction (112-114). These factors can also work through suppressor of cytokine signaling (SOCS) proteins, which directly bind to the insulin receptor to inhibit phosphorylation at activating tyrosine residues (115). In contrast to WAT, brown adipose tissue (BAT) has been found to be protective and capable of increasing the basal metabolic rate and improving insulin sensitivity. Several studies in both mouse and humans suggest BAT may be a promising anti-diabetic tissue (116, 117).

Whereas increased peripheral insulin resistance is a key factor pathogenic state contributing to the development of T2D, insufficient insulin secretion has been suggested as the key determining factor leading to the development of frank diabetes (118, 119). During a stage known as compensation, the β cells are continually challenged to match insulin resistance with increased insulin output by expansion in both mass and secretory capacity (120). For example, with short-term glucose infusions in rats, β cell mass doubled within 6 days, leading to augmented insulin production (13). Another commonly used animal model for this compensation stage is HFD fed mice. After 16-20 weeks of HFD with 42-60% calories from fat, β cell mass was found expanded to over 2-fold in C57BL6 mice (121). Insulin output was also increasing progressively by time during HFD while maintaining euglycemia (122). Interestingly, in this relatively short period of challenge, the expression of key β cell genes remained normal and the function of individual β cell remained almost unchanged (123). It is the failure to maintain the compensation that leads to development of diabetes, and this will be discussed in detail in section 1.3.2.

In adults, pancreatic β cell mass is controlled by several mechanisms, including β cell replication, neogenesis, hypertrophy, and survival (124). β cell proliferation has been

relatively well studied in rodent models. Multiple stimuli from other organs coordinate to trigger initiation of the cell cycle leading to hyperplasia of β cells. These factors include glucose, insulin, incretins, adiponectin, cytokines (125) and hepatocyte growth factor (126, 127). These factors activate a large number of cellular signaling pathways involved in β cell proliferation and the list continues to grow (128, 129). For example, glucokinase (Gck) and IRS-2 was found to be critical in this compensatory response, as mice with β cell–specific haploinsufficiency of Gck or knock out of IRS-2 lose the capacity to expand their β cell pool in response to obesity (121, 130). Mechanistically, target of rapamycin (mTOR) is also a classic signaling pathway that contributes to up-regulation of β cell proliferation (131, 132). Incretins, including GLP-1 and GIP, facilitate β cell proliferation by up-regulating the anti-apoptotic-cell lymphoma 2 (BCL-2) gene through cAMP response element binding protein (CREB) signaling (133). cyclin D2 is also suggested to be key in controlling β cell proliferation upon HFD challenge (134, 135). On the other hand, adult human β cells do not appear to replicate in response to the same growth factors and nutrients that induce rodent β cell replication (128). Whereas age is certainly a critical factor determining the division potential of β cells, proliferation is still quite rare in human β cells. Notably, only 2-3% of human β cells were found to proliferate even during infancy. This number further dropped to 0.2% in adult β cells (136). Thus, targeting proliferation in adult humans under physiological conditions is challenging. Nevertheless, human β cells can be stimulated to replicate when cyclins and cyclin-dependent kinase (CDKs) are overexpressed (137). A recent proteomic study in human pancreatic cells revealed the critical role of cdk6 and cyclin D1(138). These discoveries indicate that a better understanding of human β cell proliferation is essential (129).

In addition to an increase in β cell proliferation and hypertrophy, hypersecretion of insulin from individual β cells also appears to contribute to this compensatory

response. Hypersecretion of insulin occurs via several mechanisms. First, secretory granule biogenesis is higher during compensation (139). Second, due to larger amplitude action potentials that increase Ca^{2+} signals, glucose-induced insulin secretion during this compensation stage is also increased (140). Third, it has been observed that a group of genes involved in glucose phosphorylation that are normally suppressed, including hexokinase 1 and glucose-6-phosphatase, were markedly upregulated during obesity, leading to higher insulin secretion at similar glucose levels (13, 141).

1.3.2 Factors contributing to β cell dysfunction and loss during T2D progression

Unfortunately, the ability of β cells to maintain this compensatory state eventually fails in certain groups of individuals and it has been estimated that 30-50% of prediabetes or with IGT will develop diabetes within 5 years (142). As the blood glucose levels increase, persons with impaired fasting glucose or glucose intolerance often progress to a state of declining β cell mass and function (13, 143, 144). A 2004 study analyzing a limited number of cadaveric donor pancreata from both non-diabetic individuals and persons with frank T2D demonstrated a nearly 50% lower islet mass in those with diabetes. T2D islets were also noted to be smaller on average and contain a higher percentage of glucagon producing cells (145). Indeed, this decreased β cell mass was confirmed in several other studies (146-148).

These failures in compensation can be attributed to both genetic and environmental factors. With regard to genetics, T2D is primarily considered to be polygenic in nature (149, 150). However, genome wide association studies (GWAS) have begun to identify T2D susceptibility loci. In two GWAS performed in 2010 and 2013, 40 loci that have strong association with higher incidence of T2D were identified. These loci can be categorized into genes that regulate insulin resistance/action (*e.g. PPARG,*

FTO and KLF14), insulin processing (e.g. *MTNR1B, GCK*) and insulin secretion (e.g. *KCNQ1, BCL11A, HNF1A, SLC30A8 and CAMK1D, TCF7L2, HHEX/IDE, CDKAL1, CDKN2A/2B*). Also, evidence of enrichment for genes involved in cell cycle regulation was found to be associated with T2D (151, 152). Notably, but not surprisingly, similar studies performed in women with GDM, which shares overlap with T2D, showed that T2D and GDM share some susceptibility loci like *KCNJ11, GCK, and HNF4a* (153). In aggregate, though, the majority of GWAS studies have highlighted a prominent role for loci that impact the β cell.

In addition to these genetic factors, extrinsic factors may also have important detrimental effects that contribute to the decompensation of β cells. These include hyperglycemia, glucolipotoxicity (GLT), pro-inflammatory cytokine stress, alterations in the redox state, accumulation of unfolded proteins in the ER, and disturbances in Ca^{2+} homeostasis (154). In the following section, the main factors involved in declining β cell function and mass in T2D will be discussed.

a. Glucotoxicity and lipotoxicity

The first and probably foremost insult during the development of T2D includes a chronic elevation of blood glucose. Multiple studies indicate that acute or prolonged hyperglycemia impairs β cell function. In non-diabetic animals, short term glucose infusions decreased the glucose sensitivity of β cell to secrete insulin, without inducing overt oxidative stress (155). However, prolonged culturing of isolated islets from cadaveric organ donors in 28 mM glucose significantly decreased insulin content, rates of glucose oxidation, proinsulin biosynthesis, and total protein biosynthesis, indicating stressed islets (156). Furthermore, hyperglycemia decreased the expression of key β cell genes including pancreas/duodenum homeobox protein 1 (*Pdx-1*),

musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*) and insulin (*Ins1* and *Ins2*) (157). This elevated glucose level also induced alterations in expression of genes involved in glucose metabolism, causing desensitization to glucose stimuli (158). Hyperglycemia can induce expression of a series of stress response genes, including those involved in oxidative stress, ER stress, hypoxia (159) and protein glycation (160). The induction of ER stress and oxidative stress signals were directly detected after *in vivo* glucose perfusion performed in rats to raise blood glucose levels to 20-22 mM (161). In addition, glucotoxicity has been shown to transcriptionally activate thioredoxin-interacting protein (TXNIP) via CREB, which induces β cell apoptosis (162). Interestingly, hyperglycemia-induced TXNIP up-regulation also activates IL-1β expression in cultured human adipose tissue (163), thus opening up the question of whether this mechanism exists in β cells under hyperglycemic conditions. In support of this idea, TXNIP overexpression in β cells resulted in increased inflammasome activation and IL-1β expression (164).

Hyperlipidemia, elevated levels of non-esterified fatty acids (NEFA), has also been associated with an increased risk of prediabetes and diabetes (165, 166). Decreased NEFA, on the other hand, improved GSIS and decreased insulin resistance in peripheral tissues (167). However, lipids may serve as a double-edged sword with regard to their effects on β cell function and survival, as the dose and specific types of NEFA or triglyceride dictate their net effects. Short term perfusion of NEFA was found to potentiate GSIS (168). However, this hypersecretion phenomenon slowly exerted detrimental effects on β cell secretory machinery and survival, potentially through nitric oxide-dependent stress (169, 170), pro-inflammatory signal activation (171, 172), alterations in the microRNA (miRNA) profiles (173) and ER stress induction (174). These alterations directly or indirectly delayed the processing by PC1/3 and PC2, increased the

proinsulin to insulin ratio and decreased overall response of glucose stimulated insulin secretion (175, 176). Different types and metabolites of NEFA showed varied effects on β cell function and whole body glucose homeostasis. For example, poly-unsaturated fatty acids resulted in a reduction in insulin secretion, while saturated fatty acids induced insulin resistance (177).

GLT is another commonly used model that mimics the combined toxic conditions of hyperglycemia and hyperlipidemia observed during obesity and T2D. In most cases, elevated levels of lipids only exert detrimental effects when glucose levels are also high (178). This combined treatment with lipids and glucose was found to have more robust deleterious effects on β cell function and survival when compared to either alone (179). However, it should also be noted that GLT induced a period of adaptation/compensation when β cell metabolism adjusted to utilizing lipid, thus augmenting insulin secretion. Genetic predisposition likely determines whether GLT results in β cell compensation or apoptosis (180). Indeed, different strains of mice demonstrate various compensatory responses under HFD-induced obesity (181).

b. Pro-inflammatory cytokines

Elevations in pro-inflammatory cytokines including IL-1β, tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) are commonly seen during the development of T1D. Interestingly, in a recent epidemiological study of persons with prediabetes or T2D, it was found that the inflammatory profile changed in parallel with disease progression, suggesting that pro-inflammatory cytokines also play an important role in the development of T2D (182). The majority of these pro-inflammatory cytokines are secreted from immune cells such as macrophages and activated T cells that reside in or invade islets (183). This idea was supported by a recent study utilizing cultured islets

overexpressing human islet amyloid polypeptide (IAPP), which aggregates to form amyloid fibrils in people with T2D and acts as a potent stimulator of IL-1β secretion from bone marrow–derived macrophages. In this study, upregulation of IL-1β was only found in resident macrophages, but not in other cell types (184). Despite inconsistent results from different groups, it has been suggested that β cells might also secrete small amount of cytokines in the context of diabetic stressors including GLT (185, 186). Interestingly, FFAs were found to increase the expression of IL-1 receptor 1 through toll like receptor-mediated signaling pathways. This pathway acts as a signal amplifier for pro-inflammatory stimuli and may serve as a mutual mechanistic connection between T1D and T2D (185, 187, 188).

Cytokines contribute to β cell death through a variety of pathways. For example, cytokines also induce expression of several miRNAs including *miR21, miR34a* and *miR146a*, which have been shown to contribute to pro-death pathways (189). The expression of p53 upregulated modulator of apoptosis (PUMA) was also up-regulated in response to cytokine treatment. These changes impaired protein chaperone profiles and changed the ratio of Bcl2-associated X protein (BAX) to Bcl-2, to induce apoptosis (190).

Cytokines also have prominent effects to activate of ER stress and apoptosis. These actions are thought to primarily be nitric oxide (NO)-dependent (191) and involve activation of NF-kB signaling pathways (192). For example, IL-1β strongly induces NO production through inducible nitric oxide synthase (iNOS), while islets isolated from iNOS null mice showed decreased apoptosis when exposed to IL-1β (193). NO was found to exert its detrimental apoptotic effects through activation of ER stress (194). Activation of JNK and attenuation of AKT also contributed to NO-dependent β cell apoptosis (195).

Lastly, cytokines are not always detrimental factors to β cells, as a very low dose of IL-1β was found to stimulate insulin secretion (196). In a recent study, in both mouse and human islets in response to metabolic stress during obesity but not late stage diabetes, IL-1β promoted insulin secretion through increasing the RRP of insulin granules, thus playing a role in compensatory hypersecretion (197).

c. Mitochondrial dysfunction, AMP-activated protein kinase (AMPK) and oxidative stress

Mitochondria also play a critical role in maintaining β cell function and survival. The commonly seen insults during the development of T2D, including GLT and inflammation, usually induce mitochondrial dysfunction that closely interacts with other stressors and causes activation of deleterious downstream pathways. Indeed, in T2D humans and in rodent models, mitochondria in β cells become disconnected, swollen, and shorter (198).

Mitochondria play a central role in metabolism–secretion coupling by generating ATP. In pancreatic β cells from individuals with T2D, uncoupling protein 2 (UCP-2) up-regulation was observed, leading to an impairment of respiratory-chain activation, loss of mitochondrial ATP production, and altered insulin secretion (199). Similarly, depletion of mitochondrial Ca^{2+} uptake 1 (MICU1) or mitochondrial Ca^{2+} uniporter (MCU) reduced mitochondrial Ca^{2+} uptake in response to glucose, thus diminishing ATP production and insulin secretion (200). In the meantime, reactive oxygen species (ROS) produced by the mitochondria during nutrient catabolism also regulates insulin secretion. Due to the constant challenge under nutrient surfeit conditions, β cells can generate large amounts of ROS from non-enzymatic glycosylation reactions, the mitochondrial electron transport chain, and the hexosamine pathway (201). Chronic accumulation of mitochondrial free-

radical production has been regarded as a result of diminished electron transport occurring when ATP production exceeds cellular energy demand. Although acute and low grade ROS potentiates insulin secretion (202), prolonged elevation of ROS triggers apoptosis (198). Given the fact that the β cell has a relatively low expression level of antioxidant enzymes, β cells are rather vulnerable to ROS induced cell damage (203, 204). Indeed, cadaveric donor islets from individuals with T2D demonstrate elevated ROS compared to individuals with normal glycemia (205). Although, it should be mentioned that unlike skeletal muscle, in response to ROS stress, global changes in oxidative metabolism gene expression was not observed, suggesting a unique ROS response in β cells (206).

The most well-studied downstream pathways activated upon ROS exposure are the JNK, p38 MAPK, and protein kinase C (PKC) pathways (114). Activation of these kinases was found to precede the decrease in *Pdx-1* and *insulin* gene expression, indicating the potential pathological role of ROS in β cell dysfunction in diabetes (201, 207). Mitochondria dysfunction in response to pro-inflammatory cytokines also occurred in islets isolated from diabetic rodent samples, which showed significantly decreased expression of Sirtuin (SIRT)3. SIRT3 is a key regulator of ROS production and has anti-inflammatory effects, while overexpression of SIRT3 induced elevation of ROS and apoptosis in cultured β cells (208).

As the most critical energy sensing node in the cell, AMPK regulates energy balance by activating ATP synthase. When cellular energy levels are low, signified by an increased ADP/ATP ratio, AMPK is activated. Whereas the AMPK activators metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) are commonly used drugs to improve insulin resistance in peripheral tissues, chronic activation of AMPK was found to cause obesity and impair β cell function (209). This finding suggests that long-term

AMPK activation can have adverse metabolic consequences. Indeed, AMPK was also activated under certain stress models like inflammation or oxidative stress induced by ROS or reactive nitrogen species (RNS), and may contribute to downstream detrimental effects (210). Clearly the AMPK pathway plays a critical role in β cell function, but remains poorly understood. One of the specific aims of my dissertation research has been to better characterize the role of AMPK in β cell Ca^{2+} homeostasis.

d. The unfolded protein response (UPR) and ER stress

A key downstream pathway activated by GLT and pro-inflammatory cytokines is the unfolded protein response (UPR). The UPR is a protective cascade that activates a series of transcription, translation, and degradation events to increase ER folding capacity, limit delivery of new proteins to the ER, and increase clearance of unfolded proteins (211-213). ER stress is a broad term that encompasses a series of complex cellular signaling events that occur in response to the accumulation of misfolded proteins in the ER lumen (154, 214). Initially, an adaptive UPR activates signaling cascades initiated by the dissociation of BiP, a calcium-dependent ER chaperone, from inositol-requiring enzyme 1 α (IRE1α), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK). This will trigger three distinct signalling cascades that synergize to restore ER health and homeostasis. IRE1α activates alternative splicing of *Xbp-1* mRNA. Spliced *Xbp-1* is a transcriptional activator of genes whose products regulate protein maturation, folding and ER export. IRE1α also works by degrading mRNAs, thereby limiting the delivery of new proteins to the ER. ATF6 is a basic leucine zipper transcription factor, which translocates to the Golgi after dissociation from BiP where it is cleaved and activated by Site-1 and Site-2 proteases. ATF6 subsequently translocates to the nucleus and binds the ER stress response element in the promoter of ER chaperones genes like *calnexin, calreticulin,* and *BiP*. The final arm of the UPR involves

activation of PERK, which phosphorylates eIF2α. Phosphorylated eIF2α inhibits 80s ribosome assembly and therefore decreases global protein synthesis. ER-associated degradation (ERAD) is also activated, which helps clear the ER of misfolded proteins (215, 216). While the goal of the adaptive UPR is to restore cellular and ER homeostasis, sustained activation of the UPR has detrimental effects. The transition to sustained activation is referred to as ER stress, and ultimately results in apoptosis and cell death. Sustained activation of IRE-1 and PERK activates JNK, pro-apoptotic BCL-2 family members like BAX and PUMA (217), and increases activation of C/EBP Homology Protein (CHOP) (218) and cleaved caspase-3 (211, 212, 219-221).

In response to the increasing demand for insulin due to peripheral insulin resistance under conditions of obesity, the β cells are driven to considerably higher levels protein synthesis and folding. This increased activity will trigger the UPR, which is initially adaptive. However, the process can culminate in terminal ER stress (211, 222, 223). Indeed, ER stress activation has been described in both rodent and human models of T2D (41, 212, 213, 224). Several key molecules involved in the UPR pathway have been studied using genetically modified mouse models to unravel their effects in regulating β cell fate under stress conditions. Overexpression of BiP in β cells protects against ER stress-induced cell death (225), while knocking out BCL2L11 (Bim) or PUMA was able to delay glucotoxicity-induced cell death (226). Cellular inhibitor of apoptosis protein-1 (cIAP1) was found to be protective from lipotoxicity-induced ER stress in β cells by increasing the degradation of CHOP (227). Together, these studies highlight the critical role that ER health plays in maintaining β cell survival, and demonstrate that a failure of adaptive UPR can contribute to apoptosis and β cell loss.

e. Calcium dyshomeostasis

The precise control of intracellular and extracellular Ca^{2+} gradients is critical in mediating a number of aspects of β cell function including stimulus-secretion coupling, insulin maturation, insulin granule transportation, exocytosis, proliferation, and activation of the UPR (30, 154, 228, 229). Dysregulated Ca^{2+} homeostasis is found in both the cytosol and organelles under T2D conditions (230). It was also found that as animals age, the function of the β cell deteriorates in parallel with impaired Ca^{2+} mobilization (231). It is well accepted that cellular Ca^{2+} overload or perturbation of intracellular Ca^{2+} compartmentalization can cause cytotoxicity, triggering either apoptotic or necrotic cell death (232). This usually manifests as a chronic elevation of cytosolic Ca^{2+}, depletion of ER Ca^{2+}, or both.

First, cytosolic Ca^{2+} overload from abnormal Ca^{2+} efflux from the ER leads to sustained elevations in cytosolic Ca^{2+}, which can cause apoptosis (233, 234). Both inborn and environmental factors may contribute to this phenomenon. For example, mutation in the gene WFS1 encoding wolframin induced an elevation in cytosolic Ca^{2+} levels, altered ER function, and caused cell death. The *WFS1* mutation is responsible for wolfram syndrome, which has the symptoms of DM, as well as optic atrophy, and deafness (77). Other Ca^{2+} regulators might also contribute to Ca^{2+} dysregulation and ER stress. In a recent study, a mouse model with mutated RyR2 with higher Ca^{2+} transportation rate caused activation of ER stress and mitochondrial dysfunction in β cells (235). Another study suggested that IP_3R is involved in ER stress activation potentially due to the subcellular localization of IP_3R in mitochondria-associated membranes (MAMs), which are areas of close contact between the ER and the mitochondria, thus controlling Ca^{2+} homeostasis in both organelles (198). Although neither RyR nor IP_3R seem to have a direct function in insulin biosynthesis, RyR2 also

serves as a protective factor to prevent calpain 10-dependent apoptosis (236). On the contrary, gain-of-function studies show that overexpression of PMCA2 or NCX1 leads to ER Ca^{2+} depletion with subsequent ER stress as well as lower proliferation and higher apoptosis (237).

Secondly, environmental factors like GLT, pro-inflammatory cytokines, oxidative stress and ER stress also cause Ca^{2+} dyshomeostasis. Acute treatment of isolated mouse islets with NEFAs elevated basal cytosolic Ca^{2+} levels, but only slightly affect glucose-stimulated Ca^{2+} increases (phase 1 amplitude) (238). Long term palmitate treatment (24 h) in human and mouse cultured β cells depletes ER Ca^{2+} and subsequently induces ER stress (239). Chronic GLT also significantly decreases Ca^{2+} mobilization in response to glucose and GSIS (240).

This Ca^{2+}-related apoptotic response occurs partially via an increase in the permeability of the outer mitochondrial membrane (233), leading to cytochrome c release and activation of a caspase cascade. In this regard, caspase 3, one of the most important caspase family members, plays a critical role in the process of apoptosis via DNA fragmentation and chromatin condensation. Caspase 3 has also been found to cleave IP_3R and PMCA leading to further disruptions of Ca^{2+} homeostasis (241). Treatment with the SERCA inhibitor, thapsigargin, induced ER Ca^{2+} leakage and decreased the expression of BiP, the ER chaperone protein that contributes to apoptosis (242). Increased cytosolic Ca^{2+} can also cause hyper-activation of calpain-2, followed by the cleavage of α-spectrin, leading to β cell death during T2D (243).

f. Rescue strategies of ER stress and calcium dyshomeostasis

Ca^{2+}-dependent apoptosis and dysfunction can be partially rescued by decreasing cytosolic Ca^{2+} levels, replenishing ER Ca^{2+} and improving of the folding

capacity of the ER. Ca^{2+} channel blockers that prevent the inward flux of Ca^{2+} from the extracellular space showed protective effects in β cell survival that were mediated by decreasing stress-induced TXNIP expression (244). Hepatic ER stress was rescued by SERCA2b overexpression or by treatment with a small molecule allosteric activator (245, 246). Chemical chaperones are small molecules that are non-selective in their ability to stabilize misfolded proteins and facilitate their proper folding. 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) are most commonly used, and are the only two chemical chaperones approved by US Food and Drug Administration (FDA) for use in humans (247). PBA was found to significantly reduce activated caspase-12 and pro-apoptotic CHOP protein levels in the liver of animals treated with an ER stress inducer (248). In pancreatic β-cells, PBA was also found to prevent the development of the aberrant ER morphology, which is characterized by a localized punctate pattern associated with ER stress, leading to an improved GSIS (248). TUDCA was shown to improve overall islet function by decreasing protein aggregates and restoring expression of UPR mediators (249).

g. Monitoring Ca^{2+} in live cells and tissues

As discussed above, intracellular Ca^{2+} homeostasis (especially ER Ca^{2+} levels) plays critical roles in a number of aspects of the β cell in both physiological and disease states. Therefore, it is essential to measure the mobilization of Ca^{2+} in different organelles in response to certain stimuli or conditions. Although technically challenging, there are several different methods available to evaluate Ca^{2+} concentrations in these different cell compartments in cultured cells and tissues (250). There are mainly 3 types of cellular Ca^{2+} indicators: photoproteins, chemical Ca^{2+} indicators/fluorescent dyes, and fluorescent protein-based biosensors (251).

The photoproteins, mainly aequorin and genetically modified versions of aequorin, were initially discovered in jellyfish and were used to provide the first visualization of Ca^{2+} waves. These experiments required single cell injection and covered a wide Ca^{2+} range from 40 nM to 40 µM. Monitoring aequorin does not require laser excitation, thus minimizing the problems of autofluorescence, photo-bleaching and high background. However, the lack of fluorescence makes this method incompatible with high-resolution imaging microscopy. Furthermore, the low intensity of signal, over-sensitivity to Ca^{2+} concentration fluctuations and difficulty of equilibration have led to decreased use of aequorin (252).

The main fluorescent dye used to monitor Ca^{2+} concentrations is based on the Ca^{2+} chelator bis-(o-aminophenoxy)-ethane-N,N,N9,N9-tetra acetic acid (BAPTA). There are a number of commercially available chemical dyes that have different affinities to Ca^{2+} and varying spectral properties. The most commonly used fluorescent dye is Fura-2-acetoxymethyl ester (Fura-2 AM). This dye has 3 useful features: 1) high Ca^{2+} affinity (thus more suitable for cytosolic Ca^{2+} measurement which is at nM level); 2) ratiometric measurements that allow a very accurate quantification of Ca^{2+} concentration that is corrected for uneven dye loading, dye leakage, photo-bleaching and changes in cell volume; and 3) the dye is provided in a acetoxymethyl ester chemical form, which is membrane permeable and thus doesn't require an invasive loading procedure (253). The removal of the ester group by intracellular esterases prevents the dye from leaking back out of the cell.

Despite the convenience of chemical fluorescent dyes, one of the biggest disadvantages of this technique is that it cannot clearly differentiate Ca^{2+} concentrations in different organelles. However, the last type of Ca^{2+} indicator, genetically encodable Ca^{2+} indicators or Ca^{2+} sensitive fluorescent protein, have been developed to be

specifically expressed in certain organelles. Recently, the most commonly used of these is the cameleon-based fluorescent protein D1ER (254). By improving Ca^{2+} affinity and inserting the insulin promoter, D1ER was engineered to be D4ER which allows for exclusive expression in the ER of β cells (255). Our studies, as well as those of others have used this approach to detect changes in ER Ca^{2+} concentrations using this probe (255, 256).

Recently, an improved Ca^{2+} indicator, Twitch-1, was developed and has been used to monitor Ca^{2+} levels in T cells. Due to the exceptionally wide dynamic range of this indicator, it can be used to study different compartments of cell at the same scale (257). The Twitch and D1/D4ER biosensor probes generate signals through Förster Resonance Energy Transfer (FRET). The sensor unit of biosensor probes contains linked donor and acceptor fluorophores, and conformational changes allow FRET between the donor to the acceptor fluorophore. The most accurate methods for detecting changes in FRET signals measure the change in the donor fluorescence lifetime that results from the quenching by the acceptor (258). This can be monitored at a microenvironment level in individual cells by fluorescence lifetime imaging microscopy (FLIM) (259).

1.4 SERCA Structure, Function, and Regulation in β Cells

1.4.1 Protein structure, kinetic features and different isoforms of SERCA

In mammals, there are 3 different SERCA genes (*ATP2A1*, *ATP2A2* and *ATP2A3* encoding the proteins SERCA1, 2 and 3) with at least 14 isoforms arising as a result of alternative splicing. Tissue-specific expression patterns suggest cell specific function of different isoforms and also indicate patterns of temporal expression during development (260). The SERCA protein contains 10-11 transmembrane α-helix domains

and 3 additional major domains that extend into the cytosolic compartment. These domains include the P domain, where phosphorylation occurs and the N domain, which serves as the site of nucleotide-binding. These two domains form the catalytic site where ATP is hydrolyzed. The A domain, or actuator domain, is involved in the transmission of major protein conformational changes during each catalytic cycle (261).

There are two major conformational statuses of SERCA pumps: the E1 form has relatively high Ca^{2+} affinity, while the E2 confirmation has a lower affinity. The function of SERCA is highly ATP dependent, and each catalytic cycle starts with the E1 conformation, where 2 Ca^{2+} molecules are bound in the absence of ATP (E1Ca2). When ATP binds to the N domain, it triggers a conformational change and phosphorylation of the P domain (E1Ca2$\rightarrow$ E1PCa2). This event decreases Ca^{2+} affinity and drives the movement of Ca^{2+} into the ER lumen (E1PCa2 $\rightarrow$ E2PCa2 $\rightarrow$ E2P), thus favoring hydrolysis of the phosphoenzyme (E2P $\rightarrow$ E2) and completion of the cycle (262).

Among SERCA isoforms, there are two general categories. The first includes isoforms mainly found in muscle and includes SERCA1 and SERCA2a. The second category consists of non-muscle isoforms and includes SERCA2b and SERCA3. These various isoforms not only have different tissue and developmental expression profiles, but the affinity and ATP turnover rates also vary. SERCA2b and SERCA3 are rather unique among all the isoforms in terms of kinetic features (263). Compared to the structure of SERCA2a (264), SERCA2b uniquely contains an extra 11^{th} transmembrane α-helix at the C-terminus, which stabilizes the Ca^{2+} bound confirmation and provides the highest Ca^{2+} affinity among all isoforms as well as the slowest ATP turnover rate (44). On the other hand, SERCA3 has the lowest affinity for Ca^{2+} with a higher optimum pH. The other isoforms have nearly identical kinetic features.

We have previously shown SERCA2b to be the most prevalent isoform expressed in the mouse pancreatic islet. The second most abundant isoform is SERCA3, which is expressed at about 50% of the level of SERCA2b. The alternatively-spliced isoform, SERCA2a, is expressed at nearly 100-fold lower levels compared to SERCA2b (265). Among all the isoforms, the role of SERCA3 in the pancreatic β cell has been previously investigated in a series of papers by Patrick Gilon and colleagues. Using whole-body SERCA3 knockout mice, this group showed that lack of SERCA3 alone does not lead to increased cytosolic Ca^{2+} in islets and is not sufficient to disturb glucose homeostasis or impair insulin secretion (266). Rather, SERCA3 KO mice exhibited normal glucose tolerance without overt evidence of ER stress. SERCA3 ablation also did not affect basal cytosolic Ca^{2+} levels or the initial glucose-induced Ca^{2+} response within islets (266). Following glucose stimulation, SERCA3 null islets exhibited a higher amplitude of Phase 2 $[Ca^{2+}]i$ oscillations, consistent with impaired ER Ca^{2+} uptake that interestingly led to increased insulin secretion (255). Together, these data suggest that a lack of SERCA3 is insufficient in itself to alter glucose homeostasis or impair insulin secretion in mice. Rather, SERCA3 has a regulatory role in Ca^{2+} oscillation.

1.4.2 Transcriptional and post-translational regulation of SERCA2

There is some evidence suggesting that SERCA2 serves as a stress response gene and is part of a compensatory response to protect against ER stress. The SERCA2 promoter contains multiple ER stress response elements. While it has not been studied in the β cell, ER stress was found to upregulate SERCA2 transcription and activity in adrenal gland phaeochromocytoma cells (267, 268) potentially through an ATF6 binding site found in SERCA promoter (269). In response to acute ischemia, SERCA2a has been shown to be upregulated in cardiac muscle cells, suggesting an adaptive response to hypoxia (270). Similarly, ischemia induced SERCA2b mRNA up-

regulation in cortical area 1 (CA1) and CA3 hippocampal neurons in a hypoxia-inducible factor 1-α (HIF-1α) dependent manner (271).

In contrast, we and others have demonstrated diminished β cell SERCA2b expression in human and rodent models of T1D and T2D, resulting in Ca^{2+} dyshomeostasis, impaired insulin secretion, activation of ER stress signaling pathways, and impaired β cell survival (77, 256, 265, 272, 273). In addition to its role in β cells, SERCA2b has also been shown to be decreased in liver in obesity and metabolic syndrome, leading to hepatic ER stess (274).

A major goal of my work in this dissertation has been to define the pathways leading to altered β cell expression of SERCA2b under pro-inflammatory and diabetic conditions. We and others have shown that loss of SERCA2b mRNA and protein is partially NO-dependent (272, 275). We have also identified peroxisome proliferator-activated receptor γ (PPARγ) and Pdx-1 as transcriptional regulators of SERCA2. Specifically, the binding ability of PPARγ to SERCA2 promoter decreased due to under phosphorylation of S273 site, and loss of Pdx-1 expression under diabetic conditions which all contribute to down-regulation of SERCA2b (256, 265).

SERCA2 expression was also lower in infarcted myocardium tissues, and a number of miRNA species were found to be elevated in these tissues as well. Through bioinformatics strategies, miRs predicted to target SERCA2 mRNA (*miR-199a* for *SERCA2b*, *miR-140* for both isoforms, and *miR-574* for *SERCA2a*) were discovered. However, this observation was based on correlation and the direct dependency of these miRNA species and SERCA2 expression regulation need to be confirmed (276).

In addition to transcriptional mechanisms, post-translational mechanisms of SERCA2 regulation have been described. Under high-glucose induced oxidative stress, SERCA2 protein undergoes a number of post-translational modifications that affect both catalytic activity and protein stability, including nitration at the cysteine-674 site which is

thought to be an early marker in heart failure (261, 277). Nitration at tyrosine-294 and tyrosine-295 were also observed in SERCA2 in skeletal muscle, and this phenomenon has been correlated with an aging-induced decrease in SERCA activity (278).

1.4.3 SERCA dysfunction and human disease

Mutations in genes encoding isoforms of SERCA have been associated with different human diseases. Mutations in in *ATP2A1* (encoding SERCA1) cause the autosomal recessive condition, Brody's myopathy (274). Mutations in *ATP2A2* (encoding SERCA2) underlie an autosomal dominant disorder, Darier disease with the symptom of vulnerability in both keratinocytes and neuronal cells (279). As an animal model of Darier disease, whole body SERCA2 haploinsufficent mice have been developed that have higher susceptibility to squamous cell tumors and heart dysfunction (280, 281). Indeed, SERCA2a expression is reduced in heart failure in humans (282). As for diabetes, SERCA2b was lower in insulin resistant macrophage foam cells, which might contribute to the development of atherosclerosis (283). In streptozotocin (STZ)-induced diabetic rats, SERCA2a was significantly decreased in the soleus muscle with peripheral neuropathy (284). However, to date, the *in vivo* role of SERCA2 in the maintenance of metabolic homeostasis especially in regulating β cell function remains incompletely characterized. One of the specific aims in my thesis will cover this area.

1.5. Hypothesis and significance

My central hypothesis is that SERCA2b plays a critical role in the maintenance of β cell function and that loss of SERCA2b expression and activity plays a pivotal role in the failed β cell compensatory response and stimulated insulin secretion during the development of T2D.

1.5.1 Post-translational regulation of SERCA2 under pro-inflammatory condition

In chapter 2, I have defined the mechanisms through which NO signaling impacts SERCA2b expression in the pancreatic β cell. I demonstrated that NO-mediated loss of SERCA2b expression and activity is primarily due to post-translational mechanisms and identified AMPK as one of the pathways that synergize with NO to regulate β cell ER Ca^{2+} homeostasis under inflammatory conditions. These results provided a novel role for chronic AMPK activation in the regulation of SERCA2b in the β cell.

1.5.2 *In vivo* role of SERCA2 in the β cell under metabolic stresses

In chapter 3, I determined how SERCA2b regulates whole body glucose homeostasis, β cell integrity and insulin secretion. I showed that SERCA2b deficiency impaired whole body glucose homeostasis and islet integrity due to the failure of β cells to compensate for metabolic stress during obesity. Abnormal Ca^{2+} management in the context of SERCA2 deficiency led to altered β cell proliferation, secretory function, and survival while SERCA2 overexpression or the application of a small molecule allosteric activator reduced effects on β cell function and survival. These discoveries have provided key information about the global metabolic phenotype arising from SERCA2b deficiency and suggest this pathway may be a potential therapeutic target for diabetes.

CHAPTER TWO

NO Stress and Activation of AMPK Impair β Cell SERCA2b Activity and Protein Stability

2.1 Introduction

Loss of β cell SERCA2b expression under pro-inflammatory and diabetic conditions is thought to occur, at least partially, through nitric oxide (NO) dependent mechanisms (272). However, whether NO-mediated loss of SERCA2b expression and activity is primarily due to a transcriptional or post-translational mechanism is not clear. Moreover, the downstream pathways that synergize with NO-signaling to alter endoplasmic reticulum Ca^{2+} have not been fully defined. In this regard, the activity and fidelity of ATPases including SERCA are highly dependent on overall cellular energy status. SERCA pumps are estimated to consume 7%-25% of total cellular ATP whereas ADP levels are closely correlated with SERCA activity (285-287). A well-recognized downstream effect of pro-inflammatory cytokine signaling and NO production is an impairment of mitochondrial function, leading to altered ATP synthesis (288). Whereas these effects contribute to β cell dysfunction and apoptosis, they also lead to activation of AMPK, which serves as the master sensor of cellular energy status (289). AMPK has a wide range of downstream substrates involved in a variety of processes including metabolism, inflammation, as well as ion transport (290). Interestingly, AMPK has been shown to directly regulate the activity, expression, and cellular localization of other highly-energy consuming protein pumps including the plasma membrane Na^+,K^+-ATPase in lung epithelial cells and the H^+ ATPase of the intercalated cells of the kidney (291-293). This level of interaction represents one mechanism through which AMPK signaling may act to limit energy expenditure under stress conditions. Interestingly, a

recent proteomics study performed in β cells suggested a direct physical interaction between AMPK and SERCA2 (294), but a functional relationship between SERCA2b and AMPK in the β cell remains unexplored.

Here, we first aimed to define the mechanisms through which NO signaling impacts SERCA2b levels in the pancreatic β cell. Our second goal was to identify pathways that synergize with NO to regulate β cell ER Ca^{2+} homeostasis under inflammatory conditions, hypothesizing a novel role for chronic AMPK activation in the regulation of SERCA2b expression and activity.

2.2 Results

The pro-inflammatory cytokine IL-1β decreases SERCA2b protein but not mRNA stability in both INS-1 cells and isolated rat islets.

Previous work by our group and others has demonstrated significant downregulation of SERCA2b mRNA and protein levels under diabetic conditions (256, 265, 272, 273). To determine whether this downregulation was secondary to alterations in either mRNA or protein stability, actinomycin and cycloheximide time-course experiments were performed under basal conditions and then following treatment with the pro-inflammatory cytokine IL-1β. Under control conditions, β cell SERCA2b mRNA exhibited a half-life of ~9 h, and IL-1β treatment had no effect mRNA stability (Figure 2A). In contrast, SERCA2 protein in INS-1 cells exhibited a half-life of ~24 h under basal conditions (Figure 2B and C), and IL-1β significantly reduced the half-life to ~19 h (Figure 2B and C). In rat islets, the protein half-life was noted to be ~17 h under control conditions, while treatment with IL-1β significantly reduced the half-life to ~11h (Figure 2D and E).

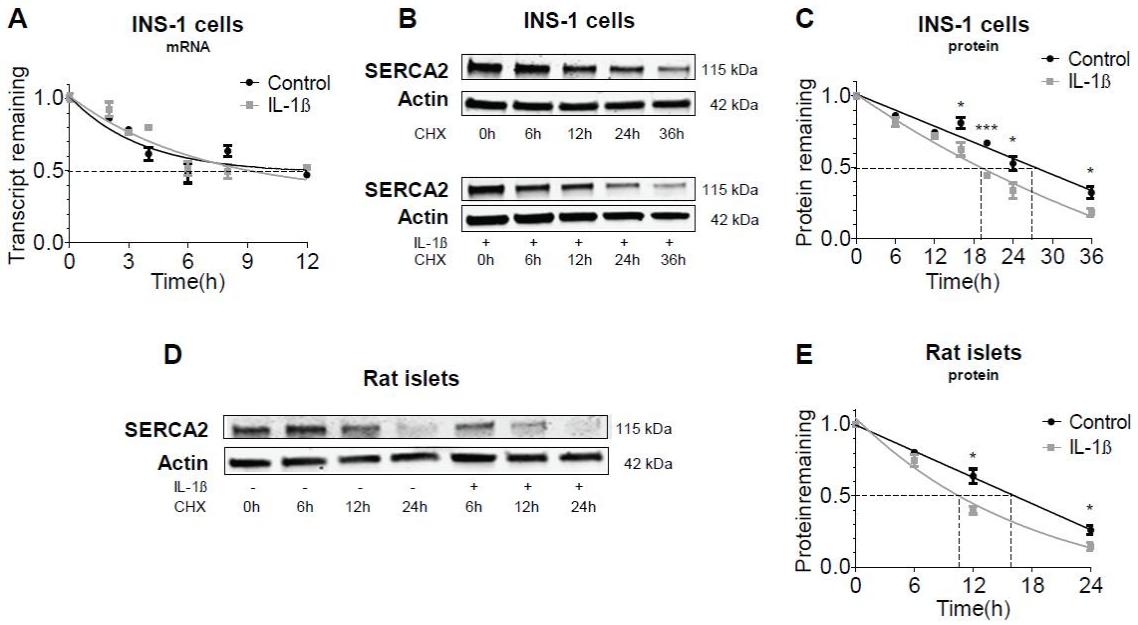

Figure 2. IL-1β treatment decreases SERCA2b protein stability in INS-1 cells and isolated rat islets.

INS-1 cells (**A-C**) or isolated rat islets (**D-E**) were treated with 1µM actinomycin or 10µM cycloheximide (CHX) combined with or without 5ng/ml IL-1β for indicated times. Total RNA and protein were isolated, and RNA was subjected to real-time qRT-PCR for quantification of SERCA2b and actin transcript levels. Immunoblot was performed using antibodies against SERCA2 and actin. Protein and mRNA levels were plotted relative to levels at time zero, and one phase decay lines for each treatment are shown. Indicated comparisons are significantly different (*$p < 0.05$, ***$p < 0.001$).

NO-dependent downregulation of SERCA2 occurs at the translational level.

The precise mechanisms underlying NO-mediated alterations in SERCA2b expression have not been fully defined. To this end, INS-1 cells were treated with IL-1β, combined with or without the nitric oxide synthase (NOS) inhibitor, NG-monomethyl L-arginine (L-NMMA), for 24 h(77). Following IL-1β treatment, loss of both SERCA2b protein and mRNA expression was observed (Figure. 3A-C). L-NMMA treatment was able to rescue SERCA2b protein levels (Figure. 3A-B). However, no effect was observed on mRNA expression (Figure. 3C). These results were confirmed in rat islets (Figure. 3D-E), where L-NMMA also resulted in a partial rescue of SERCA2 expression following treatment with the pro-inflammatory cytokine IL-1β.

INS-1 cells were treated next with IL-1β, combined with or without the specific NO scavenger Carboxy-PTIO potassium salt (C-PTIO) (Figure. 3F-H) (285). Similar to effects observed with L-NMMA, C-PTIO rescued SERCA2 protein but not transcript levels (Figure. 3H), suggesting IL-1β-mediated effects on protein expression were indeed NO-dependent. To define whether NO was then sufficient to exert this effect, INS-1 cells were treated with the NO donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP). As expected, SNAP decreased SERCA2 protein to a level almost equivalent to that observed with IL-1β, while mRNA levels were not significantly altered (Figure. 4A-C). Nitrite levels in the culture media were measured under each experimental condition and found to increase with IL-1β and SNAP treatment, while L-NMMA exhibited the expected effect of decreased nitrite production following IL-1β treatment (Figure. 4D). Finally, to confirm these results in primary cells, rat and cadaveric human islets were treated with SNAP. Consistent with results observed in INS-1 cells, SERCA2 protein expression was significantly decreased compared to control conditions in both rat and human islets (Figure. 4E-H).

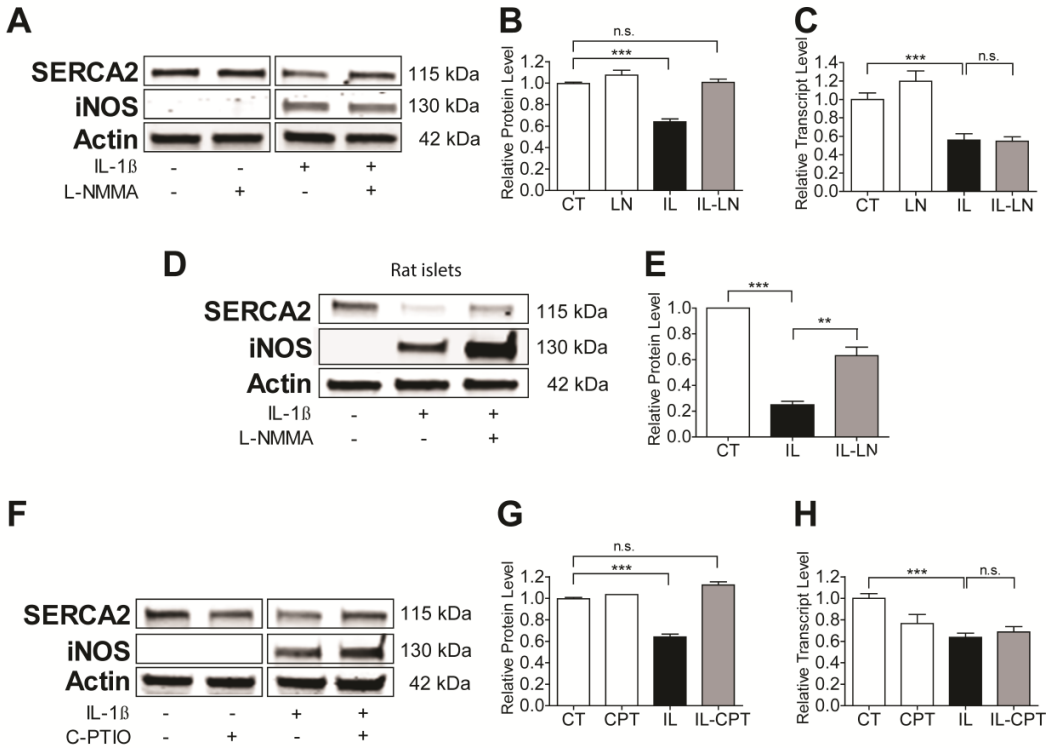

Figure 3. NO-dependent loss of SERCA2 expression occurs at a translational level.

INS-1 cells (**A-C, F-H**) or isolated rat islets (**D-E**) were treated with DMSO (CT) or IL-1β (IL) combined with or without 0.5 mM of the NOS inhibitor L-NMMA (LN) or 100µM of the NO scavenger C-PTIO (CPT) for 24h. Total protein was isolated and immunoblot was performed using antibodies against SERCA2, inducible NO synthase (iNOS), and actin. Quantitative protein levels of SERCA2 are shown graphically (**B, E, G**). Total mRNA was isolated from the INS-1 cells treated with CT, LN, IL, IL-LN or IL-CPT, and reverse-transcribed RNA was subjected to real-time PCR for quantification of SERCA2b and actin transcript levels (**C**). Indicated comparisons are significantly different (**$p < 0.01$, ***$p < 0.001$).

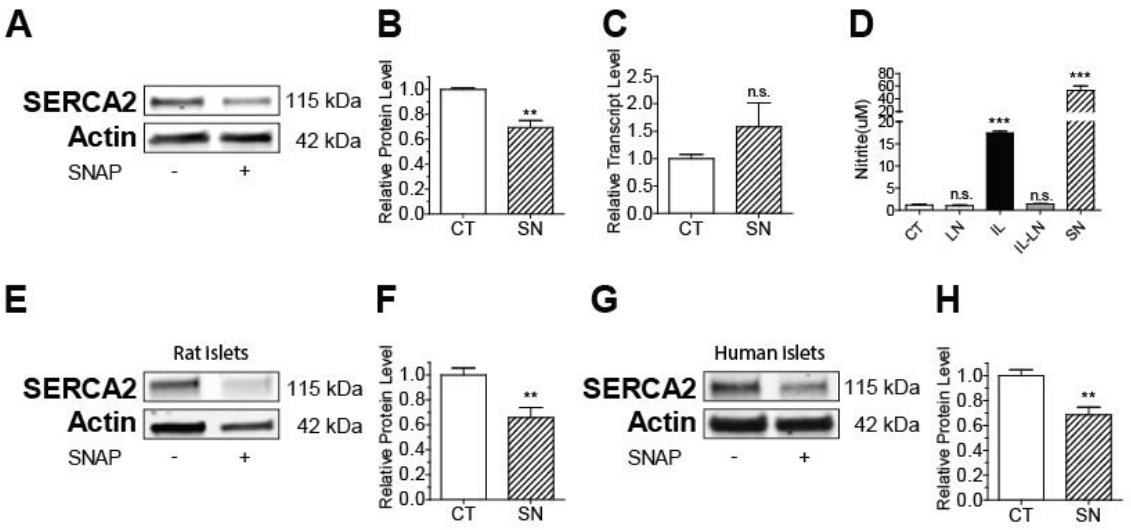

Figure 4. Direct effects of NO impair SERCA2 expression at the translational level.

INS-1 cells, isolated rat islets, or human islets were treated with or without the NO donor SNAP (SN) at 300mM for 24h (**A-C, E-H**). Total protein and mRNA were isolated. Immunoblot was performed using antibodies against SERCA2 and actin and quantitative protein levels are shown graphically (**B, F, H**). Reverse-transcribed RNA was subjected to real-time PCR for quantification of SERCA2b and actin transcript levels (**C**). INS-1 culture media was collected at treatment end, and nitrite concentration were measured as described in Materials and Methods (**D**). Results are statistically different from control conditions (**B, D, F, H**), or indicated comparisons are significantly different (**$p < 0.01$, ***$p < 0.001$).

Phosphorylation of AMPKα at Th173 contributes to SERCA2 downregulation at the translational level.

To define novel downstream pathways that synergized with NO to influence SERCA2b expression and the overall regulation of ER Ca^{2+} homeostasis and test whether AMPK-mediated effect is involved, INS-1 cells and isolated rat islets were treated with IL-1β combined with or without the AMPK inhibitor, compound C (CC). Increased levels of phosphorylated AMPKα on Th173 were observed following treatment with IL-1β, confirming previous findings that pro-inflammatory cytokines lead to the activation of AMPK signaling (289). Likewise, AMPK activation was blocked by CC (Figure. 5A). Interestingly, CC was also capable of fully reversing IL-1β-mediated loss of SERCA2 protein (Figure. 5A-B). Again, these effects appeared to be primarily restricted to protein expression as no significant change in transcript levels were observed (Figure. 5C). To confirm this relationship, rat islets were treated with IL-1β and CC. Similar to results obtained in INS-1 cells, altered SERCA2 protein expression under inflammatory conditions was prevented by CC (Figure. 5D-E).

Next, to study whether direct activation of AMPK was sufficient to decrease SERCA2 expression, INS-1 cells were treated with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) for 24 h. Results demonstrated that AICAR indeed decreased SERCA2 protein expression to a level similar to that observed with IL-1β and SNAP treatment (Figure. 5F-G). Consistent with previous results observed with SNAP, mRNA levels were again unaffected (Figure. 5H). Decreased SERCA2 protein expression with AICAR mediated-AMPK activation was confirmed in isolated rat islets and cadaveric human islets (Figure. 5I-L). In aggregate, these results indicate that β cell SERCA2 protein half-life is significantly altered under inflammatory conditions and loss of protein expression occurs concomitantly through NO and AMPK-dependent mechanisms.

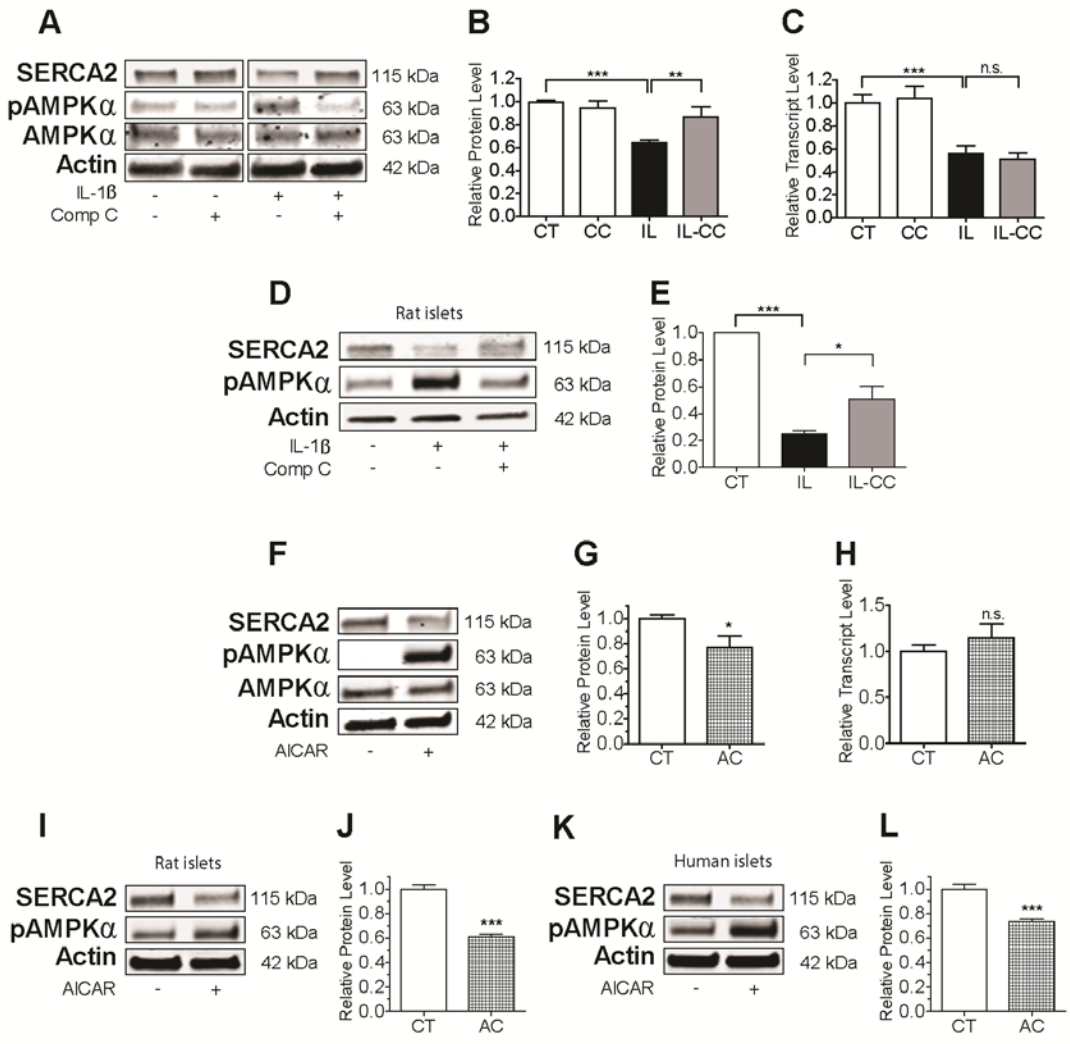

Figure 5. Activation of AMPKα Th173 leads to a loss of SERCA2 protein expression.

INS-1 cells (**A-C**) or isolated rat islets (**C-D**) were treated with DMSO (CT) or 5ng/ml IL-1β (IL) combined with or without 10μM of the AMPK inhibitor compound C (CC) for 24h. Total protein was isolated and immunoblot was performed using antibodies against SERCA2, phosphorylated AMPKα Th173 (pAMPKα), total AMPK and actin. Quantitative protein levels of SERCA2 are shown graphically (**B, E**). Total mRNA was isolated from the INS-1 cells treated with CT, IL and IL-CC, and reverse-transcribed RNA was subjected to real-time PCR for quantification of SERCA2b and actin transcript levels (**C**). Next, INS-1 cells, isolated rat islets or human islets were treated with and without AMPK activator AICAR (AC) at 2mM for 24h (**F-L**). Total protein and mRNA were isolated, and immunoblot was performed using antibodies against SERCA2, pAMPKα, AMPK and actin. Quantitative protein levels of SERCA2 are shown graphically (**G, J, L**). Reverse-transcribed RNA was subjected to real-time PCR for quantification of SERCA2b and actin transcript levels (**H**). Indicated comparisons are significantly different (**p*<0.05, ***p* < 0.01, ****p* < 0.001), or results are statistically different from control conditions (G, H, J, L).

NO and activation of AMPK decrease SERCA2 protein stability

We next addressed whether NO and AMPK-mediated loss of SERCA2 expression was specifically resulted from alterations in protein stability or not. INS-1 cells were co-treated with CHX combined with or without SNAP or AICAR. Results showed that treatment with both SNAP and AICAR significantly reduced SERCA2 protein stability (Figure 6A-C).

AMPK activation is required for IL-1β-induced downregulation of SERCA2 protein

INS-1 cells, rat islets, and human islets were next transduced with an HA-tagged AMPK-DN or luciferase expressing control adenovirus to further test the relationship between AMPK signaling and SERCA2 expression. Reduced AMPK signaling with the AMPK-DN construct was confirmed by reductions in the level of phosphorylated Acetyl-CoA carboxylase (pACC), a key downstream target of AMPK (Figure. 7A,C-D) (290). Virally transduced INS-1 cells and rat islets were treated with IL-1β whereas human islets were treated with a combination of IL-1β, INF-γ, and TNF-α. Interestingly, the AMPK-DN adenovirus increased basal expression of SERCA2 in rat and human islets (Figure. 7C-D). Moreover, in AMPK-DN transduced INS-1 cells (Figure. 7A-B), rat islets (Figure. 7C), and human islets (Figure. 7D), cytokine-induced reductions in SERCA2 protein expression were prevented. Taken together, these data demonstrate that AMPK is necessary for pro-inflammatory-induced downregulation of SERCA2 protein expression.

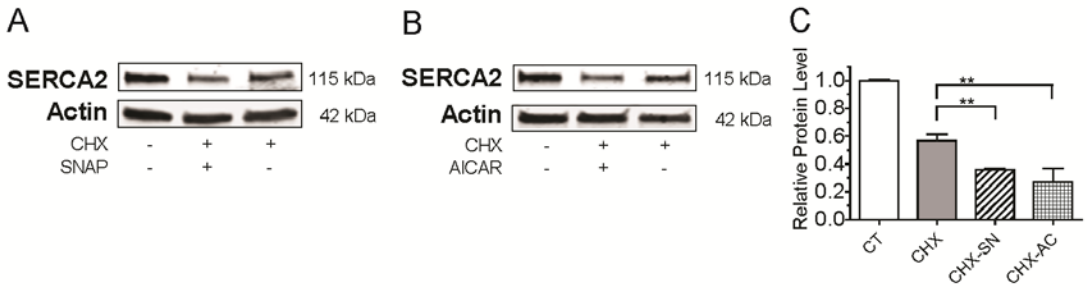

Figure 6. SERCA2 protein stability is decreased by NO-dependent signaling and AMPK activation.

INS-1 cells were treated with DMSO (CT) or 10μM cycloheximide (CHX) combined with or without 300 mM of SNAP (SN) or 2mM AICAR (AC) for 24 h. **A-B**, Total protein was isolated; immunoblot was performed using antibodies against SERCA2 and actin. **C.** Quantitative protein levels of SERCA2 are shown graphically. Indicated comparisons are significantly different (**$p < 0.01$).

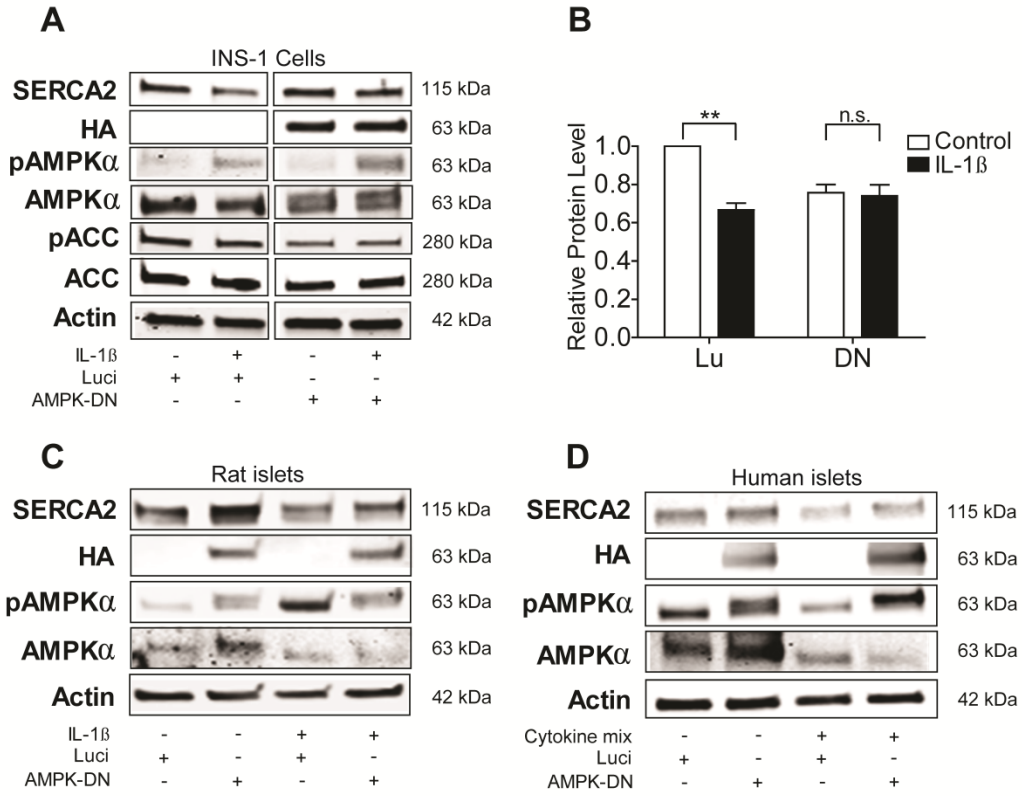

Figure 7. AMPK activation is required for IL-1β-induced loss of SERCA2 protein expression.

INS-1 cells (**A-B**), isolated rat islets (**C**) or non-diabetic cadaveric human islets (**D**) were transduced with an HA-tagged AMPK dominant negative (AMPK-DN or DN) or control adenovirus (Luci or Lu) before 24-h treatment with or without 5 ng/ml IL-1β (for INS-1 cells and rat islets) or a combination of 5ng/ml IL-1β, 1 ng/ml TNF-α and 100 ng/ml IFN-γ in human islets. Total protein was isolated, and immunoblot was performed using antibodies against SERCA2, pAMPKα, total AMPKα, phosphorylated ACC(pACC), total ACC, HA, and actin. **B**. Quantitative protein levels of SERCA2 in INS-1 cells are shown graphically. Indicated comparisons are significantly different (**$p < 0.01$).

AMPK activation modulates IL-1β induced iNOS expression and it is required in SNAP induced down-regulation of SERCA2.

To determine the relationship between AMPK and NO signaling following cytokine stress, INS-1 cell and rat islets were treated with CC concurrently with IL-1β. Interestingly, iNOS protein expression was significantly decreased in the presence of CC (Figure. 8A-C). Similarly, decreased iNOS gene expression was observed in INS-1 cells treated with CC and IL-1β (Figure. 8H). To rule out nonspecific effects from the use of pharmacological inhibitors, INS-1 cells and rat islets were again transduced with the AMPK-DN or control adenovirus and subsequently treated with IL-1β. In AMPK-DN transduced INS-1 cells and rat islets, a similar reduction in iNOS expression was noted following IL-1β treatment, and reduced iNOS gene expression was observed (Figure. 8D-F, I). This relationship was confirmed by measuring nitrite production, where results showed that IL-1β-mediated increases in nitrite production were dramatically reduced with compound C and partially reduced in INS-1 cells transduced with the AMPK-DN adenovirus (Figure. 8G). To define whether AMPK activation was then necessary for SNAP-induced loss of SERCA2 expression, INS-1 cells were treated with SNAP combined with or without CC. Interestingly, SERCA2 protein expression was indeed preserved by CC treatment (Figure. 9A-B).

In aggregate, our data showed that AMPK is activated under inflammatory conditions and IL-1β-mediated reductions in SERCA2 expression were partially AMPK-dependent. However, our results also showed that AMPK signaling led to an amplification of NO-mediated inflammatory responses in the pancreatic β cell. To investigate this further, INS-1 cells were treated with 1 mM and 2 mM of AICAR for 24 h. Results showed that AICAR decreased IκBα protein levels in a dose-dependent manner, suggesting that AMPK activation leads to reduced retention of NFκB in the cytosol (Figure. 9C-D).

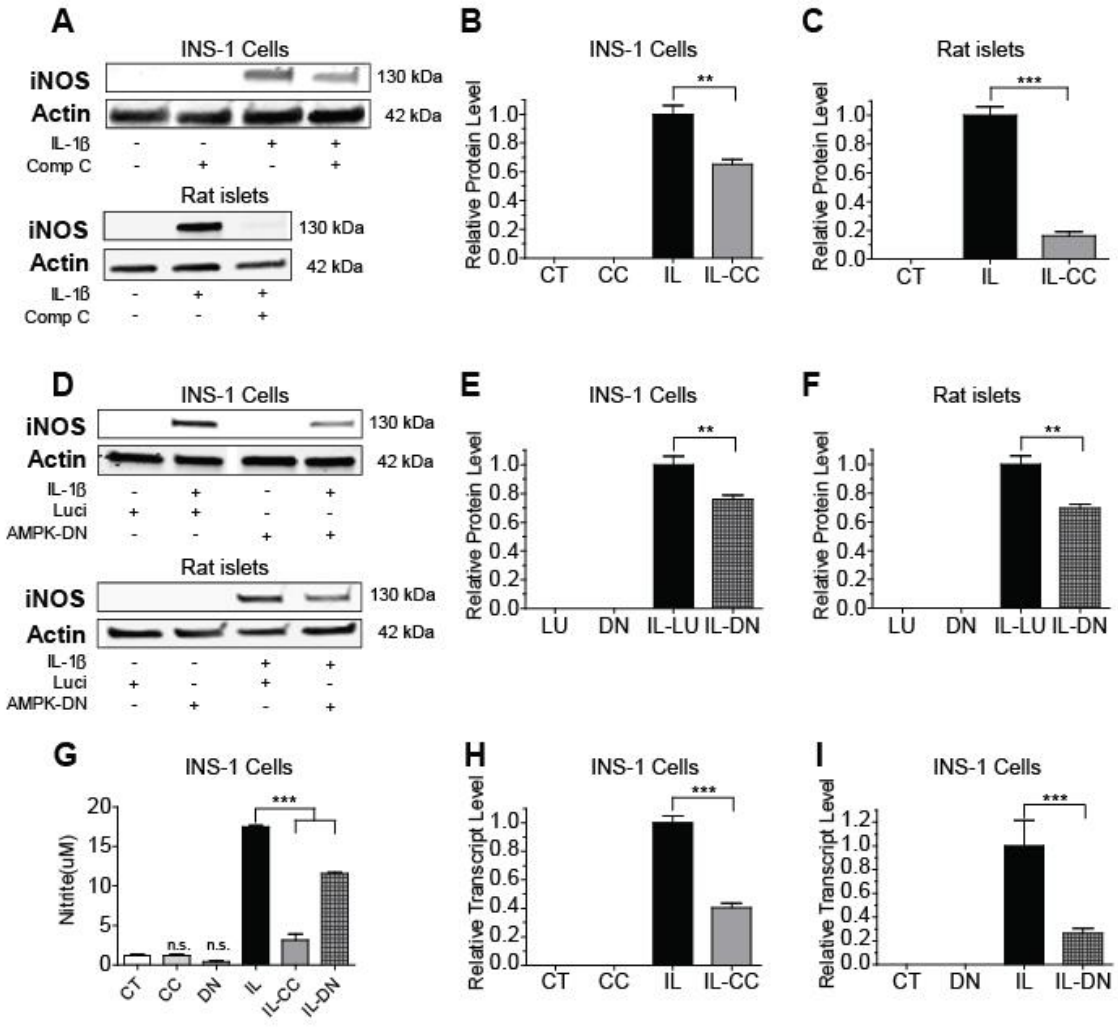

Figure 8. AMPK activation modulates IL-1β induced iNOS expression.

A-I, AMPK activation modulates IL-1β-induced iNOS expression and is required for SNAP-induced downregulation of SERCA2. INS-1 cells or isolated rat islets were treated with dimethyl sulfoxide (DMSO) (CT), CC, 5 ng/ml IL-1β (IL) with or without CC (IL-CC) or transduced with an HA-tagged AMPK-DN or DN or control adenovirus (Luci or Lu) before treatment with or without 5 ng/ml IL-1β. **A and D,** Total protein was isolated, and immunoblot was performed using antibodies against iNOS and actin. **B, C**, **E, F,** Quantitative protein levels of iNOS are shown graphically. **G**, INS-1 culture media was collected at treatment end, and nitrite concentration measurement was performed. **H and I** Total mRNA was isolated from INS-1 cells, and reverse-transcribed RNA was subjected to real-time PCR for quantification of iNOS and actin transcript levels. Indicated comparisons are significantly different (**p*<0.05, ***p*<0.01 and ****p*<0.001)

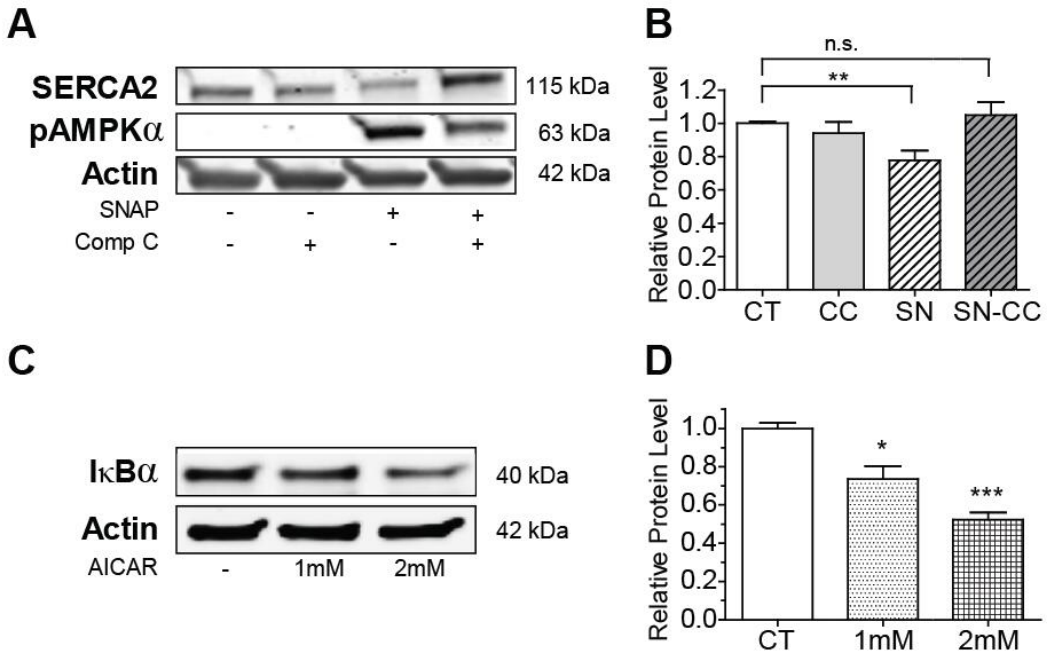

Figure 9. AMPK activation is required in SNAP induced down-regulation of SERCA2.

A-D, INS-1 cells were treated with DMSO (CT), 300 mM of SNAP (SN) combined with or without 10 μM of CC, or AICAR at the indicated doses for 24 h. Total protein was isolated, and immunoblot was performed using antibodies against SERCA2, pAMPKa, IκBα and actin. Quantitative protein levels of SERCA2 (**B**) or IκBα (**D**) are shown graphically. Indicated comparisons are significantly different (*p<0.05, **p<0.01 and ***p<0.001)

AMPK activation alters β cell Ca^{2+} homeostasis and SERCA2 activity.

To explore the functional effects of pro-inflammatory signaling on β cell Ca^{2+} homeostasis, the FLIPR Calcium 6 Assay Kit was used to measure basal cytosolic Ca^{2+} levels and Ca^{2+} mobilization from the ER in response to the indicated compounds (Figure. 10A-D). Experiments were performed both in the presence (Figure. 10A-B) and absence of extracellular Ca^{2+} (Figure. 10C-D), and showed that IL-1β, SNAP, and AICAR significantly increased basal cytosolic Ca^{2+} levels and decreased ER Ca^{2+} levels (as assessed by changes in the ΔF/F0 ratio following caffeine treatment). Notably, the effects of IL-1β were reversed by co-treatment with L-NMMA. In aggregate, these results demonstrate that pro-inflammatory NO-mediated signaling as well as AMPK activation alter β cell Ca^{2+} compartmentalization, resulting in decreased ER Ca^{2+} levels and a reciprocal increase in basal cytosolic Ca^{2+}.

Next, INS-1 cells were incubated with the Ca^{2+} dye Fura-2AM. Basal Ca^{2+} levels within the cytosolic compartment and changes in Ca^{2+} transit following ER Ca^{2+} depletion with caffeine were analyzed according to the schematic indicated in Figure. 11A. To provide an additional estimate of SERCA pump activity, the slope of the change in the Fura-2AM ratio following withdrawal of caffeine and closure of RyR was calculated using linear regression (Figure 11B). INS-1 cells were then treated with AICAR for 0, 3, and 16 h. Whereas 3 h of AICAR treatment did not alter the ΔF/F0 ratio (Figure 11C), AICAR treatment for 16 h significantly reduced the ΔF/F0 ratio. Interestingly, both short-term (3 h) and long-term (16 h) AICAR led to decreased Ca^{2+} reuptake following removal of caffeine. Together, these results were consistent with observed changes in SERCA2b expression and suggest that AMPK activation impairs SERCA activity (Figure. 8D), while chronic AMPK treatment leads to a reduction in ER Ca^{2+} storage.

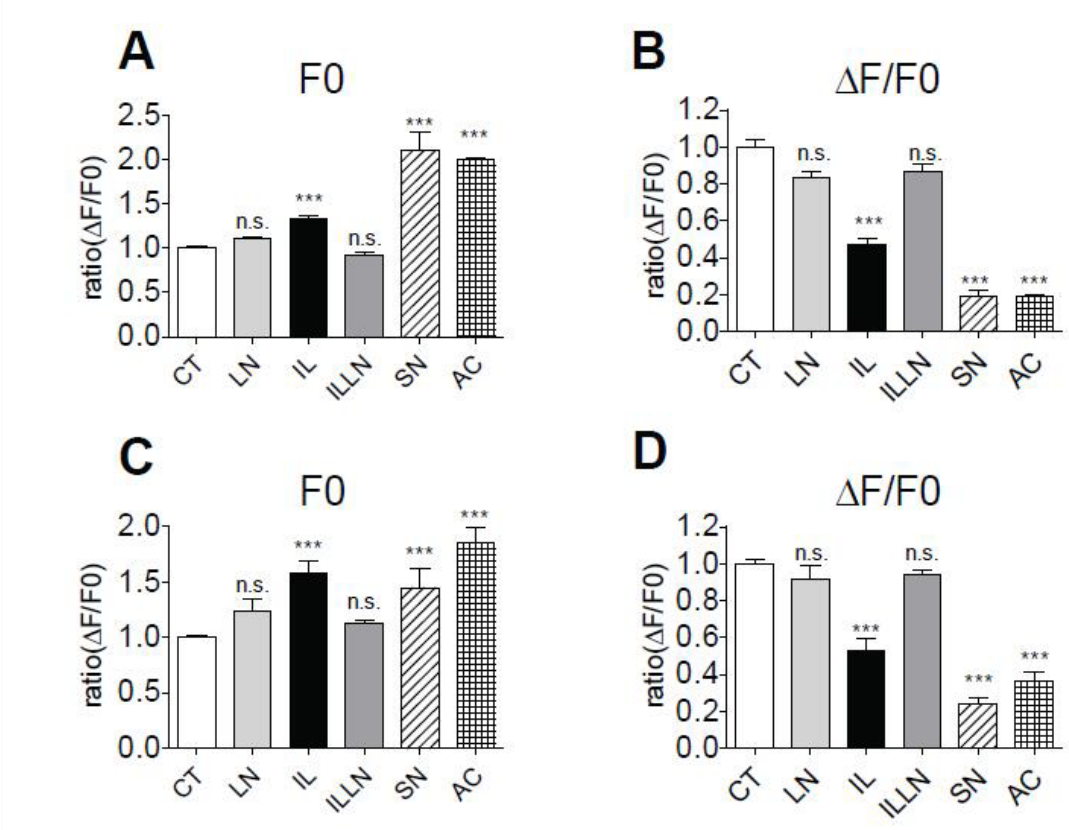

Figure 10. IL-1β, SNAP and AICAR alter β cell Ca^{2+} homeostasis.

To assess cytosolic Ca^{2+} levels, Calcium 6 fluorescence were measured as described under Materials and Methods. **A-D.** Calcium 6 measurements in INS-1 cells pre-treated with DMSO (CT), 5 ng/ml IL-1β (IL) combined with or without 0.5 mM L-NMMA (LN), 300 mM SNAP (SN) or 2 mM AICAR (AC) for 24 h. Indicated comparisons are significantly different (**p*<0.01 ****p*<0.001).

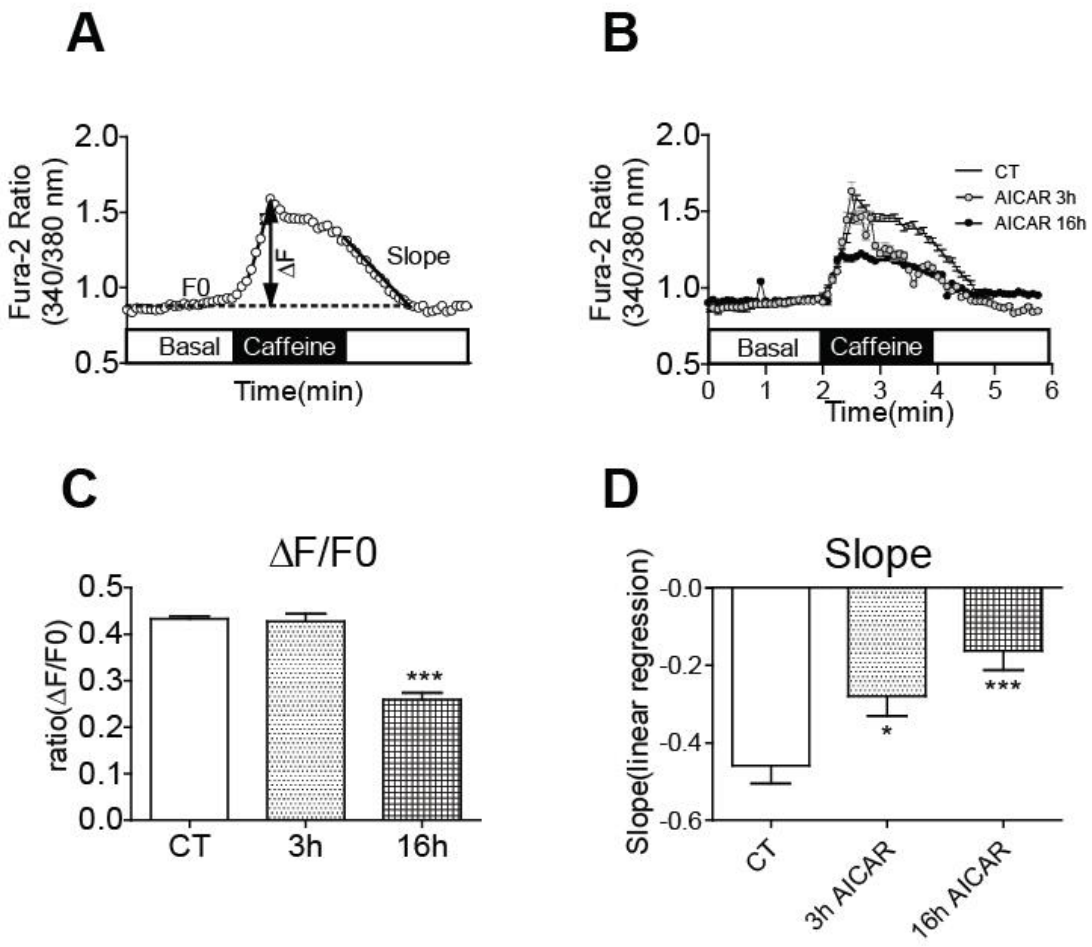

Figure 11. Direct activation of AMPK impairs SERCA2 activity.

To further assess cytosolic Ca^{2+} levels, fura-2/AM fluorescence ratios were measured as described under Materials and Methods. INS-1 cells were pre-treated with DMSO (CT) or 2 mM AICAR for 3 h or 16 h. (**A**) Schematic illustrating the calculation of ΔF/F0 ratios from fura-2/AM imaging experiments. The Ca^{2+} clearance rate was used as an estimate of SERCA2 activity and was analyzed using linear regression to calculate the slope of the fura-2/AM ratio following withdrawal of caffeine. (**B**) Representative trace from untreated INS-1 cells (CT) and INS-1 cells treated with AICAR for 3 h or 16 h. (**C-D**) Quantitative results of the ΔF/F0 ratio and slope from INS-1 cells untreated (CT) or treated with AICAR for 3 h or 16 h. Indicated comparisons are significantly different (*p<0.01 ***p<0.001).

Inhibition of iNOS and AMPK protect INS-1 cells from IL-1β-induced apoptosis

Finally, to determine whether the observed rescue of SERCA2 expression also influenced cell survival, INS-1 cells were treated with L-NMMA, C-PTIO or CC alone or combined with IL-1β, and the ratio of cleaved caspase 3 to total caspase 3 expression was measured by immunoblot. Cleaved caspase 3 expression was significantly increased with IL-1β treatment, but reduced to control levels in the presence of L-NMMA, C-PTIO, and CC (Figure. 12A-B). Next, the CellTiter-Glo Luminescent Cell Viability Assay was employed in INS-1 cells treated with the same combination of compounds above as well as SNAP and AICAR alone. Viability was similarly reduced with IL-1β, SNAP, and AICAR, while L-NMMA, C-PTIO, and CC were able to rescue IL-1β-mediated cell death (Figure. 12C), indicating that loss of β cell survival under pro-inflammatory conditions occurs through NO- and AMPK-dependent pathways and is closely correlated with changes in SERCA2b expression and activity.

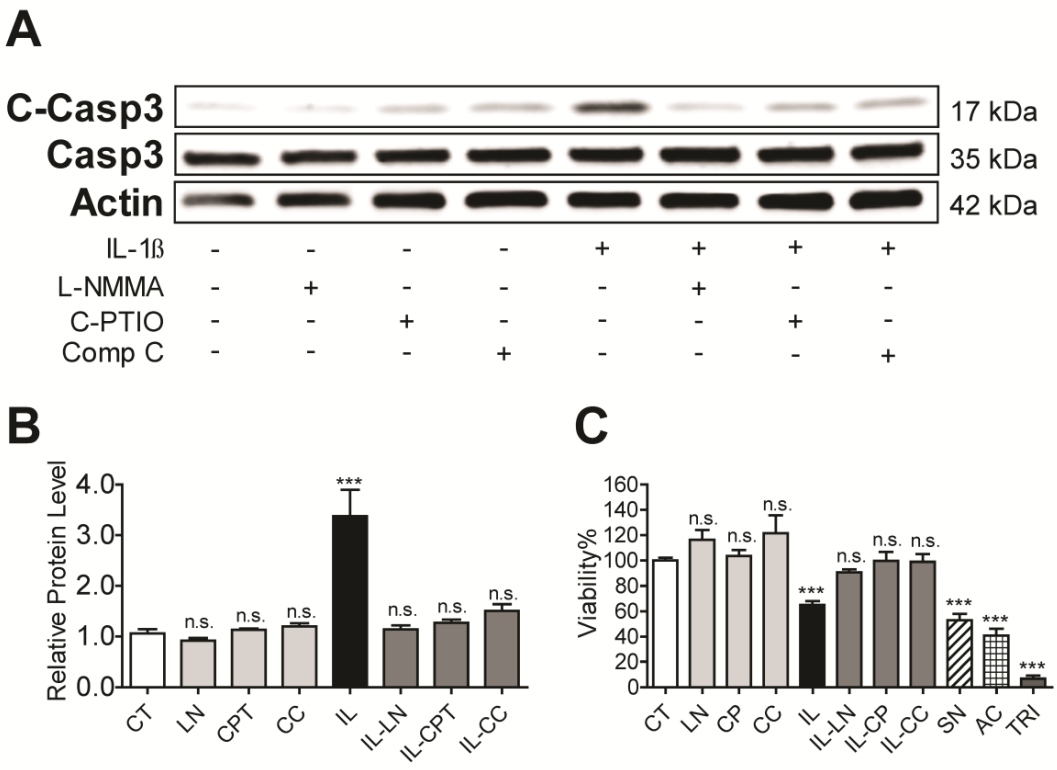

Figure 12. Inhibition of iNOS and AMPK protect INS-1 cells from IL-1β induced apoptosis

A-C. INS-1 cells were treated with DMSO (CT) or 5 ng/ml IL-1β (IL) combined with or without 0.5 mM of the NOS inhibitor L-NMMA (LN) or 100 µM of the NO scavenger C-PTIO (CPT) for 24 h. Total protein was isolated, and immunoblot was performed using antibodies against cleaved caspase 3, total caspase 3 and actin. **B.** Ratios of the relative expression of cleaved caspase 3 to total caspase 3 are shown graphically. **C**. Cell viability assays were performed as described in the Materials and Methods section; 10 % Triton treatment for 10 min was used as positive control for cell death. Indicated comparisons are significantly different (***$p < 0.001$).

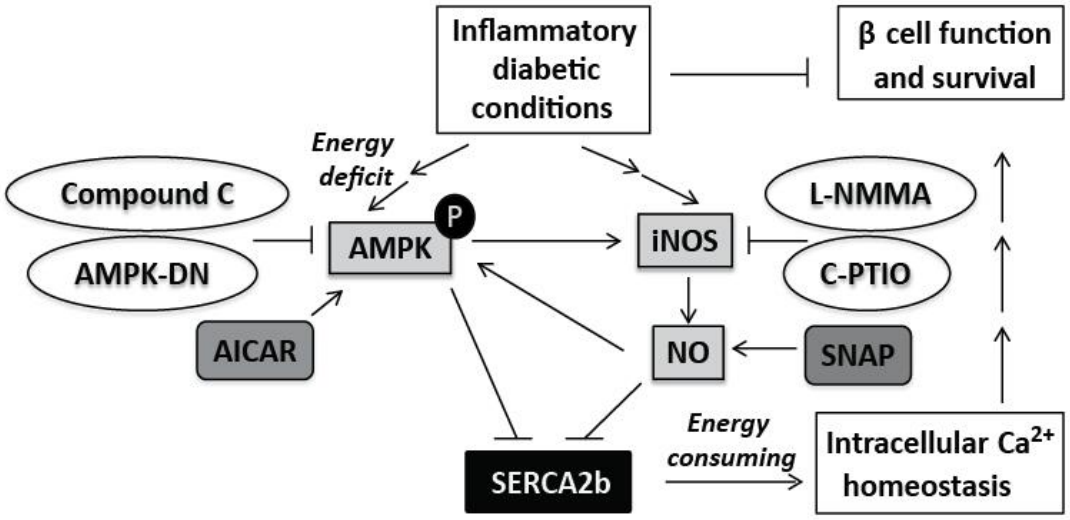

Figure 13. Overall model of NO-and AMPK-dependent down-regulation of SERCA2

Together, our data suggest that under pro-inflammatory and diabetic conditions, activation of NO-mediated signaling in the pancreatic β cell leads to an alteration in cellular energy status and activation of the master cellular energy sensor AMPK. The convergence of NO and AMPK signaling leads to a reduction of SERCA2b activity and alters SERCA2b protein stability, leading to reduced SERCA2b expression. The reduction of SERCA2b causes dysregulation of intracellular and ER Ca^{2+} homeostasis that ultimately leads to a loss of β cell function and survival. Whereas NO may initiate the activation AMPK, our data also suggest that AMPK amplifies the inflammatory response of the pancreatic β cell.

2.3 Discussion

We and others have demonstrated diminished β cell SERCA2b levels in human and rodent models of T1D and T2D (51-53), with alterations in SERCA2b expression leading to impaired Ca^{2+} homeostasis and insulin secretion, activation of ER stress signaling pathways, and altered β cell survival (68, 256, 265, 273). Cardozo et al previously described a role for pro-inflammatory cytokines and induction of the NFκB-dependent gene, inducible nitric oxide synthase (iNOS), in loss of β cell SERCA2b mRNA and protein expression (295). However, whether NO-mediated changes in SERCA2b expression and/or activity are primarily due to a transcriptional or post-translational mechanism remains unexplored. Moreover, the downstream pathways that synergize with NO to alter endoplasmic reticulum Ca^{2+} in the β cell have not been fully defined.

To address this, we first determined the half-life of SERCA2 protein and mRNA under basal and pro-inflammatory conditions. Data from both INS-1 cells and rat islets demonstrate that treatment with the cytokine IL-1β led to decreased SERCA2 protein stability without significantly altering mRNA half-life. Interestingly, treatment with the iNOS inhibitor, L-NMMA, rescued SERCA2 protein stability under inflammatory conditions, but was unable to restore mRNA levels, suggesting divergent regulation of mRNA and protein expression under diabetic and inflammatory conditions. Indeed, our previous work is consistent with this notion and has shown that reductions in SERCA2b mRNA in models of diabetes may arise instead from loss of key transcriptional regulators such Pdx-1 and PPARγ rather than through decreased transcript stability (256, 265).

While temporally regulated and controlled increases in NO have important signaling effects in the pancreatic β cell (296), chronically elevated NO generated under pathologic conditions plays a central role in cytokine-induced β cell death through a variety of effects including, decreased PKB/AKT signaling, potentiation of JNK activation, and induction of irreversible DNA damage (194, 297, 298). However, a dominant effect of NO is to impair mitochondrial oxidation and altered ATP production, which occurs through inhibition of iron-sulphur enzymes such as aconitase (299, 300). These effects are quite potent in the pancreatic islet, where treatment with IL-1β is associated with at least a four-fold reduction in ATP levels (301).

AMPK is a multisubstrate, heterotrimeric serine/threonine kinase with a role in a variety of cellular pathways. AMPK is activated directly through phosphorylation by one of three well-described upstream kinases, liver kinase B1 (LKB1), Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK), or Transforming growth factor β-activated kinase 1(TAK1), while a second major pathway of activation involves allosteric modulation by AMP and ADP. Thus AMPK activity increases in response to any process that decreases ATP levels, including NO-induced stress, hypoxia, and glucose deprivation (302). Interestingly, AMPK has also been shown to regulate ion transport as well as cellular ATPase activity. In this regard, Alzamora et al. demonstrated direct phosphorylation of the kidney vacuolar H^+-ATPase by AMPK, and this modification led to changes in cellular localization and decreased activity of the protein pump (293). Similarly, hypoxia in alveolar cells leads to AMPK-triggered inactivation and endocytosis of the basolateral Na^+,K^+-ATPase, with resulting impairments in lung fluid clearance (291).

With these relationships in mind, we investigated a novel link between SERCA2b and AMPK signaling, hypothesizing that restraint of SERCA activity may be one way that

AMPK alters β cell energy expenditure under inflammatory conditions. ATPases are energetically expensive to maintain and SERCA pumps alone have been estimated to consume upwards of 7%-25% of cellular ATP under normal conditions (285-287). In support of our hypothesis, a recent interactome study involving large-scale affinity purification -mass spectrometry of the AMPKα subunit was performed in INS-1 β cells, and identified SERCA2 as a potential AMPKα binding partner (294). Indeed, our results show that chronic AMPK activation decreases SERCA2 protein expression in INS-1 cells, rat islets, and human islets through a mechanism involving a reduction in protein half-life. Moreover, pharmacologic as well as genetic inhibition of AMPK rescued cytokine-induced loss of SERCA2 expression and cytokine-induced cell death. We explored a functional effect of this relationship using intracellular Ca^{2+} imaging and demonstrate that both short and long-term AMPK activation alters SERCA activity levels, leading reductions in ER Ca^{2+} with a reciprocal increase in cytosolic Ca^{2+}.

While beneficial metabolic effects of AMPK have been well described in peripheral tissues including the liver and skeletal muscle (302), the effects of AMPK activation in the β cell remain somewhat controversial. Divergent effects of AMPK appear to be dependent on both the level of activation as well as the chronicity of activation in experimental models. In the short term, AMPK stimulates insulin secretion and may promote recovery from stress. In contrast, chronic or long-term activation, similar to paradigm applied in our study, has been linked to impairments in insulin secretion as well as altered β cell survival (303-305). Indeed, a pro-apoptotic role for AMPK has been described in other cell types and is linked with a variety of mechanisms including cell cycle arrest, activation of p53, and JNK signaling (306). Specifically in the β cell and consistent with our findings, previous studies suggest that pharmacologic activation of AMPK with AICAR or virally induced constitutive activation of AMPK

induced islet and β cell death, while a dominant negative form of AMPK was capable of protecting against cytokine-induced apoptosis (289, 307). Similarly, constitutive activation of AMPK within transplanted islets decreased insulin secretion and β cell survival in STZ-treated diabetic mice (308).

Our study raises a number of interesting questions for future investigation. While we suggest an initiating role for ATP depletion in our model, there are likely other pathways that lead to AMPK activation under inflammatory conditions. In fact, a study by Gordon et al. showed that NO modulates AMPK activity in the β cell through an IRE-1 dependent mechanism as part of the unfolded protein response (304). It is also tempting to speculate that increased cytosolic Ca^{2+} resulting from initial perturbations in SERCA activity may augment AMPK activation through a CaMKK-dependent pathway, though this remains to be tested. Likewise, an interesting relationship uncovered by our data is that pharmacologic inhibition of AMPK with compound C and treatment with the AMPK-DN construct attenuated IL-1β-induced iNOS gene and protein expression. This result was further confirmed by measuring nitrite concentrations in the culture media. Similar to our results, Santos and colleagues have shown that knockdown of AMPK in MIN-6 β cells blocked cytokine or lipopolysaccharide induced expression of iNOS (305, 309). These data and ours suggest that AMPK is not only activated by NO signaling but may also be responsible for amplifying the inflammatory response. AICAR has previously been shown to activate NFκB in a neuroblastoma cell line through degradation of IκBα (306). We investigated this possibility in INS-1 cells, and similarly found that AICAR treatment led to a dose-dependent reduction in IκBα expression in the pancreatic β cell. Notably, a recent study discovered that a mouse model with chronic activation of AMPK has also been found to induce obesity and impair β cell function (209)

In aggregate, these data provide evidence for a novel pathway that links NO and AMPK signaling with altered β cell Ca^{2+} homeostasis and provide additional insight into the regulation of β cell survival under inflammatory conditions that typify both Type 1 and Type 2 diabetes.

CHAPTER THREE

SERCA2 Deficiency Impairs Pancreatic β Cell Function in Response to Diet-Induced Obesity

3.1 Introduction

During the development of T2D and in the face of advancing peripheral insulin resistance, the β cell undergoes a functional and proliferative compensatory response to increase insulin output and maintain euglycemia. The ability of the β cell to continue in this extended state of compensation is finite for a substantial proportion of individuals, and the typical evolution to T2D is characterized by loss of pancreatic β cell function, mass, and possibly identity by de-differentiation and trans-differentiation (147, 310).

To define the role of SERCA2 in the regulation of whole body glucose homeostasis during this compensatory state, we utilized whole body SERCA2 heterozygous mice (S2HET) challenged with HFD, and performed metabolic analysis on these animals. Here, we show that SERCA2 deficiency leads to glucose intolerance, which occurs secondary to altered β cell insulin secretion, reduced β cell proliferation, and increased β cell ER stress. In contrast, no differences in weight gain, insulin sensitivity or peripheral insulin signaling were observed between S2HET and WT controls after HFD. Finally, we show that modulation of SERCA2 activity with a small molecule allosteric activator rescued ER stress and restored ER Ca^{2+} levels, and prevented β cell apoptosis. Together, these data suggest a critical role for SERCA2 activity and the maintenance of β cell ER Ca^{2+} homeostasis in the compensatory response to diet-induced obesity. Importantly, our results raise the possibility that persons with Darier White Disease may have an increased susceptibility to metabolic disease.

3.2 Result

Whole body SERCA2 haploinsufficiency leads to impaired glucose tolerance in response to diet-induced obesity

To directly assess the role of SERCA2 in the maintenance of *in vivo* glucose homeostasis, S2HET mice bred congenic on a C57BL6/J background and WT littermates controls were studied. To determine the role of SERCA2 in the compensatory response to diet-induced obesity, S2HET and WT mice were challenged with a HFD containing 45% of kilocalories from fat beginning at 8 weeks of age. Islet SERCA2 levels remained suppressed by the expected 50% before and after HFD, and no changes in SERCA3 protein or mRNA expression were observed (Figure 14A-C). Temporal patterns of weight gain and the percentage of lean and fat mass were identical between groups (Figure 14D-E). However, compared to WT HFD-fed controls, S2HET mice demonstrated significantly higher fasting blood glucose levels and lower fed insulin levels (Figure 14F-G). HFD-fed S2HET and WT controls were next challenged with either oral or intraperitoneal glucose (OGTT and IPGTT), administered at a dose of 2g/kg of body weight. Glucose tolerance in both S2HET and WT mice during IPGTT was significantly altered after HFD. Furthermore, in response to both IP and oral glucose challenge, S2HET mice exhibited significantly increased glucose excursions and reduced glucose tolerance compared to WT controls, as measured by the area under the curve analysis of IPGTT and OGTT (Figure. 15A-D).

While insulin tolerance tests revealed significantly decreased insulin sensitivity after HFD in both S2HET and WT, no differences were observed between genotypes (Figure 16A-B). Levels of AKT phosphorylation at serine 473 in the liver, epididymal adipose tissue, and gastrocnemius muscle were measured under basal conditions and following acute insulin injection. No differences were observed between genotypes (Figure 16C-E).

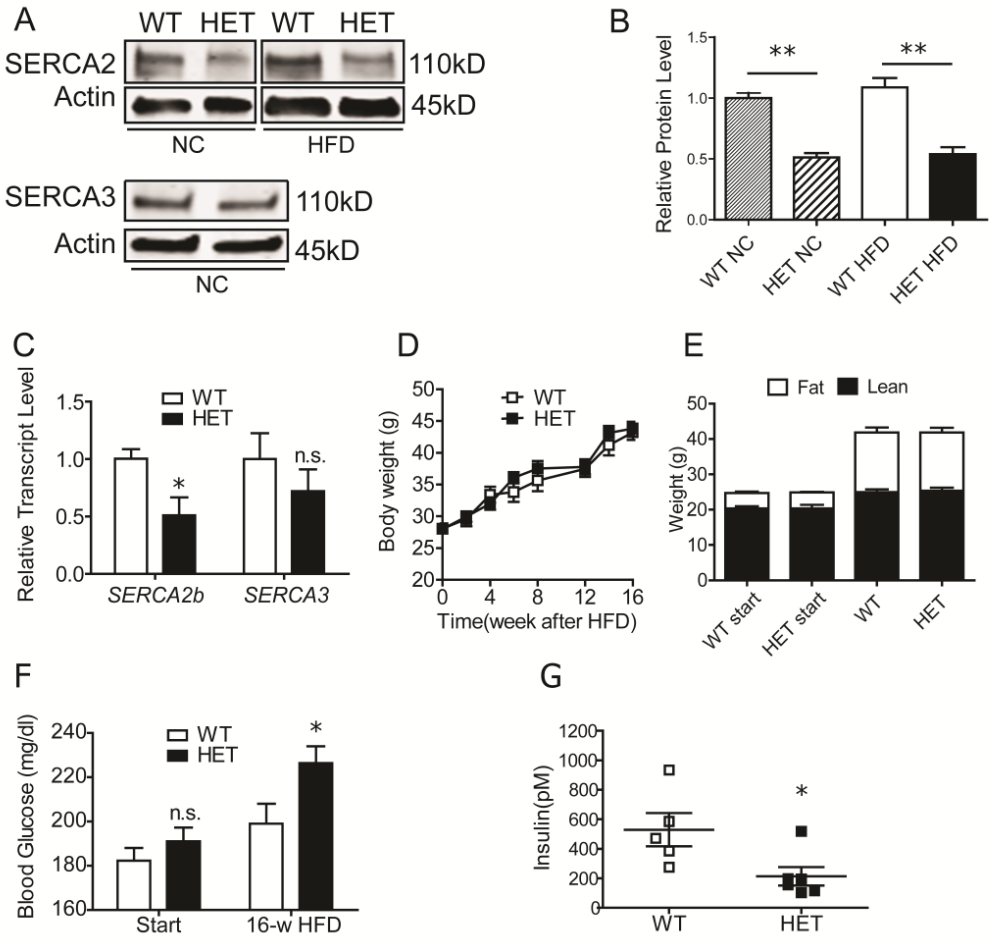

Figure 14. S2HET mice exhibit glucose dyshomeostasis following HFD.

SERCA2 haploinsufficient mice (HET) and wild-type littermate controls (WT) were fed high fat diet (HFD) containing 45% of kilocalories from fat for 16 wks starting at 8 wks of age. **A-C**, Protein and RNA were isolated from HET and WT islets before (normal chow or NC) and after 16 wks of HFD (WT-HFD and HET-HFD). Immunoblot analysis was performed using antibodies against SERCA2, SERCA3, and Actin (**A**). Quantitative SERCA2 protein levels are shown graphically (**B**). Reverse-transcribed RNA was subjected to real-time quantitative RT-PCR (qRT-PCR) to measure SERCA2b and SERCA3 transcript levels (normalized to Actb) (**C**). **D-E**, Longitudinal changes in body weight were measured, and DEXA analysis was performed in HET and WT mice at the start and after 16 wks of HFD. **F**, Blood glucose in 6 hr-fasted WT and HET mice before and after 16 wks of HFD. **G**, Random-fed serum insulin levels following 16 wks of HFD. Indicated comparisons are significantly different; *, $p<0.05$; ***, $p<0.001$.

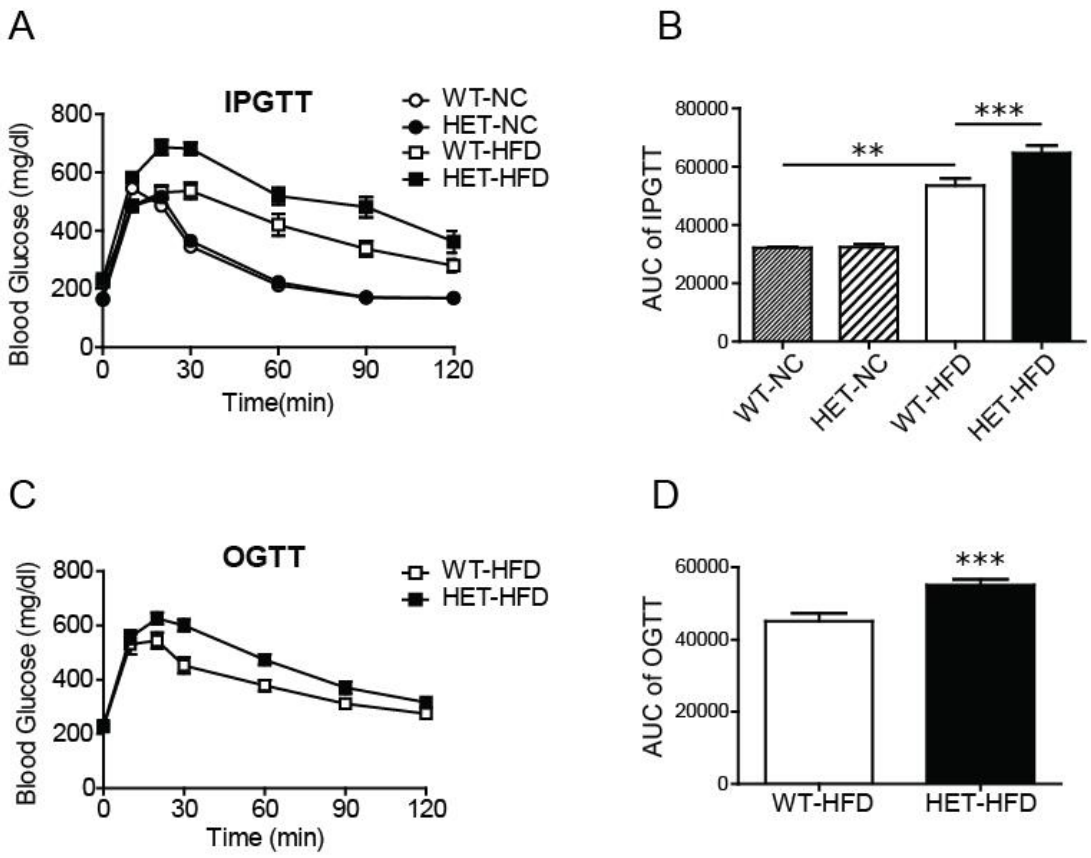

Figure 15. S2HET mice manifest impaired glucose tolerance following HFD challenge.

S2HET and WT were fed HFD containing 45% of kilocalories from fat for 16 weeks starting at 8 weeks of age. I.P. glucose tolerance tests (IPGTT) (**A-B**) or oral glucose tolerance tests (OGTT) (**C-D**) were performed before or after 16 wks of HFD treatment in HET and WT mice; area under the curve (AUC) analysis is shown graphically. Open squares, WT mice after HFD; solid squares, S2HET mice after HFD; Open circles, WT mice before HFD; solid circles, S2HET mice before HFD. Results are displayed as means ± S.E.M; n=at least 8 per group. Indicated comparisons are significantly different; *, $p<0.05$; ***, $p<0.001$.

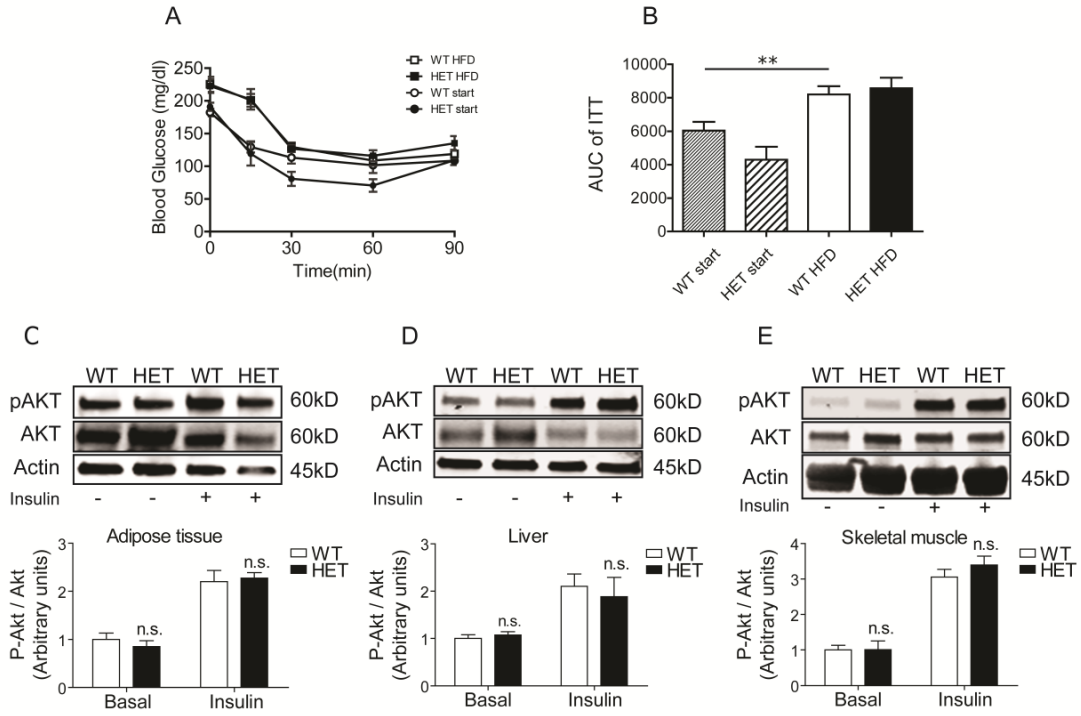

Figure 16. HFD-fed S2HET and wild-type mice exhibit comparable insulin sensitivity and levels of insulin induced AKT phosphorylation in adipose, liver, and skeletal muscle.

A-B, Insulin tolerance tests were performed before and after 14 weeks of HFD, and the AUC analysis is shown graphically. Open squares, WT mice after HFD; solid squares, S2HET mice after HFD; open circle, WT mice before HFD; solid circle, S2HET mice before HFD Adipose (**C**), liver (**D**) or skeletal muscle (**E**) protein homogenates were obtained from saline (basal) or insulin injected S2HET and WT fed HFD for 16 wks. Immunoblot analysis was performed using antibodies against phospho-AKT (ser473), total AKT, and actin. Relative protein levels are shown graphically. Results are displayed as means ± S.E.M; n=6 per group; n.s. indicates that no significant differences were observed between groups. Indicated comparisons are significantly different; **, $p<0.01$

SERCA2 deficiency results in impaired GSIS

Initial analysis of HFD-fed S2HET mice revealed hyperglycemia, reduced serum insulin levels, and impaired glucose tolerance without apparent alterations in insulin sensitivity or adiposity, suggesting a defect in β cell function. To define further the β cell phenotype associated with *in vivo* SERCA2 deficiency, insulin levels were measured in S2HET and WT controls after a 5 hour fast (time 0) and 10 min after i.p. glucose injection. In response to glucose challenge, serum insulin levels were significantly lower in HFD-fed S2HET mice compared to those of HFD-fed WT controls (Figure 17A). Similar to *in vivo* findings, results from *ex vivo* glucose stimulated insulin secretion assays performed in isolated islets revealed a significant decrease in fractional insulin secretion from HFD-fed S2HET islets (Figure 17B).

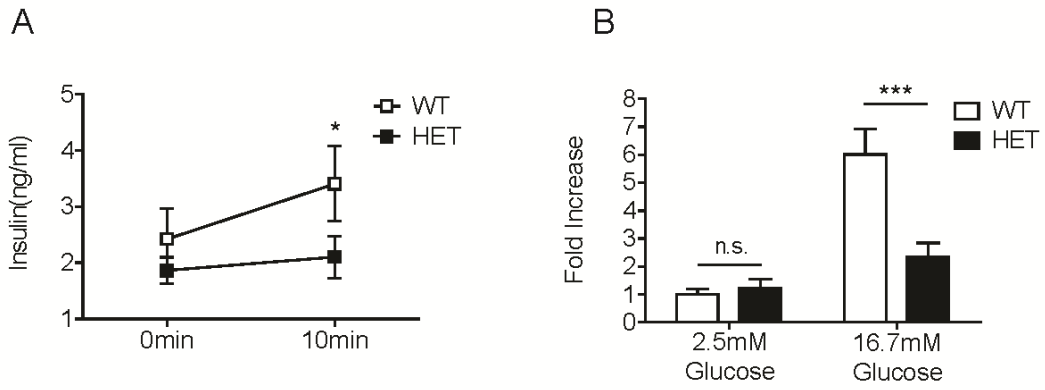

Figure 17. SERCA2 deficiency leads to impaired β cell insulin secretion.

A, Serum insulin levels were measured following a 5 hr fast and 10 minutes after i.p. injection of 2 mg/kg glucose in S2HET(HET) and WT fed HFD for 16 weeks. Open square, wild-type mice; solid square, S2HET mice. **B,** Islets were isolated from HFD-fed HET and WT mice. Glucose stimulated insulin secretion was measured and normalized to total insulin content. Results are displayed as the means ± S.E.M; n = at least 4 independent experiments for each group. Indicated comparisons are significantly different; *, $p<0.01$; ***, $p<0.001$.

SERCA2 deficiency results in impaired islet Ca^{2+} homeostasis

Next, Fura-2 AM calcium imaging experiments were performed in islets isolated from normal chow and HFD-fed S2HET and WT mice. Baseline cytosolic Ca^{2+} levels were increased after HFD-fed in genotypes, while S2HET mice exhibited a significantly larger increase in baseline Ca^{2+} (Figure 18A-C). Similarly, HFD induced a significant delay in the onset of steady state cytosolic Ca^{2+} oscillations in response to glucose (phase 1 duration). Compared to WT under both normal chow and HFD conditions, S2HET islets exhibited a further lengthening of the phase 1 duration. (Figure 18D), while the initial glucose stimulated Ca^{2+} response (labeled as the phase 1 amplitude) was also significantly reduced in HFD-S2HET islets (Figure 18B, E). The amplitude of the oscillatory response (phase 2 amplitude) was not significant different between groups (Figure 18F). Notably, Ca^{2+} response to KCl was also impaired (Figure 19A-B), suggesting these defects did not arise as a result of altered glucose sensing.

FLIM was next used to directly monitor ER Ca^{2+} in dispersed normal chow-fed WT and S2HET islets that had been transduced with the ER-directed calcium biosensor D4ER adenovirus and then treated with or without GLT Results revealed lower ER Ca^{2+} levels in S2HET β cells that was exacerbated by chronic treatment with 25 mM glucose and 500 μM BSA-conjugated palmitate (Figure 19C-D).

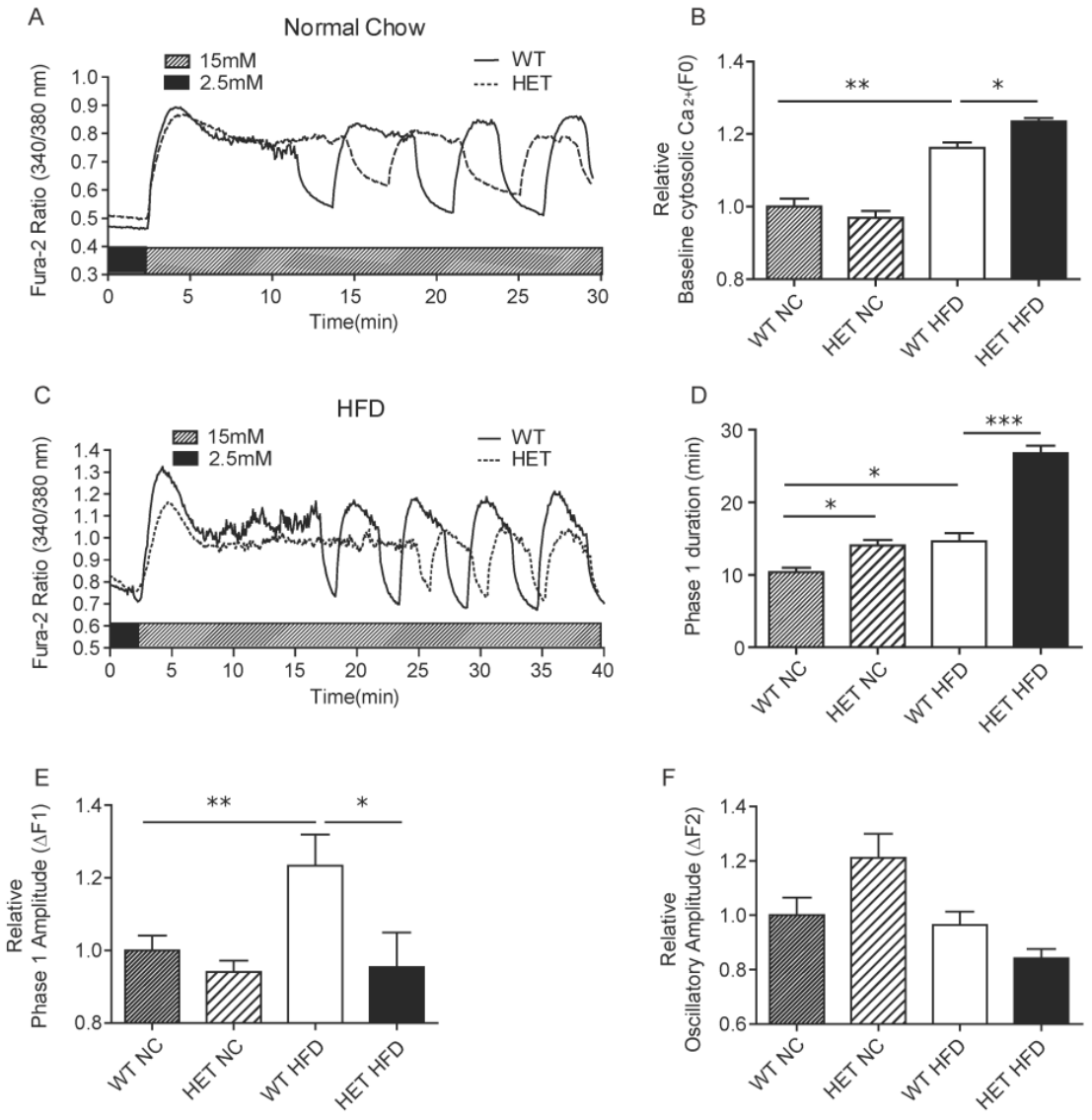

Figure 18. Islets isolated from HFD-fed S2HET mice exhibit impaired cytosolic Ca^{2+} homeostasis and oscillation in response to glucose.

Islets isolated from normal chow or HFD-fed WT (solid line) and S2HET (dashed line) mice were loaded with Fura-2AM and calcium imaging performed. **A and C**, Representative $[Ca^{2+}]_i$ recording of islets following stimulation with 15 mM glucose. B. Quantitation of the relative basal $[Ca^{2+}]_i$ (F0) in WT and HET islets. **D**, Phase 1 duration in WT and S2HET(HET) islets. **E,** Quantitation of the phase 1 $[Ca^{2+}]_i$ amplitude. **F,** Quantitation of the average Phase 2 $[Ca^{2+}]_i$ amplitude from five continuous oscillatory cycles per islet. Results are displayed as mean ± S.E.M, and indicated comparisons are significantly different. *, $p<0.05$; ***, $p<0.001$; n=26 islets from 3 biological replicates per group.

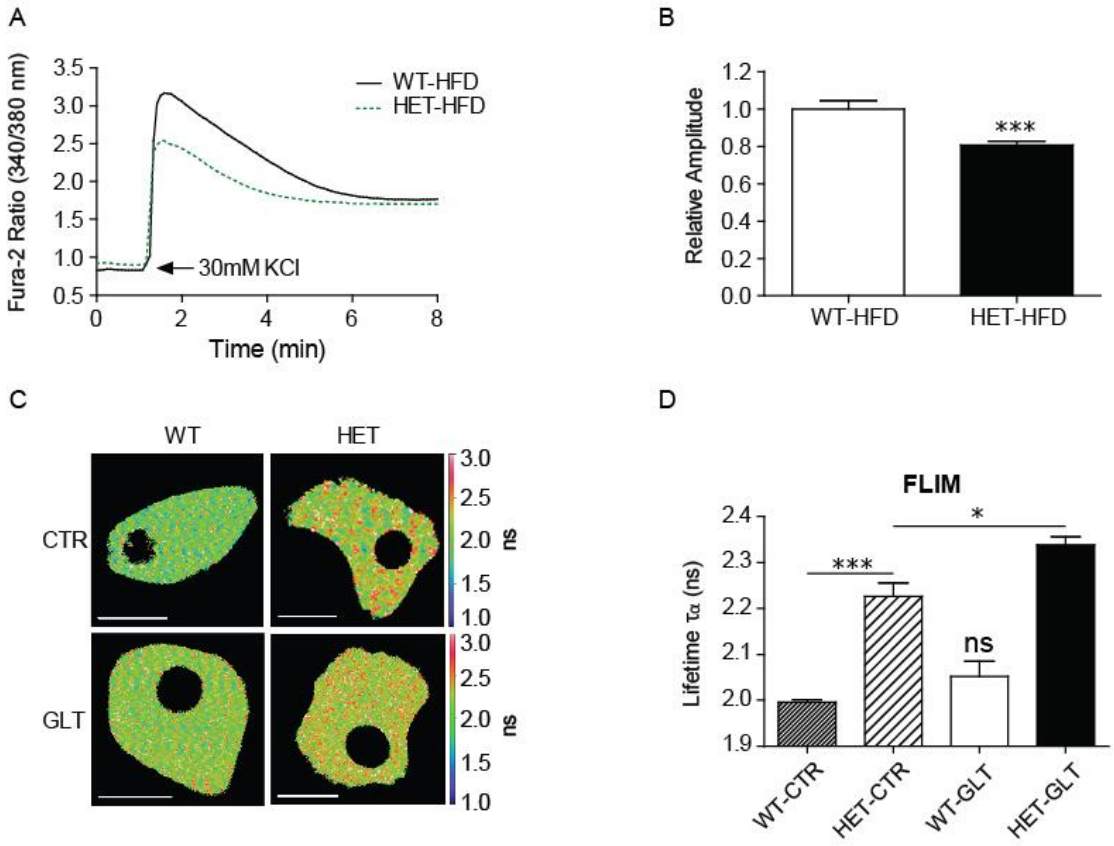

Figure 19. Islets isolated from HFD-fed S2HET mice exhibit impaired ER Ca^{2+} homeostasis.

Islets isolated from normal chow or HFD-fed WT (solid line) and S2HET (dashed line) mice were loaded with Fura-2AM and calcium imaging performed. **G**. Representative $[Ca^{2+}]_i$ recording of islets following stimulation with 30 mM KCl. **H**. Quantitation of the phase 1 $[Ca^{2+}]_i$ amplitude in response to KCl. **I-J**. Dispersed islets were transduced with a D4ER adenovirus and FLIM was used to measure endoplasmic reticulum Ca^{2+}. **I**, Representative lifetime map with lookup table indicating donor lifetime in ns (scale bar is 10 µm) and average donor lifetime (**J**) in WT and S2HET β cells treated with or without GLT (n=at least 10 cells were quantitated per treatment group). Results are displayed as mean ± S.E.M, and indicated comparisons are significantly different. *, $p<0.05$; ***, $p<0.001$; n=26 islets from 3 biological replicates per group.

SERCA2 deficiency results in impaired islet insulin production, processing and packaging

To test whether insulin biosynthesis was impaired, total insulin content and insulin mRNA levels were measured in isolated islets from HFD-fed animals. The levels of both were found to be significantly decreased in S2HET islets compared to WT controls (Figure 20A-B). Compared to HFD-fed wild-type controls, HFD-fed S2HET mice exhibited a nearly 2-fold increase in the serum proinsulin/insulin ratio (Figure 20C), suggesting a parallel decrease in proinsulin maturation.

Insulin granule morphology was next assessed by quantitative analysis of islet electron micrographic images. Typical mature insulin granules were defined according to previously published protocols and exhibited a dense homogenous core with a clear halo, while immature granules exhibited an empty or lighter core and the absence of a defined halo (Figure 20D) (311). The percentage of immature granules was nearly 2-fold higher in HFD-fed S2HET mice compared to controls. In addition, S2HET islets displayed a significantly higher percentage of rod-like granules, indicating defective insulin crystallization and packaging (Figure 20E). Paired with defects in insulin processing, both PC1/3 protein and transcripts levels were decreased in islets isolated from HFD-fed S2HET mice (Figure 20F-H). These observations in the S2HET islets are confirmed and extended by western blot performed in SERCA2 knockout INS-1 cells (described in Figure 26A-C, below).

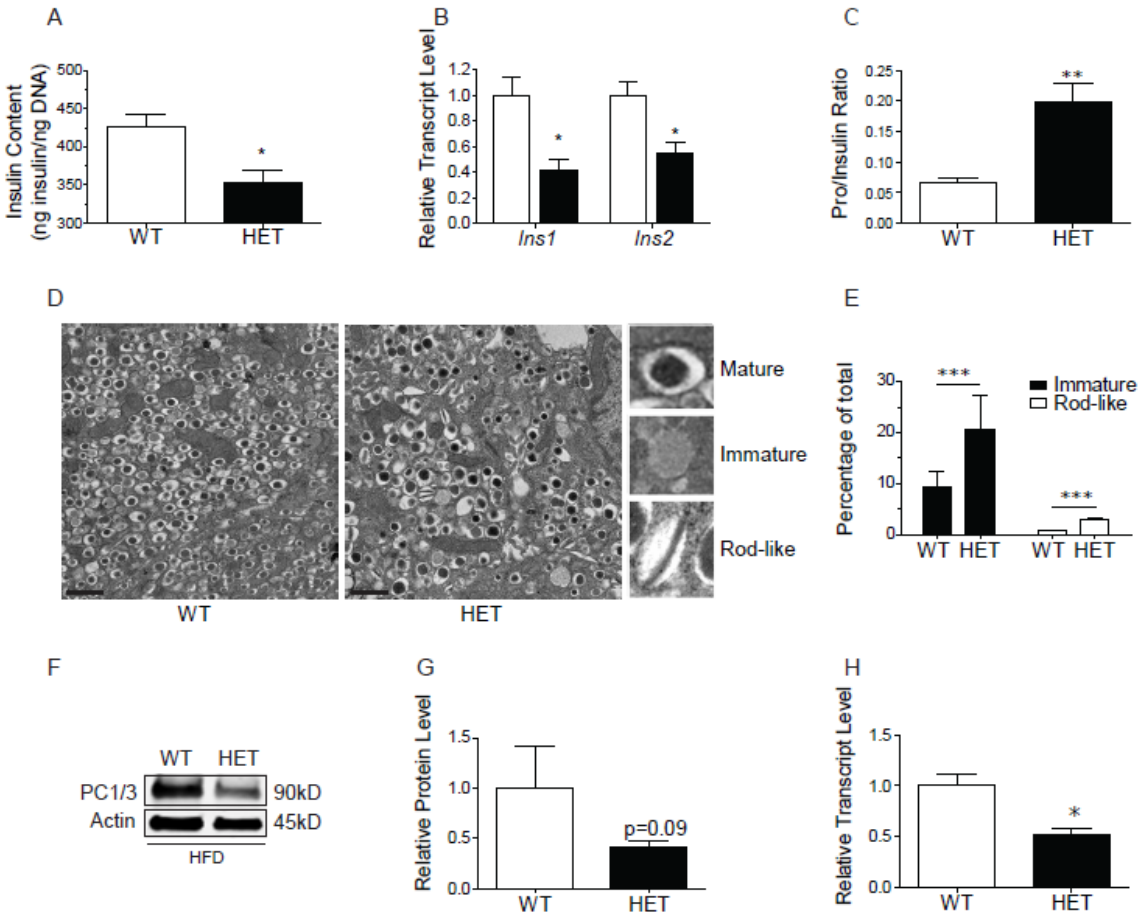

Figure 20. S2HET mice fed HFD exhibit impaired insulin biosynthesis

Islets were isolated from HFD-fed S2HET(HET) and WT mice. **A,** Total islet insulin content was normalized to islet DNA content. **B,** *Ins1* and *Ins2* transcript levels were measured by qRT-PCR and normalized to *Actb* levels. **C**, Serum proinsulin levels were measured after 16 weeks of HFD in WT and S2HET mice. Results were expressed as the ratio of serum proinsulin to insulin; n=5 biological replicates for panel A-C. **D-E**, Islets from 3 WT or S2HET mice before and after 16-wks HFD were pooleed and analyzed by electron microscopy. Representative EM images of β cells and insulin granule morphology from HFD-fed mice are shown. Panel E indicates quantitiative analysis of 20 images per group. Scale bar = 1 µm. **F-H**, Protein and RNA were isolated from islets of WT and HET mice fed 16-wks HFD. Immunoblot analysis was performed using antibodies against PC1/3 and actin. Quantitative protein and transcripts levels are shown graphically. Results are displayed as mean ± S.E.M. The indicated comparisons are significantly different. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

SERCA2 haploinsufficient mice demonstrated lower β cell proliferation, reduced β cell mass, and increased β cell death in response to HFD

Next, β cell mass was quantitated at 8 weeks of age prior to HFD initiation, and following 16 weeks of HFD exposure. At baseline, there was no significant difference in β cell mass between chow-fed S2HET and WT control mice. In contrast, after HFD, S2HET mice demonstrated significantly lower β cell mass than that of age-matched controls (Figure 21A-B). However, no significant difference in the number of alpha cells per islet was noted between groups after HFD (Figure 21C-D). To assess β cell proliferation, pancreatic sections were stained with antibodies against insulin and proliferating cell nuclear antigen (PCNA). Double-positive cells were counted and the results indicated a significant reduction in the percentage of proliferating β cells in S2HET mice in response to HFD (Figure 22E-F). Finally, to assess β cell death, droplet digital PCR was used to measure circulating levels of unmethylated cell-free insulin DNA at baseline and during HFD treatment (312). At baseline, S2HET mice exhibited a trend of higher unmethylated DNA levels compared to WT controls. After 8 weeks, S2HET mice had significantly higher circulating levels of unmethylated insulin DNA, while no difference between groups was noted after 17 weeks of HFD (Figure 22G).

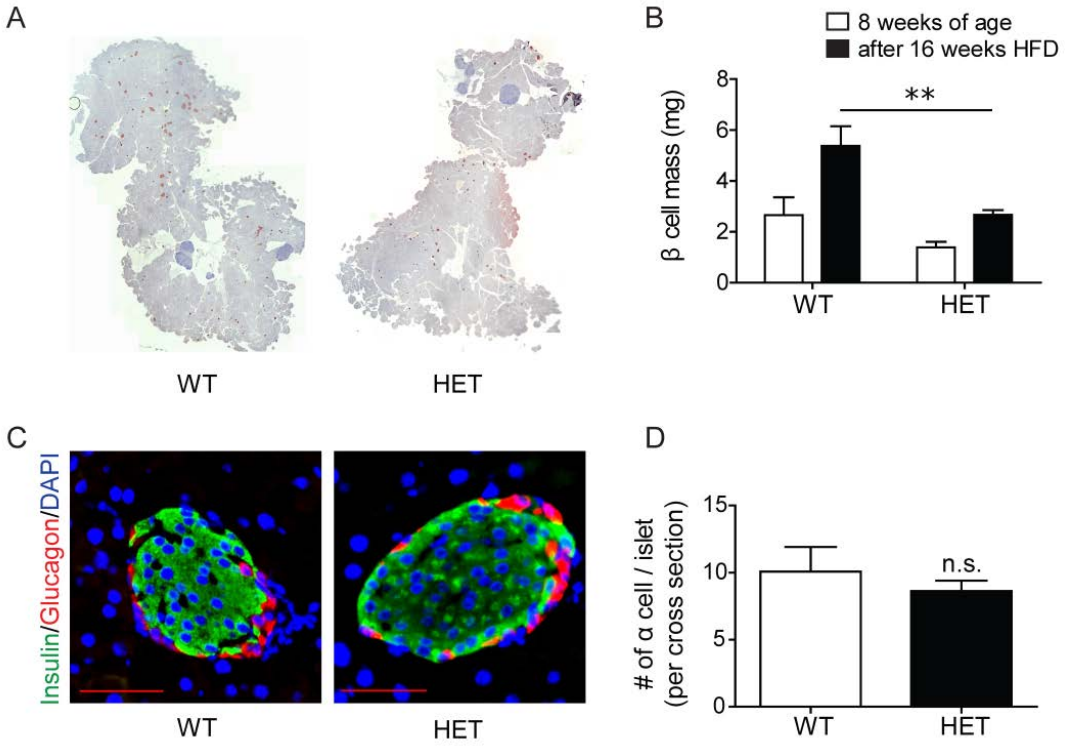

Figure 21. S2HET mice fed HFD exhibit decreased β cell mass

A, Representative images of insulin immunohistochemistry in pancreata harvested from HFD-fed S2HET(HET) and WT. **B**, β cell mass was quantified in WT and HET mice before and after HFD treatment. **C and D**, Representative images of insulin and glucagon immunofluorescence in pancreata harvested from HFD-fed WT and HET mice. Scale bar = 50 µm. Quantification of the number of alpha cells per islet is shown graphically. Results are displayed as means ± S.E.M; n=at least 4 biological replicates per group. The indicated comparisons are significantly different. *, $p<0.05$; **, $p<0.01$.

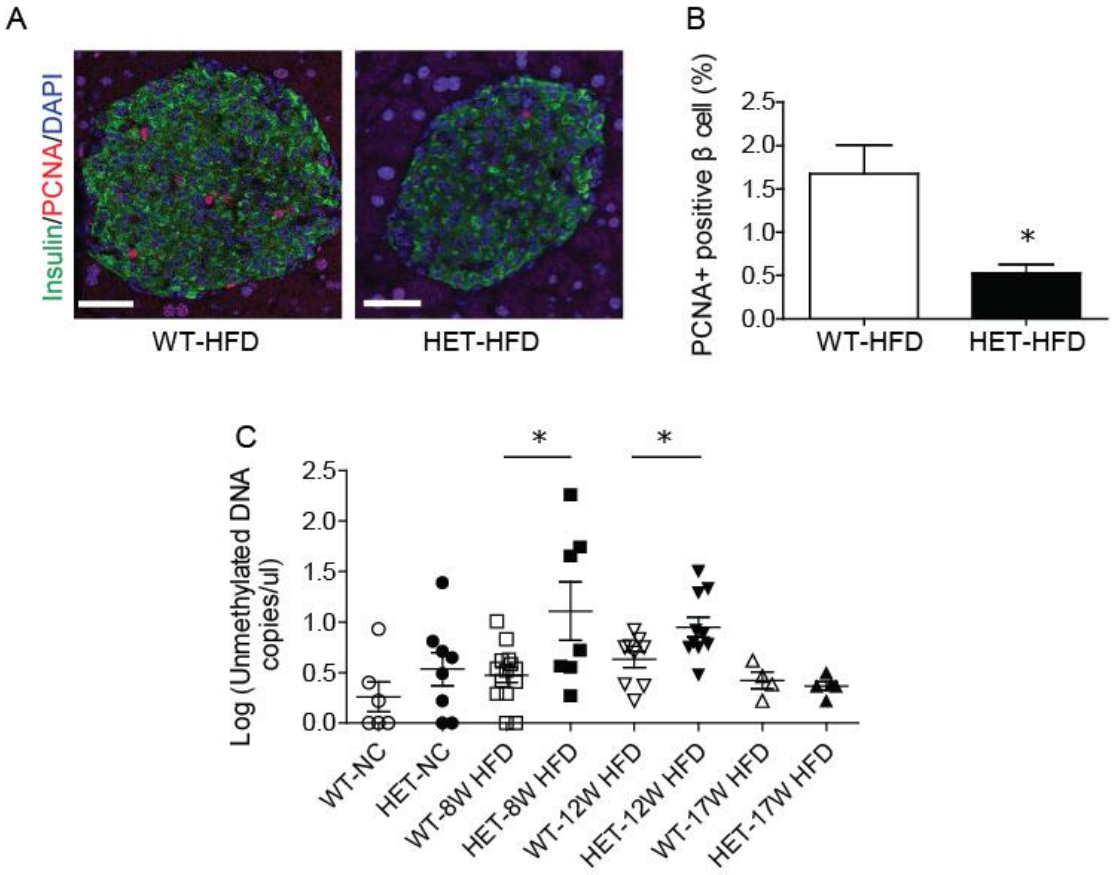

Figure 22. S2HET mice fed HFD exhibit decreased β cell proliferation with increased cell death

A-B, Quantification of the percentage of PCNA-positive β cells. **C**, Random fed serum was collected from WT and HET mice before and during HFD feeding at indicated times. Levels of circulating unmethylated mouse *Ins2* DNA levels were measured by ddPCR and depicted as Log (copies/μL). Open symbols, wild-type mice; solid symbols, S2HET mice. NC=normal chow at 8 wks of age. Results are displayed as means ± S.E.M; n=at least 4 biological replicates per group, while n values are indicated by scatterplot in Panel C. The indicated comparisons are significantly different. *, $p<0.05$; **, $p<0.01$.

SERCA2 deficiency increases ER stress pathways

Our results suggest that SERCA2 haploinsufficiency reduced the ability of pancreatic islets to compensate for the metabolic challenge of HFD, which occurred secondary to impaired β cell secretory function, decreased β cell proliferation, and increased β cell death. Next, ER morphology was analyzed from electron micrographic images of islets isolated from HFD-fed S2HET and WT mice. In contrast to the regularly spaced stacks of ER sheets observed in β cells from WT mice, analysis of S2HET β cells revealed swollen and fragmented ER morphology (Figure 23A). Expression of genes encoding proteins involved in ER stress signaling, including *Grp78, Dnajc3, Hsp90b1 and Pdia4* were significantly increased, while the spliced *Xbp-1* to total *Xbp-1* ratio (S/T *Xbp-1*) was higher in S2HET islets (Figure 23B). To confirm these findings, islets were next isolated from chow-fed S2HET and WT control mice and treated *ex vivo* with 25 mM glucose and 500 μM palmitate for 24 h (glucolipotoxicity; GLT) to mimic HFD conditions. Expression of *Grp78, Dnajc3, Hsp90b1* and *Pdia4* were increased at baseline in S2HET islets and further elevated in response to GLT stress, while the spliced *Xbp-1* to total *Xbp-1* ratio was significantly increased in GLT-treated S2HET islets compared to levels observed in WT controls (Figure 23C).

To confirm a β cell-autonomous defect with SERCA2 deficiency, a SERCA2 knockout INS-1 832/13 cell line (S2KO) was generated. While SERCA2b mRNA and SERCA2 protein were reduced in S2KO cells, no significant alterations in RyR2 or IP_3R expression were observed (Figure 24A-B). *SERCA3* transcript levels were decreased by ~40%, but no significant alteration in SERCA3 protein expression was seen. Consistent with expression analysis performed in S2HET islets, expression of genes involved in ER stress signaling, including *Grp78, Dnajc3, Hsp90b1, Pdia4* and *S/T Xbp-1,* were significantly elevated in S2KO INS-1 cells under basal conditions and in response to GLT stress (Figure 24C).

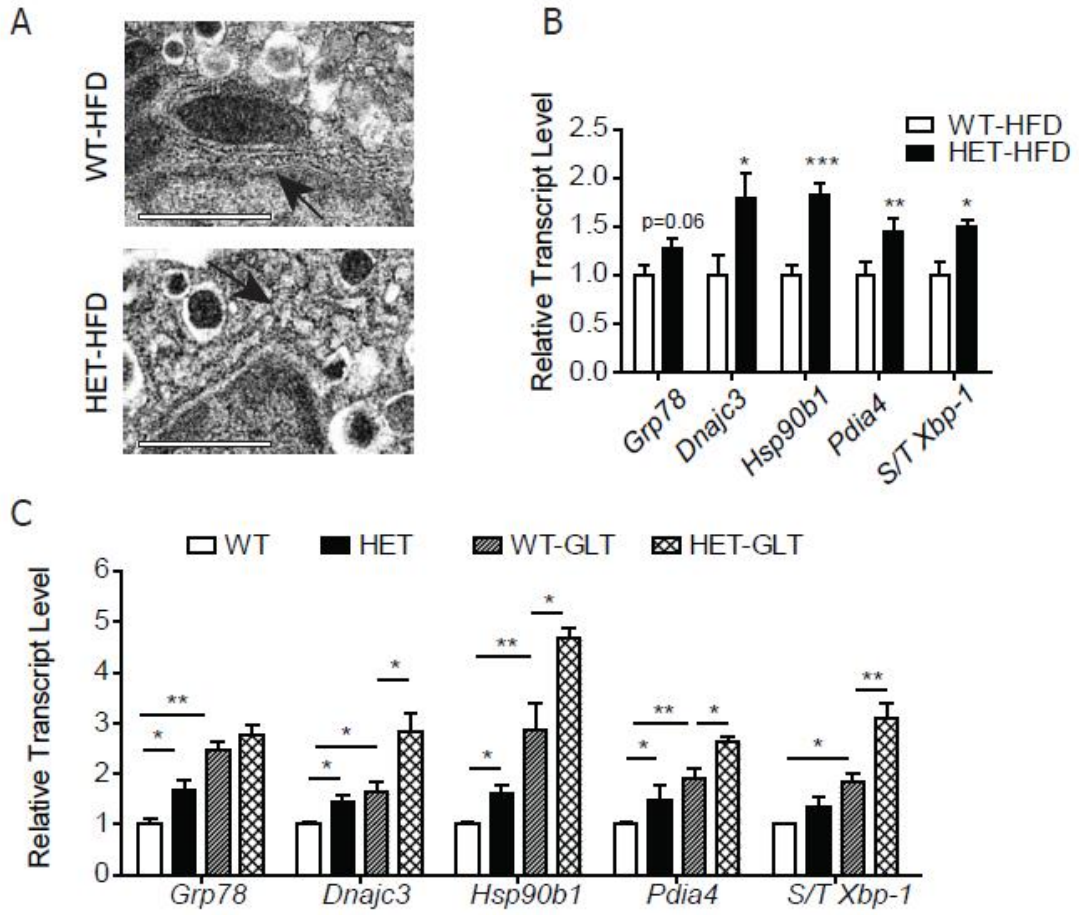

Figure 23. S2HET islets exhibit higher ER stress

A, Islets from 3 WT or 3 S2HET mice fed HFD for 16 weeks were pooled, fixed and analysed by electron microscopy. Representative images of the β cell ER structure are shown. **B**, Islets were isolated from 16-week HFD-fed S2HET mice or WT controls (n=5-6). Reverse-transcribed RNA was subjected to qRT-PCR to measure *Grp78, Dnajc3, Hsp90b1, Pdia4, spliced Xbp-1, total Xbp-1* and *Actb* transcript levels. **C**, Islets freshly isolated from 10-week-old male mice fed normal chow were treated with or without 25 mM glucose $^{+}$ 500 µM palmitate (GLT) for 24 h. Reverse-transcribed RNA was subjected to qRT-PCR to measure indicated gene transcripts levels (n=9 biological replicates analysed over three individual experiments). Results are displayed as means ± S.E.M. The indicated comparisons are significantly different. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

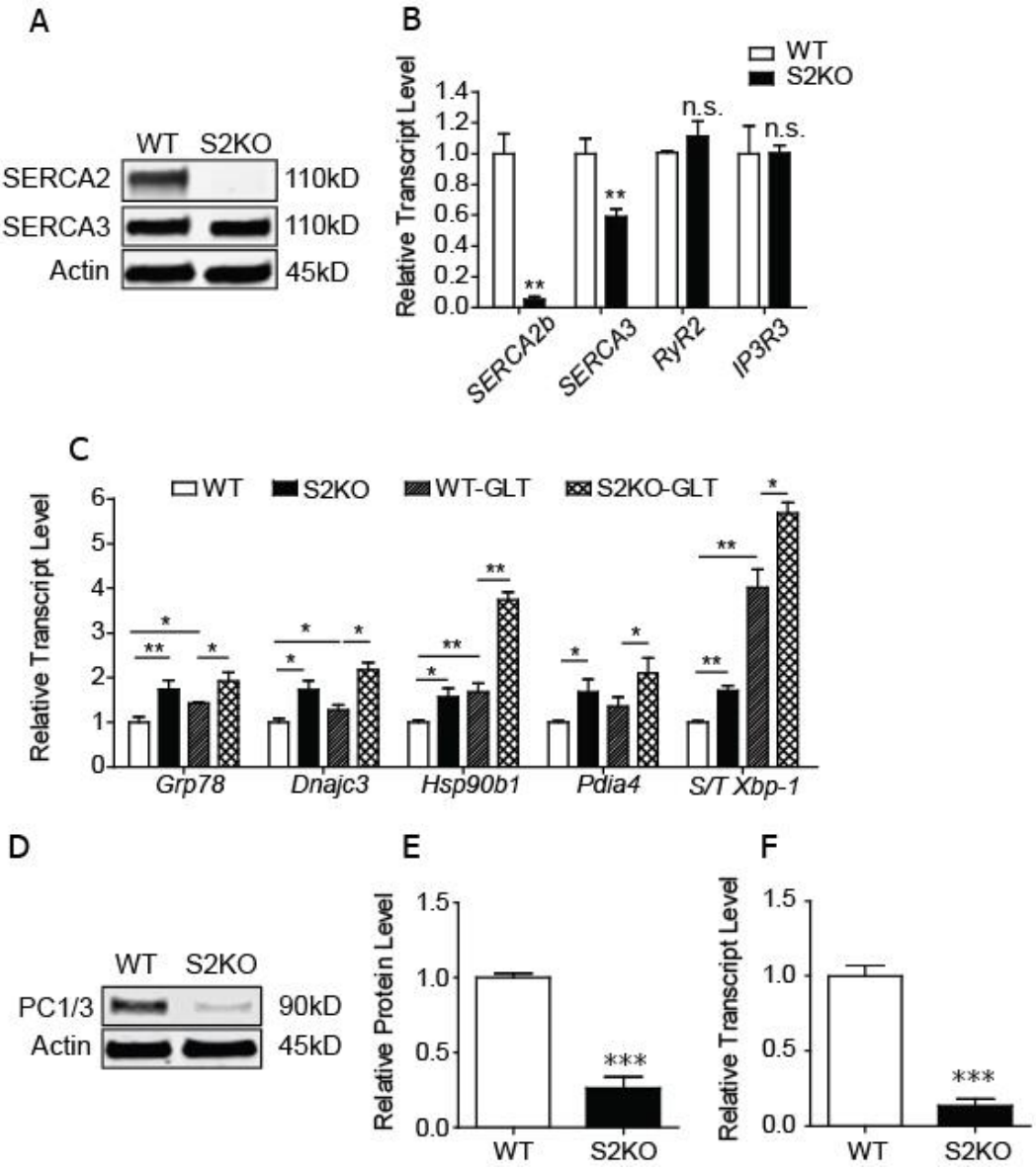

Figure 24. Cultured β cells with SERCA2 deficiency exhibit higher ER stress

A, Immunoblot analysis was performed in S2KO using antibodies against SERCA2, SERCA3, and actin. **B**, Reverse transcribed RNA isolated from S2KO and WT INS-1 cells was subjected to real-time quantitative RT-PCR for quantification of *SERCA2b* and *SERCA3, RyR2* and *IP3R3*, and results were normalized to *Actb* transcript levels. **C**, Reverse transcribed RNA isolated from S2KO and WT INS-1 cells treated with or without GLT for 24 h was subjected to real-time quantitative RT-PCR for quantification of indicated transcript levels. **D-F**. Protein and RNA were isolated from WT and S2KO INS-1 cells. Immunoblot analysis was performed using antibodies against PC1/3 and actin. Quantitative protein and transcripts levels are shown graphically. Results are displayed as means ± S.E.M. The indicated comparisons are significantly different. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

SERCA2b reconstitution protects against β cell death and Ca^{2+} dyshomeostasis in response to ER and glucolipotoxic stress

S2KO and WT cells were next treated with tunicamycin (TM) to perturb protein folding by inhibiting protein glycosylation. Under control conditions and in response to TM, cleaved caspase-3 protein levels were significantly higher in S2KO cells (Figure 25A-B). Similarly, S2KO cells exhibited decreased cell viability with TM and GLT-induced stress (Figure 25C). To test whether SERCA2 reconstitution was sufficient to reverse these effects, cells were transduced with SERCA2b or LacZ expressing adenovirus (Figure 25D). SERCA2b overexpression improved viability in both WT and S2KO cells treated with GLT and TM (Figure 25E-F).

Calcium imaging experiments were next performed in S2KO and WT cells that had been treated with GLT or TM and transduced with either SERCA2b or LacZ expressing adenovirus. Carbachol and caffeine were used to stimulate IP_3 and RyR-mediated ER Ca^{2+} release, respectively, providing an indirect assessment of ER Ca^{2+} storage (275). Results were analyzed as the change in Ca^{2+} (ΔF) in response to caffeine or carbachol, normalized to the baseline cytosolic Ca^{2+} level (F0). At baseline, S2KO cells exhibited a significant reduction in the $\Delta F/F0$ ratio. The $\Delta F/F0$ ratio was further decreased in both GLT and TM-treated WT and S2KO cells, while SERCA2b overexpression rescued the carbachol and caffeine response in S2KO cells and the carbachol response in WT cells (Figure 26A-B). Similar to results obtained in S2HET islets, FLIM performed in S2KO cells also revealed a significant reduction in the ER Ca^{2+} concentration (Figure 26C). Next, INS-1 cells were treated with CDN1163, which is a small molecular allosteric SERCA2 activator. FLIM analysis revealed that CDN1163 was capable of increasing ER Ca^{2+} within 2 h with no further increases observed after 24 h. Interestingly, CDN1163 was also able to partially rescue TM-induced cleaved caspase-3 expression (Figure 26D-F.)

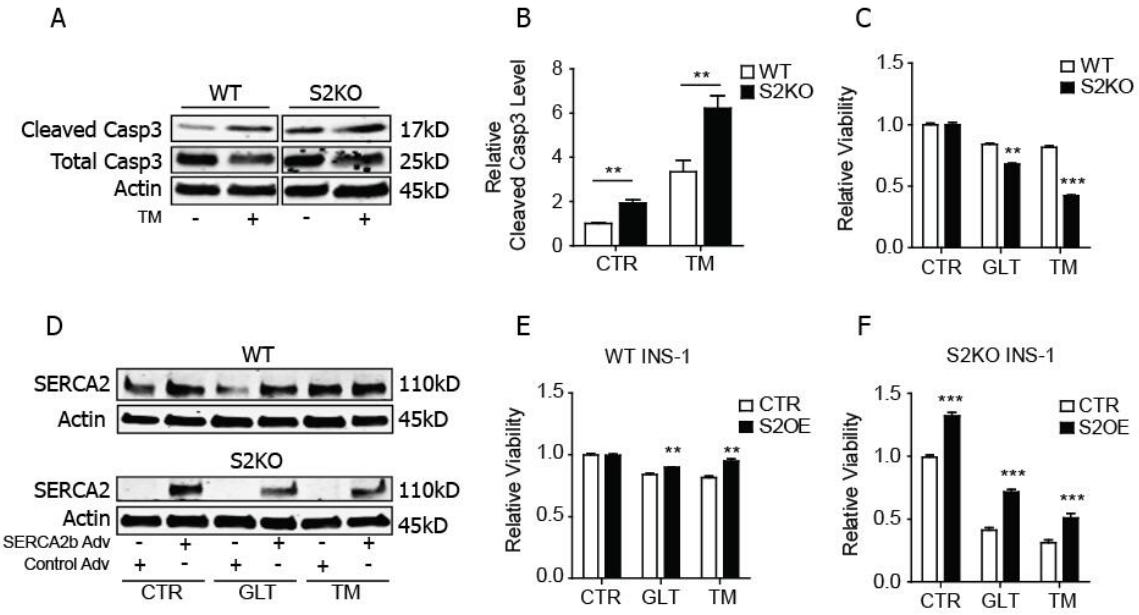

Figure 25. SERCA2 KO β cells manifest increased susceptibility to stress-induced death

A-B, WT and S2KO INS-1 cells were treated with or without 10 μM of tunicamycin (TM) for 24 hrs. Immunoblot analysis was performed using antibodies against cleaved caspase-3, total caspase-3 and Actin. Quantitative protein levels are shown graphically. **C**, WT and S2KO INS-1 cells were treated under control conditions (CTR) or exposed to GLT or TM for 24 hrs, and viability was measured using the CellTiter-Glo assay. Results were normalized to results obtained in WT cells under control conditions. **D-F**, SERCA2b was overexpressed (S2OE) via adenoviral transduction in WT and S2KO INS-1 cells and then treated with GLT or TM for 24 hrs. Immunoblot analysis was performed using antibodies against SERCA2 and Actin: (**D**); CellTiter-Glo viability tests were performed in WT (**E**) and S2KO cells (**F**). Results were normalized to cells transduced with LacZ virus under control conditions. SERCA2b was overexpressed via adenoviral transduction in WT (WT-OE) and S2KO (KO-OE) INS-1 cells followed by exposure to GLT for 24 hrs. Immunoblot analysis was performed using antibodies against cleaved caspase-3 and Actin, and quantitative protein levels are shown graphically. Results are displayed as means ± S.E.M, and indicated comparisons were significantly different. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$;

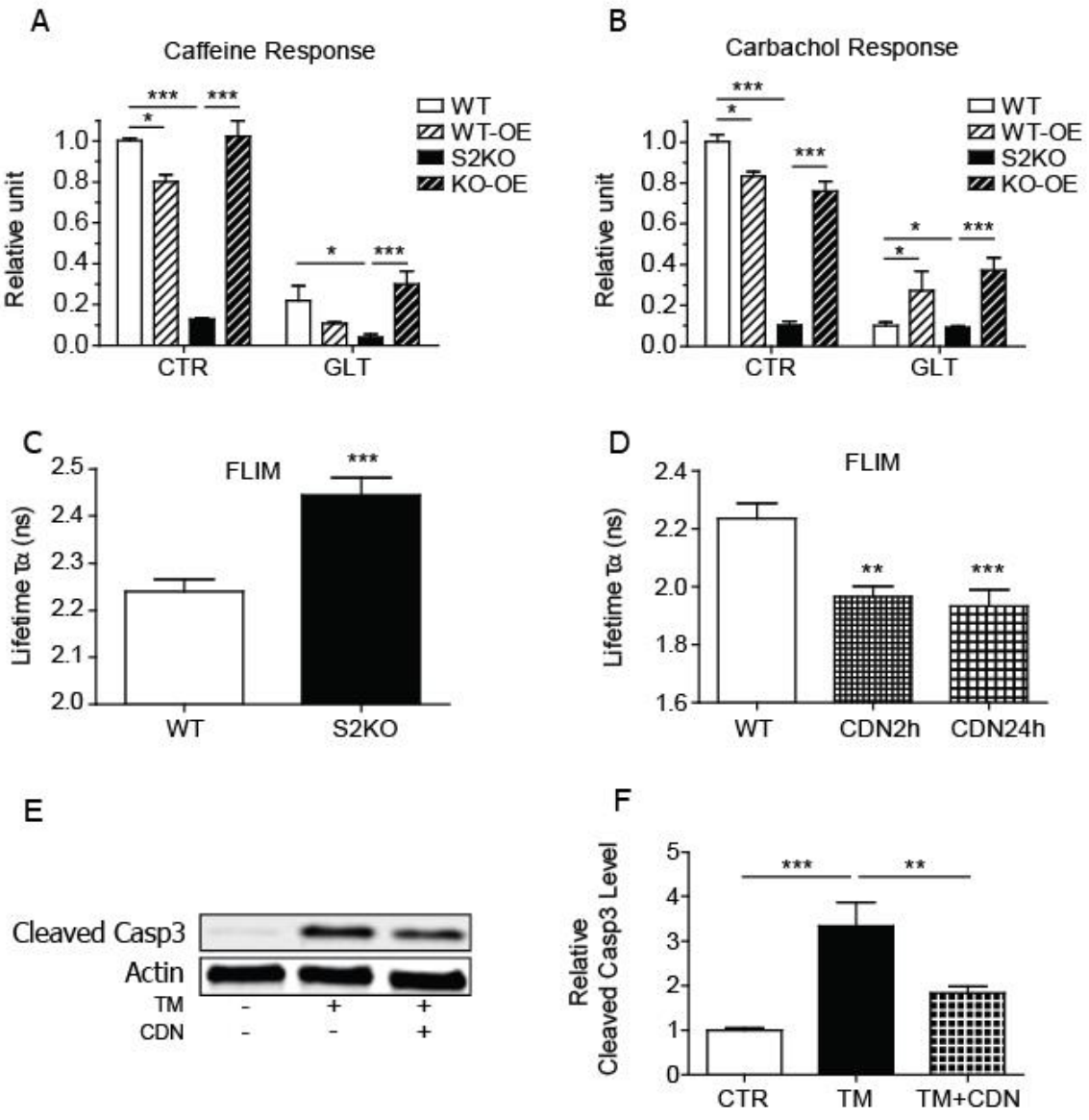

Figure 26. SERCA2 KO β cells manifest impaired Ca^{2+} storage

A and B, To assess cytosolic Ca^{2+} levels, Calcium 6 fluorescence was measured under Ca^{2+} free conditions. **C and D**, INS-1 cells were transduced with a D4ER adenovirus and FLIM was used to measure endoplasmic reticulum Ca^{2+}. Shown is the average donor lifetime in WT and S2KO INS-1 cells (**C**) or WT INS-1 cells treated with 10 μM CDN1163 for 2 or 24 hrs (**D**), n=at least 10 regions of interest per cell type or treatment. **E and F**, WT and S2KO INS-1 cells were treated with or without TM combined with or without 10 μM CDN1163 for 24 hrs. Immunoblot analysis was performed using antibodies against cleaved caspase-3 and Actin, and quantitative protein levels are shown graphically. Results are displayed as means ± S.E.M, and indicated comparisons were significantly different. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$;

3.3 Discussion

In rodent and human models of diabetes, acquired loss of β cell SERCA2 expression and activity under inflammatory conditions has been correlated with altered β cell Ca^{2+} homeostasis, reduced insulin secretion, and impaired survival. Furthermore, SERCA2 restoration has been shown to improve these parameters (256, 265, 275). However, whether *in vivo* deficiency of SERCA2 is sufficient to impair systemic metabolic function and/or β cell health has never been addressed. Because homozygous loss of SERCA2 is embryonically lethal (282), we used the S2HET model to analyzed β cell function following a HFD challenge. Our results show that SERCA2 deficiency leads to impaired glucose tolerance and hyperglycemia upon metabolic challenge. In contrast, S2HET and WT mice exhibited indistinguishable patterns of weight gain, body composition, and systemic and tissue-specific insulin sensitivity. This is notable since *in vitro* experiments have shown that SERCA2 and SERCA1 interact with IRS-1 and IRS-2 in skeletal muscle in an insulin dependent manner (313). Previous studies have shown that SERCA2 overexpression in the liver or treatment with CDN1163 attenuated liver ER stress and improved in diabetic models (245, 246). However, our data suggest that the glucose intolerance observed in HFD-fed S2HET mice does not arise from perturbations in adiposity or insulin sensitivity. Rather, we conclude that SERCA2 deficiency leads to an impaired β cell compensatory response to diet-induced obesity. In support of this conclusion, we have shown that SERCA2 deficiency leads to decreased β cell insulin production, insulin secretion, and proliferation, which likely arise because of increased β cell ER stress, and perturbed β cell Ca^{2+} signaling.

The ER is a key intracellular Ca^{2+} store with the intraluminal Ca^{2+} concentration is estimated to be at least several orders of magnitude higher than cytosolic. The SERCA family of ATPases is the only known group of transporters tasked with Ca^{2+} uptake into

the ER lumen, while ER Ca^{2+} release occurs via RyRs and IP_3Rs, which become activated in response to specific ligands or intracellular signaling pathways (314, 315). To date, the *in vivo* role of IP_3R in the pancreatic β cell has yet to be studied. However, a single point mutation in the RyR, leading to an unregulated ER Ca^{2+} leak, results in decreased insulin secretion and impaired glucose tolerance as well as β cell ER stress and mitochondrial dysfunction (235). These results and our study emphasize a pivotal role for ER Ca^{2+} homeostasis in the maintenance of β cell function and health.

Among all the 14 isoforms within SERCA family, SERCA3 function in the islet has been previously investigated in series of elegant studies by Gilon and colleagues with whole-body deletion of SERCA3. Under chow-fed conditions, SERCA3 KO mice exhibited normal glucose tolerance without overt evidence of ER stress. SERCA3 ablation also did not affect basal cytosolic Ca^{2+} levels or the initial glucose-induced Ca^{2+} response within islets (266). Following glucose stimulation, SERCA3 null islets exhibited a higher amplitude of Phase 2 $[Ca^{2+}]i$ oscillations, consistent with impaired ER Ca^{2+} uptake that interestingly led to increased insulin secretion (255). Whereas SERCA3 mice have never been challenged with diet-induced obesity, somewhat limiting direct comparisons, our results still suggest non-overlapping functions for the two isoforms. We show that SERCA2 haploinsufficiency leads to increased basal cytosolic Ca^{2+} levels, impaired glucose and KCl-stimulated Ca^{2+} responses, and a delayed onset and reduced amplitude of glucose-induced Ca^{2+} oscillations. This suggests a broader role for SERCA2 and the ER Ca^{2+} pool in patterning β cell Ca^{2+} architecture, independent of glucose sensing and ATP generation. In contrast to SERCA3 null islets, perturbations in Ca^{2+} signaling in SERCA2-deficient islets were sufficient to impair glucose-stimulated insulin secretion both *in vivo* and *ex vivo*. Notably, no compensatory up-regulation of SERCA3 protein expression in either our mouse model or in the clonal SERCA2 KO β cell line was observed.

SERCA2 deficiency also had a significant impact on β cell ER function and health. S2HET islets exhibited altered ER morphology and increased expression of genes involved in ER stress signaling, while SERCA2 deficient INS-1 cells were more susceptible to glucolipotoxic and tunicamycin-induced cell death. Previous work has shown that ER stress in β cell results in insulin mRNA degradation through activation of IRE1α, whereas PERK activation suppressed insulin translational (316-318). Consistent with these studies, islet insulin mRNA and protein levels were decreased and circulating levels of insulin were lower in S2HET mice compared to WT controls. Given that the S2HET mice have global SERCA2 deficiency including liver, the impaired glucose tolerance we observed might be partly due to hepatic ER stress in response to obesity, as was shown in a previous study (246). However, further studies are necessary using our model system to determine the specific contributions of hepatic function to impaired glucose tolerance.

In addition, S2HET islets also exhibited impaired insulin processing and decreased insulin granule maturation after HFD. Proteolytic cleavage of proinsulin into mature insulin requires the activity of prohormone convertase (PC) 1/3, PC 2 and carboxypeptidase E (CPE) within secretory granules. Ca^{2+} is also required to direct PC 1/3 into dense core secretory granules (319), while further serving as an essential cofactor for both PC 1/3 and 2 activity (31). The Ca^{2+} content within secretory granules is patterned by ER Ca^{2+} levels, suggesting a relationship between SERCA2 deficiency and convertase enzyme activity (320). A supplemental finding in our study was that PC 1/3 mRNA and protein expression were also decreased in S2HET islets and S2KO cells in both normal chow and after HFD. Regarding this point, a recent study in GLUTag murine enteroendocrine cells, which secrete glucagon-like peptide, also revealed decreased PC1/3 protein with palmitate-induced ER stress (321). In β cells, a similar effect was observed after 7 days of palmitate and oleate treatment in MIN-6 cells (176).

Our data also show that SERCA2 deficiency results in decreased β cell proliferation in response to diet-induced obesity. Increased SERCA2b expression has been shown in several cancer models of unrestrained proliferation, while a number of Ca^{2+}-regulated pathways play an important role in β cell proliferation. For example, NFAT and CREB are both activated in response to a rise in cytosolic Ca^{2+} (322, 323). Furthermore, a direct relationship between ER stress and β cell proliferation has been suggested by two recent studies. Pascoe *et al.* showed that mild ER stress favored β cell proliferation (324), while Szabat *et al.* showed that reduced insulin production relieved β cell ER stress and induced β cell proliferation (325). Precisely how altered SERCA2 activity in our model impacts known Ca^{2+}-dependent proliferative pathways and integrates with the above studies will be the subject of future investigation.

In summary, we provide the first evidence using a genetic model that loss of SERCA2 plays a pivotal role in the pathogenesis of diabetes, specifically leading to a cell autonomous defect in β cell secretory function, Ca^{2+} homeostasis, proliferation, and survival. Additional studies employing tissue specific mouse models of SERCA2 deletion are needed to unravel the relative contributions of SERCA2 loss in the β cell versus peripheral tissues. In the meantime, a growing body of evidence suggest that strategies to prevent loss of SERCA2 expression by exogenous overexpression (246) and/or modulation of SERCA2 activity via a small molecule approach represent viable strategies to improve glucose homeostasis (245). Finally, these data also have relevance for humans with Darier disease, in which one copy of the *ATP2A2* gene is defective. Although metabolic effects of SERCA2 haploinsufficiency have not been reported in these individuals, a selective predisposition to diet-induced metabolic disease and other conditions, such as heart disease (281), could easily be overlooked in this rare population and should be further studied.

CHAPTER FOUR

Summary and Perspective

4.1 Summary

DM is a disease of increasing prevalence that greatly influences quality of life. All three major types of diabetes (T1D, T2D and GDM) are the result of a relative deficiency in insulin secretion. Insulin is the only hormone that decreases blood glucose, which it does by activating the insulin receptor signaling pathway in peripheral tissues to induce glucose uptake by GLUT4 translocation. Insulin is secreted by a specialized endocrine cell type, the β cell, which resides in pancreatic islets. Similar to other endocrine cells, β cells possess specialized machinery to guarantee highly efficient hormone biosynthesis, storage and secretion in response to physiologic stimuli. These adaptations include a robust ER with high Ca^{2+} storage capacity, well packaged secretory granules and low basal proliferation rate. The most unique feature of β cells is the highly regulated orchestration between nutrient stimuli and insulin secretion, a process that occurs mainly through Ca^{2+} mobilization triggered by both metabolism and PKA-dependent pathways.

Under normal conditions, efficient Ca^{2+} mobilization in the β cell leads to healthy Ca^{2+} oscillations that are synchronized with insulin secretion and nutrient metabolism. However, as environmental insults like GLT and pro-inflammatory cytokines challenge the β cells during the pre-diabetic state, β cells undergo a transition from a state of compensation, characterized by upregulation of UPR and cellular proliferation, to a state of decompensation, which includes terminal ER stress and apoptosis. During the development of T2D, insulin biosynthesis is greatly reduced in later stages of the disease, and the responsiveness to nutrient stimuli is blunted. These impairments occur

concurrently with Ca^{2+} dyshomeostasis, manifested as a decrease in ER Ca^{2+} storage and sustained high levels of cytosolic Ca^{2+}.

Ca^{2+} homeostasis is maintained both by Ca^{2+} channels and Ca^{2+} pumps that control ion gradients between different cellular compartments. As one of the most important cellular Ca^{2+} pools, ER Ca^{2+} homeostasis is regulated primarily by the SERCA pump, which transports 2 Ca^{2+} ions into the ER lumen with each catalytic cycle. SERCA2 is the most abundant isoform expressed in the β cell, and it also has the highest affinity for Ca^{2+}. Previous studies from our lab discovered that SERCA2 is greatly reduced in rodent and human diabetic β cells and in cultured β cells treated with conditions that simulate diabetic conditions. Through promoter analysis and *in vitro* and *in vivo* confirmation, we found that this decrease in SERCA2 expression during diabetes is partly due to down regulation of the transcription factors, PPAR-γ and Pdx-1. Overexpression/restoration of either of these factors was able to rescue the expression of SERCA2 at both the transcript and protein level.

Previous studies have suggested that pro-inflammatory cytokines induce down-regulation of SERCA2 through NO-dependent pathways. However, it was still unclear that in the context of inflammatory signaling, SERCA2 is regulated at transcriptional or translational levels. Thus, we first performed assessment of SERCA2 protein and mRNA stability in INS-1 cells, rat islets and human islets treated with pro-inflammatory cytokines combined with actinomycin D or cycloheximide to block global transcription or translation. IL-1β treatment led to increased iNOS gene and protein expression that occurred concurrently with activation of AMPK. IL-1β led to decreased SERCA2b mRNA and protein expression, while time-course experiments revealed a reduction in protein half-life with no change in mRNA stability. Moreover, SERCA2b protein, but not mRNA, levels were rescued by treatment with the NOS inhibitor L-NMMA, whereas the NO

donor SNAP and the AMPK activator AICAR recapitulated the effects of IL-1β on SERC2b at post-translational level. Similarly, IL-1β-induced reductions in SERCA2b expression were rescued by pharmacological inhibition of AMPK with compound C or by transduction of a dominant negative form of AMPK, while β cell death was prevented in parallel. Finally, to determine a functional relationship between NO, AMPK signaling and SERCA2b activity, Fura-2AM Ca^{2+} imaging experiments were performed in INS-1 cells. Consistent with observed changes in SERCA2b expression, IL-1β, SNAP, and AICAR increased cytosolic Ca^{2+} and decreased ER Ca^{2+} levels, suggesting congruent modulation of SERCA activity under these conditions. In aggregate, the results in Chapter 2 show that SERCA2b protein stability is decreased under inflammatory conditions through NO and AMPK-dependent pathways, and provide novel insight into pathways leading to altered β cell calcium homeostasis and reduced β cell survival in diabetes.

Next, to study the consequences of losing SERCA2 in β cells, we utilized a whole body SERCA2 heterozygous mouse model to analyze the *in vivo* function of SERCA2. To this end, SERCA2 heterozygous mice (S2HET) were challenged with HFD containing 45% of kilocalories from fat. After 16 weeks of HFD, S2HET mice were found to have lower serum insulin concentrations, higher blood glucose levels and significantly reduced glucose tolerance compared to wild-type controls. Adiposity and systemic and tissue-specific insulin sensitivity were not different between S2HET mice and controls, suggesting an impairment in insulin secretion rather than a change in insulin action in the periphery. Consistent with this indication of β cell dysfuntion, S2HET islets and SERCA2-deficient INS-1 cells exhibited increased ER stress, altered insulin production and proinsulin processing, and reduced β cell mass and proliferation. Furthermore, glucose stimulated insulin secretion and cytosolic Ca^{2+} oscillations were reduced in

HFD-fed S2HET islets. Notably, application of a small molecule activator of SERCA2 was able to rescue the oscillatory defects in S2HET islets, and GSIS was also greatly improved in cadaveric human islets from both healthy donors and donors with T2D when treated with this activator. In aggregate, the data in Chapter 3 suggest a critical role for SERCA2 and the maintenance of ER Ca^{2+} stores during the *in vivo* β cell compensatory response to diet induced obesity.

In summary, our data suggest that SERCA2 is a target of diabetic stresses in β cells at transcriptional and translational levels. This impairment in the function of SERCA2 leads to a loss of ER Ca^{2+} homeostasis, which plays an important role in the dysfunction and death of pancreatic β cells under diabetogenic conditions. Specifically, SERCA expression influences the adaptive compensatory response in β cells, including insulin biosynthesis, regulated insulin secretion and hyperplasia. Whereas the transition between compensation and decompensation during development of T2D is usually characterized by compromised β cell function, loss of SERCA function might be one of the key contributing factors in this transition point. Furthermore, correcting these defects by increasing SERCA2 function might have protective effects in β cells. Our data indicate that SERCA might be a therapeutic target with great potential in the treatment of diabetes. The small molecule activator of SERCA2 applied in this dissertation could be a promising candidate to clinically rescue ER stress-induced β cell apoptosis and improve insulin secretion during the progression of T2D.

4.2 Limitations and future studies

The work presented in this dissertation suggests an important role for SERCA2 in ER Ca^{2+} homeostasis in β cell function and survival. However, there are still a number of topics and areas that we did not have time to fully elucidate. To promote SERCA2 as a

potential therapeutic target, there are multiple important aspects that need further analysis.

4.2.1 Post-translational regulation of SERCA2 under diabetic stresses

a. Degradation of SERCA2

The first half of our study provided a preliminary discovery of SERCA2 post-translational regulation in pancreatic β cells in normal and diabetic conditions. These mechanisms include NO- and AMPK-dependent down-regulation of protein stability and enzyme activity induced by pro-inflammatory cytokines and a pharmacological small molecule activator of AMPK. However, the specific degradation mechanisms relevant to SERCA2 stability in β cells are still unclear. While we were the first group to address SERCA2 protein stability and activity in physiological conditions in β cells, the role of SERCA has been studied in other tissues or cell types. For example, in cardiomyocytes, ischemia-reperfusion-induced calpain activation resulted in SERCA2a degradation through ER associated degradation which was mediated by the proteasome (326). Meanwhile, a mass spectrometry study showed that irreversible oxidation on cysteine-674 of SERCA2a was associated with its degradation. The end product of this oxidation reaction is a 60-70 kD protein segment (327). These findings suggest that diabetogenic stress may result in the loss of full length SERCA protein.

The protein degradation mechanisms in cell mainly include proteasome-dependent and lysosome-dependent. To elucidate the degradation mechanisms of SERCA2 in β cells, the classic methods is treating cultured β cell lines or isolated islets with small molecule inhibitors of degradation machinery, such as proteasome inhibitors (MG132) and lysosome inhibitors chloroquine (328). However, considering SERCA2 is a relatively stable protein with a half-life of about 24 h, chronic treatment with these

inhibitors might generate cytotoxicity. Alternatively, we could transduce cells with adenovirus encoding kinase dead enzymes or targeted knockdown by RNAi to block the endogenous degradation pathways. Then, we could elucidate how SERCA2 is degraded in β cells and how it might be influenced in the diabetic models we used in this dissertation. Notably, we have observed a smaller second protein band around 60-70 kD that could be detected by the SERCA2 antibodies reactive to the segment, which is consistent with the study in cardiomyocytes. Furthermore, this band accumulates under stress conditions, suggesting a specific modification-dependent degradation. Finally, using unbiased approaches, we could perform mass spectrometry to unravel different sites of post-translational modifications of SERCA2 in β cells. Despite previous studies proposing several phosphorylation, glycosylation and glutathione sites on SERCA2a, none of these modifications have been confirmed for SERCA2b in β cells.

b. SERCA2 binding proteins

Previous studies focusing on SERCA interacting proteins have been mostly performed in muscle cells or human embryonic kidney cell lines (HEK 293), or on isolated proteins produced in *E. coli*. These isolated proteins usually bind to SERCA2 to inhibit its activity, including Bcl-2 (329), S100a (330), short peptide encoded by small open reading frames (331), presenilin (332), phospholamban or sarcolipin (333). Interestingly, SERCA2 monomers also appear to interact with each other to form dimers in HEK 293 cells. This dimer was shown to be important in regulating SERCA2 function in this cell type (334). Thus, the down-regulation of SERCA2 expression might work as a feedforward mechanism to inhibit its own activity. However, considering the differences in β cells, especially given the fact that the most well-studied phospholamban and sarcolipin are not expressed in β cells, indeed more experiments are required to explore the interacting proteins of SERCA2 in β cells.

These unanswered questions highlight the importance of prioritizing immunoprecipitation of SERCA2 and followed by mass spectrometry and interactome analysis. Furthermore, this could help elucidate pathways that regulate SERCA2 activity under different diabetogenic stressors by performing these experiments following treatments with GLT, pro-inflammatory cytokines and ER stress inducers. To increase the abundance and pull down efficiency of SERCA with immunoprecipitation, we could overexpress tagged SERCA2 in β cells. This approach, however, could be limited by potential problems associated with overexpression.

4.2.2 SERCA2 in regulating β cell proliferation

The second half of the study in this dissertation focused on the *in vivo* effects of SERCA2 in regulating whole body glucose homeostasis. Our data suggest that β cells are heavily dependent upon SERCA2 during the compensatory response to diet induced obesity. The defects associated with SERCA2 haploinsufficiency involved decreased β cell mass with lower proliferation rates, higher susceptibility to ER stress induced cell death, impaired insulin processing and decreased regulated Ca^{2+} oscillation in response to glucose.

Ca^{2+} mobilization was found to be the pacemaker of the cell cycle as early as 1990 (228). The ER Ca^{2+} depot also plays a key role in many additional Ca^{2+}-mediated signalling pathways. A number of studies have shown that increased cytosolic Ca^{2+} emanating from the ER is required for cell cycle initiation and cell proliferation (228, 323). SERCA2 has previously been found to be related to cell cycle and proliferation. In prostate cancer cells, epidermal growth factor (EGF)-, insulin-like growth factor (IGF)- and TNF-α-induced cell mitosis were all SERCA dependent (335, 336). Meanwhile, it was also observed that SERCA2 gene mutation in humans or haploinsufficiency in mice

results in a higher susceptibility to colon cancer, lung cancer and squamous cell carcinomas of oral mucosa, esophagus, and forestomach and papillomas in keratinized epithelial cells (337-340). These seemingly contrasting observations might due to cell type specificity, which might be further related to isoform expression profiles during proliferation and apoptosis. Indeed, smooth muscle cells express both SERCA2a and SERCA2b isoforms, and it was observed that an isoform expression pattern shift occurs in parallel with cell proliferation status. i.e., SERCA2b expression was more dominant during proliferative stages (341). Nevertheless, an efficient ER Ca^{2+} buffering system is crucial for the maintenance of the balance between mitosis and cellular function in many cell types (342, 343).

In the current study, we did not elucidate the specific mechanisms by which SERCA2 directly or indirectly regulates the cell cycle. However, in the SERCA2 knockout β cell line (S2KO) that we generated, we performed some preliminary studies and were able to see a correlation between lower cyclin D2 expression and decreased G1-S phase transition (Figure 27). To further pursue the hypothesis that SERCA2 directly regulates cyclin D2, *in vivo* and *in vitro* models could be utilized. First of all, we could confirm the causation between SERCA2 deficiency and cyclin D2 deficiency by measuring the cyclin D2 expression levels in our S2HET mouse model. Secondly, we could also overexpress cyclin D2 in cultured SERCA2 knockout β cells to determine whether the decreased cell cycle progression is indeed cyclin D2-dependent.

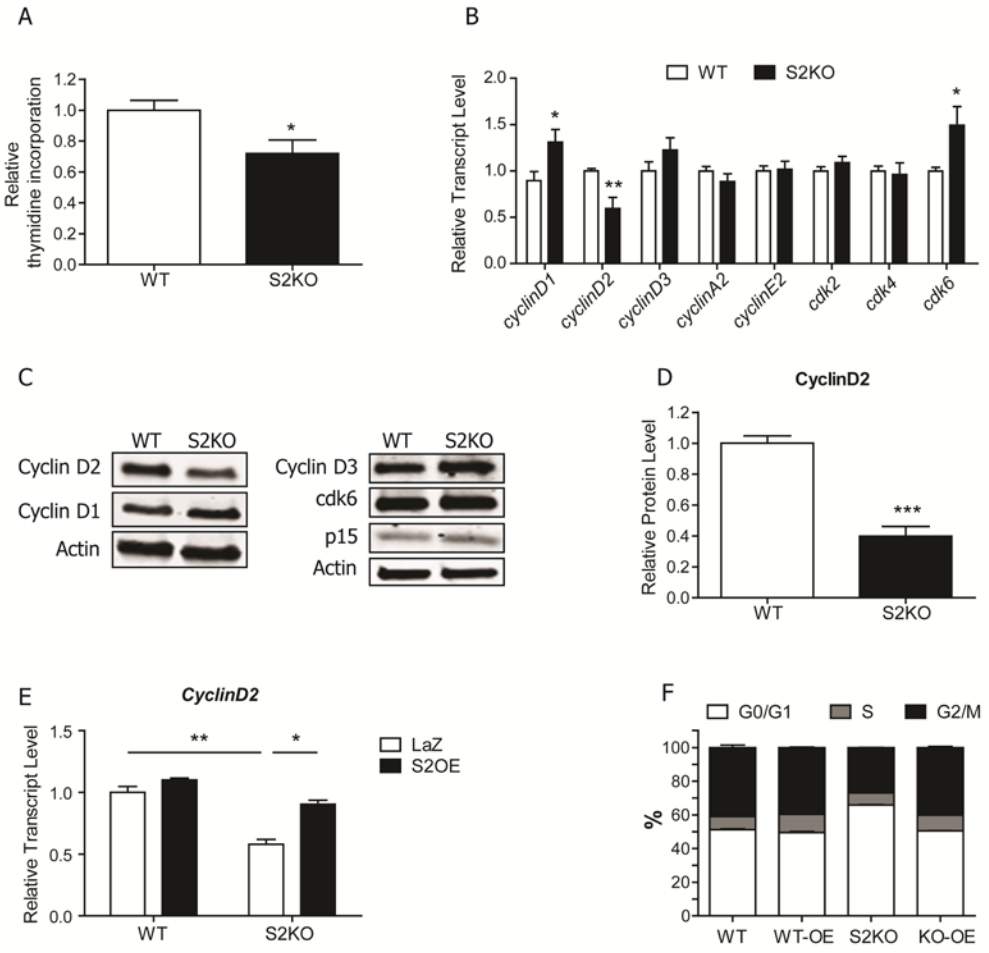

Figure 27 SERCA2 deficiency in cultured β cells induced lower proliferation with cyclin D2 involvement.

A. Thymidine incorporation experiments were performed in WT and S2KO INS-1 cells after 24h of seeding. **B-D.** Protein and RNA were isolated from WT and S2KO INS-1 cells, and qRT-PCR was used to measure, and analysis revealed decreased expression of cyclin D2 in S2KO cells. Reverse-transcribed RNA was subjected to qRT-PCR to measure genes involved in cell cycle regulation transcript levels (normalized to *Actb*) (A, C); Immunoblot analysis performed using antibodies against cyclin D1, cyclin D2, cyclin D3, cdk6, p15 and actin (B).; Quantitative protein levels of cyclin D2 are shown graphically (C); WT and S2KO cells were transduced with a SERCA2b expressing adenovirus or a LacZ control adenovirus. **E**, qRT-PCR was performed to measure *cyclin D2* transcript levels. And **F,** Propidium iodide (PI) staining was performed. Results are displayed as the mean ± S.E.M. n = 3/group. *p<0.05 **p<0.01 ***p<0.001

There are several other Ca^{2+} dependent molecules that might also be involved in SERCA2-mediated proliferative events (323). For example, a long-lasting increase in cytosolic Ca^{2+} (at least 1–2 h) is required for activation of the transcription factor, nuclear factor of activated T-cells (NFAT), which is activated by calcineurin and serves as the mediator of proliferation in almost all cell types. Depleted ER Ca^{2+} storage might contribute to blunted NFAT activation. Specifically, NFATc1 and NFATc3 might be closely involved since they were found to regulate cyclin D1 and D2 expression (344). Meanwhile, NFATc1 was also found to directly regulate IRS-2 expression in islets (345). Thus the study of SERCA's relationship with NFAT might worth further study.

Another possible link between SERCA and regulation of cell proliferation is Notch1, one of the receptors in the Notch signaling pathway that is important for cell-cell communication to control multiple cell differentiation processes during embryonic and adult life. In a recent study utilizing cell-based high-throughput screens in T cell acute lymphoblastic leukemia, SERCA inhibition was found to rescue abnormal Notch1 signaling due to its leukemia associated mutations. Interestingly, this might also be due to a direct interaction between SERCA, Notch1 and presenilin (346).

In addition to diet induced obesity models, another physiologic stimuli known to induce β cell hyperplasia is pregnancy (347). In mice, previous work has shown that there is an approximate 1.5 to 4-fold increase in maternal β cell mass during gestation, while failure in β cell mass expansion contributes to GDM. The increase in β cell mass during pregnancy was found to be mainly regulated by placental lactogen and prolactin mediated signaling pathways (348, 349). These types of molecules bind to prolactin receptors and activate downstream cascades of AKT, ERK, signal transducer and activator of transcription (STAT5) and Forkhead box protein M1 (FoxM1) (350). To study how SERCA2 deficiency may play a role in maintaining β cell function and proliferation during pregnancy, we could set up a gestational model using wildtype and S2HET

female mice. I would hypothesize that S2HET female mice would develop worsened glucose tolerance during late gestational stages due to lower β cell mass. Important analyses would include determination of whole body glucose homeostasis, β cell mass and adaptive hypersecretion. Early studies also applied pancreatic duct ligation as a model to induce β cell proliferation and regeneration. However, recent studies report that this model induces several irregular signals found to cause massive injury in pancreas (351, 352). These observations suggest that pancreatic duct ligation might not be an ideal model for β cell compensatory hyperplasia under physiological conditions of insulin resistance.

4.2.3 β cell specific SERCA2 knock out mouse model

Studies in this dissertation demonstrate that S2HET mice fed HFD had defects in β cells without a change in peripheral insulin resistance, suggesting that SERCA2 deficiency is mostly influencing β cells. However, to further prove this, it is necessary to test the effect of a β cell specific knockout mouse model.

During the early stages of the current study, we developed a β cell specific SERCA2 knockout mouse model by crossing mice with a floxed SERCA2 gene with the Mip-Cre^{ERT} line (conditional knockout created by tamoxifen administration). The β cell specific knockout animal is termed as MipCre-SERCA2 flox and SERCA2 flox mice would be used as littermate controls. Interestingly, we noticed the that MipCre-SERCA2 flox control mice demonstrated a better glucose tolerance compared to the SERCA2 flox, even before tamoxifen administration. Indeed, this protective effect was recently reported in several studies and has been shown to result from the presence of the human growth hormone (GH) emanating from GH mini gene insert on the *Cre* transgene. Thus, this background is not an ideal model to test the effects of SERCA2 loss (353-355).

Recently, another β cell specific Cre mouse line, *Ins1*Cre^{ERT2}, was developed, which has shown more equivalent metabolic features compared to non-Cre controls.

This line was generated by inserting the *CreERT2* recombinase gene in the second exon of the *Ins1* gene so that the coding region of the recombinase starts at the initiation codon and replaces the *Ins1* coding sequence (356). Currently, we are crossing the *Ins1*Cre^{ERT2} to SERCA2 flox mice to generate the knockout colonies. We plan to start by confirming there is no overt protective effect of this *Ins1*Cre^{ERT2} background, and testing the knock out efficiency and specificity of SERCA2 in β cells. Next, whole body glucose tolerance and insulin tolerance tests will be performed to determine the β cell specific role of SERCA2.

4.2.4 SERCA2 as a potential therapeutic target

A well-organized report was published recently, which collectively summarized the research on β cell dysfunction in T2D from different fields, limitations of these studies and future directions. This paper pointed out several critical directions, including but not limited to: identification and characterization of genetic loci for T2D; pathways that improve β cell function and mass; identification of circulating factors and details of the metabolic environment arising from the communication of other organs and the β cells in regulating their function and health (357).

The studies in this dissertation have demonstrated that β cell health and function rely heavily upon correct Ca^{2+} homeostasis. SERCA2 is critical for proper control of ER Ca^{2+} balance and is therefore a potential therapeutic target in other tissues including liver and cardiac muscle (245, 358). We now propose that activating SERCA2 in β cells may also protect against diabetes progression. Indeed, in the last part of our studies, we observed exciting effects of a SERCA2 small molecule activator, as this compound was able to rescue tunicamycin induced cell death, increase ER Ca^{2+} storage, and improve glucose stimulated Ca^{2+} oscillations and insulin secretion (Figure 26 and 28).

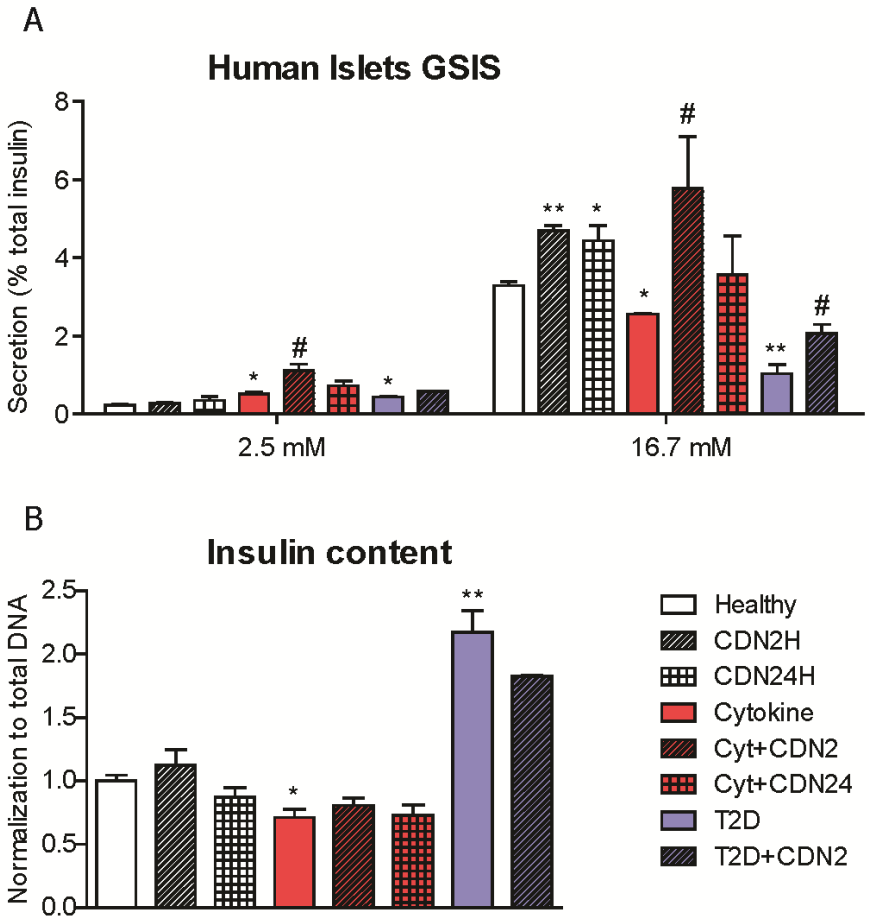

Figure 28. Small molecule of SERCA2 activator improves glucose stimulated insulin secretion without alterating insulin content

Human islets from healthy cadaveric donor were treated with 10 µM of CDN for 2 h or 24 h with or without a cytokine mix of IL-1β and TNF-α. Islets from cadaveric donor of T2D were treated with 10 µM of CDN for 2 h. **A**. Glucose stimulated insulin secretion (GSIS) was measured and normalized to total insulin content. **B**. Relative insulin content was measured after treatment and GSIS normalized to DNA content. Results are displayed as the mean ± S.E.M; n = at least 3 samples per group. Indicated comparisons are significantly different; *, $p<0.05$ compared to healthy group; **, $p<0.01$ compared to healthy group. #, $p<0.05$ compared to cytokine-treated alone group or T2D group.

All in all, the discoveries in this dissertation have improved our understanding of the importance of ER Ca^{2+} homeostasis in pancreatic β cells. SERCA2 was found to be down regulated under diabetic conditions and was identified to play a critical role in the compensatory response of the β cell to diet induced obesity. Overexpression of SERCA2 or small molecule SERCA2 activator were able to improve ER Ca^{2+} storage, Ca^{2+} mobilization and insulin secretory profiles, suggesting that SERCA2 could be a promising potential therapeutic target for the prevention of diabetes.

CHAPTER FIVE

Materials and Methods

5.1 Materials

5.1.1. Animals, islets isolation and human islets preparation

a. Mice

C57BL/6J (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 12 weeks of age. SERCA2 heterozygous (S2HET) mice were developed by Gary E. Shull of the University of Cincinnati, backcrossed onto a C57BL6/J background for at least 10 generations, and maintained under protocols approved by the Indiana University Institutional Animal Care and Use Committee, the U.S. Department of Agriculture's Animal Welfare Act (9 CFR Parts 1, 2, and 3), and the Guide for the Care and Use of Laboratory Animals

b. Rats

Male Wistar rats (250–300 g) were purchased from Harlan Lab in Indianapolis, IN, USA, and maintained under protocols approved by Indiana University Institutional Animal Care and Use Committee.

c. Human islets

Human cadaveric islets isolated from non-diabetic donors were obtained from the Integrated Islet Distribution Program or the National Disease Research Interchange. Upon receipt, human islets were hand-picked and allowed to recover overnight in Dulbecco's modified essential medium (DMEM) medium containing 5.5mM glucose, 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin.

5.1.2 Cell culture, islets isolation and culture

INS-1 832/13 rat insulinoma cells were cultured in regular 11mM glucose Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1mM sodium pyruvate, 50μM β-macaptoethanol and, 10% FBS, penicillin and streptomycin.

C57BL6/J WT, S2HET mice or rat pancreatic islets were isolated by collagenase digestion as described previously (359). The islets were hand-picked, and allowed to recover overnight in regular RPMI supplemented with 10% FBS, 100U/ml penicillin, and 100μg/ml streptomycin.

5.1.3. Primers, antibodies and reagents

Standard polymerase chain reaction (PCR) reactions for genotyping (Table 1) and quantitative reverse transcription-PCR (qRT-PCR) for gene expressions were performed using oligonucleotides synthesized by Invitrogen (Table 2).

Antibodies used in western blotting, immunofluorescent and immunohistochemistry staining are listed in Table 3.

Chemicals and reagents used in this dissertation are listed in Table 4

Table 1 Genotyping Primers

Gene	Orientation	Sequence
ATP2A2 (SERCA2)	Forward (5'-3')	CGGCCTTCTAGAATTGCCGGCTG
	Reverse (5'-3')	CTTACGAAAGATATACATGCTGCCAGCAG (for wildtype)
	Reverse (5'-3')	GCATGCTCCAGACTCCCTTG (for $ATP2A^{2+/-}$)
Mip-Cre^{ERT}	Forward (5'-3')	GCGGTCTGGCAGTAAAAACTATC
	Reverse (5'-3')	GTGAAACAGCATTGCTGTCACTT
$SERCA2^{flox/flox}$	Forward (5'-3')	TCTTCATAACACACGCCAATTT
	Reverse (5'-3')	CCCTTTGCTGCCAATTAACTATT

Table 2 Quantitative RT-PCR Primers

Gene	Species	Forward (5'-3') Reverse (5'-3')
SERCA2b	*Rattus rattus*	GTGGAACCTTTGCCACTCAT TGTGCTGTAGACCCAGACCA
SERCA3	*Rattus rattus*	TGTCTCTGCCTGTCATCCTG CTAGAAATGGCCGGTGTTGT
RyR2	*Rattus rattus*	ACGGCGACCATCCACAAAG AAAGTCTGTTGCCAAATCCTTCT
IP3R3	*Rattus rattus*	TGACCTGGGCAGCCAGCC GTCTCCAAGGTCTGGATGAC
DNAjc3	*Rattus rattus*	TCCCGAATCTGCTGATCGTT GGACTATGAAGCTGCCCAGG
Pdia4	*Rattus rattus*	GAGTCGGTGACCCAGGCTAC TTTCCCCAGGGAGACTTTCA
Hsp90b1	*Rattus rattus*	ACACCACAGAAGACACCACAGATG GGGAGAGGGAGGCTTGGG
Grp78	*Rattus rattus*	CCACCAGGATGCAGACATTG AGGGCCTCCACTTCCATAGA
Total Xbp-1	*Rattus rattus*	AGCACTCAGACTACGTGCGCCTC CCAGAATGCCCAAAAGGATATCAG
Spliced Xbp-1	*Rattus rattus*	CTGAGTCCGCAGCAGGT TGTCAGAGTCCATGGGAAGA
SERCA2b	*Mus musculus*	GATCCTCTACGTGGAACCTTTG CCACAGGGAGCAGGAAGAT
SERCA3	*Mus musculus*	AGGGGAAGCTAAGAAGCCAG CCCTCAGACTCCTCCTACCC
DNAjc3	*Mus musculus*	GACAGCTAGCCGACGCCTTA GTCACCATCAACTGCAGCGT
Pdia4	*Mus musculus*	TGACCCGGCCTACTTGCA GTGTGGTGAAACTTGTAATCTTCTCTCA
Hsp90b1	*Mus musculus*	TGTATGTACGCCGCGTATTCA TCGGAATCCACAACACCTTTG
Grp78	*Mus musculus*	TTCAGCCAATTATCAGCAAACTCT TTTTCTGATGTATCCTCTTCACCAGT
Total Xbp-1	*Mus musculus*	TGGCCGGGTCTGCTGAGTCCG GTCCATGGGAAGATGTTCTGG
Spliced Xbp-1	*Mus musculus*	CTGAGTCCGAATCAGGTGCAG GTCCATGGGAAGATGTTCTGG
Pcsk1	*Mus musculus* *Rattus rattus*	CCTCCTACAGCAGTGGTGATTACA GGGTCTCTGTGCAGTCATTGT
Ins1	*Mus musculus* *Rattus rattus*	AGCAAGCAGGTCATTGTTCC GACGGGACTTGGGTGTGTAG
Ins2	*Mus musculus* *Rattus rattus*	TCTTCTACACACCCATGTCCC GGTGCAGCACTGATCCAC

Table 3 Antibodies for Western Blotting and Immuno-staining

Targeted protein	Host Species	Manufacturer	Dilution
Western blotting			
Actin	Mouse	MP bioscience	1:10000
AKT	Mouse	Cell Signaling	1:1000
Inducible Nitric Oxidesynthase (iNOS)	Rabbit	Millipore	1:1000
IκBα	Rabbit	Santa Cruz	1:1000
Phospho-AKT (Th473)	Rabbit	Cell Signaling	1:1000
PC1/3	Rabbit	Cell Signaling	1:500
Phospho-Acetyl-CoAcarboxylase (ACC)	Rabbit	Cell Signalling	1:1000
Phospho-AMPKα (Th172)	Rabbit	Cell Signalling	1:1000
SERCA2	Goat	Santa Cruz	1:1000
SERCA3	Rabbit	Sigma-Aldrich	1:1000
Total-ACC	Rabbit	Cell Signalling	1:1000
Total AMPKα rabbit	Rabbit	Cell Signalling	1:1000
Total caspase 3	Rabbit	Cell Signalling	1:1000
Mouse IgG (IRDye 800CW)	Donkey	Li-cor	1:10000
Goat IgG (IRDye 800CW)	Donkey	Li-cor	1:10000
Rabbit IgG(IRDye 680RD)	Donkey	Li-cor	1:10000
Immune-staining			
Glucagon	Rabbit	Santa Cruz	1:500
Insulin	Guinea pig	Invitrogen	1:250
Insulin	Rabbit	Santa Cruz	1:250
PCNA	Rabbit	Santa Cruz	1:100
Rabbit IgG 488	Goat	Molecular probe	1:5000
Guinea pig IgG 555	Goat	Molecular probe	1:5000

Table 4 Chemicals, and Reagents

Name	Manufacturer
Cycloheximide	Sigma-Aldrich
Actinomycin D	Sigma-Aldrich
Compound C	Sigma-Aldrich
5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)	TRC
Recobinant mouse and human interleukin-1 β (IL-1β)	Life Technology
Tumor necrosis factor α (TNF-α)	Life Technology
Interferon γ (IFN-γ)	Life Technology
Caffeine	Santa Cruz
NG-monomethyl L-arginine (L-NMMA)	Santa Cruz
Carboxy-PTIO potassium salt (C-PTIO)	Cayman Chemical
S-nitroso-N-acetyl-D,L-penicillamine (SNAP)	Cayman Chemical
D-Glucose	Sigma-Aldrich
Palmitic acid	Sigma-Aldrich
Hanks' Balanced Salt Solution (HBSS)	Lonza
Carbachol	Millipore
Recombinant human insulin	Novo Nordisk Pharmaceutical Industres Inc.

5.2 Methods

5.2.1 Animal study

Male mice with heterozygous deficiency of SERCA2 and wild-type littermate controls were fed high fat diet containing 45% of kilocalories from fat (Harlan Laboratory, Indianapolis, IN) beginning at 8 weeks of age. Intraperitoneal glucose tolerance tests (IPGTT) and oral glucose tolerance tests (OGTT) were performed after 6 h of fasting and administration of glucose at a dose of 2 g/kg total body weight. Glucose was intraperitoneal injected or gavaged by 20 GA gavaging needle (Kent Scientific Corp, Torrington, CT) followed by fasting blood glucose measurements. Insulin tolerance tests (ITT) were performed after a 5-6 h fast and intraperitoneal administration of regular human insulin at a dose of 0.75 IU/kg total body weight. All blood samples were collected from the tail vein and glucose levels were determined using AlphaTRAK glucometer (Abbott Laboratories, Abbott Park, IL).

At 25 weeks of age, *in vivo* glucose stimulated insulin secretion (GSIS) assays described in section 5.2.4 were performed, and blood samples were collected at baseline and then 10 min after intraperitoneal glucose injection (2 g/kg body weight). Serum insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) (Crystal Chemical, Chicago, IL).

Plasma was obtained at 6 h fasting time point and fed time point (at termination point in the morning between 9-10am) for insulin, proinsulin and c-peptide content measurement. The blood was collected by EDTA coated collection tubes through tail vein for fasting and heart puncture for terminal points. After centrifugation and collection of the supernatant as the plasma sample, the insulin content was measured by a mouse insulin, proinsulin and c-peptide ELISA kit (ALPCO Diagnostics, Salem, NH).

Serum insulin and proinsulin levels were measured using ELISAs from Crystal Chemical (Chicago, IL) and ALPCO Diagnostics (Salem, NH), respectively. Dual X-ray Absorptiometry (DEXA) analysis was performed to estimate body composition using the Lunar PIXImus II (GE Medical Systems) mouse DEXA.

At treatment end and euthanasia, pancreata were rapidly dissected, weighed, fixed, embedded in paraffin, and sectioned longitudinally at 100-micron intervals. β cell mass and proliferation were assessed, as detailed in previous publications (181), using antibodies outlined in Supplemental Table 1. Isolated islets were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer and transferred to the Advanced Electron Microscopy Facility at the University of Chicago (Chicago, IL) to generate transmission electron micrographic images. The relative percentages of mature, immature and rod-like secretory granules were quantitated manually as outlined previously and using ImageJ software (311) .

To assess insulin signaling, mice were fasted for 6 h and injected intraperitoneally with insulin (10 IU/kg) or saline. After 10 min, liver, epididymal adipose, and gastrocnemius skeletal muscle were harvested. Tissues were sonicated in 2% SDS lysis buffer and immunoblotting was performed to measure AKT phosphorylation at serine 473.

5.2.2 Immunofluorescence and immunohistochemistry staining

Pancreata from at least five mice per treatment group were weighted, fixed by Z-fix (ANATECH LTD, Battle Creek, MI) overnight, paraffin embedded, and sectioned longitudinally at 100 microns intervals. Immunohistochemical analysis were applied to assess β cell mass using rabbit anti-insulin antibody, A Zeiss Axio Observer Z1 inverted microscope equipped with an Orca ER CCD camera (Hamamatsu Photonics,

Hamamatsu City, Japan) was used to acquire digital images of the entire stained longitudinal pancreatic section.

Immunofluorescent imaging using was applied to evaluate β cell proliferation and α cell composition. Antibodies used in these experiments are listed in Table 3.

5.2.3 Cell culture, islets culture and *in vitro* treatment

INS-1 832/13 rat insulinoma cells and islets isolated from C57BL6/J WT, S2HET mice or rat were used as *in vitro or ex vivo* model.

To measure the ER Ca^{2+} content in individual β cell from isolated mouse islets, Accutase (Innovative Cell Technologies, Inc. San Diego, CA) was used to disperse intact islets. In brief, 500µl of Accutase was added to ~100 islets pre-washed with PBS. After incubation at 37C for 20min with gentle pipetting occasionally, 10^5 of cells were seeded in a two-chamber imaging dish pre-treated with Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO).

A CRISPR/Cas 9 based technique was utilized to produce a SERCA2 knockout (S2KO) INS-1 832/13 cell line in the Genome Engineering Center at Washington University (St. Louis, MO).

For half-life study, time-course experiments were performed to determine the half-life of SERCA2b protein or mRNA using 10µM cycloheximide or 1µg/ml actinomycin D, respectively.

5.2.4 GSIS in INS-1 cells and isolated islets

GSIS assays were performed in INS-1 cells and isolated mouse islets or human islets from cadaveric donor. In brief, fully confluent INS-1 cells were pre-incubated in

secretion buffer (SAB, including 14 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 2.5 mM $CaCl_2$ in 0.2M HEPES buffer, pH7.2) with 2.5mM glucose for 1 h followed by another 2 h of either 2.5mM or 16.7 mM glucose SAB. For groups of islets, the incubation was 1) pre-incubation in 2.5mM glucose SAB for 1 h; 2) 2.5mM glucose SAB for 2 h and collect the supernatant; 3) 16.7 mM glucose SAB for 2 h and collect the supernatant. After the series of incubation, cells or islets were lysed in 10% NP40 lysis buffer. Insulin content in the SAB collected or cell lysate were determined by human insulin-specific radioimmunoassay/RIA (HI-14K, Millipore. Billerica, MA), and data were expressed relative to total cell protein concentration for INS-1 cells or total DNA content for islets. The protein concentration was determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and the DNA content was determined using Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Florence, KY)

5.2.5 Stress simulation

To simulate the inflammatory milieu of diabetes, cells and rat islets were treated in RPMI media containing 5 ng/mL of mouse IL-1β for indicated times. For human islet studies, DMEM media containing 5 ng/mL of mouse or human IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ was used.

To mimic the glucolipotoxicity in diabetic milieu, INS-1 cells or mouse islets were cultured in RPMI containing 25 mM glucose and 500 µM Bovine serum albumin (BSA)-conjugated palmitate for 24 h.

5.2.6 Viability assay and nitrite assay

A CellTiter-Glo Luminescent Cell Viability Assay (Promega; Madison, WI, USA) was used according to the manufacturer's instructions.

Nitric oxide (NO) generation was measured using cultured media after cytokine treatment using the Promega Griess Reagent System. (Promega; Madison, WI, USA)

5.2.7 Adenovirus treatment

A dominant-negative AMPK (AMPK DN) recombinant adenovirus expressing an HA-tagged human α1 subunit with a D159A mutation in the ATP binding domain was purchased from Eton Biosciences (San Diego, CA, USA). An adenovirus encoding firefly luciferase under control of the cytomegalovirus promoter was used as a control. For loss of function studies, INS-1 832/13 cells were transduced with 10^7 pfu/ml of control of AMPK DN adenovirus when cells reached 60-70% confluency. Rat, mouse, or human islets were transduced on the day of isolation or receipt using $5*10^7$ pfu/ml of control or AMPK DN adenovirus. For SERCA2b rescue studies, S2KO and WT INS-1 cells were transduced with an adenovirus expressing SERCA2b or LacZ with 10^7 pfu/ml. After overnight incubation, the culture media was replaced with fresh media followed by an additional 24-32 h of culture.

5.2.8 Cell cycle analysis by [3H]-thymidine incorporation assay and Propidium Iodide (PI) staining

INS-1 cells proliferation was assessed by measuring the incorporation of [3H]-thymidine into genomic DNA. [3H]-thymidine was added to INS-1 cells cultured in 12-well 24h after seeding at a final concentration of 1 µCi/ml medium. After 2 h, the DNA was precipitated in 500 µl of cold 10% tricholoroacetic acid and solubilized by the addition of 80 µl of 0.3 N NaOH. The amount of [3H]-thymidine incorporated into DNA was measured by liquid scintillation counting (PerkinElmer, Waltham, MA) and normalized by total cellular protein.

For PI staining, at the end of the treatment, INS-1 cells were digested by trypsin, collected and resuspended in PBS. After being fixed by adding 100% ethanol (final percentage at ~66%) and incubated for 15 min on ice, the cells were then washed with PBS and incubated in PI solution containing 50 μg/ml PI. After being incubated for 40 min at 37°C, the imaging cytometry analysis was performed in Cellometer^{TM} K2 (Nexcelom Bioscience, Lawrence, MA)

5.2.9 Total RNA isolation and quantitative real-time PCR (qRT-PCR)

Cultured cells or isolated islets were processed for total RNA isolation using RNeasy Mini plus or Micro plus kits (Qiagen, Valencia, CA), according to manufacturer's instructions For reverse transcription-PCR experiments, total RNA was processed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Grand Island, NY). Subsequently, qRT-PCR was performed using JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO), SYBR Green I dye and primers listed in Table 2.

5.2.10 Immunoblot analysis

Cells were lysed in 1% IGEPAL reagent supplemented with 10% glycerol, 16 mM NaCl, 25 mM HEPES, Sigma-Aldrich, St. Louis, MO), 60 mM n-octylglucoside (Research Products International Corp.), phosphatase inhibitor cocktails (PhosSTOP tablets, Roche) and phosphatase inhibitor cocktails (EDTA-free cOmplete tablets, Roche). Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and a SpectraMax M5 multiwell plate reader (Molecular Devices, Sunnyvale, CA). Equal concentrations of proteins were suspended in 10% SDS solution and heated to 70C for 5 minutes. Protein lysates were electrophoresed and transferred to methanol-activated PVDF membrane (Immobilon-FL Transfer Membrane from Millipore). Membranes were then blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) prior

to incubation with primary antibodies listed in Table 3. Subsequently, membranes were incubated with IRDye 800 or 680 fluorophore-labeled secondary antibodies from LI-COR. Protein bands were visualized using the Odyssey System (LI-COR, Lincoln, NE) and quantified with Image J software (NIH). The phosphorylated protein levels presented in bar graphs were normalized by total protein levels, and the total (e.g., non-phosphorylated or un-cleaved form) protein levels were normalized to actin protein levels.

5.2.11 Cytoplasmic calcium (Ca^{2+}) imaging

Intracellular cytosolic Ca^{2+} was measured using the ratiometric Ca^{2+} indicator Fura-2-acetoxymethylester (Fura-2 AM) from Life Technologies. In brief, INS-1 832/13 cells were seeded in glass bottom 50 mm plates for 48 h and then treated as indicated. Prior to imaging, INS-1 cells were incubated at 37ºC and 5% CO_2 in 4 μM Fura-2 AM and 0.02% pluronic F127 (Life Technologies) for 1 h, and then washed and incubated with Hank's Balanced Salt Solution (HBSS, Life Technologies) supplemented with 0.1% BSA and 2.5mM $CaCl_2$. A small-volume chamber from Warner Instruments was mounted on the microscope stage; INS-1 cells were perfused with a gradient pump (Bio-Rad) and maintained at 37°C and 5% CO_2. To activate ryanodine receptors and reversibly empty ER Ca^{2+} stores, 10 mM caffeine in basal HBSS was employed. Fura-2 AM fluorescence was measured with excitation at 340 nm and 380 nm and emission at 510 nm, and images were captured using a Zeiss Z1 microscope with a 10x objective. Results were analyzed with Zen Blue software (Zeiss).

To measure intracellular Ca^{2+} mobilization, the FLIPR Calcium 6 Assay Kit (Molecular Devices) was used according to the manufacturer's instructions. In brief, INS-1 832/13 cells were plated at a density of 5000 cells/well in black wall /clear bottom 96 multi-well plates (Costar, Tewksbury, MA) and cultured for 2 days prior to treatment.

After ~22 h of treatment, Calcium 6 reagent was directly added to cells and incubated for an additional 2 h at 37 °C and 5% CO_2. Data acquisition on the FlexStation 3 system (Molecular Devices) was acquired at room temperature using a 1.52-sec reading interval throughout the experiments. To empty ER Ca^{2+} stores, caffeine dissolved in HBSS was injected into each well to achieve a final concentration of 10 mM and fluorescence was recorded for an additional 120 sec. The increase of intracellular Ca^{2+} was determined as the increase in Calcium 6 dye fluorescence. The ratio of Max-Min Ca^{2+} levels over basal Ca^{2+} levels were calculated as an indirect estimation of ER Ca^{2+} storage. In Ca^{2+} free situations, after 2h incubation with Calcium 6 reagent, media was substituted with HBSS without Ca^{2+} prior to data acquisition.

5.2.12 Fluorescence Lifetime Imaging Microscopy (FLIM) for ER Ca^{2+} imaging

A D4ER adenovirus described previously was used for direct analysis of ER Ca^{2+} levels (256). Briefly, INS-1 cells or dispersed islets cells were cultured for 18 h with the D4ER adenovirus. Cells were then allowed to recover for 24 h. FLIM was carried out in accordance with a protocol published previously. In brief, the Alba Fast FLIM system (ISS Inc., Champaign, IL) was coupled to an Olympus IX71 microscope using a 60 water-immersion lens (Olympus, Tokyo, Japan). Confocal scanning was controlled by Build 143 VistaVision software (ISS Inc.) at 530/43 nm acceptor and 480/40 nm receptor wavelengths. Regions of interest were selected with 10 count averages. The lifetime determination was obtained by analyzing the first 11–12 modulation frequencies (10–120 MHz). τα was used to calculate the average D4ER lifetime in different cells/regions.

5.2.13 Digital droplet PCR

To assess β cell death, droplet digital PCR was used to measure serum levels of unmethylated insulin DNA using the QX200 Droplet Digital PCR System from Bio-Rad Laboratories, as previously described (312).

5.2.14 Statistical analysis

Differences between groups were analyzed for statistical significant differences using unpaired Student's t test or one-way analysis of variance with a Tukey-Kramer post hoc test. Results are displayed as the means±S.E.M. GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was employed for data analysis, and a p value < 0.05 was taken to indicate the presence of a significant difference between groups.

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CURRICULUM VITAE

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EDUCATION

Doctor of Philosophy .....August 2016

Cellular & Integrative Physiology, Indiana University, Indianapolis, IN

Bachelor of Science..... June 2011

Molecular Biology and Biochemistry, China Agricultural University, Beijing, China

RESEARCH EXPERIENCES

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Indiana University school of Medicine (IUSM), Indianapolis, IN, USA

Project: Regulation of Endoplasmic Reticulum Calcium Homeostasis in Pancreatic β cells

- *Study of post-translational regulation of SERCA2 in pancreatic β cells*
- *Determine the mechanisms of glucose intolerance in SERCA2 deficiency mice under diabetogenic conditions*
- *Exploring the role of ER calcium homeostasis in regulating pancreatic β cell cycle*

Structural Biology and Biochemistry .....Jan. 2010 - Jul. 2011

Laboratory of Dr. Can Xie

Peking University, Beijing, China

Project: The structure and evolution of plant ethylene receptors

- *Improving protein expression and purification methods of plant ethylene receptors*

- *Purification, enzymatic and stability testing of HRV3C Protease*

Cancer Biology ..... Jul.2010 - Aug.2010

Laboratory of Dr. Tianyan Gao

Markey Cancer Center, University of Kentucky, Lexington, KY, USA

Project: The effect of PH domain Leucine-rich repeats Protein Phosphatase (PHLPP) on p70S6K

- *Analyzing dephosphorylation sites of p70S6K by PHLPP in vitro*
- *Building mutant PHLPP expression construct*
- *Examining the direct interaction of wildtype or mutant PHLPP with p70S6K*

Bioinformatics ..... Jul. 2010

Laboratory of Dr. Zhihua Liu

The Institute of Medicinal Plant Development, Beijing, China

Project: A molecular phylogenetic study of Oryzeae (Poaceae)

- *Extracting regions in 17 Oryzeae's chloroplast genome to build phylogenetic tree*
- *Basic alignment of the chloroplast genome sequences*

AWARDS AND FELLOWSHIPS

DeVault Fellowship.....2014-2016

Indiana University Diabetes and Obesity Research Training Program, DK064466

Mead Johnson Research Award for Endocrinology & Metabolism section.....2016

American Physiological Society, Experimental Biology conference

Outstanding Poster Presentation—3rd place.....2016

9th Annual Midwest Islet Club meeting

Graduate Student Travel Award, IUSM.....2015

Excellent Study Award and fellowship, China Agricultural University (CAU) 2009-2011

Excellent Student Award and fellowship, CAU.....2010

Hekang scholarship for excellent performance in academic and research, CAU 2009

PUBLICATIONS

1. **Xin Tong**, Tatsuyoshi Kono, Emily K. Anderson-Baucum, Wataru Yamamoto, Gary E. Schull and Carmella Evans-Molina, SERCA2 Deficiency Impairs Pancreatic β Cell Function in Response to Diet Induced Obesity, Diabetes, Accepted

2. **Xin Tong**, Tatsuyoshi Kono, Carmella Evans-Molina, Pancreatic β Cell SERCA2b protein stability is regulated via Nitric Oxide-and AMPK- dependent pathways under inflammatory diabetic conditions, Cell Death and Disease, 2015;6:e1790

3. Emily Sims, Alexander Lakhter, Emily Anderson-Baucum, Tatsuyoshi Kono, **Xin Tong**, and Carmella Evans-Molina, β Cell MicroRNA 21 Increases Apoptosis Via Inhibition of B Cell Lymphoma 2(BCL-2) Translation, Diabetologia, Submitted

4. Xiwen Xiong, Gaihong Wang, Rongya Tao, Pengfei Wu, Tatsuyoshi Kono, Kevin Li, Wen-Xing Ding, **Xin Tong**, Sarah A. Tersey, Robert A. Harris, Raghavendra G. Mirmira, Carmella Evans-Molina, X. Charlie, Sirtuin 6 regulates glucose-stimulated insulin secretion in mouse pancreatic β cells. Diabetologia, 2015;59(1):151-60

5. Emily Sims, Ivan Restrepo, **Xin Tong**, Tatsuyoshi Kono, Raghavendra Mirmira, Carmella Evans-Molina, β Cell Derived MicroRNA 21 Increases Apoptosis and Could Serve as a Biomarker of Type 1 Diabetes Mellitus, Diabetes (Conference paper), 2015;64:A35-A36

6. Justin S. Johnson, Tatsuyoshi Kono, **Xin Tong**, Wataru Yamamoto, Matthew J. Merrins, Leslie S. Satin, Patrick Gilon, Richard N. Day, and Carmella Evans-Molina, Pancreatic and Duodenal Homeobox Protein 1 (Pdx-1) Modulates Endoplasmic Reticulum Calcium Levels Through Transcriptional Regulation of Sarco-endoplasmic Reticulum Calcium ATPase 2b (SERCA2b) in the Islet β Cell, JBC, 2014

7. Tatsuyoshi Kono, Emily K. Sims, Dan R. Moss, Wataru Yamamoto, Geonyoung Ahn, Julie Diamond, **Xin Tong**, Kathleen H. Day, Paul R. Territo, Helmut Hanenberg, Dmitry O. Traktuev, Keith L. March, Carmella Evans-Molina, Human adipose derived stromal/stem cells (hASCs) protect against STZ-induced hyperglycemia; analysis of hASC-derived paracrine effectors. Stem Cells. 2014;32(7):1831-42.

8. Xu Zeng, Zhengrong Yuan, **Xin Tong**, Qiushi Li, Weiwei Gao, Minjian Qin, Zhihua Liu, Phylogenetic study of Oryzoideae species and related taxa of the Poaceae based on atpB-rbcL and ndhF DNA sequences, Molecular Biology Reports, 2011;39(5):5737-44

INVITED PRESENTATIONS

University of Texas Southwestern Medical Center ..... Jun. 2016

Division of Hypothalamic Research

Title: Regulation of Endoplasmic Reticulum Calcium Homeostasis in Pancreatic β Cells Mediated by SERCA2b

Indiana University School of Medicine ..... Apr. 2016

Center for Diabetes and Metabolic Diseases Seminar Series

Title: Regulation of Endoplasmic Reticulum Calcium Homeostasis in Pancreatic β Cells

The Institute of Medicinal Plant Development (IMPLAD), Beijing, China.....Dec. 2015

Novel Drug Development and Pharmacology seminar

Title: Exploring The Function of SERCA2b in Diabetic Pancreatic β Cells

Indiana University School of Medicine .....Nov. 2014

Center for Diabetes and Metabolic Diseases Seminar Series

Title: SERCA2b is Critical in Maintaining Pancreatic β Cell Function and Mass in Diet Induced Glucose Intolerance

Indiana University School of Medicine ..... May. 2014

Cellular and Integrative Physiology Department Student Seminar

Title: Exploring The Function of SERCA2b in Diabetic Pancreatic β cells

Peking University, Beijing, China Aug. 2013

Student Research Symposium

Title: SERCA(Sarco/endoplasmic reticulum Ca^{2+} ATPase) in Pancreatic β Cells—What do we know now?

ORAL AND ABSTRACTS PRESENTATIONS

Oral abstract presentation ..... Apr. 2016

Experimental Biology conference, San Diego, CA

Oral presentation ..... May. 2015

8th The Midwest Islets Club, Chicago, IL

Title: SERCA2b Plays A Critical Role in The Maintenance of Pancreatic β Cell Function and Mass in Response to Diet Induced Obesity

Guided Audio Poster Tour ..... Jun. 2014

74th scientific sessions of ADA, San Francisco, CA

Title: β Cell SERCA2b Protein Stability is Regulated Via NO- and AMPK-Dependent Pathways Under Inflammatory Diabetic Conditions

Oral presentation ..... Feb. 2014

4th Annual Meeting of the Indiana Physiological Society, Evansville, IN

Title: Post-transcriptional Regulation of SERCA2b Expression in The Pancreatic β Cell

Poster presentation

9th The Midwest Islets Club, Indianapolis, IN..... May. 2016

75th scientific sessions of American Diabetes Association, Boston, MA ..... Jun. 2015

5th Annual Meeting of the Indiana Physiological Society, Indianapolis, IN. Feb. 2015

7th The Midwest Islets Club, Birmingham, AL..... May. 2014

73rd scientific sessions of American Diabetes Association, Chicago, IL ..... Jun. 2013

6th The Midwest Islets Club, Ann Arbor, MI ..... May. 2013

3rd Annual Meeting of the Indiana Physiological Society, Indianapolis, IN. Feb. 2013

TEACHING AND SERVICE ACTIVITIES

Student Ambassador Volunteer ..... Jan. and Feb. 2016

IUSM graduate program campus recruits .....

Invited Lecturer for Endocrinology BIOL559 ..... Feb. and Oct. 2015

Biology Department, Indiana University Perdue University in Indianapolis (IUPUI)

Journal Club Coordinator..... Oct. 2015

IUSM,Center for Diabetes and Metabolic Diseases Seminar Series .....

Symposium Assistant Volunteer .....Dec. 2014

Chinese Academy of Medical Sciences, Diabetes Research Center.....

Student Mentor.....2013-2014

IUSM, Mentoring IBMG (Integrated BioMedical Gateway program) 1st year students

President of Student Organization.....Jun. 2013 –Jul. 2014

Foodies Guild in IUPUI

Hospitality Assistant Volunteer .....Jun. 2012 and Jun. 2013

National Championships & World Championship Trials, Indianapolis

PROFESSIONAL AFFILIATIONS AND CERTIFICATE

American Physiological Society ..... 2014 - present

Indiana Physiology Society ..... May 2012 - present

Executive Certificate in the Business of Life Sciences ..... May 2016

Indiana University Kelly School of Business