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REGULATION OF PANCREATIC β-CELL FUNCTION BY THE RENIN-ANGIOTENSIN SYSTEM IN TYPE 2 DIABETES

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REGULATION OF PANCREATIC β-CELL FUNCTION BY THE RENIN-ANGIOTENSIN SYSTEM IN TYPE 2 DIABETES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

REGULATION OF PANCREATIC β-CELL FUNCTION BY THE RENIN-ANGIOTENSIN SYSTEM IN TYPE 2 DIABETES

Diet-induced obesity promotes type 2 diabetes (T2D). Drugs that inhibit the renin-angiotensin system (RAS) have been demonstrated in clinical trials to decrease the onset of T2D. Previously, we demonstrated that mice made obese from chronic consumption of a high-fat (HF) diet have marked elevations in systemic concentrations of angiotensin II (AngII). Pancreatic islets have been reported to possess components of the renin-angiotensin system (RAS), including angiotensin type 1a receptors (AT1aR), the primary receptor for AngII, and angiotensin converting-enzyme 2 (ACE2), which negatively regulates the RAS by catabolizing AngII to angiotensin-(1-7) (Ang-(1-7)). These two opposing proteins have been implicated in the regulation of β-cell function. We hypothesized that the RAS contributes to the decline of β-cell function during the development of T2D with obesity. To test this hypothesis we first examined the effects of whole-body deficiency of ACE2 in mice on β-cell function *in vivo* and *in vitro* during the development of T2D. Whole-body deficiency of ACE2 resulted in impaired β-cell adaptation to insulin resistance with HF-feeding and a reduction of *in vivo* glucose-stimulated insulin secretion (GSIS) associated with reduced β-cell mass and proliferation. These results demonstrate that ACE2 plays a role in the adaptive response to hyperinsulinemia with obesity. In islets from HF-fed mice, AngII inhibited GSIS. In mice with pancreatic-specific deletion of AT1aR, AngII-induced inhibition of GSIS *in vitro* from islets of HF-fed mice was abolished. However, there was no effect of pancreatic AT1aR-deficiency on glucose homeostasis *in vivo* in HF-fed mice exhibiting pronounced hyperinsulinemia. Notably, pancreatic weight, insulin content and basal and glucose-stimulated insulin secretion from islets were decreased in mice with pancreatic AT1aR deficiency. These results suggest that AT1aR may contribute to pancreatic cell development, and also contribute to AngII-induced reductions in GSIS from islets of HF-fed mice. Overall, these studies suggest a role for the RAS in the regulation of β-cell function in T2D.

Keywords: Angiotensin II, angiotensin-converting enzyme 2, diabetes, β-cell, obesity

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ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; *Ace2*$^{+/y}$, angiotensin converting enzyme 2 wild-type littermate mice; *Ace2*$^{-/y}$, angiotensin converting enzyme 2-deficient mice; ADA, American Diabetes Association; ADP, adenosine triphosphate; AGE, advanced glycation end products; AGT, angiotensinogen; AKT, protein kinase B; Ang-(1-7), angiotensin-1-7; Ang-(1-9), angiotensin-1-9; AngA, angiotensin A; AngI, angiotensin I; AngII, angiotensin II; AngII, angiotensin 2-8; AngIV, angiotensin 3-8; ARB, angiotensin type 1 receptor blocker; AT1aR, angiotensin II type 1a receptor; *AT1aR*$^{fl/fl}$, angiotensin II 1a receptor floxed mice; *AT1aR*^{pdx}, pancreatic angiotensin II type 1a receptor-deficient mice; AT1bR, angiotensin II type 1b receptor; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; AT4R, angiotensin 4 receptor; ATP, adenosine triphosphate; AUC, area under the curve; BMI, body mass index; cAMP, cyclic adenosine monophosphate; CDC, Center for Disease Control; CDK, cyclin-dependent kinase; CoA, coenzyme A; CVD, cardiovascular disease; DAG, diacylglycerol; DIO, diet-induced obesity; DPP, Diabetes Prevention Program; DPP-4, dipeptidyl peptidase-4; DREAM, Diabetes Reduction Assessment with Ramipril and Rosiglizatone Medication; ER, endoplasmic reticulum; FBG, fasting blood glucose; FFA, free fatty acid; FLP, flippase; FPG, fasting plasma glucose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, glucokinase; GLP-1, glucagon-like peptide-1; GLUT2, type 2 glucose transporter; GLUT4, type 4 glucose transporter; GSIS, glucose-stimulated insulin secretion; HbA1C, fraction of glycated hemoglobin;

HETE, hydroxyeicosatetraenoic acids; HF, high fat; HNF, hepatic nuclear factor; HOPE, Heart Outcomes Prevention Evaluation; IAPP, islet amyloid polypeptide; IFG, impaired fasting glucose; IGF-1, insulin-like growth factor-1; IGT, impaired glucose tolerance; IL, interleukin; i.p, intraperitoneal; IP_3, inositol triphosphate; IRAP, insulin-regulated aminopeptidase receptor; IRS-2, insulin receptor subtrate-2; IUGR, intrauterine growth restriction; K^+_{ATP}, ATP-sensitive potassium channels; KCNJ11, potassium inwardly-rectifying channel, subfamily J, member 11; KLF11, Kruppel-like factor 11; LF, low fat; LO, lipoxygenases; Los, losartan; MasR, Mas receptor; MCP-1, Monocyte chemoattractant protein-1; MODY, maturity onset diabetes of youth; NAVIGATOR, Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research; NO, nitric oxide; NOD, new-onset diabetes; NZO, New Zealand Obese; OGTT, oral glucose tolerance test; $p21^{Cip1}$, cyclin-dependent kinase inhibitor 1; pAKT; phosphorylated protein kinase B; PCG1α, peroxisome proliferator-activated receptor γ coactivator 1-α; pdx-1, pancreatic and duodenal homeobox 1; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLA, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PPAR-γ, peroxisome proliferator-activated receptor-γ; PPR, (pro)renin receptor; RAS, renin-angiotensin system; RCT, randomized controlled trial; ROS, reactive oxygen species; S1P, sphingosine-1 phosphate; SEM, standard error of the mean; SGLT, sodium-glucose cotransporter; STZ, Streptozotocin; SubQ, subcutaneous; T2D, type 2 diabetes mellitus; TLR, toll-like receptor; TMEM27, transmembrane protein 27; TNF-α, tumor necrosis factor-α; TZD, thiazolidinediones; UCP2, uncoupling protein 2; UKPDS, United Kingdom

Prospective Diabetes Study; UPR, unfolded protein response; VEGF, vascular endothelial growth factor; Veh, vehicle; ZDF, Zucker diabetic fatty; ZF, Zucker fatty

Section I. BACKGROUND

1.1 Type 2 diabetes (T2D): Overview

Type 2 Diabetes (T2D) is a metabolic disorder characterized by hyperglycemia. The disease is manifest as a progressive worsening of glucose homeostasis over time as a result of insulin resistance and defects in pancreatic β-cell function. Largely as a result of the loss of normal glucose homeostasis, T2D is associated with long-term complications such as retinopathy, nephropathy, peripheral neuropathy, and cardiovascular disease (CVD) (264).

1.1.1 Diagnostic criteria

Clinical diagnosis of T2D can be made from any of three assessments of glycemia: fraction of glycated hemoglobin (HbA1C), fasting plasma glucose (FPG), or oral glucose tolerance test (OGTT). HbA1C is a measure of the fraction of hemoglobin that has been non-enzymatically glycated by exposure to glucose in the plasma. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin is increased correspondingly and the A1C reliably reflects the mean blood sugar concentration over a 2-3 month time period (159). Patients with A1C values between 5.7% and 6.4% are considered to be at-risk for developing diabetes, and values $\geq$ 6.5% marks a diagnosis of T2D (1).

FPG is indicative of hepatic glucose production and is measured in the morning following an 8 hour fast. Patients with a FPG between 100-125 mg/dL have impaired fasting glucose (IFG) and are at risk for developing T2D. A value greater than 126 mg/dL, confirmed by repeating the test on another day, is a positive diagnosis for overt T2D (1). The OGTT is performed by measuring

plasma glucose levels two hours after oral ingestion of 75 grams of glucose. Patients with plasma glucose between 140mg/dL and 199 mg/dL are said to have impaired glucose tolerance (IGT) and values $\geq$ 200 mg/dL meet the criteria for a diagnosis of T2D (1). Those patients who are at risk for developing T2D are said to have pre-diabetes.

1.1.2 Prevalence of T2D

The prevalence of diabetes in adults worldwide in 2010 was 6.4% and 439 million adults are predicted to have diabetes by 2030 (251). In 2012, 37% of the adult US population was estimated to have pre-diabetes, while 12.3% were estimated to have diabetes, and in persons over 65 years old the incidence increased to 25.9% (73). The incidence of diabetes is increasing; from 1990 to 2010, the annual number of new cases in the US almost tripled. The rise in incidence in T2D cases is associated with a rising prevalence of obesity, decreased physical activity, and the aging US population (199). According to the US 2012 Census Bureau, the population of people over 65 in the US is the largest in history (and expected to double by 2050). It is clear that diabetes is a public health epidemic – and a costly one; the estimated cost of diabetes in the US in 2012 by the Center for Disease Control (CDC) was $245 billion.

1.1.3 Causes of T2D

The development of T2D results from a combination of genetic and environmental factors. Evidence for a genetic component of T2D has come from studies in twins, first degree relatives of those with T2D, and epidemiological studies in which certain populations, such as the Pima Indians, have been found

to have an extremely high prevalence of T2D (19) (189). Several single gene mutations have been identified as contributing to T2D. For example, mutations in hepatic nuclear factor (HNF) 1α or 4α are associated with severe impairments in insulin secretion and characterize a form of T2D termed maturity onset diabetes of youth (MODY) (252) and T2D patients with mutations in the insulin-receptor gene exhibit extreme insulin resistance (273). However, monogenic forms comprise only a minority of T2D cases.

In general, the cause of T2D is polygenic and results from an interaction between genes and the environment. Gene association studies have identified polymorphisms in genes expressed in adipose tissue, skeletal muscle, and β-cells relating to glucose uptake and metabolism, insulin signaling, and insulin secretion that may confer increased risk to T2D (19). Slight variations affecting amino acid sequences, gene expression, or gene regulation can lead to an increased susceptibility of T2D, but are not associated with the dramatic phenotypes that are typical with monogenic cases (22). The frequency of such polymorphisms and the subsequent interaction with environmental factors such as age, body mass index (BMI), diet, and physical activity level can dramatically influence an individual's risk for developing T2D. The prevalence of obesity is strongly linked to the prevalence of T2D (197). Since most patients with T2D are obese (2), it is likely that obesity is the most influential environmental risk factor.

1.2 Pathogenesis of T2D: Natural history

A positive diagnosis for T2D is made when hyperglycemia becomes evident, but the pathogenesis of diabetes begins prior to rising glycemia. The

development of T2D occurs on a continuum over which pre-diabetes progresses to overt diabetes (Figure 1.1). Three basic metabolic defects contribute to the progression of T2D: insulin resistance, defects in insulin secretion, and increased hepatic glucose production (80). The effects of these factors are manifest during different stages but the combined effects contribute to disease progression throughout the continuum.

1.2.1 Pre-diabetes

Both cross-sectional and longitudinal studies indicate that, especially in obese patients, decreased peripheral sensitivity to insulin precedes deficits in insulin secretion in the pathogenesis of T2D (82). The prolonged period of onset, termed pre-diabetes, is characterized by insulin resistance, in which the skeletal muscle, liver, and adipose tissue become increasingly less sensitive to the actions of insulin (223). Insulin resistance is strongly associated with obesity, but is present even in lean patients with T2D (81). Reduced insulin action in skeletal muscle, normally responsible for the majority of peripheral glucose uptake, results in a significant impairment in overall glucose disposal (79). Insulin inhibits lipolysis in adipocytes, which plays an important role in glucose homeostasis. Regulation of lipolysis is disturbed when adipose tissues become insulin resistant which can lead to increased plasma levels of free fatty acids (FFA). This can augment hepatic glucose production, which is normally suppressed with postprandial decline of plasma FFA (32). Thus, the combined effects of insulin resistance markedly disturb glucose homeostasis.

Obesity is closely correlated with insulin resistance (227), which implicates obesity as a key etiological factor in the development of T2D. Plasma FFA concentrations are chronically increased with obesity, mainly due to expansion of fat mass (222). Fatty acids and their metabolites are thought to serve as signaling molecules that inhibit insulin signaling pathways (119). In addition, pro-inflammatory adipokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL) 6, can interfere with insulin signaling either directly or via activation of inflammatory mediators (297) (295).

1.2.2 β-cell compensation

As insulin sensitivity declines, a higher level of insulin is needed to achieve normal glucose homeostasis. A feedback loop exists between insulin-sensitive tissues and β-cells and variations in insulin sensitivity modulate insulin release (148). So long as insulin secretion is increased in proportion to the severity of insulin resistance, glucose homeostasis is maintained (Figure 1.2). Thus, the pre-diabetes phase is characterized by hyperinsulinemia but an individual may remain asymptomatic with respect to glycemia for some time. This adaptive response, termed β-cell compensation, is characterized by enhanced β-cell function and increased insulin release as well as expansion of β-cell mass (96).

1.2.2.1 Increased β-cell function

The major pathway for glucose stimulation of insulin release is triggered when rising blood glucose levels reach a certain threshold and glucose enters β-cells through type 2 glucose transporters (GLUT2) (Figure 1.3). Oxidative metabolism

of glucose results in an increase in the ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), followed by closure of ATP-sensitive K^+ (K^+_{ATP}) channels. This depolarizes the plasma membrane and facilitates opening of voltage-dependent Ca^{2+} channels, upon which Ca^{2+} stimulates exocytosis of insulin granules. Modulation of various aspects of this pathway contributes to β-cell adaptation to insulin resistance, including increased glucose metabolism and potentiation of insulin release by secretagogues and other signaling molecules (Figure 1.3). Evidence from human studies indicates that β-cell compensation is distinctly due to increased secretory capacity of β-cells, and not increased β-cell glucose sensitivity (148). However, animal studies suggest that increased glucose metabolism may contribute to enhanced β-cell function. Specifically, increased oxidation and utilization of glucose was observed in islets of insulin resistant Zucker fatty rats (179). An increase in the activity of the rate-limiting enzyme glucokinase (GK) was reported in islets isolated from hyperinsulinemic rats (56) and chronic activation of GK improved glucose tolerance in insulin resistant rats (303). The importance of GK is further emphasized by the fact that knockout of pancreatic GK in mice is lethal (118) and mutations in the GK gene are associated with MODY (125).

Increased stimulation of the insulin secretory pathway by FFA and gut peptides, called incretins, are thought to play a role in the adaptive β-cell response. FFAs amplify glucose-stimulated insulin release in several ways. FFA binding to the G-protein coupled receptor GP40 initiates cellular signaling leading to an increase in intracellular Ca^{2+}, which in turn facilitates insulin granule

exocytosis (140). Additionally, fatty-acyl coenzyme A (CoA), generated by intracellular metabolism of fatty acids, can facilitate granule exocytosis directly or via protein kinase C (PKC) stimulation of Ca^{2+} release (147). The incretin hormone glucagon-like peptide-1 (GLP-1) is produced in the intestinal mucosa and upon binding to the GLP-1 receptor, a G-protein coupled receptor expressed in β-cells, potentiates the insulin response to oral (versus intravenous) glucose administration (17). The effect of GLP-1 to increase glucose-stimulated insulin secretion was further augmented in obese, insulin resistant mice (10). An increase in both plasma GLP-1 and expression of GLP-1 receptor in the pancreas was observed in a canine model of hyperinsulinemic compensation for high-fat feeding, suggesting the importance of this peptide in the β-cell adaptive response (284).

Pancreatic islets are highly innervated by both sympathetic and parasympathetic neurons (5) and the insulin secretory pathway can be augmented by acetylcholine activation of the M2 receptor or increased activity of the β-adrenergic receptor (9). Evidence from animal studies suggests that increased parasympathetic activity contributes to β-cell adaptation to insulin resistance. Potentiation of insulin release with carbachol stimulation is augmented in obese, insulin resistant mice (8). Additionally, vagal hyperactivity as a result of ventromedial hypothalamic lesions caused hyperinsulinemia in rats, and was reversed with vagotomy (26).

1.2.2.2 Increased β-cell mass

In addition to enhanced β-cell function, the adaptive response to insulin resistance is mediated by expansion of β-cell mass. In humans, β-cell volume is positively correlated with weight (156) and β-cell mass is increased in obese versus lean non-diabetic individuals (45) (93). This is strongly supported by animal studies, in which obesity is associated with increased β-cell mass in Zucker fatty (ZF) rats (144) and Zucker diabetic fatty (ZDF) rats (213), *ob/ob* mice (31, 280), *db/db* mice (104) (292) and in rodents with diet-induced obesity (133) (209).

The majority of β-cell mass formation and expansion occurs in utero and neonatally (40). Although the postnatal β-cell mass is relatively stable, the β-cell population is dynamic and β-cell mass is a tightly regulated balance between formation of new cells (via proliferation and neogenesis) and apoptosis of old cells (35). Evidence from animal studies suggests that the adaptive response to obesity and insulin resistance is mediated by nutrient and/or mitogen modulation of cell cycle regulation and pro-survival pathways.

Glucose and lipids, which are increased in the bloodstream with obesity, may act as stimulants for β-cell mass expansion. In rats, short-term infusion of glucose (36) (281) (261) or lipids (261) results in a significant increase in β-cell replication and β-cell mass. Interestingly, compensatory β-cell mass expansion is impaired in obese mice with haploinsufficiency of β-cell specific GK (274). The effects of nutrients to modulate β-cell proliferation may be mediated in part through GLP-1. GLP-1, released in the presence of enteric nutrients, stimulates

the insulin secretory pathway but can also act as a mitogen and suppress apoptosis in pancreatic cells (42). Infusion of GLP-1, or an analog, has been shown to increase β-cell mass in normal or diabetic mice (262) (236) (152). In streptozotocin (STZ) -treated mice, administration of the GLP-1 receptor agonist, exendin-4, reduced β-cell apoptosis which was associated with improved glycemia (175).

In addition to GLP-1, other mitogens that facilitate β-cell mass expansion include both insulin and insulin growth-factor-1 (IGF). Insulin signaling in β-cells can activate Akt, resulting in regulation of β-cell proliferation through activation of cell cycle regulators such as cyclin D, cyclin-dependent kinase inhibitor 1 ($p21^{Cip1}$), and cyclin-dependent kinase (CDK)-4 (92). Activation of insulin-receptor substrate-2 (IRS-2) by insulin and IGF-1 has been identified as a critical factor for β-cell proliferation and survival (128). The effects of IRS-2 are mediated through activation of Akt and increased expression of pancreatic and duodenal homeobox 1 (pdx-1) (144). IRS-2 signaling is also stimulated by the GLP-1 receptor via the cyclic adenosine monophosphate (cAMP) response element protein (145). The importance of IRS-2 is underscored by the fact that deletion or disruption of the IRS-2 receptor in mice leads to eventual β-cell failure (162).

1.2.3 Progression to overt T2D

Insulin resistance is the initiating pathogenic factor in the development of T2D, but failure of β-cells to maintain compensation to insulin resistance ultimately determines the onset of T2D. The transition from pre-diabetes to overt

T2D is marked by the appearance of mild post-prandial hyperglycemia, or IGT. Declining β-cell function leads to a relative insulin deficiency and both postprandial and fasting blood glucose begin to rise. However, the timeframe over which pre-diabetes progresses to overt T2D varies widely and in some insulin resistant individuals, β-cell compensation is maintained indefinitely.

According to data from clinical trials, approximately one-third of patients with IGT will progress to T2D (223). The identification of a population in which this shift is most likely to occur is therefore of great interest. Data from longitudinal studies indicate that in those destined to progress to overt T2D, β-cell abnormalities exist prior to the emergence of hyperglycemia. The earliest functional defect of β-cells is thought to be a decrease in the acute insulin response to glucose (also called first-phase insulin secretion). Among Pima Indians, those who progressed from normal glucose tolerance to IGT to T2D exhibited progressive decreases in the acute insulin response compared to nonprogressors (301). Other studies have similarly reported that changes in acute insulin response in follow-up visits were indicative of both glucose tolerance status and progression to T2D. Compensation to insulin resistance was associated with an increase in the acute insulin secretion, whereas unchanged or decreasing acute insulin secretion was associated with IGT or overt T2D (193) (95). These data indicate that declining insulin response to glucose is an early indicator of β-cell dysfunction.

Following the initial appearance of impaired glucose homeostasis, progression from pre-diabetes to diabetes is marked by progressive

hyperglycemia. In patients with IGT, conversion to T2D is associated with marked increases in fasting blood glucose (FBG) in follow-up visits (93). Also, the degree of abnormal fasting glucose is correlated with the rate of progression. In one study, 8.1% of subjects whose fasting glucose levels were between 100 and 109 mg/dL developed diabetes over an average of 29 months; of those with fasting glucose values between 110-124 mg/dL the percentage increased to 24.3% (201). The presence of other risk factors, such as family history, dyslipidemia, high blood pressure, and obesity greatly influences the rate of disease progression.

Following the onset of overt T2D, the disease is characterized by declining insulin secretion. Hyperglycemia is exacerbated by inadequate suppression of hepatic glucose production and further impairment of glucose uptake in insulin resistant tissues (78). The progressive loss of both β-cell function and mass contributes to the loss of glycemic control over time and the eventual need for glucose-controlling medications.

1.2.4 Factors leading to β-cell failure

At the time of diagnosis of T2D, β-cell function is 25% or less of functional capacity (234). In humans with T2D, intravenous infusion of glucose does not result in a rapid release of insulin, despite the fact that the β-cells do contain insulin (234). Observations in rodent models of T2D are consistent with those in humans, in which glucose-stimulated insulin secretion is reduced both *in vivo* (7) and in isolated islets (15). Impaired insulin secretion is further characterized by a reduction in first phase insulin release, as referenced above, as well as

abnormal pulsatile insulin release, and an increase in the circulating molar ratio of proinsulin to insulin (163).

While changes in β-cell function during the progression to T2D are fairly well characterized, far less is known about β-cell mass in patients with T2D due to the difficulty in obtaining samples. In 2003, a landmark study using autopsy pancreata reported that β-cell volume was reduced by 40% and 63% in obese patients with IFG and T2D, respectively, and by 41% in lean type 2 diabetics compared to non-diabetic controls (45). Further, the frequency of β-cell apoptosis was increased in patients with T2D (10-fold in lean and 3-fold in obese), but there was no difference in β-cell proliferation or neogenesis with T2D compared to non-diabetic controls. It was concluded that β-cell mass is decreased with T2D, and that the cause is increased β-cell death (rather than failure to compensate).

In a post-hoc analysis, a curvilinear relationship between β-cell volume and FBG was reported in obese subjects with T2D or IFG in which a steep increase in FBG was observed over a narrow window of β-cell volume (230). This is consistent with findings from other studies in which progression of IGT to T2D often occurred rather rapidly. This suggests that there exists a tipping point or threshold at which β-cells collectively become overwhelmed by conspiring forces. The mechanisms leading to β-cell failure in humans have not been fully elucidated, but many factors that promote initial β-cell defects also contribute to β-cell failure and eventual β-cell death. These defects have both genetic and acquired aspects and are confounded by environmental factors, such as obesity.

β-cell failure is not caused by a single factor, but occurs due to cumulative effects of prolonged β-cell dysfunction in the face of genetic susceptibilities (Figure 1.4).

Factors initiating β-cell dysfunction include impaired nutrient-secretion coupling, mitochondrial dysfunction, and β-cell exhaustion. Increased carbohydrate and fat intake and subsequent nutrient availability initially serve to instigate β-cell compensation, but over time are also implicated in leading to acquired defects in β-cell function. Prolonged exposure of β-cells to high concentrations of glucose can lead to impaired insulin gene transcription, subsequently reducing insulin secretion and can also deplete insulin granule stores (233). Increased mitochondrial oxidation of glucose and FFA can lead to increased production of superoxide and upregulation of uncoupling protein 2 (UCP2) (160). The uncoupling of oxidative phosphorylation has important consequences on ATP production, and therefore insulin secretion. Systemic and pancreatic concentrations of reactive oxygen species (ROS) are increased with hyperglycemia (232) (136) and ROS have been shown to both impair insulin gene transcription (231) and interfere with the insulin secretory machinery (111).

Short-term exposure to FFAs stimulates insulin secretion, but, as with glucose, chronically elevated levels of FFA may be detrimental to β-cell function. Lipid infusion into rats resulted in an increase in basal insulin release and a decrease in pancreatic insulin content, suggesting that elevated FFAs lead to depletion of insulin stores (33). It has also been suggested that elevated FFA is more detrimental to β-cell function with concomitant hyperglycemia due to dysregulation of glucose and fatty acid oxidation. Via the Randle cycle, glucose

metabolism, and thus glucose-stimulated insulin secretion, could be impaired (81). Since malonyl-CoA levels are also high if glucose is abundant, inhibition of FFA oxidation results in accumulation of FFA-derived acyl-CoA esters in the cytoplasm. This pool may serve to stimulate insulin release (217), and this is likely to occur during the earlier phase of β-cell compensation. However, acyl-CoA esters may contribute to formation of inflammatory signaling molecules like ceramide, whose cumulative downstream effects on insulin secretion are inhibitory (255).

Both chronic demand and waning production of insulin may lead to the depletion of β-cell insulin stores. The theory that the imbalance of insulin need with production is a factor contributing to impaired insulin secretion is termed β-cell exhaustion. This theory is supported by studies in which “resting” of β-cells in type 2 diabetics results in increased stimulatory response (117) and improved first phase insulin secretion and reduced molar ratio of proinsulin to insulin in type 2 diabetics (163). Inadequate production of insulin is thought to be a consequence of endoplasmic reticulum (ER) dysfunction. Chronic demand, resulting in ER stress, may lead to an increase in misfolded proinsulin (14). Initiation of the unfolded protein response (UPR) resulting in delayed translation may contribute to the reduction in insulin secretion. Long-term effects of ER stress may eventually be deleterious to β-cell viability in the later stages of T2D.

Chronic metabolic stress and prolonged β-cell dysfunction precipitate β-cell failure through purported mechanisms such as gluco- and lipotoxicity, inflammation, islet fibrosis, ER stress, and amyloid deposition. Persistent

exposure of human and rodent islets to high concentrations of glucose and saturated fatty acids are shown to have toxic effects, although the variability is large depending on concentration, species and culture conditions (87). The mechanisms by which high levels of glucose and saturated fatty acids lead to apoptosis are likely due to the initiation of an inflammatory response. Glucose induces β-cell production of the cytokine IL-1β and the FAS receptor in islets, leading to apoptosis of β-cells (186). Chronic hyperglycemia can lead to the formation of advanced glycation end products (AGE) which can cause tissue damage through activation of inflammatory mediators (196).

Fatty acid activation of toll-like receptors (TLR) in β-cells results in the production of cytokines, including IL-1β (34). Thus, the effects of IL-1β may be exacerbated due to induction via glucose and fatty acids. Further, other TLR agonists, such as certain gut flora present in obese humans and rodents may potentiate the production of IL-1β (86). Certain active lipids can promote inflammatory damage directly. Various hydroxyeicosatetraenoic acids (HETEs), produced by lipoxygenases (LO) expressed in β-cells are deleterious to both islet function and viability (184). Conversely, inhibition of the effects of ceramide by sphingosine-1 phosphate (S1P) both enhances glucose-stimulated insulin secretion (GSIS) (47) and protects β-cells from cytokine-induced apoptosis (168) in rodent studies.

In addition to initiation of apoptosis, inflammation may mediate β-cell failure by causing islet fibrosis. It is well known that organ fibrosis is a hallmark of chronic inflammatory diseases and leads to impaired tissue architecture and

function. In both humans with T2D and animal models, pancreatic tissue sections show marked fibrosis associated with loss of β-cell function (153) (158) (277). Disruption of islet architecture leading to impaired insulin secretion and loss of β-cells may also result from amyloid deposits.

Islet amyloid deposits are considered a pathological hallmark of T2D and are composed of fibrils containing aggregated islet amyloid polypeptide (IAPP, or amylin) (134). IAPP is a 37 amino acid peptide that is co-secreted in equimolar amounts with insulin during exocytosis from β-cells, and is inhibitory on both insulin and glucagon secretion (298). The role of IAPP, both in normal physiology and in β-cell failure is not very well understood. In post-mortem immunohistochemical analysis of pancreatic sections, significant amyloid deposits were identified in patients with T2D, corresponding with disrupted islet architecture, whereas no amyloid deposits were found in control subjects (64). A growing body of literature suggests that the role of IAPP in β-cell failure extends beyond structural disruption and is cytotoxic. IAPP fibrils or small oligomeric aggregates have been shown to be toxic to human and rat islets *in vitro* (180) and this is consistent with human studies in which amyloid deposition is associated with reduced β-cell mass (134). However, mechanistic insight is limited because pathological deposition of islet amyloid does not occur in the rodents, which are commonly used in diabetes research (299). IAPP aggregates have been shown to disrupt cell membranes (143), and it has been suggested that IAPP aggregates crowd the ER membrane, facilitating ER stress and dysfunction (83).

Induction of the UPR due to ER stress may initially result in reduced insulin biosynthesis, but chronic activation of the ER stress response has far more detrimental effects. Chronic ER stress promotes apoptosis via the UPR-related transcription factor CHOP, and CHOP deletion improves β-cell mass and glycemic controls in mouse models of T2D (257). In addition, ER stress has been shown to activate IL-1β independently of the UPR *in vitro* (194).

It is clear that individual factors contributing to β-cell damage are compounded to create an effect that severely impacts β-cell sustainability. Slight genetic susceptibilities can dramatically influence the tipping point at which β-cell function can no longer be maintained. In humans, several polymorphisms in genes that have varying roles in regulation of adipocyte function, cellular energy metabolism, and insulin secretion and gene expression have been associated with increased risk for T2D. Notable genes include peroxisome proliferator-activated receptor gamma (PPAR-γ), PPAR-γ coactivator 1-α (PCG1α), transcription factor Kruppel-like factor 11 (KLF11), and potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11) (218). Polymorphisms in genes whose function contribute to metabolic adaptation, coupled with the metabolic pressures of obesity, lead to increased risk for T2D.

1.3 Mouse models of T2D

Several mouse models of T2D are routinely used in basic science research, but none exactly mimic the human condition due to the complex nature of the pathogenesis of diabetes. Mouse models for T2D typically involve the development of obesity, either through diet or due to genetic manipulation.

1.3.1 Genetic mouse models

The most widely used genetic mouse models are *ob/ob* and *db/db* mice, which lack the genes for leptin and the leptin receptor, respectively. Although monogenetic forms of T2D represent the minority in humans, these strains are useful in research because they develop robust obesity, which reflects the human condition in which obesity is closely related to the development of T2D. In a 1978 publication by Coleman of The Jackson Laboratory (70), the phenotypes of these two mouse models are described. Both strains develop insulin resistance, hyperinsulinemia, and impaired glucose homeostasis within the first month of age. However, the two strains display a marked phenotypic difference in β-cell function. *Ob/ob* mice exhibit rather robust β-cell compensation, characterized by increased β-cell mass and sustained insulin secretion. In contrast, compensation to insulin resistance is not maintained in *db/db* mice. By 3-4 months of age, *db/db* mice exhibit progressive loss of β-cell function and β-cell mass, eventually manifest as hypoinsulinemia and hyperglycemia. The mice develop ketosis and die by 8-10 months of age. Use of the *db/db* mouse is therefore suitable for research focused on β-cell failure, while *ob/ob* mice are better suited for studies targeting insulin resistance.

Since the etiology of T2D in humans is most polygenetic, use of polygenetic strains, such as KK, New Zealand Obese (NZO), and TallyHo, has become more widespread. These strains develop obesity and display varying degrees of glucose intolerance and β-cell dysfunction (154). Polygenetic models of T2D have been used in studies investigating the interplay of obesity and T2D or to

study diabetic complications. However, a major drawback of these types of models is the lack of a wild-type control.

1.3.2 Diet-induced obesity

Diet-induced obesity (DIO) in the absence of genetic modifications is widely used to identify the environmental effects of obesity on the development of T2D. DIO, first described in 1988 in C57BL/6 mice, leads to robust obesity accompanied by insulin resistance, hyperinsulinemia, and impaired glucose homeostasis (266). DIO is also referred to as high-fat (HF) feeding because diets are comprised of a very large percentage of calories that are derived from fat (45-60%, compared to 15-20% as in standard rodent diets). In addition, diets are usually high in simple sugars, such as maltodextrin and/or sucrose, and sometimes contain added cholesterol to mimic diets that lead to obesity in humans. DIO is often used in combination with a specific gene knockout in order to evoke the emergence of a phenotype in response to metabolic challenge. This approach is useful for the identification of candidate genes that cause susceptibility to T2D in humans. However, consideration of the background strain of the mouse is important since there can be wide variations in response to DIO between mouse strains.

DIO in C57BL/6 mice is widely used in studies of T2D and other metabolic disorders. Obese C57BL/6 mice become markedly insulin resistant and glucose intolerant compared to non-obese controls. This strain also demonstrates a robust capacity for compensation to diet-induced insulin resistance as evidenced by increased β-cell mass and islet insulin content (71) and hyperinsulinemia that

is sustained through at least 10 months on diet (7). No evidence has been published suggesting that obese C57BL/6 mice exhibit β-cell failure, although HF-feeding has been shown to reduce the acute secretory response to glucose both *in vitro* and *in vivo* (7), (212).

C57BL/6 mice are said to be moderately prone to diet-induced diabetes compared to other strains. HF-fed C57BL/6 mice exhibit intermediate phenotypes with respect to GSIS *in vitro* and *in vivo*, as well as an intermediate phenotype for the development of glucose intolerance and insulin resistance compared to DBA, FVB, and 129 strains (23). The C57NLKS/J strain, a variation of C57BL/6, has a greater susceptibility to β-cell failure, especially when crossed onto the *db/db* strain (66). The A/J strain is considered to be "diabetes-resistant" in that HF-feeding is not associated with the development of insulin resistance and glucose intolerance (66).

1.4 Therapies for T2D

The overall objective for treatment of T2D depends on the level of disease progression. Randomized controlled trials (RCTs) have shown that in individuals with pre-diabetes, the rate of T2D onset can be significantly decreased with particular interventions (264). The Diabetes Prevention Program (DPP) was a major clinical research study in the US in which lifestyle intervention or treatment with the diabetes drug metformin could prevent or delay the onset of T2D in overweight persons with pre-diabetes. Intensive counseling and a modest reduction of body weight through dietary changes coupled with increased physical activity was shown to reduce the risk of developing diabetes by 58%,

and metformin decreased risk by 31% (157). Follow-up from this and other studies indicate that lifestyle modification is cost-effective and that risk reduction associated with weight loss is sustained over a number of years. Since β-cell failure is degenerative, the first line of treatment for T2D is prevention. However, despite preventative therapies, many patients still progress to T2D. Moreover, effectiveness of lifestyle interventions to delay or prevent the onset of T2D is limited since both program compliance and maintenance following the interventions are notoriously difficult to achieve.

Following diagnosis, the primary treatment objective is glycemic control, achieved using pharmacological therapy in addition to healthy eating, weight control, and increased physical activity. Mono-drug therapy with metformin is the preferred initial pharmacological agent per the American Diabetes Association (ADA) but glycemic control often requires two- or three-drug combinations. Metformin improves glycemia by suppressing hepatic glucose output and it also improves insulin sensitivity (110). Metformin poses a low risk for hypoglycemia and for weight gain, however it has no effect to improve β-cell function. Thiazolidinediones (TZDs), such as pioglitazone and rosiglitazone, are also effective insulin sensitizers. TZDs act by agonizing PPAR-γ to improve insulin sensitivity (289) and have a low risk for hypoglycemia, but may cause weight gain and fluid retention (264). However, in addition to acting as effective insulin sensitizers, TZDs may preserve β-cell function and morphology (108).

Some diabetes medications lower blood glucose by acting as insulin secretagogues. Sulfonylureas stimulate insulin release from β-cells and work by

binding to β-cell K_{ATP} channels and facilitating membrane depolarization (3). These drugs, however, do promote weight gain and some studies suggest they may be deleterious to long-term β-cell viability (96). GLP-1 mimetics or GLP-1 receptor agonists are emerging as a promising class of drugs for the treatment of T2D. These drugs stimulate post-prandial insulin release, inhibit glucagon secretion and delay gastric emptying. In addition to being low risk for hypoglycemia, these drugs may beneficially augment β-cell function. A related class of drugs are those that prevent the cleavage of GLP-1, called dipeptidyl peptidase-4 (DPP-4) inhibitors, which thereby promote the beneficial effects of GLP-1 on β-cell function (90).

Other anti-hyperglycemic drugs include α-glucosidase inhibitors and sodium-glucose cotransporter (SGLT)-2 inhibitors. α-glucosidase inhibitors inhibit carbohydrate absorption through competitive inhibition of carbohydrate digestive enzymes, and have recently been shown to augment incretin secretion (316). SGLT-2 inhibitors reduce hyperglycemia by increasing urinary glucose excretion independently of insulin secretion or action (75). Despite the variety of pharmacological treatment options available, most clinical trials indicate that loss of glycemic control over time is inevitable, due to the progressive nature of β-cell failure, and insulin therapy is eventually needed for many patients with T2D (96).

1.5 T2D: Link to the renin-angiotensin system (RAS)

Patients with T2D are at an increased risk for CVD morbidities and mortalities. According to the CDC, diabetes is an independent risk factor for CVD and among individuals with diabetes, the risk for stroke and other

cardiovascular events is doubled. Further, CVD is the leading cause of death in diabetics. Pre-diabetes or T2D is often concomitant with CVD risk factors, such as obesity, hypertension, and dyslipidemia (264). Interestingly, all of these conditions have been associated with increased activity of the renin-angiotensin system (RAS).

1.5.1 Overview of the RAS

The RAS is an endocrine system classically associated with systemic regulation of blood pressure and fluid homeostasis mediated by various angiotensin peptides. An overview of the components of the RAS is depicted in Figure 1.5. Activation of the RAS occurs when the renal-derived protease, renin, cleaves angiotensinogen (AGT), a large, primarily hepatic-derived protein, to generate the biologically inactive decapeptide angiotensin I (AngI). Hydrolysis of AngI by the lung-derived carboxy-dipeptidase angiotensin-converting enzyme (ACE) results in the formation of angiotensin II (AngII). AngII, considered to be the main bioactive peptide of the RAS, exerts its effects through the two G-protein coupled angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors.

In addition to AngII, several other peptides can be generated that have biological activity. AngII can be catabolized by aminopeptidases to generate angiotensin 2-8 (AngIII) and angiotensin 3-8 (AngIV) (161) and by decarboxylation to generate angiotensin A (AngA) (142). AngIII and AngA have been demonstrated to have pressor responses capable of affecting blood pressure similar to AngII (226) (142) (308). AngIV, which binds to the angiotensin II type 4 receptor (AT4R), also known as the insulin-regulated

aminopeptidase receptor (IRAP), is thought to play a role in facilitation of learning and memory (53). Interestingly, IRAP has been shown to play a role in the trafficking of glucose transporter type 4 (GLUT4) vesicles, although the role of IRAP in glucose uptake in insulin-sensitive tissues has not been well defined (293).

1.5.1.1 Angiotensin II (AngII) receptors

The AT1R and AT2R share 34% homology at the protein level and AngII binds to each with similar affinity (206). However, the tissue distribution and expression density of AT1R is diverse, while that of AT2R is limited (and higher during development). The majority of the well-known cardiovascular effects of AngII are mediated by AT1R. These include vasoconstriction of vascular smooth muscle, stimulation of aldosterone release from the adrenal cortex, and activation of the sympathetic nervous system. These actions of AngII contribute to its ability to increase blood pressure and fluid volume, but AngII also acts at AT1R to stimulate the release of prostaglandins, inflammatory mediators, and ROS, and is involved in cellular growth and proliferation.

Rodents express two subtypes of the AT1R, AT1a and AT1b, which share 94% homology (126). The rodent AT1aR subtype is thought to be most similar to the human AT1R based on tissue distribution and physiologic effects. Rat AT1aR shares 95% homology with human AT1R (24) and the tissue distribution of AT1R in humans and rodents is similar. In humans and rodents, AT1R/AT1aR expression includes kidney, lung, liver, gonads, adrenal gland, brain, adipose tissue, and vascular smooth muscle (44) (52) (85). Rodent AT1bR distribution is

more limited and includes kidney, testes, adrenal and anterior pituitary glands, and mesenteric resistance vessels (44) (85) (318). In rodents, deletion of AT1aR is associated with reduced blood pressure, which is consistent with pharmacological blockade of AT1R in humans (139). Deletion of AT1bR in rodents has no effect on blood pressure (57).

The functional role of the AT2R is not well understood. The expression of AT2R is high in fetal tissues but declines prior to birth, suggesting a role for AT2R during fetal development, (115). However, growth and development are not impaired in AT2R-knockout mice although the drinking response is decreased and the vasopressor response to AngII is increased (126) (135). Post-natal expression of AT2R has been demonstrated in brain, heart, adrenal medulla, kidney, and reproductive tissues (282). Although AT2R knockout animals lack a strong phenotype, exposure to disease models tends to result in a more severe response suggesting that AT2R may function in a protective manner (290) (265). Additionally, AT2R stimulation has been shown to promote neuronal differentiation and regeneration after injury (182).

The signal transduction pathways and subsequent physiological effects of the angiotensin receptors differ markedly. AT1R (including AT1aR and AT1bR subtypes) is antagonized by biphenylimidazoles, such as losartan, candesartan, and valsartan, which selectively bind the AT1R with high affinity (43). These drugs, also called angiotensin type 1 receptor blockers (ARBs) are used clinically to inhibit the effects of AngII on blood pressure and fluid retention. In contrast,

tetrahydroimidazolpyridines, such as PD123319 and PD123177, selectively antagonize the AT2R (85).

The most well-described signal transduction pathway for the classical effects of AT1R includes $G_{q/11}$ coupled activation of phospholipase C (PLC) with subsequent inositol triphosphate (IP_3)-mediated release of intracellular calcium stores leading to vasoconstriction in vascular smooth muscle cells (210). Vasoconstriction is also mediated by PLC via diacylglycerol (DAG), by G_i-mediated inhibition of adenylate cyclase, and by facilitating opening of cAMP-L-type calcium channels (85). G_q coupling also simulates prostaglandin release via activation of phospholipase A_2 (PLA) and phospholipase D (PLD), and mediates growth and proliferation via mitogen-activated kinases (85). Many of the effects of AT2R are thought to be contrary to that of AT1R. These include vasodilation via stimulation of nitric oxide (NO) production (72) and inhibition of proliferation, (263) possibly via G-coupled protein-mediated activation of phosphatases (85).

1.5.1.2 Angiotensin-converting enzyme 2 (ACE2)

In 2000 two groups described a newly discovered component of the RAS, a carboxy-peptidase called angiotensin converting enzyme 2 (ACE2) that shares 42% sequence homology with ACE (88) (278). ACE2 is a zinc metalloprotease that cleaves the c-terminal amino acid from AngII to generate the heptapeptide angiotensin 1-7 (Ang-(1-7)) (278). The effects of Ang-(1-7) are mediated by the Mas receptor (MasR) (240), and are generally thought to oppose that of AngII (94). ACE2 can also hydrolyze AngI, although with less affinity than for AngII, to

generate angiotensin 1-9 (Ang-(1-9)) (288) with subsequent hydrolysis by ACE resulting in Ang-(1-7) (296). Also shown to have effects that oppose AngII is the newly characterized peptide alamandine, which is generated either directly from Ang-(1-7) or by hydrolysis of AngA by ACE2 (167). The discovery of ACE2 and the characterization of the ACE2/Ang-(1-7)/MasR axis has led to the concept that the RAS has two active arms whose effects oppose each other, with ACE2 acting as a negative physiological regulator.

1.5.2 Pharmacologic inhibition of the RAS: Effect on new-onset diabetes (NOD)

Pharmacological inhibition of the RAS is achieved either by preventing the formation of AngII with either renin or ACE inhibitors, or blocking the effects of AngII with ARBs. Drugs that inhibit the RAS are first-line treatments for hypertension. Over the last decade, findings from RCTs support a benefit of ACE inhibitors or ARBs to improve cardiovascular outcomes beyond that of blood pressure control in both diabetic and non-diabetic patients (313) (76) (97). In addition to improved cardiovascular protection, some studies suggest that ACE inhibitors or ARBs may protect against the development of T2D (Table 1.1).

One of the first studies to report a beneficial effect of RAS inhibition on development of diabetes was the Heart Outcomes Prevention Evaluation (HOPE) study. In patients at high risk for cardiovascular events, the ACE inhibitor ramipril was associated with reduced rates of death, myocardial infarction, and stroke compared to placebo; as a secondary outcome ramipril was associated with fewer cases of new onset diabetes (NOD) (313). A post-hoc analysis was

published the following year reporting a 34% reduction in risk of developing NOD in patients treated with ramipril versus placebo (312). Similar findings were reported in several other RCTs in which the efficacy of ACE inhibitors or ARBS to improve cardiovascular outcomes was the primary endpoint. However, results are mixed as some trials reported no effect of RAS inhibition to reduce the incidence of T2D. Further, few RCTs have been done in which prevention or delay of NOD with use of ACE inhibitors or ARBs was a primary endpoint.

The Diabetes Reduction Assessment with Ramipril and Rosiglizatone Medication (DREAM) study was designed on the heels of the HOPE trial to evaluate whether ramipril (and, separately, rosiglizatone) reduces the risk of diabetes in people with IFG or IGT but who are at low risk for CVD. Results of the study were disappointing in that ramipril was shown to have no significant effect versus placebo on the incidence of NOD; a moderate improvement of glycemia with ramipril treatment was the only positive finding after three years of follow-up (37). Roziglitazone, however, was shown to substantially reduce the incidence of NOD (109).

The Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) study was the second RCT in which diabetes was the primary outcome. Patients displaying IGT and one or more cardiovascular risk factors were given valsartan or placebo (and separately, nateglinide or placebo) with concomitant lifestyle modification and follow-up occurred over a mean of 5 years. Valsartan was shown to have a 14% reduction in risk of developing NOD compared to placebo (192) and nateglinide did not reduce the incidence of NOD

(192). Results from this study with respect to valsartan's efficacy to reduce NOD were positive but modest. The actual effects of valsartan are difficult to interpret since lifestyle modification was a goal for both groups and concomitant use of additional ACE inhibitors and ARBs at the last visit was significantly greater in the placebo group.

1.5.3 RAS and glucose homeostasis

The underlying mechanisms linking the RAS to diabetes are incompletely understood, but evidence from human and animal studies supports that activation of the RAS is associated with insulin resistance and β-cell dysfunction. Components of the RAS, including those required for the synthesis of AngII, have been identified in a myriad of tissue types including heart, brain, kidney, skeletal muscle, adipose tissue, and pancreas. This has given rise to the concept of a local tissue RAS that may play a role in normal organ function and AngII has been shown to have paracrine or autocrine effects resulting in the promotion of inflammation, fibrosis, oxidative stress, cell proliferation, and apoptosis (220). Further, activation of the RAS has been implicated in organ dysfunction and changes in organ structure in a variety of diseases, including diabetes, independent of blood pressure. Local AngII production has been shown to induce blood-pressure independent cardiac hypertrophy (191) and overactivity of the renal RAS contributes to fibrosis and end-organ damage (286). The pro-fibrotic and pro-inflammatory actions of AngII have been linked to the macrovascular complications of diabetes (113) as well as diabetic nephropathy (317).

1.5.3.1 The RAS and insulin sensitivity

Studies in humans, animals, and cells support a role for AngII in the development of insulin resistance through impaired function of both skeletal muscle and adipose tissue. Early studies implicating the RAS in impaired insulin resistance were focused on the hemodynamic effects of AngII. In animals, pressor doses of AngII increased insulin resistance and decreased glucose uptake (229) (205). Conversely, in human studies improvements in glucose disposal and insulin sensitivity with ACE inhibitors were attributed to a reduction in the vasoconstrictive effects of AngII (198) (208) (268). More recently, AngII has been implicated in impaired insulin sensitivity and reduced glucose disposal through direct inhibition of insulin signaling in skeletal muscle (129) (256) (129). Several studies suggest that AngII-mediated insulin resistance is due to AngII activation of NADPH oxidase and the production of ROS (30) (165) which inhibits phosphatidylinositol 3-kinase (PI3K) recruitment of GLUT4 (294).

1.5.3.2 Obesity and the RAS

Rodent and human adipose tissue expresses all the components of the RAS required for the synthesis of AngII (Table 1.2). This local RAS is upregulated with obesity (39) and may contribute to adipose dysfunction. Both increased production of adipokines and impaired adipocyte differentiation leading to adipocyte hypertrophy are thought to be deleterious to insulin sensitivity. The role of the RAS on adipocyte differentiation remains controversial. It has been postulated that AngII has an inhibitory effect on lipolysis and adipogenesis via AT1R (114) (100) while other studies report AngII enhances adipocyte

differentiation (239) and is AT1R-mediated (244). Interestingly, in a mouse model of adipocyte-specific deficiency of AT1aR, deletion of AT1aR had no effect on adipocyte differentiation or lipolysis in obese mice, and did not improve glucose tolerance, but was associated with reduced differentiation and increased adipocyte hypertrophy in lean mice (220). In rodents, improved insulin sensitivity with ARB treatment was associated with increased adipocyte differentiation as well as reduced inflammatory markers in adipose tissue and increased gene expression of adiponectin and PPARγ (169). It is possible that the differential effects of genetic versus pharmacological inhibition of the AT1R may be attributed to the fact that some ARBs have PPAR-γ agonistic effects.

Perhaps more impactful on the development of diabetes than possible effects of local adipose RAS on insulin sensitivity is the fact that obesity is associated with an increase in the systemic RAS. In obese rodents, systemic levels of AGT and angiotensin peptides (including AngII) are increased, corresponding with the development of hypertension (39) (121). Further, adipocyte-specific deficiency of AGT ablated obesity-hypertension and was associated with a reduction in circulating AngII (309). These results suggest that adipose tissue is a major source of systemic AngII in obesity. This has significant implications in the role of the RAS to promote the development of T2D given the association between obesity and T2D.

1.5.4 A tissue pancreatic RAS

The endocrine pancreas expresses several components of the RAS (Table 1.2), including AT1R, suggesting that AngII may act directly at the pancreas to

regulate β-cell function. Further, components of the RAS are upregulated with hyperglycemia in rodent and human islets (61) (185). In mice, AngII has been shown to reduce insulin release and biosynthesis in islets (166) (61) and infusion of AngII promoted hyperglycemia and reduced GSIS (58). Conflicting reports of the effects of AngII on insulin secretion do exist, as AngII has been demonstrated to increase insulin release both *in vitro* and *in vivo* (224) (112). However, blockade of the RAS using ARBs or ACE inhibitors in rodent models of T2D consistently improves overall glucose homeostasis (277) (61) (69) (235) (98). Taken together, these data support a role for AngII to modulate glucose homeostasis, however the role of the pancreatic RAS in progressive β-cell failure in T2D is unknown.

1.5.4.1 Regulation of β-cell function by AngII

AngII has been implicated in β-cell dysfunction *in vitro* and in animal studies *in vivo* by increasing oxidative stress, inflammation, and fibrosis in islets. In mouse islets, as in skeletal muscle, AngII induced superoxide generation via NADPH oxidase activation (132). In *db/db* mice, AT1R antagonism reduced islet mRNA expression of both NADPH oxidase and UCP2 and was associated with improved insulin secretion and reduced apoptosis of β-cells (62). In addition to increasing the production of ROS, AngII is also purported to stimulate the release of inflammatory mediators, such as IL-1β, monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-8 in mouse islets (59) (246). Recently, the deleterious effects of AngII infusion into HF-fed mice to induce islet inflammation and decreased GSIS were reversed with inhibition of IL-1β (246). Given the

previously published role of IL-1β as a mediator of the negative effects of gluco- and liptoxicity on β-cell function and survival, this study provides an important link between AngII and the inflammatory response associated with glucotoxicity. Further, in human islets cultured in high glucose, where mRNA expression of AT1R and AGT was increased, losartan prevented deleterious effects of glucotoxicity (185). Finally, blockade of the RAS attenuated islet fibrosis and improved insulin secretion in both ZDF rats (277) and *db/db* mice (250).

Although most of the effects of AngII to regulate β-cell function are demonstrated as AT1R-mediated, there is some evidence for a role of AT2R. In contrast to that of AT1R, AngII stimulation of AT2R may function as an insulinotropic mediator. In rats, insulin secretion was increased with administration of AngII, AngII plus losartan, or an AT2R agonist, and this effect was abolished with AT2R antagonism (249). Recently AT2R has also been reported to regulate the development of the endocrine pancreas. In human fetal pancreatic progenitor cells, AT2R was shown to be a key mediator of AngII-induced upregulation of transcription factors important in β-cell development (171). The same group further reported that AT2R, but not AT1R, blockade during embryonic development in mice impaired β-cell development and was associated with impaired insulin secretion in islets isolated from neonatal pups (172).

1.5.4.2 Regulation of β-cell function by ACE2

While AngII acting through the AT1R is generally thought to reduce pancreatic function and overall glucose homeostasis, recent studies suggest that

the opposite many be true of Ang-(1-7). In a transgenic rat model expressing an Ang-(1-7)-producing fusion protein, increased circulating Ang-(1-7) enhanced glucose tolerance and insulin sensitivity (242). Further studies in rat models of T2D demonstrated that an oral formulation of Ang-(1-7) improved peripheral glucose uptake and insulin resistance resulting in improved glycemia (207) (243). Thus, where AngII may promote the development of T2D, Ang-1-7 may be protective. Since ACE2 is the critical regulator of the relative abundance of these two peptides, this suggests that ACE2 may play a role in glucose homeostasis.

ACE2 was identified in islets of mice (203), rats (91), and humans (211). Mice with a whole body deficiency of ACE2 become progressively glucose intolerant with age and display impairments in insulin secretion, yet remain sensitive to insulin (203). In addition, MasR expression was demonstrated in mouse endocrine and exocrine pancreas (29), suggesting a role for the Ang-(1-7)/MasR axis in the regulation of β-cell function. Adenoviral-mediated overexpression of ACE2 in pancreas of *db/db* mice increased islet insulin content, reduced β-cell apoptosis, and improved glucose tolerance (28). These effects were attributed to ACE2-mediated production of Ang-(1-7), since the beneficial effects of ACE2 overexpression were prevented with blockade of the MasR.

Adenoviral overexpression of ACE2 in the pancreas also prevented the deleterious effects of AngII infusion on glucose homeostasis into mice (58). Therefore, the protective effects of ACE2 may be a result of both increased Ang-(1-7) and decreased AngII. Interestingly, protein expression of ACE2 was

reduced in islets of AngII-infused mice with impaired glycemia (58). A reduction in ACE2 protein was also observed in islets from *db/db* mice at 16 weeks of age compared to 8 weeks of age (28). These data suggest that reduced function of the ACE2/Ang-(1-7)/MasR axis may be one component by which RAS over-activity facilitates the development of T2D.

Table 1.1 Effects of RAS antagonists on the development of T2D		
Study name	*Effect*	*Reference*
HOPE	Ramipril treatment reduces the risk of new-onset diabetes (NOD) in 5720 patients at risk for CV events over 4.5 years (relative risk 0.66; ramipril vs. placebo treatments).	(312)
DREAM	Ramipril treatment had no significant effect on the development of NOD, however it did increase regression to normoglycemia compared to placebo in study of 5269 patients	(37)
NAVIGATOR	Valsartan treatment reduced the development of NOD by 14% in a study including 9306 patients with impaired glucose tolerance and other cardiovascular risk factors over 5 years	(192)
SOLVD	Of the 291 non-diabetic paitents included in the SOLVD trial, 5.9% of those given enalapril compared to 22.4% of those given placebo developed NOD	(287)
CAPPP	Of the 10,413 non-diabetic patients included in the CAPPP trial, there was a 14% reduction in NOD in captopril-treated patients	(202)
ALLHAT	Patients without diabetes at baseline receiving lisinopril instead of chlorthalidone had reduced fasting glucose and through 2 years of follow-up had significantly reduced NOD (odds ratio 0.55)	(20)
LIFE	Over 4.8 years, losartan treatment significantly reduced the incidence of NOD compared to atenolol (relative risk 0.75) in 7998 hypertensive patients with left ventricular hypertrophy	(178)

Table 1.2 Pancreatic components of the RAS			
RAS Component	*Species*	*Cell Type/Location*	*Reference*
Renin	Human	β-cells Islets: connective tissue surrounding blood vessels and in reticular fibers	(271)
	Rat	AR42J – A pancreatic acinar cell line	(54)
AGT	Canine	Pancreas	(55)
	Rat	α-cells	(228)
ACE	Rat	Islets: microvasculature, islet periphery	(277)
	Rat	Pancreas	(138)
ACE2	Rat	Acini and islets	(277)
	Rat	Islets	(91)
	Mouse	Islets	(28)
AT1Receptor	Human	β-cells	(271).
	Mouse	β-cells	(166)
AT2Receptor	Rat	Colocalized with somatostatin-producing cells	(304)
	Mouse	Islets (β-cells)	(60)
Mas Receptor	Mouse	Endocrine and exocrine	(29)

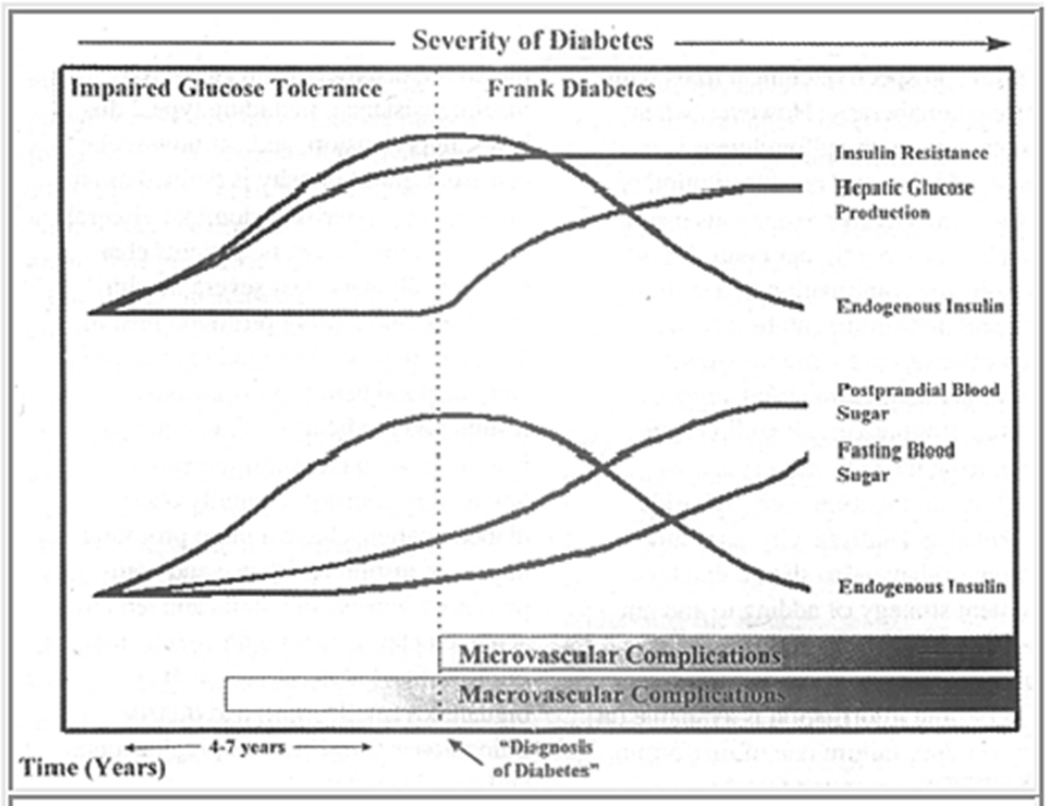


Figure 1.1 The natural history of the development of T2D: progression from impaired glucose tolerance to overt T2D, from Ramlo-Halsted and Edelman, 2000

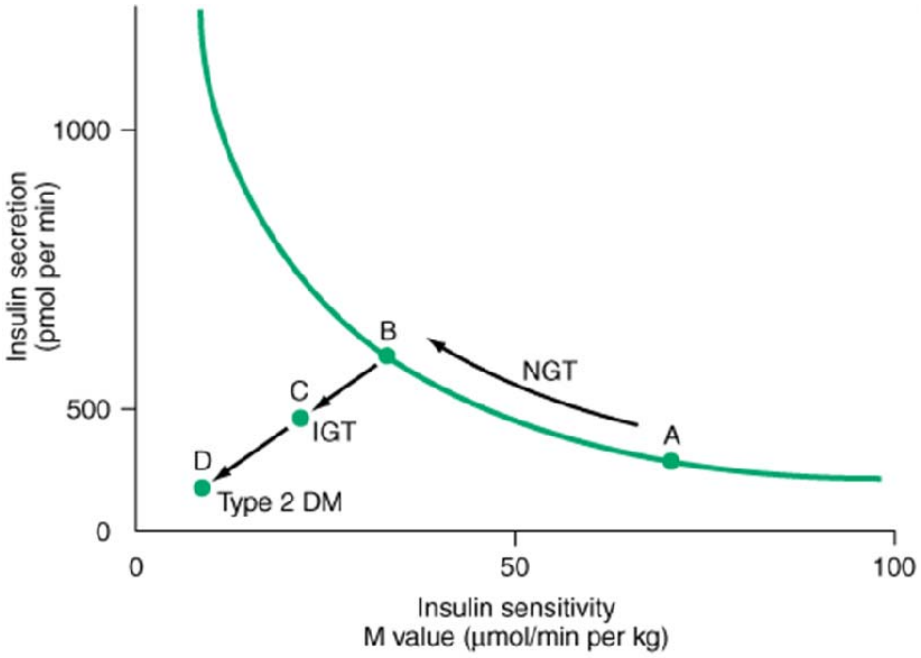
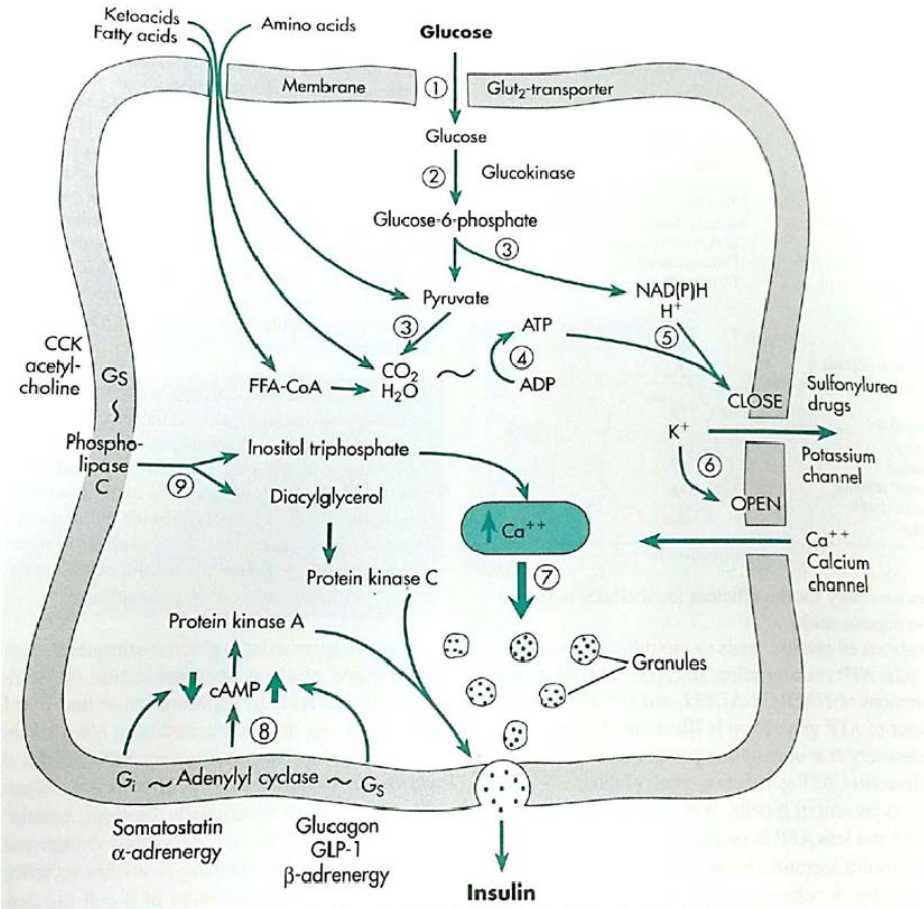


Figure 1.2 Metabolic changes during the development of T2D. Insulin secretion and insulin sensitivity are related. As an individual becomes more insulin resistant (by moving from point A to point B), insulin secretion increases and normo-glucose tolerance (NGT) is maintained. A failure to compensate by increasing the insulin secretion results initially in impaired glucose tolerance (IGT; point C) and ultimately in type 2 DM (point D). From Fauci et al, *Harrison's Principles of Internal Medicine, 17th Edition,* McGraw-Hill, 2008



■ **Fig. 41-5** Current concepts of regulation of insulin secretion by the β cell. Glucose transport *(1)* and glucokinase-catalyzed phosphorylation *(2)* raise glucose-6-phosphate levels. Metabolism *(3)* subsequently leads to increased adenosine triphosphate (ATP) levels *(4)* and NAD(P)H levels *(5)* that inhibit or close a potassium channel *(6)* and open a calcium channel *(6)*. Increased calcium levels then trigger exocytosis of insulin granules *(7)*. Other modulators of secretion act via the adenylyl cyclase–cAMP–protein kinase pathway *(8)* and the phospholipase-phosphoinositide pathway *(9)*. *GLP-1*, Glucagon-like peptide-1; *CCK*, cholecystokinin; *NAD(P)H*, reduced nicotine adenine dinucleotide phosphate; *ADP*, adenosine diphosphate.

Figure 1.3 Regulation of insulin secretion; From Berne et al, *Physiology 5th Edition,* Mosby, 2004

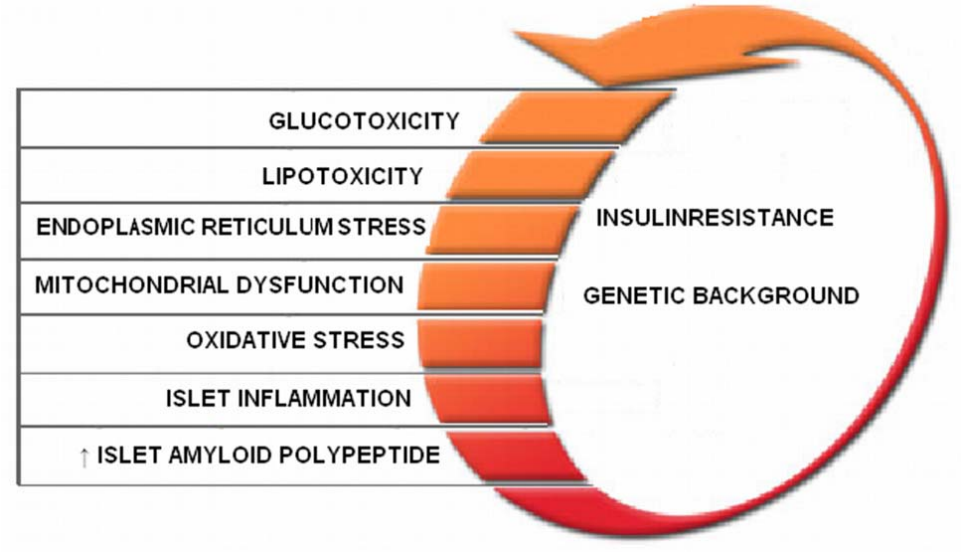


Figure 1.4 Factors contributing to β-cell failure; from Popa et al, *Beta-Cell Function and Failure in Type 2 Diabetes*, *Type 2 Diabetes* Prof. Kazuko Masuo (Editor), 2013

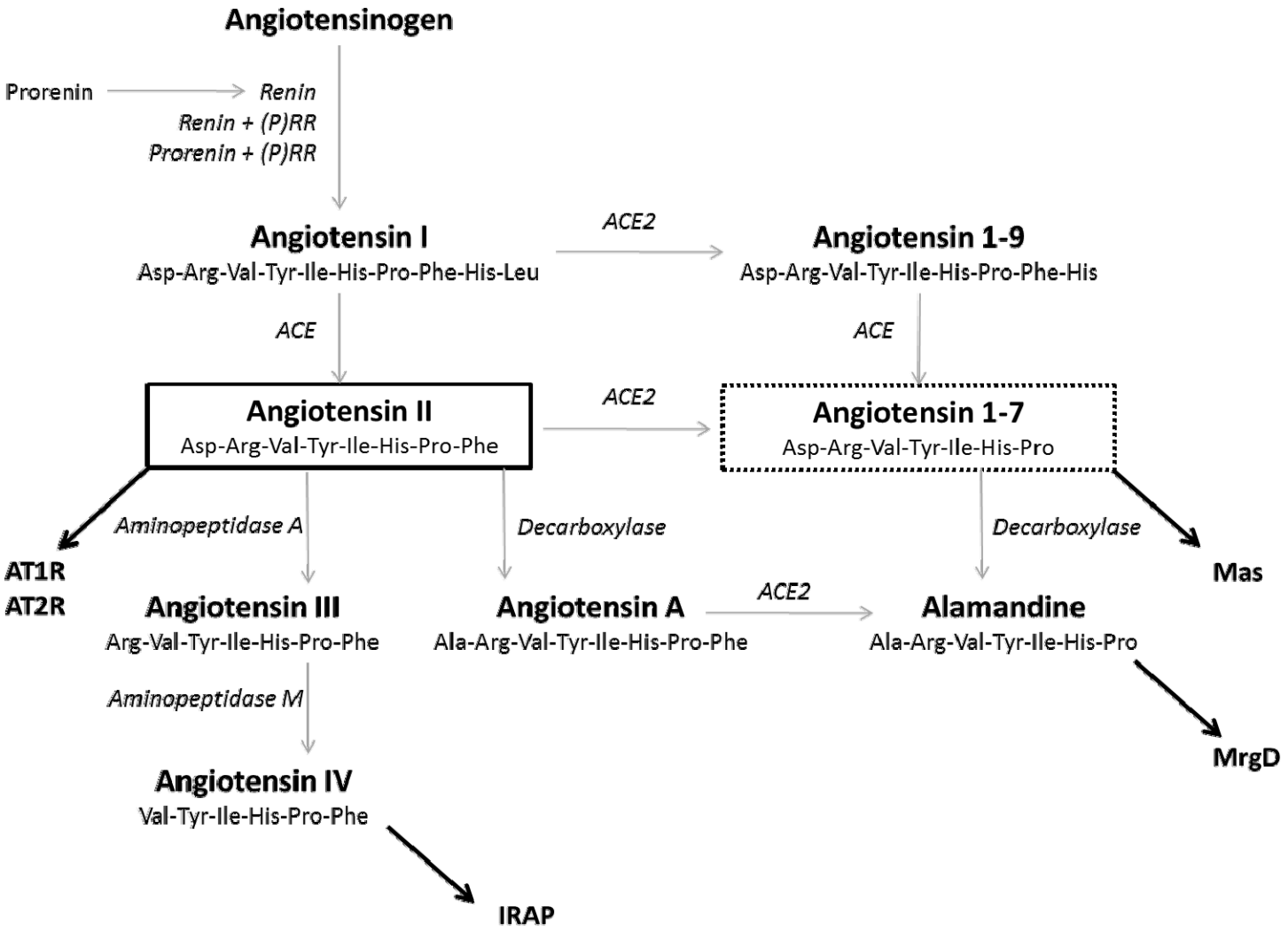


Figure 1.5 Schematic overview of the renin-angiotensin system.

Figure 1.5 Schematic overview of the renin-angiotensin system. The precursor peptide, angiotensinogen, is cleaved by renin to form the decapeptide angiotensin I. The catalytic activity of renin increases when bound to the (pro)renin receptor [(P)RR] and furthermore, the otherwise inactive prorenin can become catalytically active when bound to the (P)RR. The dipeptidase angiotensin converting enzyme (ACE) cleaves angiotensin I to form the octapeptide angiotensin II (AngII), the central active component of this system. AngII can be catabolized by angiotensin converting enzyme 2 (ACE2) into angiotensin1-7 (Ang1-7), another active peptide of this system which typically opposes the actions of AngII. AngII can also be cleaved into smaller fragments, such as angiotensin III and angiotensin IV by aminopeptidases A and M, respectively. Angiotensin A (AngA) is generated from AngII by decarboxylation. ACE2 can convert AngA into alamandine, which acts at the Mrg receptor. Most effects of AngII are mediated by the angiotensin type 1a receptor (AT1R), however AngII can also bind to the angiotensin type 2 receptor (AT2R) which generally exhibits opposing effects to those at the AT1R. Ang1-7 acts via the Mas receptor and angiotensin IV can bind to the insulin-regulated aminopeptidase receptors (IRAP). Adapted from Putnam et al 2012**.**

STATEMENT OF THE PROBLEM

Type 2 diabetes (T2D) is a metabolic disorder characterized by an initial adaptive increase in insulin secretion in response to insulin resistance followed by a progressive decline in cell function leading to loss of glycemic control. Obesity plays a significant role in T2D by contributing to the development of insulin resistance and both facilitating and exacerbating β-cell dysfunction. In 2012, the number of adults in the United States with diabetes or pre-diabetes was estimated at 29 million and 86 million, respectively, and more than 78 million American adults are obese. People with T2D are at an increased risk for cardiovascular disease (CVD), and CVD is the major cause of death in diabetics. T2D is a public health epidemic and the identification of mechanisms that link obesity, T2D, and CVD is a major focus of current research.

In clinical trials, drugs that inhibit the renin-angiotensin system (RAS) have been associated with a decrease in the risk of developing T2D. However, mechanisms by which the RAS contributes to the development of T2D remain unclear. Our lab and others have demonstrated that obesity is associated with an increase in the plasma concentrations of angiotensin II (AngII). The endocrine pancreas expresses several components of the RAS, including angiotensin type 1 receptors (AT1Rs). AngII has been demonstrated in animal studies to impair glucose homeostasis, inhibit insulin secretion, and promote the formation of reactive oxygen species and inflammatory mediators in isolated pancreatic islets. Pharmacologic blockade of the RAS in animal models of T2D improves glucose tolerance and insulin resistance. However, many of these

studies employ whole body AT1R blockade, making it difficult to define the direct effect of AngII actions at AT1R on pancreatic islets. Moreover, mechanisms for the observed benefits of RAS blockade, specifically whether they improve insulin sensitivity, as opposed to improving β-cell function, have not been well defined.

Recent studies indicate a role for angiotensin converting-enzyme 2 (ACE2) in the regulation of β-cell function. ACE2 is a negative RAS regulator in that the enzyme cleaves the vasoconstrictor, AngII, to generate the vasodilator, angiotensin-1-7 (Ang-1-7). Mice with whole body deficiency of ACE2 display moderate impairments in glucose homeostasis. Conversely, overexpression of ACE2 in the pancreas was shown to improve insulin secretion and glycemia by increasing Ang-(1-7) effects at Mas receptors in the endocrine pancreas of *db/db* diabetic mice. In separate studies, ACE2 overexpression prevented the deleterious effects of AngII infusion on glucose homeostasis in mice. While these results implicate ACE2 in the regulation of whole body glucose homeostasis, the role of ACE2 in the regulation of β-cell function in the progression of T2D is not well defined.

It is well accepted that activation of the local and systemic RAS contribute to CVD. An emerging body of evidence suggests that over-activity of the RAS is a common thread in the pathophysiology of obesity, CVD, and T2D. Our focus was to define the role of two opposing proteins of the RAS, namely AT1R and ACE2, on the regulation of β-cell function and glucose stimulated insulin secretion (GSIS) *in vitro* and *in vivo*. We chose these RAS components as they

have both been implicated in T2D, and since both may serve as potential therapeutic targets (AT1R blockade, ACE2 activation) in the treatment of T2D.

We used high fat (HF) diet-induced obese mice with whole body ACE2 deficiency to define the enzyme's effects on *in vivo* and *in vitro* GSIS. For studies on AT1R, we created a mouse model of pancreas-specific deficiency of the AT1aR to be used in conjunction with diet-induced obesity to gain a better understanding of the AngII/AT1aR-mediated effects on β-cell function. The central hypothesis of these studies is that the RAS contributes to the decline of β-cell function during the development of T2D. Specifically, in Aim 1 we tested the hypothesis that ACE2 deficiency impairs *in vitro* and *in vivo* GSIS through deficits in β-cell function in HF-fed mice. In Aim 2, we tested the hypothesis that pancreatic AT1aR deficiency will protect against impaired *in vitro* and *in vivo* GSIS in HF-fed mice. The following aims were designed to test this hypothesis:

Specific Aim 1: Determine the effect of whole body deficiency of ACE2 on glucose homeostasis in mice during the development of T2D.

- **A.** Characterize the temporal effect of ACE2 deficiency on β-cell function in lean and obese mice.
- **B.** Determine the relative roles of increased AngII and decreased Ang-1-7 on glucose homeostasis in obese mice with ACE2 deficiency.

Specific Aim 2: Determine the role of pancreatic AT1aRs on β-cell function in mice with diet-induced obesity.

- **A.** Define the effect of AngII on insulin secretion in isolated islets in obese mice.

B. Determine the effect of pancreas-specific AT1aR deficiency on glycemic control and insulin secretion from islets in obese versus lean mice.

Section II. SPECIFIC AIM 1

2.1 Summary

Pancreatic islets express angiotensin-converting enzyme 2 (ACE2), which catabolizes angiotensin II (AngII) to generate angiotensin-(1-7) (Ang-(1-7)). The role of ACE2 in β-cell function and glucose homeostasis in type 2 diabetes (T2D) has not been well-defined. In this study, we determined the effect of ACE2-deficiency on the adaptive β-cell hyperinsulinemic response to obesity. Male mice with a whole-body deficiency of ACE2 (*Ace2*$^{-/y}$) and their wild-type littermate controls (*Ace2*$^{+/y}$) were fed a high fat (HF) or control low fat (LF) diet for 4 months. On standard mouse diet, 8 week-old *Ace2*$^{-/y}$ mice had reduced plasma insulin compared to wild-type controls resulting in mild hyperglycemia during a glucose tolerance test. Plasma insulin remained diminished in *Ace2*$^{-/y}$ mice compared to *Ace2*$^{+/y}$ mice over 4 months of HF-feeding. Moreover, with chronic HF-feeding, ACE2-deficient mice became obese and developed insulin resistance to the same degree as wild-type controls, but were not able to maintain an adaptive increase in insulin secretion. This was associated with hyperglycemia in the fed, but not fasted state, indicating that deficits in the adaptive hyperinsulinemic response are manifest as impaired glucose homeostasis. Since plasma insulin was reduced in *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice as early as 1 month of HF-feeding, early β-cell dysfunction was further defined at this time point. 1-month HF-fed *Ace2*$^{-/y}$ mice displayed impaired glucose-stimulated insulin secretion (GSIS) *in vivo* compared to *Ace2*$^{+/y}$ mice which was associated with increased plasma glucose and no difference in body

weight or insulin resistance. Since deficiency of ACE2 could influence pancreatic function either by increasing AngII (and AT1R functions) or by reducing Ang-(1-7), we defined if initial *in vivo* deficits in glucose-stimulated insulin secretion (GSIS) of HF-fed ACE2 deficient mice could be prevented by AT1R blockade or by infusion of Ang-(1-7). Antagonism of the AT1R improved glucose tolerance in both genotypes, likely through improvements in insulin sensitivity, however *in vivo* deficits in GSIS of HF-fed ACE2 deficient mice could not be overcome by AT1R blockade or by Ang-(1-7) infusions, suggesting mechanisms that are unrelated to angiotensin peptide balancing properties of ACE2. Immunohistochemical analysis of pancreatic sections revealed that while HF-feeding increased β-cell mass and average islet area in both genotypes, obese ACE2-deficient mice had significantly less β-cell mass and smaller islet area than obese wild-type mice. Further, ACE2-deficiency was associated with significantly reduced proliferation of β-cells in response to HF-feeding. These results suggest that ACE2 is a critical regulator of β-cell compensatory responses to HF-induced hyperinsulinemia.

2.2 Introduction

Drugs that inhibit the renin-angiotensin system (RAS) have been shown to delay the onset of type 2 diabetes (T2D) (312) (192). Efficacy of RAS blockade in T2D may relate to improvements in insulin sensitivity or β-cell function during the adaptive phase of hyperinsulinemia. Several components of the RAS are present in rodent and human pancreatic islets (55) (166) (271) including the monocarboxypeptidase angiotensin-converting enzyme 2 (ACE2) that cleaves angiotensin II (AngII) to generate angiotensin-(1-7) (Ang-1-7) (74). Local pancreatic ACE2 may control AngII levels at islets and serve as an endogenous mechanism to limit RAS activity. Indeed, recent studies suggest that ACE2 is a positive regulator of pancreatic function (58) (124) as mice with whole body deficiency of ACE2 became progressively glucose intolerant due to impairments in insulin secretion (203). Moreover, in mice fed a high fat (HF) diet, whole body ACE2 deficiency deteriorated islet function through a mechanism involving impairment of islet microvasculature (311). Conversely, adenoviral overexpression of ACE2 in the pancreas of db/db mice improved glycemic control through Ang-(1-7) effects at Mas receptors (28). Notably, over-expression of ACE2 in pancreas increased β-cell proliferation and reduced β-cell apoptosis in 8-week old db/db mice, but had no effect in 16 week old db/db mice when glycaemia was severely impaired. These findings suggest that ACE2 may play a role in the adaptive β-cell hyperinsulinemic phase of T2D.

Previously, we demonstrated that plasma and tissue concentrations of AngII are increased in glucose intolerant mice with HF diet-induced obesity (39). Moreover, our previous studies suggested that deficits in ACE2 may contribute to an increase in the balance of AngII versus Ang-(1-7) in obese mice (122). In this study, we hypothesized that deficiency of ACE2 impairs the adaptive β-cell hyperinsulinemic response to HF diet in the progression to T2D. We first quantified plasma insulin secretion in wild type and ACE2 deficient mice chronically fed a low fat (LF) or HF diet. Since deficiency of ACE2 could influence pancreatic function either by increasing AngII (and AT1R functions) or by reducing Ang-(1-7), we defined if initial *in vivo* deficits in glucose-stimulated insulin secretion (GSIS) of HF-fed ACE2 deficient mice could be prevented by AT1R blockade or by Ang-(1-7). Finally, we focused on the regulation of β-cell mass in HF-fed ACE2 deficient mice as a mechanism for deficits in the adaptive hyperinsulinemic response.

2.3 Materials and Methods

2.3.1 Experimental animals and diets.

All studies were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ littermates on a C57BL/6 background (2 months of age; bred from male *Ace2*$^{-/y}$ and female *Ace2*$^{+/-}$ breeding pairs) were fed either LF (10% kcal as fat; D12450B; Research Diets, New Brunswick, NJ) or HF diets (60% kcal as fat; D12492; Research Diets) ad libitum with free access to water for 5 weeks (n=5-6 per group) or 17 weeks (n=5-11 per group). In a separate study, male *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice (2 months of age; n=12-16 per group) were infused via osmotic minipump (Alzet, model 2006) with losartan (Los; 17 μg/kg/min, Merck, Whitehouse Station, NJ) (181), Ang-(1-7) (0.4 μg/kg/min, Bachem, Torrence, CA) (276) or saline (Sal) and fed a HF diet for 28 days. Plasma Ang-1-7 concentrations were quantified by ELISA using a commercial kit (Peninsula Laboratories, San Carlos, CA). At study endpoint, mice were anesthetized with ketamine/xylazine (100/10 mg/kg) for exsanguination and tissue harvest.

2.3.2 Glucose tolerance, insulin tolerance, and plasma glucose/insulin quantification.

Following a 6 hour fast, blood glucose concentrations were quantified using a glucometer (Freedom Freestyle Lite, Abbott Laboratories, Abbott Park, IL) immediately before and 15, 30, 60, 90, and 120 minutes following intraperitoneal (i.p.) administration of glucose (2 g/kg body weight). Insulin tolerance was

assessed following a 4 hour fast by quantifying blood glucose concentrations at 0, 30, 60, and 90 minutes after administration of human insulin (Regular®, 0.5 U/kg body weight, i.p., Novo Nordisk, Princeton, NJ). Both glucose tolerance and insulin tolerance are expressed as area under the curve (AUC). Plasma insulin and glucose concentrations were quantified from blood samples (10-15 µl) collected via tail stick in conscious mice (n=5 mice per group) following a 6 hour fast and again after i.p. administration of glucose. Plasma insulin concentrations were quantified by ELISA using a commercial kit (Crystal Chem, Downers Grove, IL).

2.3.3 Glucose-stimulated insulin secretion (GSIS) from isolated pancreatic islets.

The method for islet isolation was adapted from published protocols (270) (51). Pancreata were perfused *in vivo* through the common bile duct with 5 mLs of collagenase (0.6 mg/mL, Collagenase P, Roche, Indianapolis, IN) immediately following exsanguination. The excised pancreata were incubated in 10 mLs of collagenase at 37ºC for 15-20 minutes, and the tissue was then mechanically separated. Islets were purified from exocrine tissue using a sucrose gradient (Histopaque 1077, Sigma-Aldrich, St. Louis, MO), allowed to recover in culture media (RPMI, 10% FBS, 1% penicillin/streptomycin) for 48 hours, and further purified from exocrine tissue and debris by handpicking with a pipette and transferring to fresh media. Purified islets (20-25) were placed in inserts in 12 well plates (Greiner Bio One, Monroe, NC) and incubated in Krebs buffer with 3 mM glucose for one hour at 37ºC. The inserts containing the islets were then

transferred to Krebs buffer with 28 mM glucose and incubated for 1 hour at 37ºC. Insulin concentrations in media and in islets (harvested in lysis buffer) were quantified by ELISA and normalized to protein content of islets (BCA assay, ThermoFisher, Rockford IL).

2.3.4 Determination of pAKT/AKT.

A subset of male *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice (n=4-6 per group) infused via osmotic minipump with losartan, Ang-(1-7) or saline and fed a HF diet for 28 days were fasted for 4 hours and injected with 10U/kg body weight insulin 15 min prior to anesthetization and exsanguination. Liver, solei, and subcutaneous adipose tissue were dissected and frozen in liquid nitrogen. Phosphorylated AKT and total AKT were assessed using a commercial ELISA kit (Cell Signaling, Danvers, MA).

2.3.5 Immunohistochemistry and immunofluorescence.

Mouse pancreata were washed in PBS, fixed in 10% formalin overnight at 4°C, dehydrated in grades of ethanol, and paraffin-embedded. Starting at a depth of 400 μm, five longitudinal sections (5 μm thick) were prepared every 100 μm. Sections were deparaffinized, rehydrated in alcohol and subjected to antigen retrieval (steam; Vector Labs Antigen Retrieval Unmasking Solution). For β-cell mass and morphometric analysis, sections were incubated with rabbit anti-insulin antibody (1:100, Abcam ab63820, Cambridge, MA) followed by incubation with biotinylated anti–rabbit secondary antibody (1:200; Vector Labs, Burlingame, CA) at 40°C for 30 minutes. A streptavidin-based ABC system and peroxidase-based red chromagen AEC (both from Vector Labs) were used to

identify the antigen-antibody reactions, after which sections were counterstained with hematoxylin. β-cell proliferation was assessed in sections incubated for 20 hours at 4°C with rabbit anti-Ki67 (Abcam ab66155, 1:100) and guinea pig anti-insulin (1:500, Dako A056401-2, Carpinteria, CA) followed by incubation with goat anti-rabbit Alexa fluor 488 and goat anti-guinea pig Alexa fluor 594 in PBS for 30 minutes at 40°C then mounted with DAPI. Isotype-matched IgG was used as controls, as was omission of primary and/or secondary antibodies. Images were captured with a Nikon Eclipse 80i microscope and analysis was performed using NIS Elements software (Nikon Instruments, Japan). A commercial kit for measuring cell death by fluorescein detection of TUNEL staining was used to measure β-cell apoptosis (Roche, Indianapolis, IN).

2.3.6 Analysis and quantification of β-cell mass, islet size, proliferation, and apoptosis.

For determination of β-cell mass, an entire pancreas tissue section was imaged at 4x under brightfield (5 sections per mouse, approximately 120 μm apart; n=3 mice per group). The total pancreas tissue area and the total insulin-positive (β-cell) area was selected for each image and β-cell mass is reported as the mean over 5 sections of the ratio of insulin-stained area/total pancreas area per mouse. Three 3 mm x 3 mm fields from two sections per animal approximately 500 μm apart (imaged at 10x) were used to obtain the average islet area/section (calculated from individual islet areas); data are reported as the mean islet area per mouse. β-cell proliferation was determined by obtaining the ratio of area positive for both Ki67 and insulin from individual islets (10-15 islets

per section imaged at 40x; analyzed from 2 sections per mouse and 3 mice per group). Apoptosis in β-cells was assessed by counting the number of TUNEL-positive cells in sections double-stained for insulin (20 islets per section were imaged at 20x; 2 sections per mouse and 3 mice per group were used for analysis).

2.3.7 Statistical analysis.

Data are presented as mean ± SEM. Data were analyzed using two-way ANOVA with diet and genotype or (treatment where appropriate) as between-group factors. If statistical differences existed between experimental groups, Holm-Sidac method was utilized for post hoc analyses. Values of $P < 0.05$ were considered to be statistically significant. All statistical analyses were performed using SigmaStat (SPSS).

2.4 Results

2.4.1 ACE2-deficient mice fed standard diet have mild impairments in glucose tolerance and insulin secretion.

ACE2-deficient mice had reduced body weight compared to controls at 8 weeks of age (Figure 2.1A, $P<0.01$). Insulin tolerance was not different between genotypes (Figure 2.1B, C). However, during a glucose tolerance test, ACE2-deficient mice exhibited increased plasma glucose concentrations at 15 and 30 minutes compared to controls (Figure 2.1D, $P<0.05$). Modest elevations in plasma glucose concentrations did not influence the area under the curve (AUC) for plasma glucose concentrations (Figure 2.1E). Notably, plasma insulin concentrations were significantly lower in *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice following a 6 hour fast (time 0, Figure 2.1F; $P<0.05$) and at 30 minutes post-glucose administration (Figure 2.1F, $P<0.05$).

2.4.2 Plasma insulin concentrations are reduced in hyperglycemic ACE2-deficient mice chronically fed a HF diet.

We examined effects of chronic (4 month) HF feeding on glucose homeostasis and the adaptive hyperinsulinemic response in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. Initial body weights of ACE2-deficient mice were lower than controls, resulting in a modest reduction in body weights of LF-fed ACE2-deficient mice at study endpoint (Figure 2.2A, $P<0.05$). HF-fed mice of each genotype had significantly increased body weights compared to LF-fed controls, with no differences between genotypes (Figure 2.2A; $P<0.001$). Insulin tolerance was significantly impaired in obese mice of each genotype compared to LF-fed

controls (Figure 2.2B, C; P<0.01). Additionally, HF-fed ACE2-deficient mice became insulin resistant to the same degree as control mice. Fasting plasma insulin concentrations were significantly reduced in ACE2 deficient mice compared to controls as early as 1 month of HF feeding, with marked reductions in plasma insulin concentrations in 4 month HF-fed *Ace2*$^{-/y}$ mice (Figure 2.2D; P<0.05). Although there were no significant differences in fasting plasma glucose concentrations between HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice at 4 months (Figure 2.2E), non-fasted plasma glucose concentrations were significantly elevated in HF-fed *Ace2*$^{-/y}$ mice compared to controls (Figure 2.2F, P<0.05).

2.4.3 ACE2-deficient mice have impaired *in vivo* GSIS after 1 month of HF-feeding.

Since plasma insulin concentrations were reduced in obese ACE2-deficient mice compared to controls after 1 month of HF-feeding, we sought to determine if β-cell dysfunction was also present at 1 month of HF feeding in ACE2-deficient mice. Both groups of mice developed hyperinsulinemia, hyperglycemia, and insulin resistance (Figure 2.3A-D; P<0.01). However, plasma insulin concentrations were significantly lower in HF-fed ACE2-deficient mice following a 6 hour fast (time 0, Figure 2.3A; P<0.05) and at 30 and 60 minutes following intraperitoneal glucose administration compared to *Ace2*$^{+/y}$ controls (Figure 2.3A, P<0.05). Moreover, plasma glucose concentrations were slightly elevated in ACE2-deficient mice of both genotypes compared to wild-type controls (time 0) and this effect was significant at 30 minutes following glucose administration (Figure 2.3B, P<0.05).

To determine if deficits of *in vivo* GSIS of ACE2-deficient mice resulted from impaired insulin secretion from pancreatic islets, we quantified GSIS from isolated pancreatic islets from mice of each genotype and diet. Under high glucose conditions, islets from HF-fed mice of each genotype exhibited significantly increased GSIS compared to islets from LF-fed mice (Figure 2.3E; $P<0.05$). However, there were no significant differences in insulin secretion from islets of HF-fed mice of either genotype.

2.4.4 Neither infusion of an AT1R antagonist nor infusion of Ang-(1-7) restore *in vivo* deficits of GSIS in HF-fed ACE2-deficient mice.

Since ACE2 cleaves AngII to Ang-(1-7), we determined if antagonism of AngII effects at AT1R (by infusion of Los), or infusion of Ang-(1-7) to restore plasma peptide concentrations in HF-fed (1 month) ACE2 deficient mice would reverse *in vivo* deficits of GSIS. Infusion of Los had no effect on plasma concentrations of Ang-(1-7) in *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ mice (Fig. 4A). However, infusion of Ang-(1-7) significantly increased plasma Ang-(1-7) concentrations in both genotypes, with no differences between genotypes (Figure 2.4A).

Administration of Los significantly improved glucose tolerance compared to Sal in mice of each genotype, with no differences between genotypes (Figure 2.4B; $P<0.05$). However, Los administration significantly decreased fasting blood glucose concentrations in *Ace2*$^{+/y}$, but not in *Ace2*$^{-/y}$ HF-fed mice (Figure 2.4C; $P<0.05$). Moreover, fasting blood glucose concentrations were significantly increased in *Ace2*$^{-/y}$ mice regardless of treatment group compared to control (Figure 2.4C; $P<0.05$). As described above (Figure 2.3A), plasma insulin

concentrations following *in vivo* glucose administration were significantly lower in HF-fed *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice (Sal groups, Figure 2.4D; P<0.05). Los administration was unable to restore plasma insulin concentrations in HF-fed *Ace2*$^{-/y}$ mice to the level of controls (Sal groups, Figure 2.4D). Since Los administration improved glucose tolerance, but had no effect on plasma insulin concentrations in mice of either genotype, we quantified tissue (skeletal muscle, adipose) concentrations of pAKT as an index of insulin sensitivity. Los administration significantly increased the ratio of pAKT to AKT in soleus muscle and subcutaneous adipose tissue of both genotypes compared to Sal controls, with no differences between genotypes (Figure 2.4E, F; P<0.05).

Despite significant elevations in plasma Ang-(1-7) concentrations in mice infused with the peptide (Figure 2.4A), Ang-(1-7) had no effect on glucose tolerance (Figure 2.4B) or fasting blood glucose concentrations (Figure 2.4C) in mice of either genotype. Moreover, infusion of Ang-(1-7) had no effect on plasma insulin concentrations in *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ HF-fed mice (Figure 2.4D).

2.4.5 HF-fed ACE2-deficient mice have reduced islet size and β-cell mass.

To define mechanisms for *in vivo* deficits in GSIS of HF-fed *Ace2*$^{-/y}$ mice, we quantified average islet area and β-cell area (mass) in pancreatic sections from 1 month LF- and HF-fed mice of each genotype (representative images in Figure 2.5A). In LF-fed mice, average islet area and β-cell mass were significantly decreased in *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice (Figure 2.5B,C; P<0.05). HF-feeding significantly increased β-cell mass in both genotypes (Figure 2.5A,B;

P<0.05). However, the magnitude of increase in β-cell mass was greater in *Ace2*$^{+/y}$ (44%) compared to *Ace2*$^{-/y}$ (30%) mice. Moreover, average islet area and β-cell mass were significantly reduced in HF-fed *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice (Figure 2.5B, C; P<0.05).

2.4.6 β-cell proliferation is decreased in HF-fed ACE2-deficient mice.

To define mechanisms for reductions in β-cell mass of HF-fed *Ace2*$^{-/y}$ mice, we measured β-cell proliferation by quantifying double-staining of pancreatic sections from 1 month LF- and HF-fed mice of each genotype for insulin and Ki67 (respresentative image, Figure 2.5D). Low proliferation of β-cells was observed in LF-fed mice, and there were no differences between genotypes. In HF-fed *Ace2*$^{+/y}$ mice, β-cell proliferation was significantly increased (3-fold) compared to LF controls (Figure 2.5E; P<0.001). In contrast, although β-cell proliferation was slightly increased in HF-fed *Ace2*$^{-/y}$ mice compared to LF controls, the effect was not significant. Notably, β-cell proliferation was significantly lower in pancreatic sections from HF-fed *Ace2*$^{-/y}$ compared to *Ace2*$^{+/y}$ mice (Figure 2.5E, P<0.01). To assess whether apoptosis contributed to decreased β-cell mass of HF-fed *Ace2*$^{-/y}$ mice, TUNEL staining was performed on pancreatic sections. Apoptosis was minimal in islets of LF or HF mice of either genotype and there was no difference in the number of TUNEL-positive islets between groups (data not shown).

2.5 Discussion

It has previously been reported that ACE2 deficiency is associated with altered glucose homeostasis (203) (25) but mechanisms for this effect have not been extensively investigated. Our findings provide new insight into the importance of ACE2 in the adaptive β-cell hyperinsulinemic response to insulin resistance. The onset of T2D in humans is characterized by a decrease in insulin secretion and an inability to maintain hyperinsulinaemia (223). A reduction in the acute insulin response to glucose, considered to be an early indicator of β-cell dysfunction, is associated with the transition from normal to impaired glucose tolerance in humans and rodents (300) (7). Our results demonstrate that HF-fed ACE2-deficient mice became obese and developed insulin resistance to the same degree as controls but were not able to maintain adaptive hyperinsulinemia, resulting in hyperglycemia in the fed state. *In vivo* GSIS deficits of HF-fed ACE2 deficient mice were not the result of impaired insulin secretory mechanisms in pancreatic islets. Rather, HF-fed ACE2 deficient mice exhibited reductions in β-cell mass associated with impaired proliferative capacity of islets. Finally, *in vivo* GSIS deficits of HF-fed ACE2 deficient mice could not be overcome by AT1R blockade or by Ang-(1-7) infusions, suggesting mechanisms unrelated to angiotensin peptide balancing properties of ACE2. These results suggest that ACE2 is a critical regulator of β-cell compensatory responses to HF-induced hyperinsulinemia.

Previous studies demonstrate ACE2-deficient mice have low insulin gene expression (25) and age-dependent impaired first-phase insulin secretion when fed a standard mouse diet (203). Conversely, ACE2 overexpression improved first-phase insulin secretion *in vivo* in *db/db* mice (28). Our results agree with and extend these findings by demonstrating that modest impairments in *in vivo* GSIS in young ACE2-deficient mice contribute to failure to maintain adaptive hyperinsulinemia in the development of T2D. AngII has been suggested to regulate insulin secretion from islet cells of various species with conflicting reported findings of a stimulatory effect on insulin secretion from human islets (224), *versus* reductions in insulin secretion from primary mouse islets (166). Our results do not support differences in insulin secretory mechanisms from pancreatic islets of HF-fed ACE2 deficient mice as contributors to the observed deficits of *in vivo* GSIS. Several studies have demonstrated that AngII can regulate islet blood flow and thereby influence insulin release (50) (151). Thus, ACE2 deficiency may have impaired *in vivo* GSIS of HF-fed mice through AngII/AT1R-mediated reductions in islet blood flow. Since administration of losartan to HF-fed ACE2 deficient mice did not reverse deficits of *in vivo* GSIS, it is unlikely that elevated systemic or local AngII concentrations in obese mice (122) contributed to deficits of *in vivo* GSIS through AngII/AT1R-mediated regulation of islet blood flow. These findings agree with a recent study in which administration of an ACE-inhibitor to ACE2-deficient mice did not correct β-cell defects (25).

AT1R antagonists have been shown to improve glucose tolerance and increase insulin secretion (61) (235), suggesting that AngII acting through the AT1R contributes to the pathogenesis of T2D. Consistent with previous findings (98), in this study losartan administration improved glucose tolerance in both genotypes. Our results suggest that losartan reduces insulin resistance, as evidenced by increased pAKT/AKT ratios in insulin sensitive tissues. Similarly, ACE2 activation of the Ang-(1-7)/MasR axis increased insulin secretion *in vivo* (28). Specifically, infusion of Ang-(1-7) (at a 4-fold lower dose) improved insulin sensitivity in non-obese ACE2-deficient mice fed a high sucrose diet (102). Moreover, in a rat transgenic model of insulin resistance, an oral formulation of Ang-(1-7) improved insulin sensitivity and glycaemia (241). Our results demonstrate that infusion of Ang-(1-7) at a dose that elevated plasma Ang-(1-7) concentrations and blunted an activated RAS (275) (276) had no effect on glucose homeostasis in HF-fed mice of either genotype. Differences in the dose or formulation of Ang-(1-7), coupled with varying models of T2D may have contributed to diverging effects of the peptide on glucose homeostasis.

Our results demonstrate that the compensatory response to increase β-cell mass with chronic HF feeding (71) (133) was blunted in ACE2 deficient mice. Compensatory β-cell mass expansion is achieved through increases in islet size (hypertrophy) or β-cell proliferation (hyperplasia) (144). In humans with T2D, a curvilinear relationship has been reported between β-cell volume and fasting blood glucose concentrations (230) Our results agree with previous findings where islet vascularization and insulin staining were reduced in chronically obese

ACE2-deficient mice (311). Moreover, our results extend previous findings by demonstrating insulin deficits at 5 weeks of HF feeding in ACE2-deficient mice that are associated with decreased β-cell mass and proliferation. Both LF- and HF-fed ACE2-deficient mice exhibited significant reductions in average islet area and β-cell mass, which may have contributed to impaired first-phase insulin secretion and loss of the adaptive hyperinsulinemic response. We found that reductions in β-cell mass of HF-fed ACE2-deficient mice were attributed to deficits in proliferation rather than apoptosis, contributing to an impaired adaptive hyperinsulinemic response to metabolic challenge.

Since neither an AT1R antagonist nor infusion of Ang-(1-7) could restore deficits in *in vivo* GSIS of HF-fed ACE2 deficient mice, it is unlikely that angiotensin peptide balance contributes to effects of ACE2 deficiency. Little is known about the function of ACE2 independent from its role to enzymatically cleave AngII in the generation of Ang-(1-7). ACE2 shares considerable homology with the membrane protein collectrin, but collectrin lacks an active dipeptidyl carboxypeptidase catalytic domain. Both collectrin and ACE2 are reported to be downstream targets of the transcription factor hepatocyte nuclear factor-1α (HNF-1α) (101) (211), which is mutated in maturity-onset diabetes of the young (MODY) (307). Collectrin has been demonstrated to increase insulin secretion *in vitro* (13) through regulation of insulin exocytosis (101). However, mixed results have been reported regarding the role of collectrin to regulate cell growth of pancreatic β-cells, as overexpression of collectrin increased β-cell mass *in vivo* (12) but had no effect on β-cell proliferation *in vitro*, while whole

body collectrin deletion had no effect on β-cell mass (188). Since ACE2 deficiency had no effect on insulin secretion from isolated islets but did regulate β-cell mass, these results do not support a role for the collectrin domain of ACE2 as the mechanism for impaired adaptive hyperinsulinemia.

In summary, results demonstrate a critical role for ACE2 in the adaptive hyperinsulinemia response to insulin resistance induced by HF feeding. Reduced hyperinsulinemia of HF-fed ACE2 deficient mice was detrimental, as ACE2 deficient mice exhibited hyperglycemia following glucose challenge. Moreover, insulin deficits did not appear to be related to imbalances in the RAS, as neither an AT1R antagonist nor infusion of Ang-(1-7) could reverse insulin deficits of HF-fed ACE2 deficient mice. Rather, ACE2 deficiency reduced the adaptive response to increase β-cell mass in HF-fed mice, associated with deficits in β-cell proliferation. These results demonstrate that ACE2 is a critical regulator of β-cell proliferation and growth, and may serve as a therapeutic target for T2D.

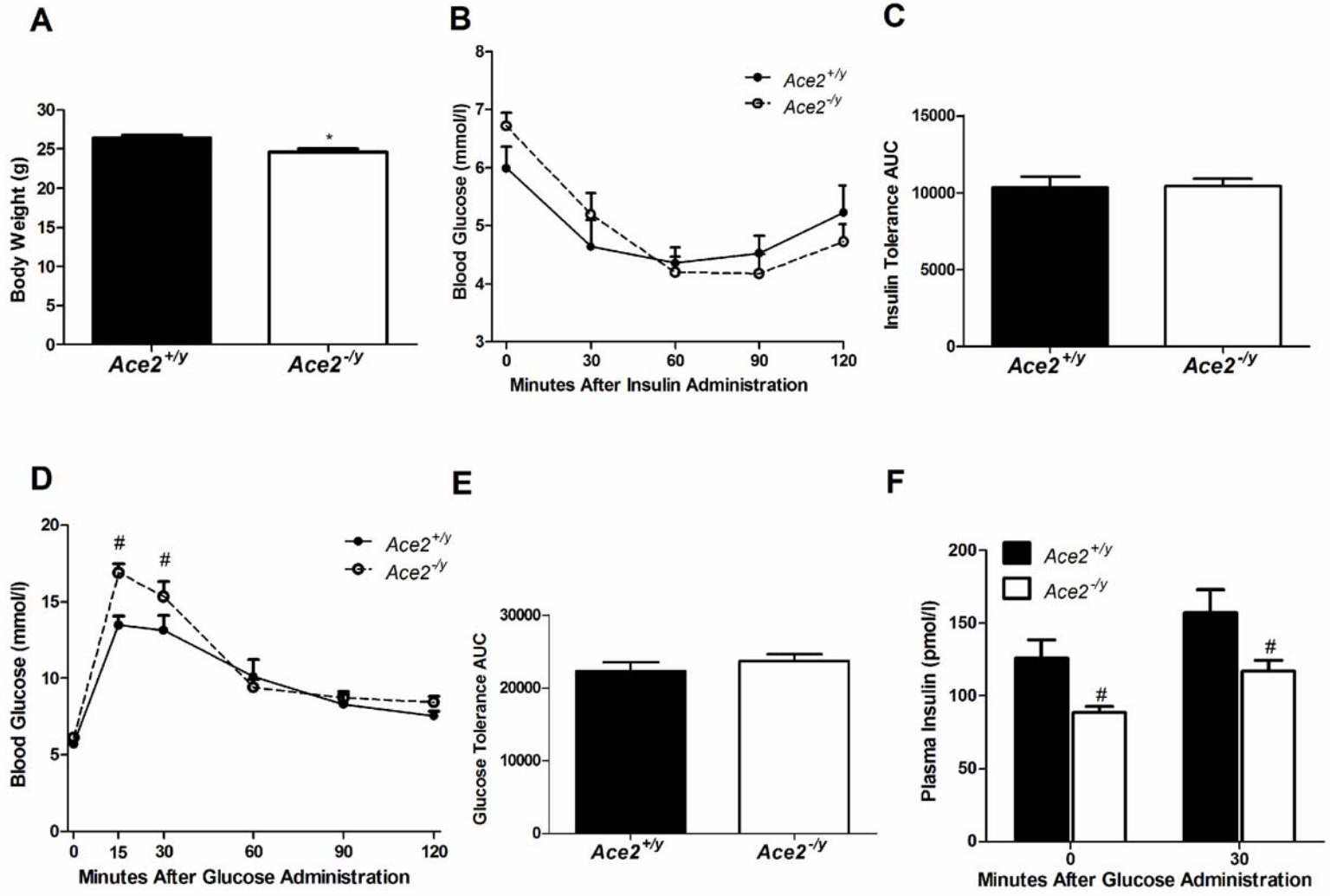

A
Body Weight (g)
30
25
20
15
10
5
0
*
Ace2+/y
Ace2-/y
B
Blood Glucose (mmol/l)
8
7
6
5
4
3
Ace2+/y
Ace2-/y
0
30
60
90
120
Minutes After Insulin Administration
C
Insulin Tolerance AUC
15000
10000
5000
0
Ace2+/y
Ace2-/y
D
Blood Glucose (mmol/l)
20
15
10
5
0
#
#
Ace2+/y
Ace2-/y
0
15
30
60
90
120
Minutes After Glucose Administration
E
Glucose Tolerance AUC
30000
20000
10000
0
Ace2+/y
Ace2-/y
F
Plasma Insulin (pmol/l)
200
150
100
50
0
Ace2+/y
Ace2-/y
#
#
0
30
Minutes After Glucose Administration

Figure 2.1 ACE2-deficient mice fed standard diet exhibit impaired glucose tolerance associated with reductions of *in vivo* GSIS. (A) Body weights of 8-week old *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice (*, P<0.01). (B) Insulin tolerance test and (C) area under the curve (AUC). (D) Glucose tolerance test and (E) AUC in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. #, P<0.05 compared to *Ace2*$^{+/y}$ within time point. (F) Plasma insulin concentrations in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice following a 6 hour fast (time = 0) and at 30 minutes after glucose administration. #, P<0.05 compared to *Ace2*$^{+/y}$ within time point. Data are mean $\pm$ SEM from n=5-11 mice/group.

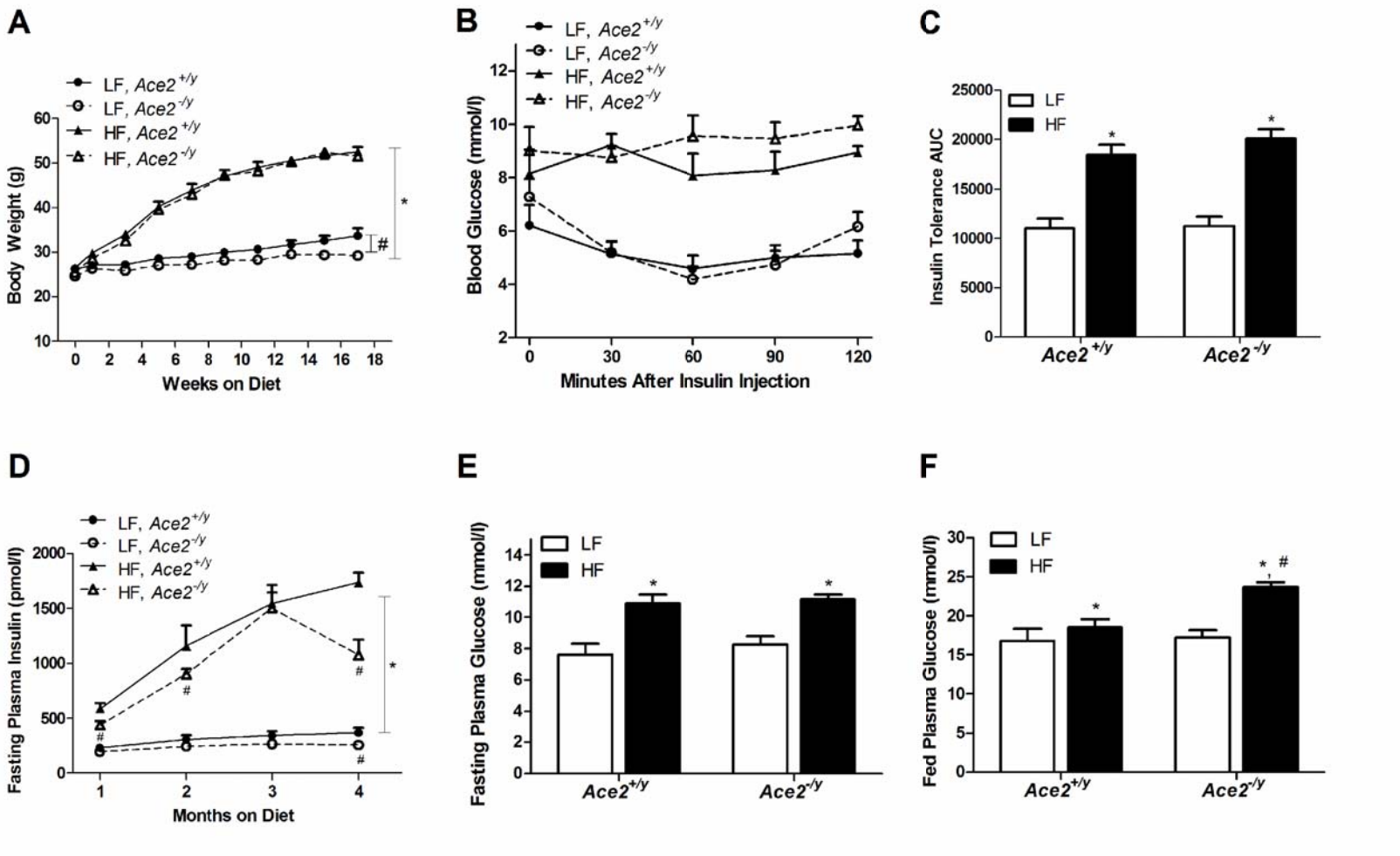

A
LF, Ace2+/y
LF, Ace2-/y
HF, Ace2+/y
HF, Ace2-/y
Body Weight (g)
Weeks on Diet
B
Blood Glucose (mmol/l)
Minutes After Insulin Injection
C
Insulin Tolerance AUC
LF
HF
Ace2+/y
Ace2-/y
D
Fasting Plasma Insulin (pmol/l)
Months on Diet
E
Fasting Plasma Glucose (mmol/l)
F
Fed Plasma Glucose (mmol/l)

Figure 2.2 ACE2-deficient HF-fed mice exhibit diminished adaptive hyperinsulinemia. *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ mice were fed a HF diet for 17 weeks. (A) Body weight progression. *, P<0.001 effect of diet and #, P<0.05 effect of genotype. (B and C) Insulin tolerance test and corresponding AUC. *P<0.01 compared to LF. (D) Fasting plasma insulin concentrations. *, P<0.01 compared to LF within genotype and #, P<0.05 compared to *Ace2*$^{+/y}$ within time point. (E) Fasted plasma glucose and (F) non-fasted plasma glucose in LF and HF-fed mice. *, P<0.01 compared to LF within genotype and #, P<0.05 compared to *Ace2*$^{+/y}$ within diet group. Data are mean $\pm$ SEM from n=5-11 mice/group.

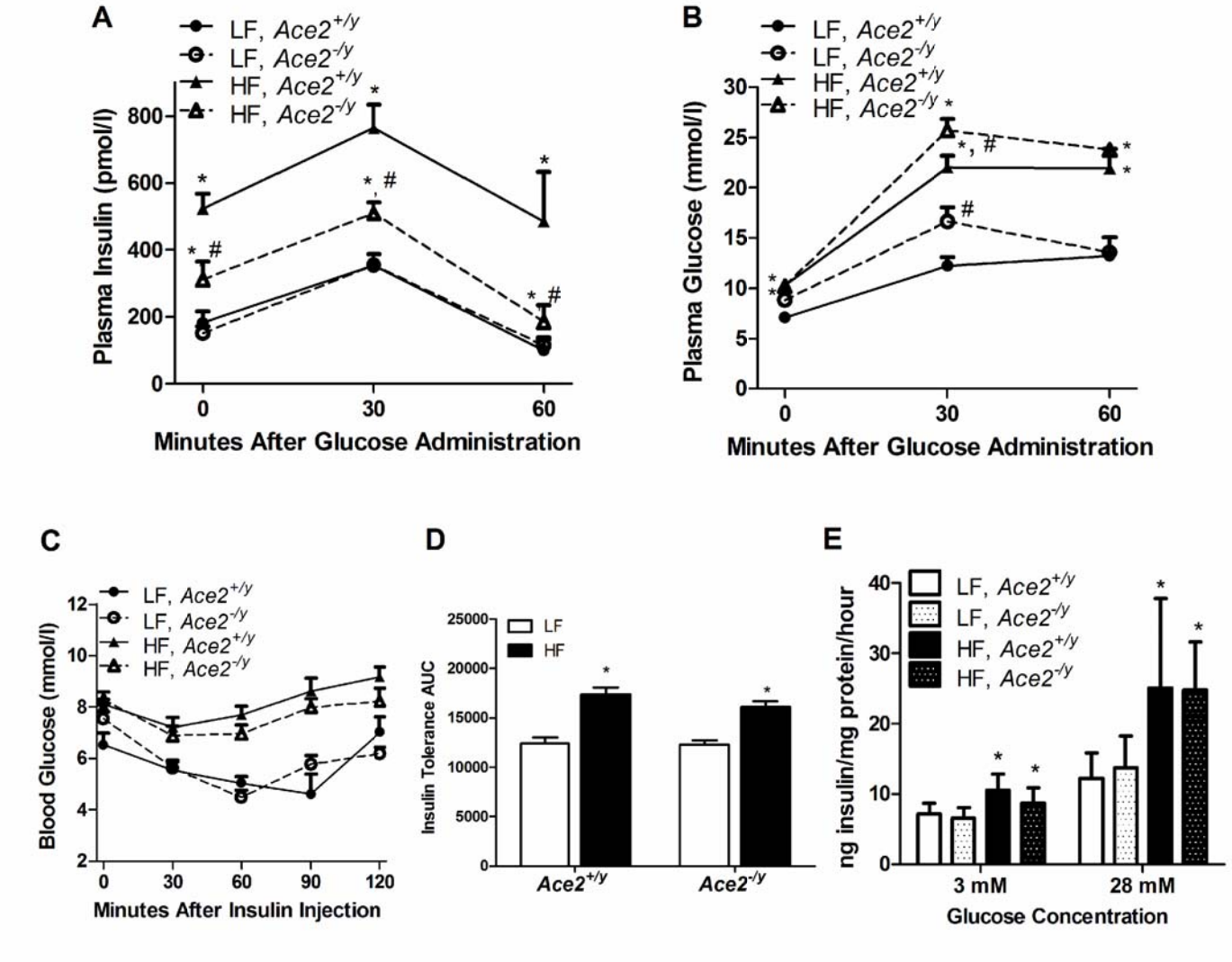

A
LF, Ace2+/y
LF, Ace2-/y
HF, Ace2+/y
HF, Ace2-/y
Plasma Insulin (pmol/l)
Minutes After Glucose Administration
B
Plasma Glucose (mmol/l)
Minutes After Glucose Administration
C
Blood Glucose (mmol/l)
Minutes After Insulin Injection
D
LF
HF
Insulin Tolerance AUC
Ace2+/y
Ace2-/y
E
ng insulin/mg protein/hour
3 mM
28 mM
Glucose Concentration

Figure 2.3 ACE2-deficient mice have impaired *in vivo* GSIS after 1 month of HF-feeding. (A) Plasma insulin concentrations and (B) corresponding plasma glucose concentrations following acute administration of glucose (2 g/kg body weight) in LF- and HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. *, P<0.01 compared to LF within time point and #, P<0.05 compared to *Ace2*$^{+/y}$ within time point. (C) Insulin tolerance test and (D) corresponding AUC in LF and HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. *, P<0.01 compared to LF. (E) Insulin release from pancreatic islets isolated from LF- and HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice (n=20 islets per mouse) at low (3mM) and high (28mM) glucose concentrations. *, P<0.05 compared to LF within treatment. Data are mean $\pm$ SEM from n=5-6 mice/group.

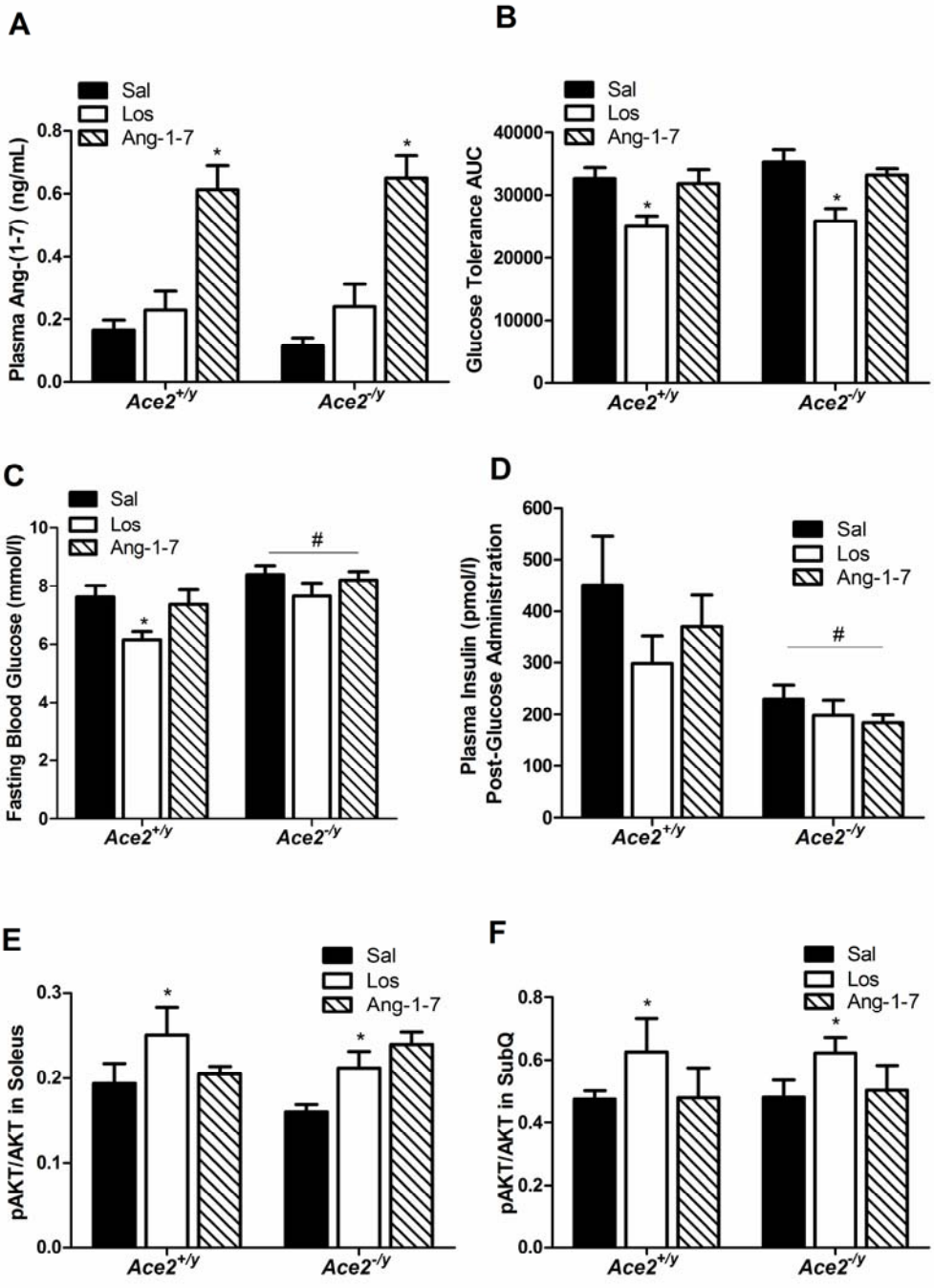

A
Sal
Los
Ang-1-7
Plasma Ang-(1-7) (ng/mL)
0.8
0.6
0.4
0.2
0.0
*
*
Ace2+/y
Ace2-/y
B
Sal
Los
Ang-1-7
Glucose Tolerance AUC
40000
30000
20000
10000
0
*
*
Ace2+/y
Ace2-/y
C
Sal
Los
Ang-1-7
Fasting Blood Glucose (mmol/l)
10
8
6
4
2
0
*
#
Ace2+/y
Ace2-/y
D
Sal
Los
Ang-1-7
Plasma Insulin (pmol/l)
Post-Glucose Administration
600
500
400
300
200
100
0
#
Ace2+/y
Ace2-/y
E
Sal
Los
Ang-1-7
pAKT/AKT in Soleus
0.3
0.2
0.1
0.0
*
*
Ace2+/y
Ace2-/y
F
Sal
Los
Ang-1-7
pAKT/AKT in SubQ
0.8
0.6
0.4
0.2
0.0
*
*
Ace2+/y
Ace2-/y

Figure 2.4 Neither AT1R antagonism nor infusion of Ang-(1-7) restore *in vivo* deficits in GSIS of HF-fed ACE2-deficient mice. (A) Infusion of Ang-(1-7) for 1 month increased plasma Ang-(1-7) concentrations in both *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ HF-fed mice compared to mice administered saline (Sal). Infusion of losartan (Los) had no effect on plasma Ang-(1-7) levels. *, P<0.05 overall effect of Ang-(1-7). (B) Glucose tolerance test in 1 month HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice administered Sal, Los, or Ang-(1-7). *, P<0.05 overall effect of Los. (C) Fasting blood glucose concentrations in mice of each genotype and treatment group. *, P<0.05 overall effect of Los. #, P<0.05 overall effect compared to *Ace2*$^{+/y}$. (D) Plasma insulin concentrations quantified at 60 minutes following glucose administration. #, P<0.05 overall effect compared to *Ace2*$^{+/y}$. (E and F) Ratio of pAKT to AKT in soleus muscle and subcutaneous (SubQ) adipose tissue, respectively, from *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice infused with Sal, Los, or Ang-(1-7). *, P<0.05 overall effect of Los. Data are mean $\pm$ SEM from n=5-8 mice/group.

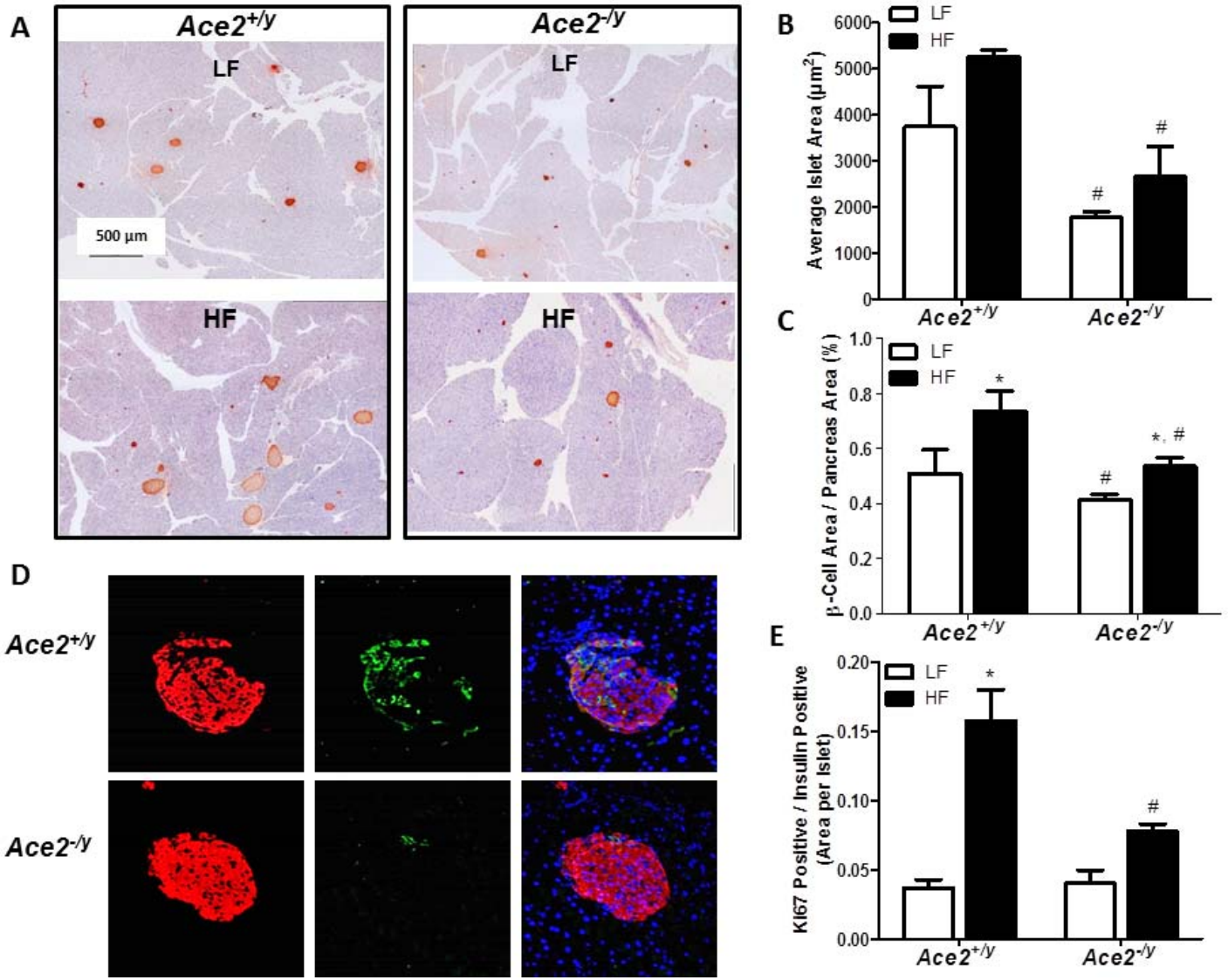

A
Ace2+/y
Ace2-/y
LF
HF
500 µm
B
Average Islet Area (µm2)
LF
HF
Ace2+/y
Ace2-/y
C
β-Cell Area / Pancreas Area (%)
D
Ace2+/y
Ace2-/y
E
Ki67 Positive / Insulin Positive (Area per Islet)

Figure 2.5 HF-fed ACE2 deficient mice have reduced β-cell mass and islet proliferation. (A) Representative 3mm x 3mm fields from pancreas tissue sections immunostained with anti-insulin antibody (red) in 1 month LF- and HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. (B) Average islet area in LF- and HF-fed mice of each genotype. #, P<0.01, compared to *Ace2*$^{+/y}$ within diet group. (C) β-cell mass as determined by the percent insulin-positive (β-cell) area per total area of pancreatic sections in LF- and HF-fed mice of each genotype. *, P<0.05, compared to LF within genotype; #, P<0.05, compared to *Ace2*$^{+/y}$ within diet group. (D) Representative immunofluorescence images in islets from HF-fed *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice – insulin (red), Ki67 (green) and DAPI staining of nuclei (blue). (E) Quantification of the Ki67^{+}/insulin^{+} ratio showing marked β-cell proliferation in LF- and HF-fed mice. *, P<0.001, compared to LF within genotype; #, P<0.01, compared to *Ace2*$^{+/y}$ within diet group. Data are mean $\pm$ SEM from n=3 mice/group.

Section III. SPECIFIC AIM 2

3.1 Summary

Diet-induced obesity promotes type 2 diabetes (T2D). Drugs that inhibit the renin-angiotensin system (RAS) have been demonstrated in clinical trials to decrease the onset of T2D. Pancreatic islets have been reported to possess components of the renin-angiotensin system (RAS), including angiotensin type 1a receptors (AT1aR). Previously, we demonstrated that mice made obese from chronic consumption of a high-fat diet (HF) have marked elevations in systemic concentrations of angiotensin II (AngII). We tested the hypothesis that pancreatic-specific deletion of the AT1aR would improve glucose-stimulated insulin secretion (GSIS) from isolated islets of mice with diet-induced obesity.

We demonstrated that GSIS from islets isolated from HF, but not low-fat (LF) mice was markedly diminished in the presence of AngII compared to vehicle (VEH). Based on these findings, we developed a mouse model of pancreatic-AT1aR deficiency. *AT1aR*$^{fl/fl}$ mice on a C57BL/6 background were bred to transgenic Cre mice expressing the pdx-1 promoter to generate pancreatic AT1aR-deficient mice (*AT1aR*^{pdx}) and littermate controls (*AT1aR*$^{fl/fl}$). AT1R mRNA expression was markedly decreased in whole pancreas, but not other tissues, in *AT1aR*^{pdx} mice. *AT1aR*^{pdx} mice had reduced pancreatic weight compared to littermate controls, but there was no difference in body weight. Further, there was no effect of pancreatic AT1aR deletion to impair glucose tolerance or GSIS *in vivo* in *AT1aR*^{pdx} mice compared to *AT1aR*$^{fl/fl}$ mice fed a standard murine diet.

Male, 8-week old *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice were fed a HF or LF diet for 16 weeks. There was no effect of pancreatic AT1aR-deficiency on the development of obesity. HF-fed mice became glucose intolerant and displayed elevated plasma levels of insulin with no significant difference between genotypes. However, there was an effect of pancreatic AT1aR-deficiency to impair GSIS *in vivo*. AngII significantly reduced insulin secretion from islets isolated from HF-fed wild-type mice. However, islets isolated from HF-fed pancreatic AT1aR-deficient mice did not exhibit significant reductions in AngII-mediated insulin secretion. Unexpectedly, pancreatic AT1aR-deficiency was associated with reduced GSIS *in vitro* and reduced islet insulin content. Taken together, these results suggest complex effects of pancreatic AT1aR deficiency, with possible developmental effects to impair pancreas growth but potential protective effects against AngII-induced β-cell dysfunction with HF-feeding. Future studies will define the relative role of developmental effects of pancreatic AT1aR deficiency from those regulating GSIS of pancreatic islets of HF-fed mice.

3.2 Introduction

According to the Centers for Disease Control and Prevention (CDC), 29.1 million adults in the United States (or 9.3% of the population) have diabetes with new cases diagnosed each year at a rate of 7.8%. Type 2 diabetes (T2D) is characterized by insulin resistance and the progressive loss of function of pancreatic β-cells. At the time of diagnosis, the functional capacity of β-cells is estimated to be about 25% or less (234) and a reduced β-cell function is evident several years prior to disease onset in individuals with impaired glucose tolerance (95). The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that declining insulin secretion over time is the main cause of failure to maintain long-term glycemic control. Further, current medical therapies for T2D, which substantially lower A1C or fasting plasma glucose compared to diet alone, were demonstrated to be ineffective against increasing loss of glycemic control over time (190) (96).

In a 2002 position statement by the American Diabetes Association (ADA), it was noted that epidemiological studies indicate a continuous association between the risk of cardiovascular complications and glycemia, as well as between systolic blood pressure and complications of diabetes such as stroke, diabetes-related deaths, heart failure, microvascular complications, and visual loss. Drugs that inhibit the renin-angiotensin system (RAS) have been used traditionally to treat hypertension and have been shown in clinical trials to improve cardiovascular outcomes beyond that of blood pressure control in both diabetic and non-diabetic patients (313) (76) (97). In addition to improved

cardiovascular protection, results from several clinical trials suggest that pharmacologic inhibition of the RAS may protect against the development of T2D (312) (192) (202) (20).

Inhibition of the RAS has been shown in animal models of T2D to improve insulin sensitivity and β-cell function (61) (235). Pancreatic β-cells express several components of the RAS, including the angiotensin type 1 receptor (AT1R), (55) (49) (166). Infusion of angiotensin II (AngII) to mice has been reported to promote hyperglycemia and reduce glucose-stimulated insulin secretion (GSIS) from isolated islets (58) (246). Conversely, administration of an AT1R antagonist to *db/db* or obese mice improved β-cell function and glycemic control (61) (68) (98). In humans, several clinical trials have demonstrated an effect of RAS blockade to reduce the risk of new-onset diabetes (NOD), although conflicting results have been reported. In the Diabetes Reduction Assessment (DREAM) study, which excluded patients with significant cardiovascular risk, the ACE inhibitor ramipril was shown to have no significant effect versus placebo on the incidence of NOD and only a moderate improvement in glycemia after three years of follow-up (37). The Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) study demonstrated 14% risk reduction in the development of T2D in patients with one or more cardiovascular risk factor (192). It is possible that the effectiveness of RAS blockade to reduce new-onset diabetes is seen only in patients with an activated RAS.

We have demonstrated that high-fat (HF) feeding in rodents is associated with development of obesity-hypertension and activation of the systemic RAS in

the form of elevated plasma concentrations of AngII (39) (121) (309). These results suggest that pancreatic β-cells of obese rodents may experience high concentrations of AngII acting at AT1R. Notably, AT1R gene expression in islets is reported to be increased by hyperglycemia (170). In this study, we hypothesized that elevated systemic or local AngII with obesity acts at pancreatic AT1aRs to reduce insulin secretion and contribute to the decline in β-cell function in obese mice. To test this hypothesis, we first examined effects of AngII on GSIS in islets from chronic HF-fed mice. Based on results from these studies, we generated mice with pancreas-specific deficiency of AT1aR and investigated the effect of AngII on insulin secretion from islets isolated from chronic HF-fed mice with or without pancreatic-AT1aR deficiency.

3.3 Materials and Methods

3.3.1 Experimental animals and diets.

All experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male C57BL/6 mice (8 weeks of age; The Jackson Laboratory, Bar Harbor, ME) were randomly assigned to receive either a low fat (LF, 10% kcal from fat; D12450B; Research Diets, New Brunswick, NJ) or high fat (HF, 60% kcal from fat; D12450B; Research Diets, New Brunswick, NJ) diet fed ad libitum for 4 months (n=10 per group).

To generate mice with pancreas-specific deletion of AT1aR, female mice with loxP sites flanking exon 3 of the AT1aR gene (*AT1aR*$^{fl/fl}$) were bred to male *AT1aR*$^{fl/fl}$ hemizygous transgenic mice expressing Cre recombinase under the control of the pancreas-duodenum homeobox-1 (pdx-1) promoter. Pdx-1, a transcription factor expressed throughout the pancreas in early embryogenesis but primarily restricted to insulin-producing β-cells in the adult pancreas, has been demonstrated as an effective promotor for use in Cre-mediated deletion of pancreatic proteins (103). The resulting offspring were either experimental animals with pancreatic-AT1aR deletion (*AT1aR*^{pdx}) or littermate controls (*AT1aR*$^{fl/fl}$). Male mice were maintained on standard murine diet (18% kcal from fat; Harlan Laboratories, Indianapolis, IN) until 8 weeks of age and then were randomly assigned to receive either a LF or HF diet fed ad libitum for 4 months (n=5-13 per group). Body weights were measured weekly. 8-week old male and

female mice were used for tissue characterization of *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice (n=9-13 per group). Pancreas, heart, liver, kidney, duodenum, stomach, spleen, lung, soleus, and perigonadal adipose tissue were dissected, frozen in liquid nitrogen and stored at -80°C until use. At study endpoint mice were anesthetized with ketamine/xylazine (100/10 mg/kg) for exsanguination and tissue harvest.

3.3.2 Extraction of DNA and RNA, quantification of mRNA abundance using real-time polymerase chain reaction.

DNA was extracted from pancreas (DNeasy; Qiagen, Alameda, CA) and cDNA was generated using the forward primer: 5'-TCTTCAAGACTGCTGATGTC and the following reverse primers: 5' – GGTTGAGTTGGTCTCAGAC (generates a 355 bp product between loxP site 1 and exon 3 of AT1aR gene demonstrating the presence of the floxed AT1aR gene) and 5' – GCAACTATGTCTGTCACTGG (generates a 423 bp sequence between loxP sites 1 and 3 demonstrating the deletion of exon 3 of the AT1aR gene). Total RNA was extracted from tissues using the SV total RNA Isolation System (Promega; Madison, WI) and from isolated pancreatic islets using TRIzol Reagent (Life Technologies; Grand Island, NY) followed by RNA Clean-up and Concentrator from Zymo Research (Irvine, CA) and quantity was assessed spectrophotometrically. Reverse transcription was performed using qScript cDNA Supermix (Quanta Biosciences; Gaithersburg, MD) and real-time PCR was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences; Gaithersburg, MD). Mouse primers used were as follows: AT1R and AT2R

primers were obtained from Qiagen (Alameda, CA); angiotensin converting enzyme 2 (ACE2), forward 5' – ACGAGATGGGACACATCCA, reverse 5' – GAAAATCGGATGGCAGAAGA; AT1b, forward 5' – ATGGGGAGCAGCCAAGAGGC, reverse 5' – CAGGGCAAGATTCAGAAGGA; 18S forward, 5' – AGTCGGCATCGTTTATGGTC, reverse 5' – CGAAAGCATTTGCCAAGAAT;. Data are expressed as ΔΔCt relative to 18S rRNA.

3.3.3 GSIS from isolated pancreatic islets.

The method for islet isolation was adapted from published protocols (270) (51). Pancreata were perfused *in vivo* through the common bile duct with 5 mLs of collagenase (0.6 mg/mL; Collagenase P; Roche, Indianapolis, IN) immediately following exsanguination. The excised pancreata were incubated in 10 mLs of collagenase at 37ºC for 15-20 minutes, and the tissue was then mechanically separated. Islets were purified from exocrine tissue using a sucrose gradient (Histopaque 1077; Sigma-Aldrich, St. Louis, MO), allowed to recover in culture media (RPMI, 10% FBS, 1% penicillin/streptomycin) for 48 hours, and further purified from exocrine tissue and debris by handpicking with a pipette and transferring to fresh media. Purified islets (20-25) were placed in inserts in 24 well plates (Greiner Bio One; Monroe, NC) and incubated in Krebs buffer with 3 mM glucose (vehicle), angiotensin II (AngII, 10 nM), or losartan (1μM) for one hour at 37ºC. The inserts containing the islets were then transferred to Krebs buffer with 28 mM glucose (vehicle), AngII (10 nM), or AngII plus losartan (1μM) and incubated for 1 hour at 37ºC. Insulin concentrations in media and in islets

(harvested in acid-ethanol) were quantified by ELISA using a commercial kit (Crystal Chem; Downers Grove, IL) and normalized to protein content of islets (BCA assay; ThermoFisher, Rockford IL) or total initial insulin concentration.

3.3.4 Glucose tolerance and plasma glucose/insulin/glucagon quantification.

Following a 6 hour fast, blood glucose concentrations were quantified using a glucometer (Freedom Freestyle Lite; Abbott Laboratories; Abbott Park, IL) immediately before and 15, 30, 60, 90, and 120 minutes following intraperitoneal (i.p.) administration of glucose (1.5 g/kg body weight). Glucose tolerance is expressed as either a time course following glucose administration or as area under the curve (AUC). Plasma insulin and glucose concentrations were quantified from blood samples (10µl) collected via tail stick in conscious mice (n=5 mice per group) following a 6 hour fast and again after i.p. administration of glucose (2 g/kg body weight). Plasma insulin concentrations were quantified by ELISA (Crystal Chem; Downers Grove, IL). Plasma glucagon concentrations were quantified in non-fasted mice by ELISA (Crystal Chem; Downers Grove, IL).

3.3.5 Statistical analysis.

Data are presented as mean ± SEM. Two-tailed Student's t-tests were used for analysis of data between two groups. Data were analyzed using two-way ANOVA with diet and treatment, diet and genotype, genotype and treatment, or genotype and time as between-group factors, where appropriate. If statistical differences existed between experimental groups, Holm-Sidac method was utilized for post hoc analyses. Values of $P<0.05$ were considered to be

statistically significant. All statistical analyses were performed using SigmaStat (SPSS).

3.4 Results

3.4.1 AngII inhibits GSIS from isolated islets of obese, glucose intolerant mice.

Mice fed a HF diet for 4 months became obese (Figure 3.1A; $P<0.001$), displayed elevated plasma concentrations of insulin compared to LF controls (Figure 3.1B; $P<0.05$), and developed glucose intolerance (Figure 3.1C, D; $P<0.01$). Pancreatic islets were isolated from LF- and HF-fed mice and stimulated with glucose to release insulin in the presence or absence of AngII, with or without losartan. In LF-fed mice, incubation of islets with AngII had no effect on GSIS (Figure 3.1E; $P>0.05$). However, in islets isolated from HF-fed mice, incubation with AngII significantly blunted insulin release compared to vehicle (Figure 3.1E; $P<0.05$). Incubation of islets with losartan totally abolished AngII-induced reductions in GSIS. Islets from HF-fed mice did not exhibit significant differences in AT1aR mRNA abundance (Figure 3.1F; $P=0.25$); however, ACE2 mRNA abundance was significantly decreased in islets from HF-fed mice compared to LF-fed controls (Figure 3.1G; $P<0.05$).

3.4.2 Development and characterization of a mouse model of pancreas-specific AT1aR deletion.

To test the hypothesis that elevated systemic levels of AngII with HF-feeding act at pancreatic AT1aR to decrease GSIS and impair glucose homeostasis, we created a mouse model of pancreas-specific AT1aR deficiency. The AT1aR gene was deleted from pancreas using the Cre-LoxP system driven by the pancreas-specific promoter, pdx-1 (Figure 3.2A). AT1aR deletion was confirmed

by PCR using DNA samples from whole pancreas (Figure 3.2B). In whole pancreas, AT1aR mRNA abundance was significantly decreased in *AT1aR*^{pdx} mice compared to *AT1aR*$^{fl/fl}$ controls (Figure 3.2C; $P<0.05$). In contrast, AT1R mRNA gene expression was not significantly different between genotypes in heart, liver, kidney, duodenum, stomach, spleen, lung, soleus, or adipose tissue (perigonadal). AT1bR gene expression in pancreas was not significantly different in *AT1aR*^{pdx} compared to *AT1aR*$^{fl/fl}$ mice (Figure 3.2D) and AT2R mRNA was not detected in either genotype.

In *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice fed standard murine diet, there was no significant difference in body weight (Figure 3.3A) or tissue weight of heart, liver, kidney, duodenum, stomach, spleen, lung, soleus, or adipose tissue (perigonadal) (Table 3.1). However, pancreas weight was significantly reduced in *AT1aR*^{pdx} mice compared to *AT1aR*$^{fl/fl}$ mice (Figure 3.3B; $P<0.01$). Reductions in pancreas weight did not alter pancreatic function, as there were no significant differences in glucose tolerance (Figure 3.3C,D), *in vivo* GSIS (Figure 3.3E), or in non-fasted plasma insulin concentrations (Figure 3.3F). In addition, non-fasted plasma glucagon concentrations did not differ between *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice (0.142 ± 0.008 *versus* 0.167 ± 0.011 ng/mL, respectively; $P=0.09$).

3.4.3 Pancreatic AT1aR deficiency has no effect on whole body glucose homeostasis in chronic HF-fed mice.

Pancreatic AT1aR deficiency had no significant effect on body weight in LF or HF-fed mice (Figure 3.4A). Chronic HF feeding resulted in significant impairment in glucose tolerance as indicated by a significant increase in area under the

curve (AUC) in both genotypes, with no significant differences between genotypes (Figure 3.4B). Plasma insulin concentrations were significantly increased in HF-fed mice of each genotype, with no significant differences between genotypes (Figure 3.4C, time 0; $P<0.05$). At 30 minutes post-glucose administration, plasma insulin concentrations were not significantly different between genotypes (Figure 3.4C, time 30; $P>0.05$).

3.4.4 Despite a lack of effect of pancreatic AT1aR deficiency to regulate *in vivo* GSIS, the effect of AngII to decrease *in vitro* GSIS from pancreatic islets is abolished in HF-fed mice with pancreatic AT1aR deficiency.

Pancreatic islets from HF-fed mice were isolated and stimulated with glucose to release insulin in the absence or presence of AngII. Similar to previous findings (Figure 3.1E), incubation of islets from HF-fed *AT1aR*$^{fl/fl}$ mice with AngII significantly inhibited GSIS, which was prevented by pre-incubation of islets with losartan (Figure 3.4D; $P<0.05$). In contrast, islets from HF-fed *AT1aR*^{pdx} mice had significantly reduced GSIS in the absence or presence of AngII compared to *AT1aR*$^{fl/fl}$ controls (Figure 3.4D; $P<0.05$). As a result, there was no effect of AngII to decrease GSIS in islets from HF-fed *AT1aR*^{pdx} mice. Insulin content of islets was significantly decreased in HF-fed *AT1aR*^{pdx} mice compared to *AT1aR*$^{fl/fl}$ controls (Figure 3.4E; $P<0.01$). As with previous findings (Figure 3.1G), HF-feeding was associated with a significant decrease in the islet mRNA abundance of ACE2 (Figure 3.4F; $P<0.05$). Notably, deletion of pancreatic AT1aR prevented HF-fed mediated reductions in ACE2 gene expression (Figure 3.4F).

3.5 Discussion

It has previously been reported that infusion of AngII in mice impairs glucose tolerance (58) (246), while blockade of the RAS improves whole body glucose homeostasis in mouse models of T2D (61) (68). However, systemic effects of AngII on insulin sensitivity *versus* effects of AngII directly at the pancreas in the regulation of β-cell function in models of T2D have not been extensively investigated. Results demonstrate that exposure of islets from HF-fed mice to AngII inhibited *in vitro* GSIS. This effect of AngII to decrease GSIS was not present in LF-fed mice, suggesting that AngII-induced β-cell dysfunction is augmented with chronic HF-feeding. We report the generation of a novel mouse model with pancreas-specific AT1aR deficiency. Notably, pancreas weight was reduced in pancreatic AT1aR deficient mice fed standard murine diet, suggesting a potential developmental effect of pancreatic AT1aR deficiency. Upon HF feeding, *in vivo* GSIS of pancreatic AT1aR deficient mice was not different from HF-fed controls. However, islets from HF-fed AT1aR deficient mice did not exhibit AngII-induced reductions in GSIS. Interestingly, HF-fed pancreatic AT1aR deficient mice exhibited reductions in islet insulin concentration. Taken together, our results suggest complex effects of pancreatic AT1aR deficiency, with potential detrimental developmental effects on the pancreas that are counterbalanced by protective effects to blunt AngII-induced β-cell dysfunction in HF-fed mice. Future studies will define the relative role of developmental effects of pancreatic AT1aR deficiency from those regulating GSIS of pancreatic islets of HF-fed mice.

AngII has been reported to regulate insulin secretion; however, results have been inconsistent. In mouse models of T2D, exposure to AngII has been reported to have an inhibitory effect on GSIS (61) (246) and blockade or silencing of the AT1R has been demonstrated to improve insulin secretion (68) (315). In contrast, AngII has been reported to stimulate insulin release in studies using chow-fed mice (112) or islets from non-diabetic human subjects (224). These data suggest potential stimulatory effects of AngII on insulin release under non-pathological conditions, but deleterious effects on β-cell function in conditions promoting T2D. The onset of T2D in humans is characterized by a decrease in insulin secretion and an inability to maintain hyperinsulinemia (223). Our results demonstrate that although HF-feeding resulted in impaired glucose tolerance, both wild-type and pancreatic AT1aR-deficient mice exhibited markedly increased plasma concentrations of insulin. Thus, the adaptive response to hyperinsulinemia with HF-feeding was maintained in both groups of mice fed a HF-diet. These data indicate that HF-fed C57BL/6 mice in this study did not progress to β-cell failure which may have prevented determination of the effects of pancreatic AT1aR-deficiency on glucose homeostasis *in vivo*. This is supported by a study demonstrating that even after 10 months of HF-feeding, total insulin secretion is sufficient to compensate for insulin resistance in C57BL/6 mice (7).

We report the novel and unexpected finding that pancreatic AT1aR deficiency reduced basal and glucose-stimulated insulin secretion *in vitro*, insulin content from isolated islets of HF-fed mice, and pancreas weight in mice fed standard

murine diet. Reductions in pancreas weight suggest that deletion of AT1aR may have a developmental effect on the endocrine pancreas. AT1R expression in the pancreas has been reported to be localized to β-cells, pancreatic vasculature, and acinar tissue (271) (173). The pdx-1 promoter is expressed during early stages of murine development throughout the entire pancreas, including both endocrine and exocrine cells, but expression is later restricted primarily to the insulin-producing β-cells (103). Thus, pdx-1 driven Cre-mediated deletion of AT1aR occurs in all pancreatic cell types. No AT1R expression has been reported in non β-cell endocrine cell types and no studies have specifically examined effects of AngII to regulate hormone release other than insulin from the endocrine pancreas. We report no effect of pancreatic AT1aR deletion on plasma glucagon concentrations, suggesting pancreatic AT1aR deletion does not affect α-cells; however, potential developmental effects of AT1aR deletion in exocrine or other islet cell types are unknown. Pancreatic progenitor cells express both AT1R and AT2R, where locally generated AngII was found to exert mitogenic and anti-apoptotic effects *in vitro* (171). Systemic blockade of AT2R, but not AT1R, in mice during the second transition in pancreatic development reduced the β-cell to α-cell ratio in neonate pups and impaired insulin secretory function and glucose tolerance *in vivo* (172). It is possible that deficiency of AT1aR in pancreas indirectly influenced pancreas development through unopposed AT2 receptor stimulation. However, as agonist effects of AngII at AT2R would be anticipated to increase, rather than decrease β-cell development, mechanisms for effects of AT1aR deficiency on pancreas weight, insulin content

and release are unclear. Future studies should utilize inducible deficiency of AT1aR from β-cells of adult mice to eliminate developmental effects of pancreatic AT1aR deficiency.

Pancreatic islets are highly vascularized and contain a functionally unique vascular network critical for rapid exchange of nutrients and hormones between the islet and the bloodstream (18). AngII is a potent stimulator of VEGF-induced angiogenesis (219) (137) (48) and islet endocrine cell-derived VEGF has been demonstrated to be a principal regulator of islet vascular development (41). Pancreas-specific deletion of VEGF-A in mice impaired development of islet capillaries, and although pancreatic-VEGF-A deficiency was not associated with a reduction in the total islet area of pancreas it was associated with a reduced pancreas weight (164). It is possible that AngII may play a role in islet endocrine cell development through modulation of VEGF-mediated development of islet vasculature. Interestingly, AngII stimulation of AT1R and AT2R have both been demonstrated to differentially regulate the VEGF driven angiogenic response in endothelial cells *in vitro* where AT1R induced and AT2R inhibited pro-angiogenic activity (48).

ACE2 has been demonstrated to improve β-cell function in animal models of T2D through increasing the activity of the Ang-(1-7)/Mas receptor axis of the RAS (28). Infusion of AngII into mice, resulting in impaired glucose tolerance, increased AT1R expression and decreased ACE2 expression in pancreas, which was prevented by pancreas-targeted ACE2 gene therapy (58). These results demonstrate an inhibitory effect of AngII on ACE2 expression in the pancreas.

Our results are consistent with these findings in that ACE2 gene expression was significantly decreased in islets isolated from HF- *versus* LF-fed mice. Moreover, pancreatic deletion of AT1aR prevented reductions in ACE2 gene expression in HF-fed mice. Increased activity of ACE2 may contribute to protective effects of pancreatic AT1aR deficiency in HF-fed mice.

In summary, results suggest complex effects of pancreatic AT1Rs with a potential role in pancreatic development, but deleterious effects during diet-induced development of T2D. AngII inhibited insulin release from islets isolated from HF-fed mice. However, in C57BL/6 HF-fed mice that did not exhibit deficits in β-cell function, there was no effect of pancreatic deficiency of AT1aR on *in vivo* β-cell function. Although pancreatic AT1aR-deficiency prevented AngII-mediated reductions in insulin secretion *in vitro*, there was an unexpected overall effect of reduced GSIS and insulin content of islets. Given that pancreas weight was reduced in pancreatic AT1aR-deficient mice, these results suggest a potential developmental defect as a result of pancreatic AT1aR deletion. Future studies will define developmental effects of pancreatic AT1aR deficiency *versus* those regulating GSIS of pancreatic islets of HF-fed mice and will focus on the effects of pancreatic AT1aR deletion to improve β-cell function in mice displaying a more pronounced β-cell failure phenotype.

Table 3.1 Tissue weights of *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice fed a standard murine diet.

Tissue Weight (g)	*AT1aR*$^{fl/fl}$		*AT1aR*^{pdx}	
	Male	**Female**	**Male**	**Female**
Heart	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
Liver	1.33 ± 0.06	1.06 ± 0.07	1.31 ± 0.12	1.22 ± 0.03
Kidney	0.39 ± 0.02	0.3 ± 0.02	0.36 ± 0.02	0.31 ± 0.01
Duodenum	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.11 ± 0.02
Stomach	0.19 + 0.01	0.19 ± 0.01	0.16 ± 0.01	0.18 ± 0.01
Spleen	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Lung	0.18 ± 0.02	0.20 ± 0.03	0.19 ± 0.01	0.18 ± 0.03
Soleus muscle	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Adipose (perigonadal)	0.46 ± 0.05	0.43 ± 0.14	0.38 ± 0.02	0.34 ± 0.07

Data are mean ± SEM from 9-13 per genotype

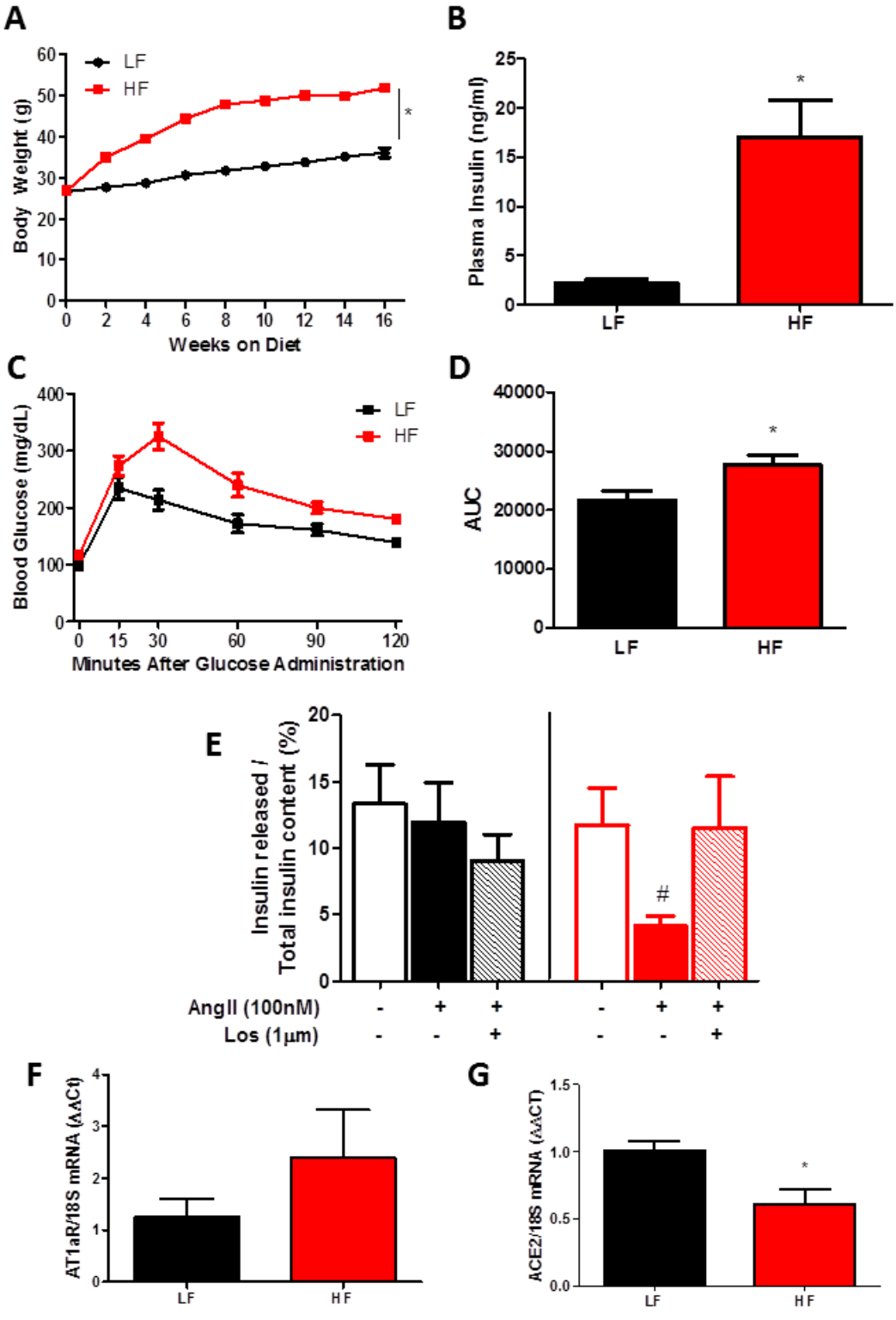

A
LF
HF
Body Weight (g)
Weeks on Diet
B
Plasma Insulin (ng/ml)
LF
HF
C
LF
HF
Blood Glucose (mg/dL)
Minutes After Glucose Administration
D
AUC
LF
HF
E
Insulin released / Total insulin content (%)
AngII (100nM)
Los (1μm)
F
AT1aR/18S mRNA (ΔΔCt)
LF
HF
G
ACE2/18S mRNA (ΔΔCT)
LF
HF

Figure 3.1 AngII inhibits GSIS from islets isolated from obese mice. (A) Body weights of mice fed a LF or HF diet for 4 months. (B) Non-fasted plasma insulin concentrations are increased in HF compared to LF-fed mice. (C) Glucose tolerance test and (D) corresponding area under the curve (AUC) indicating that HF-fed mice become glucose tolerant. (E) GSIS from islets isolated from LF- and HF-fed mice. Treatment of isolated islets with AngII blunted GSIS in HF-fed mice and the effect could be prevented by pretreatment with losartan. Data are represented as percent of initial total islet insulin content. (F and G) AT1R and ACE2 mRNA expression, respectively, in isolated pancreatic islets. Data are mean $\pm$ SEM from n=10 mice per group. *, $P<0.01$, compared to LF; #, $P<0.05$ effect of AngII compared to vehicle.

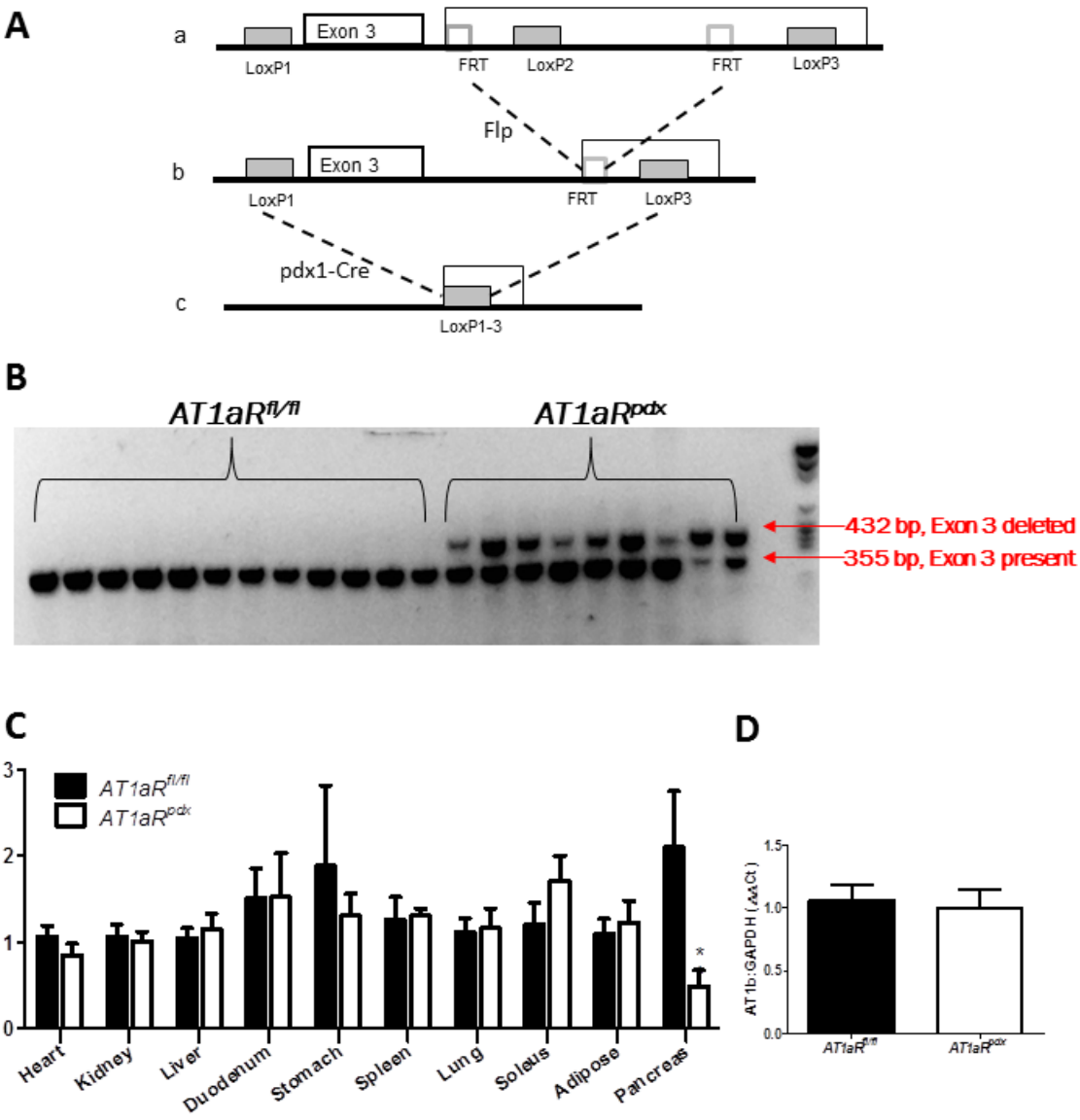

A
a
Exon 3
LoxP1
FRT
LoxP2
FRT
LoxP3
Flp
b
Exon 3
LoxP1
FRT
LoxP3
pdx1-Cre
c
LoxP1-3
B
AT1aRfl/fl
AT1aRpdx
432 bp, Exon 3 deleted
355 bp, Exon 3 present
C
AT1aRfl/fl
AT1aRpdx
AT1R:18S mRNA (ΔΔ Ct)
Heart
Kidney
Liver
Duodenum
Stomach
Spleen
Lung
Soleus
Adipose
Pancreas
*
D
AT1b:GAPDH (ΔΔCt)
AT1aRfl/fl
AT1aRpdx

Figure 3.2 Development and characterization of a mouse model of pancreas-specific AT1aR deletion. (A) Mice with loxP sites flanking exon 3 of the AT1aR gene *(a)* were bred to mice expressing flippase (FLP) which recognizes the FRT sites to remove the neocassette *(b)* $AT1aR^{fl/fl}$ mice were bred to transgenic mice expressing Cre recombinase driven by the pdx-1 promoter to generate AT1aR deficient mice ($AT1aR^{pdx}$) and non-transgenic littermate controls ($AT1aR^{fl/fl}$) *(c).* (B) PCR reactions were performed with DNA samples extracted from pancreas. Primers were designed to detect the deleted portion of exon 3 of the AT1aR gene. Exon 3 was deleted as demonstrated by the presence of a 432 base pair product. (C) Tissue characterization of AT1R mRNA gene expression demonstrating AT1R deletion is specific to pancreas. (D) AT1b mRNA expression is not different between $AT1aR^{fl/fl}$ and $AT1aR^{pdx}$ mice. Data are mean $\pm$ SEM from n= 9-13 mice per group. *, $P<0.05$ versus control.

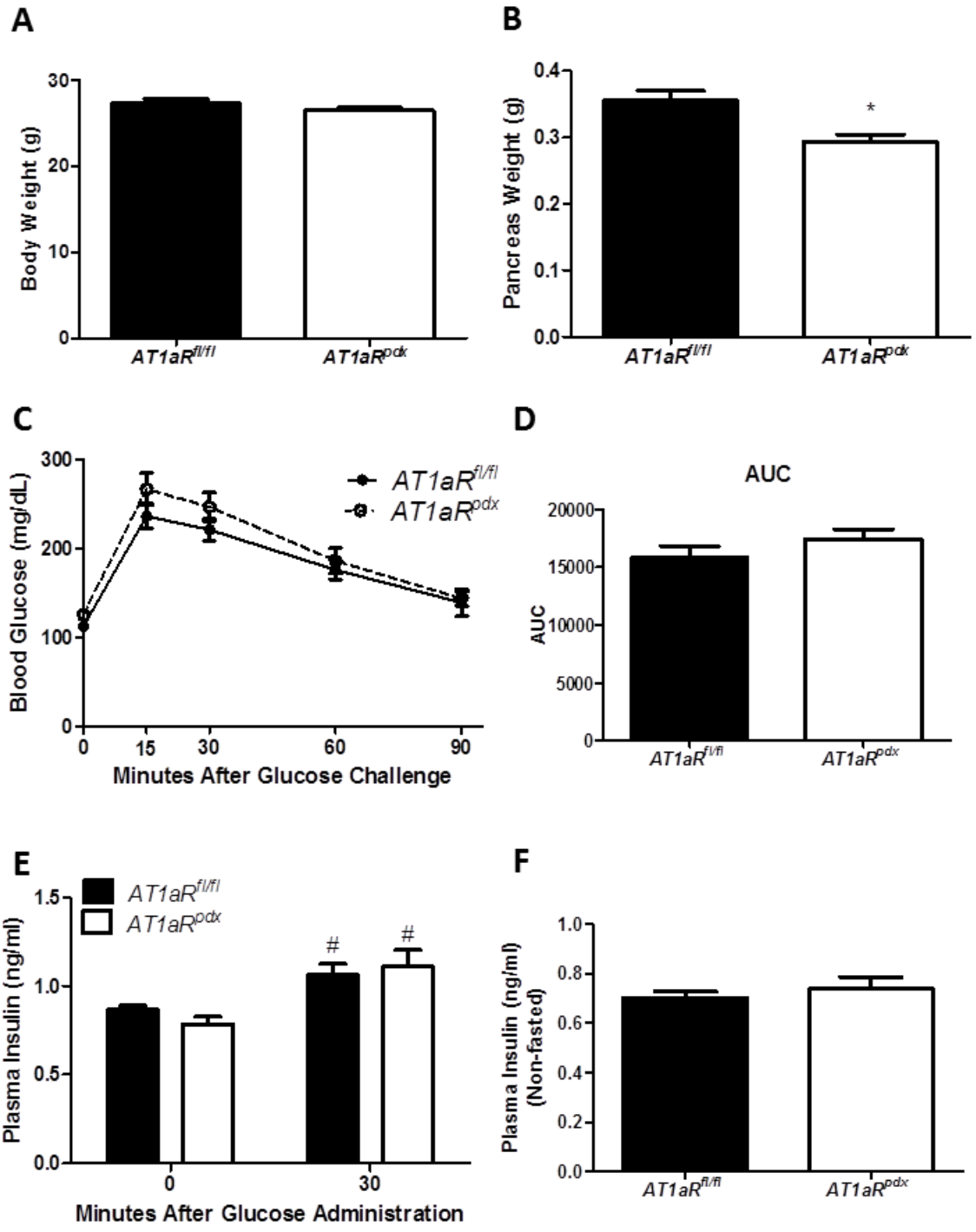

A
Body Weight (g)
30
20
10
0
AT1aR^fl/fl
AT1aR^pdx
B
Pancreas Weight (g)
0.4
0.3
0.2
0.1
0.0
*
AT1aR^fl/fl
AT1aR^pdx
C
Blood Glucose (mg/dL)
300
200
100
0
AT1aR^fl/fl
AT1aR^pdx
0
15
30
60
90
Minutes After Glucose Challenge
D
AUC
AUC
20000
15000
10000
5000
0
AT1aR^fl/fl
AT1aR^pdx
E
Plasma Insulin (ng/ml)
1.5
1.0
0.5
0.0
AT1aR^fl/fl
AT1aR^pdx
#
#
0
30
Minutes After Glucose Administration
F
Plasma Insulin (ng/ml) (Non-fasted)
1.0
0.8
0.6
0.4
0.2
0.0
AT1aR^fl/fl
AT1aR^pdx

Figure 3.3 8-week old pancreatic AT1aR-deficient mice fed a standard diet exhibit normal glycemia. (A) Body weight and (B) pancreas weight of 8-week old *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice. (C) Glucose tolerance and (D) corresponding AUC indicating no difference in glucose tolerance between *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice. (E) Plasma insulin following a 6 hour fast (0 minute time point) and 30 min following an intraperitoneal glucose challenge. Both genotypes respond to glucose with an increase in plasma insulin but there is no different between genotypes. (F) Non-fasted plasma insulin. Data are mean ± SEM from n=9-12 mice per group. *, P<0.05 compared to *AT1aR*$^{fl/fl}$; #, P<0.01 compared to 0 minute time point.

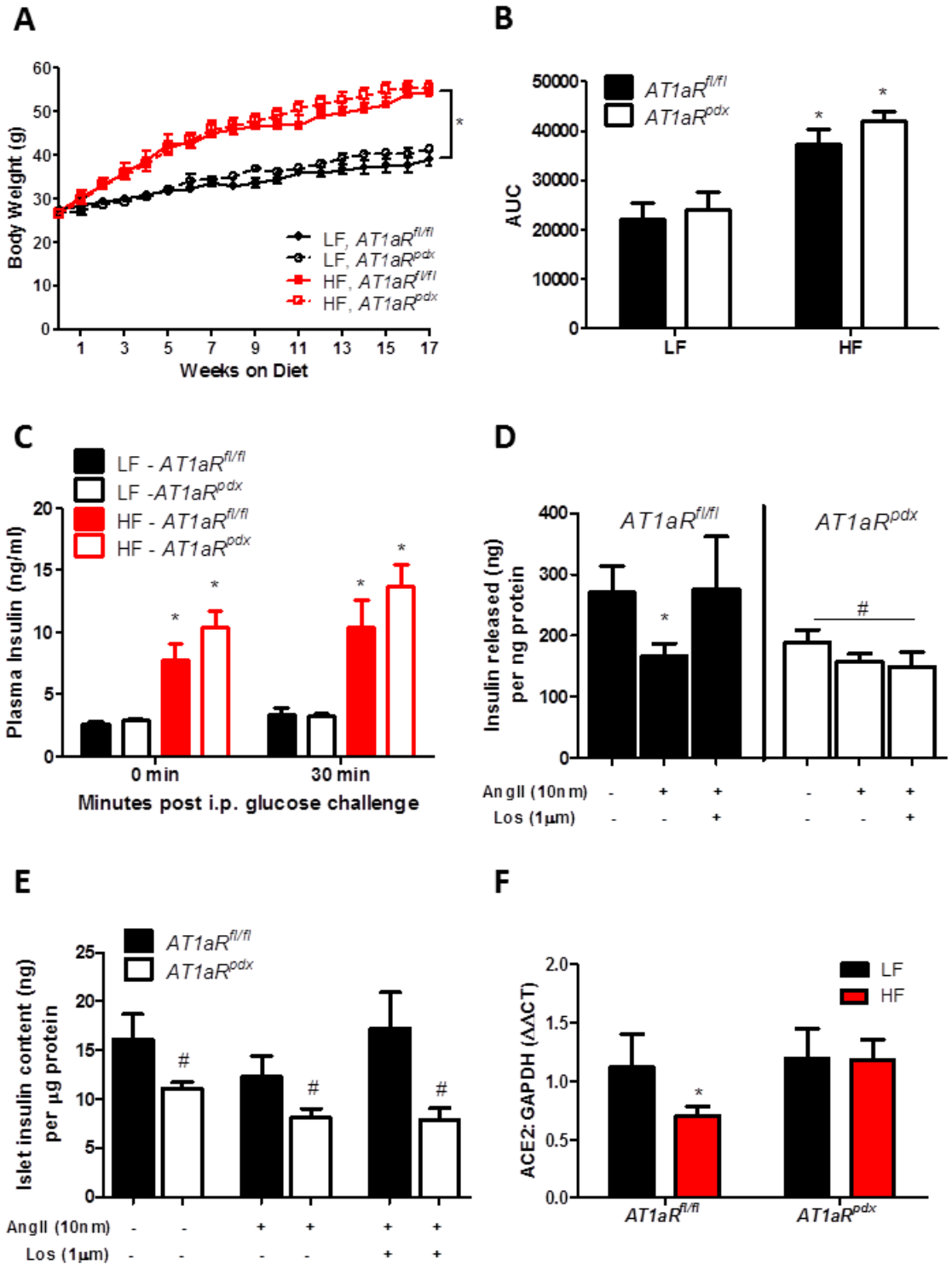

A
Body Weight (g)
Weeks on Diet
LF, AT1aR fl/fl
LF, AT1aR pdx
HF, AT1aR fl/fl
HF, AT1aR pdx
B
AUC
AT1aR fl/fl
AT1aR pdx
LF
HF
C
Plasma Insulin (ng/ml)
LF - AT1aR fl/fl
LF -AT1aR pdx
HF - AT1aR fl/fl
HF - AT1aR pdx
0 min
30 min
Minutes post i.p. glucose challenge
D
Insulin released (ng) per ng protein
AT1aR fl/fl
AT1aR pdx
AngII (10nm)
Los (1µm)
E
Islet insulin content (ng) per µg protein
AT1aR fl/fl
AT1aR pdx
AngII (10nm)
Los (1µm)
F
ACE2: GAPDH (ΔΔCT)
LF
HF
AT1aR fl/fl
AT1aR pdx

Figure 3.4 AngII-induced reductions in insulin secretion from islets are prevented in mice with pancreatic-deletion of AT1aR, however this is not manifest as improved glucose homeostasis

(A) Body weight progression in LF-and HF-fed *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice. (B) GSIS from islets isolated from obese *AT1aR*$^{fl/fl}$ and *AT1aR*^{pdx} mice. Insulin secretion is normalized to total protein content. AngII has no effect on insulin secretion from islets isolated from HF-fed *AT1aR*^{pdx} mice, whereas insulin secretion is significantly reduced with AngII treatment in wild-type mice. There is an overall effect of genotype to reduce insulin secretion in *AT1aR*^{pdx} mice. (C) Glucose tolerance represented as AUC. (D) Plasma insulin following a 6 hour fast and 30 min following an intraperitoneal glucose challenge Although pancreatic AT1aR-deficiency prevented AngII-mediated reductions in insulin secretion *in vitro*, this did not translate to reduced insulin levels *in vivo* (either fasting or post-glucose challenge). Importantly, both groups of mice fed the HF-diet display marked hyperinsulinemia, indicating a lack of progression to β-cell failure. Data are mean $\pm$ SEM for n=3-4 mice per group for LF and n= 7-11 mice per group for HF. *, $P<0.01$ overall effect of diet; *, $P<0.05$ effect of AngII compared to vehicle; #, $P<0.05$ overall effect of genotype.

Section IV. GENERAL DISCUSSION

4.1 Summary

The purpose of the studies described in this dissertation was to test the hypothesis that the RAS contributes to the decline of β-cell function in T2D. We examined the role of two opposing proteins, ACE2 and AT1aR, in the regulation of β-cell function and glucose stimulated insulin secretion (GSIS) *in vitro* and *in vivo*. Specifically, we hypothesized that deficiency of ACE2, which would increase the balance of angiotensin II (AngII) to angiotensin-(1-7 (Ang-(1-7)), contributes to the decline in β-cell function with T2D. Conversely, we hypothesized that in mice with HF diet-induced obesity, elevated systemic or local concentrations of AngII act at pancreatic AT1Rs to inhibit insulin secretion.

Whole-body ACE2 deficiency was associated with a reduction in the adaptive hyperinsulinemic response to chronic obesity, which was manifest as hyperglycemia in the fed state. β-cell dysfunction was evident as early as 1 month of HF-feeding, as HF-fed ACE2-deficient mice had impaired plasma GSIS. Reductions in plasma insulin secretion *in vivo* were not a result of a secretory defect, but rather due to reduced β-cell mass associated with impaired proliferative capacity of islets. Notably, reduced β-cell mass was evident in both LF- and HF-fed mice with ACE2-deficiency. Finally, neither AT1R blockade nor administration of Ang-(1-7) could restore deficits in *in vivo* GSIS in HF-fed ACE2-deficient mice. These results demonstrate that ACE2 regulates β-cell mass and is a critical regulator in the β-cell compensatory response to HF-diet induced insulin resistance (Figure 4.1).

The contribution of AngII acting at pancreatic AT1aRs to regulate β-cell function with T2D was also studied. In pancreatic islets isolated from HF-fed mice, incubation with AngII markedly diminished GSIS and this could be prevented by pre-treatment with losartan. Based on these findings, a novel mouse model of pancreas-specific deletion in AT1aR was developed to investigate the role of pancreatic AT1R to regulate insulin secretion in mice with diet-induced obesity. Upon HF-feeding, there was no effect of pancreatic AT1aR-deficiency to increase *in vivo* GSIS or improve glucose tolerance. However, islets from HF-fed mice deficient in pancreatic AT1R did not exhibit AngII-mediated reductions in insulin secretion (Figure 4.2). Interestingly, pancreatic AT1aR deficiency was associated with reduced GSIS *in vitro* and reduced islet insulin content. Notably, pancreas weight was reduced in pancreatic AT1aR-deficient mice fed standard murine diet. Taken together, these results suggest complex effects of pancreatic AT1aR deletion, with potential deleterious effects on pancreatic development that are counterbalanced by protective effects against AngII-mediated β-cell dysfunction with HF-feeding.

Overall, the results of these studies suggest complex roles of ACE2 and AT1aR to regulate β-cell function both during normal pancreas development and in response to chronic obesity. Loss of ACE2 may have potential developmental effects to decrease β-cell mass that are not severe enough to impact glucose homeostasis under normal conditions. However, in the face of a metabolic challenge like obesity, the reduced adaptive capacity of β-cells becomes manifest as hyperglycemia. While loss of ACE2 seems to exert a deleterious

effect during development and also reduces β-cell function of adult obese mice, loss of AT1aR may have differential effects on developing versus adult β-cell function. Reductions in pancreas weight, insulin secretion, and insulin content with pancreatic-AT1aR deficiency suggest a role for AngII/AT1aR in normal pancreas development. However, an ability of AngII to reduce insulin secretion in islets from HF-fed (but not LF-fed) mice suggest detrimental effects of AngII/AT1aR on β-cell function during the development of T2D.

4.2 Mechanisms of RAS-mediated impairment in β-cell function

Traditionally, studies of the role of the RAS to regulate β-cell function have focused on effects of AngII mediated by the AT1R that may contribute to a decline in β-cell function with T2D. Purported mechanisms of AngII-mediated β-dysfunction include reduced insulin secretion due to impaired islet blood flow, increased inflammation and fibrosis, increased oxidative stress, and increased apoptosis. Recently, focus has broadened to include favorable effects conferred by the opposing protein, ACE2. Interestingly, beneficial effects of ACE2 are not always attributed to the mere counterbalance of the deleterious effects of AngII. The following sections highlight findings from our studies in relation to previously reported effects of AngII or ACE2 on the regulation of pancreatic function.

4.2.1 Effects on islet blood flow

Previous studies suggest reductions in insulin secretion may be mediated by reduced islet blood flow due to vasoconstrictor properties of AngII on the microvasculature in the pancreas (50). We have demonstrated that chronic HF feeding in mice is associated with elevated plasma concentrations of AngII that

contribute to the development of obesity-hypertension (122). Whether improvements in glucose tolerance associated with systemic pharmacologic inhibition of the RAS are due to suppression of an over-active pancreatic RAS versus improved pancreatic blood flow due to blockade of systemic vasoconstrictor properties of AngII has been debated. Recent results demonstrated that blood pressure normalization in AngII-infused HF-fed mice did not restore AngII-induced deficits in β-cell function, demonstrating that AngII directly regulates β-cell function independent of its vasoconstrictor properties (246). Our data agrees with these findings in that incubation of islets with AngII *ex vivo* directly reduced GSIS.

A contribution of ACE2 to regulate β-cell function by influencing islet blood flow has not been well studied. In mice infused with AngII, overexpression of ACE2 in the pancreas did not improve islet blood flow but did improve glucose tolerance (58), suggesting that improved blood flow is not part of the mechanism by which ACE2 improves glucose homeostasis. In contrast, deficiency of ACE2 may negatively impact islet vascularization. VEGF expression and islet vascularization were reported to be reduced in obese ACE2-deficient mice with impaired plasma GSIS (311). The authors concluded that reduced islet function in ACE2-deficient mice was due to reduced compensation of islet vascularization with long-term HF-feeding. Given that AngII is an established positive regulator of VEGF-driven angiogenesis (219) (137) (48), the finding that islet VEGF expression and vascularization are reduced in mice with ACE2 deficiency (with presumed elevations in AngII) is counter-intuitive. Our results demonstrate that

short-term HF-feeding (1 month) impairs β-cell mass and islet proliferation in ACE2-deficient mice, but we did not define whether impaired islet vascularization may have contributed to reductions in β-cell proliferation. Alternatively, previously observed reductions in vascularization of islets in ACE2 deficient mice may be a reflection of reduced β-cell mass, as demonstrated in this study. Notably, neither administration of an AT1R antagonist or infusion of Ang-(1-7) reversed the effects of ACE2 deficiency to impair GSIS *in vivo*. As systemic delivery of these agents would be anticipated to influence their ability to regulate islet blood flow, these results also suggest that regulation of islet blood flow is not the primary mechanism for ACE2-mediated regulation of β-cell mass.

4.2.2 Effects on oxidative stress and inflammation

The deleterious effects of AngII to promote β-cell dysfunction may be driven in part by increased oxidative stress. It is well accepted that AngII-stimulation of NADPH contributes to increased production of superoxide and contributes to the pathology of cardiovascular diseases (107). AngII has been demonstrated to induce superoxide via NADPH oxidase activation in isolated pancreatic islets (132) and antagonism of AT1R downregulated components of NADPH oxidase in β-cells of db/db mice resulting in improved β-cell function (250) (62). In addition to antagonism of the AT1R, overexpression of ACE2 in the pancreas was also reported to reduce AngII-mediated increases in oxidative stress (58).

AngII has been recognized as a key mediator of inflammation in a number of diseases, including chronic kidney disease (237) and vascular diseases (77). Obesity and T2D are associated with chronic activation of the innate immune

system (214) and systemic concentrations of proinflammatory cytokines are positively correlated with progression of T2D (260) (130). Local inflammation of pancreatic islets has gained recognition as a contributing factor to progressive β-cell dysfunction in T2D (86). In human and mouse islets, as well as murine β-cell lines, exposure to AngII increases mRNA expression of proinflammatory cytokines (59) (246). Blockade of the RAS in rodent models of obesity reduced serum and adipose levels of proinflammatory cytokines (68) and reduced gene expression of inflammatory markers in pancreatic islets (310). Recently, the effects of AngII infusion to induce islet inflammation in HF-fed mice were demonstrated to be mediated by IL-1β, a cytokine previously shown to play a key role in obesity-associated β-cell dysfunction (246). Reduced plasma GSIS with AngII infusion was reversed with subcutaneous injection of anti-IL-1β antibodies. We report no difference in plasma insulin secretion or islet mRNA expression of IL-1β in HF-fed mice with pancreatic AT1aR deficiency. However, HF-fed mice of both genotypes in our study displayed marked hyperinsulinemia, suggesting 4 months of HF-feeding in C57BL/6 mice was insufficient to induce progression to β-cell failure. We anticipate that under experimental conditions promoting β-cell failure, pancreatic AT1aR-deletion would diminish IL-1β-mediated islet inflammation.

Effects of ACE2 to counter the negative effects of AngII on islet inflammation have not been reported; however, one study reported that Ang-(1-7) reduced expression of inflammatory factors in a pancreatic cell line *in vitro* (291). Interestingly, there are other deleterious effects of AngII that are not reported to

be countered by ACE2. For example, AT1R blockade has been shown to reduce apoptosis in β-cells (62). However, overexpression of ACE2 in the pancreas of db/db mice had no effect on apoptosis, but rather increased proliferation of β-cells (28). These results are consistent with our finding that ACE2 deficiency markedly impaired β-cell proliferation but had no effect on apoptosis. AT1R antagonism has been demonstrated to improve islet fibrosis in mouse models of T2D (277) (250). However, neither ACE2 deficiency nor ACE2 overexpression have been reported to improve fibrosis in β-cells. Taken together, these results suggest that ACE2 may have beneficial effects on β-cell function that extend beyond merely reducing the actions of AngII.

4.2.3 Role of the ACE2/Ang-(1-7)/MasR axis

Many of the beneficial effects of ACE2 have been attributed to increased activity of the Ang-(1-7)/MasR axis. For example, in db/db mice with pancreatic overexpression of ACE2, improvements in β-cell proliferation were abolished by administration of a MasR antagonist (28). However, most reported effects of Ang-(1-7) to improve glucose homeostasis have been ascribed to peripheral improvements in insulin sensitivity. Infusion of Ang-(1-7) improved insulin sensitivity in non-obese mice fed a high-sucrose diet (272) and an oral formulation of Ang-(1-7) improved glycemia and insulin sensitivity in rodent models of T2D (241) (241) (207). Our results demonstrate that in obese ACE2-deficient mice, infusion of Ang-(1-7) at a dose that elevated plasma Ang-(1-7) concentrations and reportedly blunted an activated RAS (275) (276) had no effect on glucose homeostasis and did not correct deficits in plasma GSIS.

Differences in the dose or formulation of Ang-(1-7), coupled with varying models of T2D may have contributed to diverging effects of the peptide on glucose homeostasis.

4.3 Other substrates of ACE2

4.3.1 Dynorphin

In addition to AngII, ACE2 is capable of hydrolyzing the peptide dynorphin-(1-13) with high catalytic efficiency (288). Dynorphins are a class of opioid peptides primarily expressed within the central nervous system. Dynorphins are thought to contribute to regulation of the hypothalamo-pituitary axis and may have a role in energy homeostasis (238). Insulin secretion from isolated rat islets increased with dynorphin incubation (116), but intravenous administration of dynorphin to mice had no effect on insulin secretion (6). No other studies have been published investigating the role of dynorphins on insulin secretion or β-cell function.

4.3.2 Apelin

ACE2 is capable of catabolizing the peptide apelin by cleaving the terminal amino acid, rendering it biologically inactive (288). ACE2 is the only enzyme known to regulate apelin metabolism (150). Apelin, the endogenous ligand of the G-protein coupled APJ receptor, has been shown to regulate cardiac contractility (269). The APJ receptor has significant homology to the AT1R, although AngII is not a ligand for the receptor (204). The effects of apelin are thought to be cardioprotective and counterbalance pathological effects of AngII. Apelin mutant mice demonstrated impaired cardiac contractility and hypertrophy, and increased

vasoconstrictor responses to AngII, which are rescued by both blockade of AT1aR or treatment with Ang-(1-7) (245). Additionally, apelin upregulates ACE2 expression *in vitro* and *in vivo* (245) further supporting a role for apelin to mitigate actions of AngII.

The APJ receptor is expressed in pancreatic islets (259) and incubation of isolated mouse islets with apelin inhibited insulin secretion (259) by activating PI3-kinase-dependent phosphodiesterase 3B with subsequent suppression of cAMP levels (120). Apelin is expressed by adipocytes and circulating levels of apelin are increased with obesity (38). Further, insulin was demonstrated to positively regulate apelin expression in adipocytes (38). This suggests that the effects of apelin to decrease insulin secretion may serve as a β-cell protective mechanism under conditions of increased insulin demand. However, the effects of apelin on insulin secretion may be exacerbated with ACE2-deficiency, especially in the context of obesity and increasing apelin concentrations. Further, ACE2 expression is upregulated by apelin, suggesting apelin is subject to negative feedback regulation, which may be disrupted with ACE2 deficiency. Thus, increased actions of apelin may contribute to decreased plasma insulin concentrations in ACE2-deficient mice. Our results demonstrate reduced β-cell mass in ACE2-deficient mice, suggesting that possible effects of apelin on insulin secretion are in addition to effects of ACE2 deficiency to impair β-cell proliferation. To date, no studies have investigated the role of apelin in the regulation of β-cell mass.

4.4 Non-enzymatic roles of ACE2

ACE2 is well known for its role to cleave AngII to generate Ang-(-1-7) and its primary function is thought to be the regulation of the relative abundance of these two opposing peptides. Considerably less is known about the non-enzymatic functions of ACE2, particularly as it pertains to regulation of glucose homeostasis. The following sections provide insight into effects of ACE2 that may contribute to regulation of β-cell function independently of regulation of peptide balance.

4.4.1 ACE2 and collectrin

ACE2 shares 47.8% sequence homology with the membrane protein collectrin, or transmembrane protein 27 (TMEM27), in the transmembrane and cytosolic regions but collectrin lacks a dipeptidyl-peptidase domain (314). Both ACE2 (211) and collectrin (101) (248) are reported to be downstream targets of HNF1-α which is mutated in MODY3 (305). Islet gene expression of collectrin is reported to be increased in obese mouse models of T2D (101) (12). Overexpression studies demonstrate that collectrin increases insulin release *in vitro* (13) and *in vivo* by regulating exocytosis (101), and mice with whole-body knockout of collectrin exhibit reduced serum insulin concentrations (188). Collectrin has been reported to regulate β-cell mass and proliferation, but mixed results have been reported as overexpression of collectrin increased β-cell mass *in vivo* (12), while whole-body collectrin deficiency had no effect on β-cell mass (188).

Gene expression of collectrin was reported to be reduced in whole pancreas of mice with whole-body deficiency of ACE2 (25). Since the gene encoding collectrin is located adjacent to, but not overlapping, the gene encoding ACE2 on the X chromosome (314), reduced expression of collectrin in ACE2-deficient mice is not likely to be an artifact of ACE2 deletion. It is not known whether collectrin is regulated by ACE2 or vice versa. However, it must be considered whether reduced GSIS *in vivo* with ACE2 deficiency may be a result of either reduced collectrin expression or mediated by loss of the collectrin domain of the ACE2 protein. We report that ACE2 deficiency had no effect on *ex vivo* GSIS of isolated islets, but rather reduced β-cell mass, suggesting that loss of the collectrin domain is not the mechanism for impaired adaptive hyperinsulinemia with ACE2 deficiency.

4.4.2 Binding of ACE2 to β1-integrin

Recent studies demonstrate that by acting as a binding partner, ACE2 can influence expression and function of other proteins. ACE2 has been shown to bind β1 integrin *in vitro* (65) and an interaction of ACE2 with β1 integrin was reported in the left ventricle of human hearts in heart failure (177). In this study, an ACE2 activity assay demonstrated that an ACE2/β1 integrin protein complex isolated by immunoprecipitation was capable of hydrolyzing AngII, but not AngI. The investigators proposed that the interaction between ACE2 and β1 integrin was a regulatory mechanism for ACE2 activity and localization. In another study, ACE2 augmented integrin-mediated cell adhesion and modulated integrin

signaling *in vitro*, suggesting that ACE2/integrin interactions may play a regulatory role in cellular attachment (65).

No studies have investigated whether ACE2 binds β1 integrin in β-cells, but β1 integrin has been documented to regulate a variety of functions in the endocrine pancreas, including adhesion and migration of progenitor cell populations (63) and secretory functions in adults islets (149). Recently, β1 integrin was demonstrated to be a crucial regulator of pancreatic β-cell expansion (84). Diaferia et al demonstrated that mice with β-cell specific deletion of β1 integrin displayed defective β-cell expansion during development, resulting in reduced β-cell mass that persisted into adulthood. Despite significantly reduced β-cell number, loss of β1 integrin did not affect differentiation or function of β-cells. Interestingly, with respect to glucose homeostasis, adult mice with β-cell-specific deficiency of β1-integrin displayed a phenotype similar to that of ACE2-deficient mice reported in this dissertation; specifically a reduced plasma insulin output in response to glucose. These data make it tempting to speculate that the effects of ACE2 deficiency to reduce β-cell mass and plasma insulin secretion may be related to a reduction in ACE2/ β1 integrin-mediated regulation of β-cell mass. In the study by Diaferia et al, ablation of β1 integrin affected expression of genes that regulate cell cycle progression. Other studies have demonstrated that deletion of critical cell cycle regulators in β-cells impairs β-cell mass and plasma insulin secretion (reviewed by Heit, et al (127)).

4.4.3 ACE2 association with the neutral amino acid transporter, B°AT1

In addition to binding integrins, ACE2 has also been demonstrated to associate with the neutral amino acid transporter, B°AT1 (Slc6a19). B°AT1 co-transports neutral amino acids with Na+ across the apical membrane in intestine and kidney (89). Mutations in this transporter lead to Hartnup disorder, an autosomal recessive disorder characterized by neutral aminoaciduria (155) (247). Both ACE2 and collectrin serve as partner proteins to B°AT1 in the intestine and kidney proximal tubule, respectively (46). ACE2 has been shown to be necessary for B°AT1 expression in luminal intestine (46) and ACE2 null mice exhibit defective intestinal amino acid transport associated with decreased weight gain in pups following weaning (254). Expression of B°AT1 has not been reported in pancreas or β-cells, and no studies have identified potential implications of ACE2 association with B°AT1 on glucose homeostasis. However, since amino acids co-transported with Na+ are capable of stimulating insulin secretion (200), it is possible that decreased B°AT1 expression with ACE2 deficiency may contribute to reduced insulin secretion in ACE2 deficient mice.

4.5 Study Limitations

4.5.1 Limitations of the model of whole-body deficiency of ACE2

4.5.1.1 Effects of ACE2 deficiency on fetal development

Maternal ACE2 deficiency has been reported to negatively impact both maternal gestational body weight gain and pup weight (27), so the effect of ACE2 deficiency to impair intestinal amino acid uptake may have a nutritional consequence on the development of the pancreas in utero. It is well documented

that intrauterine growth restriction (IUGR) as a consequence of maternal under-nutrition or protein restriction results in low birth weight and increases the risk for impaired glucose homeostasis or T2D in both animals and humans (216). In particular, because fetal β-cell mass is primarily established during gestation, β-cell mass is particularly sensitive to alterations in the intrauterine environment (40). Fetal malnutrition or IUGR in rodents is associated with reduced β-cell mass in adult offspring (106) (253) and while β-cell mass may be sufficient to maintain glycemia under normal conditions, the effects of perinatal malnutrition may limit adaptive expansion of β-cell mass under conditions of increased demand later in life (105). Thus, effects of ACE2 deficiency to reduce the adaptive response to obesity in adults may be a consequence of inadequate establishment of β-cell mass in utero as a result of maternal malnutrition. Alternatively, IUGR affecting fetal development of β-cell mass with ACE2 deficiency could be due to increased placental ischemia due to vasoconstriction, as increased concentrations of AngII were reported in the placenta of pregnant ACE2-deficient mice (27).

4.5.1.2 ACE2 and insulin resistance

It has been reported that ACE2 deficiency affects insulin resistance, although results have been conflicting. In one study, ACE2 deficient mice were reported to be insulin resistant compared to wild-type controls due to a reduction in GLUT-4 in skeletal muscle (102). In contrast, a recent study reported that ACE2 deficiency shifts energy metabolism towards glucose utilization and this was associated with increased insulin sensitivity and an increase GLUT4 mRNA

abundance in skeletal muscle in ACE2 deficient mice compared to wild-type (25). We report no effect of ACE2 deficiency on insulin resistance and no difference in pAKT/AKT ratios in insulin-sensitive tissues with HF-feeding. Such varying results could be due to differences in the generation or source of ACE2-deficient mice, differences in diet compositions, and differences in the strain of wild-type controls. Differences in insulin sensitivity with ACE2 deficiency could impact β-cell adaptive responses to obesity. Use of a model of tissue specific deletion of ACE2 in β-cells would allow for dissemination between potential peripheral versus β-cell specific effects of ACE2 deficiency to regulate β-cell function.

4.5.2 Limitations of the model of pancreatic-AT1aR deficiency

The model of pancreatic AT1aR deficiency used in these studies was developed using the Cre/LoxP system where Cre-mediated deletion of exon 3 (the coding region) of the AT1aR gene was driven by the pdx-1 promoter. Pdx-1 is expressed in the vertebrate posterior foregut endoderm in a region destined to become the antral stomach, pancreas, and rostral duodenum (258) (103). AT1R receptor expression has been reported in gastric (123) (123) and duodenal (146) tissues and AngII was shown to impair intestinal absorption of water and ions *in vitro* (174). This has implications for possible pdx-1 driven deletion of AT1aR in the gastrointestinal tract in our mouse model to affect nutrient absorption or fluid homeostasis. However, AT1aR mRNA abundance was not significantly different in pancreatic-AT1aR deficient mice compared to controls. Although no further investigation was made into possible effects of AT1aR deletion in the

gastrointestinal tract, these data suggest there is no Cre-mediated deletion of the AT1aR gene in stomach or duodenum.

During the early stages of embryogenesis, pdx-1 is expressed in both endocrine and exocrine cells of the pancreas, although the primary abundance in adults is in insulin-producing β-cells (103). Thus, pdx-1 driven Cre-mediated deletion of AT1aR occurs in all pancreatic cell types. Validation of cell-specific deletion of AT1aR at the protein level is difficult due to lack of a specific AT1aR antibody (225) (131). However, AT1R expression in the pancreas has been reported to be localized to β-cells, pancreatic vasculature, and acinar tissue (271) (173) and no AT1R expression has been reported in non β-cell endocrine cell types.

4.5.3 Limitations of the use of diet-induced obesity (DIO) as a model for T2D

DIO in C56BL/6 mice is widely used as a model for T2D. In this dissertation, this model was selected based on previous findings that plasma concentrations of AngII are elevated in C57BL/6 mice fed a HF diet for 16 weeks (121) which provide a basis for the central hypothesis that activation of the RAS with HF-feeding contributes to β-cell dysfunction with T2D. While DIO in C56BL/6 mice has been reported to be a good model for the development of insulin resistance (266), results from this study indicate that this model is not well-suited for the study of β-cell failure with T2D. We report month-to-month increases in plasma glucose levels at up to 4 months of HF-feeding, indicating steady compensation to insulin resistance. This is consistent with other reports that decreases in

insulin secretion contributing to glucose intolerance are not evident in HF-fed C57BL/6 mice until 10 months of HF-feeding (7). Future studies should consider using a model better suited for study of β-cell failure, such as *db/db* mice, which display evidence of β-cell failure by 3-4 months of age (70).

4.5.4 Limitations of measuring glucose *in vivo*

Plasma and blood glucose measurements in this dissertation were performed using the Freedom Freestyle Lite glucometer (Abbott Laboratories). It should be noted that blood glucose can be rapidly modulated by the SNS (16) and that care should be taken to ensure blood glucose measurements are taken under conditions designed to minimize stress to the animal. In this study, all measurements were taken by the same investigator in the same procedural manner to increase accuracy of glucose measurements. Further, accuracy of the glucometer, as published by Abbott Laboratories, is reported to be $\pm$ 20%.

4.6 Clinical Significance

4.6.1 Inhibition of the RAS as a treatment for T2D

Causes of T2D vary widely from monogenic to polygenic forms of T2D and the risk for development of T2D is complicated by environmental factors, like obesity, and concomitant health conditions, like cardiovascular disease. Current therapeutics for T2D are focused on the management of glycemia, and treatment is based on where an individual lies on the continuum of T2D, rather than the pathology of individual patients. Clinical trials have demonstrated that RAS blockade reduced the risk of new onset diabetes in patients with one or more cardiovascular risk factors (312) (178) (287) (202) (20). Results from meta-

analyses suggest that treatment with an ARB or ACE inhibitor reduces the incidence of T2D by 16-30% (141) (4) (279). However, in most of the trials where risk for developing T2D was reduced with RAS blockade, T2D was not a major endpoint.

Results from the two large prospective studies specifically designed to investigate the effect of RAS inhibition on the development of T2D were less favorable than anticipated. In the DREAM trial, 3 years of ramipril treatment reduced the incidence of T2D by 9%, but the finding was not significant (37); the incidence of T2D was significantly reduced by rosiglitazone (109). However, regression from IGT to normoglycemia was increased in patients given ramipril versus placebo (37), suggesting a positive effect of RAS inhibition to control glycemia. Notably, the patient population in the DREAM trial was free from cardiovascular diseases. In the NAVIGATOR trial, valsartan and concomitant lifestyle modification reduced the incidence of T2D by 14% versus lifestyle modification alone, and patients exhibited reduced fasting and post-load glucose following an oral glucose test (192). Although findings from meta-analyses suggest blockade of the RAS is favorable for reducing the incidence of T2D, findings vary depending on the patient population, study endpoint, pharmacologic agent, and duration of study. Such distinctions may be important for identifying patient populations most likely to benefit from pharmacologic inhibition of the RAS to improve diabetes outcomes.

In the majority of clinical trials indicating an effectiveness of RAS blockade to reduce the risk for T2D, the patient population displayed some form of CVD. The

RAS is upregulated in many cardiovascular pathologies including hypertension (39), cardiac hypertrophy (11), and heart failure (283) and clinical trials demonstrate that blockade of the RAS improves cardiovascular outcomes (67) (21). Incubation of human islets in high glucose conditions increased gene expression of RAS components (183) (185) and treatment with ARBs or ACE inhibitors has been demonstrated to improve β-cell function in humans patients with hypertension (215) (267) (285). Further, obesity is associated with an activation of the systemic RAS (221), and most patients with T2D are obese. Taken together, these results suggest that the beneficial effects of RAS inhibition to reduce the incidence of T2D may be most effective in patient populations most likely to have an increased systemic RAS.

Since T2D is a multi-organ disease, effects of systemic RAS blockade may have multiple effects beyond direct action at β-cells that ultimately contribute to improved β-cell function. RAS inhibitors have been demonstrated to improve insulin resistance in animals and humans (141) (220) (285) and some ARBs, such as telmisartan, may provide additional insulin sensitizing effects by agonizing PPAR-γ (176). Improvements in insulin sensitivity may improve β-cell function by reducing the demand for insulin and thereby protecting against β-cell exhaustion. Both ACE inhibitors and ARBs have been demonstrated to have systemic anti-inflammatory effects (176), which may serve to reduce the exposure of β-cells to the harmful milieu that can potentiate β-cell dysfunction. RAS inhibition as a therapeutic option is attractive due to the beneficial effects

systemically, at peripheral tissues and β-cells, and because of effectiveness to mitigate dangerous co-existing cardiovascular diseases.

4.6.2 ACE2 as a novel therapeutic treatment

In addition to pharmacologic inhibition of the RAS, studies in animal models of T2D demonstrate that ACE2, the physiological negative regulator of the RAS, may be a therapeutic target for treatment of T2D. Interestingly, ACE2 is downstream target of HNF1-α (211), and mutations in this transcription factor are responsible for the most frequent monogenic form of diabetes, MODY3 (99). MODY3, a distinct subtype of T2D, is characterized by progressive β-cell dysfunction requiring insulin treatment with onset occurring by about 25-35 years of age (195). Lehto et al demonstrated that individuals with MODY3 exhibited decreased fasting and incremental plasma insulin concentrations compared to individuals with non-insulin-dependent T2D. Compared to individuals with non-insulin-dependent T2D, a key characteristic of MODY3 is the lack of an insulin resistance phenotype, and importantly, low insulin responses to glucose in patients with MODY3 were not a function of increased insulin sensitivity.

Mouse models with mutated HNF1-α in β-cells exhibit hyperglycemia, reduced β-cell mass and proliferation rate, and reduced insulin response to glucose (306). This phenotype is similar to that observed in our studies where ACE2 deficient mice displayed hyperglycemia and reduced β-cell mass and proliferation compared to wild-type controls. ACE2 overexpression in the pancreas of db/db mice improved glycemia, β-cell mass and proliferation (28).

Taken together, these data specifically suggest a role for ACE2 as a therapeutic target in individuals with MODY3.

4.7 Future Directions

4.7.1 Exploration of ACE2 as a downstream target of HNF-1α

The exact role of ACE2 in the regulation of glucose homeostasis has yet to be elucidated and there are many possible options for mechanisms by which ACE2 contributes to the regulation of β-cell mass. One area that has yet to be fully investigated is the relative roles of the catalytic versus collectrin-homologous domain of ACE2. ACE2 expression or activity has not been characterized in human patients with MODY3 or animal models with mutations of HNF1-α. However, decreased expression of collectrin has been documented in mice with HNF1-α-deficiency (101). Deletion of collectrin in mice impairs insulin secretion, but does not impair β-cell mass (188), suggesting effects of ACE2 in the regulation of β-cell function extend beyond effects conferred by the collectrin domain. The potential dual function of ACE2 to regulate β-cell mass and proliferation as well as to increase insulin secretion as a function of the collectrin domain makes ACE2 activation an attractive therapeutic target for patients with MODY3 or other forms of T2D characterized primarily by defects in β-cell function.

4.7.2 Implications of an ACE2/β1-integrin association in the regulation of β-cell mass

HF-fed ACE2 deficient mice exhibit impaired GSIS as a result of reduced β-cell mass due and impaired β-cell proliferation; however, mechanisms by which

ACE2 regulates cell proliferation are unknown. In adult mice, antagonism of the AT1R or infusion of Ang-(1-7) in ACE2 deficient mice to restore the peptide balance did not restore plasma GSIS, suggesting mechanisms unrelated to the enzymatic function of ACE2. No studies have reported direct effects of ACE2 in the regulation of the cell cycle. However, ACE2 has been reported to bind with β1-integrin and influence integrin signaling (65) and β1-integrin has been demonstrated to regulate β-cell mass possibly through signaling cascades regulating cell cycle proteins (84). These data suggest a novel role for ACE2 in the regulation of β-cell proliferation mediated by protein-protein interactions with β1-integrin. Future studies would demonstrate co-localization between ACE2 and β1-integrin in β-cells and investigate the role of an ACE2/β1-integrin association in the regulation of cell cycle proteins and β-cell proliferation.

4.7.3 Developmental versus post-natal roles of ACE2 to regulate β-cell function

Evidence suggests an effect of ACE2 deficiency to impair β-cell development, confounding the role of ACE2 in the adaptive β-cell response to obesity in adults. Future directions include disseminating the relative roles of ACE2 in the development of β-cell mass versus post-natal regulation of β-cell mass. This includes not only discerning between maternal effects of ACE2 deficiency to alter the intrauterine environment versus the role of ACE2 to regulate β-cell mass during fetal embryonic development, but determining if effects of ACE2 are related to angiotensin peptide balance versus other, non-enzymatic roles of ACE2. Our results suggest a role for ACE2 in the post-natal, adaptive β-cell

response to obesity. Future studies would be best carried out using an inducible Cre-driven β-cell-specific mouse model.

4.7.4 Use of conditional models of cell-specific AT1aR deletion

We report reduced pancreatic weight and reduced insulin content and GSIS *in vitro* in mice with pancreatic AT1aR deficiency, suggesting protective effects of AT1aR during development of T2D may be masked by a potential developmental effect. A mouse model where Cre-mediated deletion of AT1aR could be induced in adulthood would be more effective to define specific effects of AT1aR deletion to protect against the detrimental effects of AngII during HF-diet-induced T2D. A variety of mouse models conferring conditional inactivation of genes in various cell types of pancreas exist and were extensively described in a recent review (187)

4.7.5 Potentiation of β-cell failure to determine effects of pancreatic-AT1aR deletion to protect against AngII-mediated β-cell dysfunction

HF-fed C57BL/6 mice are commonly used as a mouse model for T2D due to the rapid development of profound insulin resistance accompanied by hyperinsulinemia (302). However, in an *in vivo* study, the effects of short-term versus long-term HF-feeding demonstrated that over three months, impaired glucose effectiveness was the primary contributor to IGT in C57BL/6 mice. Decreased acute insulin secretion was observed after 10 months of HF-feeding in this strain, although total insulin secretion was still sufficient to compensate for insulin resistance (7). We report marked hyperinsulinemia in pancreatic-AT1aR-deficient mice after 4 months of HF-feeding, indicating a lack of progression to β-

cell failure. Since we did not observe β-cell failure in chronic HF-fed C57BL/6 mice, this most likely precluded an ability to detect a significant effect of pancreatic AT1aR deficiency on glucose homeostasis. In a recent study, infusion of AngII via osmotic pump for 28 days to 12-week HF-fed C57BL/6 mice decreased GSIS *in vivo* (246). We plan to follow a similar study design using our mouse model of pancreatic AT1aR-deficiency. We anticipate that infusion of AngII will augment detrimental effects of HF-feeding on β-cell function and hypothesize that AngII-infused mice with pancreatic AT1aR-deficiency will have improved insulin secretion and glucose tolerance *in vivo*.

4.8 Concluding remarks

Overall these studies demonstrate a protective role for ACE2 in the adaptive β-cell response to hyperinsulinemia and a detrimental effect of AngII/AT1R on β-cell function during the development of diet-induced T2D. ACE2-deficiency reduced the adaptive response to increase β-cell mass in HF-fed mice, which did not appear to be a result of imbalances in the RAS. These data suggest a critical role for ACE2 to regulate β-cell proliferation and growth and that ACE2 activation may be a novel therapeutic option to improve β-cell function. Pancreatic-deletion of AT1aR prevented AngII-mediated decreases in insulin secretion, but may be associated with potential developmental defects in pancreatic development. Future studies should define the developmental versus post-natal effects in cell-specific models of β-cell failure of ACE2 and AT1aR deletion.

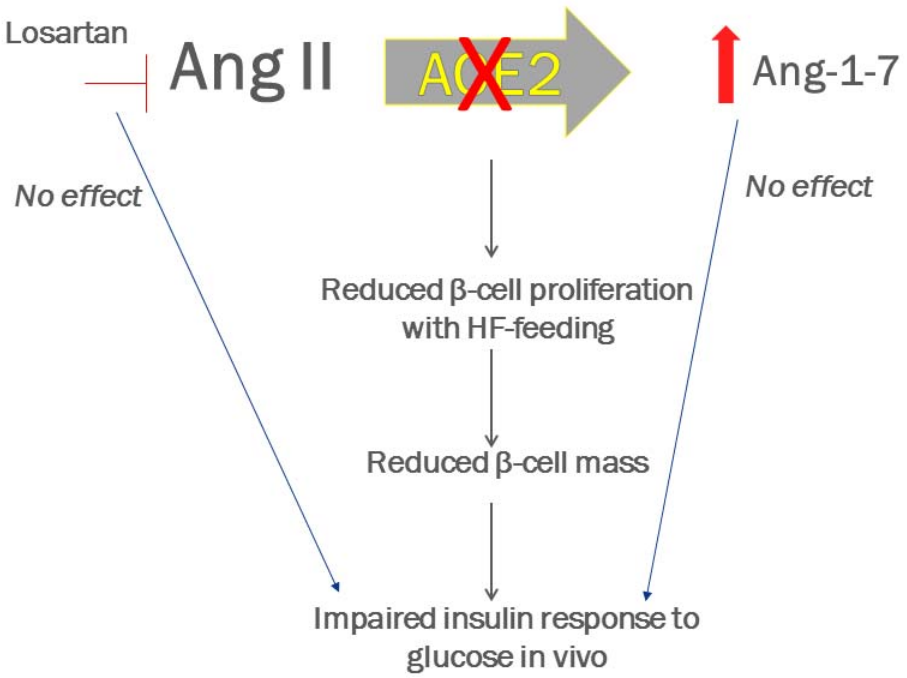


Figure 4.1 ACE2 plays a role in β-cell adaptation in response to obesity. ACE2 deficient mice fed a HF diet have reduced hyperinsulinemia and impaired *in vivo* GSIS. In adult mice, insulin deficits cannot be corrected by AT1R antagonism or infusion of Ang-(1-7) to restore the peptide balance in ACE2 deficient mice. Rather, ACE2 deficiency reduced the adaptive response to increase β-cell mass associated with deficits in β-cell proliferation. These results demonstrate that ACE2 is a critical regulator of β-cell proliferation and growth.

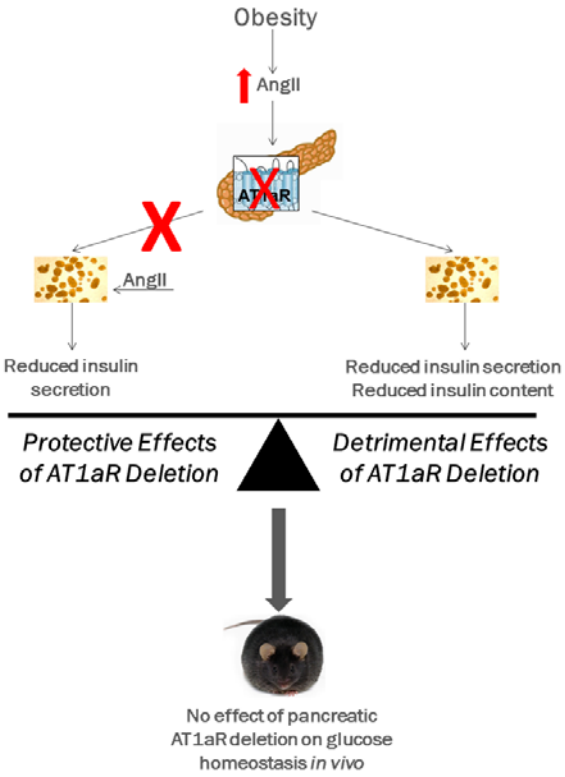


Figure 4.2 Pancreatic-AT1aR deficiency prevents AngII-mediated reductions in insulin secretion *ex vivo* in islets from HF-fed mice but has no effect on glucose tolerance *in vivo*. Deletion of AT1aR from pancreas prevents AngII-mediated reductions in GSIS from islets isolated from HF-fed mice. However, pancreatic AT1aR deficiency is associated with reduced GSIS and reduced insulin content from isolated islets, which may be due to a detrimental effect of AT1aR deletion on normal pancreas development. Taken together, these results suggest complex effects of pancreatic AT1aR deficiency, with potential detrimental developmental effects on the pancreas that are counterbalanced by protective effects to blunt AngII-induced β-cell dysfunction in HF-fed mice. These offsetting factors may contribute to the lack of observed effect of pancreatic AT1aR deletion on glucose homeostasis *in vivo.*

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Publications

Shoemaker, R; Yiannikouris, F; Thatcher, S; Cassis, L. ACE2 Deficiency Reduces Beta-Cell Mass and Impairs Beta-Cell Proliferation in Obese C57BL/6 Mice. (Manuscript under review *AJP Endocrinology and Metabolism*, 2015)

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