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Abigail Tice

West Virginia University, altice@mix.wvu.edu

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The Effects of Obesity, Chronic Stress, and Exercise on Pancreatic Health

Abigail Lynn Tice

Thesis to be submitted to the School of Medicine at West Virginia University in partial
fulfillment of the requirements of the degree:

Masters of Science in Exercise Physiology

Randall W. Bryner, Ed.D., Committee Chair/Research Advisor

Paul D. Chantler, Ph.D.

I. Mark Olfert, Ph.D.

Division of Exercise Physiology

Morgantown, West Virginia

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Oxidative stress, apoptosis, thioredoxin-interacting protein (TXNIP)

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Abstract
The Effects of Obesity, Chronic Stress, and Exercise on Pancreatic Health

Abigail L. Tice

Obesity is concomitant with hyperglycemic conditions, increased proinflammatory cytokines, and increased oxidative stress. Under hyperglycemia, thioredoxin interacting protein (TXNIP) is increased and causes increased oxidative stress and pancreatic β -cell apoptosis. Chronic stress is associated with increased glucocorticoid levels and a proinflammatory environment. The purpose of this study was to examine the effects of obesity, chronic stress, and exercise on pancreatic health.

Lean Zucker Rats (LZR) and Obese Zucker Rats (OZR) were randomly assigned to four groups: 1) sedentary controls (C: n=8 per); 2) unpredictable chronic mild stress (UCMS) (S: n=8); 3) exercise (E: n=8); and 4) combined UCMS and exercise (ExS: n=8). Exercise rats underwent 8 weeks of treadmill running for 60 minutes. Stress rats were subjected to UCMS that included cage tilt, no bedding, damp bedding, bath, social stress, and altered light-dark cycles for 8 hours a day, 5 days a week for 8 weeks. Pancreas tissue was obtained from rats around 20 weeks of age. Total pancreatic TXNIP protein was quantified, including total pancreatic cytokine expression for IL-1 β , TNF- α , IL-4, and IL-10. Using immunohistochemistry, β -cell and α -cell counting, islet area measurements, and β -cell to α -cell ratio were examined. TUNEL staining, to determine apoptosis, was examined in pancreatic lysates.

OZR had higher insulin, glucose, and body mass compared to LZR, as well as higher β -cell and α -cell number and islet area ($p<0.05$). TXNIP in pancreas had an increasing trend in OZR-C compared to LZR-C ($p=0.10$); TNF- α and IL-4 were higher in OZR-C compared to LZR-C ($p<0.05$). Little to no differences in morphology or TXNIP in either genotype of stressed animal. OZR-S had lower TNF- α , IL-4, and IL-6 when compared to OZR-C ($p<0.05$). Islet area and α -cell numbers were lower with exercise in obese animals, while β -cell to α -cell ratio was higher ($p<0.05$). TXNIP was not different, but IL-4 and IL-6 were lower in OZR-EX compared to OZR-C ($p<0.05$). β -cell and α -cell numbers and islet area were higher in LZR-EXS compared to LZR-S ($p<0.05$), but no differences were seen in the obese model of stress and exercise. TXNIP was lower in LZR-EXS compared to LZR-S ($p<0.05$), and TXNIP trended to decrease in OZR-EXS compared to OZR-S ($p=0.10$). No differences were observed in cytokines in these groups. No differences were observed in any groups with apoptosis.

In a model of obesity and metabolic syndrome, the pancreas appeared to be stimulated to grow as indicated by greater numbers of β - and α -cells and larger islets even in the presence of slightly higher pancreatic TXNIP and TNF- α . Obesity resulted in an increased level of inflammation and reduced antioxidant potential within the pancreas as indicated by significantly higher TXNIP, IL-1 β , and TNF- α . Eight weeks of treadmill running reversed this effect. Exercise training was also able to reduce TXNIP within the pancreas as a result of stress.

Dedication

To my family, lab mates, teammates, and many mentors for guiding me every step of the way in
and out of the lab and classroom.

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In the six years I have spent at WVU and in the Exercise Physiology program, I have had the privilege of having many distinguished mentors and making some of the greatest friends. Within the Exercise Physiology family, I have had the opportunity to be guided by many intelligent minds, such as Randy, Paul, Mark, Ed, Lori, Emily, Danny, Jim, and many others. I was inspired by Dr. Pistilli's lectures in EXPH 386 to try out research, and thanks to the efforts of my committee, I have fallen in love with this path. I have many thanks to give to Guy and my weightlifting family for being my home away from home and helping me workout through the stresses of my masters to become a stronger (mostly physically, but a little bit mentally) person. I have learned the importance of discipline and time management... and not waiting until two days before a meet to try to cut six kilos.

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ABBREVIATIONS

T2D Type 2 diabetes

GC Glucocorticoid

HPA-axis Hypothalamus pituitary adrenal axis

SNS Sympathetic nervous system

GLUT4 Glucose transporter 4

TXNIP Thioredoxin-interacting protein

Trx Thioredoxin

ASK-1 Apoptosis signaling kinase-1

Cyt-C Cytochrome-C

NLRP3 Nod-like receptor protein 3

UCMS Unpredictable chronic mild stress

ROS Reactive oxygen species

NO Nitric oxide

OZR Obese Zucker rat

LZR Lean Zucker rat

C Control

EX Exercise

EXS Exercise and stress

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Metabolic Syndrome, which affects approximately 23 percent of adults in the United States, is comprised of a cluster of risk factors that can lead to many chronic diseases, including cardiovascular diseases, cancer, and diabetes^{1,2,3}. The criteria for the Metabolic Syndrome includes increased waist circumference (obesity), hypertension, high circulating triglycerides, low high-density lipoprotein-cholesterol (HDL-C), and high fasting glucose.² The risk factors for Metabolic Syndrome are similar to risk factors related to the development of type 2 diabetes (T2D), such as obesity, high circulating triglycerides, and high fasting glucose, and the condition often leads to insulin resistance and hyperinsulinemia.^{4,5} There are various modifiable risk factors that can lead to insulin resistance, including physical inactivity, poor nutrition, obesity, and stress.¹ Obesity is an epidemic affecting nearly one-third of the adult population in the US, and it is proposed that by 2030, there will be 65 million more obese adults in the US.^{6,7} Obesity is the accumulation of excess adipose tissue, an active endocrine tissue, which is a major storage site for triglycerides, and a major site of pro-inflammatory cytokine production.^{7,8} Initially, obesity and a high fat diet is associated with an increase in pancreatic islet mass due to hypertrophy of the β -cells and the α -cells to compensate for the hyperglycemic state caused by increased levels of circulating free-fatty acids (FFA).⁹ Chronically high levels of circulating FFA are associated fatty acid and ceramide accumulation in peripheral tissues such as the liver and skeletal muscle which can result in insulin resistance and hyperinsulinemia, a process known as lipid toxicity.⁹ It can also result in β -cell dysfunction and eventual decreased insulin secretion, as a result of an interference with the insulin peptide folding causing an impairment of proper insulin processing.⁹ Obesity-induced adipocyte hypertrophy leads to altered visceral adipose tissue (VAT) homeostasis which causes an elevation of circulating proinflammatory cytokines,

such as IL-6 and TNF- α , through the proliferation of proinflammatory cells within the VAT.¹⁰ These proinflammatory cytokines are also seen to promote insulin resistance and place the body in a proinflammatory state by negatively affecting the insulin cascade.^{7,8,11,12,13}

Stress, another modifiable risk factor for obesity, can lead to the hypersecretion of glucocorticoids (GCs) and proinflammatory cytokines, such as IL6 and TNF- α .¹⁴ An upregulation of proinflammatory cytokines can lead to insulin resistance and an increased risk for the development of T2D.¹⁵ When the body senses stress, the hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) are activated to release GCs and catecholamines (norepinephrine and epinephrine), respectively.¹⁶ In the presence of chronic stress, the HPA axis is subject to dysregulation, which in turn dysregulates inflammation with an associated increase in proinflammatory cytokines. Immune cells become desensitized to GCs that normally terminate an immune response, inducing a proinflammatory environment.¹⁷ Chronically elevated levels of circulating GCs promote increased adiposity and increase stress on pancreatic islet cell function.⁹ Upregulated GC exposure over time leads to increased insulin secretion and induces an increase in caloric intake, preference for calorically-dense foods, and circulating FFAs, promoting insulin resistance.⁹ The excessive secretion of GCs that is seen with chronic stress can lead to depression.¹⁴ Depression has been associated with poor health behaviors, including physical inactivity, smoking, and excessive caloric intake which can lead to obesity.¹⁵ Therefore, the co-morbidities are often linked in humans.

Physical inactivity is another modifiable risk factor of T2D, yet 80 percent of the US adult population does not meet recommendations for physical activity.¹⁸ A single bout of moderately intense exercise is associated with increased insulin sensitivity for 12-48 hours post exercise bout.¹⁹ Insulin sensitivity and β -cell function homeostasis are maintained through the

balance of β -cell replication, neogenesis, and apoptosis, and regular exercise stimulates β -cell neogenesis. Chronic, daily, physical activity not only improves insulin sensitivity and action, but also works to improve GLUT4 translocation in skeletal muscle. This in turn reduces insulin requirements and improves peripheral insulin action. Regular physical activity preserves β -cell mass through the prevention of β -cell exhaustion that is seen through overuse in T2D. Acute exercise increases the release of GCs through the stimulation of the HPA axis, but with regular exercise, HPA axis activity can be normal or even reduced at rest.²⁰ In addition, regular exercise lowers elevated GCs, mainly cortisol, brought about by chronic stress, therefore increasing insulin sensitivity and reducing insulin resistance.²¹

The thioredoxin system is a key component of the cellular anti-oxidant mechanism.²² Thioredoxin-interacting protein (TXNIP), a proapoptotic factor stimulated by hyperglycemia, acts as an inhibitor of thioredoxin (Trx), interfering with the anti-oxidant response and promoting oxidative stress.^{22,23,24,25} Hyperglycemia enhances the interaction between TXNIP and Trx, and TXNIP is essential for glucose toxicity-induced β -cell apoptosis.^{22,23,24} TXNIP interferes with Trx through a variety of pathways, including mitochondrial interaction, cytoplasmic interference, and nuclear mechanisms.^{22,23,24,25,26} Within the mitochondria, TXNIP binds to Trx2, releasing apoptosis signal regulating kinase-1 (ASK1) from its inhibited state, increasing the release of Cytochrome C (Cyt-C) and cleaved caspase-3, which leads to β -cell apoptosis.^{23,25,26} The TXNIP/Trx interaction in the cytoplasm causes the anti-oxidative mechanisms of Trx1 to be inhibited, increasing oxidative stress and leading to β -cell apoptosis.^{25,26} Through increased TXNIP expression, insulin transcription and β -cell function are reduced through the increased expression of microRNAs that interfere with the expression of target genes.^{24,25} Along with these increases in apoptosis, increased TXNIP expression also leads to the activation of the nod-like

receptor protein 3 (NLRP3) inflammasome, a major producer of IL-1 β , a proinflammatory cytokine associated with insulin resistance.^{22,27,28} ER stress induces increased TXNIP expression through unfolded protein response pathways IRE1- α and PERK, and therefore ER stress regulated NLRP3 production of IL-1 β through TXNIP pathway, increasing the proinflammatory environment in the pancreas and other parts of the body.²⁸

PURPOSE AND SPECIFIC AIMS

The purpose of this study was to assess the effects of obesity, chronic stress and exercise training on pancreatic health in a model of metabolic syndrome. We hypothesized that obesity would cause a decrease in pancreatic β -cell number and islet area because of an increase in TXNIP, the proinflammatory environment, and overall apoptosis-related proteins in the pancreas. We also hypothesized that stress would further increase and exercise training decrease these effects, through altered TXNIP and pro- and anti-inflammatory cytokines within the pancreas.

Specific Aim 1.1: To determine the effects of obesity on pancreatic morphology

Hypothesis 1. Obesity alone (OZR-C) will decrease the number of pancreatic β -cells, the β -cell to α -cell ratio, and islet area when compared with lean animals (LZR-C).

Specific Aim 1.2: To determine the effects of stress alone and in combination with obesity on pancreatic morphology

Hypothesis 1. Eight weeks of UCMS (LZR-S) will decrease the number of pancreatic β -cells, the β -cell to α -cell ratio, and islet area when compared with lean animals (LZR-C).

Hypothesis 2. Eight weeks of UCMS in combination with obesity (OZR-S) will augment the obesity-related decreases in the number of pancreatic β -cells, the β -cell to α -cell ratio, and islet area when compared with obese (OZR-C) animals.

Specific Aim 1.3: To determine the effects of exercise training on the obesity and stress related changes to pancreatic morphology.

Hypothesis 1. Eight weeks of treadmill running in combination with obesity (OZR-EX) will attenuate the obesity-related decreases in pancreatic β -cell number, the β -cell to α -cell ratio, and islet area compared with obese animals (OZR-C).

Hypothesis 2. Eight weeks of treadmill running in combination with eight weeks of UCMS (LZR-EXS) will attenuate the stress-related decreases in pancreatic β -cell number, the β -cell to α -cell ratio, and islet area compared with lean stressed animals (LZR-S).

Hypothesis 3. Eight weeks of treadmill running will attenuate the combined effects of obesity and UCMS (OZR-EXS) on pancreatic decreases in β -cell number, the β -cell to α -cell ratio, and islet area compared with obese stressed animals (OZR-S).

Specific Aim 2.1: To determine the effects of obesity on the pancreatic pro-inflammatory environment and level of apoptosis

Hypothesis 1. Obesity alone (OZR-C) will result in an increase in pancreatic TXNIP, pro-inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF α) and apoptosis and a decrease in anti-inflammatory cytokines (IL-4 and IL-10) compared with lean animals (LZR-C).

Specific Aim 2.2: To determine the effects of stress alone and in combination with obesity on the pancreatic pro-inflammatory environment and level of apoptosis

Hypothesis 1. Eight weeks of UCMS (LZR-S) will result in an increase in pancreatic TXNIP, pro-inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF α) and apoptosis and a decrease in anti-inflammatory cytokines (IL-4 and IL-10) compared with lean animals (LZR-C).

Hypothesis 2. Eight weeks of UCMS in combination with obesity (OZR-S) will augment the obesity-related increase in pancreatic TXNIP, pro-inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF α) and apoptosis and a decrease in anti-inflammatory cytokines (IL-4 and IL-10) compared with obese animals (OZR-C).

Specific Aim 2.3: To determine the effects of exercise training on the obesity and stress related changes to the pancreatic pro-inflammatory environment and level of apoptosis

Hypothesis 1. Eight weeks of treadmill running in combination with obesity (OZR-EX) will attenuate the obesity-related increase in pancreatic TXNIP, pro-inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF α) and apoptosis and a decrease in anti-inflammatory cytokines (IL-4 and IL-10) compared with obese animals (OZR-C).

Hypothesis 2. Eight weeks of treadmill running in combination with eight weeks of UCMS (LZR-EXS) will attenuate the stress-related increase in pancreatic TXNIP, pro-inflammatory cytokines (e.g., IL-1 β , IL-6, and TNF α) and apoptosis and a decrease in anti-inflammatory cytokines (IL-4 and IL-10) compared with lean stressed animals (LZR-S).

Hypothesis 3. Eight weeks of treadmill running will attenuate the combined effects of obesity and UCMS (OZR-EXS) on pancreatic increases in TXNIP, pro-inflammatory cytokines (e.g., IL-

1 β , IL-6, and TNF α) and apoptosis and a decreases in anti-inflammatory cytokines (IL-4 and IL-10) compared with obese stressed animals (OZR-S).

CHAPTER 2: LITERATURE REVIEW

Pancreas

The pancreas is an organ in the body responsible for maintaining glucose homeostasis through the counter-regulatory actions of insulin and glucagon.^{29,30} There are many types of cells in the pancreas that make up the islet of Langerhans, including β -cells that produce insulin, α -cells that produce glucagon, somatostatin, ghrelin, and polypeptide-secreting endocrine cell types that each produce a unique hormone. Eight-five percent of the pancreatic tissue is made up of exocrine cell types, such as acinar cells that produce digestive enzymes and duct cells that produce a conduit to the gut for digestive enzymes.^{31,32} The remaining fifteen percent is made up of the pancreatic islets, which consist of approximately seventy-five percent β -cells and twenty percent α cells.³³

Blood glucose is important for the stimulation of β -cell growth in fetal and adult life.³⁴ Blood glucose levels modulate the release of insulin from the pancreatic β -cells when blood glucose is high, a process known as glucose-stimulated insulin secretion.^{29,35} When glucose levels are low, glucagon is released from the pancreatic α -cells, and this triggers glycogenolysis and gluconeogenesis by the liver to increase glucose levels.³⁰ A rise in blood glucose level triggers glucose oxidation via glycolysis and the Krebs cycle within the pancreas and an increase in cytosolic ATP/ADP ratio that leads to an influx of free Ca^{2+} , triggering a signal for insulin secretion from pancreatic β -cells (Figure 1).^{29,35,36}

Figure 1 shows the process of glucose entering the human β -cell through the GLUT 1 (GLUT 2 in rodent) receptor and proceeding through the Krebs cycle to produce ATP. Potassium channels then close off, depolarizing the membrane to allow calcium channels to open, triggering the release of insulin from the β -cells.³⁶

The development of pancreas tissue involves apoptosis, replication, and neogenesis. During the neonatal period, the pancreas undergoes substantial remodeling, and apoptosis is a major process determining the final β -cell mass.³⁷ There are two process by which pancreatic β -cells develop: differentiation and proliferation.^{34,38,37} Neoformation of pancreatic β -cells occurs from the differentiation of precursor cells.³⁴ Proliferation of pre-existing pancreatic β -cells is a key component in regeneration.³⁸ It has been suggested that pancreatic β -cells derive from α -cells, in that α -cells develop first in the embryo. Figure 2 shows the process of development of the cells in the pancreatic islets.³⁹ When insulin resistance leads to T2D, it is suggested to be a

bi-hormonal disease with hypoinsulinemia (β -cells) and hyperglucagonaemia (α -cells). Though this could suggest changes to α -cell mass in the pancreas with the progression to T2D, the α -cell to β -cell ratio increases with T2D when compared to non-diabetic pancreas islets due to decreases in β -cell numbers, with no significant changes occurring to the α -cell number.⁴⁰

Figure 2 shows the development of pancreatic β -cells and α -cells.³⁹

Insulin resistance is a process that develops gradually as pancreatic β -cells overproduce insulin to compensate for the onslaught of blood glucose until these cells begin to experience cell death. The periphery becomes resistant to the effects of insulin after circulating insulin remains consistently elevated.⁴¹ This is characterized by diabetes, which is subdivided into two pathways of dysfunction: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is an autoimmune disease that results in complete exogenous insulin dependence due to pancreatic β -cells being destroyed and unable to sufficiently produce insulin. T2D is a progressive disease that is also known as insulin-independent diabetes. Under extreme conditions, like morbid obesity, T2D can progress into total insulin-dependence, though this is rare.^{42,43,44}

There are five stages to the development of insulin resistance: compensation, stable adaptation, unstable early decompensation, stable decompensation, and severe decompensation.⁴² The compensation stage is found to be highly associated with obesity and involves upregulated insulin secretion and increased GSIS following a glucose challenge.^{42,43} This increase in insulin is suggested to be brought about by increased β -cell mass as has been observed postmortem in obese patients.^{42,45} The stable adaptation stage involves pancreatic β -cells unable to compensate for the high glucose levels. This stage is considered stable because those individuals who adhere to a healthy diet and regular exercise regimen progress at a much slower rate to T2D.^{42,46} Stage 2 is characterized by fasting glucose levels between 5.0 and 7.3 mmol/l, the loss of GSIS, and changes in β -cell differentiation.^{42,47} Patients can remain in stage two for years, but once β -cells can no longer compensate and β -cell mass declines, glucose levels rise substantially over a short period of time. This has been characterized as the unstable early decompensation stage (stage three). Stage four will most likely be maintained throughout the lifetime of a patient of T2D. Those individuals with T1D have a rapid autoimmune destruction of β -cells that lead to stage five, which is severe decompensation. Stage five is rarely reached with T2D.⁴²

Two contributors to the development of T2D include chronic stress and obesity.^{48,49,50} Both of these processes promote inflammation, increased oxidative stress, and apoptosis, including but not limited to pancreatic β -cell death. Chronic stress has been associated with an increase in obesity, and obesity can promote chronic stress.⁵¹ Both of these comorbidities are associated with increased GCs, hyperglycemia, hyperinsulinemia, and increased FFA, along with others, all of which can be associated with insulin resistance.^{4,7, 8,9,11,12,13,15}

Metabolic Syndrome

The Metabolic Syndrome is a noninfectious pandemic that involves organs such as the heart, liver, adipose tissue, pancreas, etc.⁵² Of the estimated 200 million overweight Americans, over 50 million are estimated to be affected by the Metabolic Syndrome.⁵² This disease is a cluster of factors that are linked to increased risk for diseases such as diabetes, cardiovascular disease, cancer, etc. These factors include abdominal obesity, high blood glucose levels (insulin resistance), dyslipidemia, high cholesterol or low HDL-C levels, and hypertension.³ It is suggested to be due mostly to the environment, with little influence from genetics.^{53,54,55} From 1970 to 2003, the caloric intake in the United States increased by 523 calories, while advances in technology have led to a more sedentary lifestyle.^{54,55} Proinflammatory markers have been shown to positively correlate with features of the Metabolic Syndrome, like insulin resistance with no correlation to the degree of obesity. These proinflammatory markers include TNF- α , IL-6, IL-1 β , and other chemokines.⁵⁶ Vozarova and colleagues⁵⁷ showed a correlation with high white blood cell (WBC) count, an indicator that the immune system is activated, and the development of diabetes. Phillips and Perry⁵⁸ showed a positive correlation with metabolically healthy individuals and lower levels of IL-6 and WBCs and higher levels of adiponectin.

Obesity

Obesity is the accumulation of adipocytes in the form of adipose tissue. Adipocytes are cells that store fat in the form of triglycerides (TGs) as energy for times of starvation.⁵³ In order to avoid lipids from accumulating in non-adipose tissue, adipocytes not only serve as the storage for TGs, but these cells also secrete leptin to signal the brain that satiety is reached and stimulate fatty-acid oxidation in those non-adipose tissues.⁵³

As energy intake increases and energy expenditure decreases, adipose tissue accumulates via hypertrophy, or in severe conditions, hyperplasia.⁵⁹ Organs involved in the Metabolic Syndrome, like the pancreatic islets, accumulate ectopic fat in both humans and rodents.⁶⁰ It was shown in overweight humans using magnetic resonance spectroscopy (MRS) that the pancreas accumulates TG deposits.⁶¹ When glucose tolerance is impaired, or in the presence of diabetes, fat accumulation in the pancreas is significantly even greater.⁶¹ This is suggested to be glucolipotoxicity, where increased glucose further promotes lipogenesis and lipotoxicity.⁶² Adipose tissue is an active endocrine organ known to secrete free-fatty acids (FFA) and adipokines, such as cytokines and chemokines.⁵⁹ This action may contribute to the development of metabolic diseases through hyperglycemia, dyslipidemia, and inflammation.⁵⁹ It has also been shown that obesity stimulates the production of reactive oxygen species (ROS) via increased FFA being released by adipose tissue into circulation.⁶³ All of these actions can lead to the development of insulin resistance.

Inflammation and Obesity

Macrophage content of adipose tissue is positively correlated with body mass and adipocyte size, and these macrophages secrete cytokines like TNF- α , IL-6, and IL-8, which are associated with insulin resistance.⁷ Macrophages are the key immune cells involved in adipose tissue inflammation in obese individuals, and during chronic exposure to inflammation, macrophages within the adipose tissue impair adipocyte function.^{64,65,66} Macrophages are cells of the innate immune system and serve as a link between the innate immune system and the adaptive immune system.^{13,64} Obesity has been shown to induce a phenotypic change in macrophage polarization, from M₂ to M₁ (M_{anti} to M_{pro}).^{13,65} M₁ are macrophages that are crucial

for fighting bacterial infections and tumors, but excess activation leads to a proinflammatory environment that can cause tissue damage. In order to fight off infections, M_1 secrete ROS, nitric oxide (NO), and proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6.¹³ TNF- α is one of the first cytokines produced in this process, and along with the proinflammatory cytokine IL-1 β , it can induce the production of IL-6, which can be both a proinflammatory cytokine and an anti-inflammatory cytokine, depending on its origin (i.e. skeletal muscle produces IL-6 in an anti-inflammatory fashion, while adipose tissue produces a proinflammatory IL-6).^{13,67,68,69} The M_2 pathway is an anti-inflammatory pathway that produces anti-inflammatory cytokines such as IL-10 to suppress inflammation.^{13,70}

Macrophages have been suggested to be linked to insulin resistance, more specifically through the release of proinflammatory cytokines such as TNF α .^{64,66} TNF- α is thought to be a major cause of the development of insulin resistance, and it has been shown that obese mice that lack either TNF- α or TNF- α receptors are protected from developing insulin resistance.^{71,69} In rodent models, mice deficient in TNF- α show decreased levels of FFA and TGs when compared to control mice, and rodents subjected to TNF- α showed impaired action of glucose uptake by the skeletal muscle.⁷² Advances in insulin sensitivity were not mimicked in humans with anti-TNF- α treatments.⁷³ TNF- α increases lipolysis in adipose tissue to release FFA into systemic circulation. FFA are then able to deposit in non-adipose tissues, like skeletal muscle.^{71,74,75} In humans, 10-35% of circulating IL-6 is housed in adipose tissue, and so hypertrophy of adipocytes is accompanied by an increased production of adipose tissue IL-6.^{76,77} It has been shown that expression of IL-6 from adipose tissue positively correlates with insulin resistance, and so when neutralization of TNF- α in humans brings about no improvement in insulin

sensitivity, it can be suggested that IL-6 makes up for the absence of the effects of TNF- α .^{78,79} Hyperglycemia has also been associated with increases in IL-6 levels from adipose tissue.⁸⁰

Oxidative Stress and Obesity

Obesity has been observed to increase oxidative stress, which is known to cause direct and indirect damage to organs that can result in many pathological processes, including the Metabolic Syndrome.^{63,81} Biomarkers for oxidative stress have been shown to be correlated directly with many factors involved with obesity, including BMI, percent body fat, LDL oxidation, and TG levels, and it has been shown that these biomarkers are increased in diets high in fat and carbohydrates.^{82,83} As stated above, obesity is associated with a proinflammatory environment through the overproduction of cytokines, which are known to increase ROS and NO. TNF- α causes damage to the mitochondria in the mitochondrial chain complex III and activates the production of ROS at the ubiquinone site.^{84,85} This was further investigated by inhibiting mitochondrial oxidative metabolism, which not only lead to the inhibition of TNF- α cytotoxicity, but also reduced TNF- α activation of nuclear-factor kappa- β (NF- $\kappa\beta$) and IL-6.^{85,86} With obesity there is a higher demand on the heart, and therefore an increase in mitochondrial respiration; this leads to the consumption of more oxygen, and the production of more ROS in the form of superoxide and hydrogen peroxide.⁶³ It has been shown that hydrogen peroxide is increased in obese mouse adipose tissue, and along with the fact that adipose tissue was the only observed organ that had increased hydrogen peroxide levels, can suggest that adipose tissue is the major producer of this oxidative stress biomarker.⁶³

The adipokine, adiponectin, has insulin-sensitizing effects that increase insulin sensitivity.⁵⁹ Adiponectin is shown to be reduced in obese individuals, and it has been shown that

plasma adiponectin levels are inversely related to oxidative stress biomarkers in nondiabetic human subjects.^{63,87} In rodent models, the absence of adiponectin is shown to induce insulin resistance, whereas overexpression of this adipokine leads to increased insulin sensitivity.

Obesity's Effects on the Pancreas

Excess circulating lipids are taken up by the pancreas and other organs, leading to lipid accumulation in those organs.⁸⁸ It was shown in obese homozygous (*fa/fa*) male ZDF rats that apoptosis in the pancreatic islets was increased as prediabetic and diabetic stages progressed. It is suggested that elevated levels of circulating FFA and lipoproteins transport more FFA than the pancreas can oxidize, leading to a lipotoxic effect on islets.⁸⁹ Excess circulating lipids were associated with diminished pancreatic β -cell function and even apoptosis in in vivo rat models and in vitro human islet cells.⁹⁰ It is suggested that ceramides are a mediator of the FFA-induction of pancreatic β -cell apoptosis.⁸⁹

Lipotoxicity and glucotoxicity in the pancreas have been shown to increase ER stress through JNK activation, leading to decreases in β -cell mass in animal studies.^{91,92} On the other hand, Saisho and colleagues⁹³ found that in human pancreas autopsies that β -cell mass was increased with obesity. Oxidative stress is known to cause damages to many organs, including impairment of insulin secretion in pancreatic β -cells and impairment of glucose transport into muscle and adipose tissue.^{94,95,96} The adverse effects of ROS on insulin production not only leads to insulin resistance, but also may lead to β -cell apoptosis.⁸⁸ ER stress in the mitochondria of the pancreatic β -cell can lead to the overproduction of ROS, and obesity has been associated with this increase in ER stress-induced oxidative stress in the mitochondria.⁸⁸

General Stress Response

Chronic stress is a modifiable risk factor for T2D and insulin resistance.¹⁴ Organisms have a primal instinct to survive, and this requires physiological adaptations that must occur when homeostasis is threatened.⁹⁷ When a stressor is perceived by the brain, a cascade of signals activates the HPA axis, and GCs are released.^{97,98,99,100,17} The stressor promotes corticotropin-releasing hormone (CRH) to initiate the stress response in the hypothalamus and flow through circulation to the anterior pituitary gland to stimulate the release of adrenocorticotrophic hormone (ACTH).^{97,99,100,16} When ACTH reaches the systemic circulation the adrenal glands are stimulated to release GCs (cortisol in humans and corticosterone in rodents) that are then pumped into the systemic circulation.^{97,99,100,16} In an acute stress response, GCs act as a negative feedback mechanism to the hypothalamus to stop the release of CRH to turn off the stress response cascade.⁹⁷ When in circulation, GCs bind to their receptors, GRs to help modulate a number of biological functions due to GRs being expressed in almost all cell types. In immune cells and other organs and tissues, including adipose tissue, GCs are one of the key regulators of energy influx in adipocytes.^{98,100}

This release of GCs allows for energy to be mobilized for one of two reasons: 1) a reactive response, in which energy is needed to meet a physical insult, or 2) an anticipatory response, in which energy is assembled to prepare for a predicted need.⁹⁷ Energy is mobilized through the binding of GCs to glucocorticoid receptors (GR) in the hypothalamus and pituitary glands of the brain that promote mobilization of energy stores for the liver, fat, and muscle and increase inflammation and neural function.¹⁰¹ GR receptors are considered to lead the bulk of feedback regulation.⁹⁷ GCs work to increase the liver glucose output and the release of FFA from

white adipose tissue along with the reduction of insulin secretion so that energy can be available for behavioral reactions to stressors.¹⁰²

Glucocorticoids and Obesity

When under chronic psychological stress, it has been shown that there is a tendency for overconsumption of energy dense foods.^{98,103} Chronic stress has been shown in both humans and rodents to trigger the desire for high fat, energy dense foods.¹⁰⁴ Even in instances in which stressed subjects do not gain weight, it is still shown that high fat and/or sweet foods are favored.¹⁰⁴ It has been shown in animal studies that under chronic mild stress, rodents saw weight gain and increased food consumption when sweet condensed milk was offered in comparison to regular chow.^{105,106} In humans, chronic stress induces an increase in cortisol that may lead to an increased consumption in energy-dense food, which could lead to weight gain.¹⁰⁷

Some key components of obesity are hyperglycemia, or high blood sugar., hyperinsulinemia, and insulin resistance. In acute stress conditions, GCs exposure can lead to decreased skeletal muscle glucose transport and increase hepatic glucose production.^{5,108} Acute exposure to GCs can improve pancreatic β -cell function to glucose sensitivity and insulin secretory capacity, but chronic exposure to the stress hormone can lead to dysfunctions including impaired peripheral insulin sensitivity and decreased GSIS.²⁰ Chronic exposure of GCs can result in damage to the pancreas that can lead to insulin resistance, hyperinsulinemia, and hyperglycemia when chronically stimulated, which can lead to deteriorated glucose homeostasis.^{98,109,5,9} Chronic stress can promote increased abdominal obesity and excess ectopic fat deposition.^{98,5,108} Humans subjected to chronic GC treatment (>14 days) showed decreases in insulin secretion when subjected to a glucose challenge. This impairment in insulin action could

suggest an indirect loss in pancreatic β -cell function or suppression of the release of insulin by pancreatic β -cells.^{110,111} This could suggest that over time, chronic exposure to stress and GCs can lead to insulin-dependent diabetes through the elimination of pancreatic β -cells. It was also shown that increased endogenous glucose production, as well as lipolysis, increased plasma free-fatty levels.^{110,111} This goes along with the belief that GCs cause metabolic and morphological attenuations to the pancreatic β -cells.^{20,112} Chronic GC exposure has been shown to increase β -cell mass due to hypertrophy and hyperplasia, which can lead to hyperinsulinemia.²⁰ GCs have been observed to favor glycogenolysis activation, gluconeogenesis, lipid mobilization, and protein synthesis inhibition, which can lead to increased blood glucose and in turn hyperinsulinemia.¹¹² These different responses in the pancreatic β -cells to chronic exposure to GCs could be due to differences in the progression of pancreatic damage brought about by the chronic exposure to stress.

Inflammation and Chronic Stress

The HPA axis is one of the main mechanisms the brain utilizes to control the immune system, and stress has been shown to have deleterious effects on the immune system.⁹⁹ GCs are known to be primarily an anti-inflammatory defense.^{17,109} In acute situations, GCs combat the immune cells; at a cellular level, GCs induce apoptosis in T lymphocytes, neutrophils, basophils, and eosinophils and modulate macrophage cytokine production by inhibiting p38 MARK at the transcriptional level.^{100,113,114} GCs are known to suppress proinflammatory cytokines such as IL-6 and TNF- α and upregulate the secretion of anti-inflammatory cytokines like IL-4, IL-10, and IL-13.^{16,115} GCs have also been shown to downregulate IL-1 β transcription.¹¹⁶ Corticosterone enters the nucleus to inhibit the transcription control pathways, including NF- κ B, mitogen-

activated protein kinases (MAPKs), and others.^{16,117} GCs have also been studied to promote the Th1 to Th2 shift, meaning they suppress proinflammatory (Th1) processes and upregulate anti-inflammatory (Th2) cytokines.^{16,118} GCs act to suppress the production of IL-12, the main inducer of the Th1, or proinflammatory, response.^{16,118}

Although GCs can be used for therapeutic benefits in conditions like rheumatoid arthritis or Addison's Disease where the adrenal glands are not producing GCs, high doses of GCs are associated with diabetes, hypertension, and other diseases risks.^{100,119,120} Cushing's Syndrome is the excess production of GCs, and like chronic stress can overstimulate the HPA axis causing immune cells to become less sensitive to GCs.^{100,17,121} Chronic stress can lead to changes in lymphocyte populations, changes in NK cell number and activity, changes in ratio of helper: suppressor T cells, impaired antibody responses, etc.⁹⁹

Lymphocytes are mediators in cell immunity, and T lymphocytes are divided into four subgroups: cytotoxic T cells (CD8+ T cells that destroy non-self-cells), helper T cells (CD4+ T cells that secrete cytokines), memory T cells (either CD4+ or CD8+ T cells that are antigen-specific), and regulatory T cells (suppressor T cells that suppress antibody production).⁹⁹ Stressors have been shown to have varying effects on the T lymphocytes, including: decreases in total T lymphocytes, helper T lymphocytes, and suppressor T lymphocytes^{122,123}; decreased helper-to-suppressor T lymphocyte ratio^{122,124}; increased levels of CD8+ cytotoxic T cells¹²⁵; and poor memory T cell proliferation.^{126,127} Natural killer cells, cytotoxic T lymphocytes that primarily kill viral infections and cancer, experience a decrease in number and activity in response to stressors; this leads to a decrease in the ability to fight infection.⁹⁹ Stress has been shown to have differing effects on immunoglobins in the absence of infection, including increasing plasma IgA without changes in IgG, IgM, and salivary IgA during examination

stress,¹²⁸ increases in IgG, IgM, and IgA during examination stress,⁹⁹ and in mice, decreased serum IgM with footshock.¹²⁹ Also, white blood cells develop a counterregulatory response to downregulate the function of receptors responsible for binding and reacting with GCs.¹²¹ This allows for a proinflammatory environment to collect with chronic stress.^{17,121}

Cytokines are divided into two subsets: Th1 cytokines, or proinflammatory cytokines, which include IL-1, IL-2, IL-6, INF γ , and TNF- α ; and Th2 cytokines, or anti-inflammatory cytokines, which include IL-4, IL-5, IL-10, and IL-13.⁹⁹ With chronic stress, studies have shown the development of glucocorticoid-resistance syndrome, where GCs can no longer suppress cytokine production,¹⁶ or glucocorticoid-receptor resistance, where a decrease in the immune cells sensitivity to GCs allows for increased for chronic inflammation.¹⁷ Studies have shown that with prolonged stress, the ability to suppress the secretion of IL-6, IL-1 β , and TNF- α is diminished, allowing for the upregulation of proinflammation.^{99,17,117,130,131} GCs have also been shown to induce the activation of the NLRP3 inflammasome, a NOD-like receptor that is a major producer of IL-1 β , a proinflammatory cytokine.¹³²

Oxidative Stress and Chronic Stress

Apoptosis is programmed cell death that, under normal conditions, helps remove damaged or aged cells and maintain homeostasis.^{133,134} Excess oxidative stress causes cell death through apoptosis.^{135,136} Too much apoptosis can lead to adverse consequences, and hyperglycemia is a pathophysiological component that induces both oxidative stress and apoptosis.²⁹ Glucocorticosteroids and TNF- α can both stimulate apoptosis through severe oxidative stress.²⁹

Oxidative stress is also thought to contribute to glucotoxicity, an effect that can be brought about by over nutrition and the steady increase in high blood glucose levels that can lead to the development of conditions such as type 2 diabetes.²⁹ In hyperglycemic conditions, more glucose is circulating through the glycolytic pathways and therefore increasing the amount of pyruvate and acetyl-CoA being produced. This process produces NADH, an electron carrier, and in hyperglycemic conditions, there is a surplus of NADH produced, creating electron pressure on the mitochondria electron transport chain, especially in the pancreatic β -cells.^{137,138,139,140} The mitochondrial complex I (MC-I) functions to recycle NADH, and when hyperglycemia overproduces NADH, a heavy burden is placed on MC-I.^{141,142} This increased cycling of NADH through the MC-I produces more superoxide, the precursor for all ROS, through the leakage of electrons that can reduce oxygen to superoxide.^{141,143,144,145} The four major sources of ROS are oxidative burst, oxidative processes, lipid peroxidation, and oxidative stress.¹⁴⁶

In depression, oxidative stress is increased and antioxidant defenses are decreased.¹⁴⁷ In patients with depressive disorders, plasma, serum, and urine samples have shown increases in total oxidative status, oxidative stress index, and markers of lipid peroxidation.¹⁴⁸ It was also shown in peripheral blood samples of depression patients that mitochondrial ROS production, nitric oxide levels, and lipid peroxidation were all increased while the antioxidant markers superoxide dismutase (SOD) and GPx were decreased.^{149,150,151,152} In the postmortem hippocampus of patients that suffered depression or bipolar disorder, there was an increased RNA oxidation observed, as well as decreases in antioxidant marker GPx in the frontal cortex.^{153,154} These results suggest that, while oxidative stress can be a consequence of depression, it also contributes to and increases the progression of depression.¹⁵⁵

UCMS Protocol

The unpredictable chronic mild stress (UCMS) protocol, also known as CUMS, CMS, etc., can be used to induce stress in the rodents to mimic stress in humans. These protocols induce stress presents similar behaviors to that of depression in rodents, presenting itself such that rodents show anhedonia-like behavior and other behaviors that suggest depression is present.^{112,146,148} The protocols success can be measured in different ways, including coat score assessment, behavior testing, and corticosterone measurements. Rodents undergo a randomized schedule of stressors, including bath, cage tilt, damp bedding, wet bedding, and social stress. These are outlined in detail in the methods section. The schedule is randomized for each day, with different stressors and different durations to make sure that the animals do not grow accustomed to the stressors. Coat scores are assessed at the end of each week by the same individual, and the blood samples taken the day of surgery are used to measure corticosterone levels.

In a study done by López-López and colleges,¹¹² the pancreas was examined in relation to oxidative stress caused by chronic, unpredictable, mild stress (CUMS). Corticosterone was increased with the stress protocol, as well as lipoperoxide concentration in the pancreas gradually from day 20 to day 60. This was shown by the increased levels of lipid peroxidation and oxidative proteins in the pancreas, along with decreases in total antioxidant capacity with the stress protocol. The pancreas is a regulator of the metabolism of carbohydrates, lipids, and proteins, and is not equipped as a major regulator of the redox state of an organism.¹¹²

The liver is the major site for antioxidant enzyme capacities in the body and is considered the regulatory organ for the redox state.^{112,148} The CUMS protocol resulted in an increase in oxidative stress markers and lipid peroxidation in the liver, though total antioxidant capacity did

not significantly decrease over the 60-day duration.¹¹² Duda and colleagues¹⁴⁸ used chronic mild stress (CMS) to show the increase markers of oxidative stress, including ROS, malondialdehyde (MDA), and non-protein sulfhydryl (NPSH), while the antioxidant markers catalase (CAT) and glutathione peroxidase (GPx) increased as well within the liver.

Exercise and Obesity

Regular exercise is associated with better quality of life and health outcomes.¹⁵⁶ Exercise has been shown to improve physical attributes of health, including reduced blood pressure, enhanced cardiovascular fitness, increased insulin sensitivity, reduced abdominal adiposity, and prevention of chronic diseases, such as diabetes and obesity.^{157,158} Even short-term exercise has been shown to improve many aspects of the Metabolic Syndrome.¹⁵⁶ A 12-week exercise program in middle-aged women resulted in decreases in blood pressure, body fat percentage, fast glucose levels, triglycerides, and cholesterol.¹⁵⁹

Exercise has been shown to enhance the loss of adipose tissue, which can in turn reduce the proinflammatory environment brought about by adipocyte hypertrophy.¹⁵⁸ In a study with severely obese human subjects, 15-weeks of hypocaloric diet and daily exercise resulted in decreased circulating inflammatory markers and decreases in adipose tissue expression of inflammatory markers, such as IL-6 and TNF- α . The lifestyle intervention also produced increases in adiponectin and improvement in metabolic status.¹⁶⁰ Though a high-fat diet was shown to increase obesity, inflammation, and insulin-resistance, voluntary exercise reduced these parameters. Voluntary exercise not only reduced the inflammation brought about by the HFD, but this intervention also reduced inflammation in the exercise-regular chow diet.¹⁶¹

Exercise and Stress

Depression has been associated with low levels of physical activity, and regular exercise has been shown to improve mental health.^{162,157} Individuals with depression spent significantly less time in light and /or moderate physical activity than did non-depressed adults, while adults that lived a physically active lifestyle showed reduced depression symptoms.^{163,164} Sedentary lifestyle has been demonstrated to have a direct relationship with depression.¹⁶² Exercise has been advocated as a means to improve mood and depressive-type mental disorders through physiological and psychological processes, including improved mood state, enhanced self-esteem, and lower stress and anxiety.¹⁵⁷ In a study done by DiLorenzo and colleagues,¹⁶⁵ aerobic exercise showed not only improvements in health parameters, but also in many aspects of psychological well-being, including depression, anxiety, self-concept, and vigor. In obese and overweight individuals, increasing moderate-to-vigorous physical activity and decreasing sedentary time resulted in lower risk for the development of depression.¹⁶⁶

In animal studies, regular exercise caused alterations in serotonergic and noradrenergic systems, which are targeted by pharmacological treatments for depression.¹⁶⁷ Treadmill running with rats was shown to increase levels of blood tryptophan, allowing the brain to produce an increased rate of synthesis of serotonin, 5-hydroxyindoleacetic acid (5-HT).¹⁶⁸ Exercise promoted increases in 5-HT turnover in the hypothalamus in rats. In rats exposed prenatally to high level of GCs, four weeks of swimming exercise resulted in decreased levels of serum corticosterone and depressive behavior.¹⁶⁹ HPA-axis activity decreased in rats exposed to low-intensity stressors with six-weeks of intermittent, voluntary wheel running.^{170 171}

In humans, ten weeks of moderate-intensity exercise produced anxiolytic effects correlated with a downregulation of postsynaptic serotonin receptors and with a blunted cortisol

response to the 5-HT_{2C} agonist, meta-chlorophenylpiperazine (m-CCP).^{172,173} In a human cross-sectional study of 269 adult subjects, regular exercise was shown to reduce anxiety and lower depression, and in 7 randomized controlled studies, regular exercise was associated with reduced stress and decreased anxiety.^{174,175} In a study done with patients with major depressive disorder (MDD), a single bout of treadmill running resulted in improved mood.¹⁷⁶

Thioredoxin System

There are two main antioxidant systems in mammalian cells, the thioredoxin (Trx) system and the GPx system.¹⁷⁷ The thioredoxin system is a pivotal component in the antioxidant defense system and is comprised of three components: NADPH, thioredoxin reductase (TrxR), and Trx.^{22,177,178,179} The Trx system defends against oxidative stress by the reduction of disulfide activity and regulating the dithiol/disulfide balance in cells.^{177,178,179} Trx1 is found in the cytosol and nucleus, while Trx2 is located in the mitochondria.^{22,178,179} The Trx system plays a role in reversing oxidative modifications, like sulfenylation and nitrosylation.¹⁵⁵ Trx1 becomes oxidized and inactivated after it reduces oxidized cysteine groups on intracellular proteins, and TrxR then can re-activate this oxidized Trx by reducing it through the use of NADPH.^{177,22,179} Trx2 binds apoptosis-signaling kinase-1 (ASK-1) in the mitochondria and renders it dormant, allowing for the inhibition of ROS, inflammation, and apoptosis.^{155,180,181,182} Trx plays a crucial role in many protective mechanisms, including limiting apoptosis, protective anti-oxidant mechanisms, like breaking down hydrogen peroxide into water, and DNA synthesis and repair.^{177,178,179}

Thioredoxin Interacting Protein

Thioredoxin-interacting protein (TXNIP) has also been referred to as vitamin D-upregulated protein and thioredoxin-binding-protein-2.²² This protein has structural characteristics that fit the α -arrestin family, and has distinct binding sites for proteins like ASK-1 ubiquitin ligase, and others.¹⁸³ TXNIP acts to inhibit the antioxidant capabilities of the Trx system.^{22,24,184,185,23} TXNIP is a proapoptotic factor that has been thought to be a key player in the glucotoxic β -cell death that is associated with insulin resistance and diabetes.^{24,185} TXNIP is localized to the nucleus in pancreatic β -cells, but “shuttles” to the cytoplasm and/or mitochondria of the pancreatic β -cells (Figure 3).^{22,23,25} Oxidative stress induced by hydrogen peroxide (H_2O_2) has been shown to lead to shuttling of TXNIP into the mitochondria by TXNIP binding to importin- α .²³ Within the mitochondria, TXNIP competes with ASK-1 for the binding site on Trx2, causing ASK-1 to be released and phosphorylated; this can further lead to mitochondrial dysfunction and apoptosis (Figure 3).^{22,23,25,186} It was shown with Trx2 knockdown in INS-1 β -cells that phosphorylation and activation of ASK-1 and activation of mitochondrial death pathways were increased. This mitochondrial death pathway was assessed via increases in activated caspase-3. It was also shown that increasing mitochondrial TXNIP resulted in increased Trx2 oxidation, decreased Trx2-ASK-1 binding, and increased phosphorylation and activation of ASK-1.²³ Within the cytosol, TXNIP binds to reduced Trx1, inhibiting the ability of the antioxidant system to reverse protein cysteine oxidative modifications.^{22,27,155,178}

Figure 3 depicts the processes by which TXNIP interferes with the thioredoxin system in the pancreatic β-cell cytoplasm, mitochondria, and nucleus.²⁵

TXNIP has been suggested to be the link between glucose toxicity and pancreatic β-cell apoptosis, such as that seen in diabetes.^{24,184,185} In both obese and non-obese diabetic mice, TXNIP expression is shown to be elevated in pancreatic β-cells.^{184,185} Incubation of human pancreatic islets in high glucose concentrations for 24 hours resulted in a five-fold increase in TXNIP expression when compared to islets incubated in low glucose concentrations.^{24,184} INS-1 β-cells incubated in high glucose concentrations resulted in increased TXNIP levels 18-fold along with a 12-fold increase in cleaved caspase-3.^{184,185} Overexpression of TXNIP resulted in increases in cellular levels of ROS and free radical-mediated stress to β-cells by H₂O₂, while overexpression of Trx inhibited the ROS increase.^{24,185} TXNIP knockout gene shows reduced hyperglycemic-induced increase in oxidative stress.²⁴ Glucose-induced TXNIP increases also showed apoptosis in pancreatic islets of INS-1 cells, including increases in the Bax (proapoptotic) to Bcl-1 (antiapoptotic) ratio, increases in cleaved caspase-3, and TUNEL positive

β -cells.^{184,185} Glucose is shown to induce TXNIP transcription via a carbohydrate response element (ChoRE).¹⁸⁴

TXNIP and Chronic Stress

A study with INS1 β -cells and human and mouse pancreatic islets demonstrated that treatment with glucocorticosteroids led to increases in TXNIP expression, and that expression was even more pronounced than in hyperglycemic conditions. Over time, these increases in TXNIP were accompanied by the induction of pancreatic β -cell death.¹⁸⁷ Chronic stress associations with increased TXNIP expression have been reported in other organs as well. In a study by Zhou and colleagues, rodents that experienced chronic stress had significantly elevated TXNIP expression in the hypothalamus and frontal cortex when compared to control mice. In addition to increases in TXNIP, increases in protein sulfenylation and nitrosylation and increases in phosphorylated ASK-1 in the hypothalamus and frontal cortex were also observed. It is proposed that increases in corticosterone due to stress lead to these increases in TXNIP and its downstream regulators.¹⁵⁵ Bharti and colleagues¹⁸⁸ showed that treatment of neuronal cells with human cortisol resulted in increased expression of TXNIP in the nucleus and cytoplasm, and that GR inhibition lead to blockage of the cortisol-induced TXNIP expression. Whether chronic stress also causes an increase in TXNIP within the pancreas remains to be determined.

NLRP3 Inflammasome

The nod-like receptor protein 3 (NLRP3) inflammasome is a major producer of IL-1 β , a proinflammatory cytokine associated with insulin resistance (Figure 4).^{22,27,189,190} This contribution to the proinflammatory environment can also promote increases in proapoptotic

proteins, cleaved caspase-3 and cleaved PARP-1, which activate the apoptotic pathway P38 MAPK-JNK.²² TXNIP has been suggested to activate the NLRP3 inflammasome, triggering the production of IL-1 β .^{22,27,189} TXNIP binds to the leucine rich repeat (LRR) region of the inflammasome, and the activation is dependent on the generation of ROS, as all NLRP3 activators are known to generate ROS.^{27,191} This is further supported by the induction of NLRP3 inflammasome activity and IL-1 β secretion by addition of H₂O₂ in THP-1 human macrophages.^{27,191} When TXNIP expression was suppressed, H₂O₂-driven inflammasome activation was blunted, as shown by suppression of IL-1 β and caspase-1.²⁷

Figure 4 explains the role of adipose tissue NLRP3 inflammasome activity and its effects on other organs. The secretion of mature IL-1 β by the adipose tissue is associated with insulin resistance.¹⁹⁰

A HFD is accompanied by significant increases in the interaction of TXNIP with the NLRP3 inflammasome, along with significant increases in cleaved IL-1 β and cleaved caspase-1

and expression of TXNIP and mature IL-1 β . Mohamed *et al* incubated human retinal endothelial cells in palmitate-BSA to mimic HFD and found that the interaction of TXNIP and the inflammasome was increased, resulting in the activation of caspase-1 and IL-1 β . To further confirm these results, TXNIP was silenced and resulted in the abolishment of the palmitate-BSA increase of cleaved IL-1 β and decreases in cell death, as shown by decreases in proapoptotic cleaved caspase-3 and larger proportion of live cells to dead cells.¹⁸⁹

CHAPTER 3: METHODS AND MATERIALS

Animals

32 male OZR and 32 male LZR, ages 7-8 weeks, were obtained from Harlan. All rats were acclimatized to their environment for one week in the West Virginia University Health Sciences Animal Facility then placed on a reverse 12 hour light-dark cycle. LZRs and OZR were then randomly assigned to four groups: 1) sedentary controls (C: n=8); 2) unpredictable chronic mild stress (S: n=8); 3) exercise (EX: n=8); and 4) a combination of UCMS and Ex (EXS: n=8). All rats received standard chow and tap water ad libitum, and protocols received approval from the WVUHSC Animal Care and Use Committee prior to the start.

UCMS Protocol

The UCMS protocol was developed to produce depression-like behaviors in rodents.^{112,146,148} This approach is the most appropriate model for clinical depression in rodents and mimics human-like depression behaviors, including anhedonia and learned helplessness.¹⁹² During the UCMS protocol, rats were singly housed, and randomly exposed to the following mild environmental stressors for 8 hours each day, 5 days per week, over the 8 week course:

1. Damp bedding – 10 oz. of water was added to each standard cage
2. Bath – all bedding was removed and ~0.5 inches of water was added to the empty cage. Water temperature was at room temperature, ~24°C
3. Cage tilt – cage was tilted to 45 degrees without bedding
4. Social stress – each rat was switched into a cage of a neighboring rat
5. No bedding – all bedding was removed from the cage

6. Alignment of light/dark cycles – turning the lights off/on in random increments for a scheduled period

The protocol was altered each day to prevent rats from habituating to the stress.¹⁹²

Exercise Training Protocol

EX and EXS groups underwent 8 weeks of treadmill running, 5 days per week on multi-lane motor driven treadmills set at a 5% grade. The animals were acclimatized during the first week by running for 20 minutes then increasing by 10 minutes per day until a sustainable duration of 60 minutes daily was achieved. A maximum speed test was conducted on each rat to determine a target-running speed of 60-70% of the determined maximum. Once acclimatized, rats exercise for 60 minute per day. During the first 15 minute, the treadmill speed was gradually increased until the targeted-running speed was reached. At that point, the rats continued running at the targeted-running speed for the remaining 45 minutes. To discourage rats from stopping, a mild electrical stimulus ($\leq 0.3\text{mA}$) was used at the rear of the treadmill. At the end of the 8-week exercise protocol, there was a 48-hour wash out period between the last bout of Ex and the terminal surgery.

UCMS and Exercise Combination Protocol

LZR and OZR assigned to the EXS group performed the treadmill running first thing in the morning (8-9am) and then were immediately subjected to the UCMS protocol, as described previously.¹⁹²

Stress Marker Analysis

Coat Score

Throughout the 8-week protocol, coat scores were recorded every week for all rodents, with 0 being clean and 1 being dirty, for eight different body parts (i.e. head, neck, forelimbs, stomach, hindlimbs, tail, genitals). A total cumulative coat score was determined across all parts, and weight was recorded each week along with grooming habits.

Circulating Cortisol

A commercially available ELISA Kit (Cayman Chemical, Item #501320) was used to examine corticosterone levels in duplicate accordingly to the manufacture's instruction. Blood samples were collected at the time of the terminal surgery and plasma was used for the ELISA Kit.

Pancreas Health Analysis

Immunohistochemistry (IHC) Protocol

Pancreatic tissue was sectioned by cryo-stating (Leica CM3050S cryostat) three tissue samples per slide and 5 slides per animal. The cryo-stat was kept as close to -23°C as possible throughout the procedure, and the thickness was set to 8µm. These slides were then stored in a freezer at -18°C.

Sectioned slides with pancreatic tissue were air-dried for 5-10 minutes before the IHC process occurred. Once the slides were dried, the pap-pen was used to draw thin but solid lines around each tissue section, and the slides were then rehydrated in 1X PBS 10 minutes. The slides were fixed in room temperature, 4% paraformaldehyde for 20 minutes. Three, five-minute

washes were done in 1X PBS before the slides were permeabilized using 0.2% Triton X-100 at 4°C. After 10 minutes, the slides were again washed in 1X PBS for three, 5-minute rounds. After the third wash, the pap pen lines were carefully dried with Kimwipes and blocked with 150µL horse serum and 10mL 1X PBS for 60 minutes. The pap pen lines were re-dried after the 60-minute blocking period, and the primary Insulin (C27C9) Rabbit mAb #3014 antibody and primary Glucagon (10988) Mouse mAb solution was incubated on slides in a damp box at 4°C overnight.

On the second day of the IHC protocol, the slides were washed three times in 1X PBS. After the final wash, the lights were turned off and remained off for the remainder of the protocol due to the use of photosensitive antibodies. Alexafluor 546 IgG anti-rabbit secondary (for insulin) and Alexafluor 488 IgG, IgM anti-mouse (for glucagon) was incubated on the slides for 45 minutes. The slides were then washed in PBS three times for 10 minutes each round. Using Kimwipes, the slides were dried as much as possible without disturbing the sections and were mounted with DAPI hard set/DAPI prolonged stain and a cover. The slides were stored in the dark at room temperature overnight. The slides were imaged using SPOT5.2 Advanced program, and the images were analyzed using ImageJ.

Protein Analysis

Protein Quantification

Homogenates were made up by combining 200µL of RIPA buffer (with added dissolved protease-inhibitor tablet) for every 1mL of pancreatic tissue into a tube with ceramic beads, and using the BeadMill system, the tubes were homogenized for 30 seconds at a speed of 4m/s. Homogenates were centrifuged for 10 minutes at 4°C at a speed of 10rpm, and the supernatant

was pipetted into fresh tubes and saved, while the rest was discarded. Standards were prepared for the DC assay (Bio-Rad, Hercules, California). Homogenates were diluted with double-distilled water at a 1:20 ratio, and samples and standards were pipetted, in duplicate fashion, into a 96-well plate. A mixture of 20 μ L of reagent S per every 1mL of reagent A was made, and 25 μ L of this mixture was pipetted into each well except the blanks in wells H1-H2. 200 μ L of reagent B was added to each well, except the blanks in wells H1-H2, and the plate was gently shaken for 30 seconds and incubated for 15 minutes before begin read at 750nm.

TXNIP Analysis

To prepare the Simple Western System Wes plate (Protein Simple®, San Jose, California), DDT powder was mixed with 40 μ L of DIY water, and Master Mix tube powder was mixed with 20 μ L DDT mix and 20 μ L 10X Sample Buffer. The Biotinylated ladder was made by adding 20 μ L of double-distilled water to the ladder powder. Samples were diluted with 0.1X sample buffer to equate 3 μ L of protein per sample. After these were made, 4 μ L of diluted lysate and 1 μ L of Master Mix were mixed in fresh tubes. After all the samples were made up in Master Mix, they were heat denatured at 95°C for 5 minutes. The samples were added after they had been allowed to cool to room temperature. The primary TXNIP (TG2596699, Invitrogen®) Rabbit IgG mAb and TXNIP (188865, abcam®) Rabbit IgG mAb were diluted to a 1:50 ratio. The secondary conjugate (anti-Rabbit, (Protein Simple®, San Jose, California) was diluted 10-fold, and 200 μ L of Luminal-S was added to 200 μ L of Peroxide to yield the Luminol-Peroxide mix added to the fifth row of the plate.

The plate was centrifuged for 5 minutes at room temperature at 1000g or 2500rpm. The Wash Buffer was added to the top three rows. The bottom foil was then removed after centrifuge,

and any bubbles that had formed in the plate were popped using pipette tips or the foil before the plate was run.

Before running the Wes plate, a Self-Test was run on the Wes System. The plate and capillaries were then loaded, and the assay tab was set to the correct setting for the plate size being ran. The stacking time was changed to 18 minutes, the separation time to 31 minutes, and the antibody times all to 30 minutes, after which the plate was run.

Cytokine Analysis

RayBiotech® (Peachtree Corners, GA) kits were used for all ELISAs (IL-4, IL-6, IL-10, IL-1 β , and TNF- α). A DC assay was done before the ELISA kits were started to get the protein concentrations. Wash Buffer (20X) was diluted to a 1X Wash Buffer, the Sample Diluent Buffer and the Assay Diluent Buffer were diluted 5-fold. Standards were made according to each different cytokine ELISA based on the manufacturer's instructions.

100 μ L of the standards and samples were added in duplicate to the wells. The plate was covered and incubated for 2.5 hours at room temperature with gentle shaking. The plate was washed four times by filling the wells with 300 μ L 1X Wash Buffer. The wells were completely dried by carefully vacuuming out all the remaining liquid. The biotinylated ladder was made by adding 100 μ L of Assay Diluent Buffer to the Detection Antibody, and the detection antibody concentrate was then diluted 80-fold. 100 μ L of the 1X biotinylated ladder was added to every well and incubated, covered for 1 hour at room temperature with gentle shaking. The washing step was then repeated, and all remaining liquid was removed from all the wells. HRP-Streptavidin concentrate was made for each ELISA as follows:

- IL-4: 600-fold dilution with 1X Assay Diluent Buffer

- IL-6: 400-fold dilution with 1X Assay Diluent Buffer
- IL-10: 140-fold dilution with 1X Assay Diluent Buffer
- IL-1 β : 200-fold dilution with 1X Assay Diluent Buffer
- TNF- α : 200-fold dilution with Assay Diluent Buffer

Each HPR-Streptavidin concentrate was made into 10mL stocks, and 100 μ L was pipetted into each well and incubated, covered at room temperature with gentle shaking for 45 minutes. The wash step was repeated, and all the liquid was vacuumed out of the wells. With the lights dimmed 100 μ L of the One-Step Substrate Reagent was added to each well, and the plate was covered and incubated in the dark at room temperature with gentle shaking for 30 minutes. 50 μ L of Stop Solution was added to the wells, and the plate was read immediately at 450nm.

Cell Death Analysis

TUNEL staining was performed using a Cell Death Detection ELISA^{PLUS} (Roche® Indianapolis, IN). Slides were sectioned with 8 μ m thick pancreas tissue and stored in -20°C freezer. After air-drying for 10-15 minutes, sections were tightly traced with the pap-pen. Slides were fixed in 4% paraformaldehyde for 10 minutes, after which three, five-minute washes were done with 1X PBS. Slides were dried and permeabilized in 0.1X Triton-X-100 for two minutes. Slides were washed twice for ten minutes in 1X PBS. Slides were dried, and TUNEL solution was made using an In Situ Cell Death Detection Kit, Fluorescein. 450 μ L of labeling solution was added to 50 μ L of enzyme solution. 50 μ L of this mixture was added to ten sections, and 50 μ L of labeling solution alone was added to two sections for negative controls. Slides were incubated again and placed in a humidifier at 37°C, or body temperature, for 60 minutes. A final ten-minute wash was done after the TUNEL incubation in 1X PBS. The dried slides were mounted

with DAPI hard set/DAPI prolonged stain and a cover slip. The slides were stored in the dark at room temperature overnight.

Pancreatic lysate homogenates were used in the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) to obtain protein concentration values for all samples. Samples were diluted to a 1:20 ratio with double-distilled water, and 10µL of standard and 10µL of sample were added to each well in two 96-well plates in a duplicate fashion. Working reagent was made by thoroughly mixing 50 parts of BCA Reagent A with 1 part BCA Reagent B, and 200µL of the working reagent was added to each well, except the duplicate blank wells. The plate was shaken for 30 seconds and left to incubate for 30 minutes in 37°C. After the plate was cooled to room temperature, the absorbance was measured at 562nm on a plate reader.

The same homogenates were used from the BCA Protein Assay for the Cell Death ELISA^{PLUS} kit (Roche® Indianapolis, IN). 20µL of samples were randomly plated in duplicate in a pre-coated 96-well Cell Death ELISA plate, along with a positive control provided by the Cell Death kit and one blank well. All samples were carefully pipetted into the center of the plate. To each well, 80µL of the Immunoreagent was added. The Microplate (MP) was covered in an adhesive cover and incubated for 2 hours, shaking at 300rpm at 25°C. The MP was washed three times by filling and emptying the wells with 250-300µL of incubation buffer. The wells were dried completely, and 100µL of ABTS solution was added. The plate incubated for approximately 15 minutes with shaking at 250rpm. 100µL of Stop Solution was pipetted into the wells, and the absorbance was measured at 405nm in a plate reader.

Oxidative Stress Analysis

Hydrogen Peroxide Analysis

Pancreatic lysate homogenates were used for a DC Assay prior to the Amplex® Red Kit (Invitrogen®), as described above. 10mM Amplex® Red reagent stock solution was made dissolving Amplex® Red reagent vial contents in 60µL of DMSO, and 1X Reaction Buffer was made by adding 4mL of 5X Reaction Buffer to 16mL of deionized water. 10U/mL of Horseradish Peroxidase (HRP) was made by dissolving the contents of the HRP vial in 1.0mL of 1X Reaction Buffer. 20mM Hydrogen Peroxidase (H₂O₂) working solution was made by diluting 22.7µL of ~3% H₂O₂ into 977µL of 1X Reaction Buffer. Standards were prepared in a serial dilution from 2.5µM to 0.02µM using the 20mM H₂O₂ working solution.

50µL of the standards were pipetted in duplicate into the first two columns of wells, and 50µL of sample were pipetted in duplicate in random order in the rest of the wells. This was done over two plates, with five samples from plate one being added to five single wells on plate two for cross-plate comparisons. An Amplex® Red reagent/HRP working solution was made by combining 50µL of 10mM Amplex® Red reagent stock solution, 100µL of 10U/mL HRP stock solution, and 4.85mL of 1X Reaction Buffer. 50µL of this Amplex® Red reagent/HRP working solution was added to each well, and the plate was incubated at room temperature for 30 minutes, protected from the light. A microplate reader equipped for excitation in the range of 530-560nm was used to read the plate after incubation.

Nitrate/Nitrite Analysis

The Sievers Nitric Oxide Analyzer (NOA 280) was used for Nitrate Analysis. Samples approximately 50µg in size were homogenized using RIPA buffer with protease inhibitors to

give volumes of approximately 100 μ g. Standards were made using 1/10 dilutions starting with 100mM NO₃⁻ of stock solution sodium nitrate (0.425g NaNO₃⁻ / 50mL H₂O). The standards were made as follows: 10mM, 1mM, 100 μ M, 10 μ M, 1 μ M, and 0.1 μ M. Figure 5 shows the set up for the NOA 208.

50 μ L injections were done for standards and samples. After samples were taken up in the syringe, the needle was wiped with a kimwipe and then injected into the needle valve. After injections, the syringe was cleaned with distilled water two to three times, and the needle was wiped with a kimwipe. The standards and samples were injected after baseline was attained after each injection. Samples were tested in a triplicate manner, and standards were retested throughout the experiment to make sure no changes were occurring in the NOA 208 environment.

Figure 5 shows the set-up of the Sievers Nitric Oxide Analyzer (NOA 280) was used for Nitrate Analysis.¹⁹³

Superoxide/Uric Acid Analysis

Homogenates from above DC Protein Assays were used. Stock solutions were prepared and the concentrations of the standards was determined using the spectrophotometer. These

concentrations were used to determine the dilutions of the stock solutions. The water bath was set to 37°C, and three tubes were labeled for each sample, with duplicates for each tube (i.e. six total tubes for each sample).

The samples were columned through G-25 columns. One tube collected the volume liquid that eludes from the column before the Blue-Dextran colored solution (pre-column). A second tube collected the blue stained solution (post-column). The meniscus were marked on each of these tubes to use as a reference to other samples collected in fresh tubes. BCA protein assays were ran before and after columning to obtain protein concentrations for a ratio pre-column to post-column to determine the dilution factor due to columning.

After Xanthine was added to the samples, the tubes were vortexed and incubated in a 37°C water bath for 60 minutes. During the incubation, 150µL solution of Allopurinol in Acetonitrile (ACN) was made. At the 55 minute mark of the incubation, the tubes were placed in the same order as the addition of Xanthine, and at the 60 minute mark, 1mL of ice cold ACN/Allopurinol solution was added in the same order as Xanthine addition. The samples were vortexed and centrifuged at 4°C at 12,600rpm for 15 minutes. The supernatant was transferred to the second set of pre-labeled tubes and store at -80°C until time of analysis.

At the time of analysis by HPLC-EC, the samples were speed-dried and resolubilized in 300µL of mobile phase. The uric acid standards were prepared and both standards and samples were ran as prepared.

Statistical Analysis

All data are expressed as means and $\pm$ standard errors. A multifactorial (analysis of variance (ANOVA) with an interaction term was performed to determine differences between

groups using SPSS program. Tukey's post hoc analysis was performed to compare between groups. Independent T-tests were used to compare differences between two groups when appropriate. Statistical significance was set to $P \leq 0.05$.

CHAPTER 4: RESULTS

Treadmill running for eight weeks significantly decreased body mass in both genotypes (LZR and OZR). In addition, UCMS alone significantly decreased body mass in both LZR and OZR. UCMS significantly increased glucose, corticosterone, and coat scores levels in LZR and OZR, which demonstrated that the UCMS protocol was successful in producing a stress response in the rats (Table 1). EX rodents had significantly lower glucose levels when compared to C and EXS animals. Insulin plasma levels were significantly lower with training and increased with UCMS in LZR. This data is shown in Table 1.

Table 1 *P<0.05 vs. control group within same strain, ‡P<0.05 vs. UCMS group within strain, §P<0.05 vs. Training group.

	LZR-C	LZR-EX	LZR-S	LZR-EXS	OZR-C	OZR-EX	OZR-S	OZR-EXS
Age (weeks)	18	18	18	18	18	18	18	18
Body Mass (g)	400 ± 20	358 ± 15*	361 ± 12*	343 ± 12*	641 ± 29	599 ± 27*	585 ± 20*	567 ± 25
Glucose (mg dl-1)	98 ± 15	101 ± 20‡	124 ± 17*	115 ± 15	184 ± 29	154 ± 29*‡	230 ± 34*	219 ± 37*§
Insulin (ng ml-1)	1.2 ± 0.5	1.4 ± 0.7	2.1 ± 1.0	1.8 ± 1.0	6.6 ± 2.7	4.6 ± 2.4*	7.8 ± 3.7*	6.2 ± 2.4
Corticosterone	6.97 ± 0.4	9.44 ± 2.6	8.78 ± 1.3*	10.67 ± 3.6*	13.82 ± 1.4	10.6 ± 2.4*	17.3 ± 3.6*	15.78 ± 3.6§

The effects of obesity on pancreatic morphology

Pancreatic β -cell numbers ($p=0.03$), α -cell numbers ($p=0.00$), and islet area ($p=0.01$) were significantly higher in OZR-C versus LZR-C (Figure 6A, C, and E), while β -cell to α -cell ratio ($p=0.07$) was trending higher in LZR-C versus OZR-C (Figure 6F). β -cell density was

decreased ($p=0.02$), while α -cell density was increased ($p=0.05$) in OZR-C compared to LZR-C (Figure 6 B and D).

Figure 6 LZR-C vs. OZR-C mean β -cell number (A), β -cell density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). $\delta p < 0.05$ vs LZR-C.

The effects of stress alone and in combination with obesity on pancreatic morphology

There were no significant differences in β -cell numbers, α -cell numbers, islet area, or β -cell to α -cell ratio in LZR-S versus LZR-C (Figure 7A, C, and E). Although β -cell density was

not significantly changed, α -cell density was increased ($p=0.01$) in LZR-S compared to LZR-C (Figure 7B and D). OZR-S had a significantly lower number of α -cells ($p=0.00$) compared to OZR-C (Figure 8C), but there were no significant differences in β -cell numbers, islet area, or β -cell to α -cell ratio in OZR-S versus OZR-C (Figure 8A, C, and E). β -cell and α -cell densities were not different in OZR-S versus OZR-C (Figure 8B and D).

Figure 7 LZR-C vs. OZR-S mean β -cell number (A), density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). * $p < 0.05$ vs. control group within same strain.

Figure 8 OZR-C vs. OZR-S mean β -cell number (A), β -cell density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). * $p < 0.05$ vs. control group within same strain.

The effects of exercise training on obesity and stress related changes to pancreatic morphology

There was no significant difference in pancreatic β -cell number, but β -cell density was trending to increase ($p=0.10$) between OZR-EX and OZR-C (Figure 9A-B). The α -cell number ($p=0.00$) was lower and the islet area ($p=0.02$) was smaller in OZR-EX compared to OZR-C

(Figure 9C and E). The α -cell density did not significantly change between these groups (Figure 9D). In addition, the β -cell to α -cell ratio was significantly higher ($p=0.04$) in OZR-EX versus OZR-C (Figure 9F).

P=0.

Figure 9 OZR-C vs. OZR-EX mean β -cell number (A), β -cell density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). * $p < 0.05$ vs. control group within same strain.

In the UCMS model, β -cell number ($p=0.00$), α -cell number ($p=0.00$), and islet area ($p=0.00$) were significantly higher in LZR-EXS compared to LZR-S, but there were no significant changes in β -cell or α -cell density or β -cell to α -cell ratio (Figure10A-F).

Figure 10 LZR-S vs. LZR-EXS mean β -cell number (A), β -cell density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). ‡ $p < 0.05$ vs. UCMS group within strain.

Pancreatic β -cell number and density, α -cell number and density, islet area, and β -cell to α -cell ratio did not show significant differences in OZR-EXS versus OZR-S (Figure 11A-F). All of the data from the above results on pancreatic β -cell number, α -cell number, islet area, and β -cell to α -cell ratio is also shown in Table 2.

Figure 11 OZR-S vs. OZR-EXS mean β -cell number (A), β -cell density (B), α -cell number (C), α -cell density (D), islet area (E), and β -cell to α -cell ratio (F). $\ddagger p < 0.05$ vs. UCMS group within strain.

Table 2 *P<0.05 vs. control group within same strain, ‡P<0.05 vs. UCMS group within strain

	LZR-C	LZR-Ex	LZR-S	LZR-ExS	OZR-C	OZR-Ex	OZR-S	OZR-ExS
β-Cell	96 ± 9	123 ± 10*	66 ± 9	147 ± 11‡	134 ± 15	146 ± 22	127 ± 16	111 ± 9
α-Cell	49 ± 4	55 ± 4	45 ± 7	88 ± 6	98 ± 15	35 ± 5	51 ± 6	36 ± 5
β-Cell to α-Cell Ratio	2.4 ± 0.31	2.3 ± 0.14	2.3 ± 0.72	1.8 ± 0.11	1.5 ± 0.16	5.4 ± 1.2	2.7 ± 0.33	4.5 ± 0.96
Islet Area	94167 ± 8708	106783 ± 9479	61746 ± 7409	129770 ± 10463	157002 ± 19128	91687 ± 15615	121704 ± 17613	106769 ± 9240

The effects of obesity on the pancreatic pro-inflammatory environment and level of apoptosis

TXNIP was trending higher (p=0.10) in pancreatic tissue of the OZR-C compared to the LZR-C (Figure 12A). As expected, TNF-α concentration was higher (p=0.03) in OZR-C versus LZR-C (Figure 12B), however, although IL-1β appeared greater in OZR-C compared to LZR-C, the difference was not significant (Figure 12C). IL-4 concentration was higher (p=0.01) in OZR-C versus LZR-C, but IL-10 and IL-6 showed no significant differences between groups, although IL-10 (p=0.08) did show a trending increase (Figure 12D-F). Hydrogen peroxide was shown to decrease in OZR-C compared to LZR-C (p=0.00), but other markers of oxidative stress were not significantly different (Figure 13A-C). No differences were noted in the level of apoptosis between groups.

Figure 12 LZR-C vs. OZR-C mean TXNIP (A), TNF- α (B), IL-1 β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. $\delta p < 0.05$ vs LZR-C.

Figure 13 LZR-C vs. OZR-C Nitrate/nitrite, NO (A), hydrogen peroxide, H₂O₂ (B), and superoxide, XO (C). * $p < 0.05$

The effects of stress alone and in combination with obesity on the pancreatic pro-inflammatory environment and level of apoptosis

Stress, alone (LZR-S) and in combination with obesity (OZR-S), did not cause a significant increase in TXNIP when compared with matched controls (Figure 14A and 16A). No cytokines, including TNF- α , IL-1 β , IL-4, IL-5, and IL10, were significantly different in LZR-S compared to LZR-C (Figure 14B-F). In OZR-S, TNF α (p=0.03), IL-4 (p=0.02), and IL-6 (p=0.01) concentrations were lower when compared to OZR-C (Figure 16B, D, and F). IL-1 β and IL-10 concentrations were not different in OZR-S compared to OZR-C (Figure 16C and E). Hydrogen peroxide was decreased in LZR-S and OZR-S when compared to their respective controls (p=0.00), although no other markers of oxidative stress were not significantly different between these groups. (Figure 15 and 17A-C). No changes in apoptosis were seen between any of these groups.

Figure 14 LZR-C vs. LZR-S mean TXNIP (A), TNF- α (B), IL-1 β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. *p<0.05 vs control group within same strain.

Figure 15 LZR-C vs. LZR-S Nitrate/nitrite, NO (A), hydrogen peroxide, H₂O₂ (B), and superoxide, XO (C). *p<0.05

Figure 16 OZR-C vs. OZR-S mean TXNIP (A), TNF-α (B), IL-1β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. *p<0.05 vs control group within same strain.

Figure 17 OZR-C vs. OZR-S Nitrate/nitrite, NO (A), hydrogen peroxide, H₂O₂ (B), and superoxide, XO (C). *p<0.05

The effects of exercise training on the obesity and stress related changes to the pancreatic pro-inflammatory environment and level of apoptosis

TXNIP was not significantly different in OZR-EX versus OZR-C (Figure 18A). IL-4 (p=0.03) and IL-6 (p=0.03) were significantly lower (Figure 18D and F), while IL-10 (p=0.06) and TNF-α (p=0.07) were trending lower in OZR-EX compared to OZR-C (Figure 18B and E). IL-1β concentration was not significantly different between these two groups (Figure 18C). Although hydrogen peroxide was shown to be lower in OZR-EX versus OZR-C (p=0.00), no other markers of oxidative stress were different (Figure 19A-C).

Figure 18 OZR-C vs. OZR-EX mean TXNIP (A), TNF- α (B), IL-1 β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. * $p < 0.05$ vs control group within same strain.

Figure 19 LZR-C vs. LZR-S Nitrate/nitrite, NO (A), hydrogen peroxide, H_2O_2 (B), and superoxide, XO (C). * $p < 0.05$

TXNIP ($p=0.01$) was significantly lower in LZR-EXS compared to LZR-S (Figure 20A), but no significant differences were seen in the proinflammatory cytokines TNF- α , IL-1 β , IL-4, IL-6, or IL-10 concentrations between LZR-EXS and LZR-S (Figure 20B-F). Nitrate/nitrite activity was lower, and hydrogen peroxide was higher in LZR-EXS compared to LZR-S ($p=0.02$), while superoxide was not different (Figure 21A-C). The mean TXNIP in OZR-EXS tended to be lower ($p=0.10$) compared with OZR-S (Figure 22A). TNF- α , IL-1 β , IL-4, IL-6, and IL-10 concentrations were not significantly different in OZR-EXS versus OZR-S (Figure 22B-F). Nitrate/Nitrite activity was lower in OZR-EXS versus OZR-S ($p=0.02$), although no other oxidative stress measures saw significant differences (Figure 23A-C). No apoptosis was seen with any exercise groups.

Figure 20 LZR-S vs. LZR-EXS mean TXNIP (A), TNF- α (B), IL-1 β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. $\ddagger p < 0.05$ vs. UCMS group within strain.

Figure 21 LZR-C vs. LZR-S Nitrate/nitrite, NO (A), hydrogen peroxide, H₂O₂ (B), and superoxide, XO (C). ‡p<0.05

Figure 22 OZR-S vs. OZR-EXS mean TXNIP (A), TNF-α (B), IL-1β (C), IL-4 (D), IL-6 (E), IL-10 (F) concentration. ‡p<0.05 vs. UCMS group within strain.

Figure 23 LZR-C vs. LZR-S Nitrate/nitrite, NO (A), hydrogen peroxide, H₂O₂ (B), and superoxide, XO (C). ‡p<0.05

CHAPTER 5: DISCUSSION

The primary purpose of this study was to determine the effects of obesity, chronic stress and exercise training on pancreatic morphology and health. We hypothesized that obesity would decrease the number of pancreatic β -cells, the β -cell to α -cell ratio, and islet area. A primary finding of this study was that obesity did not result in a decrease in β -cell number but instead was associated with increases in β -cell number, α -cell number, and islet area. With pancreatic β -cell and α -cell numbers increasing, this led to no change in the β -cell to α -cell ratio. Islet area may have increased to compensate for increases in both β -cell and α -cell numbers, and did so at a greater magnitude as indicated by the lower β -cell density in the obese animals. Previous research has shown that there are five stages to developing insulin resistance: compensation, stable adaptation, unstable early decompensation, stable decompensation, and severe decompensation.⁴² Given that pancreatic β -cell numbers were higher with obesity in this study, it can be suggested that the obese animals were in an early compensation stage of insulin resistance. This is further supported by the fact that plasma insulin was higher in the obese animals. The insulin producing pancreatic β -cells may have been undergoing hyperplasia to control for the higher levels of glucose also seen in the obese animals. Although the glucose measurement, 184 mg dl^{-1} could suggest that the rats were further along than the compensation phase, this was not a fasted measurement and was only taken at the time of the termination surgery. In order to compensate for increases in β - and α -cell numbers, islet area also increased in the obese animals. Previous research has suggested that obesity leads to a reduced β -cell to α -cell ratio due to β -cell apoptosis with no real change to α -cell numbers.⁴⁰ However, this would most likely occur during later phases of decomposition, when the pancreatic β -cells begin experiencing elevated apoptosis and greater sustained fasted glucose levels than observed in the

present study.^{40,42} Our results showed very little apoptosis in the obese animals and no differences compared with controls again indicating an earlier stage of decompensation. Although these results differ from those of Shimabukuro *et al*⁸⁹ who reported that apoptosis occurred during the early stages of diabetes development in obese homozygous (*fa/fa*) male ZDF, they are consistent with those found that in human pancreas from obese individuals during autopsies.⁹³ In the latter study, β -cell mass was actually increased with obesity.⁹³

We also hypothesized that chronic stress would negatively alter the morphology of the pancreas. However, no significant differences were observed between stress and controlled rats in the number of β - and α -cells, islet area, or the β -cell to α -cell ratio. The α -cell density did increase in the lean stress environment, which can be a result of the small decrease seen in islet area, though that decrease was not significant. We further hypothesized that stress in combination with obesity would have a combined deleterious effect on the pancreatic β -cell and α -cell numbers, islet area, and β -cell to α -cell ratio. However, the combined effect only had a deleterious effect on the α -cell numbers. The fact that obesity and chronic stress had little negative effects on pancreatic morphology was further confirmed by the absence of pancreatic apoptosis in each of these conditions. Plasma insulin levels at the time of surgery were higher in the stressed, obese animals, again suggesting that β -cells were still able to compensate for the elevated glucose plasma levels that were seen in the stressed, obese animals. Altogether this can suggest that these animals were in the early compensation phase of the pathway to insulin resistance.⁴²

Chronic stress which leads to elevated levels of GCs can result in damage to the pancreas that can lead to insulin resistance, hyperinsulinemia, and hyperglycemia.^{5,9,98,109} Humans subjected to chronic GC treatment (>14 days) showed decreases in insulin secretion when

subjected to a glucose challenge. This impairment in insulin action could suggest an indirect loss in pancreatic β -cell function or suppression of the release of insulin by pancreatic β -cells.^{110,111} Rats exposed to eight weeks of UCSM did in fact, have significantly elevated corticosteroid and glucose levels. However, the UCMS protocol used in the current study produced very few morphological changes to the pancreas and was not associated with a loss of β -cells. These results go against the belief that GCs cause metabolic and morphological attenuations to the pancreatic β -cells.^{20,112} It is possible that the level of stress used in the current study was too low to produce the morphological changes to the pancreas often seen in other models. The stress method used by Zhou *et al*¹⁵⁵ that resulted in increases in TXNIP within the hippocampus and frontal cortex with stress, utilized a much more intense protocol, including foot shock, tail clamp, and cold water swimming. Give that our UCMS model was a mild stress model, this may suggest that a longer duration is required to see these negative effects at the level of the pancreas.

Chronic exercise has been shown to improve physical attributes of health, including reduced blood pressure, enhanced cardiovascular fitness, increased insulin sensitivity, reduced abdominal adiposity, and prevention of chronic diseases, such as obesity.^{157,158} We hypothesized that the addition of eight weeks of treadmill running would attenuate obesity-related changes in β -cell numbers, the β -cell to α -cell ratio, and islet area. Exercise training in the obese animals was associated with decreases in α -cell numbers and islet area, but increases in β -cell to α -cell ratio. Eight weeks of treadmill running was associated with lower body weights in both obese and lean animals compared with controls. This was associated with slightly lower glucose and corticosterone levels, both of which are known to stimulate β -cell proliferation and islet growth. It is possible that many of the deleterious physiological effects associated with obesity were

blunted in the trained rats, which provided less stress on the pancreas leading to reduced cell and islet proliferation.

It was also hypothesized that eight weeks of treadmill running would attenuate the stress-related morphological changes to the pancreas. The UCSM protocol used in this study, shown to be effective by increased coat scores in the stress animals, produced only a few measured morphological changes to pancreas even when combined with obesity. However, when combined with exercise, stressed animals had greater numbers of β - and α -cells and a larger islet area when compared with the stressed only rats. This effect may represent either a reversal of the minor stress-induced decreases to the pancreas (e.g., smaller islet area) or more likely, a greater stimulation for pancreatic growth. Although exercise training has been shown to reduce stress, the model used in the current study required animals to be stimulated to run.^{169,172,173} Animals, which simultaneously underwent the UCMS protocol, may have been conditioned to perceive the treadmill running as an additional stressor. The UCSM protocol induces stress that presents similar behaviors to that of depression in rodents, presenting itself such that rodents show anhedonia-like behavior and other behaviors that suggest depression is present.^{112,146,148} Although HPA-axis activity decreased in rats exposed to low-intensity stressors with six-weeks of intermittent, voluntary wheel running, forced treadmill running when combined with the UCMS may have caused greater levels of corticosteroid release and a greater stimulation of pancreatic growth.^{170,171} Interesting, corticosteroid was slightly higher in the LZR-EXS compared with the LZR-EX rats, supporting this idea.

Obesity and chronic stress have been shown to result in a proinflammatory environment within the host.^{7,17,99,117,130,131} TXNIP is a proapoptotic protein associated with increased oxidative stress and apoptosis, specifically β -cell apoptosis within the pancreas.^{24,25,185}

According to previous research, obesity and chronic stress are associated with increases in TXNIP throughout different parts of the body.^{155,184,185,187,188,189} Based on this research, we hypothesized that TXNIP would increase with obesity in the pancreas, and our findings indicated that TXNIP was elevated, although not significantly, in the obese versus lean rats. We also hypothesized that obesity would be associated with an increased proinflammatory environment. Our results showed that the proinflammatory cytokine TNF- α was significantly elevated in the obese animals, although IL-1 β was not. Previous research has suggested that both TNF- α and IL-1 β increase in conjunction with obesity.^{7,10,22,27,28} IL-1 β did appear to be somewhat elevated with obesity in the present study, but this elevation did not reach significance compared with controls. IL-1 β is a proinflammatory cytokine, primarily produced by the NLRP3 inflammasome and triggered by TXNIP.^{22,27,189,190} Since we did not see a significant increase in TXNIP, it can be suggested that IL-1 β would also not be significantly increased. IL-4, a cytokine that can act acutely as an anti-inflammatory cytokine and chronically as a proinflammatory cytokine, was increased with obesity; IL-4 works to increase macrophage activation to combat foreign substances, but IL-4 can chronically stimulate inflammation.¹⁹⁴ IL-6, a cytokine acting as either anti-inflammatory or proinflammatory depending on the tissue of origin, was not effected by obesity.^{13,67,68,69} Finally, IL-10 concentrations, an anti-inflammatory cytokine, had an increasing trend in the obese animals.^{13,70} These results may suggest that there was an increase in the proinflammatory environment of the obese animals, and that an anti-inflammatory response was reacting to reduce this inflammation. Obesity has been shown to induce a phenotypic change in macrophage polarization, from M₂ to M₁.^{13,65} During this shift, it would be reasonable to see both proinflammatory and anti-inflammatory cytokines upregulated as the environment is shifting from M₁ to M₂.

We hypothesized that eight weeks of UCMS alone and in combination with obesity would increase pancreatic TXNIP, proinflammatory cytokines, and apoptosis and decrease anti-inflammatory cytokines. TXNIP was not shown to be increased in the pancreas by either chronic stress alone, or in combination with obesity. The fact that we did not see a difference in TXNIP between the LZR-S and LZR-C indicates that the UCMS protocol alone was either not stressful enough or too short to produce an effect within the pancreas. Previous research has reported that rodents that experienced chronic stress had significantly elevated TXNIP expression in the hypothalamus and frontal cortex when compared to control mice. It was proposed that increases in corticosterone due to stress lead to these increases in TXNIP and its downstream regulators.¹⁵⁵ A study done with INS1 β -cells and human and mouse pancreatic islets demonstrated that treatment with glucocorticosteroids led to increases in TXNIP expression, and that expression was even more pronounced than in hyperglycemic conditions.¹⁸⁷ Although circulating corticosteroid and glucose were higher in the LZR-S versus LZR-C animals, these values may not have reached the level necessary to cause an elevation in pancreatic TXNIP in the stressed animals. Furthermore, no cytokines, including TNF- α , IL-1 β , IL-4, IL-6, and IL10, were changed with chronic stress alone. These results were further confirmed when comparing OZR-S to OZR-C. In combination with obesity, TNF- α , IL-4, and IL-6 were actually decreased in the obese stressed rats, while IL-10 and IL-1 β were not changed. These results were highly surprising and seem to indicate a unique interaction between the two treatments. It must be noted that the cytokines were measured within the pancreas lysates. Future research should examine the level of stress necessary to see changes to the proinflammatory environment within the pancreas independent of other conditions.

Although we hypothesized that eight weeks of treadmill running would decrease TXNIP, proinflammatory cytokines concentrations, and indices of apoptosis in combination with obesity, our results showed that TXNIP and IL-1 β was not different between OZR-EX and OZR-C. However, obese animals which underwent eight weeks of treadmill running did have lower TNF- α , IL-4 and IL-6, cytokines that can be proinflammatory, and lowering trends in IL-10, an anti-inflammatory cytokine.^{13,56,70} When combined with the fact that treadmill running was associated with reduced α -cell numbers and smaller islet, this seems to indicate that our exercise training was sufficient to limit some of the deleterious effects of obesity. Brunn *et al* showed that 15 weeks of hypocaloric diet and daily exercise resulted in decreases in circulating proinflammatory cytokines and decreases in adipose tissue expression of inflammatory markers, like IL-6 and TNF- α .¹⁶³ Given our exercise protocol was only eight weeks, it is promising to see that there are already lowering cytokine levels.

Treadmill running in combination with chronic stress was hypothesized to attenuate the stress-induced increases in TXNIP, proinflammatory cytokines, and apoptosis. However, as previously mentioned, stress alone produced very few negative effects within the pancreas. When combined with treadmill running, only TXNIP was lower in LZR-EXS compared with LZR-S animals. No differences were seen with any cytokine concentrations, including TNF- α , IL-1 β , IL-4, IL-6, or IL-10. It is interesting that no differences were observed in the proinflammatory environment between those animals who underwent eight weeks of UCMC and treadmill running verses UCMS alone. These results do seem to go along with our findings that the combined treatment caused enhanced pancreatic growth. We speculate that the latter was caused by a combination of the stimulatory effects of enhanced circulating glucocorticoids and possibly glucose, and a pancreatic environment conducive to growth. Associated with this idea

was the fact that TXNIP was lowered in the combined group. This would have led to a reduced environment for apoptosis, possibly allowing β cells to proliferate and islets to grow. The fact that the oxidative stress marker H_2O_2 was higher in LZR-EXS animals may indicate that the combined treatment was more stimulatory to the pancreas. However, these results must be taken with caution as oxidative stress was measured on frozen not fresh tissue.

We further hypothesized that eight weeks of treadmill running would attenuate the combined effects of obesity and UCMS on pancreatic increases in TXNIP, proinflammatory cytokines, and apoptosis and decreases in anti-inflammatory cytokines. TXNIP concentration did show a decreasing trend with exercise, although there were no differences in any of the cytokines, including $TNF-\alpha$, $IL-1\beta$, $IL-4$, $IL-6$, and $IL-10$. This would again indicate that the UCSM protocol used in the present study may have been ineffective in causing negative changes within the pancreas.

The stress-induced increases in TXNIP in the hippocampus and frontal cortex in mice, was associated with increases in corticosterone, a key regulator of TXNIP.¹⁵⁵ Corticosterone followed the TXNIP pattern in the current study, increasing with obesity and decreasing with exercise training. UCMS also increased corticosterone significantly in both genotypes although TXNIP was not significantly increased with chronic stress. This again would indicate that obesity, more so than the UCMS used in the current study, was able to alter specific hormones and proteins known to have an effect on pancreatic morphology and health.

Although TXNIP, a proapoptotic protein, was increased in some incidences in this study, there were no signs of apoptosis, as confirmed through multiple methods. This could suggest that downstream markers of this proapoptotic pathway, such as p-ASK-1, were not yet activated in the cascade to cell death.

CONCLUSION

In conclusion, in a model of obesity and metabolic syndrome, the pancreas appeared to be stimulated to grow as indicated by greater numbers of β - and α -cells and larger islets even in the presence of slightly higher pancreatic TXNIP and TNF- α . Since apoptosis was not observed, it can be suggested that the proapoptotic-TXNIP pathway in the pancreas may take more time than we allotted to reach a true proapoptotic phase. Eight weeks of treadmill exercise training was shown to attenuate some of the obesity-induced morphological and proinflammatory changes within the pancreas. It did not however appear to reduce TXNIP. Eight weeks of UCMS alone produced no morphological changes in the pancreas, no alterations in TXNIP, and no changes in the measured proinflammatory cytokines compared with controls. When combined with obesity, we also very few deleterious effects within the pancreas. Exercise training did appear to lower TXNIP in both lean and obese stressed mice. It would appear from this study that a greater stress load or a longer period of stress is necessary to show detrimental effects within the pancreas.

LIMITATIONS AND FUTURE RESEARCH

A limitation to this study is that data was collected at a single time-point in the progression of the chronic stress and obesity protocol. When forming a hypothesis, it is impossible to know at what point in the progression of pancreatic health decline the animals may be. Future research should examine the longitudinal effects of both obesity and stress. Another limitation to this study is that pancreatic lysates were made of the whole pancreas tissue homogenates. Although we had desired to isolate the β -cells and mitochondria of the β -cells, these procedures could not be done on previously frozen tissue. Results for many of the measured oxidative stress markers were quite surprising and opposite to what has been reported

in the literature. A major limitation to this data was the fact that these measures were made on frozen not fresh tissue, the preferred method for this analysis. Unfortunately, fresh tissue was unavailable and having only samples from a previous study limits experiments that require fresh tissue. Lastly, it would benefit this study to have more animals, as some of the groups only had 3 or 4 pancreases available.

Future research can be done to evaluate the training relationship with TXNIP and the thioredoxin system. A longevity study can be done to assess at what point pancreatic health (along with other organs) begins to decline and β -cell numbers begin to change and the role that TXNIP and its downstream agonists play in the development of insulin resistance with this model. Using a model, such as Zucker Fatty Diabetic (ZDF) rat, would be better to assess the effects of TXNIP, given that this model is known to develop pancreatic β -cell dysfunction and apoptosis. Using the ZDF model in a longevity study, could provide a better understanding of TXNIP's relationship with β -cell apoptosis and how high feeding, chronic stress fat, and exercise influence outcomes. A TXNIP knockout or overexpressed mouse could be used to better understand the role of TXNIP in apoptosis in the pancreas and other organs and how chronic stress and training influence these effects, though this would be more useful after studies were done with the ZDF rats. These proteins and relationships can also be studied on other organs. Skeletal muscle expression of TXNIP levels and other downstream markers of the TXNIP pathway can be measured to assess if training is changing the periphery environment in a model of metabolic syndrome and chronic stress.

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