Heat Shock Protein 72 Regulation of Metabolism

By

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Heat Shock Protein 72 Regulation of Metabolism

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Abstract

The prevalence of metabolic disease continues to rise in the United States, leading to conditions such as metabolic syndrome, diabetes, and NAFLD. Heat shock proteins (HSPs) are molecular chaperones which aid in protein folding during cellular stress. These proteins are also important in metabolic function, through inhibiting inflammation and increasing oxidative capacity in skeletal muscle. Reduced HSPs may also lead to metabolic disease, demonstrated by reduced HSP72 expression in skeletal muscle of diabetic patients and in the liver with the progression of NAFLD.

Our studies further demonstrate that disruption in the HSP response could be an underlying commonality in various metabolic conditions and tissues. First, we found that a reduction in HSPs in skeletal muscle of a post-menopausal rat model is associated with reduced mitochondrial protein expression, increased lipid storage, and reduced exercise capacity. We also investigated the role of HSP72 in the liver. A loss of HSP72 in hepatocytes led to a reduction in fatty acid oxidation, mitochondrial dysfunction, and increased lipid storage. A reduction in HSPs in both the skeletal muscle and the liver may increase susceptibility to the development of metabolic disease.

Our work also demonstrates that activation of HSPs has potential to protect from metabolic dysfunction. We found that a heat treatment intervention increases HSP72 in the liver, reduces hepatic triglyceride storage, and improves whole-body glucose homeostasis in rodents fed a high-fat diet. We also found that acute and chronic exercise increase HSP72 protein expression in the liver. The induction of HSPs with exercise was associated with changes in autophagy and mitophagy protein expression, which may protect hepatocytes from accumulation of damaged organelles. This work strongly suggests that HSP72 maintains whole-body metabolic homeostasis through protecting against lipid accumulation in both skeletal muscle and liver. Therapies which activate HSP72 may be the key to protecting against metabolic disease.

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Table of Contents

Table of Figures

CHAPTER 1

Introduction

1.1 Obesity and Metabolic Syndrome

1.1.1 Epidemiology

Obesity is a significant health concern in the United States that is predicted to affect half of Americans by 2030 [1]. This places an immense burden on our health care system, as the yearly cost in the United States for obesity is $147 billion dollars [1]. Metabolic syndrome develops over time and is a collection of factors including abdominal obesity, dyslipidemia, hypertension and glucose intolerance stemming from metabolic dysfunction [2]. Obesity and metabolic syndrome put people at risk for many other health complications such as type 2 diabetes, cardiovascular disease, and stroke. This health crisis is not slowing, as one in six children in the United States are obese [3]. Due to the high prevalence, the medical community is in need of novel and more effective strategies for prevention and treatment of metabolic syndrome.

Insulin resistance is a major component of metabolic dysfunction. Due to high blood glucose, inflammation and lipid toxicity, metabolic tissues such as skeletal muscle and the liver become insulin insensitive over time. This results in dysregulation of gluconeogenesis by the liver, and decreased ability of skeletal muscle to perform glucose uptake [4, 5]. At the beginning of metabolic dysfunction development, pancreatic β-cells overproduce insulin in order to compensate for insulin resistance in metabolic tissues. However, the hormone no longer effectively clears glucose from the blood. Eventually with the progression into type 2 diabetes, β-cells are not able to produce sufficient insulin [6].

1.1.2 Risk Factors and Current Treatment

There are many factors that affect a person’s susceptibility to metabolic syndrome. Obesity, excess nutrition, sedentary behavior, insulin resistance and family history of the disease are all risk factors for development of metabolic syndrome. Certain racial groups, such as Hispanics, are more susceptible to developing metabolic syndrome [7]. The first treatment

option for metabolic syndrome involves lifestyle changes such as physical activity and improved nutrition [7]. If lifestyle changes are not sufficient, surgery and medication are also options. Bariatric surgery has become a common strategy to decrease obesity [8]. Medications which control blood pressure, cholesterol levels, and blood glucose are also typically used. Insulin sensitizers, such as metformin and thiazolidinediones (TZDs), are a main form of treatment in patients with advancing diabetes [9, 10].

1.2 Metabolic Dysfunction in Postmenopausal Women

The development of insulin resistance in skeletal muscle and systemically is a critical step towards diabetes, and often occurs in post-menopausal women [11]. Estrogen's most studied role is in reproductive tissues, but estrogen is also important in many non-reproductive tissues [12, 13]. Estrogen therapy can ameliorate the increased risk of diabetes in postmenopausal women through improving metabolic homeostasis systemically and in skeletal muscle [14]. Clinical studies have been essential in identifying the role of estrogen in metabolism, but rodent models have allowed us to identify tissue-specific effects of estrogen action including the importance of estrogen action in skeletal muscle. Rodent models, such as ovariectomy and estrogen receptor knockout, have also deepened our understanding of the estrogen-mediated mechanisms.

1.2.1 Susceptibility to Insulin Resistance in Male and Female Rodents

Males and females exhibit variations in metabolic regulation and response to metabolic stressors. Although these variations have been observed in rodent and human research, studies investigating the effect of a high-fat diet (HFD) on weight gain in male versus female rodents has yielded inconsistent results. Some studies have found that female rodents gain significantly less body weight [15] and adipose tissue weight [15, 16] than male rodents on a HFD. Other studies have found that male and female rodents gain similar weight [16, 17] or that female rodents gain more body weight and adipose tissue than male rodents on a HFD [18-20].

Variables that could significantly change the outcome in these studies are the species of rodent and also the duration and composition of HFD. Also, it is understandable that estrogen can be protective from metabolic dysfunction to only a certain extent before diet can overcome that protection.

Although many studies are not in agreement with changes in weight gain with a HFD, one difference that has been consistently observed in past studies is decreased susceptibility to insulin resistance in female rodents following a HFD [17, 18, 21-25]. For example, Yakar et al. found that a 10-week HFD equally increased body weight and fat percentage in both male and female rodents, however male rodents demonstrated increased insulin resistance as measured by glucose and insulin tolerance tests, while females did not [22]. Other studies have found similar results regarding protection from insulin resistance in female rodents even without reduced weight gain compared to male rodents [17, 18]. In addition to reduced susceptibility to insulin resistance, female rodents show greater whole-body oxygen consumption compared to males [16, 26]. Both enhanced insulin sensitivity and oxygen consumption contribute to improved whole-body metabolism in female rodents.

Insulin sensitivity and systemic metabolic homeostasis may be better maintained in female rodents on a HFD due to enhanced skeletal muscle metabolism. Many studies have demonstrated that a HFD in male rodents impairs skeletal muscle glucose metabolism [27-29], but not as many studies have compared males and females. In two different studies, researchers found metabolic differences in skeletal muscle that may protect female rodents from HFD-induced insulin resistance. Gomez et al. found that female rodents had higher triglyceride storage capacity in skeletal muscle [17] and adipose tissue and increased expression of fatty acid utilization genes in skeletal muscle [18]. Additionally, they found that stress and antioxidant enzyme activity in muscle was similar between genders, although female rodents demonstrated slightly higher skeletal muscle oxygen consumption and significantly higher cytochrome C oxidase (COX, the last enzyme in the respiratory electron transport chain)

activity than male rodents in response to a HFD. Skeletal muscle from female rodents also had increased Glut4 protein content, the main glucose transporter in skeletal muscle, and UCP3 protein content, an uncoupling protein, in response to a HFD [17]. Uncoupling proteins (UCP) transfer hydrogen ions to the intermembrane space and can protect mitochondria from oxidative stress [30] with increased flux into the electron transport chain (ETC). Another study by Catala-Niell et al. found that female rodents have reduced hydrogen peroxide production and increased antioxidant activity in skeletal muscle [16]. Although these studies found varying results, overall improved skeletal muscle metabolic function and flexibility in female rodents could be a defining factor in the protection from HFD-induced insulin resistance.

1.2.2 Skeletal Muscle Metabolic Dysfunction following Ovariectomy

Ovariectomy (OVX) in rodents is a model of human ovarian hormone loss that is seen in postmenopausal women. Like in postmenopausal women, ovariectomy in rodents leads to increased metabolic dysfunction which results in increased weight gain and body fat [15, 22, 31-42]. In addition to body weight changes, insulin sensitivity is impaired with a loss of estrogen [22, 39, 43, 44]. Yakar et al. observed increased body weight and body fat percentage, as well as reduced glucose tolerance as measured by a glucose tolerance test in OVX rodents [22]. Additionally, Kumagai et al. found that OVX rodents do not demonstrate changes in body weight or fat, but were insulin resistant as measured by a euglycemic hyperinsulinemic clamp [43]. Skeletal muscle also becomes insulin resistant with a loss of estrogen as demonstrated by reduced insulin-stimulated glucose uptake [40, 43, 45, 46]. When OVX rodents are placed on a high-fat diet (HFD), metabolic dysfunction is further exacerbated. A high caloric diet for 10 weeks in OVX rodents leads to further increased body weight, body fat percentage, insulin resistance as demonstrated by an insulin tolerance test, glucose tolerance test and high leptin levels [22]. Later studies found similar results in the exacerbation of weight gain, food intake [37] and insulin resistance with a HFD [35, 36].

Reduced metabolic signaling in muscle from OVX rodents may account for reduced insulin sensitivity in skeletal muscle as well as systemically. Muscle from OVX mice demonstrate reduced phosphorylation of AMP-activated protein kinase (AMPK), which is a master regulator of energy homeostasis that activates glucose and fatty acid oxidation pathways [47, 48]. Additionally, changes in glucose uptake following a loss of estrogen may be due to reduced insulin signaling. Phosphorylation of Akt [40, 48] and IRS-1 in the insulin signaling pathways of skeletal muscle are reduced following ovariectomy [48]. Insulin receptor substrate 1 (IRS-1) is a signaling adaptor protein, and Protein kinase B (Akt) is a serine/threonine-specific protein kinase. Both are required for the insulin-induced translocation of Glut4 to initiate glucose uptake. Although ovariectomies in rodents reduce skeletal muscle glucose uptake, the majority of studies have found no difference in Glut 4 protein content [37, 46, 49]. Reduced translocation of Glut4 may be more important than changes in protein content with a loss of estrogen [46].

Along with reductions in insulin signaling, other metabolic signaling pathways are also reduced in OVX rodents. Whole-body oxygen consumption is decreased in OVX rodents [38, 40], which may be due to downregulated fatty acid oxidation signaling pathways specifically in skeletal muscle. Impaired fatty acid oxidation signaling in skeletal muscle of OVX rodents includes various aspects of fat metabolism including fatty acid uptake, β-oxidation, and mitochondrial biogenesis. Specifically, Campbell et al. found that a loss of circulating estrogen resulted in a 20% reduction of CPT-1 and β-HAD activity in skeletal muscle [50]. CPT-1 (carnitine palmitoyltransferase) is a protein that transports fatty acids into the mitochondria for oxidation, and β-HAD (β-3-hydroxyacyl-CoA dehydrogenase) is a part of the β-oxidation pathway. Additionally, Kamei et al. found reduced expression of genes (PPARGC1A, ERR1, ACO, and MCAD) which regulate fatty acid oxidation in OVX rodents. [34]. Other studies have found similar reductions in PGC-1α and downstream effector TFAM gene expression with ovariectomy [48, 51]. PGC-1α is a transcriptional coactivator that activates various energy metabolism pathways and is a master regulator of mitochondrial biogenesis. Therefore,

reductions in this protein as well as reductions in numerous aspects of fatty acid oxidation can have dramatic negative effects on skeletal muscle metabolism.

Along with reductions in expression of various metabolic genes, mitochondrial function is also impaired in skeletal muscle with ovariectomy. Skeletal muscle oxygen consumption was observed to be reduced in OVX rodents in multiple studies [33, 41, 51]. Cavalcanti-de-Albuquerque et al. found that by 8 weeks post-ovariectomy, there was a decrease in PGC-1α expression and oxygen consumption of lipid substrates in both fast and slow twitch muscle. Additionally, these animals had higher lactate levels following intense exercise, possibly indicating increased reliance on glycolysis [41]. Studies have also observed increased H_20_2 generation [51] in ovariectomized rodents, and a reduction in uncoupling protein expression which can worsen damage from oxidative stress [48, 51]. Deficiencies in mitochondrial oxygen consumption, mitochondria content, and increased oxidative stress contribute to skeletal muscle metabolic dysfunction in ovariectomized rodents.

In addition to the abovementioned mitochondrial deficiencies observed with a loss of estrogen, impaired mitochondrial function with ovariectomy may also be due to alterations in mitochondrial dynamics. Capllonch et al. demonstrated that OVX decreases expression of proteins which regulate mitochondrial dynamics including MFN1, MFN2, and DRP1 [51]. Mitofusin (MFN) proteins are mitochondrial fusion proteins, while Dynamin-related protein 1 (DRP1) is a mitochondrial fission protein. Activation of DRP1 and fission of the mitochondria is required for mitophagy (degradation of dysfunctional mitochondria). Mitophagy is important in the maintenance of high mitochondrial quality, and reduction in mitophagy may contribute to obesity and diabetes [52, 53].

A loss of mitochondrial function and accumulation of dysfunctional mitochondria can significantly contribute to impaired lipid handling in various tissues, including skeletal muscle. OVX mice have increased skeletal muscle triglyceride (TAG) storage [33, 34, 40, 42], and diacylglycerol (DAG) content [40], a lipid intermediate, which contributes to insulin resistance.

Additionally, Camporez et al. observed increased protein content of lipid transporter proteins, CD36 and FABP. CD36 (also called FAT, fatty acid translocase) and FABP (fatty acid binding protein) both transport fatty acids across the cell membrane to be oxidized or stored [40]. Together these studies demonstrate that ovariectomy reduces fatty acid oxidation, impairs mitochondrial function, and increases fatty acid uptake which may contribute to increased lipid accumulation and insulin resistance that are seen following a loss of estrogen.

Many studies investigating ovariectomy or estrogen treatment fail to evaluate or consider fiber type in their results looking at skeletal muscle. It is important to remember when investigating skeletal muscle-specific differences that findings may vary depending on the fiber type investigated. These muscle types are described as oxidative (type I), glycolytic (type II), or intermediate (type IIa) based on the metabolic pathways they typically depend on. Oxidative muscle fibers contain more mitochondria, higher levels of myoglobin, and increased expression of oxidative enzymes. Glycolytic muscles contain few mitochondria and greater expression of glycolytic enzymes. The effect of ovariectomy on skeletal muscle outcomes may be due to a fiber type shift, since diabetic patients demonstrate a shift in their muscle types to more type II fibers [54, 55].

1.2.3 ERα as a Primary Mediator of Metabolic Function

The physiological action of estrogen is mediated through two receptors, ERα and ERβ. In the classical genomic pathway, ligand-bound estrogen receptors bind to estrogen response elements (ERE) in gene promoters and regulate transcription [56]. In non-classical pathways, ERs (such as GPER) localize at the plasma membrane and are activated/inhibited via post-translational modifications through various signaling pathways [57-60]. Although ovariectomized models have been valuable in investigating metabolic dysfunction with a loss of estrogen, estrogen receptor knockout models have also provided important evidence towards how estrogen acts metabolically. ERα is the primary player in the protective metabolic effects of estrogen, and this was clearly demonstrated in ERα$^{-/-}$ models. Male and female ERα$^{-/-}$ mice

exhibit increased adiposity and insulin resistance, as well as impaired fatty acid β-oxidation and accumulation of bioactive lipids in muscle [61, 62]. Additionally, muscle-specific ERα$^{-/-}$ mice also demonstrate impaired glucose homeostasis, diminished skeletal muscle oxidative metabolism, and impaired mitochondrial function and dynamics [63]. Furthermore, ERα pharmacological activation improves metabolic homeostasis through increasing insulin sensitivity [64], oxygen consumption and reducing weight gain and fat accumulation [65]. These past studies have been imperative in identifying the role of ERα in metabolic homeostasis. Similar studies investigating ERβ and metabolism have found that ERβ knockout animals do not have impaired metabolism [62, 66, 67]. Additionally, activation of ERβ did not improve skeletal muscle insulin sensitivity like activation of ERα [68]. However, one study identified that a reduction in ERβ may protect against insulin resistance and glucose intolerance in adipose tissue [67]. Future studies are needed to identify the role of ERβ in regulating metabolism.

Further investigation into estrogen-agonists has also advanced the field's understanding of possible treatment methods. Hormone replacement therapy is known to alleviate conditions in postmenopausal women such as osteoporosis and cardiovascular disease [69]. In the Women's Health Initiative clinical trials, hormone therapy alleviated some of these conditions, but they also observed increased risk of breast cancer, stroke, and heart disease [70]. Since this trial, there has been a demand for the development of safe estrogen mimetic compounds. There is promise in the use of selective estrogen receptor modulators (SERMs) and tissue-selective estrogen complexes (TSEC) which have agonistic and antagonistic activity in a tissue-specific manner [71-74]. Kim et al. demonstrated that the SERM bazedoxifene (BZA) alone, and in combination with conjugated estrogen, acts in an ERα specific manner to improve glucose tolerance and energy expenditure in mice [73] and can reduce body weight gain caused by ovariectomy [75-77]. Further studies continue to investigate SERMs [74, 78, 79] and will be important in the treatment of postmenopausal women.

1.2.4 Exercise as Treatment for Postmenopausal Women

Exercise is a possible treatment approach to prevent or reverse metabolic dysfunction that occurs during menopause [80-83]. With a loss of estrogen, rodents and humans demonstrate a loss of physical activity [31, 32, 84-87]. This is consistent in ERα knockout mice [88]. This reduction in physical activity is also extended to exercise capacity. Ovariectomized [31, 32, 84, 89] and ERα knockout animals (unpublished observations) demonstrate a reduction in capacity to perform exercise. This may be partially due to the observed impairments in whole-body and skeletal muscle oxygen consumption and skeletal muscle mitochondrial function [33, 38, 40, 41, 51, 61]. Impairments in exercise capacity, whole-body oxygen consumption and skeletal muscle metabolism imply that with a loss of estrogen it's likely that there is also a reduction in cardiorespiratory fitness. Cardiorespiratory fitness, or aerobic capacity, describes the ability of the body to deliver and utilize oxygen in peripheral tissues. Low aerobic capacity is a prominent risk factor for metabolic dysfunction, hypertension, cardiovascular disease and mortality [90-93].

Work from the laboratory of Vieira-Potter recognizes the importance of aerobic capacity in protection against ovariectomy-induced insulin resistance. Her laboratory has studied the relationship between aerobic capacity and a loss of estrogen by using a rat that is artificially selected for low and high running capacity. These rodents are only exposed to a single running test to separate them into two groups; the low and high capacity runners (LCR and HCR) [94]. These animals have stark differences in aerobic capacity. Her laboratory found that rodents with enhanced aerobic capacity are protected against increased adipose accumulation and the development of insulin resistance with ovariectomy [95]. This is most likely due to the enhanced energy expenditure [95] and increased insulin-stimulated glucose uptake in skeletal muscle and adipose tissue in the rodents with high aerobic capacity [96]. Additionally, Park et al. from the Vieira-Potter laboratory investigated if rodents with low aerobic capacity could improve their metabolic homeostasis following ovariectomy by using exercise. They found that exercise

increased insulin sensitivity, energy expenditure, mitochondrial content and skeletal muscle AMPK activation in both low and high-capacity runners following ovariectomy [97].

Although aerobic capacity has very strong genetic components [94], low physical activity also reduces aerobic fitness, which demonstrates that participation in exercise can improve one's aerobic fitness [98]. This observation is supported by a study from Earnest et al. and others which demonstrate that aerobic exercise protects postmenopausal women from the development of metabolic syndrome and is dose-dependent [80, 99]. In addition to aerobic capacity, some past studies have shown that exercise training can improve metabolism in ovariectomized rodents [32, 100-106]. Researchers have found that exercise can mitigate some of the loss in metabolic function that occurs with a loss of estrogen. Exercise and estrogen act similarly to improve whole-body and skeletal muscle metabolism [95, 100, 103, 107-113] through improving insulin action, lipid handling, and oxidative capacity and increasing skeletal muscle expression of ERα [107, 108, 114]. It is clear from these past studies that reduced aerobic capacity contributes to the development of metabolic syndrome in postmenopausal women. These studies also demonstrate that exercise should continue to be considered as a strong treatment option in postmenopausal women.

1.3 Non-Alcoholic Fatty Liver Disease

Metabolic disease is very common, and can affect various age-groups, organs, and both genders. Fatty-liver, or non-alcoholic fatty liver disease (NAFLD) is the liver component of obesity and metabolic disease. As discussed above, skeletal muscle is very important in glucose homeostasis through its regulation of glucose uptake and the effects of skeletal muscle mitochondrial function on whole-body metabolic homeostasis. The liver is also very important in systemic metabolism, specifically through its regulation of gluconeogenesis. With metabolic disease, the liver displays various metabolic defects including mitochondrial dysfunction, excess

lipid storage, inflammation, and insulin resistance all of which contribute to systemic dysregulation of glucose.

1.3.1 Prevalence and Diagnosis of NAFLD

NAFLD is defined as excessive accumulation of fat in the liver which is also referred to as steatosis. This is defined by the liver triglyceride content as being more than 5 % of liver weight [115]. Currently, it is estimated that 34% of the general population and 75-100% of obese and extremely obese individuals have NAFLD [116, 117]. Due to this connection, NAFLD patients have a high risk of developing hepatic insulin resistance, ultimately contributing to hyperglycemia, dyslipidemia (high plasma triglycerides or low HDL cholesterol) and development of type 2 diabetes [118, 119]. In about 25% of cases increased hepatic lipid storage in NAFLD can progress on to more severe liver disease called non-alcoholic steatohepatitis (NASH) [120]. In addition to steatosis, hepatocyte ballooning, lobular inflammation, and mega mitochondria development occur with NASH. This can further progress to fibrosis, cirrhosis and hepatocarcinoma [120].

NAFLD is diagnosed by identifying the presence of steatosis through imaging or histology, along with reports of little alcohol consumption by the patient [121]. Imaging procedures can be used (CT scans, magnetic resonance imaging, or transient elastography) to detect steatosis and possible fibrosis [122]. The severity of NAFLD is difficult to diagnose since liver biopsy is the best method to properly assess the liver histologically [123]. NAFLD is further diagnosed through abnormal liver tests (such as serum aspartate transaminase (AST) and alanine transaminase (ALT), and hepatitis blood tests) and the presence of metabolic risk factors [124]. A NAFLD fibrosis score can be determined by a physician through identifying a combination of characteristics such as hyperglycemia, body mass index and high AST/ALT levels [125].

1.3.2 Development and Treatment of NAFLD

Liver fat accumulation occurs when fatty acid uptake, storage, and de novo lipogenesis is greater than fatty acid oxidation and efflux of lipids. Inflammation, lipotoxicity, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and variations in the microbiome are all part of the development of NAFLD. NAFLD is thought to progress to liver injury through the "two hit hypothesis". The first hit is the excessive accumulation of triglycerides in the liver. The second hit involves oxidative stress, mitochondrial dysfunction and inflammation which can lead to further liver damage [126]. Kupffer cells are macrophages that are present in the liver through which inflammatory signaling and cytokine production are mediated [127, 128]. Alterations in cytokine production (e.g. TNFα, IL6) and hormone production (e.g. adiponectin) in obesity contribute to NAFLD development [129, 130]. The main therapeutic approach to treat NAFLD is a change in lifestyle. Modest weight loss around 5-10% can decrease inflammation, improve steatosis and histology, and lower risk of disease progression [131].

Research from Dr. Thyfault and others has shown that exercise training can effectively prevent and treat hepatic steatosis [132-134]. Exercise and intrinsic aerobic capacity in rodents leads to improved hepatic mitochondrial function (enzyme activities, respiration and oxidation) [135, 136]. Fitness and physical activity are also important in improving liver function and decreasing hepatic triglyceride levels in humans [137-139]. For example, Kantartzis et al. observed that high cardiorespiratory fitness increased effectiveness of a lifestyle intervention to decrease liver fat [137]. Exercise also alleviates insulin resistance and NAFLD symptoms in patients independent of weight loss [140]. Further research is necessary to allow clinicians to prescribe exercise that is specific to treat NAFLD and NASH.

Surgery and pharmacological interventions are also possible treatments for NAFLD. Bariatric surgery improves glucose production, insulin sensitivity, fibrosis, and decreases VLDL-TG secretion [141, 142]. It is also common for physicians to prescribe insulin sensitizers, hypertension medication, or medication to manage dyslipidemia such as statins [143].

Pharmacological agents to treat weight loss have not been adopted as the main therapeutic approach by physicians due to negative side effects or overall little improvement [144-146]. There has also been a push for researchers and clinicians to find an effective and safe medication that can treat NAFLD and NASH directly. Various pharmacological compounds have been investigated to treat NASH such as vitamin E [147], pentoxifylline [148] and ursodeoxycholic acid [149]. Although there are many promising pharmacological agents currently being studied, as of yet there is no medication that can effectively and safely treat NAFLD and NASH.

1.3.3 Relationship between NAFLD and Metabolic Syndrome

It is continually debated whether NAFLD is a cause or result of whole-body metabolic dysfunction and insulin resistance. Increased steatosis is often associated with whole body insulin resistance [150-152]. However, there are also numerous animal studies that have found a disconnect between steatosis and insulin resistance, in which they found the presence of steatosis but not insulin resistance [153-155]. In fact, in some cases steatosis is a mechanism which guards the liver from the development of insulin resistance through protection from exposure to lipid intermediates [156, 157].

Still, other studies have found a strong connection between steatosis and insulin resistance. Insulin resistance may precede and encourage the development of fatty liver through promoting lipid synthesis. In healthy individuals, insulin activates hepatic insulin signaling and suppresses gluconeogenesis [158]. Insulin resistance in the liver decreases the liver's ability to regulate gluconeogenesis, therefore hyperglycemia is increased [130].Throughout the progression of insulin resistance, there is increased production of insulin by the pancreas in an attempt to compensate for the decreased whole-body sensitivity and increased circulating glucose. Hyperinsulinemia stimulates de novo lipogenesis and triglyceride synthesis pathways in the liver, therefore contributing to the development of fatty liver [159].

NAFLD also contributes to systemic insulin resistance and may occur before insulin resistance. One of the liver's primary functions is to maintain whole-body metabolic homeostasis through regulation of blood sugar levels. Increased storage of hepatic lipids due to increased circulation of free fatty acids in obesity leads to dysfunction in hepatic glucose and fatty acid metabolism [159]. Current evidence suggests that dyslipidemia also induces insulin resistance through increased intracellular generation of toxic lipid intermediates (DAG, ceramides) which can inhibit insulin signaling and activate inflammation [160, 161]. Due to the shared mechanisms in the development of NAFLD and metabolic syndrome, it is nearly impossible to discern the cause and effect [162] and mostly likely both equally contribute to whole-body metabolic dysfunction.

One commonality in the pathogenesis of NAFLD and insulin resistance is inflammation. One main pro-inflammatory pathways that is activated in various tissues with insulin resistance and NAFLD progression is the JNK pathway [163, 164]. The cJUN-N-teriminal-kinase (JNK) pathway is not only an important player in the cell stress response, but it also is a major signaling transducer in obesity and insulin resistance [165]. Deletion of certain isoforms of JNK protects mice from insulin resistance induced by a HFD [166], and increased JNK activity occurs in in the muscle, liver, and adipose tissue of various rodent models of obesity [163]. Specifically, JNK decreases insulin signaling in skeletal muscle through inhibiting the insulin receptor substrates 1 and 2 [165, 167]. Additionally, JNK promotes the increase in production of pro-inflammatory cytokines from adipose tissue in obese individuals [168]. JNK also contributes to the development of NAFLD through dysregulating hepatic mitochondrial function. Fatty acid oxidation and respiration are impaired while reactive oxygen species (ROS) production in the liver is increased with the activation of JNK [163, 166, 167, 169, 170]. JNK is an essential part of the molecular mechanism connecting obesity to metabolic syndrome and NAFLD.

1.3.4 Liver Metabolism and Mitochondrial Dysfunction

The liver is a central organ involved in metabolic regulation. The liver takes up free fatty acids from circulation, oxidizes free fatty acids and glucose, synthesizes triacylglycerol, produces ketones, and assembles and secretes VLDL particles. Dysregulation in any of these pathways can lead to metabolic dysfunction and NAFLD [171]. In simple terms, NAFLD occurs when there is an imbalance in hepatic fatty acid input (uptake, storage, and synthesis) and fatty acid output (oxidation and secretion). Dysfunction of various hepatic metabolic pathways leads to NAFLD (Figure 1).

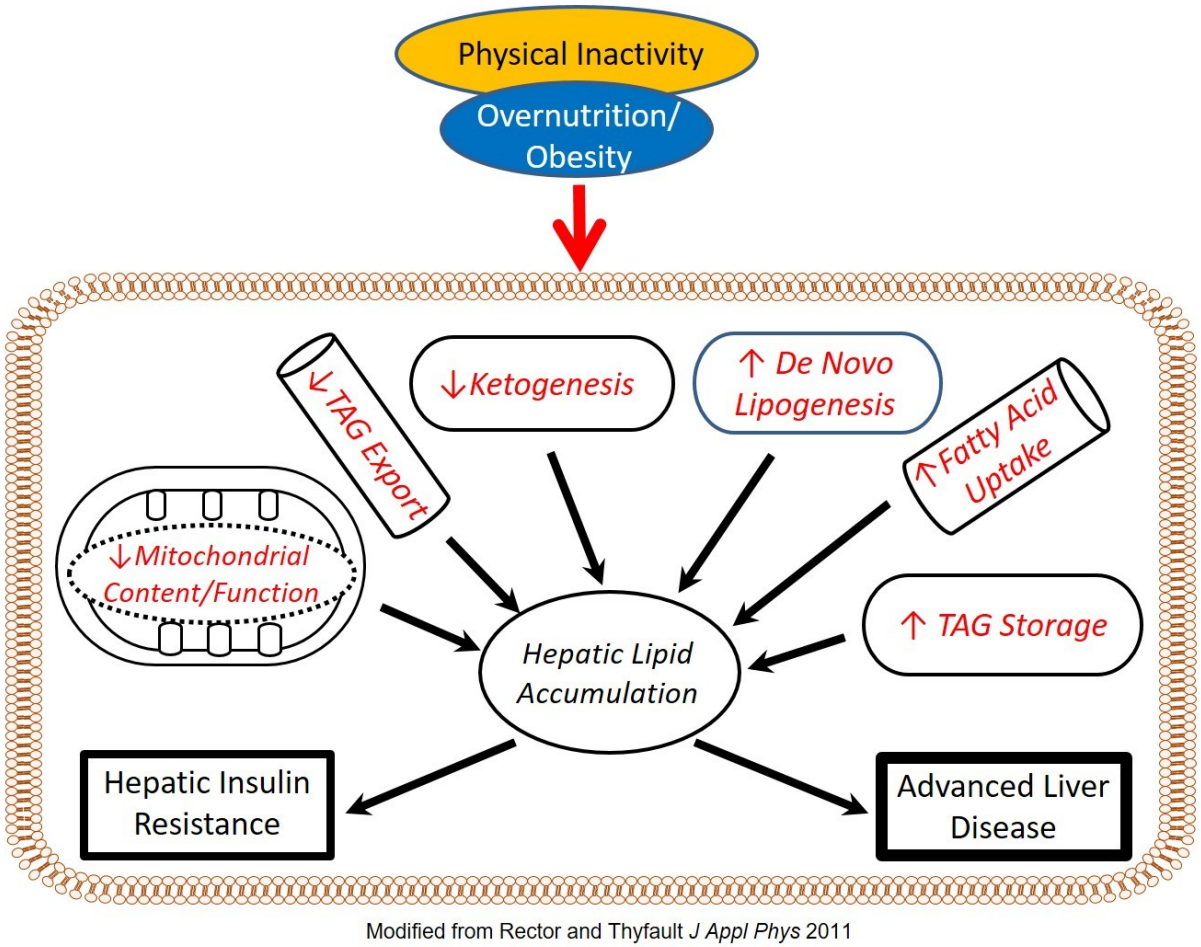

Figure 1. **Hepatic metabolic dysfunction contributes to excess lipid accumulation.** Physical inactivity and overnutrition/obesity lead to dysfunction in various hepatic metabolic pathways. Reduced lipid efflux or oxidation, and increased uptake, storage, and de novo lipogenesis together result in the excess accumulation of lipids in the liver. This leads to further metabolic dysfunction, insulin resistance, and advanced liver disease.

Uptake of Fatty Acids

Hepatocytes obtain fatty acids from various sources: lipolysis of adipose tissue to non-esterified fatty acids (NEFA), hydrolysis of chylomicrons coming from the gut, de novo lipogenesis, and hydrolysis of intracellular triglycerides. Fatty acids that are absorbed in the gut are converted to chylomicrons as triacylglycerol (TAG) and sent out to the lymphatic system. Chylomicrons reach the liver through the hepatic artery and portal vein and lipoprotein lipase (LPL) at the hepatic cells releases the non-esterified fatty acids. In order for lipids to enter the liver, membrane transport proteins such as cluster of differentiation 36 (CD36), fatty acid binding protein (FABP), fatty acid transport proteins (FATP), and calveolin proteins allow for fatty acids to cross the hepatic membrane [171, 172]. Once inside the cell, long chain fatty acids are activated to acyl-coenzyme A (CoA) through attachment of a CoA by acyl-CoA synthetases [173]. Long-chain fatty acids and fatty acyl-CoAs bind to fatty acid binding protein (FABP) or acyl-CoA synthetases (ACS) and then are carried to various cellular compartments.

Fatty acid transporters have a role in the development of NAFLD. FAT/CD36 is a fatty acid transporter that is expressed in tissues that are important for lipid metabolism [174]. Obese rodent models have increased hepatic steatosis and expression of CD36 [151, 175]. Additionally, FATP5 [176] and FABP1 knockout animals [177, 178] exhibit protection from triglyceride storage and NAFLD. The repetitive mechanisms that are used by liver cells to take in lipids from circulation demonstrates the dependence of the liver on free fatty acids, and the complications in studying how fatty acid uptake contributes to NAFLD.

Triglyceride Storage

Fatty acyl-CoAs are mainly shuttled to three different pathways which are oxidation, ketogenesis, or conversion into triglycerides. Fatty acids that are converted to triglycerides can be stored or secreted as very low-density lipoproteins. The main pathway for construction of triglycerides from fatty acyl-CoAs is the glycerol-3-phosphate pathway [179]. There are numerous enzymatic steps for acyl-CoA to be combined with glycerol-3-phosphate (G3P) and

form TAG. The production of phospholipids is also intertwined in this pathway, and some of the intermediates produced can branch off for phospholipid production. The steps happen sequentially through 1) glycerol-3-phosphate acyltransferase (GPAT), 2) 1-acylglycerol-3-phsophate acyltransferase (AGPAT), 3) the phosphatidate phosphatase action of lipin, and the 4) giacylglycerol acyltransferase (DGAT) enzyme [180].

Increased activation of the TAG synthesis pathway may lead to the development of NAFLD. This phenomenon has been demonstrated in studies utilizing models which target TAG synthesis enzymes such as GPAT and DGAT. Knockout rodent models for GPAT1 are protected from steatosis and insulin resistance in the liver when placed on a high-fat diet [181], and have decreased circulating TAG and VLDL cholesterol [182]. Also, GPAT1 liver overexpression results in the reversal of these measures [183], which further supports the importance of GPAT1 for fatty liver disease development. The last step for TAG synthesis involves diacylglycerol acyltransferase (DGAT) 1 or 2 which adds an acyl moiety to glycerol [184]. Silencing or knockout of each isoform has been demonstrated to reduce steatosis and increase oxidation and energy expenditure [185, 186]. It is clear that DGAT proteins are involved in the development of NAFLD, although the consequences of increased lipid storage are still unclear. Monetti et al demonstrated that DGAT1 and 2 overexpression in the liver resulted in increased steatosis without changes in insulin signaling [153]. These results demonstrate the need for further investigation into whether steatosis is deleterious or protective in different situations.

Lipid Export

In addition to intracellular storage, triglycerides are released as VLDL from the liver in order to deliver cholesterol and triglycerides to peripheral tissues. VLDL particles contain apolipoprotein B (ApoB), cholesterol esters and triglycerides and are formed by the microsome triglyceride transfer protein (MTTP). In NAFLD patients, increased VLDL is formed due to increased adipose tissue lipolysis, hepatic de novo lipogenesis and increased circulating

triglycerides [187, 188]. Additionally, patients with NAFLD have decreased high density lipoprotein cholesterol (HDL) [189]. HDL particles aid in the elimination of excess cholesterol in the body by transporting it to the liver for conversion into bile. VLDL-TG secretion is one mechanism to decrease triglyceride storage in the liver, however in patients with NAFLD increased VLDL synthesis is unable keep up with intracellular triglyceride production and fatty acid uptake [190]. In patients with NAFLD, increased VLDL secretion rate eventually plateaus, which may contribute to the progression and worsening of NAFLD [190].

Fatty Acid Oxidation

The liver requires a high amount of energy to perform its tasks. One of the liver's main functions is to maintain systemic metabolic homeostasis. During fasting or exercise, the liver maintains metabolic homeostasis by breaking down glycogen stores through glycogenolysis and also making glucose through gluconeogenesis. Hormonal signals regulate these processes. For example, glucagon increases hepatic gluconeogenesis in times of low glucose, and insulin decreases hepatic gluconeogenesis in the liver [191]. Metabolites that come from skeletal muscle and adipose tissue (lactate, amino acids, NEFAs and glycerol) are taken up in the liver and used for gluconeogenesis.

The liver mainly fuels its processes through oxidizing fatty acids. However, during hyperglycemia, the liver uses glucose as its main fuel source through glycolysis and oxidative phosphorylation, stores glucose as glycogen, and also converts glucose into fatty acids through de novo lipogenesis. In times of lower glucose, the liver depends on fatty acids. Once inside hepatocytes, fatty acyl-CoAs are primarily oxidized in the mitochondria. Fatty acyl-CoAs enter the mitochondrial matrix through conversion to acyl carnitines by CPT-1 and 2 and are transported across the mitochondrial membrane by carnitine-acylcarnitine translocase. This is the rate limiting step of fatty acid oxidation (FAO) [192]. Once inside the lumen, the acyl-carnitines are once again converted to acyl-CoAs and subsequently go through β-oxidation. Overexpression of CPT-1 in hepatocytes results in increased FAO and lower TAG storage

[193]. In NAFLD patients, CPT gene expression is decreased in the liver compared to people without NAFLD [194], thus ultimately reducing fatty acid oxidation.

β-oxidation involves multiple enzymatic steps including dehydrogenation, hydration, and cleavage. These reactions are catalyzed by four enzymes; acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. This process shortens a fatty-acyl CoA by two carbon units each cycle. The product of carbon cleavage is the molecule acetyl-CoA which thus can enter (tricarboxylic acid cycle) TCA cycle for complete oxidation and production of reducing equivalents that enter the Electron Transport Chain.

Peroxisomes and microsomes also partially contribute to fatty acid oxidation. About 10% of β-oxidation occurs in peroxisomes, although this is increased with high lipid exposure [195]. Peroxisomal β-oxidation breaks down very long chain fatty acids and dicarboxylic acid and is different from mitochondrial oxidation due to the production of H_2O_2 in the first dehydrogenation step [196]. Peroxisomes do not contain an electron transport chain and send intermediates to the mitochondria. In situations with high lipid exposure, like obesity and NAFLD, peroxisome function is important for the cell to face metabolic challenges. One study found that when β-oxidation is not functional in hepatic peroxisomes, there is increased steatosis [197]. Additionally, overutilization of peroxisomal β-oxidation, which occurs with high lipid exposure and metabolic disease, leads to H_2O_2 production from peroxisomes and can further contribute to NAFLD progression. Microsomal oxidation (ω-oxidation) also contributes minimally overall fatty acid oxidation, but like peroxisomal oxidation, microsomal oxidation is increased with high lipid exposure and produces H_2O_2 [171, 194].

Ambiguities exist as to how the rate of hepatic fatty acid oxidation may be linked to obesity and NAFLD. Previous research has shown that a reduction of β-oxidation enzymes in the livers of rodents leads to increased steatosis in the liver [198], and an increase in β-oxidation enzymes reduces hepatic steatosis [199]. However, a reduction of FAO does not seem to contribute to the development of NAFLD in humans. With increased exposure to fatty

acids in NAFLD, initially there are increased levels of fatty acid oxidation [200, 201]. However, other deficits are seen in the mitochondria such as morphological changes, impaired ATP production and also increased ROS production [202-205]. Although oxidation may not be reduced as NAFLD develops, there could be uncoupling of fatty acid oxidation process to ATP production which would alter overall hepatic metabolism. Additionally, impaired FAO may depend on the stage of NAFLD, and only occur in later stages or with a transition to NASH.

Ketogenesis

Ketogenesis is another important process for the liver to maintain metabolic homeostasis. Ketone bodies (acetoacetate and β-hydroxybutyrate) are primarily produced in liver mitochondria and production is increased when glucose is not available or following exposure to high levels of fatty acids [206]. Ketones are formed from acetyl-CoA produced during β-oxidation of fatty acids. Various studies have observed an increase, no change, or a decrease of hepatic ketogenesis during metabolic disease [207-214]. However, not all of these studies specifically identified the stage of liver disease development, which may contribute to the inconsistencies. When studies have focused on the specific stage of liver disease, more clarity is achieved. In early insulin resistance or hepatic steatosis either no change or an increase of ketogenesis has been observed [207-212]. This is similar to what has been observed with changes in fatty acid oxidation in early liver metabolic dysfunction [207, 211-214]. With more advanced liver disease and the development of NASH, studies have found a reduction in ketogenesis [209, 210, 212].

Ketogenesis is often considered as a fuel source for other tissues, but this process is important for the maintenance of hepatic metabolic function as well. About two-thirds of the fat that comes to the liver is converted into ketones [215], therefore the majority of fatty acid oxidation is directed to ketogenesis [211]. When the liver is exposed to excess lipids it may compensate by increasing ketogenesis, β-oxidation, and cholesterol export to prevent excess hepatic lipid accumulation and lipotoxicity. As liver disease advances towards steatohepatitis,

increased lipid storage and lipotoxicity occurs since these pathways cannot compensate for the increased influx of substrates due to lipid oversupply. Therefore, ketogenesis can be a mechanism of lipid disposal, and dysfunction in this pathway could promote the development of NAFLD into NASH. Cotter et al. studied the importance of ketogenesis to prevent steatohepatitis by using a knockout mouse for mitochondrial 3-hydroxymethylglutaryl CoA synthase (HMGCS2), which is the first enzyme involved in ketogenesis. On a chow diet, these animals had increased gluconeogenesis and de novo lipogenesis, but when placed on a HFD they developed severe inflammation and impaired gluconeogenesis [209]. This study demonstrated that even in non-fasting conditions ketogenesis is critical for normal metabolic flexibility in the liver.

De Novo Lipogenesis

In addition to transporting lipids across the hepatic membrane, the liver also synthesizes lipids through de novo lipogenesis. Acetyl-CoA can be made from citrate, a TCA cycle intermediate. This process is enhanced with increased TCA cycle flux, such as in times of increased energy intake or with dysregulation of lipid or glucose metabolism [187, 188]. Acetyl-CoA is then targeted for de novo lipogenesis through conversion to malonyl-CoA by acetyl-CoA carboxylase (ACC). The final enzyme in this pathway, fatty acid synthase (FAS), converts malonyl-CoA and NADPH into palmitic acid.

Numerous enzymes and transcription factors regulate the rate of lipogenesis including SREBPs, ChREBPs, LXR, FXR and PPARs [171, 194, 216, 217]. Hormones such as insulin and glucagon control de novo lipogenesis through regulation of these enzymes. Specifically, insulin increases expression of ACC and SREBP-1c, and activates insulin signaling (Akt, mTOR) to promote de novo lipogenesis [217, 218]. Glucagon, which increases in times of fasting and exercise, opposes insulin action through activating AMPK. AMPK is a master regulator of energy homeostasis. Glucagon stimulates AMPK to increase hepatic fatty acid oxidation and inhibit de novo lipogenesis [219, 220].

De novo lipogenesis intermediates also regulate fatty acid oxidation through inhibiting entrance of fatty acids into the mitochondria. McGarry et al. showed that malonyl-CoA is an inhibitor of CPT-1 [221]. Thus, ACC, which converts acetyl-CoA to malonyl-CoA, not only increases de novo lipogenesis but also decreases CPT-1 activity [222]. This system allows for an extra layer of regulation and rapid shifts in substrate utilization. Dysregulation of hepatic de novo lipogenesis is mainly due to increased expression of lipogenic genes in patients with metabolic disease [159, 194, 223]. This increase in de novo lipogenesis can further promote insulin resistance and elevate circulating LDL cholesterol and triglyceride [224].

Role of the Mitochondria

Mitochondria produce energy through the oxidation of nutrients and are highly important to the function of hepatocytes. Hepatocytes have a higher density of mitochondria and faster mitochondrial turnover than skeletal muscle [225]. Substrates come to the mitochondria mainly from glucose and lipid metabolism. Glucose is broken down into pyruvate which is brought into the mitochondria and converted to acetyl-CoA for entrance into the TCA cycle. Fatty acids are brought to the mitochondria, are broken down in β-oxidation and then enter into the TCA cycle also as acetyl-CoA. Mitochondria are the coordinators of hepatic lipid metabolism and as a result, mitochondrial dysfunction is considered central to the development of NAFLD [226, 227]. The mechanisms leading to mitochondrial dysfunction in NAFLD are complex and can be attributed to abnormalities in FAO (as mentioned above), mitochondrial biogenesis, and oxidative phosphorylation. Mitochondrial dysfunction can alter lipid metabolism, increase ROS production and lipid peroxidation, and increase the release of cytokines [228]. All of these factors contribute to liver disease development.

Various animal models have provided evidence linking hepatic mitochondrial dysfunction to NAFLD and insulin resistance. Rector et al. studied a rodent model with a genetic defect in mitochondrial β-oxidation to determine the effect on insulin action. This impairment of β-oxidation resulted in hepatic steatosis and systemic insulin resistance [229]. In a separate

study, Rector et al. showed that in a hyperphagic rodent model (obese OLETF rat) a reduction of fatty acid oxidation and mitochondrial content occurs before the progression to hepatic steatosis or insulin resistance [230]. These studies demonstrate the importance of mitochondrial dysfunction in animal models that develop hepatic steatosis and insulin resistance.

The Electron Transport Chain and ATP Production

Reducing equivalents (NADH and $FADH_2$) are produced throughout fatty acid and glucose metabolism for entrance into the electron transport chain. NADH and FADH2 donate electrons to the ETC which is coupled to proton pumping into the inner membrane space. Pumping of protons across the inner mitochondrial membrane creates an electrochemical gradient. Energy is produced through coupling electron transfer down the ETC to ATP production. ETC complex function can be compromised with metabolic dysfunction [228, 231]. Past research has demonstrated reduced activity of ETC complex proteins with NAFLD and NASH in rodent and human studies [203, 232, 233]. This can lead to impaired mitochondrial function through reduced respiration. Mechanisms behind ETC dysfunction are still being studied, but may be due to increased reactive oxygen species production [234, 235].

Oxidative stress

Increased ROS production occurs with NAFLD and can lead to damage of cellular components including mitochondrial DNA, lipids, and protein [232, 235]. Reactive lipid mediators can be formed with oxidative stress, such as 4-hydroxy-2-nonenal (4HNE), which can increase uncoupling of the mitochondria thus reducing ATP production [236]. ROS can also further increase cytokine production in the liver such as TNFα which can activate apoptotic pathways [237]. In addition to increased oxidative stress, patients with NAFLD have an impaired antioxidant capacity through a reduction in antioxidant enzyme activity such as glutathione and SOD [238]. Low antioxidant capacity increases mitochondrial damage and impairs the liver's ability to face a metabolic challenge. Various antioxidants have been investigated as treatments for NAFLD. Vitamin E is an antioxidant which was shown to improve fibrosis and progression of

liver disease, although this treatment had negative side effects and is only recommended for NASH [147, 239]. Other antioxidations that possibly prevent the ROS damage are betaine [240], N-acetylcysteines [241], vitamin C [242], and resveratrol [243]. Although there have been promising results in studies investigating these antioxidants, they have had minimal success in clinical trials or require further study.

Biogenesis and Degradation of Mitochondria

Mitochondria are able to adjust to their environment and high or low substrate exposure through regulating ATP production, mitophagy, and apoptosis [244]. They achieve this through rapid post-translational modifications and cellular signaling, as well as longer term adjustments in mitochondrial mass. Mitochondrial content is maintained through the balance of biogenesis and mitophagy [245]. Mitochondrial biogenesis is regulated by transcription factors and signaling proteins. PGC-1α is a transcriptional coactivator and one of the master regulators for mitochondrial biogenesis. This coactivator is important in tissues with high oxidative capacity such as skeletal muscle, liver, and brown adipose tissue [246, 247].

PGC-1α interacts with transcription factors such as PPARα and TFAM to activate target gene expression thus increasing not only mitochondrial biogenesis, but also fatty acid oxidation, ketogenesis, and ATP production [248, 249]. PGC-1α also increases gluconeogenesis through activating the transcription factors hepatocyte nuclear factor 4a (HNF4a) and FoxO1 [250]. Rapid cell signaling through AMPK occurs with exercise and fasting which then increases mitochondrial gene expression through AMPK-mediated phosphorylation of PGC-1α [251]. Additionally, exercise and fasting increases NAD+ levels which leads to activation of Sirtuin 1 (SIRT1). SIRT1 is an energy sensor and deacetylase, which deacetylates PGC-1α and FOXO1, further activating them in order to increase gluconeogenesis and fatty acid oxidation. These pathways are highly important in mitochondrial biogenesis and function, and can prevent excess liver triglyceride storage [252].

Mitochondrial biogenesis is also balanced with mitochondrial degradation through mitophagy.

With metabolic dysfunction, mitochondrial morphology is altered as evidenced by a loss of cristae and swelling of the mitochondria [202, 205]. This has been observed with obesity, type 2 diabetes [253, 254] and NAFLD [202, 205]. One reason behind the accumulation of damaged mitochondria is an impairment in mitochondrial dynamics. Mitophagy occurs to remove damaged mitochondria through the cellular degradation process of autophagy. This is achieved through the sequestration of damaged mitochondria in autophagosomes which then fuse with lysosomes for degradation [255]. Mitophagy requires a coordinated interaction of various autophagy proteins (ex: LC3II, p62) and mitophagy proteins (ex: Parkin, BNIP3, Mfn2, DRP1) which aid in the fission, targeting, and tethering of the mitochondria to the autophagosome. Impaired mitophagy can result in retention of damaged, ROS producing mitochondria [53, 256]. The balance between mitophagy and mitochondrial biogenesis may be essential in lipid metabolism [257-261]. More investigation needs to be done to understand the role of mitophagy in the development of NAFLD.

Impairments in an overall cellular degradation pathway, autophagy, also seems to contribute to NAFLD. Autophagy defects have been implicated in liver disease, neurodegenerative diseases, and metabolic syndrome [52]. Additionally, autophagy has been demonstrated to be important for maintenance of glucose homeostasis [262]. Autophagy can specifically degrade lipids through lipophagy. Thus, impaired autophagy could cause lipid accumulation in NAFLD [259]. Novel strategies to protect cellular and mitochondrial integrity and function through upregulation of mitophagy and autophagy could be effective in treating and preventing NAFLD.

1.4 Heat Shock Proteins

1.4.1 Role of Heat Shock Proteins

Growing evidence suggests the heat shock response and/or heat shock proteins (HSPs) could play an important role in preventing insulin resistance and the development of type 2 diabetes. HSPs are a highly conserved family of proteins best identified for their role as molecular chaperones [263]. They play a critical role in maintaining cellular function via regulation of protein folding and degradation. Not surprisingly, changes in their expression profile and cellular localization are linked to numerous disease states. Several studies suggest that induction, transcription and translation of these cytoprotective HSPs decline with chronic disease such as NAFLD [264], Huntington's Disease [265], and type 2 diabetes [266]. Conversely, induction and/or transgenic overexpression of HSPs results in ample metabolic benefit in animal models of obesity/metabolic disease [266-272]. Less clear, however, are the factors that regulate HSP expression in the pathological development of metabolic disease. In particular, very little is known regarding skeletal muscle HSP expression levels throughout the progression of obesity, insulin resistance and type 2 diabetes.

As skeletal muscle is the primary tissue responsible for insulin-stimulated glucose uptake [273], many researchers have investigated changes in skeletal muscle HSP expression during obesity, insulin resistance and type 2 diabetes. Skeletal muscle HSP72 (a member of the HSP70 family expressed in animals) expression is inversely related to body fat percentage and blood glucose in healthy subjects [270, 274]. Additionally, both HSP72 mRNA and protein expression are significantly reduced in the skeletal muscle of type 2 diabetic patients and subjects with insulin resistance [266, 275-278]. Therefore, many have asserted that HSP72 expression levels are tightly correlated to adiposity and decrease through the progression from obesity to metabolic disease (i.e. insulin resistance and type 2 diabetes).

Interestingly, multiple studies using animals fed a HFD highlight that this relationship is much more complex. For instance, investigations in primates and rodents show that short-term

high-fat feeding (16- and 6-12 weeks, respectively) results in hallmark symptomology of insulin resistance but does not significantly reduce skeletal muscle HSP72 expression [267, 268, 271, 279-281]. In fact, HSP72 expression may increase after short-term high-fat feeding, suggesting a possible compensatory response to combat metabolic dysfunction [279, 280]. However, long-term high-fat feeding (6 years) appears to cause significant reductions in skeletal muscle HSP72 expression similar to the phenomenon described in type 2 diabetics [279]. Therefore, it is possible that skeletal muscle HSP72 expression can be characterized as an inverted parabolic relationship wherein initial increases in skeletal muscle HSP72 combat metabolic dysfunction, but these levels will eventually peak and decline depending on the severity and time spent under metabolic strain (Solid green line, Figure 2).

Discrepancies in the data regarding skeletal muscle HSP72 reductions during obesity, insulin resistance and type 2 diabetes may also be due to the model being used and the muscle type analyzed. For example, investigations reporting significant reductions in HSP72 expression during obesity, insulin resistance and type 2 diabetes primarily analyzed the vastus lateralis muscle from human subjects [275-278]. Alternatively, primate and rodent investigations observing no significant reductions in HSP72 expression in response to short-term high-fat feeding analyzed the biceps femoris, soleus, and extensor digitorum longus muscles [267, 268, 271, 279-281]. Thus, it is also possible that organismal differences and/or muscle fiber type differences, variations in muscle oxidative capacity, and muscle size could contribute to inter-study data variations. It is critical that future investigators address these inconsistencies when designing studies to address the role of HSP72 expression in metabolic disease. A greater understanding of the regulation of skeletal muscle HSPs during insulin resistance will allow future development of targeted therapies to maintain and even increase HSP expression to prevent metabolic disease (Figure 2, dashed green line).

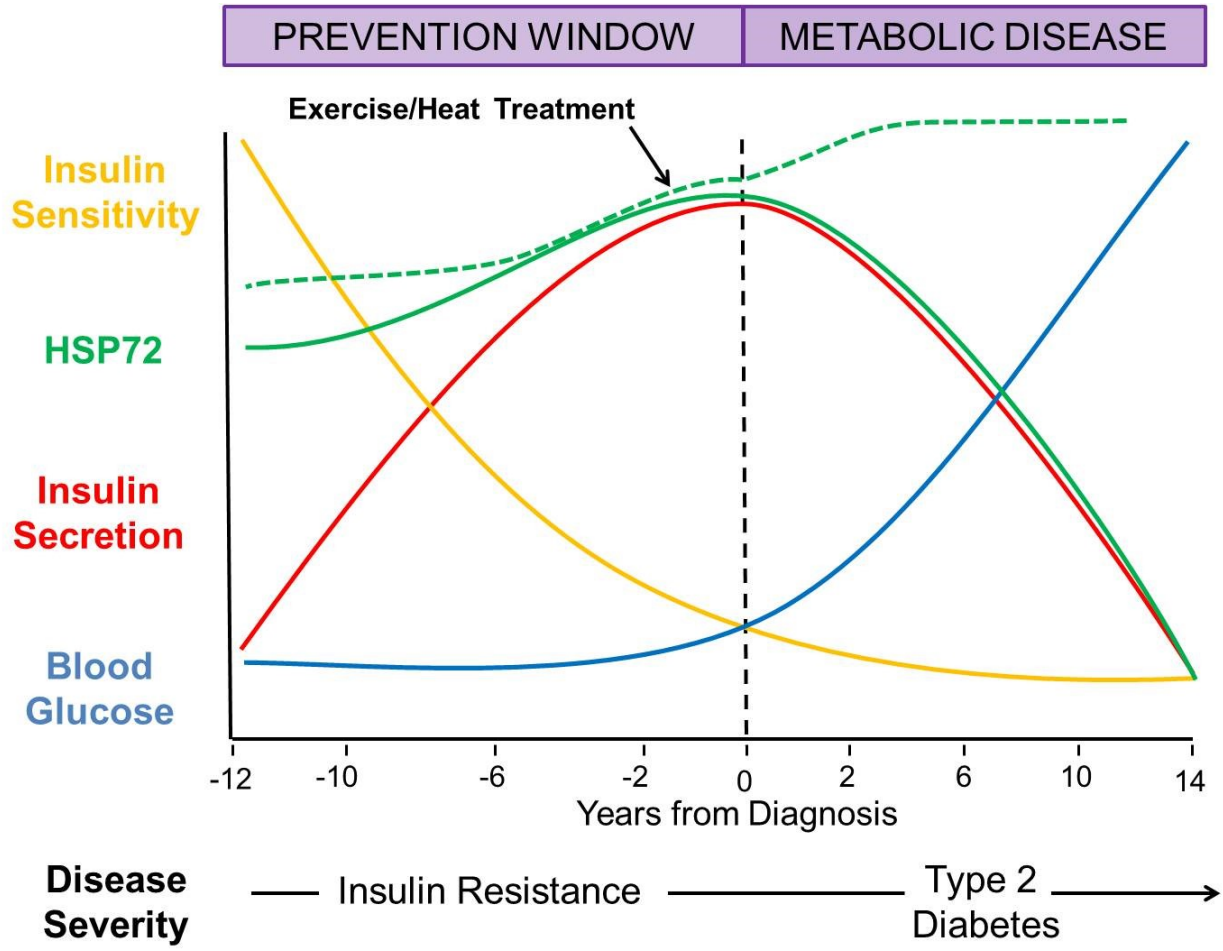

Figure 2. Targeting heat shock proteins in the prevention of insulin resistance. Schematic depicting the timeline of metabolic disease from insulin resistance to type 2 diabetes. Insulin resistance can persist for 10-12 years prior to clinical diagnosis of type 2 diabetes, a time period that represents an increased risk for cardiovascular disease, obesity and type 2 diabetes. During insulin resistance, insulin secretion (red line) from pancreatic beta cells increases in an effort to maintain blood glucose (blue line). Insulin sensitivity declines (yellow line) resulting in a gradual increase in blood glucose and the development of type 2 diabetes. Insulin resistance represents a window of time when progression towards more severe metabolic disease can be prevented by lifestyle interventions like diet and exercise. HSPs are robustly induced by exercise and HSP72 mRNA and protein expression are significantly reduced in the skeletal muscle of type 2 diabetic patients. However, very little is known about HSP expression patterns and regulation during insulin resistance. We hypothesize that HSP72 expression, in particular, may demonstrate an inverted parabolic relationship wherein initial increases in HSP72 combat metabolic dysfunction, but expression levels eventually peak and decline with disease severity and time spent under metabolic strain (solid green line). Exercise and heat treatment represent potential targeted therapies that could maintain and even increase HSP expression to prevent metabolic disease (dashed green line).

1.4.2 HSP Mechanisms of Action in Insulin Resistance

The complex, integrative and multi-organ nature of the HSP response makes the identification of specific mechanisms of action difficult. For instance, the most widely known HSP, HSP72, has varying roles and mechanisms of action in heart muscle, skeletal muscle, adipose tissue and the liver. Recent studies suggest decreasing inflammation, improving mitochondrial function/oxidative capacity, and maintaining proteostasis could be viable mechanisms of action for HSPs in metabolic tissues.

Anti-Inflammatory Properties of HSP72.

The ability of HSPs to decrease inflammation has centered on the proinflammatory protein JNK. Importantly, JNK activation is increased with the progression of insulin resistance and diabetes [166, 167, 169, 170, 282, 283], while HSP72 expression is correspondingly decreased [266, 275, 284, 285]. This inverse relationship between JNK activation and HSP expression also occurs during the progression from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) [264]. This relationship is of no coincidence. JNK activation indirectly inhibits HSP expression by maintaining heat shock factor 1 (HSF1), the primary HSP transcription factor, in its inactive monomeric state [286, 287]. Beyond inactivation of HSF1 and HSP expression, there are other downstream targets of JNK that potentiate insulin resistance.

JNK is thought to drive insulin resistance through inhibitory phosphorylation of insulin receptor substrate1- (IRS-1), a key protein in the insulin signaling cascade [288]. In addition, JNK can downregulate peroxisome proliferator-activated receptor α/fibroblast growth factor 21 (PPARα/FGF21) signaling in hepatocytes, leading to reduced fatty acid oxidation and the development of insulin resistance [289]. JNK activation also inhibits mitochondrial respiration, increases ROS production and causes apoptosis [290-294]. Previous studies suggest that HSP72 induction directly inhibits JNK activation, thereby improving insulin sensitivity and glucose tolerance at both skeletal muscle-specific and systemic levels [266, 267, 269, 295,

296]. For example, work by our laboratory has demonstrated that in vivo heat treatments decrease JNK activation in skeletal muscle of aged and HFD-fed rats [268, 297]. Pharmacological activation of HSP72 also causes reduced JNK activation in skeletal muscle and liver [267, 295]. Finally, overexpression of HSP72 in skeletal muscle decreased JNK activation in mice fed a HFD and was associated with beneficial metabolic outcomes [266]. In each instance, lowering of JNK activation resulted in improvements in insulin sensitivity and glucose tolerance, highlighting the importance of this HSP-mediated mechanism for insulin action.

HSP72 is proposed to regulate JNK activation through multiple mechanisms including direct inhibition via protein-protein interaction with JNK [298], and/or inhibition of upstream JNK signaling pathways [299, 300]. Evidence also exists suggesting that activation of HSP72 in the liver may decrease inflammation independent of JNK inhibition. Specifically, pharmacological activation of HSP72 decreases steatosis without decreasing JNK activation in HFD-fed rodents [301]. Although no change in JNK activation was observed, increased HSP72 expression resulted in inhibition of tumor necrosis factor α (TNFα) in the liver of rodents fed a HFD.

HSP72 may also play additional anti-inflammatory roles extracellularly or via localization in macrophages. For instance, HSP72 decreases during NAFLD progression in human Kupffer cells, which are liver-specific macrophages [264]. Interestingly, heat-induced upregulation of HSP72 in Kupffer cells coincides with suppression of TNFα [302, 303]. Additionally, in myeloid cells, JNK activity is considered essential for activation of macrophages and a release of pro-inflammatory cytokines [168, 304]. The ability of extracellular HSP72 to inhibit pro-inflammatory cytokine release from macrophages, lymphocytes, and other immune cells [305-310] could be critical in decreasing local inflammation and attenuating the development of insulin resistance.

HSP72 Regulation of Mitochondrial Integrity and Function.

Mitochondrial dysfunction is a primary contributor to the development of metabolic disease and is therefore a possible target for therapy [311-313]. Our laboratory and others have

shown that heat treatment improves skeletal muscle mitochondrial function by improving fatty acid oxidation [268], increasing mitochondrial enzyme activity [268, 314, 315], and increasing mitochondrial biogenesis [316]. Transgenic overexpression of HSP72 in skeletal muscle also increases mitochondrial enzyme activity, mitochondrial content, and endurance running capacity [266, 269]. Thus, it is possible that the beneficial mitochondrial adaptations stemming from heat treatment are a result of HSP72 induction.

HSP72 induction may mediate mitochondrial improvements by regulating mitophagy, the targeted degradation of mitochondria through autophagy. For instance, mice lacking skeletal muscle HSP72 demonstrate a reduced ability to degrade mitochondria through mitophagy [317]. Additionally, these mice exhibit enlarged, dysmorphic mitochondria with reduced muscle respiratory capacity and increased lipid accumulation. Thus, activation of HSP72 may improve mitochondrial quality by enhancing the degradation of dysfunctional mitochondria.

Heat Shock Transcription Factor Regulation of Oxidative Capacity.

One of the most important heat shock response functions in metabolic tissue may actually lie upstream of HSP72. HSP72 overexpression leads to an increase in mitochondrial content, oxidative capacity, and insulin sensitivity [266, 311-313, 318]. Similarly, the absence of HSP72 expression results in mitochondrial dysfunction and insulin resistance [317]. In addition to increasing HSP72 content, and thereby the ability to enhance mitochondrial quality control, exercise also increases peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α) expression [319-321]. PGC1α is the primary transcriptional coactivator for mitochondrial biosynthesis [322, 323]. Interestingly, recent investigations reveal that the upstream regulatory elements of the *PPARGC1A* gene contain a heat shock element (HSE) binding sequence. This HSE sequence provides a docking site for the primary HSP transcription factor, heat shock factor 1 (HSF1). Indeed, chromatin immunoprecipitation analyses show that HSF1 and PGC1α co-occupy the HSE sequence on the promoter of the *PPARGC1A* gene [324]. Through a myriad

of HSF1 activation and knockdown experiments, the Mueller lab provides compelling evidence that HSF1 is a primary regulator of mitochondrial biogenesis, enzymatic function, and whole-body metabolism [324, 325]. These data exemplify the elegant coordination of HSF1 downstream targets (i.e. HSPs and PGC1α) in regulating mitochondrial biogenesis, quality control, and enzymatic function under conditions of metabolic demand and/or chronic disease. Importantly, future research is needed to delineate the specific contributions of varying downstream HSF1 targets, as well as potential direct effects of HSF1 itself, with regard to metabolic outcomes. This information, combined with a greater understanding of HSP mechanisms of action in metabolic tissue, may provide novel therapeutic targets to ameliorate metabolic dysfunction.

1.4.3 Exercise-Induced HSP Response

Exercise is a primary treatment modality for patients exhibiting symptoms of metabolic dysfunction. Specifically, regular exercise training is known to decrease metabolic and cardiovascular disease risk factors in patients suffering from obesity and metabolic dysfunction [326, 327]. Exercise is also a potent inducer of HSP expression [328], with HSP72 showing the most robust and consistent upregulation with exercise. HSP72 induction via heat treatment, pharmacologic intervention, and transgenic overexpression result in metabolic effects similar to exercise in models of obesity and insulin resistance [266-268, 297, 328]. Thus, exercise-induced HSP72 expression may contribute to the beneficial metabolic effects observed with exercise training. There is already a significant amount of information available about exercise and HSPs, however little is known regarding the role of exercise-induced HSP72 expression in treating metabolic disease.

Complexity of the Exercise HSP Response

The direct cause of exercise-induced HSP upregulation, primarily HSP72, remains unknown. It is hypothesized that a variety of biochemical, metabolic, and/or physical stressors may stimulate HSP72 expression post-exercise. For instance, common challenges to tissues

during exercise such as mechanical stress, acidosis, hypoxia, ischemia, reactive oxygen species formation, and calcium signaling changes are shown to independently cause HSP induction [329-337]. Additionally, increased metabolic stress via depletion of bioenergetic substrates (i.e. glycogen) is shown to potentiate exercise-induced HSP72 expression [338]. A similar potentiation effect is observed when exercise bouts are completed in a hot environment, but this effect is blunted in a cold environment [339]. Thus, it appears that elevations in HSP72 expression post-exercise are not a result of one, but many physiologic stressors associated with exercise.

Adding complexity is the understanding that exercise-induced HSP expression is training modality, intensity, and duration dependent. In skeletal muscle, elevations in HSP72 expression occur with both aerobic and resistance training [340, 341]. Importantly, HSP72 expression is dependent on exercise intensity. For instance, HSP72 expression displays a positive relationship with exercise intensity during both aerobic and resistance training [338, 340, 342, 343]. This relationship also exists when comparing exercise intensity and metabolic outcomes [344], supporting the potential contribution of HSP72 induction to the metabolic benefits associated with exercise.

HSP72 expression also varies based on the duration of the training regimen (i.e. acute versus chronic training). Acute exercise bouts cause dramatic elevations in HSP72 within 24h [340], while chronic training regimens typically result in minimal elevations in HSP72 post-exercise [336]. Similarly, untrained subjects exhibit lower basal HSP72 expression and a higher degree of change in HSP expression post-exercise compared to fit subjects [336, 345]. The minimal degree of change in HSP72 expression observed during long-duration training protocols and in fit subjects is likely a result of adaptation to exercise. This phenomenon, referred to as the repeated bout effect [346, 347], is exemplified by the lack of potentiated HSP induction in recurring exercise bouts (specifically HSP72 and HSP27) [348]. However, cessation

of exercise in trained subjects will cause basal HSP expression to return to levels comparable to those observed pre-exercise [349].

Aerobic Capacity and Exercise Training Impact HSP Expression and Induction.

Recently, our lab has published data suggesting that intrinsic aerobic capacity, or the ability of the body to take up and utilize oxygen, is coupled to HSP induction and metabolic flexibility [271]. Low aerobic capacity increases susceptibility to developing metabolic dysfunction. Importantly, it is estimated that 50-70% of one's aerobic capacity is attributable to inheritable traits [350]. This genetic/phenotypic phenomenon is exemplified by rodent models selectively bred for high-capacity or low-capacity running (HCR and LCR respectively) [351]. Specifically, these models have drastic differences in susceptibility to metabolic complications [136, 352-354]. For instance, the HSP72 response is blunted in LCR rodents after heat treatment and they require the heat intervention to maintain metabolic flexibility/protection when acutely challenged with a HFD [271]. Conversely, HCR rodents maintain the ability to upregulate HSP72 expression in skeletal muscle via heat treatment and display metabolic flexibility/protection independent of intervention when metabolically challenged. These data suggest that intrinsic aerobic capacity is coupled to the HSP72 response in skeletal muscle and that these two factors are primary contributors to whole-body metabolic health. As mentioned, unfit subjects with metabolic dysfunction, and most likely low aerobic capacity, have markedly low levels of HSP72 expression compared to healthy controls [266, 275, 276]. Thus, chronic exercise may restore basal HSP72 expression levels to that of healthy subjects. The restoration of basal HSP72 expression via exercise may directly impact organ-specific insulin sensitivity.

Tissue-Specific HSP Expression and Induction.

As mentioned, exercise increases skeletal muscle HSP72 expression. However, the levels of both basal HSP72 expression and exercise-induced HSP72 expression are dependent on muscle fiber type. For instance, muscles predominantly composed of type I fibers have higher basal HSP72 expression compared to muscles composed of type II fibers [355, 356].

Furthermore, the magnitude of HSP72 upregulation is much greater in type II muscle fibers post-exercise compared to type I fibers [340, 357]. This may explain the intensity dependent increases in HSP72 expression post-exercise, as higher intensity activities cause the recruitment of fast-twitch muscle fibers, resulting in a greater overall change in HSP72 expression. As type II muscle fibers are inherently glycolytic and have a high dynamic range of HSP72 expression, it invites the possibility that the positive metabolic effects seen with HSP72 overexpression may be primarily mediated by changes in type II fast-twitch muscles.

Exercise is also known to increase HSP72 expression in the liver, kidney, lungs, heart, and brain [358-360]. Interestingly, exercise also results in the release of extracellular HSPs (eHSPs) from the hepatosplanchnic viscera and brain into circulation [338, 361], and other potential sites of origin include epithelial cells [362] and immune cells [363, 364]. During states of metabolic dysfunction, HSP72 expression in the liver is of primary concern due to the organ's role in maintaining whole-body metabolic homeostasis. Pharmacologic HSP72 induction in the liver is shown to improve insulin sensitivity and glucose tolerance in models fed a high-fat diet [295]. This protective effect may stem from the enhancement of HSP72-mediated mitochondrial quality control and the restoration of the insulin signaling pathway in hepatocytes - both of which occur with exercise and HSP72 upregulation in skeletal muscle. Thus, exercise-induced HSP72 expression in the liver may act to restore liver insulin sensitivity by mechanisms similar to those observed in skeletal muscle. However, future studies are needed to confirm this notion.

1.5: Research Questions

It is established that HSPs regulate metabolic function systemically and specifically in skeletal muscle. Impaired HSP induction in skeletal muscle has been studied in the prevention of obesity and diabetes, although information is still needed about the role of HSPs in the prevention of other metabolic-related conditions. Two specific metabolic conditions which may

be prevented by activating HSPs are metabolic syndrome in postmenopausal women and NAFLD.

Past studies using ERα$^{-/-}$ mice have aided in the understanding of metabolic dysfunction with a loss of estrogen in postmenopausal women. However, the metabolic characteristics have not been characterized in the ERα$^{-/-}$ rat model which could further define estrogen-mediated metabolic regulation. Additionally, the effect of these metabolic changes on exercise capacity has not been investigated. We aim to identify the metabolic effects of ERα$^{-/-}$ in a rat model, as well as investigate how ERα loss affects exercise capacity. One past study in ERα$^{-/-}$ mice has identified an impairment of HSP induction [88]. We also aim to investigate heat shock protein expression in the rat ERα$^{-/-}$ model and speculate on a connection between ERα and the heat shock response. These data could identify similarities between ERα and HSP regulation of metabolism and identify future areas of investigation for an ERα/HSP72 mechanism in skeletal muscle.

Past studies looking at the metabolic effects of heat shock proteins have mainly focused on the skeletal muscle. Due to the need for new therapeutic approaches for NAFLD, identifying the role of HSP72 in hepatic metabolism is necessary. We aim to identify the hepatocyte-specific role of HSP72 in preventing hepatic steatosis. Additionally, we have previously observed the induction of HSPs in the liver [268], and others have identified hepatic HSP72 induction with exercise [358, 365, 366]. However, HSP72 induction across multiple exercise modalities and with acute and chronic exercise has not been established in the liver. We aim to investigate HSP72 induction with acute and chronic exercise and identify how this could improve hepatic metabolic function. These studies will widen the understanding of HSP72 in the development of metabolic dysfunction and advance its use as a therapeutic target in various disease states.

CHAPTER 2

The Role of Estrogen Receptor Alpha in Systemic and Skeletal Muscle Metabolism of a Rat Model

2.1 Abstract

Postmenopausal estrogen deficiency increases the risk for metabolic syndrome, obesity, and type 2 diabetes. Estrogen treatment improves metabolic function in postmenopausal women, and estrogen receptor α (ERα) is thought to be the primary player in these protective metabolic effects through increasing insulin sensitivity and mitochondrial function. However, there is not still not a complete understanding of ERα-mediated mechanisms. Additionally, the effect of these metabolic deficits on exercise capacity have not been elucidated. The purpose of this study was to investigate the impact of ERα loss on whole-body metabolic homeostasis, skeletal muscle metabolic pathways, and exercise capacity in a rat model. 14 wk-old female Wildtype (WT) and ERα knockout (ERα $^{-/-}$) rats were exercised to exhaustion and one week later a glucose tolerance test was performed. At sacrifice, body weight and adipose tissue depot weights (subcutaneous, retroperitoneal and periuterine) were measured. Adipocyte cell size, mitochondrial protein content, and heat shock protein expression were assessed by immunohistochemistry and western blot, respectively. Intramuscular triglyceride storage was also assessed. ERα deficient rats had increased body weight and subcutaneous adipose tissue mass compared with WT rats. Although there was no increase in retroperitoneal adipose tissue mass, adipocyte size was increased in ERα deficient rats. HOMA-IR values were increased in ERα $^{-/-}$ rats indicating increased insulin resistance. ERα deficiency also decreased exercise time to exhaustion when compared with WT rats. Mitochondrial proteins Complex IV, cytochrome c, and mitochondrial regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) were significantly decreased in white gastrocnemius muscle, and intramuscular triglyceride storage was increased in red and slightly increased in white gastrocnemius skeletal muscles from ERα deficient rats. Lastly, basal HSP72 and HSP60 protein expression was reduced in white gastrocnemius of ERα $^{-/-}$ rats. Our data demonstrate the potential regulatory role of ERα to enhance aerobic capacity and improve energy metabolism both systemically and in skeletal muscle in a rat model. Exercise as treatment in postmenopausal women has the

potential increase aerobic capacity and preserve high metabolic function. Increased aerobic capacity maintains heat shock protein and mitochondrial protein expression in skeletal muscle, thus acting to prevent metabolic disease.

Key words: Estrogen receptors, postmenopausal women, skeletal muscle, insulin resistance, metabolic syndrome, heat shock proteins, mitochondria.

2.2 Introduction

Metabolic syndrome is a cluster of metabolic disorders affecting 23% of adults [367]. This condition puts people at risk for other health problems such as cardiovascular disease, type 2 diabetes, and stroke [367]. Post-menopausal women are at increased risk for metabolic syndrome and type 2 diabetes. With a loss of circulating estrogen, women experience weight gain, reduced insulin sensitivity, and decreased energy expenditure and fat oxidation [14, 368-372]. Ovariectomized rodents demonstrate similar metabolic impairments such as weight gain, increased adiposity, and insulin resistance [15, 33, 34, 38]. Estrogen treatment improves insulin sensitivity and glucose tolerance [43, 44, 373-375]. This is due, in part, to estrogen activation of skeletal muscle metabolic pathways at a cellular level [40, 376-379].

Estrogen acts through two receptors in tissues, estrogen receptor (ER)α and ERβ. The receptor that is most responsible for the metabolic actions of estrogen is ERα, which is highly expressed in insulin sensitive tissues [88, 380]. ERα knockout models have allowed for deeper understanding of estrogen-mediated mechanisms. Whole-body ERα ablation in a mouse model results in obesity and reduced glucose tolerance [62, 88, 375, 381]. These mice also develop skeletal muscle insulin resistance, increased lipid storage, and reduced markers of fatty acid oxidation [62, 88, 375]. Additionally, muscle-specific ERα ablation (MERKO) in a mouse model results in a similar phenotype of increased adiposity, insulin resistance, and skeletal muscle metabolic dysfunction [63]. Ribas et al. found that skeletal muscle mitochondrial dysfunction, specifically in the mitochondrial degradation pathway of mitophagy, contributes to increased lipid accumulation and impaired oxidative metabolism in MERKO mice [63]. ERα can also be activated with pharmacological compounds, which increases insulin sensitivity and energy expenditure and reduces weight gain in rodents [64, 65, 77, 382]. Knockout models and ERα activation studies have allowed for a better understanding of estrogen regulation of metabolism at a tissue and cellular-level, however metabolic characterization has not been done in other available $ER\alpha^{-/-}$ species, specifically the rat [383]. Further characterization of the effect of ERα

knockout will continue to better define these mechanisms and help identify best treatment approaches in postmenopausal women.

Exercise is one treatment modality which could improve insulin resistance and reduce adiposity caused by a loss of estrogen during menopause [80-83]. Past research has shown that ovariectomized mice run less than control animals [31, 32, 84], and that whole-body ERα$^{-/-}$ mice demonstrate reduced ambulatory movement [88], however exercise capacity in the ERα knockout models has not been investigated. The purpose of this study is to 1) characterize metabolic dysfunction in the ERα$^{-/-}$ rats and 2) identify the impact of ERα loss on exercise capacity. Our results demonstrate that ERα$^{-/-}$ rats have whole-body and skeletal muscle-specific metabolic deficits including a reduction in expression of mitochondrial proteins and regulators. Additionally, a whole-body loss of ERα results in reduced exercise capacity. These findings identify other pathways through which ERα could act in skeletal muscle. This study also identifies that a loss of ERα negatively impacts the ability for rodents to tolerate a metabolic stressor such as exercise.

2.3 Methods

Experimental Animals. Twelve-Fifteen week-old female Wildtype (WT) and ERα$^{-/-}$ rats were from the laboratory of Dr. Michael Soares and Dr. MA Karim Rumi [383]. They were housed in a temperature-controlled facility (22 ± 2˚C) with 12:12 h light:dark cycles. Animals were allowed *ad libitum* access to water and were fed a standard chow diet (Envigo TekLad 8604). Following an overnight fast, animals were anesthetized with pentobarbital sodium and tissues dissected for experimental procedures. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Glucose tolerance test. One week prior to sacrifice, both WT and ERα$^{-/-}$ animals went under an intraperitoneal glucose tolerance test (IPGTT). Following an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (2.5 mg/100 g body

weight) and injected with a glucose load of 2 g/kg body wt. Tail blood was removed every 30 min and glucose levels were assessed using a glucometer and test strips (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN, USA). Blood was allowed to clot for 30 min on ice, spun at 3,000 g for 60 min at 4˚C, and serum was drawn off and frozen at -80˚C. Serum was analyzed for concentration of insulin using an insulin ELISA (Alpco, Salem, NH, USA).

Immunohistochemistry adipose tissue. Retroperitoneal adipose tissue (rpWAT) was fixed overnight in 4% paraformaldehyde, placed in 70% ethanol for 48-72 hr, processed and paraffin embedded. Ten μM sections were places on slides and subsequently hematoxylin and eosin (H & E) stained. Images were taken on a Nikon 80i microscope and images were quantified using Image J.

Glucose Transport. Insulin-stimulated glucose transport into extensor digitorum longus (EDL) and soleus muscle was determined as previously [268, 297, 384, 385]. After dissection, muscle strips were placed in vials in a shaking incubator (35˚C) for 60 min containing Krebs-Henseleit bicarbonate (KHB) buffer with 8 mM glucose and 32 mM mannitol. Non-insulin treated muscles stayed in the same vials for 30 more minutes. Insulin-treated muscles were transferred to new vials for 30 min in the same buffer with the addition of insulin (1 mU/ml) at 35˚C. Muscle strips were then transferred to new vials containing 2 ml of KHB and 40 mM mannitol, with or without insulin (1 mU/ml) for 10 min at 29˚C. Muscle strips were again transferred to new vials containing 2 ml of KHB and 4 mM 2-[1,2-^{3}H] deoxyglucose (1.5 μCi/ml) and 36 mM [^{14}C] mannitol (0.2 μCi/ml), with or without insulin (1 mU/ml) for 20 min. During all incubation steps, muscle strips were exposed to a gas phase of 95% O_2-5% CO_2 at 29˚C. Finally, muscle strips were blotted, clamp-frozen at -80˚C, and processed for determination of intracellular and extracellular accumulation of 2-deoxyglucose.

Western blots in muscle. Muscles were processed for Western blotting by methods previously described [268, 297, 386]. Briefly, muscle was homogenized in a 12:1 (volume-to-weight) ratio of ice-cold cell extraction buffer (Biosource, Invitrogen) containing 10 mM Tris·HCl

(pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na3VO4; 20 mM Na4P2O7; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 µl/5 ml protease inhibitor cocktail. Homogenates were rotated for 30 min at 4˚C, and then centrifuged for 20 min at 3,000 rpm at 4˚C. The supernatant was removed and protein concentration determined by Bradford assay. Samples were diluted in HES buffer and Laemmli buffer containing 100 mM dithiothreitol (DDT) (Thermo Scientific, Rockford, IL, USA) based on protein concentration to generate samples containing equal concentration of protein. Samples were heated in a boiling water bath 5 min. For assessment of mitochondrial complexes, samples were diluted in HES buffer, non-reducing lane marker buffer not containing DDT (Thermo Scientific, Rockford, IL, USA), and were not boiled.

Protein (40-80 µg) was separated on SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 90 min at 200 mA. Membranes were blocked in Tris-buffered saline (TBS), 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 5% bovine serum albumin (BSA) followed by incubation with the appropriate primary antibodies. Following three washes with TBST, blots were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in TBST 1% nonfat dry milk or BSA at a concentration of 1:10,000 for 1 hr at room temperature. Blots were then washed twice with TBST and once with TBS, dried, and visualized by enhanced chemiluminescence (ECL). Bands were quantified using Image J or Image Lab (Bio-Rad) densitometry. Blots were then stripped for 15-20 min at 55˚C in buffer containing 62.5 mM Tris·HCl, 2% SDS, and 100 mM 2-mercaptoethanol and re-probed for α-tubulin as a loading control. Ponceau (Sigma) was used as a loading control for Complex IV.

Primary antibodies used included HSP72 (cat no. SPA-810, Enzo Life Sciences, Farmingdale, NY), HSP60 (cat no. SPA-807, Enzo Life Sciences), PGC-1α (cat no. 516557, Cal-Biochem, Darmstadt, Germany), MitoProfile Total OXPHOS (cat no. ab110413, Abcam, Cambrdige, MA), LC3B (cat no. 2775, Cell Signaling Technology, Inc., Danvers, MA), cytochrome c (cat no. AAM-175, Enzo Life Sciences), pParkin (phospho s65) (cat no.

ab154995, Abcam) Parkin (cat no. sc-32282, Santa Cruz Biotechnology, Dallas, Texas), and α-tubulin (cat no. ab7291, Abcam). Secondary antibodies used included goat anti-mouse (cat no. 170-5047, BioRad, Hercules, CA) donkey anti-rabbit (cat no. 711-035-15, Jackson, Immuno-Research, Inc., West Grove, PA), and goat anti-rabbit (cat no. sc-2004; Santa Cruz Biotechnology).

Exercise capacity. Animals were acclimatized to the treadmill for two days at 12 m/min speed. The exercise test on the third day began at a speed of 12m/min at 0% incline. The speed was increased every 3 min by 3 m/min until 24 m/min was reached. The exercise was stopped when rodents stayed on the shock grid three times for 5 seconds. Exercise time was recorded for each animal.

Triglyceride assay. Intramuscular triacylglycerol concentration was determined based on the methods by Frayn and Maycock (123 bob diss). The white and red gastrocnemius muscle were homogenized in 3 ml of 2:1 chloroform:methanol, transferred to 13x100 mm borosilicate glass tubes, vortexed, and incubated overnight at 4˚C. The following day, 3 ml of 4 mM MgCl2 was added to each tube, vortexed, and centrifuged at 1,000 g for 1 hr at 4˚C. The bottom organic layer (1.5 ml) was drawn off and placed into clean borosilicate glass tubes, allowed to dry overnight, reconstituted with 500 µl of ethanolic KOH, and heated at 75˚C for 20 min. Following heating, 1 ml of 0.15 mM MgSO4 was added to each tube, centrifuged at 1,000 g for 1 hr at 4˚C, and supernatant removed and assayed for triglyceride and free glycerol concentration using a commercially available colorimetric assay (F6428, Sigma, St. Louis, MO).

Statistical analyses. Results are presented as mean ± SEM. Statistical significance was set at $P < 0.05$. Analysis was performed using Sigma Plot for Windows, version 12.0 (Systat Software Inc., Chicago, IL, USA). Data were compared by unpaired t-test or two-way analysis of variance (ANOVA) with Fisher's post-hoc differences performed where appropriate. Where raw values did meet the assumptions of equal variance or normal distribution, values were logarithmically, square root, or reciprocal transformed.

2.4 Results

Body weight and adipose changes in the ERα null rat

Body weight (WT: 307.6 ± 8.2 g, ERα$^{-/-}$: 428.7 ± 11.3 g) was 39% greater in ERα$^{-/-}$ rats than WT rats (P<0.001, **Figure 3A**), and energy efficiency was 2.7-fold greater in ERα null animals (P<0.05, **Figure 3B**). This was calculated as the change in body weight divided by kilocalories consumed. When normalized to body weight, subcutaneous adipose tissue weight (WT: 1299.4 ± 131.3 mg, ERα$^{-/-}$: 3505.8 ± 466.8 mg) was significantly increased in ERα$^{-/-}$ animals (90%, P<0.001, **Figure 3C**) while other fat pad weights (retroperitoneal and periuterine) were not significantly different than the WT animals (not shown). Adipose cell size was also evaluated in the retroperitoneal adipose tissue. Although adipose weight was not different, cell size of retroperitoneal adipocytes was increased by 62% in the ERα$^{-/-}$ animals (P<0.05, **Figure 3D and 3E**), which is associated with impaired systemic insulin sensitivity [387]. Increased adipocyte size has also been observed in ovariectomized animals [38, 373].

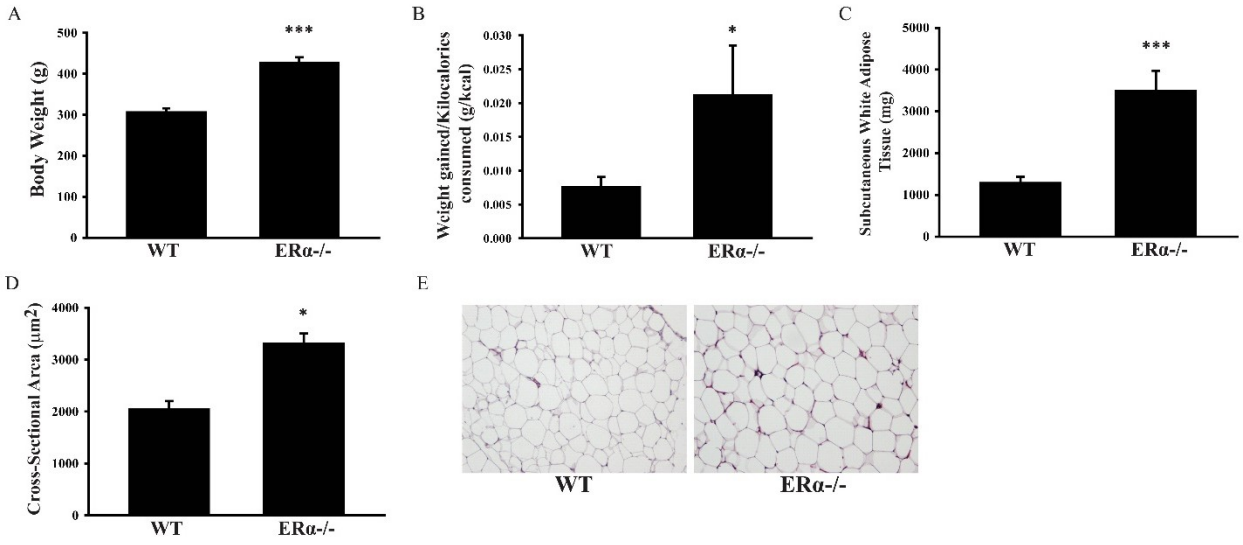

Figure 3.Whole-body ERα -/- in rats results in increased body weight and adipose tissue. Body-weight related changes were compared between groups. A) Body weight, B) food efficiency C) and subcutaneous adipose tissue (normalized to body weight) were evaluated. N=11-14 for body weight and adipose tissue. N=5 for energy efficiency. D) Quantification of retroperitoneal (rpWAT) adipocyte size. E) Representative images of rpWAT cross-sections. N=5 per group, ~500 cells counted per animal. Values are reported as the mean ± SE. All were analyzed by t-test. *P < 0.05,***P < 0.001.

Effect of ERα knockout on insulin resistance

Fasting blood glucose levels were similar between WT and ERα$^{-/-}$ rats (**Figure 4A**). However, fasting serum insulin levels were 51% greater in ERα rats (P<0.05, **Figure 4B**). This resulted in a 59% greater HOMA-IR in the ERα rats (P<0.05, **Figure 4C**), suggestive of increased insulin resistance. Glucose tolerance was also compared between strains via intraperitoneal injection of glucose and tracking of glucose and insulin values over two hours. ERα null and WT rats demonstrated similar glucose concentrations throughout the glucose tolerance test (**Figure 4D**) and similar glucose area under the curve (AUC) values (**Figure 4F**). Insulin concentrations were also similar throughout the glucose tolerance test between groups, other than a significant increase in insulin values in the ERα$^{-/-}$ at the zero and sixty-minute time points (**Figure 4E**). There was a strong trend for an increased insulin AUC in ERα$^{-/-}$ rats (45%, P=0.07, **Figure 4G**). This is suggestive of increased insulin secretion in order to maintain similar blood glucose values as the WT animals. We also compared insulin-stimulated glucose transport in the soleus and EDL between WT and ERα$^{-/-}$ rats. In the soleus muscle, there was no significant difference in insulin-stimulated glucose uptake between the WT and ERα$^{-/-}$ animals. (data not shown). However, in the EDL muscle, insulin significantly increased glucose transport in the WT (60%, P<0.05, **Figure 4H**), but not in the ERα$^{-/-}$ animals.

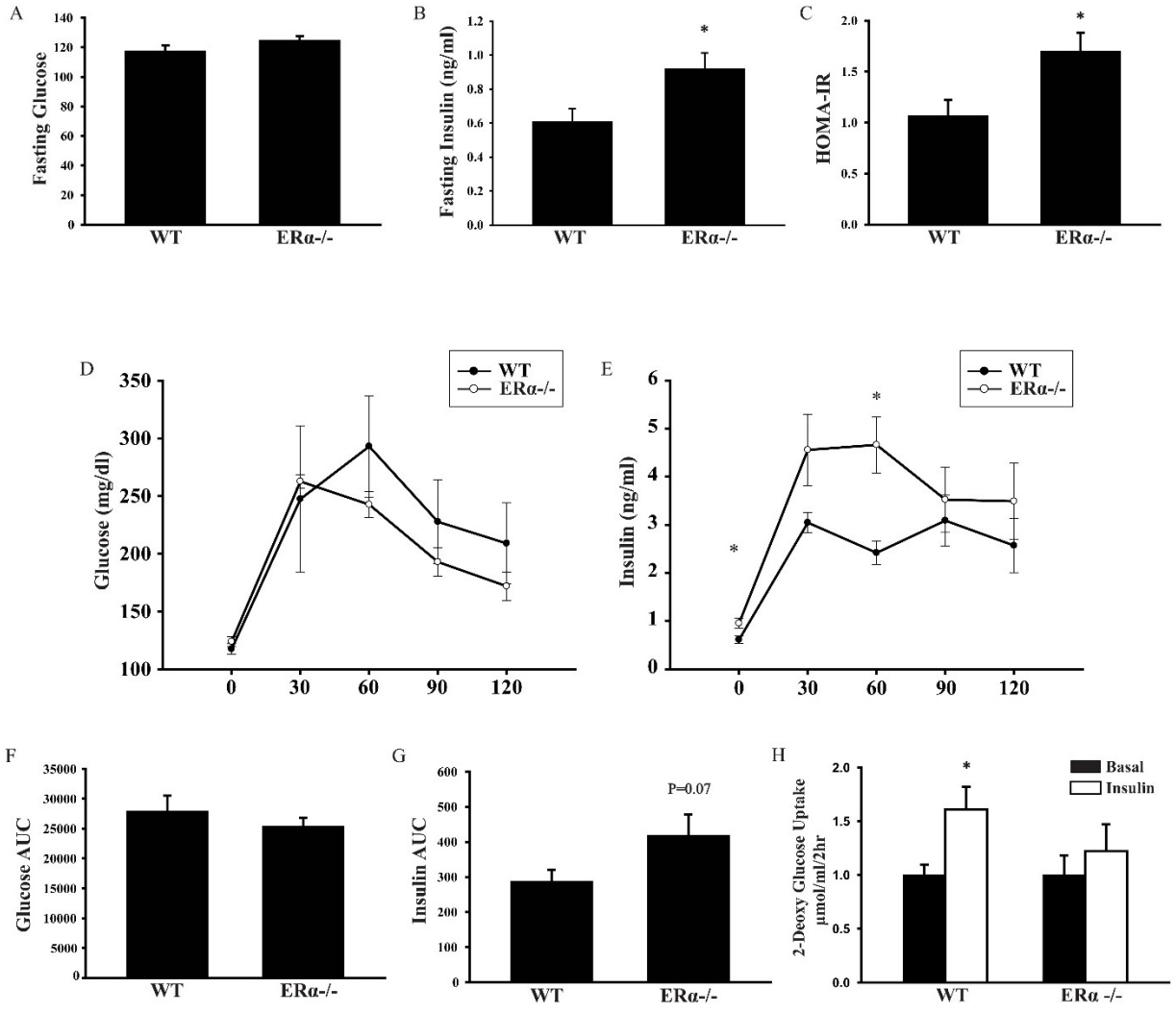

Figure 4. Loss of ERα-/- leads to systemic and skeletal muscle insulin resistance. A) Fasting glucose, and B) insulin were evaluated from serum, and C) HOMA-IR was calculated from these values. D) Glucose concentrations, E) insulin concentrations, F) glucose AUC, G) and insulin AUC were calculated in response to an intraperitoneal glucose injection. Rats were fasted overnight one week prior to sacrifice and were injected with a glucose load of 2 g/kg body weight intraperitoneally. Blood glucose was measured prior to, and 30, 60, 90, and 120 min following injection using a glucometer. N=6-8 per group. H) Insulin-stimulated glucose uptake was evaluated from EDL muscles. Insulin-stimulated glucose uptake was determined by incubating muscles in the presence or absence of 1 mU/ml insulin for 20 min. N=8-10 per group. Values are reported as the mean ± SE. All were analyzed by t-test, besides the glucose transport which was analyzed by two-way ANOVA. *P < 0.05.

Mitochondrial protein expression

In order to identify differences in regulation of mitochondrial biogenesis, we measured expression of the mitochondrial proteins and regulators. We evaluated changes in peroxisome proliferator-activated receptor gamma co-activator-alpha (PGC-1α), electron transport chain complexes, and cytochrome C in red gastrocnemius and white gastrocnemius. There was over an 85% reduction in PGC-1α ($P<0.001$, **Figure 5A**), an 82% reduction in Complex IV ($P<0.001$, **Figure 5B**), and a 38% reduction in cytochrome c protein content ($P<0.05$, **Figure 5C**) in white gastrocnemius of ERα$^{-/-}$ rodents. No significant differences were observed in the other mitochondrial complex proteins, or in any of the abovementioned proteins in the red gastrocnemius muscle (data not shown). Due to past findings related to mitophagy dysfunction in the MERKO mouse [63], we also evaluated autophagy/mitophagy protein expression in the ERα$^{-/-}$ rat. Autophagy is a major cellular degradation pathway, while mitophagy is specifically mitochondrial degradation through autophagy. We found a 45% decrease in the expression of microtubule-associated protein 1 light chain 3 (LC3-II) with a loss of ERα ($P<0.05$, **Figure 5D**). LC3 is lipidated to form LC3-II when autophagosomes are forming [388], thus is a marker of increased autophagy. We also evaluated Parkin, the E3 ubiquitin ligase which targets mitochondria for degradation through mitophagy. Parkin is recruited to the mitochondria and activated by Pink1, which phosphorylates Parkin at serine 65 (Ser 65). We observed a 50% decrease in Ser 65 phosphorylation of Parkin in the ERα$^{-/-}$ rats ($P<0.05$, **Figure 5E**).

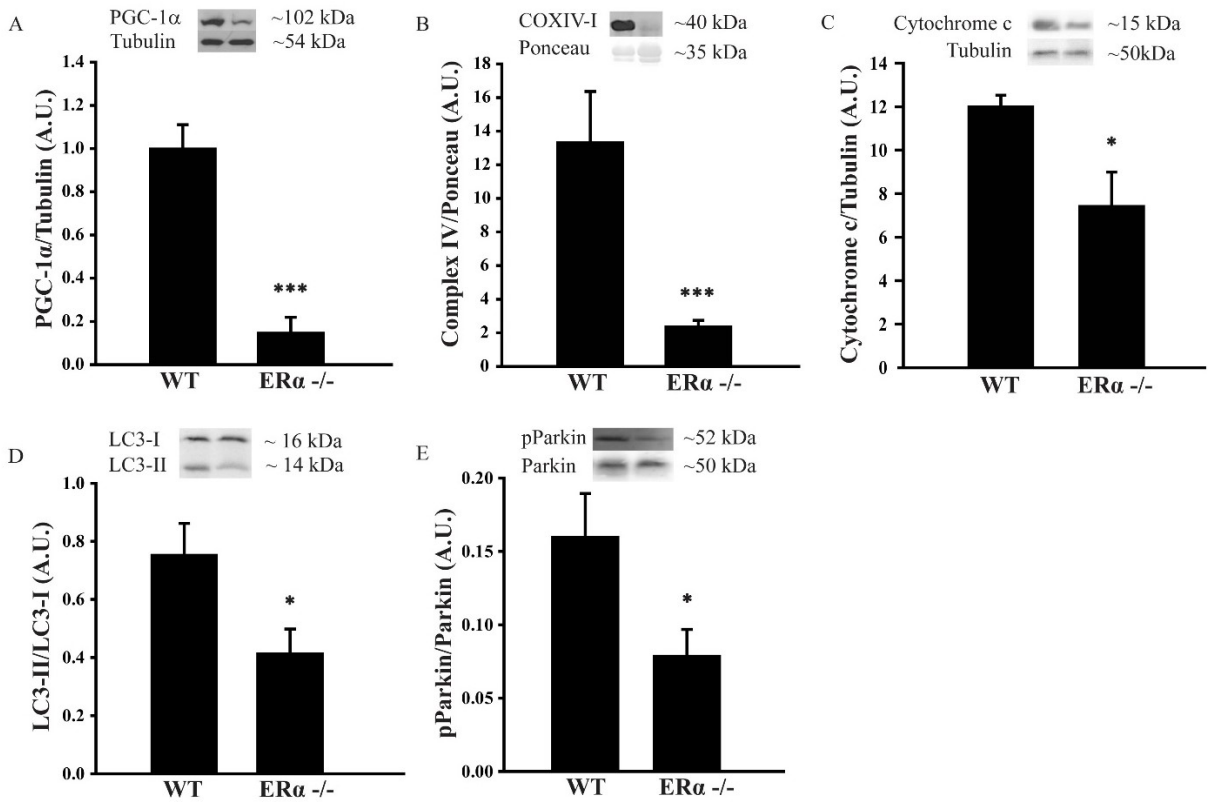

Figure 5. Mitochondrial protein expression is reduced with a loss of ERα.
A) Protein expression of PGC-1α, B) Complex IV, and C) cytochrome C, D) LC3II/LC3I E) and phosphorylation of the Parkin protein expression were quantified in the white gastrocnemius muscle. N=4-5 per group. Values are reported as the mean ± SE. All were analyzed by t-test. *P < 0.05, ***P < 0.001.

Heat shock protein expression and triglyceride storage with a loss of ERα

Heat shock proteins (HSP), which are cellular chaperone proteins that protect the cell from stress, were also evaluated. There was over a 95% reduction in HSP72 ($P<0.001$, **Figure 6A**) and a 25% reduction in HSP60 ($P<0.05$, **Figure 6B**) in white gastrocnemius of ERα$^{-/-}$ rodents compared to WT. We observed no significant difference in heat shock protein expression in the red gastrocnemius. Due to the observed reduction in mitochondrial and heat shock protein expression, we also evaluated triglyceride storage in white and red gastrocnemius. We observed a trend towards an increase in triglyceride storage in the white gastrocnemius (52%, $P=0.09$, **Figure 6C**), and a significant increase in triglyceride storage in the red gastrocnemius (48%, $P<0.05$, **Figure 6D**) of ERα$^{-/-}$ rats.

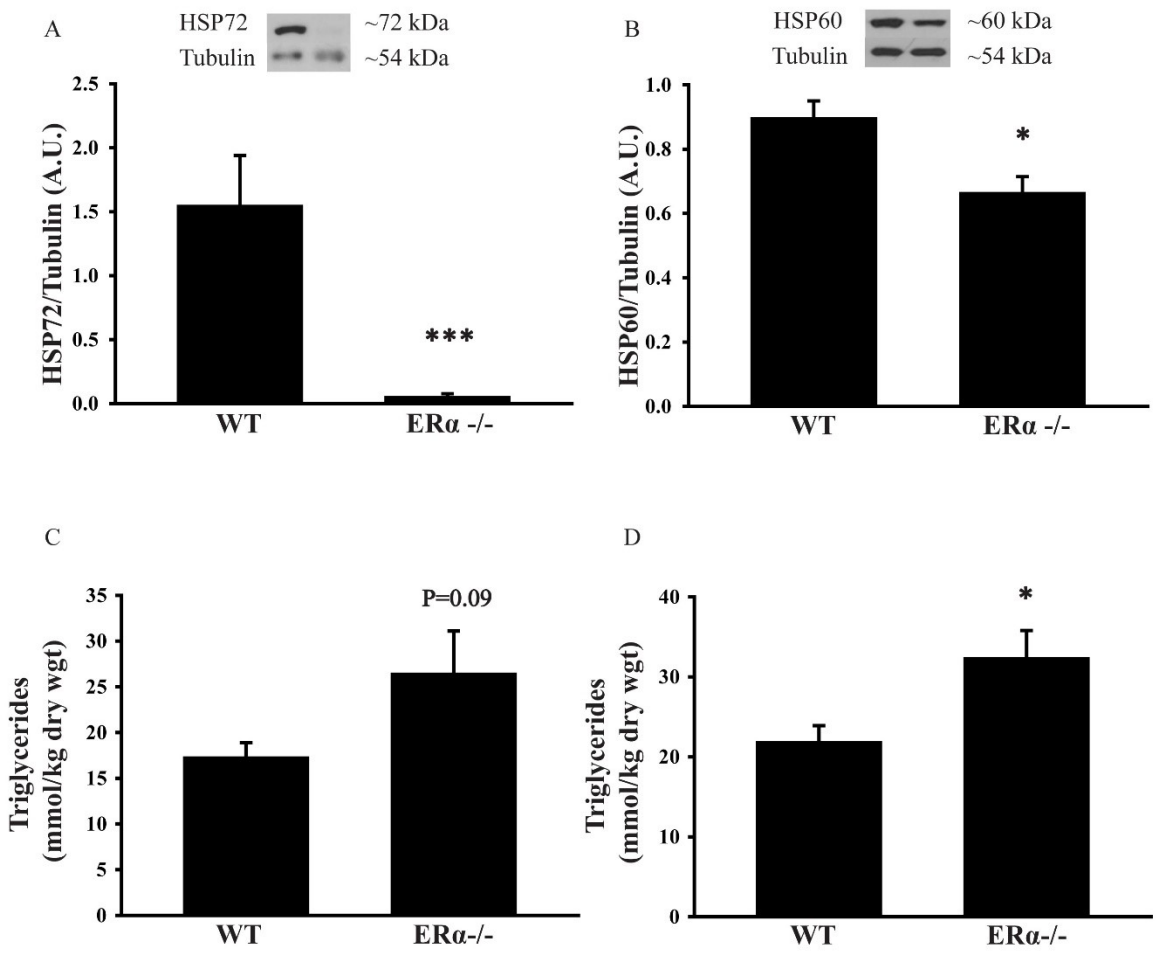

Figure 6. Basal heat shock protein expression is reduced and triglyceride storage is increased in skeletal muscle of ERα$^{-/-}$ rats.
A) HSP72 and B) HSP60 protein expression were compared between groups in white gastrocnemius. N=4-5 per gruop. C) Triglyceride content in the white gastrocnemius D) and red gastrocnemius were also evaluated. N=9-11 per group. Values are reported as the mean ± SE. All were analyzed by unpaired t-test. *P < 0.05, ***P < 0.001.

Effects of ERα knockout on exercise capacity

Due to changes in mitochondrial and heat shock protein expression, we hypothesized that ERα$^{-/-}$ rats would have reduction in exercise capacity. In untrained animals, WT animals ran twice as long as ERα$^{-/-}$ animals in an exhaustive exercise treadmill test ($P<0.05$, **Figure 7**). This result suggests that the metabolic deficits in skeletal muscle affect exercise capacity in the ERα$^{-/-}$ rats.

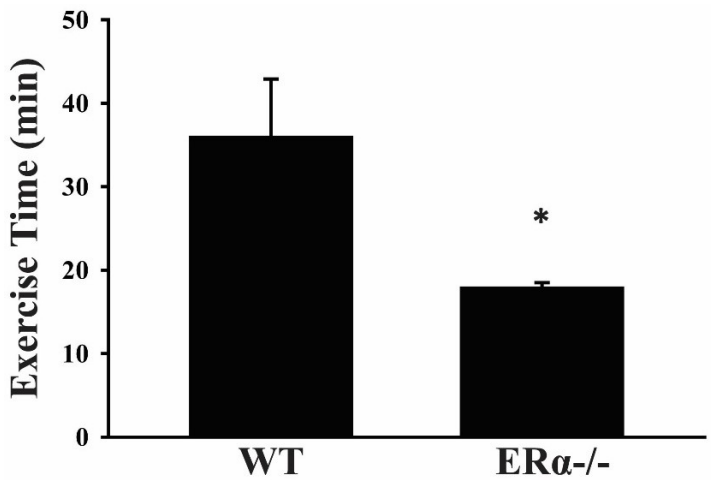

Figure 7. Impaired exercise capacity in the ERα$^{-/-}$ rat.
After two days of acclimation, untrained WT and ERα$^{-/-}$ animals performed an exhaustive exercise bout on a treadmill. N=6-7 per group. Values are reported as the mean ± SE. Results were analyzed by unpaired t-test. *P < 0.05.

2.5 Discussion

Various studies have identified ERα as a therapeutic target to prevent the development of metabolic syndrome in postmenopausal women. Past studies have discovered mechanisms through which ERα acts to maintain skeletal muscle and whole-body metabolism. However, the loss of ERα and its effect on metabolism in a rat model had not been characterized. Additionally, the effect of ERα loss on exercise capacity was previously unknown. This work further defined ERα mechanisms in a new model, and identified the importance of ERα in the ability to perform exercise. These results will allow for a better understanding of the development of metabolic syndrome with a loss of estrogen which will lead to better treatment approaches.

In this study, we evaluated exercise capacity in a rat with whole-body knockout of ERα through a treadmill exercise test to exhaustion. We identified that whole-body ERα$^{-/-}$ results in a reduced running time compared to WT animals. These results agree with past work which demonstrated reduced exercise capacity with a loss of estrogen following ovariectomy [31, 32, 84, 89]. Ovariectomized rodents also demonstrate reductions in whole-body oxygen consumption [38, 40], skeletal muscle fatty acid oxidation and mitochondrial biogenesis signaling [34, 48, 50, 51], and skeletal muscle oxygen consumption [33, 41, 51]. The observed reduction in exercise capacity in ERα$^{-/-}$ rats may be partially due to the loss of PGC1-α, the major regulator of mitochondrial biogenesis, in skeletal muscle. Rodents with mitochondrial biogenesis deficiencies through skeletal muscle-specific PGC1-α knockout also demonstrate reduced endurance capacity [389]. Additionally, PGC-1α overexpression in the muscle leads to enhanced exercise capacity [390, 391]. These past studies demonstrate the importance of PGC-1α in exercise capacity.

Another observation from our study was a reduction in basal HSP72 and HSP60 expression in the white gastrocnemius muscle in ERα$^{-/-}$ rats. In whole-body ERα$^{-/-}$ mice, Ribas et. al did not observe a reduction in basal HSP levels, however they did find a decrease in HSP induction with a high-fat diet [88]. Past studies have found that a skeletal muscle HSP

expression is associated with exercise and aerobic capacity [271, 392]. Muscle-specific overexpression of HSP72 results in a 2-fold increase in endurance exercise capacity and increased mitochondrial content and enzyme activity [266, 269]. With the large reductions in PGC-1α and HSP72 protein expression in skeletal muscle of ERα$^{-/-}$ rats, it is likely that these rodents have reduced aerobic capacity. Cardiorespiratory fitness, or aerobic capacity, describes the body's ability to deliver and use oxygen in peripheral tissues. Low aerobic capacity increases susceptibility to developing metabolic dysfunction, hypertension, and cardiovascular disease [90-92].

This study suggests that ERα is important in maintaining aerobic capacity through skeletal muscle-specific mechanisms. Reduced aerobic capacity through decreased PGC-1α and HSP72 protein expression may be what leads ERα$^{-/-}$ rodents to develop insulin resistance. ERα may be acting through heat shock proteins and the mitochondria in order to maintain metabolic health. Past studies have shown that exercise training and aerobic capacity improve metabolism in ovariectomized rodents [95, 100-105]. Researchers have also identified that exercise and estrogen act similarly to improve whole-body and skeletal muscle metabolism [95, 100, 103, 107-113]. These data imply that exercise should continue to be considered as viable treatment modality in postmenopausal women. It would be interesting for future studies to investigate the ability of exercise training to improve metabolic parameters in the whole-body and muscle-specific ERα$^{-/-}$ rodents. These studies would identify the ability of exercise to improve metabolic dysfunction that is seen with a loss of ERα.

Due to the similarities in metabolic regulation between heat shock proteins and PGC-1α, more studies have begun to investigate links between these proteins. A recent study identified a heat shock element (HSE) binding sequence upstream from the gene for PGC-1α (PPARGC1A). This allows the major HSP transcription factor, heat shock factor 1 (HSF1), to bind and regulate PPARGC1A expression [324]. Ma et. al also found that HSF1 and PGC-1α bind to each other at the HSE on the PPARGC1A gene. Based on the interaction and

coregulatory activity of HSF1 and PGC1-α, and the loss of both PGC1-α and HSP72 in ERα$^{-/-}$ rats, it is possible that ERα is also a part of this mechanism. Past studies have identified estrogen or estrogen receptor regulation of heat shock proteins in skeletal muscle and cardiomyocytes [88, 393-395]. However, the relationship between estrogen receptors and heat shock proteins is unclear. Work from our lab did not find a reduction of HSP expression with ovariectomy [37], and another study found that estrogen attenuates the HSP72 response after exercise [396] . Future studies should investigate the relationship between HSF1 and ERα, and the possibility that ERα could be an upstream regulator of HSF1, thus affecting downstream expression of PGC1-α and HSP72.

In addition to investigating exercise capacity in these rodents, we are the first to characterize metabolic deficiencies in the ERα$^{-/-}$ rat. Metabolic outcomes can vary between species, even between species as close as the mouse and the rat [397-400]. For example, mice have a higher basal metabolic rate compared to rats in order to maintain body temperature, as well as increased gluconeogenesis and circulation of free fatty acids [397, 400, 401]. These differences could cause variability in metabolic outcomes, and highlight the importance of characterizing both species in order to further define metabolic molecular mechanisms with a loss of ERα.

Similar to previous reports in ERα knockout mice, we observed metabolic dysfunction with a loss of ERα in the rat model. We observed whole-body changes such as increased body weight, adipose tissue, and markers of insulin resistance. We also observed changes in mitochondrial protein expression, however, they were unique from past findings [63, 88]. In white skeletal muscle, along with a significant decrease the mitochondrial biogenesis regulator PGC-1α, we observed a reduction in complex IV, and cytochrome C protein expression. Cytochrome c and complex IV are a part of the electron transport chain. There was no change in gene expression for these proteins in the MERKO mouse [63], and they were not evaluated in the whole-body knockout mice [88]. Although there was no change in protein expression, Ribas

et. al did observe a reduction in mitochondrial DNA replication in the MERKO mice [63]. Since PGC-1α is a major factor controlling energy homeostasis through regulating mitochondrial biogenesis and FAO enzymes, ERα's metabolic effects could be mediated through this protein. Future work investigating changes in mitochondrial content through electron microscopy would further define the implications of these protein changes.

Another major finding by Ribas et. al in the MERKO mice was a reduction in the mitochondrial degradation pathway, mitophagy [63]. They found that ERα is important in preserving mitochondrial health through maintaining mitochondrial turnover. This is in agreement with past studies which have found the importance in autophagy and metabolic health [388, 402-404]. In the $ERα^{-/-}$ rats also saw a reduction in autophagy and mitophagy proteins through reduced phosphorylation of Parkin and LC3-II protein content in the $ERα^{-/-}$ rats. In the mouse model, Ribas et. al did not evaluate Parkin phosphorylation, although they observed a reduction in Parkin protein content [63], which we did not observe. They also found a reduction in PINK protein content, which phosphorylates and activates Parkin [63]. Based on our findings and the findings from the MERKO model, it's possible that of ERα regulates Parkin activation through regulating its phosphorylation. These data along with the other observed mitochondrial protein changes confirm that there are mitochondrial deficits in the rat model, although they seem to be uniquely regulated compared to the mouse models.

Another unique perspective from our study is looking at fiber-type specific effects. The observed deficits in mitochondrial protein expression, heat shock protein expression, and glucose transport were observed in either the EDL or white gastrocnemius muscles, which are both mainly comprised of type II muscle fibers [405]. We did not observe these changes in the red gastrocnemius which is mainly comprised of type I muscle fibers. However, triglyceride storage in red gastrocnemius was significantly increased, and there was a trend towards increased storage in the white gastrocnemius. Diabetic patients often undergo a shift from type I, oxidative muscle fibers to more type II, glycolytic muscle fibers [54, 55], while the opposite

occurs with endurance exercise training [406]. A limitation in this study is that we did not evaluate fiber type changes through staining of the myosin heavy chain, however past evidence has shown that PGC-1α and HSP72, as well as various mitochondrial proteins, are increased in type I, oxidative muscle fibers compared to type II fibers [355, 356, 407-411]. These data suggest that with a loss of ERα, there could be a shift away from oxidative characteristics in skeletal muscle. These results further suggest that exercise training could prevent some of the negative metabolic shifts that occur with a loss of estrogen during menopause.

In this study, we utilized a whole-body ERα$^{-/-}$ rat to evaluate metabolic changes and exercise capacity. Future studies to investigate metabolic characteristics in muscle-specific ERα$^{-/-}$ rats would be necessary to further identify skeletal muscle-specific changes with a loss of ERα. Additionally, since we utilized a whole-body knockout, it is possible that part of the reduction in exercise capacity could be due to a loss of ERα in the brain, leading to reduced physical activity [114, 412]. However, due to the metabolic dysfunction and insulin resistance seen in both whole-body and MERKO mice in past studies [62, 88, 375, 381], the skeletal muscle metabolic deficits seen in our model could be due to loss of ERα$^{-/-}$ specifically in the skeletal muscle. Additionally, past research has shown the importance of skeletal muscle-specific PGC-1α and HSP72 in exercise capacity [266, 269, 271, 389-392]. Therefore, it is conceivable that the skeletal muscle metabolic impairments in the ERα$^{-/-}$ rat also contribute to the reduction in exercise capacity. Future studies using a muscle-specific knockout would further support this work.

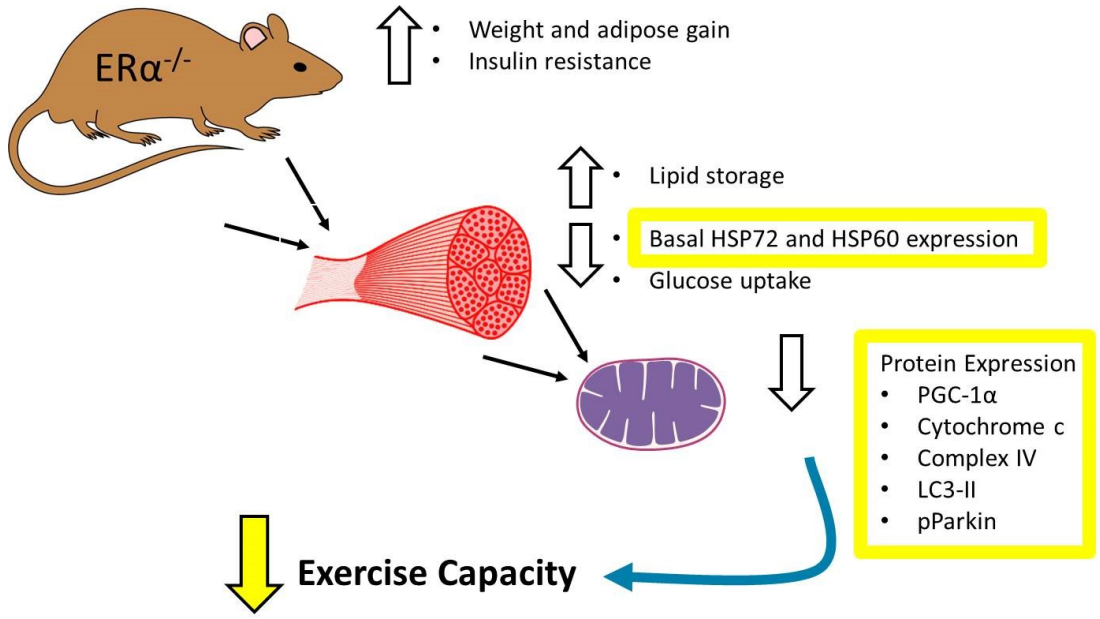

Figure 8. Systemic and skeletal muscle metabolic dysfunction in the ERα$^{-/-}$ rat. Summary of the results describing findings in the ERα-/- rat model. Unique findings are highlighted in yellow.

Conclusion

Systemic and skeletal muscle metabolic dysfunction in the ERα$^{-/-}$ rat contribute to a reduction in exercise capacity. Skeletal muscle reductions in mitochondrial, autophagy/mitophagy, and heat shock protein expression likely contribute to this decrease in exercise capacity (**Figure 8**). This study characterized unique metabolic deficits in the whole-body ERα$^{-/-}$ rat, which identified that basal HSP expression and deficits in mitochondrial proteins and regulators could contribute to the skeletal muscle metabolic dysfunction. This suggests that therapies that improve skeletal muscle metabolic signaling with a loss of estrogen could be effective in maintaining exercise capacity and metabolic homeostasis.

CHAPTER 3

Heat Shock Protein 72 Regulates Hepatic Lipid Accumulation

Portions of this chapter have been accepted for publication. It is printed here with adaptations since publication with permission. Archer, A.E., Rogers, R.S., Von Schulze, A.T., Wheatley, J.L., Morris, E.M., McCoin, C.S., Thyfault, J.P., andGeiger, P.C. (2018). *Heat Shock Protein 72 Regulates Hepatic Lipid Accumulation.* Am J Physiol Regul Integr Comp Physiol.

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3.1 Abstract

Induction of the chaperone Heat Shock Protein 72 (HSP72) through heat treatment, exercise, or transgenic overexpression improves glucose tolerance and mitochondrial function in skeletal muscle. Less is known about HSP72 function in the liver where an accumulation of lipids can result in inflammation, hepatic insulin resistance and Nonalcoholic Fatty Liver Disease (NAFLD). The purpose of the current study was 1) to determine whether weekly *in vivo* heat treatment (HT) induces HSP72 protein expression in the liver and improves glucose tolerance in rats fed a high-fat diet (HFD) and 2) to determine the ability of HSP72 to protect against hepatic lipid accumulation and mitochondrial dysfunction in primary hepatocytes. Male Wistar rats were fed a HFD for 15 weeks and were anesthetized and given weekly HT (41°C for 20 min) or sham-treatments (ST, 37°C for 20 min) for the last 7 weeks. A glucose tolerance test and insulin-stimulated glucose uptake in skeletal muscle were performed. The impact of an acute loss in HSP72 via siRNA on lipid handling and mitochondrial integrity was examined in primary hepatocytes. Weekly in-vivo HT increased glucose tolerance and HSP72 protein content and triglyceride storage was decreased in skeletal muscle and liver. In primary hepatocytes, mitochondrial quality was reduced in the absence of HSP72 while protein expression of PGC-1α, and mitochondrial ETC complexes were unchanged. Primary hepatocytes also demonstrated reduced fatty acid oxidation and increased lipid accumulation following palmitate treatment compared with control siRNA-treated primary hepatocytes. This data suggests that in vivo HT significantly improves systemic metabolism with robust induction of HSP72 in the liver. Acute loss of HSP72 in primary hepatocytes significantly impacts mitochondrial quality and lipid handling. These findings suggest future therapies targeting HSP72 in the liver may prevent hepatic insulin resistance and NAFLD.

Key words: liver metabolism, steatosis, non-alcoholic fatty liver disease, heat shock proteins, mitochondria

3.2 Introduction

The heat shock response is a highly conserved defense system to combat cellular and oxidative stress [413] comprised of a family of heat shock proteins (HSPs) identified by molecular weight [263, 414, 415]. Best known for their chaperone functions, HSPs also have cell signaling functions and more recently identified roles in regulation of metabolism [269]. HSP72 is of great interest in relation to metabolic disease as it is highly induced in response to stress and with endurance exercise [266, 268, 269]. Kurucz et al. first demonstrated that HSP72 expression was markedly decreased in skeletal muscle of insulin resistant and type 2 diabetic patients [276]. Subsequent studies from our lab and others showed that heat treatment (HT), transgenic overexpression of HSP72, and pharmacological induction of HSP72 effectively prevent high-fat diet-induced glucose intolerance and skeletal muscle insulin resistance [266-269, 295, 297, 416, 417]. HSP72 is also important in skeletal muscle mitochondrial function. Loss of HSP72 results in retention of dysmorphic mitochondria, but transgenic overexpression of skeletal muscle HSP72 results in an increase in mitochondrial content and activity of mitochondrial enzymes [269, 317]. As a result, HSP72 has a well-established role in regulating glucose homeostasis, insulin sensitivity and oxidative capacity in skeletal muscle.

Two recent studies have indicated a potential role for HSP72 in liver metabolism. Zeng et al. demonstrated the small molecule drug matrine, used for treatment of chronic viral infections and tumors in the liver, has hepatoprotective effects that involve the activation of HSP72 in the liver [301]. In addition, Di Naso demonstrated a correlation between decreased HSP72 protein levels in obese patients with progression of insulin resistance and nonalcoholic fatty liver disease (NAFLD) [264]. NAFLD is characterized by an excessive accumulation of lipids in the liver that ultimately contribute to the development of hepatic insulin resistance, hyperinsulinemia, hyperglycemia, and type 2 diabetes. Mitochondria are critical to liver function and considerable evidence indicates decreased mitochondrial function contributes to the pathobiology of metabolic diseases. However, the mechanisms that govern hepatic mitochondrial function and alter

susceptibility for hepatic insulin resistance and steatosis are poorly understood. To date, no studies have examined the direct effect of HSP72 modulation on hepatic lipid metabolism.

The purpose of the present study was two-fold. First, to determine whether *in vivo* HT intervention would sufficiently induce hepatic HSP72 expression and improve insulin sensitivity in rats following 8 wk of high-fat feeding. Previously we had noted that 12 wks of weekly HTs in conjunction with 12 wk high-fat feeding protected against the development of insulin resistance [268], however studies have not investigated the ability of HT to be used as an intervention in rodents. Our second aim was to examine the impact of direct HSP72 modulation on primary hepatocytes in vitro. Prior studies provide strong evidence that induction of HSP72 increases oxidative capacity and mitochondrial function in skeletal muscle. Given that the liver has high mitochondrial density and fast mitochondrial turnover [225], it is likely that HSP72 plays an important role in maintaining hepatic mitochondrial function. We hypothesize that HSP72 exerts beneficial metabolic effects in hepatocytes by supporting fatty acid oxidation and preventing lipid storage. Targeting HSP72 could be an effective strategy for reducing hepatic insulin resistance, NAFLD and type 2 diabetes.

3.3 Methods

Experimental animals and in vivo heat treatment. Eight wk old male Wistar rats (~150 – 180 g each) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature controlled facility (22 ± 2˚C) with 12:12 h light:dark cycles. Animals were allowed *ad libitum* access to water. Rats were fed a modified Kraegen high-fat diet (HFD) (60% of kilocalories from fat) for 15 wk [268, 418]. During the last 7 wk of the HFD, rats received either weekly *in vivo* heat (HT) or sham treatment (ST, n=9/group). All animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) prior to ST or HT. HT consisted of lower body immersion in a 42°C water bath to gradually increase body temperature to between 41°C and 41.5°C where it was maintained for 20 min. ST

consisted of immersion in a 37°C water bath and maintaining body temperature at 37°C for 20 min [268, 386]. Body temperature was monitored by a rectal thermometer. Following treatment, 0.5 ml of 0.9% saline was injected intraperitoneally to aid in recovery. Forty-eight h following HT or ST, and following a 10-hr overnight fast, animals were again anesthetized with pentobarbital sodium and tissues dissected for experimental procedures. All protocols and procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Glucose tolerance testing and other blood measures. One week prior to sacrifice, and 48 h following HT or ST, rats underwent an intraperitoneal glucose tolerance test (IPGTT). Following an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) and injected with a glucose load of 2 g/kg body wt. Tail blood was removed every 30 min and assessed for blood using a glucometer and the manufacturer's test strips (Accu-Chek Active, Roche Diagnostics, Indianapolis, IN). Blood was allowed to clot for 30 min on ice, spun at 3,000 *g* for 60 min at 4°C, and serum drawn off and frozen at -80°C. Serum was analyzed for concentration of insulin using an insulin ELISA (Alpco, Salem, NH, USA). Serum triglycerides and NEFAs were also determined by colorimetric assays using the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI and Wako Diagnostics, Richmond, VA, respectively).

Glucose Transport. Insulin-stimulated glucose transport into extensor digitorum longus (EDL) and soleus muscle was determined as previously [268, 297, 384, 385]. After dissection, muscle strips were placed in vials in a shaking incubator (35˚C) for 60 min containing Krebs-Henseleit bicarbonate (KHB) buffer with 8 mM glucose and 32 mM mannitol. Non-insulin treated muscles stayed in the same vials for 30 more minutes. Insulin-treated muscles were transferred to new vials for 30 min in the same buffer with the addition of insulin (1 mU/ml) at 35˚C. Muscle strips were then transferred to new vials containing 2 ml of KHB and 40 mM mannitol, with or without insulin (1 mU/ml) for 10 min at 29˚C. Muscle strips were again transferred to new vials

containing 2 ml of KHB and 4 mM 2-[1,2-^{3}H] deoxyglucose (1.5 µCi/ml) and 36 mM [^{14}C] mannitol (0.2 µCi/ml), with or without insulin (1 mU/ml) for 20 min. During all incubation steps, muscle strips were exposed to a gas phase of 95% O_2-5% CO_2 at 29˚C. Finally, muscle strips were blotted, clamp-frozen at -80˚C, and processed for determination of intracellular and extracellular accumulation of 2-deoxyglucose.

Adipose tissue imaging. Epididymal white adipose tissue was fixed overnight in 4% paraformaldehyde, placed in 70% ethanol for 48-72 h, processed and paraffin embedded. Ten µM sections were places on slides and subsequently hematoxylin and eosin (H & E) stained. Images were taken on a Nikon 80i microscope and images quantified using Image J.

Hepatocyte Primary Cell Culture. Hepatocytes from C57Bl6/J mice (~8-20 weeks) were isolated by collagenase perfusion. Animals were anesthetized with 1-2 ml of isoflurane. The hepatic portal vein was cannulated and the liver was infused with a perfusion buffer (1xHBSS Ca++/Mg++ free, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES) at 8.2 ml/min. The portal vein and diaphragm were cut and the superior vena cava was clamped. After 10 minutes with the first perfusion buffer, the buffer was changed to a second perfusion buffer (1xHBSS with Ca++/MG++, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 0.025 mg/ml collagenase, Roche Liberase TM). Colleganase digestion continued for ~7-10 minutes until signs of digestion were observed. The perfusion pump was stopped and the liver was excised. The liver was placed in 100 ml sterile beaker containing 20-30 mls of a cold third buffer (1xHBSS Ca++/Mg++ free, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 1x10-7 M Insulin). The liver was chopped with scissors and forceps and filtered through cell filters beginning with 100 µM, then 70 µM, and the 30 µM. Cells were collected into a 50 ml conical tube. Cells were centrifuged in a volume of 50 ml at 50 g for 5 min at 4 degrees. The media was aspirated and the wash step was repeated 2 more times using the cold third buffer. After the third wash the cells were resuspended in Williams E Media (Sigma, St. Louis, MO) and viability was determined using trypan blue. Hepatocytes were plated on collagen-coated plates (rat tail

collagen type I, Corning) in hepatocyte growth media (Williams E, 10% FBS, 4 mM L-glutamine, 100 U penicillin/100 mg streptomycin, 2 ng/mL rat EGF, 100 nM insulin, 100 nM dexamethasone, 0.1% BSA, 10 mM sodium pyruvate). For fatty acid oxidation experiments hepatocytes were plated into a 12-well plate ($2x10^5$ cells/well). For all other experiments, hepatocytes were plated in 6 well plates ($1x10^6$ cells/well). For lipid exposure experiments, cells were treated with 250 μM Palmitate for 24 h. Palmitate was conjugated to 1% BSA in hepatocyte growth media. Further cell experiments are described below.

siRNA transfection. Hepatocytes were transfected with 15 nM HSP72 siRNA (NM_212504; Sigma) or MISSION® siRNA Universal Negative Control #1 using Mission siRNA transfection reagent (Sigma) according to the manufacturer's protocol in hepatocyte growth media. Hepatocytes were harvested 24-48 h after transfection, and knockdown was confirmed by western blot.

Heat treatment in primary hepatocytes. Hepatocytes were heat treated (42°C) or sham treated (37°C) in a water bath for 30 minutes, and growth media was changed immediately after. Experiments continued 24 hours following heat treatment.

Lipid droplet detection. Along with siRNA or heat treatment, cells were treated with Palmitate or Vehicle as described above. Cells were then treated with 3.8 μM Bodipy 493/503 (D3922; Thermo Fisher Scientific, Rockford, IL) for 15 minutes in serum-free William's E media and then washed with 1xPBS. Cells were then imaged live using confocal imaging. Images were acquired with the Nikon A1 Confocal Live Cell Scanning Microscope (Melville, NY).

Triglyceride content. Triglyceride content from various tissues was evaluated as done previously [133, 136]. Intramuscular triacylglycerol concentration was determined based on the methods by Frayn and Maycock [419]. The tibialis anterior, consisting of mostly glycolytic muscle fibers [420], was homogenized in 3 ml of 2:1 chloroform:methanol, transferred to 13x100 mm borosilicate glass tubes, vortexed, and incubated overnight at 4˚C. The following day, 3 ml of 4 mmol/l $MgCl_2$ was added to each tube, vortexed, and centrifuged at 1,000 *g* for 1 h at 4˚C.

The bottom organic layer (1.5 ml) was drawn off and placed into clean borosilicate glass tubes, allowed to dry overnight, reconstituted with 500 µl of ethanolic KOH, and heated at 75°C for 20 min. Following heating, 1 ml of 0.15 mmol/l $MgSO_4$ was added to each tube, centrifuged at 1,000 *g* for 1 h at 4°C, and supernatant removed and assayed for triglyceride and free glycerol concentration using a commercially available colorimetric assay (F6428, Sigma). Liver was processed similarly except that after drying overnight, samples were reconstituted in butanol – Triton X-110 and assayed directly afterwards [133].

Liver triacylglycerol concentration was also detected from primary hepatocytes. Cells were harvested using lysis buffer (0.03% SDS in PBS), followed by addition of 1 ml of 2:1 chloroform/methanol to each tube of lysate. The tubes were centrifuged for 1 h (1000 *g*, 4 C) and the organic layer was transferred to a clean Eppendorf tube. Tubes were then transferred to a fume hood for 48-72 h to evaporate the organic phase, and then each sample was reconstituted in 75 ul of butanol: Triton X-114 (3:2). Glycerol content was evaluated to measure lipid content and was determined by using the Glycerol Free Reagent kit (F6428; Sigma). Optical density was evaluated at 540 nm.

Mitochondrial quality measurement. Hepatocytes were treated with 100 nM MitoTracker Green FM (M7514; Thermo Fisher) in William's E Media, a green fluorescent mitochondrial stain which localizes to mitochondria regardless of mitochondrial membrane potential. Cells were also stained with 600 nM tetramethylrhodamine, ethyl ester (TMRE) (T669; Thermo Fisher) in William's E Media, which is a red florescent stain sequestered by active mitochondria. Cells were washed with warm PBS, stained with both stains for 30 min, and then washed again with warm PBS. Cells fixed with 3.7% PFA and imaged with confocal imaging. Images were acquired with the Nikon A1 Confocal Live Cell Scanning Microscope.

Fatty acid oxidation. Fatty acid oxidation (FAO) was determined in primary hepatocytes based on previous protocols [252]. Primary hepatocytes in 12-well plates were serum starved and then washed with warm PBS. Cells were then incubated in FAO reaction medium

containing DMEM-low glucose (Invitrogen), 0.5 μCi/ml [1-^{14}C] palmitate, 100 μM palmitate ,0.25% BSA, 1 mM carnitine, and 12.5 mM HEPES (pH ~7.4) at 37°C for 3 h in triplicate. To identify carnitine palmitoyltransferase-1 (CPT-1)-mediated FAO, some wells were treated with the CPT-1 inhibitor etomoxir (100 μM). CPT-1-mediated FAO is calculated as the difference between total FAO and FAO in the presence of etomoxir. After 3 h, the medium from each well was collected, and an aliquot of medium was put into the sealed trapping device. The $^{14}CO_2$ was driven from the media aliquot by addition of perchloric acid and trapped in NaOH, which was collected and analyzed by liquid scintillation counting for determination of complete FAO to CO_2. The acidified media was collected, refrigerated, and centrifuged (16,000 g, 4°C). An aliquot was analyzed by liquid scintillation counting for determination of the acid-soluble metabolites (ASMs) of FAO. ASMs are radiolabeled fatty acids which have not been completely oxidized to CO_2, thus represent incomplete FAO. The cells were rinsed three times with ice-cold Krebs-Henseleit buffer and lysed with SDS lysis buffer. The protein concentration of the lysate was determined by BCA assay.

Transmission Electron Microscopy. The tissue was fixed with 2% glutaraldehyde in 0.1M Cacodylate buffer. Tissue was rinsed 2Xs 10 min each with Cacodylate buffer. Tissue was post fixed in 1% osmium tetroxide buffered, for 1 hour. Tissue was rinsed 3Xs 10 min each with distilled water. Tissue was dehydrated in a graded series of ethanol as follows: 50%, 70%, 80%, 95%, 100%,100% 10 min each. Tissue was placed into propylene oxide 2Xs 20 min each then transferred to a half/half mixture of propylene oxide/Embed 812 medium mixture resin (Electron Microscopy Sciences, Fort Washington PA) samples were left to infiltrate overnight. Tissue was placed into 100 Embed 812 resin for 1 h. They were then placed into BEEM capsules size 00 and cured overnight in a 65°C oven. The individual sample blocks were sectioned using a Diatome diamond knife on a Leica UC-7 ultramicrotome at 80nm thick and picked up on 200 mesh copper grids. Samples were examined using a J.E.O.L. JEM-1400 TEM operated at 100KV.

Western blotting. Muscles and liver were processed for Western blotting by methods previously described [268, 297, 386]. Briefly, muscle and liver tissue were homogenized in a 12:1 (volume-to-weight) ratio of ice-cold cell extraction buffer containing 10 mmol/l Tris·HCl (pH 7.4); 100 mmol/l NaCl; 1 mmol/l each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mmol/l Na_3VO_4; 20 mmol/l $Na_4P_2O_7$; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 µl/5 ml protease inhibitor cocktail. Homogenates were rotated for 30 min at 4°C, and then centrifuged for 20 min at 3,000 rpm at 4°C and the supernatant was removed. Before harvesting for western blots, hepatocytes were serum starved for 6 h. Cells for western blotting were rinsed with PBS scraped, collected, and pelleted. The cell pellets were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1mM NA_3VO_4, and protease inhibitors (Thermo Fisher)) and were centrifuged at 12,000 rpm x 12 min 4°C. The protein content of the supernatant for tissue homogenates and cell culture determined by Bradford assay and lysates were stored at -80°C until analysis.

Samples were diluted in HES buffer and Laemmli buffer containing 100 mmol/l dithiothreitol (DDT) (Thermo Fisher) based on protein concentration to generate samples containing equal concentration of protein. Samples were heated in a boiling water bath 5 min. For assessment of mitochondrial complexes, samples were diluted in HES buffer, non-reducing lane marker buffer not containing DDT (Thermo Fisher), and were not boiled. Protein (20-80 µg) was separated on SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 1.5-4 h at 200-250 mA. Membranes were blocked in Tris-buffered saline (TBS), 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 5% bovine serum albumin (BSA) followed by incubation with the appropriate primary antibodies. Following three brief washes with TBST, blots were incubated with an appropriate HRP-conjugated secondary antibody in TBST 1% nonfat dry milk or BSA at a concentration of 1:10,000 for 1 h at room temperature. Blots were then washed twice with TBST and once with TBS, dried, and visualized by Enhanced chemiluminescence

(ECL). Bands were quantified using Image J or Image Lab densitometry. Blots from liver and muscle were then stripped for 15-20 min at 55°C in buffer containing 62.5 mmol/l Tris·HCl, 2% SDS, and 100 mmol/l 2-mercaptoethanol and re-probed for α-tubulin or β-actin as a loading control. Ponceau (Sigma) was used as a loading control from cell culture studies.

Antibodies. HSP72 primary antibody (cat. no. SPA-810) and Cytochrome c (cat no. AAM-175) were purchased from Enzo Life Sciences (Farmingdale, NY). PGC-1α (cat. no. 516557) was purchased from Cal-Biochem (Darmstadt, Germany). MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (cat. no. 110413) was purchased from MitoSciences (Eugene, Oregon). Bradford protein quantification reagent was purchased from Bio-Rad (Hercules, CA). Secondary antibodies used included goat anti-mouse (cat no. 170-5047, BioRad), donkey anti-rabbit (711-035-152, Jackson Immuno-Research, Inc., West Grove, PA), and goat anti-rabbit (cat no. sc-2004; Santa Cruz Biotechnology). Enhanced chemiluminescence reagents were purchased from Thermo Fisher.

Statistical Analysis. Results are presented as mean ± SEM. Statistical significance was set at $P < 0.05$. Analysis was performed using Sigma Plot for Windows, version 12.0 (Systat Software Inc., Chicago, IL). Data were compared by an unpaired t-test, one-way ANOVA, or two-way ANOVA using Fisher's post-hoc differences performed where appropriate. Where raw values did meet the assumptions of equal variance or normal distribution, values were logarithmically, square root, or reciprocal transformed.

3.4 Results

At the end of the initial 8 wk period of high-fat feeding, body weight (Sham: 498.8 ± 19.1 g, Heat: 492.1 ± 15.7 g) and daily food intake (Sham: 19.2 ± 0.8 g/d, Heat: 19.4 ± 1.1 g/d) were similar prior to beginning HT or ST. During the 7 wk HT or ST period there was a strong trend for HT rats to gain less weight on the high-fat diet (HFD) compared to ST animals ($P = 0.056$, **Figure 9A**). Food intake was not significantly different following HT (**Figure 9B**). Energy

efficiency, calculated as the change in body weight divided by the kilocalories consumed during this period, was 19% lower in the HT rats compared to ST ($P < 0.05$, **Figure 9C**). Coinciding with modest changes in body weight, the weight of the eWAT (Sham: 17.1 ± 2.0 g, Heat: 14.3 ± 1.2 g), subcutaneous white adipose tissue (SCAT) (Sham: 9.3 ± 1.5 g, Heat: 6.7 ± 1.1 g), and brown adipose tissue (BAT) (Sham: 729.4 ± 67.3 mg, Heat: 700.0 ± 38.7 mg) were not significantly different between HT and ST rats. However, adipocyte size in the eWAT was 28% lower in HT rats compared to ST ($P < 0.001$) (**Figure 9D & 9E**).

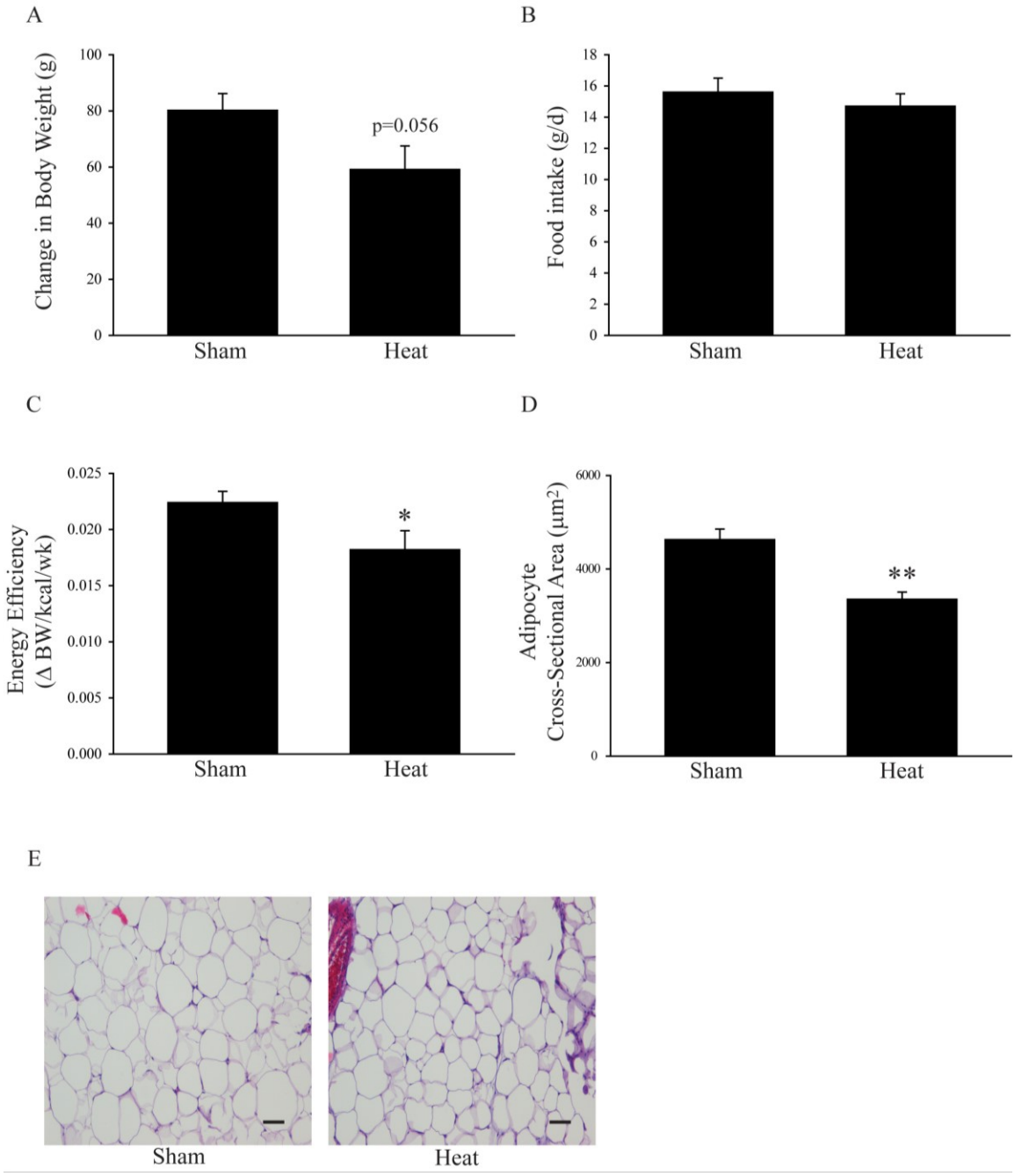

Figure 9. Body weight related changes following weekly heat treatment in rats fed a HFD. (A) Change in body weight, (B) food intake, (C) energy efficiency, and (D) cross-sectional area of adipocytes from eWAT in rats fed a HFD for 15 kw and receiving weekly *in vivo* ST (37˚C, 20 min) or HT (41˚C, 20 min) during the last 7 wk of the HFD. (E) Representative images of 10 μm thick sections of eWAT stained with H&E. Bar represents 50 μm. * $P < 0.05$, *** $P < 0.001$ denotes a significant difference between groups determined by unpaired t-test. Values are mean ± SEM. N=8-9 animals/group.

Heat treatment, glucose tolerance, and insulin sensitivity

Whole-body insulin resistance has been consistently shown in rats after just 3-6 wk of high-fat feeding [418, 421-423]. Fasting blood glucose concentration was not significantly different between ST and HT rats (**Figure 10A**), and fasting insulin concentration was only modestly reduced following HT ($P = 0.07$, **Figure 10B**). Following an intraperitoneal injection of glucose one wk prior to the final ST/HT, and 48 h after the last ST/HT, HT rats had significantly lower blood glucose concentrations compared to ST (main effect of treatment $P < 0.05$, **Figure 10C**), as well as 20% lower glucose area under the curve (AUC) values ($P < 0.05$, **Figure 10D**). Insulin concentration was significantly lower 30 min following glucose injection in HT rats compared to ST, but was not significantly different 60 min, 90 min, or 120 min following injection (**Figure 10E**). Insulin AUC values were not significantly different between HT and ST rats (**Figure 10F**). Serum triglycerides were not significantly different between ST (120.7 ± 11.3 mg/dl) and HT rats (114.3 ± 17.1 mg/dl), and serum NEFA concentration were not significantly different between ST (0.469 ± 0.04 mmol/l) and HT animals (0.421 ± 0.04 mmol/l).

We also evaluated skeletal muscle insulin sensitivity in both the soleus and EDL muscle of ST and HT rodents. The EDL is a composed of primarily glycolytic type II muscle fibers while the soleus is composed primarily of oxidative type I muscle fibers [420]. In the EDL of HT rats, we observed significantly greater insulin-stimulated glucose uptake compared to ST rats ($P < 0.05$) (**Figure 10G**). In the soleus, we did not observe significant differences in insulin-stimulated glucose uptake between HT and ST rats (data not shown).

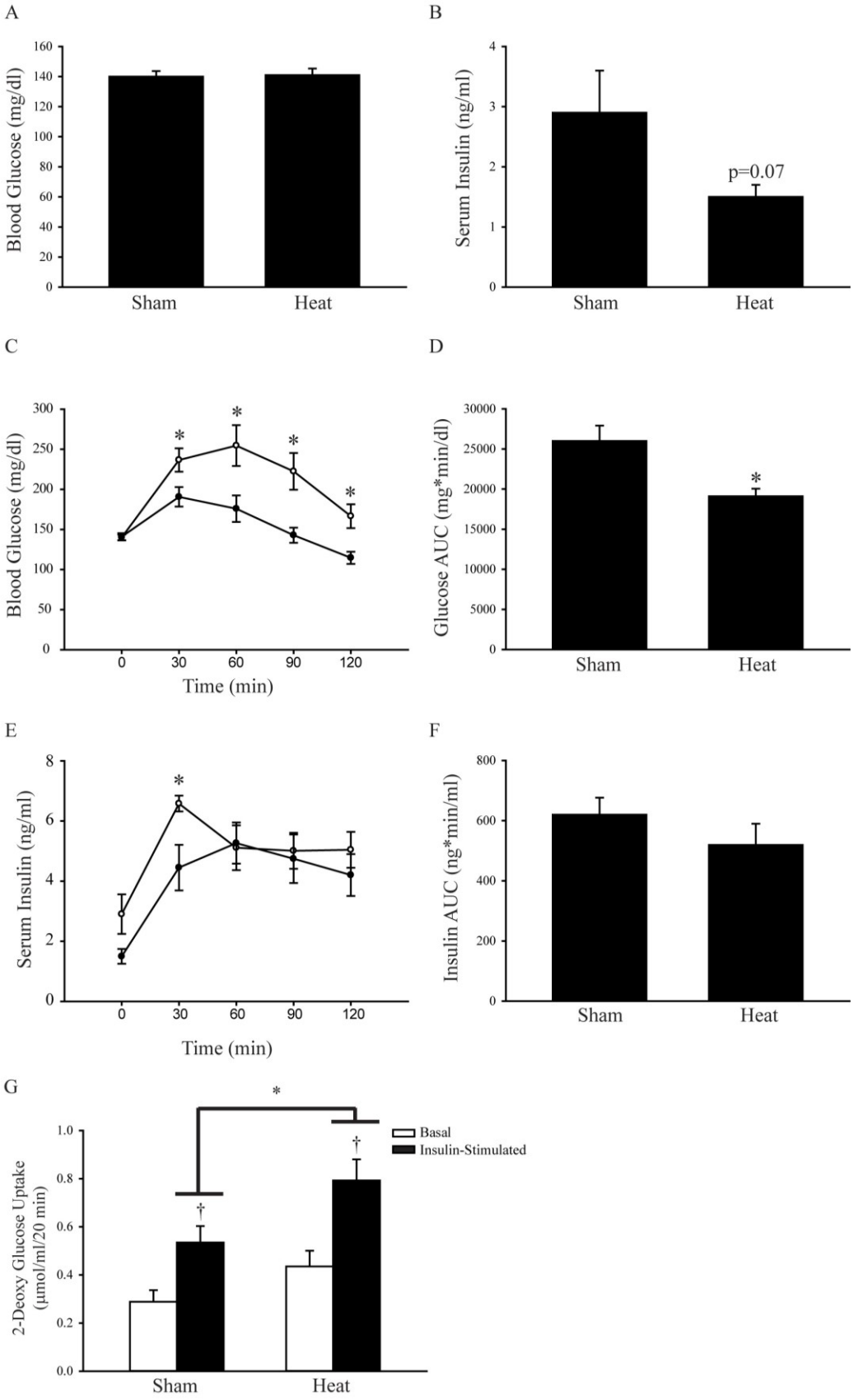
A
Blood Glucose (mg/dl)
160
140
120
100
80
60
40
20
0
Sham
Heat
B
Serum Insulin (ng/ml)
4
3
2
1
0
p=0.07
Sham
Heat
C
Blood Glucose (mg/dl)
300
250
200
150
100
50
0
*
0
30
60
90
120
Time (min)
D
Glucose AUC (mg*min/dl)
30000
25000
20000
15000
10000
5000
0
*
Sham
Heat
E
Serum Insulin (ng/ml)
8
6
4
2
0
*
0
30
60
90
120
Time (min)
F
Insulin AUC (ng*min/ml)
800
600
400
200
0
Sham
Heat
G
2-Deoxy Glucose Uptake (µmol/ml/20 min)
1.0
0.8
0.6
0.4
0.2
0.0
*
†
†
Basal
Insulin-Stimulated
Sham
Heat

Figure 10. Heat treatment improves glucose tolerance and insulin-stimulated glucose uptake in rats fed a HFD.
(A) Fasting blood glucose and (B) serum insulin concentration in rats fed a HFD for 15 wk and receiving weekly *in vivo* ST (37˚C, 20 min) or HT (41˚C, 20 min) during the last 7 wk of the HFD. Blood glucose (C) and insulin concentrations (E) in response to an intraperitoneal (i.p.) glucose injection, and AUC of glucose (D) and AUC (F) of insulin following i.p. glucose injection. Rats were fasted overnight one week prior to sacrifice and were injected with a glucose load of 2 g/kg body weight intraperitoneally. Blood glucose was measured prior to, and 30, 60, 90, and 120 min following injection using a glucometer. Differences between groups were determined by unpaired t-tests at each GTT time point and also when comparing AUC and fasting glucose and insulin levels. * $P < 0.05$ (G) Insulin-stimulated glucose uptake in the EDL muscle in ST and HT rats. Insulin-stimulated glucose uptake was determined by incubating muscles in the presence or absence of 1 mU/ml insulin for exactly 20 min. † $P < 0.05$ denotes insulin-stimulated glucose uptake is significantly greater than basal determined by one-way ANOVA with Fisher's post hoc analysis performed where appropriate. Values are mean ± SEM. N=6-9 animals/group.

Liver and skeletal muscle responses to heat treatment

Induction of HSP72 by HT was then determined in skeletal muscle and liver. HSP72 levels were 315% greater in the EDL of HT rats compared to ST ($P < 0.001$, **Figure 11A**), but HSP72 levels were not significantly different between HT and ST rats in the soleus (**Figure 11B**). Hepatic HSP72 protein expression was 54% greater following HT compared to ST ($P < 0.01$, **Figure 11D**). In addition to HSP72 induction, we also evaluated triglyceride storage in muscle and liver. We observed that in the tibialis anterior, a primarily glycolytic muscle [420], triglyceride content was 20% lower in HT rats compared to ST ($P < 0.05$, **Figure 11C**). Hepatic triglyceride content was reduced to an even greater extent, by 50%, in HT, HFD-fed rats compared to ST rats ($P < 0.05$, **Figure 11F**).

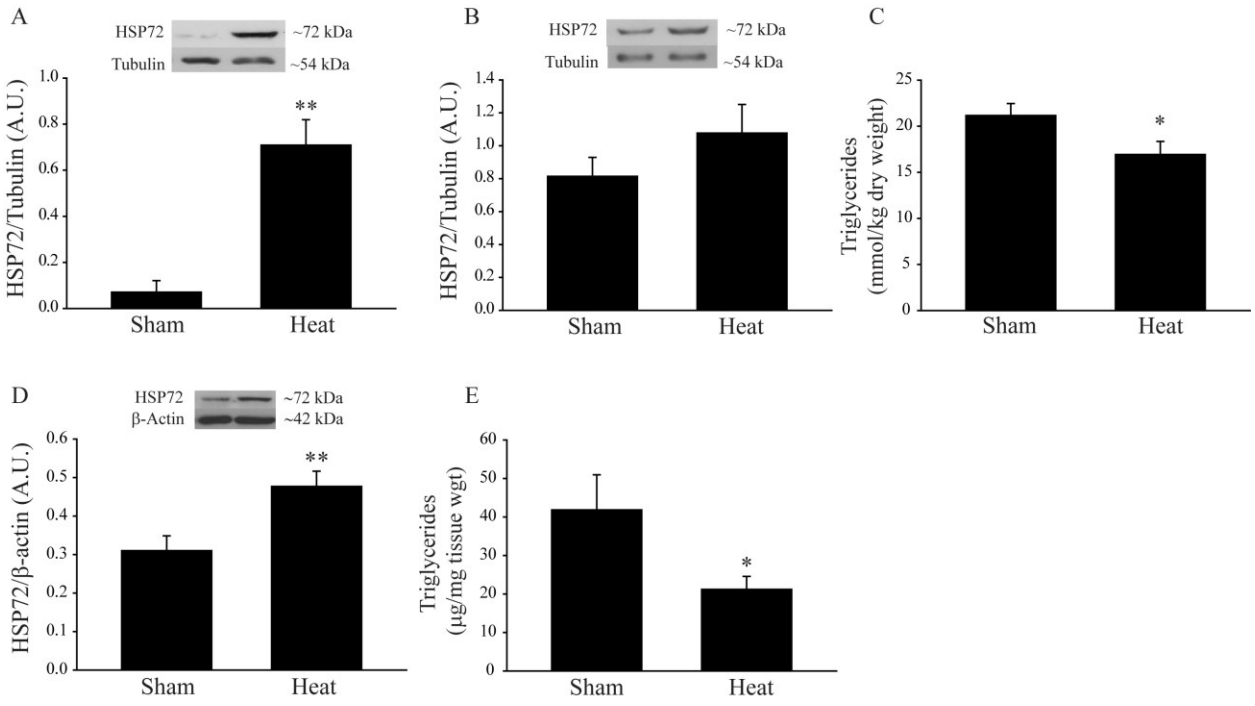

Figure 11. Heat treatment induces HSP72 and decreases triglyceride content in muscle and liver.
ST and HT rats were fed a HFD for 15 wk and received weekly *in vivo* ST (37˚C, 20 min) or HT (41˚C, 20 min) during the last 7 wk of the HFD. (A,B) HSP72 protein expression in the EDL and soleus, (C) triglyceride content in tibialis anterior, (D) hepatic HSP72 expression, (E) and hepatic triglyceride content were compared between ST and HT rats. * $P < 0.05$, ** $P < 0.01$ denotes significantly different between treatment groups determined by unpaired t-test. Values are mean ± SEM. N=8-9 animals/group.

Loss of HSP72 in primary hepatocytes disrupts mitochondrial integrity

Exposure to siRNA for HSP72 in primary hepatocytes resulted in a 58% knockdown of protein expression following HT ($P < 0.01$, **Figure 12A**). Control and HSP72 siRNA treated hepatocytes were examined by transmission electron microscopy to evaluate differences in mitochondrial morphology. Mitochondria treated with siHSP72 were larger and swollen compared to control siRNA treated cells (**Figure 12B**). Hepatocytes treated with control siRNA or siHSP72 were also stained with MitoTracker green, a dye taken up by both functional and nonfunctional mitochondria (**Figure 12C**, middle images in green labeled B and E) and tetramethylrhodamine ethyl ester, (TMRE) a poteniometric dye taken up only by functional mitochondria (**Figure 12C** images in red labeled A and D). We observed reduced TMRE staining in siHSP72 treated cells without observing changes in MitoTracker green staining (**Figure 12C**, images C and F). Lower TMRE staining demonstrates a decrease in functional mitochondria relative to total mitochondria in hepatocytes lacking HSP72.

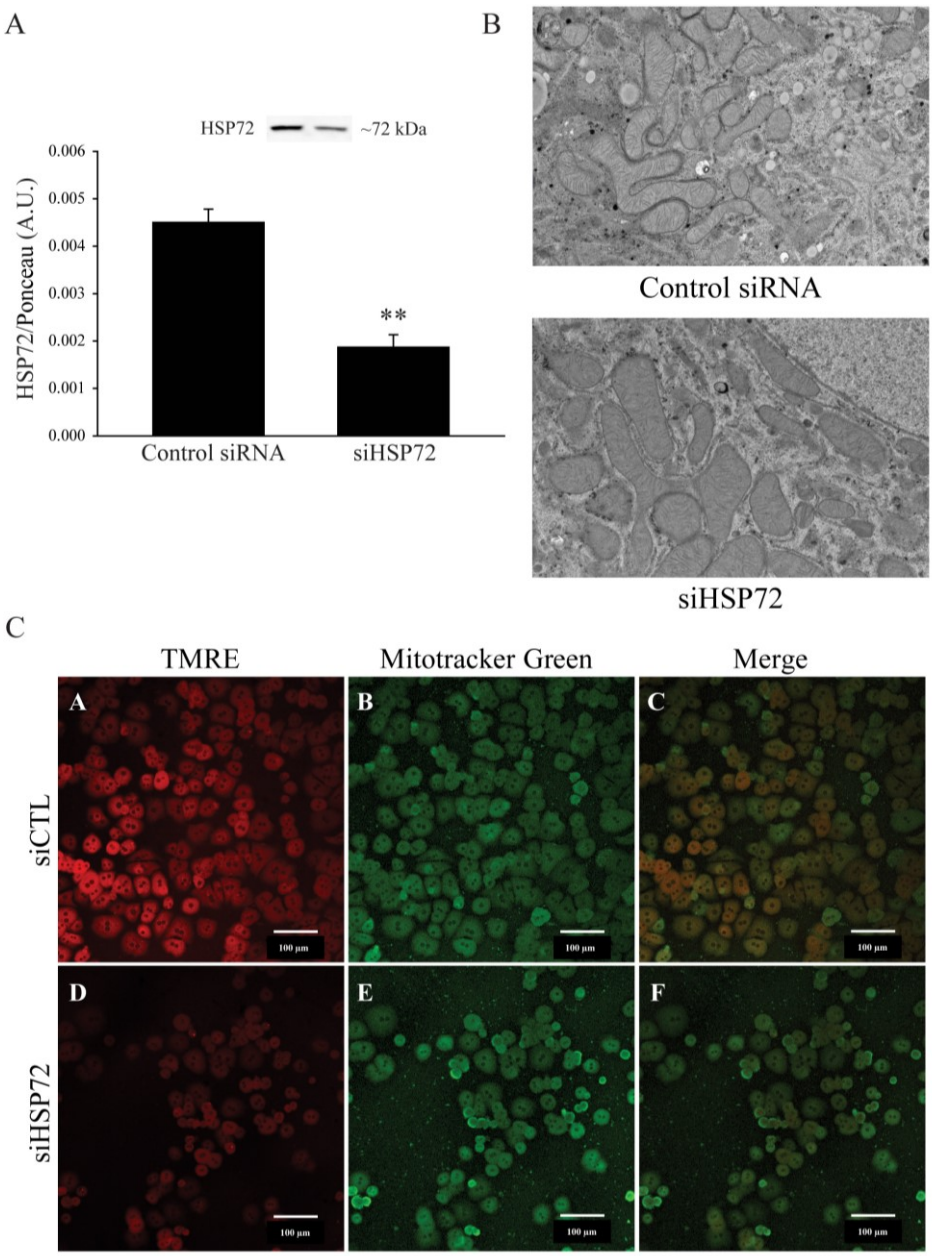

Figure 12. HSP72 knockdown in primary hepatocytes disrupts mitochondrial integrity. (A) Primary hepatocytes from C57/Bl6 mice were transfected with HSP72 siRNA or control siRNA and heat-treated 24 hours later. A 58% knockdown of HSP72 protein expression was observed 24 hours following HT. ***P* < 0.01, denotes significantly different between treatment groups determined by unpaired t-test. Values are mean ± SEM. N=3. (B) Primary hepatocytes in the presence or absence of HSP72 were imaged through Transmission Electron Microscopy. Representative images are shown. C) Primary hepatocytes treated with control siRNA or siHSP72 were stained with MitoTracker Green (middle images in green) to stain all mitochondria, and TMRE (left images in red) which stains only mitochondria with intact membrane potentials. The right images are merged MitoTracker/TMRE images. Representative images are shown.

Mitochondrial protein expression was then evaluated to determine if reductions to mitochondrial integrity were paralleled by reductions in mitochondrial protein expression. There was no significant difference in protein expression of PGC-1α or Electron Transport Chain (ETC) Complexes between control siRNA treated and HSP72 siRNA treated hepatocytes (**Figure 13, A, C**). However, cytochrome c protein expression increased by 20% with a loss of HSP72 ($P < 0.05$, **Figure 13B**). These data suggest that an acute loss of HSP72 does not reduce mitochondrial protein expression or biogenesis pathways.

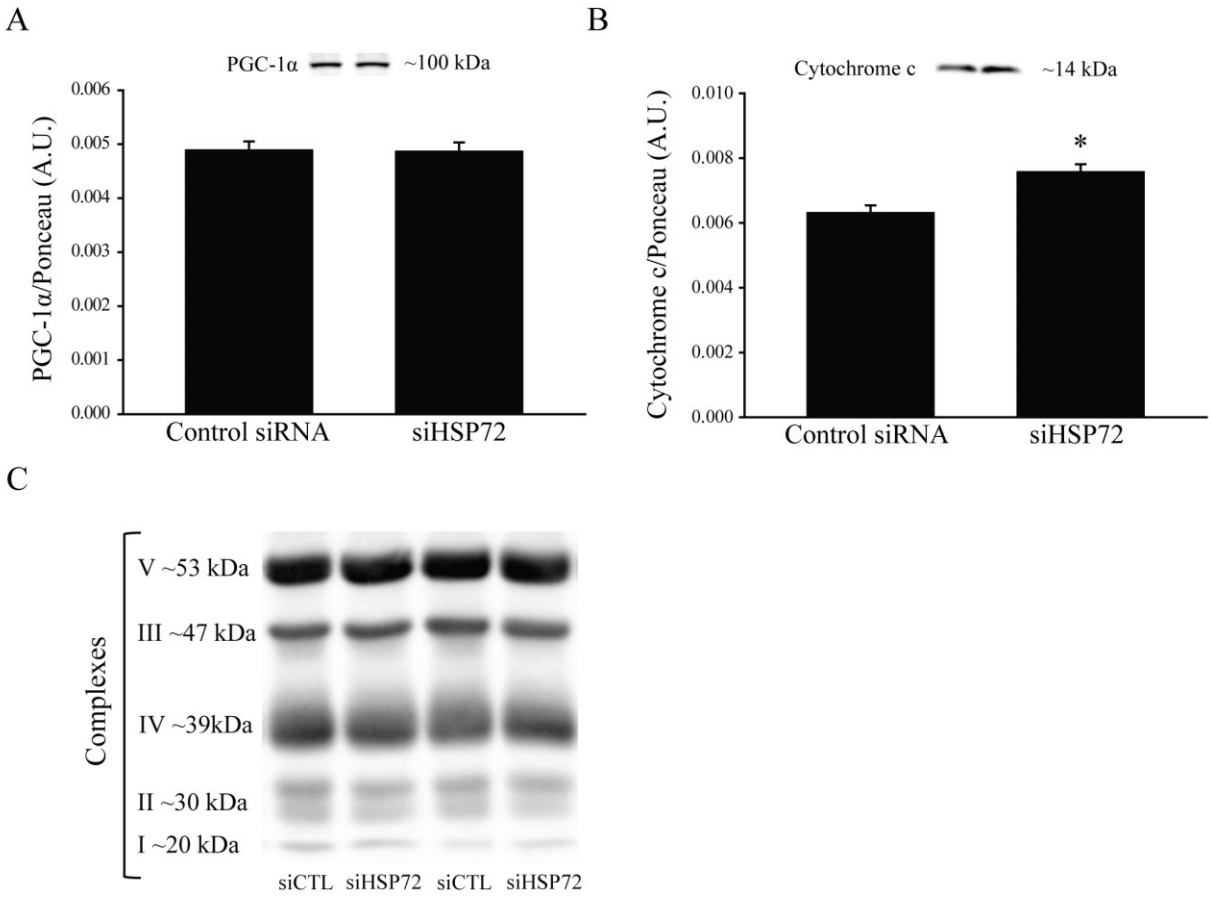

Figure 13. The effect of HSP72 reduction on mitochondrial protein expression.
Primary hepatocytes from C57/Bl6 mice were transfected with HSP72 siRNA or control siRNA. Protein expression levels of (A) PGC-1α and (B) Cytochrome c. * $P < 0.05$, denotes significantly different between treatment groups determined by unpaired t-test. Values are mean ± SEM. N=3-4. C. Representative blots of Complexes I-V.

A loss of HSP72 in primary hepatocytes reduces fatty acid oxidation

To determine if mitochondrial function was altered by an acute loss in HSP72 expression, fatty acid oxidation (FAO) of ^{14}C palmitate was assessed in control siRNA and siHSP72-treated primary hepatocytes. Incomplete FAO (**Figure 14A**), complete FAO through the TCA cycle to CO_2 (**Figure 14B**), and total FAO (incomplete + complete FAO to CO_2) (**Figure 14C**) were evaluated. A separate set of hepatocytes were also treated with etomoxir, an inhibitor of CPT-1, which allows for determination of differences between mitochondrial FAO and non-mitochondrial FAO mediated by other organelles.

Loss of HSP72 resulted in a 20% reduction of incomplete FAO ($P<0.001$, **Figure 14A**) and a 19% reduction in total FAO ($P < 0.01$, **Figure 14C**). Complete FAO to CO_2 was not different between groups (**Figure 14B**). Mitochondrial incomplete FAO (19%, $P<0.01$) and non-mitochondrial incomplete FAO (23%, $P < 0.01$) were significantly decreased with a loss of HSP72. Reductions in mitochondrial total FAO (16% $P < 0.05$) and non-mitochondrial total FAO (22%, $P < 0.01$) were also observed in siHSP72 treated hepatocytes. Additionally, siHSP72 treatment resulted in a 26% decrease in non-mitochondrial complete FAO ($P < 0.05$). No significant difference in mitochondrial complete FAO was observed with a reduction in HSP72. Collectively, these data suggest that a loss of HSP72 decreases total FAO in primary hepatocytes due to decreased mitochondrial and non-mitochondrial incomplete FAO.

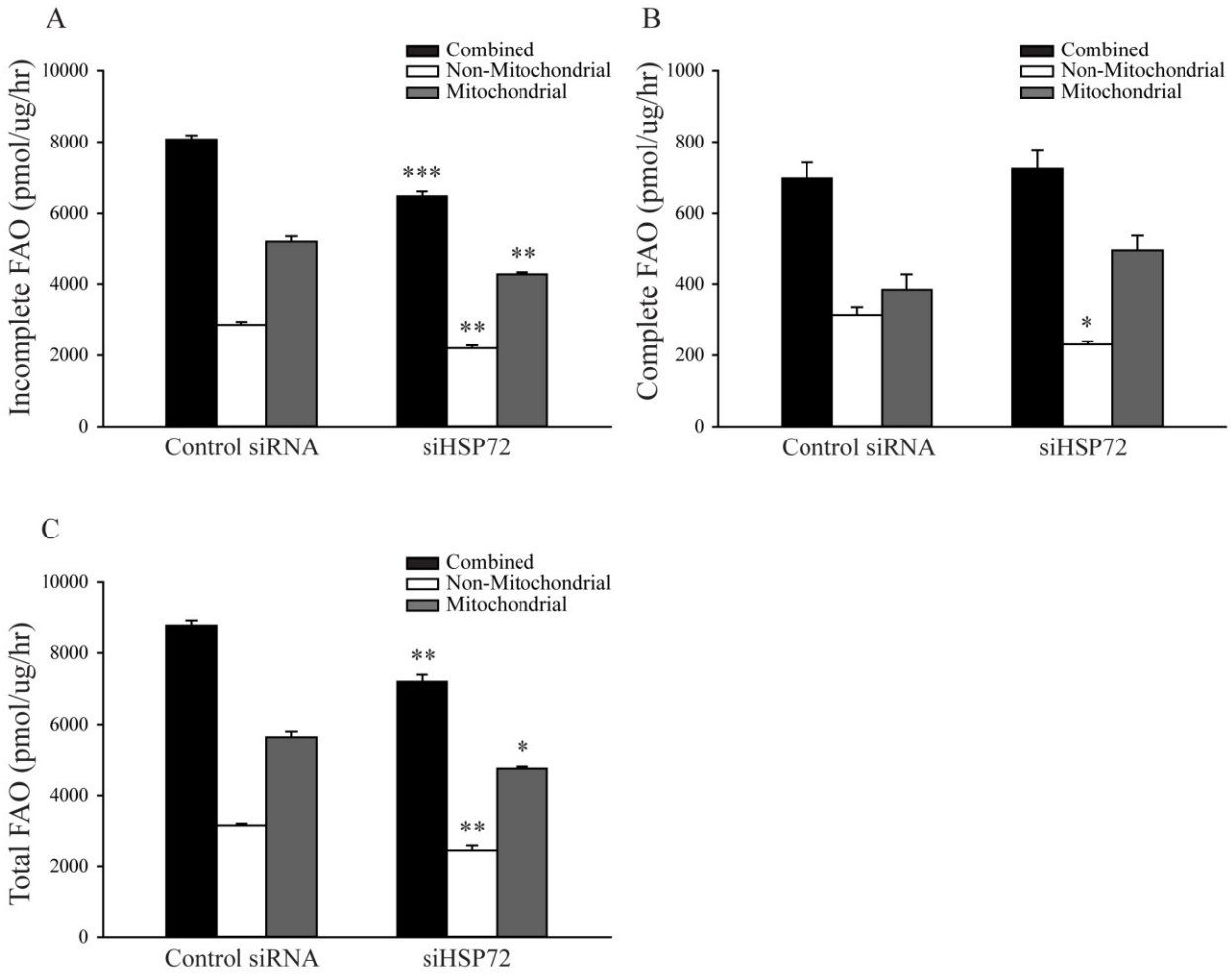

Figure 14. Knockdown of HSP72 alters primary hepatocyte fatty acid oxidation. ^{14}C-radiolabeled FAO of palmitate (100μM) in primary hepatocytes was evaluated in the presence and absence of the CPT-1 inhibitor etomoxir (100 μM) allowing for the evaluation of mitochondrial and non-mitochondrial mediated FAO separately and together (combined FAO). Incomplete FAO, complete FAO to CO_2, and total FAO were determined. A) Incomplete, combined FAO (black bars), non-mitochondrial incomplete FAO (white bars) and mitochondrial incomplete FAO (gray bars) in control siRNA and siHSP72 treated hepatocytes. B) Complete, combined FAO to CO_2 (black bars), non-mitochondrial complete FAO to CO_2 (white bars), and mitochondrial complete FAO (gray bars). C) Total FAO (determined by adding incomplete and complete FAO to CO_2) (black bars), non-mitochondrial total FAO, and mitochondrial total FAO (gray bars). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ denotes significantly different between treatment groups determined by unpaired t-test between control siRNA and siHSP72 groups. Values are mean ± SEM. N=3.

HSP72 expression in primary hepatocytes modulates lipid storage

The impact of altering HSP72 protein expression on lipid accumulation was evaluated in primary hepatocytes. Palmitate treatment increased triglyceride (TAG) 161% in control siRNA treated hepatocytes ($P < 0.001$, **Figure 15A and 15B**). When compared to control siRNA treated hepatocytes, siHSP72-treated hepatocytes had 77% greater lipid accumulation following 24 h of palmitate treatment (250 μM) ($P < 0.001$, **Figure 15A and 15B,** black bars). In addition, non-palmitate, siHSP72 treated hepatocytes demonstrated a 177% increase in lipid accumulation compared to control ($P<0.001$, **Figure 15B,** white bars).

A separate set of hepatocytes were exposed to ST (37°C) or HT (42°C) for 30 minutes in order to increase HSP72 protein expression. HT was performed after 24 h of palmitate treatment. Palmitate treatment increased hepatic TAG by 95% in ST hepatocytes ($P < 0.001$, **Figure 15C and 15D**). However, HT blunted lipid accumulation in primary hepatocytes. TAG accumulation was 29% lower in HT hepatocytes compared to ST hepatocytes following 24 h palmitate exposure ($P < 0.01$, **Figure 15C and 15D,** black bars).

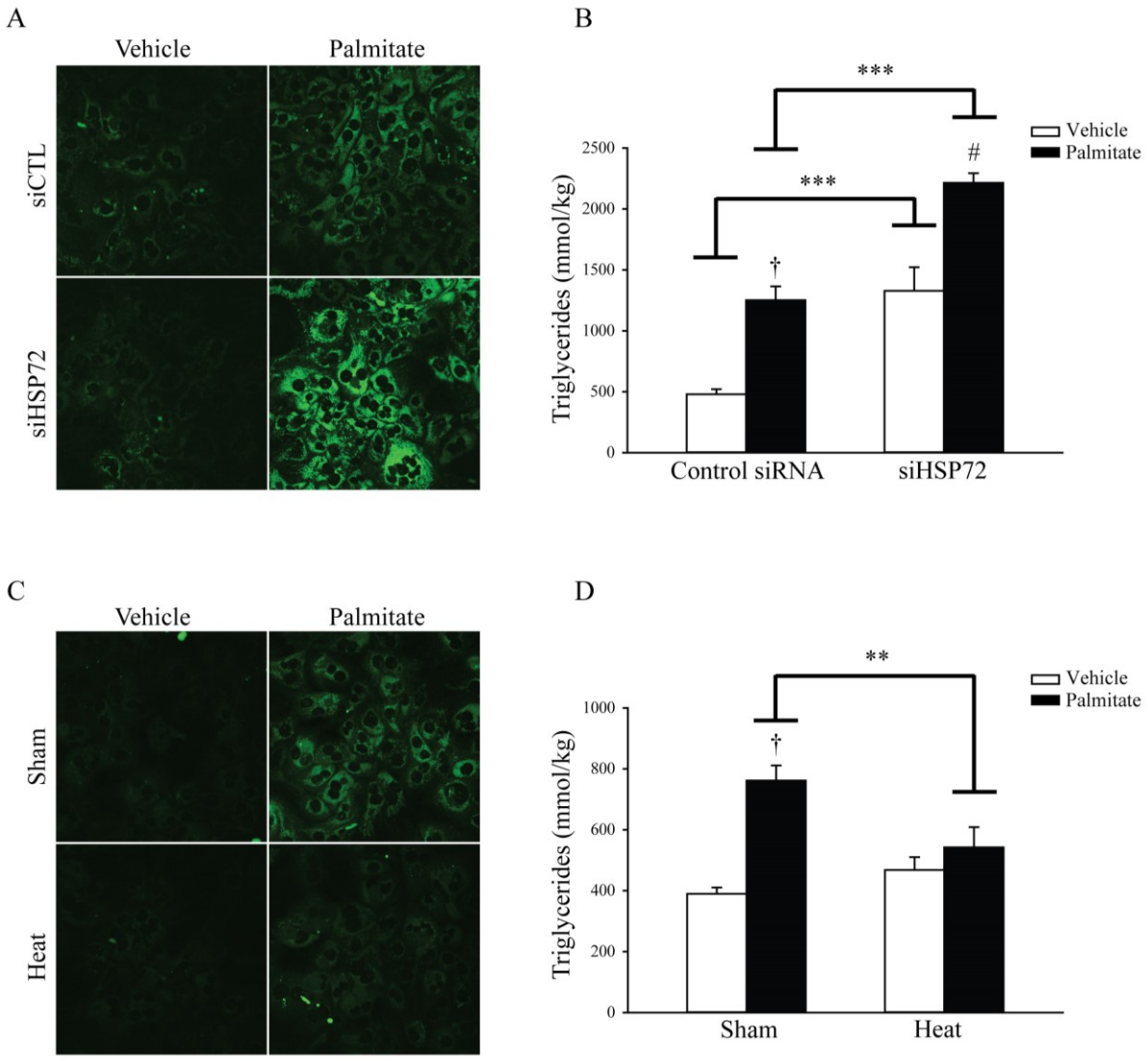

Figure 15. HSP72 modulates lipid storage in primary hepatocytes.
A) Primary hepatocytes from C57/Bl6 mice were first treated with control siRNA or siRNA for HSP72 for 24 h and then with 250 μM Palmitate or Vehicle for 24 h. Cells were then stained with Bodipy for 20 min prior to imaging at 40X to assess triacylglycerol content. B) Liver triacylglycerol (TAG) content was also determined biochemically through a colorimetric assay. C) In separate experiments, cells were treated with Palmitate (250μM) or Vehicle for 24 h, and then exposed to ST (37ºC) or HT (42ºC) for 30 min. Following these treatments, cells were stained with Bodipy and imaged. D) Biochemical liver TAG was also determined. ** $P < 0.01$ and *** $P < 0.001$ denotes significant differences between siRNA treatment groups. # P< 0.01 and † P< 0.001 denotes significant differences between vehicle and palmitate treatments. Significant differences were determined by two-way ANOVA with Fisher's post hoc analysis performed where appropriate. Values are mean ± SEM. N=6 wells.

3.5 Discussion

NAFLD is the liver component of metabolic disease and is highly prevalent [424]. Currently, mechanisms underlying this condition are not well understood. HSPs are known to play important roles in skeletal muscle and systemic metabolism, but their potential roles in liver metabolism are unclear. In this study, we investigated the effect of in vivo HT intervention on whole-body and liver-specific metabolic outcomes. We also investigated the effect of HSP72 modulation on mitochondrial integrity and lipid handling in primary hepatocytes. Through a combination of these in vivo and in vitro experiments, we demonstrated that in vivo HT induces hepatic HSP72 expression and reduces lipid storage in the liver. We also found that HSP72 is important in maintaining mitochondrial integrity and FAO, as well as preventing lipid storage in primary hepatocytes (**Figure 16**).

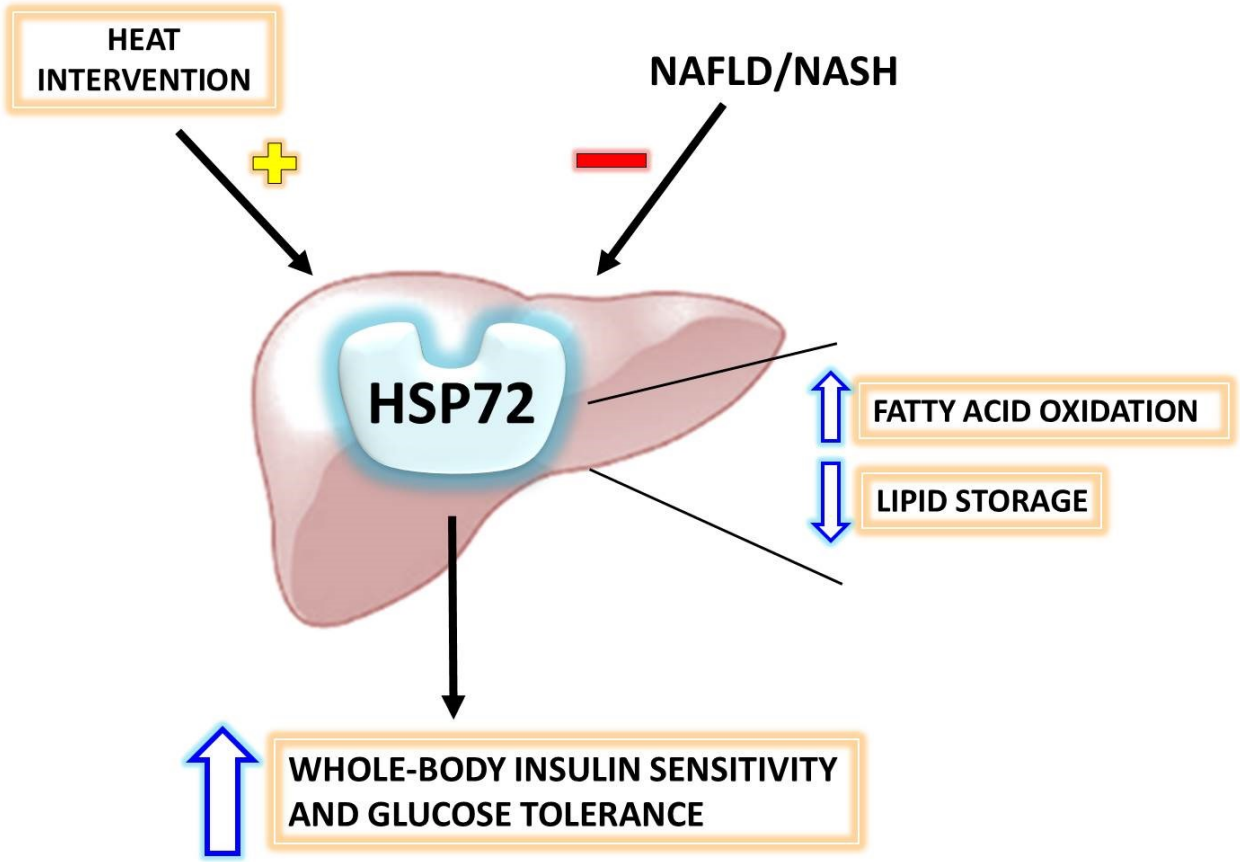

Figure 16. The role of hepatic HSP72 on lipid handling and whole-body metabolic homoeostasis.
HSP72 was found by Di Naso et al. to be reduced with the progression of NAFLD and NASH. Based on the results of this study, we propose that heat treatment intervention and other treatments that induce hepatic HSP72 can improve lipid handling in the liver through increasing fatty acid oxidation and reducing lipid storage. This can lead to improved whole-body insulin sensitivity and glucose tolerance.

In addition to identifying liver-specific outcomes in this study, these are the first findings to indicate the ability of HT to reverse the damaging metabolic effects of a prior HFD. Previously we showed that 12 wk of weekly *in vivo* HT prevented HFD induced whole-body and skeletal muscle insulin resistance [268]. In the present study, we expand upon these findings by showing that after 8 wk of high-fat feeding, weekly *in vivo* HT improved whole-body glucose tolerance and increased insulin sensitivity in glycolytic skeletal muscle. In addition, weight gain was blunted by weekly HT and energy efficiency was reduced, indicating that energy utilization had been altered. Adipocyte size was reduced by weekly HT in the eWAT, although fat pad mass was not significantly reduced. Our results agree with prior studies showing HT, transgenic overexpression, or GGA administration to induce HSP72 levels reduces WAT mass and adipocyte size [266, 268, 295]. Adipocytes are known to play a large role in whole-body glucose homeostasis [425], and smaller adipocytes are generally more insulin sensitive [426]. Like reduced triglyceride storage in the liver, we also observed reduced triglyceride storage in skeletal muscle with HT. Increased lipid storage in the skeletal muscle has been shown to generate secondary messengers (i.e., DAGs and ceramides) that inhibit insulin signaling [427, 428]. In the present study, we observed lower triglyceride content in glycolytic skeletal muscle of HT rats fed a HFD in association with increased insulin-stimulated glucose uptake.

As excess lipid accumulation in the liver is a hallmark of NAFLD, reductions in triglyceride storage observed here represent a potential treatment modality that has not been widely explored. Past work by our laboratory and others has identified increased hepatic HSP72 protein expression with heat and exercise [268, 365, 366, 429], however, these studies did not investigate the ability of HSP72 to affect liver steatosis. Adachi *et al.* reported that induction of HSP72 by administration of the compound GGA ameliorated hepatic insulin resistance and reduced JNK phosphorylation [295], but hepatic triglyceride content was not measured. Reports using mild electrical stimulus coupled with heat stress have shown reduced triglyceride storage, reduced inflammation, and increased insulin responsiveness in the liver of high-fat fed mice and

db/db mice [430, 431]. In these reports, HSP levels were not measured. Work from Di Naso et al. also supports that HSP72 activation may be important in the prevention of NAFLD, since they found that decreased HSFP72 levels correlated with NAFLD progression in humans [264]. Maybe one of the most promising studies linking HSP72 to liver metabolism is one by Zeng et al. which investigated pharmacological activation of HSP72 by the small molecule drug matrine. In mice, matrine increased hepatic HSP72 protein content and also reduced liver triglyceride storage and glucose intolerance [301]. In the current study, we observed that both HSP72 and HSP25 protein expression levels are induced in the liver by weekly HT in rats fed a HFD. Weekly HT also reduced hepatic triglyceride content. Our results expand on past research through identifying HT as a treatment modality to induce HSP72 and reduce triglyceride storage in the liver.

Based on our results from weekly in vivo HT, we then aimed to identify if HSP72 has a more direct role in the protection against lipid accumulation in hepatocytes. We found with a loss of HSP72, primary hepatocytes demonstrated a reduction in mitochondrial integrity as shown by reduced mitochondrial membrane potential and changes in morphology. We also found that mitochondrial total FAO of palmitate was reduced with a decrease in HSP72 expression. The most interesting results of this study are the robust effects of HSP72 modulation on lipid storage in primary hepatocytes. We found that with an acute loss of HSP72, lipid accumulation was increased following palmitate exposure, while HT effectively prevented lipid accumulation. The consistency in results from our in vivo HT experiments and in vitro studies are very promising to identify HSP72 induction as a strategy to prevent NAFLD.

Identifying the effects of HSP72 loss on mitochondrial integrity and FAO is important due to the strong mitochondrial component in the development of NAFLD [226, 227]. Similar to our observations with a loss of HSP72 in primary hepatocytes, mitochondrial morphology is altered and a loss of cristae and swelling of the mitochondria is observed with diabetes and NAFLD [202, 205, 253, 254]. Past research in rodent models also provides strong evidence that

FAO contributes to the progression of NAFLD. FAO in the liver is an essential part of hepatic lipid metabolism, and dysregulation of this process results in hepatic steatosis. Studies have shown that a reduction or genetic defect of hepatic β-oxidation enzymes leads to increased steatosis in the liver of rodents [198, 229], and an increase in β-oxidation enzymes reduces hepatic steatosis [199]. Additionally, Rector et al. also showed that in a hyperphagic rodent model a reduction of FAO occurs before the progression to hepatic steatosis or insulin resistance [230]. However, there isn't evidence that decreased FAO contributes to the development of NAFLD in humans. Actually, some studies have found increased FAO or mitochondrial oxidation gene expression in patients with NAFLD and NASH [194, 200, 201, 205, 432], although hepatic FAO in humans is difficult to accurately measure and often has small sample sizes [433]. Studies have found other mitochondrial deficits such as impaired ATP production and increased ROS with NAFLD and NASH [202-205]. It's possible that HSP72 regulation of FAO contributes to lipid storage, however, future research must identify the significance of this finding in the context of NAFLD or NASH development in humans.

Reduced hepatic mitochondrial FAO that we observed with a loss of HSP72 is consistent with past work investigating HSP72 and skeletal muscle mitochondrial function. Our laboratory and others have shown that HT improves skeletal muscle mitochondrial function by improving FAO [268], increasing mitochondrial enzyme activity [268, 314, 315], and increasing mitochondrial biogenesis [316]. Modulation of HSP72 levels through overexpression or knockout also regulates skeletal muscle mitochondrial function. Whole body and skeletal muscle HSP72 overexpression increases mitochondrial enzyme activity in rodents [266, 269]. Skeletal muscle specific overexpression of HSP72 increases skeletal muscle FAO [269] and reduces skeletal muscle lipid storage. This was consistent with a global knockout of HSP72 which demonstrated reduced skeletal muscle FAO, β-oxidation enzyme activity, and increased lipid storage [317]. Similar to our findings in primary hepatocytes, mitochondria were enlarged and dysmorphic in skeletal muscle of HSP72 knockout animals. Drew et al. also found that that the mitochondrial

degradation pathway, mitophagy, was impaired and contributed to the accumulation of damaged mitochondria in skeletal muscle of HSP72 knockout animals [317]. It is probable that impaired mitophagy occurs with a loss of HSP72 in the liver, and future studies will need to investigate this interaction.

Fatty acids are mainly oxidized in the mitochondria, through the subsequent reactions of β-oxidation. Acetyl-CoA is produced which then may enter the TCA cycle and be completely oxidized to CO2, which is called complete FAO [173]. Incomplete oxidation of fatty acids can also occur and form acid-soluble metabolites such as ketone bodies, acyl-CoAs, and acylcarnitines. In this study, we observed reduced total mitochondrial FAO with a loss of HSP72 in primary hepatocytes which was driven by reductions in incomplete hepatic FAO. About two-thirds of the fat that comes to the liver is converted into ketones [215], therefore the majority of FAO is directed to ketogenesis from incomplete FAO [211]. Cotter et al. showed that ketogenesis can be a mechanism of lipid disposal, even in non-fasted conditions, and dysfunction in this pathway promotes the development of NAFLD into NASH [209]. With the reductions we observed in incomplete FAO with a loss of HSP72, further research is needed in order to identify if HSP72 could be involved in regulation of ketogenic pathways and if that is connected to steatosis. Another aspect of FAO we evaluated was non-mitochondrial FAO. FAO occurs in the mitochondria and also partially in peroxisomes and microsomes [171, 194, 195]. With a loss of HSP72, we observed reduced complete and incomplete non-mitochondrial FAO, which implies a decrease in FAO in other organelles. It's possible that HSP72 can regulate both mitochondrial and non-mitochondrial FAO processes, and future research should address this possible role of HSP72.

In this study we also evaluated PGC-1α, cytochrome c, and mitochondrial complexes and found no difference in protein expression of PGC-1α and complex proteins with a loss of HSP72 in primary hepatocytes, and a slight increase in cytochrome c protein content. The increase in cytochrome c may be a reflection of compensation by the mitochondria. Past work in

skeletal muscle of a whole-body HSP72 knockout animals did not evaluate PGC-1α, but they found no difference in cytochrome c expression or protein levels of the electron transport chain proteins in skeletal muscle [317]. They also found no difference in citrate synthase activity or mtDNA between WT and knockout animals in skeletal muscle, which are both markers of mitochondrial content. However, Henstridge et al. found with whole-body overexpression of HSP72, mitochondrial number was increased by 50% in skeletal muscle, without any observed changes in in PGC-1α expression. Although in this current study we did not evaluate mitochondrial number, based on similar or slight increase in mitochondrial protein content between control siRNA and siHSP72, it is likely that mitochondrial content is not affected by a loss of HSP72 in primary hepatocytes. This would be consistent with observations from skeletal muscle of whole-body HSP72 knockout animals [317]. It is also possible that other proteins or post-translational modifications not evaluated in this study, such as PGC-1α acetylation, could be affected by a loss of HSP72. These outcomes will be important to evaluate in future studies.

It is possible that HSP72 is not only important in steatosis, but also in the advancement of NAFLD. In about 20% of individuals, NAFLD eventually develops into a more severe liver disease called non-alcoholic steatohepatitis (NASH). NASH includes inflammation and cellular damage that can lead to fibrosis and cirrhosis [424]. Molecular metabolic defects occur in stages as NAFLD and NASH progress, and these changes continue to be investigated. For example, Sanyal et al. found the presence of various mitochondrial defects and insulin resistance with both NAFLD and NASH patients, but only observed mitochondrial structural defects in NASH patients [205]. Studies have also found that ketogenesis is increased in early hepatic steatosis [207-212], but eventually is reduced with advancement into NASH [209, 210, 212]. Additionally, Di Naso et al. found that decreased HSP72 protein content and HSF1 content, the main transcription factor for HSP72, correlated with the advancement of NAFLD and NASH. It is possible that a loss of HSP72 induction contributes to the transition of NAFLD into NASH. Hepatic HSP72 protein content could be a potential marker of NAFLD stage,

although this would be difficult to measure in vivo. Heat shock proteins can also be released extracellularly into circulation [338, 361-364, 434]. As suggested by Di Naso et al. and others, future work needs to identify if extracellular HSP72 in circulation could serve as a marker of hepatic metabolic disease stage [264, 285, 435, 436].

In this study, we identified that HSP72 is important in lipid handling in hepatocytes specifically by modifying FAO and lipid storage. One limitation of this study is the fact that we did not investigate more aspects of lipid handling such as lipid secretion as VLDL, which is an important mechanism for the liver to export lipid out of hepatocytes [190]. Our findings strongly support that HSP72 has a direct role in hepatocytes. However, HSP72 protein in Kupffer cells could also be important in the development of NAFLD and NASH. Di Naso et al. suggested that impaired HSP72 in Kupffer cells could increase liver inflammation as liver disease progresses [264]. This is important since pro-inflammatory activity of Kupffer cells seems to be a part of NAFLD advancement into NASH, fibrosis and cirrhosis [437-439]. Interestingly, heat-induced upregulation of HSP72 in Kupffer cells coincides with suppression of TNFα [302, 303]. Although outside the scope of this work, it is important to recognize the dual role HSP72 could have in hepatocytes and Kupffer cells to prevent NAFLD development. Our work utilized in vivo and in vitro HT and siRNA treatment in primary hepatocytes to address our research questions. To further identify the impact of hepatic HSP72 mechanisms in whole-body metabolic homeostasis, the development of a liver-specific HSP72 knockout model will be necessary in future investigation of these mechanisms.

Conclusion

In this study, we identified that HT improves glucose tolerance and skeletal muscle insulin action in HFD-fed rodents. Weekly HT also specifically improves the metabolic profile of the liver by inducing HSP72 and reducing lipid storage. To our knowledge, this is the first study to directly modulate hepatic HSP72 levels and identify the importance of HSP72 in FAO and

prevention of steatosis. Thus, treatments that induce the expression of HSP72 should be explored as treatment options for other metabolic conditions, including NAFLD.

CHAPTER 4

Heat Shock Protein 72 Activation in the Liver with Exercise

4.1 Abstract

Heat shock proteins (HSPs) are molecular chaperones that aid in the recovery from stress and guard cells from future insults. HSP72, in particular, plays an important role in maintaining skeletal muscle insulin sensitivity and glucose homeostasis and is induced in skeletal muscle with heat or exercise. Less is known about HSP72 induction with exercise in the liver. The liver also goes through numerous metabolic adaptations following exercise, including possible activation of the degradation pathways autophagy and mitophagy. The purpose of this study was to examine the ability of various chronic and acute exercise modalities to induce HSP72 in the liver as well as autophagy and mitophagy pathways. Male Sprague Dawley rats were divided into sedentary (SED), voluntary wheel running (VWR), treadmill endurance (TM-END) or treadmill interval sprint training (TM-IST) groups for four weeks. We found that with endurance treadmill training, HSP72 protein content was robustly increased in the liver. Interval sprint training also increased HSP72 in the liver, while there was not a significant increase in HSP72 with voluntary wheel running. In response to an acute bout of exercise, increased HSP72 and reduced inhibitory phosphorylation of transcription factor HSF-1 were observed. Increased hepatic HSP72 in response to chronic and acute exercise were paralleled by increased autophagy (LC3-II). An increase in phosphorylation of a mitophagy protein, Parkin, was also observed with acute exercise. Our findings demonstrate that HSP72 expression in the liver is increased with both chronic and acute exercise in a time and intensity dependent manner. Additionally, mitophagy and autophagy protein changes could be a part of HSP72 action with exercise. Among the many benefits of exercise, increased hepatic HSP72 expression with exercise could play an important role in improving hepatic metabolic function.

Key words: heat shock proteins, exercise, autophagy, mitophagy, liver metabolism

4.2 Introduction

Heat shock proteins (HSPs) are molecular chaperones that aid in the recovery from stress and guard cells from future insults [263, 415]. HSP72 is the highly inducible heat shock protein that can be activated in skeletal muscle with stressors such as heat and exercise [263, 366, 440-442]. Additionally, Kurucz et al found that HSP72 is decreased in skeletal muscle of insulin resistant and type 2 diabetic patients [284]. Recent work by Di Naso et al. found that HSP72 could also be important in liver metabolism, due to decreased expression of HSP72 with non-alcoholic fatty liver disease progression [264].

Our lab and others have demonstrated that increased HSP72, for example through heat treatment, transgenic overexpression, and pharmacological treatment, improves glucose tolerance, skeletal muscle insulin resistance, and mitochondrial function [266-269, 295, 297, 416]. Mice lacking skeletal muscle HSP72 have enlarged, dysmorphic mitochondria with reduced muscle respiratory capacity and lipid accumulation in the skeletal muscle [317]. These mice also demonstrate a reduced ability to degrade mitochondria through mitophagy. This is important since degradation pathways such as mitophagy and autophagy are important in the maintenance of lipid homeostasis and mitochondrial function [257, 259, 260]. This evidence suggests that HSP72 could be a possible target for therapies to improve insulin sensitivity and hepatic mitochondrial function. Although much has been revealed in the importance of heat shock protein activation to improve metabolic function in skeletal muscle, there is a lack of knowledge in the role of heat shock proteins in the liver following exercise. Some studies have found increased HSP induction in the liver with exercise or heat treatment [268, 365, 366, 429], although the role of HSPs in the liver has not been investigated.

The liver is essential for whole-body metabolic function metabolism and exercise tolerance through maintaining whole-body glucose homeostasis. The liver goes through its own exercise-induced set of acute and chronic adaptations such as improved fat handling [220]. This adaptation seems to be due to increased markers of mitochondrial function and content which

improve lipid metabolism [132, 133, 443-445]. Recently, a publication by Fletcher et al. compared the effect of various exercise modalities on hepatic mitochondrial metabolism [135]. In this study, mice performed voluntary wheel running, endurance treadmill running, or interval sprint training. Regardless of the training modality, exercise significantly improved hepatic mitochondrial respiration and also improved other markers of mitochondrial metabolism which were not always dependent on increased mitochondrial content [135]. These adaptations in the liver are important in the maintenance of hepatic metabolic dysfunction, although the role of HSPs in this process are not clear.

The purpose of this investigation is to continue the work by Fletcher et al and evaluate hepatic HSP72 induction across these multiple exercise modalities and with chronic and acute exercise. Additionally, we also aim to identify changes in autophagy/mitophagy protein expression with various exercise modalities. Past studies have shown that degradation pathways such as autophagy increase with exercise [404, 446-450]. We hypothesize that HSP72 will be increased with various exercise modalities, and that we will also observe increased expression of autophagy and mitophagy proteins. HSP72 may be essential in the liver to increase mitochondrial function and activate degradation pathways to dispose of damaged organelles such as mitochondria.

4.3 Methods

Experimental Animals – Chronic Exercise.. Twelve-week old Sprague Dawley male rats (n=46) were split into into sedentary (SED), voluntary wheel running (VWR), treadmill endurance training (TM-END), or treadmill interval sprint training (TM-IST) groups (n=8-10 per group). The exercise intervention lasted 4 weeks. Rodents were individually housed in temperature controlled rooms (21°C) with 12:12 light:dark cycles. Rats were allowed *ab libitum* access to food and water as well as a standard rodent chow (Formulab 5008; Purina Mills, Brentwood, MO). Animals were sacrificed following 24 hours after the last bout of exercise or 24

hours after locking the wheels of the VWR animals. Animals from all groups were also fasted starting 18 hours prior to exercise. At the end of the experiment (16 weeks old) the rats were anesthetized with pentobarbital sodium (100 mg/kg) and the heart was removed. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia [135].

Experimental Animals – Acute Exercise. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Twelve male Sprague Dawley rats were purchased from Charles River Laboratories and were randomly assigned (n=2-5 per group) into fed sedentary (SED), fed acute treadmill exercise (EX-FED), or fasted acute treadmill exercise (EX-FASTED). All animals were housed in a temperature controlled facility (22 ± 2˚C) with 12:12 hr light:dark cycles. Animals were allowed *ad libitum* access to water and a standard chow diet (9604; Harlan Teklad, Madison WI, USA). Two hours post-exercise, rats were anesthetized with pentobarbital sodium (100 mg/kg) and then exsanguinated by removal of the heart.

Chronic exercise protocol. Rats were randomly assigned into four groups of SED, VWR, TM-END, or TM-IST rats. VWR animals had running wheels available in their cages. Wheel revolutions of the wheels were counted throughout the intervention period using VitalView software (VitalView, Version 4.2, 2007; Mini Mitter Company, Inc., Bend, OR). Animals in the treadmill groups (TM-END or TM-IST) went through an acclimation period for five days 15 m/min, 5-10 min/day). During the first 1.5 weeks of treadmill exercise, the animals gradually worked up to a higher speed. The TM-END group of rats began at 20 m/min for 10 minutes (12% gradient) and progressively increased to 30m/min for 60 minutes (12% gradient). They maintained this speed for 5 days/week during the entire four weeks. Similarly, the TM-IST group began with 6 short, intense bouts of exercise at 35 m/min for 1 minute (12 % gradient, 4.5 minutes rest in between exercise bouts). They slowly increased to six 50m/min sprints for 2.5

minutes (12% gradient, 4.5 minutes rest in between exercise bouts). Treadmill groups performed exercise in the morning.

Acute exercise protocol. Rodents were separated into three groups of SED, EX-FED, or EX-FASTED (~515 g). Animals were acclimatized for four days on the treadmilll prior to exhaustive exercise bout. Acclimatization was for ~5 min at 0 m/min on day one, 15 m/min on day two, 15 m/min one day three, and 20 m/min on day four. EX-FASTED animals were fasted for 12 hours prior to exhaustive exercise bout. The exhaustive exercise bout began for 5 min at 20 m/min and increased to 25 m/min until exhaustion (~45-50 min). The exercise was stopped when rodents stayed on the shock grid three times for 5 seconds. SED animals were placed on locked treadmill throughout the same amount of time as exercised animals during acclimatization and during the exhaustive exercise bout.

Western blot. Liver was processed for Western blotting by methods previously described [268, 297, 386]. Briefly, liver was homogenized in a 12:1 (volume-to-weight) ratio of ice-cold cell extraction buffer (Biosource, Invitrogen) containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, and phenylmethylsulfonyl fluoride; 2 mM Na3VO4; 20 mM Na4P2O7; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate; and 250 µl/5 ml protease inhibitor cocktail. Homogenates were rotated for 30 min at 4˚C, and then centrifuged for 20 min at 3,000 rpm at 4˚C. The supernatant was removed and protein concentration determined by Bradford assay. Samples were diluted in HES buffer and Laemmli buffer containing 100 mM dithiothreitol (DDT) (Thermo Scientific, Rockford, IL, USA) based on protein concentration to generate samples containing equal concentration of protein. Samples were heated in a boiling water bath for 5 min.

Protein (40-80 µg) was separated on SDS-PAGE gels, followed by a wet transfer to a nitrocellulose membrane for 90 min at 200 mA. Membranes were blocked in Tris-buffered saline (TBS), 0.1% Tween 20 (TBST), and 5% nonfat dry milk or 5% bovine serum albumin (BSA) followed by incubation with the appropriate primary antibodies. Following three washes with

TBST, blots were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in TBST 1% nonfat dry milk or BSA at a concentration of 1:10,000 for 1 hr at room temperature. Blots were then washed twice with TBST and once with TBS, dried, and visualized by enhanced chemiluminescence (ECL). Bands were quantified using Image J or Image Lab (Bio-Rad) densitometry. Blots were then stripped for 15-20 min at 55˚C in buffer containing 62.5 mM Tris·HCl, 2% SDS, and 100 mM 2-mercaptoethanol and re-probed for β-actin as a loading control.

Primary antibodies used included HSP72 (cat no. SPA-810, Enzo Life Sciences, Farmingdale, NY), HSP25 (cat no. SPA-801, Enzo Life Sciences), HSP60 (cat no. SPA-807, Enzo Life Sciences), LC3B (cat no. 2775, Cell Signaling Technology, Inc., Danvers, MA), p62 (cat no. 5114, Cell Signaling), pParkin (phospho s65) (cat no. ab154995, Abcam, Cambridge, MA), Parkin (cat no. sc-32282, Santa Cruz Biotechnology, Dallas, Texas), HSF-1 (cat no. NB300-730, Novus Biologicals, Littleton, CO), and pHSF-1 serine 303 (cat no. ab47369, Abcam) and β-actin HRP-conjugated (cat no. ab20272, Abcam). Secondary antibodies used included goat anti-mouse (cat no. 170-5047, BioRad, Hercules, CA) donkey anti-rabbit (cat no. 711-035-15, Jackson, Immuno-Research, Inc., West Grove, PA), and goat anti-rabbit (cat no. sc-2004; Santa Cruz Biotechnology).

4.4 Results

Rodent characteristics and mitochondrial function

As describe in the published work by Fletcher et al. [135], following four weeks of exercise, TM-END and TM-IST groups had reduced body weight compared to the SED and VWR groups. All exercise groups had significantly reduced body fat compared to the SED group. Additionally, both SED and VWR animals had increased feeding efficiency compared to the other exercise groups. Fletcher et al. also measured mitochondrial function through evaluating mitochondrial respiration. All exercised animals regardless of exercise modality had

increased oxidative phosphorylation as demonstrated by increased flux through complex I and also increased maximal uncoupled mitochondrial respiration (above data no shown) [135].

Chronic exercise and hepatic HSP72 induction

The rodents performed four weeks of voluntary wheel running (VWR), treadmill endurance exercise (TM-END), or treadmill interval sprint training (TM-IST). These groups were compared to a sedentary (SED) group of animals (**Figure 17**).

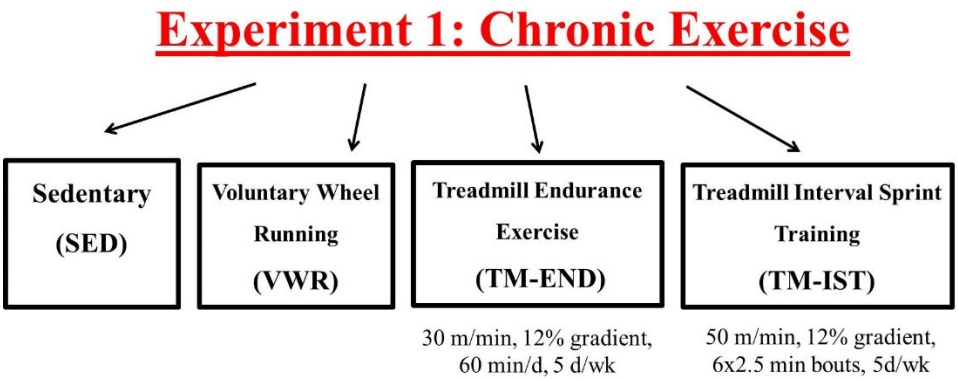

Figure 17. Various exercise modalities in rodents.
Rats were split into four groups: Sedentary (SED), Voluntary Wheel Running (VWR), Treadmill Endurance Exercise (TM-END), and Treadmill Interval Sprint Training (TM-IST). Interventions were performed for four weeks.

We compared hepatic induction of HSP72 between these various types of chronic exercise (**Figure 18**). Following four weeks of exercise, there was no difference in HSP72 protein induction between SED and VWR rats, however we did see a significant increase in HSP72 induction in the treadmill exercise groups. Four weeks of TM-IST resulted in an 8-fold increase in HSP72 protein expression ($P < 0.001$). TM-END had a robust effect on HSP72 compared to the other groups. There was a 37-fold increase in liver HSP72 protein expression with four weeks of endurance training (TM-END) ($P < 0.001$). This was a ~350% greater increase in HSP72 induction with the TM-END group compared to TM-IST group ($P < 0.001$).

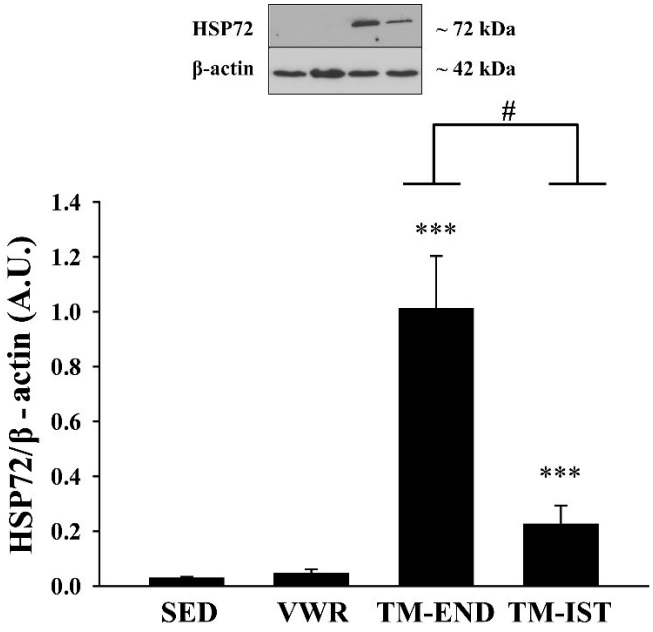

Figure 18. Hepatic HSP72 induction with various exercise modalities.
Effects of various exercise modalities (VWR, TM-IST, and TM-END) on HSP72 induction in the liver. n=6-9 per group. Analyzed by one-way ANOVA.***P < 0.001 compared to SED animals. # P < 0.001 between TM-END and TM-IST groups.

Heat shock and autophagy protein induction with endurance chronic exercise

Due to the drastic increase in HSP72 protein expression in the liver with chronic endurance exercise (**Figure 19A**), we also evaluated expression other two other heat shock proteins, HSP25 and HSP60. HSP25 was increased by 32-fold ($P < 0.001$, **Figure 19B**), and HSP60 increased by 27% with endurance exercise ($P < 0.05$, **Figure 19C**). Due to past work linking exercise and autophagy [404, 446-450] and also possibly HSPs and autophagy/mitophagy [317], we also evaluated autophagy protein expression with endurance chronic exercise. We found a 56% increase in the expression of microtubule-associated protein 1 light chain 3 (LC3-II) with endurance exercise ($P < 0.05$, **Figure 19D**). LC3 is lipidated and becomes LC3-II when autophagosomes are forming [388], thus is a maker of increased autophagy. The protein p62 is a selective cargo adaptor that is important for sequestration of organelles for autophagy. This protein is degraded with increased autophagy. We observed no difference in p62 protein expression with endurance exercise (**Figure 19E**).

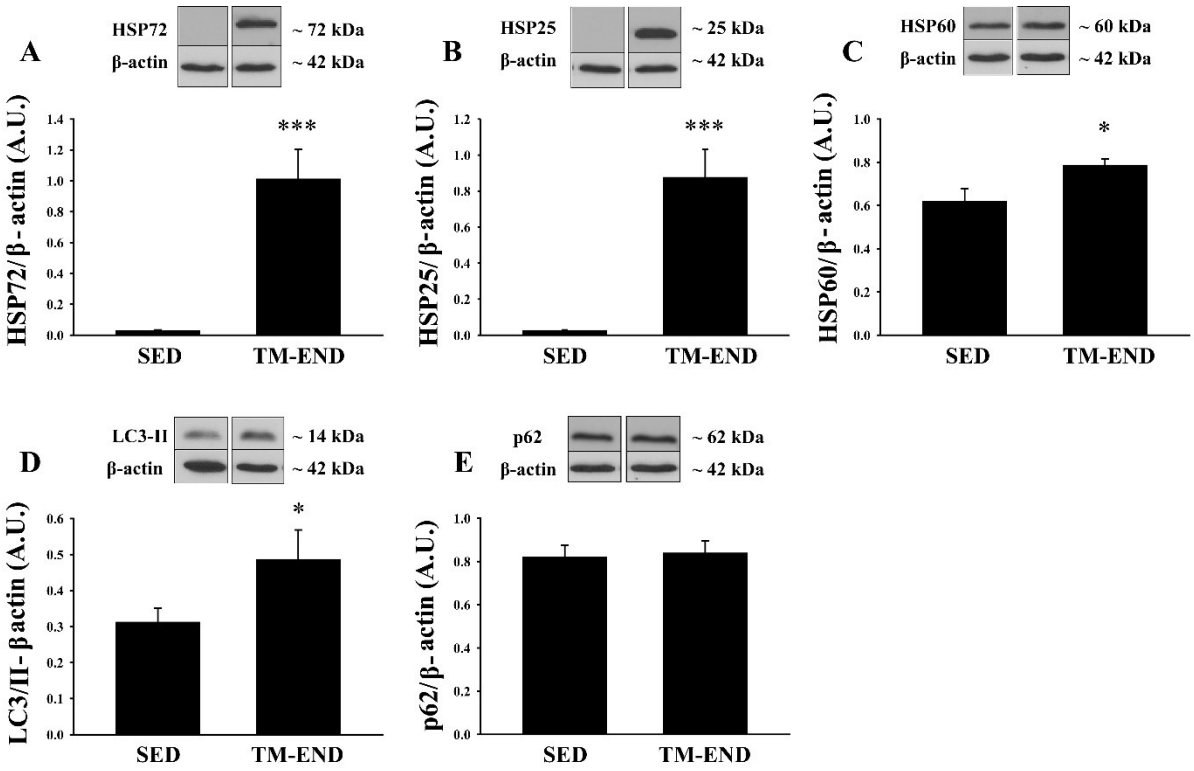

Figure 19. Endurance exercise induces the heat shock response and alters autophagy protein expression in the liver.
Following four weeks of endurance exercise training A) HSP72, B) HSP25 and C) HSP60 were evaluated. Autophagy protein expression of D) LC3-II and E) p62. n=6-10 per group. All were analyzed by unpaired t-test. *P<0.05, ***P < 0.001.

Acute exercise increases HSP72 induction and mitophagy protein expression

Based on the differences we observed in hepatic HSP72 induction with chronic endurance treadmill exercise, we were interested in the timing of HSP72 induction with treadmill exercise. We performed acute treadmill exercise and collected tissues 2 hours following exercise to identify if HSP72 was induced rapidly following an exercise bout. In addition to investigating acute exercise, we also added a fasted, exercised group to our study (**Figure 20**). Fasting has been shown to induce HSPs [451, 452] and also increase autophagy [453, 454]. We hypothesized that fasting in addition to exercise would increase HSP72 and autophagy protein expression more than exercise alone. Two groups of Sprague Dawley rodents were exercised and fed (EX-FED) or exercised and fasted (EX-FASTED). The animals performed acute treadmill exercise to exhaustion. We also had a third sedentary, fed group (SED) that were placed on a locked treadmill during the same amount of time as the exercised animals. We observed a 34-fold increase in HSP72 induction in the exercised, fed group ($P < 0.05$) and a 50-fold increase in HSP72 induction in the exercised, fasted group ($P < 0.01$, **Figure 21A**). There was a 48% greater increase in the exercise fasted group compared to exercise alone.

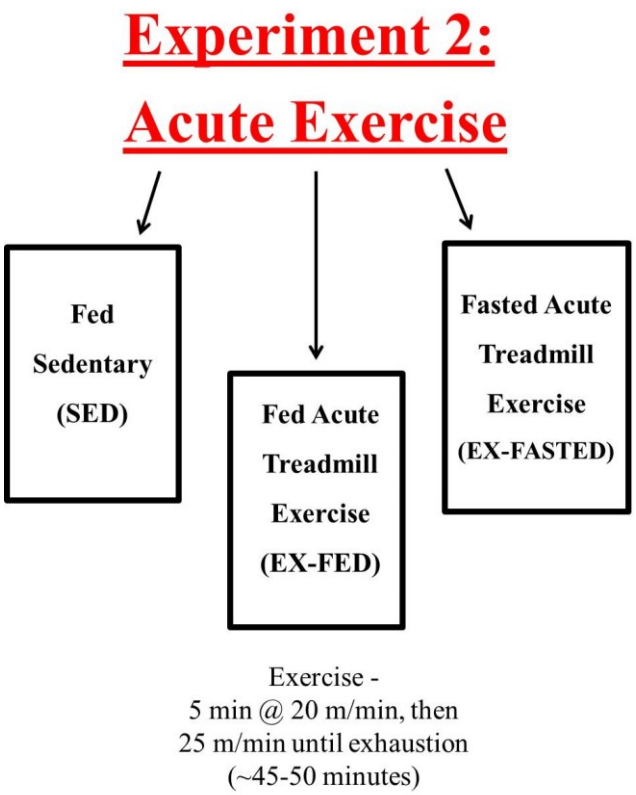

Figure 20. Acute exercise in rodents.
Rats were either sedentary (SED) or performed acute treadmill exercise. The exercised animals were either fed (EX-FED) or fasted (EX-FASTED). Rats were fasted or fed for 12 hours and then underwent one bout of treadmill exercise until exhaustion. Tissues were collected post-exercise following a 2-hour recovery.

We were interested in the mechanism of how acute exercise was increasing HSP72 in the liver. We evaluated changes in protein expression of the transcription factor for HSP72, Heat Shock Factor-1 (HSF-1). HSF-1 has various phosphorylation sites including serine 303 which is an inhibitory phosphorylation site on HSF-1. We observed a 68% reduction (P=0.09) in HSF1 phosphorylation on serine 303 in the exercise, fasted group (**Figure 21B**), indicating a reduction in inhibitory phosphorylation of HSF-1.

Lastly, we evaluated changes in protein expression of Parkin, the E3 ubiquitin ligase which targets mitochondria for degradation through mitophagy. Parkin is recruited to the mitochondria and activated by Pink1, which phosphorylates Parkin at serine 65 (Ser 65). Therefore, we evaluated Parkin phosphorylation at serine 65 as a way to look at Parkin activation with exercise. We found a slight increase in Parkin phosphorylation ($P = 0.07$) in the exercised, fed group and a 58% increase in the exercise, fasted group ($P < 0.01$, **Figure 21C**).

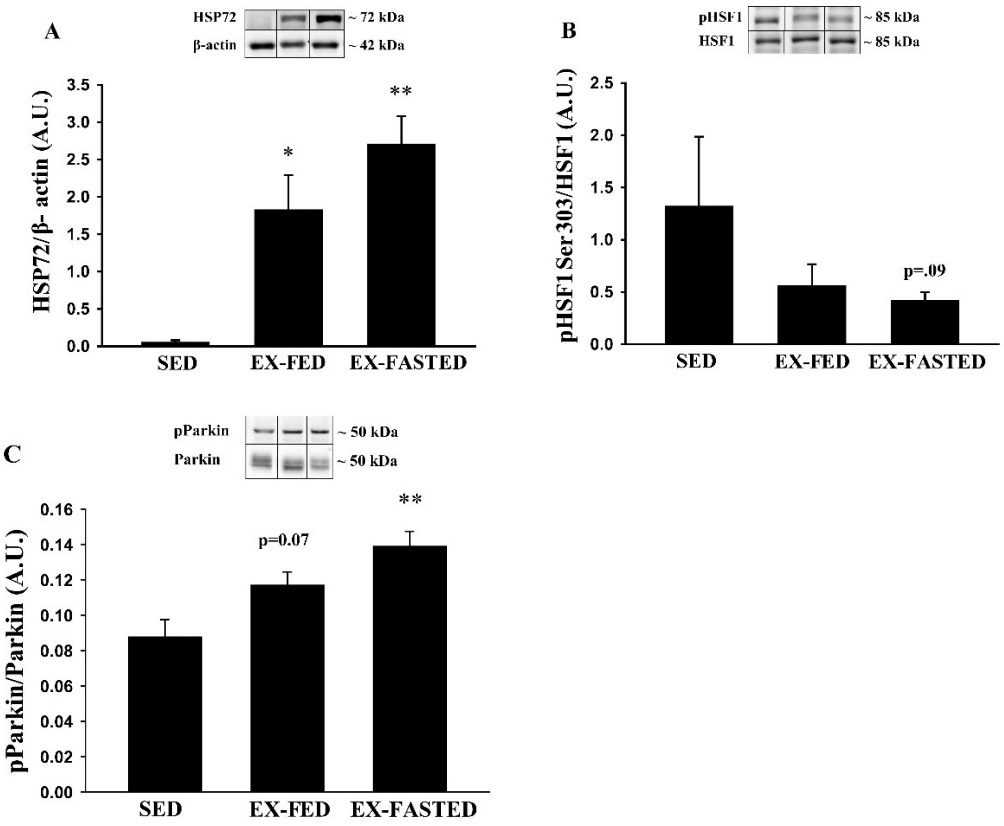

Figure 21. Fasting combined with acute treadmill exercise increases HSP72 induction and phosphorylation of mitophagy-associated ubiquitin ligase, Parkin in the liver.
In rodents that performed acute exercise (fed or fasted) we evaluated A) HSP72, B) pHSF1 Ser 303, and C) pParkin Ser 65 protein expression. All were analyzed by one-way ANOVA. n=2-5. *P<0.05 **P < 0.01, ***P < 0.001 when compared to SED.

4.5 Discussion

The liver is a major metabolic organ which maintains whole-body glucose homeostasis. This metabolic organ goes through various acute and chronic adaptations to exercise which are important in the prevention of metabolic disease. Currently, differences in the hepatic metabolic response to various exercise modalities are not well understood. Identification of the variability in metabolic responses to exercise, including the heat shock response, will facilitate the development of exercise protocols as therapy.

In this study, we evaluated heat shock protein induction in the liver with chronic and acute exercise. Additionally, with acute exercise, we investigated the combination of exercise and fasting. With chronic exercise, rodents performed various exercise modalities to compare differences in heat shock protein induction. New findings from the present study indicate that the HSP72 protein expression is increased with acute and chronic treadmill endurance exercise. HSP72 was also induced to a lesser degree with chronic interval sprint training, but not voluntary wheel running. With acute treadmill exercise, fasting and exercise resulted in a further increase in HSP72 induction compared to exercise alone. Additionally, a reduction of inhibitory phosphorylation on HSP72's major transcription factor, HSF1, was also observed with exercise and fasting combined.

The mechanism behind HSP72 activation requires activation and translocation of the transcription factor, heat shock factor 1 (HSF1). When HSF1 is activated it moves to the nucleus and binds to heat shock elements which allows for transcription of HSP72. Elevated levels of both HSP90 and HSP70 act as a feedback mechanism and negatively regulate HSF1. Once there is a stressor such as exercise, HSPs dissociate from HSF1 which is then free to translocate to the nucleus and bind to HSEs which allows for increased transcription of HSP genes [455]. The HSP response is rapid and powerful, but also acts through feedback inhibition, as eventually increased HSPs re-bind to HSF1 which inhibits transcription. HSF1 is also regulated by multiple post translational modifications. Constitutive phosphorylation of serines

303 and 307 have an important role in the negative regulation of HSF1 transcriptional activity [456], while phosphorylation sites serines 230 and 326 contribute to activation of HSF1 in times of stress [457, 458]. Once heat shock occurs, phosphorylation of HSF1 soon follows, while acetylation is more delayed and coincides with the feedback inhibition of HSF1. Increased expression and activity of SIRT1 which deacetylates HSF1 increases the binding of HSF1 to DNA [458]. These intricate mechanisms allow for regulation of duration and intensity of the heat shock response. Studies have not investigated these mechanisms in relationship to HSP72 activation with exercise, especially in the liver. This study has identified the reduction of inhibitory phosphorylation at serine 303 that occurs following acute exercise, further specifying the mechanism of HSP72 activation in the liver.

Multiple types of exercise can increase the heat shock response, and the majority of past research has focused on skeletal muscle. Heat shock protein increases can be detected 24 hours or up to a week after an exhaustive bought of exercise in skeletal muscle, and chronic endurance or resistance training protocols have also been shown to increase HSP72 in skeletal muscle [336, 340-343, 459-463]. Many of these effects are intensity-dependent. HSP72 induction is increased with exercise intensity with aerobic and resistance exercise [338, 340, 342, 343]. It was also found that treadmill endurance training results in an increase in HSP72 and HSF1 expression in the liver [429]. Additionally, acute exercise has also been shown to increase HSP72 in the liver, with multiple waves of HSP72 induction within 48 hours of exercise [366]. These studies identified the importance of heat shock proteins as a part of the complex molecular and metabolic adaptations following exercise. However, this is the first study to compare various exercise modalities along with HSP72 induction in the liver. In this study, the 37-fold increase in HSP72 induction observed with chronic treadmill exercise and the 34-50-fold increase (fasted and fed) in HSP72 induction observed with acute treadmill exercise is equal to or much greater than induction that has been observed in skeletal muscle [429]. These results

suggest that robust HSP72 induction could have a major role in post-exercise hepatic adaptation.

HSP72 is the highly inducible heat shock protein and has been identified to be important in metabolic function, however we also observed induction of other heat shock proteins (HSP25 and HSP60) with chronic endurance exercise. HSP25 works as a molecular chaperone and protects against oxidative and thermal stress [464-466]. This protein also seems to be important for skeletal muscle to recover from exercise [333, 463, 467, 468]. Additionally, HSP60 is important for mitochondrial function through aiding in correct folding of proteins into the mitochondria [440, 469, 470]. HSP60 has also been shown to be induced by heat and exercise [440, 471, 472]. Future work would need to delineate the importance of these individual proteins in relation to HSP72 in the liver.

In addition to studying the heat shock response following acute and chronic exercise, we also identified changes in autophagy and mitophagy protein expression with exercise. Autophagy involves a complex set of mechanisms which leads to the targeted degradation of organelles. Organelles are essentially tagged and marked for degradation, and are attached to the forming autophagosomes. LC3 lipidation which then forms LC3-II is a part of this process [388]. Additionally, p62, a polyubiquitin-binding protein, aids in the tethering of organelles to the autophagosome. Autophagosomes then fuse with the lysosomes resulting in the degradation of p62 and targeted organelles. Studies have shown that increased LC3-II and reduced p62 protein expression indicate the presence of increased autophagy [53, 403]. LC3-II is also increased with increased mitophagy [53], the targeted degradation of mitochondria.

In response to four weeks of chronic endurance treadmill training we observed an increase in LC3-II accumulation with no change in p62. This suggests a possible increase in autophagosome formation following four weeks of chronic treadmill exercise. Autophagy may be an important mechanism in the prevention of excess hepatic steatosis, as autophagy can be important for the degradation of lipids, called lipophagy [473]. Additionally, autophagy has been

demonstrated to be essential for glucose homeostasis [262]. Due to its effect on LC3-II accumulation, exercise may be a potential treatment modality which could treat liver steatosis. With acute and chronic treadmill exercise we also evaluated changes in mitophagy protein expression. Parkin is a major regulator of mitophagy through being an E3 ubiquitin ligase and marking mitochondria for degradation. Parkin is regulated by the protein Pink1 through the post-translational modification. Pink1 finds dysfunctional mitochondria that have lost membrane potential and recruits and activates Parkin through phosphorylation of serine 65 [53]. With chronic exercise, we observed no difference in Parkin or phosphorylation of Parkin (data not shown). However, with acute exercise we observed an increase in phosphorylation of Parkin. This pattern was very similar to our observations of HSP72 with acute exercise, with a slight increase of each protein with the fed, exercised group and a further increase in the fasted, exercise group.

This is the first study to both identify the response of HSP72 and autophagy and mitophagy proteins with exercise. Additionally, to our knowledge, this is the only study to identify these changes with exercise and the combination of fasting and exercise. This is important since HSP72 has been linked to autophagy and mitophagy processes in past work [269, 317]. Overexpression of HSP72 in skeletal muscle was shown to increase autophagy in response to fasting [269]. Additionally, Drew et al showed that HSP72 can bind to Parkin, aid in Pink1 binding and increase mitochondrial degradation through mitophagy. Mice with a loss of HSP72 have Parkin that is non-functional. This is demonstrated through increased accumulation of damaged mitochondria. Therefore, HSP72 activation with exercise, and possibly through a combination of exercise and fasting, may activate autophagy and mitophagy pathways and maintain a group of higher-functioning mitochondria in the liver.

It is important to connect the results from this study to past results from Fletcher et al. [135]. Their group found that all types of chronic exercise (VWR, TM-END, and TM-IST) increased mitochondrial respiration and also individually demonstrated their own unique

improvements in mitochondrial function. It is interesting that in our study, we found robust increases in LC3-II and also HSP72 mainly in the TM-END group, and not in all exercise groups as they observed with mitochondrial respiration. We also observed increased HSP72 and phosphorylation of the mitophagy protein, Parkin, with acute treadmill endurance exercise. These results could be important when prescribing exercise to patients with non-alcoholic fatty liver disease or other metabolic conditions that already have mitochondrial damage and impairments [202, 205, 226, 227, 253, 254]. It may not be the best approach initially to perform types of exercise that increase respiration through damaged, ROS producing mitochondria. Initially, maybe the best treatment approach for this population would be a type of exercise that would activate the degradation pathways autophagy and mitophagy in order to first degrade unhealthy mitochondria and other organelles. This study would suggest that endurance treadmill exercise may be the best exercise modality to activate both HSP72 and degradation pathways acutely and chronically.

There are a few limitations to this study that limit study conclusions. Future studies will be needed to further identify the effects of acute and chronic exercise on hepatic autophagy and mitophagy protein expression. Specifically, the usage of a lysosomal inhibitor such as leupeptin would be needed to fully identify an increase in autophagy with exercise. This compound allows the identification of true autophagy flux. In future studies, the use of leupeptin will be necessary to recognize that autophagy or mitophagy is increased with both chronic and acute exercise. The addition of fasting to exercise in this study is a novel approach, especially since studies have not identified the effect of this combination on HSP72 or autophagy protein expression. However, the lack of a fasting sedentary group limits the experiment's conclusions. It is unknown if exercise and fasting were additive like suggested above, or if fasting alone would increase HSP72 expression and phosphorylation of Parkin in the liver to maximum levels. Future work will need the addition of a fasting, sedentary group to answer this question.

In addition to HSP72, past work has found a link between reduced HSF1 protein content to the development non-alcoholic fatty liver disease [264]. HSF1 has also been linked to regulation of mitochondrial biogenesis and enzyme function [324]. It would be necessary to identify if it is the HSP72 activity, or activity of its upstream regulator, HSF1, that has the primary effect on liver metabolism. The development of a liver-specific HSP72 knockout model would aid in answering this question. Future experiments using an HSP72 liver-specific knockout along with leupeptin during acute and chronic exercise would identify if this protein is essential in post-exercise metabolic modifications, specifically with autophagy and mitophagy.

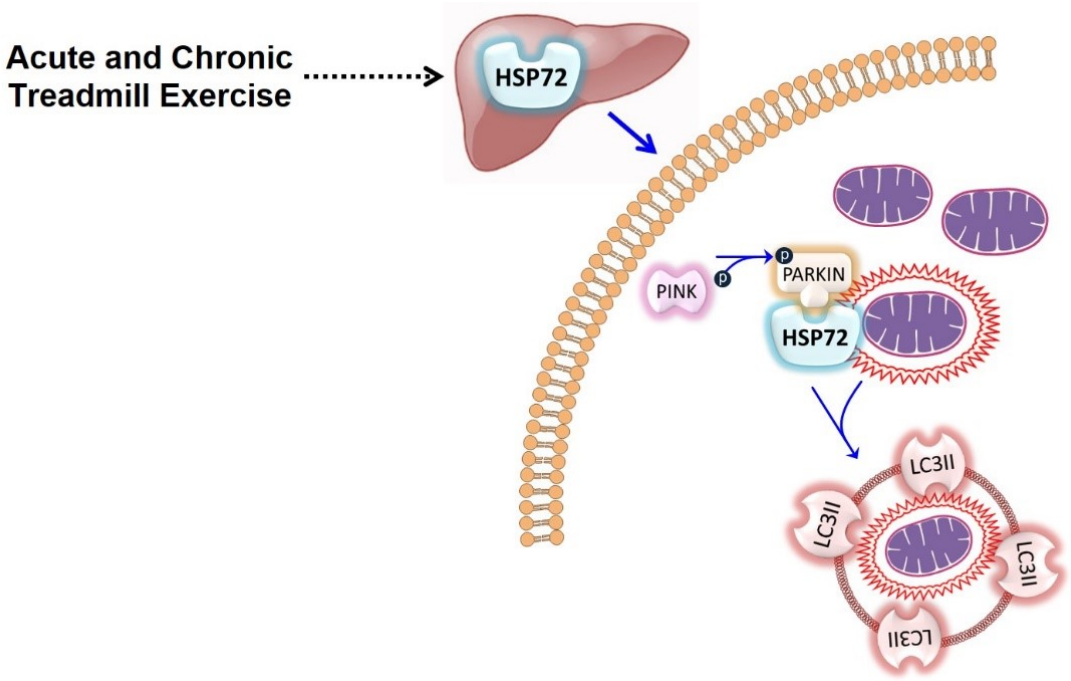

Figure 22. HSP72 induction in the liver and activation of mitophagy.
Summary of the proposed mechanism of HSP72 action following induction in the liver with exercise.

Conclusion

The activation of HSPs has been proposed as a potential target in preventing and treating metabolic dysfunction [318]. Based on our data, exercise could be an appropriate approach to activate HSPs in the liver and improve hepatic metabolism through autophagy and mitophagy (**Figure 22**). Acute and chronic exercise may improve hepatic mitochondrial quality through inducing HSP72. This approach could be specifically effective as treatment for non-alcoholic fatty liver disease and hepatic insulin resistance.

CHAPTER 5

Discussion and Future Directions

Past work has solidified that HSP72 induction is essential in skeletal muscle and whole-body metabolism. Our recent work has identified that HSP72 is also important for lipid metabolism in skeletal muscle and liver in two different populations with metabolic dysfunction: in a postmenopausal model with metabolic syndrome and also in NAFLD. We have also identified that HSP72 can be induced in the liver with exercise, and may be linked to changes in autophagy/mitophagy. These experiments further solidify the importance of HSP72 in lipid metabolism and mitochondrial health in various metabolic tissues.

5.1 HSPs, JNK, and Fatty Acid Oxidation

Metabolic disorders, including metabolic syndrome in postmenopausal women and NAFLD, lead to increased inflammation throughout the body which exacerbates insulin resistance [163, 165, 167]. Pro-inflammatory stress kinases such as PKC, IKKβ, and JNK are active in insulin resistent rodents [163, 164]. This occurs in various tissues including skeletal muscle, adipose, and liver [165, 167, 474]. One source of JNK activation is increased lipid and glucose exposure that occurs with metabolic dysfunction [475, 476]. Our laboratory and others have demonstrated an inverse relationship between HSP72 induction through heat or pharmacological activation and JNK activation in the skeletal muscle and liver [267, 268, 295, 297]. Di Naso et al. also observed an inverse relationship between HSP72 and JNK activation with NAFLD and NASH progression [264]. This is important since JNK can lead to dysregulation of lipid handling and mitochondrial function, thus inhibiting mitochondrial respiration and increasing ROS [163, 166, 167, 169, 170]. Prevention of JNK activation may be an approach to prevent metabolic disease not only through maintaining insulin signaling but also through increasing fatty acid utilization. This is especially important since it has been shown very recently in rodents that pharmacological inhibition of JNK relieved NAFLD [477]. Both JNK and HSPs have been shown to inhibit each other [286, 287, 298-300]. Activation of HSPs through

exercise, heat or pharmacological interventions may be a necessary strategy to prevent pro-inflammatory pathway activation.

Past work and our current studies have shown that HSPs increase mitochondrial function in the skeletal muscle [269, 317] and in the liver. Specifically, in our study, we observed reduced fatty acid oxidation with a loss of HSP72 in primary hepatocytes. The mechanism through which HSP72 can protect mitochondrial function, specifically in the liver, is still not well understood. We propose that mitochondrial function could be partially maintained through HSP induction and prevention of JNK activation. We believe this mechanism occurs in the liver, however skeletal muscle mechanisms could be similar.

The contribution and mechanism of FAO impairment in NAFLD development continues to be unclear. Past research has identified that JNK may contribute to reduced fatty acid oxidation in liver and skeletal muscle. Specifically, knockdown of JNK in hepatocyte culture and in vivo was shown to reduce lipid synthesis, increase fatty acid oxidation, and improve insulin sensitivity [478, 479]. This mechanism seems to be present in skeletal muscle as well, since inhibition of JNK activation increases fatty acid oxidation in muscle [480].

A more recent study by Vernia et al. demonstrated that JNK regulates fatty acid oxidation through PPARα [289]. PPARα is a transcription factor that is highly expressed in metabolic tissues including the liver and regulates many aspects of fatty acid metabolism, ketogenesis, as well as fibroblast growth factor 21 (FGF21) gene expression [481-487]. FGF21 is a hepatokine and myokine which improves systemic metabolic function through many peripheral tissues. Importantly, it regulates fatty acid oxidation and ketogenesis gene expression in hepatocytes [484]. Whole-body as well as liver-specific deletion of PPARα in mice results in increased steatosis, impaired fatty acid metabolism and ketogenesis, as well as a reduction in expression of FGF21 in the liver [488-493]. Increased lipid storage, oxidative stress and lipid peroxidation that occur with hepatic steatohepatitis can be avoided through PPARα activation [490, 494]. PPARα activity allows the liver to adjust to increased influx of lipids in response to

high lipid exposure, and is a possible pharmacological target to treat liver disease. Therefore, PPARα-mediated activation of FGF21 may be essential in hepatic lipid handling and prevention of steatosis [484].

PPARα is regulated by many signaling pathways, including pro-inflammatory molecules. Vernia et al showed that JNK inhibits hepatic fatty acid oxidation through the PPARα and FGF21 pathway [289]. They found that JNK activity is necessary to maintain expression of the PPARα transcriptional corepressors NCOR1 and NRIP1. These corepressors act by binding to PPARα and reducing transcription, thus reducing expression of FGF21, fatty acid oxidation and ketogenesis genes [289]. This group also demonstrated that JNK deficiency in the liver of HFD-fed mice results in increased FGF21 expression and improved whole-body metabolism [289]. These improved metabolic outcomes were dependent on FGF21 expression. This pathway may be important in other tissues besides the liver, since FGF21 has also been shown to be a powerful myokine affecting whole body metabolism [495, 496], and JNK is also increased in the muscle with metabolic syndrome and diabetes [282]. Inhibition of PPARα -related pathways by JNK could contribute to a reduction of fatty acid oxidation during the progression of liver disease and skeletal muscle metabolic dysfunction.

A close co-regulatory relationship between HSPs and JNK has been established in skeletal muscle, [165, 167, 267, 268, 286, 287, 295, 297-300] and studies are beginning to corroborate this relationship in the liver [264, 295]. In future studies, it will be necessary to recognize 1) is JNK activation a major contributor to increased steatosis in NAFLD through inhibition of the PPARα/FGF21 pathway, and 2) can HSP induction prevent JNK-mediated inhibition of fatty acid oxidation and protect from the development of steatosis. Our findings demonstrate in primary hepatocytes that following the loss of HSP72, there is a reduction in fatty acid oxidation and increased lipid accumulation. It is possible that with a loss of HSP72, JNK activation increases and inhibits PPARα/FGF21 pathway, reduces fatty acid oxidation and

increases lipid storage. Further research is needed to determine these HSP72-mediated mechanisms are present in hepatocytes and can protect against the development of NAFLD.

5.2 Autophagy and HSP72

Autophagy is an important cellular degradation process which degrades organelles and maintains cellular function. Defects in autophagy have been implicated in the development of metabolic syndrome and glucose insensitivity in skeletal muscle [52, 262]. Additionally, autophagy degradation of lipids (lipophagy) has also been shown to be important for liver homeostasis [259]. Autophagy is also impaired in livers of obese rodents which leads to ER stress and insulin resistance [260]. However, another study found that autophagy deficiency in skeletal muscle can lead to FGF21 induction and overall protection from insulin resistance [495]. Therefore, the role of autophagy is unclear, and the mechanisms that lead to autophagy dysfunction in metabolic disease are unknown.

In our studies, we found changes in autophagy gene expression in skeletal muscle and liver in two different models. We found that ERα$^{-/-}$ rats develop insulin resistance and skeletal muscle metabolic dysfunction, similar to what is observed in postmenopausal women. We also observed a reduction LC3-II protein content in skeletal muscle, which suggests a reduction in autophagosome accumulation in the insulin-resistant muscle. This reduction in autophagy may contribute to cellular organelle dysfunction and skeletal muscle insulin resistance. Additionally, in a separate study, we investigated expression of autophagy proteins in the liver following chronic exercise. Four weeks of chronic endurance treadmill exercise increased LC3-II protein content in the liver and suggests that chronic exercise increased autophagosome accumulation. This is in agreement with other studies in skeletal muscle which have shown that exercise increases autophagy [497].

In both studies, when we saw changes in autophagy protein content, we also observed a similar pattern of change in HSP72 content. It is logical that heat shock proteins and autophagy

would work together to maintain proteostasis and cellular health. In order to reach proteostasis there has to be a balance of protein folding and protein degradation [498]. Heat shock proteins are important in maintaining protein function through correct protein folding, while autophagy degrades dysfunctional proteins. It is possible that HSP72 could play a role in autophagic regulation in both skeletal muscle and liver.

Heat stress induces heat shock proteins, and an early study showed that heat exposure in primary hepatocytes induced heat shock proteins, but reduced autophagy protein levels [499]. However, a separate study showed that heat exposure actually increases autophagy in various cell types including hepatocytes [500-503]. Recent studies have demonstrated an inverse relationship between HSF1 levels (the primary transcription factor for HSP72) and LC3-II levels [500, 502]. However, HSP72 overexpression in skeletal muscle increases LC3-II during fasting compared to fasted control animals [269]. Additional research is needed to clarify the relationship between HSPs and autophagy.

It is well known that exercise increases heat shock proteins. The research area of exercise and autophagy activation has recently had renewed interest. Exercise has been demonstrated to increase autophagy in skeletal muscle [446, 504, 505]. Following exercise, many pathways of degradation and repair are activated, and both autophagy and heat shock protein regulation may be important in order to adapt to exercise [498]. Dokladny et al. proposed that autophagy is likely important in the initial degradation response following exercise, while the heat shock response is important for cellular building and protein synthesis that occurs in the days following exercise [498]. The coordination of these two systems likely allow for optimal adaptation and improvements of cellular physiology following exercise. Our studies showed that autophagy and heat shock proteins likely interact, especially during exercise, but more research is needed to fully understand autophagy regulation by the heat shock response in disease states.

In preliminary experiments, we studied the effect of HSP72 overexpression on autophagy flux in primary hepatocytes. First, we overexpressed HSP72 (adenoviral transfection) for 24 hours in growth media, and then with chloroquine (10 μM) in low serum media (0.5% FBS) for an additional 24 hours. Chloroquine treatment allows for assessment of autophagy flux by inhibiting lysosomal acidification and subsequent quantification of the upstream autophagy proteins that accumulate. We evaluated LC3-I which during increased autophagy flux is lipidated and becomes LC3-II [388]. The protein p62 is a selective cargo adaptor that is important for sequestration of organelles for autophagy. This protein is degraded with increased autophagy. As expected, chloroquine treatment increased LC3-I, LC3-II, and p62 (**Figure 23A, B, and C**). Additionally, following HSP72 overexpression, we saw a reduction in LC3-I and p62 protein content with chloroquine treatment compared to control (**Figure 23B**, $P < 0.001$, and **Figure 23C**, $P < 0.01$). We did not see any change in LC3-II accumulation, a classical marker for autophagosome accumulation (**Figure 23A**). In summary, HSP72 may regulate expression of certain autophagy proteins.

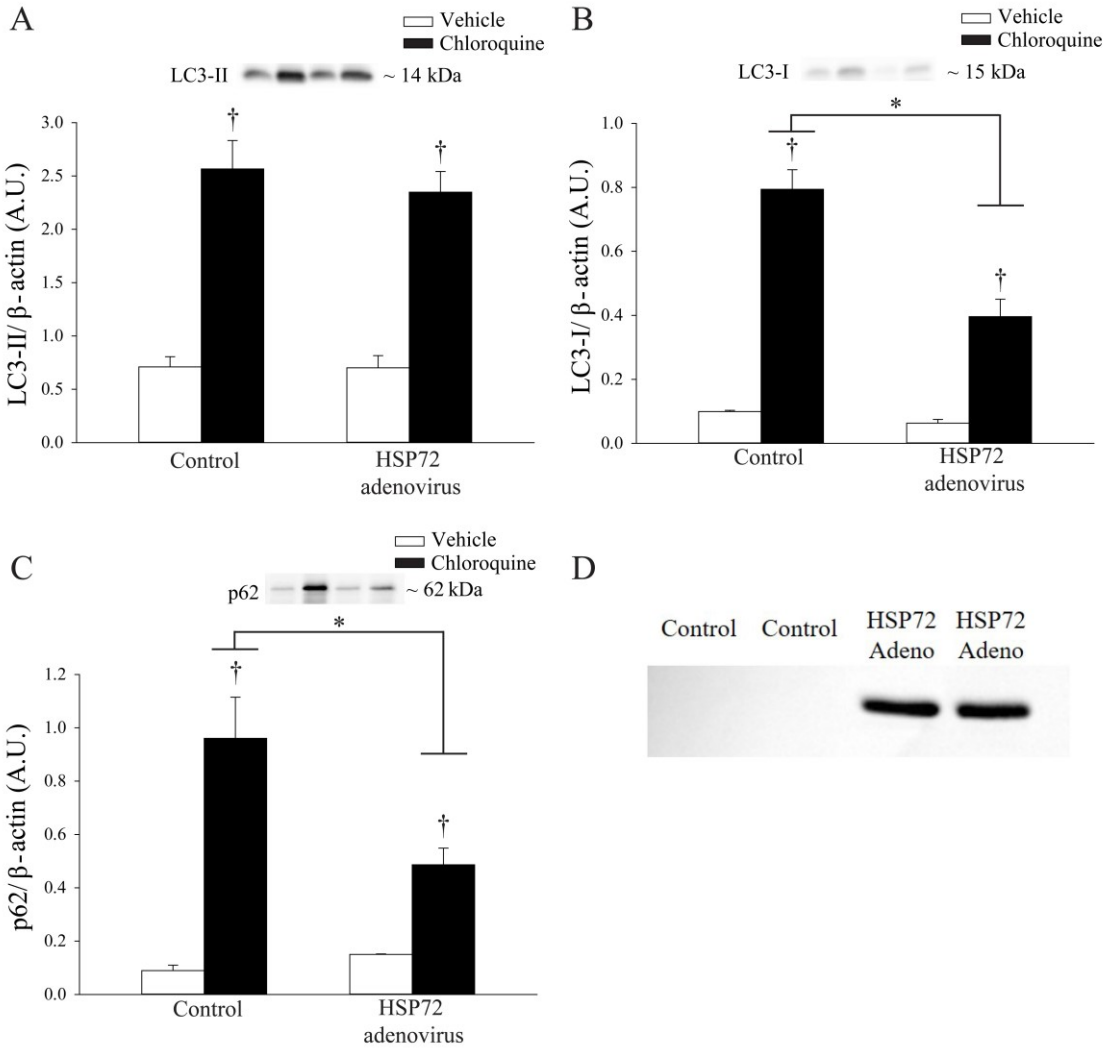

Figure 23. HSP72 overexpression reduces autophagy protein expression.
Primary hepatocytes were treated with an HSP72 overexpressing adenovirus for 24 hours in Williams E growth media, and then with chloroquine (10 μM) in Williams E media (0.5% FBS) for 24 hours A) LC3-II, B) LC3-I and C) p62 were compared between groups. D) Representative blot of HSP72 overexpression. n=3 wells per group from one animal. Analyzed by one-way ANOVA. *P < 0.05 between control and HSP72 adenovirus groups, and † P < 0.05 between vehicle and chloroquine treated groups.

Although impairments in autophagy occur with metabolic disease, the mechanism of how this develops is largely unknown. Increased cellular lipid exposure has been proposed as a possible cause for reduced autophagy with obesity and metabolic diseases [506]. Saturated fatty acids are abundant in the Western diet. They may contribute to lipotoxicity and development of NAFLD through activation of JNK, ER stress, oxidative stress, and mitochondrial dysfunction [507]. However, findings from studies investigating the effect of a lipid challenge on autophagy are inconsistent. Koga et al. found chronic exposure to a lipid challenge through a 16-wk HFD in rodents resulted in a reduction in autophagy rate in the liver [506]. However, Mei et al. found that acute exposure of palmitic acid, a saturated fatty acid, had no effect on autophagy or slightly suppressed autophagy, while the unsaturated fatty acid oleic acid induced autophagy [508]. More research is needed to fully understand lipid regulation of autophagy.

In preliminary experiments, we aimed to identify the effect of acute palmitate exposure and HSP72 loss on autophagy flux in primary hepatocytes. Hepatocytes were treated with siHSP72 for 48 hours to suppress HSP72 levels. All cells were subsequently treated with chloroquine for 30 min before treatment with palmitate plus chloroquine for an additional 24 hours in order to assess autophagy flux. We evaluated LC3-II, LC3-I and p62 protein expression following a loss of HSP72. Similar to the findings of Wei et al., we observed no significant difference in autophagy protein expression following palmitate exposure (**Figure 24A, B and C**) [508]. Additionally, we did not observe differences in protein expression between control siRNA and siHSP72 treated hepatocytes. Combined treatment with palmitate and siHSP72 treatment had no effect on LC3-II and LC3-I (**Figure 24A, and B**), but significantly increased p62 protein content ($P < 0.05$, **Figure 24C**). We observed variability between groups, and the presence of outliers which may have affected our results and should be addressed in future repeated experiments.

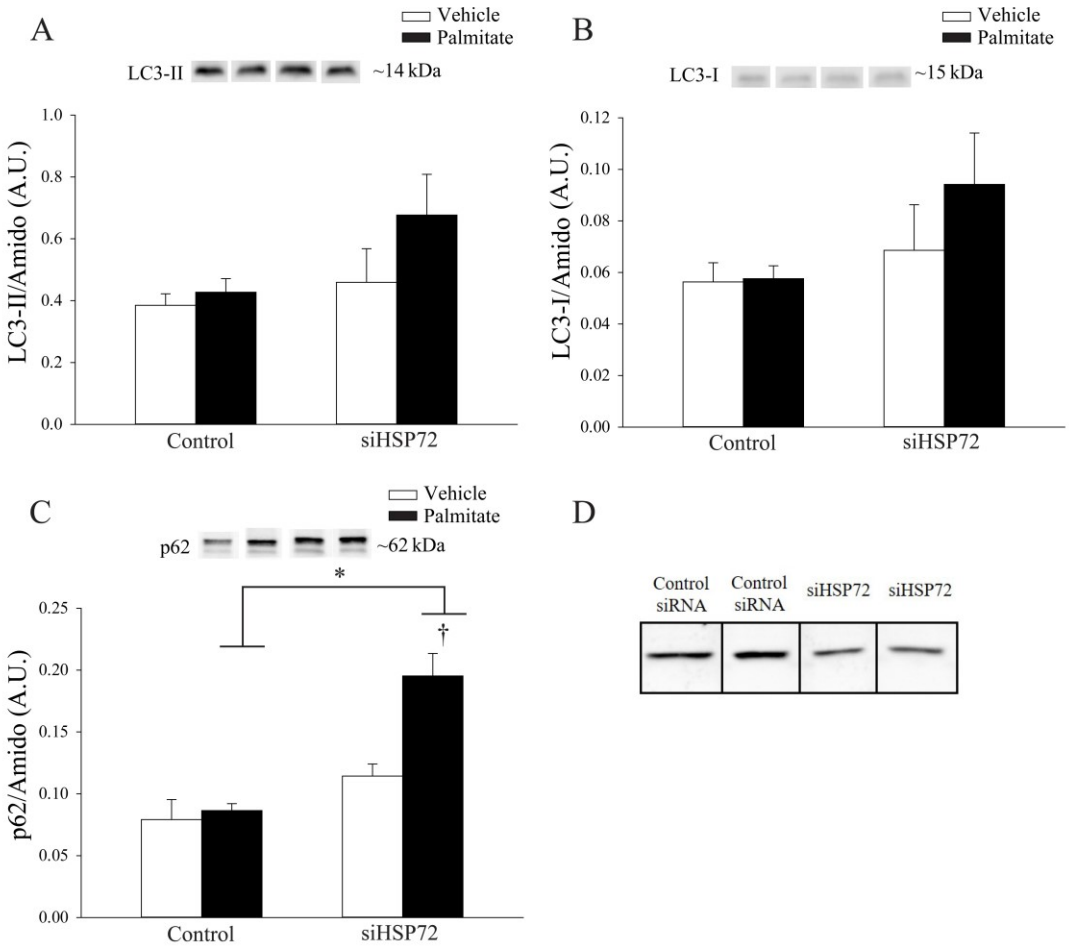

Figure 24. A loss of HSP72 and palmitate treatment increases autophagy protein expression.
Primary hepatocytes were treated with siHSP72 for 48 hours (15 nM) and then palmitate for 24 hours (250 μM). All hepatocytes were treated with chloroquine (10 μM) for 30 min before treatment of palmitate plus chloroquine for 24 hours. A) LC3-II, B) LC3-I and C) p62 were compared between groups D) Representative blot of HSP72 knockdown of ~60%. n=3-6 wells per group from one animal. Analyzed by one-way ANOVA. *P < 0.05 between control and siHSP72 groups, and † P < 0.05 between vehicle and palmitate treated groups.

Expression of proteins involved in autophagy may increase in primary hepatocytes following a combination of siHSP72 and palmitate exposure in order to manage organelle damage that both stressors put on the cellular components. It is likely that following a loss of the chaperone HSP72 hepatocytes become more vulnerable to cellular stressors associated with metabolic disease. An increase in JNK may also contribute to altered autophagy that occurs during metabolic disease progression. Inhibition of JNK in rodents has been shown to relieve NAFLD, and also suppress autophagy [477]. Yan et al. suggested that inhibition of JNK and suppression of autophagy in the liver contributed to improved insulin sensitivity [477]. A loss of HSP72 could be a key step towards further disease progression due to the inability to induce the heat stress response associated with increased cellular stress and inflammation.

5.3 Liver-Specific HSP72 Knockout Model

To date, no studies have examined direct effects of HSP72 on hepatic lipid metabolism. The use of a novel liver-specific HSP72 knockout animal in our laboratory will be key in identifying the importance of HSP72 in hepatic lipid metabolism. We believe that a loss of HSP72 will lead to steatosis and impaired whole-body glucose homeostasis. We also expect that a loss of HSP72 will lead to mitochondrial dysfunction and inflammation, which will be further exacerbated with a HFD. Additionally, we expect that a loss of HSP72 will impact autophagy and mitophagy pathways. Re-introduction of HSP72 through overexpression in this model should rescue steatosis and inflammation that occurs with a HFD. Using this model, we will be able to identify the importance of both HSP72 and its transcription factor, HSF1, to liver metabolism. Past research has demonstrated that HSF1 and PGC-1α can work together to regulate mitochondrial biogenesis and whole-body metabolism [324, 325]. In the liver-specific

HSP72 knockout rodent, we will be able to determine if those mechanisms are dependent on HSP72.

5.4 HSP72 Regulation of the Unfolded Protein Response and Proteostasis.

Cellular stress causes unfolded proteins to accumulate in the endoplasmic reticulum (ER), which activates the unfolded protein response (UPR) [509-511]. This response is important for cellular adaptation to ER stress and prevention of ER-stress induced apoptosis [512, 513]. ER stress and chronic activation of the UPR causes inflammation and contributes to the development of insulin resistance [514-523].

Although other HSP family proteins have been shown to be a part of the UPR [455, 524, 525] new evidence has also identified cytoplasmic HSP72 as part of the UPR. Specifically, HSP72 interacts with and upregulates inositol requiring enzyme 1 α (IRE1α) signalling to the ER. Activation of IRE1α by HSP72 enhances cell survival through prevention of ER-stress induced apoptosis [526]. This mechanism may be important in HSP72- mediated metabolic improvements, since activation of IRE1α also has been shown to suppress lipogenesis [527].

HSP72 is known to regulate mitochondrial function, but it's possible it regulates both the ER and the mitochondria. Additionally, ER and mitochondria can be tightly linked through interactions at mitochondria-associated membranes [528]. At these interactions, the organelles exchange lipids and calcium which is important for regulation of cellular homeostasis and adaption to metabolic stressors. Future work is needed to identify the role of HSP72 at the ER and between the ER and mitochondria.

HSP72 also likely impacts metabolic health through the protein's additional responsibilities as a cellular chaperone. During stress, HSP72 is essential to refold misfolded proteins and to maintain proteostasis. One way that HSP72 may maintain proteostasis is by regulating proteosomal degradation and autophagy [529, 530]. Degradation pathways via

proteasomes and autophagy are well established, but it was recently demonstrated that mitochondria also function as sites for protein degradation [531]. Specifically, the chaperone HSP104 detangles protein aggregates allowing mitochondrial transporters to import proteins into the outer and inner mitochondrial membrane. Proteases in the mitochondrial matrix are then able to degrade the newly imported unfolded proteins. Importantly, defects in HSP70 activity result in increased transport of misfolded proteins into the mitochondria, causing increased mitochondrial damage and ROS production. This phenomenon has been confirmed in both yeast and human retinal pigment epithelium cells [531]. It is tempting to speculate that defects in HSP72 activity could contribute to mitochondrial dysfunction by trigging this alternative mitochondrial-dependent degradation pathway. This alternative pathway may contribute to the swollen, rounded appearance of the mitochondria during metabolic disease, as well as decreased mitochondrial function as a respiratory organelle. Additional investigation of this mechanism in metabolic organs should be the focus of future research.

5.5 HSP72 as a Biomarker of Metabolic Health

Researchers have shown that HSPs can be released extracellularly (eHSP), specifically during exercise [338, 361-364]. HSP release could allow for organ crosstalk and the spread of HSPs to various tissues. eHSP72 function in general is associated with activation of the immune system [434], and in contrast to the anti-inflammatory actions of intracellular/cytosolic HSP72 (iHSP72), can induce activation of proinflammatory pathways. Based on this antagonistic action of HSP72 on the inflammatory response, the Chaperone Balance Hypothesis contends that the balance between eHSP72 and iHSP72 (eHSP72/iHSP72) could determine the extent of tissue inflammation, and thereby influence the pathogenesis of insulin resistance and type 2 diabetes [532]. According to this hypothesis, an intervention which lowers the eHSP72/iHSP72 ratio could in effect improve insulin sensitivity. Long term exercise training results in decreased eHSP72 and increased iHSP72 expression (as in skeletal muscle, and as we have observed in

the liver), supporting this hypothesis. In addition to the research supporting the many metabolic benefits of exercise in post-menopausal women [80-83] and also in patients with NAFLD [137-139], the effect of exercise on the eHSP72/iHSP72 ratio could be key in preventing metabolic dysfunction.

Importantly, the eHSP72/iHSP72 ratio could be a valuable biomarker for assessment of the inflammatory response in metabolic disease. In women, these ratios could be assessed pre- and post-menopause in order to identify the development of metabolic syndrome. It equally could be used in patients who have risk of diabetes or NAFLD development.

5.6 Conclusion

These current studies have identified the importance of HSPs in various tissues and metabolic diseases. In this work, we identified the importance of HSPs in exercise capacity of a postmenopausal model and possible mechanisms of HSP and estrogen receptor interaction in skeletal muscle. We also investigated HSPs in an understudied organ, the liver. We identified the importance of HSP72 in prevention of steatosis, and the ability of acute and chronic exercise to induce HSPs in the liver. Intracellular and extracellular HSP levels could be a valuable marker of overall and tissue-specific metabolic health. Clinicians should consider safe and effective treatments that induce the heat shock response (e.g. heat and exercise) as viable treatments for various metabolic diseases in humans.

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