HEAT SHOCK PROTEINS: NOVEL THERAPEUTIC TARGETS AGAINST INSULIN RESISTANCE AND TYPE 2 DIABETES

BY

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ABSTRACT

Impaired insulin action, termed insulin resistance, is characteristic of type 2 diabetes, obesity and aging. Given the rising epidemic of diabetes, efforts to understand the mechanisms of insulin resistance and discover effective therapeutic interventions are urgent. Considerable evidence now implicates oxidative stress in the patho-physiology of insulin resistance, a condition prevalent in the elderly and obese. Oxidative stress is known to activate several signaling cascades. This includes pathways that activate the stress kinases c-Jun N-terminal kinase (JNK) and the inhibitor of kappa B kinase β (IKKβ), which interact with and inhibit the insulin signaling cascade. The heat shock proteins HSP72 and HSP25 have been recently identified as natural inhibitors of JNK and IKKβ, respectively, and therefore represent novel therapeutic targets against insulin resistance. Overexpression of HSPs has been shown to protect against obesity-induced insulin resistance as well as age-related muscle damage. Skeletal muscle, the largest glucose disposing tissue, also contains large amounts of inducible HSPs. We hypothesized that heat shock protein overexpression in skeletal muscle could protect against insulin resistance in obesity and aging. We tested this hypothesis using aged male Fischer 344 rats (24-month-old) as the aging model of insulin resistance and male Wistar rats given a high fat diet (60% calories from fat) as the model of diet induced-insulin resistance. We examined the role of HSPs in insulin resistance by inducing HSP expression with both *in vitro* and *in vivo* heat treatments and anti-oxidant administration. Our results showed that

reduced HSP expression in the aging muscles is associated with a higher degree of stress kinase activation and insulin resistance in fast-twitch muscles compared to slow-twitch muscles. Increasing HSP72 expression in the muscles of young and old animals via heat treatment inhibited JNK activation. Heat-mediated JNK inhibition was specific to HSP72 induction, as determined by HSP72-inhibition studies, and was mediated by a direct interaction between HSP72 and JNK. In contrast to the muscle, brain sections from aging rats showed a robust increase in HSP25 expression, suggesting a tissue-specific regulation of HSPs in aging. In the high fat diet model, alpha-lipoic acid (LA), a potent antioxidant, was administered to relieve oxidative stress associated with high fat feeding. LA treatment improved insulin signaling and glucose transport, reduced stress kinase activation and increased HSP expression. As another method of HSP-induction, heat treatment, given in parallel with a high fat diet, improved glucose tolerance, reduced hyperinsulinemia, and reduced epididymal fat storage. In skeletal muscles, heat treatment induced HSP72 expression, improved insulin sensitivity, and reduced stress kinase activities. Heat treatment also enhanced mitochondrial function in fast-twitch muscles, normalizing the compensatory changes in mitochondrial protein expression seen with high fat feeding. Studies in L6 myotubes showed that heat treatment improved oxygen consumption and fatty acid oxidation. Mechanistically, our results indicate that heat shock proteins can 1). improve insulin sensitivity, 2). directly inhibit stress kinase activities, and 3). protect and enhance mitochondrial function. Our studies provide strong evidence that HSP

induction in skeletal muscle could be a potential therapeutic treatment for age-related and obesity-induced insulin resistance.

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LIST OF ABBREVIATIONS:

AD: Alzheimer's Disease
ADA: American Diabetes Association
AICAR: 5-Amino-Imidazole-4-Carboxamide-Riboside
ALS: Amyotrophic Lateral Sclerosis
AMPK: AMP dependent Protein Kinase
AS160: Akt Substrate 160 (KDa)
ATP: Adenosine Tri-Phosphate
ADP: Adenosine Di-Phosphate
BMI: Body Mass Index
CAMK: Ca2+/calmodulin dependent protein Kinase
CDC: Center for Disease Control and prevention
CoA: Coenzyme A
CPT1: Carnitine Palmitoyl Transferase-1
CRP: C-reactive protein
Ctx: Cortex
CVD: Cardiovascular Diseases
2-DG: 2-Deoxyglucose
cAMP: cyclic Adenosine Mono-Phosphate
DAG: Diacylglycerol
DMEM: Dulbecco's Modified Eagle Medium
DPM: Disintegrations Per Minute
ECL: Electrochemiluminescence
EDL: Extensor Digitorum Longus (muscle)
Epi: Epitrochlearis (muscle)
ER: Endoplasmic Reticulum
ETC: Electron Transport Chain
FA: Fatty Acid
FAO: Fatty Acid Oxidation
FBS: Fetal Bovine Serum
FCCP: p-trifluoromethoxy carbonyl cyanide phenyl hydrazone
FFA: Free Fatty Acids
GGA: Geranylgeranylacetone
GLUT4: Glucose Transporter-4
GP: Globus pallidus
GSH: (reduced) glutathione
GSK3: Glycogen Synthase kinase 3
GSSG: (oxidized) glutathione
GSV: Glut4 Storage Vesicles
GTP: Guanosine Tri-Phosphate
HDL: High Density Lipoprotein
HF: High Fat
HRP: Horse Radish Peroxidase

HS: Heat Shock
HSF-1: Heat Shock Factor-1
HSL: Hormone Sensitive Lipase
HSPs: Heat Shock Proteins
HT: Heat treatment
IKK: Inhibitor of Kappa B Kinase
IL-6: Interleukin-6
iP: inorganic phosphate
I.P.: Intraperitoneal
IPGTT: Intraperitoneal Glucose Tolerance Test
IR: Insulin Receptor
IRE1α: Inositol Requiring Kinase -1
IRS-1: Insulin Receptor Substrate-1
JNK: c-Jun N-terminal Kinase
KHB: Krebs-Henseleit Bicarbonate (buffer)
LA: Lipoic Acid
LCFA: Long Chain Fatty Acid
MAPK: Mitogen Activated Protein Kinase
MKP-1: MAPK phosphatase-1
MHC: Myosin Heavy Chain
mTOR: mammalian Target of Rapamycin
NCEP: National Cholesterol Education Program Adult Treatment Panel
NFκB: Nuclear Factor κ B
OXPHOS: Oxidative Phosphorylation
PAI-1: Plasminogen Activating Factor-1
PBS: Phosphate Buffered Saline
PD: Parkinson's Disease
PDE-3: Phosphodiesterase Enzyme
PDH: Pyruvate Dehydrogenase
PDK1: Phosphoinositide-Dependent protein Kinase-1
PGC-1α: Peroxisome proliferator-activated receptor-γ Coactivator
PH: Pleckstrin Homology (domains)
PI3K: Phosphatidylinositol 3 Kinase
PIP3: Phosphatidylinositol tri Phosphate
PKB: Protein Kinase B
PKCζ: Protein Kinase C-ζ
PTB: phosphotyrosine binding (domains)
RBP-4: Retinoic Acid Binding Protein-4
ROS: Reactive Oxygen Species
SDS-PAGE: Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SN: Substantia nigra
SNAP23: Soluble NSF-Attachment Protein
Ser473: Serine (residue) 473
SGLT: Sodium dependent Glucose Transporters

ST: Sham Treatment
Str: Striatum
T1D: Type 1 diabetes
T2D: Type 2 diabetes
TBST: Tris Buffered Saline with Tween-20
TCA: Tri-Carboxylic acid (cycle)
Thr308: Threonine (residue) 308
TNF-α: Tumor Necrosis Factor- α
UCP-3: Uncoupling Protein -3
v-SNARE: Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptors
VAMP: Vesicle-Associated Membrane Protein

Chapter 1

INTRODUCTION

INTRODUCTION

1.1. Diabetes and insulin resistance

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, resulting from either impaired insulin action (insulin resistance) or impaired insulin secretion (insulin deficiency). It is this hyperglycemia that gives the disease its name: *Diabetes* meaning "passing through" and *mellitus* meaning "honey sweet", in reference to the abundance of sugar detected in blood and urine.

Diabetes mellitus is regarded as the 7th leading cause of deaths in the US (CDC, 2006). It is manifested in two types: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D constitutes 5-10% of the diabetic population and is characterized by insulin deficiency. T1D has an autoimmune pathology, in which a cell-mediated autoimmune response destroys the insulin-secreting beta cells in the pancreas (Knip and Siljander 2008). It is most commonly diagnosed in childhood. Type I diabetes has strong genetic factors, but also has some environmental factors that are yet to be completely defined. Insulin administration for life has been the most important treatment regime (Cohen and Shaw 2007), and thus it is often referred to as *'Insulin-dependent diabetes mellitus'*.

T2D, which is the most predominant form of diabetes (90-95% cases), is characterized by insulin resistance. It was previously referred to as 'Non-insulin-dependent diabetes' or 'adult onset diabetes', but now these terms are seldom used.

Chronic insulin resistance results in a compensatory increase of insulin secretion by β cells in the pancreas in an effort to maintain euglycemia (Weir and Bonner-Weir 2007). However, prolonged compensation by β cells can subsequently lead to β cell-dysfunction and hyperglycemia ensues. Therefore, advanced T2D can also be characterized by insulin dependency (Prentki and Nolan 2006).

T2D has emerged as one of the most serious health concerns across the globe. The Center for Disease Control and prevention (CDC) estimates that nearly 8% of the population of the US alone (23.6 million people) currently suffers from diabetes. Because of this prevalence, diabetes is often referred to as an *'epidemic'*. A very recent study by Novo Nordisk estimated the economic burden of diabetes in 2008 as $218 billion in the US (ADA, 2008). T2D has a close association with aging, and therefore, more than half the economic burden of diabetes is incurred by people 65 years of age or older (Hogan et al. 2003). Diabetes is also tightly associated with the epidemic of obesity. More than 30% of American adults are obese (body mass index, BMI$\geq$30) and more than 65% are overweight (BMI$\geq$25). Furthermore, the average age of onset of T2D has now decreased due to an alarming rise in the incidence of childhood obesity (Klein et al. 2004). In the US 15.8% of children between the ages of 6 and 11 years and 16.1% of adolescents have a BMI in the range of overweight for their age (Hedley et al. 2004).

Abdominal obesity and insulin resistance are 2 of the 5 factors defining the *'metabolic syndrome'*. The *'metabolic syndrome'* or *'syndrome X'* was first described by Reaven in 1988 (Reaven 1988). Ever since, the definition of "metabolic

syndrome" has been evolving, but broadly encompasses a set of metabolic abnormalities. According to the National Cholesterol Education Program Adult Treatment Panel (NCEP), patients are considered to have the metabolic syndrome if they meet 3 of the 5 criteria: 1.) abdominal obesity, 2.) hypertriglyceridemia, 3.) low HDL cholesterol, 4.) high blood pressure or the use of antihypertensive medication, 5.) insulin resistance or glucose intolerance (or the use of antidiabetic medication) (NCEP 2001). In general, the risk for cardiovascular events is doubled and the risk for type 2 diabetes is increased about 5 fold in patients with the metabolic syndrome compared to those without it (Grundy 2008).

T2D is known to be one of the main causes of mortality and morbidity worldwide (Saltiel and Kahn 2001), although, mortality due to diabetes occurs mainly from the associated complications. Poorly-managed diabetes leads to complications such as cardiovascular diseases (CVD), nephropathy, neuropathy, neurodegenerative diseases, cerebrovascular diseases and retinopathy (Tripathi and Srivastava 2006). But recent evidence suggests that chronic whole body insulin resistance, even before the onset of full blown diabetes, could be the basic pathophysiological disturbance that underlies atherogenic risk factors (Park et al. 2005; Ferrannini and Iozzo 2006).

Development of T2D begins with insulin resistance and glucose intolerance. Skeletal muscle is responsible for the majority (75%) of insulin-mediated glucose disposal in the body (Bjornholm and Zierath 2005), making this tissue vital in maintaining insulin action and glucose homeostasis. Consequently, insulin resistance in muscle quickly leads to global dysregulation of glucose metabolism in the body.

Thus, treatments that prevent muscle insulin resistance may improve whole-body insulin action and significantly minimize the associated risks of T2D and cardiovascular disease.

1.2. Risk factors for insulin resistance

1.2.1. Advancing age:

Insulin sensitivity is known to decrease with advancing age. The decline begins in the third or fourth decade of life and progressively reduces throughout an individual's lifespan (DeFronzo 1981). Although several factors have been proposed to be responsible for age-related insulin resistance, the exact mechanism remains to be elucidated.

It is suggested that reactive oxygen species produced during metabolic processes such as cellular respiration accumulate over the life time of an individual (Frisard and Ravussin 2006). Systemic oxidative stress is prevalent in the elderly as indicated by increased levels of protein carbonyls with age (Berlett and Stadtman 1997; Levine 2002), increased glycated proteins (Baynes 2001), increased formation of oxidizing species (Schoneich 1999) and reduced proteasomal activities required to selectively degrade the oxidized proteins (Petropoulos et al. 2000). In addition, there is a reduction in anti-oxidant levels with age, including vitamin E, glutathione and HSP70 (Hall et al. 2000; Hall et al. 2001). Collectively, increased oxidative stress and reduced anti-oxidant mechanisms are characteristic of aging and a potential cause of

insulin resistance (Asghar and Lokhandwala 2006). Aging is also associated with mitochondrial dysfunction (Short et al. 2005), as a result of increased reactive oxygen species (ROS) (Sohal and Orr 1992), decreased mtDNA abundance (Barazzoni et al. 2000; Welle et al. 2003) and cumulative mtDNA damage (Melov et al. 1995) in the skeletal muscle. Reductions in mitochondrial activity, which contribute to dysregulated intracellular lipid metabolism, have been implicated in the increased incidence of insulin resistance and subsequent T2D that occurs with aging (Petersen et al. 2003).

The underlying insulin resistance poses increased risks for several diseases in the elderly, including diabetes, CVD, hypertension, neurodegenerative diseases, etc. As baby boomers grow older, the number of people over the age of 65 years is expected to double in the next 25 years (CDC), making the rise of diabetes within this population an increasingly critical health issue. As seen by the rise in the percentage of nursing facility residents with type 2 diabetes, it is thought that an age trend and an epidemic are converging (Adler 2008; Marquess 2008).

1.2.2. Obesity:

- ***Adiposity:*** Adiposity plays a central role in causing insulin resistance. Likewise, weight loss and bariatric surgery have shown significant reversal of metabolic derangements and insulin resistance associated with obesity (Leichman et al. 2008). Although obesity is a serious health concern, a significant proportion of obese individuals can achieve longevity without developing any morbidity such as T2D and CVD. One hypothesis to explain this observation is that total body fat is not

the sole source of the adverse health complications of obesity; rather the fat distribution or the relative proportion of lipids in various lipid deposition compartments is what determines the metabolic risk of the individual. It is now recognized that abdominal (visceral) obesity is a greater risk factor for insulin resistance than subcutaneous adiposity (Hamdy et al. 2006). Adipose tissue distribution is significantly altered in T2D patients. Compared to subcutaneous adiposity, visceral fat and intramuscular adipose depots are more closely associated with exacerbated insulin resistance in persons with T2D (Gallagher et al. 2009). A proposed mechanism by which visceral fat may cause its adverse effects is related to secretion of inflammatory cytokines. When examined *in vitro*, visceral fat has been shown to secrete increased amounts of inflammatory mediators, including C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), retinoic acid binding protein-4 (RBP-4) and plasminogen activating factor-1 (PAI-1), compared to subcutaneous fat (Fain et al. 2004). Similarly, obese individuals with increased visceral adiposity have increased markers of systemic inflammation compared to equally obese subjects with increased subcutaneous fat (Tsigos et al. 1999).

- Free fatty acids (FFA) and adipokines: Adipocytes decrease insulin action in peripheral tissues by releasing fatty acids and adipokines. In humans, even short term infusion of fatty acids is shown to induce insulin resistance and reduce insulin stimulated muscle glucose uptake (Dresner et al. 1999). It has been suggested that fatty acids and the associated oxidative stress activate several inflammatory stress kinases including JNK, IKKβ, PKCθ, mTOR and GSK3β (reviewed in detail in

section 1.4.1). Adipose tissue, previously considered only a storage tissue, is now recognized as an endocrine tissue, secreting several adipokines which regulate insulin sensitivity and global metabolism (Kershaw and Flier 2004). While adipokines such as leptin (Friedman and Halaas 1998), adiponectin (Berg et al. 2002) and visfatin positively regulate insulin action, inflammatory cytokines secreted by adipocytes such as TNF-α (Hotamisligil et al. 1993), IL-6, IL-1β, PAI-1 (Shimomura et al. 1996), resistin (Steppan et al. 2001) and RBP4 (Yang et al. 2005) inhibit insulin action.

- ***Intramyocellular lipid accumulation:*** Skeletal muscle triglyceride levels are increased in obese individuals and are inversely related to insulin action (Pan et al. 1997). Muscles from obese individuals exhibit increased rates of fatty acid uptake (Luiken et al. 2001) and extremely obese individuals have reduced capacity for lipid oxidation through lowered activity of key mitochondrial enzymes (Thyfault et al. 2004). Thus, the imbalance between fatty acid uptake and fatty acid oxidation is thought to cause accumulation of lipids in the insulin resistant muscle.

The relationship between intramuscular lipid content and reduced insulin action is not always consistent since elite athletes, who are very insulin sensitive, also have very high levels of intramuscular lipid content (Goodpaster et al. 2001). Further, improvements in muscle insulin sensitivity can happen with little or no change in muscle lipid levels (Phillips et al. 1996). The nature of this metabolic paradox, also called the *'athlete's paradox'*, seems to indicate that it is not the size of the intramyocellular pool, but rather the balance between fatty acid availability, cellular uptake, oxidation (lipid turnover) and cellular energy demand.

- ***Intrahepatic lipid accumulation:*** Non-alcoholic fatty liver disease (NAFLD) is caused by fatty infiltration of the liver without excessive alcohol consumption (Adams and Angulo 2006). Factors that contribute to the hepatic fatty acid pool include circulating FFAs, *de novo* lipogenesis within the liver and dietary factors, including intake of both fatty acids and carbohydrates that serve as precursors for lipogenesis (Diraison et al. 2003). The balance between lipogenesis and lipolysis in the liver is mainly affected by the ratio of insulin to glucagon. In the case of insulin resistance, fatty acid flux to the liver is increased due to increased lipolysis in adipose tissue, which further leads to increased fatty acid uptake. This, in turn, increases hepatic glucose output and triggers increased insulin secretion, in order to maintain euglycemia. Finally, increased concentrations of insulin in the liver induce *de novo* lipogenesis, thus creating a vicious cycle (Diraison et al. 2003).

1.2.3. Genetic susceptibility:

Although determining the genetic basis of a polygenic, multifactorial disease such as T2D has been very challenging, some putative genetic links have been made. Genes such as *CAPN10, TCF7L2, PPARG, KCNJ11, TCF2/HNF1B* and *WFS1* have been implicated in T2D (Ridderstrale and Groop 2008). Since not all obese individuals develop diabetes, and not all diabetic individuals are obese, genetic susceptibility is a prerequisite, but determining susceptibility to T2D with genetic testing is still premature. Maturity Onset Diabetes in the Young (MODY) is a monogenic disease which has a phenotypic expression similar to the symptoms of T2D. Future studies will indicate the role of genetic susceptibility in diabetes.

1.2.4. Sedentary lifestyle

Exercise has been long recognized as the best known treatment against insulin resistance. The imbalance between increased caloric intake and reduced physical activity causes storage of the unutilized calories as fat, which is the underlying cause of insulin resistance. Studies have shown that clinical measures of physical fitness, or the lack thereof, can be used to predict insulin resistance in people at risk for diabetes (Chen et al. 2008), and even short term physical inactivity can have adverse metabolic effects (Hamburg et al. 2007). In contrast, even a single session of exercise increases glucose uptake into the muscles during exercise, and increases glycogen accumulation and the ability of insulin to promote glucose uptake in the muscles after the exercise; all of which are important for glucose metabolism (Turcotte and Fisher 2008). The pathways mediating glucose uptake with exercise include activation of AMPK, p38 MAPK and CAMK and will be discussed in detail in section (1.3.6.) below. Exercise also induces expression of GLUT4 (Ren et al. 1994), increases fatty acid oxidation (Thyfault et al. 2007) and increases mitochondrial biogenesis (Wright et al. 2007). Muscle from insulin-resistant individuals exhibit decreased fatty acid oxidation rates and it is thought that aerobic exercise may alleviate insulin resistance by correcting the mismatch between fatty acid uptake and fatty acid oxidation (Koves et al. 2005; Koves et al. 2008).

1.2.5. Psychological stress:

Recent evidence suggests that psychosocial stress also exerts a profound impact on glucose metabolism (van Dijk and Buwalda 2008). Stress can also alter

eating habits and thereby affect insulin metabolism indirectly. A study examining a cohort of 425 middle aged women showed that women who exhibited high levels of psychological stress including depression, tension and anger had elevated risk for developing the metabolic syndrome as seen in the follow up screening (Raikkonen et al. 2002). Thus, reduction of psychological stress may prevent the development of metabolic abnormalities.

Clearly, there are multiple factors that can predispose individuals to develop insulin resistance and T2D. The multifactorial nature of this disease makes it a complex disease in need of future studies designed to investigate basic molecular mechanisms and identify molecular targets for treatment.

1.3. Physiological functions of insulin

1.3.1. Structure, synthesis and release of insulin:

Insulin is a small protein secreted by the β-cells of the pancreas in response to increasing blood glucose concentrations. The insulin gene is highly conserved (Philippe 1991) and has two polypeptide chains A and B (21 and 30 amino acids, respectively), linked together by a pair of disulphide bonds. During synthesis of insulin, pre-proinsulin is first converted to proinsulin by proteolytic action, followed by folding of the protein into its stable configuration with the addition of three disulphide bonds. Proinsulin is then cleaved into C-peptide and mature insulin in the golgi. Insulin, C-peptide, Zn++ and amino acids are packaged into vesicles within the

golgi (Halban 1994). Release of the insulin vesicles requires increased uptake of glucose by pancreatic β-cells, which, leads to a concomitant increase in metabolism. Increased metabolism leads to an elevation in the ATP/ADP ratio, and ATP is responsible for the inhibition of an ATP-sensitive K^+ channel. The net result is a depolarization of the cell, leading to Ca^{2+} influx, and fusion of vesicles with the membrane in a Ca-calmodulin-dependent process. Insulin is secreted from the beta cells into the portal vein in a pulsatile fashion, and impairment of the pulsatility of insulin secretion is observed in type 2 diabetes (Porksen 2002).

1.3.2. Glucose metabolism:

The levels of circulating glucose are maintained within a narrow range of 4 mM to 7 mM (80-100 mg/dL) by insulin in normal individuals (Saltiel and Kahn 2001). Insulin maintains blood glucose levels by increasing glucose uptake in adipose tissue and striated muscle and by inhibiting hepatic glucose release. Once secreted into the blood stream, insulin binds several insulin responsive tissues that express the insulin receptor. Insulin signaling mediates several other functions beside glucose uptake in most tissues in the body, but the primary glucose disposing tissue is the skeletal muscle, responsible for almost 75% of glucose disposal in the body (Bjornholm and Zierath 2005). The adipose tissue accounts for less than 10% of glucose disposal (Klip et al. 1990). Insulin signaling in the liver controls gluconeogenesis by the inhibition of the chief gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Barthel and Schmoll 2003). Increasing evidence suggests that insulin also impacts glucose metabolism in several

other tissues, including the brain. Impaired insulin signaling in the brain has been recently implicated in diabetes (Reagan 2005).

1.3.3. Other metabolic roles of insulin:

The role of insulin in metabolism is not limited to glucose homeostasis. Insulin is a very potent hormone, with functions in almost all areas of metabolism in the body. Insulin regulates vital processes such as lipogenesis, protein expression, protein synthesis, cell survival, cell differentiation and proliferation. It also acts to inhibit lipolysis, glycogenolysis and protein breakdown. For example, in adipose tissue, insulin inhibits the hormone sensitive lipase (HSL) by activating phosphodiesterase enzyme (PDE-3). This in turn decreases cAMP levels required to activate HSL-activating-PKA (protein kinase A), which inhibits lipolysis and stimulates lipogenesis (Large and Arner 1998).

Two main branches of the insulin signaling mitogenic cascade have been characterized, one controlled by phosphatidylinositol 3 kinase (PI3K) and another controlled by the small GTP-binding protein $p21^{ras}$ (Ras) (Tsakiridis et al. 1996). The downstream effectors of PI3K activation are mTOR and p70S6K (ribosomal S6 kinase), which initiate protein synthesis by activating translation machinery including eukaryotic initiation factors (eIFs) and eukaryotic elongation factors (eEFs) (Chung et al. 1994). Insulin-activated Akt inhibits glycogen synthase kinase 3 (GSK3β) and thus inhibits GSK3β-mediated eIF2B inactivation. Akt also phosphorylates the TSC1 (tuberous sclerosis complex 1)–TSC2 complex to relieve its inhibitory action on mTOR. mTOR controls translation initiation and elongation via p70S6K and eEF2K

(Proud 2006). Activation of small GTP binding proteins by insulin also turns on the proliferative signal through the sequential activation of Raf, MEK1/2, ERK1/2 and p70S6K (Iijima et al. 2002). Insulin also activates the stress activated mitogen activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) acutely (Lee et al. 2003).

1.3.4. Insulin signal transduction:

Insulin signaling is mediated by a highly complex and intricate integrated network which is also highly regulated (reviewed in (Taniguchi et al. 2006)). The binding of insulin to its dimeric receptor (insulin receptor, IR) on the cell surface activates the receptor tyrosine kinase activity of the IR. The receptor phosphorylates itself and also results in recruitment and phosphorylation of insulin receptor substrates. There are at least 11 intracellular substrates of the IR, and 6 of these belong to the IRS family (IRS-1 to 6) (Cai et al. 2003). Other substrates include Gab-1 (Lehr et al. 2000), Cbl (Baumann et al. 2000) and isoforms of Shc (Gustafson et al. 1995). The IRS proteins have both pleckstrin homology (PH) domains and phosphotyrosine binding (PTB) domains which account for the high affinity of these substrates for the insulin receptor. The center and C terminus of IRS contain up to 20 potential tyrosine phosphorylation sites (Gual et al. 2005), which upon phosphorylation are capable of binding to molecules containing Src-homology-2 (SH2) domains, such as the phosphatidylinositol 3 kinase (PI3K). IRS-1 is the predominant isoform in skeletal muscle and IRS-1 deficiency results in impaired insulin action primarily in the muscle (Araki et al. 1994). Along with tyrosine

phosphoryation sites, the IRS-1 has several potential serine phosphorylation sites. Serine phosphorylation of IRSs seems to negatively regulate IRS signaling and has been implicated in the pathogenesis of insulin resistance (Gual et al. 2005; Zick 2005).

Tyrosine phosphorylated IRS recruits and activates PI3K, which consists of a regulatory and a catalytic subunit, each occurring in several isoforms (Myers et al. 1992). PI3K catalyzes the formation of the lipid second messenger phosphatidylinositol tri phosphate (PIP3). PIP3 binds to proteins containing the PH domains, and allows such proteins to localize to the membrane and be activated. Activation of PH domain-containing 3-phosphoinositide-dependent protein kinase-1 (PDK1) causes phosphorylation of Akt/PKB on Thr308 (Alessi et al. 1997) and PKCζ on Thr410 (Le Good et al. 1998), thereby activating these kinases. For complete activation, Akt requires phosphorylation on Ser473 as well, which is proposed to be mediated by mTOR (Sarbassov et al. 2005). Downstream targets of Akt include GSK3 (which leads to glycogen synthesis), tuberin (TSC2, inhibition of which by Akt activates mTOR), FOXO (regulates expression of gluconeogenic and lipogenic genes) and the Rab-GTPase-activating protein, AS160. Phosphorylation of AS160 triggers the activation of Rab small GTPases that are involved in the cytoskeletal reorganization required for the translocation of the glucose transporter GLUT4 to the plasma membrane (Sano et al. 2003). PKCζ, one of the atypical PKCs, is also activated in the PI3K axis and has been shown to have a role in insulin stimulated glucose uptake (Farese 2002).

1.3.5. Redistribution of glucose carriers by insulin:

The lipid bilayer of cells is impermeable to hydrophilic molecules such as glucose. Thus, glucose has to be transported across the plasma membrane by means of membrane associated carrier proteins, glucose transporters. The two types of glucose transporters are: the sodium dependent glucose transporters (SGLT) and the facilitative glucose transporters (GLUT) (Bell et al. 1990). SGLT co-transporters, or symporters, are essentially $Na^{+}K^{+}$ ATPase pumps that mediate the energetically favored movement of a sodium ion across the plasma membrane into the cell, driven by a concentration gradient and a membrane potential; and this is coupled to the movement of the glucose molecule (Scheepers et al. 2004).

The facilitative glucose transporter, or GLUT family, comprises of 13 members and all catalyze hexose transport across membranes in an ATP-independent manner (reviewed in (Joost and Thorens 2001)). All members have a striking tissue specific expression and variable hexose affinities. The facilitative glucose transporters are predicted to have 12 membrane spanning regions with intracellular amino- and carboxy-termini (Scheepers et al. 2004). GLUT1 is ubiquitously expressed and its highest expression is seen in erythrocytes and endothelial cells of brain. GLUT2 is primarily expressed in pancreatic β cells, liver, kidney and intestine. GLUT3 is expressed in neurons (Joost and Thorens 2001).

GLUT4 is primarily present in insulin responsive tissues such as skeletal muscle, adipose tissue, brain, and heart and is the insulin responsive glucose transporter. Insulin acts by stimulating the translocation of specific GLUT4

containing vesicles (GLUT4 storage vesicles, GSV) from intracellular pools to the plasma membrane resulting in glucose transport (Bryant et al. 2002). Impaired mobilization of GLUT4 via insulin signaling and decreased expression of GLUT4 in adipose tissue has been implicated in insulin resistance and diabetes (Shepherd and Kahn 1999).

Under basal conditions, GLUT4 vesicles reside in the intracellular storage vesicles and a small fraction (4-5%) are present at the plasma membrane (Klip et al. 1990). GLUT4 vesicles travel to the plasma membrane at a very slow rate even in the absence of insulin, but the rate of endocytosis is greater than that of exocytosis (Li et al. 2001). Insulin reverses this rate profile. Insulin signaling changes the cellular dynamics of GLUT4 vesicles via the actin cytoskeleton (Guilherme et al. 2000) and increases the plasma membrane distribution of GLUT4. The organization of the actin cytoskeleton aids the movement of GLUT4 vesicles to the plasma membrane and their subsequent fusion (Guilherme et al. 2000).

GLUT4 storage vesicles have a perinuclear location in 3T3L1 adipocytes and L6 myocytes and co-localize with v-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) vesicle-associated membrane protein (VAMP)2 (Malide et al. 1997). VAMP2 is a selective marker of GSVs and is required for insulin-stimulated fusion of GSVs to the plasma membrane, but not in the basal state. Despite several studies, the characterization of GLUT4 vesicles and the regulation of their translocation in response to insulin still remain controversial. The fusion process is mediated by interaction of v-SNARE (VAMP2) on the vesicles with cognate

receptors t-SNARE (soluble NSF-attachment protein, SNAP23) on the target membrane, orchestrated by several accessory proteins such as Munc18c, Synip and Tomosyn (Watson and Pessin 2006).

1.3.6. Insulin independent GLUT4 translocation:

Although insulin-stimulated GLUT4 translocation to the plasma membrane constitutes the major pathway of increasing glucose transport, several other mechanisms have been described. These insulin-independent mechanisms are receiving great attention with the prospect of improving glucose uptake in insulin resistant patients. Muscle contraction and exercise (Lund et al. 1995; Kennedy et al. 1999), osmotic pressure (Chen et al. 1997), hypoxia (Mu et al. 2001; Wright et al. 2005) and acute oxidative stress (Kim et al. 2006; Dokken et al. 2008) each stimulate glucose transport in muscle. The PI3K-independent signaling pathways mediating glucose uptake are not clearly defined, although Ca2+/calmodulin dependent protein kinase (CAMKII) (Wright et al. 2004), AMP dependent protein kinase (AMPK) (Mu et al. 2001; Fujii et al. 2007), conventional protein kinase C isoforms (α,β,γ,ζ) (Perrini et al. 2004) and p38 MAPK (Geiger et al. 2005) have been implicated. Contraction-associated changes in intramyocellular calcium concentrations activate CAMKII, which, by yet unclear mechanisms, induces glucose uptake. Inhibition of CAMKII with its inhibitor KN62 decreases contraction-induced glucose transport by about 50% (Wright et al. 2004). p38 MAPK is activated by muscle contraction (Wretman et al. 2001) but its role in glucose uptake is under debate (Fujishiro et al. 2001). While some studies show inhibitory effects of p38 MAPK (Ho et al. 2004), others support

its role in glucose uptake. Recent studies indicate a role for p38 MAPK in GLUT4 activation rather than GLUT4 translocation (Somwar et al. 2001).

AMPK, a sensor of cellular energy status, has been implicated in exercise and contraction mediated glucose transport (Kurth-Kraczek et al. 1999; Mu et al. 2001). AMPK is a heterotrimeric protein consisting of a catalytic α subunit and two regulatory subunits (β and γ). AMP activates AMPK allosterically and induces phosphorylation of a threonine residue (Thr172) within the activation domain of the α subunit by upstream kinases such as LKB-1 and CAMKK β (Hurley et al. 2005; Kahn et al. 2005). AMPK can also be activated by an adenosine analog (AMP mimetic) 5-aminoimidazole-4-carboxamide-riboside (AICAR) in resting muscle to stimulate glucose uptake (Merrill et al. 1997). The downstream signaling from AMPK leading to glucose uptake is not clearly understood, but AS160 is thought to be one of its substrates. Likewise, in human skeletal muscle, insulin (Karlsson et al. 2005) and exercise (Deshmukh et al. 2006) stimulate phosphorylation of AS160. Thus, the two pathways (insulin-PI3K-Akt and AMPK signaling) are thought to converge at the point of AS160 leading to glucose uptake (Thong et al. 2007).

The AMPK pathway also has profound effects on the regulation of fatty acid metabolism. Fatty acid oxidation in the muscle involves a rate-controlling step that is regulated by carnitine palmitoyltransferase 1 (CPT1). CPT1 transfers long chain acyl-CoA into the mitochondria and this process is inhibited allosterically by malonyl-CoA (Ruderman et al. 1999), synthesized by acetyl CoA carboxylase (ACC) (Trumble et al. 1995). The activity of ACC is inhibited by AMPK via reversible

phosphorylation (Winder et al. 1997). During exercise or in presence of AICAR, AMPK inhibits ACC to reduce malonyl CoA concentrations, thereby driving long chain acyl-CoA into the mitochondria for beta oxidation to restore energy balance (Merrill et al. 1997). Thus the ability of AMPK to induce lipid oxidation and thus lower muscle lipid deposition is considered an important feature for the insulin sensitizing effects of AMPK.

1.4. Mechanisms of insulin resistance

1.4.1. Oxidative stress:

Increasing evidence points at chronic tissue oxidative stress as the underlying factor in the etiology of insulin resistance (Houstis et al. 2006). In high fat feeding, increased oxidative stress has been shown to precede the onset of insulin resistance (Matsuzawa-Nagata et al. 2008). Furukawa and colleagues have shown that fat accumulation in obesity correlates with systemic oxidative stress in humans and mice (Furukawa et al. 2004). Oxidative stress can result from endoplasmic reticulum stress, mitochondrial dysfunction, nutrient excess, hyperinsulinemia and inflammatory cytokines such as TNF-α. Aging is also associated with oxidative stress and accumulation of reactive oxygen species over the years is believed to in fact contribute to the aging process itself (Beckman and Ames 1998). Insulin resistance appears to be a manifestation of metabolic overload in obesity, and obesity/high fat

feeding is associated with high levels of reactive oxygen species formed during incomplete oxidation of fatty acids (Muoio and Newgard 2008).

The connecting link between oxidative stress and insulin resistance are the stress activated kinases which can phosphorylate the IRSs on serine residues (Figure 1). The pivotal point in the switch to insulin resistance is believed to be at the level of the IRSs, where serine phosphorylation of IRS causes a conformational change in the IRS, preventing insulin induced tyrosine phosphorylation (Liu et al. 2004; Draznin 2006). Specifically, phosphorylation on the Serine 307 (Ser307 in mouse, rat; Ser312 in humans) residue of IRS-1 is thought be the crucial inhibitory signal leading to insulin resistance (Aguirre et al. 2000; Jiang et al. 2003). Transgenic mice with a muscle-specific serine to alanine substitution in IRS-1 are protected from fat-induced insulin resistance, as seen by improved glucose tolerance, increased insulin stimulated glucose uptake and increased IRS-1-PI3K association (Morino et al. 2008). IRS-1 serine phosphorylation occurs in response to insulin stimulation as well, primarily as a feedback mechanism to contain insulin action (Hers and Tavare 2005). But it has been shown that insulin/Insulin like growth factor-1 (IGF-1) and TNF-α associated phosphorylation of IRS-1 Ser307 occur by distinct pathways, indicating that normal regulatory negative feedback mechanisms and mechanisms of insulin resistance are distinct (Rui et al. 2001). Chronic serine phosphorylation of IRS-1 can lead to proteasomal degradation of the protein, and low protein levels of IRS-1 are observed in patients with diabetes (Carvalho et al. 1999; Zhande et al. 2002). On the other hand, some evidence also argues against the sole involvement of IRS-1 in causing

insulin resistance (Potashnik et al. 2003). Using a heterologous system involving a platelet derived growth factor pathway that recapitulates many aspects of insulin action independent of IRS-1, Hoehn et al. showed that the major nodes of insulin resistance are independent of IRS-1 (Hoehn et al. 2008).

The kinases implicated in serine phosphorylation of IRS-1 include c-Jun N-terminal kinase (JNK) (Aguirre et al. 2000), inhibitor of kappa B kinase (IKKβ) (Yuan et al. 2001; de Alvaro et al. 2004; Sriwijitkamol et al. 2006), mammalian target of rapamycin (mTOR) (Carlson et al. 2004), protein kinase C theta (PKCθ) (Griffin et al. 1999; Haasch et al. 2006) and glycogen synthase kinase-3 (GSK3) (Dokken et al. 2005; Henriksen et al. 2008). Of these kinases, the roles of JNK, IKKβ and PKCθ have been the most extensively characterized in causing insulin resistance and an orchestrated effect of all these kinases is suggested (Gao et al. 2004). The activation stimuli for JNK and IKKβ include cellular stresses such as oxidative stress, UV radiation, heat, proinflammatory cytokines like TNF-α, IL-1β (Ip and Davis 1998; Hacker and Karin 2006), while PKCθ is primarily activated by the breakdown products of fatty acids, namely diacylglycerols and ceramide (Griffin et al. 1999; Idris et al. 2002).

FIGURE 1.

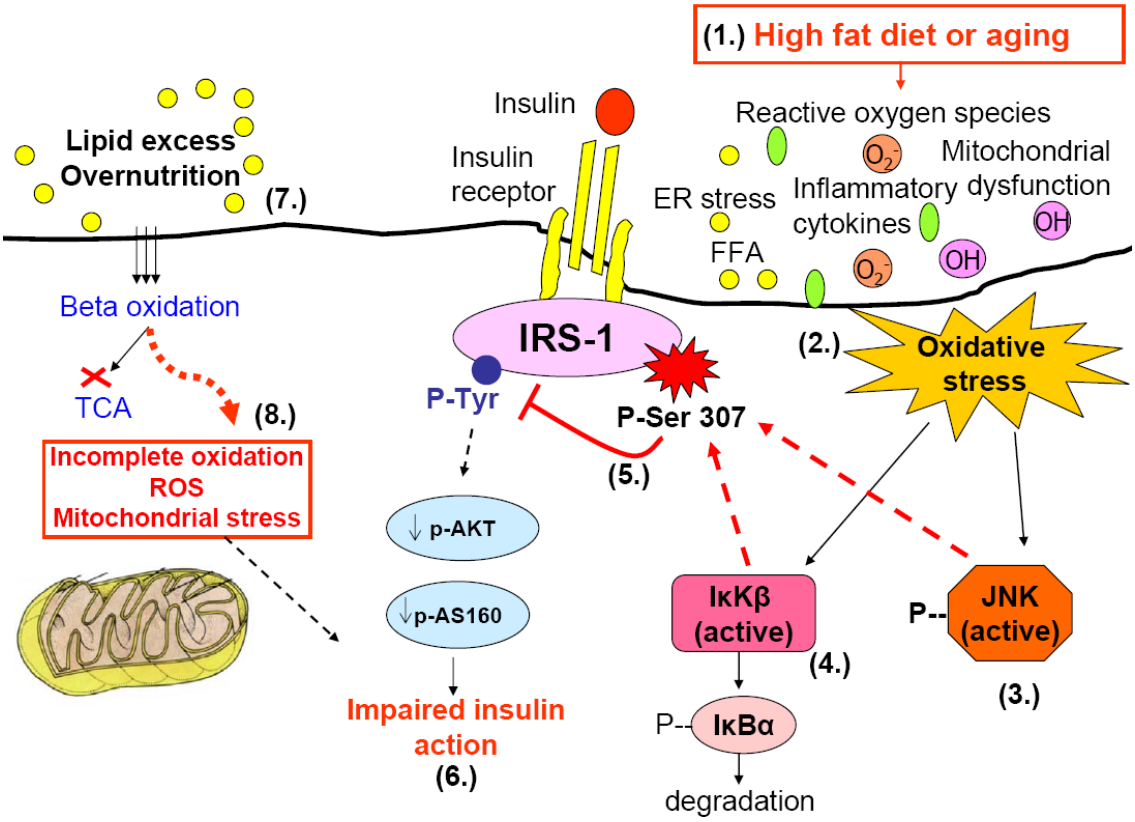

Figure 1. Mechanisms of insulin resistace: role of oxidative stress, IRS-1 inhibition by JNK and IKKβ, and mitochondrial stress. High fat diet and aging **(1.)** are associated with accumulation of reactive oxygen species and free fatty acids, mitochondrial dysfunction and endoplasmic reticulum stress. Cumulatively, these factors cause chronic oxidative stress **(2.)** in the skeletal muscle. Oxidative stress activates stress kinases JNK **(3.)** and IKKβ **(4.)**, which, in turn, phosphorylate the insulin receptor substrate IRS-1 on Ser307. (p)-Ser307-IRS-1 inhibits the insulin stimulated tyrosine phosphorylation of IRS-1 **(5.)**, and thereby reduces the action of insulin to mediate glucose transport **(6.)**. Lipid excess or overnutrition seen in obesity **(7.)** leads to incomplete beta oxidation, and thereby results in accumulation of intermediate products of oxidation, increased reactive oxygen species (ROS) and mitochondrial stress **(8.)**. In aging, mtDNA damage and reduced enzyme activities cause mitochondrial dysfunction. Mitochondrial stress and the associated oxidative stress are believed to contribute in causing insulin resistance.

1.4.2. Role of JNK in insulin resistance:

JNK activity levels are elevated in liver, muscle, and adipose tissues of diet induced- and genetic models of obesity, and its involvement in causing insulin resistance has been confirmed by showing that JNK-/- mice are resistant to insulin resistance (Hirosumi et al. 2002). Overexpression of a dominant negative JNK mutant in the liver of obese mice resulted in dramatic improvements in insulin sensitivity and reduced IRS-1 serine phosphorylation (Nakatani et al. 2004). Further, a recent study proposed the use of a cell permeable JNK inhibitory peptide as an effective treatment to reverse diabetes by reducing (p)-Ser307-IRS-1 (Kaneto et al. 2004). Also, silencing of the upstream MAP4K4 in human skeletal muscle prevented TNF-α-JNK-mediated insulin resistance (Bouzakri and Zierath 2007). Inversely, activation of JNK with free fatty acids/TNF-α/western diet (Nguyen et al. 2005; Prada et al. 2005) or with obesity in human skeletal muscle (Bandyopadhyay et al. 2005) increased (p)-Ser307-IRS-1 and reduced insulin signaling. Taken together, the JNK pathway is implicated in the development of insulin resistance, and JNK-mediated (p)-Ser307-IRS-1 is implicated as the molecular mechanism (Figure1).

1.4.3. Role of IKKβ in insulin resistance:

Oxidative stress also activates the inhibitor of kappa B kinase β (IKKβ), the kinase that activates the NFκB pathway (Ogihara et al. 2004; Arkan et al. 2005). Activity of IKKβ has been reported to be increased in insulin resistant muscles of obese animal models, and inhibition of IKKβ with salicylates has reversed this insulin resistance (Shoelson et al. 2003). Targeted disruption of IKKβ reversed obesity and

diet induced-insulin resistance (Yuan et al. 2001). IkBα/β are the downstream targets of IKKβ and get phosphorylated and degraded upon activation of IKKβ, further activating the NFkB pathway (Mercurio et al. 1997). Protein levels of IkBβ are reduced in the skeletal muscle of diabetic patients, indicating increased activity levels of the inflammatory kinase IKKβ (Sriwijitkamol et al. 2006). The insulin resistance-inducing inflammatory cytokine TNF-α induces activation of IKKβ in obesity, but sodium salicylate has been shown to inhibit this activation (Schwenger et al. 1998; de Alvaro et al. 2004) (Figure1).

The involvement of IRS-1 serine phosphorylation in insulin resistance, and the role of JNK and IKKβ in mediating (p)-Ser307-IRS-1, has been extensively characterized in obesity models of insulin resistance. However, the mechanisms of insulin resistance in the aging muscle are poorly understood. Studies addressing the involvement of stress kinases and IRS-1 phosphorylation status are needed to get a better understanding of age-related impairment of insulin signaling.

1.4.4. Mitochondria and insulin resistance:

Mitochondrial dysfunction is characteristic of aging and has been proposed to be a cellular malfunction that contributes to insulin resistance and diabetes even in the obese (Figure 1). Reduced mitochondrial numbers are seen in patients with diabetes and insulin resistant individuals with glucose intolerance (30% less than the insulin sensitive control subjects) (Lowell and Shulman 2005; Morino et al. 2006). Along with reduced numbers, diabetic patients, insulin resistant individuals, and insulin resistant offsprings of patients with T2D have impaired mitochondrial function,

downregulation of OXPHOS metabolism, and impaired mitochondrial enzyme activities (Kelley et al. 2002; Patti et al. 2003; Petersen et al. 2004; Sparks et al. 2005; Asmann et al. 2006). The master regulator of mitochondrial biogenesis PGC-1α (Peroxisome proliferator-activated receptor-γ Coactivator-1α), and the corresponding PGC-1 responsive genes are downregulated in diabetes and obesity (Mootha et al. 2003; Crunkhorn et al. 2007). Mitochondrial dysfunction appears to result from chronic oxidative stress (Bonnard et al. 2008). Therefore, interventions that protect mitochondrial function in insulin resistance could have potential benefits in protecting against insulin resistance. Studies exploring such interventions are important and could lead to novel approaches for the prevention of diabetes.

Although mitochondrial depletion and dysfunction have been believed to be intrinsic to the pathology of insulin resistance, recent evidence argues against it. Hancock et al. show that a high fat diet is associated with insulin resistance despite increased mitochondrial number and activity (Hancock et al. 2008). Another study by Turner et al. showed that increased mitochondrial number, increased enzyme activities, increased fatty acid oxidation, and increased PGC-1α are seen in rodents given a high fat diet (Turner et al. 2007). An increase in the expression of citrate synthase protein has been reported in epitrochlearis muscles from rats with high plasma fatty acid levels, along with cytochrome c, COX1, COX4 and others (Garcia-Roves et al. 2007). Thus, further studies are warranted to resolve the controversy of mitochondrial function in insulin resistance.

1.4.5. Muscle type differences in insulin action:

The mammalian skeletal muscle comprises the following four different fiber types characterized by specific myosin heavy chain (MHC) isoforms (Delp and Duan 1996):

1. slow-twitch fiber type (type I, oxidative, MHC1β),
2. fast-twitch fiber types (type II)
    - type IIA, oxidative/glycolytic, MHCIIa
    - type IIB, non-oxidative/glycolytic, MHCIIb
    - type IID/X, MHCIId/x, which is in between type IIA and IIB fibers

Humans do not typically express MHCIIb in locomotor skeletal muscles (Spangenburg and Booth 2003). Types IIA, IIX, and IIB fibers are found in skeletal muscle of other mammals (e.g., rodents and cats). The rat has muscles with highly specific fiber types and therefore provides a good model for studying their specific responses. For example, the soleus muscles in the hind limb consist predominantly of slow-twitch red fibers (84% type I, 16% type II) (Ariano et al. 1973), while the Epitrochlearis muscle in the fore limb is comprised of primarily fast-twitch white fibers (15% type I, 20% type IIA, 65% type IIB) (Nesher et al. 1980).

Type I fibers seem to be more responsive to insulin, exhibiting greater insulin binding capacity and increased activation of insulin signaling intermediates compared with type II fibers (Song et al. 1999). Aging-associated muscle atrophy and loss of muscle mass, termed as *'sarcopenia',* and is thought to occur almost exclusively in the type II myofibers. Sarcopenia develops focally along the length of the fiber in

regions that are characterized by compromised respiratory function, mitochondrial DNA deletions and oxidative damage (Lopez et al. 2000; Wanagat et al. 2001; Bua et al. 2002). Anderson et al. found that mitochondrial free radical leak (H_2O_2 produced/O_2 consumed) is two- to three-fold higher in type II fibers than in type I fibers during basal respiration. Further, when normalized for mitochondrial content, the H_2O_2 scavenging capacity is lower in the type II fibers compared with type I soleus muscle fibers, indicating that there are muscle fiber type-specific differences in the capacity of handling oxidative stress (Anderson and Neufer 2006). In contrast, obese subjects tend to exhibit fewer type I fibers and increased percentage of type II fibers than do lean subjects (Hickey et al. 1995). In light of these observations, it would be important to determine whether the susceptibility of the two fiber types to insulin resistance with age or high fat feeding is different.

The mitochondria in the skeletal muscle are morphologically and functionally heterogenous because of the metabolic demands and substrate preferences associated with each specific muscle fiber type (Kushmerick et al. 1992; Jackman and Willis 1996). Thus it would also be important to determine how the mitochondria in the different muscle fiber types respond to a change in diet (example, a high fat diet) and an intervention such as heat treatment.

1.5. Heat shock proteins

1.5.1. Chaperone function of HSPs:

Heat shock proteins have been extensively characterized in their role of being chaperones. As chaperones, HSPs maintain proteins in correctly folded conformation by: 1.) prevention of aggregation, 2.) promotion of folding to native state, 3.) solubilization of aggregates and refolding of proteins, and 4.) targeting severely damaged proteins for degradation (reviewed in (Mayer and Bukau 2005)). The best characterized heat shock protein is HSP72, the inducible member of the HSP70 family. HSP72 contains 3 major functional domains:

1.) N-terminal ATPase domain, which functions to bind ATP and hydrolyzes it. Unfolded proteins are bound in the ATP-bound state.

2.) Substrate binding domain that contains a binding site for about 7 hydrophobic amino acid residues. Hydrolysis of ATP locks the substrate in the cavity.

3.) C-terminal domain, which serves as a 'lid' for the substrate binding domain. Release of ADP and inorganic phosphate, iP at the end of the ATP cycle allows the release of substrate and subsequent binding of ATP.

The exact mechanism by which protein refolding occurs is still not clearly understood, but it is thought that binding and release cycles allow local refolding of the substrate, and help overcome the kinetic barriers for proper folding (reviewed in (Slepenkov and Witt 2002; Mayer and Bukau 2005)). Although the chaperone function of HSPs may be one of the factors involved in protection from insulin

resistance, evidence suggests that there may be other poorly understood roles of the HSPs in the cells (Yaglom et al. 1999) as discussed below.

1.5.2. Evidence of involvement of HSPs in insulin resistance:

The first direct evidence of the association of HSPs with insulin resistance, to our knowledge, came from a study by Kurucz et al. (Kurucz et al. 2002). They showed that HSP72 expression was decreased in skeletal muscle of type 2 diabetic patients and the reduction in HSP72 expression correlated with the degree of insulin resistance. The study also showed that HSP expression correlates with the rate of glucose uptake and other measures of insulin-stimulated carbohydrate and lipid metabolism. Shortly after, another study by Bruce et al. (Bruce et al. 2003), also showed that intramuscular HSP72 mRNA expression was reduced in patients with type 2 diabetes. Furthermore, in a study comparing 5600 genes of nondiabetic subjects with diabetic subjects, *HSP701A* was one of only 12 genes that were significantly lower (by 42%) in individuals with diabetes and whose expression correlated inversely with fasting glucose levels (Patti et al. 2003). These studies therefore brought up the idea that loss of HSPs may be involved in the pathogenesis of insulin resistance.

Experimental diabetes induced with streptozotocin, an alkylating agent that selectively destroys pancreatic beta cells (Wang and Gleichmann 1998), also shows altered heat stress response, seen as reduced HSP expression in response to heat (Najemnikova et al. 2007). Along with other anti-oxidant mechanisms, HSP expression is reduced in the serum from subjects with metabolic syndrome (Armutcu

et al. 2008). Taken together, it is clear that reduction of HSP expression is somehow related to insulin resistance. Consequently, increasing HSP expression could be considered a therapeutic strategy to protect from insulin resistance and diabetes.

In light of HSP induction as a therapeutic strategy, a pilot study in patients with type 2 diabetes found that hot tub therapy can improve glycemia control in these patients (Hooper 1999). Kokura et al. showed similar findings in obese db/db mice subjected to heat therapy (Kokura et al. 2007). HSP induction as been proven beneficial in several other conditions, including protection from ischemia reperfusion injury (Ooie et al. 2001; Chen et al. 2005), reducing transplantation rejects (Ghavami et al. 2002), attenuation of skeletal muscle damage by cryolesioning (Miyabara et al. 2006), and in protection from age-related muscle damage during contractile activity (Broome et al. 2006). In their anti-oxidant function, HSPs are known to protect against oxidative stress (Escobedo et al. 2004; Selsby and Dodd 2005; Broome et al. 2006).

The strongest evidence of HSP induction as a protective mechanism came from the study by Chung et al., who showed that HSP72 overexpressing mice were protected from insulin resistance (Chung et al. 2008). A combination of a mild electric stimulation and heat shock was shown to improve insulin signaling and reduce lipid accumulation in the liver (Morino et al. 2008).

Clearly, it is now being recognized that HSPs might play a major role in glucose homeostasis. The mechanisms of action and therapeutic interventions to reap

the benefits of HSP-overexpression are yet to be identified. Further, the role of HSPs in muscle-specific glucose uptake has not been established.

1.5.3. Mechanisms of HSP benefits:

The mechanisms underlying the beneficial effects of HSPs are poorly understood, but several hypotheses have been proposed:

1.5.3.1. Inhibition of stress activated kinases:

Studies in the apoptosis area of research have shown that HSP72 induction is associated with reduction in JNK-associated apoptosis (Mosser et al. 1997). Cell culture studies have shown that HSP72 can specifically interact with JNK and inhibit activation of JNK (Park et al. 2001). Using siRNA silencing techniques, this study confirmed the role of HSP72 in JNK inhibition and HSP72-associated apoptosis inhibition. Interestingly, this novel role of HSP72 in JNK inhibition is independent of HSP72's role in prevention of protein damage (Yaglom et al. 1999). Further evidence suggests that HSP25 can bind and inhibit activation of IKKβ (Park et al. 2003) and HSP25 overexpression is protective against apoptosis (Lee et al. 2004). Thus, HSP72 and HSP25 have been suggested to function as *natural inhibitors* of the stress kinases JNK and IKKβ respectively. But the effects of HSP-mediated stress kinase inhibition on insulin action are not known. Novel information regarding this novel role of HSPs may lead to discovering novel therapeutic targets and interventions.

Some evidence suggests that HSP72 interaction with JNK directly inhibits activation of JNK by upstream kinases (Park et al. 2001). HSP72 interacts with and inhibits the Dual Leucine zipper bearing kinase-1 (DLK-1), known as a kinase

responsible for activating JNK (Daviau et al. 2006). Other studies suggest that HSP72-JNK interaction activates a phosphatase such as the MAPK phosphatase-1 (MKP-1), which dephosphorylates and inactivates JNK (Meriin et al. 1999; Lee et al. 2005). Indeed, overexpression of MKP-1 can increase insulin sensitivity (Emanuelli et al. 2008). Nevertheless, either mechanism results in reduced JNK activity despite the presence of a JNK-activating stimulus (oxidative stress, anisomycin etc). The characterization of the interaction of HSPs with other kinases such as IKKβ has been very limited and warrants further study.

1.5.3.2. Removal of oxidative stress:

Exercise is a potent inducer of HSPs and is associated with modest amounts of oxidative stress. It is believed that HSP overexpression in exercise could be a mechanism of protection against oxidative stress (Smolka et al. 2000). In support of this possibility, heat stress and HSP induction are known to reduce oxidant damage in the muscle as seen by reduced 4-hydroxy-2-nonenol levels and reduced nitrosylated tyrosine residues (Selsby and Dodd 2005).

1.5.3.3. Prevention of stress-induced apoptosis:

JNK is involved in both intrinsic as well as extrinsic pathways of apoptosis and functions to suppress several inhibitors of apoptosis including p53, Bcl-2, Bax and Bak, while HSPs can prevent this suppression (reviewed in (Beere 2005)). HSP70 can prevent the activation of stress activated JNK (Gabai et al. 1997) and prevent TNF-induced Bid-dependent apoptosis pathways (Gabai et al. 2002). Inversely, reduced thermotolerance in aged rats is thought to result from a loss of HSP72-

mediated control of JNK pathway (Volloch et al. 1998). The relationship between reduced apoptosis and insulin sensitivity is not clear but HSPs would play a role in maintaining healthy cell structures that would be crucial in facilitating efficient signaling.

1.5.3.4. Mitochondrial protection:

Heat shock proteins have been shown to protect mitochondrial function from hypoxia/reoxgenation injury (Williamson et al. 2008), from oxidative stress (Downs et al. 1999) and from ischemic injury (Volobоueva et al. 2008; Xu et al. 2008). In fact, mitochondria have been suggested to be the selective targets for the protective effects of heat shock treatment against oxidative stress (Polla et al. 1996). Heat shock proteins have been implicated in maintaining mitochondrial function by reducing oxidative stress (Ouyang et al. 2006), mediating protein folding in stress conditions (Kregel 2002), increasing mitochondrial enzyme activities (Sammut et al. 2001) and improving protein import (Young et al. 2003). HSPs are known to aid transport of nuclear encoded proteins into the mitochondria via interaction with the mitochondrial protein import receptor protein Tom70 (Young et al. 2003). Sammut et al. showed that heat stress enhances the activity of the mitochondrial complex, affording prolonged preservation of the cardiac cells for transplantation (Sammut et al. 2001). It is thought that the protection by heat stress is primarily due to enhancement of mitochondrial energetics in conditions of stress. Further, the target of one HSP72 inducing drug, geranylgeranylacetone (GGA), is cardio-protection in the mitochondria. GGA preserves mitochondrial function and reduces apoptosis in

response to hypoxia-reoxygenation (Shinohara et al. 2007). HSP72 can directly enhance the activity of superoxide dismutase during myocardial ischemia-reperfusion and thereby protect the mitochondria from injury (Suzuki et al. 2002).

Taken together, mitochondria have been repeatedly implicated in insulin resistance and HSPs are thought to protect mitochondrial function. Thus, it would be crucial to determine whether HSPs modulate mitochondrial function in an insulin-resistant animal model and whether such modulations have benefits on reversing insulin resistance.

Mitochondrial function is also regulated by the uncoupling protein-3 (UCP-3), which is expressed almost exclusively in the skeletal muscle (Gong et al. 1997). UCP-3 has been implicated in increasing fatty acid oxidation capacity (Wang et al. 2003; Bezaire et al. 2005), and mitigating ROS production by providing a negative feedback loop to reduce transmembrane proton motive force (Echtay et al. 2002). But unlike UCP-1, UCP-3 may not play a significant role in non shivering thermogenesis, unless there is supraphysiological adrenergic stimulation (Mills et al. 2003). Acute exercise (Schrauwen et al. 2002) and high fat feeding (Matsuda et al. 1997) have both demonstrated increased UCP-3 expression in rodents and humans. The effect of heat treatment on mitochondrial UCP-3 expression is not known and whether the beneficial effects of heat of mitochondrial function are associated with UCP-3 expression needs to be investigated.

1.5.4. Tissue-specific regulation of HSPs:

The skeletal muscle is a suitable tissue for studying HSP regulation and glucose metabolism, since it exhibits high levels of inducible HSPs and is a major site for glucose metabolism. But other tissues involved in glucose homeostasis, such as the brain, adipose tissue, liver and the pancreas also show HSP expression, although the HSP expression levels are highly variable from tissue to tissue in conditions of stress. For example, in diabetic monkeys, Kavanagh et al. (Kavanagh et al. 2008) found that HSP72 expression was reduced by 32% and 50% in serum and liver, respectively, although HSP72 expression was increased in the pancreas. The latter has been speculated as a compensatory response to protect the vulnerable beta cells from the stress of insulin hypersecretion. It is not well understood if HSP upregulation in tissues such as the brain, can be beneficial and even less is known about the regulation of HSP in these tissues in response to aging, high fat diet and oxidative stress.

1.5.5. HSP induction:

1.5.5.1. The heat shock response:

HSP induction is part of the cellular stress response and is controlled at the transcriptional level by the transient activation of the heat shock transcription factor HSF-1 (reviewed in (Wu 1995)). Under normal conditions, HSF-1 is maintained as latent non-DNA binding monomers, which upon receiving a stress stimulus, assemble into active homotrimers capable of interacting with heat shock elements upstream of HSP genes. In the unstressed cells, HSP90 serves as the principal cellular repressor of

HSF-1, maintaining HSF-1 in the inactive monomeric form. Upon stress, HSP90 is sequestered into protein aggregates and HSF-1 is free for activation and trimerization (Zou et al. 1998). Complete activation is a multistep process involving hyperphosphorylation and de-phosphorylation of certain residues. Upon removal of the stress stimulus, or after achieving critical levels of HSP expression, the trimers of HSF-1 are disassembled back to inactive monomers in a process called *attenuation* (Chu et al. 1996; Winegarden et al. 1996; Kline and Morimoto 1997; Xia and Voellmy 1997).

Previous studies have demonstrated that serine residues 303, 307 and 363 in the regulatory domain of human HSF-1 are constitutively phosphorylated by GSK3, ERK1/2, and JNK/PKC respectively, and *in vivo* mutational analyses have shown that these phosphorylations function to repress the DNA-binding and transcriptional activity of HSF-1 (Chu et al. 1996; Knauf et al. 1996; Kline and Morimoto 1997; Chu et al. 1998; Dai et al. 2000; Xavier et al. 2000). On the other hand, phosphorylation on Serine 230, 326 and 419 has been shown to promote transcriptional activity (Holmberg et al. 2001; Westerheide and Morimoto 2005). Aging is associated with a decrease in HSF-1 transcriptional activity as seen in cell free systems and isolated hepatocytes (Heydari et al. 2000).

1.5.5.2. Manipulating heat shock protein expression:

Most of the pioneering work describing the protective role of HSPs has been done in cell culture, but to study the physiological effects of HSP upregulation and its potential in therapy, various methods of HSP induction are being actively pursued in

laboratory animals (reviewed by (Tolson and Roberts 2005)). Physical methods include water immersion, where anaesthetized animals are immersed in a water bath set at 41.5°C and core body temperature is maintained between 41°C and 41.5°C for 20 min. Upregulation of HSPs is seen as early as 6 hours, peaking around 48 hr, and persists till 96 hours. A similar treatment can be given using a heating pad (Demirel et al. 2003). When HSP upregulation is needed in a specific region of the body, local heating is used by using small heating steel cylinders (Oishi et al. 2002), diathermy (Touchberry et al. 2008), photoirradiation with an 815 nm diode lazer (Souil et al. 2001), ultrasound (Madio et al. 1998) or microwave radiation of 2.06 GHz (Walters et al. 1998). Genetic alteration approaches have produced several HSP overexpression mouse models (Marber et al. 1995), some of which are now commercially available. Exercise increases HSP expression in several tissues including the skeletal muscle, myocardial tissue, adrenal gland, brain and liver (Skidmore et al. 1995; Powers et al. 1998; Campisi et al. 2003; Gonzalez and Manso 2004). Cold treatment and psychological stress has shown HSP induction in a few tissues (Matz et al. 1996; Hoekstra et al. 1998), but results from these applications have been limited. Lastly, pharmacological methods inducing HSPs include amphetamine (Salminen et al. 1997), aspirin (Ghavami et al. 2002), bimoclomol/arimoclomol/BRX-220 (Kurthy et al. 2002; Lubbers et al. 2002), geranylgeranylacetone (Ikeyama et al. 2001; Ooie et al. 2001; Fujiki et al. 2003; Yasuda et al. 2005), stannous chloride (House et al. 2001), β-estradiol (Papaconstantinou et al. 2003), resveratrol (Putics et al. 2008), curcumin (Kanitkar and Bhonde 2008), glutamine (Wischmeyer et al. 2001), HSP90-inhibitors

such as geldanamycin (Kiang et al. 2004) and many more. Modified derivatives of several of the above drugs are being actively investigated for lower toxicity and better efficacy.

1.5.5.3. Drug trials:

Several HSP-inducing drugs are being actively pursued in bench research as well as clinical trials for treatment of a variety of diseases from neurodegenerative diseases to cancer and autoimmune diseases. The non-toxic hydroxylamine derivative Bimoclomol or its related conjugates BRX-220 and BGP15 have been explored in treatment against insulin resistance, diabetes and diabetic neuropathy in animal models (Vigh et al. 1997; Kurthy et al. 2002; Sebokova et al. 2002). Substances in this class of therapeutics are thought to function as co-activators of the heat shock protein response (Torok et al. 2003). N-Gene Research Laboratories, Inc. has reported positive Phase II results with BGP-15 functioning as an insulin sensitizer. In 42 nondiabetic individuals with insulin resistance given the agent for 28 days, whole-body glucose utilization and mitochondrial function was significantly improved (Kolonics et al. 2006).

1.6. Antioxidant treatments to alleviate oxidative stress and reverse insulin resistance

Oxidative stress has been implicated as one of the important mechanisms of insulin resistance (Houstis et al. 2006). Consequently, antioxidant treatments to alleviate oxidative stress are expected to improve insulin sensitivity. Several anti-oxidants have been shown to successfully improve insulin sensitivity in insulin resistance and diabetes, including alpha-lipoic acid (LA) (Jacob et al. 1995; Bitar et al. 2004), vitamin E (Laight et al. 1999), Tempol, a compound that mimics the action of superoxide dismutase (Blendea et al. 2005), or a combination of vitamin E, vitamin C and lipoic acid (Vinayagamoorthi et al. 2008). Of these antioxidants, LA presents a very potent effect and has unique properties that other anti-oxidants lack. Apart from its anti-oxidant properties that reduce oxidative stress, LA also possesses certain pro-oxidant properties (Konrad 2005) that enable it to directly induce the insulin signaling cascade and stimulate glucose uptake (Estrada et al. 1996; Konrad et al. 2001). LA can also induce glucose uptake through insulin independent pathways via the activation of AMPK (Khanna et al. 1999; Lee et al. 2005). Besides increasing the GSH/oxidized glutathione GSSG ratio, LA also enhances GSH biosynthesis (Packer et al. 2001).

LA is a short chain fatty acid that functions as a cofactor in the pyruvate dehydrogenase complex (Konrad et al. 1999). Natural synthesis of LA occurs in the liver. Two enantiomers of LA are seen: R(+) is the naturally occurring stereoisoform,

more potent that the S(+) for insulin sensitivity actions. In fact, S(+) LA could be inhibitory to insulin action (Klip et al. 1994). Synthetic LA occurs as a racemic mixture.

LA has both hydrophilic as well as hydrophobic properties, another unique characteristic among anti-oxidants. Thus LA can elicit its action in both the cytosol as well as the plasma membrane; in serum as well as in lipoproteins (Kagan et al. 1992). Three activities of LA and its reduced form, dihydrolipoic acid have been observed: 1.) direct ROS scavenging, 2.) capacity to regenerate endogenous anti-oxidants such as glutathione and Vitamin C and E due to its redox potential, and 3.) metal chelating activity resulting in reduced ROS production through a metal-catalyzed chemical reaction (Konrad 2005).

Lipoic acid protects against oxidative stress induced impairment in insulin stimulation of protein kinase B and glucose transport in 3T3-L1 adipocytes (Rudich et al. 1999). In L6 muscle cells, LA protects against oxidative stress-induced insulin resistance when used in micromolar concentrations (Maddux et al. 2001). At higher concentrations (millimolar), LA can directly engage the insulin pathway to stimulate glucose transport (Yaworsky et al. 2000). *In vivo* anti-diabetic effects of LA have been reported in several animal models, including Goto-Kakizaki rats (Bitar et al. 2004), streptozotocin injected rats (Khamaisi et al. 1997), obese zucker fatty rats (Saengsirisuwan et al. 2004), high fructose fed rats (Thirunavukkarasu et al. 2004), diabetes prone OLETF rats (Song et al. 2005) and high fat diet-fed rats (Yang et al. 2008). In humans, LA has been used in Germany for more than 30 years to treat

diabetic neuropathy and liver cirrhosis (Ziegler et al. 1999). Oral administration of alpha-lipoic acid improves glucose effectiveness in lean and obese patients with type 2 diabetes (Jacob et al. 1999; Konrad et al. 1999; Strokov et al. 2000; Kamenova 2006). Other reports suggest that oral administration of LA has shown only marginal effects, while intravenous infusion has the best effects on insulin mediated glucose transport in T2D patients (Evans and Goldfine 2000).

Despite the beneficial effects of LA repeatedly seen in humans and animal models, the exact mechanism of action of LA remains unclear. Further studies are needed to understand if the benefits of LA are from a combination of its anti-oxidant and pro-oxidant properties.

1.7. Research questions

With the background of the above literature, our studies attempted to fill the knowledge gaps in the understanding of insulin resistance and identify potential therapeutic targets against insulin resistance. Our primary goals were to determine the roles of heat shock proteins and their association with insulin action in two different models of insulin resistance- aging and diet induced-insulin resistance. We examined the mechanisms of insulin resistance and their alleviation with HSPs using interventions such as anti-oxidant treatment and heat treatment. HSPs were examined in fast- and slow-twitch muscle types and in various regions of the brain.

Chapter 2

AGE-RELATED DIFFERENCES IN SKELETAL MUSCLE INSULIN SIGNALING: THE ROLE OF STRESS KINASES AND HEAT SHOCK PROTEINS

Anisha A. Gupte, Gregory L. Bomhoff, and Paige C. Geiger

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2.1. Abstract

Aging is associated with an increase in insulin resistance in skeletal muscle, yet the underlying mechanism is not well established. We hypothesize that with aging, a chronic increase in stress kinase activation, coupled with a decrease in oxidative capacity, leads to insulin resistance in skeletal muscle. In aged (24 month-old) and young (3 month-old) Fischer 344 rats, 2-deoxy glucose uptake and insulin signaling (as measured by phosphorylation of IRS-1, Akt and AS160) decreased significantly with age. Activation of c-Jun NH2-terminal kinase (JNK), glycogen serine kinase-3β (GSK-3β), and degradation of IkBα by the upstream inhibitor of kappa B kinase (IKKβ), as measured by Western blot analysis, were increased with age in both soleus and Epi muscles. However, much higher activation of these kinases in Epi muscles from young rats compared to soleus results in a greater effect of these kinases on insulin signaling in fast-twitch muscle with age. Heat shock protein 72 (HSP72) expression and phosphorylation of HSP25 were higher in soleus compared to Epi muscles and both parameters decreased with age. Age and fiber type differences in cytochrome oxidase activity are consistent with observed changes in HSP expression and activation. Our results demonstrate a significant difference in the ability of slow-twitch and fast-twitch muscles to respond to insulin and regulate glucose with age. A greater constitutive HSP expression and lower stress kinase activation may account for the ability of slow-twitch muscles to preserve the capacity to respond to insulin and maintain glucose homeostasis with age.

2.2. Introduction

Aging is associated with a decrease in the body's ability to respond to the hormone insulin, a condition referred to as insulin resistance. Insulin resistance represents an independent risk factor in age-related diseases and insulin resistance of skeletal muscle glucose transport represents a key defect in the development of type 2 diabetes. The underlying mechanism for age-related insulin resistance remains poorly defined, yet increased oxidative stress, inflammation and impaired defense against stress are likely involved.

The initial step in the insulin-signaling pathway is binding of insulin to its receptor at the cell surface of tissue such as muscle, fat or liver. Insulin binding stimulates the intrinsic tyrosine kinase activity of the insulin receptor resulting in the tyrosine phosphorylation of its cytosolic substrate, insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) (White 1998). After tyrosine phosphorylation, IRS-1 and IRS-2 bind and activate the enzyme phosphatidylinositol 3-kinase (PI3K). PI3K activation results in serine phosphorylation of Akt/protein kinase B (Akt), and ultimately, stimulation of glucose transport in skeletal muscle and adipose tissue. Previous studies indicate the development of insulin resistance with age occurs downstream of the insulin receptor (Arias et al. 2001).

IRS-1 is the primary IRS expressed in skeletal muscle and acts as a metabolic switch for the insulin signaling pathway (Tamemoto et al. 1994). Serine/threonine phosphorylation of IRS-1 can result in suppression of the insulin signaling pathway

and increased serine phosphorylation of IRS-1 has been reported in animal models of insulin resistance (Werner et al. 2004) and in muscles of patients with insulin resistance (Bandyopadhyay et al. 2005). Previous studies have shown a decrease in tyrosine IRS-1 phosphorylation (Carvalho et al. 1996) and a decrease in total IRS-1 protein (Arias et al. 2001) in aged rats. To our knowledge, only one other study has assessed chronic serine phosphorylation of IRS-1 with age and they showed an increase in serine IRS-1 phosphorylation in 30 month-old Brown Norway/Fischer 344 rats compared to 6 month-old rats (Haddad and Adams 2006). These investigators examined the mixed fiber type medial gastrocnemius muscle and therefore could not make any correlations between muscle fiber type specific insulin signaling and age. Skeletal muscle displays a fiber type specific response in the activation of key insulin signaling intermediates (Song et al. 1999), and given the preferential atrophy of fast-twitch fibers that occurs with age (Lexell 1995), we might expect insulin signaling to be compromised to a greater extent in fast-twitch muscles compared to slow. The current study examined the effect of age on IRS-1 function in fast-twitch and slow-twitch rat skeletal muscles with age.

Obesity-induced insulin resistance is characterized by increased oxidative stress, inflammation and impaired defense against stress (Wellen and Hotamisligil 2005; Chung et al. 2008). Obesity is linked to a state of chronic inflammation resulting in TNF-α mediated activation of serine threonine kinases, primarily c-Jun NH2-terminal kinase (JNK), inhibitor of kappa B kinase (IKKβ) and PKCθ (Wellen and Hotamisligil 2005). These serine/threonine kinases phosphorylate IRS-1 on serine

residues and suppress the insulin signaling pathway. The important role of JNK and IKKβ in obesity models of insulin resistance is well established and genetic models that alter JNK and IKKβ expression offer protection against obesity induced insulin resistance (Hirosumi et al. 2002; Arkan et al. 2005). Aging is also characterized by chronic inflammation and oxidative stress (Broome et al. 2006), yet the extent to which inflammatory mediators affect insulin signaling in aged skeletal muscle is not known.

A decline in the body's best known endogenous defense system, the heat stress response, could play an important role in the development of insulin resistance. Aging, hyperlipidemia, and type 2 diabetes are all associated with a diminished heat stress response, as measured by decreased expression of heat shock protein 72 (HSP72) (Hooper and Hooper 2005; Selsby et al. 2005; Najemnikova et al. 2007) and recent studies of individuals with type 2 diabetes demonstrate that reduced skeletal muscle HSP72 mRNA correlates with the degree of insulin resistance (Kurucz et al. 2002; Bruce et al. 2003). Although expression levels of heat shock protein 25 (HSP25) were not reduced in streptozotocin-induced diabetic rats, the ability to increase HSP25 with heat stress in these animals was attenuated (Najemnikova et al. 2007). In addition to their chaperonin functions, HSPs have been shown to confer resistance to oxidative stress and inhibit stress kinase activation (Gabai et al. 1997; Park et al. 2001). Consequently, HSP72 expression levels and HSP25 activation may play a significant role in the development of age-related insulin resistance in skeletal muscle. HSPs have also been shown to have a protective effect on mitochondrial

function (Polla et al. 1996) and therefore could have a significant role in preserving muscle oxidative capacity with age. In order to examine potential mediators of insulin resistance of glucose uptake with age, we measured insulin signaling intermediates, stress kinases, and HSPs in fast-twitch and slow-twitch skeletal muscles from young and aged rats. We hypothesize that with aging, a chronic increase in stress kinase activation, coupled with a decrease in oxidative capacity, leads to insulin resistance in skeletal muscle. Comparing the oxidative soleus muscle and the more glycolytic Epi muscle will allow us to determine the extent to which the muscle's oxidative capacity impacts the development of age-related insulin resistance.

2.3. Materials and methods

Materials

[^{14}C] Mannitol was obtained from ICN Radiochemicals (Irvine, CA). 2-Deoxy [1,2-^{3}H] glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies against phospho-SAPK/JNK(T183/Y185), total SAPK/JNK, phospho-Akt (S473), total Akt, IkBα , phospho-GSK-3 α/β (Ser21/9), and phospho-PKCθ (T538) were purchased from Cell Signaling (Beverly, MA). Anti-HSP72, anti-phospho-HSP25 (S82) and anti-HSP25 were obtained from Stressgen (Victoria, BC, Canada), and anti-tubulin was obtained from Sigma (St.Louis, MO). Anti-PKCθ and goat-anti-rabbit HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Anti-phospho-Ser307-IRS-1, phospho-AS160 (Thr 642) and total AS160 antibodies were purchased from Upstate (Lake Placid, NY), anti-phospho-Tyr612-IRS-1 from Biosource (Camarillo, CA) and anti-IRS-1 was from BD Biosciences (Franklin lakes, NJ). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma (St.Louis, MO).

Experimental animals and muscle dissection

The Fischer 344 rat strain provides a suitable aging model due to its relatively short life span (senescence is reached at 24 months) (Yu et al. 1985). In addition,

considerable background data exists for this particular strain, including the extensive characterization of age-associated changes under many environmental and genetic conditions. Finally, barrier-reared aging colonies of Fischer 344 rats, maintained under the supervision of the NIA, are readily available for purchase from reputable commercial breeders. Male Fischer 344 rats were purchased at 3 (n=6, body mass 238.50 ± 5.11 g) and 24 (n=6, body mass 393.0 ± 17.72 g) months of age via the National Institutes of Health. Rats were housed in pairs in large cages in a temperature controlled facility (24+1 ^{o}C) with a 12 h light/dark cycle. All animals were given free access to Purina Rat Chow and water *ad libitum*. A total of 5 rats per age group were used in the current study. The rats were fasted for 12 h overnight prior to the following morning's experiment to remove effects of endogenous insulin on the experiments. During this time, the normal 12 h dark cycle was maintained. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) followed by the removal of the soleus and epitrochlearis (Epi) muscles. Soleus muscles consist predominantly of slow-twitch red fibers (84% type I, 16% type II) (Ariano et al. 1973), while the Epi is comprised of primarily fast-twitch white fibers (15% type I, 20% type IIa, 65% type IIb) (Nesher et al. 1980). Soleus muscles were split prior to incubation to allow adequate diffusion of oxygen and substrates. In spite of the sarcopenia associated with age, due to the greater body weight of the aged rats compared to the young rats, the soleus muscles of the aged rats remained larger than those from the young rats. The slightly larger soleus muscles of aged rats were carefully trimmed to remain comparable in size with those

of the young soleus. Muscle wet weight was used to determine equivalent size. The thin Epi muscles are well suited for glucose transport and the Epi does not significantly increase in thickness with age (Cartee et al. 1993), so the intact Epi is used for all experiments. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Muscle incubations

Following dissection, muscles recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O_2/5% CO_2. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, muscles were either treated with 1 mU/ml insulin or left untreated for 30 min and then clamp frozen in liquid nitrogen. For measurement of basal MAP and stress kinase phosphorylation, soleus and Epi muscles from young and aged F344 rats were removed and rapidly frozen for Western blot analysis.

Measurement of glucose transport activity

The muscles were rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with or without insulin. After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2[1,2-^{3}H] deoxyglucose (2-DG) (1.5 μCi/ml) and 36 mM [^{14}C] mannitol (0.2 μCi/ml), with or without insulin, with a gas phase of 95% O_2/5% CO_2, in a shaking incubator. The muscles were then blotted, clamp-frozen, and processed for determination of

intracellular 2-DG accumulation and extracellular space as described previously (Young et al. 1986; Geiger et al. 2006).

Measurement of cytochrome oxidase activity

Mitochondria were isolated from soleus and epitroclearis muscles as described previously (Ghosh et al. 2007). Briefly, muscles were dissected out and homogenized in a mitochondria isolation medium (0.32 M sucrose, 2 mM K^+EDTA, 10 mM Tris Base, 0.3% BSA, 1 mM ATP, pH 7.4). After 3 low speed centrifugations (two at 3200 rpm and one at 11,000 rpm), the final supernatant was layered on a Ficoll gradient (10-7.5%) and subjected to ultracentrifugation at 27,500 rpm for 45 min. The mitochondrial pellet was resuspended in BSA-free isolation buffer. Protein concentrations were determined with a Bradford assay. For the cytochrome oxidase assay, a cuvette containing 50 μg of protein, potassium phosphate buffer (20 mM, pH 7.0), and dodecyl maltoside (20 μl of 10 mg/ml stock solution) was warmed to 30°C for three min. The reaction was initiated by addition of 25 μM reduced cytochrome *c*, which brought the total cuvette volume to 1 ml. The oxidation of the reduced cytochrome *c* was followed for 2 min at 550 nm on a DU series spectrophotometer (Beckman Coulter, Fullerton, CA). To facilitate calculation of enzyme activity, the maximally oxidized cytochrome *c* absorbance was determined by adding a few grains of potassium ferricyanide. The activity was calculated and normalized to the amount of protein (seconds^{-1} per milligram of protein).

Western Blotting

Clamp frozen soleus and Epi muscles were homogenized in a 12:1 (volume to weight) ratio of ice cold buffer from Biosource containing: 10 mM Tris-HCL (pH 7.4), 100 mM NaCl, 1 mM each of EDTA, EGTA, NaF, PMSF and 2 mM Na_3VO_4, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 10% glycerol, 0.1 % SDS, 0.5% deoxycholate, 250 μl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes and then centrifuged for 20 minutes at 13,000 rpm at 4°C. The protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 2X Laemmli buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 5 minutes.

30-100 μg of protein was subjected to SDS-PAGE (6.25-10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA/tank). Membranes were blocked for 1 hour at room temperature in TBST 5 % non-fat dry milk followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1000. Antibodies were diluted in Tris-buffered saline, 0.1% Tween-20 (TBST), 5% BSA. Following 3 brief rinses in TBST, blots were incubated in TBST 1% non-fat dry milk supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 hour at room temperature. Bands were visualized by ECL and quantified using densitometry. To allow direct comparisons between age groups and muscle types, young and aged, soleus and Epi samples were run on the same gel and identical conditions (including transfer time, washing conditions, antibody dilutions and chemiluminescence detection) were maintained

across gels. Equal amounts of protein were loaded onto each gel lane and to ensure accuracy, protein concentration was determined in triplicate prior to gel loading. To verify transfer of proteins and equal loading of lanes, the membranes were stained with ponceau S (Figure 2). From the ponceau stain, a protein band was selected at random (~60kDa) and quantified in each lane via densitometry. This band was directly proportional to the amount of protein loaded and there was no significant difference in band intensity across soleus and Epi muscles. Blots were stripped and probed for tubulin to further assess equal protein loading and transfer. Tubulin expression does not change with age in soleus and Epi muscles, making this an accurate loading control for age comparisons within each muscle. However, tubulin expression did differ between soleus and Epi muscles and therefore could not be used as a loading control for soleus and Epi muscle protein comparisons.

FIGURE 2.

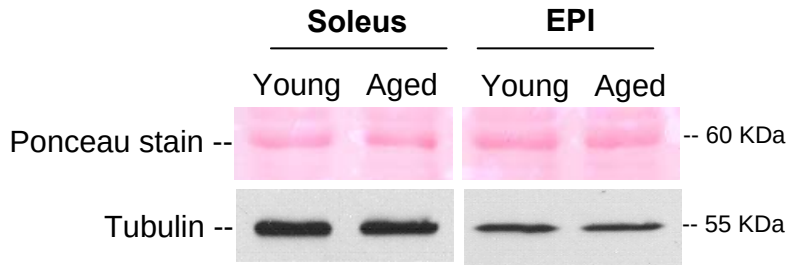


Figure 2. Western blot loading controls. Samples from both young and aged soleus and Epi muscles were run together on gels to allow comparison across groups. Following the ponceau S stain, a protein band was selected at random (~60kDa) and quantified in each lane via densitometry. This band was directly proportional to the amount of protein loaded and there was no significant difference in band intensity across soleus and Epi muscles. Blots were stripped and probed for tubulin to further assess equal protein loading and transfer. Tubulin expression does not change with age in soleus and Epi muscles, making this an accurate loading control for age comparisons and further verifying equal loading.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used when both age and muscle fiber type differences were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P<0.05$.

2.4. Results

Insulin-stimulated glucose uptake

Glucose uptake in isolated soleus and Epi muscles was measured using 2-DG uptake analysis. Figure 3 shows the basal (non-insulin stimulated) and insulin-stimulated 2-DG response in soleus and Epi muscles from young and aged rats. Basal 2-DG uptake was higher in soleus muscles compared to Epi from both young and aged rats. Basal 2-DG uptake was maintained with age in the soleus muscle, while a significant decrease in basal glucose uptake was observed in Epi muscles with age (32% decrease from young). To determine the muscle glucose uptake in response to insulin, soleus and Epi muscles were incubated in the presence of 1 mU/ml insulin. As shown in Figure 3, insulin-stimulated glucose uptake was higher in young soleus compared to young Epi muscles. In aged rats, insulin-stimulated glucose uptake was significantly decreased in both the soleus and Epi muscles. Although the decrease in insulin-stimulated 2-DG uptake with age was slightly greater in the soleus muscles (35% decrease from young) compared to Epi muscles (26% decrease from young), the insulin-stimulated 2-DG uptake remained greater in soleus muscles compared to Epi.

FIGURE 3.

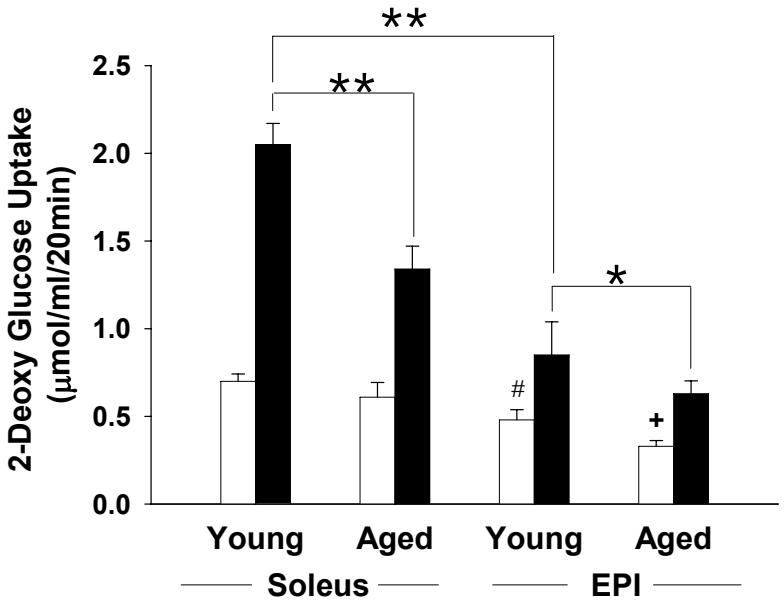


Figure 3. Insulin stimulated glucose transport in muscles from young and aged rats. Young (3 mo) and aged (24 mo) F344 rats were fasted overnight and the soleus and Epi muscles were dissected out. Muscles recovered in KHB for 1 hr and were then incubated without (open bars) or with (filled bars) insulin (1 mU/ml) in flasks containing KHB with 2[1,2-^{3}H]-deoxy glucose (1.5 μCi/ml) and [^{14}C] mannitol. Insulin stimulation resulted in a significant increase from basal glucose uptake for all muscles shown ($P<0.005$). Intracellular 2-DG and extracellular space were quantified in a scintillation counter. Values are means of $\pm$ SE for muscles from 10-14 muscles per group. Symbols for basal 2-DG values, open bars: Significance between young Epi and soleus basal glucose uptake #$P<0.05$. Significance between young and aged Epi basal glucose uptake +$P<0.05$. Symbols for insulin-stimulated glucose uptake, filled bars: *$P<0.05$, **$P<0.001$.

Insulin-stimulated phosphorylation of Akt

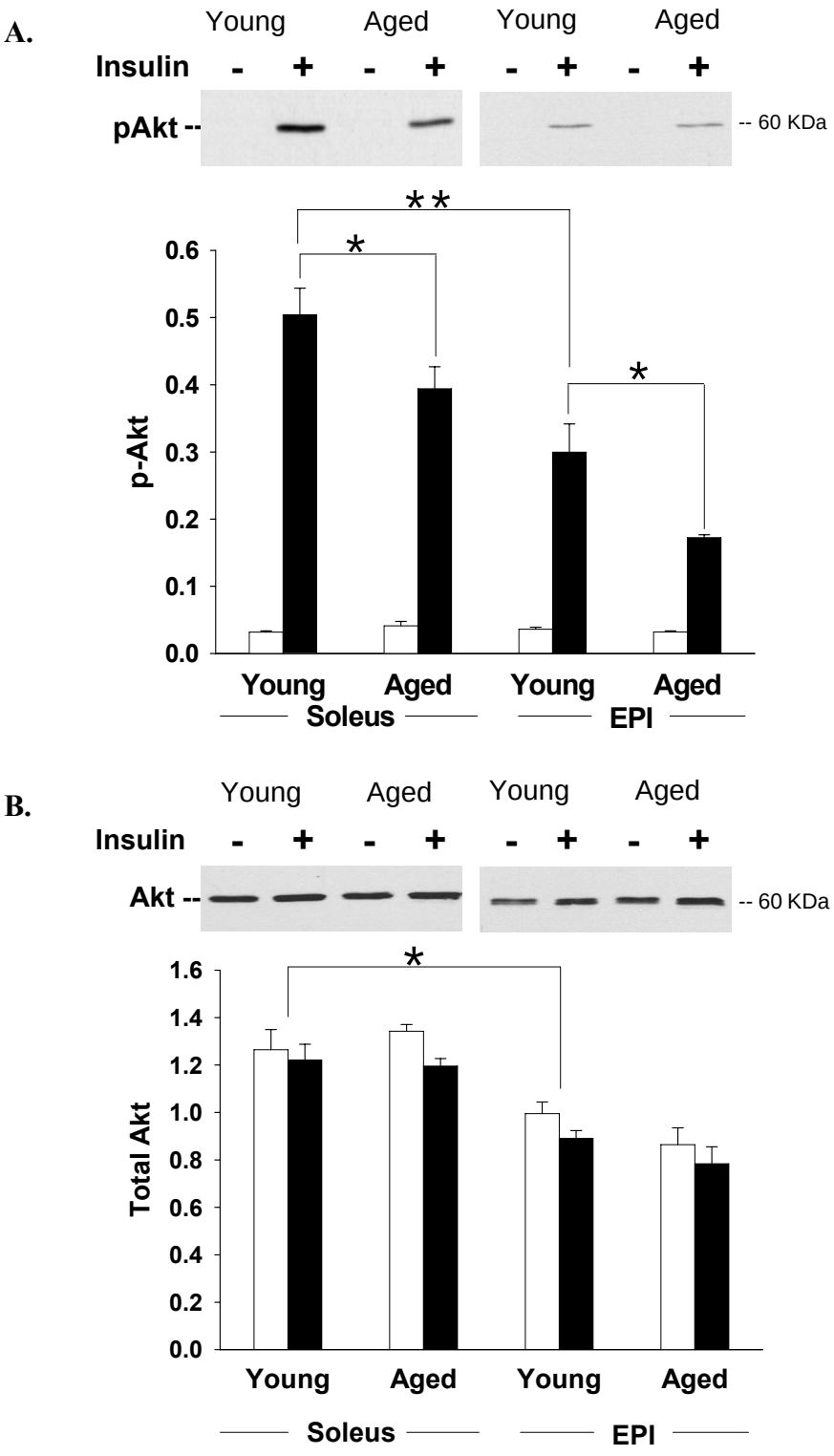
Akt is a serine kinase located downstream of IRS-1/PI3K in the insulin signaling pathway. Akt phosphorylation in the absence and presence of 1 mU/ml insulin was measured by Western blot analysis with a phosphospecific antibody that recognizes Akt when phosphorylated on Ser 473. Basal, non-insulin stimulated, Akt phosphorylation levels were similar between soleus and Epi muscles from both young and aged rats (Figure 4A). The pattern of Akt phosphorylation in response to insulin displayed a similar pattern to the 2-DG transport in soleus and Epi muscles. As shown in Figure 4A, Akt phosphorylation on Ser 473 was increased with insulin to a greater extent in the soleus muscle compared to the Epi in both young and aged muscles. Insulin-stimulated Akt phosphorylation was significantly decreased with age in both soleus and Epi muscles. The relative decrease in Akt phosphorylation with age is greatest in Epi muscles (35% decrease from young) compared with soleus muscles (22% decrease from young). Basal and insulin-stimulated total Akt protein expression was measured and as shown in Figure 4B, there were no differences in total Akt expression in response to insulin. However, muscle fiber type differences in total Akt expression were observed with soleus muscle Akt expression greater than Epi in young rats. Somewhat surprisingly, there was no decrease in total Akt expression with age in either the soleus or Epi muscles.

Insulin-stimulated phosphorylation of AS160

Akt substrate of 160 kDa (AS160) is a Rab GTPase-activating protein previously shown to be activated by insulin and muscle contraction, the latter most

likely via AMPK (Bruss et al. 2005; Kramer et al. 2006). We measured AS160 phosphorylation in the absence and presence of 1 mU/ml insulin by Western blot analysis with a phosphospecific antibody that recognizes AS160 when phosphorylated on Thr642. Basal, non-insulin stimulated, phosphorylation of AS160 did not differ between soleus and Epi muscles (Figure 4C). Although basal phosphorylation of AS160 increased slightly with age in the soleus muscle, no significant age differences in basal AS160 phosphorylation were observed in either muscle fiber type ($P<0.08$ between young and aged basal AS160 phosphorylation). In response to insulin, phosphorylation of AS160 was increased to the same extent in soleus and Epi muscles. An age-related decrease in insulin-stimulated AS160 phosphorylation was observed in both soleus and Epi muscle (37% and 37.5% in soleus and Epi, respectively). Figure 4D demonstrates basal and insulin-stimulated total AS160 protein expression and similar to Akt, there were no effects of insulin on total protein expression of AS160 in soleus and Epi muscles. Total AS160 protein expression did not differ between soleus and Epi muscles, however there was a significant effect of age in both muscles. Total AS160 protein expression decreased by 22% and 33% in soleus and Epi muscles, respectively.

FIGURE 4.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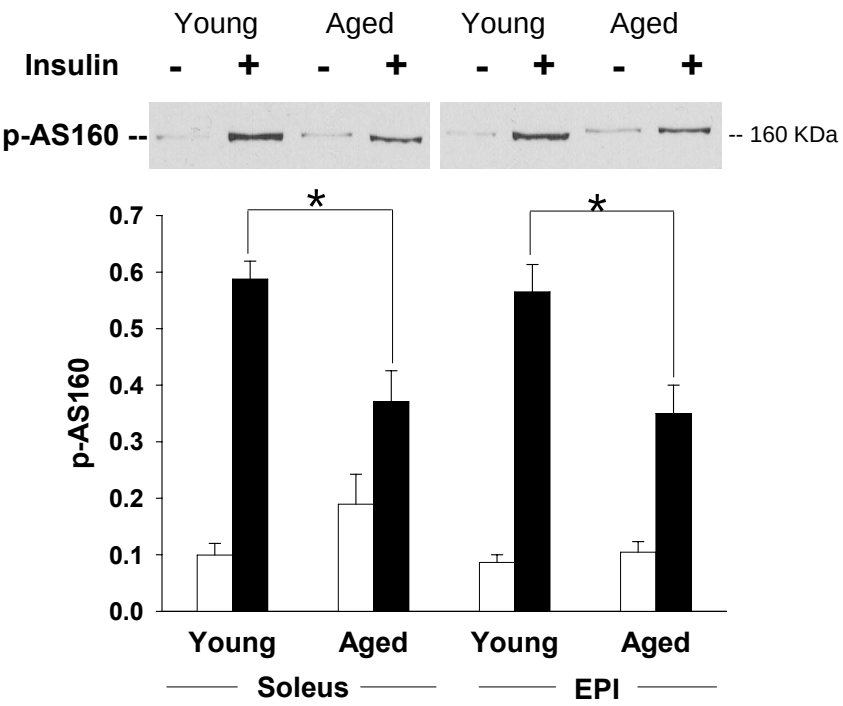
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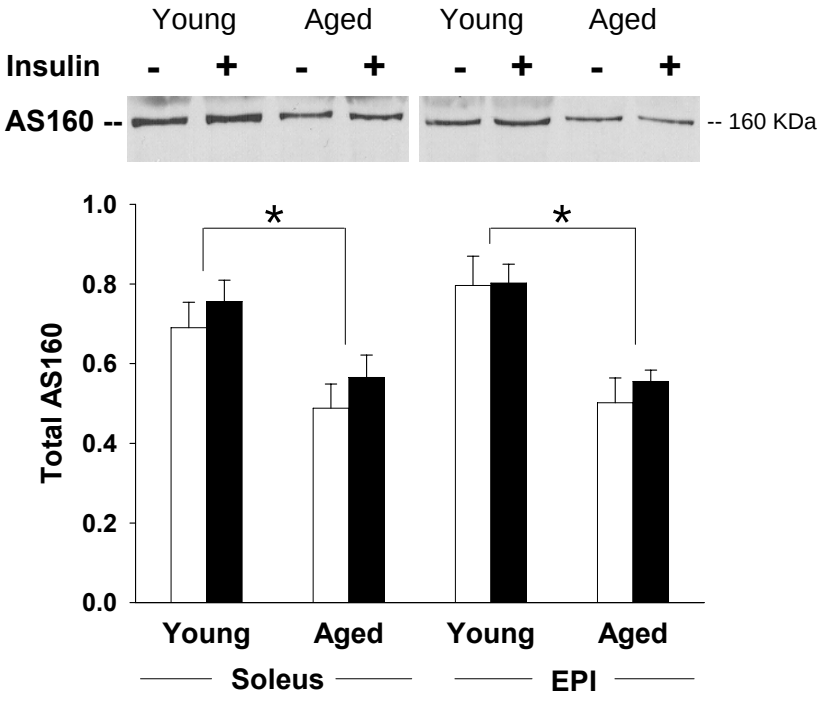
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Soleus
EPI

Figure 4. Effect of insulin on phosphorylation of Akt and AS160 in young and aged muscles. Soleus and Epi muscles were incubated in the absence (open bars) or presence (filled bars) of insulin (1 mU/ml) for exactly 10 min. Homogenates of clamp frozen muscles were used for measurements of phosphorylation of Akt on serine 437 **(A)**, total Akt (**B),** phosphorylation of AS160 **(C)**, and total AS160 **(D)**, with Western blot analysis. Insulin stimulation resulted in a significant increase from basal phosphorylation of Akt and AS160 in all muscles shown ($P<0.05$). The effect of insulin on total Akt and AS160 values was not significant. Values are means of $\pm$ SE for 4-9 muscles per group. *$P<0.05$ and **$P<0.001$.

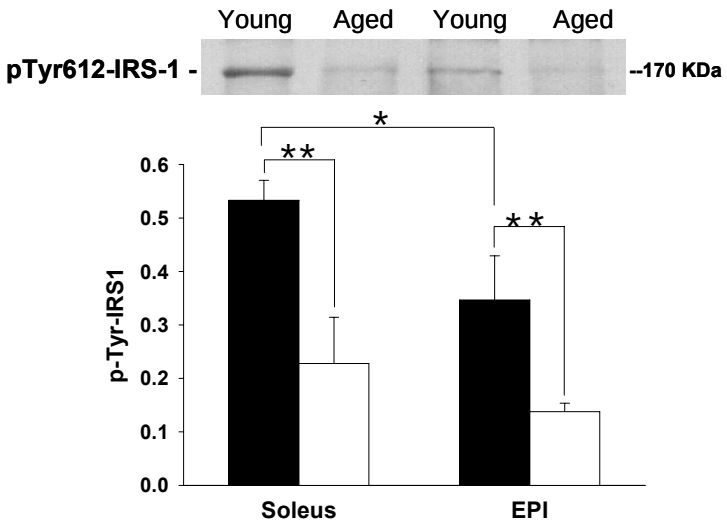
Insulin-stimulated tyrosine and basal serine phosphorylation of IRS-1

Our observations of a decrease in glucose uptake and phosphorylation of Akt and AS160 with age suggest a defect in insulin signaling. IRS-1 functions as a metabolic switch in skeletal muscle, with tyrosine phosphorylation resulting in downstream activation of PI3K and serine phosphorylation of Akt. To determine whether differences in insulin-stimulated IRS-1 tyrosine phosphorylation exist between young and aged soleus and Epi muscles, muscles were exposed to 1 mU/ml insulin for 30 min. Western blots were performed on muscle homogenates with an antibody that recognizes IRS-1 tyrosine 612 phosphorylation. Basal, non-insulin stimulated, tyrosine phosphorylation of IRS-1 is negligible in skeletal muscle and therefore this data is not shown. Figure 5A demonstrates that insulin-stimulated phosphorylation of IRS-1 at tyrosine 612 was significantly greater in soleus muscles compared to Epi for both young and aged muscles. Insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased with age in both soleus and Epi muscles (57% and 60% decrease from young, respectively). Serine phosphorylation of IRS-1 inhibits tyrosine IRS-1 phosphorylation and chronic increased serine phosphorylation could prevent activation of the normal insulin signaling cascade. For this reason, we measured basal, non-insulin stimulated, serine phosphorylation of IRS-1 to determine the extent to which serine phosphorylation of IRS-1 could impact insulin action. Basal IRS-1 serine phosphorylation was measured with an antibody that recognizes IRS-1 when serine 307 is phosphorylated (Figure 5B). Serine phosphorylation of IRS-1 was significantly increased with age in both the soleus and Epi muscles. The

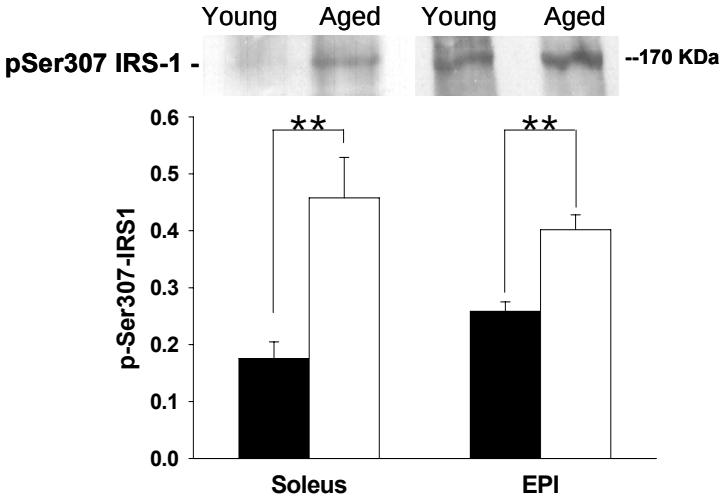
increase in serine 307 phosphorylation in soleus muscles with age was significantly greater than that observed in the Epi muscles (160% and 56% increase above young, respectively). Our findings also reveal a significant decrease in total IRS-1 levels in both muscles with age (Figure 5C). Although total IRS-1 expression is greater in soleus compared to Epi in young rats, the relative decrease in IRS-1 expression with age is similar in both muscles (65% and 64% decrease from young, respectively).

FIGURE 5.

A


Young
Aged
Young
Aged
pTyr612-IRS-1 -
--170 KDa
*
**
**
p-Tyr-IRS1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Soleus
EPI

B.


Young
Aged
Young
Aged
pSer307 IRS-1 -
--170 KDa
**
**
p-Ser307-IRS1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Soleus
EPI

C.

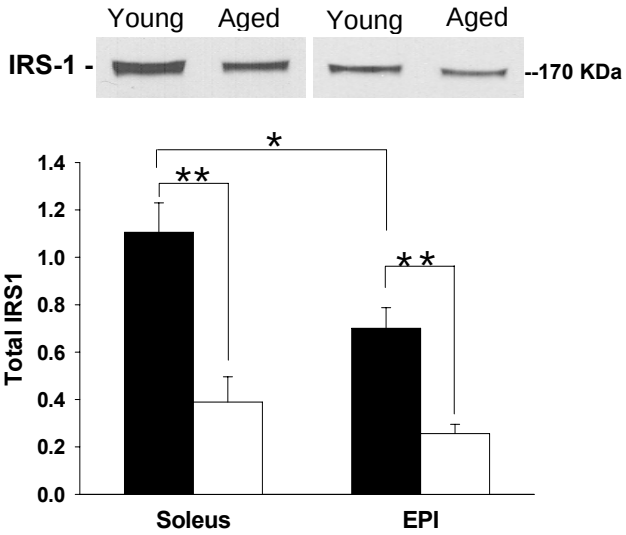

Young
Aged
Young
Aged
IRS-1 -
--170 KDa
*
**
**
Total IRS1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Soleus
EPI

Figure 5. Insulin-stimulated tyrosine and basal serine phosphorylation of IRS-1 in young and aged muscles. Soleus and Epi muscles were dissected from overnight fasted rats and incubated with insulin (1 mU/ml) for 30 min and muscle homogenates were then used to determine the insulin-stimulated tyrosine phosphorylation of IRS-1 **(A)**. Alternatively, muscles were incubated in KHB without insulin to assess basal serine phosphorylation of IRS-1 **(B)**, and total IRS-1 **(C)**. Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of $\pm$ SE for 4-12 muscles per group. *P<0.05 ** P<0.001.

Modulation of serine/threonine kinases

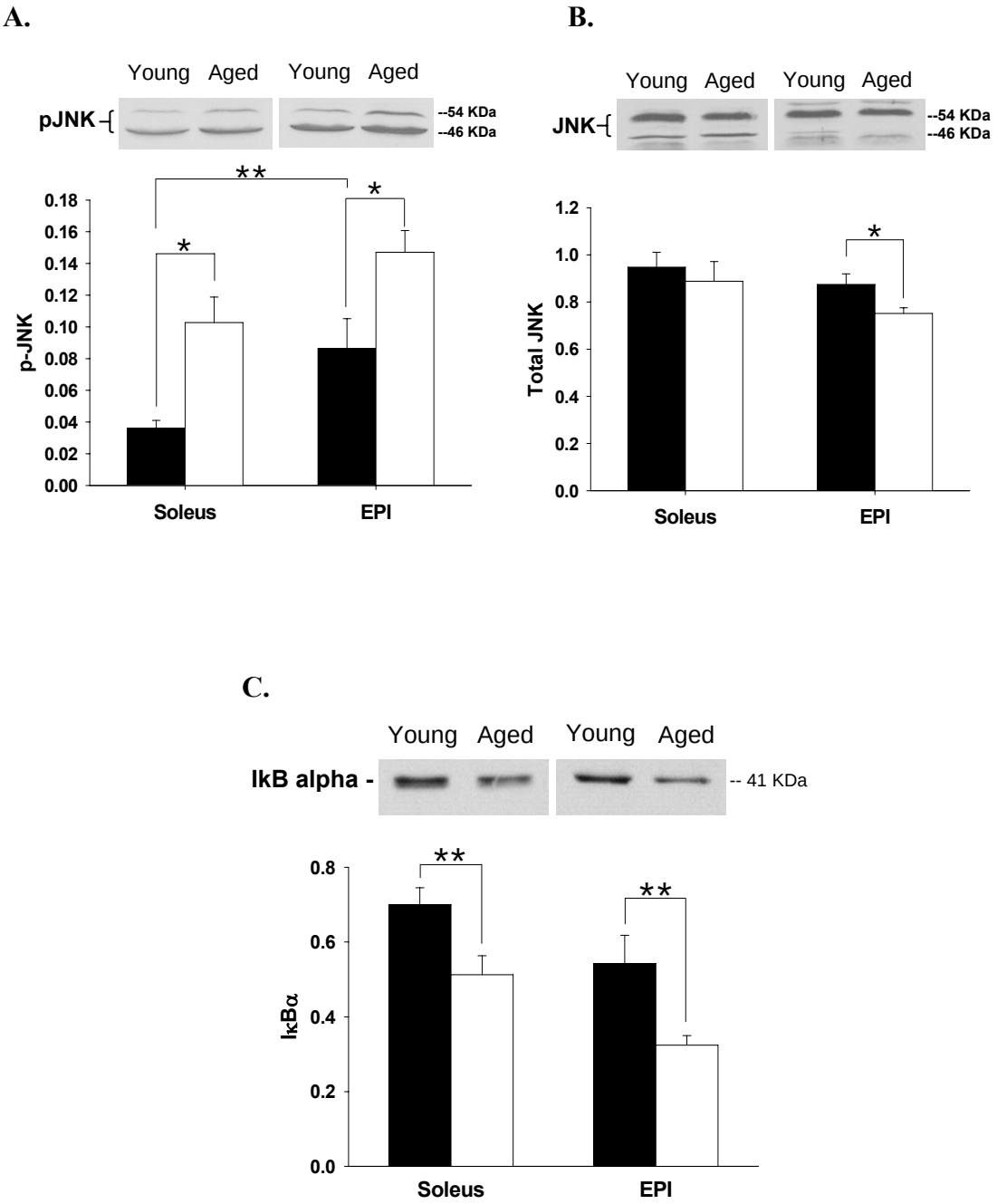
A number of serine/threonine kinases have been identified with the potential to serine phosphorylate IRS-1, with JNK and IKKβ being the most commonly associated with insulin resistance. We measured basal phosphorylation levels of JNK with Western blot analysis in soleus and Epi muscles from young and aged rats (Figure 6A). JNK phosphorylation was significantly lower in soleus muscles compared to Epi muscles in young and aged rats. In both soleus and Epi muscles, JNK phosphorylation increased with age, but to a much greater extent in the soleus compared to the Epi (184% and 70%, respectively). Total JNK protein expression was measured and no significant differences exist between soleus and Epi muscle total JNK expression (Figure 6B). Total JNK decreased significantly with age only in the Epi muscles. These data demonstrate that, regardless of age, JNK phosphorylation is greater in the fast Epi muscles compared to the soleus.

IKKβ activation results in degradation of the downstream target IkBα and IkBα degradation is considered a marker of increased IKKβ activity. Figure 6C demonstrates that IkBα expression levels were higher in soleus muscles compared to Epi muscles from both young and aged rats, indicating a lower level of activation of IKKβ in soleus. With age, a decrease in IkBα protein levels occurred in both soleus and Epi muscles, with a greater degradation in the Epi (27% and 40% decrease, respectively).

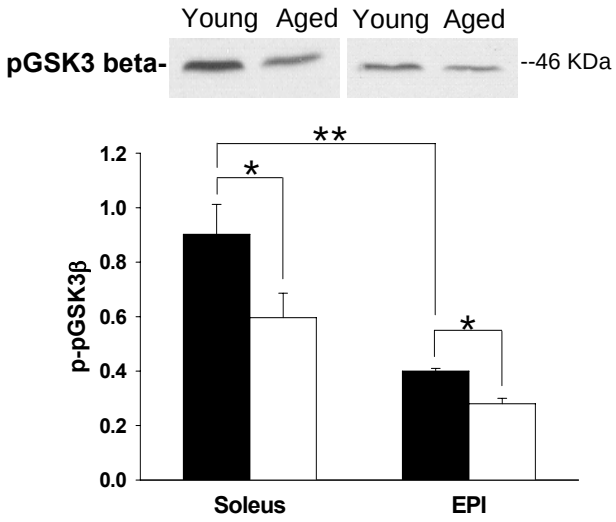
We measured phosphorylation of GSK-3β on serine 9, a measure inversely related to GSK-3 activity (Dokken et al. 2005). As previously shown with JNK and

IKKβ activation, GSK-3β activation is lower in the slow soleus muscle, indicated by increased GSK-3β phosphorylation in young and aged soleus compared with young and aged Epi muscle (Figure 6D). Phosphorylation of GSK-3β on serine 9 is decreased with age in both soleus and Epi muscles to a similar extent (34% and 31% decrease, respectively). Total GSK-3β protein expression is dramatically higher in soleus muscle compared to Epi in both young and aged rats (Figure 6E). GSK-3β protein expression did not change with age in either muscle type. Phospho-PKCθ levels were similar across soleus and Epi muscles from both young and aged rats (data not shown). There was no change in PKCθexpression with age in either muscle type.

FIGURE 6.


A.
Young
Aged
Young
Aged
pJNK
--54 KDa
--46 KDa
p-JNK
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
**
*
*
Soleus
EPI
B.
Young
Aged
Young
Aged
JNK
--54 KDa
--46 KDa
Total JNK
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Soleus
EPI
C.
Young
Aged
Young
Aged
IkB alpha -
-- 41 KDa
IκBα
0.8
0.6
0.4
0.2
0.0
**
**
Soleus
EPI

D.



E.

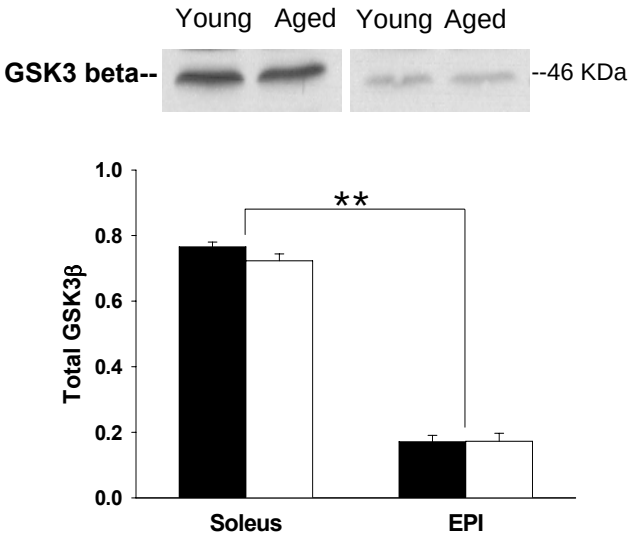


Figure 6. Modulation of serine/threonine kinases in young and aged muscles. Soleus and Epi muscles from young and aged rats were dissected, clamp frozen and homogenized. Lysates were analyzed by Western blot for JNK phosphorylation **(A)**, and total JNK **(B)**, inhibitor I kappa B alpha (IkBα, **C**), and phosphorylation of GSK-3β **(D)** and total GSK-3β **(E)**. Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of ± SE for 4-6 muscles per group. *P<0.05 ** P<0.001.

Modulation of HSPs

In the present study, HSP72 expression was significantly greater in soleus muscles compared to Epi muscles from both young and aged rats (Figure 7A). This is consistent with other results indicating HSP72 expression correlates with muscle oxidative capacity (Locke et al. 1991). HSP72 expression was significantly reduced in both soleus and Epi muscles with age, although to a greater degree in the Epi (10% and 41% decrease, respectively). The phosphorylation state of HSP25 is thought to play an important role in modifying its function (Huey 2006). HSP25 is phosphorylated by MAPKAPK2, a kinase downstream of p38 MAPK (Stokoe et al. 1992). Similar to our findings for HSP72 expression, phosphorylation of HSP25 was greater in soleus muscle compared with Epi from both young and aged rats (Figure 7B). Phosphorylation of HSP25 decreased with age in both soleus and Epi muscles to the same extent (32% and 29% in soleus and Epi, respectively). Total HSP25 expression was greater in the soleus muscle compared to Epi from young rats and aged rats (Figure 7C) but there was no significant change with age in either muscle. This supports previous findings of greater HSP25 expression in oxidative compared with glycolytic muscle (Huey 2006).

FIGURE 7.

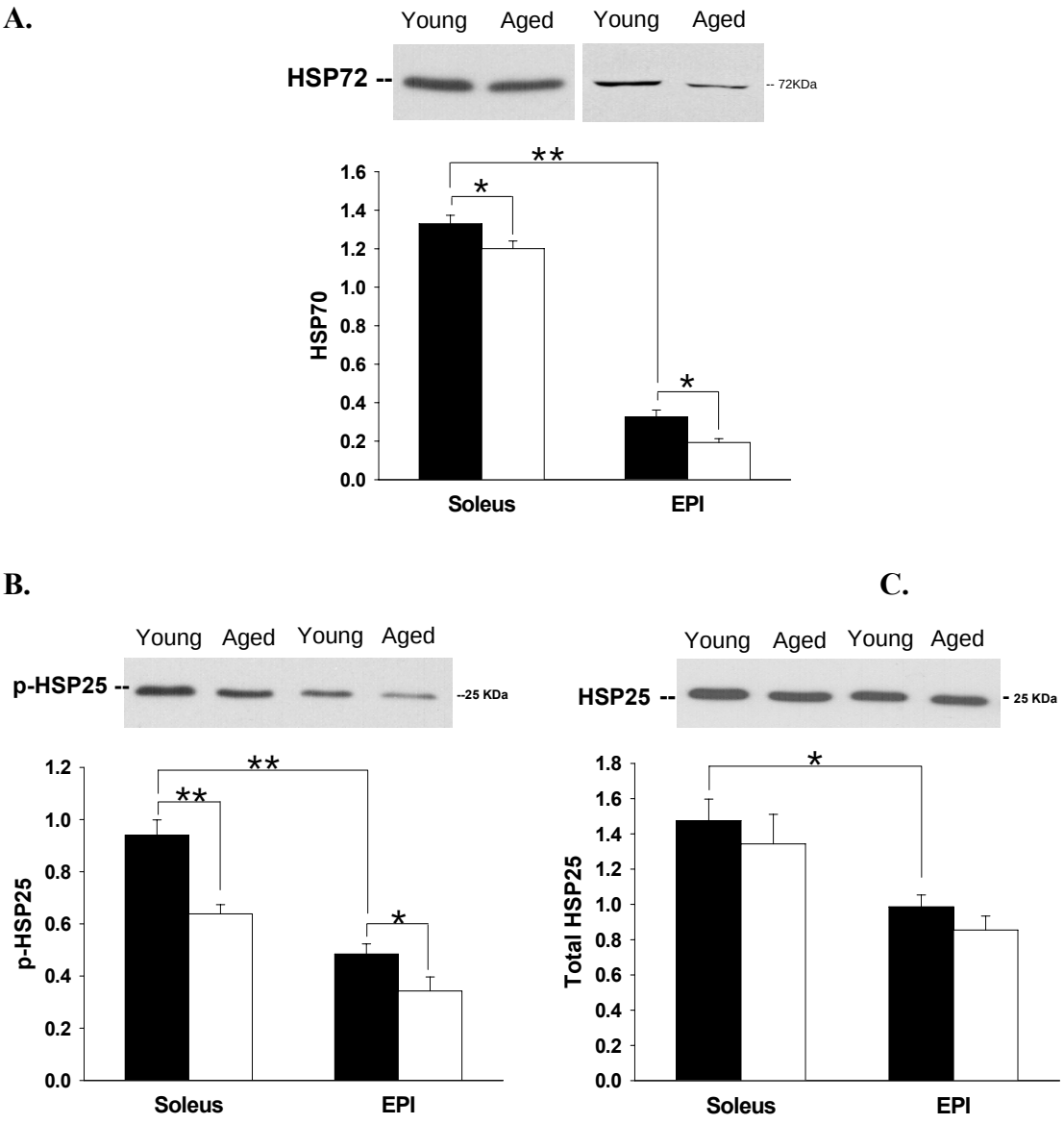


Figure 7. Heat shock protein expression in young and aged muscles. Soleus and Epi muscles from young and aged rats were dissected, clamp frozen and homogenized. Lysates were analyzed by Western blot for HSP72 **(A)**, phosphorylation of HSP25 **(B),** and total HSP25 **(C)**. Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of ± SE for 8-15 muscles per group. *P<0.05 ** P<0.001.

Cytochrome oxidase activity

Cytochrome oxidase activity was measured in soleus and Epi muscle homogenates (Figure 8). Enzyme activities in soleus muscle were 48% higher than Epi muscle from both young and aged rats. Cytochrome oxidase activity decreased with age in both soleus and Epi muscles by approximately 15%. A decrease in cytochrome oxidase activity is indicative of a reduced mitochondrial content per milligram of muscle.

FIGURE 8.

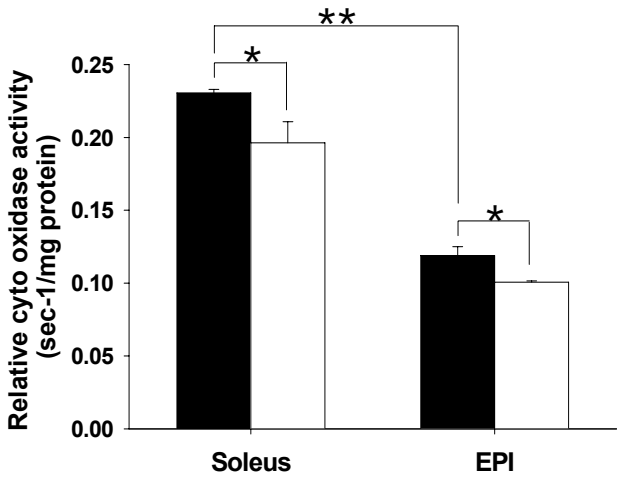


Figure 8. Cytochrome oxidase enzyme activity in young and aged muscles. Mitochondria were isolated from soleus and Epi muscles from young and aged rats. Cytochrome oxidase activity was determined spectrophotometrically. Filled bars represent young muscle values and open bars represent aged muscles values. *P<0.05 ** P<0.001.

2.5. Discussion

The purpose of this study was to determine the role of stress kinases and HSPs in mediating age-related insulin resistance. By comparing the slow-twitch oxidative soleus muscle and the fast-twitch glycolytic Epi muscle, we assessed the impact of muscle insulin and oxidative capacity on the development of insulin resistance with age. Our results demonstrate that decreased insulin-stimulated glucose uptake in soleus and Epi muscles occurs primarily due to decreased total protein expression and increased serine phosphorylation of IRS-1. However, the combination of higher stress kinase activation and lower HSP expression in the Epi muscle compared to the soleus muscle, results in fast-twitch muscle being more susceptible to a decline in insulin action with age. Examination of the mechanisms through which slow-twitch muscles preserve the capacity to respond to insulin and maintain glucose homeostasis with age could have implications for future treatment of insulin resistance and diabetes.

Insulin-stimulated glucose uptake was greater in young soleus compared to young Epi muscles, consistent with previous studies indicating insulin-stimulated glucose transport activity is positively correlated with the percentage of oxidative muscle fibers (Henriksen et al. 1990; Zierath et al. 1996). Similarly, insulin signaling intermediates are present and activated to a greater extent in oxidative muscle fibers (Song et al. 1999). Our present findings demonstrating greater phospho-Akt, phospho-tyrosine IRS-1 and total IRS-1 in the oxidative soleus muscle compared with the Epi muscle agree with these previous studies. Oxidative muscles appear to have a

greater reserve for insulin signaling and glucose uptake that is not utilized under normal conditions. However, the aging condition may be one in which the reserve capacity for insulin signaling in slow muscles serves a valuable purpose. In the current study, although insulin-stimulated glucose transport decreased significantly with age in both soleus and Epi muscles, glucose transport in aged soleus muscles was still greater than that seen in young Epi muscles. This occurred in spite of a similar decrease in tyrosine IRS-1 phosphorylation in both soleus and Epi muscles with age, and an even greater increase in serine IRS-1 phosphorylation in soleus muscles with age compared to Epi muscles.

The decrease in basal glucose uptake observed in Epi muscles with age further demonstrates the decline in glucose homeostasis that occurs in fast-twitch muscle. In a previous study by Cartee et al. (Cartee et al. 1993), Epi muscles from 25 month-old male Fischer 344/Brown Norway hybrid rats demonstrated a pattern of lower basal 3-methyl glucose uptake values compared to 3.5 month-old rats, although the differences were not statistically significant. We did not measure the levels of glucose transporters in the current study, however, a decrease in basal glucose uptake with age could result from decreased GLUT1 protein expression (Buse et al. 2002). Similarly, a decrease in GLUT4 protein expression with age as previously reported (Cartee et al. 1993), could also contribute to the observed decrease in insulin-stimulated glucose uptake with age in this study. Interestingly, fiber type differences in insulin-stimulated phosphorylation of AS160 were not observed in the present study. Although AS160 phosphorylation decreased with age in both muscles in a

manner similar to that seen with Akt, muscle fiber type differences in insulin signaling are not maintained downstream of Akt. The lack of a fiber type difference in total AS160 between soleus and Epi muscles, potentially allows for a greater insulin-stimulated phosphorylation of AS160 in Epi muscles. These findings indicate fiber type differences in insulin-stimulated glucose uptake occur upstream of AS160.

Results from the present study reveal a significant decrease in total IRS-1 levels in soleus muscle from aged rats compared with young rats (Figure 5C). While serine phosphorylation of IRS-1 is often thought of as a short-term inhibitory mechanism, degradation of IRS-1 protein could result from long-term insulin resistance. Cellular and oxidative stress and proinflammatory cytokines can induce the degradation of IRS-1 through proteasome-dependent or -independent processes (Gual et al. 2003). Serine phosphorylation of IRS proteins is considered the primary means of suppressing IRS-1 activity and contributing to insulin resistance (White 1998; Werner et al. 2004; Wellen and Hotamisligil 2005). Previous studies have shown a decrease in tyrosine IRS-1 phosphorylation (Carvalho et al. 1996), a decrease in total IRS-1 protein (Arias et al. 2001) and no change in Akt in aged rats, findings consistent with our results. Although not measured in the present study, Arias et al. (Arias et al. 2001) previously demonstrated that muscle levels of insulin receptor (IR) and PI3K were increased in aged rats, a finding that would suggest these proteins are not involved in age-related insulin resistance. These studies measured a mixture of hindlimb muscles (Carvalho et al. 1996) and exclusively plantaris (Arias et al. 2001) or Epi muscle (Cartee et al. 1993) and thus could not assess muscle fiber

type differences in insulin signaling with age. Haddad et al.(Haddad and Adams 2006) showed an increase in serine IRS-1 phosphorylation with age in the medial gastrocnemius muscle from 30 month-old Fischer/Brown Norway rats. Our findings are the first to show an increase in serine IRS-1 phosphorylation in both slow-twitch and fast-twitch skeletal muscle with age.

There are a number of serine/threonine kinases capable of inducing serine IRS-1 phosphorylation and we demonstrated several that are increased with age in the current study. Recent investigations of oxidative stress and inflammatory pathways on insulin signaling have focused on obesity and genetic models of insulin resistance. This has resulted in a gap in the literature concerning the primary inflammatory mediators of age-related insulin resistance. Our findings suggest that JNK, IKKβ and GSK-3β could play a role in the development of insulin resistance in aged skeletal muscle. Although increased activation of PKCθ and extracellular regulated kinase (ERK) have been implicated in other animal models of insulin resistance, findings from our laboratory indicate these serine/threonine kinases are not increased in skeletal muscles from aged rats (data not shown). As PKCθ has been primarily associated with insulin resistance in the presence of increased lipid availability (Wellen and Hotamisligil 2005), the lack of an increase in PKCθ in aged, insulin-resistant skeletal muscle potentially differentiates the development of insulin resistance in aging and obesity models of insulin resistance. Future studies designed to inhibit inflammatory-mediated pathways in aging skeletal muscle could profoundly impact the prevalence of insulin resistance and diabetes in this population.

Results from this study are the first to characterize these potential mediators of insulin resistance in fast-twitch and slow-twitch skeletal muscles with age. A previous study demonstrated an increase in basal JNK phosphorylation in soleus and the fast-twitch extensor digitorum longus (EDL) muscles from 30 month-old and 36 month-old compared with 6 month-old Fischer 344/Borwn Norway rats (Mylabathula et al. 2006). However these investigators did not compare JNK activation or expression levels between the two muscle types. In the current study, we show dramatically greater phosphorylation levels of JNK in Epi muscles compared to soleus (Figure 6A), suggesting JNK may play an important inhibitory role in Epi muscle insulin action. In our findings, IKKβ (as measured by increased degradation of IkBα) and GSK-3 follow this same pattern of increased activation in fast-twitch compared to slow-twitch muscle, further emphasizing the role these inhibitory stress kinases may play in regulating insulin signaling in fast-twitch muscle. Atherton et al. (Atherton et al. 2004) support our findings of greater GSK-3β expression in soleus muscles compared to the fast-twitch EDL, although these investigators did not measure serine 9 phosphorylation as an estimate of GSK-3 activation in these muscles. Dokken et al. (Dokken et al. 2005) measured GSK-3β serine 9 phosphorylation in soleus and Epi muscles from lean and obese Zucker rats. The results of this study showed a greater GSK-3 activity in obese soleus compared to obese Epi muscles from Zucker rats. These findings along with a study of the spontaneous nonobese model of GK rats suggest insulin resistance is more pronounced in skeletal muscle composed predominantly of oxidative fibers (Song et al. 1999; Dokken et al. 2005).

Our findings in an aging model suggest a different fiber type effect with a greater impact of insulin resistance on fast-twitch glycolytic muscle. One possible explanation for divergent fiber type specific effects of insulin resistance with aging and obesity models could be due to age- and obesity-specific metabolic changes that occur in skeletal muscle. A greater impact on insulin signaling in fast-twitch muscle in the aging model could be due to the greater sarcopenia, muscle atrophy (Lexell 1995) and decrease in expression of insulin signaling intermediates that occurs with age in fast-twitch muscle (Carvalho et al. 1996; Arias et al. 2001). In contrast, a theory of obesity-induced insulin resistance suggests the metabolic overload and influx of fatty acids that occurs in skeletal muscle with overnutrition results in an increase in β-oxidation without a subsequent increase in tricarboxylic acid cycle flux (Muoio and Newgard 2008). This dysregulation results in accumulation of metabolic by-products of incomplete fat oxidation (acylcarnitines, ROS) in the mitochondria. Given the greater mitochondrial content of oxidative muscle fibers, this lipid stress could have a greater impact on oxidative fibers compared to glycolytic fibers.

Our findings regarding fiber type differences in HSP72 and HSP25 expression support what has been previously shown in the literature, with more oxidative fiber types expressing higher levels of HSPs (Locke et al. 1991; Huey 2006). Previous studies demonstrate that skeletal muscle HSP72 expression correlates with the degree of insulin resistance (Kurucz et al. 2002; Bruce et al. 2003) and this is also supported by our data demonstrating a decrease in HSP expression in aged, insulin resistant muscle. The HSP72 content in skeletal muscle has previously been shown to decrease

with age in soleus muscle from male Fischer 344 rats (Selsby et al. 2005) and to remain unchanged in the soleus and plantaris muscles of aged female Fischer 344 rats (Naito et al. 2001).The lack of a decrease in HSP72 with age in the study by Naito et al. (Naito et al. 2001) could be due to the ability of estrogen to upregulate HSP72 as previously shown in female rat skeletal muscle (Voss et al. 2003). While few studies have examined fiber type specific changes in HSP25 with age, HSP25 expression increased with age in rat mixed gastrocnemius muscle (Chung and Ng 2006) and decreased with age in rat cardiac muscle (Colotti et al. 2005). The downregulation of HSP25 that occurs with unloading suggests activity plays a role in regulation of HSP25 expression in skeletal muscle (Lawler et al. 2006), a theory that is consistent with an age-related decrease in HSP25 phosphorylation shown here.

Previous studies have demonstrated a role for HSPs in protecting mitochondria from oxidative stress (Polla et al. 1996) and a correlation between HSP72 mRNA expression and mitochondrial enzyme activity (Bruce et al. 2003). Our findings of age and fiber type differences in cytochrome oxidase activity are consistent with observed changes in HSP expression and activation. Muscle fiber type differences in cytochrome oxidase activity demonstrated in the present study are consistent with previous findings. Chabi et al. (Chabi et al. 2008) recently reported that soleus muscle cytochrome oxidase activity was approximately 40% higher than the fast-twitch plantaris muscle. In addition, they reported a 30% decrease in cytochrome oxidase activity in 36-month-old male Fischer 344 Brown Norway hybrids as compared to 6-month-old rats. Different muscle and animal strain account

for the slight difference in age effect, however the pattern of cytochrome oxidase changes is evident. Future studies are needed to determine the exact mechanism by which HSPs could protect mitochondria from oxidative damage.

Although the reason for a decrease in HSP expression with age and insulin resistance is not known, it appears that stress kinase activation may play a role. For example, GSK-3, and the MAP kinases, ERK and JNK, are known to negatively regulate the primary HSP transcription factor, HSF-1. Phosphorylation of HSF-1 by GSK-3, ERK and JNK on serine residues 303, 307, and 363, respectively (He et al. 1998; Dai et al. 2000) holds HSF-1 in an inactive state under normal physiological growth conditions. Consequently, overactivity of stress kinases capable of phosphorylating HSF-1 on serine residues may repress HSF-1 activation, and subsequent HSP72 transcription and HSP25 activation, in insulin resistant tissue.

Induction of HSPs may serve as a powerful tool for preventing insulin resistance with age. A recent publication demonstrated, for the first time, that increased HSP72 expression protects against obesity-induced insulin resistance (Chung et al. 2008). In the study by Chung et al. (Chung et al. 2008) heat shock therapy, as well as transgenic and pharmacologic means of overexpressing HSP72, all resulted in improved glucose tolerance and insulin action in skeletal muscle. In all cases, this corresponded with a decrease in JNK phosphorylation. It has been proposed that HSP72 binds to JNK, preventing activation of JNK by upstream kinases SEK1 and MKK7 (Park et al. 2001). Similar studies revealed that HSP72 inhibits IKKβ, either by direct binding or through an, as yet, uncharacterized

signaling intermediate (Park et al. 2003). This natural inhibitory effect of HSP72 on JNK, and of HSP25 on IKKβ could serve as a powerful tool for targeting stress kinase inhibition in the treatment of skeletal muscle insulin resistance. The ability of HSPs to protect against age-related insulin resistance has not yet been tested. In conclusion, our results demonstrate a significant difference in the ability of slow-twitch and fast-twitch muscles to respond to insulin and regulate glucose with age. While a decrease in insulin signaling and glucose uptake occurs in both slow-twitch soleus muscles and fast-twitch Epi muscles with age, the greater reserve capacity of the slow-twitch soleus muscle results in a far smaller decrement in insulin capacity in this muscle with age. Our findings suggest the proportionately lower activation of JNK, GSK-3β degradation of IkBα by IKKβ and greater expression and phosphorylation of HSP72 and HSP25, play a role in protecting slow-twitch muscle from further insulin resistance with age. Future studies are needed to examine the relationship between HSP expression and stress kinase activation and the impact of these pathways on insulin signaling in skeletal muscle.

3.6. Acknowledgements

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Chapter 3

HSP72 INHIBITS JNK ACTIVATION AND IMPROVES INSULIN SIGNALING IN AGED SKELETAL MUSCLE

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and Paige C. Geiger

Manuscript in preparation.

3.1. Abstract

Aging is associated with impaired insulin action and reduced expression of heat shock proteins. Recently, induction of heat shock proteins has been implicated in protection from obesity-related insulin resistance, however, the ability of heat shock proteins to protect against age-related insulin resistance is not known. Heat treatment to 24-month-old Fischer 344 rats showed improved insulin-stimulated glucose uptake in soleus muscles. Induction of HSP72 with an *in vitro* heat treatment to muscles from young and old rats was associated with reduced anisomycin-induced JNK activation. But heat treatment had no effect on p38 MAPK, which is a related kinase that is also activated with anisomycin. The heat-mediated JNK inhibition was specifically associated with HSP72 upregulation since inhibition of HSP72 with KNK437 reversed the heat-induced inactivation of JNK. In the heat treated muscles, HSP72 co-immunoprecipitated with JNK, indicating a direct protein-protein interaction. In conclusion, an acute bout of heat treatment can alleviate age-related insulin resistance with HSP72-mediated JNK inhibition as the potential mechanism.

3.2. Introduction

Type 2 diabetes is known to be one of the main causes of mortality and morbidity worldwide (Saltiel and Kahn 2001). Currently, diabetes, affects 7% of the U.S. population and 20% of the elderly population suffers from diabetes (CDC). The estimated economic burden of diabetes in United States is $132 billion per year, of which 51.8% is incurred by people 65 years of age or older (Hogan et al. 2003). As the baby boomers grow older, the number of people over the age of 65 years is expected to double in the next 25 years, increasing the demands for better treatments for diabetes.

Insulin resistance, an early defect in the development of type 2 diabetes, is strongly associated with the aging process itself (DeFronzo 1981). Exercise training is known to improve insulin sensitivity, but even endurance-trained individuals exhibit a decline in insulin action with age, indicating that the aging process itself limits insulin sensitivity (Clevenger et al. 2002). Aging is associated with chronic accumulation of the reactive oxygen species and several age-related pathologies, including diabetes, are thought to occur due to oxidative stress (Houstis et al. 2006). Oxidative stress can increase activation of inflammatory mediators such as c-jun terminal kinase (JNK) and inhibitor of kappa B kinase β (IKKβ) (Shoelson et al. 2003; Kaneto et al. 2007). Activation of these kinases results in serine phosphorylation of the insulin receptor substrate 1 (IRS-1), ultimately leading to impaired insulin signaling and insulin resistance. Insulin resistance in skeletal muscle, the tissue responsible for 75% of

glucose utilization in the body, is an important risk factor for the development of type 2 diabetes (Bjornholm and Zierath 2005).

One of the body's major endogenous defense systems against the increased production of reactive oxygen species in skeletal muscle is the highly conserved family of proteins known as heat shock proteins (HSPs) (McArdle et al. 2001). The role of HSPs in tissue protection and repair against a number of pathological conditions has been well documented (Welsh et al. 1995; Powers et al. 2001). Expression of HSPs and their upregulation in response to stress are significantly reduced with aging and diabetes (Kregel et al. 1995; Vasilaki et al. 2002; Atalay et al. 2004). We have previously shown that HSP expression is reduced in aging rat muscle and is associated with increased stress kinase activities and reduced insulin sensitivity (Gupte et al. 2008). But the mechanism of HSP-mediated stress kinase inhibition is not clear.

Enhanced heat shock protein 72 (HSP72) expression can protect cells and tissue from injury, modulate inflammatory signaling by directly inhibiting JNK, and decrease injurious oxidative stress associated with normal aging in skeletal muscle (Broome et al. 2006). Studies show decreased expression of HSPs in patients with type 2 diabetes correlates with reduced insulin sensitivity (Kurucz et al. 2002). Conversely, increased HSP72 expression with heat therapy has been shown to improve clinical parameters in patients with type 2 diabetes (Hooper 1999). Lifelong overexpression of HSP72 in skeletal muscle protected mice from the age-associated accumulation of oxidative damage and preserved muscle function (Smolka et al.

2000; Broome et al. 2006). We and others have shown previously that induction of HSPs with heat treatment can protect against obesity-related insulin resistance (Kokura et al. 2007; Chung et al. 2008; Gupte 2008; Morino et al. 2008), but the effects of HSPs on aging muscle are not known.

The purpose of the current study was to examine the specific involvement of HSP72 in the benefits of heat treatment in aging, insulin resistant muscle. Earlier studies from our lab have shown that weekly repeated heat treatments given in parallel with a high fat diet protects skeletal muscle from insulin resistance (Chapter 6, (Gupte 2008)). We were interested in knowing whether a single bout of heat treatment can improve insulin sensitivity in an insulin resistant animal. We examined the benefits of *in vivo* heat treatment in soleus muscles from insulin resistant F344 rats and then examined the importance of HSP72 upregulation in young and aged soleus muscle with *in vitro* muscle incubation.

3.3. Materials and methods

Materials

[^{14}C] Mannitol was obtained from ICN Radiochemicals (Irvine, CA). 2-Deoxy [1,2-^{3}H] glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies against phospho-SAPK/JNK(T183/Y185), total SAPK/JNK, phospho-p38 MAPK (T180/Y182) , total p38 MAPK, were purchased from Cell Signaling (Beverly, MA). Anti-HSP72 antibody was obtained from Stressgen (Victoria, BC, Canada), and anti-tubulin was obtained from Sigma (St.Louis, MO). Goat-anti-rabbit HRP-conjugated secondary antibodies and Protein A/G PLUS agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). KNK437 and Anisomycin were obtained from Calbiochem (San Diego, CA). Insulin was purchased from Humalog (Elli Lilly, Indianapolis, IN). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma (St.Louis, MO).

Experimental animals and muscle dissection

The Fischer 344 rat strain provides a suitable aging model due to its relatively short life span (Yu et al. 1985), extensive characterization of age-associated changes and the availability of barrier-reared aging colonies of Fischer 344 rats, maintained under the supervision of the NIA. Male Fischer 344 rats were purchased at 3 (body

weight 238.50 ± 5.11 g) and 24 (body weight 393.0 ± 17.72 g) months of age via the National Institutes of Health. Rats were housed in pairs in large cages in a temperature controlled facility (24+1 ^{o}C) with a 12 h light/dark cycle. All animals were given free access to Purina Rat Chow and water *ad libitum.* The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) followed by the removal of the soleus which consists predominantly of slow-twitch red fibers (84% type I, 16% type II) (Delp and Duan 1996). Soleus muscles were split longitudinally prior to incubation to allow adequate diffusion of oxygen and substrates. The slightly larger soleus muscles of aged rats were carefully trimmed to remain comparable in size with those of the young soleus. Muscle wet weight was used to determine equivalent size. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

In vivo heat treatment

Animals were lightly anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight). While in supine position, a thermal blanket (Chattanooga group, Hixson, TN) was wrapped around the lower body such that only the hind legs are completely covered with the blanket. As previously described (Tolson and Roberts 2005), the blanket temperature was set to 112°F and core body temperature was monitored using a rectal probe from ThermoWorks (Braintree scientific, Braintree, MA). The rats took between 40-55 min to get to 41°C, after which the core temperature was maintained between 41^{o}C and 41.5^{o}C for 20 min by opening and closing the blanket. For the sham treatment, rats were anesthetized

and their core temperature was maintained at 35°C. After the heat or sham treatments, the rats were administered 5 ml of 0.9 % saline, I.P. to prevent dehydration. Rats were fasted overnight (12 h) and anaesthetized once again 24 h post heat treatment for assessment of glucose transport.

Measurement of glucose transport activity

Twenty four following heat treatment, isolated soleus muscle strips were recovered for 60 min in Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O_2/5% CO_2. The muscles were rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with (1 mU/ml) or without insulin. After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2[1,2-^{3}H] deoxyglucose (2-DG) (1.5 μCi/ml) and 36 mM [^{14}C] mannitol (0.2 μCi/ml), with (1 mU/ml) or without insulin, with a gas phase of 95% O_2/5% CO_2, in a shaking incubator. The muscles were then blotted, clamp-frozen, and processed for determination of intracellular 2-DG accumulation and extracellular space as described previously (Young et al. 1986; Geiger et al. 2006).

In vitro heat treatment and muscle incubations

Following dissection, isolated soleus muscles were incubated in flasks containing 2 ml MEM (Invitrogen, Carlsbad, CA) supplemented with 0.01% BSA, 2.2 g/L NaHCO3, 5 mM mannitol, 2.54 mM CaCl2, 10% FBS, 1 ml/L Pen/Strep (all from Sigma) and 50 μU/ml of insulin (Elli Lilly) and supplied with a gas phase of 95% O_2/5% CO_2 at 35°C (Henriksen et al. 1990; Geiger et al. 2005). The flasks were

incubated in a temperature-controlled shaking water bath. For the *in vitro* heat shock treatment, muscles were incubated at 42°C for 30 minutes while non-heat shock treated muscles remained in recovery buffer at 35°C. Following 30 min at 42°C or 35°C, muscles were allowed to recover for 12 h in the MEM recovery medium at 35°C. For subsequent treatments, the muscles were transferred to fresh flasks containing anisomycin (10 μg/ml) for 30 min in Krebs-Henseleit bicarbonate buffer (KHB). When KNK437 (100 μM) was used, muscles were pre-incubated with KNK437 for 3 h, followed by heat treatment in presence of KNK437 and then incubated in fresh media containing KNK437 for 8 h. Finally the muscles were rapidly frozen in liquid nitrogen and processed for Western blot analysis.

Immunoprecipitation assay:

Anti-JNK antibody (BD Biosciences, San Jose, CA) was coupled to 50 μl of Protein A/G PLUS agarose beads in 200 ul of 1X PBS with constant rotation at 4°C. Coupled beads were spun down briefly, washed a couple of times with 1X cold PBS and mixed with 1 mg of protein lysate which was diluted to a final volume of 1 ml. After an overnight incubation on a rotator at 4°C, pellets were separated with a brief centrifugation and washed thoroughly 3 times with ice cold PBS. Pellets were resuspended in 40 μl 1X Laemmli buffer and analyzed for HSP72 with Western blot analysis. Pellets were also analyzed for total JNK pulled down in the assay.

Western Blotting

Clamp frozen soleus muscles were homogenized in a 12:1 (volume to weight) ratio of ice cold buffer while L6 myotubes were scraped in 100 μl per well of

homogenization buffer from Biosource (Invitrogen). The buffer contains: 10 mM Tris-HCL (pH 7.4), 100 mM NaCl, 1 mM each of EDTA, EGTA, NaF, PMSF and 2 mM Na_3VO_4, 20 mM $Na_4P_2O_7$, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 250 μl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes and then centrifuged for 20 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 1X Laemmli buffer (final) containing 100 mM dithiothreitol and heated in a boiling water bath for 5 minutes.

Protein samples (30-100 μg) were subjected to SDS-PAGE (6.25-10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA/tank). Membranes were blocked for 1 hour at room temperature in TBST 5% non-fat dry milk followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1000. Antibodies were diluted in Tris-buffered saline, 0.1% Tween-20 (TBST), 5% BSA. Following 3 brief rinses in TBST, blots were incubated in TBST 1% non-fat dry milk supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 hour at room temperature. Bands were visualized by ECL and quantified using densitometry (Image J, NIH). Blots were stripped and normalized for the total protein or tubulin as loading controls.

Citrate synthase assay

Citrate synthase activity was assessed in muscle lysates, using a modified protocol (Smirnova et al. 2006) by Srere et al. (Srere 1969). The absorbance was recorded at 405 nm every 20 sec for 3 min at 30°C, using a MRXII microplate reader

and kinetic software package (Dynex Technologies, Chantilly, VA). The linear portion of the reaction curve was used to calculate activity levels of citrate synthase, in μmol/g/min.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used when both age and treatment differences were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Values are expressed as means of ± SE. Differences between young and aged muscles are stated in the figure legends. Statistical significance was set at $P<0.05$.

3.4. Results

Reversal of insulin resistance with heat treatment in aged rats

Heat treatment given in parallel with an insulin resistance-inducing diet, has been shown to protect against insulin resistance (Chapter 6, (Chung et al. 2008; Gupte 2008)). Whether heat treatment can improve insulin sensitivity in already insulin resistant animals is not known. Previous studies from our lab have shown that 24 month old F344 rats are insulin resistant as reflected by decreased glucose uptake into soleus and epitrochlearis muscles (Chapter 2, (Gupte et al. 2008)). We administered lower body heat treatment to the aged rats with a thermal blanket (41 ^{o}C for 20 min), while the controls were given a sham treatment (35 ^{o}C for 20 min). Twenty four hours post heat treatment, soleus muscles were dissected and 2-deoxyglucose uptake was measured. Insulin stimulated glucose uptake was significantly improved in the heat treated rats compared with the sham treated rats (Figure 9). Thus, a single dose of mild heat treatment can effectively improve insulin sensitivity in the insulin resistant aged muscles.

In vitro heat shock treatment induces HSP72 expression in soleus muscles from young and aged rats.

To dissect the mechanisms and the specificity of HSP72 involvement in the heat induced benefits of insulin sensitivity, we developed an *in vitro* model of heat treatment. The *in vitro* model allows us to manipulate temperature, add/remove activators/inhibitors in a time dependent manner. This system allows us to study

FIGURE 9.

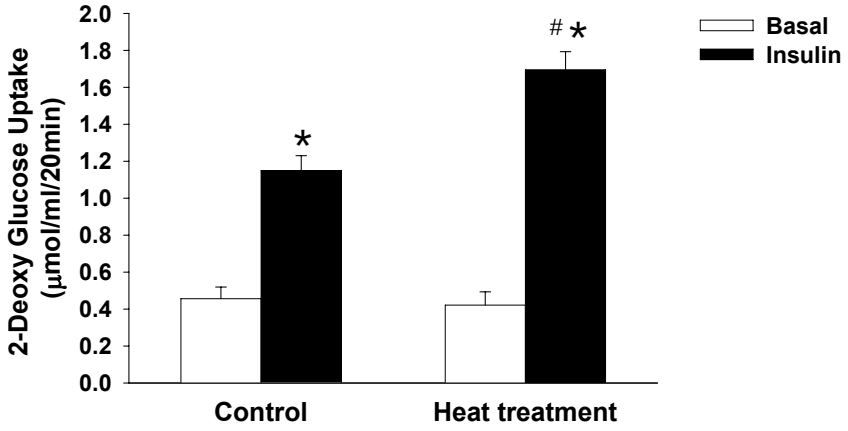


Figure 9. *In vivo* heat treatment improves glucose uptake in insulin resistant soleus muscles from aged rats. 24 mos old F344 were given a either a sham treatment (Control, 35 ^{o}C for 20 min) or a heat treatment (41 ^{o}C for 20 min) using a thermal blanket. Soleus muscles were removed 24 hours after the treatments and analyzed for basal (- insulin, open bars) and + Insulin (1 mU/ml, dark bars) glucose transport. (*P<0.001 Insulin vs. Basal and #P<0.001 Heat treatment vs. control for +Insulin). Values are means of ± SE for 4-7 muscles per group.

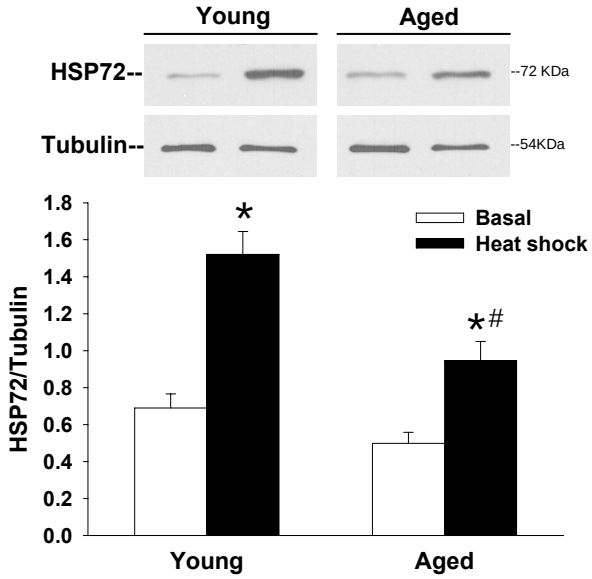
effects of heat treatment in the muscle independent of the whole body physiological changes that an *in vivo* heat treatment will have. Previous studies examining the *in vivo* heat shock response in muscle have found that maximal HSP72 expression occurs 4-48 h post heat treatment, depending on the muscle type (Oishi et al. 2002). We found that the maximal response to *in vitro* heat treatment to soleus muscles is seen 12 h post heat shock (data not shown). Both young and aged soleus muscles showed a robust induction of HSP72 protein expression (120% increase in young soleus, 90% increase in aged soleus, compared to the respective basals) (Figure 10A). The aged soleus muscles have slightly less HSP72 expression and the heat shock response is considerably dampened in the aged muscle compared to the young soleus.

In vitro heat shock treatment prevents JNK activation with Anisomycin.

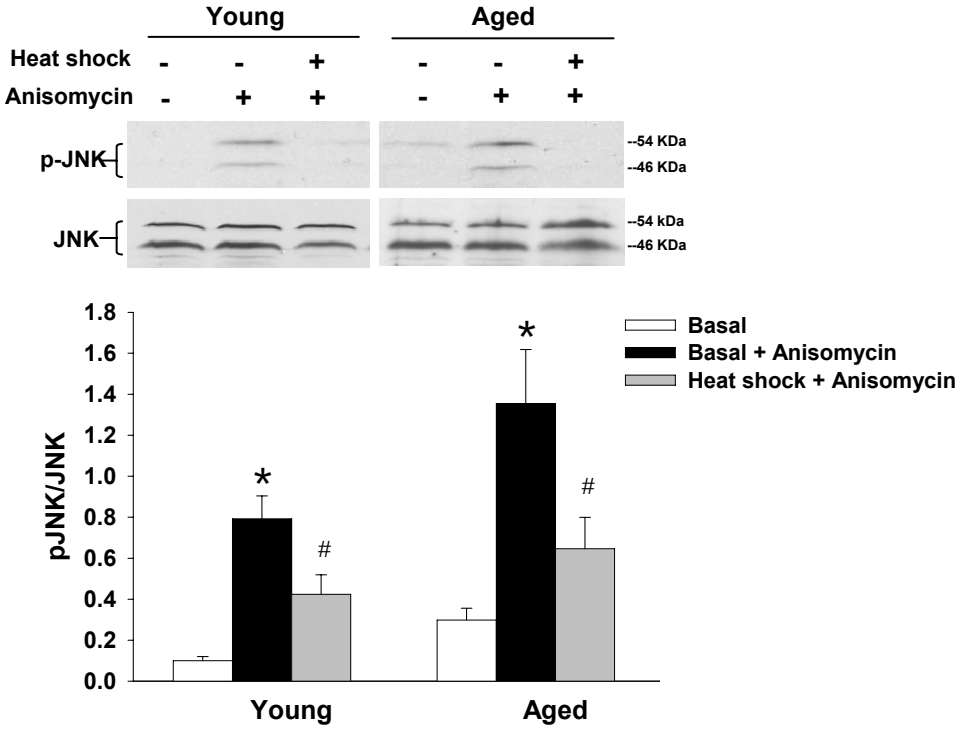
Basal (35^{o}C for 30 min) or heat treated (42^{o}C for 30 min) muscles were allowed to recover for 12 hr and then treated with anisomycin (10 μg/ml) for 30 min. Anisomycin is a potent activator of stress activated protein kinases JNK and p38 MAPK and previous studies have shown that incubation at low concentrations and for a short period as used in this study do not have any adverse effects on protein stability (Geiger et al. 2005). In basal muscles, anisomycin induced a robust activation of JNK as seen by increased phosphorylation levels (Figure 10B). Activation of JNK was much greater in the aged muscles than the young soleus muscles, given that aged muscles have greater basal phospho-JNK levels due to oxidative stress (Mylabathula et al. 2006). But in heat treated muscles, the same concentrations of anisomycin could not fully activate JNK in both young and aged muscles (gray bars).

FIGURE 10.

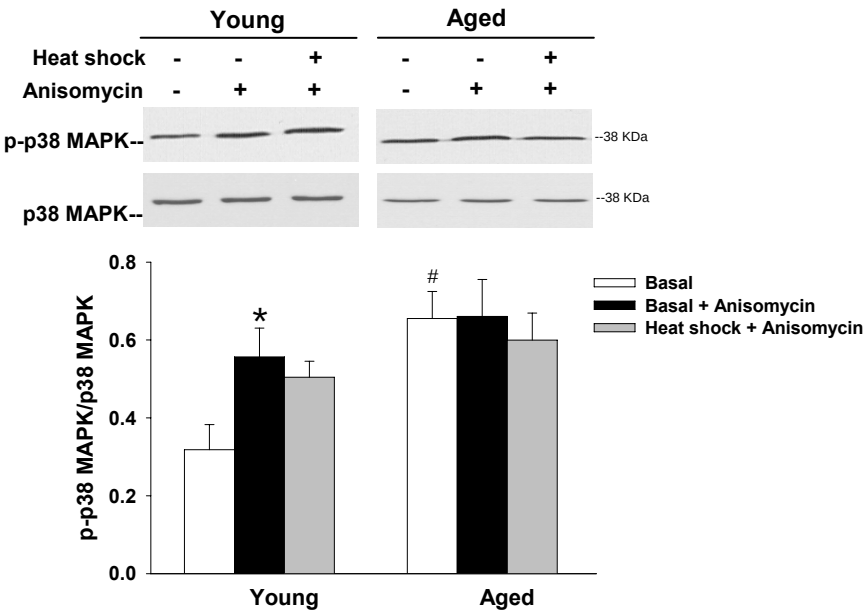
A.


Young
Aged
HSP72--
--72 KDa
Tubulin--
--54KDa
HSP72/Tubulin
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*#
Basal
Heat shock
Young
Aged

B.


Young
Aged
Heat shock - - + - - +
Anisomycin - + + - + +
p-JNK
--54 KDa
--46 KDa
JNK
--54 kDa
--46 KDa
pJNK/JNK
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
*
#
Basal
Basal + Anisomycin
Heat shock + Anisomycin
Young
Aged

C.


Young
Aged
Heat shock - - + - - +
Anisomycin - + + - + +
p-p38 MAPK--
--38 KDa
p38 MAPK--
--38 KDa
p-p38 MAPK/p38 MAPK
0.0
0.2
0.4
0.6
0.8
*
#
Young
Aged
Basal
Basal + Anisomycin
Heat shock + Anisomycin

D.

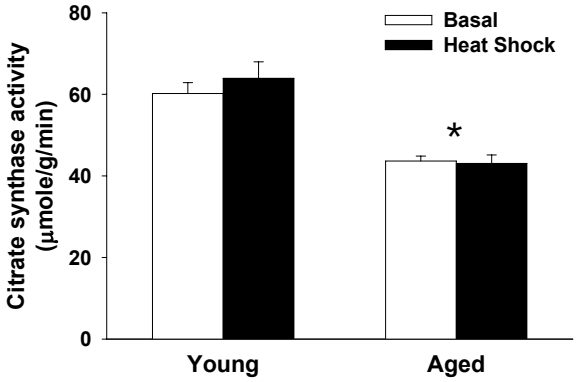

Citrate synthase activity (µmole/g/min)
0
20
40
60
80
*
Young
Aged
Basal
Heat Shock

Figure 10. *In vitro* heat treatment induces HSP72 expression and inhibits Anisomycin induced JNK activation. Soleus muscles from young (3 mos) and aged (24 mos) old F344 rats were heat treated at 42 ^{o}C for 30 min (HS) or sham (basal) at 35 ^{o}C for 30 min. Muscles recovered for 12 hours, were then frozen and analyzed for HSP72 expression **(A)** with Western blots. Blots were stripped and probed for tubulin as a loading control. (*P<0.01 Heat shock vs. Basal and #P<0.001 aged vs. young for Heat shock treatment; n=8-17 muscles/group). A subset of the muscles was stimulated with anisomycin (10 μg/ml) for 30 min after the 12 h recovery and then snap-frozen. Muscle lysates were analyzed for (p)-JNK/JNK **(B)**. (*P<0.001 Anisomycin vs. Basal, #P<0.001 HT+Anisomycin vs. Basal+Anisomycin, P<0.01 for all aged vs. all young groups; n=7-20 muscles/group). Lysates were also analyzed for (p)-p38 MAPK/p38 MAPK **(C)** (*P<0.05 Anisomycin vs. Basal, #P<0.01 young vs. aged basal; n=8-14 muscles/group) and for citrate synthase activity **(D)** (*P<0.001 aged vs. young; n=3 muscles/group).

This indicates that heat shock treatment prevents JNK activation even in the presence of a strong activator like anisomycin. Anisomycin also activates p38 MAPK, a MAP kinase with properties similar to JNK. Therefore we examined whether heat treatment affects p38 MAPK activation as well. Anisomycin induced activation of p38 MAPK as seen by increased phosphorylation levels in the young soleus muscles (Figure 10C). Basal phospho-p38 MAPK was much greater in the aged soleus and therefore did not increase further with anisomycin treatment. Heat treatment had no effect on the activation of p-38 MAPK in either the young or aged soleus muscles.

To further verify that heat shock mediated JNK inhibition was not an artifact of the heat treatment itself, we examined citrate synthase activity, routinely measured to assess muscle viability. Citrate synthase activity levels were greater in the young soleus muscles than the aged soleus muscles as has been reported before (Figueiredo et al. 2008), but no reduction in activity levels were seen with the heat treatment (Figure 10D).

KNK437 inhibits heat induced HSP72 expression

KNK437 is a specific inhibitor of inducible heat shock proteins (Yokota et al. 2000; Koishi et al. 2001). Its mechanism of action is believed to be inhibition at the transcriptional level (Yokota et al. 2000). Muscles were pre-incubated with KNK437 (100 μM) for 3 h, followed by an *in vitro* heat or sham treatment as described above. Since KNK437 is a reversible inhibitor, it was included during most of the recovery period as well (8h). HSP72 induction with heat treatment was significantly dampened in muscles from both young and aged rats in the presence of KNK437 (Figure 11A).

FIGURE 11.

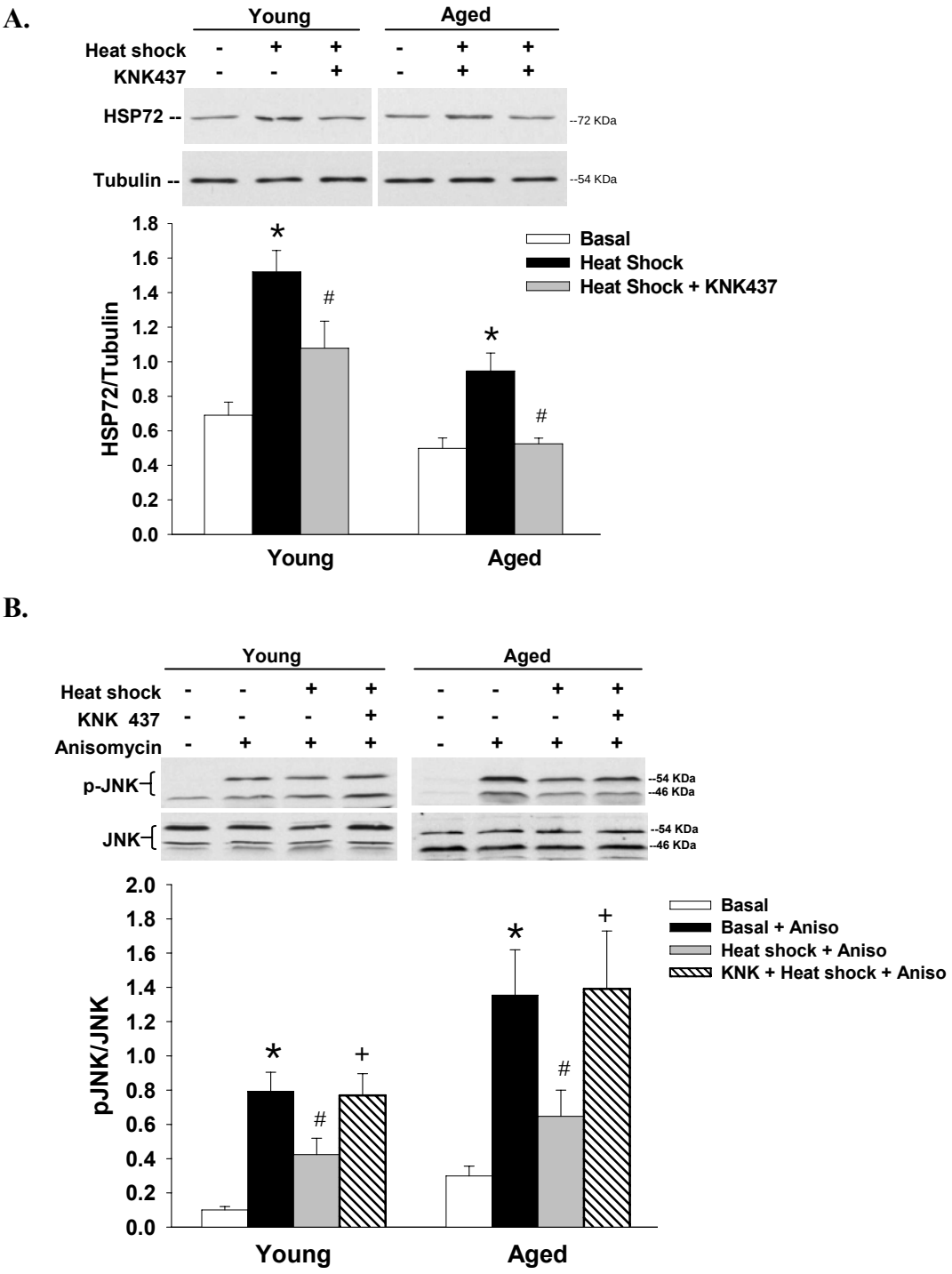

A.
Young
Aged
Heat shock
KNK437
HSP72 --
--72 KDa
Tubulin --
--54 KDa
HSP72/Tubulin
Basal
Heat Shock
Heat Shock + KNK437
Young
Aged
B.
Young
Aged
Heat shock
KNK 437
Anisomycin
p-JNK
JNK
--54 KDa
--46 KDa
pJNK/JNK
Basal
Basal + Aniso
Heat shock + Aniso
KNK + Heat shock + Aniso
Young
Aged

Figure 11. Pharmacological inhibition of HSP72 induction eliminates heat stress-mediated inhibition of JNK. Isolated soleus muscles from young and old F344 rats were exposed to sham or heat shock (HS) at described above. A reversible pharmacological inhibitor of HSP, KNK437 (100 μM), was included in the incubation buffer 2 h prior to, during and 8 h post HT for a subgroup of muscles. Following recovery for a total of 12 h at 35°C, muscles were assessed for HSP72 **(A)**. JNK activation in response to anisomycin (Aniso, 10 μg/μl, 30 min), was tested at the end of the recovery period **(B)**. (*P<0.001 HS vs. Basal and Basal+Aniso vs. Basal, #P<0.001 HS+KNK vs. HS and HS+Aniso vs. Basal+Aniso, +P <0.001 HS+KNK+Aniso vs. HS+Aniso, P<0.001 for all aged vs. all young groups). Values are means of ± SE for 4-7 for the aged muscles and 7-20 for young muscles per group.

KNK437 had no significant effect on the muscles without heat treatment (data not shown).

Inhibition of HSP72 with KNK437 reverses heat shock mediated JNK inhibition:

Heat treatment induces several molecular and physiological changes in cells, including membrane fluidization (Nagy et al. 2007), upregulation of chaperone proteins, reducing oxidative stress etc. To verify that HSP72 has a specific role in the observed JNK inactivation, we examined JNK activity levels in the presence of the HSP72 inhibitor KNK437. Anisomycin induced JNK activation was reduced with heat shock, but in the muscles incubated with KNK437, heat shock did not decrease JNK activation with anisomycin (Figure 11B). Thus, pharmacological inhibition of the HSP72 response to heat shock reversed the heat-mediated inhibition of JNK. This data indicates that JNK inactivation was specifically mediated by HSP72.

Heat induced HSP72 interacts with JNK

Homogenates from basal and heat treated muscles were subjected to an immunoprecipitation assay using anti-JNK antibody. HSP72 co-immunoprecipitated with JNK only in the heat treated muscles, from both young and aged rats (Figure 12). This indicates that HSP72 interacts with JNK in the muscles receiving heat treatment. This could indicate a potential mechanism by which HSP72 could inhibit JNK activation.

FIGURE 12.

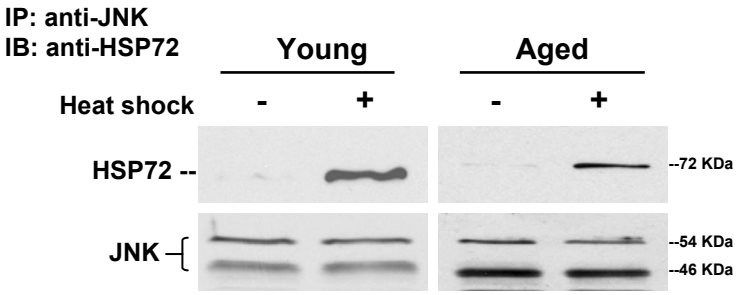


Figure 12. HSP72 and JNK co-immunoprecipitate in heat shock treated muscles. Lysates of heat shock treated (42^{o}C 30 min, 12 h recovery) and basal (35^{o}C 30 min, 12 h recovery) soleus muscles were subjected to an immunoprecipitation assay using an anti-JNK antibody coupled to Protein A/G PLUS agarose beads. Pellets were analyzed for HSP72 and total JNK with Western blot analysis. Blots are representative of 3 independent experiments, 4-5 muscles per condition.

3.5. Discussion

Age related-insulin resistance is a serious health concern since it represents an underlying risk factor for several age-related diseases including diabetes, Alzheimer's disease and cardiovascular disease. The role of HSPs in protecting insulin sensitivity in muscle is under recognized. Although previous studies, including ours, have shown that HSP induction can protect against insulin resistance (Chung et al. 2008; Gupte 2008), we now show that HSP induction can also reverse pre-existing insulin resistance in the aged rats. We also report that HSP72 induction is associated with JNK inactivation, that JNK inactivation is specific to HSP72 induction and is mediated by a direct protein-protein interaction. Our results suggest that HSP72 inducing treatments may have potential therapeutic benefits for insulin resistance in the elderly.

Aging and insulin resistance are associated with an inter-dependent cycle of reduced heat shock proteins, chronic inflammation and reduced insulin sensitivity. Therapeutic upregulation of HSPs is thought to forestall this cycle (Hooper and Hooper 2008), but the precise mechanisms are not clear. Overexpression of HSPs could potentially protect skeletal muscle from oxidative stress-induced insulin resistance by decreasing muscle susceptibility to stress (chaperone role of HSPs). Alternatively, increased HSP expression could more directly improve insulin resistance and mediate insulin action by inhibiting stress kinase activation. In NIH3T3 cells, HSP72 has been shown to bind to JNK and prevent its activation by

the upstream kinases SEK1 and MKK7 (Park et al. 2001). While other investigators suggest HSP72 prevents stress-mediated inactivation of a phosphatase that targets JNK, both proposed mechanisms effectively inhibit JNK activation (Meriin et al. 1999; Gabai et al. 2000). This role of HSP72 as a natural inhibitor of JNK has been shown to be independent of its chaperone function since removal of the ATPase domain of HSP72 still maintains JNK inactivation (Yaglom et al. 1999). In agreement with previous studies in NIH3T3 cells (Park et al. 2001), we report here that HSP72 interacts with JNK in heat treated muscles. Future studies are required to understand whether binding of HSP72 to JNK prevents phosphorylation of JNK by upstream kinases or facilitates its de-phosphorylation by MAPK-phosphatases. Other mechanisms proposed recently are mitochondrial protection (Polla et al. 1996; Williamson et al. 2008), reduction of oxidative stress (Selsby and Dodd 2005), and inhibition of DLK-1, a kinase upstream of JNK (Daviau et al. 2006).

Heat shock protein expression is vastly different between fast- and slow-twitch muscles, with slow-twitch muscles expressing several fold more HSP72 (Locke et al. 1991). The degenerative loss of muscle mass and energy with aging, known as *sarcopenia*, occurs primarily in type II glycolytic fibers (Grimby 1995). Thus type I muscle fibers such as those in the soleus muscles are more abundant and relatively healthier in aged animals, and treatments designed for improving age-related insulin resistance should factor in this fiber type difference. It is for this reason that we chose the soleus muscles for our experiments. Inhibition of JNK with HSP72 overexpression could not only protect the aging muscle from insulin

resistance but also from age-related muscle loss and oxidative stress (Broome et al. 2006). Thus HSP inducing treatments in the aging muscle could have several beneficial effects.

Aging is associated with a reduced heat shock response(Kregel et al. 1995) and in our hands, we also find that HSP72 induction with heat was dampened in the soleus muscles from aged rats compared with young rats. We also found that the *in vivo* heat treatment was stressful on the old rats, particularly if the temperature was raised to 41°C very quickly (ie <30 min). If the temperature was raised slowly over a period of 45 min or more by opening and closing the blanket, the stress was reduced. To remove the effects of whole body stress and hone into the mechanisms of HSP function, we used the *in vitro* muscle heat treatment. A solution to reducing the stress of heat and other potential complications in the elderly would be to use milder heat stress along with co-inducers such as bimoclomol and its derivatives. Nevertheless, our studies show that HSP72 upregulation can improve insulin action in aged muscle, indicating that HSP72 is a very promising therapeutic target in reversing advanced insulin resistance. Several HSP72-inducing compounds have been explored for treating diabetic neuropathy (Kurthy et al. 2002), myocardial infarction (Lubbers et al. 2002) etc. and now could be potentially investigated in treatment of advanced insulin resistance.

In conclusion, our studies show that HSP72 can specifically inhibit JNK in the aging muscle and protect against insulin resistance. HSP-inducing treatments like

exercise, heat treatments and BGP-15 could be explored as interventions to alleviate insulin resistance in the elderly.

3.6. Acknowledgements

The authors would like to thank the KUMC Biomedical Research Training Program award to Gupte A.A. for research support.

Chapter 4

AGE-RELATED CHANGES IN HEAT SHOCK PROTEIN EXPRESSION IN THE BRAIN

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and John A. Stanford

Submitted.

4.1. Abstract

The aging process of the brain is associated with chronic oxidative stress. However, neuronal cells are thought to actively utilize compensatory defense mechanisms such as the heat shock proteins (HSPs) to protect themselves from persisting stress over time. Pharmacologically induced HSP expression has been shown to protect against age-related mitochondrial stress and oxidative stress. Despite the protective role of HSPs, little is known about the HSP response in the aging brain and the time point of its activation. The purpose of this study was to examine HSP expression in 3 different age-points: 6-months, 18-months and 30-months in the striatum, substantia nigra, globus pallidus and cortex regions from Male Fischer 344/Brown Norway hybrid rats. Phosphorylated HSP25 and total HSP25 expression showed robust increases with age in the 30-month-old animals. But p38 MAPK, a protein previously implicated in activating HSP25 did not change with age. Although HSC70 and HSP60 expression did not change with age in any brain sections, HSP72 was reduced with age only in the striatum. In conclusion, our results suggest that HSP25 is the main HSP responding to age-related pathology in the brain. Understanding the patterns of endogenous HSP protection in the aging brain may have potential implications in designing therapeutic interventions to prevent age-related neurodegenerative disorders.

4.2. Introduction

In the brain, normal aging is associated with oxidative stress, reduced antioxidant levels (including vitamin E and glutathione) (Hall et al. 2000; Hall et al. 2001), and mitochondrial dysfunction (Hiona and Leeuwenburgh 2008). These changes are thought to contribute to motor and cognitive impairment (Weissman et al. 2007; Sekler et al. 2008), as well as to age-related diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Heneka et al. 2003; Mosconi et al. 2008). One mechanism used by neurons to counteract pathogenic conditions is to induce the expression of heat shock proteins (HSPs) (Calabrese et al. 2004). These proteins serve as biomarkers for stress conditions that accompany neurodegenerative diseases (Goldbaum and Richter-Landsberg 2001). HSC70, the constitutively expressed member of the HSP70 family and HSP72, the inducible HSP70 have been shown to increase with age in the brain (Unno et al. 2000; Calabrese et al. 2004).

Small HSPs such as the HSP25 have been shown to increase in the reactive astrocytes of brains undergoing neurodegeneration (Brzyska et al. 1998), although HSP25 expression and phosphorylation of HSP25 in various regions of the aging brain has not been characterized. A positive correlation has been reported between the decrease in reduced glutathione levels (indicating increased oxidative stress) and increase in HSP72 expression levels (Calabrese et al. 2004). HSP25 has been shown to protect mitochondrial function, specifically complex I activity in murine PC12 cells (Downs et al. 1999). HSP25 inhibits actin polymerization, (Wieske et al. 2001)

and also functions as a chaperone, preferentially interacting with unfolded actin and preventing formation of aggregates (Panasenko et al. 2003). Phosphorylation and supramolecular organization of HSP25 reduces its inhibition of actin polymerization (Benndorf et al. 1994).

Despite their importance as intracellular protectors in aging, the time point at which changes in the activation and expression of HSPs occur is not known. For example, it is not known whether these changes occur progressively with age or whether they are an indicator of senescence. The purpose of this study was to determine if the changes in HSP expression occur progressively over age and whether they differ in the different regions of the brain.

4.3. Materials and methods

Animals and dissection

Male Fischer 344/Brown Norway hybrid rats were purchased at 6 (n=3, body weight 466.67 ± 5.77 g), 18 (n=3, body weight 518.67 ± 32.02 g) and 30 (n=3, body weight 625 ± 43.00 g) months of age from NIA colonies. Rats were housed in pairs in large cages in a temperature controlled facility with a 12 h light/dark cycle, with ad libitum access to food and water. The rats were fasted for 12 h overnight prior to sacrificing. Rats were deeply anesthetized with sodium pentobarbital and decapitated for brains removal. The following sections were collected in tubes and frozen on dry ice: striatum (Str), substantia nigra (SN), globus pallidus (GP) and cortex (Ctx). All protocols were approved by the KUMC Institutional Animal Care and Use Committee and procedures adhered to the *Guide for the Care and Use of Laboratory Animals*.

Western blotting

Antibodies against HSP72 (SPA-810), phospho-HSP25 (S82), total HSP25, HSC70 (SPA-815) and HSP60 were obtained from Stressgen (Victoria, BC, Canada), and antibodies against Actin were obtained from Abcam (Cambridge, MA). Antibodies against phospho-p38 MAPK and total p38 MAPK were purchased from Cell Signaling (Beverly, MA). Goat-anti-rabbit HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules,

CA). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma (St.Louis, MO).

Frozen brain sections were homogenized using a hand held homogenizer in a 12:1 (volume to weight) ratio of ice cold buffer from Biosource (Invitrogen, Carlsbad, CA) as described before (Morris et al. 2008). Homogenized samples were rotated for 30 minutes and then centrifuged for 30 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 1X Laemmli buffer (final) containing 100 mM dithiothreitol and denatured in a boiling water bath for 5 minutes.

Protein samples (30-100 μg) were subjected to SDS-PAGE (6.25-10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA/tank). Membranes were blocked for 1 hour at room temperature in TBST 5 % non-fat dry milk followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1000. Antibodies were diluted in Tris-buffered saline, 0.1% Tween-20 (TBST), 1% milk. Following 3 brief rinses in TBST, blots were incubated in TBST 1% non-fat dry milk supplemented with the respective HRP-conjugated anti-rabbit or anti-mouse secondary antibody at a concentration of 1:10,000 for 1 hour at room temperature. Bands were visualized by ECL and quantified using Image J densitometry. Blots were then stripped and probed for actin as a loading control.

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate differences between age groups within each brain section. This was followed by a post hoc comparison using the Student-Newman-Keuls test when appropriate. Statistical significance was set at $P<0.05$.

4.4. Results

HSP25 phosphorylation and expression

Robust increases in phosphorylated HSP25 (pHSP25) occurred with age in Ctx, SN, GP and striatum sections (Figure 13A-D). The greatest increase in pHSP25 was in the GP, with a 1356% increase in the 30-month-old animals compared to the 6-month-olds. While pHSP25 peaked at 30 months in each brain section examined, significant pHSP25 increase in 18-month-old animals was seen only in the SN. Total HSP25 expression also increased markedly in all 4 brain sections in the 30-month-old old compared to the 6-month-old animals. Like pHSP25, the increase in total HSP25 in the 30-month-old animals was greatest in the GP and least in the striatum. When pHSP25 is normalized to total HSP25, changes in pHSP25 were no longer seen, indicating that most of the total HSP25 protein present is in the phosphorylated state (data not shown).

p38 MAPK phosphorylation and activation

Activation of p38 MAPK, as indicated by its phosphorylation, did not differ significantly with age in any of the brain sections examined (Table 1). Neither the total p38 MAPK protein expression, nor phosphorylated to total protein ratio, differed significantly with age.

HSP72 expression

HSP72, the inducible member of the HSP70 family, was examined using an antibody (SPA-810) that specifically recognizes the inducible HSP72 and does not

cross-react with HSC70. Although HSP72 expression was very low compared to HSC70 in the brain sections under investigation, the striatum showed a significant decrease with age (Table 1). Compared to the 6-month-old group, HSP72 expression was 42% less in the 18-month-old animals and 49% less in the 30-month-old animals. The GP, SN and Ctx did not show significant changes in HSP72 expression in either age group.

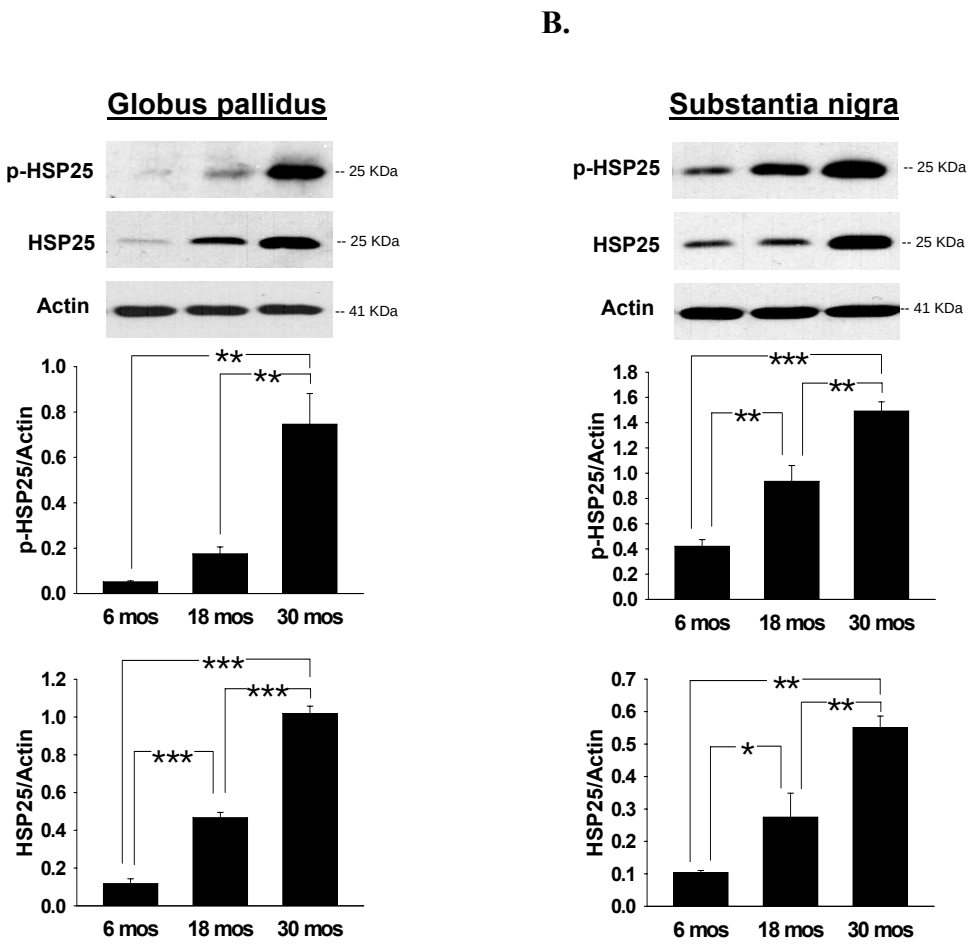
HSC70 expression

HSC70, the constitutively expressed HSP70 family member was examined in the 4 regions of the brain using an antibody (SPA-815) that specifically reacts with HSC70 and does not cross react with HSP72. HSC70 was abundantly expressed in each of the brain regions, but did not vary significantly with age (Table 1).

Mitochondrial HSP60 expression

Impaired mitochondrial function is associated with age-related neurodegenerative diseases. Because HSP60 is associated with the mitochondrial heat shock response, we quantified its expression. HSP60 expression did not change with age in any of the brain sections examined (Table 1).

FIGURE 13.


A.
B.
Globus pallidus
Substantia nigra
p-HSP25
HSP25
Actin
-- 25 KDa
-- 25 KDa
-- 41 KDa
p-HSP25/Actin
HSP25/Actin
6 mos
18 mos
30 mos

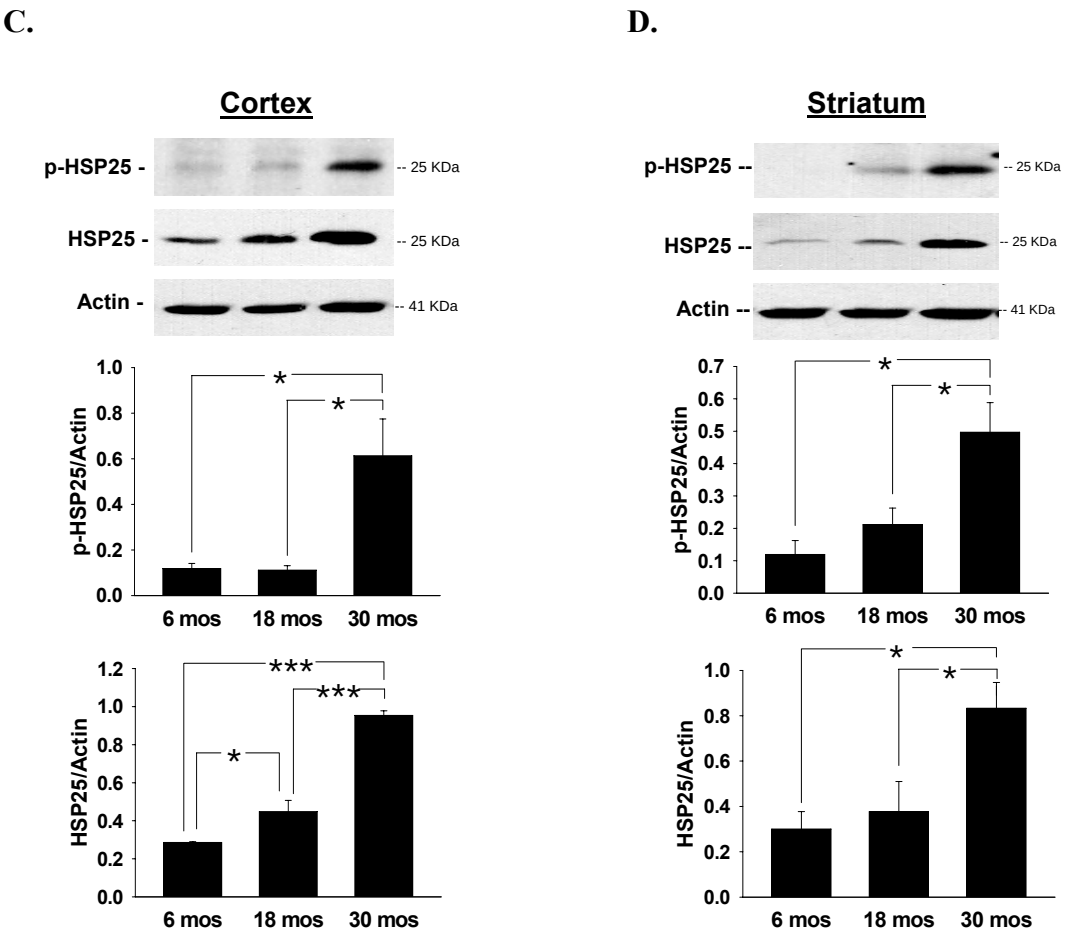


Figure 13. Increased expression of phospho-HSP25 and total HSP25 with age in the brain. Expression levels of p-HSP25 and total HSP25 were measured with Western blot analysis in the Globus pallidus **(A)**, Substantia nigra **(B)**, Cortex **(C)** and Striatum regions **(D)** from 6-month-old (6 mos), 18-month-old (18 mos) and 30-month-old rats (30 mos). All blots were normalized to actin expression as a loading control. (***P<0.001, **P<0.01, *P<0.05). Values are means of ± SE for 3 rats per group.

TABLE 1. Age-related changes in expression of HSPs and p38 MAPK in the brain.

	Globus pallidus			Substantia nigra		
	6 mos	**18 mos**	**30 mos**	**6 mos**	**18 mos**	**30 mos**
HSC70	0.588±0.06	0.618±0.08	0.600±0.04	0.459±0.02	0.471±0.02	0.442±0.04
HSP72	0.365±0.04	0.400±0.05	0.370±0.07	0.287±0.01	0.340±0.05	0.335±0.03
HSP60	0.417±0.07	0.388±0.08	0.422±0.03	0.511±0.05	0.539±0.08	0.587±0.04
p-p38 MAPK	0.145±0.02	0.150±0.01	0.188±0.03	0.170±0.041	0.268±0.08	0.285±0.03
p38 MAPK	0.272±0.06	0.322±0.03	0.421±0.06	0.258±0.03	0.299±0.04	0.308±0.19

	Cortex			Striatum		
	6 mos	**18 mos**	**30 mos**	**6 mos**	**18 mos**	**30 mos**
HSC70	0.817±0.05	0.741±0.08	0.755±0.03	0.414±0.06	0.496±0.05	0.435±0.05
HSP72	0.432±0.04	0.360±0.02	0.382±0.03	0.175±0.11	0.067±0.05*	0.068±0.02*
HSP60	0.656±0.02	0.693±0.14	0.594±0.05	0.665±0.06	0.726±0.06	0.746±0.11
p-p38 MAPK	0.139±0.02	0.131±0.03	0.214±0.04	0.111±0.05	0.139±0.04	0.192±0.03
p38 MAPK	0.323±0.03	0.318±0.04	0.393±0.02	0.411±0.11	0.544±0.03	0.488±0.06

*P<0.05 when compared with 6-mos. Values are means of ± SE for 3 rats per group.

4.5. Discussion

Endogenous defense systems such as the heat shock proteins function to protect the body against stressors throughout the lifetime of an organism and stressors related to the aging process provide the ultimate test of these systems. In this study we examined the changes in HSP expression with age, using 3 age time points in 4 different regions of the brain that are commonly associated with age-related neurodegenerative diseases. We found that HSP25 is markedly upregulated in all the brain regions examined and this change is most robust in the globus pallidus region. On the other hand, HSP72 expression was reduced only in the striatum. Understanding the patterns of endogenous HSP protection in the aging brain can help in designing therapeutic interventions to prevent neurodegenerative disorders.

The most robust age-related changes in our study are seen in HSP25 expression/phosphorylation. HSP25 has been shown to protect NADH:ubiquinone oxidoreductase and NADH dehydrogenase activity (Complex I) in submitochondrial vesicles during heat and oxidative stress (Downs et al. 1999). Additional evidence of mitochondrial protection by HSP25 includes HSP25 inhibition of 6-hydroxydopamine-induced cytochrome c release and apoptosis in PC12 cells (Gorman et al. 2005). Upregulation of HSP25 has also been reported in the spinal cords of a mouse model of familial amyotrophic lateral sclerosis and is accompanied by increases in the antioxidant peroxiredoxin 6 (Strey et al. 2004). Thus, increased HSP25 could be a possible anti-oxidant defense mechanism that the neuronal cells

resort to in a desperate attempt to survive oxidative stress. Further studies will be needed to see if removal of age-related oxidative stress can reverse this large increase in HSP25 expression.

We have previously reported that aging is associated with a decrease in HSP expression in the skeletal muscle (Gupte et al. 2008) and others have shown decreases in cardiac muscles (Colotti et al. 2005). But the pattern of HSP25 expression with aging in the brain appears to be just the opposite. This suggests that the response to age-related stress is different in different tissues, possibly dependent on multiple parameters, including rate of turnover of proteins, rate of regeneration of tissue, exposure levels of stressors etc. Studies in the aging model of *C.elegans* revealed that, a progressive deterioration of most tissue occurs with aging, except for the nervous system, which remarkably preserves its integrity even in advanced old age (Herndon et al. 2002). Thus, it is likely the neurons protect themselves against aging with strong defense mechanisms such as the heat shock proteins seen in our study. Failure of these mechanisms would make these neurons susceptible to neurodegenerative diseases such as PD and AD. In support of this idea, it has been observed that only after HSP25 compensation fails, neurodegeneration follows in the ALS-SOD1 mice (Maatkamp et al. 2004).

Different HSPs are induced in response to different stressor such as heat stress (induces HSP72) and oxidative stress (induces HSP32) (Goldbaum and Richter-Landsberg 2001). Thus, it is likely that age-related stress in the brain is associated primarily with the induction of HSP25, but not any other HSPs. In our hands, we did

not see any changes in HSC70 but saw decreases in the inducible HSP72 with age, only in the striatum. But in the brain regions that we examined in this study, HSP72 expression is extremely low whereas the constitutive HSC70 expression is abundant. Therefore, the relative effect of reduced HSP72 may not be a significant aberration at this stage of aging but may serve as a starting point of deterioration of the overall defense systems of the brain.

HSP72 did not change significantly in any other section but the striatum, where it reduced with age. The cortex showed trends of HSP72 reduction but did not make it to significance. These two regions also showed the smallest increases in HSP25 among the regions studied. Thus, it could be speculated that the striatum and cortex of the brain lose the HSP protection earlier in the progression of age-related deterioration and could be the regions most susceptible to age-related neurodegenerative diseases.

Using immunohistochemical and immunoblot analyses, Murashov et al. showed that HSP25 is localized primarily to the facila, trigeminal, ambiguous, hypoglossal and vagal motor nuclei of the brain stem (Murashov et al. 1998). Astrocytes strongly express HSP25 and astrocytes are known to increase up to 20% in the aged cortex and other brain regions (Peinado et al. 1998), and PD and AD are both associated with reactive gliosis (Renkawek et al. 1999). Also, the occurrence of spheroids with age is seen predominantly in the globus pallidus and substantia nigra pars reticulata of aged rhesus monkeys, and the spheroids are associated with overexpression of HSPs (Fukuda et al. 2005). In our study, we see the largest

induction of HSP25 with age in these two regions. Therefore, future studies will be needed to see whether HSP25 expression in the aging brain increases in the neurons or is contributed by the increasing number of astrocytes.

p38 MAPK has been suggested as the primary upstream kinase of HSP25, which phosphorylates and activates HSP25 in response to stress (Yuan and Rozengurt 2008). In this study, although we saw large increases in phosphorylated HSP25, we did not see significant changes in p38 MAPK activation. One likely possibility is that phosphorylation of HSP25 in the aging brain is mediated by other kinases such as PKD, PKD2 and ERK, which have also been indicated as HSP25-kinases (Geum et al. 2002; Yuan and Rozengurt 2008).

In conclusion, our findings suggest that HSP expression is regulated differently in different regions of the brain. Maintaining their regulation or upregulating their expression may be an important strategy of preventing aging-associated neurodegenerative diseases.

4.6. Acknowledgements

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Chapter 5

LIPOIC ACID INCREASES HEAT SHOCK PROTEIN EXPRESSION AND INHIBITS STRESS KINASE ACTIVATION TO IMPROVE INSULIN SIGNALING IN SKELETAL MUSCLE FROM HIGH FAT-FED RATS

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J Appl Physiol. **(2009 Jan 29): [Epub ahead of print]**

5.1. Abstract

The anti-oxidant alpha-lipoic acid (LA) has been shown to improve insulin action in high fat-fed animal models, yet little is known about its underlying mechanisms of action. We hypothesize that LA acts by inducing heat shock proteins which then inhibit stress kinases known to interfere with insulin signaling intermediates. Male Wistar rats were fed a high fat diet (HF diet, 60% calories from fat) for six weeks, while controls received a chow diet (10% calories from fat). Half of the rats in each group received daily LA injections (30 mg/kg body wt). In rats fed a HF diet, LA increased expression of heat shock protein 72 (HSP72) and activation of HSP25 in soleus muscle, but had no effect on heat shock proteins in muscle from chow-fed rats. LA treatment reduced phosphorylation of c-Jun N-terminal kinase (JNK) and Inhibitor of Kappa B Kinase beta (IKKβ) activity (IκBα protein levels) in rats fed a HF diet and effectively restored insulin responsiveness, as seen by insulin-stimulated pAkt/Akt and 2-deoxyglucose uptake in soleus muscle. LA also induced activation of p38 MAPK and AMPK, proteins previously implicated in insulin-independent glucose uptake. In addition, acute LA treatment induced heat shock proteins *in vitro* in L6 muscle cells and prevented the activation of JNK and IKKβ with stimulants such as anisomycin and TNF-α, respectively. In conclusion, our results suggest chronic LA treatment results in stress kinase inhibition and improved insulin signaling through a heat shock protein-mediated mechanism.

5.2. Introduction

High fat diet-induced insulin resistance is associated with impaired insulin signal transduction and reduced glucose transport in skeletal muscle. Stress kinases such as c-Jun N-terminal Kinase (JNK) and the Inhibitor of Kappa B Kinase beta (IKKβ) are activated by oxidative stress and activity levels of these kinases are high in skeletal muscles from insulin resistant animals (Yuan et al. 2001; Hirosumi et al. 2002; Ropelle et al. 2006; Gupte et al. 2008). Chronically increased stress kinase activation is implicated in dysregulation of the insulin signaling pathway. JNK and IKKβ interfere with normal insulin signaling by phosphorylating the insulin receptor substrate (IRS-1) on serine-307, reducing its interaction with downstream effector phosphatidylinositol 3-kinase (PI3K) (Zick 2005).

Specific inducible heat shock proteins (HSPs) have the potential to inhibit JNK and IKKβ (Park et al. 2001; Park et al. 2003). Heat shock proteins are chaperone proteins, extensively studied in their role in mediating protein refolding, tissue protection, tissue repair and cellular homeostasis (Bukau et al. 2006). A study by Park et al. showed that heat shock protein 72 (HSP72) can also function as a natural inhibitor of JNK by direct binding and subsequent prevention of its activation by upstream kinases (Park et al. 2001). Similarly, the small heat shock protein HSP25 binds and inhibits the stress kinase IKKβ (Curry et al. 1999; Park et al. 2003; Alford et al. 2007) and can regulate TNF-α-induced NF-κB activation (Park et al. 2003). We have recently demonstrated that HSP induction with heat treatment can improve

whole body glucose tolerance and muscle specific insulin sensitivity while reducing stress kinase activation in rats fed a HF diet (Gupte 2008). Another *in vivo* study by Chung et al. demonstrated that increased HSP72 expression can protect against obesity-induced insulin resistance, and this was tightly associated with the prevention of JNK phosphorylation (Chung et al. 2008). Based on this evidence, a treatment that could induce HSPs in skeletal muscle has the potential to inhibit JNK and IKKβ and improve insulin action.

Alpha lipoic acid (LA) is a short chain fatty acid that acts as a cofactor in the pyruvate dehydrogenase complexes and is a potent biological anti-oxidant (Packer et al. 1995; Lovell et al. 2003). Administration of LA has demonstrated beneficial effects in several disease states associated with oxidative stress such as diabetic neuropathy, heavy metal poisoning and liver cirrohosis in humans (Ziegler et al. 1999). A recent study showed that high fat diet-induced (p)-ser307-IRS-1 in skeletal muscle was reduced by treatment with an anti-oxidant mixture containing LA (Vinayagamoorthi et al. 2008). In addition, increased skeletal muscle glucose uptake following LA treatment in obese zucker rats was associated with an increase in IRS-1 content and insulin stimulated PI3K activation (Saengsirisuwan et al. 2004). Although improvements in IRS-1 function have been shown in previous studies with LA treatment, the mechanisms by which LA improves IRS-1 function are unknown. A recent study showed that LA treatment can increase production of HSPs in diabetic kidney and plasma (Strokov et al. 2000; Oksala et al. 2007) yet its effect on skeletal muscle HSPs is unknown. Therefore, the purpose of this study is to determine

whether chronic LA treatment increases the expression of HSPs in skeletal muscle, and if this induction can effectively inhibit stress kinases and improve insulin action.

5.3. Materials and methods

Materials

(±)-Alpha Lipoic acid was obtained from Sigma (St. Louis, MO) and dissolved in Tris-HCl (120 mM, pH 7.4). [^{14}C] Mannitol and 2-Deoxy [1,2-^{3}H] glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). Dulbecco's modified Eagles's medium (DMEM; 4500 mg/L glucose), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Sigma. Antibodies against phospho-SAPK/JNK (T183/Y185), total SAPK/JNK, phospho-Akt (S473), total Akt, IkBα, and phospho-PKCθ (T538), phospho-p38 MAPK (T180/Y182) , total p38 MAPK, phospho-AMPK (T172), total AMPK were purchased from Cell Signaling (Beverly, MA). Antibodies against HSP72, phospho-HSP25 (S82) and total HSP25 were obtained from Stressgen (Victoria, BC, Canada); an antibody against tubulin was obtained from Sigma and the actin antibody was obtained from Abcam Inc. (Cambridge, MA). Total PKCθ antibodies and goat-anti-rabbit HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). KNK437 was purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma .

L6 cell culture and treatment

L6 myoblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured (37°C, 5 % CO2) in DMEM supplemented with 10 % FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were differentiated into myotubes by changing culture media to DMEM + 2 % FBS 4-6 days after plating (at 80 % confluence). Myotubes were used 5-6 days later upon full differentiation. Cells were incubated with 300 µM Lipoic acid for 18 hr, washed and stimulated with either 10 µg/µl anisomcin (30 min) or 20 ng/ml TNF-α (6 h) before harvesting. For the HSP inhibition experiments, an additional set of myotubes was incubated with 300 µM Lipoic acid and KNK437 (10 µM) for 18 hr. Cells were washed and then incubated with either anisomycin or TNF-α as described above.

Experimental animals and treatment

Male Wistar rats (100-120 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and were housed in a temperature controlled ($22 \pm 2\ ^{o}C$) room with 12 h light and dark cycles. Animals were randomly divided into 4 groups (n=8 rats/group): 1) Chow+Tris (Chow), 2) Chow+Lipoic acid (Chow+LA), 3) High fat (HF), 4) High fat+Lipoic acid (HF+LA). Chow rats were fed *ad libitum* (Harlan Teklad, Madison, WI, 10% calories from fat), while HF rats received a modified- Kraegen diet (Storlien et al. 1986) (60% calories from fat and 20% calories from carbohydrates (Storlien et al. 1986) for 6 weeks, Table 2.)

TABLE 2. High fat diet recipe

Ingredient	g/kg
Casein	254
Sucrose	85
Cornstarch	169
Vitamin mix	11.7
Choline Chloride	1.3
Mineral mix	67
Bran	51
Methionine	3
Gelatin	19
Corn oil	121
Lard	218

Subgroups were injected with Lipoic acid daily, intraperitoneally at a dose of 30 mg/kg body weight or with vehicle Tris (8.3 ml/kg body weight, pH 7.4). The rats were fasted 12 h prior to all experimental procedures to lower and normalize basal plasma glucose levels prior to insulin stimulation. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) for the removal of the soleus muscle. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Intraperitoneal glucose tolerance test (IPGTT)

Overnight fasted rats were anesthetized and given a glucose load of 2 g/kg body weight. Blood glucose was measured at 0, 15, 30, 60, 90 and 120 min post injection using a glucometer. To prevent dehydration, 5 ml of 0.9 % saline was administered after the GTT.

Muscle incubations

Following dissection, the soleus muscles were carefully trimmed and split longitudinally to allow adequate diffusion of substrates as described previously (Henriksen and Holloszy 1991; Gupte et al. 2008). Two muscle strips per rat were assessed for glucose transport and two strips for Western blot analysis. Muscles strips designated for western blot analysis, recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O_2/5% CO_2. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, one muscle strip was treated with 1 mU/ml insulin and the other muscle strip was left untreated in recovery medium (basal) for exactly 10 min and then clamp frozen in liquid nitrogen.

Measurement of glucose transport activity

The muscles recovered after dissection in recovery medium for 1 h at 35°C and then rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with or without insulin (1 mU/ml). After the rinse step, muscles were incubated for 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2[1,2-^{3}H] deoxyglucose (2-DG) (1.5 μCi/ml) and 36 mM [^{14}C] mannitol (0.2 μCi/ml), with or without insulin (1 mU/ml), with a gas phase of 95 % O_2/ 5 % CO_2, in a shaking incubator. The muscles were then blotted, clamp-frozen, and processed as described previously (Young et al. 1986; Geiger et al. 2006) for determination of intracellular 2-DG accumulation (^{3}H DPM) and extracellular space (^{14}C DPM) on a scintillation counter.

Western Blotting

Clamp frozen soleus muscles were homogenized in a 12:1 (volume to weight) ratio of ice cold buffer from Biosource (Invitrogen, Camarillo, CA) containing: 10 mM Tris-HCL (pH 7.4), 100 mM NaCl, 1 mM each of EDTA, EGTA, NaF, PMSF and 2 mM Na_3VO_4, 20 mM $Na_4P_2O_7$, 1 % Triton X-100, 10 % glycerol, 0.1 % SDS, 0.5 % deoxycholate, 250 μl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes and then centrifuged for 30 minutes at 3,000 rpm at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 2 X Laemmli buffer containing 100 mM dithiothreitol and boiled in a water bath for 5 minutes.

30-100 μg of protein was separated on a SDS-PAGE (6.25-10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA). To verify transfer of proteins and equal loading of lanes, the membranes were stained with ponceau S (data not shown). Membranes were blocked for 1 hour at room temperature in TBST 5 % non-fat dry milk followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1000. Antibodies were diluted in Tris-buffered saline, 0.1% Tween-20 (TBST), 5% BSA. Blots were incubated in TBST 1% non-fat dry milk supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 hour at room temperature. Bands were visualized by ECL and quantified using Image J densitometry.

Statistical Analysis

Two way analysis of variance (ANOVA) was used when differences between both diet and lipoic acid treatments were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P<0.05$.

5.4. Results

Animal characteristics.

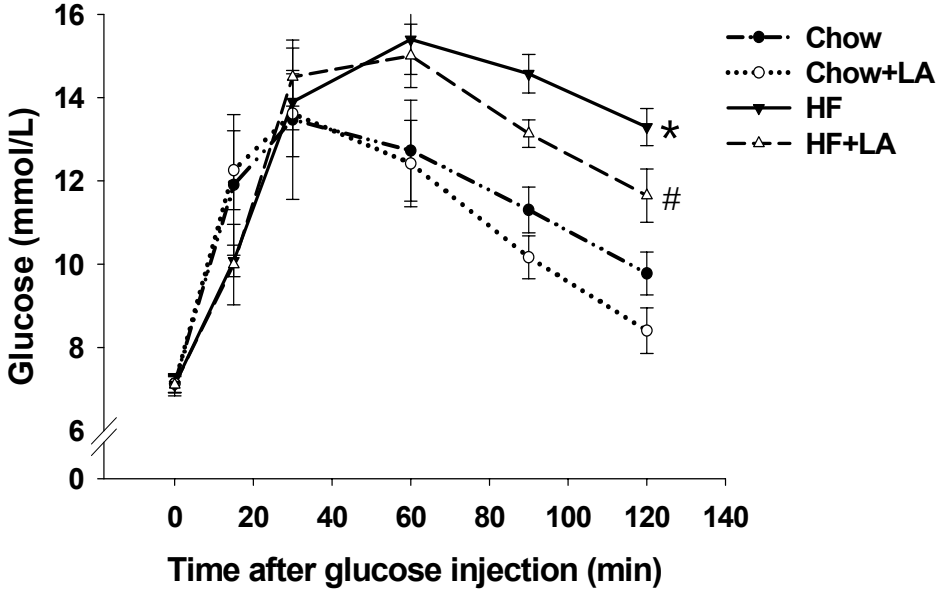
Six weeks of the HF diet induced a small increase in body weight with a trend for a decrease in body weight with LA treatment, though neither change was significant (Chow 369 ± 35.4 g, Chow+LA 360.5 ± 36.8 g, HF 397.25 ± 27.45 g, HF+LA 391 ± 30.07 g, P=0.09). An intraperitoneal glucose tolerance test was performed to assess whole body glucose utilization in response to a glucose challenge. The HF-fed rats showed decreased glucose tolerance compared to both chow groups and did not effectively clear the glucose bolus at the end of the 2 h period (Figure 14A). LA treatment administered in parallel with the HF diet effectively improved glucose tolerance compared to non-treated HF-fed rats in agreement with previous work (Saengsirisuwan et al. 2001). This is further reflected by a decrease in the glucose area under the curve (Figure 14B).

Effects of LA treatment on insulin action in the soleus muscle.

Skeletal muscle accounts for the largest amount of insulin-stimulated glucose transport and consequently, insulin resistance in skeletal muscle can reflect whole body changes in glucose homeostasis (Bjornholm and Zierath 2005). To investigate the effects of a HF diet and LA on skeletal muscle glucose uptake, we performed 2-deoxyglucose uptake assays on rat soleus muscles. The HF diet resulted in reduced insulin-stimulated glucose transport compared to the chow-fed rats (54.7 % reduction in HF compared with chow, Figure 15A). LA had no effect on glucose uptake in

FIGURE 14.

A.



B.

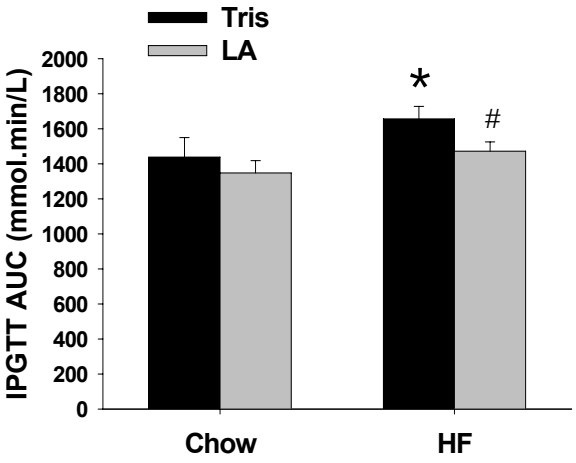


Figure 14. Lipoic acid reverses HF diet-induced whole body insulin resistance. IPGTT **(A)**: Overnight fasted rats were injected with a glucose load of 2 g/kg body weight, intraperitoneally. Blood glucose was measured at 0, 15, 30, 60, 90 and 120 min post injection using a glucometer. IPGTT is expressed as glucose area under the curve **(B)**. (*P <0.05 HF vs. Chow, #P <0.05 HF+LA vs. HF. Values are means of ± SE for 6-8 rats per group).

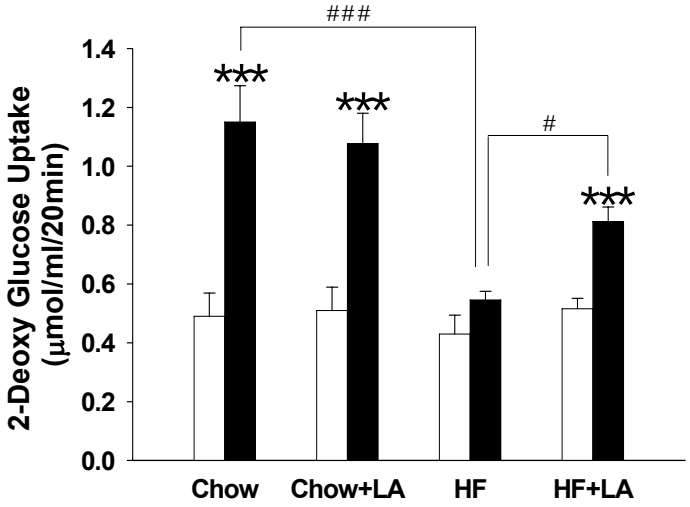
chow-fed rats. However, LA treatment significantly improved 2-deoxyglucose uptake in the HF-fed rats (55.7 % increase in HF+LA over HF). Akt is a serine threonine kinase in the insulin/IRS-1/PI3K signaling cascade, activation of which is crucial for insulin-stimulated glucose transport. Activation of Akt in response to insulin stimulation, as measured by phosphorylation of Akt on Serine 473, was reduced in the muscles from rats fed a HF diet (47.8 % reduction in HF compared with chow, Figure 15B). LA treatment did not induce further Akt activation in chow-fed rats, however, it partially restored Akt phosphorylation in the HF diet rats (74 % increase in HF+LA over HF). No significant changes were seen between groups for basal (non-insulin stimulated) glucose uptake or Akt activation.

LA induces activation of AMPK and p-38 MAPK

Apart from improving glucose metabolism through insulin-sensitive mechanisms, there is evidence that LA can improve glucose uptake by activating insulin-independent mechanisms (Henriksen et al. 1997; Khanna et al. 1999). AMP-activated protein kinase (AMPK) is an enzyme that is activated in response to a decrease in cellular energy (Hardie and Carling 1997) and plays an important role in insulin-independent glucose uptake. AMPK activation has been reported in beta cells and in muscles with 3 days of LA treatment (Lee et al. 2005; Targonsky et al. 2006) and we wanted to assess the effect of long-term LA treatment on AMPK in skeletal muscle. Our results indicate chronic LA treatment also induces AMPK activation, again only in HF-fed rats (57.6 % increase in HF+LA over HF, Figure 16A).

FIGURE 15.

A.


###
#
***
***
***
2-Deoxy Glucose Uptake
(µmol/ml/20min)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Chow
Chow+LA
HF
HF+LA

B.

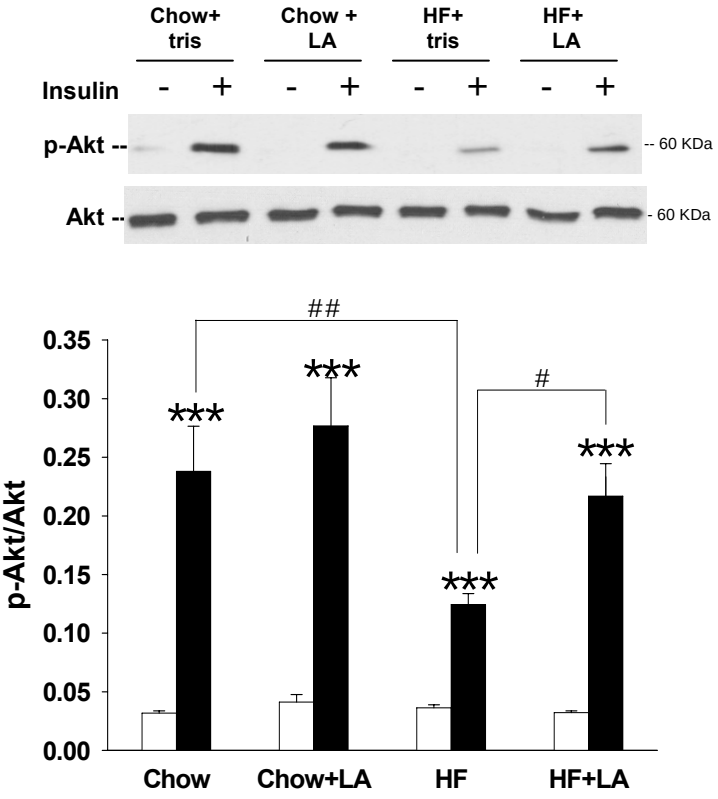
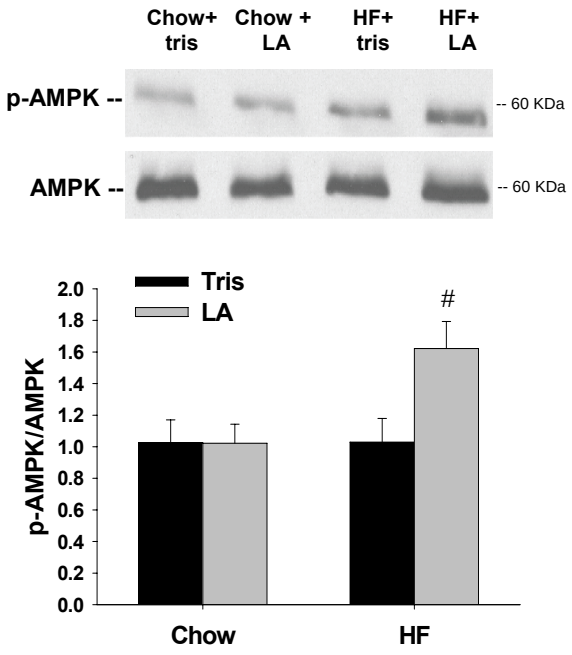

Chow+ tris
Chow + LA
HF+ tris
HF+ LA
Insulin - + - + - + - +
p-Akt --
-- 60 KDa
Akt --
- 60 KDa
##
#
***
***
***
***
p-Akt/Akt
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Chow
Chow+LA
HF
HF+LA

Figure 15. Lipoic acid improves glucose uptake and phosphorylation of Akt in soleus muscles from HF-fed rats. Insulin stimulated glucose transport **(A):** Soleus muscles were incubated in presence of insulin (1 mU/ml, filled bars) or in the absence of insulin (open bars) along with 2[1,2-^{3}H]-deoxy glucose and [^{14}C] mannitol. Insulin stimulated 2-deoxyglucose uptake into the soleus muscles was determined. (***P<0.001 Insulin vs Basal, ### P<0.001 HF vs. Chow, #P<0.05 HF+LA vs. HF. Values are means of ± SE for 6-8 muscles per group). Insulin stimulated (p)-Ser473-Akt **(B):** Soleus muscles were incubated with insulin (1 mU/ml, filled bars) or without insulin (open bars) for exactly 10 min. Muscle lysates were separated with SDS-PAGE and blots were analyzed for (p)-Akt. Blots were then stripped and probed for total Akt. (***P<0.001 Insulin vs. Basal, ## P<0.01 HF vs. Chow, #P<0.05 HF+LA vs. HF. Values are means of ± SE for 4-6 muscles per group)

FIGURE 16.

A.



B.

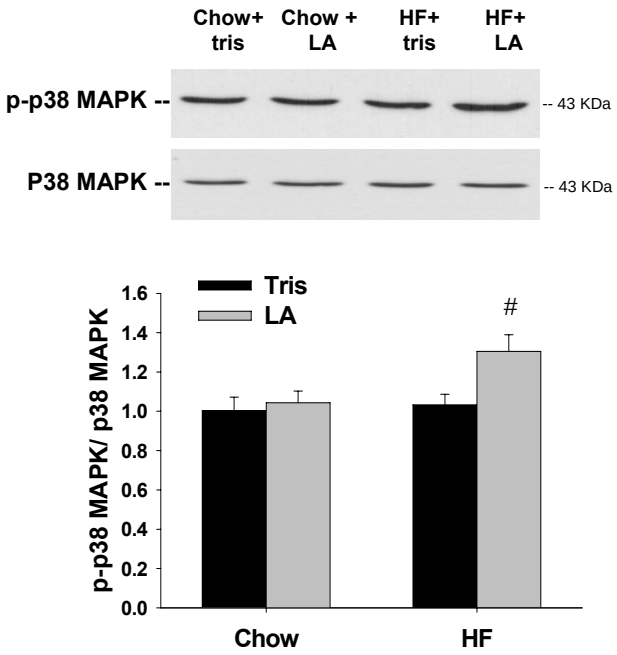


Figure 16. Lipoic acid leads to activation of p38 MAPK and AMPK in soleus muscles from HF-fed rats. Soleus muscles from Chow and HF-fed animals, treated with either Tris or LA, were analyzed for (p)-AMPK **(A)** and then stripped and probed for total AMPK; (p)-p38 MAPK **(B),** then stripped and probed for total p38 MAPK with Western blot analysis. (# $P < 0.05$ HF+LA vs. HF. Values are means of ± SE for 4 samples per group).

Studies in L6 cells have shown that acute LA treatment induces mitogen activated protein kinase p38 (p38 MAPK) activation which is potentially important in activation of GLUT4 (Konrad et al. 2001). In our study we report that even long term LA treatment administered in parallel with a HF diet results in activation of p38 MAPK (26.4 % increase in HF+LA over HF, Figure 16B).

Induction of HSPs with LA treatment.

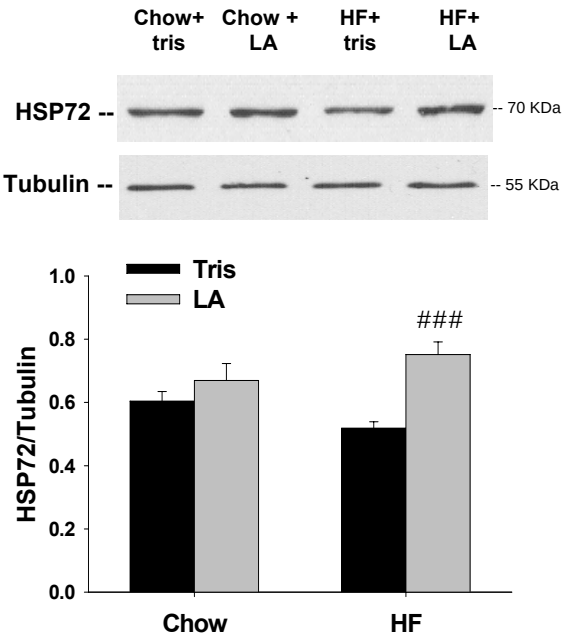
We examined levels of HSPs in the soleus muscles from chow- and HF-fed rats treated with either tris or LA using Western blot analysis. The HF diet showed a slight trend for a reduction in HSP72 expression (14 % reduction in HF compared with chow, P= 0.1, Figure 17A). LA treatment did not affect HSP72 expression in the chow-fed rats, while LA induced HSP72 in rats fed a HF diet (44.9 % increase in HF+LA over HF). Phosphorylation or activation of HSP25 also showed a significant increase with LA treatment in rats fed a HF diet (44.5 % increase in HF+LA over HF, Figure 17B). As shown in Figure 17B, p38 MAPK, a kinase upstream of HSP25 (Yuan and Rozengurt 2008), is activated by LA and could play a role in its activation. The most likely mechanism for HSP induction would be activation of the primary HSP transcription factor, heat shock factor 1 (HSF-1). In the current study, activation of HSF-1, as examined by its phosphorylation on Serine 230, tended to increase with LA treatment (data not shown, P=0.07).

Effects of LA treatment on stress kinase activation.

JNK1/2 activity levels were measured with Western blot analysis using a phospho-specific antibody. While rats fed a HF diet showed a significant increase in

FIGURE 17.

A.


Chow+ tris
Chow + LA
HF+ tris
HF+ LA
HSP72 --
-- 70 KDa
Tubulin --
-- 55 KDa
Tris
LA
###
HSP72/Tubulin
1.0
0.8
0.6
0.4
0.2
0.0
Chow
HF

B.

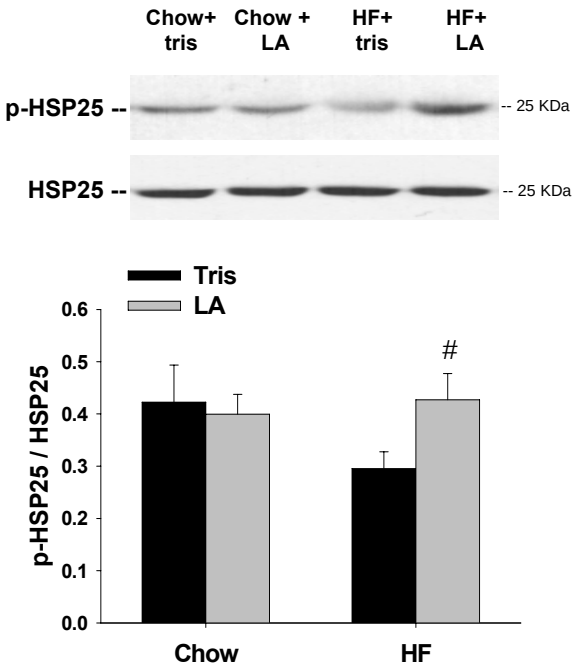

Chow+ tris
Chow + LA
HF+ tris
HF+ LA
p-HSP25 --
-- 25 KDa
HSP25 --
-- 25 KDa
Tris
LA
#
p-HSP25 / HSP25
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Chow
HF

Figure 17. Lipoic acid induces expression of heat shock proteins in soleus muscles from HF-fed rats. Soleus muscles from Chow and HF-fed animals, treated with either Tris or LA, were analyzed for HSP72 **(A)** and then stripped and probed for tubulin (###P<0.001 HF+LA vs. HF. Values are means of ± SE for 8 muscles per group). Blots were analyzed for (p)-HSP25 **(B)** then stripped and probed for total HSP25 with Western blot analysis. (#P<0.05 HF+LA vs. HF. Values are means of ± SE for 8 muscles per group).

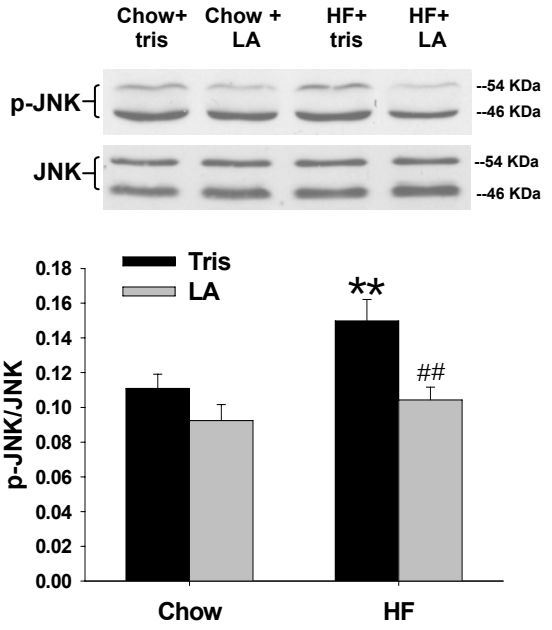
phosphorylated JNK levels (35 % increase in HF over chow), LA treatment reversed this to the levels seen in chow-fed rats (30.4 % decrease in HF+LA compared with HF, Figure 18A). No changes were detected in total JNK expression (representative blot in Figure 18A, data not shown). IKKβ activation results in degradation of its downstream target IκBα and therefore, IκBα degradation is considered a marker of increased IKKβ activity (Hacker and Karin 2006). Figure 18B demonstrates that IκBα protein levels were reduced in the soleus muscles from rats fed a HF diet (19.44 % reduction in HF compared with chow), but increased to levels in the muscles of chow-fed rats when treated with LA (44.8 % increase in HF+LA over HF). High levels of diacylglycerol (DAG) resulting from HF feeding are known to induce Protein Kinase C-θ activity. PKCθ is implicated in causing DAG-induced insulin resistance by phosphorylation of the insulin receptor substrate IRS-1 on serine residues (Haasch et al. 2006). In our study, a HF diet resulted in increased phosphorylation of PKCθ in the soleus muscles (84 % increase in HF over chow, Figure 18C). However, LA treatment did not significantly reduce activation of PKCθ. This suggests some specificity for LA to decrease JNK and IKKβ activation in skeletal muscle.

LA pretreatment induces HSPs and prevents activation of JNK and IKKβ in L6 muscle cells

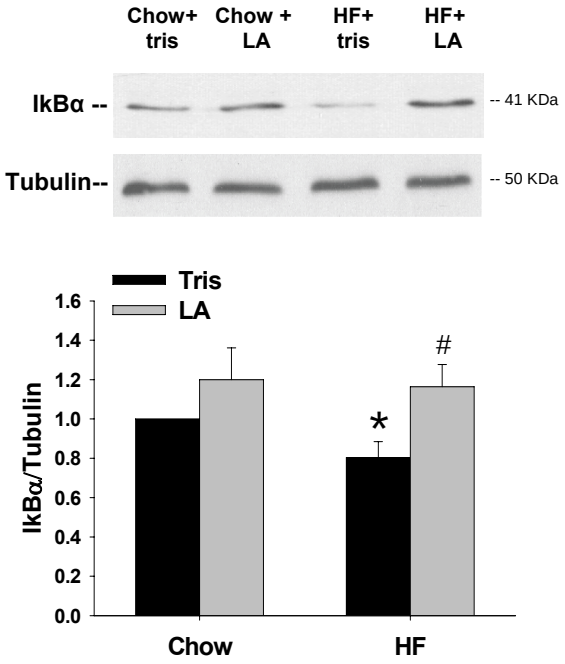
To verify the effects of chronic LA on induction of heat shock proteins and inhibition of stress kinases seen in HF-fed animals, we examined the effect of acute LA treatment on L6 muscle cells. The cytokine TNF- α was used to induce stress

FIGURE 18.

A.


Chow+ tris
Chow + LA
HF+ tris
HF+ LA
p-JNK
JNK
--54 KDa
--46 KDa
--54 KDa
--46 KDa
Tris
LA
p-JNK/JNK
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
**
##
Chow
HF

B.


Chow+ tris
Chow + LA
HF+ tris
HF+ LA
IkBα --
Tubulin--
-- 41 KDa
-- 50 KDa
Tris
LA
IkBα/Tubulin
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Chow
HF

C.

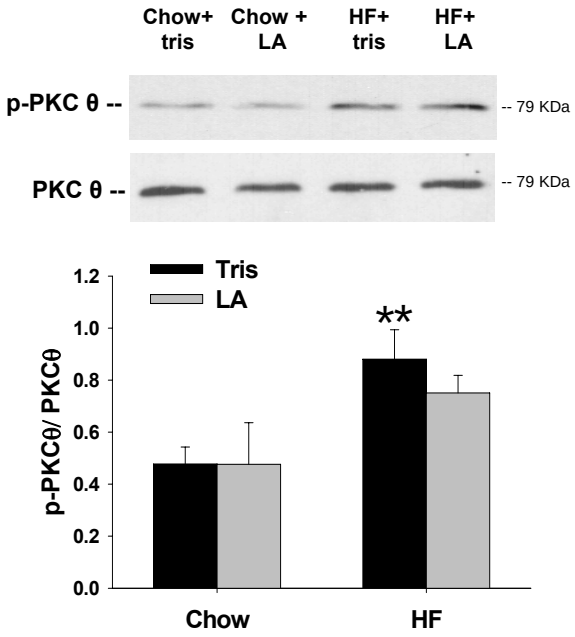
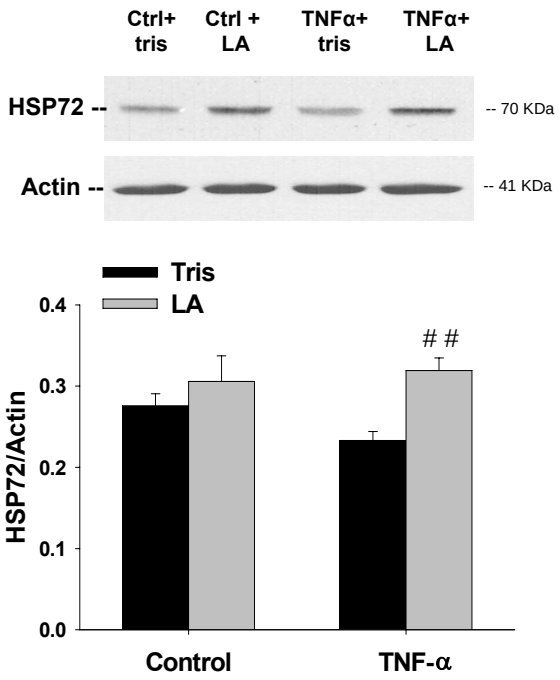


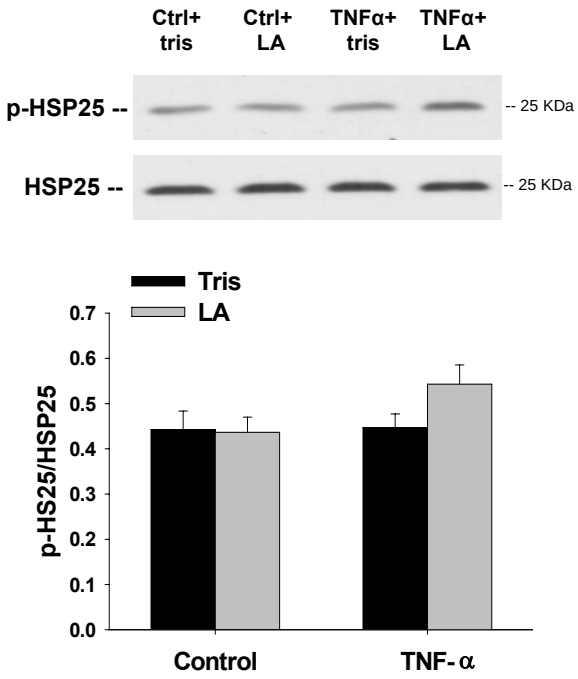
Figure 18. Lipoic acid prevents HF diet-induced stress kinase activation in soleus muscles from HF-fed rats. Soleus muscles from Chow and HF-fed animals, treated with either Tris or LA, were analyzed for (p)-JNK **(A)**, then stripped and probed for total JNK. (**P=0.01 HF vs. Chow, ##P<0.01 HF+LA vs. HF. Values are means of ± SE for 8 muscles per group). IKKβ activation **(B):** Western blots were analyzed for IkBα and then stripped for tubulin as a loading control (*P<0.05 HF vs. Chow, #P<0.05 HF+LA vs. HF. Values are means of ± SE for 8 muscles per group). (p)-PKCθ **(C):** Soleus muscles were analyzed with western blotting using an antibody specific for phospho-T538-PKCθ. Blots were normalized to total PKCθ. (**P<0.01 HF vs. Chow. Values are means of ± SE for 4-5 muscles per group).

FIGURE 19.

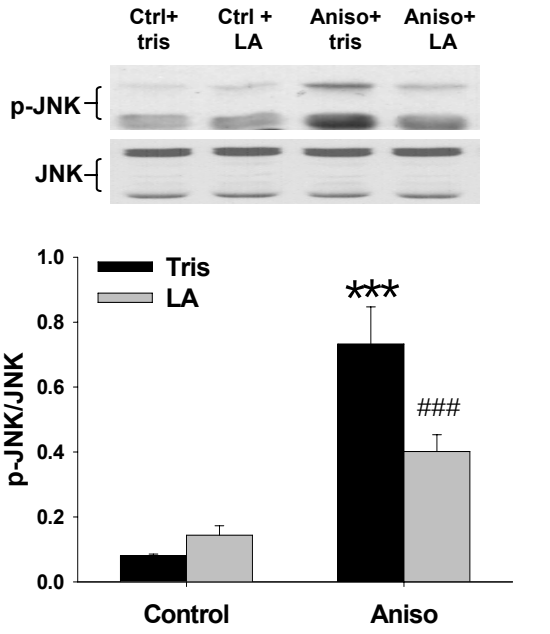
A.


Ctrl+ tris
Ctrl + LA
TNFα+ tris
TNFα+ LA
HSP72 --
-- 70 KDa
Actin --
-- 41 KDa
Tris
LA
HSP72/Actin
0.4
0.3
0.2
0.1
0.0
# #
Control
TNF-α

B.


Ctrl+ tris
Ctrl+ LA
TNFα+ tris
TNFα+ LA
p-HSP25 --
-- 25 KDa
HSP25 --
-- 25 KDa
Tris
LA
p-HS25/HSP25
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
TNF-α

C.


Ctrl+ tris
Ctrl + LA
Aniso+ tris
Aniso+ LA
p-JNK
JNK
Tris
LA
***
###
p-JNK/JNK
1.0
0.8
0.6
0.4
0.2
0.0
Control
Aniso

D.

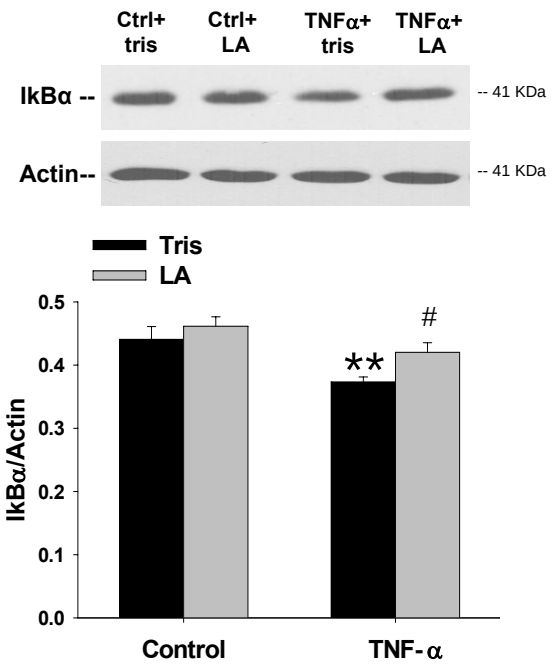

Ctrl+ tris
Ctrl+ LA
TNFα+ tris
TNFα+ LA
IkBα --
-- 41 KDa
Actin--
-- 41 KDa
Tris
LA
**
#
IkBα/Actin
0.5
0.4
0.3
0.2
0.1
0.0
Control
TNF-α

Figure 19. Lipoic acid induces heat shock proteins and prevents stress kinase activation in L6 muscle cells. L6 cells were pre-treated with Lipoic acid (300 μM) or Tris for 18 h. Cells were then washed briefly and treated with TNF-α (20 ng/mL) for 6 hr or with Anisomycin (Aniso, 10 μg/mL) for 30 min. Homogenates of the cells were analyzed for HSP72/Actin **(A),** (p)-HSP25/HSP25 **(B),** (p)-JNK/JNK **(C)** and IkBα/Actin **(D).** (***P <0.001 Aniso vs. Ctrl., ### P=0.001 Aniso+LA vs. Aniso, **P<0.01 TNF-α vs. Ctrl , #P<0.05, ##P<0.01 TNF-α+LA vs. TNF-α. Values are means of ± SE for 6-13 samples per condition).

kinase activation in muscle cells. Treatment of L6 myotubes with LA for 18 h, followed by TNF-α for 6 h, resulted in an increase in HSP72 expression and phosphorylation of HSP25 (Figure 19A and 19B). Consistent with our findings for LA treatment in chow-fed animals, LA did not increase HSP expression/activation in control cells not exposed to TNF-α. Anisomycin, a potent inducer of JNK (Grollman 1967; Aguirre et al. 2000), induced a robust activation of JNK in L6 myotubes, however, pretreatment with LA for 18 h suppressed this anisomycin induced JNK activation (45.2 % decrease from Anisomycin, Figure 19C). Similarly, TNF-α induced activation of IKKβ as seen by reduced IκBα levels, but pretreatment with LA protected the myotubes from the TNF-α induced IKKβ activation (Figure 19D). These data further suggest a role for LA in inducing HSPs as a potential mechanism of stress kinase inhibition.

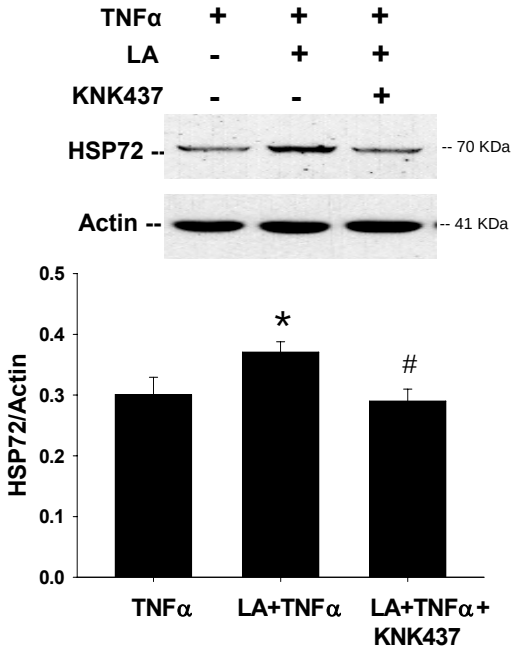
Inhibition of HSP72 abolishes the protective effective of LA on JNK

To examine the specificity of HSPs in the effects of LA, we inhibited HSP72 and assessed its effects on JNK activity. L6 myotubes were treated with the HSP72 inhibitor, KNK437, along with LA for 18 hours, followed by incubation with TNF-α for 6 hours. KNK437 inhibits the induction of HSP72 at mRNA levels and prevents transcription of new HSP72 (Yokota et al. 2000). As seen before (Figure 19A), LA treatment induced HSP72 expression, but this was blunted when incubated in the presence of 10 μM KNK437 (24% reduction from LA+TNF-α, Figure 20A). Higher concentrations of KNK437 caused greater suppression of HSP72 but also resulted in a small non-specific decrease in JNK activation. In the presence of KNK437, LA

treatment did not prevent anisomycin-induced JNK activation (43% increase over LA+Aniso, Figure 20B). This indicates that in the absence of HSP72 induction, LA does not effectively suppress JNK activation.

FIGURE 20.

A.


TNFα + + +
LA - + +
KNK437 - - +
HSP72 --
-- 70 KDa
Actin --
-- 41 KDa
HSP72/Actin
0.5
0.4
0.3
0.2
0.1
0.0
*
#
TNFα
LA+TNFα
LA+TNFα+ KNK437

B.

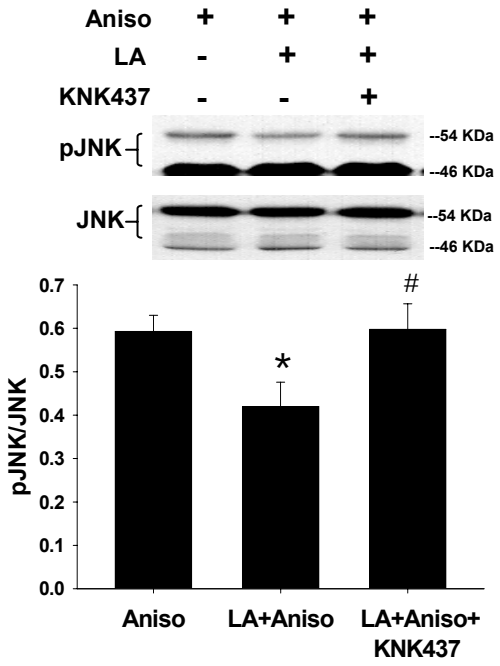

Aniso + + +
LA - + +
KNK437 - - +
pJNK
--54 KDa
--46 KDa
JNK
--54 KDa
--46 KDa
pJNK/JNK
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
#
Aniso
LA+Aniso
LA+Aniso+ KNK437

Figure 20. In the absence of HSP72 induction, LA does not prevent JNK activation. L6 cells were treated with either Tris or LA (300 μM) or a combination of LA (300 μM) +KNK437 (10 μM) for 18 h. Cells were then washed and treated with TNF-α (20 ng/mL) for 6 hr or with Anisomycin (Aniso, 10 μg/mL) for 30 min. TNF-α treated groups were examined for HSP72/Actin **(A)** and Aniso treated groups were analyzed for (p)-JNK/JNK **(B).** (*P <0.05 TNF-α+LA vs. TNF-α and Aniso+LA vs. Aniso, #P<0.01 TNF-α+LA+KNK437 vs. TNF-α+LA, #P<0.05 Aniso+LA+KNK437 vs. Aniso+LA. Values are means of ± SE for 5-6 samples per condition for (p)-JNK and 9-10 samples for HSP72).

5.5. Discussion

The purpose of this study was to determine whether chronic LA treatment increases the expression of HSPs in skeletal muscle, and if this induction can effectively inhibit stress kinases and improve insulin action. We demonstrate the ability of LA to induce HSPs in skeletal muscle *in vivo* and in L6 cells in culture with a concomitant reduction in the activation of stress kinases JNK and IKKβ. While the effects of LA in skeletal muscle are likely multi-factorial, HSP-mediated stress kinase inhibition is a previously unexplored mechanism for the improvement in insulin action with LA.

Kurucz et al. were the first to establish that in patients with type 2 diabetes, subjects with impaired glucose tolerance, and in nondiabetic identical twins whose twin had type 2 diabetes, very little HSPs are expressed in skeletal muscle (Kurucz et al. 2002). Low tissue HSP expression could play a critical role in the induction of insulin resistance and diabetes. Hooper et al. (Hooper 2007; Hooper and Hooper 2008) propose that type 2 diabetes is a vicious cycle stemming from low HSP expression that results in an impaired stress response, metabolic inflammation, and subsequent insulin resistance. Our results suggest that increasing HSPs with LA could be one of the important factors that forestall this cycle, thereby improving insulin sensitivity and diabetes. A number of *in vitro* and *in vivo* studies demonstrate the anti-inflammatory action of HSPs (Yenari et al. 2005; Kim et al. 2006). Previous studies have shown that NFκB activation is suppressed by HSP inhibition of IKKβ and that

HSP72 protects against TNF-α induced IL-6 production, shock, apoptosis and death. In addition, HSP72 has been shown to inhibit JNK, and low levels of HSP72 result in JNK activation (Chung et al. 2008). The recent study by Chung et al. showed that overexpression of HSP72 was tightly associated with the prevention of JNK activation in models of diet- and obesity- induced insulin resistance (Chung et al. 2008). The activation of cytokines as a result of low HSP expression in insulin resistant tissue is a relatively new theory regarding the development of insulin resistance and type 2 diabetes. Given the number of ways in which HSPs can be induced via physiological stressors (exercise, heat, calorie restriction), small molecules (BGP-15, geranylgeranylacetone, resveretrol), and antioxidants (lipoic acid), therapeutic induction of HSPs could be a promising new approach to the treatment of insulin resistance.

To our knowledge, this is the first study to report that LA can induce HSP expression in skeletal muscle. Although our findings did not demonstrate a significant increase in HSF-1 activation with LA, a strong trend was seen. Thus, an effect of LA on HSF-1 cannot be ruled out. LA has been shown to increase HSF-1 mRNA in diabetic kidneys (Oksala et al. 2007). Additional time points following LA treatment may be necessary to observe activation of HSF-1 in skeletal muscle. Also, HSF-1 activation is known to vary depending on the muscle fiber type (Najemnikova et al. 2007) and further studies will be needed to determine if the effect of LA on HSF-1 is evident in fast-twitch muscles. Activation of HSF-1 is tightly regulated by the inhibitory phosphorylation of HSF-1 by JNK, ERK and GSK3 on Ser 363, 307, 303

respectively (Chu et al. 1996; Dai et al. 2000; Xavier et al. 2000). Increased activation of these inflammatory kinases with HF feeding, diabetes and aging is known to downregulate the HSF-1 machinery and thereby reduce HSP expression. In our hands, six weeks of HF feeding was not sufficient to reduce HSP expression significantly in soleus muscles. Longer duration HF diet regimes may be needed to determine the role of LA on maintaining HSP expression.

Data in L6 cells demonstrates that acute treatment with LA also results in an induction of HSPs and a concomitant inhibition of JNK and IKKβ. These results support our observations in the animal model and validate our findings regarding the proposed mechanism of LA action. Several mechanisms of HSP-induced stress kinase inhibition have been suggested. HSP72 has been shown to bind to JNK and prevent its activation by the upstream kinases SEK1 and MKK7 (Park et al. 2001)**.** Further, interaction of HSP72 with a co-chaperone CHIP is thought to negatively regulate activity of Dual leucine zipper-bearing kinase (DLK), a kinase upstream of JNK (Daviau et al. 2006). While other investigators suggest HSP72 prevents stress-mediated inactivation of a phosphatase that targets JNK (Meriin et al. 1999; Lee et al. 2005), both proposed mechanisms effectively inhibit JNK activation. Similarly, IKKβ could be inhibited by HSP25, either by direct binding or an as yet uncharacterized signaling intermediate (Park et al. 2003). Future studies are needed to determine the mechanism of HSP inhibition of stress kinases in insulin resistant tissue.

It is possible that LA influences insulin signaling via oxidant effects independent of HSP induction. However, research indicating LA has no effect on

insulin sensitive tissue argues against a direct activation of insulin signaling intermediates as the mode of LA action. LA treatment has little or no effect on cells that were not subjected to oxidative stress (Maddux et al. 2001) or even on lean insulin sensitive animals (Saengsirisuwan et al. 2002; Lee et al. 2005). Consistent with these studies, we saw no significant changes with LA treatment of chow-fed rats in insulin sensitivity measures, stress kinase activation, AMPK/p38 MAPK activation or HSP expression. AMPK plays an important role in insulin-independent glucose uptake and is activated with 3 days of LA treatment (Lee et al. 2005). In our study, although we did see a robust increase in phosphorylation of AMPK with LA in high fat-fed rats, we did not see a statistically significant increase in basal glucose transport. It is likely that the observed increase in AMPK activation was not sufficient to result in increased basal glucose uptake or that chronic activation of AMPK with LA, as in the present study, might have a greater impact on fatty acid oxidation than glucose transport. Chronic AMPK activation can result in increased fatty acid oxidation and thus reduced accumulation of fatty acids and reactive oxygen species, contributing to the overall insulin sensitizing effects of LA. Also, it has been reported that intramuscular triglycerides are reduced with LA treatment (Saengsirisuwan et al. 2004), potentially as a result of AMPK activation.

The ability of LA to display both reducing (anti-oxidant) and pro-oxidant properties makes its action in skeletal muscle multi-factorial (Konrad 2005). A number of previous studies using LA suggest a mechanism of action primarily attributed to its antioxidant properties. For example, *in vivo* studies show that LA

treatment reduces protein carbonyl levels in skeletal muscle and significantly improves insulin sensitivity in insulin resistant animal models such as the Goto-Kakizaki rats (Bitar et al. 2004; Bitar et al. 2005). Additional anti-oxidant properties of LA include the capacity to scavenge ROS and to regenerate anti-oxidants such as reduced glutathione, vitamin C and E (Packer et al. 1995). The antioxidant properties of LA could certainly improve the overall metabolic state in the muscle and indirectly improve insulin action. The pro-oxidant properties of LA include an ability to activate insulin signaling; cell culture studies have shown that high concentrations of LA (2.5 mM) can directly induce glucose uptake by activating insulin signaling intermediates in a PI3K dependent manner (Yaworsky et al. 2000; Konrad et al. 2001). The induction of HSPs in skeletal muscle can also be added to the list of LA pro-oxidant properties. The finding that LA had a slight but insignificant effect on PKC θ levels suggests that the action of LA is targeting primarily JNK and IKKβ in soleus muscle, likely due to the induced HSP72 and HSP25, respectively. Our observation that HSP72 inhibition with KNK437 abolishes LA's ability to inhibit JNK, further supports the idea that HSPs constitute a key mechanism of LA action. Thus, the beneficial effects of chronic LA treatment in skeletal muscle could result from both an overall reduction in tissue oxidative stress and, as our results suggest, from a complementary increase in the content of stress protective HSPs in skeletal muscle.

Apart from just increasing glucose uptake into the muscle, LA may also facilitate the efficient oxidation of glucose, since LA is a co-factor of the pyruvate dehydrogenase (PDH complex). Although we did not measure PDH activity in this

study, increased LA treatment has been shown to increase PDH activity and improve glucose effectiveness in lean and obese patients with type 2 diabetes (Konrad et al. 1999).In conclusion, the current findings demonstrate, for the first time, that LA treatment increases expression of HSP72 and activation of HSP25 in skeletal muscle. Our study provides new information regarding the inhibition of JNK and IKKβ with chronic LA treatment and suggests HSP-mediated stress kinase inhibition is one mechanism by which LA improves insulin signaling in skeletal muscle. Future studies are needed to examine the direct mechanisms by which HSPs inhibit stress kinases.

5.6. Acknowledgements

The authors wish to thank the KUMC Biomedical Research Training Program award to A.A. Gupte for financial support.

Chapter 6

HEAT TREATMENT IMPROVES GLUCOSE TOLERANCE AND PREVENTS SKELETAL MUSCLE INSULIN RESISTANCE IN RATS FED A HIGH FAT DIET

Anisha A. Gupte, Gregory L. Bomhoff, Russell H. Swerdlow

and Paige C. Geiger

Diabetes **(2008 Dec 10): [Epub ahead of print]**

6.1. Abstract

Objective: Heat treatment and overexpression of heat shock protein 72 (HSP72) have been shown to protect against high fat diet-induced insulin resistance, but little is known about the underlying mechanism or the target tissue of HSP action. The purpose of this study is to determine if *in vivo* heat treatment can prevent skeletal muscle insulin resistance.

Research Design and Methods: Male Wistar rats were fed a high fat diet (60% calories from fat) for 12 weeks and received a lower-body heat treatment (41°C for 20 min) once per week.

Results: Our results show that heat treatment shifts the metabolic characteristics of rats on a high fat diet toward those on a standard diet. Heat treatment improved glucose tolerance, restored insulin-stimulated glucose transport, and increased insulin signaling in soleus and extensor digitorum longus (EDL) muscles from rats fed a high fat diet. Heat treatment resulted in decreased activation of JNK and IKK-β, stress kinases implicated in insulin resistance, and upregulation of HSP72 and HSP25, proteins previously shown to inhibit JNK and IKK-β activation, respectively. Mitochondrial citrate synthase and cytochrome oxidase activity decreased slightly with the high fat diet, but heat treatment restored these activities. Data from L6 cells suggest that one bout of heat treatment increases mitochondrial oxygen consumption and fatty acid oxidation.

Conclusions: Our results indicate heat treatment protects skeletal muscle from high fat diet-induced insulin resistance and provide strong evidence that HSP induction in skeletal muscle could be a potential therapeutic treatment for obesity-induced insulin resistance.

6.2. Introduction

Insulin resistance is associated with many related health complications including type 2 diabetes and heart disease. A recent study demonstrated induction of the body's natural defense system, heat shock proteins (HSPs), protects against obesity-induced insulin resistance (Chung et al. 2008). Earlier studies in patients with type 2 diabetes showed hot tub therapy improved glycemic control (Hooper 1999) and an inverse correlation between HSP72 mRNA expression and the degree of type 2 diabetes (Kurucz et al. 2002). Currently, several HSP-inducing drugs are under investigation or in clinical trials for diabetic neuropathy and neurodegenerative diseases (Kurthy et al. 2002; Westerheide and Morimoto 2005) and could be considered for prevention of insulin resistance. However, little is known about the mechanism behind this newly discovered role of HSP72, whether other inducible HSPs could be protective against insulin resistance, or the primary target tissue of HSP action.

Skeletal muscle is the major tissue responsible for whole body insulin-mediated glucose uptake (DeFronzo 1981; Song et al. 1999). HSPs are expressed in skeletal muscle and are strongly induced with exercise training (Morton et al. 2006; Tupling et al. 2007). Overexpression of HSP72 has been shown to reduce skeletal muscle atrophy and oxidative stress with age (Broome et al. 2006). Therefore, skeletal muscle is a logical choice as the target tissue for the benefits of HSP overexpression. Previous studies indicate basal levels of HSPs differ between muscle

fiber types with slow-twitch oxidative muscles having higher constitutive expression of HSPs than fast-twitch glycolytic muscles (Gupte et al. 2008). In contrast, fast-twitch muscles possess greater capacity for HSP induction in response to physiological stressors and exercise (Oishi et al. 2002; Gupte et al. 2008). It is uncertain whether HSPs would be equally effective as mediators of insulin action in slow- and fast-twitch muscle.

The purpose of the present study was to determine if weekly *in vivo* heat treatment could prevent skeletal muscle insulin resistance in rats fed a high fat diet and elucidate mechanisms of HSP function in skeletal muscle. We hypothesized that heat treatment allows skeletal muscle to adapt and resist the development of insulin resistance as a result of increased HSP expression. Our findings indicate that heat treatment prevents skeletal muscle insulin resistance and stress kinase activation, while increased oxygen consumption and fatty acid oxidation in L6 cells suggests heat treatment can improve mitochondrial function.

6.3. Materials and methods

Materials

[^{14}C] Mannitol and 2-Deoxy [1,2-^{3}H] glucose were purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies used include: phospho-Thr183/Tyr185 and total JNK , phospho-Ser473 and total Akt, IkBα (Cell Signaling, Beverly, MA); HSP72, phospho-Ser82 and total HSP25, HSP60, cytochrome c (Stressgen, Victoria, BC, Canada); tubulin (Sigma, St.Louis, MO); cytochrome oxidase IV subunits I and IV (Molecular probes, Eugene, OR); citrate synthase (Alpha diagnostic, San Antonio, TX); UCP-3 (Chemicon International, Temecula, CA); PGC-1α (Calbiochem, San Diego, CA); phospho-Tyr612-IRS-1 (Biosource, Camarillo, CA) and IRS-1 (BD Biosciences, Franklin lakes, NJ). ^{3}H-Palmitate was purchased from Perkin Elmer (Waltham, MA), insulin ELISA kits from Alpco diagnostics (Salem, NH) and all other reagents from Sigma.

Experimental animals and treatment

Male Wistar rats (100-130 g) from Charles River Laboratories, Inc. (Wilmington, MA) were housed in a temperature controlled (22 + 2°C) room with a 12:12 light/dark cycle. Animals were fed ad libitum for 12 weeks with a standard chow diet (Harlan Teklad, Madison, WI, 8604) or high fat (HF) diet (60% calories from fat comprised of lard and corn oil, and 20% calories from carbohydrates (Storlien et al. 1986)). Experiments were conducted 48 hours after the last heat or sham treatment and rats were fasted 12 h prior to experimental procedures. All

protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

In vivo heat treatment

Once per week, HF-fed animals were anesthetized with pentobarbital sodium (5 mg/100 g body weight) and the lower body was immersed in a water bath. Body temperature was gradually increased and maintained between 41 - 41.5°C for 20 min as monitored with a rectal thermometer. Sham treatment maintained core temperature at 36°C. Following treatment, 5 ml of 0.9 % saline was administered to prevent dehydration. Preliminary experiments in our laboratory established that one heat treatment per week maintains an increase in HSP72 expression and avoids potential HSP inhibition by repeated heat treatment (Lee et al. 2005).

Intraperitoneal glucose tolerance test (IPGTT)

An IPGTT was performed in week 11, 48 hr after the last heat/sham treatment. Overnight fasted rats were anesthetized and given a glucose load of 2 g/kg body weight. To prevent dehydration, 5 ml of 0.9 % saline was administered after the GTT.

Immunoblotting, Glucose Tranport and Kinase Assay

In week 12, rats were anesthetized for the removal of soleus and extensor digitorum longus (EDL) muscles. Muscles were split longitudinally to allow adequate diffusion of substrates (Henriksen and Holloszy 1991; Gupte et al. 2008). Two muscle strips per rat were assessed for glucose transport and two strips were incubated with or without 1 mU/ml insulin for 20 min and frozen for Western blot

analysis as previously described (Gupte et al. 2008). Western blots were first probed for phosphorylated proteins and then stripped for total protein expression. Glucose transport activity was determined using 2[1,2-^{3}H] deoxyglucose (2-DG) (1.5 μCi/ml) and [^{14}C] mannitol (0.2 μCi/ml) (Young et al. 1986; Geiger et al. 2006; Gupte et al. 2008). Activity levels of IKKβ in whole cell lysates were assayed as previously described (Tse et al. 2007). Phosphorylated IκBα levels were detected by Western blot analysis.

KNK437 incubation

Soleus muscles were isolated from 3 month-old Fischer 344 rats and subjected to 42ºC heat or 35ºC sham treatment for 30 min *in vitro*. Subsets of muscles were incubated in 100 μm HSP70 inhibitor KNK437 (Calbiochem, San Diego, CA) and stimulated with anisomycin (10 μg/ml, Calbiochem). HSP72 and p-JNK/JNK were detected by Western blot analysis.

Mitochondrial enzyme activity

Citrate synthase activity was assessed in muscle lysates (prepared in the cell extraction buffer used for immunoblotting, Biosource), using a modified protocol (Smirnova et al. 2006) by Srere et al. (Srere 1969). The absorbance was recorded at 405 nm every 20 sec for 3 min at 30°C, using a MRXII microplate reader and kinetic software package (Dynex Technologies, Chantilly, VA). The linear portion of the reaction curve was used to calculate activity levels of citrate synthase, normalized to citrate synthase protein expression levels, in μmol/g/min.

For the cytochrome oxidase assay, 140 μg of muscle lysate in potassium phosphate buffer (20 mM, pH 7.0) and dodecyl maltoside (0.2 mg) was warmed to 30°C (Ghosh et al. 2007). The reaction was initiated by addition of 25 μM reduced cytochrome *c* and oxidation of reduced cytochrome *c* was followed for 2 min at 550 nm on a DU series spectrophotometer (Beckman Coulter, Fullerton, CA). Maximal oxidization of cytochrome *c* was determined by adding potassium ferricyanide. Activity was calculated and normalized to Cox-4 protein expression levels (sec^{-1} / mg protein).

Measurement of oxygen consumption rates

L6 myoblasts from American Type Culture Collection (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. 5-6 days after differentiation, L6 cells were treated with 30 ng/ml TNF-α alone or in combination with heat treatment (43°C for 20 min) and experiments were performed 24 h later. O_2 consumption rate was determined using an Oroboros Oxygraph-2K high resolution respirometer (Innsbruck, Austria). After baseline stabilization, respiratory chain inhibitors were sequentially injected: oligomycin (1 μg/ml), FCCP (3 μM), rotenone (1 μM) and myxothiazol (2 μM). All values were normalized to protein content and non mitochondrial respiration rates were subtracted.

Fatty acid oxidation rates

Fatty acid oxidation was performed as previously described (Djouadi et al. 2003). Briefly, L6 myotubes were heat or sham treated and exposed to 200 μM

palmitate coupled with 7.5% BSA (wt/v) for 72 hours. A second heat treatment was given in the last 24 h of palmitate treatment. Cells were washed with PBS and incubated with 200 µl of 125 µM ^{3}H-labelled-Palmitate-BSA solution (1 mCi/ml stock), supplemented with 1 mM carnitine for 2 hours at 37°C. Following incubation, solution from each well was added to 200 µl of cold 10 % TCA and centrifuged at 3,300 rpm for 10 min. After neutralizing with 6 N NaOH the mixture was run through a DOWEX resin column to separate the fatty acid oxidation intermediates and ^{3}H – labeled water. Scintillation fluid was added to the flow-through and ^{3}H-CPM was counted. Fatty acid oxidation rate was normalized to protein content and expressed as nmole/hour/mg.

Statistical Analysis

Analysis of variance (ANOVA) and two-way ANOVA were used to study differences between groups. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P<0.05$.

6.4. Results

Body composition, serum insulin and glucose tolerance

Twelve weeks of HF feeding resulted in an increase in body weight compared with chow-fed rats (Figure 21A). Weekly heat treatment in HF-fed animals did not significantly reduce body weight and there was no difference in food intake between the HF-fed groups (Chow 106.05 ± 2.72 kcal/d; HF 114.75 ± 1.41 kcal/d; HF+HT 116.93 ± 1.57 kcal/d, $P<0.05$ Chow vs HF). Epididymal fat pad weight increased with HF feeding while this increase was blunted in rats subjected to heat treatment (Figure 21B). Fasting blood glucose levels did not change significantly with either diet or heat treatment (Figure 21C). In contrast, fasting insulin levels increased robustly in HF rats and heat treatment significantly blunted this increase (Figure 21D). While HF-fed rats showed decreased whole body glucose clearance (Figure 21E), heat treatment effectively improved glucose clearance as further indicated by a decrease in glucose area under the curve (Figure 21F).

Insulin-action is improved with heat treatment

We examined the effect of heat treatment on glucose uptake, for the first time, in representative slow-twitch soleus (84% type I, 7% type IIA, 9% type IIB) and fast-twitch EDL muscle (20% type IIA, 38% type IIB, 38% type IID, 4% Type I) (Delp and Duan 1996). In rats fed a HF diet, insulin-stimulated glucose transport increased above basal (non-insulin stimulated) values in both muscle types (Figure 22A,

FIGURE 21.

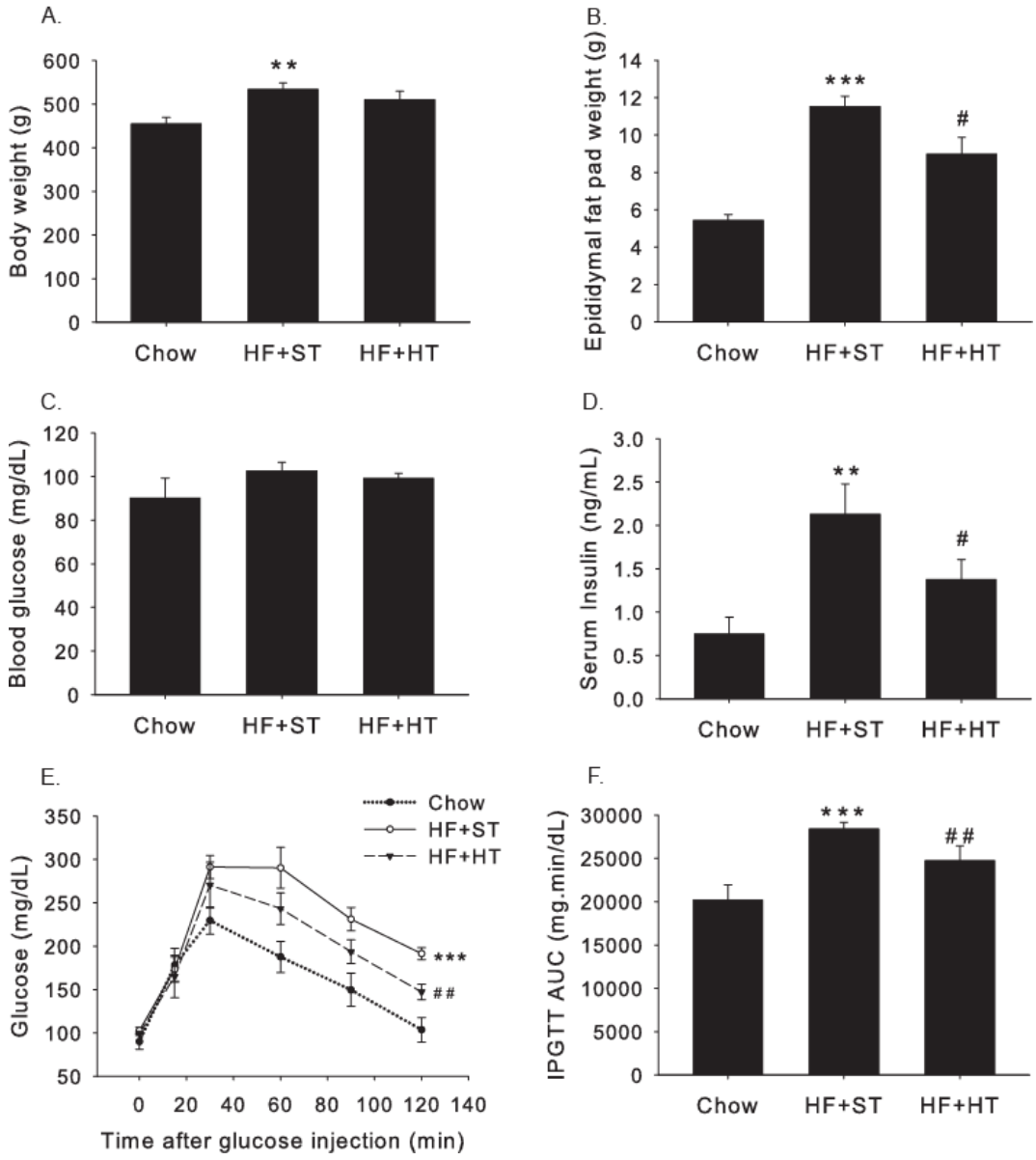

A.
Body weight (g)
600
500
400
300
200
100
0
**
Chow
HF+ST
HF+HT
B.
Epididymal fat pad weight (g)
14
12
10
8
6
4
2
0
***
#
Chow
HF+ST
HF+HT
C.
Blood glucose (mg/dL)
120
100
80
60
40
20
0
Chow
HF+ST
HF+HT
D.
Serum Insulin (ng/mL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
#
Chow
HF+ST
HF+HT
E.
Glucose (mg/dL)
350
300
250
200
150
100
50
Chow
HF+ST
HF+HT
***
##
0
20
40
60
80
100
120
140
Time after glucose injection (min)
F.
IPGTT AUC (mg.min/dL)
30000
25000
20000
15000
10000
5000
0
***
##
Chow
HF+ST
HF+HT

Figure 21. Weekly heat treatment improves characteristics of HF diet-induced insulin resistance. Male Wistar rats were given a chow or high fat (HF) diet for 12 weeks. The HF rats received either a lower body heat treatment (HF+HT, 41^{o}C for 20 min) or sham treatment (HF+ST, 36^{o}C for 20 min) once per week during the diet regimen. At the end of the 12 week study, body weight **(A)** and epididymal fat pad weights **(B)**, fasting glucose **(C)** and fasting serum insulin **(D)** were measured. IPGTT **(E)**: Overnight fasted rats were injected with a glucose load of 2 g/kg body weight, intraperitoneally. Blood glucose was measured at time 0, 15, 30, 60, 90 and 120 min post injection using a glucometer. IPGTT is expressed as glucose area under the curve in **(F)**. (***P<0.001, **P<0.01 HF+ST vs. Chow and ##P <0.01, #P <0.05 HF+HT vs. HF+ST). Values are means of ± SE for 5-7 rats per group.

HF+ST). However, the insulin effect was significantly reduced compared to chow-fed insulin sensitive muscles. With heat treatment, insulin-stimulated glucose transport was significantly increased above sham treated soleus and EDL muscles from HF rats.

Insulin phosphorylates and activates the insulin receptor substrate (IRS-1) on tyrosine residues (reviewed in (Karlsson and Zierath 2007)). Phosphorylation of Tyr612 on IRS-1 was reduced with the HF diet (not significantly in soleus muscle), but rescued fully with heat treatment in both muscles (Figure 22B). Downstream of IRS-1, insulin-stimulated activation of Akt by phosphorylation on Ser473 was reduced in soleus and EDL muscles with HF feeding (Figure 22C). Akt activation was fully rescued with heat treatment in soleus muscle and showed a non-significant improvement in the EDL. HF feeding reduced phosphorylation and activation of AS160 in EDL muscles (non-significant decrease in soleus muscle Figure 22D); while heat treatment improved AS160 activation in both muscles.

Induction of HSP72 and inhibition of JNK with heat treatment

HSP72 expression was unchanged in soleus and EDL muscles with HF feeding. Heat treatment resulted in a much greater increase in HSP72 expression in EDL muscle compared to soleus (Figure 23A). Heat treatment also induced an increase in HSP72 expression in adipose tissue and liver (adipose: Chow 0.258 ± 0.06, HF+ST 0.287 ± 0.09, HF+HT 0.760 ± 0.11, $P<0.005$ HF+HT vs. HF+ST; liver:

FIGURE 22.

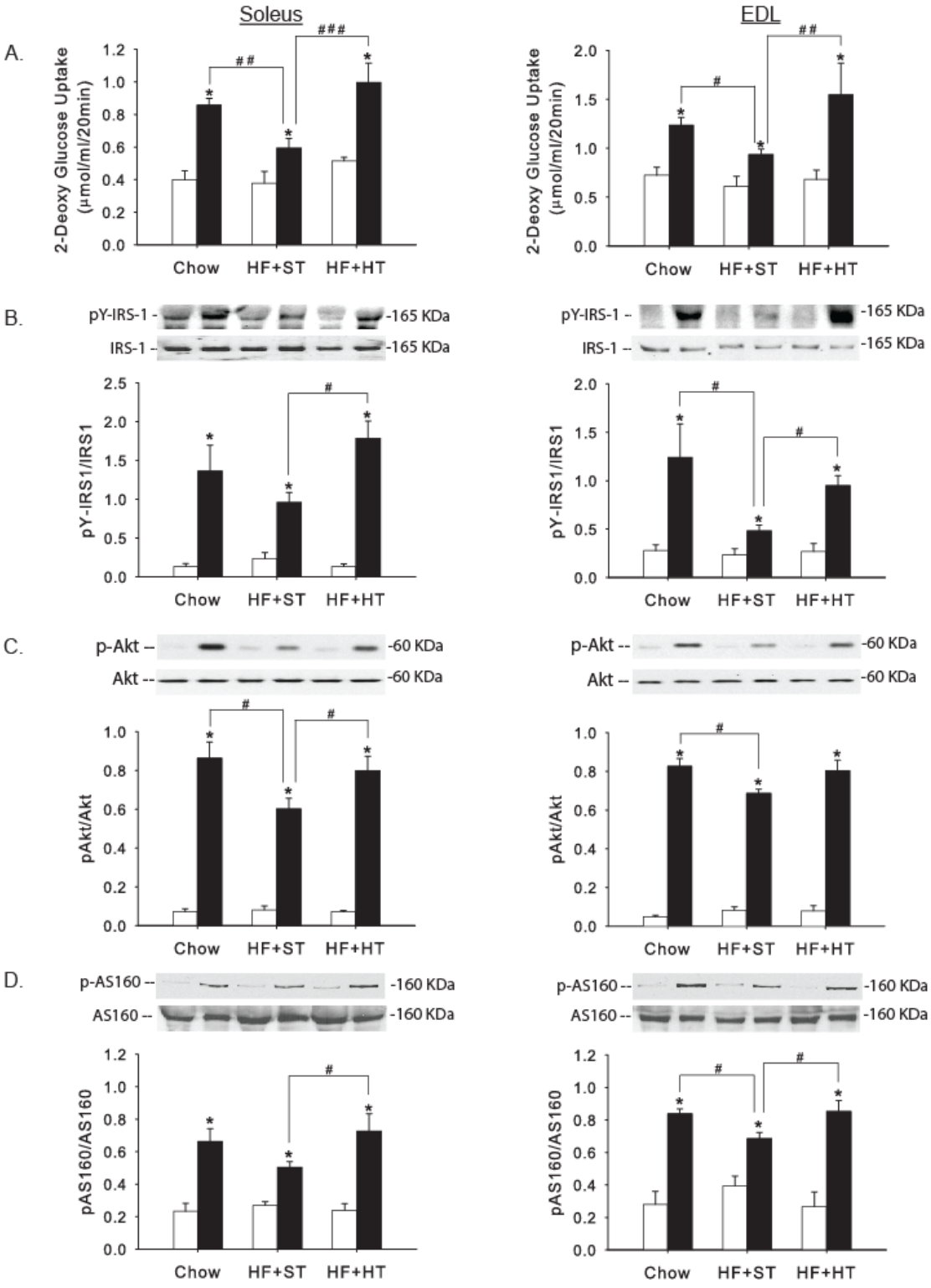

Soleus
EDL
A.
2-Deoxy Glucose Uptake (µmol/ml/20min)
Chow
HF+ST
HF+HT
B.
pY-IRS-1
IRS-1
-165 KDa
pY-IRS1/IRS1
C.
p-Akt
Akt
-60 KDa
pAkt/Akt
D.
p-AS160
AS160
-160 KDa
pAS160/AS160

Figure 22. Heat treatment increases insulin stimulated glucose uptake and phosphorylation of IRS-1, Akt, and AS160. Insulin stimulated glucose transport **(A)**: Soleus and EDL from Chow, HF+ST and HF+HT rats were incubated in presence or absence of 1 mU/ml insulin with 2[1,2-^{3}H]-deoxyglucose and [^{14}C]-mannitol and 2-deoxyglucose uptake into the muscles was determined. Values are means of ± SE for 3-5 muscles for the HF and HF+HT groups, 11-12 muscles per group for chow. Insulin signaling: Soleus and EDL muscles were incubated in the presence or absence of insulin (1 mU/ml) for exactly 20 min. Muscle lysates were separated with SDS-PAGE and blots were analyzed for (p)-Y612-IRS-1/IRS-1**(B)**, (p)-S473-Akt/Akt **(C)** and (p)-T642-AS160/AS160 **(D)**. Open bars represent basal (non-insulin stimulated) measurements and filled bars represent insulin-stimulated measurements. (*P<0.05 Insulin treated vs. Basal, ### P<0.001, ## P <0.01 and # P<0.05). Values are means of ± SE for 5-6 muscles per group.

FIGURE 23.

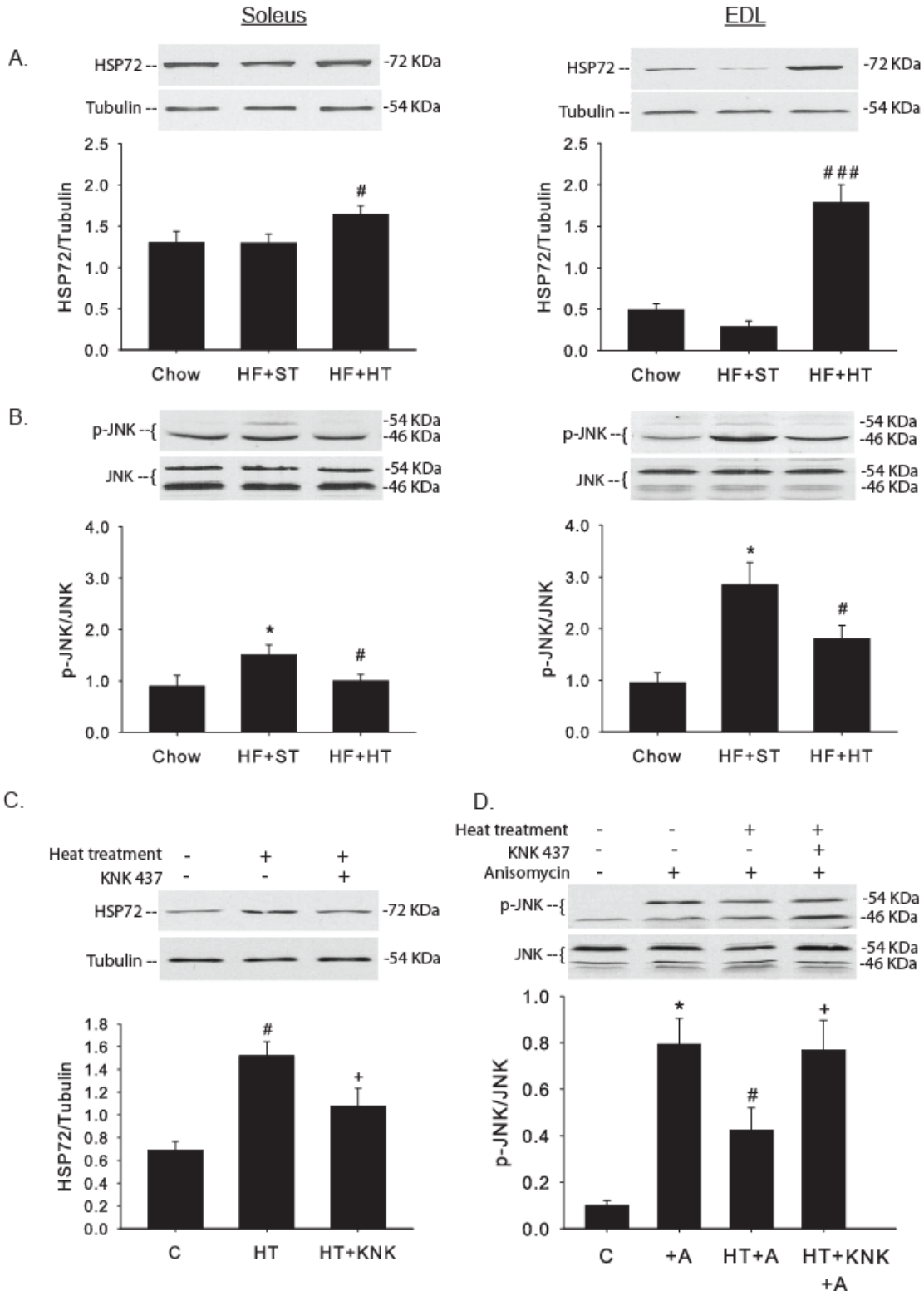

Soleus
EDL
A.
HSP72 --
Tubulin --
-72 KDa
-54 KDa
HSP72/Tubulin
Chow
HF+ST
HF+HT
B.
p-JNK --{
JNK --{
-54 KDa
-46 KDa
p-JNK/JNK
C.
Heat treatment
KNK 437
C
HT
HT+KNK
D.
Anisomycin
+A
HT+A
HT+KNK +A

Figure 23. Heat treatment induces HSP72 expression and a concomitant JNK inactivation. Soleus and EDL muscles were dissected from Chow, HF+ST and HF+HT rats and snap frozen in liquid nitrogen. Lysates were subjected to Western blot analysis for HSP72/Tubulin **(A)** and (p)-JNK/JNK **(B)**. (*P<0.05 HF+ST vs. Chow, ###P<0.001, #P <0.05 HF+HT vs. HF+ST). Values are means of ± SE for 5-7 muscles per group. Pharmacological inhibition of HSP72 prior to heat treatment eliminates heat stress-mediated inhibition of JNK. Isolated soleus muscles from F344 rats were incubated in KHB in a water bath at 35°C and then exposed to sham (35°C) or heat treatment (HT, 42°C for 30 min). A reversible pharmacological inhibitor of HSP72, KNK437 (100 μM), was included in the incubation buffer prior to, during and post HT for a subgroup of muscles. Following recovery for 12 h at 35°C, muscles were assessed for HSP72 **(C)**. JNK activation in response to Anisomycin (A, 10 μg/μl, 30 min), a potent inducer of JNK, was tested at the end of the recovery period **(D)**. (*P<0.001 A vs. C, #P<0.001 HT vs. C and HT+A vs. A, +P<0.001 HT+KNK vs HT and HT+KNK+A vs HT+A). Values are means of ± SE for 7-20 muscles per group.

Chow 0.061 ± 0.01, HF+ST 0.058 ± 0.01, HF+HT 1.157 ± 0.21, P<0.001 HF+HT vs. HF+ST). JNK activation, as measured by phosphorylation levels, increased in both soleus and EDL muscles with a HF diet (Figure 23B), although to a greater extent in EDL muscles. With heat treatment there was a significant reversal of JNK activation in both muscles. As shown in Figure 23C, the ability of heat treatment to increase HSP72 expression in isolated soleus muscle was blunted in the presence of the HSP72 inhibitor, KNK437 (Yokota et al. 2000). KNK treatment alone did not inhibit HSP72 activation (data not shown). Muscle incubation in anisomycin results in an increase in JNK phosphorylation that can be directly inhibited by prior heat treatment (Figure 23D). The presence of the HSP72 inhibitor KNK437 in the incubation medium eliminated the ability of heat treatment to decrease JNK phosphorylation.

Activation of HSP25 and inhibition of IKKβ with heat treatment

Phophorylation of HSP25 occurs in response to heat stress. (p)-HSP25 was unaffected with diet or heat in the soleus, but was significantly increased in EDL muscles with heat treatment (Figure 24A). Phosphorylation of HSP25 was not increased in adipose tissue (Chow 0.331 ± 0.1, HF+ST 0.359 ± 0.09, HF+HT 0.563 ± 08), however, there was a dramatic increase in the liver (Chow 0.030 ± 0.00, HF+ST 0.031 ± 0.00, HF+HT 0.379 ± 0.15, P<0.05 HF+HT vs. HF+ST). Activity of IκKβ was assessed by measuring the protein levels of its downstream target IkBα, which upon activation of IKKβ gets phosphorylated and targeted for degradation (Hacker and Karin 2006). Protein levels of IkBα were reduced with HF in both muscles

FIGURE 24.

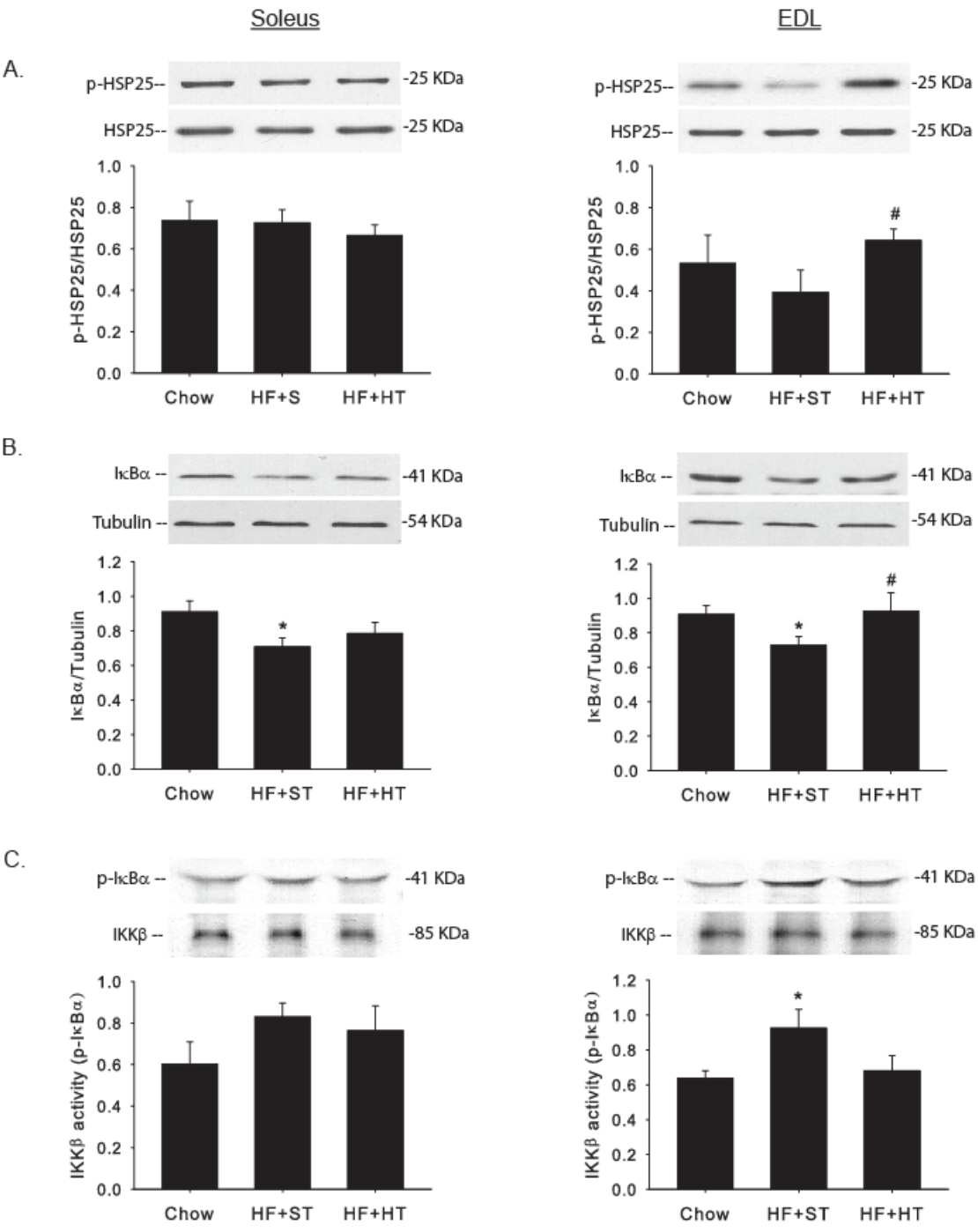

Soleus
EDL
A.
p-HSP25--
HSP25--
-25 KDa
-25 KDa
p-HSP25/HSP25
1.0
0.8
0.6
0.4
0.2
0.0
Chow
HF+S
HF+HT
HF+ST
#
B.
IκBα --
Tubulin --
-41 KDa
-54 KDa
IκBα/Tubulin
1.2
*
C.
p-IκBα --
IKKβ --
-85 KDa
IKKβ activity (p-IκBα)

Figure 24. Heat treatment prevents HF-induced IKKβ activation in skeletal muscles in a fiber type specific pattern. Snap frozen soleus and EDL muscles from Chow, HF+ST and HF+HT rats were analyzed by Western blot for (p)-HSP25/HSP25 **(A)** and IκBα/Tubulin as a measure of IKKβ activity **(B)**. Kinase activity of IKKβ was assessed by measuring phosphorylation of exogenous IκBα substrate **(C)**. Equal amounts of total IKKβ were immunoprecipitated and subjected to a kinase assay in the presence of ATP and IκBα substrate. (*P<0.05 HF+ST vs Chow and #P <0.05 HF+HT vs HF+ST). Values are means of ± SE for 5-7 muscles per group.

indicating increased IKKβ activation (Figure 24B). However, heat treatment reversed this activation only in the EDL muscle. Direct assessment of IKKβ kinase activity showed the same pattern of IKKβ activation in soleus and EDL muscles (Figure 24C).

Effects of heat treatment on mitochondrial protein expression and enzyme activity

Proteins involved in mitochondrial metabolism including cytochrome c, citrate synthase, cytochrome c oxidase subunit-1 (Cox-1, encoded by the mitochondrial genome) and subunit-4 (Cox-4, encoded by the nuclear genome) were assessed in the current study. Our results indicate these mitochondrial proteins increased with the HF diet exclusively in the EDL muscle (Figure 25A-D). However, an increase in mitochondrial proteins did not occur when heat treatment was administered in parallel with the HF diet. The mitochondrial HSP, HSP60, was slightly reduced with a HF diet and showed a significant increase with heat treatment (Chow 0.971 ± 0.047, HT+ST 0.834 ± 0.033, HF+HT 1.117 ± 0.113; $P<0.05$ for HF+ST vs HF+HT). The HSP60 expression pattern differs from other mitochondrial proteins, but is consistent with the pattern of other HSPs in the current study. Expression of uncoupling protein-3 (UCP-3), regulated by peroxisome proliferator-activated receptor (PPAR) (Wang et al. 2003), increased with the HF diet in EDL muscles and heat treatment further potentiated this increase (Figure 25E). While HF feeding resulted in an increase in the master regulator of mitochondrial biogenesis, Peroxisome Proliferator-Activated receptor-γ Coactivator 1α (PGC-1α), this effect was not observed in HF muscles given heat treatment (Figure 25F). Mitochondrial

protein expression was not significantly altered in the soleus muscle with diet or heat treatment (data not shown).

In spite of the increase in mitochondrial protein expression seen with a HF diet, mitochondrial enzyme activity was not maintained in EDL muscle. A 12 week HF diet resulted in a slight decrease in citrate synthase and cytochrome oxidase activity in EDL muscles (Figure 26A & B). In muscles given weekly heat treatment, enzyme activity levels were maintained. Soleus muscle mitochondrial enzyme activity was unchanged with diet or heat treatment.

FIGURE 25.

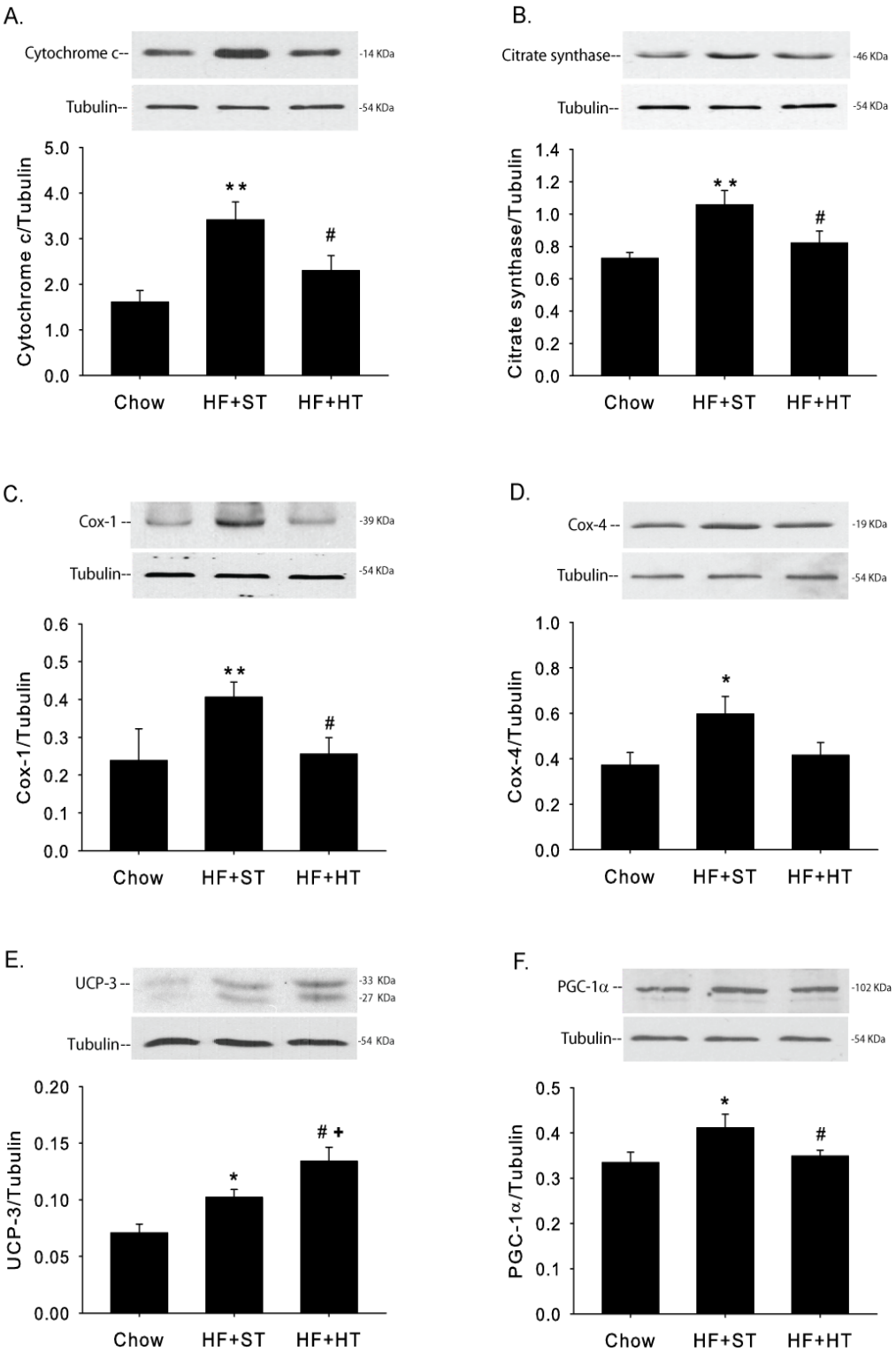

A.
Cytochrome c--
-14 KDa
Tubulin--
-54 KDa
Cytochrome c/Tubulin
5.0
4.0
3.0
2.0
1.0
0.0
**
#
Chow
HF+ST
HF+HT
B.
Citrate synthase--
-46 KDa
Tubulin--
-54 KDa
Citrate synthase/Tubulin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
#
Chow
HF+ST
HF+HT
C.
Cox-1 --
-39 KDa
Tubulin--
-54 KDa
Cox-1/Tubulin
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
#
Chow
HF+ST
HF+HT
D.
Cox-4 --
-19 KDa
Tubulin--
-54 KDa
Cox-4/Tubulin
1.0
0.8
0.6
0.4
0.2
0.0
*
Chow
HF+ST
HF+HT
E.
UCP-3 --
-33 KDa
-27 KDa
Tubulin--
-54 KDa
UCP-3/Tubulin
0.20
0.15
0.10
0.05
0.00
*
+
Chow
HF+ST
HF+HT
F.
PGC-1α --
-102 KDa
Tubulin--
-54 KDa
PGC-1α/Tubulin
0.5
0.4
0.3
0.2
0.1
0.0
*
#
Chow
HF+ST
HF+HT

Figure 25. HF-feeding results in an increase in mitochondrial protein expression but this increase does not occur when HF-fed rats are given heat treatment. EDL muscles from Chow, HF+ST and HF+HT rats were analyzed for mitochondrial protein expression using Western blot analysis. Cytochrome c **(A)**, citrate synthase **(B)**, cytochrome c oxidase subunit-1 (Cox-1) **(C)** and cytochrome c oxidase subunit-4 (Cox-4) **(D)** were increased with HF feeding in EDL muscles but not in rats given heat treatment. UCP-3 expression was increased with HF feeding and further potentiated by HT **(E)**. The long form of UCP-3 is detected at 33 KDa and short form is detected at 27 KDa. Expression of the master regulator of mitochondrial biogenesis, PGC-1α **(F)** was also increased in EDL with HF, but no increase was seen with HT. (**P<0.01, *P<0.05 HF+ST vs Chow; #P <0.05 HF+HT vs HF+ST, +P<0.05 HF+HT vs Chow). Values are means of ± SE for 5-7 muscles per group.

FIGURE 26.

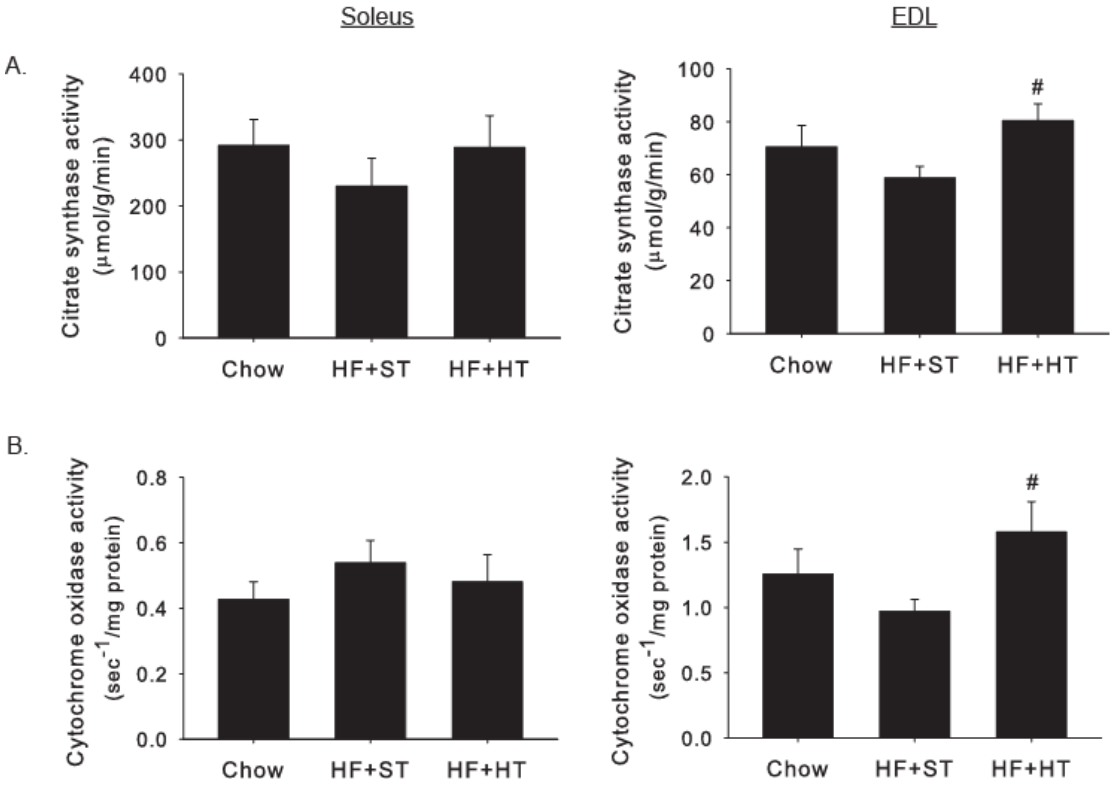


Figure 26. Heat treatment maintains citrate synthase and cytochrome oxidase activities in the presence of a HF diet. Soleus and EDL muscle homogenates were assessed for citrate synthase **(A)** and cytochrome oxidase **(B)** activity levels as described in the methods section. Citrate synthase activity was normalized to total protein levels of citrate synthase and cytochrome oxidase activity was normalized to total Cox-4 protein present in EDL and soleus muscles. (#P <0.05 HF+HT vs HF+ST). Values are means of ± SE for 5-7 muscles per group.

Acute effects of heat treatment on glucose uptake and mitochondrial function

We measured, for the first time, the effects of a single heat treatment on skeletal muscle glucose uptake and insulin signaling. As shown in Figure 27, 24 h after heat treatment, insulin-stimulated glucose uptake was increased in EDL muscles above that seen with insulin alone, with no effect of heat treatment in soleus muscle. Phosphorylation levels of the insulin signaling intermediates Akt and AS160 were not increased in either muscle 24 h after one heat treatment (data not shown). Additional time points may be necessary to determine effects of acute heat treatment on insulin signaling.

FIGURE 27.

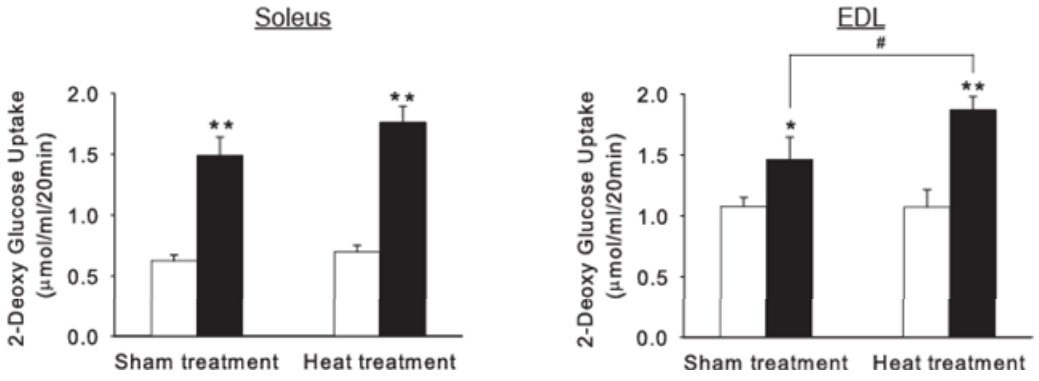


Figure 27. A single bout of heat treatment improves insulin stimulated glucose uptake in EDL muscle. Male Wistar rats received one heat bout of 41^{o}C for 20 min or a sham treatment of 36^{o}C for 20 min. Twenty-four hours post treatment, insulin-stimulated glucose uptake was assessed in isolated soleus and EDL muscles. (**P<0.001, *P<0.05 Insulin vs. Basal and #P <0.05 Heat treatment vs. Sham). Values are means of ± SE for 7-10 muscles per group.

To determine the impact acute heat treatment may have on mitochondrial function, we measured oxygen consumption in L6 muscle cells (Figure 28A). L6 muscle cells treated with TNF-α, a cytokine known to induce mitochondrial dysfunction and insulin resistance (Mariappan et al. 2007), show reduced ATP-coupled oxygen consumption compared with untreated cells (Figure 28B). In contrast, cells given one heat treatment in the presence of TNF-α show preserved ATP-coupled oxygen consumption. Maximal respiratory rate and proton leak showed a non-significant increase with heat treatment (Figure 28C and 28D). As another assessment of mitochondrial function, fatty acid oxidation in response to palmitate and heat treatment was measured in L6 cells. Exposing L6 cells to chronic palmitate results in decreased fatty acid oxidation and insulin resistance (Pimenta et al. 2008) and decreased mitochondrial enzyme activity (Bonnard et al. 2008). In the current study, chronic exposure of L6 cells to palmitate reduced the fatty acid oxidation rate, while heat treatment resulted in a small but significant increase in fatty acid oxidation (Figure 28E). These findings suggest acute heat treatment can positively enhance glucose uptake and mitochondrial function.

FIGURE 28.

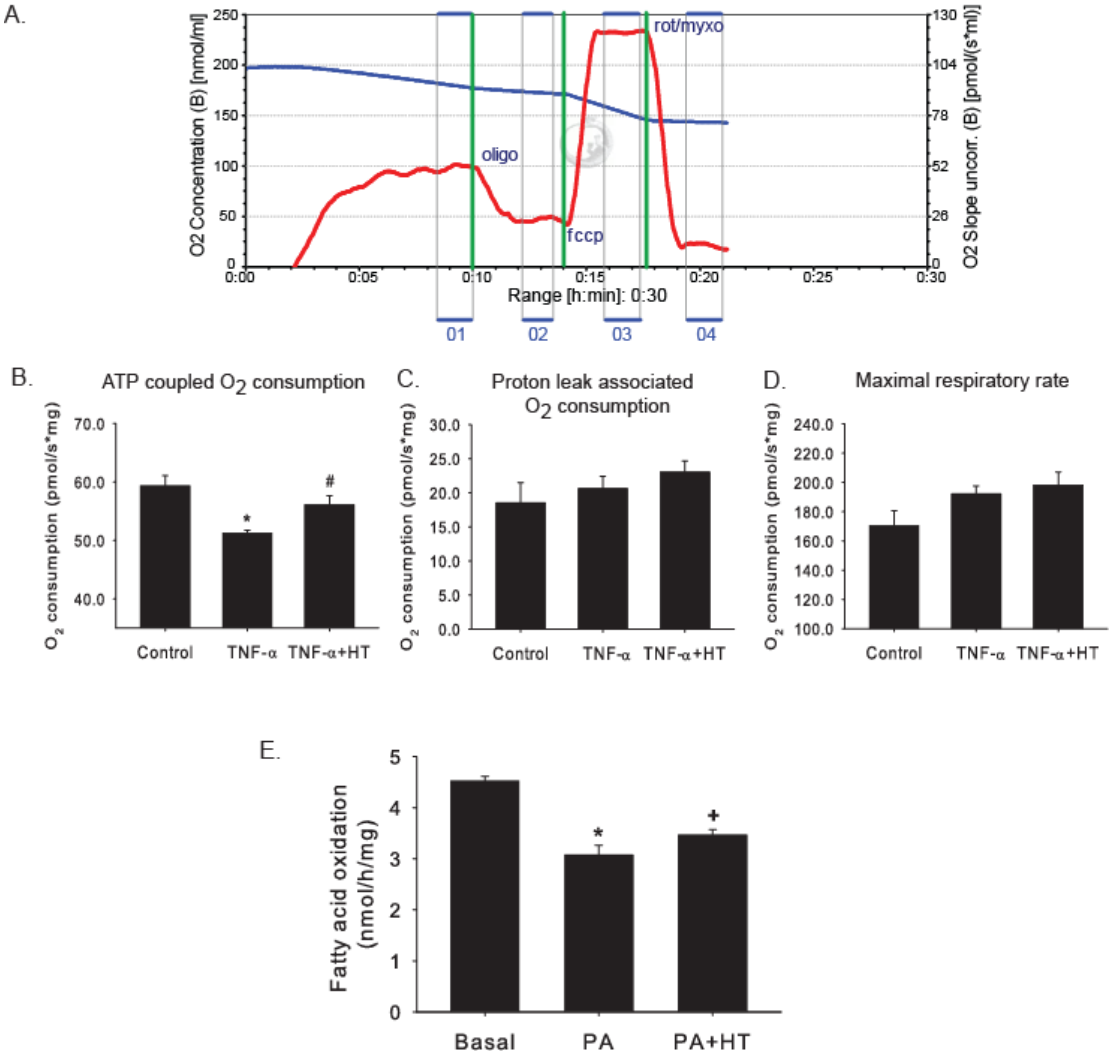

A.
O2 Concentration (B) [nmol/ml]
O2 Slope uncorr. (B) [pmol/(s*ml)]
oligo
fccp
rot/myxo
Range [h:min]: 0:30
01
02
03
04
B.
ATP coupled O2 consumption
O2 consumption (pmol/s*mg)
Control
TNF-α
TNF-α+HT
C.
Proton leak associated O2 consumption
D.
Maximal respiratory rate
E.
Fatty acid oxidation (nmol/h/mg)
Basal
PA
PA+HT

Figure 28. Heat treatment improves oxygen consumption and fatty acid oxidation in L6 muscle cells. (A) Representative tracing of O_2 consumption rates in L6 myotubes in a respirometer. Rates were determined by measuring O_2 consumption in time frames O1 for Baseline, O2 for rates after oligomycin addition (1 μg/ml), O3 for rates after FCCP (3 μM) addition, O4 for rates after rotenone (1 μM)/myxothiol (2 μM) addition. O_2 consumption rates of L6 cells were measured under control conditions, after treatment with TNF-α (20 ng/mL, 24 hr) and treatment with both TNF-α (20 ng/mL, 24 h) and one bout of HT (43°C, 20 min, recovery 24 h). ATP-coupled oxygen consumption **(B)** was calculated as the mitochondrial rate sensitive to oligomycin, proton leak **(C)** as the mitochondrial rate insensitive to oligomycin and maximal uncoupled respiration rate **(D)** as the O_2 consumption rate after FCCP addition (Amo et al. 2008). Rates were normalized to cellular protein, calculated in pmol/s·mg and expressed as percentage of basal (**P*< 0.001 TNF- α vs Control, #*P*<0.01 TNF-α+HT vs TNF- α). Values are means of ± SE for 5-6 samples per group. **(E)** L6 cells were treated with 200 μM palmitic acid coupled with BSA for 72 hours (PA) while control cells received BSA only (Basal). A subgroup of cells (PA+HT) received two heat treatments (43°C, 20 min), one right before the palmitate incubation and one after 48 hours. Cells were washed thoroughly and fatty acid oxidation was assayed using ^{3}H-palmitate-BSA (125 uM). (*P<0.001 PA vs. Basal and $^{+}$P=0.05 PA+HT vs. PA). Values are means of ± SE for 4 wells per group.

6.5. Discussion

New data in the present study demonstrates the ability of heat treatment to restore glucose uptake and improve insulin signaling in skeletal muscle from rats fed a HF diet. Our results indicate heat treatment can prevent an increase in JNK phosphorylation and IKKβ activation, possibly through direct interaction with the inducible HSPs, HSP72 and HSP25, respectively. Heat treatment increases mitochondrial HSP60 and UCP-3 expression and maintains mitochondrial enzyme activity in the presence of a HF diet. Our findings in L6 muscle cells indicate acute heat treatment increases mitochondrial oxygen consumption and fatty acid oxidation. These results provide strong evidence that HSP induction in skeletal muscle could be a potential therapeutic treatment for obesity-induced insulin resistance.

Twelve weeks of a HF diet resulted in increased overall body weight and epididymal fat pad weight. Epididymal fat pad weight was reduced with heat treatment although body weight and food intake remained the same between the HF sham and heat treated rats. These changes suggest an increased level of metabolism in the animals receiving heat treatment. Whole body hyperthermia has been shown to reduce plasma triglycerides and free fatty acids in db/db mice (Kokura et al. 2007), suggesting heat treatment improves fatty acid utilization and reduces their accumulation. Data from the current study demonstrating increased fatty acid oxidation in palmitate treated muscle cells with acute heat treatment support this idea. Future studies are needed to measure the effects of chronic heat treatment on fatty

acid oxidation in skeletal muscle. Heat treatment, like exercise, could increase the cells' demand for energy such that fatty acid uptake and utilization are matched, reducing the accumulation of free fatty acids and adipose tissue in the presence of a HF diet.

We demonstrate for the first time that heat treatment can restore insulin-stimulated glucose transport and increase activation of insulin signaling intermediates in skeletal muscles from HF-fed rats. Increased oxidative stress has been strongly implicated as a cause of diet induced-insulin resistance (Houstis et al. 2006; Matsuzawa-Nagata et al. 2008). Activation of stress-induced kinases such as JNK, glycogen synthase kinase (GSK3β), IKKβ and protein kinase C (PKC-θ) (Wellen and Hotamisligil 2005; Vinayagamoorthi et al. 2008) can cause inhibitory phosphorylation of IRS-1 on serine 307. A decrease in muscle oxidative stress with heat treatment could result in decreased stress kinase activation and improved insulin signaling. Alternatively, HSP72 and HSP25 have been implicated as natural inhibitors of JNK and IKKβ, respectively (Park et al. 2001; Park et al. 2003).

In soleus and EDL muscles, an increase in HSP72 expression with heat treatment was accompanied by a concomitant decrease in JNK activity. Our findings combined with Chung et al. (Chung et al. 2008) indicate HSP72 protection is strongly associated with prevention of JNK phosphorylation. HSP25 phosphorylation was increased in the EDL muscles with heat treatment, and a corresponding reduction in IKKβ was observed. However, without an induction of HSP25 in soleus muscle, IKKβ was unchanged with heat treatment. This suggests IKKβ may not play a

significant role in insulin resistance in the soleus muscle, since insulin signaling and glucose uptake were restored despite the lack of effect on IKKβ. Higher constitutive levels of HSPs in the soleus muscle may provide protection from stress kinase activation, as evidenced by much smaller activation of JNK in soleus compared to EDL. Muscle fiber type differences and the potential role of HSP25 were not explored in the previous study by Chung et al. (Chung et al. 2008) and demonstrate the importance of fiber type and pre-existing HSP levels in determining HSP function in skeletal muscle.

Changes in mitochondrial number and function with obesity and insulin resistance are currently controversial in the literature (Petersen et al. 2004; Morino et al. 2006; Turner et al. 2007; Bonnard et al. 2008; Hancock et al. 2008; Koves et al. 2008). Our findings agree with previous studies demonstrating an increase in mitochondrial proteins with a HF diet (Garcia-Roves et al. 2007; Turner et al. 2007; Hancock et al. 2008). Proteins involved in mitochondrial metabolism increased with a HF diet in EDL muscle, however this increase did not occur in HF-fed rats given heat treatment. It is hypothesized that skeletal muscle mitochondrial biogenesis occurs as an adaptation to an increased supply of fatty acids with a HF diet. An increased muscle energy demand, as a result of heat treatment given in parallel with a HF diet, could reduce the accumulation of free fatty acids and preclude the stimulus for mitochondrial biogenesis with a HF diet in the EDL muscle. Interestingly, mitochondrial biogenesis occurs in response to a HF diet in fast-twitch glycolytic and not in slow-twitch oxidative muscle. To our knowledge, this is the first study to make

this direct comparison in fast- and slow-twitch muscles and could explain contradictory findings in the literature using mixed muscle types. Oxidative muscle has greater potential to fully oxidize lipid substrates (Koves et al. 2005) and therefore may not undergo adaptive mitochondrial biogenesis in the presence of a HF diet. In spite of HF diet-induced mitochondrial biogenesis, citrate synthase and cytochrome oxidase activities decreased in EDL muscle. Bonnard et al. (Bonnard et al. 2008) suggest a decrease in citrate synthase activity in mice with 4 weeks of HF feeding, despite any other indications of mitochondrial dysfunction, could reflect initiation of mitochondrial dysfunction subsequently observed with 20 weeks of HF feeding. Other studies have demonstrated an increase in mitochondrial function, as measured by oxidative capacity, with a HF diet in parallel with increased mitochondrial protein expression (Garcia-Roves et al. 2007; Turner et al. 2007; Hancock et al. 2008). It is unknown whether muscle oxidative capacity could increase due to enhanced fatty acid oxidation even in the presence of decreased citrate cycle and respiratory chain enzyme activity. Interestingly, Koves et al. (Koves et al. 2008) found that in spite of high rates of fatty acid catabolism with HF feeding, moderate depletion of several citrate cycle intermediates occurred in insulin resistant muscle. These authors hypothesize that an increase in fatty acid oxidation that is not matched by increased flux through downstream mitochondrial pathways results in an accumulation of incomplete fatty acid oxidation by-products and reactive oxygen species (ROS) that could activate stress kinases (Koves et al. 2008; Muoio and Newgard 2008).

In this context, heat treatment, like exercise training (Koves et al. 2008), could result in a coordinated increase between fatty acid oxidation and downstream mitochondrial pathways in skeletal muscle. In support of this idea, citrate synthase and cytochrome oxidase activity levels were restored in EDL muscles of rats given heat treatment in the present study. In addition, data from L6 muscle cells suggest heat treatment can increase fatty acid oxidation and mitochondrial oxygen consumption. This data is supported by previous studies demonstrating HSPs can protect and enhance mitochondrial function (Polla et al. 1996; Sammut et al. 2001). Future studies are needed to assess chronic heat treatment on specific steps in fatty acid metabolism in conjunction with a HF diet.

Mitochondrial proteins HSP60 and UCP-3 were increased in response to heat treatment in the current study. HSP60 has been shown to protect complex IV activity in mitochondria (Veereshwarayya et al. 2006) while heat treatment enhanced mitochondrial complex I-V activity (Sammut et al. 2001). Based on previous findings and those of the current study, the mitochondria may be a primary target site for the protective effects of heat treatment from oxidative stress (Polla et al. 1996). UCP-3 expression increased in response to a HF diet and to a greater extent with heat treatment in the EDL muscle. Although efforts to define UCP-3 function are ongoing, a recent study suggests UCP-3 induction may limit ROS emission in conditions of lipid excess (Anderson et al. 2007). Increased UCP-3 with heat treatment could reduce ROS and enhance metabolic flux through mitochondrial pathways. Future

studies are needed to investigate these potential heat shock-mediated mechanisms in the prevention of insulin resistance.

In conclusion, our study shows that weekly *in vivo* heat treatment can mitigate HF diet-induced skeletal muscle insulin resistance. Our findings indicate heat treatment decreases JNK phosphorylation and IKKβ activation, significantly improves insulin signaling and glucose uptake, and preserves oxidative phosphorylation capacity. Exercise, resveratrol, the small molecule BGP-15, and lipoic acid have all been shown to induce HSP expression. Targeted induction of HSPs for the prevention of insulin resistance is a realistic approach for future therapeutic applications.

6.6. Acknowledgements

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Chapter 7

SUMMARY OF RESULTS AND DISCUSSION

7.1. Summary of results

Our studies examined the role of stress kinases in insulin resistance in aging and high fat diet models, and determined the role of heat shock proteins in protecting against insulin resistance. The ***principle findings*** and ***future implications*** of our studies are as follows:

1.) Age-related insulin resistance is associated with stress kinase activation and reduced HSP expression. Stress kinase activities are exacerbated in fast-twitch muscles compared to slow-twitch muscles, the latter showing greater HSP expression and being less affected by insulin resistance.

- These observations raise an important question as to whether or not high HSP expression provides the slow-twitch muscles with a greater *'reserve capacity'* to handle oxidative stress. Our studies highlight the importance of fiber type and preexisting HSP levels in determining insulin action.

2.) We show for the first time that HSP72 overexpression via heat treatment in the aging soleus muscle directly inhibits JNK activation.

- This role of HSP72, as a natural inhibitor of JNK, could be potentially explored to design treatments that alleviate JNK-induced insulin resistance. Additional potential mechanisms of heat shock protein action and its applications in other tissues also need to be investigated. For example, heat-induced HSP72 may have '*hormetic'* effects, protecting the body from various stressors of aging/HF feeding.

3.) Normal aging in the brain is associated with oxidative stress, but, in contrast to the muscles, the neurons in the brain exhibit high levels of compensatory defense mechanisms. We report that HSP25 expression in the brain is robustly increased with age.

- We hypothesize that, in situations where this protection is lost, the brain could become more susceptible to neurodegenerative disorders like AD and PD.

4.) The anti-oxidant lipoic acid alleviates high fat diet-induced insulin resistance, and we show that the underlying mechanisms of action include inhibition of stress kinases JNK and IKKβ, activation of AMPK, p38 MAPK and importantly, induction of HSPs.

- To our knowledge, this is the first report of HSP protein induction by LA, which supports the theory that LA has both pro-oxidant and anti-oxidant properties that make it a very versatile therapeutic agent.

5.) Our studies show that weekly heat treatment protects against high fat diet-induced insulin resistance. For the first time, we show that heat treatment protects muscle-specific insulin signaling and glucose uptake, and reduces stress kinase activities. Heat treatment resulted in systemic improvements of glucose tolerance, reductions in fat deposition and hyperinsulinemia. Further, we demonstrate that the benefits of heat treatment also come from its protective actions in the mitochondria, which are stressed with lipid overload due to high fat feeding.

- These results provide strong evidence that HSP induction in skeletal muscle could be a potential therapeutic treatment for obesity-induced insulin resistance. Our studies

also give an insight into the muscle-specific mitochondrial protein changes in insulin resistance and propose a potential role for HSPs in enhancing mitochondrial function to more effectively cope with lipid overload and its detrimental effects. HSP-inducing treatments such as heat, exercise and pharmacological agents need to be investigated for therapeutic applications.

Taken together, our studies show that age-related and obesity-related insulin resistance are associated with increased stress kinase activities and that HSP upregulation can prevent insulin resistance in both cases. The following discussion attempts to explain the rationale behind using HSP-inducing stressors for treatment, identifies similarities and differences in the two models of insulin resistance studied here, explains the variations in observations in the different muscle fiber types and provides possible explanations to some controversies in the field.

7.2. Discussion

7.2.1. Heat treatment, a mild stress: *the concept of hormesis:*

In our studies, we found that mild heat treatment induces HSP expression and decreases insulin resistance. One might wonder how heat stress, a stress in itself, can protect against stressors that cause insulin resistance. The prevailing theory is that mild heat stress, as well as other stresses discussed below, function in a *'hormetic'* fashion to prepare the body to combat larger stresses.

'Hormesis' refers to the beneficial effects of a toxin or stress at low doses, which at higher doses would have an opposite and detrimental effect (Mattson 2008). Several phytochemicals are believed to reduce the risk for diseases such as cancer, CVD, neurodegenerative diseases, and delay the aging process. Their primary mechanism of action is believed to be their roles as anti-oxidants, but recent evidence suggests that they have a hormetic effect and prepare the body to protect against the above risks. In a plant, these phytochemicals function as toxins to protect against predators such as insects. However, in the small doses that humans consume them, these phytochemicals trigger an adaptive cellular stress response mechanism that enables protection from a variety of adverse conditions (Son et al. 2008). Resveratrol and curcumin are some examples of phytochemicals shown to improve glucose metabolism and increase lifespan (Baur et al. 2006; Lagouge et al. 2006; Weisberg et al. 2008). Interestingly, both of these chemicals also induce HSP72 expression (Kanitkar and Bhonde 2008; Putics et al. 2008). It has been proposed that

phytochemicals mediate their action by stimulating production of antioxidant enzymes and protein chaperones as a protective mechanism against even larger stressors to come.

Therefore, although heat treatment is a stress in itself; mild heat treatment has hormetic mechanisms of action. We propose that mild heat treatments, like the phytochemicals, induce cellular stress response elements (such as the HSPs) and thus protect against the insult of high fat feeding and age-related oxidative stress. In support of this idea, it has been shown that repeated mild heat stress to human skin fibroblasts undergoing aging *in vitro* has an anti-aging hormetic effect. The effects include maintenance of stress protein profiles, reduction of oxidatively damaged proteins, enhanced levels of antioxidant enzymes and stimulation of proteasomal activities to degrade abnormal proteins (Rattan 2005). We found that lipoic acid, commonly studied as an anti-oxidant, also induces HSPs. Therefore, the HSP-inducing ability of LA might be a hormetic effect that has yet to be formally recognized as such. This concept of *hormesis* is receiving increasing attention in elucidating the therapeutic role of phytochemicals in improving disease resistance in neurological disorders (Son et al. 2008), and could have future implications in designing treatments to protect against insulin resistance.

7.2.2. Aging versus obesity:

In our studies, we examined insulin resistance in aging and obesity, but whether the underlying causes of insulin resistance are the same in the two cases is not clear. We believe that the connecting link between the two models is the common

pathology of increased oxidative stress (Berlett and Stadtman 1997; Levine 2002). In aging, a lifelong accumulation of ROS through metabolic processes, as well as increasing mtDNA mutations (hence mitochondrial dysfunction) are responsible for oxidative stress (Melov et al. 1995; Short et al. 2005; Frisard and Ravussin 2006). In the case of obesity, excessively large lipid load and reduced metabolic demand for ATP results in incomplete lipid oxidation and ROS production (Furukawa et al. 2004; Muoio and Newgard 2008). It is this chronic oxidative stress that is becoming increasingly recognized as the underlying trigger for insulin resistance (Houstis et al. 2006).

Aging is often associated with adiposity, which raises the question of whether age-related insulin resistance is really a consequence of the aging process or of excess fat accumulation. To that end, abdominal adiposity has been shown to be a better predictor of insulin resistance than fitness levels in the elderly (Racette et al. 2006). But the strongest evidence for involvement of muscle-insulin resistance versus adiposity induced-insulin resistance in aging came from a study by Petersen et al. (Petersen et al. 2003). In this study, young and elderly subjects were matched for lean body mass and percent fat mass. The selected subjects in the study were all lean (BMI<25 m^2/kg), and young (18 to 39 years old) subjects were compared with old subjects (61 to 84 years old). Despite removing the adiposity variable, the study found that the elderly were more insulin resistant than the fat mass-matched young individuals. Petersen et al. concluded that the insulin resistance was associated with reduced insulin-stimulated muscle glucose metabolism, which was, in turn, associated

with increased intramuscular lipid accumulation and a 40% reduction in mitochondrial oxidative and phosphorylation activity (Petersen et al. 2003). Therefore, when studying age-related insulin resistance, it would be important to use the appropriate animal model and take the adiposity into account.

7.2.3. Choosing the right animal models:

- ***Aging models:*** In our studies, the choice of animal model with respect to aging was influenced by the adiposity component, in an effort to determine whether the adiposity or pathogenic changes due to aging are responsible for the age-related insulin resistance. The Fischer 344XBrown Norway hybrid rat model gains significant abdominal fat mass, lives longer and exhibits significant sarcopenia, and is thus regarded as a superior model for studying age-related diseases (Larkin et al. 2001). We used this rat model to study age-related changes in HSP expression in the brain. But for studying age-related insulin resistance, we used non-hybrid Fischer 344 rats, in order to reduce the adiposity variable in our studies. The Fischer 344 rats exhibit muscle insulin resistance, but do not exhibit as much adiposity as the Fischer 344XBrown Norway hybrids. To confirm age-related muscle pathology and sarcopenia, we measured the muscle mass of young and old Fischer 344 rats. When muscle mass is compared with body weight, Fischer 344 rats also exhibit significant sarcopenia (Young soleus 0.530±0.03 g, Aged soleus 0.397±0.02 g, n=3, P=0.01).

- ***High fat feeding models of insulin resistance:*** Several models of obesity-induced insulin resistance have been reported in the literature, including genetically altered models such as the ob/ob mice, db/db mice, zucker fatty rats (White and

Martin 1997; Lindstrom 2007), and diet induced obesity models (Buettner et al. 2007). Although genetically altered (leptin deficient/mutated leptin receptor) mice exhibit strong insulin resistance and hyperglycemia, these models do not sufficiently represent the majority of obesity cases in humans. Obesity due to leptin deficiency is extremely uncommon in humans (Friedman and Halaas 1998). For this reason, we removed the variable of genetic susceptibility from our studies and used a high fat diet model to mimic the obesity-inducing Western diet. Male Wistar rats were given a fat-rich diet, in which 60% of the calories came from fat. This model and diet composition (modified from the Kraegen diet (Storlien et al. 1986)), has been extensively used in previous studies, and results in whole body and muscle specific insulin resistance (Kim et al. 2000). Recent studies report that effects of the high fat diet vary with sex, strain and age of the animals at the start of the diet regime (Nishikawa et al. 2007). Therefore it would be important to extensively characterize the specific model used within each study.

7.2.4. Muscle fiber type differences:

The skeletal muscle is highly heterogeneous and differences in the metabolic properties of the muscle fiber types are striking. Therefore, it became important in our studies to examine the effects of insulin resistance and interventions to alleviate it in both the fast- and slow-twitch muscles. Insulin's action of glucose uptake and metabolism occurs in a muscle fiber type-specific pattern, with a greater response of insulin signaling and glucose uptake seen in type I versus type II (Song et al. 1999). Sarcopenia in aging is associated with a loss of primarily type II muscle fibers

(Grimby 1995), but in contrast, it is the percentage of type I muscle fibers that is reduced with extreme obesity (Hickey et al. 1995; Gaster et al. 2001) and inactivity (Hjeltnes et al. 1998).

In our studies, we see that fast-twitch epitrochlearis muscles show reduced insulin sensitivity compared with slow-twitch soleus muscles, and further reduction occurs with age (Chapter 2). In contrast, high fat feeding showed slightly more insulin resistance in slow-twitch soleus muscles than in fast-twitch EDL muscles (Chapter 6), a finding also supported by previous observations (Song et al. 1999). But upregulation of mitochondrial proteins in response to HF feeding occurred only in the EDL muscles. These observations indicate that compensatory mechanisms activated to rescue insulin action are different within muscle types and between aging and obesity.

One possible explanation is the source of oxidative stress in the two cases. In the case of aging, it is likely that the cumulative mtDNA mutations leading to mitochondrial dysfunction cause oxidative stress. The soleus muscles appear to have a *'reserve capacity'* (large number of mitochondria, greater expression of insulin signaling intermediates and more GLUT4) that protects them from becoming severely insulin resistant. High levels of oxidative stress in the type II muscle fibers with age (Anderson and Neufer 2006) cause dysfunction to the smaller number of mitochondria in these fibers. Also, greater mitochondrial dysfunction with age is evident in muscles with higher type II fiber content in humans (Conley et al. 2007). Therefore, in terms of insulin action, the fast twitch muscles do worse with aging.

In the case of high fat feeding, a mitochondrial compensation to handle the excess lipid load is seen only in the fast-twitch fibers and this compensation could be protecting these fibers from insulin resistance. Since fast-twitch fibers have less oxidative capacity, lipid overload in high fat feeding forces a fuel switch which, in turn, forces a mitochondrial biogenesis response. Previous studies showing robust mitochondrial biogenesis in response to HF feeding have also reported this observation in fast-twitch epitrochlearis muscle (Hancock et al. 2008) or mixed quadriceps (Turner et al. 2007); but neither of these studies examined slow-twitch muscle types. We believe that this compensation may eventually fail as the duration of the diet increases and the disease becomes more severe. Further studies using several different age-points, durations of HF diet and fiber-type specific analyses will be needed to see whether insulin resistance occurs before or after mitochondrial dysfunction in aging and obesity.

7.2.5. Mitochondrial controversies: the chicken or the egg?

In our studies, we observed mitochondrial dysfunction in the aging skeletal muscle (Chapter 2) and increased mitochondrial protein expression with high fat feeding (Chapter 6). As illustrated in the introduction (Chapter 1, section 1.4.4.), strong evidence suggests that mitochondrial depletion and dysfunction is associated with insulin resistance (Petersen et al. 2004; Lowell and Shulman 2005; Morino et al. 2006), while opposing evidence suggests that insulin resistance can occur in the presence of elevated mitochondrial number and activity (Turner et al. 2007; Hancock et al. 2008). Clearly, future studies are required to fully elucidate the association of

mitochondrial function and insulin resistance. It would be important to investigate whether these discrepancies arise from the type of diet regimes, duration of diet, the tissue under study, severity of the disease or animal models versus human tissue under study. Several of these studies have used mixed muscles such as the quadriceps muscle. Because the slow-twitch and fast-twitch muscles have distinctively different mitochondrial capacity and metabolic responses, one would expect differential responses to a HF diet in the two muscle types. Further, given the vastly different HSP levels in the two muscle types, it would be important to understand the muscle type-specific responses to both a high fat diet as well as heat-induced mechanisms that improve mitochondrial function.

It has been suggested that mitochondrial dysfunction occurs over time. Chanseuame et al. (Chanseaume et al. 2007) showed that a HF diet induces an initial increase in mito-OXPHOS activity, followed by a decrease. Respiration rates and ATP production increased with HF diet at day 14, and then were significantly reduced at day 40, compared with the controls. The authors report that mitochondrial dysfunction in the oxidative muscle occurs late and most likely occurs after the onset of insulin resistance within the muscle. One could hypothesize that this result might be due to the greater *'reserve oxidative capacity'* of this muscle type. Although there is an initial compensation for processing the excess free fatty acids, the mitochondria eventually lose the ability to keep up in the face of prolonged excess fat delivery. So, before getting 'victimized,' the mitochondria attempt to compensate and thus, any intervention to increase mitochondrial OXPHOS activity should be advantageous to

the prevention of frank diabetes (Chanseaume et al. 2007). We show that heat treatment increases the mitochondrial OXPHOS activity, thereby protecting the muscle from lipid accumulation and insulin resistance.

To understand the mitochondrial biogenesis response to prolonged HF feeding, we examined PGC-1α expression in 2 different diet regimes and in db/db diabetic mice (preliminary data, Figure 29). Our data shows PGC-1α expression increased by 60% in rats fed a high fat diet for 6 weeks, but only a mere 25% increase was observed in a more severe model of 12 weeks of high fat feeding. Further, db/db diabetic mice showed a reduction in PGC-1α expression. Thus, these data support the idea that severity or duration of the high fat diet affects mitochondrial compensation, which eventually fails in frank diabetes.

FIGURE 29.

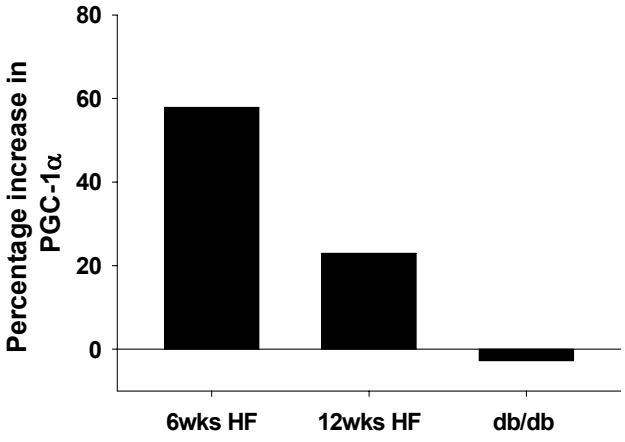

Figure 29. PGC-1α expression changes with longer duration of HF diet and obesity. PGC-1α expression increases with HF feeding, potentially to compensate for the lipid overload. However, increase in PGC-1α is less with longer duration of the HF diet and obesity. Muscles from male Wistar rats fed a high fat diet for 6 weeks or 12 weeks and from ob/ob obese mice were analyzed for PGC-1α expression with Western blot analysis. Within each group, values were compared with the respective controls (chow-fed rats for HF diet and wild type mice for db/db) and values were expressed as percentage change from controls. N=5-7 per group.

In our studies, we see increased mitochondrial protein expression with high fat feeding in EDL muscles only. Heat treatment normalized the protein levels and increased mitochondrial function to the levels of the controls, again only in the EDL muscles. This muscle-type specific response to high fat diet gives an interesting insight into the importance of pre-existing oxidative capacity of the muscle. In support of this idea, it has been previously reported that UCP-3 levels are lower in oxidative than in glycolytic fibers (Hesselink et al. 2001), and we were not able to detect any expression of UCP-3 in slow-twitch soleus muscle (Chapter 6). It has been proposed that fibers already equipped with an increased capacity for handling fatty acids would not require UCP-3-mediated fatty acid-handling pathways. Anderson et al. showed that induction of UCP-3 by exercise suppresses mitochondrial oxidant emission during fatty acid-supported respiration (Anderson et al. 2007). Thus, UCP-3 could control ROS production in heat treated muscles from HF rats, in spite of increased fatty acid oxidation.

Increased capacity for fatty acid oxidation is potentially a compensatory response to elevated fatty acid substrate availability. For example, mice with muscle-specific overexpression of lipoprotein lipase, which increases influx of fatty acid into the muscle, show extensive mitochondrial proliferation (Levak-Frank et al. 1995). Despite the increases in fatty acid oxidation seen in the studies by Turner et al. and Hancock et al. (Turner et al. 2007; Hancock et al. 2008), intramuscular lipid is a characteristic feature of these animal models. Increased fatty acid uptake in these muscles may cause accumulation of the lipids, but the compensatory increase in fatty

acid oxidation is not capable of completely eliminating the fatty acids. These models also show increased adipose mass and circulating free fatty acids (Hegarty et al. 2002).

Using transcriptomics and targeted metabolic profiling tools based on mass spectrometry it was suggested that lipid-induced insulin resistance is a condition in which upregulation of PPAR-targeted genes and high rates of beta-oxidation are not supported by a commensurate upregulation of the tricarboxylic acid (TCA) cycle activity (Koves et al. 2008). The authors suggest that high fat feeding results in a persistent burden of high lipid load to the mitochondria, and this lipid load increases beta-oxidation. The disconnection between the rates of oxidation and TCA result in accumulation of incompletely oxidized acyl-carnitine intermediates. This group further found that exercise brings about tighter coupling between beta-oxidation and TCA (Reviewed in (Muoio and Koves 2007)). We hypothesize that heat treatment, like exercise, increases the coupling between beta-oxidation and TCA, such that flux through the mitochondria is increased. Increased mitochondrial flux then reduces fatty acid accumulation and deposition, as seen in our heat treated animals. In the future, it would be important to study the metabolic rates and oxygen consumption of these heat treated/HF-fed animals using metabolic chambers. Lipid accumulation is also reflective of the fact that the insulin-resistant muscle has '*metabolic inflexibility*', such that its ability to select substrates for fuel metabolism and to transition between carbohydrates and fatty acid oxidation is lost in insulin resistance (Kelley et al. 2002).

Heat treatment could also have potential roles in reversing this '*metabolic inflexibility*', a theory that needs experimental validation.

Chapter 8

FUTURE DIRECTIONS

8.1. Mechanisms

Future studies following this project, should focus on the mechanisms of HSP action using HSP knock out animals and or siRNA-knockdown techniques. While direct stress kinase inhibition is one of the putative mechanisms of action, the mechanism of mitochondrial protection by HSPs in insulin resistance is not very clear at this point. We see in our studies that heat treatment maintains mitochondrial function such that the muscles remain closer to homeostasis, in spite of high fat feeding, and exhibit characteristics similar to those of chow fed rats. However, the molecular target(s) of HSPs in the mitochondria are unknown. HSPs are known to facilitate protein import into the mitochondria, improve enzyme function and reduce mitochondrial oxidative stress (Sammut et al. 2001; Young et al. 2003; Ouyang et al. 2006). Also, whether the observed mitochondrial effects are due to heat shock proteins or the actual heat treatment is not known. One speculation is that heat treatment, like exercise, increases the flux of activated fatty acids through beta oxidation, TCA and the electron transport chain, and facilitates complete oxidation of the fatty acids. This reduces accumulation of fatty acids as adipose depots, as well as free fatty acids that impair insulin signaling. Despite high levels of fatty acid oxidation flux through the mitochondria, ROS levels might be maintained by UCP-3 and the HSPs themselves; a theory that needs to be experimentally verified in future studies. An important follow-up experiment will be to feed HSP knockout (*hspa1a*, *hspa1b-/-*) mice a high fat diet and then determine whether or not insulin resistance is

worse than that of wild type mice. Such an experiment will determine the specific beneficial role of HSP72 on insulin action and mitochondrial function.

In our studies, we saw that HSP72 showed the most robust effects in preventing insulin resistance, but other HSPs such as HSP25 and HSP60 also contributed. Studies examining aging effects in the brain show that HSP25 has more robust effects in the brain than HSC70, HSP72 and HSP60. Small HSPs like HSP32 are known to increase with oxidative stress in neurons (Goldbaum and Richter-Landsberg 2001), and may have potential roles in combating oxidative stress-associated neuronal damage. In addition, it would be important to determine the cellular localization of the HSPs, especially HSP25, in response to age, diet and heat. HSP25 activation is accompanied by phosphorylation, oligomerization and nuclear localization, and HSP25 is thought to perform different functions in these three states (Benndorf et al. 1994). Thus, future studies would be important to characterize the functions of each of these major HSPs in insulin resistance and their induction with heat in different tissues.

8.2. HSPs as therapeutic targets against insulin resistance

Current therapeutic agents administered for controlling insulin resistance mainly consist of the thiazolidinediones (TZDs). TZDs function as insulin sensitizing drugs, by activating the transcription factor peroxisome proliferator-activate receptor γ (PPAR γ) axis and are thought to improve metabolism in both adipose tissue and liver (Bhatia and Viswanathan 2006). Although TZDs have so far been the best in improving glycemia control, several undesirable side effects have been reported,

including fluid retention (Berlie et al. 2007), weight gain (Boden and Zhang 2006), worsening of coronary artery disease and an increased risk of myocardial infarctions (Lincoff et al. 2007). Given the complications associated with the current therapeutics, it is highly crucial to discover novel therapeutic targets and therapeutic agents to improve glucose homeostasis with minimal side effects.

HSPs represent a very important treatment target for insulin resistance and associated diseases. The importance of this under-appreciated role of HSPs is being increasingly recognized in the scientific community (Hooper and Hooper 2005; McCarty 2006). We report the role of HSPs in protection against insulin resistance, but the scope of this mechanism may not be limited to insulin resistance. HSPs also protect against disuse muscle atrophy in senescence (Dodd et al. 2008), as well as age-related muscle damage (Broome et al. 2006) and the mechanism involves inhibition of NF-κB and Foxo3a transcriptional activities (Senf et al. 2008). HSP70 overexpression reduces symptoms of peripheral neuropathy in diabetes (Kurthy et al. 2002) and increases beta-cell survival during chronic hyperglycemia (Laybutt et al. 2002). Thus, sufficient evidence exists to show that HSPs may serve as a beneficial target for the treatment of insulin resistance and several associated complications. Current drug trials including that of BGP-15-like compounds should soon validate the use of HSPs as a therapeutic target (Kolonics et al. 2006). KU-32, a non-toxic small molecule, is an HSP90-inhibitor and therefore, functions as an HSP72-activator (Lu et al. 2008). KU-32 inhibits the interaction between HSP90 and HSF-1 such that free HSF-1 can then bind the heat shock elements and promote transcription of heat shock

genes. Preliminary data from our lab showed that intraperitoneal administration of KU-32 induces HSP72 in the epitrochlearis muscle (Figure 30). Future experiments to determine the effects of this drug on insulin sensitivity will be important.

While novel HSP-inducing drugs such as BGP-15 are being investigated, it would also be important to recognize the HSP-inducing properties of naturally occurring compounds such as glutamine (Wischmeyer et al. 2001), resveratrol (found in grapes (Putics et al. 2008)) and curcumin (found in curry spice turmeric (Kanitkar and Bhonde 2008)). These compounds have also shown beneficial effects in maintaining glucose homeostasis (Baur et al. 2006; Weisberg et al. 2008), and thus, warrant more attention as potential therapeutic agents. Including these compounds as dietary supplements may offer protection from insulin resistance. Novel resveratrol-like compounds that are more efficacious are being investigated in clinical trials to treat metabolic disorders and age-related pathologies (Milne et al. 2007).

FIGURE 30.

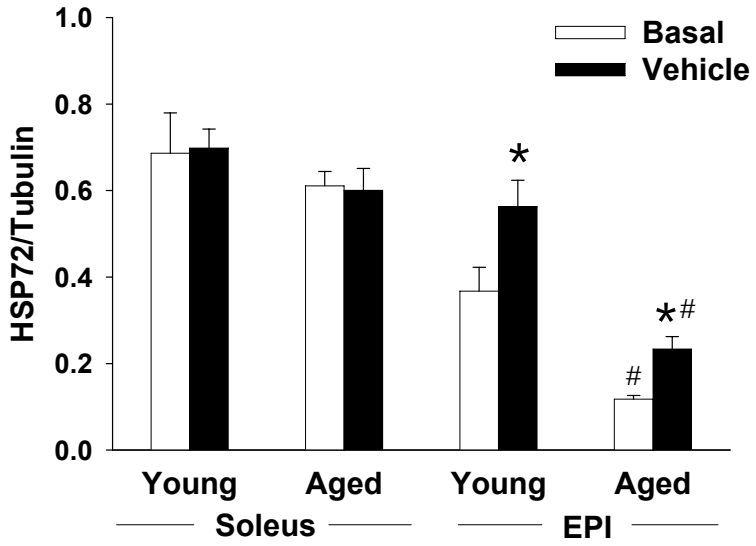

Figure 30. The HSP90-inhibitor, KU-32 induces HSP72 expression in the epitroclearis muscle. KU-32 (2 mg/kg body weight) was administered via intraperitoneal injection to young (6-month-old) and aged (30-month-old) F344XBrown Norway hybrid rats. HSP72 expression in the soleus and epitrochlearis muscles was determined 1 week after injection. KU-32 induced HSP72 expression only in the epitrochlearis (fast-twitch) muscle but not in the soleus (slow-twitch) muscle. KU-32-induced increase in HSP72 expression was dampened in the aged rats. *P <0.05 KU-32 vs. vehicle, #P<0.01 aged vs young for KU-32 and vehicle; n=4-5 rats per condition.

8.3. HSP function in non-muscle tissues

Most of the HSP benefits reported here were observed in the skeletal muscle. We focused on the skeletal muscle because of its crucial function in glucose disposal and its ability to respond to HSP-inducers. However, preliminary data from our studies also suggest beneficial effects of HSPs in the liver and adipose tissue (data not shown). In response to heat treatment, both adipose tissue and liver were protected from HF diet-induced JNK activation, adipose tissue depots were reduced and triacylglycerol accumulation was reduced in the liver. Previous studies have repeatedly shown the benefits of heat pretreatment in recovery from ischemia-reperfusion (Ooie et al. 2001). HSP70 overexpression protects mitochondria of the heart from hypoxia/reoxygenation injury (Williamson et al. 2008). HSPs are protective against nerve damage in neurodegenerative diseases. Gifondorwa et al. (Gifondorwa et al. 2007) have shown that exogenous delivery of HSP70 increases the lifespan in a mouse model of amyotrophic lateral sclerosis (ALS). HSP72 attenuates renal tubular cell apoptosis and interstitial fibrosis in obstructive nephropathy (Mao et al. 2008). Taken together, one can say that the benefits of HSPs are not limited to the skeletal muscle and could be potentially extrapolated to several other tissues. However, caution should be exercised in this approach, since different tissues appear to have different thresholds for HSP induction and have varied responses in aging and oxidative stress. For example, the fast-twitch muscles induce higher levels of HSPs with heat treatment than slow-twitch muscles. Further, our data shows that HSP expression decreases with age in the skeletal muscle whereas the brain actually shows

an age-related increase in HSP expression. We believe that this is because the neurons in the brain possess stronger anti-oxidant defense systems related to their essential functions in the body, and because they are one of the longest living cells in the body. Nevertheless, future translational use of HSPs must consider that some tissues will respond to HSP treatments to a greater extent than others.

8.4. Exercise and heat shock proteins

Exercise is a potent inducer of heat shock proteins (Noble et al. 2008), although the precise mechanism(s) are currently poorly understood. The general consensus is that several factors, activated by exercise, act as stimuli for the HSP response. The stimuli include exercise-induced increase in muscle temperature (Oishi et al. 2002), reactive oxygen species generated during exercise (Kayani et al. 2008), ATP depletion (Chang et al. 2001) and activation of signaling pathways (Noble et al. 2008). Exercise induces HSP expression in diabetic animals but the HSP response is severely blunted (Atalay et al. 2004). But if exercise training is started in the insulin resistant state (before diabetes), it can protect the HSP response and potentially prevent diabetes. One of the postulated mechanisms of exercise-induced HSPs is that exercise activates Akt (Sakamoto et al. 2003) and inhibits GSK3-β activity, which in turn regulates HSF-1 activation (Xavier et al. 2000). Therefore, exercise can positively regulate HSF-1 activity and prevent reduction of HSP response in diabetes and aging.

Our studies suggest that heat treatment and exercise have several similar effects. Both induce robust HSP expression and improve glucose uptake. But the

underlying mechanisms could be different. AMPK activation, mitochondrial biogenesis and increased GLUT4 (Holloszy 2005) are major mechanisms implicated in the insulin-sensitizing effects of exercise. None of these changes were observed in the heat-treated HF-fed animals. Heat treatment has direct effects on activation of the insulin signaling cascade, but like exercise, heat also increased mitochondrial function and mitochondrial flux. We therefore propose a combination therapy for those who cannot derive the benefits of exercise. A co-therapy of heat, followed by exercise, might help in increasing the HSP response. Also, potentially lower heating temperatures and less intense exercise protocols could be employed to get the same beneficial effects of a single treatment of heat or exercise alone.

8.5. Potential problems with HSP induction as a therapeutic intervention

The role of HSPs in protection from neurodegenerative diseases has long been recognized, but none of the potential HSP-inducing drugs have had sufficient success. Geldanamycin was rejected for its toxicity, while its conjugates that followed are still being verified for toxicity (Sharp and Workman 2006). A large part of the concern regarding HSP drugs comes from the cancer literature. While we are trying to induce HSPs for protection from diabetes, some cancer researchers are trying to inhibit HSPs to reduce drug resistance, thermotolerance or increase immunosuppression (Yokota et al. 2000). So it would be imperative to use HSP treatment cautiously and design the treatment regime in a case-specific manner.

Mild heat treatment used by our laboratory and others in diabetes research has shown significant benefits, but one has to be vigilant about the potential hazards of

heat therapy in treatment. In our study model, local heat treatment restricted to the lower body showed systemic benefits, suggesting that a treatment targeting just the skeletal muscles of the lower limbs can have whole body benefits. However, in elderly individuals or in individuals with advanced diabetes, the heat shock response ability is diminished (Hooper and Hooper 2005; Najemnikova et al. 2007; Kayani et al. 2008). Hence, a heat treatment of sufficient temperature and duration for promoting systemic benefits in such individuals may be very stressful and may cause other complications. Thus, use of HSP-inducing drugs may be more beneficial.

Several drugs belonging to the bimoclomol class of drugs are believed to act as co-inducers of HSPs rather than direct inducers of HSPs (Hargitai et al. 2003). Preliminary data from our lab suggests that resveratrol may serve to potentiate the heat shock response (Figure 31). Future studies examining the mechanism of HSP-induction by resveratrol, its use as an HSP-co-inducer and its use as an insulin-sensitizing drug could yield exciting information. Such drugs could be used in combination with a very mild heat therapy, where lower temperature or shorter duration of heat treatment may achieve an optimal HSP induction required for improving insulin sensitivity. Alternatively, such drugs could be used along with mild exercise. Therefore, a combination therapy involving a co-inducer and a low dose inducer could be a safer strategy of treatment.

FIGURE 31.

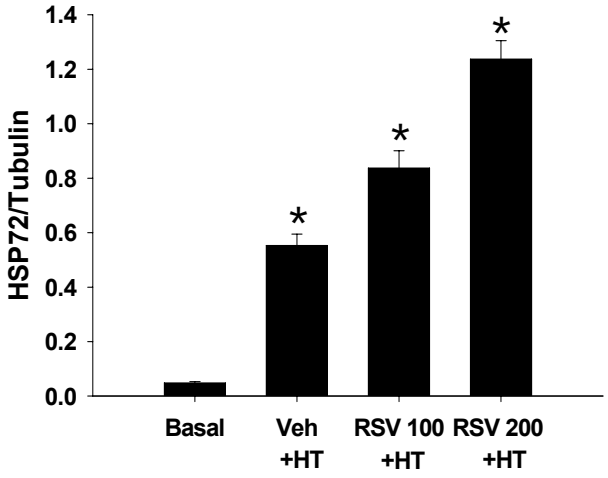

Figure 31. Resveratrol potentiates the effect of heat treatment on HSP72 expression. L6 myotubes were pretreated with vehicle (DMSO, Veh) or 100 μM and 200 μM resveratrol (RSV) for 3 h. All cells except basals were then heat treated for 20 min at 43 ºC. Cells were recovered for 24 h at 37 ºC with fresh RSV or vehicle. Cells were harvested to measure HSP72 expression with Western blot analysis. *P <0.01 compared with all other groups, n=3-5 samples per condition.

8.6. Protecting HSP expression in diabetes and aging: a potential therapeutic strategy

In our study of heat treatment and high fat diet, we administered the heat treatment in parallel with the high fat diet, such that their HSP levels were maintained throughout their diet regime (Chapter 6). Since loss of HSP expression is a common finding in aging, obesity and frank diabetes, an important strategy of treatment could be aimed at maintaining tissue HSP expression. One thought is that insulin action is involved in maintaining the HSP response and this is mediated by PI3K-dependent Akt activation (Shinohara et al. 2006). Thus, HSP treatment benefits may be limited in the case of full blown diabetes. HSP expression is tightly controlled by GSK-3β, ERK and JNK via inhibitory phosphorylation of the transcription factor HSF-1 (Chu et al. 1996; Dai et al. 2000). Activation of these kinases is increased in insulin resistance (Aguirre et al. 2000; Dokken et al. 2005; Henriksen et al. 2008) and thus, HSF-1 is maintained in its inactive state. HSPs are, in turn, responsible for inhibiting stress kinases which means that regulation of HSPs and kinases is interdependent. Inhibition of GSK-3β, ERK and JNK may be especially important in individuals that are more susceptible to insulin resistance, such as first degree relatives of T2D patients, children in families with hereditary obesity or diabetes and people from high T2D-risk ethnic communities such as within the Hispanic, Native American and Asian Indian populations.

While all of these proposed methods of HSP-based treatments may provide promising results of protection from insulin resistance, managing a healthy diet will

be of prime importance to their success, and such treatments should not be an excuse for leading an unhealthy lifestyle.

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