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The impact of Heat Shock Protein 72 expression
on mitochondrial function and insulin action

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Physiological Science

by

Xiao Song

2014

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2014

ABSTRACT OF THE THESIS

The impact of Heat Shock Protein 72
on mitochondrial function and insulin action

by

Xiao Song

Master of Science in Physiological Science

University of California, Los Angeles, 2014

Professor Andrea L. Hevener, Co-Chair

Professor David William Walker, Co-Chair

Cellular stress resistance against inflammatory and metabolic insult is critical for disease prevention and longevity. The heat shock response is an evolutionarily conserved defense system engaged during stress to maintain cellular homeostasis. Basal HSP72 levels and induction response to cellular stress are diminished in muscle from obese and type 2 diabetic patients and overexpressed during endurance exercise in healthy lean individuals. Recently, studies in HSP72 knockout mice have shown that HSP72 is a critical regulator of stress-induced mitochondrial triage signaling via Parkin, a ubiquitin ligase that tags mitochondrial membrane proteins for degradation. However, the underlying mechanisms of HSP72 on mitochondrial dynamics remain largely unresolved.

The purpose of this investigation was to determine the effect of restoring muscle HSP72 in male HSP72^{+/-} mice on mitochondrial dynamics including fusion, fission, and mitophagy. Considering previous observations, it is hypothesized that HSP72 overexpression improves mitochondrial health by regulating Parkin and downstream proteins associated with fission and fusion. HSP72^{+/-} mice expressing an inducible HSP72 transgene (HSP72-Tg) or mice with a global null mutation of HSP72 genes (HSP72-KO) were studied and compared phenotypically to aged-matched wildtype (WT) animals. Western blots revealed increased Parkin and decreased pDRP1 S637 protein in HSP72-KO muscle, however these changes in muscle protein and phospho-protein events were prevented by restoration of muscle HSP72 in HSP72-Tg mice. Furthermore, LC3 II/I was increased in muscle from HSP72-Tg mice over WT, suggesting that HSP72 promotes autophagy. Finally, restoration of muscle HSP72 in HSP72-Tg mice improved insulin-stimulated glucose uptake, which was significantly reduced from WT in HSP72-KO mice. Collectively these data support that HSP72 is a viable therapeutic target to combat insulin resistance and metabolic dysfunction associated with type 2 diabetes.

The thesis of Xiao Song is approved.

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2014

TABLE OF CONTENTS

	Page
I. List of Acronyms.....	vi
II. Acknowledgments.....	vii
III. Introduction	
a. Cellular Stress and Heat Shock Protein 72.....	1
b. Mitochondrial Function.....	3
c. Insulin Action.....	5
IV. Materials and Methods	
a. Animals.....	8
b. Genotyping.....	8
c. Muscle strip glucose uptake.....	8
d. Immunoblot analysis.....	9
e. Statistics.....	10
V. Results.....	11
VI. Discussion.....	15
VII. Figures.....	18
VIII. References.....	21

LIST OF ACRONYMS

HSP72: Heat shock protein 72

WT: wildtype mouse line of C57BL/6J background

HSP72-KO: HSP72-knockout mouse line with global null mutation of Hspa1a and Hspa1b

HSP72+/-: HSP72 heterozygous mouse line bred from cross between HSP72-KO and WT

HSP72-Tg: HSP72-muscle transgenic mouse line with chimeric rat inducible HSP72 gene on HSP72+/- background

HSP72-Tg^{KO}: HSP72-muscle transgenic mouse line with chimeric rat inducible HSP72 gene on HSP72-KO background

OMM: outer mitochondrial membrane

IMM: inner mitochondrial membrane

CMV: cytomegalovirus promoter

MFN2: mitofusin 2, an OMM GTPase involved in mitochondrial fusion

DRP1: dynamin-related protein 1, a GTPase involved in mitochondrial fission

LC3 II/I: ratio of microtubule-associated protein 1B/1A-light chain 3, a marker of Atg5/Atg7-mediated macroautophagy

OPA1: optic atrophy 1

PINK1: PTEN induced putative kinase 1

BGP-15: pharmacological inducer of HSP72

IRS-1/IRS-2: insulin-receptor substrate type 1 or 2

PIP₂/PIP₃: Phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol (3,4,5)-trisphosphate

Akt/PKB: a serine threonine kinase

GLUT4: glucose transporter type 4

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INTRODUCTION

I. Cellular Stress and Heat Shock Protein 72

Environmental stressors, such as extreme temperature elevation and toxic chemicals exposure, pathophysiological states, and cell growth processes trigger acute and chronic stress in the cell [1]. To maintain homeostasis, the cell induces production of a family of stress response proteins called heat shock proteins (HSPs). HSPs are named for their activation by heat shock, a sub-lethal, temporary elevation in temperature [2]. As molecular chaperones, HSPs aid in refolding misfolded proteins, solubilizing denatured protein aggregates, and transporting immature proteins to their target organelles, which protect cells from damage [3]. Elevation of HSPs significantly reduces the level of pathology and cell death, which could potentially affect the course of disease [1].

Although the HSP family consists of various molecular weight proteins, HSP72, a 72 kDa heat shock protein, deserves particular attention because it is greatly reduced in obese and type 2 diabetic individuals [4, 5] and overexpressed during endurance exercise [6]. The production of HSP72, which is transcribed from two genes, Hspa1a and Hspa1b, is mediated by a transcription factor, heat shock factor 1 (HSF1). In unstressed cells, HSF1 monomers are sequestered by HSP72 in the cytoplasm, preventing gene transcription. During heat shock or stress, HSF1 monomers assemble into trimers, dissociating from HSP72, and accumulating in the nucleus. HSF1 then binds to DNA recognition sequences flanking Hspa1a/Hspa1b to induce HSP72 production. Upon removal of cellular stressors or adaptation, HSP72 rebinds to HSF1, disassembling HSF1 from DNA and halting HSP72 gene transcription. In this way, the accumulation of HSP72 from induction prevents its own gene transcription via a negative feedback mechanism.

Previous studies in HSP72-knock out (HSP72-KO) and HSP72-muscle transgenic mice on HSP72-KO background (HSP72-Tg^{KO}) suggest that eliminating or overexpressing HSP72 has a profound effect on metabolism. Loss of HSP72 in HSP72-KO mice with global null mutations in Hspa1a/Hspa1b genes increased body weight and fat pad mass and promoted insulin resistance. These mice also had impaired fatty acid oxidation and increased intramuscular diacylglycerol and triacylglycerol levels [7]. Importantly, knockout of both Hspa1a and Hspa1b genes ensured that there was no compensation by any other gene that encodes the same protein, strengthening a role for deletion of HSP72 in metabolic regulation.

On the other hand, overexpression of HSP72, by heat therapy or genetic induction in HSP72-Tg^{KO} mice, improves metabolic homeostasis and ameliorates insulin resistance associated with high fat diet (HFD) and genetic obesity. Furthermore, heat therapy decreases hyperinsulemia and hyperglycemia and prevents high fat diet-induced JNK phosphorylation, a marker of muscle inflammation, in skeletal muscle [4]. Compared to wildtype (WT) mice on a high fat diet, HSP72-Tg^{KO} mice on a HFD have lower basal glucose and insulin levels, decreased body and fat pad weight, and enhanced insulin sensitivity [4]. HSP72-Tg^{KO} mice also perform superiorly on treadmill running due to increased energy expenditure, whole body fat oxidation, and mitochondria number [8]. These findings suggest that HSP72 plays a significant role in regulating metabolic homeostasis and insulin action. Thus, further investigation into HSP72 function will provide important insight into novel therapeutic strategies to combat insulin resistance and metabolic dysfunction associated with type 2 diabetes.

II. Mitochondrial Function

Mitochondria, the primary energy generators of eukaryotic cells, undergo dynamic processes of fusion and fission critical for maintaining mitochondrial function. The balance between fission and fusion controls mitochondria morphology, size, number, and organelle integrity, indicators of organelle health. During high rates of oxidative phosphorylation, reactive oxygen species (ROS), normally produced by the mitochondria in metabolism, accumulate and damage the mitochondria, promoting increased ROS production and further damage [9]. Fusion and fission processes mitigate mitochondrial damage to preserve mitochondria quality [10]. Previous studies by my laboratory have shown that HSP72 is a protein chaperone of Parkin, an E3 ubiquitin ligase [7] and a mediator of mitochondrial dynamics [11].

Fusion is thought to protect the mitochondria from damage by diluting toxic contents across the mitochondrial network. Two families of GTPases regulate outer and inner membrane fusion, both of which are required for mitochondria to fully merge. Mitofusin 1 (MFN1) and Mitofusin 2 (MFN2) mediate outer membrane fusion while Optic Atrophy1 (OPA1) mediates inner membrane fusion. During OMM fusion, MFN1 and MFN2 on adjacent mitochondria tether the two outer membranes together by forming homo- and hetero-oligomers [12]. MFN 2 plays an important role in fusion because mitochondria in mouse embryonic fibroblasts (MEFs) lacking MFN1 and MFN2 cannot fuse together [13], and homozygous MFN2-knockout is embryonic lethal [10]. Furthermore, knockout of MFN1, MFN2, or OPA1 in MEFs produces small, fragmented mitochondria [10], highlighting the role of these proteins in maintaining mitochondrial morphology.

Similar to fusion, fission also plays a protective role in the mitochondria in response to mitochondrial damage. During fission, mitochondria divide and become highly fragmented. In

mammals, dynamin-related protein 1 (DRP1) is thought to mediate this process by assembling and constricting mitochondrial tubules, much like the role of dynamin in endocytosis [14]. DRP1 resides in the cytosol and is recruited to the mitochondria via several potential receptors on the OMM, including Fis1, Mff, MiD49, and MiD51 [12]. Phosphorylation of DRP1 at Ser616 (pDRP1 S616) activates fission activity, while phosphorylation at Ser 637 (pDRP1S637) inhibits fission [15]. Phosphorylation of DRP1 at S637 is thought to prevent mitophagy during exercise and starvation conditions when macroautophagy is activated [12].

Fission of mitochondria appears to be a prerequisite for mitophagy, a quality control process of mitochondrial degradation by the autophagosome, in order to reduce the mitochondrial size for autophagosome engulfment. Mitochondrial damage-inducing stimuli alter the inner membrane proton gradient, reducing membrane potential promoting an accumulation of PINK1, a serine/threonine kinase, on the OMM. [12]. PINK1 recruits Parkin, which polyubiquitinates MFN1 and MFN2, marking them for degradation through the ubiquitin-proteasome system (UPS). Degradation of MFN1/MFN2 inhibits mitochondrial fusion, so the mitochondria become fragmented due to fission and segregated from the healthy mitochondrial network [12]. These mitochondrial fragments are later degraded by autophagosomal fusion with a lysosome in mitophagy.

LC3-I and LC3-II are important marker proteins for macroautophagy, the overarching degradation process that includes mitophagy. LC3-I, which exists in the cytosol, is converted to LC3-II by conjugation with phosphatidylethanolamine. LC3-II is then recruited to autophagosomal membranes, where it is degraded along with autophagosomal components. Thus, an increased LC3-II to LC3-I ratio (LC3II/I) is an accepted index of autophagy [16].

Previous studies on HSP72 have shown that HSP72 is involved in mitochondrial dynamics [7]. Muscle from HSP72-Tg^{KO} mice display upregulated Tfam, AMPK, and SIRT1, all of which mediate the mitochondrial biogenesis pathway [8]. HSP72, usually found in the cytoplasm, translocates to the mitochondria upon activation by cell stress. One of the central targets of HSP72 is Parkin, a ubiquitin ligase shown to tag mitochondrial membrane proteins for degradation to promote mitochondrial isolation and subsequent degradation by the lysosome [7, 17]. HSP72-deletion promotes the accumulation of nonfunctional Parkin protein in the cytosol and prevents the translocation of Parkin to damaged mitochondria [7, 17]. Furthermore, DJ-1, an oncogene whose loss leads to mitochondrial fragmentation and oxidative stress [18], has been shown to colocalize with HSP72 [19]. These suggest that HSP72 may contribute to regulation of mitochondrial quality control processes of biogenesis, autophagy, and fission.

III. Insulin Action

Two prominent developments in the pathogenesis of type 2 diabetes are insulin resistance in liver and skeletal muscle and the failure of pancreatic β -cells to secrete insulin from glucose stimulation, leading to hyperglycemia [20]. Prediabetes patients become insulin resistant 1-2 decades before manifestation of type 2 diabetes. Insulin resistance is clinically defined as the inability of an individual to uptake as much glucose as a healthy individual in response to physiological insulin levels [21]. Reduced cellular responsiveness to the hormone insulin is typically quantified by glucose disposal into glucoregulatory tissues. Both insulin resistance and β -cell failure can be explained by the unifying hypothesis of mitochondrial dysfunction [20].

Insulin action in skeletal muscle requires insulin to first bind to insulin receptor (IR), causing IR β subunits to autophosphorylate and recruit insulin receptor substrate 1 and 2 (IRS-1

and IRS-2). IRS-1 and IRS-2 themselves become phosphorylated and activate phosphoinositide 3-kinase (PI3K), leaving PI3K free to convert membrane-bound second messenger PIP₂ to PIP₃. PIP₃ activates the Akt/PKB cascade, signaling the translocation of GLUT4, a glucose transporter, to the plasma membrane for glucose uptake [22]. However, in the skeletal muscle of diabetic individuals, mitochondrial dysfunction prevents fatty acid oxidation, leading to intracellular fatty acyl CoA and diacylglycerol accumulation [23]. This is thought to activate novel PKCs that phosphorylate IRS-1 and inhibit PI3K, preventing GLUT4 translocation and glucose uptake [20].

Manipulating HSP72 expression has profound effects on insulin action. Previous studies by our laboratory have shown that in HSP72-KO mice compared to WT mice, insulin-stimulated glucose disposal rate (IS-GDR), as measured by hyperinsulinemic-euglycemic clamp, is significantly reduced. Furthermore, in isolated soleus muscles of HSP72-KO mice, insulin-stimulated glucose uptake (IS-GU) is highly reduced, insulin signaling is impaired, and Akt phosphorylation is reduced compared to WT [7]. Diacylglycerol and triacylglycerol, which inhibit insulin action, are significantly elevated in HSP72-KO muscles [23]. These results are recapitulated in HSP72-KO myotubes, which show reduced cellular glucose uptake, impaired insulin-stimulated phosphorylation of Akt, and reduced oxidation compared with WT [7].

HSP72-Tg^{KO} mice (HSP72-Tg on HSP72-KO background), in contrast, are protected from insulin resistance, hyperglycemia, and hyperinsulemia, and display increased mitochondria numbers, improved fatty acid oxidation, and enhanced treadmill running capacity [8]. These data indicate that HSP72 induction increases oxidative capacity and prevents HFD-induced insulin resistance adaptations that could benefit human subjects susceptible to metabolic dysfunction induced by diet and inactivity.

Although the positive effects of HSP72 can be seen in HSP72 overexpression and knockout mice, the mechanism underlying HSP72's action is still unknown. Based on previous observations, we hypothesize that HSP72 overexpression improves mitochondrial health and insulin action by regulating Parkin and downstream proteins associated with mitochondrial quality control processes fission, fusion, and mitophagy. By evaluating the mediators of mitochondrial dynamics, we hope to answer the question: how does HSP72 improve insulin action and mitochondrial function, features that underlie type 2 diabetes?

MATERIALS AND METHODS

Animals. Male wildtype (WT), HSP72-knockout (HSP72-KO) mice, and muscle HSP72-transgenic (HSP72-Tg) were obtained from the Mutant Mouse Regional Resource Center Repository (MMRRC) at 8 weeks of age. WT, HSP72-KO, and HSP72-Tg mice were of pure C57BL/6J background, confirmed by Jackson Laboratories. HSP72-KO mice had whole-body null mutation of Hspa1a and Hspa1b genes. HSP72-Tg mice had basal overexpression of an inducible rat HSP70 gene in skeletal muscle and were bred as previously described [24].

To obtain HSP72^{+/-} (heterozygous) animals, HSP72-KO mice were backcrossed to WT mice. To obtain HSP72-Tg mice, HSP72^{+/-} mice were crossed to muscle HSP72-transgenic mice. Animals were fasted for 6 hours prior to harvest of quadriceps and soleus muscles. All procedures follow the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Animal Subjects Committee of the University of California, Los Angeles.

Genotyping. Mice were genotyped using tail DNA extracted with Bioland Easy Genotyping Kit. Primers used were CMV for HSP72-Tg, Neo for HSP72-KO, and WT for WT. PCR supermixes include Econotaq (Lucigen) for HSP72-Tg and HSP72-KO and Phire II Hot Start for WT. PCR products were run on 2% agarose gel and visualized with 1:10000 dilution Sybr Safe (Invitrogen) in 1X TAE buffer. 100bp DNA ladder (Invitrogen) was run with the samples to determine product size.

Muscle strip glucose uptake. All buffers, preincubation (pre-inc), preincubation + insulin (pre-inc+ins), incubation (inc), and incubation + insulin (inc+ins), were prepared with 100 mL gassed

Krebs-Henseleit buffer (KHB), 100 mg fatty acid free BSA, 1 mL sodium pyrophosphate, and 300 μ L mannitol, with the addition of 5 μ L insulin in pre-inc+ins and inc+ins buffers or 75 μ L [3 H]-2-deoxyglucose and 119.3 μ L 14 C-mannitol in inc and inc+ins buffers. Soleus muscles were incubated at 35°C in 2 phases: first, 30 min in pre-inc (basal) or pre-inc+ins (insulin-stimulated) buffers to equilibrate muscle, and second, 20 min in inc (basal) and inc+ins (insulin-stimulated) buffers to determine glucose uptake. After incubation, muscles were washed on a KHB-wetted Kimwipe, dried on a Kimwipe, frozen in liquid nitrogen, and stored at -60°C. After removal from storage, muscles were homogenized in 500 μ L RIPA buffer, and 150 μ L of the supernatant and 100 μ L dilute (1:10) inc or inc+ins buffer were placed in liquid scintillation vials. Glucose uptake was analyzed as the accumulation of [3 H]-2-deoxyglucose by liquid scintillation counting and displayed as IS-GU in μ mol/g.

Immunoblot analysis. To prepare for immunoblotting, mouse tissues, flash frozen in liquid nitrogen, were homogenized in 1mL RIPA lysis buffer with a glass dowel at 400rpm. Approximately a quarter of quadriceps were used in the homogenization. RIPA lysis buffer contained 10mM EGTA, 100mM NaF, 100mM sodium pyrophosphate, 100mM β -glycerophosphate, 100mM Na_3VO_4 , 100mM PMSF, phosphatase inhibitor (Roche), and protease inhibitor (Sigma) in 1X RIPA buffer. After homogenization, lysates were mixed by orbital rotation at 4°C for 1 hr and cell debris pelleted at 12000rpm for 5 min at 4°C. Supernatant was analyzed for protein concentration using BCA Pierce assay (Thermo Scientific).

Samples, containing 35 μ g protein diluted in 4X Laemmli sample buffer and 20% β -mercaptoethanol, were clarified and resolved by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked in 3% BSA, washed 3 times in PBST (0.05% Tween),

and probed with the following primary antibodies overnight at 4°C: HSP72 (Stressgen), GAPDH (Abcam), Parkin (#2132 & #4211, Cell Signaling), LC3B (Novus), DRP1 (Cell Signaling), phospho-DRP1 Ser637 (Cell Signaling), and MFN2 (Sigma, m9073). Following primary incubation, membranes were washed in PBST and incubated in HRP-conjugated goat anti-mouse/rabbit secondary antibodies (BioRad). Membranes were again washed in PBST and imaged with BioRad Universal Hood II. BioRad Chemidoc Quantity One software was used to perform densitometric analyses.

Statistics. Means \pm SEM were calculated with GraphPad software. Alpha level was set at $P < 0.05$. Ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test with singled pooled variance was performed to identify significance between and within multiple groups. Multiplicity adjusted P value was chosen to calculate p value between groups.

RESULTS

HSP72-KO mice exhibit undetectable HSP72 protein levels.

HSP72-KO mice are characterized by global null mutations in Hspa1a and Hspa1b genes by insertion of a Neomycin sequence (Neo). Genotyping of tail DNA with Neo primers confirms the presence of Neo sequence in mice genome, while indicating lack of wildtype HSP72 sequence (Figure 1B). In contrast, WT mice express only wildtype HSP72 sequence and no Neo sequence (Figure 1A). Furthermore, western blot analysis of HSP72-KO muscle confirms the HSP72 null mutation as HSP72-KO mice exhibit undetectable levels of HSP72 protein (Figure 1D).

HSP72-Tg mice are heterozygotes that overexpress HSP72 protein.

HSP72-Tg mice are bred with a rat HSP72 transgene driven by the CMV promoter in skeletal muscle cells, as previously described [24]. Genotyping with CMV primers confirms the presence of the transgene in the mice genome (Figure 1C). Furthermore, genotyping indicates that mice are of whole body heterozygote background (HSP72^{+/-}) due to the presence of Neo and wildtype bands (Figure 1C), while in WT or HSP72-KO mice, only one or the other band is present (Figure 1A, 1B). Immunoblotting indicated that HSP72-Tg mice express HSP72 protein, approximately 270-330-fold times higher than WT and HSP72-KO (Figure 1D).

Introduction of the HSP72 transgene restores Parkin protein levels in male HSP72^{+/-} mice.

HSP72 is a critical regulator of Parkin, an E3 ubiquitin ligase that tags proteins on the mitochondrial outer membrane and nonmitochondrial targets, including p53, for degradation [7]. To test the hypothesis that HSP72 overexpression improves mitochondrial health by regulating

Parkin, we restored HSP72 in HSP72^{+/-} mice and immunoblotted for Parkin protein. In WT mice, basal Parkin protein turnover is high so muscle levels are low, while in HSP72-KO mice, Parkin protein level is approximately 14 times higher (Figure 2A) as previously shown by Drew et al. [7]. However, when HSP72 is increased in HSP72-Tg mice, Parkin protein is decreased to approximately half of Parkin levels in HSP72-KO mice (Figure 2A). Since previous evidence shows that Parkin activity in HSP72-KO mice is impaired [7], these results suggest that HSP72 may provide a beneficial effect to muscle by promoting the restoration of overexpressed, nonfunctional Parkin to normal levels.

Mitophagy is increased in HSP72-KO and HSP72-Tg mice, which correlates with Parkin function.

Mitophagy involves fusion of the mitochondria with an autophagosome and subsequent fusion with a lysosome to degrade autophagosomal contents. The fusion is mediated by LC3, which exists in two forms: I, a cytosolic protein, and II, an autophagosome-associated membrane protein [16]. LC3 I is cleaved into LC3 II during Atg5/Atg7-dependent autophagy, and thus the ratio of LC3 II to LC3 I is viewed as a marker of macroautophagy [25]. In HSP72-KO mice, LC3 II/I is increased 1.5 fold, demonstrating that autophagy is increased in HSP72-KO mice compared to WT (Figure 2B). Since Parkin is known to regulate mitochondrial triage signaling [7] and Parkin is also increased in HSP72-KO mice (Figure 2A), this increase of autophagy in HSP72-KO mice correlates with Parkin function. Interestingly, LC3 II/I is increased more in HSP72-Tg mice (2.5 fold) than in HSP72-KO mice, demonstrating even higher levels of autophagy under conditions of HSP72 overexpression (Figure 2B). While unexpected, since Parkin levels are reduced in HSP72-Tg mice (Figure 2A), the reintroduction of HSP72 can

promote the production of functional Parkin, which should enhance autophagy. The change in autophagy in HSP72-KO and HSP72-Tg mice support our hypothesis that HSP72 is a regulator of autophagic processes such as mitophagy via Parkin.

Mitochondrial fission may be decreased in HSP72-KO mice.

Fission and fusion of mitochondrial are dynamic processes that maintain mitochondrial quality in healthy cells [26]. DRP1 mediates mitochondrial fission by cleaving the mitochondrial membrane, and its activity is regulated posttranslationally via phosphorylation on various serine sites. Phosphorylation of DRP1 on Ser637 blocks mitochondria fission, while phosphorylation on Ser616 promotes it [27]. In HSP72-KO mice, there is a decrease in muscle pDRP1 S637, but the decrease did not reach statistical significance ($p=0.28$, Figure 2C). This suggests that fission may possibly be enhanced with global knockout of HSP72. As well, replacement of HSP72 in HSP72-Tg mice tends to increase pDRP1 S637 to approximately WT levels (Figure 2C). This demonstrates that reintroducing HSP72 may counteract the effect of null HSP72 mutations on mitochondrial fission.

Mitochondrial fusion may be increased in HSP72-KO and HSP72-Tg mice.

As in mitochondrial fission, several membrane proteins, including MFN2, mediate fusion. In HSP72-KO mice, MFN2 is increased by ~2.5 fold, suggesting that lack of HSP72 promotes mitochondrial fusion (Figure 2D). Indeed, evidence from previous studies shows mitochondria from HSP72-KO muscle display a dysmorphic morphology of elongation and hyperfusion [7]. Additionally, MFN2 is increased more in HSP72-Tg (Figure 2D), suggesting that HSP72 restoration promotes mitochondrial fusion. This increase in MFN2 is supported by the

observation that HSP72 seems to decrease fission while increasing fusion. However, the data is difficult to interpret because MFN2 may be responsible for other undiscovered functions in the cell. Finally, elevation of MFN2 in HSP72-KO mice correlates well with previous findings, indicating that Parkin action is diminished in HSP72-KO mice, since one of Parkin's functions is to target MFN2 for degradation [7]. A novel finding is that HSP72-Tg mice also exhibit increased MFN2, which may suggest that Parkin was unable to mark MFN2 for degradation, and further studies on Parkin activity need to be performed to clarify the degree of Parkin functionality restored by HSP72.

HSP72 overexpression restores insulin sensitivity of HSP72 knockout muscle.

In a separate cohort of 3 month-old male mice, soleus muscles of HSP72-KO mice displayed 81% less insulin-stimulated glucose uptake than WT mice ($p = 0.03$; Figure 3). In contrast, soleus muscles of HSP72-Tg mice displayed a restored level of IS-GU, which was comparable to that of WT mice (Figure 3). These findings were consistent with previous glucose tolerance and euglycemic-hyperinsulinemic clamp studies, which demonstrated the same pattern of insulin resistance in HSP72-KO mice [7] and improved insulin action in HSP72-Tg mice [8]. These observations strongly suggest that HSP72 induction ameliorates insulin resistance and may be of therapeutic benefit to mitigate complications associated with type 2 diabetes.

DISCUSSION

Mitochondria are dynamic organelles in a constant state of flux, undergoing fusion and fission to maintain quality. HSP72 is shown to affect mitochondrial health via its regulation of Parkin, a protein that plays a role in the quality control process known as mitophagy. The findings of this thesis demonstrate that HSP72 restores elevated Parkin protein levels in HSP72-KO mice back to half that of WT mice, suggesting that HSP72 is beneficial for regulating Parkin protein turnover. Decreased Parkin levels in HSP72-Tg may indicate improved functionality of Parkin, as LC3 data reflect autophagy and macroautophagy is a requisite for mitochondrial turnover [28] and Parkin has been shown to mediate mitophagy downstream of PINK1 [17]. Furthermore, pDRP1 S637 protein expression is approximately the same between HSP72-Tg and WT, though it is decreased in HSP72-KO, suggesting that mitochondrial fission decreases in HSP72-KO, but the decrease is restored to normal in HSP72 overexpression. It is thought that mitochondrial fission is a requisite for mitophagy to facilitate engulfment by the autophagosome [28]. Thus, the return of fission to WT levels in HSP72-Tg suggests that HSP72 could possibly restore conditions favoring mitophagy. Finally, LC3 II/I is increased in HSP72-Tg compared to HSP72-KO and WT (Figure 2B), suggesting that autophagy may be increased in HSP72-Tg, which could be explained by restored Parkin activity. Interestingly we observed an increase in the Parkin target MFN2 in muscle from HSP72-Tg. This could be a consequence of increased MFN2 gene expression and may not be a particularly reflective readout of Parkin action. Considering that MFN2 is elevated with exercise training [29] and in our heterozygotes with overexpression of HSP72 (Figure 2D), MFN2 may play an important role in maintaining mitochondrial function and health.

Since Parkin inactivation promotes insulin resistance [7], it is reasonable to speculate that HSP72-improvement in Parkin function is insulin sensitizing. Indeed, insulin stimulated glucose uptake is restored in HSP72-Tg mice vs. HSP72-KO mice (Figure 3), and this finding is paralleled by a restoration of Parkin protein. These results support the conclusion that HSP72 may enhance mitochondrial health through restoration of Parkin protein (Figure 2A) and DRP1 signaling (Figure 2C) which appears linked to improved insulin sensitivity.

The ability of HSP72 to improve mitochondrial health and insulin action has several important implications. First, since mitophagy is a process that controls mitochondrial quality, HSP72 can be regulated for the maintenance of mitochondrial health and function. If muscle retains poorly functioning mitochondria that cannot oxidize substrate properly, oxidative damage builds up and causes structural damage to nucleotides, leading to DNA mutation. Lipid products also accumulate near the mitochondria, interfering with cell signaling [30]. We propose that HSP72 is a potential therapeutic target to improve mitochondrial health and tissue oxidative function. Second, patients suffering from type 2 diabetes and obesity exhibit smaller and fewer subsarcolemmal mitochondria with reduced NADH oxidoreductase and citrate synthase activity compared to nondiabetic individuals [31, 32]. Mitochondrial dysfunction is often associated with impaired fatty acid oxidation and accumulation of intracellular fatty acyl-CoA and diacylglycerol, shown to impair insulin action. Intracellular lipids activate a variety of stress kinases thought to impair signaling through the insulin-receptor substrate type 1 (IRS-1), PI3-Kinase, and Akt cascade thus dampening insulin action [20]. Since mitochondrial dysfunction is believed by many to underlie insulin resistance and decreased insulin secretion that characterizes type 2 diabetes, elevation of HSP72 by pharmacological means may serve as an effective target to ameliorate the features of type 2 diabetes [8]. Proof of concept trials are currently ongoing

using the small molecule activator of HSF-1, BGP-15, shown previously to reverse insulin resistance associated with consumption of a HFD or genetic obesity [4, 33].

The underlying theme of mitochondrial dysfunction unifies muscle and liver insulin resistance and impaired pancreatic insulin secretion [20], primary features of type 2 diabetes. Our findings show that HSP72 induction improves mitochondrial function and is protective against insulin resistance possibly through improved Parkin action. Type 2 diabetes is the most frequently occurring metabolic disease in the world, affecting 382 million people in 2013 [34]. In the United States, type 2 diabetes is the leading cause of blindness, end-stage renal disease, and non-traumatic limb amputation, as well as neuropathy and cardiovascular disease [35]. Based on this study, HSP72 remains a viable therapeutic target for exploitation in type 2 diabetes treatment.

FIGURES

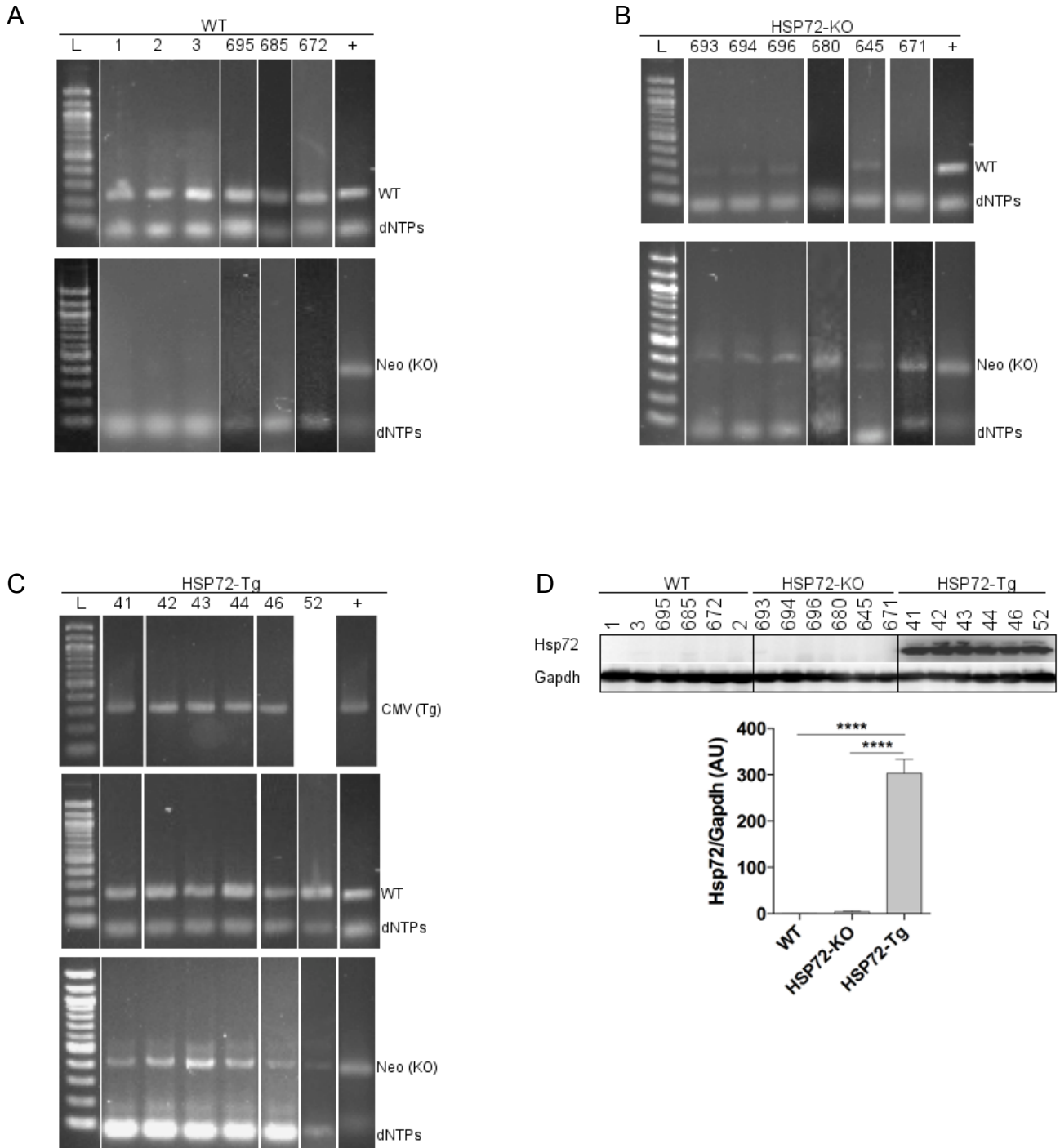


Figure 1. Genotyping of WT, HSP72-KO, and HSP72-Tg mouse lines from tail DNA. A) WT mice. Bands indicate the presence of WT sequence and no HSP72-KO sequence (Neo) on agarose gels. B) HSP72-KO mice. Bands indicate Neo sequence and no WT sequence. C) HSP72-Tg mice. Bands indicate HSP72 transgene (CMV), WT, and Neo sequence, suggesting heterozygote background with transgene. D) immunoblot for HSP72 in mouse lines, confirming presence of HSP72 protein in HSP72-Tg mice. The graph displays immunoblot quantification. n=6 mice/genotype. Values are expressed as means \pm SEM. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

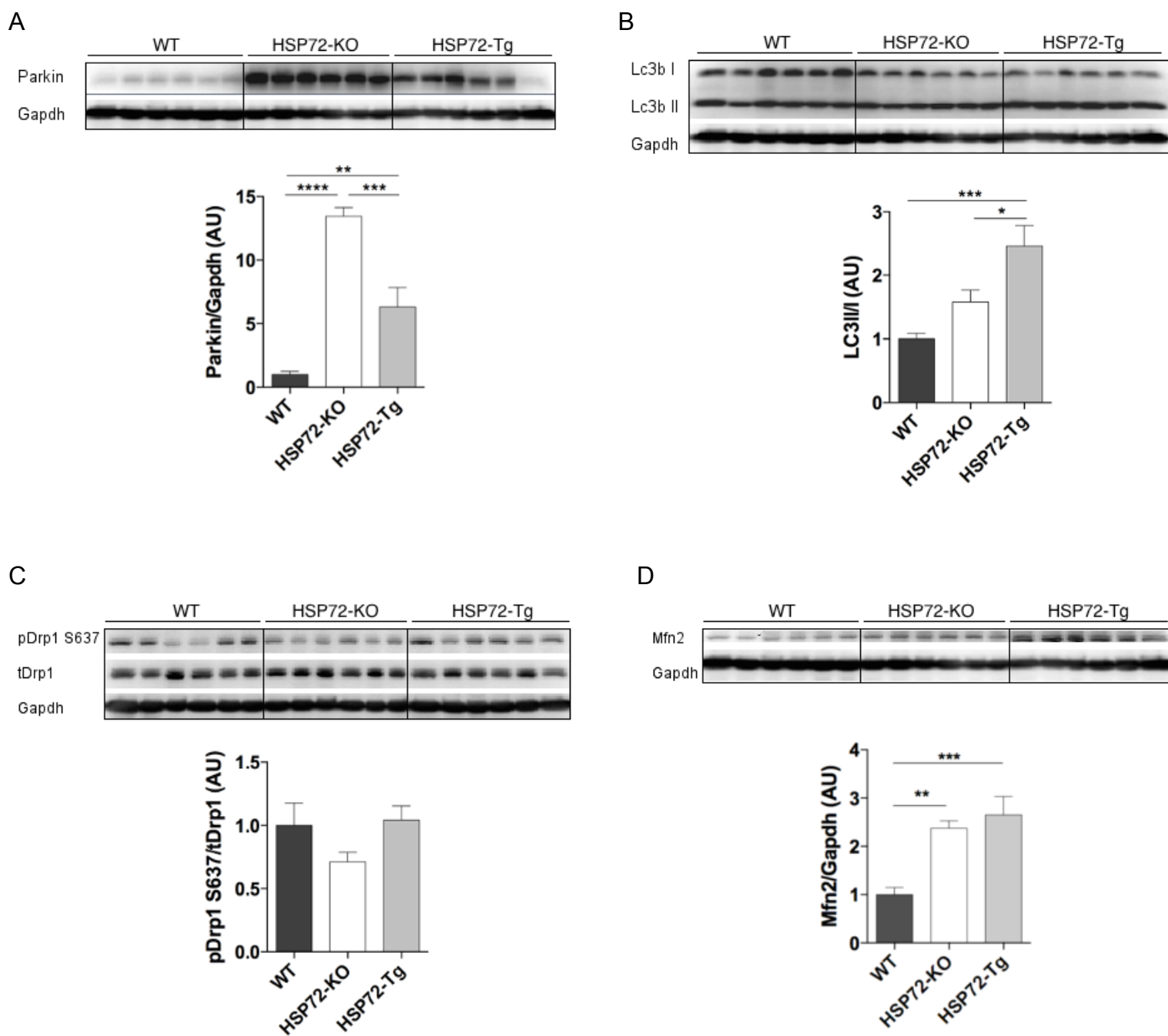


Figure 2. Immunoblots of WT, HSP72-KO, and HSP72-Tg mouse muscle. A) immunoblot of Parkin shows Parkin overexpression in HSP72-KO, but diminished overexpression in HSP72-Tg. B) immunoblots of LC3-I and LC3-II show increased LC3 II/I in HSP72-KO and HSP72-Tg. C) immunoblots of pDRP S637 show decreased pDRP S637 in HSP72-KO and restoration in HSP72-Tg. D) immunoblot of MFN2 displays increases in MFN2, a marker for mitochondrial fusion, in HSP72-KO and HSP72-Tg. n=6 mice/genotype. Values are expressed as means \pm SEM. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

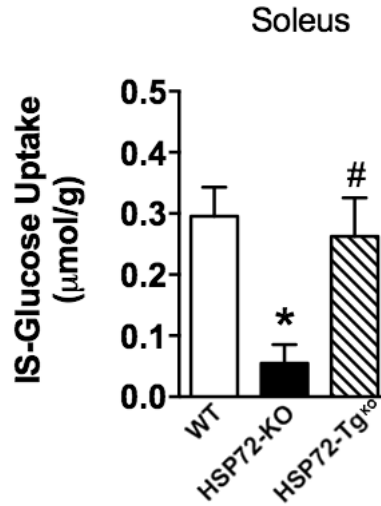


Figure 3. Muscle strip glucose assay of WT, HSP72-KO, and HSP72-Tg^{KO} soleus muscles treated with 60 μ U/ml physiological insulin dose. HSP72 deletion (HSP72-KO) reduces insulin-stimulated glucose uptake (IS-glucose uptake), while HSP72 overexpression (HSP72-Tg^{KO}) restores sensitivity to insulin (n=8 mice/WT and HSP72-KO, n=5 mice/HSP72-Tg^{KO}). Values are expressed as means \pm SEM, * = significance, P<0.05, between genotypes. # = significance, P<0.05, within genotype, between treatments.

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