Activating HSP72 in rodent skeletal muscle increases mitochondrial number and oxidative capacity and decreases insulin resistance

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Short title: Activation of Hsp72 protects against diet-induced obesity.

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Abstract

Induction of heat shock protein 72 (HSP72) protects against obesity-induced insulin resistance, but the underlying mechanisms are unknown. Here we show that HSP72 plays a pivotal role in increasing skeletal muscle mitochondrial number and oxidative metabolism. Mice overexpressing HSP72 in skeletal muscle (HSP72Tg) and control wildtype (WT) mice were fed either a chow or high fat diet (HFD). Despite a similar energy intake when comparing HSP72Tg with WT mice, the HFD increased body weight, intramuscular lipid accumulation (triacylglycerol (TAG), diacylglycerol (DAG) but not ceramide) and insulin resistance in WT mice alone. Whole body oxygen consumption, fatty acid oxidation and endurance running capacity were markedly increased in HSP72Tg mice. Moreover, HSP72Tg mice exhibited an increase in mitochondrial number. In addition, the HSP72 co-inducer BGP-15, currently in human clinical trials for type 2 diabetes, also increased mitochondrial number and insulin sensitivity in a rat model of type 2 diabetes. Together, these data identify a novel role for activation of HSP72 in skeletal muscle. Thus, the increased oxidative metabolism associated with activation of HSP72 has potential clinical implications, not only for type 2 diabetes, but for other disorders where mitochondrial function is compromised.
**Introduction**

Although obesity is associated with insulin resistance, the exact mechanism by which increased adiposity contributes to this disorder is unresolved. It is well established, however, that insulin resistance is associated with excess ectopic expression of lipid in liver and skeletal muscle (1). Moreover, the accumulation of deleterious lipid species such as diacylglycerol (DAG) (2; 3) and ceramide (4) are known to impair insulin action. Apart from the role of excess ectopic lipid accumulation, it has become apparent that insulin resistance may also be associated with defective oxidative metabolism in skeletal muscle (5). A coordinated reduction in the expression of genes encoded by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is observed in skeletal muscle of patients with type 2 diabetes (6; 7) and healthy subjects with a family history of diabetes (7). In addition, mitochondrial function in skeletal muscle plays a vital role in the pathogenesis of type 2 diabetes (8; 9). Importantly, activation of key pathways involving AMP-activated protein kinase (AMPK) (10), Peroxisome proliferator-activated receptor delta (PPARδ) (11), sirtuin 1 (SIRT1) (12) and carnitine palmitoyltransferase-1 (CPT1) (13), increase fatty acid oxidation (FAO), thereby decreasing lipid esterification and, in doing so, ameliorate insulin resistance. Hence, activators of these pathways are thought to have therapeutic potential for the treatment of type 2 diabetes.

We have identified an essential role for heat shock protein (HSP) 72 (the inducible form of the 70kDa family of heat shock proteins) in preventing insulin resistance in the context of high-fat feeding or genetic obesity in mice (14). In this (14) and subsequent (15; 16) studies, overexpression of HSP72 was accompanied by a marked reduction in high fat diet (HFD)-induced activation of pro-inflammatory signaling, i.e. phosphorylation of c-Jun N-terminal kinase (p-JNK) in skeletal muscle, which is implicated in insulin resistance (17). However,
whether this decrease in p-JNK could account for all of the whole-body protective effects of HSP72 overexpression is debatable. Since HSP72 transgenic mice are protected from developing obesity in the absence of hyperphagia, it is possible that they have increased energy expenditure (14). In addition, markers of oxidative metabolism in skeletal muscle are increased in HSP72 transgenic (HSP72Tg) mice (14), and in C2C12 muscle cells (18) and rats (15) undergoing heat treatment to induce HSP72. These data are in agreement with our previous observations that correlate increased HSP72 expression with mitochondrial enzyme activity in human skeletal muscle (19). Together, these studies raise the hypothesis that the mechanism of HSP72 action may be in regulating energy balance via enhanced oxidative metabolism in skeletal muscle. To test this hypothesis, we fed HSP72Tg and control mice either a standard chow or HFD. We also treated type 2 diabetic (Goto-Kakizaki) rats with the HSP72 co-inducer BGP-15, currently in human clinical trials for type 2 diabetes (20). In both experimental models, overexpression or induction of HSP72 increased mitochondrial number.

Methods

Mouse studies. Male wildtype (WT) and HSP72Tg mice were used as previously reported (14). In addition, to test whether the HSP72Tg phenotype was penetrative across different genetic strains of mice, additional studies were performed in HSP72Tg and littermate control mice, back-crossed at least 10 times onto a C57Bl/6 background. All experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee. All experiments commenced when mice were 8 weeks of age. Mice were fed either a chow diet (5% of total energy from fat) or a HFD (45% of total energy from fat) for 10 wk. Animals were given their prescribed diet and water ad libitum and housed in a controlled environment with a 12 hour light–dark cycle.
Glucose tolerance test, analysis of insulin signaling and insulin-stimulated glucose clearance. Glucose tolerance tests (1g/kg i.p.) were performed in 5 h fasted mice as previously described (21). Insulin stimulated glucose clearance was examined as previously described (22). The rate of tissue metabolism of [\(^3\)H]-2-deoxyglucose (K) was calculated by use of the single-injection model (23).

Indirect calorimetry and exercise tests.

Oxygen consumption (VO\(_2\)) was measured using a twelve-chamber indirect calorimeter (Oxymax series; Columbus Instruments, Columbus, OH) with an airflow of 0.6 l/min as previously described (24). For exercise tests, mice were subjected to a three day familiarization protocol that consisted of progressively increasing the intensity and duration of treadmill running (Columbus Instruments, Columbus, OH) before experimental testing. All experiments were performed at 1000 h and food was withdrawn from mice 4 h prior to running. Mice performed two exercise tests separated by three days. Initially, an incremental exercise test was performed which consisted of mice running at 10 m/min for 2 min. The velocity was increased by 2 m/min every 2 min. Once treadmill speed reached 20 m/min, the gradient was increased by 5% every 2 min until fatigue. This was defined as spending >10 sec at the base of the treadmill despite manual encouragement. Three days later, endurance capacity was assessed by running mice at 16 m/min at a 5% grade until they reached fatigue.

Ex vivo muscle incubations. For palmitate activation of JNK experiments, Soleus muscles from WT or HSP72Tg mice were carefully dissected tendon-to-tendon and placed in sealed flasks containing pregassed (95\% O\(_2\)/5\% CO\(_2\)) Krebs-Henseleit buffer, pH 7.3, supplemented with 4\% BSA and 4 mM pyruvate. Muscles were allowed to recover for 30 min and were
incubated with 0.5 mM or 1.5 mM palmitic acid for 6 h. Palmitate oxidation studies were performed as previously described (25).

**Western blotting.** Muscle samples were lysed and protein concentration determined as previously described (26). Immunoblotting was performed using the following primary antibodies: pAkt serine 473; pAkt threonine 308; total Akt; pSAPK/JNK Thr183/Tyr185; total JNK; SIRT1 (mouse specific); acetylated lysine, Parkin, LC3, ATG12, PKC-alpha, PKC-delta, PKC-theta (Cell Signaling, USA), lipoprotein lipase (LPL) (Abcam, USA), CD36 (R&D Systems, USA), p62 (ProGen), PINK1 (Cayman Chemicals), PGC1α purchased from (Millipore, USA) and α-tubulin from (Sigma-Aldrich, USA). For PKC studies subcellular fractionation was carried out as previously described (13).

**RNA extraction and Real time quantitative PCR.** Total RNA was isolated from skeletal muscle tissue with Tri Reagent® (Sigma Aldrich) and reverse transcribed to cDNA with the use of random hexamers. Real-time PCR was performed on a 7500 fast sequence detector (Applied Biosystems). Each assay included a no-template control, a no-reverse transcriptase control. Oligos for PGC1a (Mm01208835_m1), SIRT1 (Mm00490758_m1), Mitochondrial transcription factor A (TFAM) (Mm00447485_m1), CD36 (Mm01135198_m1), CPT1b (Mm00487200m1), FABPpm (Mm02342495_m1), HSL (Mm00495359_m1), LPL (Mm00434770), Nuclear respiratory factor 1 (NRF1) (Mm00447996_m1), FATP1 (Mm00449511_m1) ATGL1 (Pnpla2) (Mm00503040_m1) DGAT1 (Mm00515643_m1), DGAT2 (Mm00499536_m1) were obtained from, Applied Biosystems, TaqMan®. Cyclophilin A Fwd: aggatgagaacctctgatagca Rev: tggcagtgcagataaaactg) from Geneworks. The relative concentrations of measured mRNA’s were determined by plotting
the threshold cycle (Ct) versus the log of the serial dilution points, and the relative expression of the gene of interest was determined after normalization to 18S or cyclophilin A.

Muscle metabolites and enzymes. TAG, DAG and ceramide content were determined in the quadriceps muscles using methods previously described (13; 21). Citrate synthase (CS) and β-hydroxyacyl CoA dehydrogenase (β-HAD) activity were measured in 5–10 mg of skeletal muscle as previously reported (14).

Electron Microscopy: HSP72Tg mice: For electron microscopy standard processing methods were used. The muscle was fixed in 2.5% Glutaraldehyde, osmicated with 2.5% Osmium tetroxide, dehydrated through a graded series of acetone solutions and embedded in Epon-araldite. Ultra-thin sections were cut on a Reichert-Jung Ultra-S microtome and collected on Nickel Grids. The sections were stained with uranyl acetate and lead citrate and viewed on a Hitachi H7500 TEM. Random images were taken at a magnification of 20K. The area of the field of view was calculated, mitochondria counted and expressed as number of mitochondria per µM². Ten different areas of muscle were calculated for each animal and averaged.

Seahorse Analyser: C2C12 myoblasts were transfected with pIRES HSP72 plasmid or empty vector (control) before undergoing selection with geneticin. After 3 weeks of selection, pooled stable myoblasts were seeded to Seahorse V7 plates and induced to differentiate by serum withdrawal. Mitochondrial function of HSP72 over-expressing and control cells was analyzed using the Seahorse XF analyzer as previously described (27).

Muscle mitochondrial isolation: Mitochondrial isolation was performed in quadriceps muscle. Fresh tissue (~300mg) was immediately placed in ice-cold isolation medium (pH7:
100mM sucrose; 100mM KCl; 50mM Tris-HCl; 1mM KH2PO4; 0.1mM EGTA; 0.2% fatty acid free BSA). After chopping the tissue in 2mL of fresh ice-cold isolation medium, samples were incubated for 2 min with 1.5µg of proteinase per mg of tissue. Samples were homogenized in 6mL of isolation medium with an electric homogenizer. After spinning down the samples for 5min at 800g, the supernatant was centrifuged again at 12000g for 10min. Mitochondria in the resulting pellet were washed in 1mL of isolation medium and centrifuged at 10000g for 10 min.

**Respiratory measures and AMPK activity** Oxygen consumption rates (OCR) were measured in isolated mitochondria from the skeletal muscle of WT and Hsp72Tg mice fed a normal chow diet using a Clarke Electrode. Basal, adenosine diphosphate (ADP)-stimulated State III (2.4mM), state IV and 2,4-dinitrophenol (DNP) stimulated uncoupled respiration (0.1mM) were measured in isolated mitochondria preps in the presence of (A) complex 1 substrates (5mM pyruvate, 2mM malate) and (B) complex II substrates (10mM succinate, 4µM rotenone). OCR was then normalized and expressed relative to citrate synthase activity levels. OCR measurements for isolated mitochondria using the Seahorse Analyzer in Supp. Fig. 7 were carried out as described in the figure legend. Total AMPK activity from whole cell lysates was measured as described (28).

**Fiber typing.** Muscle fiber type was determined in quadriceps muscle by incubation of slides in acidic (pH 4.3) solution containing potassium acetate and CaCl2 2H2O. Following 5 min in this solution, slides were then incubated at 37°C in ATP solution followed by room temperature incubations in 1% CaCl2.2H2O, 2% CoCl2.6H2O and 1% ammonium sulphide before being dehydrated in ascending alcohol stocks. Sections were viewed under light microscopy.
Plasma analysis. Plasma measurements were performed on blood collected from the chest cavity and centrifuged at 14,000g for 10 min. Insulin concentrations were measured by ELISA (Linco Research, St. Louis, MO). The concentration of free fatty acids was determined using a colorimetric kit (Wako Pure Chemical Industries, Osaka, Japan). Plasma acylcarnitines were measured as previously described (13).

Statistics. Data were analyzed by two-way analysis of variance and Tukey post-hoc tests. * indicates a diet effect, † indicates a genotype effect, ‡ indicates a treatment effect unless otherwise indicated. An unpaired Student’s t-test was also used for comparison of relevant groups where # indicates a significant difference. All data are presented as mean ± standard error of the mean (SEM) unless indicated. Statistical significance was set at P<0.05.

Results

HSP72Tg mice are protected from high fat diet-induced obesity and insulin resistance. We previously observed that HSP72Tg mice are protected from HFD-induced activation of JNK in vivo (14). To confirm this observation in an acute setting with exposure to higher fatty acid concentrations, we examined whether overexpression of HSP72 could inhibit fatty acid-induced JNK activation in skeletal muscle ex vivo. Consistent with our previous observation, high dose palmitate treatment resulted in a robust increase in JNK phosphorylation in soleus muscles from WT mice, but this effect was markedly blunted in HSP72Tg mice (Supp. Fig. 1A). Therefore, although it is clear that increasing HSP72 can block JNK activity, whether blockage of pro-inflammatory signaling is the primary mechanism by which HSP72 protects against HFD-induced insulin resistance is not known. In our previous study (14), mice were fed a diet containing 60% of total energy from fat for 16 wk, which resulted in an
upregulation of JNK phosphorylation in the skeletal muscles from the WT cohort. In the current investigation, we deliberately chose to use a diet containing significantly less lipid (45% of energy from fat) for a shorter time (10 wk). Contrary to our previous study (14), this dietary intervention did not cause JNK activation in skeletal muscle from either WT or HSP72Tg mice (Supp. Fig. 1B). Nonetheless, this dietary intervention increased body weight (Fig. 1A) and fat pad mass (Fig. 1B) and induced fasting hyperglycemia (Fig. 1C) and hyperinsulinemia (Fig. 1D) in WT mice. Importantly, however, these HFD-induced alterations in metabolic homeostasis were prevented in HSP72Tg mice (Fig. 1 A-D). Consistent with these findings, the HFD promoted glucose intolerance (Fig. 2A,B) and impaired insulin-stimulated phosphorylation of Akt (Fig. 2C-E) in WT mice, whereas the HSP72Tg mice were largely protected from these deleterious effects of the diet. Intriguingly, even on a normal chow diet HSP72 Tg mice showed enhanced glucose tolerance (Fig. 2A). To assess this finding in more detail, we measured insulin-stimulated glucose clearance into skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) in normal chow fed mice using tracer methodology (for radioactivity and blood glucose graphs during insulin stimulation see Supp. Fig. 2A,B). Insulin-stimulated glucose uptake into the Tibialis Anterior muscle was increased in HSP72Tg compared with WT mice (Fig. 2F) demonstrating that nutrient overload is not necessary for HSP72 overexpression to improve insulin action. We also observed the same increase in insulin-stimulated glucose uptake in both WAT (Fig. 2G) and BAT (Fig. 2H), even though HSP72 was not overexpressed in these peripheral tissues. Interestingly, further analysis of the WAT also revealed increased rates of lipolysis and oxidative enzymes citrate synthase and β-HAD (Supp. Fig. 3A-D). These initial studies were performed in HSP72Tg and WT mice on a balb/c background. To determine whether the metabolic phenotype observed in these mice was penetrative across different genetic strains of mice, we back-crossed the mice at least 10 generations onto a C57BL/6...
background. We showed that under HFD conditions, heterozygous HSP72Tg mice on such a
background were leaner and more glucose tolerant compared with littermate control mice
(Supp. Fig. 4A-F). Taken together, these findings suggest that HSP72 protects against the
development of diet-induced insulin resistance by mechanisms other than blocking JNK
activation in skeletal muscle and possibly by indirect action on metabolic tissues that do not
express the transgene. In addition, the effect of HSP72 overexpression on metabolic
homeostasis is preserved across mice with different genetic backgrounds using littermate
mice as controls.

*Hsp72 transgenic mice exhibit increased energy expenditure, whole body fat oxidation and
skeletal muscle fat oxidation and are resistant to HFD-induced ectopic lipid deposition.*

HSP72Tg mice were leaner than WT animals (Fig. 1B), and this finding could not be
explained by food consumption as energy intake was similar between the genotypes,
suggesting that HSP72Tg mice exhibit enhanced energy expenditure compared with WT
mice. To examine this further, we measured whole-body VO$_2$ by indirect calorimetry.
HSP72Tg mice exhibited elevated VO$_2$ compared with WT animals (Fig. 3A) with
accompanying increased in energy expenditure (Fig 3C). Importantly, this was not due to
merely having a lower body weight, since we observed a markedly higher VO$_2$ in HSP72Tg
mice on a chow diet (Fig. 3A), when bodyweight was identical (Fig. 1A). In addition, the
respiratory exchange ratio (RER) was reduced in HSP72Tg mice, implying that the increased
energy expenditure was supported by an increased rate of fat oxidation (Fig. 3B). HSP72Tg
mice were resistant to HFD-induced weight gain, and displayed increased whole body VO$_2$
and fat oxidation. As skeletal muscle is an important tissue for lipid metabolism, we next
assessed whether skeletal muscle FAO was enhanced in these animals. The HFD increased
skeletal muscle FAO irrespective of genotype (Fig. 3D) as previously observed (29).
Importantly, however, skeletal muscle FAO was markedly elevated in HSP72Tg mice compared with WT animals irrespective of diet (Fig. 3D). β-HAD activity was elevated in Hsp72Tg mice (Fig. 3E), and consistent with increased whole body fat oxidation (Fig. 3A) and skeletal muscle FAO (Fig. 3D), we observed a decrease in circulating free fatty acids in HSP72Tg mice compared with WT (Normal Chow: WT 1.45±0.17, HSP72 Tg 0.63±0.13µM, HFD: WT 1.29±0.15, HSP72 Tg 0.88±0.08 µM). Considering that the accumulation of bioactive lipids in skeletal muscle impairs insulin action, we next examined levels of triacylglycerol (TAG), DAG and ceramides in the skeletal muscles from HSP72Tg and WT mice fed a normal chow or HFD. The HFD caused an increase in both DAG (Fig. 3F) and TAG (Fig. 3G) in WT, but this increase was prevented in HSP72Tg mice (Fig. 3F,G). Ceramide concentrations were neither altered by the HFD, nor were they different between genotypes (Fig. 3H). As alterations in protein Kinase C (PKC) isoforms have been linked to lipid induced alterations in insulin signaling (30), we analyzed three PKC isoforms (theta, alpha and delta). While a HFD tended to increase the membrane/cytosol ratio in the WT animals, but not in the HSP72 Tg mice, there was no significant difference between groups (Supp. Fig. 5A-D). Together, these data suggest that overexpression of HSP72 in skeletal muscle enhances muscle oxidative metabolism, thereby preventing ectopic lipid accumulation in skeletal muscle when animals are challenged with a HFD.

As we had observed reductions in TAG and DAG when HSP72Tg mice were fed a HFD, we next assessed whether HSP72 overexpression affected fatty acid transporters and the lipolytic enzyme machinery of the skeletal muscle. Western blotting analysis revealed a significant increase in the expression of the lipolytic enzyme LPL in the HSP72Tg mice, while no changes were observed for the fatty acid transporter CD36 (Fig. 4A-C). We also analyzed the mRNA expression of the genes encoding these two proteins, as well as others involved in
fatty acid lipolysis, uptake and synthesis (Fig 4D). While many of these genes were altered with HFD (PNPLA2, LPL, SLC27A1, Got2, CPT1B and DGAT2), only two were affected by HSP72 overexpression. These were SLC27A1, which was significantly decreased in the HSP72Tg mice on a HFD compared with chow, and DGAT1 where a main effect was detected for a decrease in expression in the HSP72Tg mice (Fig 4D). As the LPL mRNA was not different between genotypes, it is likely that the overexpression of HSP72 increased LPL protein expression post-translationally in a chaperone dependent manner.

**HSP72Tg mice have increased number of mitochondria and superior running performance.**

To determine the gene expression signature that underlies the enhanced metabolic phenotype observed in the HSP72Tg mice, we next performed microarray analyses on skeletal muscle from both genotypes of mice. We employed Gene Set Enrichment Analyses, previously described (21), but no differences were observed for genes associated with the tricarboxylic acid cycle, electron transport chain or oxidative phosphorylation (data not shown) when comparing the cohorts. Slow oxidative muscle fiber content was also not different in the quadriceps muscle between HSP72Tg and WT mice (Supp. Fig. 6A). Next, we investigated the functional capacity of mitochondria from quadriceps muscle of the HSP72Tg and WT mice by measuring relative oxygen consumption rates in response to ADP and the chemical uncoupler (DNP). We observed no difference in OCR, per unit of mitochondria, between the HSP72Tg mice and WT in the basal state, in response to ADP or DNP (Supp. Fig. 6B,C), or in the HSP72 Tg backcrossed mice on a normal chow or HFD (Supp. Fig. 7A-D). As there was no difference in muscle fiber type or mitochondrial respiratory capacity (per unit mitochondria), we next tested whether muscles from HSP72Tg mice contain a greater number of mitochondria. Recent work suggests that heat stress-induced HSP72 expression stimulates mitochondrial biogenesis (18). Accordingly, we performed electron microscopy
studies to quantify the number of mitochondria in the *quadriceps* muscles of the HSP72Tg and WT mice. We observed an approximate 50% increase in the number of mitochondria in the HSP72Tg relative to WT mice (Fig. 5A). Thus, although functional capacity per unit mitochondria was not different between the genotypes, HSP72 overexpression in muscle increased the abundance of mitochondria in that tissue. Increased mitochondrial density does not necessarily equate to increased mitochondrial function. Since increased functional mitochondrial number and muscle oxidative metabolism are usually associated with increased exercise endurance, we next examined running performance. Treadmill running including both an incremental exercise test (Fig. 5B) and time to voluntary exhaustion (Fig. 5C) were assessed. Irrespective of the test type, HSP72Tg mice exhibited a markedly enhanced exercise capacity (Fig. 5B,C). Interestingly, while impaired mitochondrial capacity is associated with the pathogenesis of insulin resistance and type 2 diabetes (8; 9), others have suggested that increasing energetic flux through the mitochondria may result in incomplete β-oxidation and alterations in plasma acylcarnitines which may lead insulin resistance (31). Accordingly, we next measured plasma acylcarnitines in our model. Consistent with previous studies (31), we observed that some acylcarnitine species increased, while others decreased when mice were placed on a HFD (Supp. Fig. 8). In addition, the total plasma acylcarnitine levels are slightly, but significantly, reduced on HFD. However, despite the marked effect of HSP72 overexpression on mitochondrial energy turnover as measured by several methods, the plasma acylcarnitine levels comparing WT and HSP72Tg animals were very similar, irrespective of diet (Supp. Fig. 8), providing solid evidence that muscle oxidative capacity is uncoupled from plasma acylcarnitine levels, at least in our model. Together, these data indicate that elevated HSP72 expression drives an increase in mitochondrial number, oxidative metabolism, enhances exercise performance and insulin action in mice.
**Hsp72Tg mice display up-regulation of Tfam transcription, enhanced AMPK activity and increased SIRT1 protein expression.** Considering that muscle from HSP72Tg mice displayed an increased mitochondria number and enhanced oxidative metabolism, we next examined known regulators of these pathways. The PGC-1 family of transcriptional coactivators, putative regulators of mitochondrial oxidative metabolism (32; 33), target the transcription factor Nrf-1 to stimulate the expression of Tfam, a matrix protein essential for the replication and transcription of mitochondrial DNA (32). Although skeletal muscle PGC-1α mRNA was not different when comparing HSP72Tg with WT, Tfam mRNA was 2-fold higher in HSP72Tg (Fig. 6A). PGC-1α is directly linked to the activity of AMPK (34), an evolutionarily conserved sensor of cellular energy status, critical for enhancing fuel metabolism (35). Indeed, AMPK phosphorylation (Thr\(^{172}\); Fig 6B) and activity (Fig. 6C) was enhanced in the muscles from HSP72Tg relative to WT mice. Another important fuel-sensing molecule is the NAD\(^+\)-dependent deacetylase Sirtuin 1 (SIRT1), an important regulator of oxidative metabolism (12; 36). AMPK and SIRT1 regulate each other and share many common target molecules (37). Accordingly, we examined whether SIRT1 expression and activation were affected by HSP72 overexpression. While SIRT1 mRNA expression tended (NS, P =0.1) to be increased in HSP72Tg mice (Fig. 6A), SIRT1 protein levels were higher in the HSP72Tg relative to WT mice irrespective of diet (Fig. 6D). Given that SIRT1 is known to deacetylate PGC-1α (38), we performed immunoprecipitation experiments to investigate PGC-1α-acetylation levels, but we failed to detect differences between HSP72 Tg and WT mice (Supp. Fig. 9A), nor did we see any difference between the groups in total PGC-1α expression from whole tissue lysates (Supp. Fig. 9B).

As PGC-1α was not altered, it was possible that HSP72 Tg overexpression could reduce the degradation of mitochondria. To address this question, we next investigated any changes in
autophagy/mitophagy in the skeletal muscle of the HSP72Tg mice. As shown in Supp. Fig. 10 (A-F), we observed a general increase in autophagy in the skeletal muscle of the HSP72Tg mice fed a HFD. Both p62, a ubiquitin-binding scaffold protein that is degraded with autophagy, and ATG12, a part of a complex involved in the recruitment of LC3A levels, were reduced in HSP72Tg animals fed a HFD (Supp. Fig. 10). In addition, LC3A, a marker for the autophagosome membrane, tended to be increased in the HSP72 Tg mice (Supp. Fig. 10). To further examine the relationship between HSP72 overexpression and autophagy, we next collected skeletal muscle from animals that had either been euthanized in the fed state or following a 24 h fast. Fasting increased the level of LC3A conversion and decreased ATG12 protein expression while p62 was unchanged (Supp Fig. 10G-K). Interestingly, the HSP72Tg mice displayed greater LC3A conversion in the fasted state and less ATG12 expression (Supp. Fig 10G-K), again indicating increased autophagy and greater turnover of organelles in the skeletal muscle. To determine whether we could detect any specific differences in mitophagy markers (the mitochondrial specific autophagic degradation process), we blotted for the E3 ubiquitin ligase Parkin and the PTEN-induced putative kinase 1 (PINK1). No significant differences were observed between HSP72Tg and WT mice for these proteins (Supp. Fig. 11A-C), however a significantly greater quantity of p53 (a tumour suppressor protein linked to both increased and decreased autophagy) was observed in the HSP72Tg muscle (Supp Fig 11D-E). Of note, p53 has recently been shown to inhibit parkin mediated mitophagy (39). Finally, we looked at mitochondrial fusion and fission factors (Supp. Fig. 11F-J). Mitochondrial morphology is a highly dynamic process regulated by the balance of fission and fusion processes (40). These processes also impact on mitophagy and, therefore, impact on mitochondrial quantity. Mitochondrial outer membrane proteins mitochondrial fission factor (MFF) and mitochondrial fission protein 1 (Fis1) are thought to regulate mitochondrial fission via recruitment of dynamin-related protein 1 (DRP1) to the
mitochondria. Interestingly MFF was decreased in the HSP72Tg mice, whereas Fis1 was increased (Supp. Fig. 11F,H,J) theoretically opposing each other's actions. Optic atrophy 1 (Opa1) a protein linked to mitochondrial fusion and the phosphorylation of DRP-1 at Ser_637 were not different between groups (Supp. Fig. 11F,G,J). Together, these data indicate that while autophagy may be elevated in the HSP72Tg muscle, mitophagy, fission and/or fusion processes do not appear to be markedly affected by HSP72 overexpression, although further research is required to clarify the role of HSP72 in these mitochondrial dynamic processes.

Overexpression of HSP72 increases cellular respiration, while the HSP72 co-inducer BGP-15 increases mitochondrial number in a diabetic rodent model. In order to determine whether the increase in mitochondrial number observed in the HSP72Tg mice was preserved across different experimental models and in an environment free of the confounding effects of any circulating factor/s, we next measured OCR in skeletal myotubes engineered to stably overexpress HSP72 (Fig. 7A). These cells displayed significantly increased basal and maximal respiration rates compared with control cells (Fig. 7B). Importantly, supportive of the interpretation that a mitochondrial phenotype drives the increase in energy expenditure and protection from diet-induced obesity and insulin resistance, the increase in basal respiration was due to increased ATP turnover rate (Fig. 7B). Furthermore, there was a trend for an increase in glycolysis (ECAR is a proxy measure of glycolysis) in the cells stably expressed with HSP72 (Supp. Fig. 12A,B), indicating an overall increase in cellular bioenergetics and substrate oxidation in the cells stably overexpressing HSP72.

We have previously demonstrated that the HSP72 co-inducer BGP-15 increases HSP72 expression in skeletal muscle (14). Moreover, BGP15 treatment improved insulin sensitivity in ob/ob mice (14) and patients with type 2 diabetes (20). Given our findings thus far, we
next examined the effect of BGP-15 treatment on mitochondrial density in skeletal muscles from Goto-Kakizaki (GK) rats, a non-obese Wistar substrain which develops type 2 diabetes early in life (41). BGP-15 treatment increased the relative mitochondrial area (Fig. 7C) in skeletal muscle of rats compared with vehicle treated. In addition, consistent with our previous observations in both mice (14) and humans (20), BGP-15 was equally as effective as either metformin or Rosiglitazone in improving insulin action in GK rats (Fig. 7D). Together, these data suggest that the increasing HSP72 expression in vivo by transgenesis, in cells stably transfected to overexpress HSP72 or via pharmacological activation of HSP72 in vivo increases mitochondrial number, function and capacity.

**Discussion**

It is clear that the induction of HSP72 in skeletal muscle can protect mice from HFD-induced insulin resistance. However, the mechanism by which this occurs has remained unclear since HSP72 can block JNK signaling in skeletal muscle and increase oxidative metabolism, both of which can protect against insulin resistance (14). Herein, we provide new evidence showing that increased skeletal muscle HSP72 expression elevated whole body energy utilization and fatty acid oxidation, thereby preventing intramuscular lipid accumulation and insulin resistance independent of changes in pro-inflammatory signaling. Moreover, increased muscle HSP72 promotes mitochondrial biogenesis and enhanced oxidative metabolism, likely via a mechanism involving increased AMPK activity and sirtuin activation.

In the current investigation, whole body oxygen consumption, skeletal muscle fatty acid oxidation and running capacity were increased in HSP72Tg mice compared with WT controls. Consistent with an enhanced oxidative metabolism phenotype, HSP72Tg mice
exhibited a reduced RER reflective of a greater reliance on fatty acid oxidation and this functional readout is consistent with the observed increases in Tfam mRNA expression, AMPK activity, SIRT1 expression and, indeed, mitochondrial number. Even though our data suggest that mitochondrial biogenesis (i.e synthesis) is responsible for the increased mitochondrial number observed in the HSP72 Tg mice or in mice treated with BGP-15, nonetheless, we cannot dismiss the potential for HSP72 to improve the maintenance of mitochondrial quality and potential slowing of organelle turnover. Indeed, we show that markers of autophagy were increased in HSP72 Tg mice (Supp. Fig. 10). HSP72 is well known to function as a chaperone-mediated stabilizer of a number of enzymes and/or nuclear receptors, and aids in the delivery of nuclear encoded proteins to the mitochondrial membrane for mitochondrial importation (42). We, therefore, cannot rule out chaperone mediated mitochondrial stabilization as a factor contributing to the observed phenotype.

Despite the fact that we observed an increase in Tfam expression and mitochondria number, we failed to detect any increases in mRNA, protein expression or acetylated lysine levels of PGC-1α in our study. This was most unexpected given the known role of PGC-1α in regulating mitochondria biogenesis. While unlikely, it is possible that HSP72 somehow regulates Tfam expression in a yet to be determined manner independently of PGC-1α. In support of this notion, exercise training in PGC-1α deficient mice induced expression of genes involved in oxidative phosphorylation, demonstrating that PGC-1α is not mandatory for at least some alterations in oxidative metabolism following endurance training (43; 44).

Interestingly, despite HFD-induced increases in TAG and DAG in skeletal muscle, ceramide levels were not elevated, suggesting that increased ceramide accumulation in skeletal muscle is not necessary to induce insulin resistance, as previously demonstrated (3). Of interest, LPL
protein expression was increased in skeletal muscle of the HSP72Tg relative to WT mice (Fig. 4A,B). LPL functions to hydrolyze serum triglycerides releasing fatty acids which can be internalized into the myocyte. It was recently demonstrated that decreased mitochondrial content in muscle of insulin resistant offspring may be due, in part, to reductions in LPL expression (45). Hence, it is possible that the effect of HSP72 overexpression on LPL protein expression could explain, in part, the improvement in insulin action in the HSP72 Tg mice.

Despite these extensive data suggesting that mitochondria play a role in insulin resistance, they are nonetheless associative and it has been argued that there is no causal link between mitochondrial dysfunction and insulin resistance (46). Although relatively understudied and often overlooked, are the studies from patients with mitochondrial myopathies that support the contention that impaired mitochondrial function and insulin resistance are causally linked. A genetic point mutation affecting position 3243 in the tRNA leucine mitochondrial gene results in poor insulin sensitivity in these patients (47) and maternally transmitted type 2 diabetes (48). In addition, patients with one of the most common mitochondrial diseases, Chronic Progressive External Ophthalmoplegia, have impaired glucose tolerance (49). Taken together, our current data and these previous studies argue that the mitochondria may be a potential target organelle for the treatment of skeletal muscle insulin resistance and type 2 diabetes. Importantly BGP-15, which activates HSP72 and results in increased mitochondria and insulin action (Fig. 7C,D), is currently the subject of a multicenter human clinical trial for the treatment of type 2 diabetes.

While our study focused on high fat diet-induced insulin resistance, our observations may have broader consequences for several conditions where mitochondrial function is compromised. It has been suggested that mitochondrial dysfunction (e.g. reduced
mitochondrial quality, excessive production of radical oxygen species and increased mitochondrial DNA mutation rate) plays a critical role in the aging process (50). Indeed lower respiratory capacity is a characteristic feature of aged human skeletal muscle (51-53). Of note, in a mouse model that phenocopies premature, human aging (the PolyG mouse), endurance exercise rescued the accelerating aging phenotype in this model by improving mitochondrial number and function (54). Although not a classical mitochondrial disorder Duchenne muscular dystrophy (DMD) is also a condition associated with mitochondrial dysfunction (55; 56). Importantly, we recently demonstrated that increasing intramuscular HSP72 expression preserves muscle strength and ameliorates the dystrophic pathology in two mouse models of muscular dystrophy (57). Moreover, when we treated a mouse model that phenocopies DMD, with BGP-15 both muscle function and lifespan were increased (57). Given the results presented herein, it would be interesting to determine if mitochondrial dynamics were altered in the dystrophic models with HSP72 overexpression or BGP-15 treatment. Unfortunately, despite the well known benefits of exercise, patients suffering these diseases either cannot (e.g. morbidly obese individuals or boys with severe muscular dystrophy), or will not, exercise. Exercise training, therefore, has limited long-term efficacy as a primary and sole strategy in treating these conditions. Importantly, our current data provide a realistic therapeutic avenue for treatment.

In summary, we demonstrate that skeletal muscle specific overexpression of HSP72 protects against obesity-induced insulin resistance via up regulation of oxidative metabolism. This heightened oxidative metabolism and reduced accumulation of deleterious lipid species following high fat feeding likely promotes insulin sensitivity and glucose tolerance in high fat fed HSP72Tg mice. Importantly, in terms of therapeutic potential, the small molecule HSP72 co-inducing compound BGP-15 mimics much of the beneficial effects of genetic
overexpression of HSP72 and exercise training including increased mitochondria area and insulin sensitivity suggesting that BGP-15 may offer therapeutic utility in the treatment of diseases associated with metabolic and mitochondrial dysfunction.

Author Contributions

DCH, CRB, and MAF designed research; DCH, CRB, BGD, KT, AK, EE, JC, NW, TG, RS L-Y, TC, MJW, KC, MH, SLM, ALH, performed and/or analyzed research; DCH, CRB, ALH and MAF wrote the paper and all authors contributed to the writing of the final submitted version.

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had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Figure Legends

**Figure 1.** Characteristics of WT and HSP72Tg mice fed a normal chow or high fat diet for 10 wk. (A) body weight, (B) epididymal fat pad mass, (C) fasting blood glucose and (D) fasting insulin; n = 6-8. * Diet effect: P<0.05 vs chow within same genotype, † Genotype effect: P<0.05 vs WT within same diet.

**Figure 2.** Glucose tolerance test in 5h fasted WT and HSP72Tg mice fed either a chow or high fat diet for 10 wk. (A) Blood glucose levels after an intraperitoneal glucose injection (1 g/kg) and (B) incremental area under the curve during the glucose tolerance test. Insulin-stimulated Akt phosphorylation in WT and HSP72Tg mice fed either a chow or high fat diet for 10 wk. (C) Representative immunoblots of phosphorylated and total Akt (D, E) quantification of Akt phosphorylation on serine site 473 and threonine 308 respectively; n = 6-8 per group. † P<0.05 main effect for genotype within diet group, †† Genotype effect within treatment (insulin-stimulation): P<0.05 vs. WT insulin-stimulated condition, † Treatment effect: P<0.05 basal vs. insulin-stimulated condition within each diet and genotype. Insulin-stimulated glucose clearance into peripheral tissues in chow fed mice. 2-Deoxyglucose clearance into (F) Tibialis Anterior skeletal muscle (G) White adipose tissue from the epididymal fat pad (H) and brown adipose tissue; n = 7-8 per group. # P<0.05 vs WT. Data are mean ± SEM.

**Figure 3.** Aspects of whole-body energy metabolism in WT and Hsp72Tg mice fed either a chow or high fat diet for 10 wk. (A) VO₂ (B) RER (C) Energy Expenditure. Data are mean ± SEM; n = 6. * P<0.05 main effect for diet, † P<0.05 main effect for genotype. Skeletal muscle lipid levels and palmitate oxidation rates in WT and HSP72Tg mice fed either a chow or high fat diet for 10 wk. (D) Palmitate oxidation in isolated soleus muscle (E) β-HAD activity in
quadriceps muscle; n = 6-8 per group † P<0.05 main effect for diet , ‡ P<0.05 main effect for genotype. (F) Muscle diacylglycerol concentration. (G) Muscle triacylglycerol concentration (H) Muscle ceramide concentration; n =6-8 per group. * Diet effect: P<0.05 vs. WT chow, † Genotype effect: P<0.05 vs. high fat fed HSP72 Tg. Data are mean ± SEM.

**Figure 4.** Markers of lipolysis and fatty acid transporters in normal chow and high fat fed mice. (A) Western blotting analysis for HSP72, CD36 and LPL. Quantification of (B ) LPL and (C) CD36 protein levels relative to GAPDH. Data are mean ± SEM; n = 6. † P<0.05 main effect for genotype. (D) RT-PCR analysis of various genes related to lipolysis, fatty acid cellular or mitochondrial uptake and fatty acid synthesis. Data is expressed relative to cyclophilin A levels and are mean ± SEM; n = 4-7.

**Figure 5.** Mitochondrial number via electron microscopy imaging in WT and HSP72Tg mice fed a chow diet. (A) Representative pictures and quantification from quadriceps muscle sections from WT and HSP72 Tg mice. The arrows indicate mitochondria; n = 10-11. † P<0.05 WT vs HSP 72 Tg. **Endurance running capacity in chow fed WT and HSP72Tg mice.** (B) Running time during an incremental running test to fatigue (C) time to fatigue during a treadmill run at fixed speed and gradient; n = 5-10 per group ‡ P<0.05 vs WT. Data are mean ± SEM.

**Figure 6.** RT-PCR analysis of skeletal muscle and AMPK phosphorylation and activity. (A) Genes related to mitochondrial biogenesis in skeletal muscle (Pgc1-α, SIRT1, Nrf1 & TFAM) in WT and HSP72Tg mice fed a normal chow diet; n = 6. ‡ P<0.05 WT vs HSP 72Tg. (B) Representative blot and quantification of phosphorylation of AMPK (Thr172), (C)
AMPK activity levels form skeletal muscle lysates; n = 4 per group for phosphorylation, n=14 per group for activity. # P<0.05 WT vs HSP72Tg. SIRT1 protein expression in skeletal muscle from WT and HSP72Tg mice fed either a chow or high fat diet for 10 wk. (D) Representative immunoblot and quantification of SIRT1 expression; n = 6 per group † P<0.05 main effect for genotype.

**Figure 7.** Oxygen consumption analysis in HSP72 stably expressed myotube cell line. (A) Representative oxygen consumption trace in (A inset) HSP72 stably overexpressed cells and wild type cells. (B) Analysis from mitochondrial function test for basal respiration, uncoupled respiration, ATP turnover, maximal mitochondrial respiration, and spare respiratory capacity; Addition of compounds at the straight lines were as follows: (a) Oligomycin, (b) FCCP and (c) Antimycin A. # indicates p<0.05 relative to control group. **BGP-15 treatment of the Goto-Kakizaki rat increases mitochondria number** (C) Representative electron micrographs and (D) quantification of BGP-15 stimulated mitochondrial biogenesis. # indicates p<0.05 relative to control group, n=36 for BGP-15 treatment and n=41 for control group. **Glucose infusion rate as measured by hyperinsulinemic euglycemic clamp after BGP-15 treatment.** (D) GK rats were administered daily oral doses of BGP-15 (5, 10, 20, 30 mg/kg), rosiglitazone (2 mg/kg), metformin (100 mg/kg) or vehicle for five days. Insulin sensitivity was evaluated after 5d using the hyperinsulinemic euglycemic clamp method. Data expressed as mean ± SEM, n =6 animals per group. * indicate p<0.05 relative to control group.
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Figure 1

(A) Body weight (grams) - Chow vs. HFD (WT vs. HSP72 Tg).
(B) Epididymal fat pad mass (grams) - Chow vs. HFD (WT vs. HSP72 Tg).
(C) Fasting blood glucose (mM) - Chow vs. HFD (WT vs. HSP72 Tg).
(D) Fasting insulin (mU/L) - Chow vs. HFD (WT vs. HSP72 Tg).
Figure 2

254x190mm (96 x 96 DPI)
Figure 3
254x190mm (96 x 96 DPI)
Figure 4

**A**

**B**

**C**

**D**

254x190mm (96 x 96 DPI)
Figure 5

254x190mm (96 x 96 DPI)
Figure 7

A

HSP72
HSP72

B

OCR (pmol/min)

WT
HSP72

C

Control
BGP-15

D

Relative Mitochondrial area (%)

CON
BGP-15

E

D Glucose infusion rate

Con 5 10 20 30 2 100

BGP-15 Rosi Metf

Figure 7
254x190mm (96 x 96 DPI)
Supplementary Figure 1. A) Levels of phosphorylated and total JNK in soleus muscle from WT and HSP72 Tg mice incubated with low (0.5 mM) or high (1.5 mM) palmitate for 6 h and (B) JNK expression in quadriceps muscle from WT and HSP72 Tg mice fed a normal chow (NC) or high fat diet (HFD) for 10 wk. Data are mean ± SEM; n = 6. * P<0.05 main effect for palmitate treatment, † P<0.05 main effect for genotype.
Supplementary Figure 2. Tracer kinetics and plasma glucose concentrations during Insulin-stimulated glucose clearance studies: (A) tritiated 2-deoxyglucose counts and (B) Blood glucose concentrations measured over 30 minutes after insulin stimulation in WT and HSP72Tg mice. n = 7-8. Data are mean ± SEM.
Supplementary Figure 3. Lipolysis assay on white adipose tissue (epididymal fat pad) explants from WT and HSP72Tg mice. Glycerol (A) and free fatty acid release was measured using manufacturers instructions in to the medium in response to basal (control) and isoproterenol stimulated conditions. † P<0.05 main effect for genotype * P<0.05 treatment effect by isoproterenol. Oxidative enzyme activities in white adipose tissue. β-HAD activity (C) and citrate synthase activity (D) † P<0.05 main effect for genotype. Data are mean ± SEM, n=4-9 per group.
Supplementary Figure 4. Characteristics of heterozygous HSP72Tg and littermate control mice on a C57Bl/6 background fed a normal chow or high fat diet for 12 wk. Body weight (A), fat mass (B), lean mass (C), percent body fat (D), glucose levels during an oral glucose tolerance test (OGTT) (E) and area under the curve for the OGTT (F). * P<0.05 main effect for diet within genotype † P<0.05 genotype effect on HFD. Data are mean ± SEM, n=17-23 per group.
Supplementary Figure 5. A) Western blots of PKC isoforms in the cytoplasmic (C) and membrane (M) fractions of skeletal muscle form WT and HSP72Tg mice. (B-D) Quantification of westerns blots for PKC isoforms (membrane to cytosol ratio). Data are mean ± SEM, n=6 per group.
Supplementary Figure 6. Skeletal muscle fibre typing in WT and HSP72 Tg mice fed a chow diet. (A) Representative cross sectional images from quadriceps muscle from WT and Hsp72 Tg mice and quantification of the slow twitch fibre number. Dark staining represents slow twitch fibres. Data are mean ± SEM; n = 5-9. Oxygen consumption rates in isolated mitochondria from WT and Hsp72 Tg mice fed a normal chow diet. Basal, ADP-stimulated State III (2.4mM), and DNP stimulated uncoupled respiration (0.1mM) were measured in isolated mitochondria preps in the presence of (B) complex 1 substrates (5mM pyruvate, 2mM malate) and (C) complex II substrates (10mM succinate, 4µM rotenone) Data are mean ± SEM; n = 3-5.
Supplementary Figure 7. Oxygen consumption rates in isolated mitochondria from WT and HSP72 Tg mice backcrossed onto a C57bl/6 background. Mice were fed a normal chow or high fat diet. Basal, ADP-stimulated State III (3mM), and FCCP stimulated uncoupled respiration (1μM) were measured in isolated mitochondria preps in an XF-24 Seahorse Bioanalyser (5µg/well centrifuged at 2000g for 15mins to adhere the mitochondria to the bottom of the plate) in the presence of (A and C) complex 1 substrates (5mM pyruvate, 2mM malate) and (B and D) complex II substrates (10mM succinate, 4µM rotenone) Data are mean ± SEM; n = 5.
Supplementary Figure 8. Analysis of plasma acylcarnitines from normal chow and high fat fed WT and HSP72Tg mice. Plasma was collected after a 5-6hr fast. n = 4-8 per group, * p =<0.05 for diet effect, † p = <0.05 for genotype effect between WT and HSP72 Tg mice. In the C4, C6 and C8 species there are significant interactions: Diet within WT, Diet within HSP72Tg and Genotype within NC (all p=<0.01). Data are mean ± SEM.
Supplementary Figure 9. A) Ac-Pgc1-α in skeletal muscle. Pgc1-α was immunoprecipitated from nuclear extracts with anti-Pgc1-α and immunoprecipitates were immunoblotted with anti-acetyl lysine (Ac-Lys) and Pgc1-α (B) Total Pgc1-α in skeletal muscle. Whole cell lysates probed for Pgc1-α: n=9-10 per group.
Supplementary Figure 10. (A-F) Analysis of markers of autophagy in skeletal muscle of WT and HSP72 Tg mice fed a HFD for 10 weeks. (A) Western blots for LC3A I and IIA II. (B) Quantification for LC3A I (C) and LC3A II relative to GAPDH. (D) p62 and ATG12 western blots and their respective quantifications (E, F) relative to GAPDH, n=4, # p=<0.05. (G-K) Analysis of markers of autophagy in skeletal muscle of WT and HSP72 Tg fed a chow diet with tissues collected in the Fed state (7am after the night cycle) or following a 24hr period of starvation (Starve). (G) Western blotting for LC3A I and II (H) Analysis of LC3A conversion ratio (I) Western blotting for p62, ATG12 and HSP72 (J, K) relative quantification of p62 and ATG12 relative to GAPDH. n=4 per group, * p=<0.05 for fasting effect, † p = <0.05 for difference between WT and HSP72 Tg mice.
Supplementary Figure 11. (A-E) Analysis of markers of mitophagy in skeletal muscle of WT and HSP72 Tg fed a chow diet with tissues collected in the Fed state (7am after the night cycle) or following a 24hr period of starvation (Starve). (A) Westerns blots for Parkin and PINK1 and their respective quantification (B, C) relative to alpha tubulin. (D) p53 protein expression and respective quantification (E) n = 4 per group, * p <=0.05 for fasting effect, † p = <0.05 for difference between WT and HSP72 Tg mice.  (F-J) Analysis of markers of mitochondrial fission and fusion in mice fed a HFD. (F) Western blotting for OPA1, MFF, phosphorylated and total DRP1, Fis 1 and HSP72. (G-J) Quantification of blots for OPA1, MFF, DRP1 and Fis1, n = 4, # p =<0.05 for significant difference between WT and HSP72 Tg mice. Data are mean ± SEM.
Supplementary Figure 12. Extracellular acidification rates (ECAR) and cellular bioenergetics in stably expressing HSP72 or control cells. (A) ECAR as a proxy measure of glycolysis (B) Cellular bioenergetic profile plotting OCR vs ECAR * Denotes statistically significant vs Con for OCR, n=5. Data are mean ± SEM.