Minireview

MOLECULAR METABOLISM

Chaperoning to the metabolic party: The emerging therapeutic role of heat-shock proteins in obesity and type 2 diabetes

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ABSTRACT

Background: From their initial, accidental discovery 50 years ago, the highly conserved Heat Shock Proteins (HSPs) continue to exhibit fundamental roles in the protection of cell integrity. Meanwhile, in the midst of an obesity epidemic, research demonstrates a key involvement of low grade inflammation, and mitochondrial dysfunction amongst other mechanisms, in the pathology of insulin resistance and type 2 diabetes mellitus (T2DM). In particular, tumor necrosis factor alpha (TNFα), endoplasmic reticulum (ER) and oxidative stress all appear to be associated with obesity and stimulate inflammatory kinases such as c jun amino terminal kinase (JNK), inhibitor of NF-κβ kinase (IKK) and protein kinase C (PKC) which in turn, inhibit insulin signaling. Mitochondrial dysfunction in skeletal muscle has also been proposed to be prominent in the pathogenesis of T2DM either by reducing the ability to oxidize fatty acids, leading to the accumulation of deleterious lipid species in peripheral tissues such as skeletal muscle and liver, or by altering the cellular redox state. Since HSPs act as molecular chaperones and demonstrate crucial protective functions in stressed cells, we and others have postulated that the manipulation of HSP expression in metabolically relevant tissues represents a therapeutic avenue for obesity-induced insulin resistance.

Scope of Review: This review summarizes the literature from both animal and human studies, that has examined how HSPs, particularly the inducible HSP, Heat Shock Protein 72 (Hsp72) alters glucose homeostasis and the possible approaches to modulating Hsp72 expression. A summation of the role of chemical chaperones in metabolic disorders is also included.

Major Conclusions: Targeted manipulation of Hsp72 or use of chemical chaperiones may have clinical utility in treating metabolic disorders such as insulin resistance and T2DM.

Keywords Hsp72; Skeletal muscle; Insulin resistance; Type 2 diabetes; Obesity; Inflammation; Oxidative capacity; Mitochondria; Chemical chaperones

1. INTRODUCTION

Heat shock proteins (HSPs) were serendipitously discovered over 50 years ago by the Italian scientist Ferruccio Ritossa. Indeed, the inadvertent application of heat shock to drosophila salivary glands (by the accidental adjustment of incubator settings) and the subsequent observation of new HSP RNA synthesis remains a striking demonstration of environmentally induced changes in gene expression [1,2]. A rather different and no doubt, more complex collective gene response is occurring consequential of today's snowballing and problematic environment of nutrient excess. Rates of obesity continue to rise and the understanding of how this leads to metabolic diseases such as type 2 diabetes (T2DM) is improving. However, effective treatments remain elusive. Given the ubiquity of the highly conserved HSPs, it is perhaps unsurprising that these chaperone proteins have been implicated in the treatment of insulin resistance and obesity associated T2DM [3,4]. Attracting most attention in this regard is the inducible isoform of HSP70, Hsp72 (Hspa1a). Experimental models indicate that Hsp72 is likely to confer protection against disturbed metabolic homeostasis via multiple modes of action including, but not limited to, reducing inflammation [3,5,6] and improving skeletal muscle oxidation [3,6–8]. Importantly, we [3,8] and others [4,9] have been conducting experiments using a small molecule activator of HSP72 (a hydroxylamine derivate termed BGP-15). This agent improves insulin sensitivity and inflammation in a genetic mouse model of insulin resistance [3], increases mitochondrial volume and improves metabolic homeostasis in a rat model of T2DM [8]. This is most promising from a clinical and translational perspective since BGP-15 has now proceeded to Phase 2b clinical trials and has previously been used in humans without any side-effects [4,10]. This review summarizes the accumulating evidence for a

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Received July 31, 2014 • Revision received August 19, 2014 • Accepted August 22, 2014 • Available online 30 August 2014

http://dx.doi.org/10.1016/j.molmet.2014.08.003

role of Hsp72 in glucose metabolism and discusses the therapeutic potential of Hsp72 raising agents in the treatment of conditions associated with insulin resistance.

2. HSP EXPRESSION IN INSULIN RESISTANCE AND DIABETES

Arguably the first link between HSPs and diabetes was derived from the observation that in insulin resistant and T2DM patients, HSP expression was markedly altered. Muscle biopsies taken from patients with T2DM showed significantly lower mRNA levels of Hsp72 than those taken from non-diabetic controls [11]. Furthermore, data collected in our laboratory supported this finding and demonstrated a marked relationship between Hsp72 mRNA and insulin stimulated glucose uptake during a hyperinsulemic-euglycemic clamp in T2DM patients [12]. We and others [13] later demonstrated that skeletal muscle Hsp72 protein expression reflected the same trend as Hsp72 mRNA, supporting the hypothesis that Hsp72 expression is decreased in T2DM [3]. Early speculation considered that Hsp72 expression might be affecting insulin sensitivity through a direct interaction with GLUT4 [11]. However, other studies found no reduction in GLUT4 gene expression in diabetic patients versus aged matched controls [12]. In the same study, we directly measured intramuscular triglyceride (IMTG) content in the muscle biopsy samples derived from T2DM patients and aged matched healthy controls. IMTG content was ~150% higher in the patient group. Allied to the finding of lowered Hsp72 expression in T2DM, these data provided a rationale for the examination of the role of HSP expression in the etiology of obesity induced insulin resistance.

3. OBESITY, INFLAMMATION AND INSULIN RESISTANCE

3.1. Low grade inflammation

Numerous lines of evidence suggest a link between obesity and inflammation. Prolonged or chronic inflammation is associated with a cluster of metabolic diseases, including T2DM and is referred to as "low grade" or meta-inflammation [14]. While the cascade of molecules involved in inflammation is complex, the pro-inflammatory cytokine, TNFα appears to demonstrate a prominent role in mediating downstream transduction cascades that affect insulin signaling. For example, TNFα is increased in the adipose tissue of obese mice [15]. Moreover, in loss-of-function experiments in obese mice, null mutations in the gene encoding TNFα and its receptors resulted in improved insulin sensitivity [16]. Significantly, a multitude of metabolic stressors appear capable of inducing inflammatory signaling pathways. In addition to extracellular TNFα., stressors originating from within the cell appear influential. For example, obesity places overload on the endoplasmic reticulum (ER) due to an accumulation of misfolded proteins, lipid oversupply and increased demand on the synthetic machinery [17]. Indeed, in both high fat diet (HFD) and genetic (*ob/ob*) models of murine obesity, indicators of ER stress such as PKR-like kinase (PERK) and eIF2α are significantly phosphorylated in liver extracts from obese versus lean animals [18]. Elevated glucose metabolism can also cause an increase in reactive oxygen species (ROS) in the mitochondria. Interestingly, gene expression analysis has suggested a role for ROS in both TNFα and glucocorticoid models of insulin resistance [19]. Given that both ER and oxidative stress are known to induce inflammatory signaling cascades [17,20], these stressors provide additional means by which obesity might disrupt insulin signaling.

3.2. Inflammatory kinases and the disruption of insulin signaling

Since the inflammatory serine/threonine kinases, c jun amino terminal kinase (JNK), inhibitor of NF-κβ kinase (IKK), and protein kinase C (PKC) disrupt insulin signaling [21–23] blocking their action provides a possible means for therapeutic intervention to treat insulin resistance. In particular, the MAPK kinase JNK has emerged as a possible key regulator of metabolic alterations in insulin sensitivity. Indeed, three lines of evidence highlight this. Firstly, JNK activity is elevated in both dietary and genetic models of obesity [24,25] and deletion of two of the three JNK isoforms, JNK1 [24,26] and JNK2 [26] protects mice from HFD-induced insulin resistance. Rats fed a high fat "western" diet for 30 days showed higher JNK activity in liver, muscle and hypothalamus, compared with controls [25]. In addition, JNK phosphorylation is elevated in liver, muscle and adipose tissues taken from leptin deficient (*ob/ob*) mice, a commonly used genetic model of murine obesity [24]. Secondly, JNK is activated by free fatty acids (FFA), TNFα, ER stress and ROS [17,20,27–29], all of which are known to contribute to insulin resistance. Finally, JNK serine phosphorylates IRS-1 (ser307) which disrupts IRS-1 and IR interaction, the proximal step in insulin signaling [30,31]. Taken together, these findings provide support for the notion that JNK1 inhibition might provide a promising therapeutic avenue for the treatment of T2DM.

4. HSPS AND INFLAMMATION

A key feature of HSPs is their ability to provide cytoprotection. Early experiments demonstrated that if cells were heat treated to 43 °C, (which increased HSP synthesis) the number of cells surviving a subsequent insult of heat shock increased [32]. Once it became understood that HSPs can provide cytoprotection against other stressors, interest in their therapeutic value increased. For example, preheating of human leukemic cells led to reduced cell death following a subsequent heat shock, which was associated with an inhibition of JNK1 and p38 activation [33]. That this effect might be mediated by HSPs was assessed using ectopic over-expression of Hsp72 in the human PEER cell line. Indeed, overexpression of Hsp72 suppressed the apoptotic and stress kinase activating effects of heat, osmotic shock, H_2O_2 and UV irradiation [33]. Experimental evidence suggests several potential mechanisms by which HSP72 can downregulate JNK. Experiments utilizing Hsp72 transfected mouse embryonic fibroblasts, suggested that Hsp72 suppresses the JNK1 signaling pathway through physical association and prevention of JNK1 phosphorylation by its upstream kinase SEK1 [34]. In addition, Daviau et al. demonstrated a role for duel leucine zipper-bearing kinase (DLK) in the mechanism by which HSP72 can down-regulate JNK. DLK is a member of the mixed linage kinase family which are known mitogen activated kinase kinases [35]. DLK is a known upstream activator of JNK. These authors [35] showed in COS-7 cells that HSP72 associates with the HSP co-chaperone CHIP, a known ubiquitin ligase, which can negatively regulate DLK expression and activity. These data suggest that the mechanism by which HSP72 blocks JNK activity is via CHIP mediated DLK ubiquitination. Finally, others [36] demonstrated a role for the upstream phosphatase MAP kinase phosphatase-1 (MKP-1) in HSP72 mediated down-regulation of JNK.

5. HSP72 AND THE PREVENTION OF INSULIN RESISTANCE

Meta-inflammation appears to disrupt insulin signaling and HSPs appear to have the potential to inhibit inflammatory kinases. Therefore, there is a strong rationale to investigate the activation and/or upregulation of HSPs as a means to treat insulin resistance. Interestingly, one preliminary report has suggested that heat therapy in general might have therapeutic potential. T2DM patients using a hot tub daily for three weeks exhibited improvements in glycaemia by unknown

mechanisms [37]. In order to more comprehensively investigate the effects of heat therapy and Hsp72 induction on insulin resistance, a number of experiments were performed [3].

5.1. Heat therapy, JNK1 phosphorylation and insulin sensitivity

Mice were subjected to either heat shock (HT) or sham therapy (control) whilst consuming a HFD. HT involved raising the core temperature to 41 °C for 15 min, once a week, for 16 weeks, which transiently increased Hsp72 expression in muscle, liver and adipose tissue. As expected, in response to the HFD, control mice developed hyperglycaemia, hyperinsulinemia and insulin resistance as indicated by the homeostatic model assessment of insulin resistance (HOMA-IR). Furthermore, intraperotineal glucose tolerance tests (IPGTT) revealed glucose intolerance in these mice. Conversely, mice exposed to HT were protected against insulin resistance, and this protection was associated with an attenuation of JNK1 phosphorylation in muscle. Importantly, this observation has been subsequently confirmed by others [5,6], demonstrating that heat treatment improves glucose tolerance and prevents skeletal muscle insulin resistance and JNK1 phosphorylation in high fat fed rodents [6]. Morino and colleagues demonstrated that a combination of mild electrical stimulation (MET) and HT in high fat-fed and *db*/*db* mice for 10 min applied twice a week for 12 weeks decreased fasting blood glucose and insulin levels and improved insulin sensitivity. These observations were associated with decreased fat mass and a decrease in the phosphorylation of JNK1 [5]. Interestingly this MET protocol also protects pancreatic β-cells from high glucose and ER and oxidative stress [38]. MET increases HSP72 in the pancreas of *db*/*db* mice. When these mice were challenged with a glucose load, an increase in insulin secretion was observed. When compared with the sham treatment control group, levels of insulin, pancreatic duodenal homeobox-1 (PDX-1), GLUT2, and insulin receptor substrate-2 were upregulated in the islets of MET-treated mice, whereas the phosphorylation of JNK, nuclear translocation of forkhead box class O-1 (FOXO1), and nuclear factor-κB p65 were reduced [38]. Apoptotic signals, ER stress, and oxidative stress markers were also attenuated [38]. Together, a summary of the effects of HT and MET are displayed in Figure 1.

5.2. Genetic over-expression of Hsp72 and HFD induced insulin resistance

While heat treatment showed improvements in insulin signaling in a HFD model of insulin resistance, the non-specific nature of heat as a global stressor precludes firm conclusions on the involvement of HSPs. Therefore, transgenic mice, overexpressing Hsp72 in skeletal muscle (Hsp72+/+) were placed on a chow or HFD and compared with wild type (WT) controls to determine the specific effects of Hsp72 expression on diet induced insulin resistance. In keeping with the heat therapy data, the development of hyperglycaemia, hyperinsulinemia, insulin resistance and glucose intolerance was prevented in Hsp72+/+ mice as opposed to the WT controls. Given the role of inflammatory kinases in the disruption of insulin signaling, JNK1 and IKK phosphorylation was assessed in Hsp72+/+ and WT mice. While neither the diet nor treatment altered IKKαβ serine phosphorylation, JNK (Thr^{183}/Tyr^{185}) phosphorylation was increased in WT mice following the HFD. Again, in keeping with the hypothesis, JNK1 phosphorylation was completely prevented in Hsp72+/+ mice. Furthermore, when stimulated with insulin, AKT phosphorylation was elevated in Hsp72+/+ but not WT mice following the HFD (see Figure 2). These data indicated, therefore,

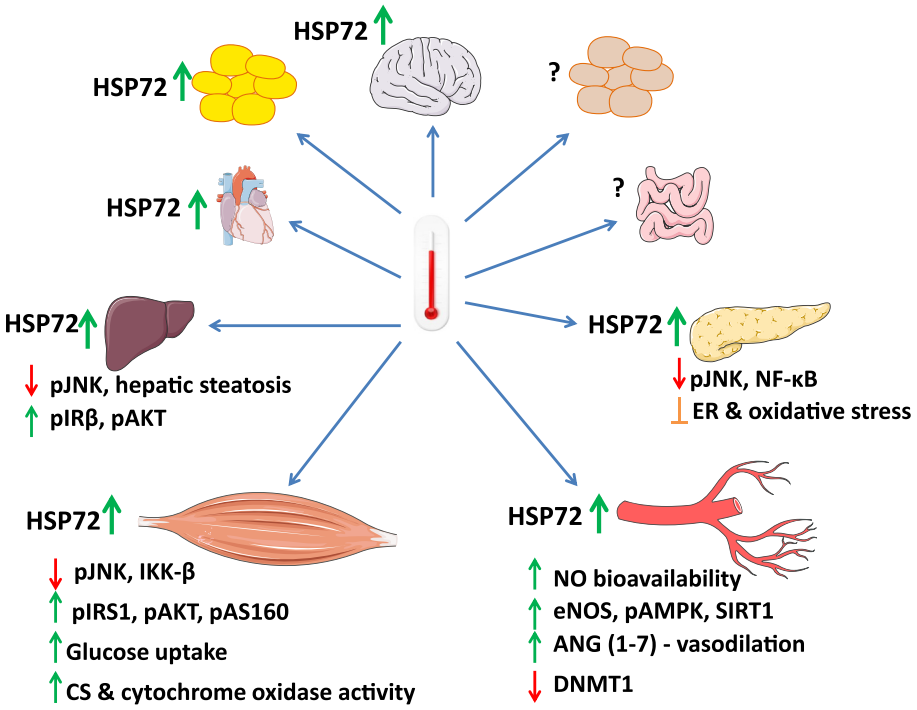

Figure 1: Impact of whole body heat shock treatment (HT) or HT plus mild electric stimulation (MET) on various organs relating to whole body metabolism in mouse models of insulin resistance or T2DM. HT typically involves acutely warming rodents to 41−42 °C acutely once per week. While it is known the Hsp72 is elevated in response to heat in white adipose tissue, the heart and brain, these organs have not been investigated thoroughly in regards to metabolism. We are not aware of reports demonstrating increases in Hsp72 brown adipose tissue and the gut with such a treatment protocol. The effects of HT on the liver, pancreas, skeletal muscle and the vasculature have been well described. The heat shock response involves the attenuation of inflammatory markers (JNK, IKK-β and NF-κB) in multiple organs, improvements in insulin stimulated signaling in muscle and liver and restoration of impaired vasodilation in vessels (see Refs. [3,5,6,38,96]).

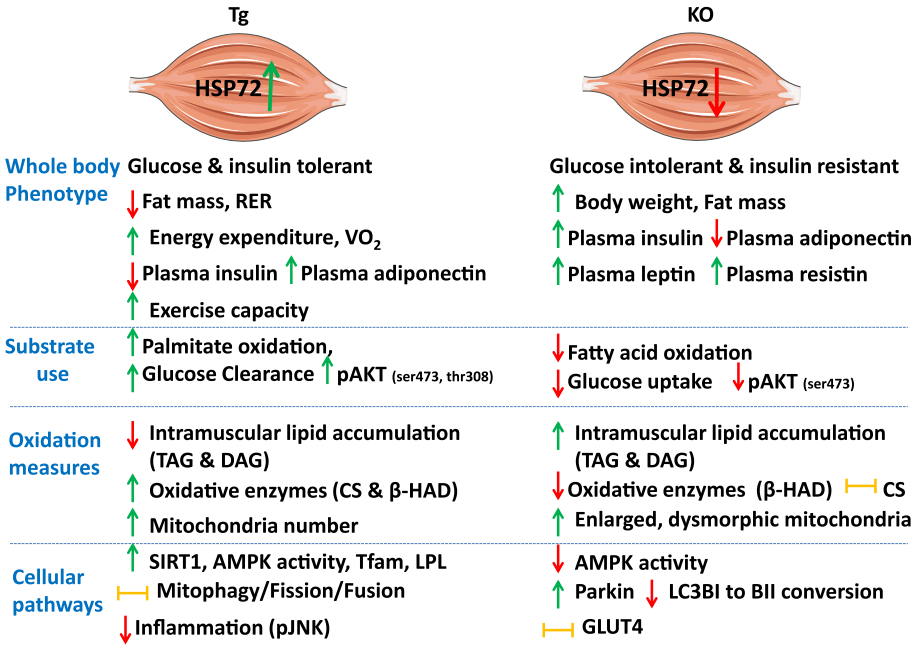

Figure 2: Comparison of mouse models where Hsp72 has been transgenically overexpressed or knocked out focusing specifically on the skeletal muscle. In many respects the opposite phenotype is observed between the two models. While Hsp72 transgenic mice are protected against obesity-induced insulin resistance, Hsp72 knockout mice are heavier and insulin resistant on a normal chow diet at 7 months of age. While indicators of oxidative capacity such as endurance capacity, β-HAD activity, mitochondria number and skeletal muscle lipid oxidation are increased in the transgenics, many of these parameters are decreased or impaired in the knock-outs. Further, while overexpression of Hsp72 resulted in a decrease in HFD intramusclular TAG and DAG lipid accumulation, the lipid levels of this class of lipids were increased in the Hsp72 knock-outs (see Refs. [3,8,83]).

that overexpression of Hsp72 inhibited fatty acid disrupted insulin signaling, through the inhibition of the JNK1 pathway of inflammation.

5.3. Pharmacological induction of Hsp72 with BGP-15

Given the significance of the presented findings, it is important from a therapeutic point of view, to determine ways in which Hsp72 can be induced. In this regard, hydroxylamine derivatives are thought to stimulate HSP expression by prolonging activation of HSF1 [39] and alteration of membrane lipid microdomains [40]. Indeed, recent studies demonstrated that the hydroxylamine derivative, BGP-15 was able to remodel cholesterol-enriched lipid platforms and that enhanced membrane fluidization was necessary for transmembrane signaling to proceed upon BGP-15 treatment [41]. The therapeutic utility of BGP-15 has been determined in a well known model of obesity and diabetes, the *ob*/*ob* mouse. Mice treated with BGP-15 by oral gavage demonstrated a significant increase in intramuscular Hsp72 compared with mice receiving control treatment (saline). In support of previous findings, the increased Hsp72 expression was associated with decreased activation of JNK1 phosphorylation. Furthermore, BGP-15 treated mice presented with improved fasting glucose and insulin concentrations than control mice and a hyperinsulinemic euglycaemic clamp revealed markedly improved glucose disposal rate in the pharmacologically treated mice. These data demonstrate that BGP-15 is able to induce heightened expression of Hsp72 in genetic models of obesity and that this increased protection is associated with improved glycaemia and insulin signaling via suppression of JNK1 activation. Importantly BGP-15 has been demonstrated to be effective in humans. In a cohort of 47 insulin resistant non-diabetic patients BGP-15 administered orally for 28 days was shown to improve whole body glucose disposal during a hyperinsulinemic euglycaemic clamp. No adverse drug effects were observed during treatment and the BGP-15 was reported as safe and well tolerated [4]. Recently, results indicate that BGP-15 inhibits multiple metabolic side effects of atypical antipsychotics, and this effect is likely to be related to its HSP co-inducing ability [9]. By way of further support for the pharmacological manipulation of Hsp72 expression, Acylic Polyisoprenoid Derivative Geranylgeranylacetone (GGA), known primarily as an anti-ulcer drug, was found to be protective against visceral adiposity and insulin resistance in high fat fed mice [42]. Four weeks of GGA administration increased Hsp72 in the liver as this treatment resulted in improved insulin sensitivity and glucose homeostasis upon glucose challenge. In line with the findings using BGP-15, activation of JNK1 was attenuated and insulin signaling was improved in the livers of high fat fed mice. These data suggest that induction of liver Hsp72 by BGP-15 or GGA is beneficial in protecting against a high fat diet in a rodent model. Encouragingly these findings were replicated in spontaneously diabetic primates, demonstrating an 85% improvement in glucose tolerance and a 42% lower and improved HOMA index for insulin resistance [43].

5.4. Are the protective effects of activation of Hsp72 in metabolic disease dependent upon blocking JNK activation?

While numerous data support the proposed role of JNK phosphorylation in the development of diet induced insulin resistance, contradictory data also exist. For example, electroporation of a constitutively active construct of JNK (CaJNK) decreased glucose clearance into the tibialis anterior muscle, but expression of a wildtype (WT-JNK) construct had no impact [44]. In a similar study, expression of constitutively active construct of JNK in skeletal muscle of mice had no effect on energy homeostasis and glucose metabolism [45]. Furthermore, since our original experimental interventions [3], we have observed no change in JNK phosphorylation in response to a shorter 10 week HFD, comprised of a lower percentage of fat, despite the development of insulin resistance [8]. Since BGP-15 administration improved insulin

sensitivity, investigation of inflammatory independent, alternative mechanisms by which Hsp72 impacts on insulin action were warranted.

5.5. Hsp72 and potential tissue crosstalk

While high level Hsp72 overexpression in the skeletal muscle of the transgenic mice provided numerous benefits to the muscle itself, analysis of other tissues from these mice suggests that they also benefited. An increase in insulin-stimulated glucose clearance into both white and brown adipose tissue was observed, even though Hsp72 was not overexpressed in these peripheral tissues [8]. Interestingly, further analysis of the white adipose tissue also revealed increased rates of lipolysis and maximal activities of the oxidative enzymes CS and β-hydroxyacyl CoA dehydrogenase (β-HAD) [8]. Recent observations suggest a similar phenomenon in the liver. High level Hsp72 expression in the skeletal muscle was associated with increased CS and β-HAD activity in the liver, while lipidomic screening via mass spectrometry revealed that although WT mice develop hepatic steatosis with high fat feeding as evidenced by significantly elevated Triacylglycerols (TAGs) and Diacylglycerols (DAGs), Hsp72+/+ are completely refractory to this occurring (Henstridge and colleagues, unpublished observations). Thus, the transgenic overexpressing of Hsp72, resulting in a ramping up of oxidative capacity or blocking of inflammation may exert a protective effect on other peripheral tissues (possible mechanisms depicted in Figure 3). In many respects, this is similar in nature to exercise training improving hepatic steatosis in humans or rodents independently of any weight loss [46–48], although it should be noted that exercise training can decrease fat mass while correspondingly increase lean mass resulting in no net change in body mass but improved body composition [49]. While the skeletal muscle is the organ manipulated (contraction with exercise, Hsp72 overexpression in Hsp72+/+) another independent organ benefits. Given these findings it is an interesting notion to consider whether targeting the skeletal muscle is a viable option to treat fatty liver disease. Alternatively, the possibility exists that Hsp72 may be eliciting some type of tissue cross talk, whereby a secreted substance from the muscle, termed a "myokine" (for review see Ref. [50]), may then act on other tissues. Hsp72 is secreted from cells independently of the common secretory pathway and instead via an exosome dependent mechanism [51]. There is growing evidence linking the expression of Hsp to changes in the lipid composition and architecture of plasma membranes [41], and it has been suggested that Hsps complex with caveolin to be released from plasma membranes via lipid rafts (for review see Ref. [52]). Recently Uyy et al. [53], demonstrated that detergent resistant membranes isolated from mice fed a high fat diet contained Hsps and caveolin-1 which positively correlated with Hsp secretion into the plasma. Together, these data suggest that Hsps are secreted from cells in a regulatory manner and give rise the possibility that Hsp72 may yet be another myokine capable of tissue cross talk. Indeed, Hsp72 is known to be released into the systemic circulation upon physical activity but it is unlikely derived from skeletal muscle (reviewed in Ref. [54]). However, it is unknown whether high level overexpression, such as that observed in the Hsp72+/+ mice can elicit such an effect.

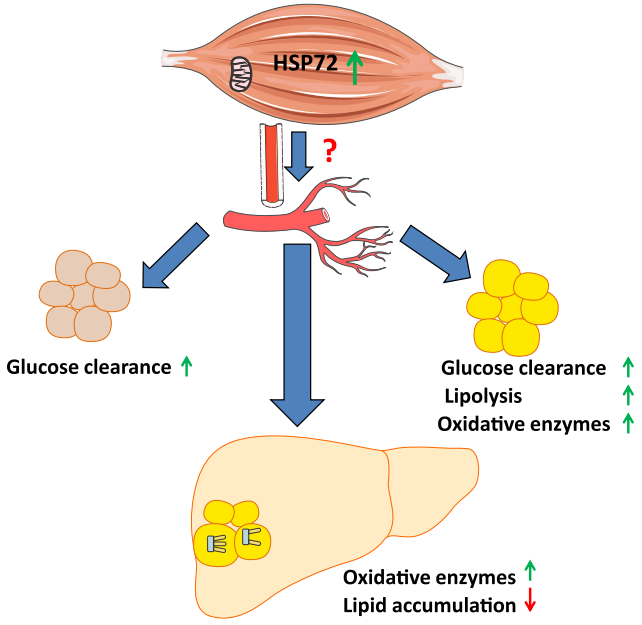

Figure 3: Schematic representation of the effects of skeletal muscle Hsp72 overexpression on other insulin sensitive peripheral tissues. Alterations include an increase rate of glucose clearance into both BAT and WAT, an elevation in oxidative enzymes in WAT and liver, increased lipolysis in WAT and a decrease in hepatic steatosis (decrease in the TAG and DAG lipid species). These observations could potentially be explained by the ramping up of oxidative capacity in the myocytes acting in a protective manner. Increases in oxidative capacity in the muscle may lead to an increased siphoning of fatty acids through this tissue, shielding the other organs from their deleterious effects. Another theory could be that a currently unknown secreted factor (myokine) is released from the skeletal muscle bed that then exerts effects on these other organs (see Ref. [8]).

6. OBESITY, MITOCHONDRIAL FUNCTION AND INSULIN RESISTANCE

6.1. Mitochondrial dysfunction in obesity induced insulin resistance: cause, consequence or other?

A great deal of research over the past few decades has investigated whether obesity and insulin resistance are linked to a defect in the energy producing organelle, the mitochondria. While it is still controversial as to exactly how mitochondrial stress impacts insulin action in muscle, whether mitochondrial dysfunction is a cause or consequence of insulin resistance, or whether mitochondrial dysfunction exists at all in parallel with insulin resistance (these issues reviewed in Refs. [55–61]), there is the distinct possibility of harnessing the function of the mitochondria to treat obesity and insulin resistance. Some of the lines of evidence suggesting insulin resistance is associated with defective oxidative metabolism in skeletal muscle are now described.

6.1.1. Mitochondrial dysfunction in metabolically impaired humans

Peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) has become known as the master regulator of mitochondrial biogenesis/oxidative metabolism due to its role as a transcription factor in regulating mitochondria biogenesis. Increased PGC-1α leads to up-regulation of target genes such as nuclear respiratory factor 1 (Nrf-1) which can, in turn, stimulate the expression of OXPHOS genes and mitochondrial transcription factor A (Tfam), a mitochondrial matrix protein essential for transcription of mitochondrial DNA [62]. A reduction in the expression of genes encoded by PGC-1α is observed in skeletal muscle of patients with T2DM [63,64] and healthy individuals with a family history of diabetes [64]. Skeletal muscle mitochondrial function may play a role, therefore, in the pathogenesis of T2DM. Skeletal muscle of insulin-resistant offspring of patients with T2DM is associated with dysregulated fatty acid metabolism, possibly due to an inherited defect in mitochondrial oxidative phosphorylation [65]. Moreover, mitochondrial oxidative activity in healthy,

lean, elderly volunteers with severe muscle insulin resistance is reduced compared with BMI and activity matched younger control subjects and this is associated with increased intramyocellular and hepatic lipid content [66]. This is suggestive of a predisposition to store lipid, leading to insulin resistance through mitochondrial dysfunction in the elderly [66].

6.1.2. Evidence from animal models and patients with mitochondrial mutations

Animal models suggest increasing oxidative metabolism may be beneficial in the setting of insulin resistance. Activation of key proteins and/or pathways involving AMP-activated protein kinase (AMPK) [67], Peroxisome proliferator-activated receptor delta (PPARδ) [68], sirtuin 1 (SIRT)1 [69] and carnitine palmitoyltransferase-1 (CPT1) [70], increase fatty acid oxidation (FAO). This has the downstream effect of decreasing lipid esterification and, in doing so, ameliorates insulin resistance. Such interventions would counteract any deficiencies in mitochondrial oxidative function that may be present in the diseased state. A caveat to such thought is the fact that high fat feeding in rodents which causes insulin resistance has been known to increase, rather than impair, the ability of the muscle bed to oxidize lipids [71]. However, it should be noted that this increase is insufficient to account for the increase in lipid uptake and as a consequence lipid esterification increases, despite the increase in lipid oxidation [70]. Metabolic studies on patients with mitochondrial myopathies caused by genetic mutation provide a link between disruption to the mitochondria and glucose homeostasis and insulin action. A genetic point mutation affecting position 3243 in the tRNA leucine mitochondrial gene results in decreased insulin sensitivity in patients with this mutation [72] and maternally transmitted T2DM [73]. In addition, patients with one of the more common types of mitochondrial diseases, Chronic Progressive External Ophthalmoplegia, have impaired glucose tolerance [74].

6.1.3. Mitochondrial shape, ROS and supply and demand

Another important consideration is the architecture of the mitochondria. If mitochondria lose their shape, their dynamics and health can change. This can result in alterations to mitophagy (the autophagic breakdown of mitochondria). Either a decrease or increase in rates of mitophagy may impact a cell negatively. Indeed, long term consumption of a HFD in mice leads to changes in the appearance of mitochondria in that they become swollen [75]. The generation of ROS, which can be associated with dysfunctional mitochondria, also leads to insulin resistance [19,76]. ROS production and ROS signaling is, therefore, another manner in which mitochondria function/dysfunction may be associated with nutrient stress in the obese or insulin resistant state. Indeed, it has been demonstrated that in skeletal muscle of both rodents and humans, a HFD increases the H_2O_2-emitting potential of mitochondria which, in turn, shifts the cellular redox environment to a more oxidized state, thereby decreasing the redox-buffering capacity. Intriguingly, this occurs without any change in mitochondrial respiratory function [77]. This line of thinking suggests skeletal muscle insulin resistance is related to redox pressures that are placed on the respiratory system when energy supply outpaces energy demand [55].

Although controversy will continue to persist as to the exact nature of the mitochondria's involvement in the obese and/or insulin resistant state, targeting of the mitochondria for therapeutics will need to take into consideration the ability of the mitochondria to oxidize and, therefore, remove lipid from the cell, the continual maintenance of normal mitochondrial architecture and the nutrient supply and demand equation.

7. HSP72, MITOCHONDRIA AND INSULIN RESISTANCE

7.1. Associations between Hsp72 and mitochondrial biology

Since mitochondrial dysfunction is associated with insulin resistance and Hsp72 is known to protect cardiac muscle against mitochondrial damage caused by ischemia reperfusion injury [78], that Hsp72 might alter mitochondrial function during nutrient excess became of interest. Significantly, heat therapy increases both mitochondrial enzyme activity and exercise endurance capacity in rats [79] along with mitochondrial biogenesis in C2C12 myocytes [7] and oxygen consumption in L6 muscle cells [6]. From a correlative perspective Hsp72, has been linked to oxidative potential due to its abundance in certain skeletal muscles. Indeed, Hsp72 expression is correlated with skeletal muscle oxidative capacity with more oxidative fiber types expressing higher levels of Hsp72 [80]. Hsp72 is expressed at higher levels in the more slow-twitch oxidative *soleus* skeletal muscle than the *epitrochlearis* muscle which is more of a mixed fiber muscle [81]. Similar to the findings with Hsp72 expression, phosphorylation of Hsp25 was also significantly higher in the more oxidative *soleus* muscle [81]. Furthermore, a significant positive correlation between the mRNA expression of Hsp72 and mitochondrial enzyme activity has been observed in human skeletal muscle [12]. It is important to note the observation of smaller fat pads in HSP72+/+ mice compared with WT mice, even though the daily food intake was the same when comparing strains.

7.2. Genetic over-expression of Hsp72 and mitochondria

The oxidative capacity in skeletal muscle of WT and HSP72+/+ mice was examined by measuring the maximal activities of two important mitochondrial enzymes, citrate synthase (CS) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD). Interestingly, the maximal activities of these enzymes was higher in HSP72+/+ compared with WT mice [3]. These data suggested that Hsp72 increases the fatty acid oxidative capacity in skeletal muscle, which may account for the protection against increases in body weight and resultant insulin resistance. By way of a more thorough investigation, we recently examined transgenic Hsp72 (HSP72+/+) and control (WT) mice fed a regular chow (chow) or high fat diet (45%) (HFD) for 10 weeks. The duration and composition of the HFD was chosen specifically so that JNK was not activated in skeletal muscle and therefore any changes observed were independent of JNK. While the HFD markedly increased body weight, epididymal fat pad mass and intramuscular lipid accumulation and induced insulin resistance in WT, no such effects were seen in Hsp72+/+ mice. Despite equivalent food intake, whole body oxygen consumption, fatty acid oxidation and oxidative enzyme activity in skeletal muscle were increased in Hsp72+/+ irrespective of diet. Furthermore, when subjected to an endurance exercise treadmill test, Hsp72+/+ mice displayed a 2-fold increase in running capacity relative to WT mice. Consistent with this oxidative phenotype, HSP72Tg mice exhibited a 50% increase in mitochondria and a significant increase in Tfam mRNA expression, a gene important for stimulation of mitochondrial biogenesis [8]. To further characterize these tissues we measured the activity of AMP-activated protein kinase (AMPK), a sensor of cellular energy status, critical for enhancing fuel utilization and metabolism. Indeed, AMPK phosphorylation (at the Thr^{172} site) and activity levels were enhanced in the muscles from HSP72+/+ relative to WT mice, via mechanisms not yet elucidated. Another important fuel-sensing molecule is the NAD^+-dependent deacetylase Sirtuin 1 (SIRT1). SIRT1 is an important regulator of oxidative metabolism and a detailed review of its metabolic actions in various mouse models has recently been described in Ref. [82]. The protein expression of SIRT1 was also

significantly increased in the skeletal muscle of HSP72+/+. Whether SIRT1 is the mechanism behind the increased oxidative capacity with HSP72 overexpression is yet to be fully elucidated. A summary of the Hsp72+/+ phenotype is illustrated in Figure 2.

7.3. Genetic deletion of Hsp72 and mitochondria

In contrast, Hsp72 knockout ($Hsp72^{-/-}$) (global null mutation of *Hspa1a*/*Hspa1b* genes) mice develop obesity and insulin resistance and display marked accumulation of lipid in skeletal muscle (see Figure 2 for summary). In addition, oxygen uptake and fatty acid oxidation rates were lower, while fatty acid esterification rates higher in primary myocytes obtained from $HSP72^{-/-}$ mice compared with WT [83] The hypothesis that Hsp72 stimulates fat oxidation with a consequent reduction in fat storage and adiposity gained further support in a clinical study, which observed lower expression of Hsp72 protein in human skeletal muscle associated with increased adiposity and decreased insulin sensitivity in healthy individuals [84]

7.4. Other HSPs?

While this review has largely focused on Hsp72, it is of note that other HSP families may also be fundamental in mitochondrial homeostasis and maintenance of insulin sensitivity. For example, Kleinridders and colleagues [85] used a murine model of T2DM, and demonstrated hypothalamic insulin resistance and mitochondrial dysfunction due to downregulation of the mitochondrial chaperone HSP60. HSP60 reduction in obese, diabetic mice was due to a disruption of leptin signaling and was restored by leptin treatment. *In vitro*, knockdown of *Hsp60* in a mouse hypothalamic cell line mimicked the mitochondrial dysfunction observed in diabetic mice [85]. The small HSP, HSP25 has also been implicated in the etiology of metabolic disease. Consistent with the pre-clinical data for HSP72, HSP25 protein expression is also reduced in the skeletal muscle of aged, insulin resistance rats [81]. Adipose tissue biopsies comparing lean versus obese patients determined that the DNAJB3 cochaperone (a member of the HSP40 family) mRNA and protein levels were decreased in the obese patients [86]. Interestingly DNAJB levels positively correlated with maximum oxygen consumption in these patients and exercise training restored the expression of DNAJB3 in obese subjects with a concomitant decrease of phosphorylated JNK [86]. Somewhat paradoxically, work from the same group, using a similar model demonstrated increased expression of HSP60, HSP90 and GRP-94 in obese adipose tissue [87].

7.5. Small molecule activators of Hsp72 and mitochondrial physiology

Since we have shown that Hsp72 expression can be manipulated pharmacologically, what evidence is there that activators of HSP expression can alter mitochondrial physiology? A recent study investigating diabetic peripheral neuropathy (DPN) in both type 1 and type 2 diabetes mouse models demonstrated that the small molecule Hsp72 modulating compound KU-32 improved mitochondrial bioenergetics and decreased DPN [88]. KU-32 is a novel, novobiocin-based Hsp90 inhibitor. Upon exposure to Hsp90 inhibitors, heat shock factor 1 dissociates from Hsp90, translocates to the nucleus, and upregulates a heat shock response that promotes synthesis of chaperones, such as Hsp72 [88]. Importantly from a mechanistic stand point, the improved mitochondrial function with KU-32 was dependent on Hsp70 as when the drug was administered to $Hsp70^{-/-}$ mice it was no longer effective [88]. There is evidence to suggest that BGP-15 may also effective at the mitochondrial level. Studies investigating BGP-15 treatment on mitochondrial density in skeletal muscles from Goto-Kakizaki (GK) rats, a non-obese Wistar substrain which develops T2DM early in life [89], revealed an increase in the relative mitochondrial area which was associated with improved glucose infusion rates during a clamp [8]. Interestingly, while BGP-15 is an Hsp72 co-activator, it may be a multi-target agent. Studies investigating the cardioprotective effect of BGP-15 have demonstrated it to be a poly(ADP-ribose)polymerase (PARP-1) enzyme inhibitor. This inhibition of PARP-1 activation leads to protection of the mitochondria from oxidative damage under the condition of ischemia-reperfusion in a rat heart model [90,91] and like the overexpression of Hsp72, leads to a reduction in the phosphorylation of JNK [92]. Recently, a clear role of PARP-1 inhibition in metabolic health was described by Auwerx and colleagues when they demonstrated that PARP inhibition improves fitness and mitochondrial function in skeletal muscle [93]. In experiments involving worms, mice and humans, the investigators were able to establish that PARP inhibition can offset genetic and acquired defective mitochondrial function. In summation of this work, PARP-1 expression negatively correlated with energy expenditure in a heterogeneous mouse population (BXD mouse genetic population). Inhibiting PARP pharmacologically was able to enhance energy expenditure, SIRT1 activity, Tfam expression, CS activity, endurance exercise capacity and mitochondrial function while improving oxidative capacity in models of reduced mitochondrial function [93]. The contribution of SIRT1 in mediating these effects were studied by repeating these experiments in muscle-specific SIRT1 deficient mice, where PARP inhibition no longer enhanced endurance capacity, respiratory capacity or CS activity [93]. As ablation of PARP-1 increases NAD^+ availability [94] and NAD^+ plays an important role in controlling metabolic health via a SIRT1 dependent mechanism [95], it seems likely that SIRT1 is a key component of this attractive potential therapeutic pathway. Intriguingly, heat treatment, which increases Hsp72 also increases SIRT1 expression in muscle cells [7] and in the vasculature of rats [96]. Furthermore, it is also increased in skeletal muscle of HSP72+/+ mice [8] suggesting similar pathways may be involved. Investigation of the effect of compounds such as BGP-15 and GGA on SIRT1 pathways are yet to be carried out but are clearly warranted.

7.6. Hsp72 and mitophagy

Since HSPs most prominent annotation is cell protection and insulin resistance and has been associated with mitochondrial dysfunction, we have considered the possible influence of Hsp72 on the fundamental preservation of this organelle during nutrient oversupply. Indeed, by manipulating HSF-1, the central regulator of Hsp72, Dokladny et al. [97] recently demonstrated a primary role of Hsp72 in autophagy, the vital process of digestion of unnecessary or dysfunctional cellular components. Significantly, in HSP72 KO mice (which lack *hspa1a* and *hspa1b* genes), loss of Hsp72 causes enlarged and damaged mitochondria which is paralleled by skeletal muscle insulin resistance and increased adiposity [83]. These series of studies were able to show that Hsp72 is a critical regulator of stress-induced mitochondrial turnover as Parkin, (an E3 ubiquitin ligase known to regulate mitophagy), was unable to ubiquitinate and regulate its own protein expression or the protein level of its central target mitofusin 2 (Mfn2) in the absence of Hsp72. Further, it was demonstrated that Hsp72 rapidly translocates to damaged mitochondria prior to Parkin recruitment and immunoprecipitates with both Parkin and Mfn2 only after specific mitochondrial insult. This data implicates Hsp72 as a mitochondrial stress sensor which is critical for the maintenance of mitochondrial and metabolic homeostasis [83]. The importance of this functional interaction between Hsp72 and Parkin was highlighted by publications from two other groups outlining similar findings from large screening based discovery platform studies [98,99]. Specific, detailed future studies are warranted to further study the parkin/Hsp72 interaction and its role in not only mitochondrial health but insulin sensitivity.

8. HSP72 AND THE VASCULATURE

8.1. Vascular defects, complications and insulin resistance

In what is probably an under-examined area of the field, it has become increasingly recognized that vascular defects (especially in the microvasculature) can contribute to insulin resistance in muscle (and perhaps other tissues such as adipose tissue and the liver). The underlying consequence of any insulin resistance induced vascular defect is impaired delivery of insulin and/or glucose to the skeletal muscle bed, which leads to insulin resistance [100]. Insulin plays an important role in the regulation of dilation of blood vessels via stimulation of nitric oxide (NO) [101]. As NO release is a critical component of normal vascular biology, any impact on this process can have consequences on whole body hemodynamic function. Indeed, essential hypertension is associated with lower NO levels [102], and decreased NO release has been observed in the setting of atherosclerosis [103]. Impairments in the vasculature can, therefore, be both a contributing factor to the development of insulin resistance and contribute to the progression of vessel disease in metabolic disease. These are important considerations given the close association between metabolic health and cardiovascular complications.

8.2. Hsp72 and insulin-resistance induced endothelial dysfunction

A recent study has now linked Hsp72 to the vascular complications associated with high fat feeding induced insulin resistance [96] (Figure 1). Karpe and Tikoo made the observation that Hsp72 expression is reduced in the aortas of HFD-fed rats and that a heat shock treatment (HT) regime could restore the Hsp72 expression back to normal levels [96]. The renin–angiotensin system (RAS) is the predominant hormone system that regulates blood pressure and importantly, from a functional perspective, while insulin resistance appears to impair the angiotensin (ANG) ANG1-7 induced vasodilator response, the induction of Hsp72 attenuates this impairment [96]. ANG-(1–7) opposes the actions of ANG II (a potent vaso-constrictor) and therefore pushes the system towards a state of vasodilation. The improvement in ANG(1–7) induced vasodilator response coincided with multiple changes in signaling events. While HFD fed rats showed attenuated endothelial nitric oxide synthase (eNOS) phosphorylation, AMPK phosphorylation and SIRT1 expression, the HT prevented the attenuation in these signaling pathways [96]. Further, implicating eNOS and SIRT1 in this pathway, inhibition of these proteins prevented the benefits of HT. Mechanistically, this is quite similar to the findings from the Hsp72+/+ mice whereby SIRT1 protein levels, AMPK phosphorylation and AMPK activity levels were increased in the skeletal muscle of the transgenic mice [8].

The induction of Hsp72 protein could, therefore, also be an approach to prevent insulin-resistance-induced vascular complications. Deciphering exactly the percentage contribution of oral agents such as BGP-15, GGA or new generation Hsp72-raising agents on improving whole body metabolism via direct impact on the skeletal muscle or improvements in the vasculature would be a difficult task, but an interesting line of scientific enquiry. From a patient's perspective, the precise mode of action it is probably inconsequential as long as they have access to a safe and effective treatment.

9. HSP72 AND AGING

While many physiological factors contribute to aging, a decline in mitochondrial function, quality or content has been demonstrated to play a key role in the aging process and is implicated in a number of age-related disorders such as, but not limited to obesity, insulin resistance, T2DM, cancer, cardiovascular disease and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. It has been shown that many tissues including skeletal muscle from aged individuals have lower respiratory function compared with those from younger individuals [104–106]. Hsp72 expression (along with phosphorylation of Hsp25) is reduced in both slow-twitch oxidative skeletal muscle (*soleus*) and mixed muscle (*epitrochlearis*) with increasing age (3-month-old versus 24-month-old rats) [81]. This decrease in HSP expression corresponded to a decline in 2-deoxyglucose uptake and insulin signaling in the muscle with age [81]. As summarized in this review, Hsp72 plays an important role in skeletal muscle mitochondrial dynamics and, as such, Hsp72 elevation may assist in maintenance of mitochondrial function in the face of functional decline with aging. Indeed, studies in both mammals and birds have revealed that Hsp70 levels in different tissues from various organs are positively correlated with lifespan [107]. Highlighting the Hsp72/aging interaction, a recent longitudinal study in non-human primates demonstrated that low baseline levels of skeletal muscle Hsp70 was a marker for developing insulin resistance within the four year study period, while a positive change in skeletal muscle Hsp70 levels was beneficial in protecting deterioration of insulin sensitivity [108]. If it can be demonstrated that Hsp72 induction is efficacious in improving various aspects and types of mitochondrial dysfunction, targeted manipulation of Hsp72 with activating compounds may have clinical utility in a number of mitochondrial related disease states including but not limited to human aging and T2DM.

10. CHEMICAL CHAPERONES

10.1. Protein folding and chaperones

Protein misfolding and aggregation are associated with a number of human diseases. Misfolded proteins can aggregate and impair signal transduction pathways or cause cell toxicity. While mutations are a common cause of misfolded proteins, they are not an absolute requirement for misfolding to occur. Protein folding involves the participation of accessory components (molecular chaperones) of which Hsp72 is one such component. The ability of chaperones to discriminate between properly folded and misfolded proteins has led to the thought process that chaperones provide the cell with a type of quality control system, recognizing, retaining and targeting misfolded proteins for degradation [109]. Molecular chaperones participate, therefore, in both the folding process of proteins and as an important component in targeting misfolded proteins to degradation.

Chemical chaperones (or pharmaceutical chaperones) are small molecules that stabilize the folding of proteins, provide improvements to protein folding and buffer abnormal protein aggregation. Like the protein molecular chaperones, the different chemical chaperones influence the rate of fidelity of the folding reaction, probably by stabilizing the properly folded form of the polypeptide [109]. Given the fundamental parallels in function between molecular protein chaperones like Hsp72 and their pharmaceutical equivalents, it is prudent to consider the therapeutic potential of these compounds.

10.2. Endogenous chaperones and insulin resistance

As mentioned earlier, ER stress has been implicated in obesity-induced insulin resistance and the impact of an increase in ER stress has largely been linked to the activation or hyperactivation of JNK. Some of the initial evidence linking chaperones and insulin resistance comes from studies which genetically altered the inducible endogenous ER chaperone protein, oxygen regulated protein 150 (ORP150). When this local ER chaperone is decreased, the resultant phenotype is insulin

resistance; while conversely, overexpression of ORP150 in mice that are obese improves insulin tolerance [110]. Furthermore, ORP150 overexpression, specifically in the liver, markedly reduces insulin resistance and ameliorates glucose intolerance in obese diabetic mice [111]. These data provided evidence, therefore, that alterations in local chaperone expression can impact on whole body insulin sensitivity.

10.3. Chemical chaperones and insulin resistance

Importantly, small molecule chemical chaperones are effective in alleviating obesity-induced ER stress and ameliorating insulin resistance and diabetes in mice [18] Ozcan and colleagues tested two types of chemical chaperones, 4-phenyl butyric acid (PBA or 4-PBA), which is a low molecular weight compound and taurine-conjugated ursodeoxycholic acid (TUDCA), an endogenous bile acid derivative. Both of these treatments had been demonstrated to modulate ER function and in this study, demonstrated strong capacity to alleviate ER stress in both cultured cells and in *in vivo* animal studies [18]. Administration of these compounds orally to a genetic model of obesity and insulin resistance (the *ob*/*ob* mouse) resulted in normalization of blood glucose levels and improvements in insulin sensitivity within a week of treatment [18]. Analysis of the organs from these mice (predominantly liver and adipose tissue) revealed that the chemical chaperone administration resulted in near complete reversal of the obesity-induced JNK activation and recovered the defective insulin receptor signaling associated with the obese condition [18]. The effect on insulin signaling was further characterized by Zhou and colleagues when they demonstrated that reductions in ER-stress by TUDCA, rescued obesity-induced insulin receptor (IR) down-regulation and insulin resistance both in *in vivo* and *in vitro* settings [112]. To complement these findings, a more recent study investigating proteasomal dysfunction as a mediator of obesity-induced ER stress and insulin resistance in the liver demonstrated that PBA administration almost completely alleviated proteasome dysfunction mediated insulin resistance [113].

While chemical chaperone enhancement has been quite thoroughly investigated in the liver and adipose tissue, not as much attention has been paid to the skeletal muscle. ER stress and the activation of the unfolded protein response (UPR) to maintain ER homeostasis under such conditions has been reported to occur in skeletal muscle [114,115]. However, dampening of ER stress signaling markers using TUDCA or PBA (or genetically by the overexpression of glucose response protein 78 Grp78), does not protect myotubes from fatty acid (palmitate) induced alterations of insulin signaling [116]. This would suggest that there may be tissue specific effects of chemical chaperone treatment or that there is dissociation between improvements in ER stress and corresponding effects on insulin signaling in skeletal muscle. This is an important consideration given the importance of skeletal muscle in insulin-stimulated whole body glucose disposal.

10.4. Chemical chaperones in the clinic

From a clinical perspective, PBA has been approved for human use and has been used in trials for multiple diseases [117,118] while TUDCA is the taurine conjugate form of ursodeoxycholic acid (UDCA), used to treat primary biliary cirrhosis [119,120] Given that both PBA and TUDCA have been safely used in humans, the potential exists for testing these or similar compounds in proof of principle trials for T2DM. To our knowledge one such trial has taken place with some degree of success. TUDCA treatment for four weeks (1750 mg/day) in obese, insulin resistant human patients increased both hepatic and muscle insulin sensitivity by 30% with no change in adipose tissue insulin sensitivity [121]. This improvement in insulin sensitivity was associated with increased muscle insulin signaling (phosphorylated insulin receptor substrate (Tyr) and Akt (Ser473) levels). Interestingly, markers of ER stress in muscle or adipose tissue did not change after treatment with either TUDCA or placebo indicating that the improvement in whole body insulin sensitivity was independent of improved ER stress in these tissues [121]. It is plausible that ER stress was improved specifically in the liver but this was unable to be determined due to the invasive nature of such testing. Further, chronic, blinded placebo controlled trials will be needed to decipher whether chemical chaperones targeting the ER stress pathway is a bone fide therapeutic for T2DM patients.

There is a gap in our understanding regarding the impact of chemical chaperones on organelles other than the ER. For example, how the chemical chaperones influence the mitochondria is largely unknown. Future studies directed at examining mitochondrial specific chemical chaperones may prove useful in identifying novel targets for metabolic conditions.

11. IN SUMMARY

Diabetes and obesity are of a major public health concern. We have summarized here proposed roles of Hsp72 in altering known pathways to insulin resistance. Regardless of the method used to overexpress Hsp72, heat treatment, genetic and pharmacological manipulation of this protein results in improved measures of insulin sensitivity in both high fat diet and genetic models of rodent obesity. Furthermore, these improvements appear to be tightly linked with a reduction in JNK1 phosphorylation and/or an increase in oxidative capacity consequential of improvements in mitochondrial homeostasis. Targeted manipulation of Hsp72 with BGP-15 (or other modified hydroxylamine derivates), GGA, as yet unidentified compounds, or simple heat treatment may have clinical utility in treating metabolic disorders. Further studies are also warranted to investigate the role of chemical chaperones as a potential future pharmacological approach to combat metabolic diseases.

ACKNOWLEDGMENTS

We thank the members of the Cellular and Molecular Metabolism laboratory for useful discussions on this manuscript. We acknowledge former laboratory members Dr Jason Chung and Dr Clinton Bruce for their contribution to work described herein. We apologize to all authors whose work in the field discussed in this review were not cited due to space limitations. The studies highlighted in this review were supported by project grants from the National Health and Medical Research Council of Australia (NHMRC grant nos. 472650, APP1004441). M.A.F. is a Senior Principal Research Fellow of the NHMRC.

CONFLICT OF INTEREST

M.A.F. is Chief Scientific Officer of N-Gene Research Laboratories Ltd. D.C.H. and M.W. have no conflict.

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