HSP72 protects against obesity-induced insulin resistance

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Patients with type 2 diabetes have reduced gene expression of heat shock protein (HSP) 72, which correlates with reduced insulin sensitivity. Heat therapy, which activates HSP72, improves clinical parameters in these patients. Activation of several inflammatory signaling proteins such as c-jun amino terminal kinase (JNK), inhibitor of κB kinase, and tumor necrosis factor-α, can induce insulin resistance, but HSP 72 can block the induction of these molecules *in vitro*. Accordingly, we examined whether activation of HSP72 can protect against the development of insulin resistance. First, we show that obese, insulin resistant humans have reduced HSP72 protein expression and increased JNK phosphorylation in skeletal muscle. We next used heat shock therapy, transgenic overexpression, and pharmacologic means to overexpress HSP72 either specifically in skeletal muscle or globally in mice. Herein, we show that regardless of the means used to achieve an elevation in HSP72 protein, protection against diet- or obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance was observed. This protection was tightly associated with the prevention of JNK phosphorylation. These findings identify an essential role for HSP72 in blocking inflammation and preventing insulin resistance in the context of genetic obesity or high-fat feeding.

inflammation | stress proteins | metabolic disorders | JNK | type 2 diabetes

It is now estimated that 10% of the world's population is overweight or obese (1). The myriad of disorders associated with obesity, including insulin resistance, glucose intolerance, and dyslipidemia, eventually lead to the development of pancreatic beta cell failure and overt type 2 diabetes. Despite major scientific advances in our understanding of the molecular pathways leading to insulin resistance and type 2 diabetes made during the last 10–15 years, current therapeutic drugs have had limited success. In the past decade, it has become apparent that obesity is linked to a state of chronic inflammation (2). Inflammation results in the secretion of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) from macrophages and/or adipocytes, which results in the activation of serine threonine kinases, namely c-jun amino terminal kinase (JNK) and inhibitor of κB kinase (IKK) in insulin responsive tissues such as adipose tissue, skeletal muscle, and liver (3). It is known that both JNK and IKK phosphorylate IRS-1 on Ser-307, rendering it a poor substrate for the activated insulin receptor (2). In addition, lipid oversupply can lead to increased deposition of lipid species such as diacylglycerol and ceramide, which can also activate JNK and IKK in liver and/or skeletal muscle, leading to insulin resistance (4). The importance of both JNK and IKK in insulin resistance is highlighted by the observation that genetic disruption of these pathways in mice confers protection against obesity induced insulin resistance (5–7).

Heat shock proteins (HSPs) function at the cellular level to protect cells against many chronically and acutely stressful conditions. Subacute activation of HSPs results in stress tolerance and cytoprotection against otherwise lethal exposures to stress-induced molecular damage (8). The induction of the HSPs, therefore, may have broad therapeutic benefits in the treatment of various types of tissue trauma and disease. Small peptides that activate HSPs are currently being investigated as therapies to treat diseases such as cancer, neurodegenerative diseases, and disorders associated with apoptosis (9). Up-regulation of HSP72 by prior heat conditioning or by ectopic expression can markedly block the activation of JNK *in vitro* (10, 11), and liposomal transfer of HSP72 and/or thermal induction of HSP72 prevents NF-κB activation and translocation, TNF-α gene transcription, and subsequent ischemia-induced renal tubular cell apoptosis (12). Importantly, the primary function of HSPs is to serve as molecular chaperones of naïve, aberrantly folded, or mutated proteins, and recent work by Hotamisligil *et al.* (13, 14) has demonstrated that small chaperone peptides that stabilize protein confirmation and facilitate the trafficking of mutant proteins can protect against insulin resistance and type 2 diabetes. Together, these previous data have led us to hypothesize that induction of HSP72 may combat insulin resistance. In this study, we tested this hypothesis by using heat shock therapy, transgenic overexpression, and pharmacologic means to overexpress HSP72 either specifically in skeletal muscle or globally in mice. We show that, regardless of the model used to achieve an elevation in HSP72 protein, protection against diet- or obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance was observed.

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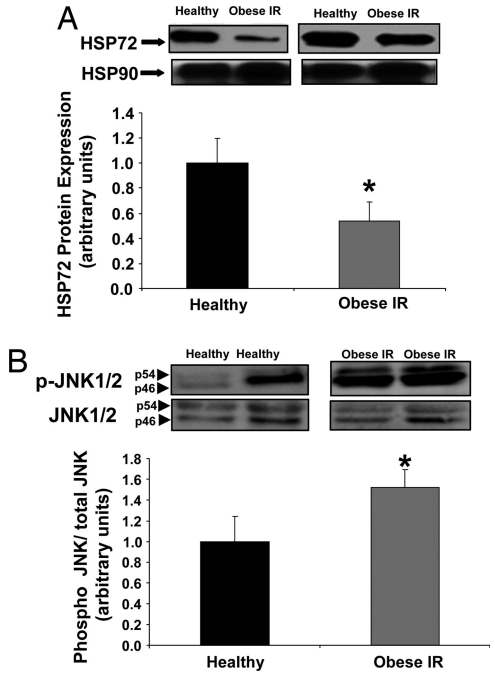

Fig. 1. Human obesity and insulin resistance are associated with decreased expression of HSP72 and increased phosphorylation of JNK in skeletal muscle. Representative immunoblots and quantification of HSP72 protein expression (*A*) and phosphorylation (Thr^{183}/Tyr^{185})/total JNK in healthy humans and obese, insulin resistant humans (*B*) (healthy, $n = 19$; obese IR, $n = 23$; *, $P < 0.05$ compared with healthy).

Results

Human Obesity and Insulin Resistance Are Associated with Decreased Expression of HSP72 and Increased Phosphorylation of JNK in Skeletal Muscle. We (15) and others (16) have previously reported that the mRNA abundance of the gene encoding HSP72 was reduced in patients with type 2 diabetes and was inversely correlated with insulin sensitivity; however, there are no data examining whether HSP72 protein expression is also reduced in insulin resistance. Here, we report that HSP72, but not HSP90, protein expression in skeletal muscle is markedly reduced in insulin resistance, and that phosphorylation of JNK is elevated in obese, insulin resistant patients relative to healthy humans [Fig. 1 and supporting information (SI) Fig. 6 *A* and *B*].

Heat Therapy Activates HSP72, but This Affect Is Blunted by Consumption of a High-Fat Diet (HFD). A preliminary report has demonstrated that hot tub therapy in humans can, by unknown mechanisms, improve glycemia in patients with type 2 diabetes (17). To examine whether heat therapy would improve hyperinsulinemia and hyperglycemia associated with an HFD, we performed heat therapy experiments. In initial experiments, we examined the effect of heat therapy, which constituted increasing body temperature to 41.5°C of 15 min (see *Methods*), on the HSP72 response. Such a treatment resulted in a transient increase in HSP72 in skeletal muscle, liver, and adipose tissue over a 24-h period (SI Fig. 7). When animals were placed on the HFD, the HSP72 response to heat therapy (HT) was reduced (SI Fig. 7), a result consistent with our observation in obese humans (Fig. 1). Transcription of HSP72 is regulated by the activation of heat shock transcription factor (HSF-1) (8), and it is also known that that glycogen synthase kinase 3 β (GSK-3 β) and extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) participate in the down-regulation of HSF-1 transcriptional activity (18). Accordingly, we examined both ERK 1/2 and GSK3β phosphorylation in chow- and high-fat-fed mice, but we observed no difference between chow and HFD for either ERK 1/2 (Thr^{202}/Tyr^{204}) or GSK3β (Ser^{9}) phosphorylation (data not shown) and, therefore, the reason for the reduced HSP72 expression in obesity-induced insulin resistance remains elusive.

Weekly Heat Therapy Prevents JNK Phosphorylation in the Skeletal Muscle of Mice, Which Improves HFD-Induced Hyperinsulinemia and Hyperglycemia. We next subjected mice to HT or sham therapy (ST) once per week for 16 weeks while consuming a standard chow diet or an HFD. The HFD-induced JNK phosphorylation observed in the skeletal muscle of ST mice was attenuated in HT mice (Fig. 2*A*). As expected, the HFD resulted in elevated fasting glucose and insulin levels and insulin resistance as measured by the homeostatic model assessment of insulin resistance (HOMA-IR) in ST mice (Fig. 2 *B–D*). In contrast, the mice subjected to HT were protected against basal hyperglycemia, hyperinsulinemia, and elevated HOMA-IR (Fig. 2 *B–D*). To determine whether mice were protected when glucose challenged, we also performed i.p. glucose tolerance tests (IPGTTs). Consistent with our basal glucose and insulin measures, the HFD induced glucose intolerance, but the severity of the HFD-induced increase in the area under the glucose

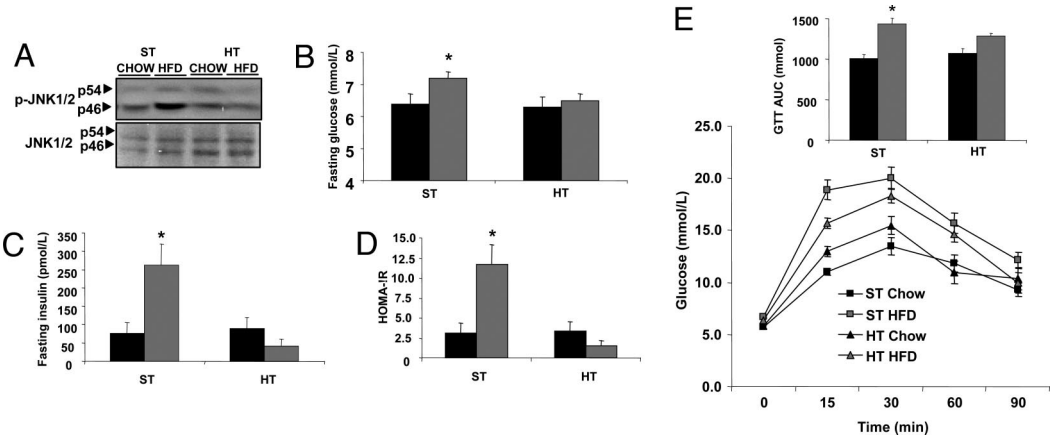

Fig. 2. Weekly heat therapy prevents JNK phosphorylation in the skeletal muscle of mice, which improves HFD-induced hyperinsulinemia and hyperglycemia. (*A*) Representative immunoblots of phosphorylation (Thr^{183}/Tyr^{185})/total JNK in mixed gastrocnemius muscle from wild-type mice subjected to ST or HT. Fasting glucose (*B*), fasting insulin (*C*), HOMA-IR (*D*), and i.p. glucose tolerance (*E*) from wild type mice placed on a standard chow diet (black bars) or an HFD (gray bars) for 16 weeks while undergoing weekly ST or HT. Experiments were completed at least 72 h after ST or HT. (n = 7–12 mice per group; *, $P < 0.05$ ST HFD vs. all other conditions.)

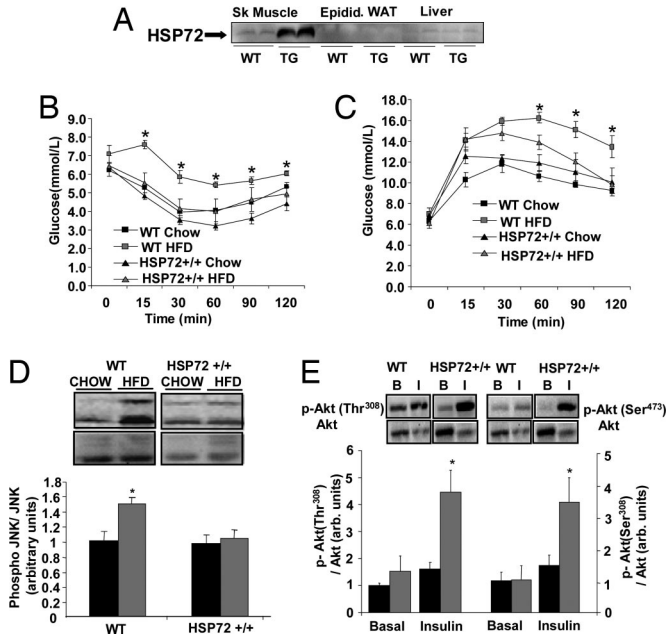

Fig. 3. HSP72 overexpression in skeletal muscle prevents high-fat-feeding-induced glucose intolerance and insulin resistance, JNK phosphorylation, and impaired insulin signaling. (*A*) Immunoblot showing enhanced HSP72 expression in mixed gastrocnemius muscle but not epididymal white adipose tissue or liver when comparing HSP72 transgenic (TG) with WT mice. I.p. insulin (*B*) and glucose tolerance (*C*) and representative immunoblots and quantification of phosphorylation (Thr^{183}/Tyr^{185})/total JNK in mixed gastrocnemius muscles (*D*) from WT and HSP72 overexpression (HSP72+/+) mice placed on a standard chow (black bars) or high-fat diet (gray bars) for 16 weeks. (*E*) Representative immunoblots and quantification of phosphorylation of Akt (Thr^{308} and Ser^{473}) in mixed gastrocnemius muscle excised 2–5 min after i.p. injection of saline (B) or 1.5 units/kg insulin (I) from WT (black bars) and HSP72+/+ (gray bars) mice after 16 weeks on the HFD. (n = 5–9 mice per group; *, $P < 0.05$ WT HFD vs. all other conditions for *B–D*; *, $P < 0.05$ HSP72+/+ insulin treated vs. all other groups for *E*.)

curve during the IPGTTs was reduced in HT compared with ST (Fig. 2*E*).

HSP72 Overexpression in Skeletal Muscle Prevents High-Fat-Feeding-Induced Elevations in Basal Glucose and Insulin, Glucose Intolerance, and Insulin Resistance. To investigate whether the reduced HSP72 expression and elevated JNK phosphorylation observed in humans (Fig. 1*A–C*) was linked and whether up-regulation of HSP72 by HT was indeed the primary mechanism responsible for the improved metabolic control in HT mice on the HFD (Fig. 2 *B–E*), we used skeletal/cardiac muscle-specific HSP72 transgenic mice (HSP72+/+) (Fig. 3*A*) and placed these and control mice (WT) on either a standard chow diet or an HFD for 16 weeks. As expected, the HFD resulted in mild fasting hyperglycemia and marked fasting hyperinsulinemia in WT mice. However, no such effect was seen in HSP72+/+ mice (SI Fig. 8 *A* and *B*). IPGTTs and i.p. insulin tolerance tests (IPITTs) were performed on the mice and, consistent with the basal glucose and insulin results, WT mice on the HFD were both insulin resistant and glucose intolerant. In contrast, HSP72+/+ mice displayed markedly improved insulin and glucose tolerance when placed on an HFD (Fig. 3 *B* and *C* and SI Fig. 8 *C* and *D*). These data indicate that HSP72 overexpression in skeletal muscle can protect against the deleterious effects of an HFD in the development of insulin resistance. Because heat therapy conferred protection against insulin resistance in a manner similar to that seen in our HSP72+/+ animals, we conclude that one mechanism by which heat therapy may improve type 2 diabetic parameters (17) is via the induction of HSP72.

HSP72 Overexpression in Skeletal Muscle Prevents High-Fat-Feeding-Induced JNK Phosphorylation and Impaired Insulin Signaling. Because HSP72 overexpression can block JNK and NFκB *in vitro* (10–12), we next tested whether the protection we observed in glucose and insulin tolerance in the HSP72+/+ mice was associated with decreased activation of these pathways. The phosphorylation of IKKαβ ($Ser^{180/181}$) was unchanged by diet or treatment in skeletal muscle (data not shown). In contrast, the HFD induced marked phosphorylation of JNK (Thr^{183}/Tyr^{185}) in WT mice, but this was completely prevented in HSP72+/+ mice (Fig. 3*D*). Because JNK is a serine/threonine kinase known to inhibit insulin signaling, we next examined tyrosine phosphorylation of IRS1 and phosphorylation of Akt in skeletal muscle. Although phosphorylation of IRS1 (Tyr^{612}) was not increased with insulin stimulation in WT mice on the HFD, it tended ($P = 0.1$) to increase in insulin-stimulated muscle from HSP72+/+ mice (SI Fig. 8*E*). Moreover, whereas insulin stimulation did not increase phosphorylation of Akt at two residues critical for activation of this protein (Thr^{308}/Ser^{473}) in WT mice on the HFD, it markedly phosphorylated Akt at these residues in the HSP72+/+ mice on the HFD (Fig. 3*E*). Together, these data suggest that overexpression of HSP72 in skeletal muscle can inhibit the lipid-induced activation of JNK, resulting in better maintained insulin signaling and improved glucose tolerance and insulin action.

HSP72 Overexpression in Skeletal Muscle Prevents High-Fat-Feeding-Induced Increases in Body Weight and Fat Pad Weight, Which Is Associated with Enhanced Mitochondrial Enzyme Activity. Whereas the HFD increased body weight in WT, no such effect was seen in HSP72+/+ mice (Fig. 4*A*). In addition, we weighed the epididymal fat pads and showed that after 16 weeks on either diet, HSP72+/+ mice had smaller fat pads relative to WT mice irrespective of diet (Fig. 4*B*). These effects were not due to hypophagia, because we observed no difference in food intake when comparing strains (Fig. 4*C*). This observation suggested that the HSP72+/+ mice may have increased energy expenditure. HSP72 is known to protect cardiac muscle against mitochondrial damage caused by ischemia reperfusion injury (19), whereas heat therapy increases mitochondrial enzyme activity and exercise endurance capacity in rats (20). In addition, we have previously observed a significant positive correlation between the mRNA expression of HSP72 and mitochondrial enzyme activity in human skeletal muscle (15). Given these associations and our observations of smaller fat pads in HSP72+/+ mice, we examined the oxidative capacity in skeletal muscle of WT and HSP72+/+ mice by measuring the maximal activities of two important mitochondrial enzymes, citrate synthase (CS) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD). In both extensor digitorium longus (Fig. 4*D*) and soleus (data not shown) muscles, the maximal activities of these enzymes was higher in HSP72+/+ compared with WT mice. These data may suggest that the fatty acid oxidative capacity was increased in the skeletal muscles of HSP72+/+ mice, which may account for the protection against increases in body weight and fat pad mass in HSP72+/+ mice on the HFD.

HSP72 Overexpression in Skeletal Muscle Prevents HFD-Induced Phosphorylation of IKKαβ in Liver, but Increases Circulating Adiponectin Levels. Previous studies have demonstrated that mice with muscle-specific genetic modifications can display phenotypic alterations in liver (21). In addition, because adipose tissue is known to secrete adipokines that can impair insulin sensitivity in tissue such as liver (22), and because we observed smaller fat pads in our HSP72+/+ mice, we next examined markers of inflammation and insulin signal transduction in liver. Whereas we observed no difference in phosphorylation of JNK in liver when comparing HSP72+/+ with WT mice (data not shown), the phosphorylation of IKKαβ ($Ser^{180/181}$) observed in WT mice on the HFD was abolished in HSP72+/+ mice (Fig. 4*E*). Despite this observation, we observed no differences in phosphorylation of Akt (Ser^{473}) in insulin stimulated liver

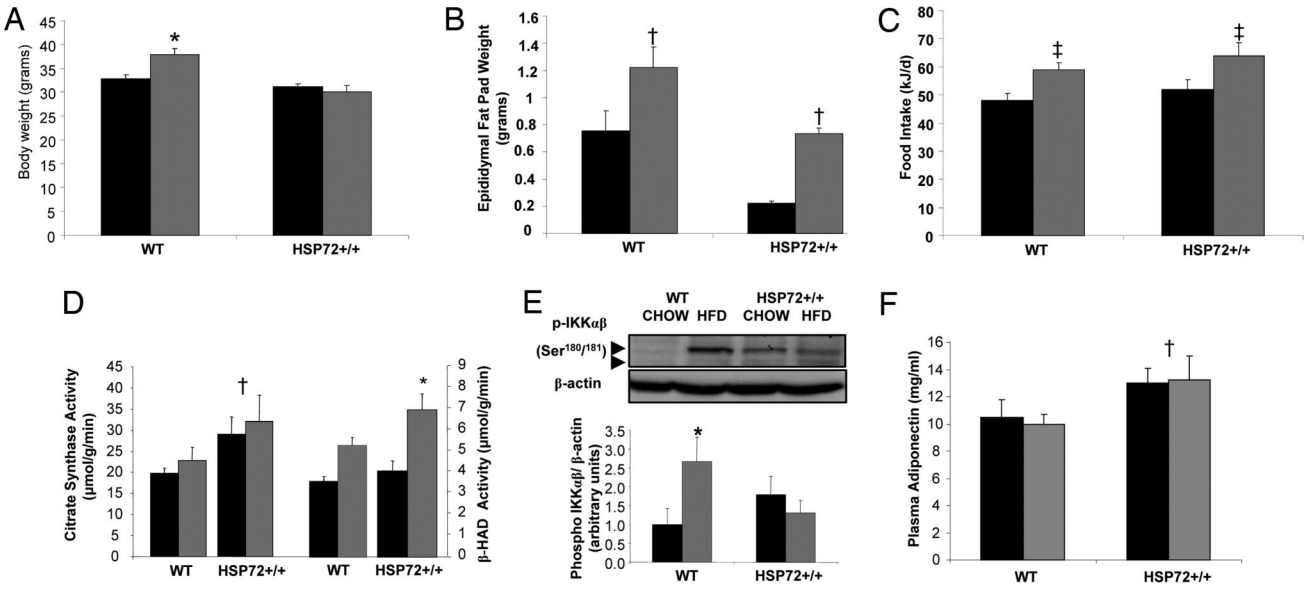

Fig. 4. HSP72 overexpression in skeletal muscle prevents high-fat-feeding-induced increases in body weight and activation of IKK in liver and results in reduced fat pad weight, increased mitochondrial oxidative enzyme activity, and increased circulating adiponectin. Body weight (*A*), epididymal fat pad weight (*B*), food intake (*C*), CS and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) in extensor digitorum longus muscle (*D*), representative immunoblots and quantification of phosphorylation (Ser^{180}/Ser^{181}) of inhibitor of κB kinase (IKK)/β-actin in liver (*E*), and plasma adiponectin (*F*) from WT and HSP72 overexpression (HSP72+/+) mice placed on a standard chow diet (black bars) or an HFD (gray bars) for 16 weeks. ($n = 4–7$ mice per group; *, $P < 0.05$ HFD CON vs. other groups for *A* and *E*, *, $P < 0.05$ HSP72+/+ HFD vs. other groups for *D*; †, main effect for genotype for *B*, *D*, and *F*; ‡, main effect for diet for *C*.)

tissue (data not shown), nor did we observe any difference in the mRNA expression of key hepatic glycogenolytic enzymes PEPCK and G-6-Pase (data not shown) when comparing HSP72+/+ with WT mice when placed on the HFD. Because these data indicate that a circulatory factor (or factors) may contribute to the differences in IKKαβ phosphorylation in the liver of HSP72+/+ and WT mice when placed on a HFD, and because adiponectin is known to down-regulate IKK activity (23), we next examined plasma adiponectin levels. Consistent with our observation of smaller fat pad weight in HSP72+/+ vs. WT mice irrespective of diet, plasma adiponectin levels were higher in HSP72+/+ compared with WT mice (Fig. 4*F*).

The Hydroxylamine Derivative BGP-15 Is a Coinducer of HSP72 *in Vitro*. Hydroxylamine derivatives such as Bimoclomol, Arimoclomol, and BRX-220 are known to be effective in the treatment of wound healing in diabetic complications in rats (24) and in delaying the progression of the fatal neurodegenerative condition amyotrophic lateral sclerosis (ALS) (25). In addition, hydroxylamine derivatives have been shown to improve insulin sensitivity in diet-induced obesity by unknown mechanisms (26, 27). As discussed, transcription of HSP72 is regulated by the activation of HSF-1 (8). Hydroxylamine derivatives are thought to activate HSP72 both via modification of membrane microdomain-associated stress-sensing and -signaling mechanisms (28, 29) and by prolonging the binding of HSF-1 to the respective DNA elements (25, 26, 30). To test whether BGP-15, a hydroxylamine derivative, activated HSP72 via this pathway, we conducted *in vitro* experiments in adipocytes and muscle cells. In preliminary experiments conducted in 3T3-L1 adipocytes, we demonstrated that BGP-15 treatment in the absence of heating cells did not activate HSF-1 or HSP72. However, when cells were heated at 41°C and cotreated with BGP-15, the phosphorylation of HSF1 and expression of HSF1 and HSP72 was markedly increased above that of heat treatment alone (data not shown). Next, fully differentiated L6 myotubes were treated with 50 μM BGP-15 or PBS (control) for 30 min and then placed at 42°C for an additional 30, 60, or 120 min. Muscle cells were then placed in a 37°C incubator for a further 7 h before being lysed. Treatment with BGP-15 increased HSF-1 at 30 min and HSP72 at 60 min but had no effect on HSP90 levels (SI Fig. 9). The data demonstrate that BGP-15 is an inducer of HSF-1/HSP72 *in vitro*, but only in the presence of cotreatment with heat.

BGP-15 Activates HSP72 in the Skeletal Muscles of *ob/ob* Mice, Preventing JNK Phosphorylation and Insulin Resistance. To test the hypothesis that pharmacological activation of HSP72 may be effective in treating obesity-induced insulin resistance, we treated leptin-deficient (*ob/ob*) mice with BGP-15 (15 mg/kg per day in 200 μl of saline) or a control (200 μl saline) for 15 days by oral gavage. After this time, mice underwent a hyperinsulinemic euglycemic clamp. The 15-day treatment had no effect on body weight in these animals [38.0 ± 1.0 vs. 36.0 ± 0.6 g for BGP-15 and control, respectively (not significant)]. BGP-15 resulted in a marked increase in intramuscular HSP72 protein expression when compared with control-treated animals (Fig. 5*A*). Accordingly, BGP-15 treatment attenuated JNK phosphorylation (Fig. 5*B*). Fasting levels of both glucose (Fig. 5*C*) and insulin (Fig. 5*D*) were markedly reduced after BGP-15 treatment. In addition, BGP-15 treatment markedly (≈2-fold) increased glucose disposal rate during the clamp (Fig. 5*E*). Moreover, whereas hyperinsulinemia did not significantly suppress hepatic glucose production (HGP) in control animals during the clamp, there was ≈50% suppression of HGP during the clamp in BGP-15-treated animals (Fig. 5*F*). These data indicate that pharmacological activation of HSP72 can improve insulin sensitivity in a genetic model of obesity in multiple insulin-responsive tissues *in vivo*, and it is likely that the mechanism for this effect is an increased expression of HSP72 via enhanced phosphorylation and expression of HSF-1 leading to a decreased activation of JNK.

Discussion

The data presented here provide compelling evidence that HSP72 is a potential target for therapeutic treatment of obesity-induced insulin resistance. Regardless of the means used to achieve an elevation in HSP72 protein, protection against diet or genetic obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance was observed. This protection was tightly associated with the prevention of JNK phosphorylation.

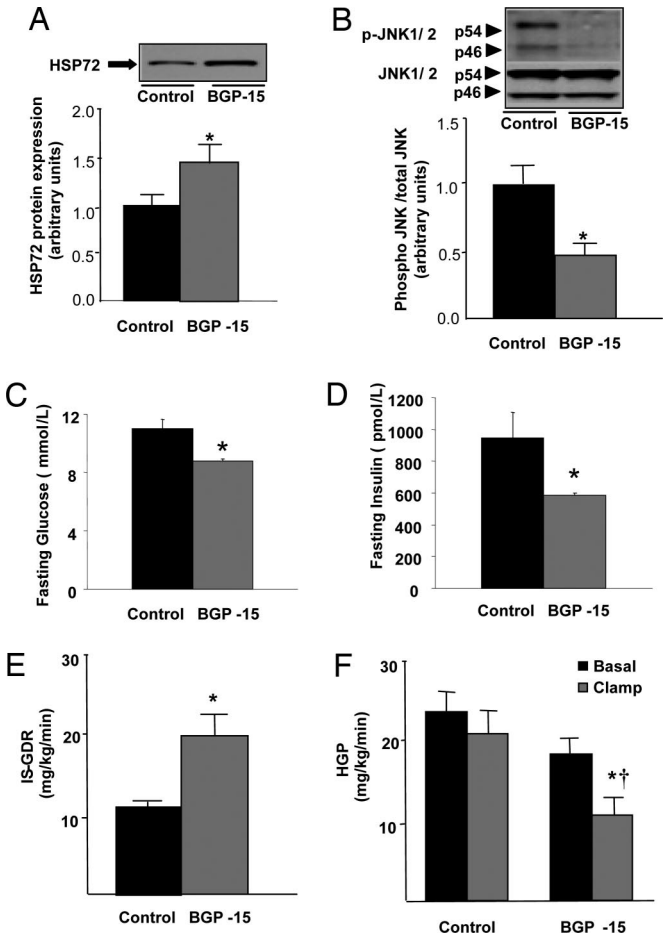

Fig. 5. BGP-15 activates HSP72 in the skeletal muscles of *ob/ob* mice, preventing JNK phosphorylation and insulin resistance. Representative immunoblots and quantification of HSP72 (*A*) and phosphorylation (Thr-183/Tyr-185)/total JNK (*B*) in quadriceps muscles. Fasting glucose (*C*), insulin (*D*), and insulin-stimulated glucose disposal rate (IS-GDR) (*E*) during a hyperinsulinemic euglycemic clamp. (*F*) Hepatic glucose production (HGP) without insulin (basal) and after a hyperinsulinemic euglycemic clamp (clamp). All experiments were performed in leptin-deficient (*ob/ob*) mice treated with saline (black bars; control, 200 μl of saline) or BGP-15 (gray bars; 15 mg/kg per day in 200 μl of saline) for 15 days by oral gavage. ($n = 5$–7 mice per group; *, $P < 0.05$ compared with control; *†, $P < 0.05$ compared with basal for BGP-15-treated group.)

These findings identify an essential role for HSP72 in blocking inflammation, which prevents insulin resistance in the context of genetic obesity or high-fat feeding.

The precise mechanism by which HSP72 protects against obesity-induced insulin resistance remains to be elucidated, but our data suggest that HSP72 acts by preventing JNK phosphorylation, which is known to inhibit insulin signal transduction (5), and/or by increasing mitochondrial enzyme activity, because a reduced mitochondrial capacity is tightly associated with insulin resistance (31). Regardless of the model we used, an enhanced HSP72 protein expression was always associated with reduced JNK phosphorylation. However, the data presented here do not provide a mechanism for how HSP72 regulates JNK activation, which is a limitation to this study. We observed no association between HSP72 and JNK *per se* in immunoprecipitation experiments (unpublished observations) and, in all of our experiments reported herein, total JNK protein content was not reduced by increased HSP72 expression. Therefore, the mechanism by which HSP72 can impair JNK activity is not likely to be via degradation of JNK. Rather, recent experiments suggest two potential mechanisms by which HSP72 can down-regulate JNK. A role for duel leucine zipper-bearing kinase (DLK) as a mechanism by which HSP72 can down-regulate JNK has recently been proposed (32). DLK is a member of the mixed lineage kinase family, which is a known mitogen-activated kinase kinase. DLK is a known upstream activator of JNK, and in this recent study (32) HSP72 was shown to associate with the HSP cochaperone CHIP, a known ubiquitin ligase that negatively regulated DLK expression and activity. Evidence also suggests, however, that the down-regulation of JNK by HSP72 is likely to involve regulation of upstream phosphatases (33). Both the upstream phosphatases MAP kinase phosphatase-1 (MKP-1) (34, 35) and MKP-3 (34) have been shown to be regulated by HSP72, mediating down-regulation of the MAP kinases. Together, it is likely that HSP72 blocks the obesity-induced increase in JNK phosphorylation via decreased DLK and/or increased MKP-1 activity. The observation that CS and β-HAD maximal activity were increased in HSP72+/+ mice was consistent with our previous studies in humans, in which we showed a correlation between the mRNA expression of HSP72 and mitochondrial enzyme activity in human skeletal muscle (15). HSP72 can enhance mitochondrial capacity and/or function via several mechanisms. It is well known that one major chaperone function of HSP72 is to aid in the mitochondrial import of nuclear encoded proteins via interaction with the mitochondrial protein import receptor protein Tom70 (36). In addition, overexpression of HSP72 in glucose-deprived cells maintains mitochondrial respiratory function and reduces ROS formation (37), the latter which has been recently linked to insulin resistance (38).

The treatment of leptin-deficient (*ob/ob*) mice with BGP-15 for only 15 days up-regulated HSP72 protein expression in skeletal muscle by ≈50% and also resulted in marked reductions in JNK phosphorylation and improvements in insulin resistance in both the liver and peripheral insulin-sensitive tissues. Importantly, hydroxylamine derivatives such as BGP-15 have been reported to be safe and well tolerated at all doses in randomized, placebo-controlled phase IIa clinical trials in patients with ALS (39). Moreover, in a preliminary report, BGP-15 administered orally for 28 days to insulin-resistant, nondiabetic patients was shown to significantly improve whole-body glucose disposal during a hyperinsulinemic euglycemic clamp (40). As discussed, a preliminary report has shown that heat therapy improves the clinical outcomes in patients with type 2 diabetes (17), and here we show that the mechanism for such an outcome is likely to be via up-regulation of HSP72. Hydroxylamines are also shown to act by perturbing membrane hyperstructures, via their highly specific lipid-interactions (28), which is sufficient for the generation and transmission of stress signals to activate HSP genes (29), and via the prolongation of the binding of HSF-1 to the heat shock elements on the DNA (30). Together, these preliminary data in humans (17, 40) and our results with respect to the insulin-sensitizing effect of BGP-15 and heat therapy in our genetic- and diet-induced models of obesity-induced insulin resistance provide a realistic therapeutic strategy to treat obesity-induced insulin resistance.

In summary, we have shown that elevating HSP72 protein by heat treatment, muscle-specific transgenic overexpression, or pharmacological means can protect mice against diet- or obesity-induced hyperglycemia, hyperinsulinemia, glucose intolerance, and insulin resistance. This protection was tightly associated with the prevention of JNK phosphorylation. We have, therefore, identified an essential role for HSP72 in blocking inflammation and preventing insulin resistance in the context of genetic obesity or high-fat feeding.

Methods

Human and Animal Experiments. Methods for human experiments are described in *SI Methods*. For diet-induced obesity studies, we used male WT and transgenic mice (HSP72+/+) that had a chimeric transgene that consisted of an inducible HSP72 gene of a rat under a β-actin promoter described in detail elsewhere (41). All experiments were approved by the Royal Melbourne Institute of Technology

Animal Ethics Committee. Experiments always commenced when mice were 8 weeks of age. Control chow diets (5% of total energy from fat) and HFDs (SF03–002; 59% of total energy from fat) were purchased from specialty feeds (Glen Forrest). Animals were given their prescribed diet and water ad libitum and housed in a controlled environment with a 12:12 light–dark cycle.

Heat Treatment Experiments. Except for the time course experiment, mice were exposed to heat treatment once per week for a total of 16 weeks. Before heat treatment, mice were anesthetized with sodium pentobarbital (0.05 mg/g body weight). While unconscious, a rectal thermometer was inserted, and mice were placed in a ventilated plastic container wrapped with an electric heating blanket that was either activated (HT) or not (ST). During HT, body temperature was allowed to rise gradually (10–15 min) to 41.5°C and maintained at this temperature for a total of 15 min by wrapping and unwrapping of the blanket. During the time course experiment, mice were killed immediately, or were allowed to recover at room temperature before being killed at 1, 4, 8, or 24 h. For metabolic testing experiments, mice recovered at room temperature, and experiments were performed at least 72 h after the final HT or ST.

Glucose and Insulin Tolerance Tests, Insulin Signaling Experiments, Protein Analysis, Oxidative Enzymes, and Plasma Cytokines. Glucose and insulin tolerance tests were performed after 16 weeks (*SI Methods*). Proteins were analyzed by SDS/PAGE and immunoblotting (4, 42). CS and β-HAD in skeletal muscle were assayed spectrophotometrically as described in ref. 15. Plasma adiponectin was assessed by using a Lincoplex mouse serum adiponectin kit (Lincoplex, MADPK-71k-ADPN; Linco Research, Millipore) as described in ref. 42.

BGP-15 Hyperinsulinemic Euglycemic Clamp Experiments and Cell Culture Experiments. Male leptin-deficient (*ob/ob*) mice were treated with vehicle (vehicle-treated; 200 μl of saline by oral gavage) or BGP-15 (15 mg/kg per day in 200 μl of saline; N-Gene Research Laboratories) for 15 days by oral gavage before undergoing a euglycemic hyperinsulinemic clamp as described in refs. 7 and 21 (*SI Methods*). Fully differentiated L6 myotubes were treated with 50 μM BGP-15 or PBS (control) for 30 min and then placed at 42°C for an additional 30, 60, or 120 min. Muscle cells were then placed in a 37°C incubator for a further 7 h before being lysed and protein analyzed as described previously (4, 42).

Statistical Analyses. Results are expressed as the mean ± SEM. Data were analyzed for differences by analysis of variance with specific differences located with a Student Newman–Keuls post hoc test, or a Student's *t* test for unpaired samples where appropriate. $P < 0.05$ was considered to be statistically significant.

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