The Role of Heat Shock Protein 72 in Preventing Obesity-induced Insulin Resistance

A Thesis Presented in Total Fulfilment of the Degree of Doctor of Philosophy

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Declaration

I, the candidate, Jason Chung, certify that:

a) except where due acknowledgement has been made, the work is that of the candidate alone;

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List of Abbreviations

Akt	Acute transforming retrovirus thymoma
AMPK	5'-adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
β-HAD	β-hydroxyacyl-CoA-dehydrogenase
Chow	Standard chow diet
Con	Control
CS	Citrate synthase
DAG	Diacylglycerol
EDL	Extensor digitorum longus muscle
EDTA	Ethylenediaminetetraacetic acid
EGP	Endogenous glucose production
EHC	Euglycemic-hyperinsulinemic clamp
EMSA	Electrophoretic mobility shift assay
FFA	Free fatty acids
Fru-1,6-P2ase	Fructose 1,6-bisphosphatase
GDR	Glucose disposal rate
GIR	Glucose infusion rate
GTT	Glucose tolerance test
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GLUT	Glucose transporter protein
HFD	High fat diet
HGP	Hepatic glucose production

HSC	Heat shock cognate
HSE	Heat shock elements
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
HOMA-IR	Homeostatic model assessment of insulin resistance
IκBα	Inhibitor of kappa B alpha
IκKα	Inhibitor of kappa B kinase alpha
IκKβ	Inhibitor of kappa B kinase beta
IκK	Inhibitor of kappa B kinase
IP	Intraperitoneal
IR	Insulin receptor
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
JNK	c-jun-N-terminal kinase
KO	Knockout
LCFA	Long chain fatty acid
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
mRNA	Messenger ribonucleic acid
mtHSP70	Mitochondrial heat shock protein 70
NEMO	Nuclear factor kappa-beta essential modulator
NFκB	Nuclear factor kappa-beta
PAI-1	Plasminogen activator inhibitor 1
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase

PI3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbol myristoylacetate
PPAR	Peroxisome proliferators-activated receptors
R_a	Rate of appearance
R_d	Rate of disappearance
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SEM	Standard error of the mean
Ser^{307}	Serine 307
TAG	Triacylglycerol
TNF-α	Tumour necrosis factor alpha
UDPG	Uridine diphosphoglucose
WT	Wild type

Abstract

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 signalling proteins such as c-jun amino terminal kinase (JNK) can induce insulin resistance but HSP72 can block the induction of these molecules *in vitro*. Whether up-regulation of HSP72 can protect against insulin resistance is not known. In experiments reported in this thesis we show that HSP72 protects against insulin resistance and blocks the activation of JNK *in vivo*. We first show that mice that underwent weekly heat shock therapy to increase intramuscular HSP72 protein expression were protected from high fat diet (HFD)-induced hyperinsulinemia, hyperglycemia and glucose intolerance, factors associated with reduced JNK phosphorylation. To determine whether the elevation in intramuscular HSP72 expression and protection from insulin resistance are causally linked, we studied muscle specific HSP72 overexpression mice ($HSP72^{+/+}$). Compared with wild-type mice, $HSP72^{+/+}$ mice were protected from hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance when placed on a HFD, factors associated with a complete inhibition of HFD-induced JNK phosphorylation in skeletal muscle. Finally, we show that $HSP72^{+/+}$ mice display greater mitochondrial enzyme activity in the liver, adipose tissue and skeletal muscle, corresponding to reduced plasma free fatty acid levels, white adipose tissue mass and alterations in circulating adipokines. These data identify HSP72 as being pivotal in protecting against obesity-induced insulin resistance possibly by blocking JNK and/or by up-regulation of mitochondrial oxidative capacity.

Chapter One

Introduction and Literature Review

1.1 Obesity and metabolic complications

Over the last 30 years, obesity has been identified as a major risk factor for the development of numerous health conditions including insulin resistance, type 2 diabetes mellitus and cardiovascular disease (Flegal et al., 2002; Hotamisligil, 2006; Must et al., 1999; Zimmet et al., 2005). Of greater concern is the dramatic increase in the prevalence of obesity related health problems, in both children and adults, leading to increased morbidity and mortality throughout the world, a state now referred to as the obesity epidemic (Harris et al., 1998; Saltiel and Kahn, 2001). According to the World Health Organization (WHO), more than 1 billion adults worldwide are overweight, of which 300 million are defined as clinically obese (body mass index (BMI) >30 kg/m^2) (WHO, 2002).

A significant portion of the obesity threat lies in its association with other metabolic complications, specifically, the development of insulin resistance and type 2 diabetes. At present, it is estimated that 190 million people worldwide have diabetes, of which 90% have type 2 diabetes (Zimmet et al., 2005). Interestingly, several decades of research in type 2 diabetes has yet to provide a clear definition of the disease and the mechanisms by which it exerts its negative effects. Currently, the WHO defines diabetes as having a fasting blood glucose of $\geq$ 7.0mmol/L or $\geq$ 11.1mmol/L two hours after oral ingestion of a 75g glucose load (WHO, 2006). Although hyperglycemia is the main criterion in defining type 2 diabetes, the dysregulation of insulin action and pancreatic beta cell dysfunction must also be considered.

1.2 Insulin action and glucose homeostasis in skeletal muscle and adipose tissue

Glucose is one of the most important energy sources available to eukaryotic cells. In animals, *in vivo* circulating glucose levels must be strictly regulated through a balance of production by the liver and uptake by skeletal muscle and adipose tissue. In humans, plasma glucose is maintained in a narrow range of 4.0 to 7.0 mmol/L, primarily through the actions of insulin, a hormone released by the beta cells of the pancreas. Insulin functions by increasing uptake of glucose from plasma into skeletal muscle and adipose tissue, whilst reducing the amount of glucose produced by the liver (hepatic glucose production or HGP) (Saltiel and Kahn, 2001).

In skeletal muscle, insulin increases glucose uptake through a cascade of signalling events, ultimately leading to the translocation of GLUT 4, a glucose transport protein, from intracellular sites to the plasma membrane (Fig.1.1) (James et al., 1989; Saltiel and Kahn, 2001; Zierath et al., 2000). Initially, insulin binds to the insulin receptor, a tetrameric protein consisting of two alpha and two beta subunits (White and Kahn, 1994). Once bound, insulin activates tyrosine kinase activity of the beta subunit, resulting in tyrosine phosphorylation of the insulin receptor and the subsequent activation of insulin-receptor-specific docking proteins, known as insulin receptor substrates (IRS) (Kasuga et al., 1983; White, 1998). Thus far, four different IRS molecules have been cloned (Virkamaki et al., 1999) and IRS-1 and IRS-2 have been identified as the predominant isoforms expressed in skeletal muscle (Zierath et al., 2000). Insulin receptor substrates function by providing docking sites for downstream signalling molecules, specifically, adaptor proteins containing SH2

domains. Phosphatidylinositol 3-kinase (PI3-K), a downstream adaptor protein, docks with IRS following phosphorylation of its tyrosine residues (White, 1998).

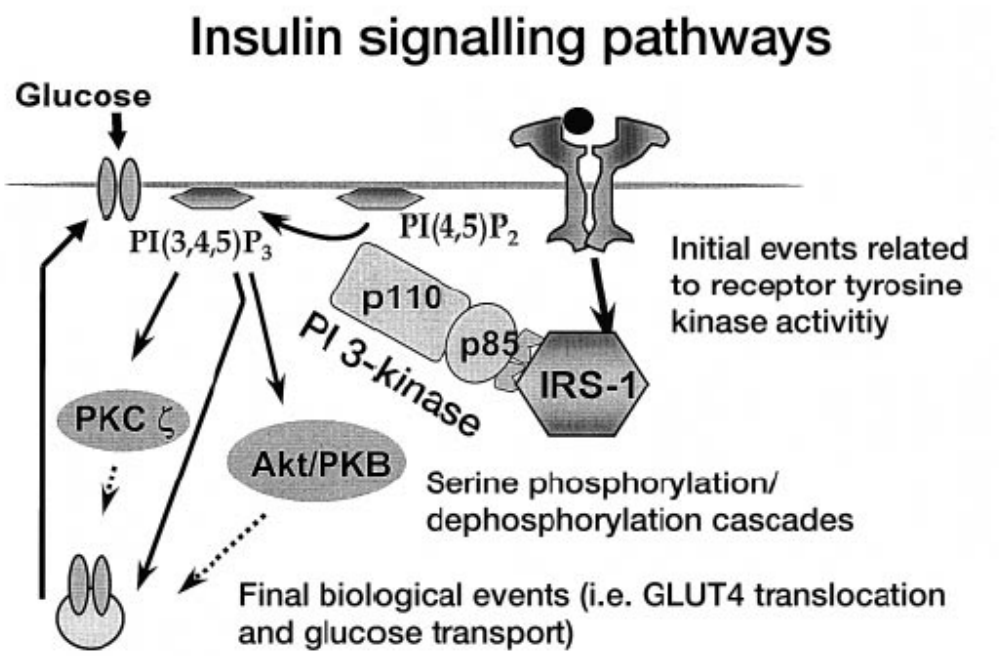


Fig 1.1 Pathway of insulin signalling from the insulin receptor to GLUT 4 translocation (Zierath et al., 2000).

Over the last decade, several studies have characterized the structure of PI3-K, along with its importance in the metabolic actions of insulin. PI3-K consists of a regulatory subunit, p85, and a catalytic subunit, p110, of which p85 contains Src-homology-2 (SH2) domains that bind with tyrosine phosphorylated motifs of IRS proteins (Cantley et al., 1991; Myers et al., 1992). The importance of PI3-K in propagating the insulin response has also been well characterized. In 3T3-L1 adipocytes, inhibition of PI3-K with the fungal metabolite, Wortmannin decreased PI3-K activity (Okada et al., 1994) and the ability of insulin to stimulate glucose incorporation into glycogen (Shepherd et al., 1995). In addition, the use of a specific PI3-K inhibitor, LY294002, effectively blocked insulin-stimulated glucose uptake by inhibiting translocation of GLUT 4 to the plasma membrane (Cheatham et al., 1994).

In conjunction with discoveries of PI3-K activity, its downstream targets have also been implicated in the process of insulin mediated glucose uptake (Saltiel and Kahn, 2001). The involvement of a serine/threonine kinase, known as Acute transforming retrovirus thymoma (Akt) or protein kinase B (PKB) has been of particular interest over the past decade. Several studies have demonstrated the ability of insulin to activate Akt, as much as 12 fold, primarily through prior activation of PI3-K and phosphorylation of its serine and threonine residues (Burgering and Coffer, 1995; Kohn et al., 1995). These studies also reported inhibition of Akt through the use of Wortmannin and coexpression of a dominant-negative mutant of PI3-K, providing evidence of PI3-K's involvement in the activation of Akt. In addition, mutation of the catalytic site of Akt, resulted in inhibition of GLUT 4 translocation in L6 myoblasts (Wang et al., 1999), highlighting the importance of PI3-K and Akt in insulin mediated glucose uptake.

Following PI3-K-mediated phosphorylation of Akt, the final signalling events in glucose uptake involve translocation of GLUT 4 from an intracellular pool (Cushman and Wardzala, 1980) to the plasma membrane (Guma et al., 1995; Hirshman et al., 1990). Recently, a novel substrate of Akt, known as AS160 (Kane et al., 2002), has been shown to play an important role in the translocation of GLUT 4 to the plasma membrane (Sano et al., 2003). In 3T3-L1 adipocytes, it was shown that in response to insulin, Akt phosphorylates AS160 on multiple sites and specific mutation of these sites significantly inhibits GLUT 4 translocation (Sano et al., 2003). In human skeletal muscle and adipose tissue, GLUT 4 is the predominant isoform expressed, playing a central role in insulin stimulated glucose uptake (Charron et al., 1989; James et al., 1989).

The series of events surrounding translocation of GLUT 4 are complex and have not yet been fully elucidated. Currently, it is believed that in the absence of stimulation, GLUT 4 cycles through a complex intracellular tubulo-vesicular network (Satoh et al., 1993) and the trans-Golgi network, both of which are excluded from the plasma membrane. Upon stimulation by insulin, transport vesicles containing GLUT 4 are tethered, docked and eventually fused to the plasma membrane. Fusion of GLUT 4 vesicles allows for exposure of its glucose binding sites to the external surface of the cell and the onset of facilitated transport (Bryant et al., 2002; Holman and Kasuga, 1997).

Taken together, the process of insulin stimulated glucose uptake in skeletal muscle and adipose tissue is complex and not fully characterized. Key signalling events include binding of insulin to its receptor and subsequent activation of IRS-1, PI3-K and Akt and eventual translocation of GLUT 4 to the plasma membrane.

1.3 Insulin action and glucose homeostasis in the liver

In this review thus far, the physiological function of insulin has emphasized its role in glucose uptake, primarily in skeletal muscle and adipose tissue. However, insulin also plays a crucial role in maintaining glucose homeostasis through its actions in the liver, an organ capable of both producing and consuming glucose.

Under normal conditions, the liver contains rate-controlling enzymes for the production of “new” glucose, a process known as gluconeogenesis. Although several enzymes are involved in this process, phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (Fru-1,6-P2ase) and glucose-6-phosphatase (G6Pase)

are considered the key rate-limiting enzymes (O'Brien and Granner, 1996; Pilkis and Granner, 1992; Postic et al., 2004). At the same time, the liver is also capable of breaking down stored glucose, termed glycogenolysis. Thus in order to maintain glucose homeostasis, stimulation of the liver by insulin results in inhibition of gluconeogenesis and glycogenolysis (Michael et al., 2000; Pilkis and Granner, 1992).

The process of hepatic gluconeogenesis involves *de novo* synthesis of glucose from non-carbohydrate precursors, such as lactate, amino acids and glycerol and can be stimulated by secretion of glucagon and glucocorticoids (Postic et al., 2004). A critical step in hepatic gluconeogenesis involves conversion of oxaloacetate to phosphoenolpyruvate, a process which is catalysed by PEPCK (Fig1.2) (O'Brien and Granner, 1996).

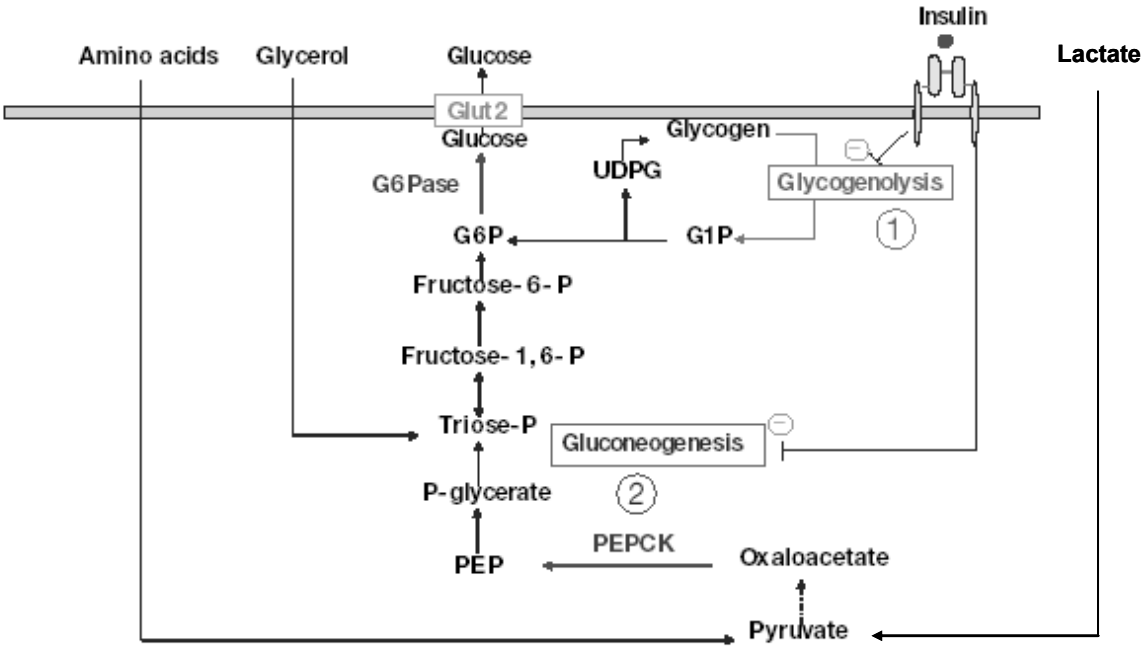


Fig. 1.2 Pathways of glucose production in the liver (Postic et al., 2004). Hepatic glucose can be produced from glycogen, termed glycogenolysis (1) or from *de novo* synthesis via gluconeogenesis (2).

In the post-prandial state or following stimulation with insulin, hepatic glucose production is reduced due to insulin's inhibitory effects on PEPCK gene transcription (Granner et al., 1983). Using a rodent hepatoma cell line, it was shown that incubation with insulin significantly decreased the amount of synthesized PEPCK, primarily due to a reduction in PEPCK mRNA (Granner et al., 1983).

Similar to studies in skeletal muscle and adipose tissue, studies examining insulin-stimulated PEPCK inhibition have revealed signalling events requiring activation of the insulin receptor and phosphorylation of IRS-1, PI3-K and Akt in the liver (Heled et al., 2004; Kotani et al., 1999; Sun et al., 2002; Sutherland et al., 1995). With the use of dominant negative mutants of PI3-K and Akt, it has been shown that insulin signalling can be disrupted, thus preventing insulin-stimulated inhibition of PEPCK. Conversely, constitutively activating PI3-K and Akt reversed this effect, resulting in a dose dependent inhibition of PEPCK gene activity (Kotani et al., 1999).

The importance of insulin to glucose homeostasis *in vivo* is highlighted when using genetic manipulation of liver insulin receptors (Michael et al., 2000). In mice with liver-specific inactivation of the insulin receptor, the ability of insulin to inhibit hepatic glucose production was severely disrupted. As a result, these mice displayed elevated plasma glucose levels, decreased tolerance to glucose and resistance to insulin in peripheral tissues (Michael et al., 2000).

Taken together, early signalling events during insulin stimulation occur through a conserved receptor-mediated pathway in the liver, skeletal muscle and adipose tissue. In the context of glucose homeostasis, insulin acts to maintain plasma

glucose in a narrow range by increasing peripheral glucose uptake and inhibiting hepatic glucose production. Disruption of these signalling events may be the primary cause of insulin resistance and the onset of type 2 diabetes.

1.4 Insulin resistance in skeletal muscle, adipose tissue and the liver

Insulin resistance has been described as a decrease in the ability of insulin to stimulate glucose disposal and inhibit hepatic glucose production (Kahn, 1994). Type 2 diabetes is the combination of insulin resistance and the inability of the pancreas to secrete enough insulin to compensate for this resistance (Kahn, 1994). Although the exact cause of type 2 diabetes is still unclear, it is now evident that insulin resistance is a major risk factor in its development (Shulman, 2000). Previous studies have shown that insulin resistance, reduced glucose clearance and hyperinsulinemia can be present as early as twenty years prior to type 2 diabetes being diagnosed (Warram et al., 1990). In addition, insulin resistance has been identified in lean offspring of diabetic patients (Perseghin et al., 1997), suggesting that insulin resistance can be present in the absence of obesity. Given that insulin resistance is consistently found in patients with type 2 diabetes (Lillioja et al., 1993), research into whole-body insulin resistance and the molecular pathways involved in its development has intensified over the last few decades.

Advances in whole-body measures of insulin resistance, including glucose tolerance tests (GTT), insulin tolerance tests (ITT), and the euglycemic-hyperinsulinemic clamp (EHC) have allowed for a greater understanding of glucose homeostasis in both rodent and human models (DeFronzo et al., 1979; Zierath et al., 2000). Interestingly, skeletal muscle has been shown to be the principal site of glucose uptake under

insulin-stimulated conditions, accounting for 75% of glucose disposal after glucose infusion (DeFronzo et al., 1981; Zierath et al., 2000).

Despite advances with *in vivo* techniques, the molecular mechanisms that cause insulin resistance in skeletal muscle, adipose tissue and the liver are still unclear. However, emerging evidence suggests that systemic insulin resistance is associated with chronic, low-grade inflammation of insulin sensitive tissues (Hotamisligil, 2006; Saghizadeh et al., 1996; Wellen and Hotamisligil, 2005). In addition, the onset of inflammation is associated with poor nutrition and lack of physical activity (Hotamisligil, 2006; Zierath et al., 2000), resulting in the accumulation of plasma free fatty acids and increased storage of fatty-acid derived metabolic products in skeletal muscle (Bruce et al., 2003a) , adipose tissue and the liver (Shoelson et al., 2007).

In addition, recent evidence suggests that immune cells, specifically macrophages, are intricately involved in obesity induced inflammation (Weisberg et al., 2003; Xu et al., 2003). Macrophages are well known for their ability to infiltrate surrounding tissues and release proinflammatory cytokines (Lin and Karin, 2007). In white adipose tissue of diet-induced and genetic models of obesity, macrophage gene transcripts and macrophage content were shown to be significantly increased, resulting in excessive proinflammatory cytokine release within the adipose tissue itself (Weisberg et al., 2003; Xu et al., 2003). Taken together, these findings suggest macrophages, and the cytokines they release, play a critical role in exacerbating the inflammatory response to obesity in adipose tissue (Fig. 1.3).

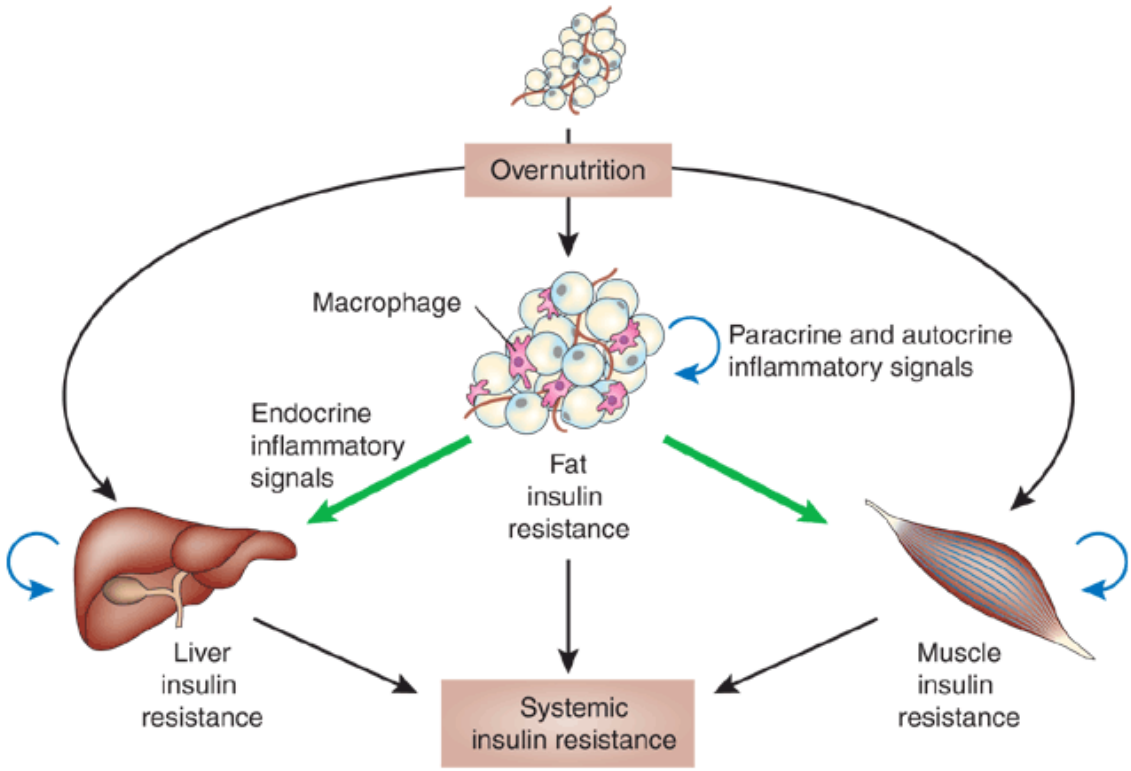


Fig. 1.3 Disruptions in adipose tissue, skeletal muscle and liver function lead to systemic insulin resistance (de Luca and Olefsky, 2006).

Given the substantial evidence linking obesity, inflammation and insulin resistance, considerable attention has been given to understanding the mechanism by which inflammation disrupts the insulin signalling cascade. Thus far, studies have revealed a key component of insulin resistance is the disruption of tyrosine phosphorylation of the insulin receptor and IRS proteins (Hotamisligil, 2006; White, 1998). Disruptions in IRS function are thought to be mediated through the actions of inflammatory cytokines and protein kinases, capable of inducing phosphorylation of serine/threonine rather than tyrosine residues of IRS proteins (Aguirre et al., 2000; Hirosumi et al., 2002; Kershaw and Flier, 2004; Qiao et al., 1999; Uysal et al., 1997). Consequently, signalling via PI3-K and Akt is prevented and glucose transport is reduced. Importantly, it has also been shown that elevated free fatty acid levels

provide a major stimulus for the initiation of inflammatory events leading to the disruption of insulin receptor signalling (Nguyen et al., 2005). In rodent models, lipid infusion and high fat feeding have become well established methods of inducing a state of insulin resistance (Kraegen et al., 2001)

1.5 Inflammation, obesity and insulin resistance

Previously, metabolic conditions such as insulin resistance were considered to be separate from states of inflammation. However, recent evidence suggests that pro-inflammatory cytokines, hormones and adipokines play a role in the mediation of insulin resistance (Guzik et al., 2006; Hotamisligil, 2006). In addition, inflammatory cytokines have been shown to activate mitogen activated protein kinases (MAPK), including c-jun N-terminal kinase (JNK) in the onset of insulin resistance (Hirosumi et al., 2002).

Amongst the pro-inflammatory cytokines, TNFα has consistently been implicated in the development of insulin resistance. In rodent and human models of obesity and diabetes, expression of TNFα is abnormally elevated in adipose tissue and skeletal muscle (Hotamisligil et al., 1993; Kern et al., 1995; Saghizadeh et al., 1996). More specifically, the expression of TNFα in adipose tissue and skeletal muscle impairs tyrosine phosphorylation of the insulin receptor (Uysal et al., 1997). This impairment is the result of TNFα mediated serine phosphorylation of IRS-1, particularly on ser^{307} residues (Rui et al., 2001). However, genetic deletion of TNFα restores insulin receptor tyrosine phosphorylation, improving whole body insulin sensitivity and glucose tolerance (Uysal et al., 1997).

These studies were amongst the first to show a molecular link between inflammation and obesity related disorders. It is now clear that TNFα is not the only inflammatory mediator associated with obesity and insulin resistance. Studies from the last 10 years have demonstrated that increased fatty acid levels can also activate protein kinases, including JNK, which has been associated with states of inflammation, (Wellen and Hotamisligil, 2005; Xia et al., 2000). JNK is a stress-induced protein kinase, controlled by three genes corresponding to the expression of three distinct isoforms JNK1, JNK2 and JNK3 (Chang and Karin, 2001). Activation of JNK is dependent upon phosphorylation of upstream kinases, MAPKK4 and MAPKK7, usually following exposure to chemical, environmental and physical stress (Chang and Karin, 2001). Phosphorylation of JNK results in downstream phosphorylation of the transcription factor, c-jun (Manning and Davis, 2003). Phosphorylation of c-jun is known to be involved in multiple cellular activities including cell development, survival and stress induced cell death or apoptosis (Fig. 1.4) (Chang and Karin, 2001; Davis, 2000; Tournier et al., 2000).

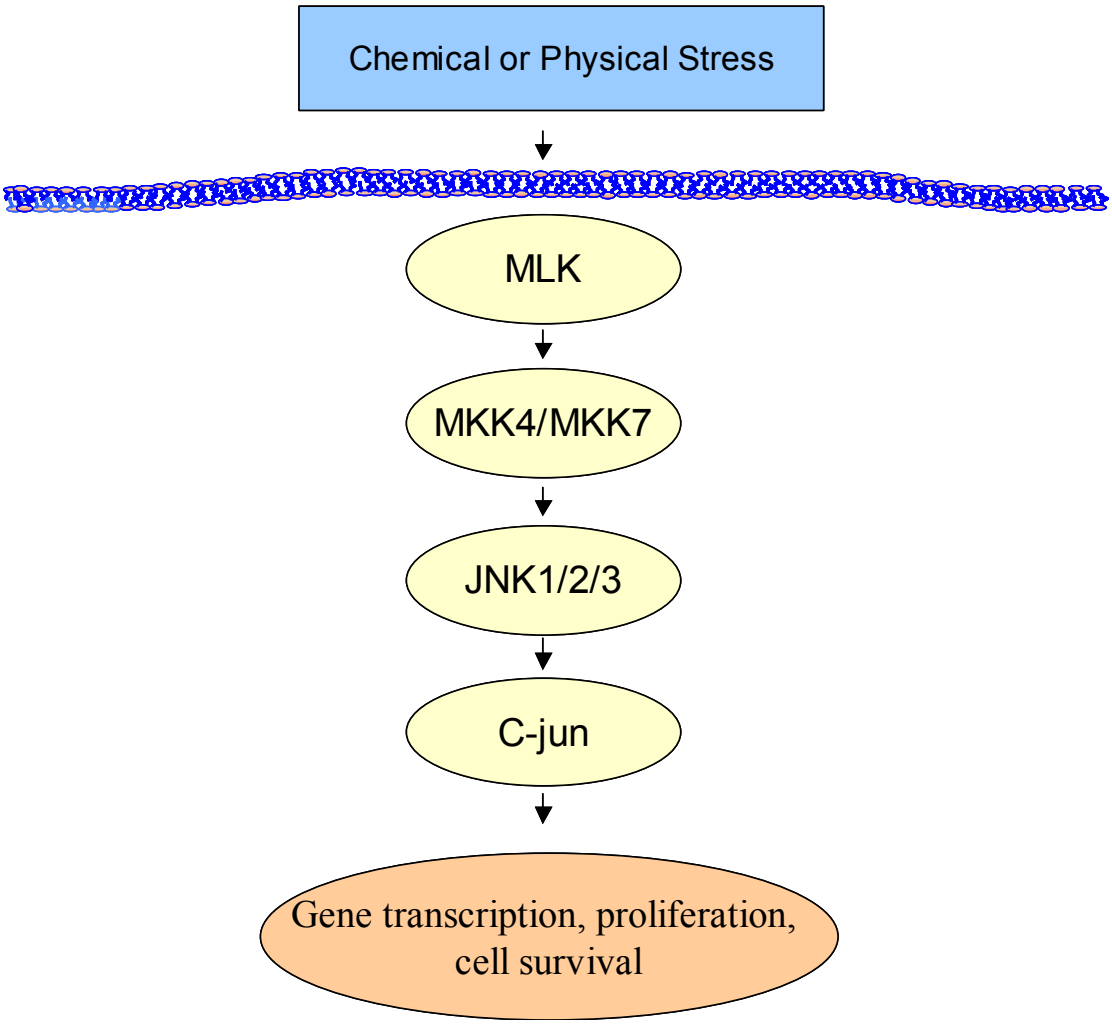


Fig. 1.4 JNK Signalling Cascade (Chang and Karin, 2001).

Recently, the expression of JNK has been implicated in states of obesity and insulin resistance (Hirosumi et al., 2002). In mice fed a high fat diet (HFD), genetic deletion (*JNK1*$^{-/-}$) and chemical inhibition of the JNK1 isoform enhanced tyrosine phosphorylation of IRS-1, while decreasing Ser^{307} phosphorylation of IRS-1 in the liver. The overall effect was an improvement in whole-body glucose tolerance and insulin sensitivity as measured by glucose and insulin tolerance tests. Furthermore, treatment of adipocytes with free fatty acids increased activation of JNK and TNFα, impairing tyrosine phosphorylation of the insulin receptor, IRS-1 and serine/threonine phosphorylation of Akt (Nguyen et al., 2005). The disruption of insulin signalling also resulted in a 50% decrease in GLUT 4 translocation and a 70% decrease in glucose

uptake. However, when JNK activity was inhibited using transfection of JNK-specific siRNA, restoration of insulin signalling and glucose uptake was observed (Nguyen et al., 2005). Taken together, it is evident that TNFα is highly expressed in models of obesity and insulin resistance. Moreover, obesity induced inflammation is also associated with JNK activation, a stress kinase capable of inducing serine phosphorylation of the insulin receptor and IRS proteins.

Interestingly, activation of JNK is not the only mechanism by which insulin signalling is disrupted. Studies of inflammation, immunity, cancer and insulin resistance have clearly established the activity of a classic inflammatory pathway, involving the inhibitor of kappa B kinase (IKK) complex and its downstream effector, nuclear factor kappa beta (NF-κB) (Gao et al., 2002; Karin, 1999; Wullaert et al., 2006). Nuclear factor kappa beta (NF-κB), is a ubiquitous transcription factor that is expressed in most cell types and is capable of mediating various biological responses including regulation of cell death and cell survival (Kim et al., 2007). Important to this review, NF-κB is also a key transcriptional regulator of inflammatory cytokines, including TNFα, IL-1, IL-2 and IL-6 (Cai et al., 2005; Ghosh and Karin, 2002; Wullaert et al., 2006). Initially, NF-κB is bound to the inhibitor of κB (IκB) protein within the cytosol of a cell. In response to inflammatory stimuli, the inhibitor of κB kinase (IKK), consisting of alpha (α), beta (β) and gamma (γ) subunits, is phosphorylated and activated (Karin and Greten, 2005). Activated IKK then targets NF-κB-bound IκB, facilitating proteosomal degradation of IκB, allowing for nuclear translocation of liberated NF-κB dimers and transcription of genes associated with cytokine production (Gao et al., 2002; Ghosh and Karin, 2002; Wullaert et al., 2006).

In the context of insulin resistance, it has been shown that inhibition of IKKβ through genetic deletion or use of anti-inflammatory agents such as aspirin and salicylates, improves fasting glucose levels, insulin sensitivity and glucose tolerance in obese rats (Yuan et al., 2001). Conversely, constitutive expression of IKKβ in mice caused increased fasting insulin levels, pronounced hepatic insulin resistance and moderate systemic insulin resistance (Cai et al., 2005). In addition, treatment of hepatoma cells with TNFα results in activation of both IKKαβ isoforms and sustained serine phosphorylation of IRS-1 (Gao et al., 2002). The mechanism by which TNFα-induces IRS-1 ser^{307} phosphorylation has been shown to involve the formation of an IKK/IRS-1 complex, specifically through the actions of the IKKαβ scaffold protein, nuclear factor-κB essential modulator (NEMO) and motor protein Myo1c (Nakamori et al., 2006). Importantly, this study showed that treatment of 3T3-L1 adipocytes with insulin induced translocation of NEMO containing the IKK complex to membrane ruffles, a process which requires the binding of NEMO to Myo1c, which then facilitates the interaction of the IKK complex and IRS-1 ser^{307}

Further investigation into the source of JNK1/2 and IKKαβ activation have led to the examination of bioactive fatty acid metabolites such as diacylglycerol (DAG) and ceramide (Schenk and Horowitz, 2007; Watt et al., 2006). It has previously been shown that oversupply of lipids through Intralipid infusion (Watt et al., 2006) or high fat diet (Schmitz-Peiffer et al., 1997) can result in the accumulation of DAG and ceramide within skeletal muscle, resulting in an impairment of IRS-1 mediated PI3K and Akt phosphorylation (Yu et al., 2002). Importantly, the accumulation of fatty acid metabolites have also been implicated in the activation of JNK1/2 and IKKαβ (Schenk and Horowitz, 2007; Watt et al., 2006). Taken together, it is evident that an increase in fatty acids is associated with increased activation of inflammatory cytokines and

stress kinases, resulting in disruption of IRS-1 mediated signalling events and ultimately glucose homeostasis (Fig. 1.5).

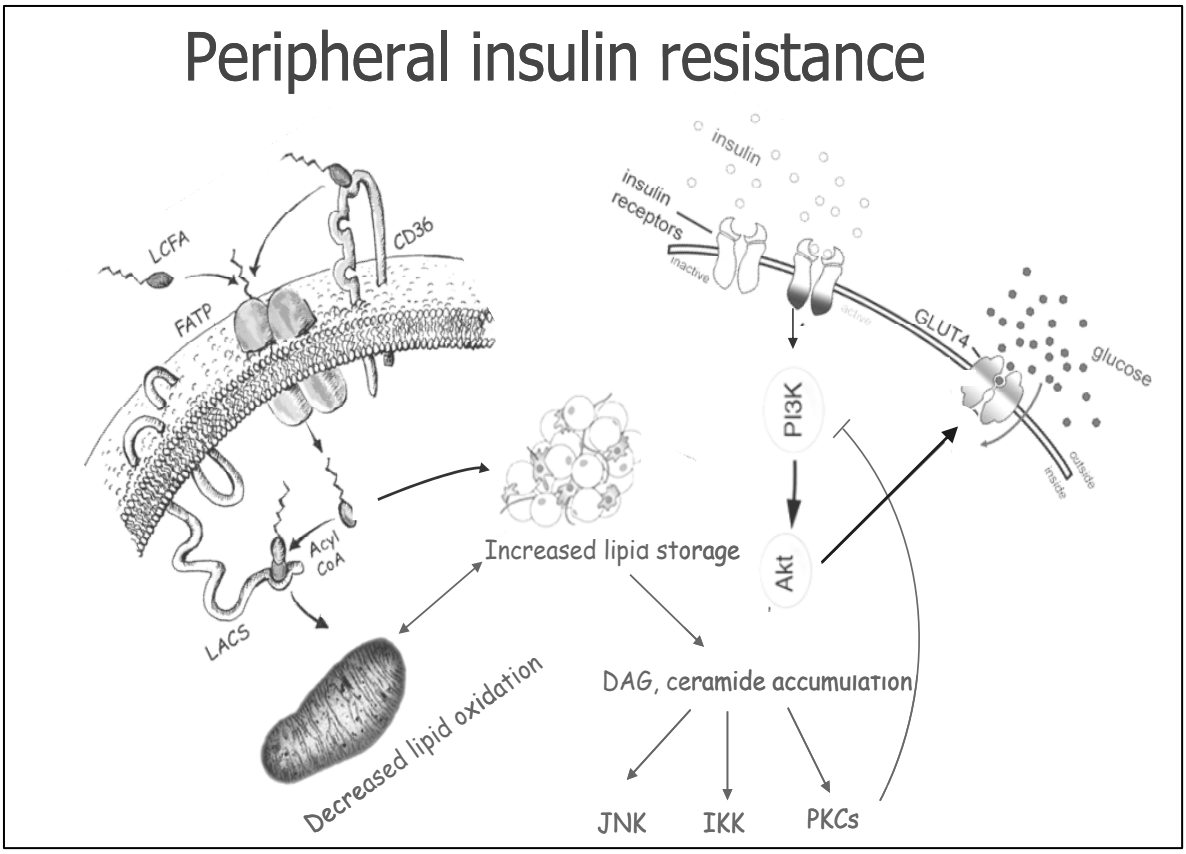


Fig. 1.5 Proposed mechanism of insulin resistance. Overnutrition leads to increased availability of long chain fatty acids (LCFA) and storage of lipid species, including diacylglycerol (DAG) and ceramide, causing increased production of inflammatory cytokines and disruption of the insulin signalling pathway amongst metabolically active tissues.

1.6 Insulin resistance, mitochondrial function and oxidative capacity

Currently, a substantial body of evidence suggests a putative role of intracellular fatty acid metabolites such as fatty acyl coenzyme As (fatty acyl CoAs), diacylglycerol (DAG) and ceramides in the onset of insulin resistance (Lowell and Shulman, 2005).

Under normal conditions, mitochondria function to convert glucose and fatty acids into ATP through oxidative processes involving β-oxidation, the citric acid cycle and electron transport chain (Alberts et al., 2002). However, during conditions of lipid oversupply, disruptions in mitochondrial content, enzyme activity and their ability to oxidize fatty acids have been suggested to lead to insulin resistance (Lowell and Shulman, 2005). Indeed, elderly subjects with an insulin resistant phenotype in skeletal muscle displayed decreased mitochondrial oxidative-phosphorylation activity by up to 40% compared to young healthy controls (Petersen et al., 2003). In addition, muscle biopsies from offspring with a family history of type 2 diabetes showed a 38% decrease in mitochondrial density, corresponding with a 50% increase in phosphorylation of serine residues of IRS-1 (Morino et al., 2005). Using electron microscopy of human skeletal muscle, it was also shown that the size of mitochondria were significantly smaller in obese and diabetic patients (Kelley et al., 2002). Furthermore, the maximal activity of mitochondrial enzymes, such as citrate synthase were shown to be reduced in patients with type 2 diabetes (Kelley et al., 2002), while β-hydroxyacyl-CoA-dehydrogenase (β-HAD) activity was significantly lower in diabetic subjects compared to well trained athletes (Bruce et al., 2003a).

These findings may be due to decreased expression of nuclear-encoded genes that regulate mitochondrial biogenesis, including those activated by peroxisome proliferator-activated receptor coactivator 1α (PGC-1α) (Mootha et al., 2003; Patti et al., 2003; Wu et al., 1999) and PGC-1β (St-Pierre et al., 2003). Using DNA microarray, expression of PGC-1 α and genes involved in oxidative phosphorylation were coordinately reduced in skeletal muscle of diabetic humans (Mootha et al., 2003; Patti et al., 2003). Results from these studies suggest that the accumulation of fatty acids observed in models of insulin resistance may be due to disrupted

mitochondrial function or reduced mitochondrial density leading to impaired fatty acid oxidation. However, uncovering the mechanism by which fatty acids induce mitochondrial defects has proven to be a significant challenge, particularly with recent studies that have shown opposing results (Garcia-Roves et al., 2007; Turner et al., 2007). In rodent models, high fat feeding was shown to increase mitochondrial biogenesis in skeletal muscle, concomitantly increasing PGC-1α expression and the activity of mitochondrial enzymes associated with fatty acid oxidation (Garcia-Roves et al., 2007; Turner et al., 2007). The upregulation of mitochondrial oxidative capacity observed with high fat feeding has been suggested to be a compensatory response to increased lipid availability (Turner et al., 2007). Nonetheless, it is evident that insulin resistance is a complex metabolic condition, involving numerous molecular targets associated with inflammation and fatty acid oxidation. As such, studies have begun to explore therapeutic targets with the potential to combat inflammation and potentially improve fatty acid oxidation. Recently, heat shock protein 72 (HSP72) has been identified as a protein with potential therapeutic benefits (Bruce et al., 2003b)

1.7 Biological role of Heat Shock Protein 72 (HSP72)

Heat shock protein 72 (HSP72) is a member of the HSP70 family, a subset of a larger family of proteins collectively known as heat shock proteins (HSP's) (Hartl, 1996; Lindquist, 1986). The HSP family consists of many members which are highly conserved in organisms ranging from bacteria to humans (Aufricht, 2005; Lindquist, 1986). Although the nomenclature can be misleading, one of the best known functions of heat shock proteins is their ability to facilitate proper folding of newly

formed proteins under normal conditions, thus they are often referred to as "molecular chaperones" (Hartl, 1996; Young et al., 2004).

A crucial step in the formation of nascent proteins involves folding of linear polypeptides into a three-dimensional, tertiary structure (Young et al., 2004). However, *in vivo*, this process is complicated by the presence of numerous other proteins and nucleic acids which readily aggregate to exposed hydrophobic sites on newly formed polypeptides (Flynn et al., 1991; Young et al., 2004). In order to prevent protein aggregation, HSP's bind to nascent polypeptides as they emerge from ribosomes, thus allowing for proper folding to occur (Hartl, 1996; Morimoto, 1993; Young et al., 2004). In higher eukaryotes, this process involves the constitutively active member of the HSP70 family, known as heat shock cognate 70 (HSC70), as well as HSP90 (Fink, 1999; Hartl, 1996; Young et al., 2004).

However, under conditions of physiological, environmental and chemical stress, cells produce another member of the HSP70 family, the stress-inducible HSP72 (Hartl, 1996). Initial observations of changes in *Drosophila* salivary glands following exposure to heat (Ritossa, 1962) inspired the discovery of HSP72 nearly 40 years ago (Lindquist, 1986). Since then, it has been shown that HSP72 expression is increased upon exposure to numerous stressors including heat, UV radiation, oxidants, toxins, ischemia and viral infections (Dorion and Landry, 2002; Hartl, 1996; Morimoto, 1993).

Extensive studies into the biological function of HSP72 have clearly established its role in the propagation of the stress response, a protective process initiated by cells to prevent excessive protein damage during stressful conditions (Erbse et al., 2004;

Lindquist, 1986; Morimoto, 1993). During states of cellular stress, proteotoxicity increases, giving rise to denatured and misfolded proteins (Aufricht, 2005). To combat this problem, HSP72 binds to damaged native proteins, as well as newly formed polypetides, ensuring proper folding and prevention of deleterious aggregation (Fink, 1999). Therefore, the combined action of HSP70 proteins ensures protein stability and function during normal conditions and conditions of cellular stress.

In mammals, expression of HSP72 is controlled at the transcriptional level by Heat Shock Factor (HSF) -1, 2 and 4. This review will focus primarily on the regulation of HSF-1. Under normal conditions, HSF-1 is bound to constitutive HSC70, therefore, HSF-1 is maintained in a monomeric, non-DNA binding form in both the cytosol and nucleus (Morimoto, 1993). Upon exposure to stress, denatured proteins also bind competitively to constitutive HSC70 (Aufricht, 2005), allowing activation of HSF-1 through its trimerization and translocation to the nucleus. Once in the nucleus, HSF-1 binds to the heat shock elements (HSE), a specific DNA sequence located in the 5'-flanking sequences of heat shock-responsive genes. At this point, the HSF-1-HSE complex is phosphorylated, transcription occurs and HSP72 levels are increased (Morimoto, 1993). The newly formed HSP70 proteins also act to attenuate the heat shock response by binding to HSF-1 in a manner that dissociates HSF-1 trimers and restores HSF-1 inert monomers (Fig. 1.6) (Morimoto, 1998).

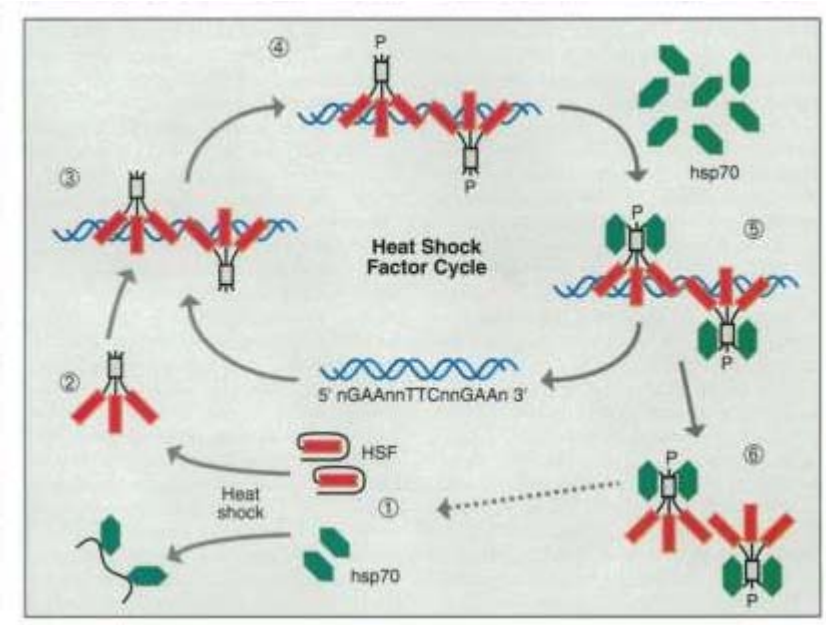


Fig. 1.6 HSF-1 regulation and production of HSP70 proteins (Morimoto, 1993)

As with any protein, an understanding of its structural conformation assists in understanding its functional properties. All HSP70 proteins, have an N-terminal ATPase domain, a substrate binding domain and a C-terminal domain, which combine to form a 70 kDa polypeptide (Erbse et al., 2004). Proper folding of new polypeptides, involving HSP70 proteins, occurs in an ATP-dependent manner (Wegele et al., 2004). Binding of ATP to HSP72 results in a conformational shift in the other two domains such that substrate binding affinity is reduced. Conversely, ADP bound HSP70 proteins display a high affinity to polypeptide substrates (Wegele et al., 2004). In the presence of newly formed polypeptides, containing exposed hydrophobic patches, HSP70 proteins readily bind via the substrate binding domain (Agard, 1993; Wegele et al., 2004). Binding of polypeptide substrates increases ATPase activity of HSP70, thus ATP is hydrolysed to ADP, resulting in a conformational shift that essentially wraps and secures HSP70 to the polypeptide substrate (Wegele et al., 2004). In this manner, HSP70 proteins occupy the

hydrophobic sites of a new polypeptide, preventing aggregation with other polypeptides.

Although the role of HSP70 proteins in protein folding and the stress response has been described at the cellular level, its protective benefits have also been characterized in whole-body, mammalian models (Haveman et al., 2004; King et al., 2002; Shinohara et al., 2006). One method of examining HSP72 *in vivo* involves the induction of whole-body hyperthermia, a process typically requiring elevation of core body temperature above 37°C (Haveman et al., 2004; King et al., 2002; Shinohara et al., 2006).

Although individual studies have used various protocols of hyperthermia, it is clear that elevation of body temperature in rodents to approximately 40°C, for periods of less than 30 minutes, is sufficient to increase HSP72 gene transcription and protein expression in numerous tissues (King et al., 2002; Shinohara et al., 2006). More importantly, hyperthermia-induced HSP72 expression in these models confers protection from various physiological disruptions, including ischemic injury, production of reactive oxygen species (ROS) and apoptotic cell death (Benjamin and McMillan, 1998; King et al., 2002).

Taken together, it is clear that HSP70 proteins have a wide range of biological activities, in both regulatory and stress-related conditions. As such, researchers have begun to analyse the potential role of HSP70 in the regulation of inflammatory responses and disease.

1.8 HSP70 proteins and inflammation

As previously described, TNFα, NF-κB and JNK are intricately involved in the cellular response to inflammation. Interestingly, recent evidence suggests that HSP70 proteins are capable of interacting with these classic inflammatory markers in a manner that produces anti-inflammatory effects (Yenari et al., 2005).

In human lymphoma cells, incubation at 43°C for 15 minutes, followed by 6 hours of recovery at 37°C, significantly increased HSP70 expression (Guzhova et al., 1997). More importantly, heat shock-induced HSP70 accumulation attenuated NF-κB activation, as compared with non-heat treated controls, following treatment with bacterial lipopolysaccharide (LPS) and the phorbol ester, phorbol myristoylacetate (PMA). Similarly, rats exposed to whole-body hyperthermia, where body temperature was raised to 41°C for 20 minutes, followed by 24 h of recovery at room temperature, displayed significant HSP70 expression in the brain (Heneka et al., 2000). Pre-treatment with heat significantly reduced NF-κB activity, as expressed by p65 subunit quantification, compared to non-heat treated controls, following LPS and cytokine injection of the brain.

Exactly how HSP70 is inhibiting NF-κB activity remains unclear, however, the use of co-immunoprecipitation techniques provides evidence of a physical association between the two proteins (Guzhova et al., 1997). From the same lymphoma cells, HSP70 was immunoprecipitated through incubation with an anti-HSP70 antibody. Interestingly, the p65 subunit of NF-κB was found to co-immunoprecipitate with HSP70, providing evidence of a physical association and possible mechanism of HSP70-mediated inhibition of NF-κB (Guzhova et al., 1997). In addition, liposomal

delivery of HSP70 in renal tubular cells exposed to ischemia displayed reduced NF-κB-DNA binding, as demonstrated by electrophoretic mobility shift assay (EMSA) (Meldrum et al., 2003). HSP70 has also been shown to directly interact with IKKγ, inhibiting its actions and subsequently inhibiting NF-κB activation (Ran et al., 2004) *In vivo,* rats exposed to whole-body hyperthermia displayed increased expression of IκB, the protein responsible for inhibition of NF-κB (Heneka et al., 2000), however, liposomal delivery of HSP70 did not show any changes in ischemia-induced IκB degradation (Meldrum et al., 2003), making the role of IκB slightly controversial.

Parallel to studies of NF-κB inhibition, HSP70 has also been shown to inhibit JNK activity, through direct protein-protein interaction or inhibition of various members of the JNK signalling pathway (Gabai et al., 1997; Hwang et al., 2005; Mosser et al., 1997; Park et al., 2001; Stankiewicz et al., 2005). In human lymphoid tumor cells, initial exposure to heat shock, 20 minutes at 43°C, prevented apoptotic cell death upon exposure to a severe bout of heat stress (60 minutes at 43°C) (Gabai et al., 1997). Importantly, pre-treatment with heat also inhibited expression of both JNK1 and JNK 2 isoforms following exposure to severe heat or 9% ethanol solution. In addition, transfection of plasmids encoding for HSP72 resulted in HSP72 overexpression, inhibition of JNK and protection from osmotic shock, H_2O_2 and UV radiation (Gabai et al., 1997).

Conversely, HSP70 deficiency leads to enhanced activation of JNK and apoptotic cell death (Lee et al., 2005a). In mouse embryonic fibroblasts, generated from *HSP70*$^{-/-}$ mice, reduced HSP70 protein expression led to earlier and more pronounced JNK expression following hyperosmotic stress treatment. In order to understand the mechanism by which HSP72 inhibits JNK activation, the physical association

between the two proteins was also examined (Park et al., 2001). As observed in previous studies, pre-treatment of cells with heat or transfection of cells with HSP72 vectors, prevented UV-induced JNK activation. Using immunoprecipitation of cell lysates, it was shown that both HSP72 and JNK1 form a direct physical association (Park et al., 2001). In addition, specific mutation of each HSP72 binding domain (ATP, substrate and C-terminal) revealed that the substrate binding domain of HSP70 is critical in the binding and inhibition of JNK (Park et al., 2001).

Recently, inhibition of JNK has also been examined through HSP72 mediated inhibition of upstream MAP Kinases (Daviau et al., 2006). It has been shown that dual leucine zipper-bearing kinase (DLK), an upstream kinase associated with JNK activation, can be negatively regulated in the presence of HSP72 and its co-chaperone, c-terminal HSP-interacting protein (CHIP) (Daviau et al., 2006). In COS-7 cells overexpressing DLK, a physical association between HSP72 and DLK was observed in the presence of functional CHIP, increasing proteosomal degradation of DLK and reduced JNK phosphorylation (Daviau et al., 2006). Additional work by others suggest that HSP72 is involved in mediation of MAP kinase phosphatase-1 (MKP-1) (Lee et al., 2005b). MAP kinase phosphatases function to inactivate MAP kinases, including JNK. Upregulation of HSP72 by heat treatment has been shown to increase phosphorylation of MKP-1, thus accelerating the inactivation of JNK following exposure to heat stress (Lee et al., 2005b).

As mentioned previously, the accumulation of fatty acid metabolites and the onset of inflammation may be the result of disrupted mitochondrial function (Lowell and Shulman, 2005). Given the diverse biological role of HSP70 proteins, numerous studies have also examined the interaction between HSP72 and mitochondrial

function. As a component of normal biogenesis, mitochondria rely on importation of nuclear encoded proteins from the cytosol to the mitochondrial matrix (Stuart et al., 1996). Recent evidence suggests HSP70 proteins mediate protein importation from the cytosol to the matrix via its interaction with the mitochondrial outer membrane import receptor, translocase of the outer membrane 70 (Tom70) (Fig. 1.7) (Young et al., 2003).

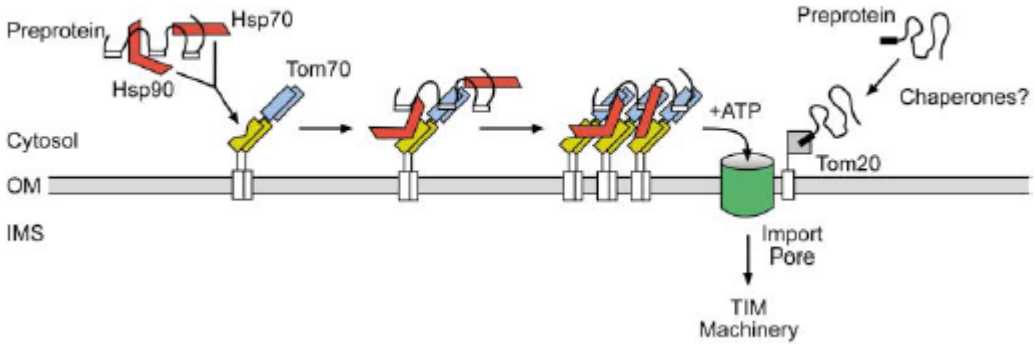


Fig. 1.7 HSP70 mediated importation of precursor proteins from the cytosol to the mitochondrial matrix via Tom70 (Young et al., 2003).

In addition, mitochondria were shown to be selective targets for the protective effects of heat shock against oxidative injury (Polla et al., 1996). During H_2O_2 treatment of human premonocytic cells, expression of HSP72 correlated with maintenance of mitochondrial membrane potential, mass and ultrastructure, thus providing protection from oxidative stress (Polla et al., 1996). Furthermore, expression of HSP72 has been shown to reduce the production of reactive oxygen species during glucose deprivation (Ouyang et al., 2006) and to preserve mitochondrial respiratory function in myocardial tissue exposed to ischemia-reperfusion injury (Jayakumar et al., 2001; Suzuki et al., 2002). As mentioned previously, reduced mitochondrial function is associated with various models of insulin resistance (Lowell and Shulman, 2005),

thus, protection from mitochondrial damage or preservation of mitochondrial activity may provide an additional mechanism by which HSP72 protects against obesity induced insulin resistance.

In summary, HSP70 proteins have been shown to inhibit markers of inflammation, including members of the NF-κB and JNK pathway. As previously reported, inflammatory signals have been implicated in the development of metabolic complications, including insulin resistance and type 2 diabetes. Moreover, expression of HSP72 has been associated with protection from mitochondrial damage and improved mitochondrial function. However, studies of HSP70 proteins, insulin resistance and type 2 diabetes have been limited.

1.9 The role of HSP70 in insulin resistance and type 2 diabetes

Although a distinct connection between HSP70 expression, inflammation and insulin resistance has not yet been established, several studies have reported associations between HSP70 and states of insulin resistance and type 2 diabetes (Atalay et al., 2004; Bruce et al., 2003b; Kurucz et al., 2002).

Interestingly, it has previously been reported that elevation of core body temperature, in patients diagnosed with type 2 diabetes, improves insulin sensitivity and glucose homeostasis (Hooper, 1999). Core body temperature was raised (mean of 0.8°C above basal) in diabetic patients via hot-tub immersion, 30 minutes a day, 6 days a week for 3 weeks. Following hot-tub therapy, patients displayed decreased body weight, fasting plasma glucose and glycosylated haemoglobin levels (Hooper, 1999). Although this study did not report any mechanism by which glucose homeostasis was

improved, it provided initial evidence of an association between heat treatment and type 2 diabetes.

Taking this idea a step further, it has been suggested that HSP70 proteins could be a key component in the protection against insulin resistance and type 2 diabetes (Atalay et al., 2004; Bruce et al., 2003b; Kurucz et al., 2002). From skeletal muscle biopsies of patients with type 2 diabetes, mRNA concentrations of HSP72 were shown to be significantly reduced (Kurucz et al., 2002), as much as 55% compared with healthy controls (Bruce et al., 2003b). In addition, patients displaying reduced HSP72 mRNA concentrations also displayed reduced glucose disposal capability as measured by euglycemic-hyperinsulinemic clamp, indicating a correlation between HSP72 and insulin resistance (Bruce et al., 2003b). In the context of mitochondrial oxidative capacity, diabetic patients also demonstrated an inverse relationship between HSP72 expression and citrate synthase and β-HAD activity (Bruce et al., 2003b).

Taken together, HSP70 proteins may have a potential role in the prevention of insulin resistance and type 2 diabetes. However, the existing body of literature does not provide evidence implicating the induction of HSP72 with the inhibition of inflammatory markers, enhancement of mitochondrial oxidative capacity and protection from insulin resistance. This thesis aims to address this paradigm.

Chapter Two

Aims of the Thesis

2.1 Aims of the thesis

The primary aim of this thesis was to determine if the expression of heat shock protein 72 (HSP72) protects against high fat diet-induced insulin resistance. This investigation involved the use of *in vivo* rodent models, whole-body measurements of glucose homeostasis and tissue examination using various analytical techniques.

HSP72, a protein involved in protection from protein denaturisation during exposure to hyperthermia and various environmental stressors, has also been implicated in insulin resistance and type 2 diabetes. Previous reports demonstrated decreased HSP72 mRNA in patients with type 2 diabetes and improvements in glucose homeostasis upon exposure to hyperthermia treatment. In addition, HSP72 has been shown to inhibit inflammatory mediators associated with insulin resistance, *in vitro*. Therefore, the aim of the first experiment (Chapter 5) was to determine if induction of HSP72 by hyperthermia could protect against diet-induced insulin resistance and to examine the potential mechanisms by which this protection occurs.

Based on results of the first study, the second experiment (Chapter 6) was designed to control the specificity of HSP72 induction through the use of a transgenic mouse model ($HSP72^{+/+}$). The aim of this study was to examine whether overexpression of HSP72, in a muscle-specific manner, led to phenotypic alterations that could protect against diet-induced insulin resistance. In addition, to determine specific differences in insulin sensitivity amongst metabolically active tissues, the euglycemic-hyperinsulinemic clamp technique was characterized and applied in this study.

Finally, based on observations of decreased adipose tissue mass and inflammation in the liver of HSP72$^{+/+}$ mice, the aim of the third experiment (Chapter 7) was to examine the possibility of enhanced mitochondrial oxidative capacity in the liver, adipose tissue and skeletal muscle of HSP72$^{+/+}$ mice and whether these differences were associated with alterations in circulating adipokines.

Chapter Three

General Methods

3.1 Animals

Eight-week old wild type (WT) male CB6-F1 (C57 X B/C) mice were purchased from The Walter and Eliza Hall Institute (WEHI, Kew, Victoria, Australia). Transgenic $HSP72^{+/+}$ mice were bred on the same background strain at Monash Mouseworks (Clayton, Victoria, Australia). Mice were housed at RMIT University Animal Facility under constant light and dark cycle (12 h) and room temperature was maintained at 21°C. All experimental procedures were approved by RMIT University Animal Ethics Committee. Following 1 week of acclimatization, mice were maintained on either standard chow diet or high fat diet (Table 3.1, Specialty Feeds, Western Australia), *ad libitum*, for a period of 16 weeks. Food consumption was measured for a period of 15 days and body mass once per week for 14 weeks.

3.2 Intraperitoneal Glucose (IPGTT) and Insulin (IPITT) Tolerance Test

Following 16 weeks of chow or high fat feeding, WT, heat-treated (HT) and $HSP72^{+/+}$ animals were exposed to an intraperitoneal glucose tolerance test (IPGTT), while only WT and $HSP72^{+/+}$ animals were given an intraperitoneal insulin tolerance test (IPITT). Prior to starting the IPGTT and IPITT, all mice were fasted for a period of 16-18h. Following the fasting period, an initial blood sample was taken via tail clip technique to assess basal glucose levels (time 0). All mice were then given an intraperitoneal injection of glucose (Baxter, 1.0 g/kg of body weight) for IPGTT or an intraperitoneal injection of human insulin (0.75 U/kg of body weight, Novolin, Novo Nordisk, New Jersey, USA) for IPITT. Blood samples were collected by reopening the tail wound at 15, 30, 60, 90 and 120 min time points. Blood glucose was

measured using a glucose analyser (Hemocue, Ängelholm, Sweden) and total area under the curve (AUC) was reported in each figure.

3.3 Tissue extraction

At the cessation of the experimental trial, all mice were anesthetized (sodium pentobarbital 0.1 mg/g). While unconscious, *gastrocnemius*, *soleus*, *extensor digitorum longus* and *vastus lateralis* muscles were removed from both hind limbs and immediately snap frozen in liquid nitrogen. In addition, the liver, epididymal and infrarenal adipose pads, kidneys, spleen, pancreas, brain and heart were removed and snap frozen.

3.4 Western Blot Analysis

Tissue samples weighing 20-30 mg were homogenized (Polytron: Brinkman Instruments, New York, NY, USA) in ice-cold homogenization buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3V0_4, 1% Triton X-100, 10% glycerol, 3 µL/mL protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride and 3 µL/mL phosphatase inhibitor cocktail). Homogenates were centrifuged (16,100 g, 30 min, 4ºC) and the supernatant removed. Protein content of tissue lysates were determined using 5 µL of tissue supernatant, diluted in 495 µL of milliQ water, incubated with protein detection reagents (Pierce, Rockford, IL, USA) and analysed on a plate reader (590 nm, Victor3, PerkinElmer, Turku, Finland) with a serial dilution of 1mg/ml bovine serum albumin (BSA) protein standard for comparison. Afterwards, 80 µg of protein was

solubilised in 4X LaemmLi buffer (40% glycerol, 8.2% Sodium dodecyl sulphate, 0.5M Tris-HCl pH 6.8, and 0.5% of 1% Bromophenol blue) and incubated at 95°C for 5 min, then stored at –80°C. Solubilized samples were then separated by SDS-PAGE on 6-12% polyacrylamide gels (1.5 M Tris, O.5 M Tris, 30% Bis-Acrylamide, 10% Sodium dodecyl sulfate, milliQ, 10% Amonium persulfate, TEMED). Gels were run at 140 V for 30 min and 150 V for approx 60 min, transferred to a nitrocellulose membrane (120 min: 0.06 Amps/membrane), washed 3 X 5 min in TBST (10% 10X Tris buffered saline pH 7.6, 0.5% Tween and 89.5% milliQ water) incubated in blocking buffer (5% skim milk in TBST) for 60 min at room temperature, washed again 4 X 5 min TBST, then incubated overnight (4°C) with appropriate antibody (for specific antibodies, see methods chapter 5 & 6). The next day, membranes were washed 5 X 5 min in TBST, then incubated in horseradish peroxidase-conjugated secondary antibody (1:2000 in 2.5% Bovine serum albumin and TBST; Amersham Biosciences, Castle Hill, NSW, Australia) and washed again 5 X 5 min in TBST. Membranes were then incubated with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, England) and immunoreactive proteins were detected using Chemidoc XRS imaging system (BIO-RAD, Hercules, USA). Protein quantification was performed using densitometry software (Quantity One 1-D).

3.5 Fasting Plasma Glucose and Insulin

Fasting plasma glucose was measured from blood samples taken at time 0 of IPGTT and IPITT (as described in 3.2 and 3.3). Fasting plasma insulin was measured by ELISA (#EZRM-13K, Linco Research, Missouri, USA).

3.6 Statistical Analysis

Results are expressed as the mean ± SEM. Data were analysed for differences by two-way analysis of variance (ANOVA) with specific differences located with a Student Newman Keuls post hoc test, $P<0.05$ was considered to be statistically significant.

Table 3.1a) Constituents of Chow Diet

Nutrient	% of total mass	Energy (MJ/kg)
Protein	19	3.89
Fat	4.6	1.68
Carbohydrate	76.4	11.07

Amino Acids Composition	% of total mass
Valine	0.87
Leucine	1.4
Isoleucine	0.8
Threonine	0.7
Methionine	0.3
Cystine	0.3
Lysine	0.9
Phenylalanine	0.9
Tyrosine	0.5
Tryptophan	0.2

Fat Composition	% of total mass
Myristic acid	0.03
Palmitic acid	0.5
Stearic acid	0.14
Palmitoleic acid	0.01
Oleic acid	1.9
Gadoleic acid	0.03
Linoleic acid	1.3
α Linolenic acid	0.3
Arachadonic acid	0.01
Eicosapentaenoic acid	0.02
Docosahexaenoic acid	0.05

Added Vitamins	
Vitamin A (Retinol)	10000 IU/kg
Vitamin D3 (Cholecalciferol)	2000 IU/kg
Vitamin K (Menadione)	20 mg/kg
Vitamin E (aTocopherol acetate)	100 mg /kg
Vitamin B1 (Thiamine)	80 mg /kg
Vitamin B2 (Riboflavin)	30 mg /kg
Niacin (Nicotinic acid)	100 mg /kg
Vitamin B6 (Pyridoxine)	25 mg /kg
Calcium Pantothenate	50 mg /kg
Biotin	300 μg /kg
Folic acid	5 mg /kg
Vitamin B12 (Cyanocobalamin)	150 μg /kg

Table 3.1b) Constituents of High Fat Diet

Nutrient	% of total mass	Energy (MJ/Kg)
Protein	19.5	3.99
Fat	36	13.17
Carbohydrate	44.5	6.45

Calculated Amino Acids Composition	% of total mass
Valine	1.3
Leucine	1.8
Isoleucine	0.9
Threonine	0.8
Methionine	0.8
Cystine	0.05
Lysine	1.5
Phenylalanine	1.0
Tyrosine	1.0
Tryptophan	0.3

Calculated Fat Composition	% of total mass
C12:0 and less	3.7
Myristic acid	1.1
Palmitic acid	7.2
Stearic acid	9.3
Arachidic acid	0.3
Palmitoleic acid	0.1
Oleic acid	11.7
Gadoleic acid	trace
Linoleic acid	1.7
α Linolenic acid	0.7
Arachadonic acid	trace
Eicosapentaenoic acid	trace
Docosahexaenoic acid	trace

Added Vitamins	
Vitamin A (Retinol)	4000 IU/kg
Vitamin D3 (Cholecalciferol)	1000 IU/kg
Vitamin K (Menadione)	1 mg/kg
Vitamin E (aTocopherol acetate)	75 mg/kg
Vitamin B1 (Thiamine)	6 mg/kg
Vitamin B2 (Riboflavin)	6 mg/kg
Niacin (Nicotinic acid)	30 mg/kg
Vitamin B6 (Pyridoxine)	7 mg/kg
Pantothenic acid	16 mg/kg
Biotin	200 μg /kg
Folic acid	2 mg/kg
Vitamin B12 (Cyanocobalamin)	100 mg/kg
Choline	1600 mg/kg

Chapter Four

The Euglycemic-Hyperinsulinemic Clamp

4.1 Introduction to the Euglycemic-Hyperinsulinemic Clamp (EHC)

The euglycemic-hyperinsulinemic clamp (EHC) is often referred to as the "gold standard" measure of insulin sensitivity. Prior to its development nearly 30 years ago (DeFronzo et al., 1979), insulin resistance was measured using various forms of glucose and insulin tolerance tests. However, the EHC is considered a superior measure as it allows for differentiation of hepatic and peripheral insulin sensitivity (Ayala et al., 2006; DeFronzo et al., 1979).

The EHC was originally designed to measure insulin sensitivity in humans, however, it has since been modified to include various animal (Camacho et al., 2005) and small rodent models (Burnol et al., 1983). An examination of studies in 2003-2004 demonstrated a tendency towards using the conscious EHC (Ayala et al., 2006), as opposed to the unconscious EHC, for mouse models of insulin resistance. Reservations towards the use of unconscious rodent EHC protocols may stem from evidence suggesting adverse effects of prolonged exposure to anesthetic agents (Clark et al., 1990; Saha et al., 2005). It has previously been shown that administration of anesthetic agents, including sodium pentobarbital, ketamine and xylazine are capable of increasing plasma glucose levels (Saha et al., 2005) and reducing hepatic glucose suppression following insulin stimulation (Clark et al., 1990). Thus, various EHC protocols have been designed to accommodate the conscious mouse model.

Briefly, the conscious EHC begins with a constant infusion of exogenous insulin, followed by exogenous infusion of glucose to maintain euglycemia (Ayala et al., 2006). Therefore, increases in the rate of exogenous glucose infusion (GIR) indicate

a greater degree of insulin sensitivity in the liver and peripheral tissues (Ayala et al., 2006; DeFronzo et al., 1981). In addition, advances in our knowledge of radioactive tracers, combined with the EHC, allows for assessment of the rate of endogenous glucose appearance (R_a) and glucose disappearance (R_d). With the advent of transgenic technology, the EHC has become particularly relevant in mouse models of insulin resistance. As the EHC in the conscious mouse has many advantages for evaluating insulin sensitivity, one major component of the work that constitutes this thesis was to establish this technique in our laboratory.

4.2 Euglycemic-Hyperinsulinemic Clamp in the Conscious Mouse: Materials and Methods

4.2.1 Cannulation of jugular vein. At 16 weeks of feeding on a chow diet, WT and HSP72$^{+/+}$ mice were implanted with dual jugular vein catheters (Micro-Renathane MRE 025, 0.025-inch outer diameter, 0.012-inch inner diameter; Braintree Scientific Inc., Braintree Massachusetts, USA) (Fig. 4.1). Initially, all mice were anesthetized with an intraperitoneal injection of a cocktail containing ketamine hydrocholoride (75 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa, USA), xylazine (14.6 mg/kg; Butler Co.), acerpromazine maleate (0.5 mg/kg; Butler Co., Columbus, Ohio, USA) and 0.9% saline. In order to isolate the jugular vein, a small incision was made on the anterior lateral surface of the neck. Once isolated, a small incision was made in the jugular vein using a 25 gauge needle and both catheters were inserted and secured with silk sutures. The catheters were then subcutaneously tunnelled to the dorsal aspect of the neck (Fig.4.2), exteriorized with another small incision and sealed with metallic plugs (Fig.4.3). During recovery from surgery, animals were given 1 mL of saline, injected subcutaneously and maintained on a heating pad for 24 h. Animals continued to recover in their cages for an additional 48 h at room temperature.

4.2.2 Basal Glucose Turnover and Tracer Infusion. At 66 h post-surgery, all mice were fasted for a period of 6 h. At 72 h, basal blood glucose was measured via tail clip technique (refer to 3.2). Basal glucose turnover was then assessed by infusion of a tracer solution (Fig. 4.4) containing 41.6 µL (4.16%) D-[3-^{3}H] Glucose (592 GBq/mmol; Amersham, Biosciences, United Kingdom), 100 µL (10%) of 8% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA) and diluted to a final volume of 1 mL with 0.9% saline. Tracer solution was infused at constant rate of 2 µL/min for

a period of 90 min. After 90 min, approximately 30 µL of whole blood was collected, separated into 2 aliquots of 15 µL and each mixed with 125 µL of zinc sulphate (0.3 N Sigma, St. Louis, Missouri, USA), 125 µL of barium hydroxide (0.3 N Sigma, St. Louis, Missouri, USA) and 25 µL of milliQ. Mixed blood samples were then centrifuged for 10 min (16,000 rpm) and supernatant was removed and stored at -80°C for analysis of basal glucose turnover.

4.2.3 Euglycemic-Hyperinsulinemic Clamp (EHC). Following tracer infusion, all mice were subjected to the EHC. Insulin infusate was created based on a 6 $mU.kg^{-1}.min^{-1}$ infusion rate and, therefore, contained 0.003 U/g insulin (Novolin R; Novo Nordisk Pharmaceutical Industries Inc., Clayton, North Carolina, USA), 41.6 µL (4.16%) D-[3-^{3}H] Glucose (592 GBq/mmol; Amersham, Biosciences, United Kingdom), 100 µL (10%) of 8% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA) and diluted to a final volume of 1 mL with 0.9% saline. Insulin infusate was infused at a constant rate of 2 µL/min for a period of 120 min. At the start of insulin infusion (Fig. 4.4), blood glucose was measured as described in 3.2 every 10 min. As blood glucose began to fall, a glucose solution containing 50% glucose (Abbott Laboratories, Chicago, Illinois, USA) was infused (Fig. 4.4) at varying rates in the remaining catheter to maintain euglycemia at a steady state of 6.4 $\pm$ 0.2 mmol/L, a value calculated from mean fasting blood glucose of all groups. Steady state was determined after 3 consecutive measures (30 min) of consistent plasma glucose were achieved. At the cessation of the EHC, whole blood samples were again extracted, mixed, centrifuged and stored as described in 4.2.2. In addition, all mice were immediately anesthetized and tissues were extracted as described in 3.3.

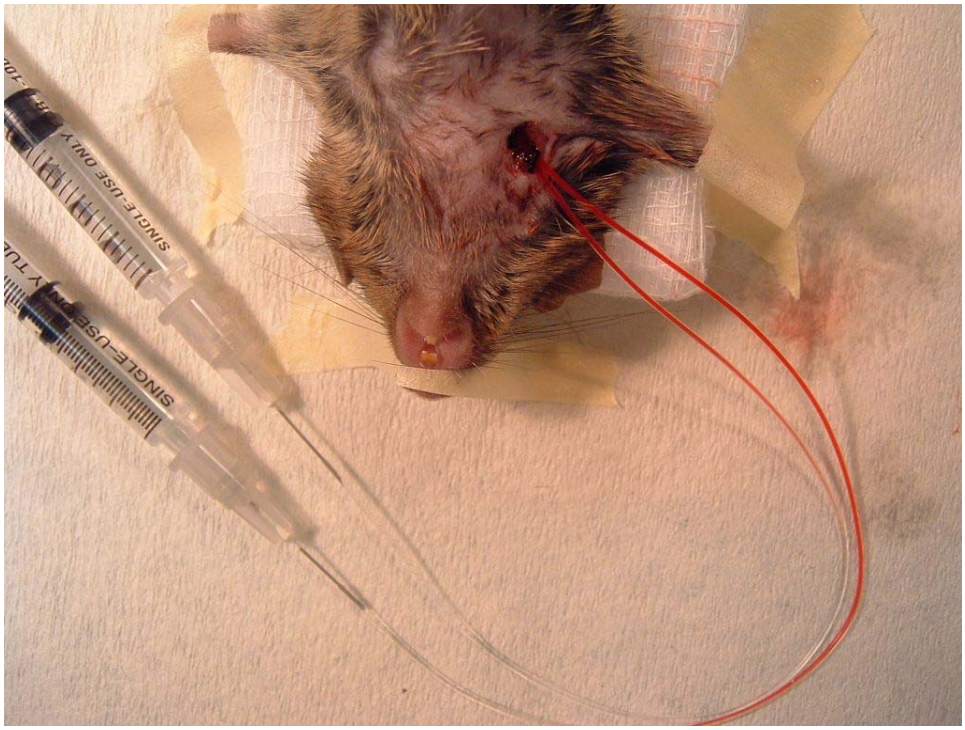


Fig. 4.1 Isolation and cannulation of jugular vein

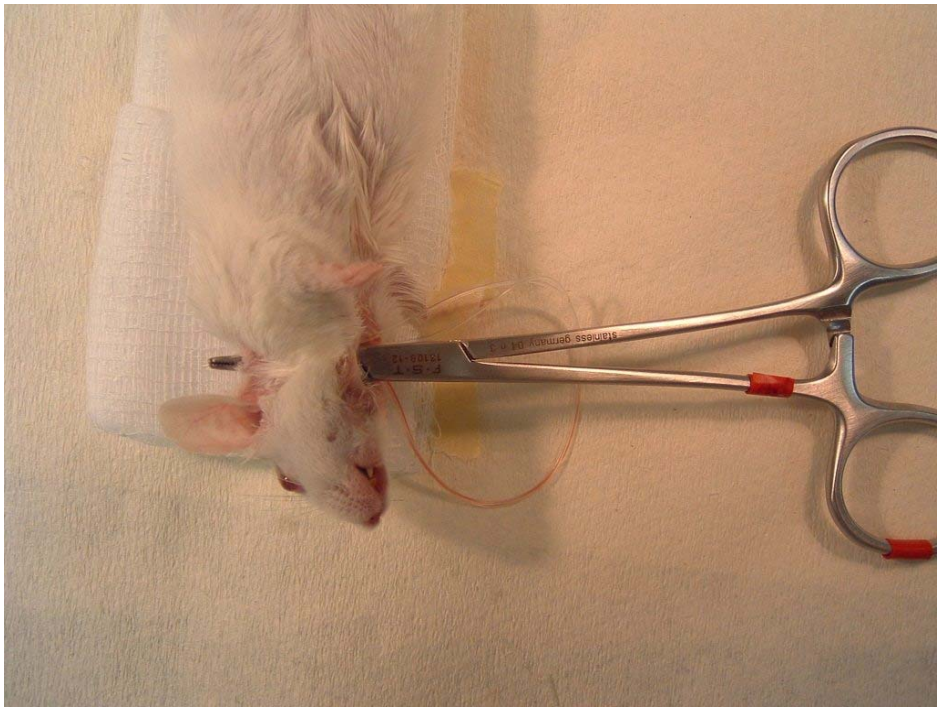


Fig. 4.2 Subcutaneous tunnelling of catheters to dorsal aspect of the neck

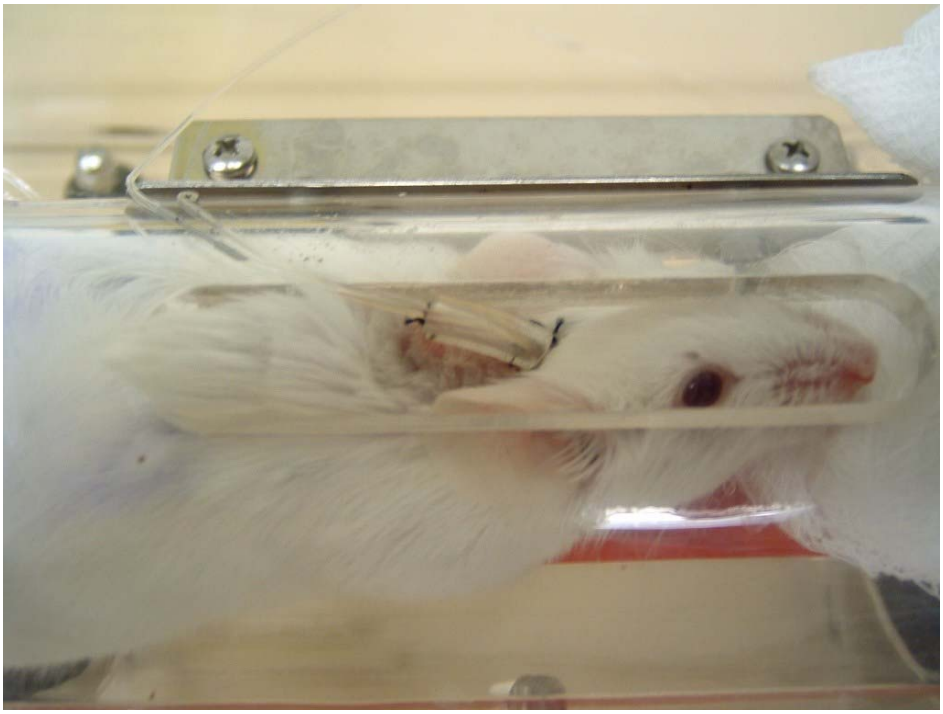


Fig. 4.3 Exteriorization and sealing of catheters

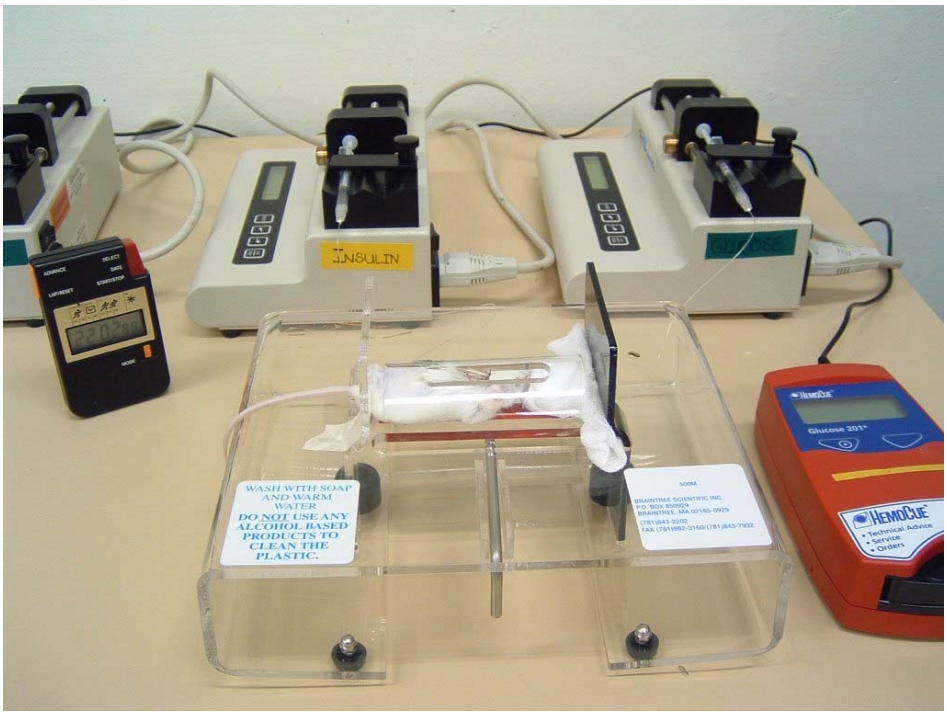


Fig. 4.4 Infusion pumps for tracer, insulin and glucose

Chapter Five

Effect of Heat Therapy on Diet Induced Insulin Resistance in Mice

5.1 Introduction

Previous studies have demonstrated that patients with type 2 diabetes have decreased HSP72 mRNA levels in skeletal muscle compared with healthy control subjects (Bruce et al., 2003b; Kurucz et al., 2002). In the study by Bruce et al. (2003), the authors also observed a significant correlation between HSP72 and insulin sensitivity, as measured by the glucose infusion rate during a euglycemic-hyperinsulinemic clamp, suggesting that HSP72 may play a role in the aetiology of insulin resistance. Interestingly, a preliminary clinical report showed that patients with type 2 diabetes exposed to regular hyperthermia via hot-tub therapy, improved their fasting glucose and glycosylated haemoglobin levels (Hooper, 1999). However, this report did not identify any molecular mechanisms by which hyperthermia led to improvements in glucose homeostasis.

The use of heat in the treatment of disease is not a new concept. Hyperthermia has been used as an adjuvant to radiation and chemotherapy amongst cancer patients for over a decade (Haveman et al., 2005; Horsman and Overgaard, 2007). As previously reported, hyperthermia has been established as a potent inducer of HSP72 in animal and cell culture models (Heneka et al., 2000; King et al., 2002; Wegele et al., 2004). The induction of HSP72 is typically associated with tolerance to environmental and chemical stressors (Gabai et al., 1997). However, its biological role has recently expanded to include inhibition of stress-related protein kinases, including c-jun N-terminal kinase (JNK) (Gabai et al., 1997; Park et al., 2001) and inhibitor of κB kinase (IKK) (Meldrum et al. 2003), both of which have been directly implicated in models of insulin resistance (Cai et al., 2005; Hirosumi et al., 2002).

In light of HSP72's role in increased stress tolerance and more specifically in blocking JNK and IKK, this project examined whether induction of HSP72 by hyperthermia could protect against high fat diet-induced insulin resistance. To test this hypothesis, a mouse model of diet-induced insulin resistance was combined with a model of hyperthermia and markers of inflammation and whole-body insulin resistance were examined.

5.2 Materials and Methods

Animals. Animals were used as described in Chapter 3.1.

Heat treatment. In order to determine whether heat treatment could induce HSP72 expression, 8 week old male WT mice, on both chow and high fat diet were exposed to a single bout of heat treatment. At the start of the treatment, all mice were lightly anesthetized with sodium pentobarbital (0.05 mg/g body weight). Once unconscious, mice were placed in a ventilated plastic container and wrapped in an electric heating blanket. Body temperature was measured using a thermal probe inserted rectally (TH-5 Thermalert Monitoring Thermometer, Physitemp Instruments Inc., New Jersey, USA). Body temperature was allowed to rise gradually for approximately 15 min and maintained at 41.5°C for 15 min by unwrapping and wrapping of the blanket. Following heat treatment, tissues were extracted immediately or at 1, 4, 8 and 24 h time points of recovery at room temperature. Once heat-induced HSP72 expression was established by western blot analysis, WT chow and WT HFD mice were subjected to sham (ST) or heat treatment (HT), once per week for 16 weeks. Mice receiving sham treatment (ST) were left at room temperature (21°C) while mice receiving heat treatment (HT) were exposed to the same heat treatment and recovery protocol as described above. At the cessation of 16 weeks of treatment, tissues were extracted 72 hours after the last heat treatment (See section 3.3).

IPGTT. Performed as previously described in Section 3.2. However, in this study a 120 minute time point was not included.

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). The homeostatic model assessment of insulin resistance (HOMA-IR) is an index of insulin resistance which requires fasting insulin and glucose levels. High HOMA-IR values indicate a greater degree of insulin resistance (Wallace et al., 2004). HOMA-IR is calculated using the following formula:

$$\frac{\text{Fasting serum insulin } (\mu\text{U/mL}) \times \text{Fasting serum glucose (mmol/L)}}{22.5}$$

Fasting insulin and fasting glucose levels were obtained as described in Section 3.5.

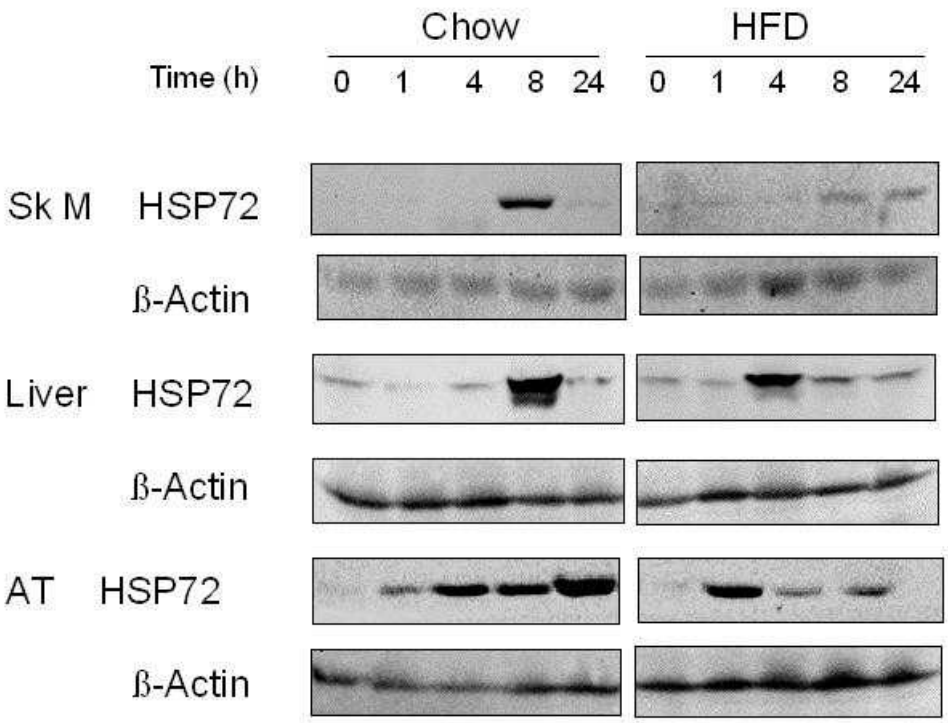
Western Blot Analysis. Protein content from tissue lysates were analysed as described in Section 3.4. Primary antibodies used in this study include HSP70 (HSP72) rabbit polyclonal antibody (Stressgen Bioreagents, Ann Arbor, Michigan, USA), HSP90 mouse monoclonal antibody (BD Transduction Laboratories, New Jersey, USA) phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) and SAPK/JNK rabbit polyclonal antibodies, phospho- IKK$\alpha\beta$ ($ser^{180/181}$) rabbit polyclonal antibody, and phospho- IκBα (ser^{32}) rabbit monoclonal antibody, phospho-GSK3β (Ser^{9}) and phospho-ERK1/2 (Thr^{202}/Tyr^{204}) (Cell Signaling Technology Inc., Massachusetts, USA).

5.3 Results

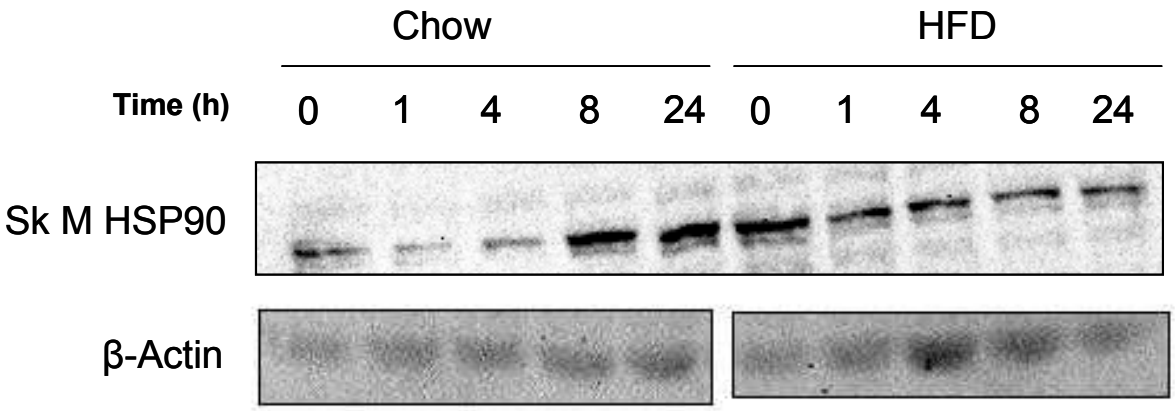
Heat Therapy Activates HSP72, but this effect is blunted by consumption of a high-fat diet (HFD). A preliminary report has demonstrated that hot tub therapy in humans can, by unknown mechanisms, improve glycemia in patients with type 2 diabetes (Hooper, 1999). To examine whether heat therapy would improve hyperinsulinemia and hyperglycemia associated with a 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 for 15 min, in WT chow and HFD mice. Such a treatment resulted in a transient increase in HSP72 in skeletal muscle, liver, and adipose tissue over a 24-h period (Fig 5.1A). When animals were placed on the HFD, the HSP72 response to heat therapy (HT) was reduced (Fig 5.1A), a result consistent with observations in obese humans (Bruce et al., 2003b). In order to examine whether heat therapy had any effect on the induction of other heat shock proteins, HSP90 expression was also measured in skeletal muscle of chow and HFD animals (Fig 5.1B). Similar to the HSP72 response, heat therapy transiently increased HSP90 expression in skeletal muscle of chow fed animals but this effect was blunted in high fat fed animals (Fig 5.1B). In an attempt to understand how a HFD diet could blunt the heat shock response, we examined regulatory factors that control HSP72 expression. Transcription of HSP72 is regulated by the activation of heat shock transcription factor (HSF-1) (Morimoto, 1993), and it is also known that that glycogen synthase kinase 3 (GSK-3) and extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) participate in the down-regulation of HSF-1 transcriptional activity (He et al., 1998). Accordingly, we examined both ERK 1/2 and GSK3 phosphorylation in chow- and high-fat-fed mice, but we observed no

difference between chow and HFD for either ERK 1/2 (Thr202/Tyr204) or GSK3 (Ser9) phosphorylation (Fig 5.1C)

A



B



C

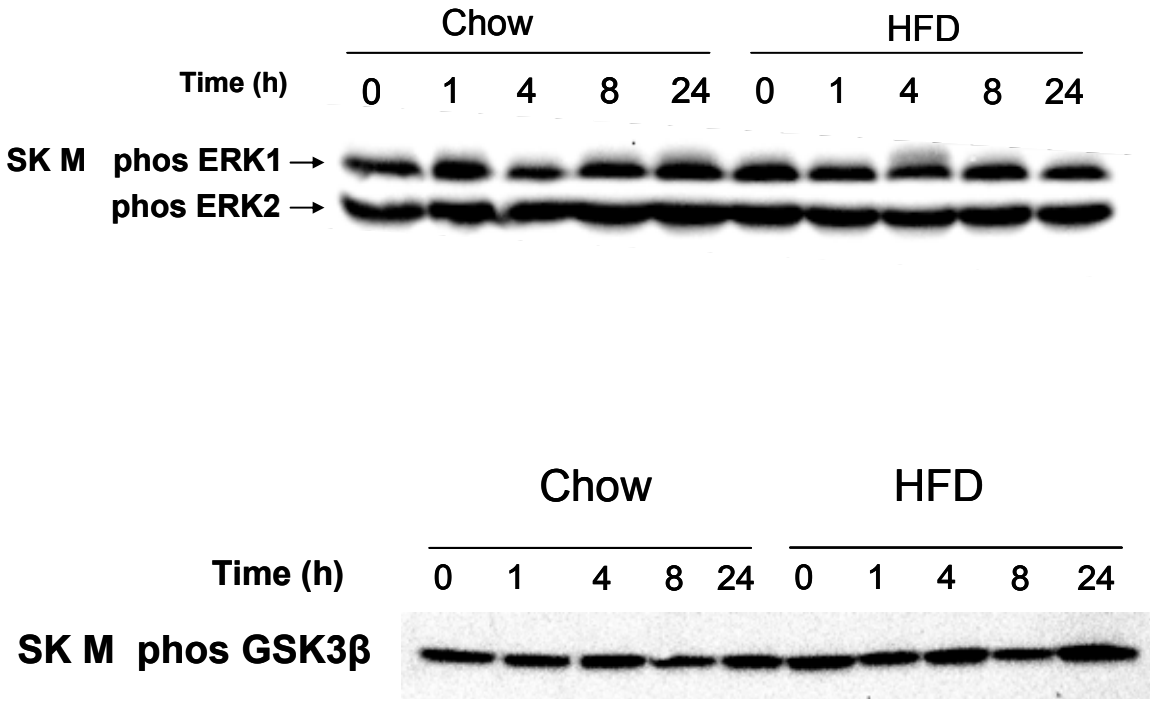


Fig 5.1 Heat therapy (HT) transiently activates HSP72 in multiple tissues (A) and HSP90 in skeletal muscle (B) in chow but not HFD animals. Representative immunoblots of HSP72 expression in skeletal muscle, liver, and adipose tissue taken from animals on a chow or high fat diet (HFD) immediately after (time 0) or 1, 4, 8 and 24 h after heat therapy (A). Representative immunoblot of skeletal muscle HSP90 from chow and HFD animals after heat therapy (B). Representative immunoblots of ERK1/2 (Th^{r202}/Tyr^{204}) and GSK3β (Ser^{9}) phosphorylation (respectively) in skeletal muscle of chow and HFD mice after heat therapy (C) (n = 3-4 mice per group)

Heat therapy improves fasting plasma glucose and insulin levels. After 16 weeks of chow diet, sham treated (ST) and heat treated (HT) mice displayed similar fasting plasma glucose (5.2A) and insulin levels (5.2B). However, after 16 weeks of HFD, ST mice displayed elevated fasting glucose (5.2A) and insulin levels (5.2B). In contrast, HT mice on HFD were protected from diet-induced hyperglycemia (5.2A) and hyperinsulinemia (5.2B).

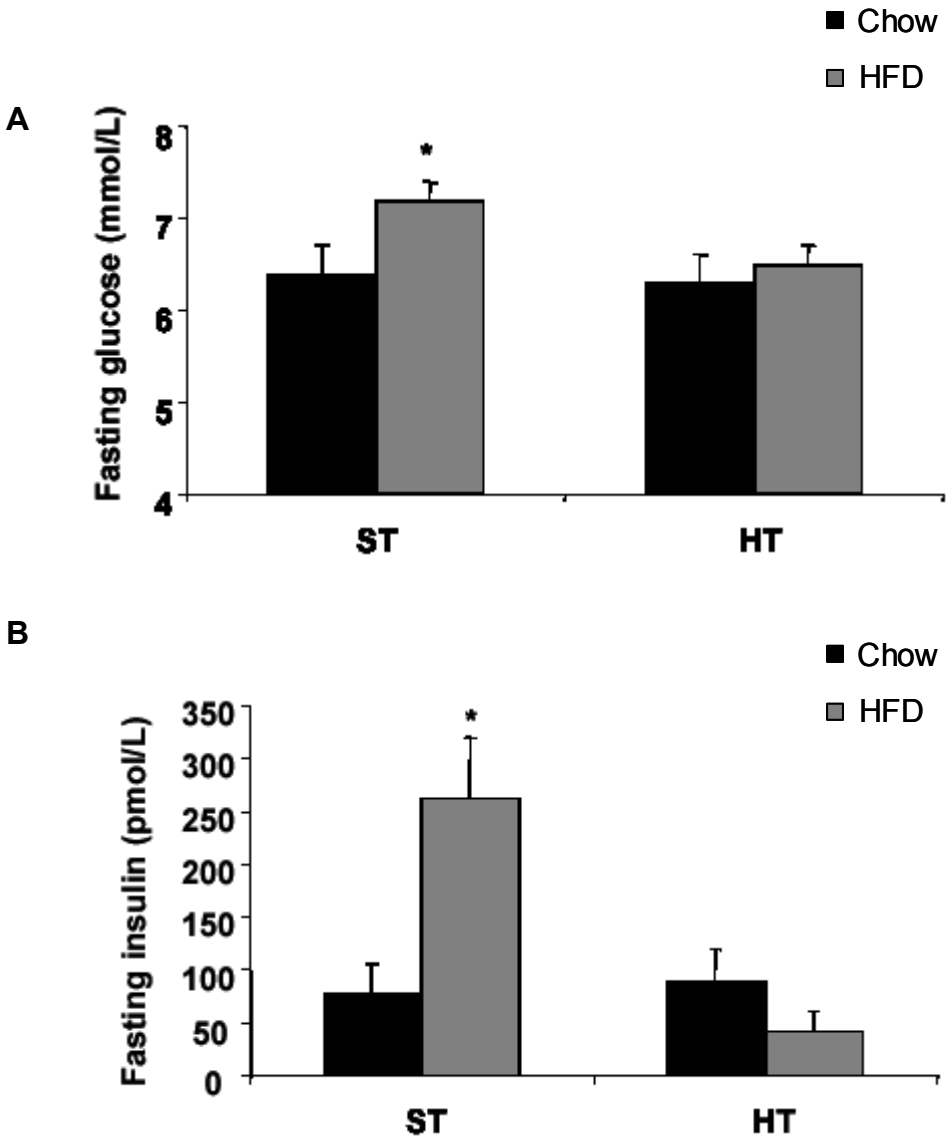


Fig. 5.2 Fasting plasma glucose (A) and fasting plasma insulin (B) of ST and HT mice on chow or HFD (n = 7-12 mice per group, *P<0.05 ST HFD vs all other conditions)

Heat therapy improves HOMA-IR. Based on calculations from fasting insulin and glucose levels, insulin resistance, as assessed by HOMA-IR, was significantly greater in ST mice on HFD compared with both ST and HT mice on chow diet and HT mice on HFD (Fig. 5.3).

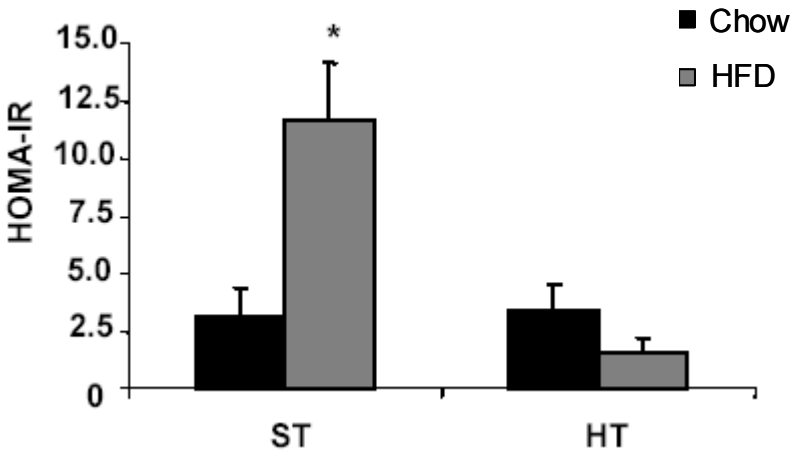


Fig. 5.3 Homeostatic model assessment of insulin resistance (n = 7-12 mice per group, *P<0.05 ST HFD vs all other conditions).

Heat therapy improves glucose tolerance. To test in vivo glucose tolerance, all mice were given an intraperitoneal glucose tolerance test (IPGTT). Consistent with basal glucose and insulin measures, the HFD induced glucose intolerance in ST mice, while both chow groups showed normal glucose tolerance (Fig. 5.4). However, HT mice on HFD showed improved glucose tolerance and a less severe response to the glucose challenge as shown in the area under the curve (Fig. 5.4)

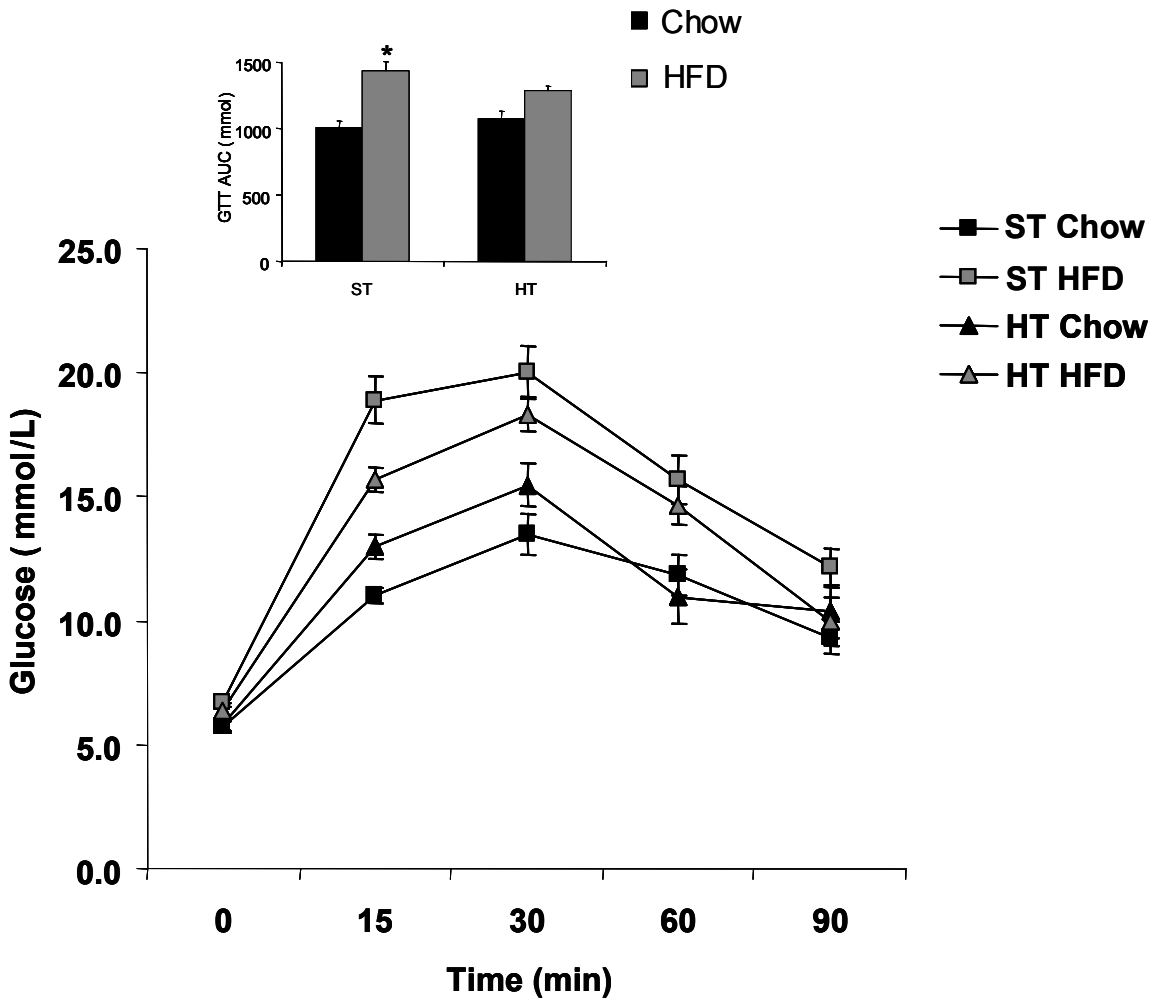


Fig. 5.4 IPGTT (1.0g/kg body weight) and area under the curve of ST and HT mice on chow or HFD (n = 7-12 mice per group, *P<0.05 ST HFD vs all other conditions).

Heat therapy decreases phosphorylation of JNK1/2 in skeletal muscle. To test whether diet and/or heat therapy had any effect on JNK activation in skeletal muscle, phosphorylation of JNK1/2 was analysed in gastrocnemius muscle of ST and HT mice on chow or HFD (Fig. 5.5). As expected, HFD increased phosphorylation of JNK1/2 in ST mice, while both chow fed groups showed lower levels of JNK phosphorylation. However, HT mice on HFD showed a significant decrease in JNK1/2 phosphorylation (Fig. 5.5).

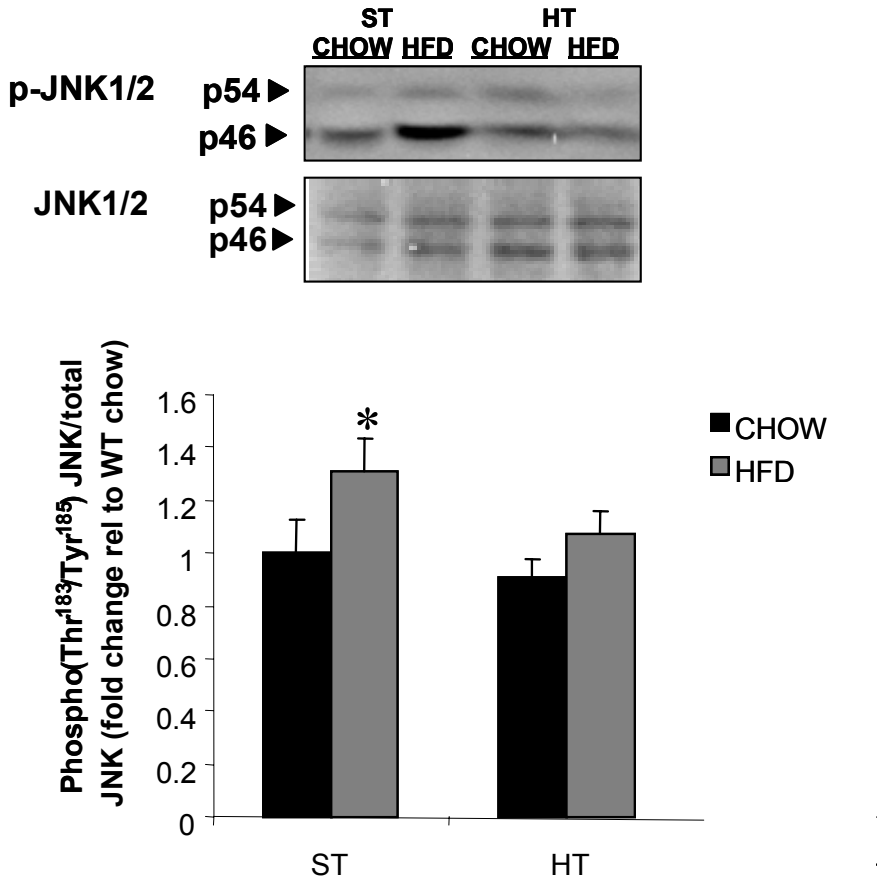


Fig. 5.5 Representative immunoblot and quantification of JNK1/2 phosphorylation (Thr183/Tyr185) and total JNK1/2 in gastrocnemius muscle from sham (ST) or heat treated (HT) mice on chow or HFD (n = 7-12 mice per group, *P<0.05 WT HFD vs all other conditions).

Heat Therapy has no effect on IKKαβ and IκBα phosphorylation in skeletal muscle. To test whether diet and/or heat therapy had any effect on IKKαβ ($ser^{180/181}$) and IκBα (ser^{32}) activation in skeletal muscle, phosphorylation of IKKαβ and IκBα were analysed in ST and HT mice on chow and HFD (Fig. 5.6). No significant differences were observed in phosphorylation of IKKαβ (1.00 ± 0.05 vs 1.02 ± 0.10 vs 0.93 ± 0.06 vs 1.03 ± 0.03 arbitrary units, fold change relative to ST chow, P>0.05: ST chow, ST HFD, HT chow, HT HFD respectively) and IκBα (1.00 ± 0.33 vs 1.05 ± 0.39 vs 0.99 ± 0.34 vs 1.08 ± 0.40, arbitrary units, fold change relative to ST chow, P>0.05: ST chow, ST HFD, HT chow, HT HFD respectively) in ST and HT animals on chow or HFD (Fig. 5.6).

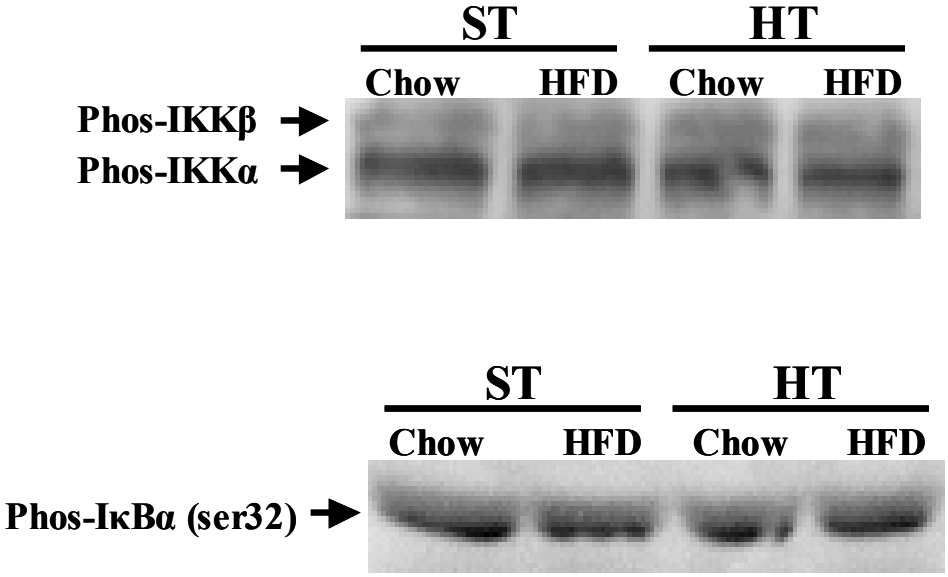


Fig. 5.6 Representative immunoblot of IKKαβ (ser180/181) (upper) and IκBα (ser32) (lower) phosphorylation in mixed gastrocnemius muscle from sham (ST) or heat treated (HT) mice (n = 7-12 mice per group).

5.4 Discussion

The present study is unique in that it combines a model of high fat diet-induced insulin resistance with a model of whole-body hyperthermia. Results from this study provide compelling evidence that heat treatment could be potentially used as a treatment for insulin resistance. These results are in agreement with a recent study demonstrating decreased plasma glucose, insulin and glucose intolerance in heat treated leptin receptor deficient (*db/db*) mice (Kokura et al., 2007). In addition, with regards to the potential mechanism by which heat treatment improved glucose homeostasis, we observed that application of heat treatment led to the induction of HSP72 and a concomitant inhibition of JNK1/2 phosphorylation.

In addition, consumption of a high fat diet produced an insulin resistant phenotype as indicated by significant increases in fasting plasma glucose and insulin levels, as well as decreased glucose clearance following exposure to an intraperitoneal glucose challenge in WT mice. Previous studies have reported that high fat fed C57/Bl6 mice and genetically obese insulin resistant mice (*ob/ob*) display increased JNK kinase activity in the liver, adipose tissue and skeletal muscle compared with control animals (Hirosumi et al., 2002). The activation of JNK1/2 by fatty acids has also been shown to disrupt insulin signalling through serine phosphorylation of IRS-1 (Aguirre et al., 2000; Solinas et al., 2006) In agreement with these findings, our study demonstrates that high-fat feeding induces JNK1/2 phosphorylation in skeletal muscle. Importantly, the application of heat therapy lead to a reduction in phosphorylation of JNK1/2 in skeletal muscle of high fat fed mice as compared with HFD sham treated animals. *In vitro*, it has been shown that hyperthermia-induced HSP72 expression inhibits JNK phosphorylation (Gabai et al., 1997), potentially through direct binding of HSP72 to

JNK1/2, as demonstrated in mouse embryonic fibroblast NIH 3T3 cells (Park et al., 2001). In this study, heat treatment led to a marked induction of HSP72 in skeletal muscle and this may contribute to the heat induced decrease in JNK1/2 phosphorylation.

Inhibition of JNK has also been examined through HSP72 mediated inhibition of upstream MAP kinases (Daviau et al., 2006). Through the interaction of HSP72 and CHIP, DLK has been shown to physically interact with HSP72 in a manner that reduces DLK's capacity to activate JNK (Daviau et al., 2006). In addition, upregulation of HSP72 by heat treatment has been shown to increase phosphorylation of MKP-1, thus accelerating the inactivation of JNK following exposure to heat stress (Lee et al., 2005b).

With regards to activation of the NFκB pathway, phosphorylation of key signalling molecules, IKKαβ and IκBα were not different in skeletal muscle regardless of diet or heat treatment. The lack of IKKαβ and IκBα phosphorylation observed in this study supports existing reports that IKKβ and NFκB do not play a substantial role in skeletal muscle insulin resistance (Cai et al., 2004; Rohl et al., 2004; Watt et al., 2006). In mice that specifically express IKKβ in skeletal muscle, fasting glucose and insulin levels as well as glucose tolerance were similar to littermate controls (Cai et al., 2004). Moreover, mice with muscle-specific IκBα suppression (Cai et al., 2004) or muscle-specific deletion of IKKβ (Rohl et al., 2004) were equally susceptible to diet-induced insulin resistance as control animals. Similarly, a two hour infusion of lipids did not result in any significant activation of the NFκB pathway in skeletal muscle of Wistar rats (Watt et al., 2006). Taken together, results from this study suggest that activation of the NFκB pathway, as assessed by IKKαβ and IκBα

phosphorylation, may not play a significant role in the development of insulin resistance in skeletal muscle.

In addition, it is important to note that the precise effects of heat treatment have not been fully characterized, thus whole body hyperthermia models are not without limitations. Although heat treatment is an effective means of inducing HSP72 expression (King et al., 2002), it also enhances induction of other HSP family members, including HSP90, HSP60 and HSP100 (Nollen and Morimoto, 2002). In addition, heat treatment is known to alter blood flow distribution in skeletal muscle (Akyurekli et al., 1997). Therefore, to fully understand the potential protective effects of HSP72 expression on insulin resistance, a specific model of HSP72 induction is required. This initial study, despite its limitations provides a solid rationale to further examine the effects of HSP72 in the prevention of diet-induced insulin resistance, using other more specific models.

Chapter Six

Effect of HSP72 Overexpression on Diet Induced Insulin Resistance in Mice

6.1 Introduction

Results from Chapter 5 demonstrated that weekly heat treatment led to transient induction of HSP72 in skeletal muscle. Importantly, the induction of HSP72 correlated with a marked reduction in diet-induced hyperglycemia, hyperinsulinemia and glucose intolerance. Furthermore, heat treatment prevented high fat diet-induced JNK1/2 phosphorylation. These findings provide additional insight into previous work demonstrating improved measures of insulin resistance in diabetic patients subjected to hot-tub therapy (Hooper, 1999). However, the results from Chapter 5 are difficult to interpret since hyperthermia leads to an increase in blood flow to skeletal muscle (Akyurekli et al., 1997), while heat stress also activates several other heat shock proteins, including HSP90, HSP60 and HSP100 (Nollen and Morimoto, 2002). In order to directly assess the effect of HSP72 on diet-induced insulin resistance, an experimental model whereby HSP72 was directly manipulated was necessary.

Recently, the construction of rat and human HSP72 transgenes have successfully allowed for tissue-specific overexpression of HSP72 in mice (Christians and Benjamin, 2005; Hutter et al., 1996; Lee et al., 2006; Marber et al., 1995; Mestril, 2005). Thus far, models of transgenic HSP72 overexpression have been characterized in myocardial infarctions (Hutter et al., 1996; Marber et al., 1995) and cerebral damage from heat stroke (Lee et al., 2006). However, to date, transgenic HSP72 mouse models have not been characterized with regards to insulin resistance. Therefore, the aim of this project was to examine the effects of muscle-specific overexpression of HSP72 on high fat diet-induced insulin resistance.

6.2 Materials and Methods

Animals. Animals were treated as described in chapter 3.1

Development of HSP72$^{+/+}$ mice. HSP72$^{+/+}$ breeding pairs were raised on a C57/Bl6 x BalbC background and generously provided by Ruben Mestril (Loyola University, Illinois, USA). For complete reference of HSP72$^{+/+}$ mouse development refer to Marber et al. (1995). Briefly, transgenic mice were created using a chimeric transgene consisting of a rat inducible HSP70 gene. This gene was then inserted into a pCAGGS vector and controlled by the addition of the human cytomegalovirus immediate early enhancer (hCMV-IE) and a chicken β-actin promoter. The chimeric transgene was then purified and inserted into fertilized eggs for development in a pregnant host. Transgene positive mice display constitutive overexpression of HSP72 in the brain, heart and skeletal muscle.

Insulin stimulation. In order to examine any changes in insulin signalling, WT (n=6) and HSP72$^{+/+}$ (n=6) mice were anesthetized with sodium pentobarbital (0.05 mg/g body weight) then administered an intraperitoneal injection of human insulin (1.5 U/kg of Body weight, Novolin, Novo Nordisk Inc., New Jersey, USA) 5 minutes prior to tissue extraction. Sham treated animals (n=6 per group) were administered saline injections (0.9% sodium chloride).

Western Blot Analysis. Protein content from tissue lysates were analysed as described in Section 3.4. Primary antibodies used in this study include HSP70 (HSP72) rabbit polyclonal antibody (Stressgen Bioreagents, Ann Arbor, Michigan, USA), phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), SAPK/JNK rabbit polyclonal antibody,

phospho-AKT (Thr^{308} and Ser^{473}), AKT rabbit polyclonal antibody, phospho-IKK$\alpha\beta$ (ser$^{180/181}$) rabbit polyclonal antibody, and phospho-IκBα (ser^{32}) rabbit monoclonal antibody, β-actin polyclonal antibody (Cell Signaling Technology Inc., Massachusetts, USA).

Euglycemic-hyperinsulinemic clamp. Refer to Chapter 4.2

6.3 Results

Muscle-specific HSP72 expression in HSP72$^{+/+}$ transgenic mice. Protein expression of HSP72 was analysed in *gastrocnemius* muscle, epididymal white adipose tissue and the liver of wild-type (WT) and transgenic HSP72$^{+/+}$ (TG) mice on a chow or HFD. As expected, there was a marked overexpression of HSP72 when comparing the skeletal muscles of HSP72$^{+/+}$ and WT mice, but there were no differences in epididymal white adipose tissue or the liver regardless of diet (Fig. 6.1).

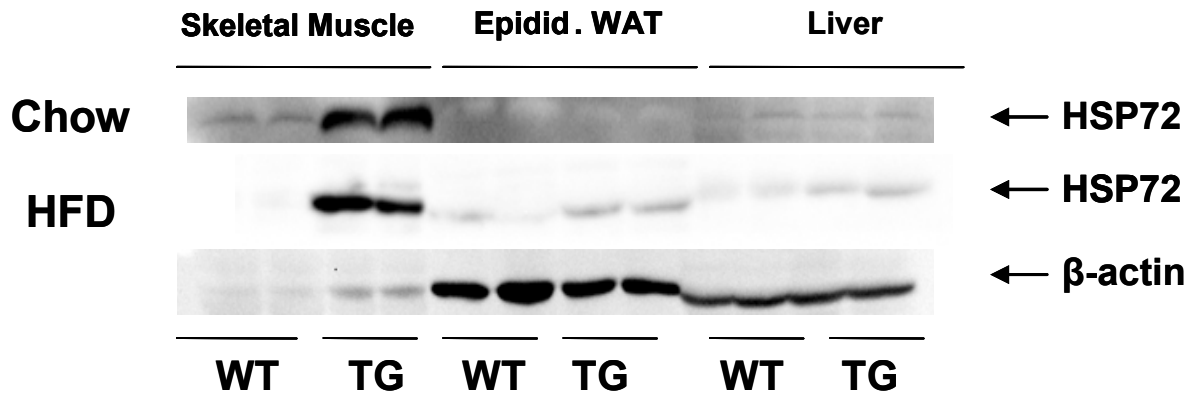


Fig. 6.1 Representative immunoblots of HSP72 expression and β-actin in gastrocnemius muscle, epididymal white adipose tissue and the liver of wild-type (WT) and HSP72$^{+/+}$ transgenic (TG) mice on chow or HFD (n = 5-9 mice per group).

Overexpression of HSP72 in skeletal muscle prevents diet-induced increases in body weight with no effect on food intake. Following 16 weeks of chow or high fat diet (HFD), WT mice on HFD diet showed a significant increase in body weight, while no such effect was observed in WT chow, HSP72$^{+/+}$ chow, and HSP72$^{+/+}$ HFD (Fig. 6.2A). Although energy intake was greater in mice on HFD from both groups, differences in body weight between WT and HSP72$^{+/+}$ mice were not due to hypophagia as no differences were observed in food intake when comparing strains (Fig. 6.2B).

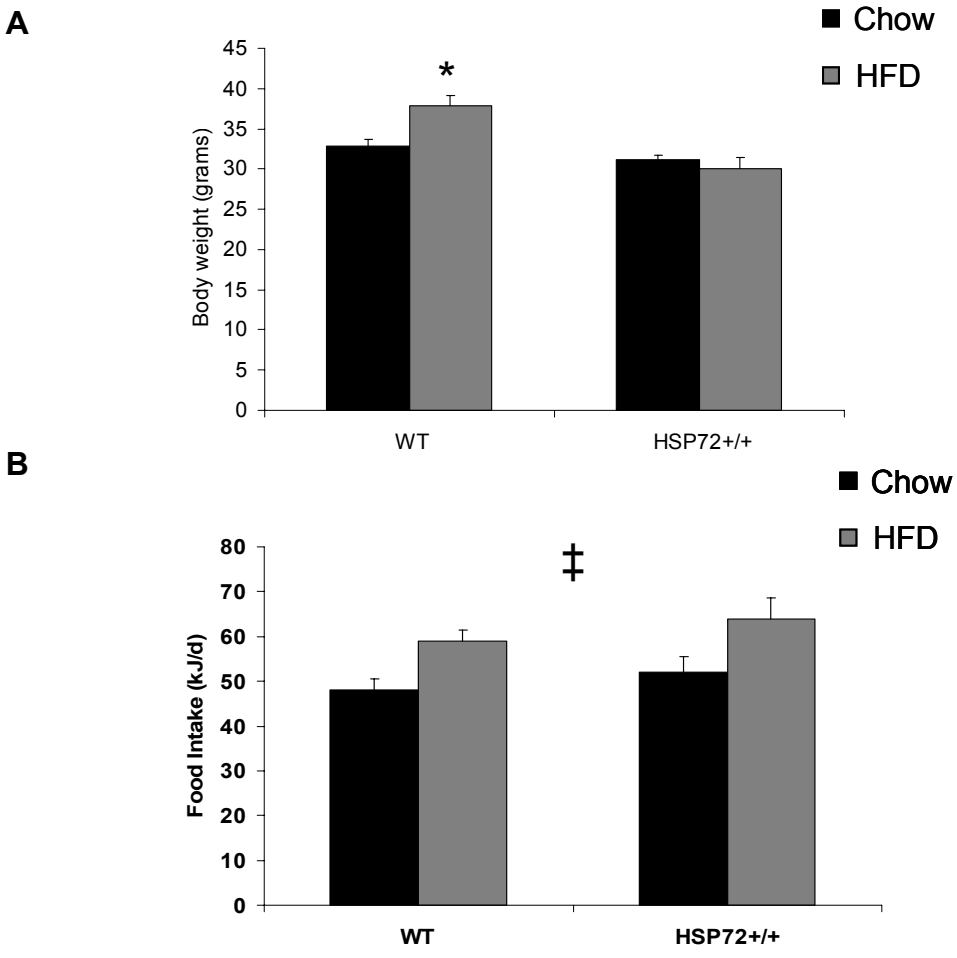


Fig. 6.2 Body weight (A) and food intake (kJ/day) (B) following 16 weeks of chow or HFD in WT and HSP72$^{+/+}$ mice (n = 4-7 mice per group, *P<0.05 WT HFD vs all other conditions, ‡P<0.05 HFD vs chow, main effect for diet).

Overexpression of HSP72 in skeletal muscle improves fasting plasma glucose and insulin levels. WT and HSP72$^{+/+}$ mice fed a chow diet displayed similar fasting plasma glucose (Fig. 6.3A) and insulin levels (Fig. 6.3B) at 16 weeks. However, after 16 weeks of HFD, WT mice displayed elevated fasting glucose and insulin levels. In contrast, HSP72$^{+/+}$ mice on HFD were protected from diet-induced hyperglycemia (Fig. 6.3A) and hyperinsulinemia (Fig. 6.3B).

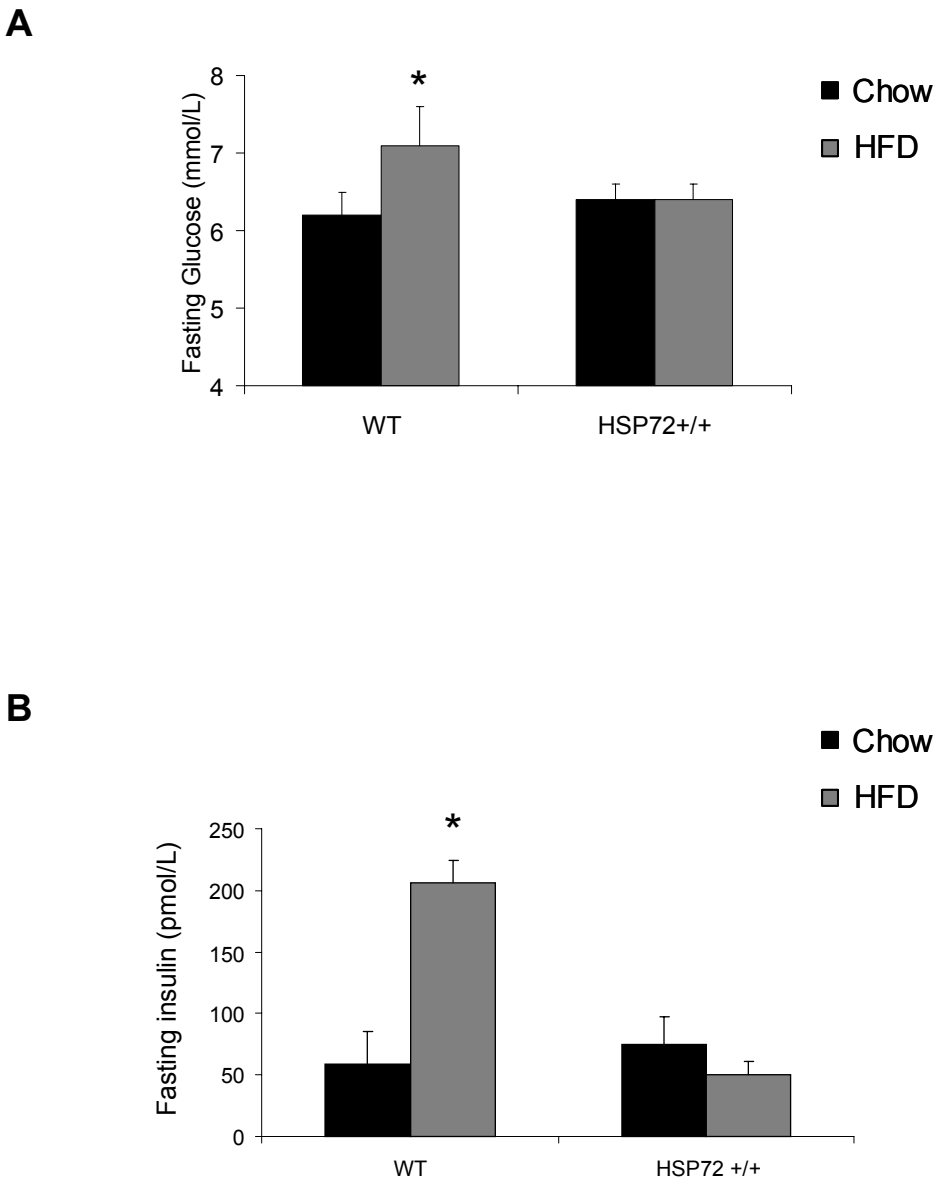


Fig 6.3 Fasting plasma glucose (A) and insulin (B) in WT and HSP72$^{+/+}$ mice on chow or HFD (n = 5-9 mice per group, *P<0.05 WT HFD vs all other conditions)

Overexpression of HSP72$^{+/+}$ in skeletal muscle improves glucose tolerance. Consistent with basal glucose and insulin measures, WT mice fed a HFD displayed significant glucose intolerance (Fig 6.4). However, HSP72$^{+/+}$ mice on HFD showed improved glucose tolerance as assessed by intraperitoneal glucose tolerance test (Fig. 6.4).

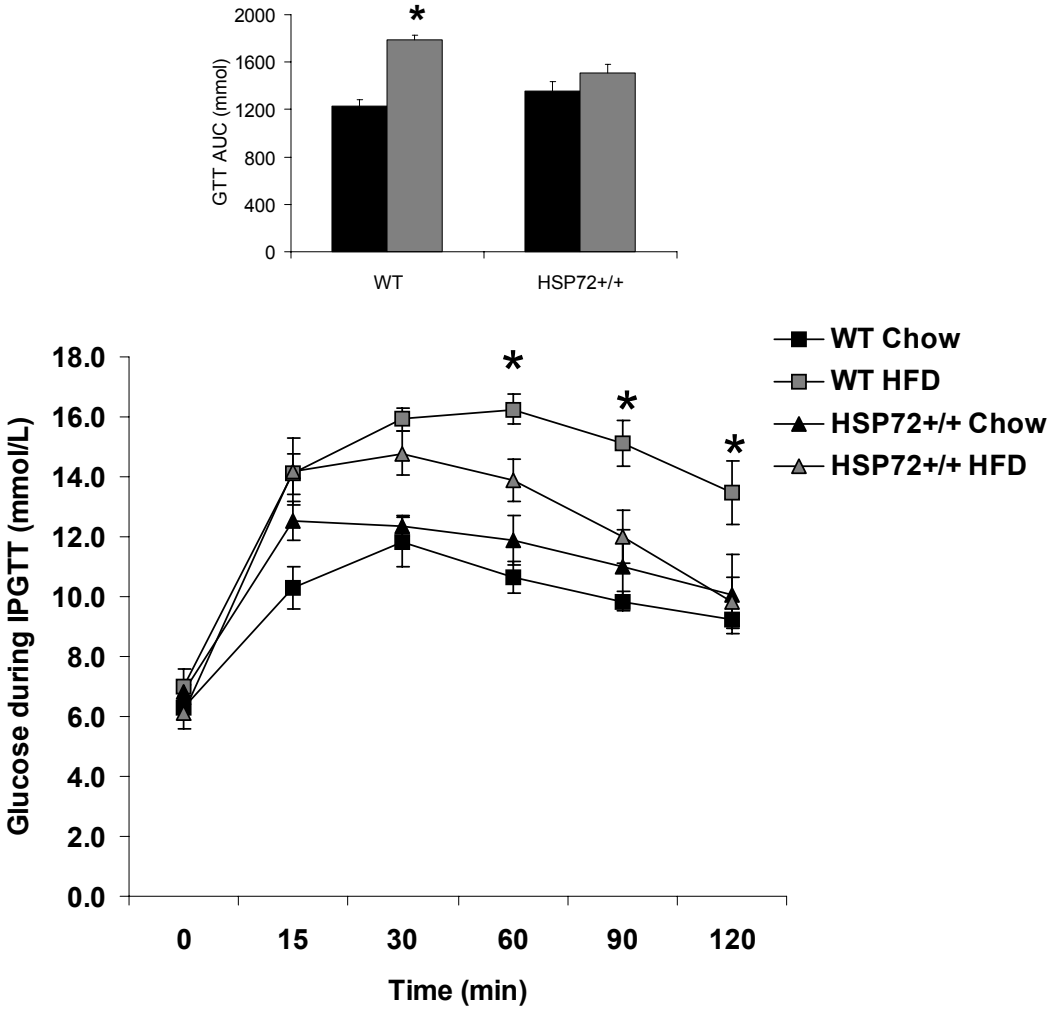


Fig. 6.4 Intraperitoneal glucose tolerance test (IPGTT, 1.0 g/kg body weight) and area under the curve (AUC) in WT and HSP72$^{+/+}$ mice on chow or HFD (n = 5-9 mice per group, *P <0.05 WT HFD vs all other conditions)

Overexpression of HSP72$^{+/+}$ in skeletal muscle improves insulin sensitivity. To test *in-vivo* insulin sensitivity, all mice were administered an intraperitoneal insulin tolerance test (IPITT). As expected, WT and HSP72$^{+/+}$ mice fed a chow diet showed efficient clearance of glucose following insulin stimulation (Fig. 6.5). Conversely, WT mice fed a HFD showed marked deficiency in glucose clearance as indicated by elevated plasma glucose levels at 15min and 30min and an overall increase in the area under the curve (Fig. 6.5). However, HSP72$^{+/+}$ mice fed a HFD showed similar glucose clearance as chow fed animals from both groups, indicating normal insulin sensitivity (Fig. 6.5).

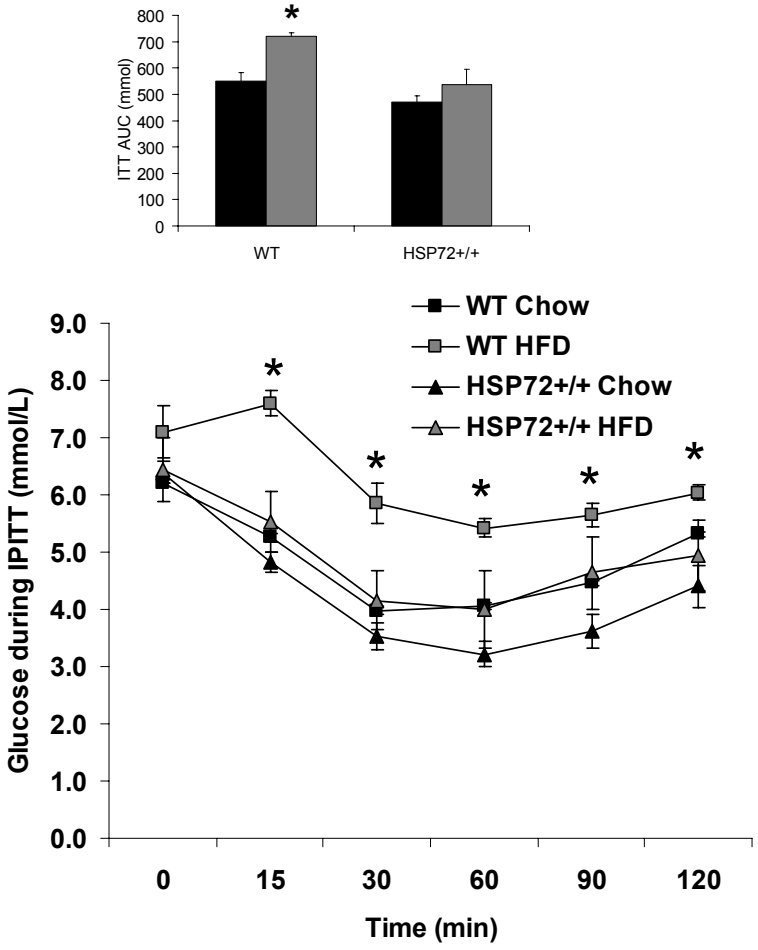


Fig. 6.5 Intraperitoneal insulin tolerance test (0.75 U/kg body weight) and area under the curve in WT and HSP72$^{+/+}$ mice on chow or HFD (n = 5-9 mice per group, *P <0.05 WT HFD vs all other conditions, values are expressed relative to WT chow = 1)

Overexpression of HSP72$^{+/+}$ in skeletal muscle decreases phosphorylation of JNK1/2. Following 16 weeks of chow or HFD, total and phosphorylated JNK1/2 (Thr^{183}/Tyr^{185}) was measured in *gastrocnemius* muscle. As expected, HFD increased JNK1/2 phosphorylation in skeletal muscle of WT mice compared with WT chow and HSP72$^{+/+}$ chow. However, HSP72$^{+/+}$ mice fed a HFD showed similar levels of JNK1/2 phosphorylation as chow fed animals (Fig. 6.6).

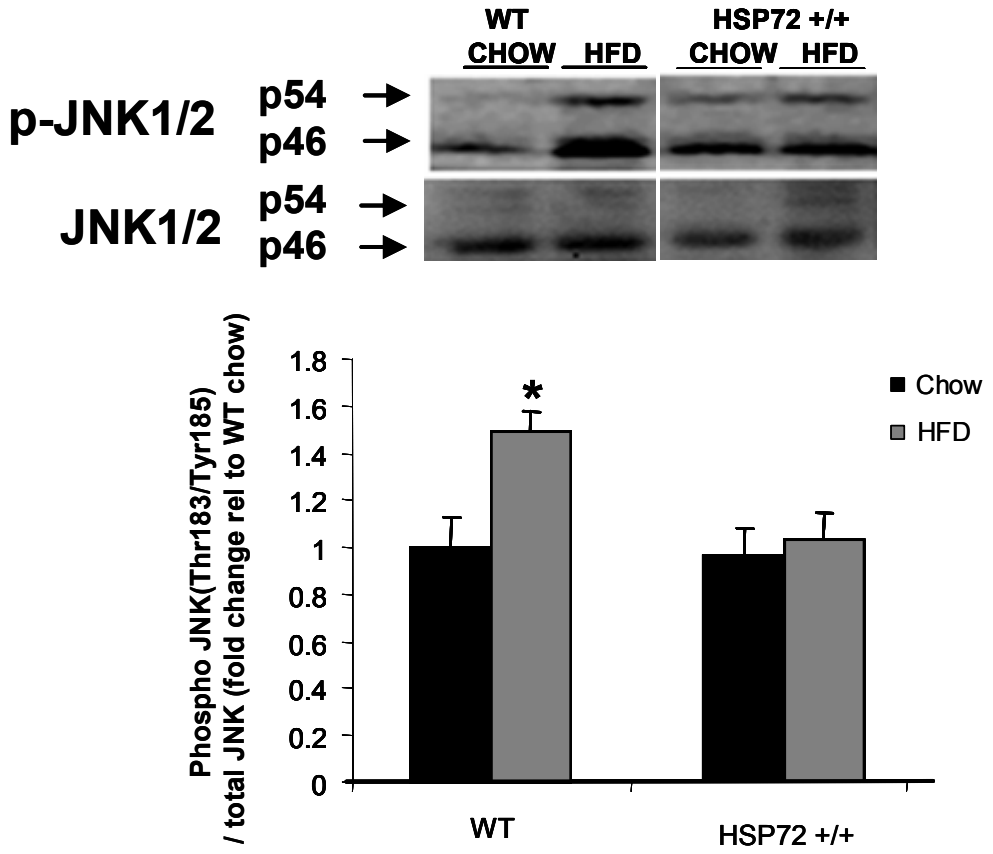


Fig. 6.6 Representative immunoblot and quantification of JNK1/2 phosphorylation (Thr^{183}/Tyr^{185}) and total JNK1/2 in mixed gastrocnemius muscle from wild-type (WT) or transgenic animals (HSP72$^{+/+}$) on chow or HFD (n = 5-9 mice per group, *P <0.05 WT HFD vs all other conditions).

Overexpression of HSP72 in skeletal muscle does not affect IKK and IκB phosphorylation. As discussed in the previous chapter, HSP72 has been shown to block the activation of NFκB signalling *in vitro* (Meldrum et al., 2003). Accordingly, we examined this *in vivo* by measuring the effect of HSP72 overexpression on IKKαβ ($Ser^{180/181}$) (1.00 ± 0.05 vs 1.02 ± 0.11 vs 0.97 ± 0.06 vs 0.94 ± 0.08 arbitrary units, fold change relative to WT chow, P>0.05: WT chow, WT HFD, $HSP72^{+/+}$ chow, $HSP72^{+/+}$ HFD respectively) (Fig. 6.7A) and IκBα (Ser^{32}) (1.0 ± 0.32 vs 1.06 ± 0.38 vs 0.99 ± 0.34 vs 1.06 ± 0.41 arbitrary units, fold change relative to WT chow, P>0.05: WT chow, WT HFD, $HSP72^{+/+}$ chow, $HSP72^{+/+}$ HFD respectively) (Fig. 6.7B) phosphorylation in skeletal muscle. Neither diet nor strain affected phosphorylation of these signalling proteins.

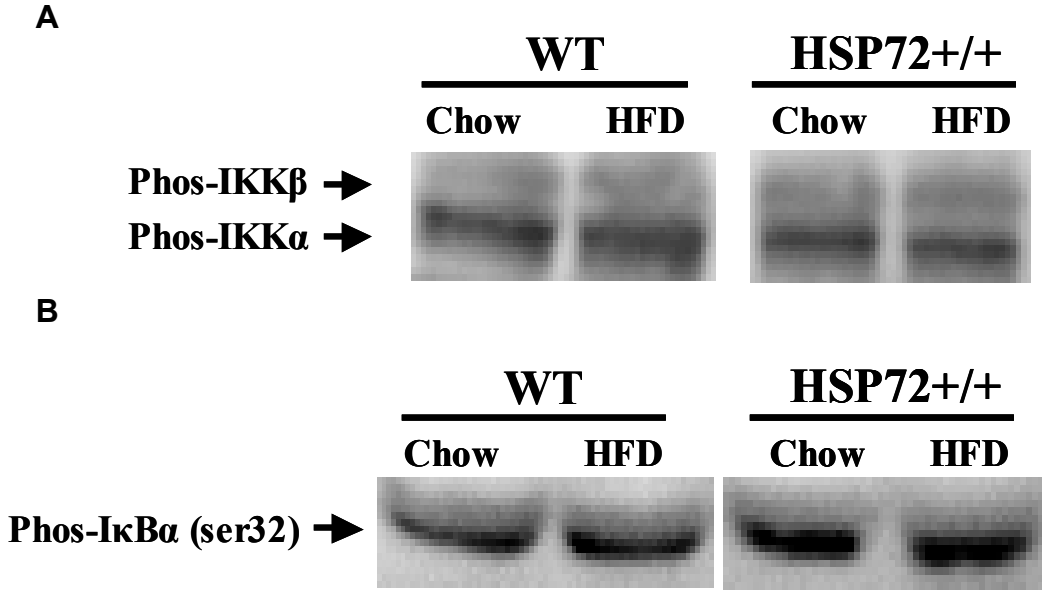


Fig. 6.7 Representative immunoblot of IKKαβ ($Ser^{180/181}$) (A) and IκBα (Ser^{32}) (B) in skeletal muscle of WT and $HSP72^{+/+}$ mice on chow or HFD (n = 4-7 mice per group)

Overexpression of HSP72 in skeletal muscle has no effect on insulin-stimulated phosphorylation of IRS-1 but increases phosphorylation of Akt. To examine the effect of high fat feeding on insulin signalling, total and phosphorylated IRS-1 (Tyr^{612}) and Akt (Ser^{473} and Thr^{308}) were measured in *gastrocnemius* muscle of WT and $HSP72^{+/+}$ mice on a HFD. Muscle samples were obtained from mice administered either insulin or saline (sham treated). Although there was a tendency toward an increase in IRS-1 (Tyr^{612}) phosphorylation in $HSP72^{+/+}$ mice compared to WT mice on a HFD, no significant differences were observed in either group (Fig. 6.8A). In addition, consistent with the magnitude of insulin resistance observed after 15 min during the IPITT (Fig. 6.5) insulin did not lead to any significant phosphorylation of Akt at either residue in WT mice (6.8B). In contrast, consistent with the improved insulin tolerance observed in the $HSP72^{+/+}$ mice, insulin markedly stimulated the phosphorylation of Akt at both residues in these animals (Fig. 6.8B).

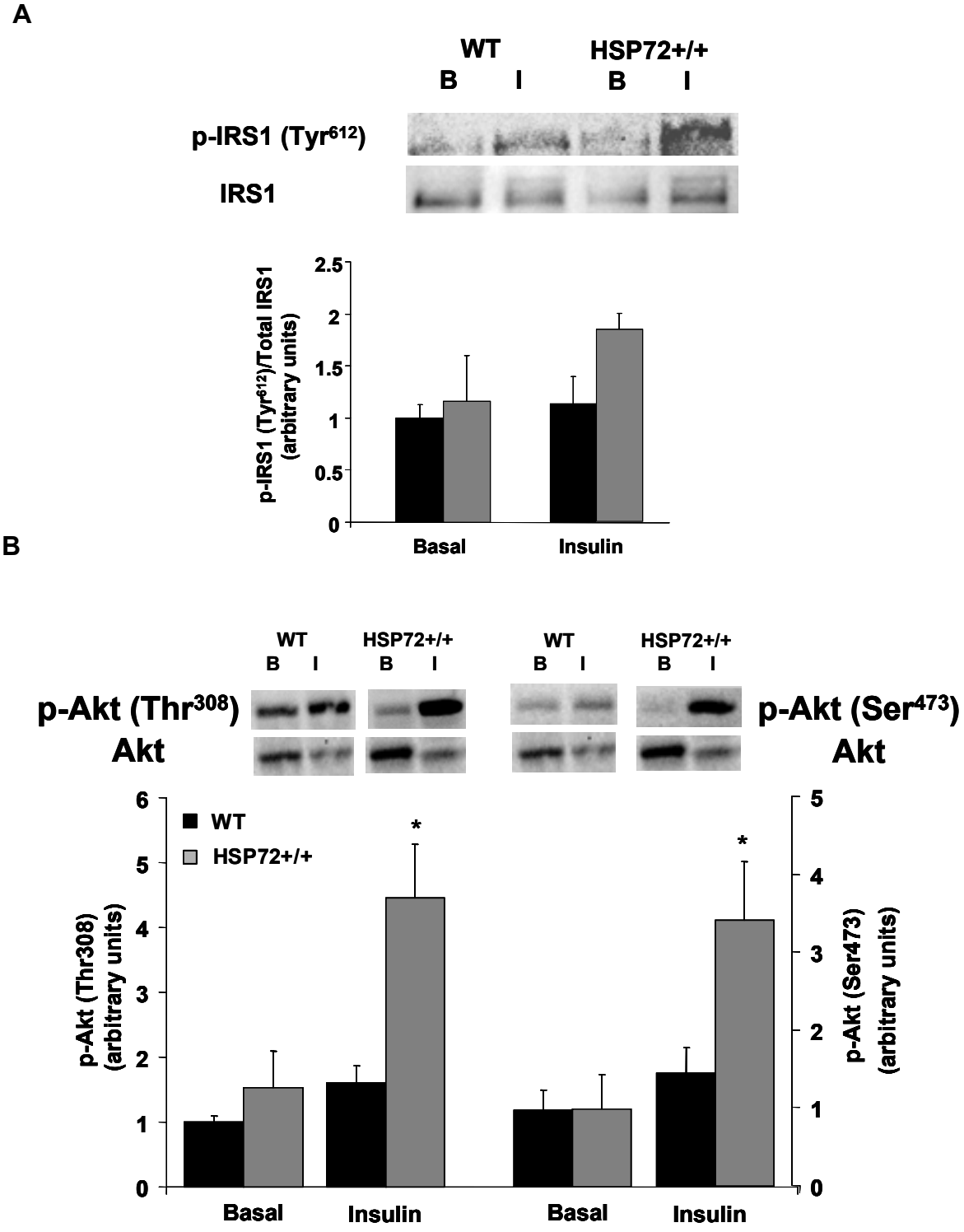


Fig 6.8 Representative immunoblots and quantification of total and phosphorylated IRS-1 (Tyr^{612}) (A) and Akt (Thr^{308} and Ser^{473}) (B) in basal or insulin stimulated gastrocnemius muscle from HSP72$^{+/+}$ mice on HFD (n = 5-9 mice per group, *P <0.05 HSP72$^{+/+}$ insulin stimulated vs all other conditions).

Overexpression of HSP72 in skeletal muscle prevents high fat-diet induced IKK phosphorylation, but not JNK phosphorylation in the liver. To examine whether HSP72 overexpression in skeletal muscle could alter the expression of inflammatory markers in the liver, an organ known to play a major role in the aetiology of insulin resistance, we measured key inflammatory signalling molecules in liver lysates. Phosphorylation of IKKαβ was significantly increased in the liver of WT mice on a HFD compared with chow fed WT and $HSP72^{+/+}$ mice, however, this elevation was abolished in the liver of $HSP72^{+/+}$ mice on a HFD (Fig. 6.9). Somewhat surprisingly, however, this was not observed when measuring phosphorylation of JNK1/2 (Thr^{183}/Tyr^{185}) in the liver of WT and $HSP72^{+/+}$ mice on chow or HFD (1.00 ± 0.08 vs 0.91 ± 0.01 vs 0.89 ± 0.02 vs 0.85 ± 0.02 arbitrary units, fold change relative to WT chow, $P>0.05$: WT chow, WT HFD, $HSP72^{+/+}$ chow, $HSP72^{+/+}$ HFD respectively) as no significant differences were observed in either group (Fig. 6.10).

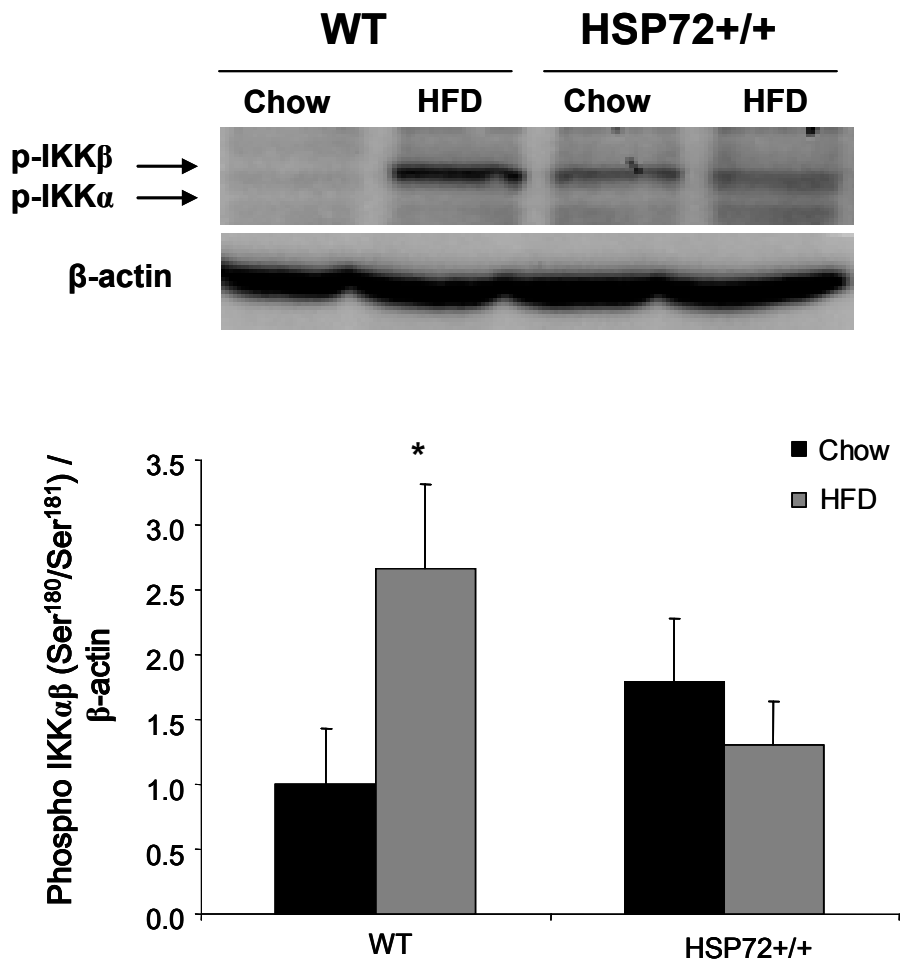


Fig. 6.9 Representative immunoblot and quantification of IKKαβ ($Ser^{180/181}$) and total β-actin in the liver of WT and $HSP72^{+/+}$ on chow or HFD (n = 4-7 mice per group, *P<0.05 WT HFD vs all other conditions).

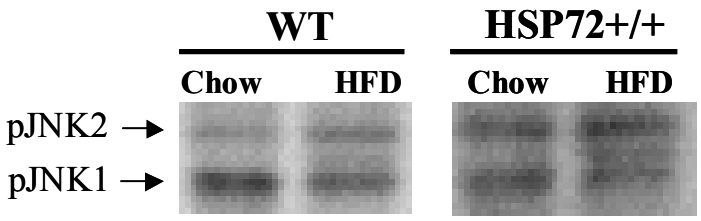
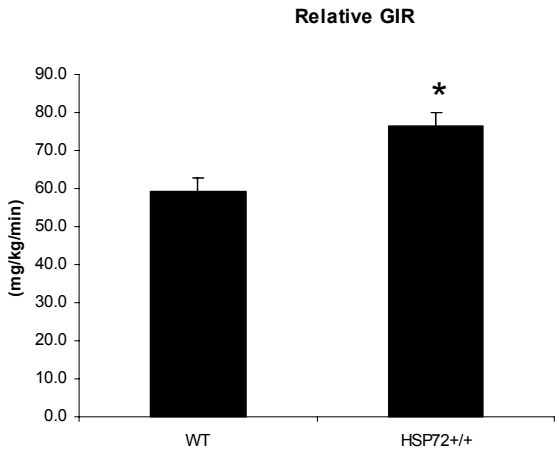


Fig. 6.10 Representative immunoblot of JNK1/2 phosphorylation (Thr^{183}/Tyr^{185}) in the liver of WT and $HSP72^{+/+}$ mice on chow or HFD (n = 4-7 mice per group).

Overexpression of HSP72 in skeletal muscle improves the glucose infusion rate (GIR) during a euglycemic-hyperinsulinemic clamp in chow fed mice. The euglycemic-hyperinsulinemic clamp (EHC) is an effective measure of *in vivo* insulin resistance as it allows for differentiation of hepatic and peripheral insulin sensitivity (Ayala et al., 2006). Although a marked difference in insulin sensitivity was observed in $HSP72^{+/+}$ mice during the IPITT (Fig. 6.5), the fact that HSP72 overexpression blunted inflammatory signalling in the liver suggested that improved hepatic insulin sensitivity could have contributed to the insulin sensitive phenotype. As this study was the first in our laboratory to establish the EHC in conscious mice, and due to its technical nature, it was not possible to examine the effect of a HFD on this parameter due to time constraints. Since we observed a tendency for improved insulin action when comparing the $HSP72^{+/+}$ with WT mice on a chow diet alone (see Fig. 6.5), we performed the EHC in a small number of mice (n=4) in both strains on a chow diet. Glucose infusion rates (GIR) were measured during the 120 minute EHC in WT and $HSP72^{+/+}$ mice on a chow diet (Fig. 6.11A). Despite the fact that the experiments were performed on standard chow diet alone, $HSP72^{+/+}$ mice displayed a significant increase in GIR compared with WT mice (Fig. 6.11). Using a radioactive tracer, we were able to measure both the glucose disposal rate (GDR), a marker of muscle/adipose tissue insulin sensitivity, and hepatic glucose production (HGP), a marker of hepatic insulin sensitivity with the EHC. While no statistical differences were observed in GDR (Fig. 6.11B) or HGP (Fig. 6.11C), we observed a tendency for an increase in GDR, while no such tendency was observed in HGP when comparing WT with $HSP72^{+/+}$ mice. Therefore results of the EHC experiments indicate that HSP72 overexpression showed a tendency towards increased muscle, but not liver, insulin sensitivity.

A



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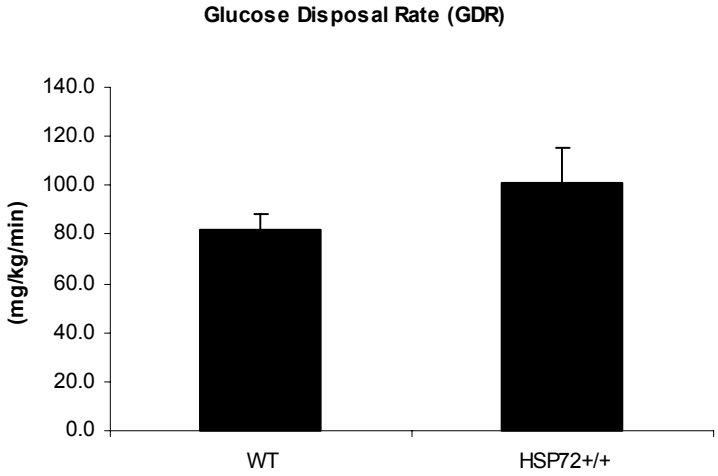


C

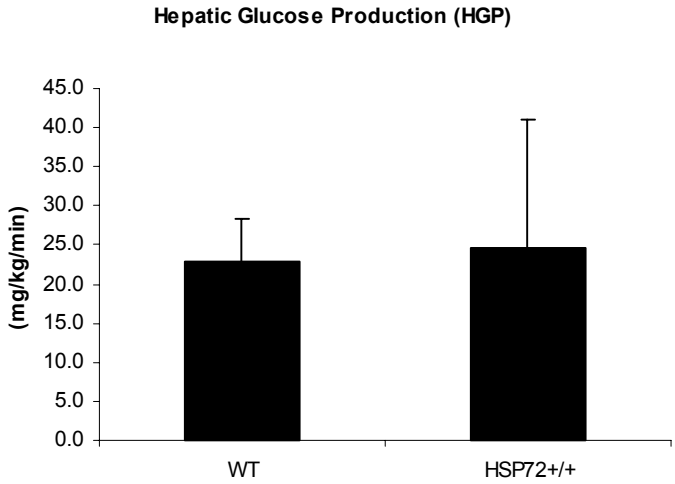


Fig. 6.11 Glucose infusion rate (GIR) (A), glucose disposal rate (GDR) (B) and hepatic glucose production (HGP) (C) in WT and HSP72$^{+/+}$ mice on chow diet (n = 4 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT).

6.4 Discussion

The aim of this study was to determine whether constitutive overexpression of HSP72 in skeletal muscle could protect against diet-induced insulin resistance. Results from this study clearly demonstrate that skeletal muscle overexpression of HSP72 protects against high fat diet-induced hyperglycemia and hyperinsulinemia. HSP72 overexpression also enhanced plasma glucose clearance following insulin stimulation during an IPITT and improves glucose tolerance during an IPGTT. In addition, data from this study suggests an association between constitutive HSP72 expression and inhibition of JNK1/2 in skeletal muscle. Importantly, these results are consistent with previous findings (Chapter 5) of hyperthermia-induced HSP72 expression. However, these results are novel in that protection from insulin resistance has not been characterized in this model of HSP72 induction.

With regards to insulin signalling, evidence suggests that in the presence of fatty acids, JNK1/2 phosphorylation is increased, resulting in disrupted insulin signal transduction through serine phosphorylation of IRS residues (Aguirre et al., 2000; Nguyen et al., 2005). Due to technical difficulties observed with the commercially available phosphospecific antibodies for IRS1 (Ser^{307}), prevention of IRS-1 serine phosphorylation was not established with HSP72 overexpression. However, serine phosphorylation of IRS-1 has been shown to inhibit phosphorylation of tyrosine residues of IRS-1 (Hotamisligil et al., 1996), thus tyrosine phosphorylation of IRS-1 can also be used as an indicator of insulin signal transduction. Examination of IRS-1 phosphorylation on the $tyrosine^{612}$ residue demonstrated a trend towards increased phosphorylation in $HSP72^{+/+}$ mice on a HFD, however this increase did not reach

statistical significance. Increased serine phosphorylation or decreased tyrosine phosphorylation of IRS-1 can have adverse effects on downstream targets, including a reduction in Akt phosphorylation (Nguyen et al., 2005; Solinas et al., 2006). Therefore, phosphorylation of Akt on serine and threonine residues was measured in WT and HSP72 mice. Corresponding to whole body measures of insulin sensitivity, increased phosphorylation of Akt (ser^{473} and thr^{308}) were observed in insulin stimulated $HSP72^{+/+}$ mice on a HFD in contrast with WT mice. In addition, $HSP72^{+/+}$ mice on a chow diet demonstrated enhanced insulin sensitivity compared with WT mice, as assessed by increased glucose infusion rate (GIR) during the EHC. As discussed above, although not statistically significant, GDR, but not HGP, showed a tendency for an increase in $HSP72^{+/+}$ mice. These results suggest that muscle-specific overexpression of HSP72 enhances skeletal muscle insulin sensitivity, possibly through inhibition of JNK1/2 phosphorylation and concomitant enhancement of insulin stimulated Akt phosphorylation. Interestingly, phosphorylation of JNK1/2 was not increased in the liver of WT mice on a HFD. The fact that these mice were bred on a C57xBalb/C strain, rather than a pure C57Bl/6 may be a potential reason for this lack of JNK1/2 phosphorylation in the liver, however, no other explanations are currently available.

It is important to note that the $HSP72^{+/+}$ mice were not only protected from insulin resistance on the HFD, they were also protected from obesity, as determined by total body weight gain. This was despite the fact that they consumed similar amounts of food as WT mice. Recent evidence demonstrates that the brain, particularly the hypothalamus, interacts with hormones such as leptin in order to maintain control of appetite and energy balance (Badman and Flier, 2007). Interestingly, the hypothalamus has also been shown to be susceptible to diet-induced insulin

resistance, resulting in an attenuation of the anorexigenic effects of insulin (Prada et al., 2005). Despite the expression of the HSP72 transgene in the brain of $HSP72^{+/+}$ mice, hyperphagia was not observed in these mice, suggesting that the improvements observed with overexpression of HSP72 may be a peripheral effect rather than a central effect.

In addition, the incorporation of a HFD led to a significant increase in IKKαβ phosphorylation in the liver of WT mice, while no significant differences were observed in skeletal muscle. These data support existing reports that IKKβ may act locally in the liver (Arkan et al., 2005) and have very little effect in mediating skeletal muscle insulin resistance (Cai et al., 2004; Rohl et al., 2004). Importantly, results from this study also indicate that muscle-specific overexpression of HSP72 prevented high fat diet-induced IKKαβ phosphorylation in the liver. Although insulin sensitive tissues, such as the liver and skeletal muscle are biologically diverse in function, it has previously been shown that genetic alterations of one tissue can alter the function of other tissues, a concept referred to as “tissue cross-talk” (Cai et al., 2005; Hevener et al., 2003). In transgenic mice selectively expressing IKKβ in the liver, glycogen synthesis and glucose uptake were reduced by 27% in skeletal muscle (Cai et al. 2005). Therefore, the reduction in liver IKKαβ phosphorylation observed in the liver of $HSP72^{+/+}$ mice suggests the potential for cross-talk between insulin sensitive tissues in these animals.

Taken together, these results provide evidence that constitutive overexpression of HSP72 in skeletal muscle can protect against diet induced hyperglycemia, hyperinsulinemia and glucose intolerance. Underlying these whole-body effects, this study also demonstrates an association between HSP72 overexpression and

inhibition of JNK1/2 in skeletal muscle. Inhibition of JNK1/2 is also associated with a concomitant increase in phosphorylation of AKT residues in skeletal muscle. Finally, differences in liver IKKαβ phosphorylation and body weight suggests the possibility of cross-talk amongst metabolically active tissues and differential energy expenditure in $HSP72^{+/+}$ mice.

Chapter Seven

Effect of HSP72 Overexpression on Markers of Oxidative Capacity in the Liver, Adipose Tissue and Skeletal Muscle

7.1 Introduction

The results from Chapter 6, while clearly demonstrating that muscle specific HSP72 transgenic mice are protected from obesity induced insulin resistance, also revealed two important findings that required further investigation. Firstly, $HSP72^{+/+}$ mice were resistant to obesity, since they did not increase body weight when placed on a high fat diet, despite the fact that energy intake on both diets was identical when compared with WT mice. Secondly, $HSP72^{+/+}$ mice displayed a reduced inflammatory phenotype in the liver despite having similar expression of liver HSP72 as WT mice. These results provided the impetus for further studies reported in this chapter.

The fact that $HSP72^{+/+}$ mice did not put on the expected weight when fed a HFD, in the absence of hypophagia, suggested that these mice may have an enhanced oxidative capacity and reduced fatty acid accumulation. Overexpression of HSP72 has been shown to protect mitochondrial respiratory function in myocardial tissue exposed to ischemia-reperfusion injury (Jayakumar et al., 2001; Suzuki et al., 2002). In addition, citrate synthase (CS) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) activity are positively correlated with HSP72 mRNA expression in human skeletal muscle (Bruce et al., 2003b).

Recently, adipokines, bioactive mediators released from adipose tissue, have been shown to be associated with improved mitochondrial enzyme activity and fatty acid oxidation in skeletal muscle, as well as decreased gluconeogenesis in the liver (Civitarese et al., 2006; Yamauchi et al., 2002). Moreover, adipokines such as adiponectin have been shown to be anti-inflammatory, resulting in the down-

regulation of the NFκB pathway (Kim et al., 2006). Therefore, it is possible that increased adiponectin, or modulation of other circulating cytokines could have contributed to the liver phenotype observed in the previous chapter. Thus, the aim of this project was to determine whether alterations in adipokine secretion and enhanced mitochondrial oxidative capacity could potentially contribute to the protection from obesity and insulin resistance observed in HSP72$^{+/+}$ mice.

7.2 Materials and Methods

Animals. Refer to chapter 3.1

Oxidative enzyme activity. *Soleus, extensor digitorum longus* (EDL) muscles, liver and adipose tissue (5-10 mg) were homogenised in 1:50 dilution (weight per volume) of a 175 mM potassium buffer solution (100 mM KH_2PO_4, 0.05% BSA). Citrate synthase activity was assayed spectrophotometrically at 37°C and measured on a plate reader (412 nm, Victor3, PerkinElmer, Turku, Finland). β-HAD activity was also assayed spectrophotometrically at 37°C, however, the degree of activity was assessed by measuring the disappearance of NADH using the same homogenate as for citrate synthase activity.

Plasma FFA concentration was measured using an enzymatic colorimetric method (NEFA C test kit, Wako, Richmond, VA).

Plasma Adipokine Concentrations. Plasma adipokines were measured using 10 µL of plasma incubated with fluorescently labelled antibody-immobilized microsphere beads (Lincoplex Kit, #MADPK-71K and MADPK-71k-ADPN, Linco Research/Millipore, USA) and read on a 96 well microtiter filter plate using a Bioplex Systems array reader (Bio-Rad Laboratories, California, USA).

7.3 Results

Overexpression of HSP72 in skeletal muscle reduces high fat diet-induced adiposity. As described in Chapter 6, HSP72$^{+/+}$ mice did not increase body weight on the HFD. To assess whether this was due to a reduction in adipose tissue mass, epididymal adipose tissue from WT and HSP72$^{+/+}$ mice were extracted and weighed (Fig. 7.1). In both groups, consumption of HFD resulted in increased adipose tissue weight when compared to chow animals of the same group, however, HSP72$^{+/+}$ mice displayed reduced adipose tissue weight when compared with WT animals, regardless of diet (Fig. 7.1)

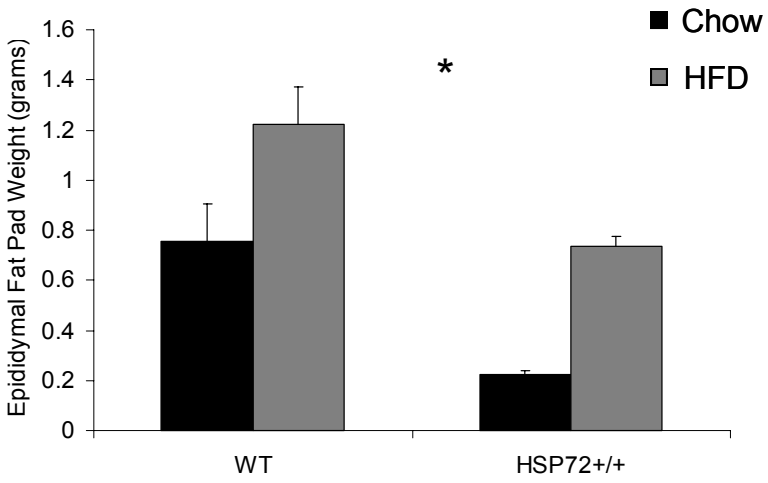
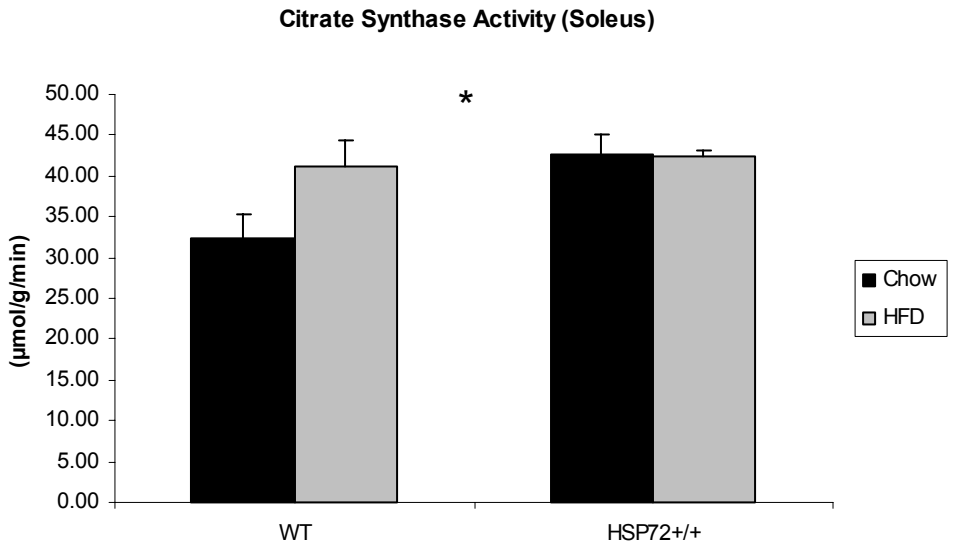


Fig. 7.1 Epididymal adipose tissue weight (g) following 16 weeks of chow or HFD in WT and HSP72$^{+/+}$ mice (n = 4-7 mice per group, *P<0.05, HSP72$^{+/+}$ vs WT, main effect for genotype)

Overexpression of HSP72 in skeletal muscle enhances mitochondrial enzyme activity. In order to examine whether HSP72 overexpression can alter markers of oxidative capacity in both oxidative and glycolytic skeletal muscle, the maximal activity of two mitochondrial enzymes, citrate synthase (CS) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) were examined in *soleus* (Fig. 7.2A and 7.2B respectively) and *extensor digitorum longus* muscle (EDL) (Fig. 7.3A and 7.3B respectively) from WT and $HSP72^{+/+}$ mice on either chow or HFD. Both CS and β-HAD enzyme activity were higher in the *soleus* (Fig. 7.2A and 7.2B respectively) and EDL (Fig. 7.3A and 7.3B respectively) of $HSP72^{+/+}$ mice compared to WT mice. In addition HFD led to a significant increase in β-HAD activity in the EDL of both WT and $HSP72^{+/+}$ mice compared to chow fed animals (Fig. 7.3B).

A



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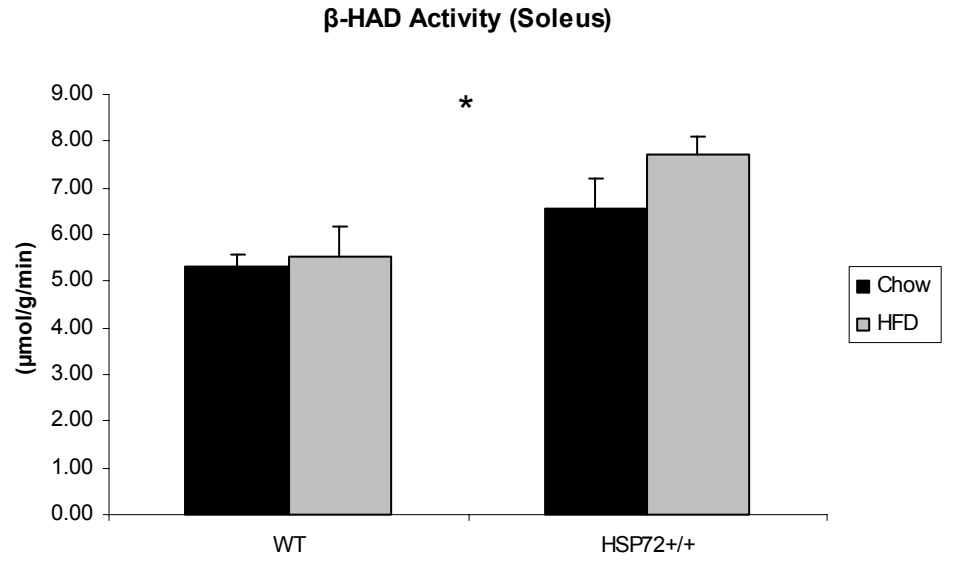
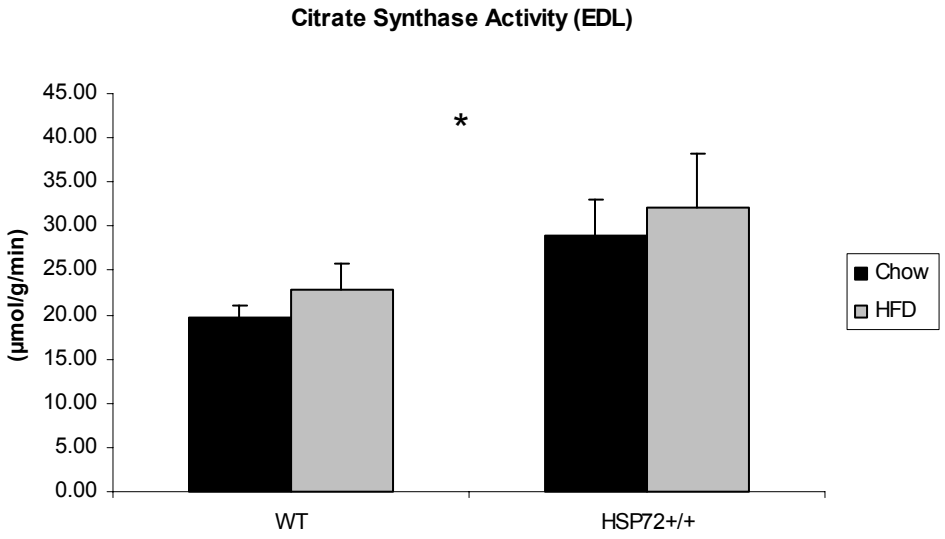


Fig. 7.2 Citrate synthase (A) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) (B) activity in soleus muscle of WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-5 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT, main effect for genotype).

A



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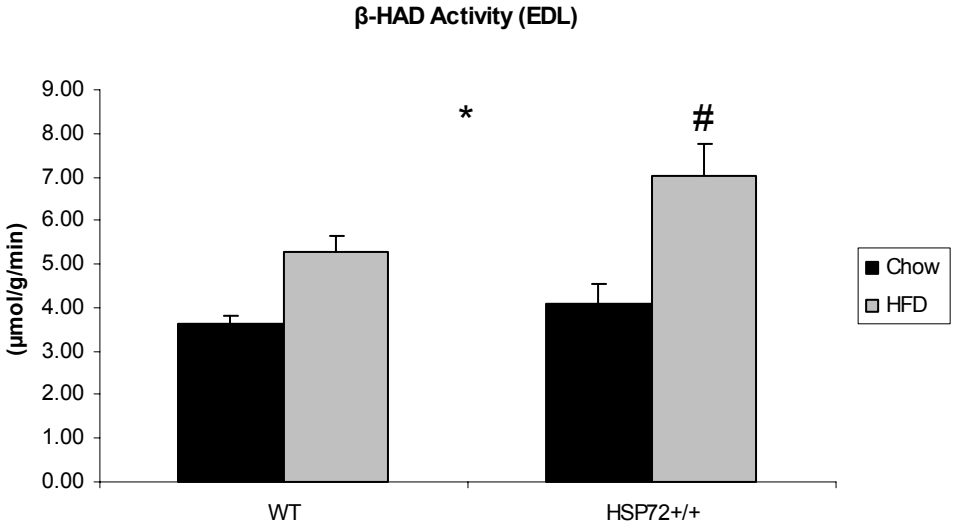


Fig. 7.3 Citrate synthase (A) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) (B) activity in extensor digitorum longus muscle of WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-5 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT, main effect for genotype, #P<0.0005 HFD vs. chow).

Overexpression of HSP72 in skeletal muscle enhances mitochondrial enzyme activity in the liver. In order to examine whether HSP72 overexpression can alter markers of oxidative capacity in other insulin sensitive tissues, the maximal activity of citrate synthase (CS) (Fig. 7.4A) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) (Fig. 7.4B) were examined in the liver of WT and HSP72$^{+/+}$ mice on chow or HFD. Both CS and β-HAD were significantly increased in the liver of HSP72$^{+/+}$ mice compared to WT mice.

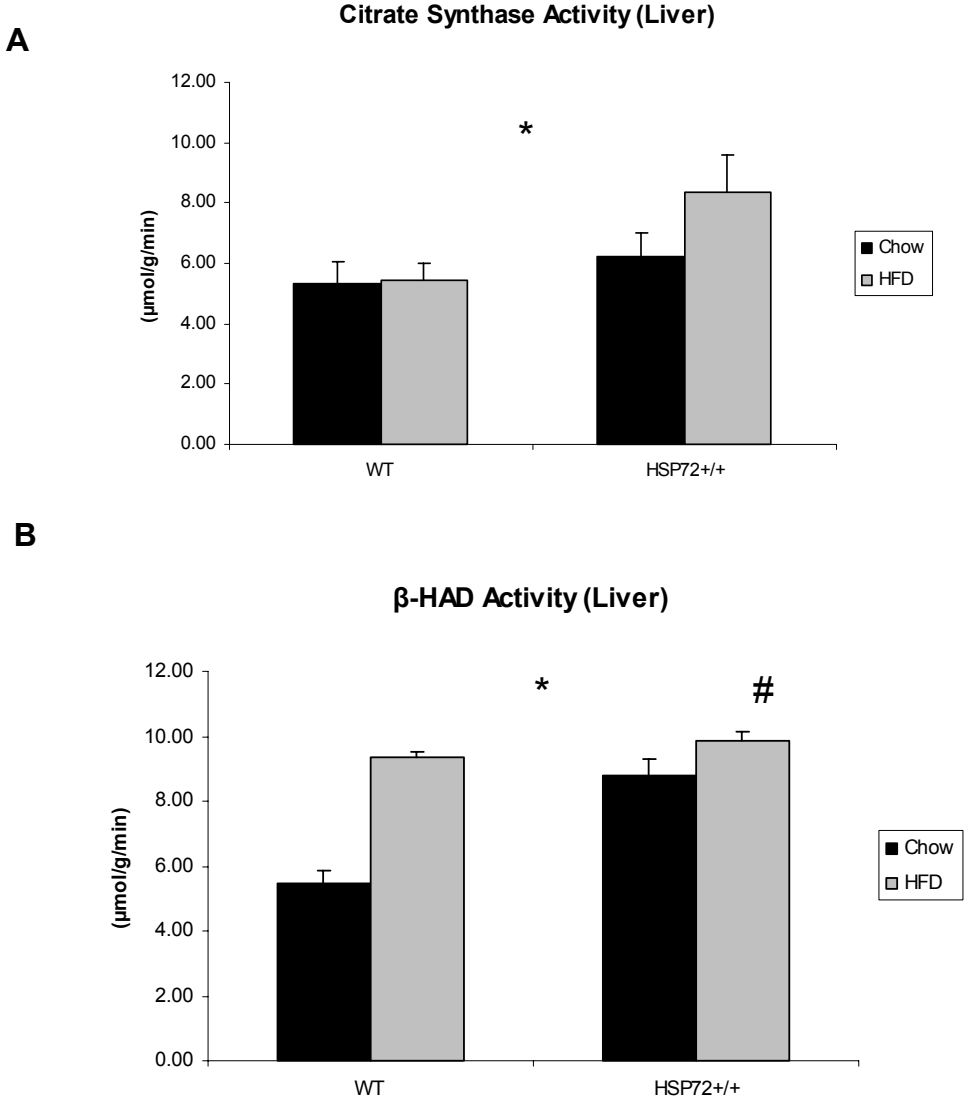


Fig. 7.4 Citrate synthase (A) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) (B) activity in the liver of WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-5 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT, main effect for genotype, #P<0.0001 HFD vs. chow).

Overexpression of HSP72 in skeletal muscle enhances mitochondrial enzyme activity in adipose tissue. Maximal activity of CS (Fig. 7.5A) and β-HAD (Fig. 7.5B) were also measured in white adipose tissue (WAT) of WT and HSP72$^{+/+}$ mice on chow or HFD. Both CS and β-HAD were significantly increased in WAT of HSP72$^{+/+}$ mice compared to WT mice.

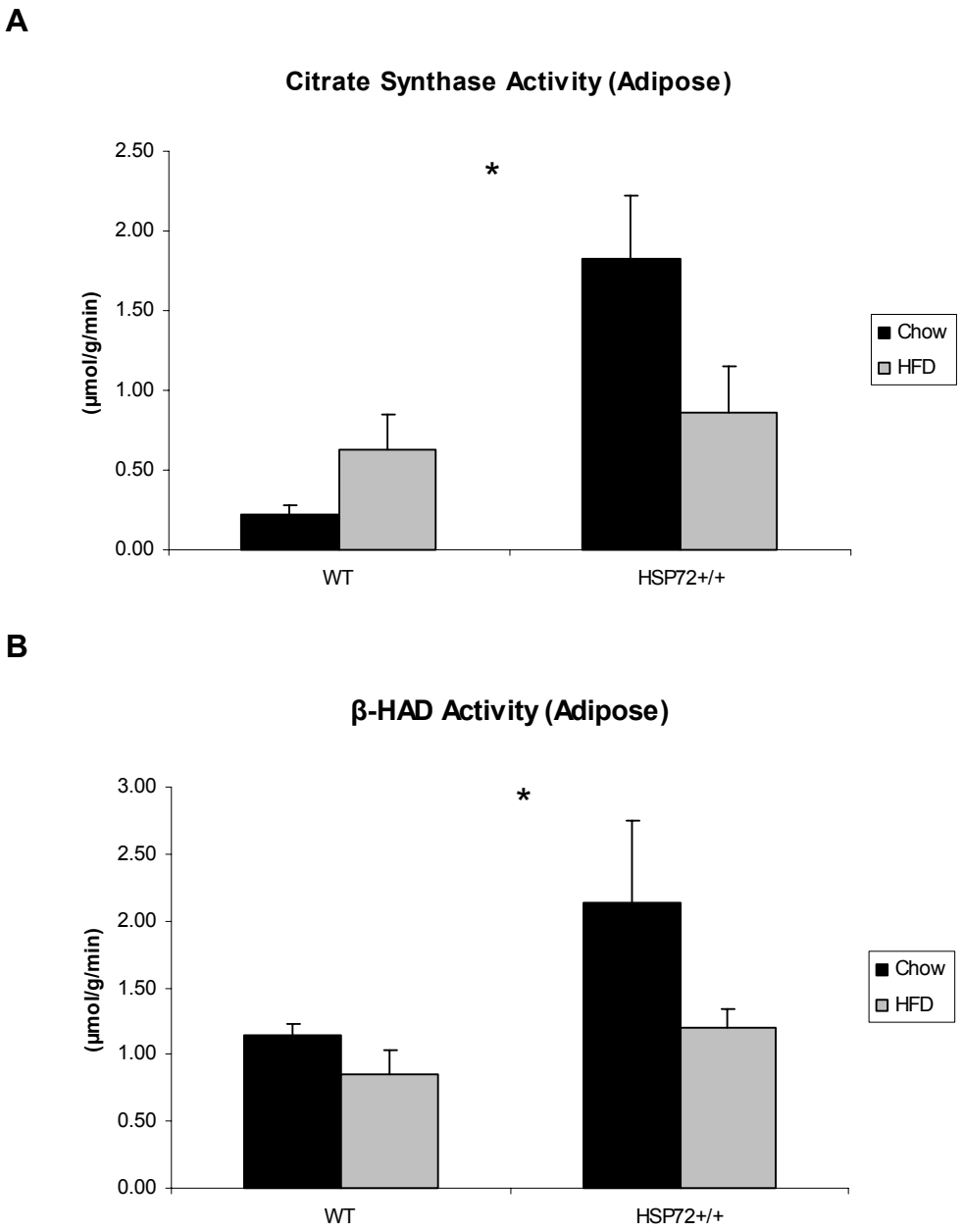


Fig. 7.5 Citrate synthase (A) and β-hydroxyacyl-CoA-dehydrogenase (β-HAD) (B) activity in white adipose tissue of WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-5 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT, main effect for genotype)

Overexpression of HSP72 in skeletal muscle reduces circulating levels of plasma free fatty acids. Based on the observed increases in mitochondrial enzyme activity in skeletal muscle, adipose tissue and the liver, as well as decreased epididymal adipose tissue mass of HSP72$^{+/+}$ mice, the level of plasma free fatty acids were measured in WT and HSP72$^{+/+}$ mice on chow or HFD (Fig. 7.6). As expected, HSP72$^{+/+}$ mice showed reduced levels of circulating plasma free fatty acids as compared to WT animals (Fig. 7.6).

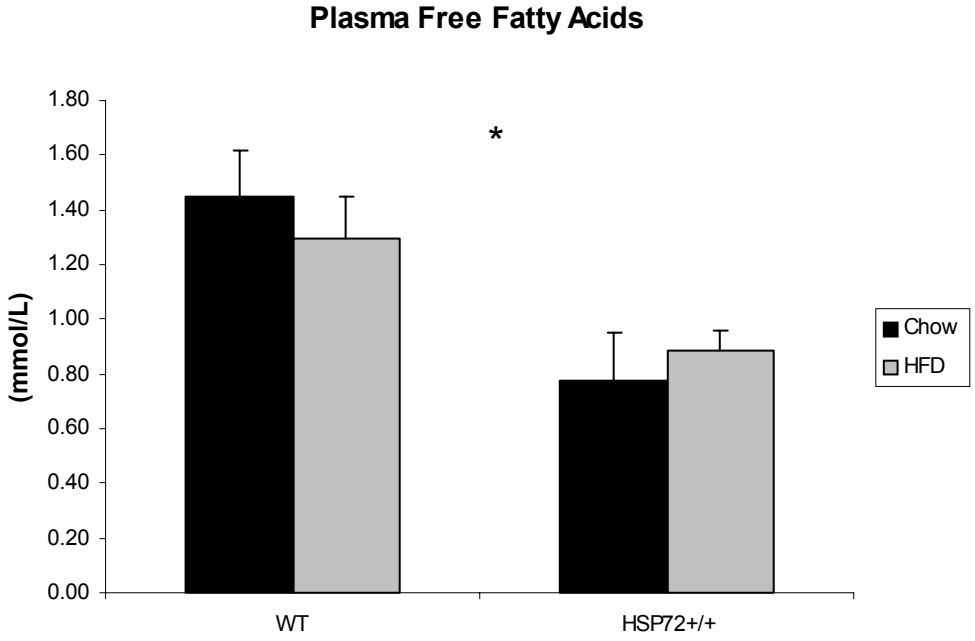


Fig. 7.6 Free fatty acid concentrations from plasma of WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-5 mice per group, *P<0.002 HSP72$^{+/+}$ vs WT, main effect for genotype).

Overexpression of HSP72 in skeletal muscle alters circulating levels of plasma adipokines. Alterations in adipokine secretion have been reported to enhance insulin sensitivity, possibly due to an increase in oxidation of fatty acids (Civitarese et al., 2006; Guzik et al., 2006; Yamauchi et al., 2002). In order to examine whether HSP72 overexpression could alter circulating adipokine levels, plasma adiponectin, leptin, MCP-1, PAI-1 and TNFα were measured in WT and HSP72$^{+/+}$ mice on either chow or HFD (Table 7.1). HSP72$^{+/+}$ mice showed increased levels of circulating adiponectin compared to WT mice (Table 7.1). HFD increased plasma leptin levels in both WT and HSP72$^{+/+}$ mice, however, HSP72$^{+/+}$ mice showed decreased leptin levels when compared to WT mice, regardless of diet (Table 7.1). PAI-1 was also decreased in HSP72$^{+/+}$ mice compared to WT, while no differences were observed with MCP-1 and TNFα could not be detected (Table 7.1).

	Wild Type		HSP72$^{+/+}$	
	Chow	HFD	Chow	HFD
Adiponectin (µg/mL)	10.47 $\pm$ 1.30	9.96 $\pm$ 0.74	12.99 $\pm$ 1.12	13.24 $\pm$ 1.77*
Leptin (pg/mL)	731 $\pm$ 336	3856 $\pm$ 2113	254.2 $\pm$ 173	763 $\pm$ 423 *†
MCP-1 (pg/mL)	9.86 $\pm$ 2.05	8.91 $\pm$ 3.08	14.21 $\pm$ 3.79	10.61 $\pm$ 1.58
PAI-1 (pg/mL)	3430 $\pm$ 674	2915 $\pm$ 542	2072 $\pm$ 470	1420 $\pm$ 268 *

Table 7.1 Plasma adipokine levels in WT and HSP72$^{+/+}$ mice on chow or HFD (n = 4-6 mice per group, *P<0.05 HSP72$^{+/+}$ vs WT, main effect for genotype, †P<0.05 HSP72$^{+/+}$ HFD vs WT HFD).

7.4 Discussion

The results from this study demonstrate that altered muscle oxidative enzyme capacity and/or circulating cytokine production could potentially contribute to the insulin sensitive phenotype described in Chapter 6. The observation that CS and β-HAD maximal activity were increased in HSP72$^{+/+}$ mice was consistent with previous studies in humans showing a correlation between the mRNA expression of HSP72 and mitochondrial enzyme activity in skeletal muscle (Bruce et al. 2003). 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 Tom70 (Young et al., 2003). In addition, overexpression of HSP72 in glucose deprived cells maintains mitochondrial respiratory function and reduces ROS formation (Ouyang et al., 2006), the latter which has recently been linked to insulin resistance (Houstis et al., 2006). Moreover, protection from mitochondrial damage by HSP72 expression has previously been shown in models of cardiac ischemia-reperfusion injury (Jayakumar et al., 2001). Overexpression of HSP72 has not only been suggested to protect mitochondrial membranes from denaturization, but has also been shown to maintain respiratory function through protection of NAD^+ and FAD linked respiration (Jayakumar et al., 2001). In addition, HSP72 has also been implicated in prevention of mitochondrial-mediated apoptosis through inhibition of cytochrome *c* release and translocation of Bax (Stankiewicz et al., 2005), a process potentially involving HSP72 mediated antioxidant expression (Suzuki et al., 2002).

Considering previous evidence of HSP72 protecting mitochondria from various defects and the observation of greater mitochondrial enzyme activity in skeletal

muscle, liver and adipose tissue of HSP72$^{+/+}$ mice, data from this study suggests that overexpression of HSP72 may potentially preserve oxidative capacity through protection of mitochondria. Previous studies have used mitochondrial enzymes such as citrate synthase and β-HAD as markers of mitochondrial density and oxidative capacity (Bruce et al., 2003a; Fitts et al., 1975; Holloszy, 1975), demonstrating an inverse correlation between oxidative enzyme activity and insulin resistance (Simoneau and Kelley, 1997). Therefore, the preservation of mitochondrial enzyme activity observed in HSP72$^{+/+}$ mice compared to WT mice may account for the reduction in epididymal fat pad weight and protection from diet-induced insulin resistance observed in our study.

Interestingly, in WT HFD mice, activity levels of citrate synthase in the soleus (Fig. 7.2A) and β-HAD in the liver were similar to HSP72$^{+/+}$ mice on the same diet. Similar to other reports using high fat fed rodents (Garcia-Roves et al., 2007; Turner et al., 2007), this effect may be due to a compensatory increase in oxidative enzymes in response to increased lipid availability in WT HFD mice, whereas HSP72$^{+/+}$ mice may have inherently higher levels of enzyme activity due to increased mitochondrial density, thus no addition enzyme activity was observed. In addition, lipid oversupply has also been shown to cause adaptations in skeletal muscle such that fatty acid uptake and clearance from circulation is increased in high fat fed animals (Hegarty et al., 2002). Using a radioactive fatty acid tracer, it was shown that in response to a high fat diet, a significant increase in fatty acid clearance into red *gastrocnemius* muscle was observed in insulin resistant rats. The authors concluded that insulin resistance induced by high fat feeding is associated with enhanced lipid utilization but may also lead to increased lipid accumulation with detrimental effects on insulin action (Hegarty et al., 2002). These results may explain the lack of difference in

plasma fatty acid levels between chow and HFD mice in our study, despite the observation that HFD led to insulin resistance.

In addition, results from this study also indicate that HSP72$^{+/+}$ mice display altered adipokine secretion compared with WT mice. The adipokine, adiponectin, has previously been associated with improved mitochondrial enzyme activity (Civitarese et al., 2006) and fatty acid oxidation (Yamauchi et al., 2002). The expression of adiponectin receptor mRNA has been shown to be positively correlated with mitochondrial DNA content in humans, while adiponectin null mice display decreased skeletal muscle citrate synthase activity and glucose intolerance (Civitarese et al., 2006). Indeed, results from this study demonstrate increased plasma adiponectin levels in HSP72$^{+/+}$ mice compared to WT mice.

In addition, leptin, an adipokine associated with control of food intake, has been shown to be positively correlated with fat mass in humans (Considine et al., 1996). Moreover, other adipokines including TNFα (Kern et al., 2001; Moller, 2000), plasminogen activator inhibitor 1 (PAI-1) (De Taeye et al., 2005) and monocyte chemoattractant protein –1 (MCP-1) (Takahashi et al., 2003) have been shown to be elevated in obese and diabetic patients (Alessi et al., 2000). Importantly, results from this study are consistent with previous findings as plasma leptin and PAI-1 levels were decreased in HSP72$^{+/+}$ mice compared with WT mice.

In conclusion, the results from this study suggest that altered muscle oxidative enzyme capacity and/or circulating cytokine production could be one mechanism by which HSP72$^{+/+}$ mice are protected from high fat diet-induced insulin resistance.

Chapter Eight

Summary, Conclusions and Future Studies

8.1 Summary and Conclusions

The primary aim of this thesis was to investigate the biological role of heat shock protein 72 (HSP72) in the prevention of obesity-induced insulin resistance.

Exposure to heat treatment is a well established method of inducing cellular HSP72 expression. The first study (Chapter 5) investigated the use of hyperthermia as a preventative treatment for diet-induced insulin resistance. This project was based on previous work demonstrating reduced expression of HSP72 mRNA in skeletal muscle of diabetic patients (Bruce et al., 2003b), while regular exposure to heat via hot-tub therapy improved measures of glucose homeostasis (Hooper, 1999). However, a specific mechanism by which these differences occurred was not established in these studies. Results from Chapter 5 demonstrated that weekly heat therapy is an effective method of inducing HSP72 in wild-type mice. In addition, it was also shown that 16 weeks of a high fat diet markedly impaired glucose homeostasis, however, weekly administration of heat therapy significantly prevented high fat diet-induced hyperglycemia, hyperinsulinemia and glucose intolerance. Previous studies have also reported an association between HSP72 induction and inhibition of inflammatory mediators, including the stress kinase JNK (Gabai et al., 1997; Park et al., 2001). Importantly, results from this study suggest an association between inhibition of both JNK1/2 isoforms and expression of HSP72 in skeletal muscle *in vivo*.

Extending from the first study (Chapter 5), the second study (Chapter 6) aimed to examine constitutive overexpression of HSP72, while eliminating confounding factors associated with heat treatment. This study employed a transgenic mouse model of HSP72 overexpression ($HSP72^{+/+}$), created using a chimeric transgene containing rat

inducible HSP72. Consistent with previous results, this study demonstrated that overexpression of HSP72 in skeletal muscle prevented high fat diet-induced hyperglycemia and hyperinsulinemia. In addition, $HSP72^{+/+}$ mice demonstrated improved glucose tolerance and enhanced insulin sensitivity as measured by intraperitoneal glucose and insulin tolerance tests. Furthermore, consistent with the first study (Chapter 5), $HSP72^{+/+}$ mice demonstrated decreased JNK1/2 phosphorylation in skeletal muscle when compared to WT mice on a high fat diet. Concomitantly, $HSP72^{+/+}$ mice on HFD showed enhanced AKT phosphorylation following insulin stimulation compared to WT HFD mice, an indication of enhanced insulin sensitivity. In an attempt to examine the degree of insulin sensitivity in WT and $HSP72^{+/+}$ mice, particularly between the liver and peripheral tissues, the euglycemic-hyperinsulinemic clamp (EHC) technique was employed in this study. Results from the EHC demonstrated enhanced insulin sensitivity through increased glucose infusion rate (GIR), as well as a tendency towards an increase in glucose disposal rate (GDR), without any difference in the rate of endogenous glucose production (EGP), confirming that the improved insulin sensitivity was due to a change in peripheral glucose disposal rather than any change in hepatic glucose production. However, it must be noted that the clamp experiments were only performed on chow fed animals, therefore different results may have been observed had the EHC been performed with animals on a high fat diet, as fat fed $HSP72^{+/+}$ mice showed a blunting of diet-induced IKKαβ phosphorylation in the liver. In addition, results from Chapter 6 showed that $HSP72^{+/+}$ mice placed on a high fat diet did not gain additional weight compared with WT mice, despite maintaining similar energy intake. In light of these findings, further investigation into the possibility of cross talk amongst insulin sensitive tissues and differential energy expenditure were explored in the next study (Chapter 7).

The aim of our third study (Chapter 7) was to determine whether protection from insulin resistance observed in HSP72$^{+/+}$ mice was associated with alterations in circulating adipokines and markers of mitochondrial oxidative capacity. Previous studies have demonstrated that increased adiponectin is associated with enhanced mitochondrial enzyme activity (Civitarese et al., 2006) and fatty acid oxidation in skeletal muscle (Yamauchi et al., 2002), while plasma leptin and PAI-1 levels are positively correlated with fat mass in humans (Considine et al., 1996; De Taeye et al., 2005). Importantly, this study demonstrated that HSP72$^{+/+}$ mice have increased circulating levels of adiponectin and decreased levels of leptin and PAI-1, correlating with decreased epididymal white adipose tissue mass and circulating free fatty acids. Furthermore, HSP72$^{+/+}$ mice showed significantly increased activity of mitochondrial enzymes, citrate synthase and β-HAD, compared to WT mice in the liver, adipose tissue and skeletal muscle, suggesting a greater capacity for mitochondrial oxidation in these mice. Results from this study provide novel insight into the relationship between adipokines, mitochondrial enzyme activity and HSP72 expression in the context of insulin resistance.

In conclusion, evidence from this thesis suggests that transient physical induction or constitutive overexpression of HSP72 is associated with protection from insulin resistance. Although the exact mechanism by which HSP72 protects against insulin resistance remains to be elucidated, results from this thesis strongly suggest that induction of HSP72 is associated with inhibition of inflammatory mediators, including JNK1/2 and IKKαβ in metabolically active tissues. In addition, expression of HSP72 is associated with alterations in adipokine secretion, decreased levels of circulating free fatty acids and enhanced mitochondrial enzyme activity, a process which may also contribute to the down-regulation of JNK1/2 and IKKαβ (Fig 8.1).

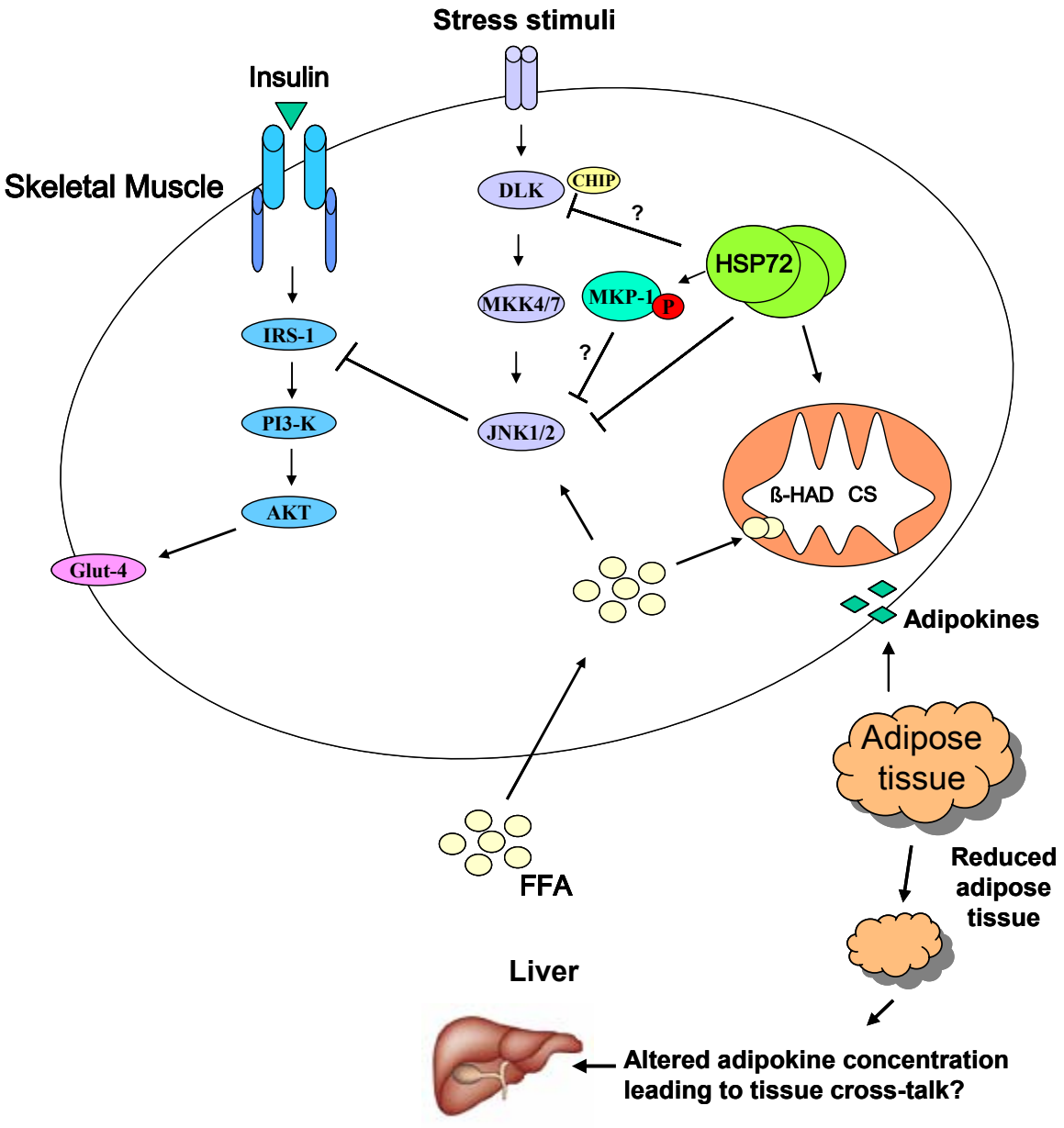


Fig. 8.1 Possible mechanisms by which HSP72 protects against obesity-induced insulin resistance. The presence of fatty acids results in the activation of JNK1/2 and disruption of insulin signalling via serine phosphorylation of IRS-1. Expression of HSP72 may act to inhibit JNK1/2 activity through chip-mediated inhibition of upstream DLK, increased phosphorylation of MKP-1, or through direct binding of JNK1/2. HSP72 may also facilitate fatty acid oxidation via enhanced mitochondrial enzyme activity while altering adipokine secretion and the possibility of tissue cross-talk.

8.2 Future directions

In order to uncover the mechanism by which HSP72 is protecting against obesity induced insulin resistance, further characterization of how HSP72 is inhibiting inflammation is required. As mentioned previously, upstream kinases of JNK, including DLK (Daviau et al., 2006) and MKP-1 (Lee et al., 2005b), appear to be attractive targets by which HSP72 may be inhibiting JNK activation. The use of immunoprecipitation techniques could provide valuable insight into the protein-protein interactions between HSP72 and DLK or MKP-1. These experiments would provide direct evidence that HSP72 is physically capable of binding to MAPK kinases, thus inhibiting JNK activation. Immunoprecipitation JNK and HSP72 can also be used to examine whether HSP72 is physically binding to JNK itself. Additionally, if HSP72 is mediating MKP-1 activation, overexpression of HSP72 may cause rapid deactivation of the JNK pathway.

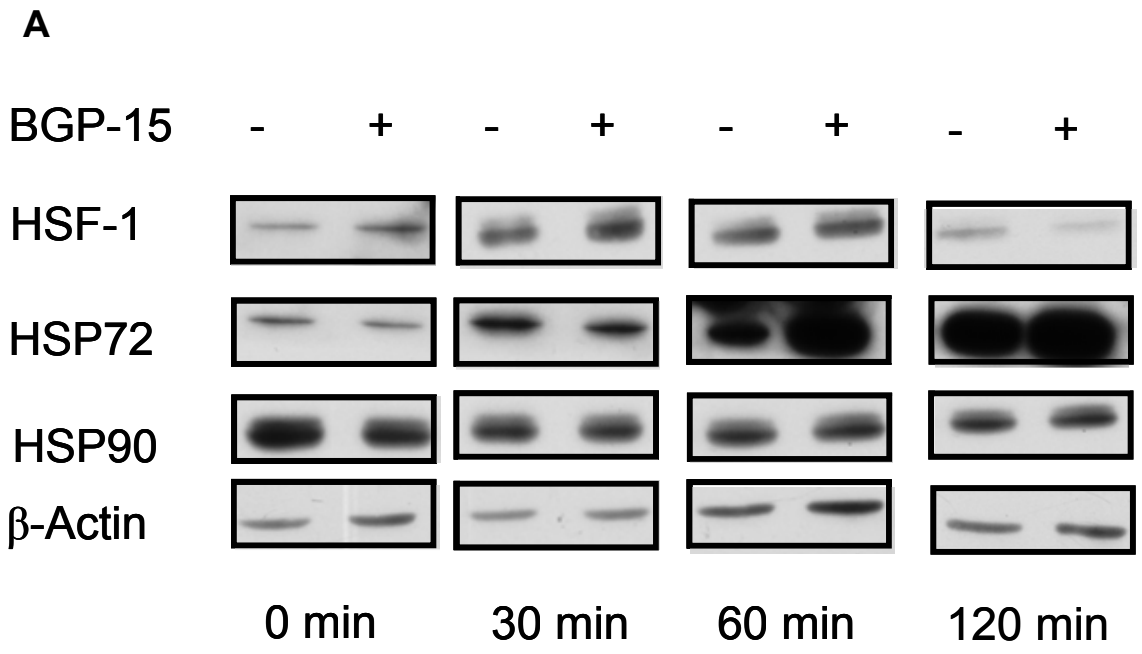
Furthermore, results from this thesis and other studies (Aguirre et al., 2000; Hirosumi et al., 2002; Solinas et al., 2006) strongly implicate JNK phosphorylation in the development of adiposity and insulin resistance. Therefore, an alternative approach to understanding whether HSP72 has a direct role in JNK inhibition would be to manipulate the expression of JNK in metabolically active tissues. Considering *JNK1*$^{-/-}$ mice exhibit improved glucose tolerance and reduced adiposity (Hirosumi et al., 2002), elimination of JNK1 in HSP72$^{+/+}$ mice or the use of heat therapy in *JNK1*$^{-/-}$ may provide further protection from the deleterious effects of inflammation. Conversely, overexpression of JNK, in metabolically active tissues may neutralize the beneficial effects of HSP72 compared to WT animals. Currently, we are examining the role of JNK1 overexpression in skeletal muscle via electroporation of a JNK1

construct into the *tibialis anterior* muscle of WT mice. Preliminary results from these studies suggest that overexpression of JNK1, via electroporation, increases IRS-1 Ser^{307} and decreases Akt Ser^{473} phosphorylation following insulin stimulation (unpublished data). Future studies may involve electroporation of the JNK1 construct into $HSP72^{+/+}$ mice. These experiments would provide insight as to whether overexpression of HSP72 can directly inhibit JNK1 *in vivo*.

With regards to mitochondrial oxidative capacity, results from Chapter 7 provided the initial foundation for further investigation into the metabolic phenotype of $HSP72^{+/+}$ mice. An examination of whole-body energy expenditure and measures of oxygen consumption, through the use of metabolic chambers, would allow for differentiation of substrate utilization in WT and $HSP72^{+/+}$ mice. Thus, a comparison of fat utilization between WT and $HSP72^{+/+}$ can be determined. In addition, measurements of fatty acid oxidation within metabolically active tissues, particularly the liver, adipose tissue and skeletal muscle, may confirm our observations of enhanced mitochondrial enzyme activity and decreased adipose tissue mass in $HSP72^{+/+}$ mice. Moreover, analysis of mitochondrial morphology, as well as mitochondrial density, would provide additional insight into the differences in oxidative capacity of WT and $HSP72^{+/+}$ mice.

Furthermore, we are currently collaborating with Dr. Andrea Hevener (California, USA) in exploring the potential of pharmacological induction of HSP72 via a novel hydroxylamine derivative, known as BGP-15. Hydroxylamine derivatives have previously been shown to improve wound healing in diabetic rats (Vigh et al., 1997) whilst delaying progression of the fatal neurodegenerative condition amyotrophic lateral sclerosis (ALS) in mice (Kieran et al., 2004). In the context of insulin

resistance, hydroxylamine derivatives have been shown to improve insulin sensitivity in diet-induced obesity, however, the mechanism by which this occurs has not been established (Kurthy et al., 2002; Sebokova et al., 2002). Hydroxylamine derivatives are also thought to activate HSF-1, a key transcription factor in HSP72 protein expression (Kieran et al., 2004; Kurthy et al., 2002). In light of these findings, we sought to examine the potential effects of BGP-15 in the induction of HSP72 and protection from obesity-induced insulin resistance. Results from this ongoing study have demonstrated that BGP-15, combined with heat treatment, is a potent co-activator of HSF-1 and HSP72 in L6 myotubes (Fig. 8.2A), while *in vivo*, BGP-15 markedly induces HSP72 in the absence of heat treatment in leptin deficient *(ob/ob)* mice (Fig. 8.2B). Corresponding to these results, treatment with BGP-15 significantly reduced fasting plasma glucose (Fig. 8.2D), insulin (Fig. 8.2E) and phosphorylation of JNK1/2 in skeletal muscle of *ob/ob* mice (Fig. 8.2C). In addition, mice treated with BGP-15 demonstrated improved whole-body insulin sensitivity as determined by measures of the EHC, including increased insulin-stimulated glucose disposal (Fig. 8.2F) and decreased hepatic glucose production (Fig. 8.2G) (Chung et al., 2008). Taken together, these preliminary results suggest an association between administration of BGP-15, induction of HSP72 and protection from insulin resistance. However, additional research is required to disseminate the mechanism by which BGP-15 exerts its beneficial effects.


A
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HSF-1
HSP72
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β-Actin
0 min
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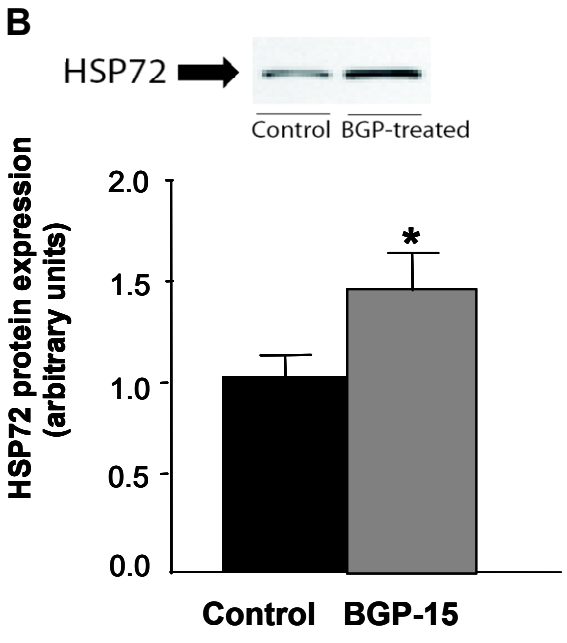
B
HSP72
Control BGP-treated
HSP72 protein expression (arbitrary units)
2.0
1.5
1.0
0.5
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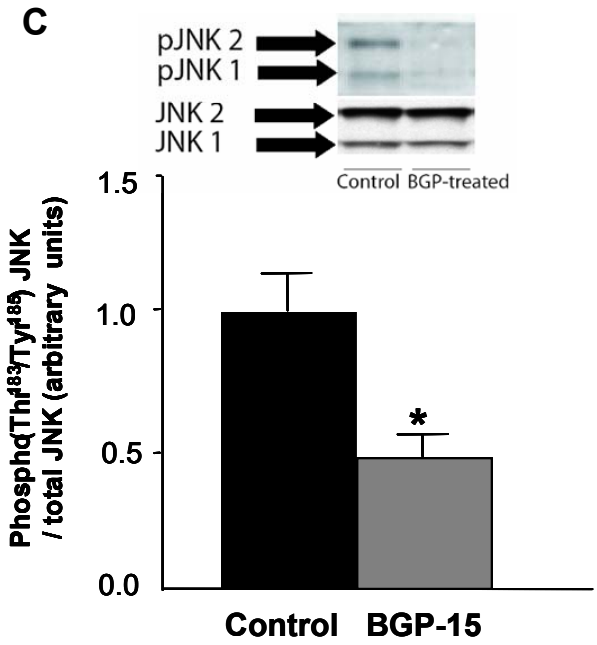
Control BGP-15


C
pJNK 2
pJNK 1
JNK 2
JNK 1
Control BGP-treated
Phosphα(Thr183/Tyr185) JNK / total JNK (arbitrary units)
1.5
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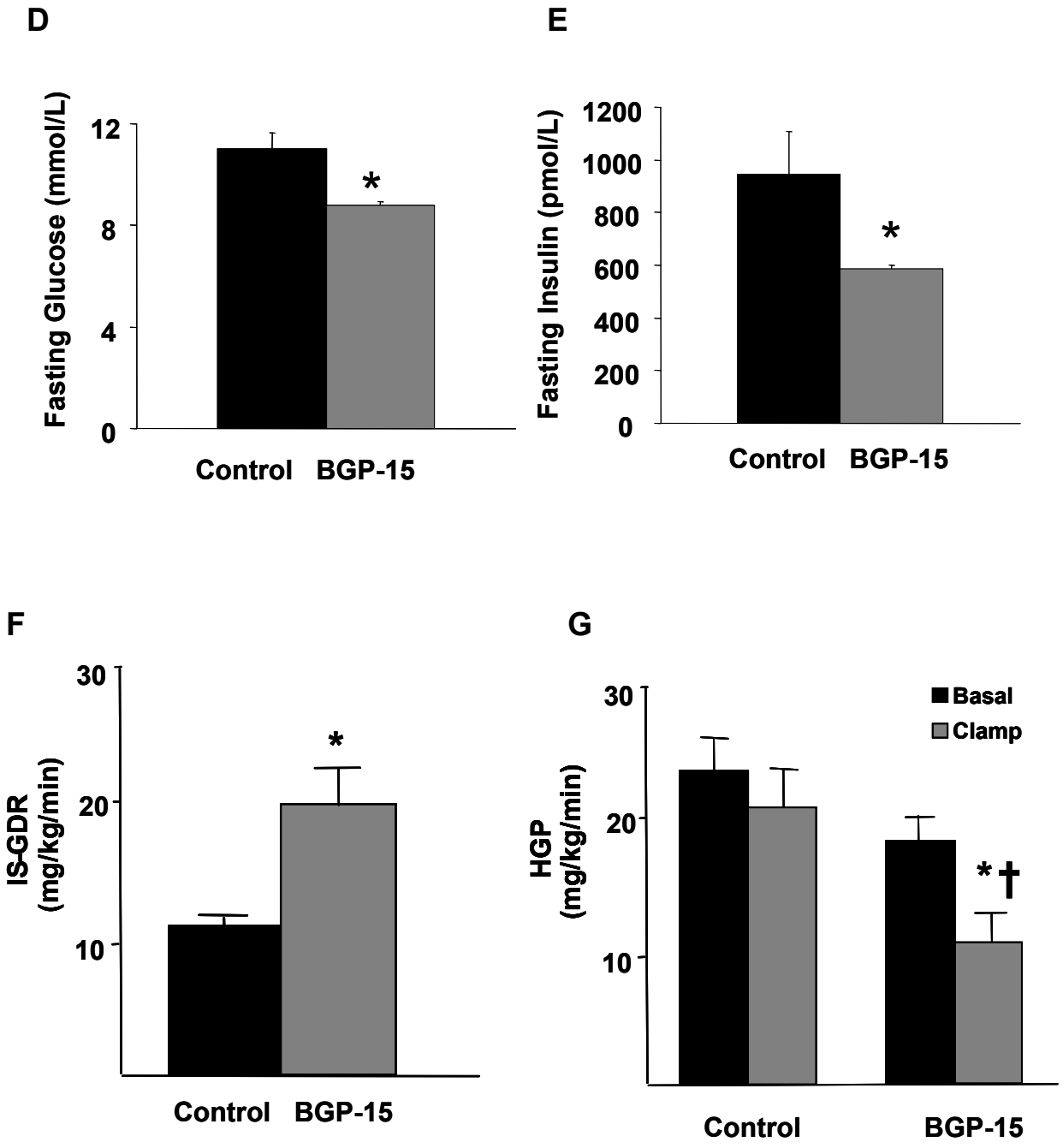

Fig. 8.2 Co-activation of HSF-1 and HSP72 by heat and BGP-15 in L6 myotubes (A) BGP-15 mediated induction of HSP72 *in vivo* (B) inhibition of JNK1/2 phosphorylation (C) fasting glucose (D) fasting insulin (E) insulin stimulated glucose disposal rate (F) and hepatic glucose production (G) (n = 3 for cell culture experiments; n = 5-7 mice per group; **P*<0.05 compared with control; †**P*<0.05 compared with basal for BGP-15 treated group).

In conclusion, further elucidation of the biological role of HSP72 may facilitate the treatment of insulin resistance and have wide ranging applications to public health. Given the current state of obesity in numerous developed nations, HSP72 may be a valuable therapeutic target for the treatment of metabolic diseases.

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