The Role of gp130 Receptor Signalling in Inflammation and Metabolism

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

By

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Declaration

I, the candidate, Bronwyn Anne Neill, certify that:

- All data included within this thesis is that obtained by the candidate alone
- Except where due acknowledgement has been made, the work is that of the candidate alone whilst enrolled as a student at The Royal Melbourne Institute of Technology.
- The work has not been submitted previously, in whole or in part, to qualify for any other academic award
- The content of this dissertation is the result of work which has been completed since the official commencement date of the approved research program

Signature of Candidate

22nd March 2010

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List of Abbreviations

α-MEM	alpha minimum essential medium
ACC	Acetyl CoA Carboxylase
ACT	α_1-antichymotrypsin
ADAM	A disintegrin and metalloprotease
Akt	v-akt murine thymoma viral oncogene homolog (Also known as Protein Kinase B)
ALT	Alanine Aminotransferase
AMPK	AMP activated mitogen protein kinase
ANOVA	Analysis of variance of analysis
AS160	Akt substrate of 160 kDa
AST	Aspartate Aminotransferase
BSF-2	Human B Cell stimulatory factor
CBM	Cytokine Binding Module
CNTF	Cilliary Neurotropic Factor
COX IV	Cyclooxygenase IV
CRP	C-Reactive Protein
EDL	Extensor Digitorum Longus
FBS	Foetal Bovine Serum
G-6-Pase	Glucose-6-phosphatase
GLUT4	Glucose Transporter 4
gp130	Glycoprotein 130
h	hour
HFD	High Fat Diet
IFN-β2	Interferon β2
IgG	Immunoglobulin G
IKK	IKappaB Kinase
IL-6	Interleukin-6
IL-6Rα	Interleukin-6 receptor α
IRS	Insulin Receptor Substrate
JAK	Janus Kinase

JNK	c-jun-N- terminal kinase
KO	Knock out
MEF-2	Myocyte Enhancer Factor-2
min	minute
MKK6	Mitogen Activated Protein Kinase Kinase 6
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtFAM	mitochondrial Transcription Factor A
mTOR	mammalian Target of Rapamycin
NRF	Nuclear Receptor Factor
OXPHOS	Oxidative Phosphorylation
p38 MAPK	p38 Mitogen Activated Protein Kinase
PEPCK	phosphoenolpyruvate carboxy kinase 1
PGC-1α	Peroxisome Proliferators Activator Receptor γ Co-activator-1α
PI3-K	Phosphatidylinositol 3 Kinase
PRMT-1	Arginine Methylation Transferase-1
RER	Respiratory Exchange Rate
rh-IL-6	recombinant human Interleukin-6
SEM	standard error of mean
sgp130	Soluble gp130
sIL-6R	soluble Interleukin-6 receptor
SIRT-1	Sirtuin-1
SOCS-3	Suppressor of Cytokine Signalling 3
SRC-2	Steroid Receptor Coactivator-2
STAT-3	Signal Transducers and Activators of Transcription-3
TNF	Tumour Necrosis Factor
UCP	Uncoupling Protein
WAT	White Adipose Tissue
WT	Wild type
VO_2	Capacity for volume of Oxygen

Abstract

Upon exercise Interleukin-6 (IL-6) is released by contracting skeletal muscle. This increased release of this cytokine increases fat and glucose oxidative metabolism within muscle and adipose and suppress hepatic glucose output. IL-6 is an unsuitable anti-obesity therapeutic agent as it is implicated in the pathogenesis of many inflammatory conditions. Classic IL-6 signal transduction occurs through a trans-membrane complex consisting of an IL-6 receptor α homo-dimer and gp130 receptor-β (gp130Rβ). Alternatively, signalling may occur through a complex of IL-6 and its soluble receptor (sIL-6R) which in turn targets membrane bound gp130Rβ. This phenomenon known as "IL-6 trans-signalling" has implications, as cells devoid of the membrane bound IL-6 receptor remain responsive to the cytokine due to the ubiquitous expression of gp130. Futhermore, it is known that the pro inflammatory effects of IL-6 are mostly mediated via IL-6 trans-signalling. Interestingly, in transgenic mice over expressing a soluble form of gp130 (sgp130), the naturally occurring competitive inhibitor of sIL-6R signalling, inflammation is prevented. Thereby, co-treatment with IL-6 and sgp130 may have therapeutic advantages for obesity related diseases, providing signalling exclusively through the membrane bound IL-6Rα/gp130β.

The primary focus of this dissertation was to examine the effect of IL-6/sIL-6R signalling in the presence and absence of sgp130 on inflammation and metabolism within skeletal muscle, adipose and liver *in vitro* and *in vivo*. Hyper IL-6, a designer cytokine consisting of rh-IL-6 and sIL-6R connected via a polypeptide linker, was utilised to mimic IL-6 trans-signalling. In vitro, phosphorylation of STAT3 (Tyr^{705}), and Akt (Ser^{473}) were elevated in both L6 myotubes and CRL1439 hepatocytes incubated with Hyper IL-6 when compared with control. Importantly, signalling was reduced upon co-treatment with sgp130Fc in CRL1439 whilst it was entirely preserved in myotubes. Moreover, Hyper IL-6 potentiated Insulin stimulated glucose uptake in the absence and presence of sgp130Fc.

Consistent with the fore mentioned data, IL-6 trans-signalling events occured in liver, muscle and epidydimal White Adipose Tissue (WAT) *in vivo*, in a tissue type dependent manner. Moreover, it was demonstrated sgp130Fc inhibited Hyper IL-6 induced signalling, however the degree to which inhibition occurred was tissue type dependent.

Finally, contrary to the transient release seen during exercise, states of disease are often associated with chronically elevated IL-6 within the circulation leading to aberrant pathway activation and inflammation within many tissues. As this paradox exists, prolonged Hyper IL-6 administration on inflammation and whole body energy metabolism was examined *in vivo*. Although sgp130Fc was able to prevent severe injury and chronic inflammation in the liver as a consequence of prolonged Hyper IL-6 treatment, it was unable to improve insulin resistance induced by this cytokine. This could reflect the enhanced bioactivity of the designer cytokine.

Collectively, these data support the proposition that in muscle, Hyper IL-6 induced trans-signalling is neutralised by sgp130Fc whilst the beneficial effects on metabolism associated with membrane bound signalling remains intact *in vitro*. *In vivo*, acute administration of this designer cytokine is able to regulate signalling pathways associated with metabolism and inflammation, an effect that sgp130Fc co-administration inhibited in a tissue type dependent manner. Due to the potency of Hyper IL-6, even when co-administered sgp130FC, it remains a poor candidate as a therapeutic tool in the war against obesity. This, however, does not discount the potential of a gp130 receptor/ligand like drug design as an anti-obesity agent.

CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

The prevalence of diabetes has severely increased world wide over recent years. In Australia alone, where the incidence of the disease is particularly high, it is estimated that 1 million people are afflicted by diabetes and that, by the year 2025, it is predicted 333 million people world wide will be suffering from this debilitating disease. This disease has great social and economical ramifications, in 2004 the cost of diabetes to the Australian community exceeded $900 million (http://www.health.gov.au/internet/main/publishing.nsf/content/pq-diabetes-stats, last updated: May 2008). Hallmarks of this debilitating disease are obesity and associated inflammation of metabolic tissues. As this is so, the need for a therapeutic and/or prophylactic agent is in high demand. One such potential therapeutic anti obesity agent may be a gp130 receptor/ligand like drug design, and such ligands have already entered clinical trials (Ettinger et al., 2003).

Upon Interleukin-6 binding to its receptor, a transmembrane complex is formed with a homo-dimer of the gp130 receptor. This causes the subsequent activation of JAK/STAT, PI3-Kinase and MAPK pathways to occur (Febbraio, 2007). This is of utmost importance as previous research has demonstrated that these pathways are important regulators of metabolism within skeletal muscle. As skeletal muscle is the major tissue involved in limiting the rate of insulin-stimulated glucose disposal (Yki-Järvinen et al., 1987) it is therefore, logical that this tissue may be a player in the development of insulin resistance. *In vitro,* co-treating L6 myotubes with IL-6 potentiates insulin induced glucose uptake and increases fatty acid oxidation (Carey et al., 2006). Furthermore, *in vivo* studies have implicated IL-6 in the oxidation of glucose and lipid (Carey et al., 2006 and Van Hall et al., 2003). However, deregulated production of this cytokine and the soluble form of its receptor is associated with many pro-inflammatory conditions, making the cytokine alone a poor candidate as a therapeutic agent. Interestingly, in transgenic mice over expressing a soluble form of gp130 (sgp130), the naturally occurring antagonist of sIL-6R signalling, inflammation is prevented (Rabe et al., 2008). Moreover, signalling mediated via the membrane bound receptor remains intact. As this is the case, co-treatment with IL-6 and sgp130 may have therapeutic

advantages for obesity related diseases, providing signalling exclusively through the membrane bound IL-6Rα/gp130β whilst maintaining the beneficial effects often associated with IL-6 signalling.

1.2 Interleukin-6

Interleukin-6, a pleiotropic cytokine, was first identified well over 25 years ago. In 1980, IL-6 was isolated in fibroblasts and cloned (Weissenbach et al., 1980). Initial indications from this study, were that IL-6 possessed anti viral activity similar to Interferon-β-1, an effect that would later be disproved (Content et al., 1982; Poupart, et al., 1987). As a result of their studies, Weissenbach and colleagues called the molecule Interferon-β-2. Over the proceeding decade, the biological function of IFN-β-2 remained controversial, a reflection of which was the numerous pseudonyms assigned to the protein. These included; B Cell Differentiation Factor (Okada et al., 1983), 26 kDa protein (Haegeman et al., 1986), Hybridoma Growth Factor (Van Damme et al., 1987a and 1987b) and Hepatocyte Stimulating Factor (Ritchie and Fuller, 1983), to name a few. This cytokine was also described as human B-Cell stimulatory factor (BSF-2) as it was capable of inducing maturation of B cells into immunoglobulin secreting cells (Hirano et al., 1986). BSF-2 was purified and the NH_2-terminal amino acid sequence encoding a novel interleukin that was “functionally and structurally unlike any other known protein” was determined. Most interestingly, it was noted that patients with Cardiac Myxoma cells produced high levels of BSF-2 and were prone to “auto immune disease like symptoms” (Hirano et al., 1986). As a consequence, these authors proposed that deregulated production of this cytokine contributes to autoimmune pathogenesis, the first evidence of a “pro-inflammatory” role for this cytokine. In 1987, Poupart confirmed that IFN-β-2 had no anti-viral activity and in support of the fore mentioned study, was highly efficacious at modulating growth and differentiation of B cells. Poupart proposed to adopt the name of “Interleukin 6”, thereby eliminating the confusion caused by the varying nomenclature.

In humans, IL-6 is a pro-inflammatory glycoprotein of 184 amino acids with a molecular weight of 21-30 kDa (May et al., 1989). The variation in mass of IL-6 is

dependent on N-glycosylation and post translational modification of this protein (May et al., 1989; Santhanam et al., 1989). A member of the IL-6 family of cytokines that includes; Cardiotrophin 1, Cardiotrophin like cytokine, Ciliary Neurotropic Factor (CNTF), Interleukin-11, Leukemia Inhibitory Factor and Oncostatin M, it consists of 4 α-helices; A, B, C and D, linked by a short chain and 2 long chain loops folded in a 'up-up-down-down' topology (Figure 1.1) (Somers, Stahl and Seehra, 1997). Although IL-6 family members recognise and bind their own specific receptor complex they are unified by the required recruitment of glycoprotein-130 (gp130) receptor complex for cellular signal transduction to occur (Heinrich et al., 2003).

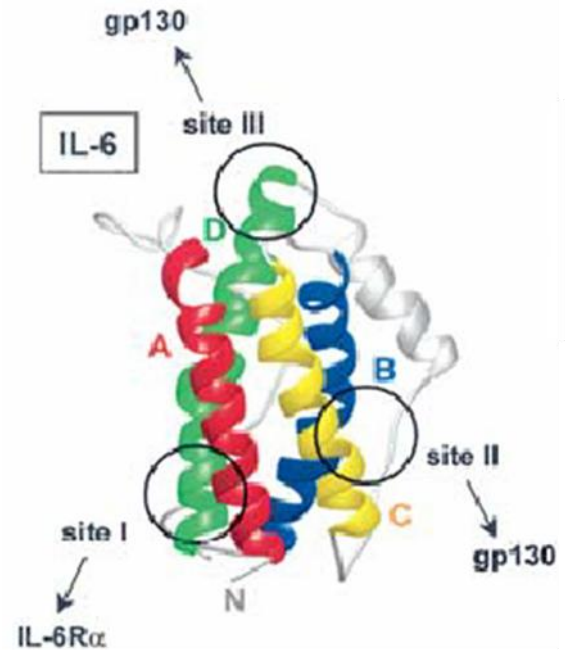


Figure 1.1 Structure of Interleukin-6. IL-6 consists of four long helices, depicted here as A (red), B (blue), C (yellow) and D (green), joined by two long chains and a short chain. The binding sites of IL-6R and gp130 are circled (Heinrich et al., 2003).

In humans, mice and rats, the IL-6 gene contains 4 introns and 5 exons (Yasukawa et al., 1987) and is located at chromosome 7p21-p14 in human (Lahn et al., 1997)

and at chromosome 5 in the mouse genome (Mock et al., 1989). A high degree of homology for the nucleotide and amino acid sequences exists across IL-6 species. Human and murine IL-6 share 60 % nucleotide and 42 % amino acid homology and similarly rat IL-6 shares 68 % and 58 %, respectively, sequence homology with that of human IL-6 (Tanabe et al., 1988, Northemann et al., 1989). Northemann (ibid) also noted that rat and murine IL-6 also share a high degree of nucleotide (92 %) and amino acid (93 %) sequence homology. Although high sequence homology exists between IL-6 species, the same can not be said for their respective receptors. As a consequence there is limited cross reactivity, however the murine IL-6 receptor is still capable of binding human IL-6.

1.3 IL-6 receptor and its interaction with gp130

The membrane bound IL-6 receptor (IL-6R) is an 80 kDa, type I membrane protein that consists of an extracellular N-terminus, a trans-membrane domain and a cytoplasmic domain (Figure 1.2). This cytokine receptor consists of 468 amino acids; including a 19 amino acid signal peptide sequence and a cytoplasmic domain of approximately 80 amino acids (Yamasaki et al., 1988). Like most cytokine receptors, it has a conserved structural feature of a cytokine binding module. The cytokine binding module (CBM) consists of an N terminal of 4 cysteine residues, two fibronectin type-III-like domains (D2 and D3) and a WSXWS motif in the C terminal (Bazan, 1990). Proximal to the CBM, an Ig-like domain (D1) of approximately 90 amino acids is located at the N-terminal (Yamasaki et al., 1988).

It is well established that IL-6 may also bind the agonistically soluble form of the IL-6 receptor, known as soluble IL-6 receptor (sIL-6R). The IL-6/sIL-6R targets the ubiquitously expressed gp130 receptor, so cells normally devoid of membrane bound IL-6R remain IL-6 responsive. This phenomenon is commonly known as “IL-6 trans-signalling”. Initially, characterisation of the sIL-6R led to the conclusion it was biologically relevant. HepG2 cells, which are naturally devoid of the membrane bound receptor, were co-treated with IL-6 and the sIL-6R receptor (Stoyan et al., 1993). As a consequence the acute phase protein α_1-antichymotrypsin (ACT) was

produced. Many studies subsequently demonstrated that the sIL-6R was capable of inducing other acute phase proteins, such as haptoglobin (Weiergräber et al., 1995) and serum amyloid A (Rabe et al., 2008). Soluble IL-6R has since been implicated in inflammatory conditions often associated with high concentrations of IL-6 within the circulation.

Initial studies demonstrated that upon challenge with bacterial toxins and chemical assault, rapid shedding of membrane bound IL-6R from human macrophages occurred (Walev et al., 1996). Importantly, sIL-6R collected from the supernatant of bacterially challenged monocytes was biologically active. In HepG2 cells, it was able to bind to its ligand and induce haptoglobin mRNA, an IL-6 dependent response. Soluble IL-6R is generated by ectodomain shedding of the receptor by metalloproteases. These proteins proteolytically cleave the receptor close to the membrane anchors, thus releasing the extracellular domains of the receptor into the extracellular space. Metalloproteases responsible for ectodomain shedding may be either endogenous, for example ADAM 10 and ADAM17 (Matthews et al., 2003) or exogenous such as microbial proteases (Vollmer et al., 1996). Secondly, sIL-6R may be generated through alternatively spliced mRNA (Lust et al., 1992). Although the soluble form of the receptor lacks the trans-membrane and cytoplasmic fragments typical of the membrane bound species, the ability to bind to its ligand remains unaffected as the extracellular CBM remains intact (Lust et al., 1992).

1.4 Interleukin 6 signalling

IL-6 binds to the cytokine binding module of IL-6R, forming a ligand/receptor complex. In turn, the IL-6/IL-6R complex binds to the cytokine binding module of gp130, ultimately leading to the homodimerisation of gp130 and internalisation of the complex (Graeve et al., 1996). gp130, a 130 kDa glycoprotein, was first isolated from mixed lymphocyte cultures (Andersson et al., 1978). gp130, a ubiquitously expressed glycoprotein, is the central player in IL-6 family cytokine signalling. The extracellular domain of gp130 consists of 597 amino acids divided into 5 fibronectin III-like domains and an Ig-like N-terminal domain. Domains 1, 2

and 3 are β-sheet sandwich domains important in cytokine engagement with the CBM (domains 2 and 3) of gp130 (Figure 1.2, Chow et al., 2001 and Heinrich et al., 2003). IL-6 and its receptor interact through the site I epitope on the B and D helical faces of the cytokine (Figure 1.1). This complex then interacts with the cytokine binding module of gp130 at Site II, forming an intermediate trimolecular complex. For signal transduction to occur the trimolecular complex must become activated. Binding of the Ig-like domain must occur at Site III of IL-6, thus forming an activated ternary complex. This ternary complex transitions to form a signalling competent, high affinity hexameric complex consisting of two IL-6, two IL-6Rα and two gp130 molecules (Boulanger et al., 2003).

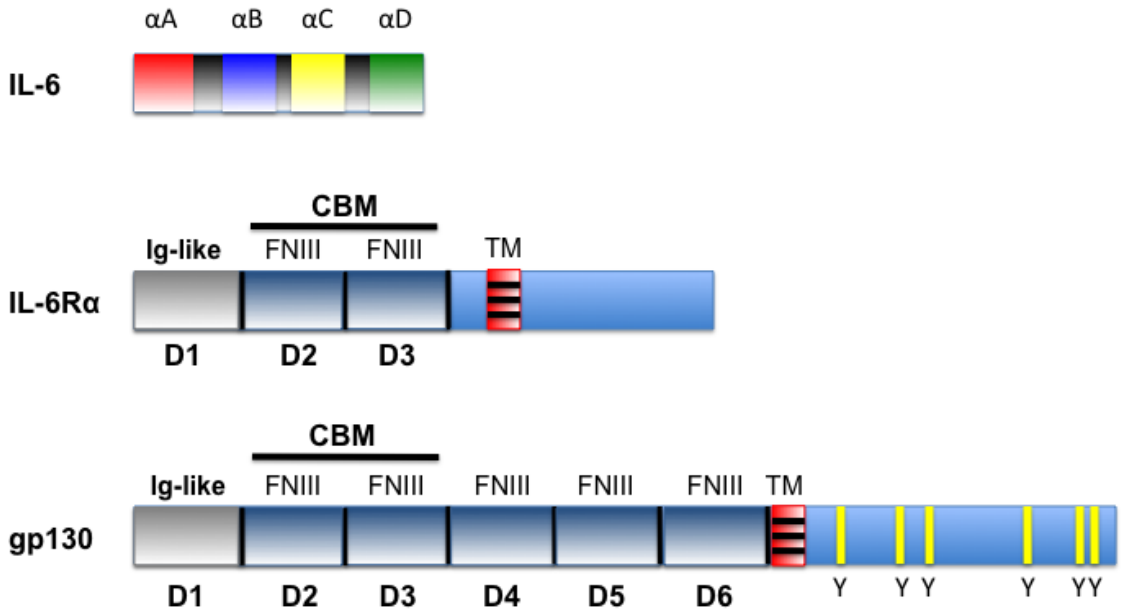


Figure 1.2 The structural organisation of IL-6, IL-6Rα and gp130. Depicted here as Ig-like: Ig like domain, FNIII: Fibronectin-Like domain, CBM: Cytokine Binding Module, TM: transmembrane, Y: tyrosine phosphorylation site (adapted from Heinrich et al., 2003)

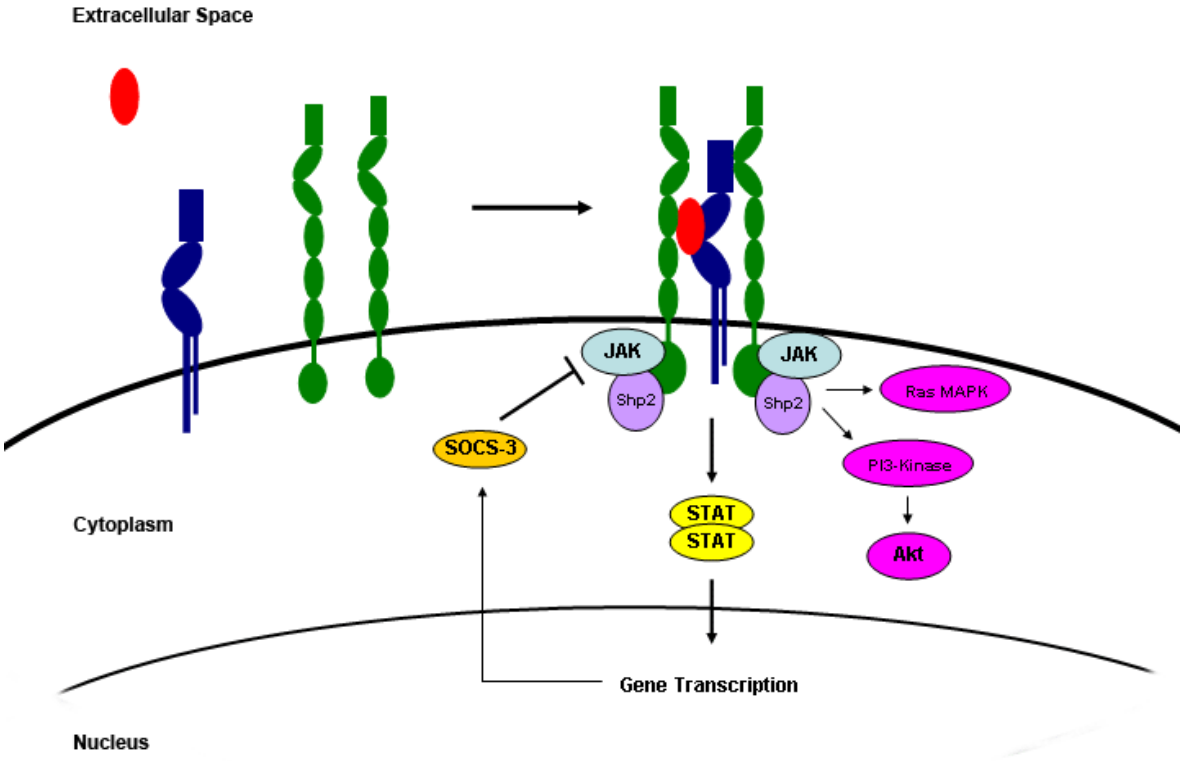
It is well established, upon homodimerisation of gp130 the intermediatory step of recruitment and activation of gp130 associated Janus Kinases (JAK-1 and JAK-2) occurs (Stahl et al., 1994; Wang and Fuller, 1994). This is sequentially important in

mediating downstream signal transduction. Upon complex formation, JAK is activated and phosphorylates the last 4 carboxyl terminal tyrosine containing motifs within the cytoplasmic tail of gp130 (Stahl et al., 1995). Tyrosine phosphorylation leads to the formation of Src Homology 2 phosphotyrosine binding domains for protein-protein interaction with Signal Transducers and Activators of Transcription (STAT-1 and STAT-3). STAT proteins, which contain SH2 binding domains, dock with the autophosphorylated tyrosine residues subsequently leading to phosphorylation of STAT-3 and homo- or hetero- dimer formation to occur (Lütticken et al., 1994). The STAT dimer then translocates to the nucleus where it initiates gene transcription. Post translocation, STAT-3 has the ability to recognise the IL-6 responsive $TT(N)_4AA$ motif within the 5' flanking region of the promoter of transcriptional targets (Hattori et al., 1990; Zhang et al., 1996). IL-6 induced transcriptional targets include those encoding proteins commonly associated with the acute phase response, such as C-Reactive Protein (CRP) (Zhang et al., 1996), α_1-antichymotrypsin (ACT) (Kordula et al., 1998), rat α_2-macroglobulin (Hattori et al., 1990) and importantly, Suppressor of Cytokine Signalling-3 (SOCS-3) (Auernhammer, Bousquet and Melmed, 1999). STAT-3 dependent activation of SOCS-3 mRNA is induced rapidly upon IL-6 stimulation and is considered to be of great importance in the negative feedback inhibition of IL-6 signalling.

SOCS-3 regulates IL-6 signalling through a negative autoregulatory loop by binding gp130 at Tyr757, thereby preventing associated JAK activity (Krebs and Hilton, 2001). This limits the autophosphorylation of gp130 and prevents STAT interaction with the cytoplasmic domain of the receptor. Alternatively, SOCS-3 may also target the receptor complex for proteosomal degradation by ubiquitination (Krebs and Hilton, 2001). The increase in SOCS-3 has further implications with regards to metabolic signalling as this protein is a known inhibitor of insulin signalling within the liver. SOCS-3 has the ability to bind to the Insulin Receptor at Tyr960, a key recognition site for IRS-1 and -2, thereby preventing phosphorylation of these proteins and subsequently suppressing insulin induced activation of PI3-Kinase and Akt (Ueki, Kondo and Kahn, 2004 and Torisu et al., 2007).

Alternatively, IL-6 induced gp130 activity may also lead to the activation of either or both of PI3-Kinase (Weigel et al., 2008) and Ras-MAPK pathway (Leu et al., 2003 and Heinrich et al., 2003). These fore mentioned signalling pathways are important mediators of many intracellular processes including protein synthesis, cell proliferation and differentiation, insulin signalling and glucose and fatty acid metabolism.

1.3a)


Extracellular Space
JAK
Shp2
JAK
Shp2
Ras MAPK
SOCS-3
PI3-Kinase
Cytoplasm
STAT
STAT
Akt
Gene Transcription
Nucleus

1.3b)

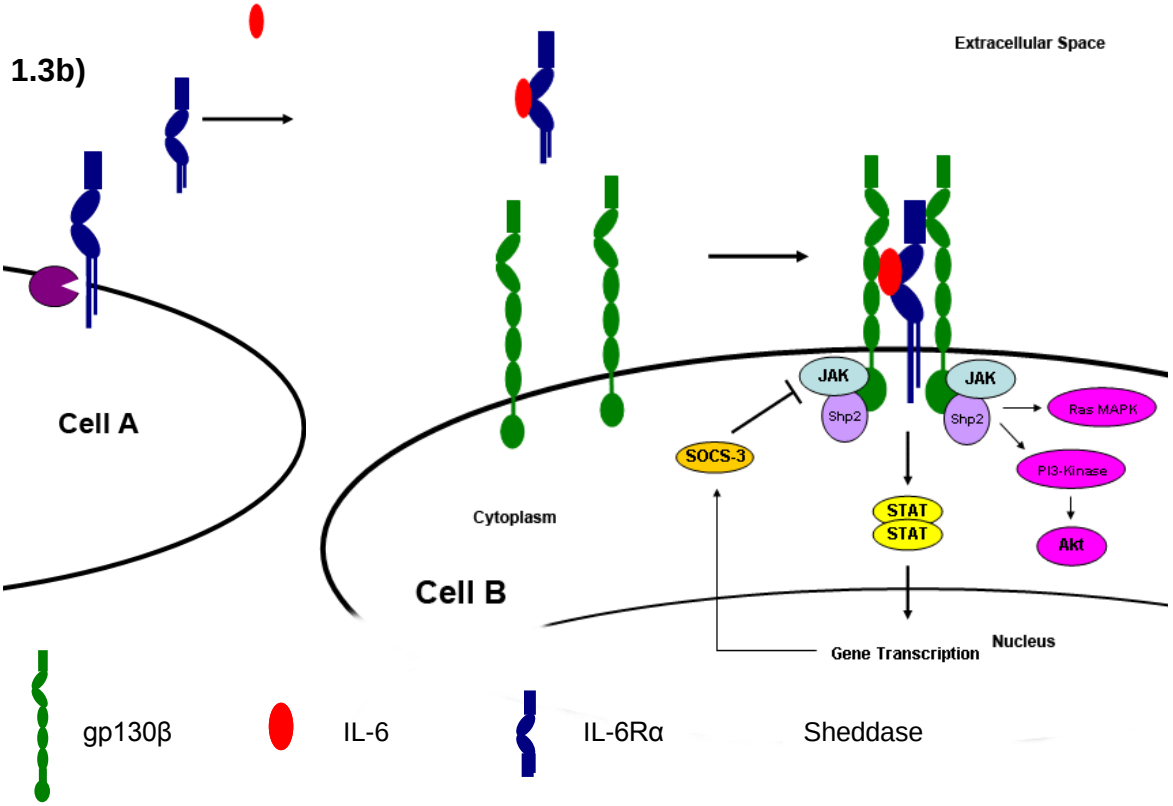

Extracellular Space
Cell A
JAK
Shp2
JAK
Shp2
Ras MAPK
SOCS-3
PI3-Kinase
Cytoplasm
STAT
STAT
Akt
Cell B
Gene Transcription
Nucleus
gp130β
IL-6
IL-6Rα
Sheddase

Figure 1.3 Mechanisms of IL-6 signal transduction – a simplified schematic. (a) Classic cognate membrane bound IL-6R signalling occurs through the transmembrane complex of IL-6 receptor α homodimer and gp130. gp130 receptor activation by the IL-6/IL-6R complex causes the intracellular tyrosine phosphorylation of its cytoplasmic tail by its associated JAKs. This leads to the docking of STAT 1 and 3 to the phospho-tyrosine sites. This causes the phosphorylation of STAT-3 and subsequently translocation of this protein to the nucleus where it initiates gene transcription. In turn, STAT-3 induced gene transcription negatively regulates IL-6 activity through inhibition of JAKs induced by increased SOCS-3 activity. Furthermore, Shp2 binds to activated gp130 resulting in pathway activation of Ras-MAPK and PI3-Kinase. (b) In a process known as "IL-6 trans-signalling", agonistic sIL-6R may be generated through alternate mRNA splicing or via cleavage of the membrane bound IL-6R by metalloproteases (referred to as "sheddase" within this figure). IL-6/sIL-6R complex then targets gp130 and results in the downstream activation of the fore mentioned pathways.

1.5 IL-6 in Health and Disease

In accordance with the true pleiotropic nature of IL-6, the release of this cytokine mediates various effects in both health and disease. Contrary to the "pro-inflammatory cytokine" description many studies have focussed on examining IL-6 production and release during exercise and the beneficial effects on metabolism often associated with this cytokine. It is well established that contracting skeletal muscle produces and releases IL-6 (Ostrowski et al., 1998 and Hiscock et al., 2004). Subsequently, increased release of IL-6 into the circulation provides a mechanism by which tissues can "cross-talk" (For review refer to Pedersen and Febbraio, 2008), whereby IL-6, produced by other tissues such as skeletal muscle, is able to bind to, and elicit effects in, tissues such as liver and adipose. This, in turn, leads to IL-6 dependent increases in hepatic glucose output and increased lipolysis of adipose (Figure 1.4). Tissue IL-6 dependent cross-talk has also been noted in states of inflammation. HFD induced insulin resistance was less in liver of

mice with ablated JNK1 in adipose tissue when compared to littermate control mice (Sabio et al., 2008). Upon further exploration it was apparent this was IL-6 dependent. JNK1 ablated mice had suppressed IL-6 production by adipose tissue and as a consequence decreased expression of SOCS-3 within the liver, an important mediator of hepatic insulin resistance.

Many studies have examined the effect of this cytokine on metabolism *in vitro* and *in vivo*. *In vitro*, IL-6 is known to increase glucose uptake in 3T3-L1 adipocytes (Stouthard, Elferink and Sauerwein., 1996). In human skeletal muscle cells glycogen synthesis is increased through IL-6 dependent activation of Akt at the ser473 residue (Weigert et al., 2005). Carey and co-workers (2006) demonstrated IL-6 leads to increased glucose uptake and fat oxidation in L6 myotubes. This study also implicated AMPK activity in IL-6 dependent increases in metabolism. In L6 myotubes infected with an AMPK DN virus, the IL-6 induced increase in glucose uptake was ablated. In further support of the notion IL-6 positively regulates metabolic processes, infusion of IL-6 into humans increased insulin stimulated glucose disposal as determined by hyperinsulinaemic-euglycaemic clamp (Carey et al., 2006). This was in accordance with previous research performed by Febbraio and colleagues (2004) who discovered recombinant human IL-6 (rh-IL-6) infusion increased the rate of contraction induced endogenous production of glucose and its rate of disposal. Recombinant human IL-6 infusion was also utilised by Van Hall et al., (2003) to determine the effect of IL-6 on human fat metabolism, *in vivo*. Recombinant human IL-6 infusion increased lipolysis, as indicated by increased appearance and elevated sera levels of fatty acids, and fat oxidation.

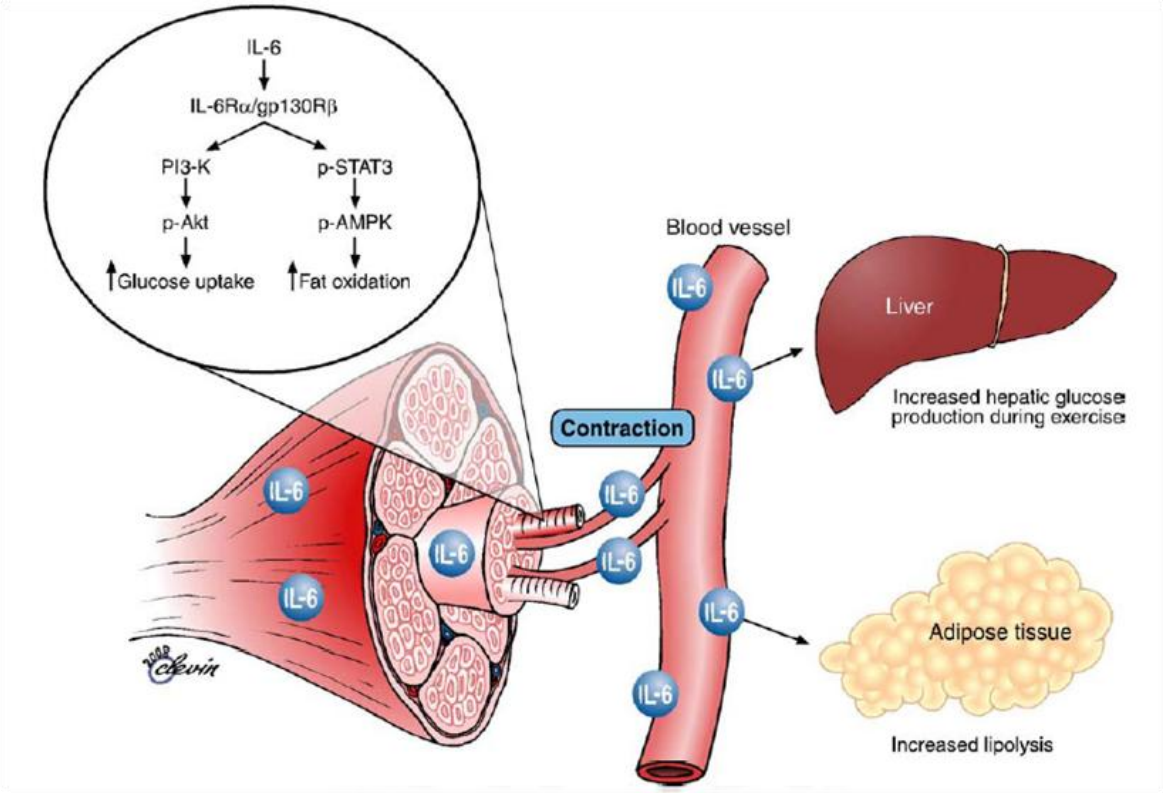


Figure 1.4 IL-6 dependent "tissue crosstalk" during exercise. Contracting skeletal muscle releases IL-6 into the circulation. IL-6 acts on skeletal muscle and other tissues in a paracrine manner, activating Akt and STAT-3 leading to increased glucose uptake and fat oxidation in muscle, increased lipolysis of adipose tissue and hepatic glucose output (Pedersen and Febbraio, 2008).

Contrary to the fore mentioned studies that demonstrate a beneficial role of IL-6 in metabolic control, this cytokine has also been implicated in insulin resistance in cultured hepatocytes *in vitro,* causing decreased glucose transport (Senn et al., 2002) and inhibited insulin stimulated glycogen synthesis (Kanemaki et al., 1998). In rats, IL-6 increases production of the acute phase protein α_2-macroglobulin and causes the degradation of glycogen within the periportal zone of the liver (Lienenlüke and Christ, 2007). These changes could possibly contribute to decreased glucose production often noted during prolonged acute phase inflammation.

Interleukin-6 has also been implicated in the pathogenesis of many inflammatory conditions. IL-6/sIL-6R complex signalling is an important mediator of such inflammatory conditions as arthritis (Nowell et al., 2003), regenerative hyperplasia and adenomas of the liver (Maione et al., 1998), AIDS associated Kaposi's Sarcoma (Miles et al., 1990) and inflammatory bowel diseases such as Crohn's disease (Jostock et al., 2001), ileitis (Sander et al., 2008) and ulcerative colitis (Atreya et al., 2000). It would appear the role of IL-6 in these conditions is through directing transition of acute phase inflammation to a chronic state of inflammation. This occurs through a change in profile of the subset of the infiltrating cells (Figure 1.5), a response that is IL-6 trans-signalling dependent as many of the infiltrating cells are naturally devoid of the membrane bound IL-6R. Cells migrate from the vasculature to the area of infection or damage in an IL-6/gp130/STAT-3 dependent manner (Sander et al., 2008). Within the first 24 to 48 hours, the cellular population present is predominately leukocytes. The termination of this innate immune response is dependent on IL-6 driven activation of STAT-3. If inflammation is not resolved within 48 hours, IL-6/gp130/STAT-3 signalling within the neutrophils leads to decreased production of chemo-attractants such as CXCL1/KC, thereby impairing trafficking of neutrophils to the area (Fielding et al., 2008). In concert with increased production of CXCL1/KC, increased neutrophilic apoptosis and shedding of the sIL-6R occurs (Chalaris et al., 2007). This leads to the trafficking and recruitment of monocytes and T Cells to the area of damaged tissue (Sander et al., 2008).

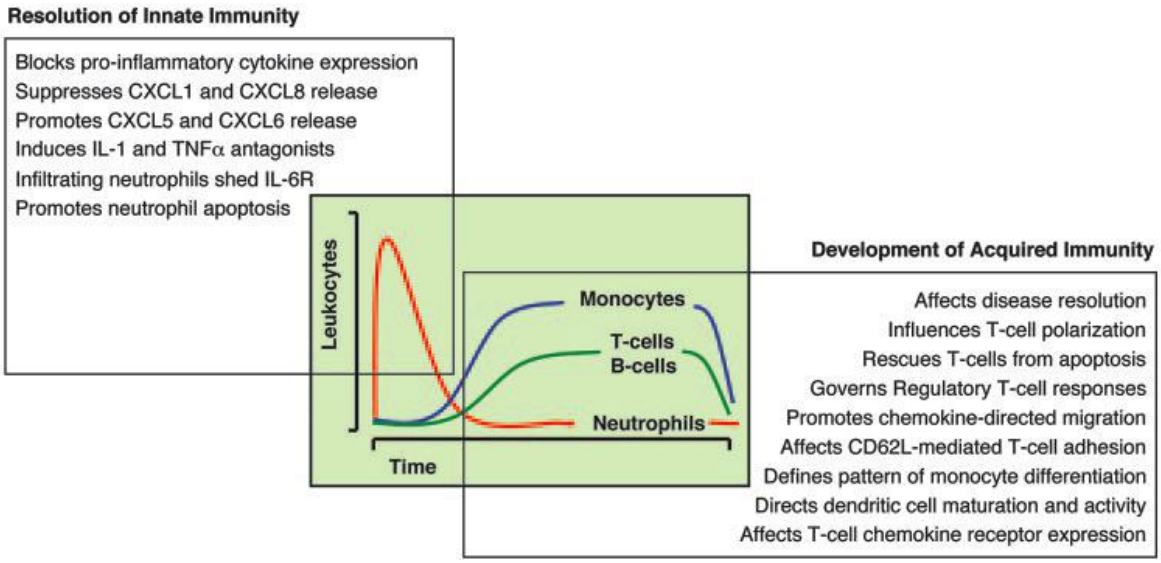


Figure 1.5 The role of IL-6 in transition from innate immunity to an acquired immune response. IL-6 is a central player in the resolution of innate immunity and progression to acquired immunity. Central to this concept is the ability of this cytokine to regulate the profile of infiltrating immune cells to the area of damage (Jones, 2005)

Most recently, a study by Briso, Dienz and Rincon (2008) proposed autoimmune diseases such as arthritis may be a consequence of sIL-6R produced by auto reactive T Cells. In support of this notion their study illustrated both naïve and memory CD4+ T Cells are also capable of shedding sIL-6R. Moreover, increases in sIL-6R production were highly correlative with expression of the metalloprotease ADAM17 within these cells. As memory CD4+ T Cells are paramount to acquired immunity this may have implications with regards to autoimmune diseases which often have deregulated T Cell expression and function.

Vital to hepatic production of acute phase proteins is the ability of sIL-6R to act in a paracrine manner. Using *in situ* hybridisation, expression patterns of IL-6 and sIL-6R were examined in transgenic mice expressing either human IL-6 only or human

IL-6 and soluble IL-6R under liver specific promoters (Peters et al., 1997). Importantly, whilst endogenous murine IL-6 levels remained unaltered, human IL-6 serum levels were increasingly elevated as the mice aged. Furthermore, human IL-6 serum concentration was highly correlative with the mRNA expression of the acute phase protein haptoglobin, an effect that was potentiated in the double transgenic mice. From this study, the investigators concluded that at low concentrations, the sIL-6R acts in a paracrine manner. However, when at higher circulating concentrations, such as that seen during immune challenge, the sIL-6R serves as an important mediator of local and systemic effects. This concept is central to the regulation of the immune response.

It is well established upon immune challenge, there is elevated shedding of the sIL-6R and increased production of IL-6 by immune cells. Importantly, by acting on cells in a paracrine manner, increased circulating concentrations of IL-6 induces the transcription and production of IL-6, sIL-6R and acute phase proteins by hepatocytes (Figure 1.6). Hepatic production of acute phase proteins is important in the regulation of the immune response and tissue repair. Acute phase proteins serve as mediators of processes central to immunity such as complement, phagocytosis, recruitment and trafficking of cellular subset populations to areas of inflammation and coagulation to name a few. IL-6 and sIL-6R are key players in mediating changes in the kinetic profile of IL-6 inducible acute phase proteins (Figure 1.6). C-Reactive Protein (CRP) circulating levels are rapidly increased in response to elevated IL-6/sIL-6R concentrations (Zhang et al., 1996), an important protein in the activation of the immunological process complement (Black, Kushner and Samols, 2004). As CRP levels fall, a concurrent increase in haptoglobin and fibrinogen occurs (for review see Gabay, 2006). Haptoglobin is responsible for the scavenging of haemoglobin produced upon haemolysis and fibrinogen is a crucial player in the tissue repair process due to its coagulation properties. Encoding genes for acute phase proteins such as haptoglobin (Oliviero and Cortese, 1989) and fibrinogen (Dalmon, Laurent and Courtois, 1993; Zhang, Fuentes and Fuller, 1995 and Liu and Fuller, 1995) possess IL-6 responsive elements within the 5' flanking region of their promoter. The acute phase immune response may be

inhibited through the use of the naturally occurring antagonist of IL-6 signalling, sgp130, preventing production of α_1-antichymotrypsin (Jostock et al., 2001), haptoglobin (Tenhumberg et al., 2008) and serum amyloid A (Rabe et al., 2008).

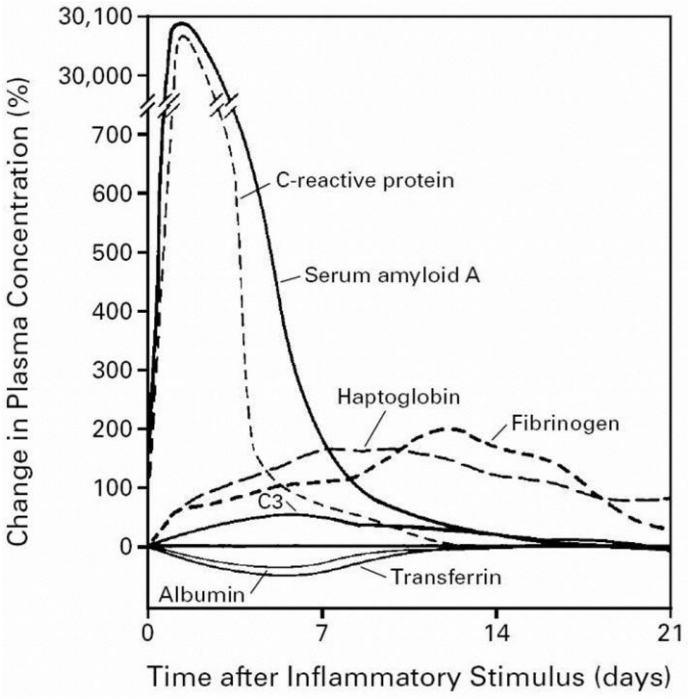


Figure 1.6 Kinetic profile of acute phase protein production induced by IL-6 (Gabay, 2006)

1.6 Soluble gp130, the natural antagonist of IL-6 signalling

In 1993, Narazaki and colleagues discovered the presence of a soluble form of the gp130 receptor (sgp130) present within human plasma (Narazaki et al., 1993). Soluble gp130 is a glycoprotein of approximately 100 kDa, which lacks the transmembrane and cytoplasmic domains the membrane bound form of the receptor possesses (Montero-Julian et al. 1997; Diamant et al., 1997; Jostock et al., 2001). Like the sIL-6R, the soluble form of the gp130 receptor is produced by proteolytic cleavage of membrane bound gp130 (Müllberg et al., 1993; Montero-Julian et al., 1997) or through alternate splicing of mRNA (Diamant et al., 1997). As the CBM of sgp130 remains intact, this receptor still has the ability to form ternary

complexes with sIL-6R bound to its ligand. Interestingly, the soluble form of the gp130 subunit, via competitive inhibition, is an IL-6 antagonist. sgp130 acts as a decoy, decreasing the bioavailability of IL-6/sIL-6R for ternary complex formation with gp130 (Figure 1.7, Jostock et al., 2001). However, sgp130 preferentially binds to IL-6/sIL-6R and is unable to bind to IL-6 alone, thus inhibiting signalling events mediated via the ternary complex only. This leaves IL-6 signalling via membrane bound IL-6R unscathed.

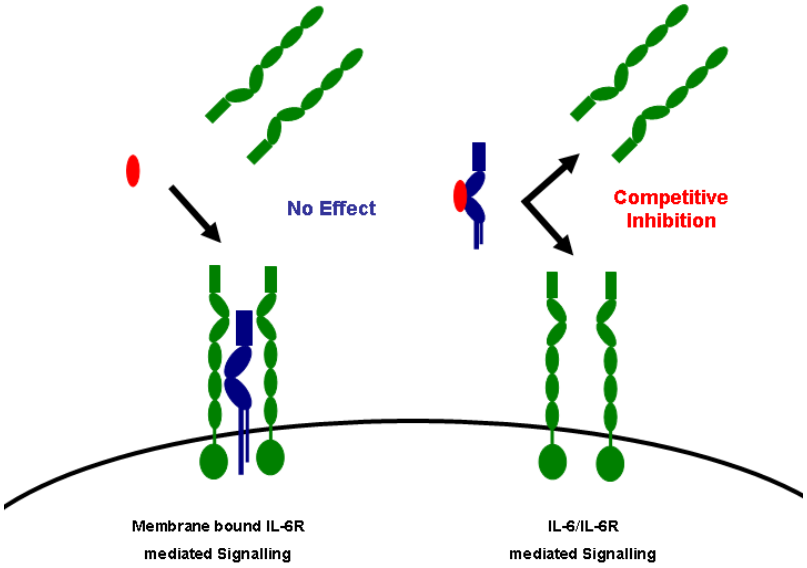


Figure 1.7 Inhibitory mechanism of sgp130. As sgp130 binds to the sIL-6R and not IL-6 alone, the cytokine is free to bind to the membrane bound IL-6Rα. Thereby eliciting a signalling response mediated via the membrane bound complex of IL-6Rα/gp130 complex. Competitive inhibition occurs when there is a molar excess of sgp130, as sIL-6R is able to bind to both soluble gp130 and its membrane bound equivalent. Symbols are as for Figure 1.3 (adapted from Jostock et al., 2001).

Many studies have demonstrated sIL-6R signalling inhibition using sgp130 to be a biologically relevant phenomenon. Initially, Narazaki et al. (1993) noted that healthy human sera contained 390 $ng \cdot ml^{-1}$ sgp130. In the murine pro B Cell line transduced with human gp130, treatment with human serum supplemented with rh-IL-6 induced DNA synthesis. Depleting the sera of sgp130 caused further DNA

synthesis. This implies naturally produced sgp130 has the capacity to negatively regulate IL-6 signalling mediated via gp130. Studies that followed demonstrated sgp130 was able to inhibit IL-6/sIL-6R driven proliferation of gp130 transduced BAF3 cells (Müller-Newen et al., 1998) but not that of IL-6 stimulated BAF3 cells expressing membrane bound IL-6R and gp130 (Jostock et al., 2001). Thereby insinuating sgp130 inhibited signalling mediated via sIL-6R. It is important to note the BAF3 cell line is unique as it is naturally devoid of the gp130 receptor (Nandurkar et al., 1996) and is therefore a useful tool for exploration of gp130 targeted therapies. In an experimentally induced form of colitis, Atreya and Colleagues (2000) demonstrated that blockade of IL-6/sIL-6R signalling using sgp130Fc, sgp130 receptor ligated to the FC portion of IgG (Figure 1.8), showed no signs of rectal prolapse, macroscopic evidence of inflammation or wasting disease when compared with untreated mice. IL-6 -/- mice induced with an experimental model of arthritis using Hyper IL-6, a designer cytokine of IL-6 linked to soluble IL-6R, had less severe joint destruction and inflammation when in the presence of sgp130 (Nowell et al., 2003). They also noted sgp130 decreased local activation of STAT-3 dependent leukocyte migration.

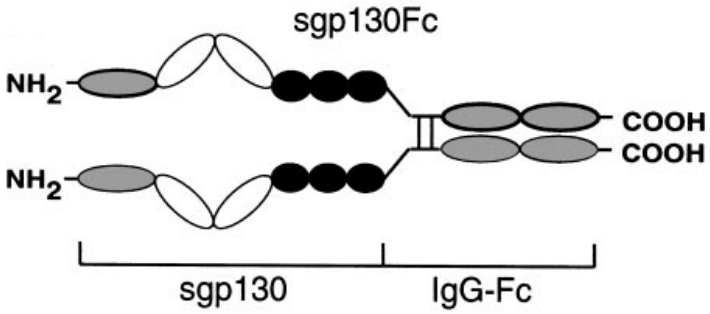


Figure 1.8 Schematic of the structure of sgp130Fc. The sgp130Fc consists of the extracellular domains; IgG like domain (grey), the cytoplasmic binding domain (white) and the Fibronectin III like domains (black) ligated to the Fc fragment of IgG via a synthetic oligonucleotide adapter (Jostock et al., 2001)

In experimentally induced murine acute peritonitis, the presence of this soluble receptor decreased monocyte and lymphocyte migration to the area of

inflammation and increased the presence of neutrophils thereby preventing the transition from an early innate immune response to that of acquired immunity (Richards et al., 2006). Importantly, using the air pouch model of inflammation in transgenic mice with over expression of sgp130 (Rabe et al., 2008), demonstrated the transition of an inflammatory response from a neutrophil dominant infiltrate to the mononuclear phase is entirely dependent on IL-6 trans-signalling rather than classic IL-6 signalling. The fore mentioned studies, compiled, provide overwhelming evidence sgp130 inhibits signalling mediated via the sIL-6R.

1.7 The designer cytokine Hyper IL-6

A great deal of the scientific community's understanding of cellular events mediated via sIL-6R and sgp130 have arisen through the use of the designer cytokine Hyper IL-6 and its antagonist sgp130Fc. In 1997, a fusion protein of recombinant human IL-6 covalently linked via a synthetic polypeptide chain to sIL-6R was expressed in and secreted from yeast (Figure 1.9, Fischer et al., 1997). This "designer cytokine" was 100 to 1000 fold more bioactive than that of IL-6 and sIL-6R unlinked. The fusion protein was able to effectively induce the expansion of the human hematopoietic progenitor cell line BAF3 transduced with gp130 and stimulate the production of the acute phase protein haptoglobin by HepG2 cells in a dose dependent manner. In a later study from the same research group (Peters et al., 1998) Hyper IL-6 induced a heightened and prolonged acute phase response, as measured by haptoglobin mRNA expression in liver of NMRI mice when compared with rh-IL-6 alone. It was hypothesised this marked difference between rh-IL-6 and the designer cytokine was due to a) the higher expression of gp130 compared to that of membrane bound IL-6R and b) slower internalisation as a result of a higher binding affinity between Hyper IL-6 and gp130 than that of IL-6 to cell surface alone. In support of the later, prolonged elevated Hyper IL-6 serum concentration was evident when compared to IL-6 alone.

The heightened and prolonged response to Hyper IL-6 would appear to be, in part, a consequence of an enhanced ability to induce nuclear translocation and DNA binding by STAT-3 (Rakemann et al., 1999). Many studies ensued exploring the

effect of Hyper IL-6 on inflammation within liver (Peters et al., 1998) and gastrointestinal derived cell lines (Jostock et al., 2001) and cellular proliferation and differentiation of hematopoietic (Fischer et al., 1997), tumourigenic melanoma (Özbek et al., 2001) and neural and glial (Islam et al., 2009) derived cell lines. However, there is a lack of literature examining the potential effect this designer cytokine has within skeletal muscle and adipose tissue. This is surprising given the prominent role IL-6 itself plays in skeletal muscle and adipose metabolism.

In summary, IL-6 is an important cytokine involved in the regulation of many cellular processes including inflammation and metabolism. It is of great importance to note, IL-6 mediated inflammation occurs through an agonistic phenomenon known as IL-6 trans-signalling, whereby IL-6 binds to the soluble form of its receptor and targets the ubiquitously expressed gp130. Hence, cells naturally devoid of the membrane bound IL-6R remain sensitive to this cytokine. Although IL-6 has many beneficial effects on metabolism, it is an unsuitable candidate for use as a therapeutic agent in the treatment of metabolic disorders such as diabetes due to the dual functionality of this protein. However, sgp130, a naturally occurring antagonist of IL-6 trans-signalling, is able to block IL-6 induced inflammation through its interaction with IL-6/sIL-6R whilst signalling mediated via the membrane receptor remains intact.

Whether it is possible to prevent IL-6 induced inflammation through blockade of IL-6 trans-signalling using sgp130 whilst maintaining any beneficial effects on metabolism mediated via the membrane bound IL-6 receptor has yet to be elucidated. Hyper IL-6, a designer cytokine has previously been used as an agent to mimic IL-6/sIL-6R signalling in various cell types but the effect this cytokine has on skeletal muscle metabolism has yet to be elucidated. Through understanding ligand / receptor interactions within metabolically active tissues such as adipose and muscle, a “designer cytokine” capable of selectively targetting various signalling components may be a useful tool in the war against metabolic diseases such as diabetes. One such potential therapeutic anti-obesity agent may be a

gp130 receptor/ligand like drug design. This forms the basis of the current dissertation.

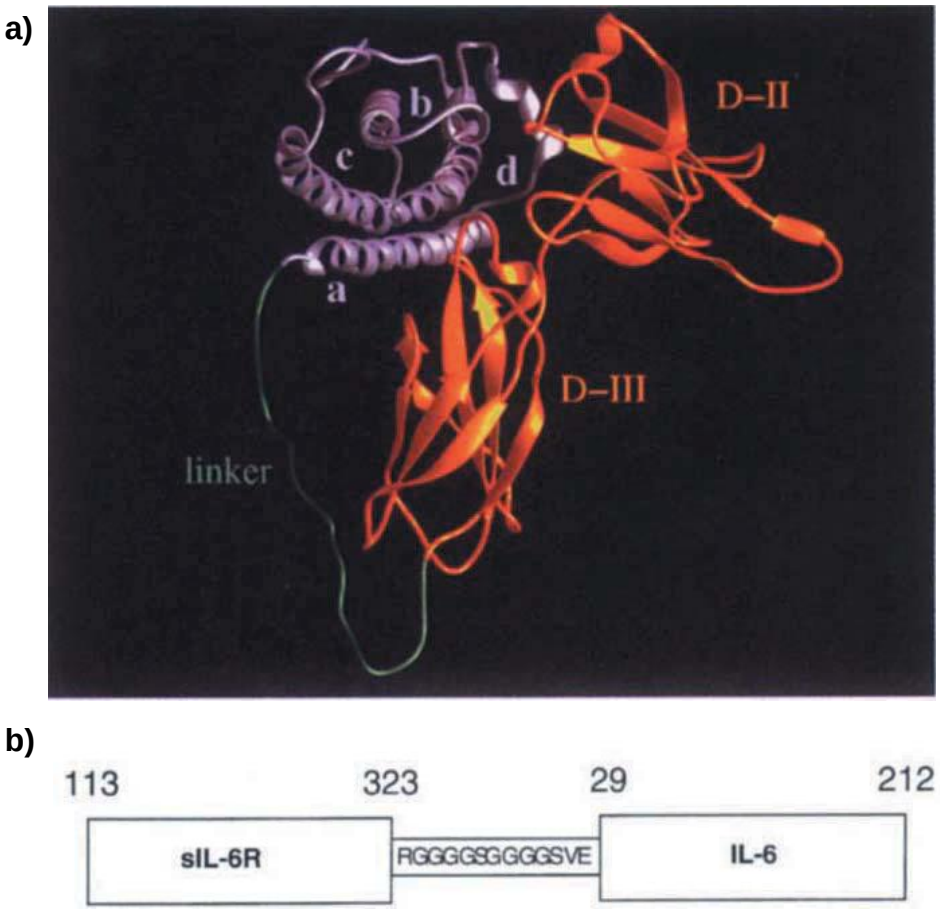


Figure 1.9 The fusion protein Hyper IL-6. a) A molecular model of Hyper IL-6 illustrating the four helices of IL-6; a,b,c and d (purple) linked via a polypeptide linker (grey) to Domain-2 and 3 (DII and DIII) of the sIL-6R CBM (orange) b) Schematic of Hyper IL-6 cDNA used for the expression and secretion of the protein in yeast. (Fischer et al., 1997)

CHAPTER TWO

Aims of the Study

2.1 Aims of the study

The significance of IL-6 in the regulation of metabolism is well established, however it is not known whether this important biological process occurs via the membrane bound IL-6 receptor or via the agonisitic soluble IL-6 receptor, a phenomenon most commonly known as “trans-signalling”. The primary aim of this study was to elucidate whether IL-6 signalling via the sIL-6R, using the designer cytokine Hyper IL-6 to mimic this scenario, is biologically relevant in the regulation of common metabolic processes such as mitochondrial oxidative phosphorylation, fatty acid oxidation, glucose utilisation and insulin sensitivity within such tissues as skeletal muscle, adipose tissue and liver. To investigate this, a variety of analytical techniques were utilised including those relevant to gene expression, cell signalling, histopathological and physiological studies.

IL-6 regulates activation of Akt, p38 MAPK and AMPK. These proteins have all been implicated in metabolism through mediation of fat oxidation, insulin signalling and glucose uptake, myogenesis and contraction-induced mitochondrial biogenesis. Importantly, PGC-1α, an important mediator of mitochondrial metabolism, possesses phosphorylation sites for all three of these proteins. The first study of this dissertation (Chapter 3) aimed to determine if sIL-6R signalling, using Hyper IL-6, was a transcriptional regulator of PGC-1α and its downstream targets in vitro. This study also examined whether intermittent and chronic IL-6 infusion in male wistar rats regulated mRNA and protein expression of PGC-1α and its downstream targets.

There is prolific evidence for IL-6 signalling mediated via the sIL-6R in many tissues including synovial fluid, the bowel and liver. However, little evidence of this phenomenon occurring in metabolically active tissues such as skeletal muscle and adipose tissue is present within the literature. The second study (Chapter 4) included in this thesis aimed to determine if this process occurs in L6 myotubes *in vitro*. IL-6 is a predominant player in regulating muscle metabolism, thus it was hypothesised that Hyper IL-6 was capable of modulating glucose uptake and fatty acid oxidation via pathways previously implicated in metabolic control. As IL-6

would appear to elicit tissue type specific responses, the secondary aim of the study included in Chapter 4 of this thesis examined the degree of bioactivity induced by membrane bound IL-6 receptor complex signalling in comparison to that of IL-6 trans-signalling within CRL1439 hepatocytes and L6 myotubes.

It was established that L6 myotubes are responsive to IL-6 trans-signalling and that this phenomena is capable of regulating important physiologically metabolic mechanisms such as glucose and fat oxidation and cellular signalling *in vitro.* It was of utmost importance to determine if this scenario translated to an *in vivo* model. This formed the basis of the cohorts in Chapters 5 and 6.

The primary focus of Chapter 5 was to examine the activation of components of various cell signalling pathways responsible for inflammation, metabolism and cell cycle, in response to acute Hyper IL-6 treatment within skeletal muscle, adipose tissue and liver.

Finally, as it was evident Hyper IL-6 was capable of activating signalling pathways typically involved in the regulation of metabolism in liver, WAT and skeletal muscle, it was proposed that Hyper IL-6 administration over a prolonged period had the ability of modulating metabolism (Chapter 6). Hyper IL-6 is often used to induce experimental states of inflammation such as arthritis, inflammatory bowel disease and hepatocellular carcinoma *in vitro*. The effect of co-treatment with a naturally occurring competitive inhibitor, sgp130Fc was examined with the idea of preventing inflammatory signalling associated with Hyper IL-6 treatment whilst maintaining the beneficial effects on metabolic signalling.

CHAPTER THREE

IL-6 does not mediate transcription and translation of the co-transcription factor PGC-1α *in vitro* or *in vivo*

3.1 Introduction

Peroxisome proliferator activator receptor γ coactivator-1 α (PGC-1α), a coactivator of transcription factors, is a key player in metabolic control through the regulation of mitochondrial proteins, resulting in increase in mtDNA and hence increased replication and translation of mitochondrial oxidative phosphorylation (OXPHOS) genes (for reviews refer to Puigserver and Spiegelman, 2003; Houten and Auwerx, 2004). PGC-1α is expressed in fat, liver, cardiac muscle, skeletal muscle and brain and its function appears to be tissue type specific and dependent on its interaction with varying transcription factors (Figure 3.1) (Houten and Auwerx, 2004).

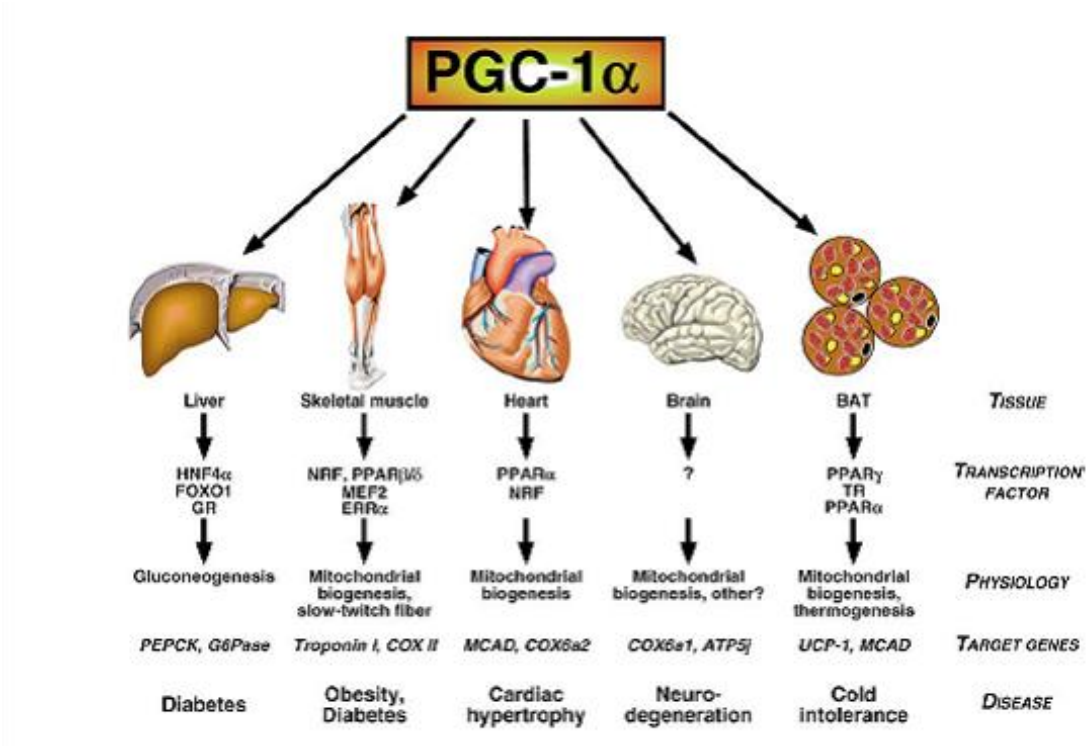


Figure 3.1 PGC-1α transcriptional targets and physiological functions in various tissues (adapted from Houten and Auwerx, 2004)

PGC-1α expression is highly responsive to meet cellular energy demands in the presence of physiological stimuli such as environmental temperature, nutritional status, physical activity and infection (for reviews refer to Puigserver and Spiegelman, 2003; Houten and Auwerx, 2004). In skeletal muscle both exercise, and pro-inflammatory cytokines increase PGC-1α regulated transcription of

mitochondrial genes for mitochondrial biogenesis, oxidative metabolism and respiration (Houten and Auwerx, 2004; Pilegaard, Saltin and Neufer, 2003 and Puigserver et al., 2001). However, the signalling pathways by which PGC-1α expression and activity are regulated in skeletal muscle remain poorly understood.

Evidence of the role of PGC-1α in metabolic disorders such as Type II Diabetes was demonstrated concurrently by Patti and colleagues (2003) and Mootha et al. (2003). These studies demonstrate that PGC-1α is significantly reduced in patients with Type II Diabetes, as are its downstream targets Nuclear Receptor Factor-1 (NRF-1), NRF-2 and other OXPHOS genes. PGC-1α mRNA expression is also decreased in subcutaneous fat of morbidly obese subjects, whereas in rodents it is thought to play a role in the conversion of white adipocytes from fat storing to fat oxidising cells (Semple et al., 2003). Cachexia, a chronic wasting of muscle mass due to an imbalance in energy expenditure, is often a hallmark of many diseases including: cancer, heart failure, HIV-AIDS and infection (for review see von Haehling et al., 2002). It is noteworthy that pro-inflammatory cytokines such as TNFα, IL-1β, IL-1α and IL-6 are often elevated in these chronic illnesses (von Haehling et al., 2002). Spiegelman and co-workers (Puigserver et al., 2001) have demonstrated that these pro-inflammatory cytokines can stimulate increased respiration and energy expenditure through the activation of PGC-1α in C2C12 myotubes. Moreover, they showed that pharmacological inhibition of the mitogen activated protein kinase p38 MAPK with SB202190 greatly reduced the stimulatory effects of cytokines on PGC-1α, thereby implicating p38 MAPK in cytokine activation of PGC-1α in states of inflammation (Puigserver et al., 2001). This study also verified p38 MAPK phosphorylates PGC-1α on residues Threonine 262, Serine 265 and Threonine 298, further insinuating a role for p38 MAPK in the regulation of PGC-1α activity (Puigserver et al., 2001).

Endurance training results in increases in PGC-1α transcription and mRNA content in human skeletal muscle (Pilegaard, Saltin and Neufer, 2003). Terada and co-workers (Terada et al., 2002) noted after a 6 h acute bout of low intensity swimming exercise, mRNA expression of PGC-1α and AMP-activated protein

kinase (AMPK) in the epitrochlearis muscle of Sprague Dawley rats was markedly higher than in unexercised, control rats (Terada et al., 2002). Moreover, skeletal muscle incubated with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an AMPK activator, resulted in a 3-fold increase in PGC-1α mRNA expression, suggesting that AMPK activation is involved in the induction of PGC-1α during exercise. In AMPK WT mice treated with β-guanidinopropionic acid (GPA), a pharmacological agent that mimics endurance exercise through the depletion of intracellular phospho-creatine and ATP concentrations, increases in PGC-1 gene expression, mitochondrial DNA and density were noted. However, these effects were abrogated in AMPK dominant negative mice (Zong et al., 2002).

A study by Jäger et al. (2007) demonstrated that in myotubes isolated from PGC-1α knock out mice treated with AICAR, the expression of the mitochondrial genes Cytochrome C, UCP-3, UCP-2, the AMPK target, GLUT-4 and oxidation was lower when compared to AICAR treated WT cells. Furthermore, it was demonstrated through co immunoprecipitation studies that AMPKα2 and PGC-1α form a complex and identified the AMPK phosphorylated residues on PGC-1α as threonine 177 and serine 578. These studies establish a role for AMPK in the regulation of mitochondrial function through the activation and regulation of PGC-1α and its activity.

A recent study (Li et al., 2007) implicated Insulin stimulated activation of Akt in the regulation of PGC-1α activity through its serine^{507} residue. When primary hepatocytes isolated from WT mice received insulin, phosphorylation of PGC-1α was increased compared to control treatment, however when Akt2 -/- mice were similarly treated this response was ablated. Moreover when cells were infected with a constitutively active Akt adenovirus, PGC-1α was increased.

It is well established, that skeletal muscle produces and releases the pro-inflammatory cytokine Interleukin-6 (IL-6) (for review see Carey and Febbraio, 2004). Elevated levels of the cytokine have also been observed in patients with Type II Diabetes (Carey and Febbraio, 2004). The effect of IL-6 on skeletal muscle

and the mechanisms by which it elicits these, remains poorly understood. Previous research from the Cellular Molecular Metabolism Laboratory has demonstrated that treating L6 myotubes with IL-6 augments AMP-activated protein kinase (AMPK) activation and p38 Mitogen Activated Protein Kinase (p38 MAPK) phosphorylation (Carey et al., 2006). Others have also implicated IL-6 in the activation and phosphorylation of AMPK and p38 MAPK in adipocytes and skeletal muscle cells (Senn et al., 2002; Geiger et al., 2005 and Carey et al., 2006). Notwithstanding, rhIL-6 infusion in healthy humans resulted in a marked increase in oxidative glucose metabolism and VO_2 (rate of O_2 consumption during exercise) (Carey et al., 2006). Interestingly, PGC-1α gene expression and its downstream targets NRF-1 and Mitochondrial Transcription Factor A (mtFAM) have been correlated with VO_2 peak (Garnier et al., 2005). The authors of this study also observed a strong correlation existed between VMax, an indication of OXPHOS activity, and PGC-1α gene expression. Thus, it is postulated that IL-6 increases OXPHOS activity through the induction of PGC-1α protein expression and activity.

Further evidence of IL-6 family cytokines in the regulation of metabolic pathways that lead to increased PGC-1α transcription and expression is provided by Watt et al. (2006). Administration of the IL-6 family cytokine member, Ciliary Neurotropic Factor (CNTF) to C57BL6 mice on a High Fat Diet resulted in increased PGC-1α protein expression and weight loss when compared to control mice. Moreover CNTF resulted in an increase in phosphorylation of ACC, an important intermediate step in fat oxidation. These data (Watt et al., 2006; Carey et al., 2006) along with those of Jäger et al. (2007) has led to the hypothesis that IL-6 family cytokines can increase fat oxidation in skeletal muscle via AMPK phosphorylation and activation of PGC-1α (Febbraio, 2007; Figure 3.2) .

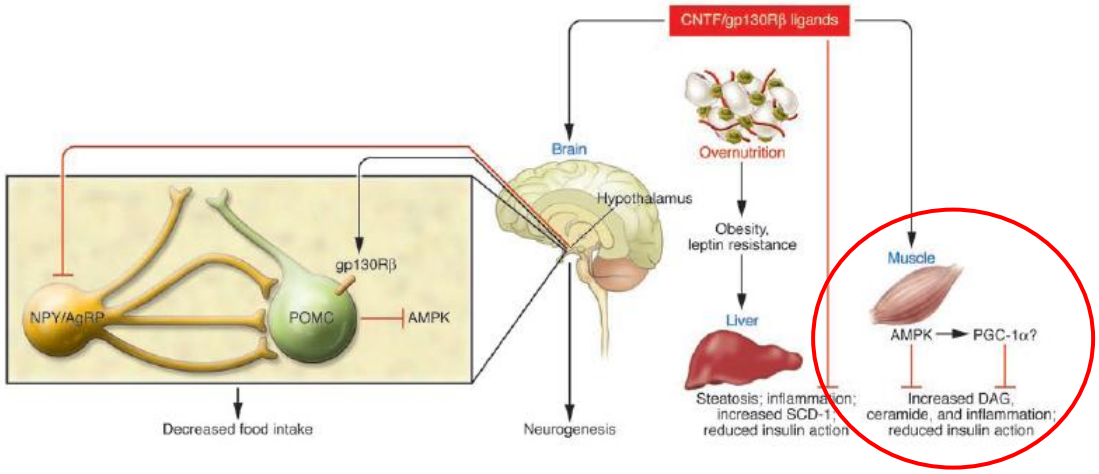


Figure 3.2 gp130 ligands mediate fat oxidation in skeletal muscle via an AMPK dependent manner (Febbraio, 2007)

As Carey et al. (2006) demonstrated that IL-6 results in increased oxidative metabolism and phosphorylation of Akt, p38 MAPK and AMPK and as PGC-1α possesses phosphorylation sites for these fore mentioned proteins, it is also hypothesised that in IL-6 treated skeletal muscle and liver cells, activity and expression of PGC-1α and its downstream targets will be increased and thus result in enhanced mitochondrial oxidation and respiration (Figure 3.3). The secondary aim of this study was to elucidate by which pathway IL-6 may regulate PGC-1α activity and expression.

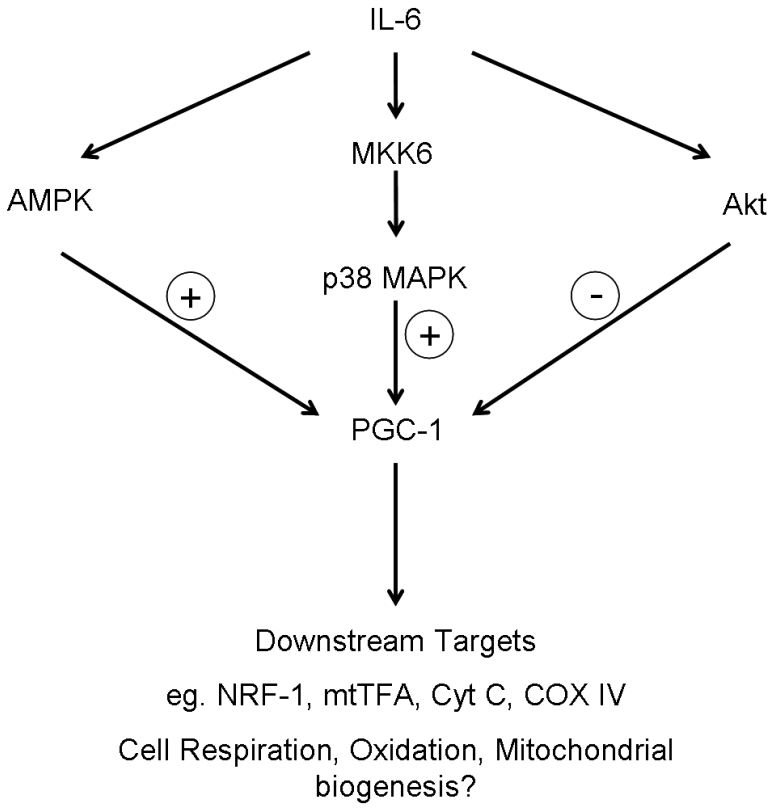


Figure 3.3 Schematic of the signalling pathways that are postulated to be involved in the regulation of PGC-1α expression and hence mitochondrial activity by IL-6.

3.2 Methods

Cell Culture - L6 myoblasts were cultured on 6 well plastic culture plates containing tissue culture growth medium consisting of alpha-Minimal Essential Medium (α-MEM, SAFC Biosciences, Sigma-Aldrich, Sydney, Australia), 10 % Foetal Bovine Serum (FBS, JRH Biosciences SAFC Biosciences, Sigma Aldrich), 1 % (v/v) Penicillin-Streptomycin-glutamine (10,000 units·ml^{-1} Penicillin G Sodium, 10,000 µg·ml^{-1} streptomycin sulphate, 0.85 % Saline, Invitrogen, Carlsbad, CA, USA). Once L6 myoblasts obtained 60 % confluency, differentiation of myoblastic cultures to myotube cultures was initiated by serum withdrawal, replacing the growth media with differentiation media (α-MEM, 2 % (v/v) Horse Serum, 1 % (v/v) Penicillin-Streptomycin-glutamine) for 6 days. After which, L6 myotubes were treated with differentiation media containing 100 ng·ml^{-1} Hyper IL-6 for 2, 6 or 24 h, after which cells were harvested for RNA quantitation. In L6 myotube signalling studies, cells were treated with 50 ng·ml^{-1} Hyper IL-6 for 1 hour, after which cells were harvested for protein determination by western blotting. CRL1439 liver cells were cultured on 6 well culture plates containing growth medium consisting of low glucose Dulbucco's Modified Eagle Medium supplemented with 10 % (v/v) FBS and 1 % (v/v) Penicillin-Streptomycin-glutamine. Once cultures had reached approximately 80 % confluency, the cells were treated with 100 ng·ml^{-1} Hyper IL-6. Post treatment cells were lysed for RNA quantitation. All cultures were stored at 37 °C in a 5 % CO_2/95 % O_2 humidified air cell incubator.

Animals - As previously described (Holmes et al., 2008), Male Wistar rats weighing approximately 220 g were housed in the RMIT University animal facility in a 12:12 hour light:dark cycle. Animals were fed a normal rat chow diet and water *ad libitum.* Animal experiments were performed as approved by the Animal Ethics Committee of RMIT University (AEEC #0407). Animals were randomly assigned to be administered Saline (control) or IL-6 in an intermittent or chronic manner.

Interleukin-6 treatment - Animals were administered IL-6 as previously described (Holmes et al., 2008). For chronic administration of IL-6 or saline, rats were surgically implanted with osmotic pumps (Alzet 2ML2; Durect, Cupertino, CA) that

had a 14 day pumping capacity. Animals were anaesthetised with 6 $mg \cdot 100g^{-1}$ sodium pentobarbital and implanted with the pumps into the intrascapular subcutaneous space.

Pumps were filled to capacity with 22.5 $\mu g \cdot ml^{-1}$ rhIL-6 diluted in 0.1 % (w/v) Bovine Serum Albumin (BSA) and 0.9 % (w/v) NaCl. Rats were administered 2400 ng rhIL-6 per day using an infusion rate 4.5 $\mu l \cdot h^{-1}$ over the 14 day treatment period. The saline control group received 0.1 % (w/v) BSA and 0.9 % (w/v) NaCl only. Animals assigned to the intermittent treatment group were administered 1200 ng rhIL-6 at 0700 and 1900 daily via intraperitoneal injection for 14 days. At day 15 rats were culled and the tissues of interest were harvested, snap frozen in liquid nitrogen and stored at -80 °C until required for further analyses.

SDS - PAGE analysis of proteins - Approximately 30 mg of White Adipose Tissue, Liver*, Extensor Digitorum Longus*, *Soleus* and *Tibialis Anterior* were homogenised, using a motorised homogeniser (Polytron; Brinkman Instruments, New York, NY, USA), in ice cold lysis buffer consisting of 50 mM HEPES, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 0.5 % Triton X-100 (v/v), 10 % glycerol (v/v), 1 mM phenylmethylsulfonyl, 50 mM phosphatase inhibitor and 50 mM protease inhibitor. For *in vitro* experiments, cells were lysed in the fore mentioned lysis buffer and the subsequent lysate collected. After which both tissue and cell lysates were centrifuged at 13000 g to remove cellular debris. Protein concentrations were determined by BCA assay. Forty micrograms of protein were solubilised in 4 x Laemmelli's Buffer and denatured at 95 °C. Samples were then stored at -20 °C until required.

Lysates were resolved using 6-14 % polyacrylamide gels (120 V; stacking gel and 150 V; resolving gel) followed by transference of protein onto nitrocellulose membrane using a semi-dry transfer unit (Biorad, Melbourne, Australia) at a constant ampere of 0.06 per gel for 120 min. Membranes were washed twice in TBST (0.05 % Tween 20 (v/v) in Tris Buffered Saline), blocked in 5 % BSA (w/v) TBST for 120 min at room temperature, washed thrice in TBST. Membranes were

then incubated in primary antibody overnight at 4 °C. The following primary antibodies were used; PGC-1α (Santa Cruz Biotechnology, CA, USA), phospho Akt^{ser473}, phospho AMPK^{thr172}, phospho p38 MAPK $^{thr180/tyr182}$, total p38 MAPK, β-actin (Cell Signaling Technology, Beverly, MA, USA) or UCP-3 (Alpha Diagnostics, San Antonia, TX, USA).

The following morning membranes were washed three times in TBST and incubated in anti rabbit horseradish peroxidase-conjugated secondary antibody (1:2000, 2.5 % BSA (w/v) TBST) for 60 min at room temperature. Membranes were then thoroughly washed with TBST (6 x 5 min). Membranes were then incubated in enhanced chemiluminescence or super chemiluminescence (Amersham, GE Health Care Lifesciences, Buckinghamshire, UK) and the immuno-reactive proteins were detected by CCD Camera (Chemidoc XRS Unit, Biorad). The digital output was analysed for densitometry using Quantity One 1-D Analysis Software Version 4.5.2 (Biorad).

Real Time PCR

Primer Design and Optimisation - Forward and Reverse Primer Sequences for β-Actin, PGC-1α, NRF-1, mtFAM, Cytochrome C and Cox-IV *(Table 3.1)* were designed using Primer Express version software (Applied Biosystems, Foster City, CA, USA) using the rat genomic sequence from GenBank/EMBL. The optimal primer concentration was determined and these concentrations were used in all subsequent gene expression experiments.

RNA Extraction - RNA was extracted from ~ 30 mg tissue, vehicle and cytokine treated L6 myotubes and CRL1439 liver cells using an RNeasy minikit as per the manufacturer's instructions (Qiagen, Melbourne, Australia). Tissue was disrupted in Buffer RLT using a hand held homogeniser after which native proteins were degraded using proteinase K (Qiagen) at 55°C for 10 min. Residual genomic DNA was removed from the sample by DNase digestion using DNase I/Buffer RDD at RT for 15 min. RNA obtained was quantified using a nano-drop 1000 spectrophotometer (Biolab) and stored at -80°C until required.

Table 3.1: Accession number and forward and reverse primer sequences used for genes of interest in real-time PCR analyses.

Target	Accession number	Forward Primer	Reverse Primer
PGC-1α	NM_031347	5'-TCTGGAACTGCAGGCCTAACTC-3'	5'-GCAAGAGGGCTTCAGCTTTG-3'
NRF-1	NM_001100708	5'-ATCGATGGGATTCCAGTCTCTGT-3'	5'-TTGAGCATCTCTCTGGGATAAATGC-3'
Cytochrome C	NM_001130491	5'-TCTGTTTGGGCGGAAGACA-3'	5'-CCCAGGTGATACCTTTGTTCTTG-3'
COX IV-1	NM_017202	5'-CAGGGATGAGAAAGTCCAATTGT-3'	5'-CATTGGTGCCCTTGTTCATCT-3'
mtFAM	NM_031326	5'-CCTCGGTCAGCATATAACATTTACG-3'	5'-TTCAATTTCCCCTGAGCTGAC T-3'
SOCS-3	NM_053565	5'-TTCTTCACACTGAGCGTCGAGA-3'	5'-CTTGAGTACACAGTCAAAGCGGG-3'
β-Actin	NM_031144	5'-GCCAACACAGTGCTGTCTGG-3'	5'-TACTCCTGCTTGCTGATCCA-3'

Reverse Transcription - Each RNA sample was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems). A 10 µl reaction contained 0.1 µg RNA, 1 µl of 10 x TaqMan RT Buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamers, 1.25 $u \cdot \mu l^{-1}$ Multiscribe™ Reverse Transcriptase, 0.5 $u \cdot \mu l^{-1}$ RNase Inhibitor. The reverse transcription reaction underwent the following conditions using a Perkin Elmer Gene Amp PCR system 2400: 10 min at 25˚C, 30 min at 48˚C and 5 min at 95˚C to stop the reaction. The subsequent cDNA was stored at -20˚C until required for Real-Time PCR.

Real time PCR - Gene expression was quantitated using fluorescent Sybr Green (Applied Biosystems) and the comparative critical threshold (CT) method using a 7500 Fast Real Time PCR System (Applied Biosystems). PCR reactions were carried out in duplicate. Reactions consisted of 5 µl 5 x Sybr Green (Applied Biosystems), 600 nM forward primer and 600 nM reverse primer for gene of interest, 20 ng cDNA and then made to a final volume of 25 µl using DEPC treated milliQ H_2O. After which the cDNA was amplified using the following conditions: 50˚C for 2 min, 95˚C for 10 min followed by 50 cycles of 95˚C for 15 s and 58.5˚C for 1 min.

For each sample β-actin C_T values were subtracted from the C_T values for the gene of interest to derive a ΔC_T. The average C_T of the saline treatment group or vehicle control was then subtracted from each sample of each treatment group, thus deriving a $\Delta \Delta C_T$ value. The expression of the genes of interest were then expressed relative to the saline group using $\Delta \Delta C_T$. Data was then expressed as fold change in mRNA for the gene of interest when compared to control (=1).

Statistical Analyses - Statistics were computed using the statistical analyses software packages SPSS or Sigma Stat. A one way ANOVA with a Tukey's Post Hoc test was used to determine significant differences between treatments in mRNA and protein expression in *in vivo* intermittent/chronic IL-6 infusion studies. Significance was accepted with a p value of < 0.05. *In vitro* mRNA expression Hyper IL-6 treatment time course studies did not undergo statistical analysis due to a lack of power as a consequence of the small sample size. Student t-tests were used to determine if significant differences in protein abundance existed between vehicle and Hyper IL-6 treatment. All data is expressed as mean ± SEM.

3.3 Results

IL-6, irrespective of mode of delivery, does not regulate PGC-1α mRNA expression in Tibialis Anterior - In *Tibialis Anterior* harvested from rats IP injected (intermittent treatment group) or infused (chronic treatment group) with rhIL-6, there was no change in mRNA levels of PGC-1α, NRF-1, mtFAM, Cox IV or Cytochrome C (Table 3.2). The mRNA encoding SOCS-3, an IL-6 inducible protein, increased ~2 fold with intermittent treatment and ~3 fold with chronic IL-6 treatment in *Tibialis Anterior* (Table 3.2). Contrary to this, SOCS-3 remained unchanged in liver (Table 3.2).

IL-6 has no effect on PGC-1α protein abundance in Soleus, EDL, Tibialis Anterior, Liver or Adipose Tissue in vivo - Intermittent and Chronic treatment with rhIL-6 caused no change in PGC-1α protein abundance in *extensor digitorum longus* (Figure 3.4b), *Tibialis Anterior* (Figure 3.4c) or liver (Figure 3.4d). Irrespective of mode of delivery, PGC-1α protein expression was decreased to approximately 60 % of that in *soleus* from saline infused rats (Figure 3.4a), however upon statistical analysis this was not significant (Saline vs. intermittent IL-6; $p = 0.2$, saline vs. chronic IL-6; $p = 0.092$). Interestingly, in white adipose tissue extracted from the intermittent treatment group, PGC-1α abundance had a tendency to be ~ 35 % less than that of tissue from saline and chronic IL-6 treated rats (Figure 3.4e). Upon statistical analysis this was not a significant difference (saline vs. intermittent IL-6; $p = 0.711$, saline vs. chronic IL-6; $p = 0.264$ and intermittent IL-6 vs. chronic IL-6; $p = 0.054$).

Table 3.2 mRNA expression of PGC-1α and downstream targets remain similar in TA from rats administered IL-6, irrespective of mode of delivery

	Saline	Intermittent	Chronic
PGC-1α	1 ± 0.17	1.1 ± 0.14	1.11 ±0.22
NRF-1	1 ± 0.13	1.05 ±0.11	1.24 ±0.24
mtFAM	1 ± 0.16	1.19 ±0.13	1.26 ± 0.25
Cox IV	1 ± 0.17	1.09 ±0.13	1.15 ±0.25
Cytochrome C	1 ± 0.17	1.05 ±0.14	1.09 ±0.23
SOCS-3 (TA)	1 ± 0.20	2 ± 0.31*	2.85 ±1.20†
SOCS-3 (Liver)	1 ± 0.23	0.88 ± 0.18	0.91 ±0.13

* $p < 0.05$ Saline vs. Intermittent IL-6 treatment † $p < 0.05$ Saline vs. chronic IL-6 treatment. Data is expressed as mean ± SEM (n=8-12).

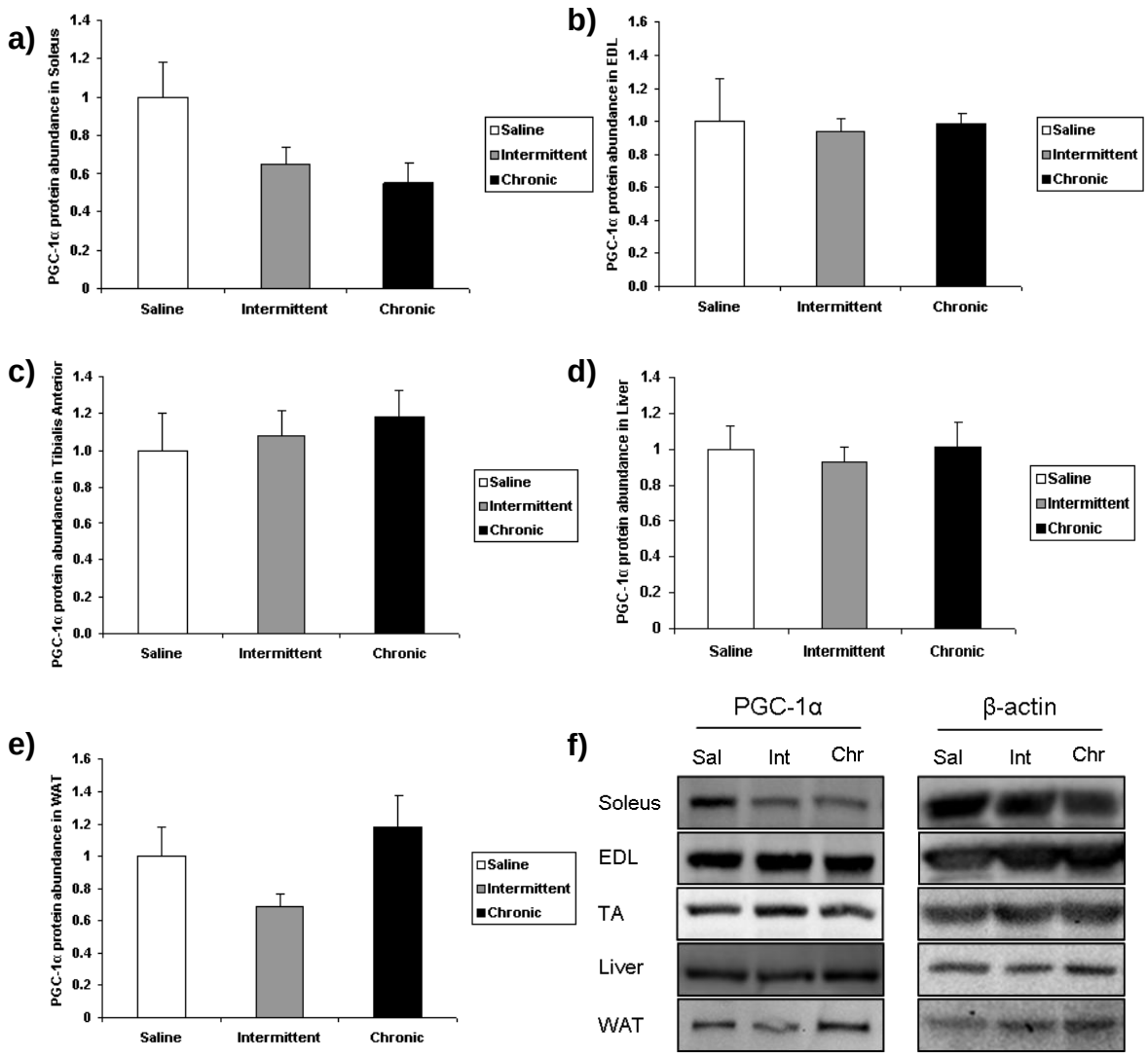


Figure 3.4 PGC-1α protein expression in tissues harvested from Intermittent and Chronic IL-6 infused Rats - Protein abundance of PGC-1α as quantitated by densitometry in a) *Soleus* b) *EDL* c) *Tibialis Anterior* d) White Adipose and e) Liver. All data are expressed as mean ± SEM, n= 8 for all tissues excluding *soleus* (n=4). Corresponding representative blots for PGC-1α and β-actin (f) are shown. Sal: Saline, Int: Intermittent IL-6 treatment, Chr: Chronic IL-6 treatment.

Soluble IL-6 receptor signalling does not regulate PGC-1α transcription in L6 Myotubes or CRL1439 liver cells - To determine if IL-6 regulates transcription of PGC-1α and its downstream transcriptional targets *in vitro*, mRNA expression was determined by Real Time PCR over a 24 hour treatment period. In L6 myotubes treated with 100 $ng \cdot ml^{-1}$ Hyper IL-6, no difference in mRNA levels of PGC-1α (Figure 3.5a), NRF-1 (Figure 3.5b), mtFAM (Figure 3.5c) and Cytochrome C (Figure 3.5d) were detected at 2, 6 and 24 hours, when compared to vehicle control. No changes were noted in mRNA expression of PGC-1α (Figure 3.6a) nor mtFAM (Figure 3.6c) and Cox IV (Figure 3.6d) in 100 $ng \cdot ml^{-1}$ Hyper IL-6 treated CRL1439 liver cells. A 0.5 fold and two fold increase in mRNA of NRF-1 (Figure 3.6b) was noted at 2 and 6 hours respectively in Hyper IL-6 treated CRL1439 liver cells when compared to vehicle control treated cells, however statistical analysis was not performed due to a lack of power as a consequence of small sample size.

Hyper IL-6 has little effect on phospho AMPK^{thr172} but increases phospho Akt^{ser473} and p38 MAPK$^{thr180/tyr182}$ – Activation of pathways hypothesised to be involved in Hyper IL-6 induced increases in PGC-1α and downstream targets was examined in L6 myotubes *in vitro*. Hyper IL-6 had minimal effect on phosphorylation of AMPK at Thr 172 residue (Figure 3.7a). However, Hyper IL-6 significantly induced Ser473 phosphorylation of Akt, with abundance approximately 18 fold that of Vehicle treated L6 myotubes (Figure 3.7b; $p < 0.001$). Furthermore, Hyper IL-6 increased phospho p38 MAPK$^{thr180/tyr182}$ ~3 fold (Figure 3.7c; $p < 0.001$).

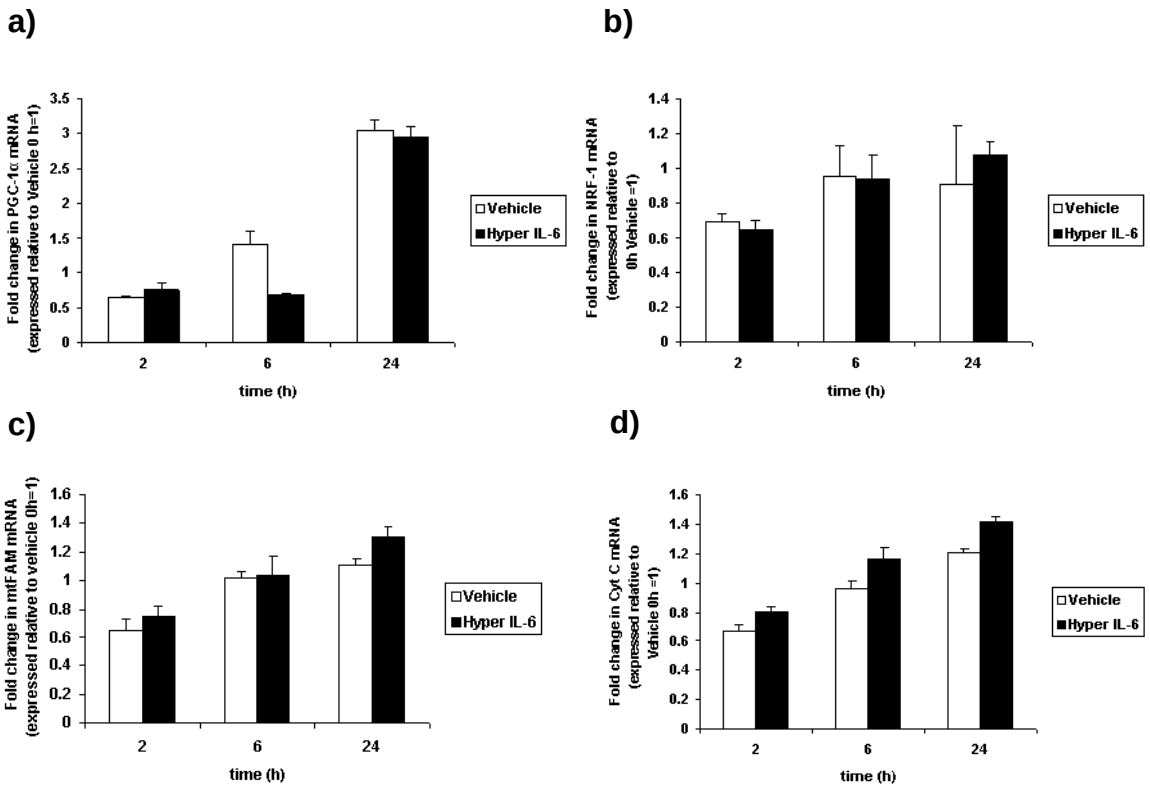


Figure 3.5 PGC-1α and downstream target gene expression in Hyper IL-6 treated L6 myotubes - mRNA expression of (a) PGC-1α and its downstream targets b) NRF-1, c) mtFAM and d) Cytochrome C in L6 myotubes in vehicle and 100 ng·ml^{-1} Hyper IL-6 treated cells. Data is expressed as mean ± SEM relative to 0 hour vehicle control (n=3 experimental replicates from one independent experiment).

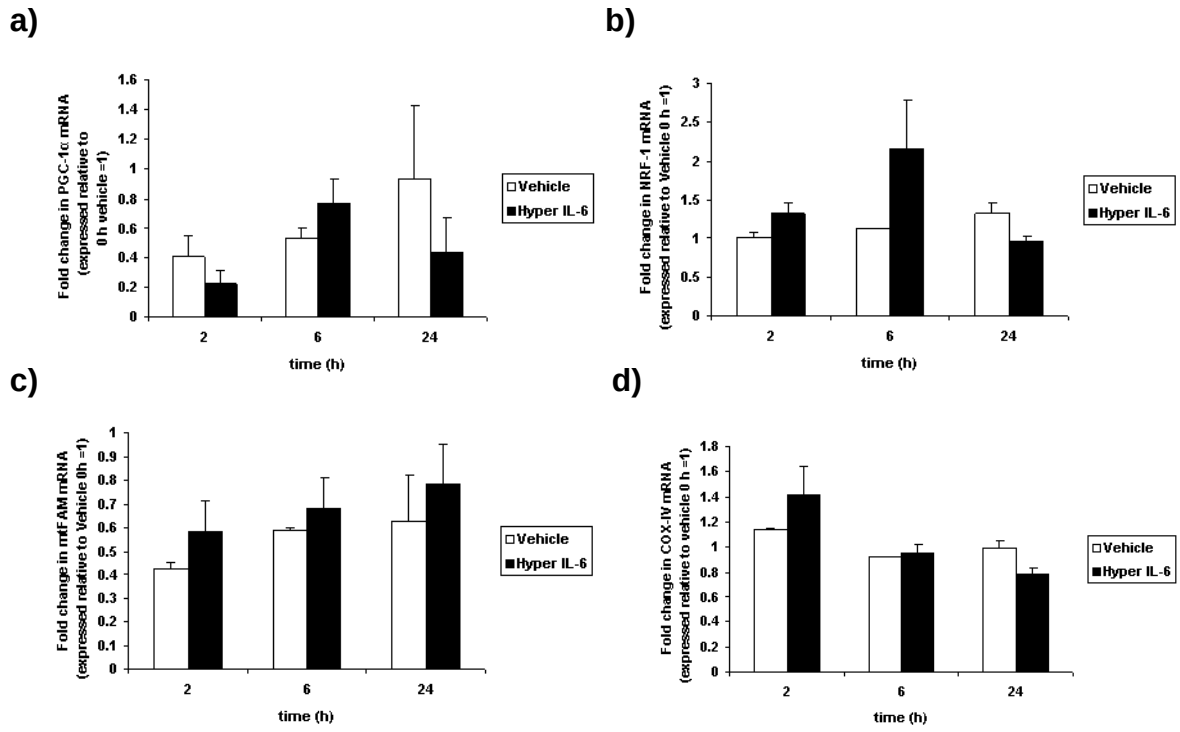


Figure 3.6 PGC-1α and downstream target gene expression in Hyper IL-6 treated CRL1439 hepatocytes - mRNA expression of (a) PGC-1α and its downstream targets b) NRF-1, c) mtFAM and d) COX-IV in CRL1439 liver cells in vehicle and 100 ng·ml^{-1} Hyper IL-6 treated cells. Data is expressed as mean ± SEM relative to 0 hour vehicle control (n=3 experimental replicates from one independent experiment).

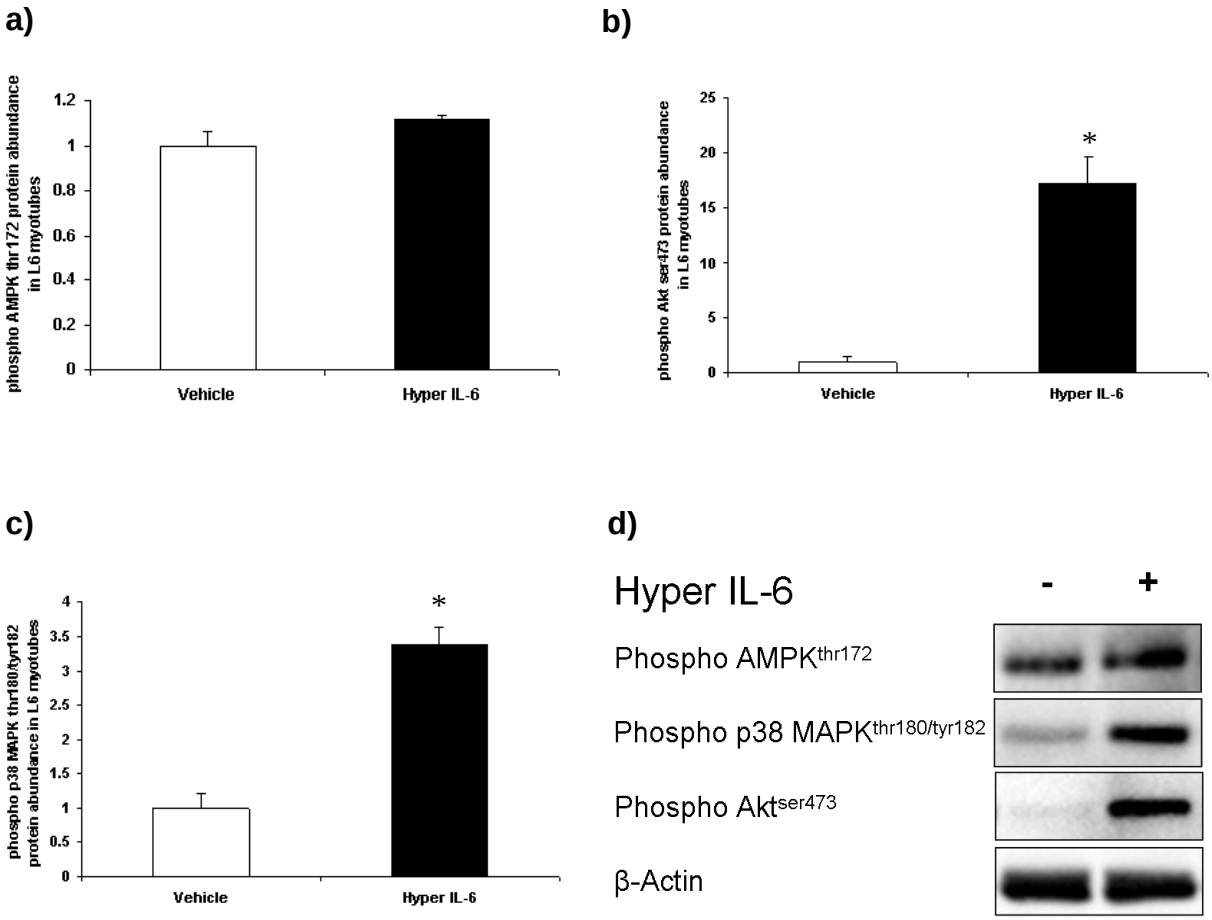


Figure 3.7 Hyper IL-6 has little effect on phosphorylation of AMPK but induces that of Akt and p38 MAPK in L6 myotubes – Protein abundance of a) phospho AMPK^{thr172} b) phospho Akt^{ser473} and c) phospho p38 MAPK$^{thr180/tyr182}$ in L6 myotubes were treated with 50 ng·ml^{-1}. d) Representative blots for the fore mentioned proteins and β-actin. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. * difference between vehicle and Hyper IL-6; $p < 0.001$. All data expressed as mean ± SEM fold change relative to vehicle; n=4 experimental replicates, representative of two independent experiments.

3.4 Discussion

PGC-1α is an important player in metabolic homeostasis through the regulation of mitochondrial functions such as mitochondrial biogenesis, β-oxidation of fatty acids, cell respiration and adaptive thermogenesis (for review refer to Handschin and Spiegelman, 2006). As previous research has also implicated IL-6 in many of these processes, this study sought to examine the effect of IL-6 signalling on PGC-1α transcription and translation.

IL-6 infused rat tissue was obtained and the PGC-1α mRNA and protein expression was examined. Contrary to our hypothesis, there was no significant difference in any of the mitochondrial genes measured in *Tibialis Anterior*. However, an increase in mRNA of the IL-6 target SOCS-3 in rats administered IL-6, irrespective of mode of delivery, was noted, an effect that did not occur in liver. The lack of measured effect of IL-6 on PGC-1α mRNA may be explained by the following: animals were culled and tissues harvested 24 hours post the last infusion of IL-6. This is concurrent with studies by Pilegaard and colleagues (2003), which showed the effect of exercise training on PGC-1α mRNA returned to normal 24 hours post exercise. It is therefore possible that any effect of IL-6 on PGC-1α transcription was also missed due to its relatively quick transcriptional turn over.

To determine if IL-6 had an effect on PGC-1α at a translational level, protein expression in several metabolically active tissues were examined. There was no change in PGC-1α protein of *Tibialis Anterior*, *EDL* or liver. By comparison IL-6, regardless of mode of delivery, had a tendency to reduce expression of PGC-1α protein in *soleus* compared to the saline counter parts. It is important to note, that upon statistical analysis this was not considered significant, a consequence of limited sample size. The *soleus* muscle of rats is described as an oxidative muscle as it predominately consists of slow twitch type I oxidative fibres, the remaining 20 % of fibres are type II glycolytic fibres, whereas *Tibialis Anterior* and *EDL* are described as fast glycolytic muscles (Pullen, 1977). *Tibialis Anterior* is known to consist of approximately 95 % type IIa and IIb fast twitch glycolytic fibres and only 5 % type I fibres (Tasić et al., 2003). It is also known that over expression of PGC-

1α in myotubes results in “fibre type switching” whereby muscle fibres become more oxidative and less glycolytic (Lin et al., 2002).

As there is a decrease in PGC-1α protein expression in *soleus* it may be feasible that IL-6 has a regulatory feedback loop thereby preventing further increases in oxidative type I fibres. However it is imperative to note this requires further investigation and any conclusion reached should be treated with caution for two reasons. Firstly, western blotting data obtained should be interpreted with caution as commercially available PGC-1α antibodies are, most often than not, of poor quality, lacking specificity and as a consequence have a high degree of non specific banding. Secondly, it is not known whether this would have been a significant difference due to the limitations on power as a result of the small amount of animal tissue available for analysis.

The current study suggests in *Tibialis Anterior*, IL-6 is not a transcriptional regulator of PGC-1α as there was no change in the abundance of PGC-1α mRNA or downstream genes. Whether IL-6 regulates transcription in other muscles remains unknown. In the current studies, the effect of IL-6 on PGC-1α and downstream targets was not examined in muscles other than *Tibialis Anterior* as there was a limited amount of tissue available for analysis. Subsequently, it may be of interest to examine PGC-1α mRNA expression in other skeletal muscle types to determine if IL-6 infusion has a fibre type effect. In support of a fibre specific effect, Ikeda and colleagues (2006) concluded mice undergoing endurance training displayed muscle type specific responses. It was noted PGC-1α was only increased in *plantaris* and not *Tibialis Anterior*. Despite the lack of increase in PGC-1α, Ikeda (et al., 2006) also noted the mitochondrial proteins Cytochrome C, Cox IV and ATP synthase were elevated in *Tibialis Anterior*. As a result, Ikeda proposed there may be mechanisms that result in increases in mitochondrial proteins independently of induction of PGC-1α. In accordance with the studies by Ikeda and colleagues, it is plausible to suggest that IL-6 may regulate mitochondrial gene expression independently of PGC-1α in some tissues, thereby accounting for changes in

oxidative capacity often associated with this cytokine (van Hall et al., 2003 and Carey et al., 2006).

Previous studies have determined that PGC-1α possesses activation sites that may be phosphorylated by AMPK, Akt and p38 MAPK. The fore mentioned players have all been implicated in metabolism through mediation of insulin signalling, glucose and fat oxidation, myogenesis and contraction induced mitochondrial biogenesis. Previously, it has been shown that IL-6 can activate these proteins in myotubes and adipocytes (Carey et al., 2006). Moreover, Akimoto et al. (2005) demonstrated that a single running bout induced a transient increase in PGC-1α transcription in the *plantaris* muscle of C57BL6 WT mice, which was concurrent with an increase in activation of the p38 MAPK pathway. Furthermore, when a constitutively active form of the p38 MAPK upstream kinase kinase, MKK6, was expressed in a muscle specific manner, protein of PGC-1α and its transcriptional target COX- IV, was significantly increased compared to that of WT littermate controls (Akimoto et al., 2005).

It has yet to be elucidated by which pathway PGC-1α expression and activity is predominately regulated. The crux of this part of the project was to determine if IL-6 regulates activity and expression of PGC-1α and its downstream targets. Subsequently, the protein and mRNA expression of PGC-1α and its known mitochondrial targets were examined in two metabolically active tissues *in vitro* and *in vivo*. In an elegant study by Wu et al. (1999), it was demonstrated that over-expression of PGC-1α in cultured C2C12 myoblasts and myotubes resulted in increases in NRF-1, mtFAM, Cox IV and Cytochrome C. As this study demonstrated the induction of these genes occurred via a PGC-1α dependent manner, mRNA expression of these were used in all subsequent studies examining the effect of IL-6 or Hyper IL-6 on PGC-1α expression and activity.

Previous research has demonstrated that after an acute bout of exercise, an increase in PGC-1α mRNA and protein was noted in the skeletal muscle from lean subjects. This occurred as soon as 30 min post exercise and remained elevated for

a further 5 hours (De Filippis et al., 2008). Importantly, the previously mentioned study also noted, the elevation in PGC-1α was less significant in obese subjects than that of lean subjects post exercise. Moreover, Pilegaard, Saltin and Neufer (2003) demonstrated that post two legged knee extensor exercises, PGC-1α mRNA expression in *vastus lateralis* was maximal at 2 hours and remained elevated for up to 6 hours, after which it returned to baseline. Increases in downstream targets such as NRF-1, mtFAM and Cox-IV occur 6 hours post treatment with Lipopolysaccharide, a known inducer of oxidative phosphorylation, and remain elevated for up to 48 hours within liver (Suliman et al., 2003). These fore mentioned studies formed the basis of the design for a preliminary experiment *in vitro*. This preliminary experiment aimed to examine PGC-1α mRNA expression in both muscle and liver cells *in vitro* in response to Hyper IL-6 over time. The intent was to use this preliminary experiment to guide the design of future studies aimed at examining the effect of Hyper IL-6 on parameters such as PGC-1α promoter activity, mitochondrial biogenesis and cell respiration. However, as it was determined Hyper IL-6 had little effect on transcription and translation of PGC-1α in both cell types examined *in vitro.* Upon closer examination of the literature, there is published evidence that cultured cells have minimal expression of PGC-1α (Puigserver et al. 2001), providing a possible explanation as to why limited levels of PGC-1 α were detected and why no change was noted. In light of this published data regarding minimal PGC-1 α expression in cultured muscle cells, these *in vitro* studies were not continued further. In future it may be of interest to explore the effect of IL-6 on PGC-1 α mRNA expression in cells either transduced or infected with PGC-1α over a more detailed time course.

An increase in PGC-1α mRNA and downstream targets was noted with time, irrespective of treatment. This is possibly an artefact of the *in vitro* model used. L6 myotubes were cultured and differentiation induced upon serum withdrawal. At day 6 post serum withdrawal, cultures were treated with Hyper IL-6 over a 24 hour period. It is important to note, that during differentiation, changes within the myogenic gene profile occur. This in turn, leads to the elongation and fusion of the cells thereby creating a post-mitotic multinucleated phenotype. In accordance with

the notion that PGC-1α may play a role in differentiation, previous research has correlated abundance of this protein with myogenic gene expression and enhanced differentiation of C2C12 myocytes (Li et al., 2007). Furthermore, it is well established that PGC-1α is paramount in fibre type switching, increased expression of this protein leads to a more oxidative phenotype (Lin et al., 2003) and muscle specific knock out leads to a more glycolytic fibre type (Handschin et al., 2007). As myoblastic cultures differentiate, increases in the oxidative capacity of the cells may be essential to this process. The fore mentioned studies lend support to the notion that, in its capacity as a player within the myogenic program, PGC-1α expression may increase as differentiation proceeds, thereby accounting for the increased mRNA expression seen in the current cohort.

Hyper IL-6 also had limited effect on phosphorylation of AMPK at the Thr172 residue *in vitro*, however this may be a consequence of determining the abundance of this protein at one treatment time point only. Secondly, phosphorylation is not necessarily a measure of intrinsic AMPK activity. Detailed experiments aimed at examining IL-6 family cytokine activation of AMPK on PGC-1α utilising either loss or gain of function may provide a definitive answer as to whether this kinase is involved in the regulation of this important mediator of mitochondrial activity in skeletal muscle. Hyper IL-6 did induce phosphorylation of Akt and p38 MAPK, proteins known to phosphorylate and activate PGC-1α. It would be of interest to determine the effect of constitutive activation and knock down of the PI3-Kinase/Akt and p38 MAPK pathways and determine the subsequent effect of Hyper IL-6 stimulated PGC-1α activity and expression. If these three proteins act in concert, the overexpression or knock out of these proteins may lead to a change in PGC-1α activity in response to this cytokine.

This study does not discount the possibility that IL-6 and/or Hyper IL-6 may regulate mitochondrial biogenesis and metabolism through post translational modification of PGC-1α by the following processes 1) phosphorylation 2) methylation or 3) reversible acetylation (For review refer to Ventura-Clapier, Garnier and Veksler, 2008). Although beyond the scope of this dissertation, future

studies aimed at exploring the effect of these cytokines on these processes may provide insight into whether increased OXPHOS associated with elevated IL-6 occurs in a PGC-1α dependent manner.

PGC-1α possesses phosphorylation sites for p38 MAPK, AMPK and Akt (Puigserver et al., 2001, Jäger et al., 2007 and Li et al., 2007). Examining whether IL-6 leads to phosphorylation of PGC-1α at these sites would provide evidence as to whether IL-6 leads to increased PGC-1α activity through phosphorylation by the fore mentioned proteins. Unfortunately, a commercial antibody against these specific residues of PGC-1α is not available and as a consequence this was not examined in this study.

Another post translational modification that can increase transcriptional activity of PGC-1α is methylation. Arginine Methylationtransferase-1 (PRMT-1) interacts with PGC-1α, causing the methylation of the co-transcription factor's arginine residues. This in turn leads to increased activation of PGC-1α target gene promoters, such as ERRα and Cytochrome C, involved in mitochondrial biogenesis (Teyssier et al., 2005). Whilst there is evidence in the osteosarcoma cell line SAOS2 that PRMT-1 is involved in increased expression of PGC-1α target genes involved in mitochondrial biogenesis, there is limited evidence of this protein's role in this process in skeletal muscle. Moreover, there are no studies in the literature examining whether IL-6 leads to increased PGC-1α activity through methylation and whether this occurs in a PRMT-1 dependent manner or not. Interestingly, PRMT-1 and CARM1, another member of the PRMT family, act synergistically to recruit steroid receptor coactivator-2 (SRC-2)/GRIP1. This leads to co-activatation of myogenic transcription factors, such as Myocyte Enhancer Factor-2 (MEF-2), involved in muscle differentiation (Chen et al., 2002). Given the prominent role IL-6 plays in myogenesis and differentiation (Baeza-Raja and Muñoz-Cánoz, 2004) and as PRMT-1 and PGC-1α have also been implicated in these processes through activation of transcription factors such as MEF-2, it may be of interest to explore the effects of this cytokine on PRMT-1 expression and methylation of PGC-1α.

Acetylation (the addition of an acetyl group) of PGC-1α inhibits its activity and causes a decrease in target genes important to nutrient and stress sensing and energy metabolism (For review refer to Ventura-Clapier, Garnier and Veksler, 2008). It is known that GCN-5, an acetyl transferase, abolishes PGC-1α dependent fatty acid utilisation and mitochondrial gene transcription in C2C12s (Lerin et al., 2006 and Gerhart-Hines et al., 2007). Conversely, deacetylation (removal of an acetyl group) of PGC-1 leads to increased Mitochondrial and fatty acid oxidation transcriptional activity (Gerhart-Hines et al., 2007). During fasting, SIRT-1, an NAD^+ dependent SIRT-1 deacetylase, expression is increased and leads to the deacetylation of hepatic PGC-1α. This in turn, promotes hepatic glucose output through inhibition of glycolytic gene transcription and activation of gluconeogenic genes (Rodgers et al., 2005). In skeletal muscle cells, SIRT-1 activity is known to increase in response to low glucose leading to the activation of PGC-1α and downstream targets specific to fatty acid oxidation (Gerhart-Hines et al., 2007). Whilst there are studies that correlate an increase in SIRT-1 activity with a decrease in IL-6 and TNFα production, there is little known about the effects of IL-6 on SIRT-1 and PGC-1α acetylation/deacetylation and its subsequent transcriptional targets. Given the associated effects of IL-6 on metabolism and the prominent role SIRT-1 has in regulation of mitochondrial and metabolic transcriptional activity, it may be of interest to examine the effect of this cytokine on SIRT-1 dependent deacetylation of PGC-1α and its transcriptional targets.

An interesting question arises from this study: as Hyper IL-6 was used, a designer cytokine that mimics IL-6/sIL-6R signalling and thought to be pro-inflammatory, is it possible that PGC-1α expression and subsequent activity may not be regulated by IL-6 trans-signalling events? Moreover is it possible that IL-6 mediates PGC-1α expression and activity through the cognate membrane bound IL-6 receptor signalling which is thought to be metabolically beneficial? To examine this possibility it may be of interest to co-treat liver and muscle *in vitro* and *in vivo* with IL-6 and the natural inhibitor of soluble IL-6 receptor signalling, sgp130, thereby allowing only membrane bound IL-6 receptor signalling to occur.

The studies described in this chapter demonstrate that IL-6 signalling through the sIL-6 receptor complex, using Hyper IL-6 treatment to mimic this phenomena, does not regulate PGC-1α at a transcriptional or translational level in cultured myotubes or hepatocytes *in vitro*. Furthermore, prolonged infusion of IL-6 in rats had little effect on mRNA or protein expression of PGC-1α *in vivo*. It is important to note, however, that there was limited tissue available for analysis of PGC-1α expression and as a consequence, this aspect to the study was only examined in *Tibialis Anterior* muscle. This does not discount the possibility that IL-6 regulates expression of PGC-1α and other mitochondrial genes in muscle types other than *Tibialis Anterior* and requires further investigation. In conclusion, the results of this study indicate that neither IL-6 nor Hyper IL-6 regulate transcription or translation of PGC-1α.

CHAPTER FOUR

Hyper IL-6 augments metabolic cell signalling and physiological parameters in myotubes and liver cells *in vitro*

4.1 Introduction

The activation of the JAK/STAT pathway is an important process in the regulation of glucose and lipid metabolism. IL-6 may activate the fore mentioned pathway via two mechanisms; In the first of the alternatives, IL-6 binds to the transmembrane complex of an IL-6 receptor α and gp130β homodimer. This causes auto-phosphorylation of the associated JAK and the recruitment of STAT-3 resulting in pathway activation (for review refer to Febbraio, 2007). Alternatively JAK/STAT activation may occur via a phenomena known as "IL-6 trans-signalling", whereby upon IL-6 interaction with the agonistic soluble IL-6 receptor (sIL-6R), a IL-6/sIL-6R complex is formed. This complex then binds to the ubiquitously expressed gp130β, resulting in JAK/STAT pathway activation (Rose-John, 2003 and 2006). IL-6 trans-signalling has broad implications, as cells naturally devoid of the membrane bound IL-6 receptor remain responsive to IL-6 due to gp130 being ubiquitously expressed (Rose-John, 2003 and 2006).

Whether trans-signalling results in subsequent metabolic signalling is not known. To examine the effects that sIL-6R activation elicits on metabolic signalling in skeletal muscle *in vitro,* IL-6 trans-signalling events were mimicked using the designer cytokine Hyper IL-6, a fusion protein of the soluble IL-6 receptor fused to recombinant human IL-6 by a flexible polypeptide linker (Fischer et al., 1997). As IL-6 is a predominant player in regulating muscle metabolism and gp130 is ubiquitously expressed it was hypothesised that acute Hyper IL-6 treatment may be more efficient than IL-6 alone in modulating glucose uptake and fatty acid oxidation via pathways previously implicated in metabolic control.

As discussed in the first chapter of this dissertation, the role of IL-6 in health and states of disease remains controversial. Many have described this cytokine as functioning in a pro-inflammatory fashion in metabolism, leading to insulin resistance within cultured hepatocytes by diminishing glucose transport (Senn et al., 2002) and decreasing glycogen synthesis through insulin desensitisation (Kanemaki et al., 1998). Contrary to this belief, others have observed that IL-6 signalling may be executed in the absence of inflammation, resulting in enhanced

metabolism and thus allowing it to be described as “anti-inflammatory”. In support of this notion, it has previously been noted that IL-6 enhances glucose uptake in 3T3-L1 adipocytes (Stouthard, Elferink and Sauerwein., 1996) and acts as an insulin sensitizer on glycogen synthesis in human skeletal muscle cells (Weigert et al., 2005). Further support of this positive metabolic regulator theory was provided by Carey and colleagues (2006). When L6 myotubes were treated with IL-6, an increase in glucose uptake and fat oxidation was observed. It was proposed that this occurred via an AMPK dependent mechanism as, when L6 myotubes were transduced with a dominant negative AMPK adenovirus, IL-6 stimulated glucose uptake was severely blunted. Notably, this study also demonstrated that infusing human subjects with IL-6 resulted in an increase in insulin stimulated glucose disposal. Further more, in a study by van Hall and colleagues (van Hall et al., 2003), IL-6 stimulated lipolysis and fat oxidation in humans. The fore mentioned studies depict IL-6 as a multi faceted cytokine that elicits metabolic responses in a tissue type dependent manner.

IL-6/sIL-6R complex signalling has been implicated in many animal models of inflammatory conditions including arthritis (Nowell et al., 2003), nodular regenerative hyperplasia and adenomas of the liver (Maione et al., 1998) and colitis (Atreya et al., 2000). This has lead to the general consensus that IL-6 signalling mediated via trans-signalling events are “pro-inflammatory”. Notably, in a study by Rabe and co-workers (2008), transgenic blockade of IL-6/sIL-6R complex by over expression of its natural inhibitor, sgp130, led to the abrogation of recruitment of mononuclear inflammatory cells in the air pouch model of inflammation. Moreover, it was determined that classic signalling via the membrane bound IL-6 receptor remained intact. Whether soluble IL-6 receptor signalling plays a part in skeletal muscle metabolism, or is purely associated with inflammation has yet to be elucidated. Thus, the primary aim of this study was to determine the significance of sIL-6R signalling within skeletal muscle *in vitro* and to examine what effect inhibition of IL-6 trans-signalling using the soluble form of gp130 (sgp130), would have within these cells.

As IL-6 would appear to elicit tissue type specific responses, the secondary aim of this study was to determine the degree of bioactivity induced by membrane bound IL-6 receptor complex signalling in comparison to that of IL-6 trans-signalling within CRL1439 hepatocytes and L6 myotubes. Furthermore, it was hypothesised that the degree of bioactivity, as determined by the activation of common signalling targets, would be conducted in an inherent cell type specific manner.

4.2 Materials and Methods

Cell Culture - L6 myotubes and CRL1439 hepatic cells were cultured as described in Chapter 3.

In vitro treatments - Six days post initiation of serum withdrawal, L6 cultures were considered to be fully differentiated myotubes as most cells had fused to form multinucleated cells. At this point the cultures were treated with the appropriate compound. Eighteen hours prior to treatment, media was replaced with fresh differentiation media. After which, vehicle (0.1 % (w/v) BSA/ PBS), 100 $ng \cdot ml^{-1}$ recombinant human IL-6 (referred to as rh-IL-6 from here in), 100 $ng \cdot ml^{-1}$ recombinant murine IL-6 (rm-IL-6), 100 $ng \cdot ml^{-1}$ recombinant rat IL-6 (rr-IL-6) (R and D Systems, Minneapolis, MN, USA), or the "designer cytokines" 100 $ng \cdot ml^{-1}$ Hyper IL-6 with or without sgp130Fc were administered via directly "spiking" the compound of interest into the media and incubating for the desired amount of time.

SDS PAGE Analysis of Protein expression - Protein analyses were performed as described in Chapter 3. Briefly, 40 µg of protein from cytokine treated lysates were subjected to SDS PAGE. The protein expression of phospho $AMPK^{thr172}$, phospho ACC^{ser79}, phospho $STAT\text{-}3^{tyr705}$, phospho p38 $MAPK^{thr180/tyr182}$, phospho Akt^{ser473}, their corresponding totals, β-actin (Cell Signaling Technology Inc, Beverly, MA, USA) and SOCS-3 (Abcam, Cambridge UK) were examined by antibody binding and enhanced chemiluminescence as described in Chapter 3.

Glucose Uptake - L6 myotubes were cultured as described above, however cells were serum starved for 4 hours prior to stimulation with 50 $ng \cdot ml^{-1}$ Hyper IL-6 and 50 $ng \cdot ml^{-1}$ sgp130Fc for 1 h, the negative control 5 µM Cytochalasin B for 30 min or 100 nM Insulin for 15 min. The treatment media was removed and cells equilibrated in fresh α-MEM media containing 100 nM D-deoxy-D-glucose for 5 min, after which the media was removed and cells were incubated in fresh media containing 100 nM D-deoxy-D-glucose and 1µCi mL^{-1} 2-Deoxy-D-[2,6-3H]glucose (Amersham, GE Healthcare Life Sciences, Buckinghamshire, UK) for 10 min. Cells were lysed in 0.3M NaOH and 400 µl of lysate was placed in 5 ml scintillation fluid

and mixed thoroughly. Radioactivity counts were determined for each sample over a 5 min period using a Packard TRICARB 1900CA Scintillation Counter (GMI Inc, Ramsey, MN, USA). For each sample, counts per minute were corrected for protein as determined by BCA assay and then expressed relative to counts for cytochalasin B control samples. Final data was expressed as fold change over vehicle.

Fatty Acid Oxidation - L6 myotubes were cultured as per above. Cells were incubated for 2 hours in differentiation media containing 0.1mM palmitate, 1.6 µCi ml^{-1} tritium labelled palmitate, 2 % (w/v) BSA and either Vehicle, 50 $ng \cdot ml^{-1}$ Hyper IL-6 or 100 nM Insulin (negative control). After which 1 ml of the media was collected and incubated with 5 ml Chloroform: Methanol (2:1 v/v), whilst vigorously shaken, for 15 min. Two millilitres of 2M KCl: HCl solution was added to the media/chloroform mix, subjected to a further vigorous shaking for 15 minutes followed by a 5 min centrifuge at 3000 rpm. This resulted in separation of the sample into an inorganic and tritium labelled palmitate containing aqueous phase, of which 0.5 ml of the latter was collected and mixed with 5 ml scintillation fluid and mixed thoroughly. Radioactivity counts were determined for each sample over a 5 min period using a Packard TRICARB 1900CA Scintillation Counter (GMI Inc, Ramsey, MN, USA). For each sample, counts per minute were corrected for protein amount as determined by BCA assay and then expressed as a function of time.

Statistics - Statistics were computed using the statistical analyses software packages SPSS 12.0.01 for Windows (Apache Software Foundation, USA) or Sigma Stat. In all experiments other than fatty acid oxidation assays, a two way (treatment*time) ANOVA was used to determine significant differences. Multiple comparisons were made using the Student-Neuman-Keuls post hoc method. For the fatty acid uptake assay a one way ANOVA with a Student-Neuman-Keuls post hoc test was used to locate significant differences between treatments. Significant differences for all experiments were accepted at $p < 0.05$. All data is expressed as mean ± SEM.

4.3 Results

Hyper IL-6 has a greater bioactivity in muscle cells than that of other recombinant IL-6 species- The bioactivity of varying species of IL-6 was determined over time in L6 myotubes treated with 100 $ng \cdot ml^{-1}$ rmIL-6, rrIL-6 and hyper IL-6. As a measure of bioactivity, the phosphorylation and subsequent activation of IL-6/IL-6R/gp130 signalling targets STAT-3 and p38 MAPK were examined after 2, 10, 30 and 120 min exposure to the fore mentioned cytokines. It was noted that in L6 myotubes treated with Hyper IL-6 for 2 min phospho STAT-3^{tyr705} expression was 20 fold greater ($p = 0.001$) than that in cells treated with other species of IL-6 (Figure 4.1a). Notably, this response peaked after 30 min exposure, to approximately 45 fold ($p < 0.001$) and was still sustained by 120 min with a 15 fold increase compared to vehicle with $p < 0.001$. Hyper IL-6 significantly induced phospho p38 $MAPK^{thr180/tyr182}$ at 30 min (Figure 4.1b), after which it returned to base line by 120 min. There were no other significant differences in phospho p38 $MAPK^{thr180/tyr182}$ observed. Hyper IL-6 also activated Akt. After 10 minutes, treatment robustly stimulated phospho Akt^{ser473} with an approximately 8 fold increase in this protein (Figure 4.2a). At 30 min phospho Akt was still 8 fold higher than that of vehicle however, by 120 min expression had returned to levels seen at 2 min exposure with Hyper IL-6. There was a trend for phospho $AMPK^{thr172}$ to be increased in the presence of Hyper IL-6 at 120 min (Figure 4.2b).

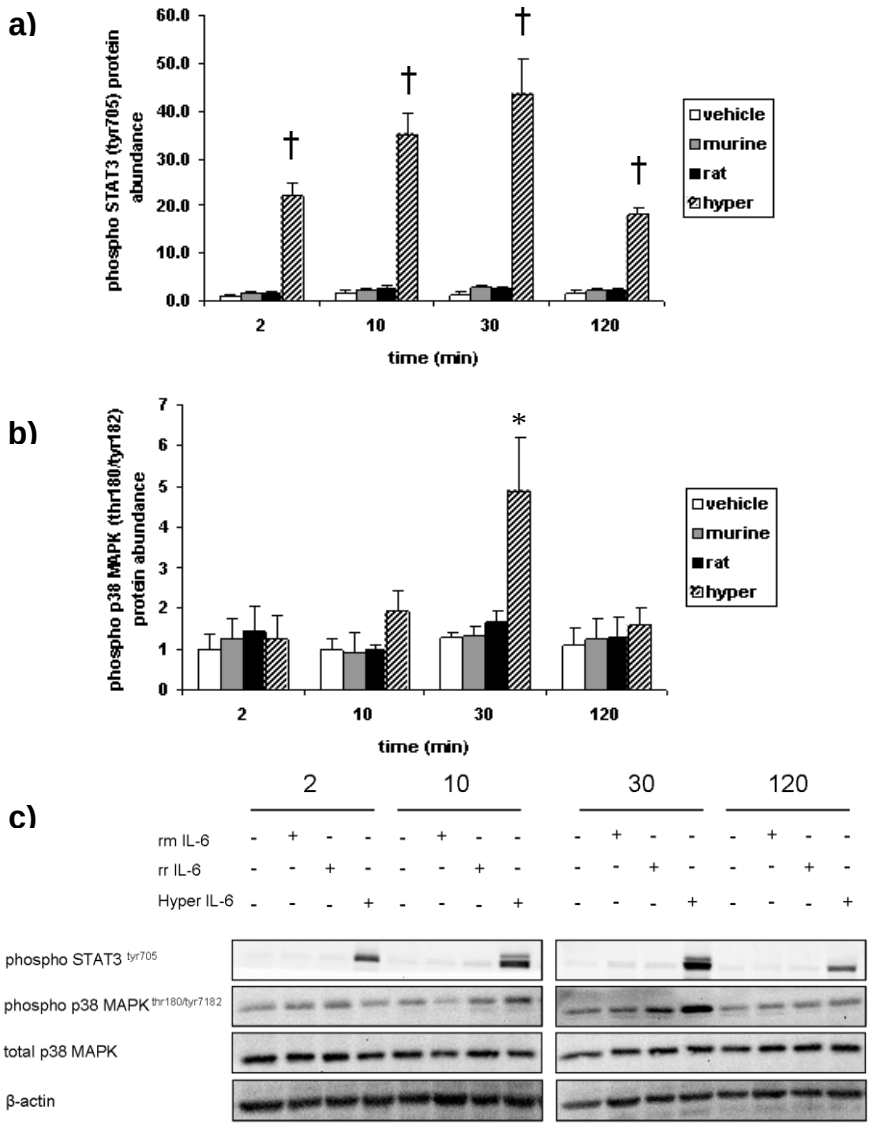


Figure 4.1 Hyper IL-6 has a greater bioactivity than that of other IL-6 species on L6 myotubes. L6 myotubes were treated with 100 ng·ml^{-1} rm IL-6, 100 ng·ml^{-1} rm IL-6 or 100 ng·ml^{-1} Hyper IL-6. When compared to vehicle control, Hyper IL-6 increased a) phospho STAT-3^{tyr705} ($p < 0.001$) and b) phospho p38 MAPK$^{thr180/tyr182}$. c) representative blots for the fore mentioned proteins. † denotes difference between vehicle and 100 ng·ml^{-1} Hyper IL-6. * Difference between vehicle and 100 ng·ml^{-1} Hyper IL-6; $p < 0.05$. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. All data expressed as mean ± SEM fold change relative to vehicle; n=6 (3+3) experimental replicates from 2 independent experiments (equal numbers from each experiment).

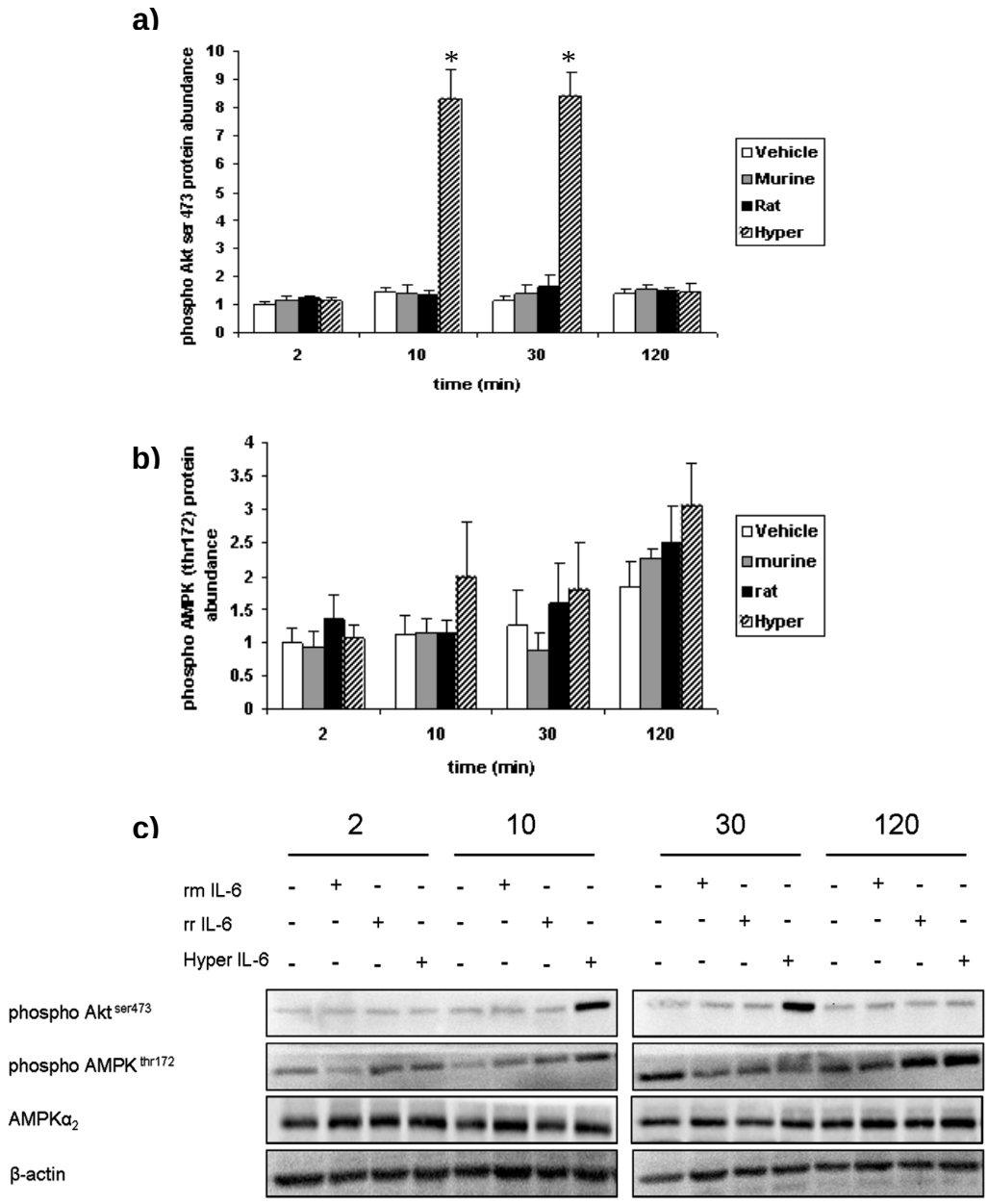


Figure 4.2 Hyper IL-6 increases a) phospho Akt^{ser473} but has no significant effect on b) phospho AMPK^{thr172}. c) Representative blots for phospho Akt ^{ser473} and phospho AMPK ^{thr172} and the corresponding totals. L6 myotubes were treated with 100 ng·ml^{-1} rm IL-6, 100 ng·ml^{-1} rm IL-6 or 100 ng·ml^{-1} Hyper IL-6. * denotes difference vehicle v Hyper IL-6 $p < 0.01$. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change from Vehicle; n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

Hyper IL-6 activates STAT-3 in a dose dependent manner in L6 myotubes – As it was evident 100 $ng \cdot ml^{-1}$ Hyper IL-6 significantly increased phosphorylation of STAT-3, the effects of time and dose were examined in L6 myotubes to determine the optimal dose of Hyper IL-6 to be used in all subsequent *in vitro* experiments. Over the timecourse, it was observed that, when compared with vehicle, concentrations of 2, 10, 50 and 100 $ng \cdot ml^{-1}$ Hyper IL-6 caused significant increases ($p=0.031$; $p=0.005$; $p=0.002$; $p=0.006$ respectively) in phosphorylation of STAT-3 (Figure 4.3a). Significant differences with time (2 min vs. 30 min; $p < 0.001$ and 30 min vs. 120 min; $p< 0.001$) were also noted. There was no significant interaction between dose and time ($p=0.836$).

In direct contrast to L6 myotubes, membrane bound IL-6R is as biologically active as sIL-6R in CRL1439 hepatocytes – To make a direct comparison between cognate membrane bound IL-6R signalling and that mediated by the sIL-6R, CRL1439 hepatocytes, which express membrane bound IL-6R, and L6 myotubes were treated with either 50 $ng \cdot ml^{-1}$ Hyper IL-6 or 100 $ng \cdot ml^{-1}$ rh-IL-6 to mimic soluble receptor complex signalling or membrane bound receptor signalling, respectively. In CRL1439, after 1 h of treatment, Hyper IL-6 induced approximately 2 fold greater phosphorylation of STAT-3 (Figure 4.4a) when compared with that of rh-IL-6 (vehicle vs. Hyper IL-6; $p<0.001$, vehicle vs. rh-IL-6; $p<0.001$, rh-IL-6 vs. hyper IL-6; $p<0.001$). However as treatment time was prolonged, hyper IL-6 and rh-IL-6 induced phosphorylation of STAT-3 to a similar degree when compared to that of vehicle treated cells, with this bioactivity being sustained for as long as 48 h post initiation of treatment. Moreover, there was a significant difference between treatments over time ($p<0.001$). By contrast, in L6 myotubes after 1 h of treatment phosphorylation of STAT-3 at the tyr705 residue was increased by 4 fold in response to rh-IL-6 (vehicle vs. rh-IL-6; $p<0.001$) and this effect was sustained for as long as the 48 h treatment period (Figure 4.4b). In the case of Hyper IL-6 treatment, increased phospho STAT-3^{tyr705} was ~20 fold ($p<0.001$) and by 12 h this had decreased to approximately a 12 fold increase when compared to vehicle ($p<0.001$). Again, this was sustained over the remaining treatment period. Co-

treatment with 100 nM Insulin had no effect on phosphorylation of STAT-3 in response to the various treatments.

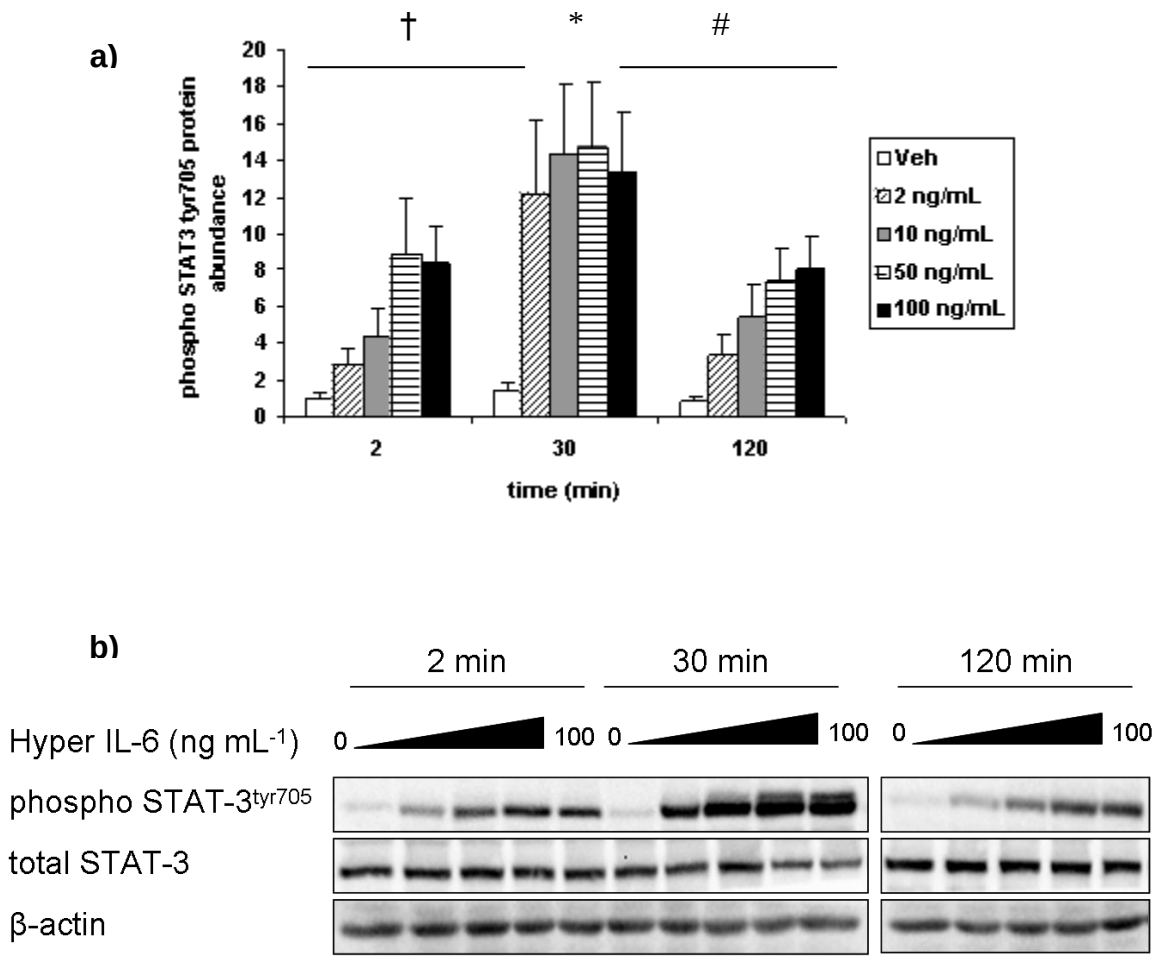


Figure 4.3 Hyper IL-6 phosphorylates STAT-3 in a dose-dependent manner. a) Quantitation of phospho STAT-3 protein abundance in L6 myotubes treated with varying concentrations of Hyper IL-6 b) Representative blots for phospho STAT-3, its corresponding total and β-actin in L6 myotubes. * at 30 min, vehicle vs. 2, 10, 50 and 100 ng·ml^{-1} Hyper IL-6; p = 0.031; p=0.005; p=0.002; p=0.006 respectively † differences with time 2 min vs. 30 min; p < 0.001 # 30 min vs. 120 min; p< 0.001. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change relative to vehicle at 2 minute time point, n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

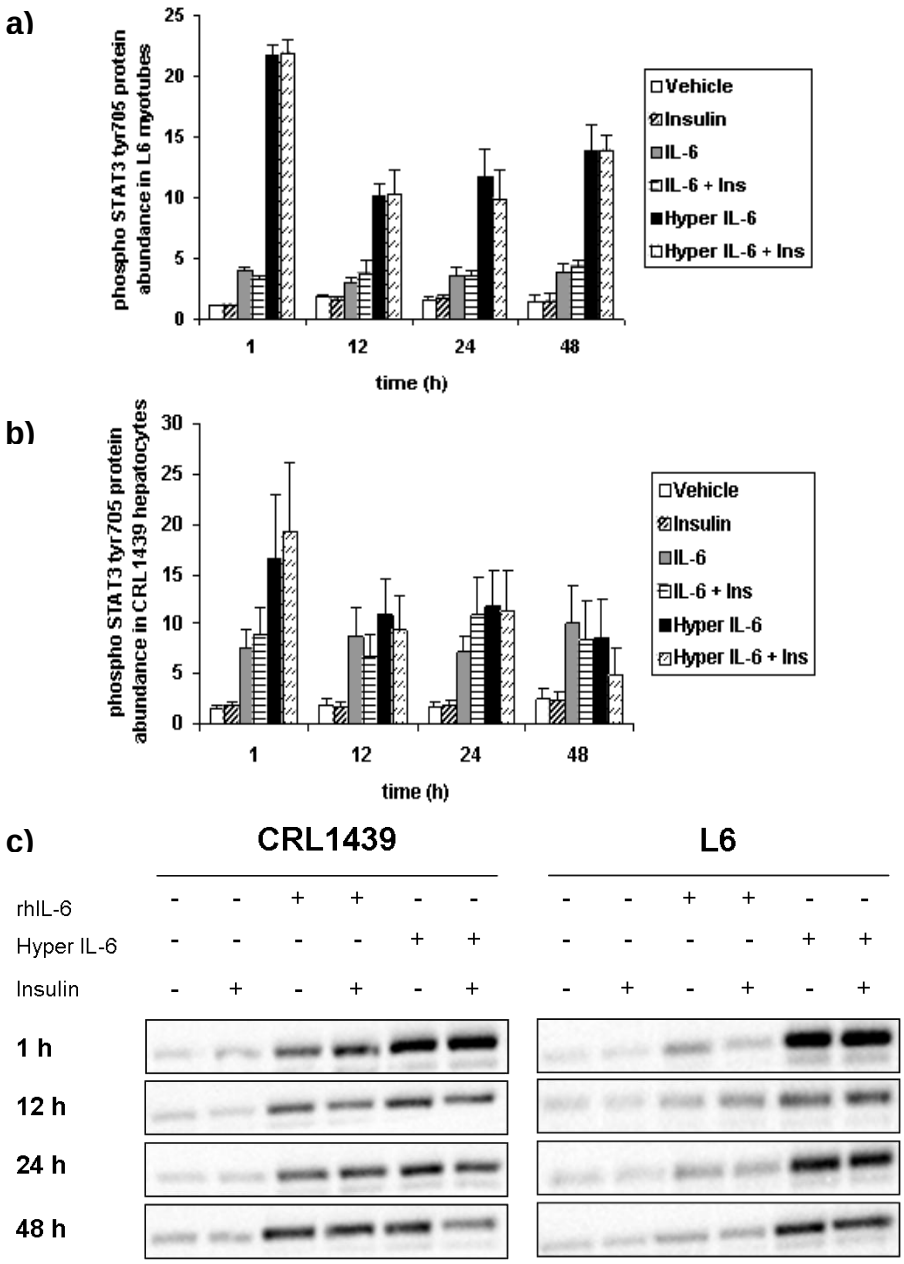


Figure 4.4 Membrane bound IL-6Rα signalling has greater bioactivity in CRL1439s than in L6 myotubes a) membrane bound versus soluble receptor signalling in myotubes and in b) CRL1439. c) phospho STAT-3^{tyr705} representative blots. L6 myotubes were treated with 100 ng·mL^{-1} rh-IL-6 or 50 ng·mL^{-1} Hyper IL-6 with or without 15 minute 100 nM Insulin stimulation. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in phospho STAT-3^{tyr705} compared with vehicle, n=3 experimental replicates from one experiment.

Hyper IL-6 co-treatment potentiates Insulin stimulated glucose uptake – Glucose uptake was significantly increased ~ 0.75 fold in L6 myotubes stimulated with 100 nM Insulin when compared to vehicle treated cells (vehicle vs. Insulin; $p < 0.001$, Figure 4.5). A similar effect on glucose uptake was seen in L6 myotubes treated with 50 ng·mL ml^{-1} Hyper IL-6 with a 0.75 fold increase in glucose uptake (vehicle vs. Hyper IL-6; $p = 0.003$). Co-treatment with 50 ng·ml^{-1} Hyper IL-6 potentiated Insulin stimulated glucose uptake by approximately 3 fold when compared to vehicle control (vehicle vs. Hyper IL-6 + Insulin; $p < 0.001$).

Hyper IL-6 induces SOCS-3 protein and potentiates the increase in insulin stimulated phospho-p38 MAPK$^{thr180/tyr182}$ in L6 myotubes. The effects of Hyper IL-6 on signalling pathway components were examined under the conditions used in the glucose uptake assay. Under these conditions, regardless of the presence of insulin, 50 ng·ml^{-1} Hyper IL-6 increased phospho-STAT3^{tyr705} approximately 13 fold (Figure 4.6a; vehicle vs. Hyper IL-6; $p < 0.001$). A protein band at a molecular weight consistent with that predicted for SOCS-3 was apparent at 25 kDa. The IL-6 inducible protein SOCS-3, was significantly increased by 5 fold (Figure 4.6b; vehicle vs. Hyper IL-6; $p < 0.001$). In cultures co-treated with 100 nM Insulin, a tendency for potentiation of Hyper IL-6 induced SOCS-3 protein abundance was apparent, albeit not statistically significant.

In L6 myotubes under basal conditions, Hyper IL-6 induced a 2 fold increase in phospho-p38 MAPK$^{thr180/tyr182}$ when compared to vehicle with $p < 0.001$ (Figure 4.7a). When stimulated with insulin, protein expression of phospho-p38 MAPK$^{thr180/tyr182}$ was augmented by 0.5 fold in vehicle treated cells ($p = 0.02$) compared to 3 fold in those cells co-treated with Hyper IL-6 (vehicle vs. Hyper IL-6 + Insulin; $p < 0.001$). In the presence of insulin, there is also a significant potentiation of Hyper IL-6 induced activation of p38 MAPK (Hyper IL-6 vs. Hyper IL-6 + Insulin; $p = 0.032$). A significant increase in insulin stimulated phosphorylation of Akt at the ser473 residue was observed ($p < 0.001$). However, there was no change in expression of this protein in response to Hyper IL-6 (Figure

4.7b). Although Hyper IL-6 had no effect on phospho-AMPK^{thr172}, Insulin reduced its expression with and without Hyper IL-6 treatment ($p = 0.037$, Figure 4.8).

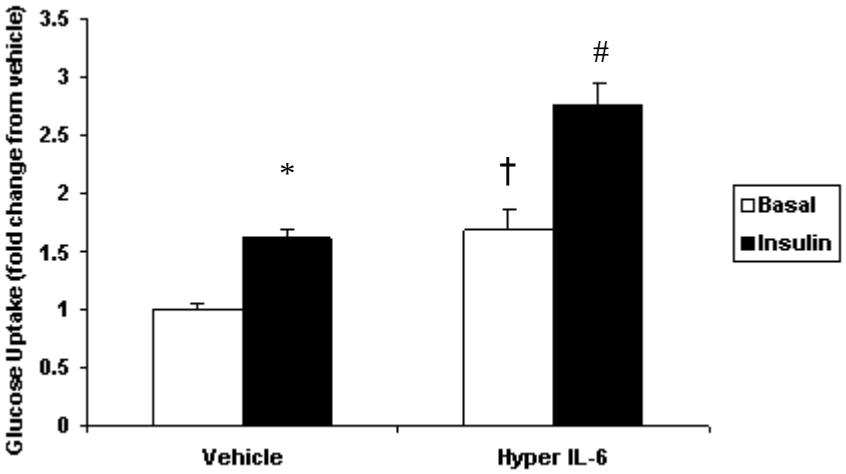


Figure 4.5 Hyper IL-6 co-treatment results in potentiation of Insulin stimulated glucose uptake in L6 myotubes. L6 myotubes were treated for 2 hours with 50 ng·mL^{-1} Hyper IL-6, during the final 15 minutes of the period cells were stimulated with 100 nM Insulin and glucose uptake determined. * denotes significant difference between Vehicle Basal v Vehicle + 100 nM Insulin; $p < 0.001$, † significant difference between Vehicle and 50 ng·ml^{-1} Hyper IL-6; $p = 0.003$, # difference between Vehicle and 50 ng·ml^{-1} Hyper IL-6 + 100 nM Insulin; $p < 0.001$. Data expressed as fold change in glucose uptake expressed relative to vehicle under basal conditions, mean ± SEM, n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

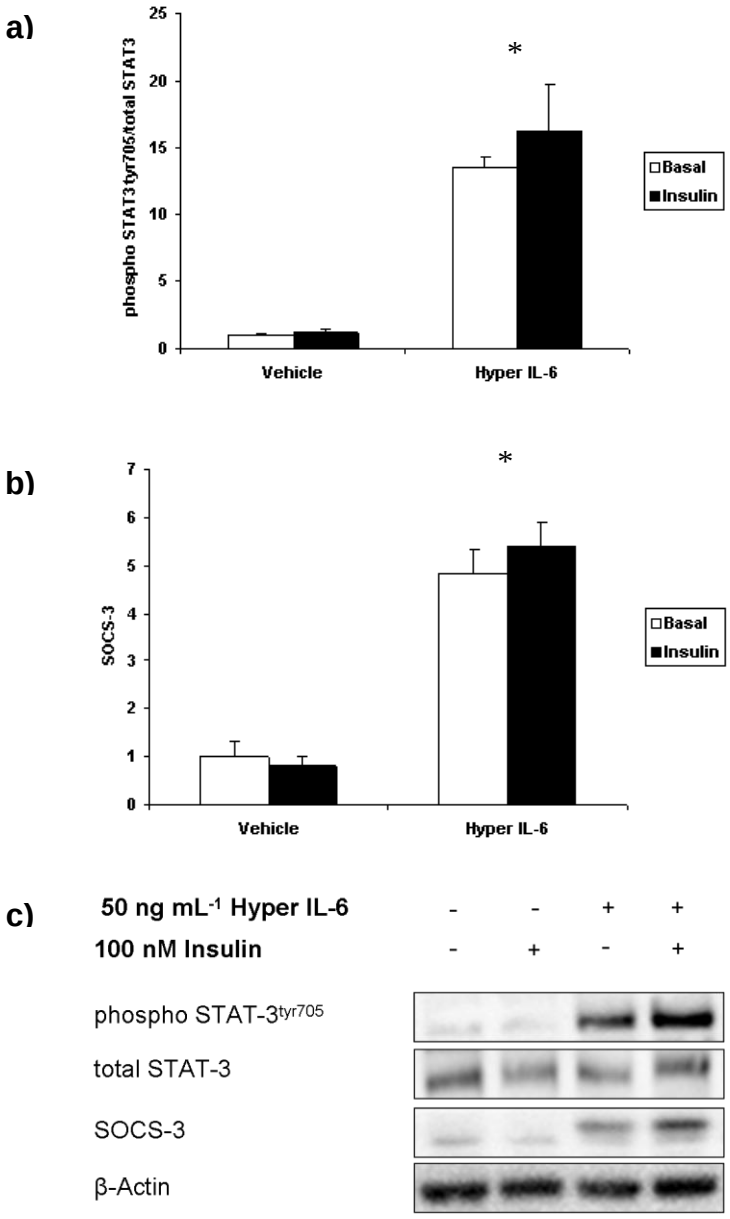


Figure 4.6 Hyper IL-6 activates STAT-3 and induces SOCS-3 protein in L6 myotubes under conditions used in the glucose uptake assay a) quantitation of phospho STAT-3^{tyr705} protein abundance b) SOCS-3 protein expression c) representative blots for protein expression of phospho STAT-3, its corresponding total, SOCS-3 and β-actin. * difference between vehicle and Hyper IL-6; $p < 0.001$. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in protein expression from vehicle; n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

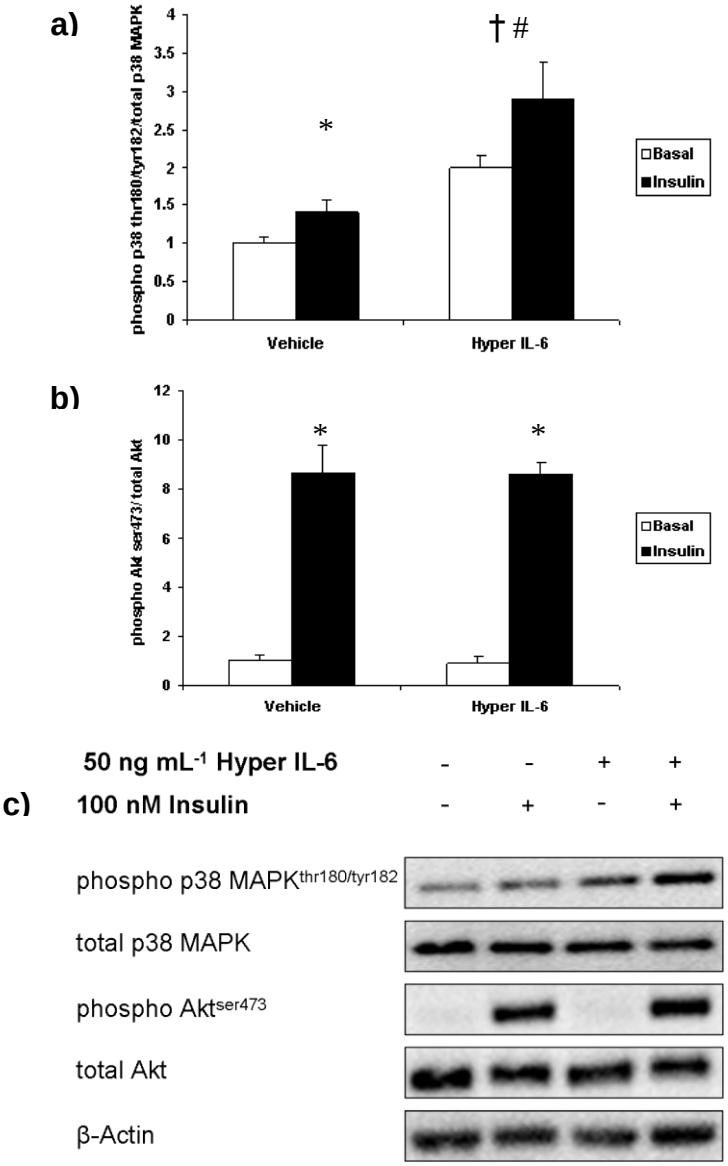


Figure 4.7 Insulin stimulated activation of Akt remains unchanged with Hyper IL-6 co-treatment where as phosphorylation of p38 MAPK is potentiated under these conditions. Quantitation of a) phospho p38 MAPK$^{thr190/tyr182}$ and b) phospho Akt^{ser473}. * Vehicle versus Insulin stimulation; p = 0.02 (phospho p38 MAPK$^{thr180/tyr182}$); p < 0.001 (phospho Akt^{ser473}), † difference between vehicle and Hyper IL-6 + Insulin; p < 0.001, # Hyper IL-6 versus Hyper IL-6 + Insulin p = 0.032. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in protein expression from vehicle; n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

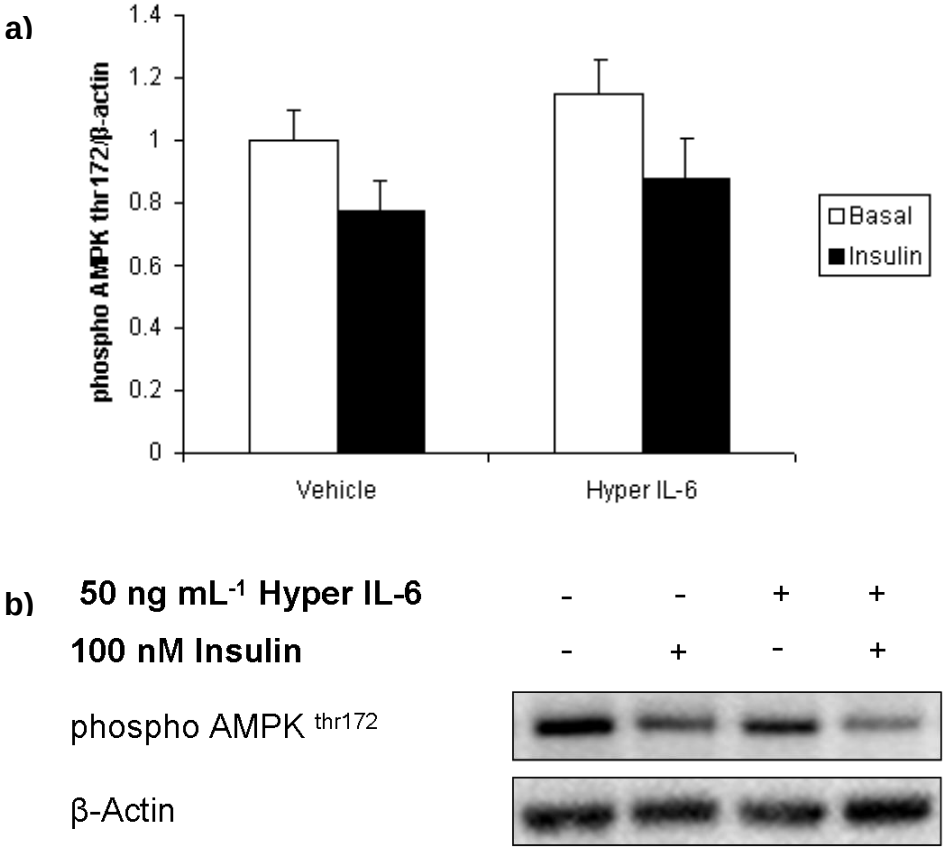


Figure 4.8 Hyper IL-6 has no effect on a) phospho AMPK^{thr172} protein abundance under conditions used in the glucose uptake assay. b) Representative blots for phospho AMPK ^{thr172} and β-actin. * basal versus Insulin; p = 0.037. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in protein expression from vehicle; n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

Hyper IL-6 attenuates fatty acid oxidation – Oxidation of palmitic acid was decreased by approximately 27 % in L6 myotubes treated with 50 ng·ml^{-1} Hyper IL-6 when compared with vehicle treated cells (Figure 4.9; vehicle vs. Hyper IL-6; $p = 0.046$). When compared with vehicle control, treatment with the negative control, 100 nM Insulin, significantly attenuated palmitate oxidation by approximately 70 % with $p < 0.001$.

sgp130Fc augments Hyper IL-6 insulin stimulated glucose uptake – Co-treating with 50 ng·ml^{-1} of the natural inhibitor of Hyper IL-6, sgp130Fc, had a tendency to attenuate Hyper IL-6 stimulated glucose uptake, however this was not considered significant (Figure 4.10). Insulin stimulated glucose uptake was increased 2 fold when compared to vehicle ($p = 0.043$), interestingly insulin stimulated Hyper IL-6 induced glucose uptake was heightened 3 fold, this was considered significant compared to that induced by the Hyper IL-6/sgp130Fc co-treatment ($p < 0.001$).

sgp130Fc has no effect on phosphorylation of STAT-3, p38 MAPK or Akt under basal conditions .The effect of 50 ng·ml^{-1} sgp130Fc on basal expression of phospho STAT-3^{tyr705}, phospho p38 MAPK$^{thr180/tyr182}$ and phospho Akt^{ser473} was examined in L6 myotubes (Figures 4.11 and 4.12). In the absence of Hyper IL-6 there was no significant effect of 50 ng·ml^{-1} sgp130Fc on basal expression of these proteins. A dose of 50 ng·ml^{-1} Hyper IL-6 caused an approximate 24 fold increase in phospho STAT-3^{tyr705} when compared to vehicle treated cells ($p < 0.001$; Figure 4.11a). Co-treatment with 50 ng·ml^{-1} Hyper IL-6 and 50 ng·ml^{-1} sgp130Fc did not decrease phosphorylation of STAT-3 at the tyr705 residue when compared to treatment with Hyper IL-6 alone. A similar observation of protein expression for phospho p38 MAPK $^{thr180/tyr182}$ and phospho Akt ^{ser473} was noted (Figure 4.12a and 4.12b, respectively).

There was no significant effect of 50 ng·ml^{-1} sgp130Fc alone on abundance of phospho p38 MAPK$^{thr180/tyr182}$ when compared to vehicle treated cells (Figure 4.12a). In L6 myotubes treated with 50 ng·ml^{-1} Hyper IL-6 a 3.5 fold increase in phospho p38 MAPK $^{thr180/tyr182}$ was evident (Vehicle vs Hyper IL-6; $p < 0.001$).

There was no significant difference in protein abundance of phospho p38 MAPK$^{thr180/tyr182}$ in cells treated with 50 ng·ml^{-1} Hyper IL-6 alone and those co-treated with 50 ng·ml^{-1} Hyper IL-6 and 50 ng·ml^{-1} sgp130Fc (50 ng·ml^{-1} Hyper IL-6 vs 50 ng·ml^{-1} Hyper IL-6 + 50 ng·ml^{-1} sgp130Fc; p = 0.399).

Cells treated with sgp130Fc alone had a similar level of phospho Akt^{ser473} abundance as that of vehicle treated cells (Figure 4.12b). A significant difference was evident between Vehicle and 50 ng·ml^{-1} Hyper IL-6, with an approximate 17 fold difference ($p < 0.001$). There was no significant difference between cells treated with 50 ng·ml^{-1} Hyper IL-6 and those treated with 50 ng·ml^{-1} Hyper IL-6 + 50 ng·ml^{-1} sgp130Fc ($p = 0.217$). As 50 ng·mL^{-1} sgp130Fc had no significant effect on Hyper IL-6 induced phosphorylation of STAT-3, p38 MAPK or Akt in L6 myotubes, the effects of sgp130Fc on Hyper IL-6 induced bioactivity were examined in a dose dependent manner in cultured myotubes and hepatocytes.

Inhibition of Hyper IL-6 bioactivity by sgp130Fc occurs in a tissue specific manner in vitro – L6 myotubes and CRL1439 hepatocytes were co treated with 25 ng·mL^{-1} Hyper IL-6 with or without varying concentrations of sgp130Fc (Figure 4.13a). Regardless of the presence of sgp130Fc at any given dose, no difference in Hyper IL-6 induced phospho STAT-3^{tyr705} was observed in L6 myotubes. Contrary to this, bioactivity was diminished in CRL1439 hepatocytes treated with 25 ng·mL^{-1} Hyper IL-6 plus 500 ng·mL^{-1} sgp130Fc as indicated by approximately a 25 % reduction in phospho STAT-3 ^{tyr705} protein abundance (Hyper IL-6 + 50 ng·mL^{-1} sgp130Fc vs. Hyper IL-6 + 500 ng·mL^{-1} sgp130Fc; $p = 0.041$, Hyper IL-6 + 100 ng·mL^{-1} sgp130Fc vs. 500 ng·mL^{-1} sgp130Fc; $p = 0.031$). When compared to cells administered Hyper IL-6 + 200 ng·mL^{-1} sgp130Fc, there was a non significant propensity for Hyper IL-6 induced phosphorylation of STAT-3 to be reduced by co-treatment with 500 ng·mL^{-1} sgp130Fc ($p=0.073$).

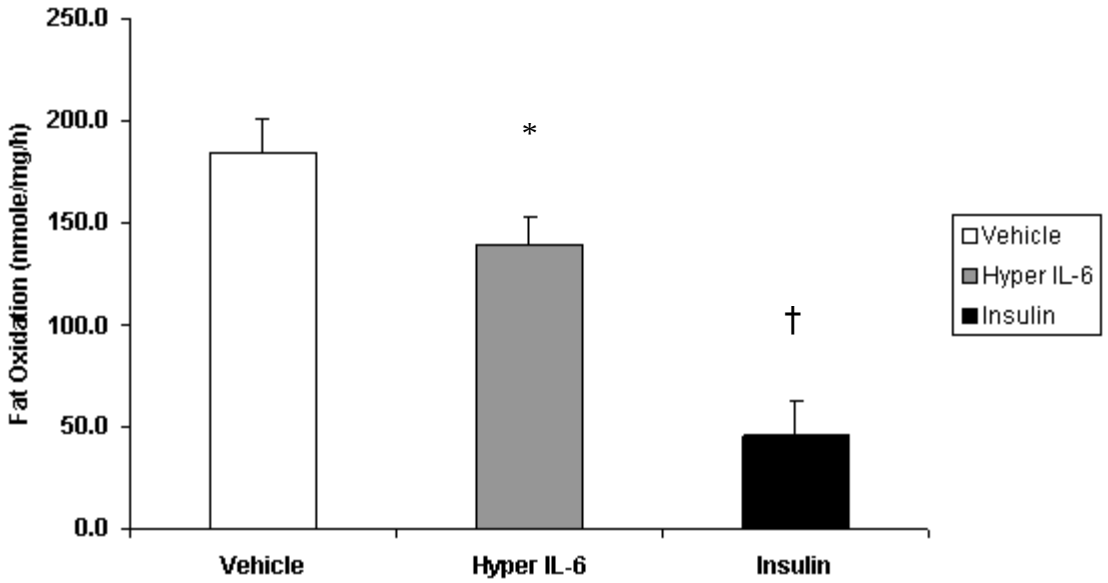


Figure 4.9 IL-6/sIL-6R signalling attenuates palmitate oxidation in L6 myotubes. ***** difference in fatty acid oxidation between vehicle and Hyper IL-6 treated myotubes; p = 0.046, † Difference between vehicle and Insulin stimulated L6 myotubes; p <0.001. Data expressed as mean ± SEM, n = 6 experimental replicates from one experiment.

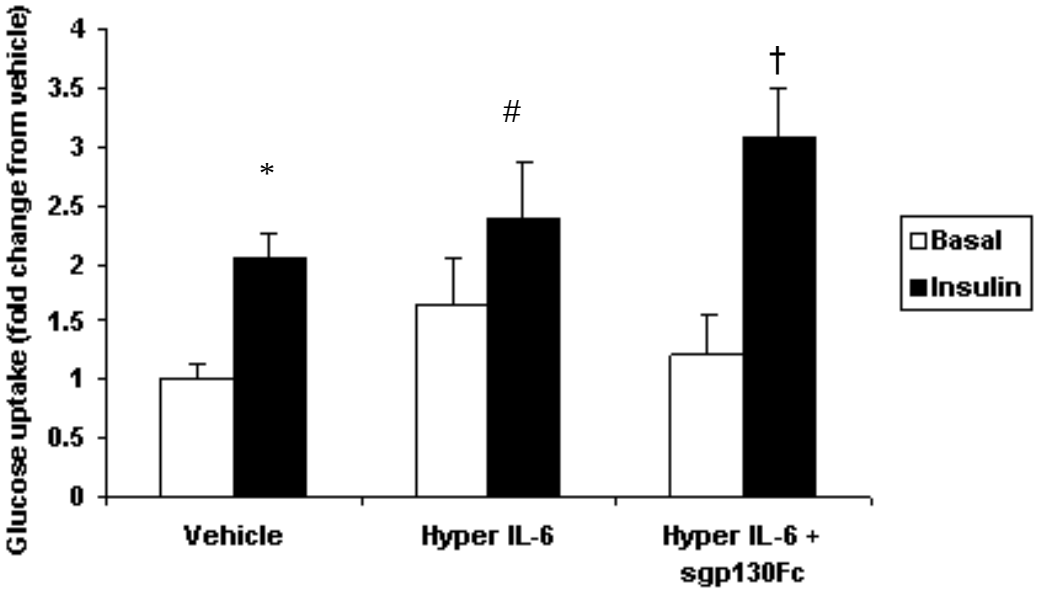


Figure 4.10 Co-treatment with sgp130Fc augments Hyper IL-6, Insulin stimulated glucose uptake. A significant difference between basal and insulin stimulation exists; $p < 0.001$. * difference between basal and insulin stimulated glucose uptake in vehicle treated cells; $p = 0.043$; # Hyper IL-6 + Insulin vs Hyper IL-6 + sgp130Fc † difference between Hyper IL-6 + sgp130Fc and Hyper IL-6 + sgp130Fc + Insulin; $p < 0.001$. Data expressed as mean ± SEM, n=6 experimental replicates from one experiment.

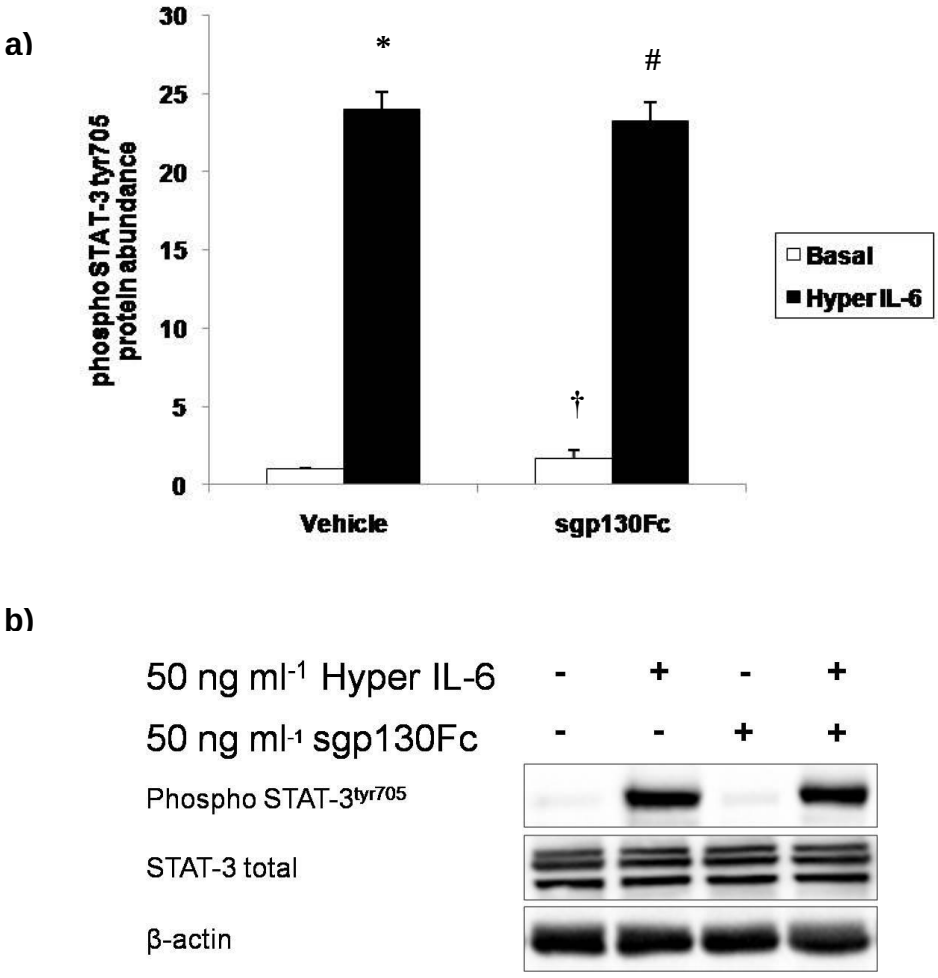


Figure 4.11 sgp130Fc has no effect on basal phosphorylation of STAT-3 in L6 myotubes. a) Quantitation of phospho STAT-3^{tyr705}. b) Representative blots for phospho STAT-3^{tyr705}, total STAT-3 and β-actin. * difference between basal vehicle and Hyper IL-6 treated cells; $p < 0.001$; † Hyper IL-6 vs sgp130Fc; $p < 0.001$; # sgp130Fc vs Hyper IL-6 + sgp130Fc; $p < 0.001$. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in protein expression from vehicle; n=4 experimental replicates from one experiment

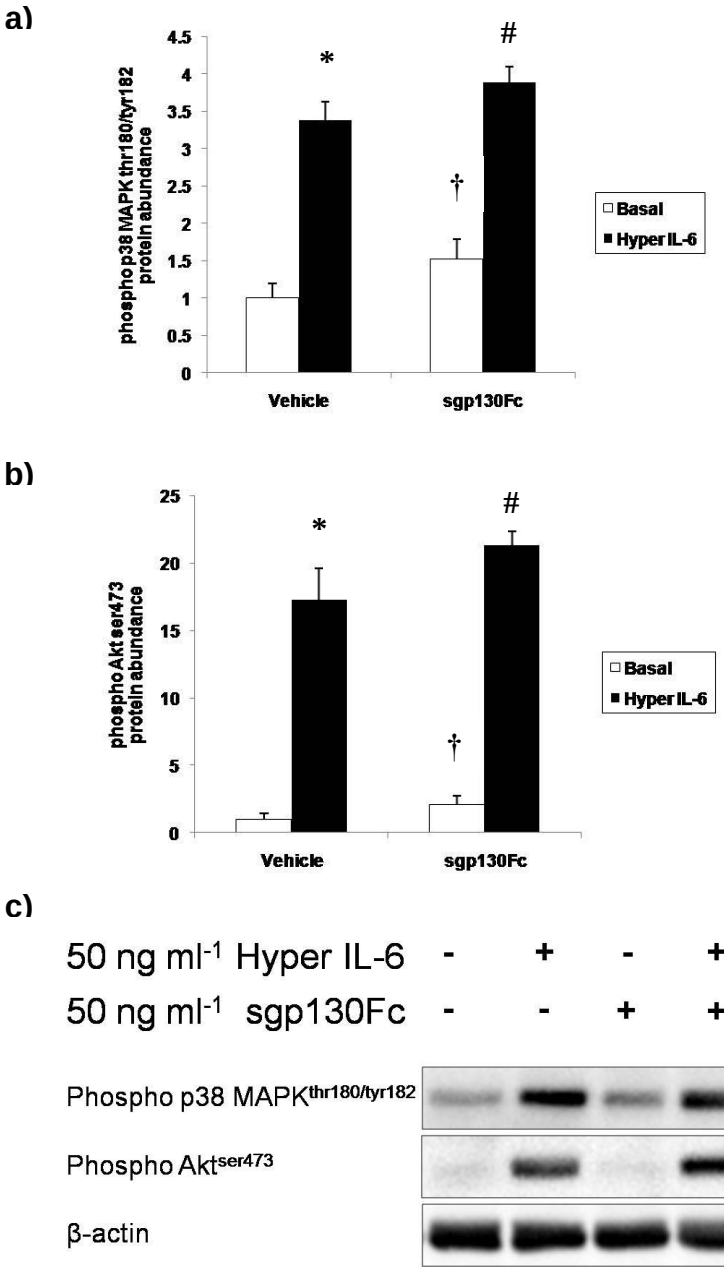


Figure 4.12 sgp130Fc has no effect on basal phosphorylation of p38 MAPK or Akt in L6 myotubes. a) Quantitation of phosphorylation of p38 MAPK in L6 myotubes treated with 50 ng·ml^{-1} sgp130Fc alone or in combination with 50 ng·ml^{-1} Hyper IL-6. b) Quantitation of phosphorylation of Akt in L6 myotubes treated with 50 ng·ml^{-1} sgp130Fc alone or in combination with 50 ng·ml^{-1} Hyper IL-6. c) Representative blots of phospho p38 MAPK$^{thr180/tyr182}$, phospho Akt^{ser473} and β-actin expression in L6 myotubes. Difference in phospho p38 MAPK and Akt between * basal vehicle and Hyper IL-6 ($p < 0.001$); † Hyper IL-6 vs sgp130Fc; $p < 0.001$; # sgp130Fc vs sgp130Fc + Hyper IL-6; $p < 0.001$. As expression of corresponding

β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data is expressed as ± SEM fold change in protein expression from vehicle; n = 4 experimental replicates from one experiment.

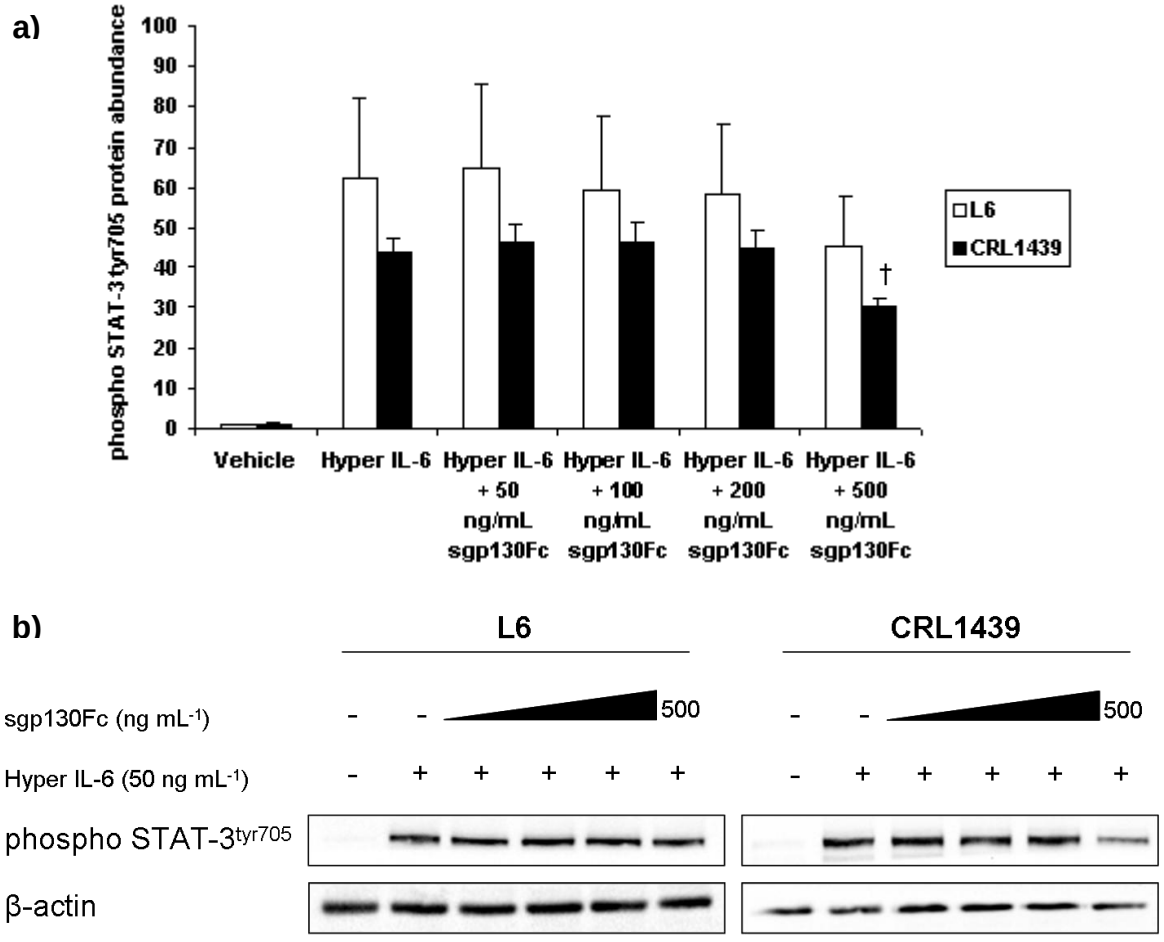


Figure 4.13 sgp130Fc inhibits Hyper IL-6 in a cell type dependent manner. a) Quantitation of phosphorylation of STAT-3 in L6 myotubes and CRL1439 myotubes treated with varying concentrations of sgp130Fc. b) Representative blots of phospho STAT-3^{tyr705} and β-actin expression in L6 myotubes and CRL1439 hepatocytes. Difference in phospho STAT-3^{tyr705} expression between vehicle and Hyper IL-6 ± sgp130Fc at varying concentrations exists in both cell lines ($p < 0.001$). * difference between Hyper IL-6 + 50 ng·ml^{-1} sgp130Fc and Hyper IL-6 + 500 ng·ml^{-1} sgp130Fc ; $p = 0.041$. † difference between Hyper IL-6 + 100 ng·ml^{-1} sgp130Fc and Hyper IL-6 + 500 ng·ml^{-1} sgp130Fc; $p = 0.036$. As constitutive expression of corresponding total proteins remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM fold change in protein expression from vehicle; n=6 experimental replicates from two independent experiments (equal numbers from each experiment).

4.3 Discussion

The current study aimed to determine whether IL-6 trans-signalling events occurred within skeletal muscle *in vitro* using the designer cytokine Hyper IL-6 to emulate the activity of the sIL-6/sIL-6R complex. Throughout the studies undertaken in this chapter it was demonstrated that Hyper IL-6 was highly bioactive within the rat skeletal muscle cell line L6, using phosphorylation of the common targets of IL-6 signalling, STAT-3 and p38 MAPK, as a measure of bioactivity. Expression of total SOCS-3 was also induced upon Hyper IL-6 treatment. SOCS-3, is an IL-6 induced protein that regulates IL-6 signalling through a negative autoregulatory loop that prevents autophosphorylation of gp130 or by targeting the receptor complex for proteosomal degradation by ubiquitination (Krebs and Hilton, 2001). This cell line was treated with either 100 $ng \cdot ml^{-1}$of recombinant IL-6 derived from mice or rats or the designer cytokine Hyper IL-6 (human IL-6). It is important to note that L6 myotubes remain responsive to the fore mentioned species of IL-6 as there is a high level of conservation of sequence between species. When compared with that of human IL-6, murine IL-6 sequence homology for the nucleotide sequence and amino acid sequence is 60 % and 42 %, respectively. Similarly, other studies have shown that Rat IL-6 shares sequence homology of 68 % for nucleotides and 58 % for amino acids (Tanabe et al. 1988; Northemann et al. 1989). Furthermore rat and murine IL-6 also share a high degree of nucleotide (92 %) and amino acid (93 %) sequence homology (Northemann et al. 1989).

Hyper IL-6 does not rely on the abundance of the membrane bound IL-6Rα but instead binds to the ubiquitously expressed gp130β receptor, resulting in pathway activation. It is plausible to suggest that in L6 myotubes the expression of the membrane bound form of the receptor is of less abundance than gp130β, thereby accounting for the heightened activity of this cytokine when compared with that of murine, rat and human IL-6 (Figures 3.1 and 3.3). This requires further examination. This supports a notion proposed by Peters and co-workers (1998) that the prolonged affect of Hyper IL-6, when compared with IL-6 alone, on haptoglobin mRNA (a downstream target of IL-6) was due to the higher expression of gp130 than the IL-6 receptor within liver. The Peters et al., (1998) study also noted that internalisation of Hyper IL-6 was impaired and it had a greater affinity

than IL-6 for its receptor, which possibly contributes to the prolonged and increased bioactivity of Hyper IL-6.

In a study by Kami et al. (2000), in skeletal muscle undergoing regeneration after contusion, mRNA of the IL-6R was only expressed in mononuclear cells and not in multi nucleated myofibres. As the L6 cultures in the present study were differentiated for 7 days ensuring a population of mostly multi-nucleated and fused myofibre colonies, this may insinuate that the IL-6 receptor is of low abundance due to the low numbers of IL-6R mRNA and protein expressing mononuclear cells present within these cultures. This may also explain the lack of response to the recombinant forms of IL-6 used in the comparative studies (Figure 4.1), as these species target cognate receptor signalling and naturally occurring trans-signalling, rather than gp130β which is more likely to be of higher abundance within L6 myotube colonies.

The lack of effect of the cytokines on AMPK phosphorylation in L6 myotubes was surprising given that every measure possible was taken to ensure the integrity of cytokines used in these experiments remained intact. This included adhering to manufacturer's instructions stringently. Before cytokine reconstitution the vial was briefly centrifuged ensuring any cytokine stuck to the walls of the vial would be pelleted at the bottom of the vial. Secondly, cytokine was reconstituted with the carrier protein BSA. Recombinant rat -IL-6 and rm-IL-6 was used immediately after reconstitution and was never freeze / thawed ensuring that the cytokine did not denature as a consequence of this process. Experiments examining the effect of rr-IL-6 and rm-IL-6 were repeated numerous times, using different batches of cytokine from different commercial companies and consistently provided similar results.

Contrary to previous studies examining IL-6 signalling (Carey et al., 2006), Hyper IL-6 did not markedly increase phosphorylation of AMPK at the thr172 residue. A possible explanation is that over time, myogenic differentiation as a result of serum starvation increases the basal level of AMPK activity, possibly due to lower

amounts of metabolic substrates for glucose utilisation available for the ever increasing energy demands of these cells. Moreover, the cells in the present study were cultured in low glucose α-MEM. This may have implications for basal phosphorylation and subsequent activation of this protein as AMPK activity is induced in glucose deprived states due to a shift in the AMP/ADP ratio. This may result in a metabolic shift allowing the cell to maintain energy homeostasis through the induction of β-oxidation. In support of this, a study by Niesler, Myburgh and Moore (2006) demonstrated that C2C12 myotubes displayed a higher basal level and stimulated activation of AMPK and this activity was greater than that seen in myoblasts. In addition, induction of apoptosis due to serum starvation was less in C2C12 myotubes than that of undifferentiated myoblasts, most likely due to the increased expression of AMPK within these cells. If IL-6 does indeed activate AMPK, one may argue that the culture conditions used within this study would be adverse for exploring this. AMPK expression and activity may be exponentially elevated with increases in post mitotic fused multi nucleated myotubes within cell populations thus masking any changes induced by cytokine treatment.

It has previously been shown that IL-6 is an important regulatory component of the myogenic differentiation program of C2C12 cells (Baeza-Raja and Muñoz-Cánoz, 2004). Under conditions considered unfavourable for C2C12 differentiation, administration of sIL-6 resulted in an increase in markers of myogenic programming including α-actin and myogenin. More recently, in a study by the same group, IL-6 release was increased over time during muscle hypertrophy (Serrano et al., 2008) and behaved in both an autocrine and paracrine manner. Importantly, this study also demonstrated that IL-6 Receptor shedding may occur in skeletal muscle both *in vitro* and *in vivo* as evident by the presence of sIL-6R in cell culture media and in *plantaris* muscle extracts, thus providing support to the current study's findings IL-6/sIL-6R induced signalling is physiologically relevant within skeletal muscle. As this is the case, it may be of interest to examine IL-6 receptor expression in both its transmembrane complex form and sIL-6R as well as metalloprotease activity, throughout the myogenic cycle.

As previous research has alluded that the physiological response to IL-6 is unique according to the cell type's intrinsic properties, it was hypothesised that activation of the JAK/STAT pathway by the two types of IL-6 receptor complexes would also be inherently different according to cell type. Thus, the response of L6 myotubes and CRL1439 hepatocytes to either recombinant human IL-6 or Hyper IL-6 was examined (Figure 4.4). It was evident that membrane bound receptor signalling was as biologically relevant as that of soluble IL-6 receptor signalling within liver cells *in vitro*. By contrast, L6 myotubes were highly responsive to Hyper IL-6, an effect that was not so apparent in cells treated with rhIL-6. It is not inconceivable that the differences in response to the cytokines are due to disparity in the relative expression of gp130 and membrane bound IL-6Rα within these two cell types and would certainly account for the heightened activity of this cytokine within L6 myotubes when compared to that of other species of IL-6. In support of this, a study by Schwabe, Zhao and Kung (1994) demonstrated that cell lines responded differently according to the relative expression of the membrane bound IL-6R.

IL-6 has previously been implicated in potentiating the dynamic cellular response of insulin stimulated glucose uptake (Carey et al., 2006). In concurrence with this study, it was determined that Hyper IL-6 stimulated glucose uptake to a similar level as that induced by Insulin. Furthermore, co-treatment with Hyper IL-6 potentiated insulin stimulated glucose uptake, suggesting that Hyper IL-6 increases glucose uptake by Insulin independent pathways. These changes in glucose uptake were associated with increases in the dual phosphorylation of p38 MAPK. This is in accordance with previous research that implicates p38 MAPK in the regulation of glucose uptake within adipocytes and skeletal muscle (Lee et al., 2008; Lemieux et al., 2003; Niu et al., 2003; Sweeney et al., 1999). The exact role of p38 MAPK in glucose uptake remains controversial. Previous research indicates that Insulin stimulated glucose uptake in L6 myotubes occurs independently of p38 MAPK (Antonescu et al., 2005). However, this does not exclude the possible effect p38 MAPK may have in IL-6 induced glucose uptake, independently of insulin.

Akt substrate of 160 kDa (AS160), a Rab GTPase binding protein, plays an integral part in Insulin and Contraction induced glucose uptake (Kramer et al., 2006). During the basal state AS160 is bound to GLUT 4, upon insulin stimulation Akt phosphorylates AS160, causing the protein to dissociate from GLUT4, leading to vesicular exocytosis to the plasma membrane and increased glucose uptake (Larance et al., 2005). It should be noted that, AS160 also possesses phosphorylation sites for AMPK (Treebak et al., 2006). These sites would appear to be important in the regulation of contraction mediated AS160 dissociation and glucose uptake (Kramer et al., 2006). Whether p38 MAPK has the ability to induce dissociation of AS160 has yet to be elucidated. It would be of great interest to determine if Hyper IL-6 potentiates glucose uptake via mediating p38 MAPK induced dissociation of AS160 and subsequently increased exocytosis of GLUT4. Alternatively, the increased glucose uptake due to Hyper IL-6 may be a result of increased intrinsic activity of GLUT4. This requires further investigation.

Contrary to previous studies, under the conditions used in the glucose uptake, AMPK activation via phosphorylation of residue thr172 did not appear to be affected by Hyper IL-6. Thus, it is proposed that AMPK is not required for sIL-6/sIL-6Rα induced glucose uptake within skeletal muscle. Further studies involving knock down of AMPK activity would provide a definitive answer to this supposition. As AMPK is activated during metabolic stress resulting in a shift of metabolism from ATP utilising to ATP generating processes and as glucose uptake was attenuated in these studies, palmitate oxidation was also examined. In support of this “metabolic switch” hypothesis, palmitate oxidation was less in L6 myotubes treated with Hyper IL-6 compared with vehicle treated cells. It is known that upon exercise, skeletal muscle releases IL-6 into the circulation thereby providing tissue cross talk which, in turn, alters metabolism within the liver by reducing hepatic glucose output (Febbraio and Pedersen, 2008) and increasing lipolysis in adipose tissue (van Hall et al., 2003). As transient increases in IL-6 occur during exercise, perhaps IL-6 trans-signalling also plays a beneficial role in an acute manner through the regulation of glucose utilisation and modulation of lipid metabolism. IL-

6 is often chronically elevated in states of disease and perhaps this chronic elevation results in aberrant metabolism and inflammation.

Unexpectedly, when co-treated with Hyper IL-6 and the natural competitive inhibitor of IL-6 trans-signalling, sgp130Fc, insulin stimulated glucose uptake was potentiated (Figure 4.10). Whether sgp130Fc is capable of mediating glucose uptake through alternate signalling pathways is not known and the mechanism by which this may occur remains elusive and requires further investigation. It is of great importance to note that classical IL-6 signalling mediated through the membrane receptor complex remains unaffected by sgp130 (Jostock et al., 2001). This was confirmed in these studies, whereby 50 $ng \cdot mL^{-1}$ sgp130Fc did not stimulate phosphorylation of STAT-3, p38 MAPK or Akt. Upon review of the literature, previous studies have demonstrated a molar excess of sgp130 is required for inhibition of sIL-6/sIL-6R complex signalling due to the comparative differences in molecular weight of the two molecules (Jostock et al., 2001). sgp130 has a molecular weight of approx 130 kDa, whereas sIL-6/sIL-6R complex is 70 kDa in size (Ward et al., 1994), thus explaining the necessity for molar excess in inhibition of the designer cytokine. Thus under the conditions used in the glucose uptake assay only partial inhibition, would occur. As it would appear that 50 $ng \cdot ml^{-1}$ sgp130Fc was unable to inhibit Hyper IL-6 potentiated insulin stimulated glucose uptake or Hyper IL-6 induced cell signalling, a cell signalling dose response was examined in both L6 myotubes and the CRL1439 cell line. It was determined that sgp130Fc was ineffective, even at higher concentrations, for inhibiting the action of Hyper IL-6 in L6 myotubes. However, contrary to the response of L6 myotubes, sgp130Fc was able to inhibit Hyper IL-6 action within CRL1439s with a 25 % reduction in activation of STAT-3 in the presence of 500 $ng \cdot mL^{-1}$ sgp130Fc. This may also be due to the relative expression of mIL-6Rα, gp130 and the action of trans-signalling in these two cell types.

In conclusion, using Hyper IL-6 to mimic the action of IL-6/sIL-6R, it was determined that L6 myotubes are indeed responsive to the IL-6 trans-signalling phenomena. Furthermore, Hyper IL-6 regulates important physiologically activities

such as glucose and fat oxidation, through the activation of the JAK/STAT pathway and its downstream targets. Moreover, these studies insinuate IL-6/sIL-6R has a cell type dependent effect causing diverse levels of bioactivity within L6 myotubes and CRL1439 hepatocytes. This is likely due to the variable expression of membrane bound IL-6Rα and gp130β within different cell types.

CHAPTER FIVE

Acute Hyper IL-6 administration has tissue specific effects on metabolic signalling pathways *in vivo*

5.1 Introduction

As discussed previously in this dissertation, it is well established within the literature that IL-6 is increased in a transient manner during exercise and is associated with increased fat oxidation and glucose utilisation within skeletal muscle, lipolysis within adipose tissue and increased hepatic glucose output (refer to Chapter 1). Contrary to the beneficial outcomes arising from transient increases in IL-6, prolonged elevated levels of this cytokine and the soluble receptor are often associated with chronic inflammatory conditions and described as a common hallmark of such diseases as arthritis, hepatocellular adenomas and colitis (Nowell et al., 2003; Maione et al., 1998, and Atreya et al., 2000)

In 1997, Fischer and co-workers designed and produced a bioactive fusion protein that consisted of 16 N terminal amino acid residues of human IL-6 linked to the sIL-6R via a flexible 13 residue polypeptide linker rich in glycine and serine. From this work and subsequent studies it was determined that this designer cytokine mimicked IL-6/sIL-6R trans-signalling and had greater bioactivity than that of IL-6, as it was required at a concentration of 100 to 1000 fold less than regular IL-6 to elicit expansion of human haematopoietic cells and induce mRNA for the acute phase protein haptoglobin (Fischer et al., 1997; Peters et al., 1998). The greater bioactivity of this designer cytokine was attributed to its distinct biochemical ability to target gp130β, which is of greater abundance and ubiquitously expressed, rather than the lowly abundant and atypically expressed membrane bound IL-6 Receptor (Rose-John et al., 2006).

Previous research has elucidated transient increases in IL-6 release from adipose tissue and skeletal muscle (Keller et al., 2003; Ostrowski et al., 1998; Hiscock et al., 2004) that occur during exercise. It is not known whether this is in concert with escalating concentrations of sIL-6R in the circulation and thus frequency of IL-6 trans-signalling events. It is known that IL-6 release leads to enhanced metabolic signalling and subsequently altered physiological function within liver, adipose tissue and skeletal muscle (Pedersen and Febbraio, 2008). Further support of the probable importance of IL-6 trans-signalling events in metabolic regulation was

provided by Chapter 4 of this dissertation whereby acute Hyper IL-6 treatment, used to mimic IL-6/sIL-6R complex signalling, regulated metabolic processes in cultured myotubes and hepatocytes *in vitro*. As a consequence, it was hypothesised that the IL-6 trans-signalling events seen *in vitro* would be biologically relevant within an *in vivo* context. Therefore, the primary aim of this study was to examine the effect of an acute administration (45 min) of 4 µg Hyper IL-6 to C57BL6 WT mice on metabolic pathway signalling within muscle, liver and adipose tissue. Furthermore, as Hyper IL-6 treatment has been implicated in progression of inflammation, inflammatory signalling pathways were examined in the fore mentioned tissues.

It is of importance to note that the sIL-6R activity may be inhibited by binding of the naturally occurring soluble form of gp130β (sgp130) (Jostock et al., 2002). Soluble gp130 is present in human sera where it binds to the soluble form of the IL-6 receptor in complex with IL-6 (Narazaki et al., 1993; Jostock et al., 2002). This prevents the IL-6/sIL-6R complex from binding to membrane bound gp130 and forming the high affinity ternary complex. An artificial form of this naturally occurring inhibitor was produced by linking the extra cellular portion of sgp130 to the heavy chain region (Fc fragment) of human IgG (Jostock et al., 2002). This recombinant protein had the ability to inhibit sIL-6R mediated signalling whilst membrane bound receptor signalling remained intact. Thus the secondary aim of the study was to explore the effects of sgp130Fc on Hyper IL-6 mediated signalling events in liver, white adipose tissue and skeletal muscle. As IL-6 trans-signalling is known to cause inflammation within liver and various other tissues, it was proposed that co-treatment with sgp130Fc would prevent inflammatory signalling mediated by Hyper IL-6, particularly within the liver, whilst maintaining the beneficial effects associated with membrane bound IL-6 receptor signalling, as previously demonstrated in Chapter 4, on metabolic signalling within skeletal muscle and WAT *in vivo*.

5.2 Methods

Animals - Male C57BL6 mice of 12 weeks of age weighing approximately 25 g were housed in the AMREP precinct animal facility in a 12:12 hour light:dark cycle. Animals were fed a standard mouse chow diet and water *ad libitum.* All animal experiments were performed as approved by the Animal Ethics Committee of the AMREP (ethics application approval number E/0679/2008/B). Animals were randomly assigned to be administered Saline (control), 4 µg Hyper IL-6 or 4 µg Hyper IL-6 + 40 µg sgp130Fc by intraperitoneal injection using a 25 gauge needle under basal or insulin stimulated conditions. Mice assigned to the fore mentioned groups that were to undergo 3 minute insulin stimulation before being culled, received 1.5 U/kg Insulin (Actrapid®, Novo Nordisk, Bagsraerd, Denmark) via intraperitoneal injection. Animals were culled by cervical dislocation, 45 minutes post administration of the assigned compounds, after which the tissues of interest were promptly excised, snap frozen in liquid nitrogen and stored at -80°C until required for further analyses.

Extraction of protein from harvested tissue - Approximately 50 mg of liver, *soleus*, EDL or white adipose tissue were cut from the excised tissues by scalpel blade whilst in a pre-chilled ceramic bowl. samples were then placed in a microfuge tube containing ice cold lysis buffer (consisting of 50 mM HEPES, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 0.5 % Triton X-100 (v/v), 10 % glycerol (v/v), 1 mM phenylmethylsulfonyl, 50 mM phosphatase inhibitor and 50 mM protease inhibitor). Samples were then ground using a plastic handheld homogeniser tip and the subsequent lysates were centrifuged at 13000 g for 60 min at 4°C. The supernatant was placed in a fresh microfuge tube and the debris containing pellet was discarded. Protein concentration was determined by BCA assay and 40 µg of protein was solubilised in Laemmelli's Buffer as described in Chapter 3.

SDS-PAGE analysis of protein expression - The expression of proteins of interest was examined using Western Blotting as previously described in Chapter 3 of this dissertation. Briefly, after proteins of each sample were separated by electrophoresis, proteins were transferred to nitrocellulose membranes by semi dry

transfer. Membranes were incubated overnight in either phospho STAT-3 tyr705, phospho Akt ser473, phospho ACC ser79, phospho AMPK thr172, PGC-1α, β-actin (Cell Signaling Technology, Beverly, MA, USA), total STAT-3 (Upstate Biotechnology Incorporated, Lake Placid, NY, USA) or SOCS-3 (Abcam, Cambridge, UK). The relative expression of the proteins of interest was determined by antibody binding and enhanced chemiluminescence as described in Chapter 3. β-actin was used as a loading control, to ensure equal amounts of protein had been analysed for each sample.

Statistics - Statistics were computed using the statistical analysis software package SPSS 12.0.01 for Windows to identify significant differences using either a one way or two way ANOVA. Multiple comparisons were made using the Tukey's post hoc test to locate significant differences between treatments. Significant differences for all experiments were accepted at $p < 0.05$. All data is expressed as mean fold change of protein expression compared to the expression of the protein of interest in tissues harvested from saline treated mice (Saline Control =1).

5.3 Results

Hyper IL-6 modulates STAT signalling in metabolically active tissues in vivo – As previous studies have determined that 4 µg Hyper IL-6 is effective in inducing an acute phase response for 48 hours in mice (Peters et al., 1998) the bioactivity of 4 µg Hyper IL-6 was examined in various tissues important in metabolic regulation. Phospho STAT-3 ^{tyr705} was used as a measure of JAK/STAT pathway activation and hence used as an indicator of efficiency of the cytokine to induce a biological response within muscle, adipose tissue and liver. This was also examined in the presence and absence of 40 µg sgp130Fc. It is important to note, administration of 40 µg sgp130Fc alone had little effect on any of the biomarkers examined in the tissues of interest and as a consequence has not been included in the current data set. To determine if Hyper IL-6 had the potential to influence insulin signalling, the level of phosphorylation of Akt at the ser473 residue was examined under basal and insulin stimulated conditions. In liver (Figure 5.1a), in the presence of Hyper IL-6, phospho STAT-3 ^{tyr705} was increased approximately 5.5 fold when compared with saline (Saline vs. Hyper IL-6, $p < 0.001$). In the presence of 40 µg sgp130Fc, the level of phosphorylation was 3.5 fold less (approx. 35 % of Hyper IL-6 induced phosphorylation) than that of Hyper IL-6 treatment (Hyper IL-6 vs. Hyper IL-6 + sgp130Fc, $p < 0.001$) only increasing phospho-STAT-3 ^{tyr705} by 2 fold when compared saline (Saline vs. Hyper IL-6 + sgp130Fc; $p = 0.042$).

Hyper IL-6, had a tendency to increase phospho Akt ^{ser473} (Figure 5.1b) and in the presence of sgp130Fc resulted in a 2 fold increase (Saline vs. Hyper IL-6 + sgp130Fc; $p < 0.01$) in liver. The difference in phospho Akt ^{ser473} between saline and saline under Insulin stimulated conditions was not quite significant (saline vs. Insulin; $p = 0.088$). Hyper IL-6 and Insulin co-administration increased phospho Akt ^{ser473} 5 fold (Saline vs. Hyper IL-6 + Insulin; $p < 0.001$, Hyper IL-6 vs. Hyper IL-6 + Insulin; $p = 0.003$). In liver harvested from Insulin stimulated mice administered Hyper IL-6 and sgp130Fc, phospho Akt ^{ser473} had a tendency to be reduced when compared to livers of insulin stimulated animals administered Hyper IL-6 (Saline vs. Hyper IL-6 + sgp130Fc + Insulin; $p = 0.005$).

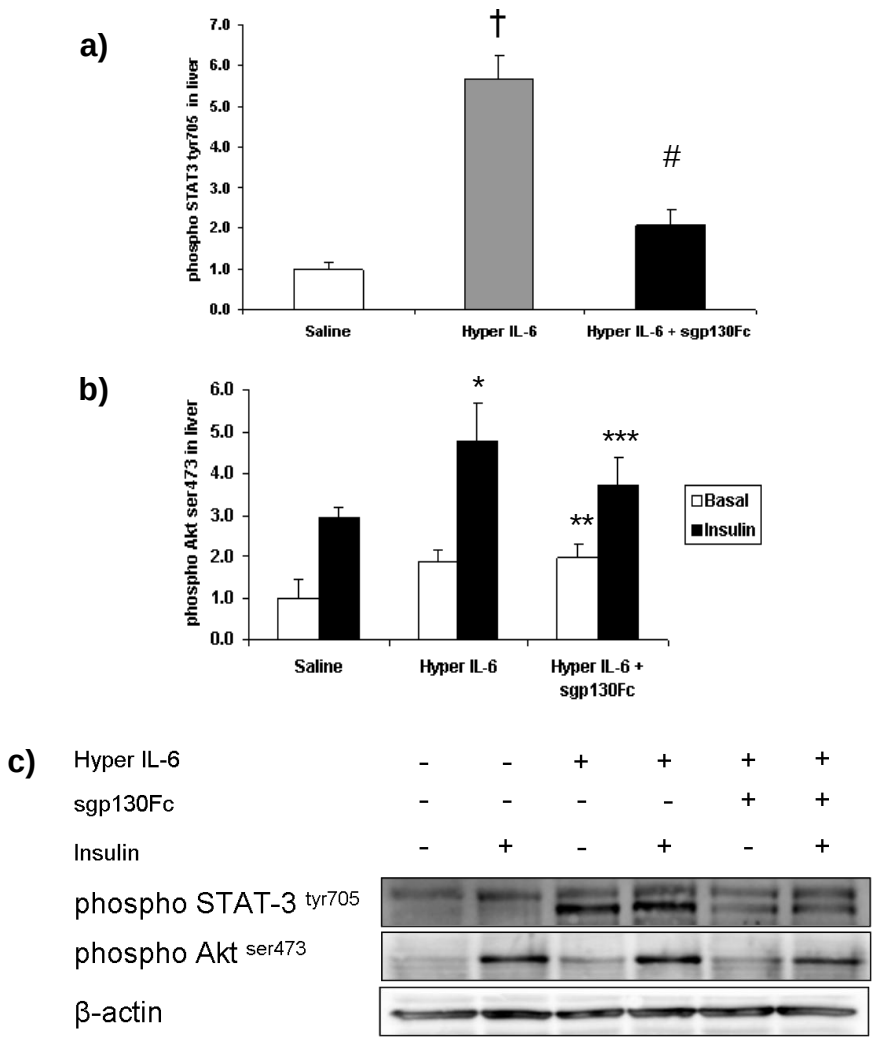


Figure 5.1 Hyper IL-6 increases a) STAT-3 activation and has a propensity to increase basal and potentiate insulin stimulated b) phospho Akt^{ser473} in liver.

c) Representative blots for proteins of interest. † denotes difference between Saline vs. Hyper IL-6; $p < 0.001$, # Saline vs. Hyper IL-6 + sgp130Fc; $p = 0.042$ and Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p < 0.001$. * denotes difference between Saline vs. Hyper IL-6 + Insulin; $p < 0.001$, Hyper IL-6 vs. Hyper IL-6 + Insulin; $p = 0.003$, ** Saline vs. Hyper IL-6 + sgp130Fc; $p < 0.01$, *** Saline vs. Hyper IL-6 + sgp130Fc + Insulin; $p = 0.005$. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM, n = 12 and n = 6 for phospho STAT-3^{tyr705} and phospho Akt^{ser473}, respectively.

In white adipose tissue (Figure 5.2a), phospho STAT-3 ^{tyr705} was significantly increased by ~11 fold (Saline vs. Hyper IL-6 p = 0.004). There was no significant difference in animals co-administered sgp130Fc (Saline vs. Hyper IL-6 + sgp130Fc; p = 0.442, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; p = 0.065). There was no significant difference between treatment groups in phospho Akt ^{ser473}, under basal or insulin stimulated conditions (Figure 5.2b). A slight tendency for this protein to be increased was noted under insulin stimulated conditions, however upon analysis this was not considered to be significant.

In skeletal muscle co-treatment with sgp130Fc diminished Hyper IL-6 induced phospho STAT-3 ^{tyr705} by approximately 40 %, regardless of the fibre type of the muscle. In *soleus* (Figure 5.3a), Hyper IL-6 elevated phospho STAT-3 ^{tyr705} ~ 4 fold (Saline vs. Hyper IL-6; p < 0.001) and this was reduced to ~ 2.5 fold in the presence of sgp130Fc (Saline vs. Hyper IL-6 + sgp130Fc; p = 0.001, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; p = 0.001). A 5 fold increase in phospho STAT-3 ^{tyr705} in the *EDL* (Figure 5.4a) of mice administered Hyper IL-6 was noted (Saline vs. Hyper IL-6; p < 0.001). Upon co-administration with sgp130Fc this was decreased by approximately 40 % to 3 fold that of phospho STAT-3 ^{tyr705} protein expression in *EDL* harvested from Saline treated animals (Saline vs. Hyper IL-6 + sgp130Fc; p = 0.023, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; p = 0.038). In *soleus* (Figure 5.3b), Hyper IL-6 + sgp130Fc increased phospho Akt ^{ser473} under basal conditions (saline vs. Hyper IL-6 + sgp130Fc; p = 0.006, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; p = 0.040). There was no difference between treatment groups under insulin stimulated conditions. There was no significant change in phospho Akt ^{ser473} under basal nor insulin stimulated conditions within *EDL* (Figure 5.4b).

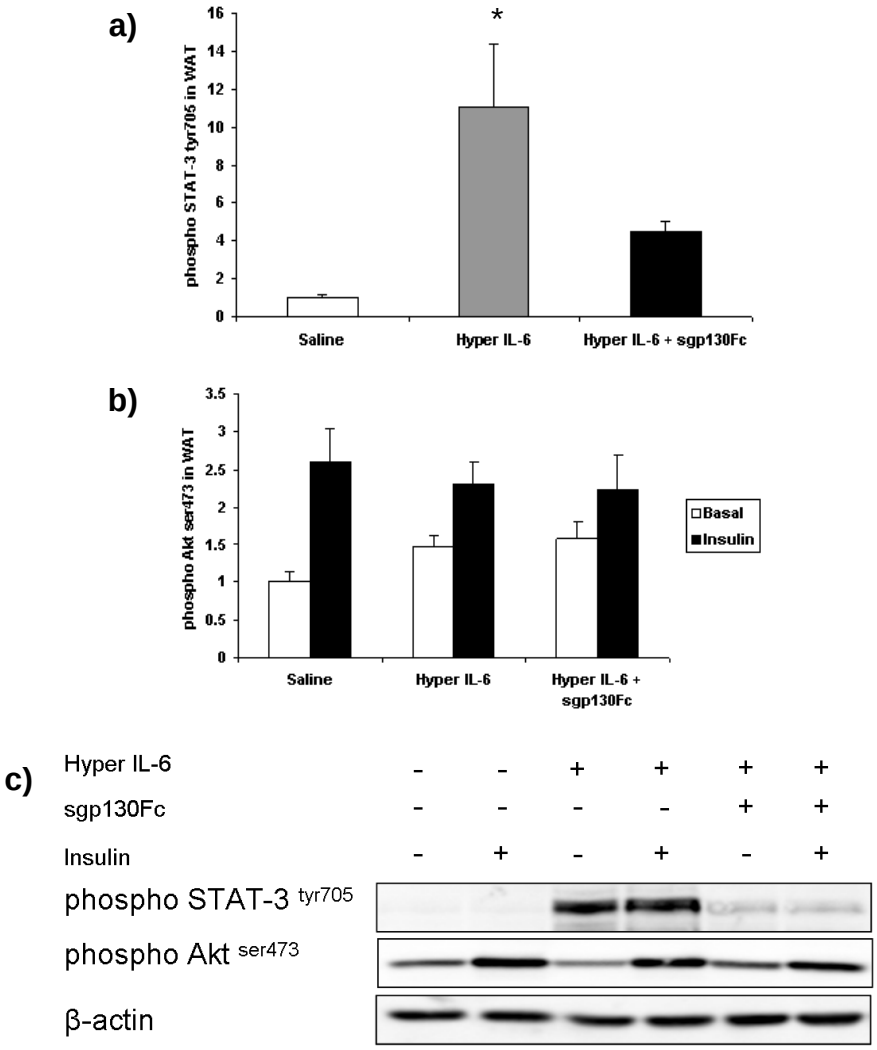


Figure 5.2 IL-6 trans-signalling events occur in WAT *in vivo* - Hyper IL-6 is bioactive in white adipose tissue, as determined by increases in a) phospho STAT-3^{tyr705} (n=12) with 4 µg Hyper IL-6 treatment, an affect that is not ameliorated in the presence of 40 µg sgp130Fc. However Hyper IL-6 does not significantly induce b) Akt activation under basal or insulin stimulated conditions (n=6) as determined by phospho Akt^{ser473} c) Representative blots for the fore mentioned proteins. * denotes Saline vs. Hyper IL-6; $p < 0.005$. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM.

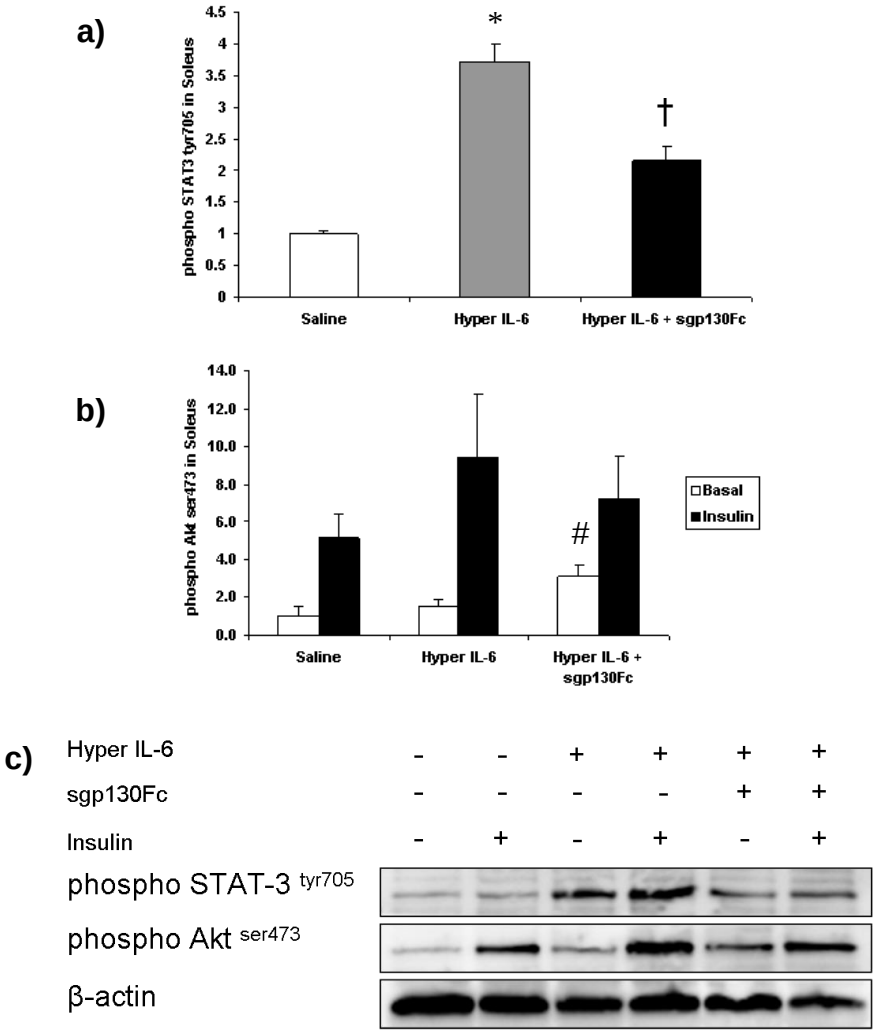


Figure 5.3 IL-6 trans-signalling occurs in *Soleus* muscle *in vivo* - a) Quantitation of phospho STAT-3^{tyr705}, which is increased in response to 4 µg Hyper IL-6 but displays a moderated increase with 40 µg sgp130Fc co-treatment (n=12). b) Quantitation of phospho Akt^{ser473} (n=6) in *soleus*. c) Representative western blots. * Saline vs. Hyper IL-6; $p < 0.001$, † Saline vs. Hyper IL-6 + sgp130Fc; $p = 0.001$, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p = 0.001$. # Saline vs. Hyper IL-6 + sgp130Fc; $p = 0.006$; Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p = 0.04$. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only. Data expressed as mean ± SEM, n = 12 and n = 6 for phospho STAT-3^{tyr705} and phospho Akt^{ser473}

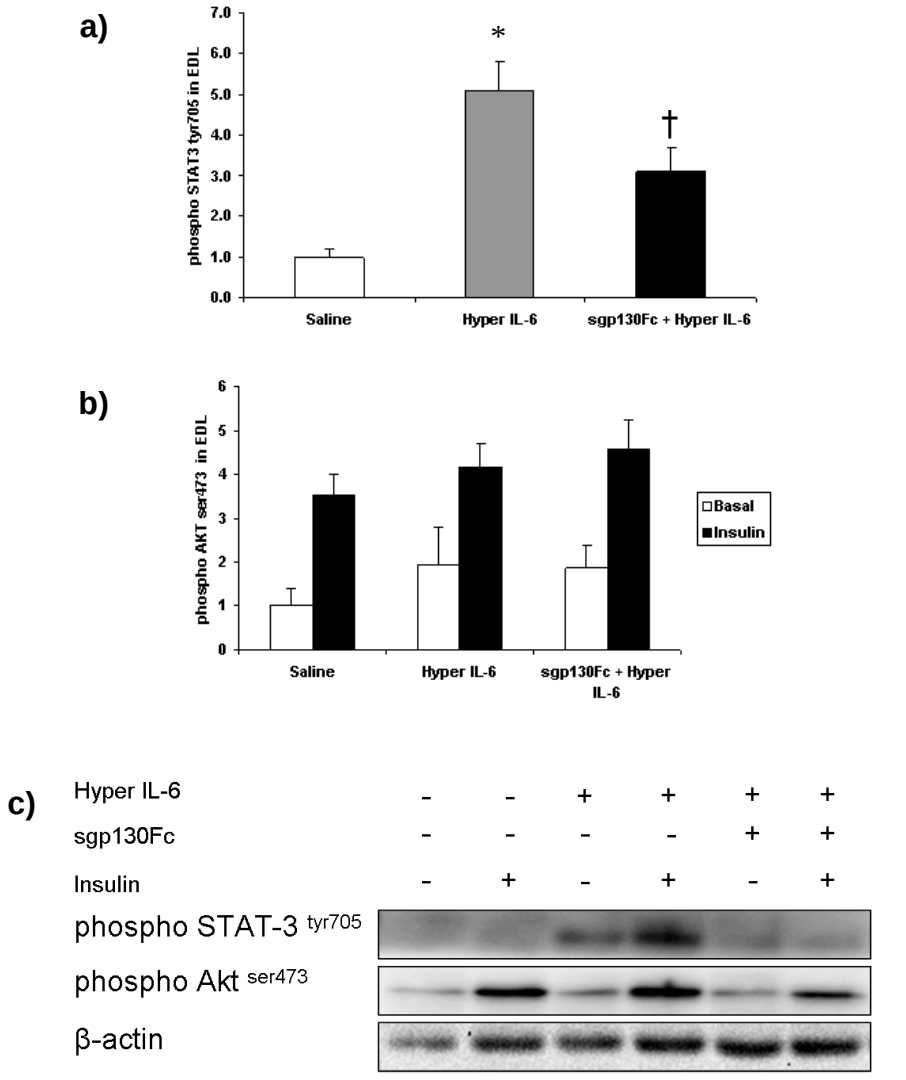


Figure 5.4 Hyper IL-6 trans-signalling is bioactive in *EDL in vivo* but displays moderated increases upon co- administration with sgp130Fc - Quantitation of a) phospho STAT-3^{tyr705} (n=12) and b) phospho Akt^{ser473} (n=6) protein expression in *EDL* and c) the corresponding representative blots for these proteins. * denotes Saline vs. Hyper IL-6; $p < 0.001$, † Saline vs. Hyper IL-6 + sgp130Fc; $p = 0.023$, Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p = 0.038$. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed

as relative expression of phosphorylated protein only. Data expressed as mean ± SEM.

Hyper IL-6 regulates proteins involved in signalling associated with fat oxidation in liver and white adipose tissue – Protein levels of important mediators of fat oxidation were measured in liver and WAT of mice that received Hyper IL-6 ± sgp130Fc. In liver, phospho ACC ^{ser79} (Figure 5.5b) was increased with Hyper IL-6 (Saline vs. Hyper IL-6; p = 0.012). Soluble gp130Fc was unable to impede Hyper IL-6 action on phospho ACC ^{ser79} (Saline vs. Hyper IL-6 + sgp130Fc; p = 0.007). There was a tendency for protein expression of phospho AMPK ^{thr172}, to be increased by 50 % in liver from mice administered Hyper IL-6, regardless of the presence of sgp130Fc (Figure 5.5a). Concomitantly, the downstream target of the fore mentioned proteins, the co-transcription factor PGC-1α (Figure 5.5c) had a tendency to be increased by 50 % in liver of animals administered Hyper IL-6 and was significantly increased by 2 fold in those animals that received Hyper IL-6 + sgp130Fc (Saline vs. Hyper IL-6; p = 0.222, Saline vs. Hyper IL-6 + sgp130Fc; p = 0.036) .

There was a trend for slight increases in phospho ACC ^{ser79} and phospho AMPK ^{thr172} in WAT, however upon statistical analysis, these were not considered to be significantly different to the levels found in WAT from control mice (Figure 5.6a and 5.6b). However, a significant increase of approximately 2 fold was noted in PGC-1α (Figure 5.7c) in WAT of C57BL6 mice administered Hyper IL-6 (Saline vs. Hyper IL-6; p = 0.013). However not statistically significant, it was of interest to note that upon co-administration with sgp130Fc, PGC-1α protein had a tendency to be decreased by approximately 25 %.

Hyper IL-6 affects mediators of fat oxidation in a skeletal muscle fibre type dependent manner – activation of proteins that are known to modulate fat oxidation were examined including phospho ACC ^{ser79}, phospho AMPK ^{thr172} and PGC-1α within *Soleus* and *EDL*. Hyper IL-6 had a tendency to increase phospho ACC ^{ser79} protein by approximately 50 % in *Soleus* (Figure 5.7b), however this was not

statistically significant. Phospho AMPK ^{thr172} (Figure 5.7a) was reduced by 25 % in *Soleus* of mice administered Hyper IL-6 (Saline vs. Hyper IL-6; p = 0.018). In *EDL*, Hyper IL-6 had a tendency to increase phospho AMPK ^{thr172} (Figure 5.8a). However, there was no significant difference in protein expression of either phospho ACC ^{ser79} (Figure 5.8b) or phospho AMPK ^{thr172}. PGC-1α was not detectable in *soleus* and *EDL*. This could reflect its rather transient nature of expression (Pilegaard, Saltin and Neufer, 2003).

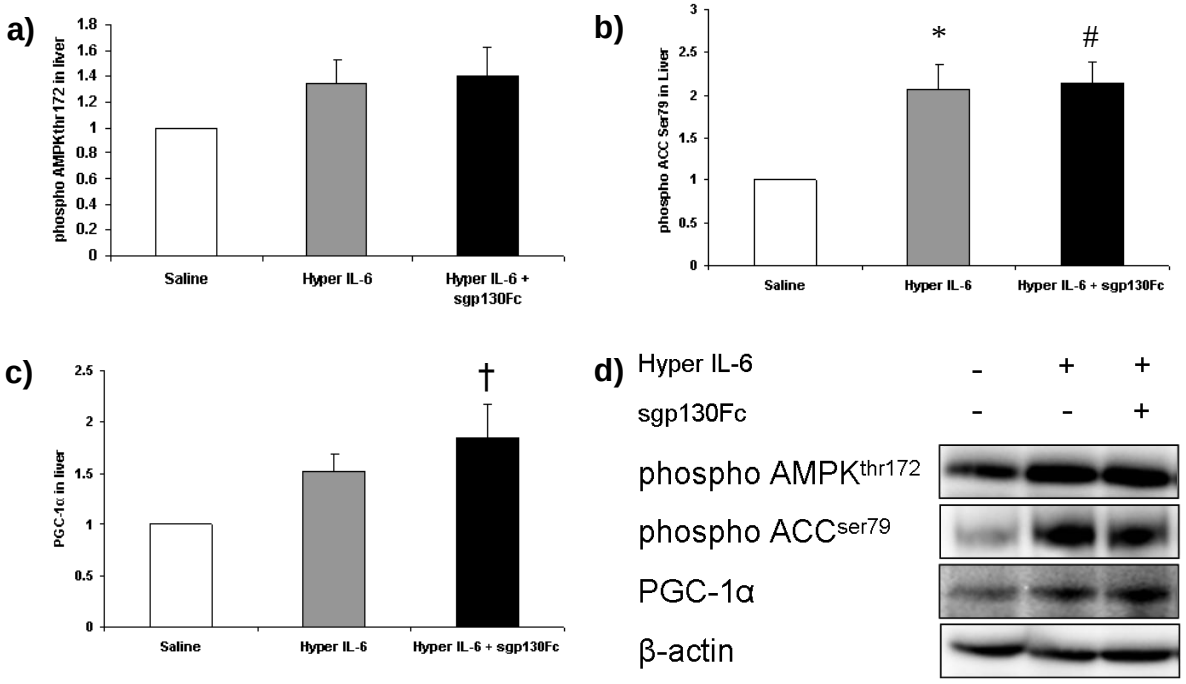


Figure 5.5 In liver, Hyper IL-6 trans-signalling has a tendency of regulating proteins that mediate fat oxidation regardless of the presence of sgp130Fc - Quantitation of a) phospho AMPK^{thr172}, b) phospho ACC^{ser79} and c) PGC-1α in liver of mice administered either 4 µg Hyper IL-6 in the presence or absence of 40 µg sgp130Fc. d) Representative western blots for phospho AMPK^{thr172}, phospho ACC^{ser79}, PGC-1α and β-actin in liver. * denotes Saline vs. Hyper IL-6; p = 0.012, # Saline vs. Hyper IL-6 + sgp130Fc; p = 0.007, † Saline vs. Hyper IL-6 + sgp130Fc; p = 0.036. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only for AMPK and ACC. All data expressed as mean ± SEM; n = 6.

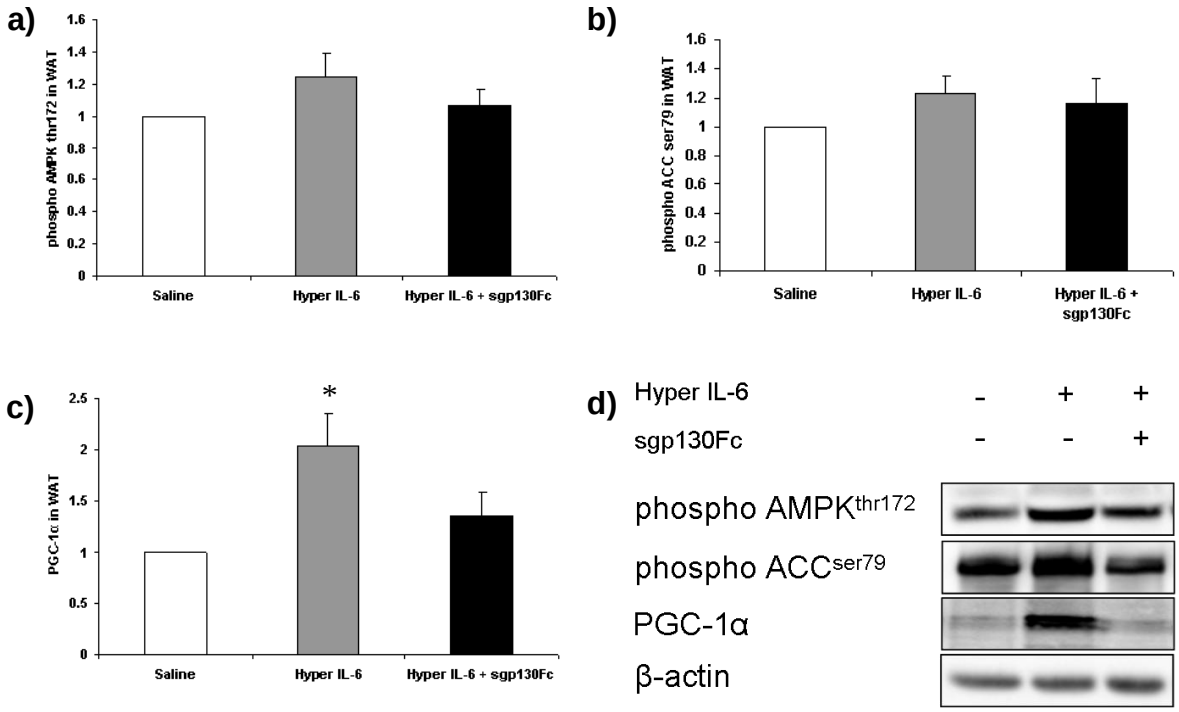


Figure 5.6 Hyper IL-6 has a tendency to increase signalling proteins associated with β- Oxidation and significantly increases protein expression of PGC-1α in WAT. Quantitation of protein levels of a) phospho AMPK^{thr172}, b) phospho ACC^{ser79} and c) PGC-1α in white adipose tissue collected from mice administered 4 µg Hyper IL-6 or 40 µg sgp130Fc. d) Representative blots for the proteins of interest. * denotes the difference between Saline vs. Hyper IL-6; p = 0.013. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only for AMPK and ACC. All data expressed as mean ± SEM; n = 6.

Hyper IL-6 induces proteins associated with inflammation in a tissue specific manner – As soluble IL-6 receptor signalling is often associated with states of inflammation and IL-6 is described as a pro inflammatory cytokine, proteins typically induced in acute phase inflammation were examined. There was no significant change in phospho IKKα ^{ser180}/IKKβ ^{ser181} or phospho JNK $^{thr183/tyr185}$ in liver (Figure 5.9a and 5.9b), *soleus* (Figure 5.11a) or *EDL* (Figure 5.11b) (data for JNK $^{thr183/tyr185}$ is not shown as it was not detectable within skeletal muscle). Of great interest is the significant increase noted in the 46 kDa subunit of phospho JNK $^{thr183/tyr185}$, an important mediator of inflammatory signalling, with Hyper IL-6 treatment (Saline vs. Hyper IL-6; $p < 0.001$) within WAT, an affect that was ameliorated with sgp130Fc co-administration (Figure 5.10b; Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p = 0.005$). Phospho IKKβ ^{ser181} had a tendency to be increased in WAT with Hyper IL-6 treatment (Figure 5.10a).

Suppressor of Cytokine Signalling-3 has a tendency to be induced with IL-6/sIL-6R complex mediated signalling – As another measure of bioactivity the protein expression of SOCS-3 was examined in the 4 tissue types. It was determined that mimicking IL-6/sIL-6R complex JAK/STAT signalling had a tendency to induce SOCS-3 protein expression in liver (Figure 5.12a), WAT (Figure 5.12b) and *EDL* (Figure 5.12d) regardless of the presence of sgp130Fc. Counter to that pattern, a trend of reduced expression of this protein upon administration of Hyper IL-6 was noted in *soleus* (Figure 5.12c). However upon statistical analyses any differences noted in protein expression of SOCS-3 were not considered significant.

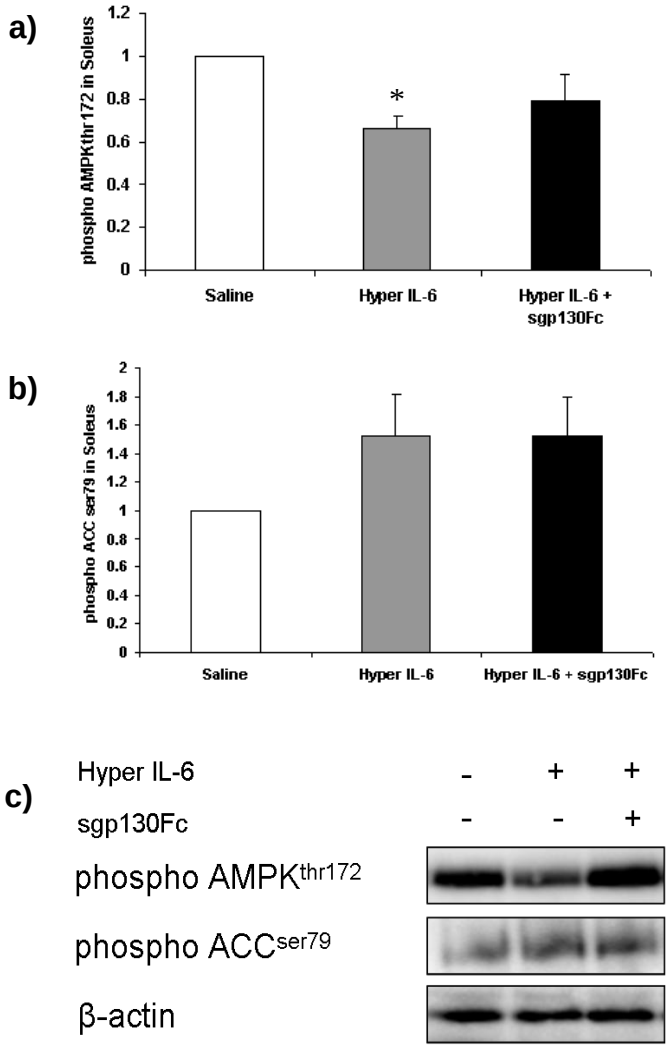


Figure 5.7 Hyper IL-6 has a tendency to increase phospho ACC^{ser79} but in contrast decreases phospho AMPK^{thr172} in *soleus* in vivo - Quantitation of protein expression of a) phospho AMPK^{thr172} and b) phospho ACC^{ser79} c) Representative blots. * denotes difference between Saline vs. Hyper IL-6; p = 0.018. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only for AMPK and ACC. All data expressed as mean ± SEM; n = 6.

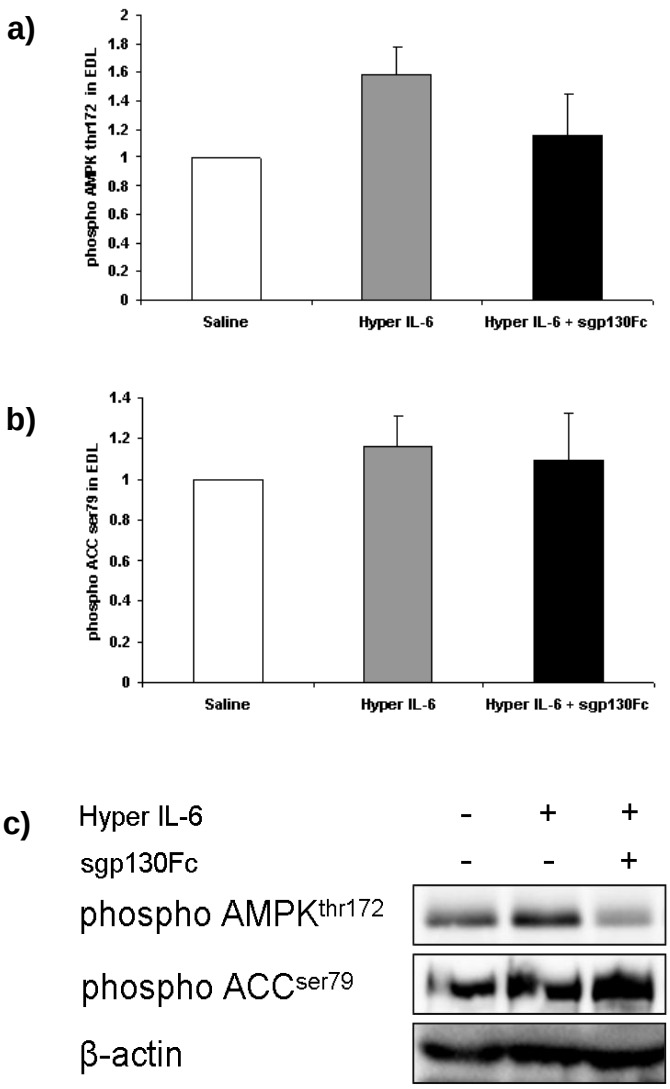


Figure 5.8 Hyper IL-6 mediates fat oxidation signalling in a fibre type specific manner as demonstrated by the contrast in signalling between *EDL* and that of *soleus* – Quantitation of protein expression of a) phospho AMPK^{thr172} and b) phospho ACC^{ser79} in *EDL*. As constitutive expression of corresponding total proteins and β-actin remained unchanged, data is expressed as relative expression of phosphorylated protein only for AMPK and ACC. All data expressed as mean ± SEM; n = 6.

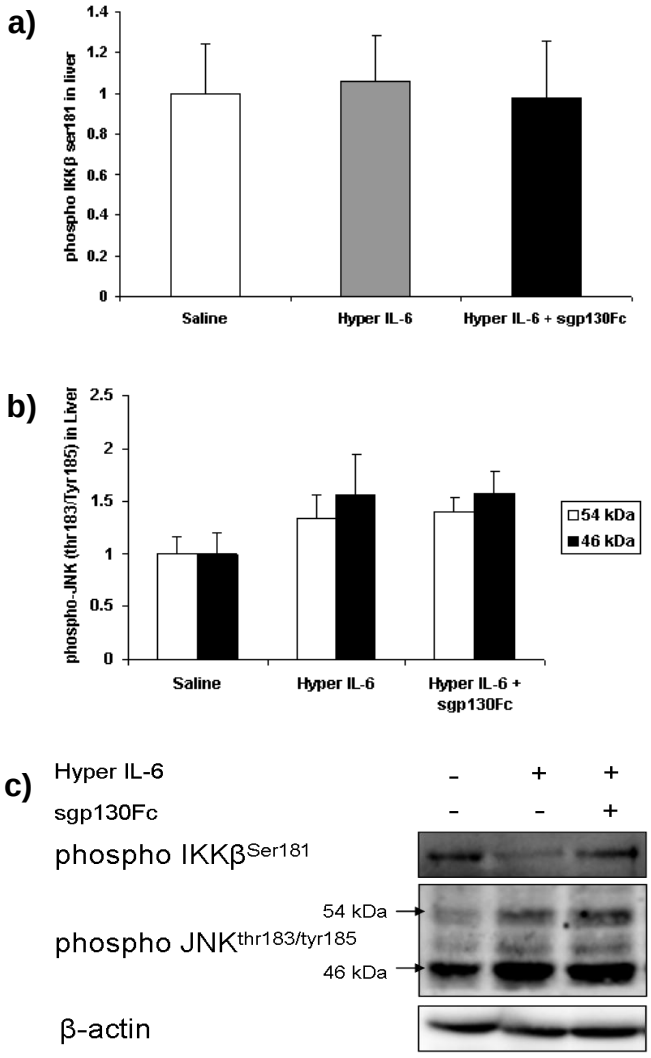


Figure 5.9 In liver, acute Hyper IL-6 treatment does not stimulate robust activation of a) IKK or b) JNK, proteins implicated in the induction of inflammation - a) phospho IKKβ^{ser181} remains unchanged in liver from mice that were administered 4 µg Hyper IL-6 ± 40 µg sgp130Fc when compared to saline. b) Four micrograms Hyper IL-6, regardless of the presence of 40 µg sgp130Fc had a tendency to increase phospho JNK$^{thr183/tyr185}$ at both the 46 kDa and 54 kDa subunits. c) Representative western blots. Data is expressed as mean ± SEM, n = 6.

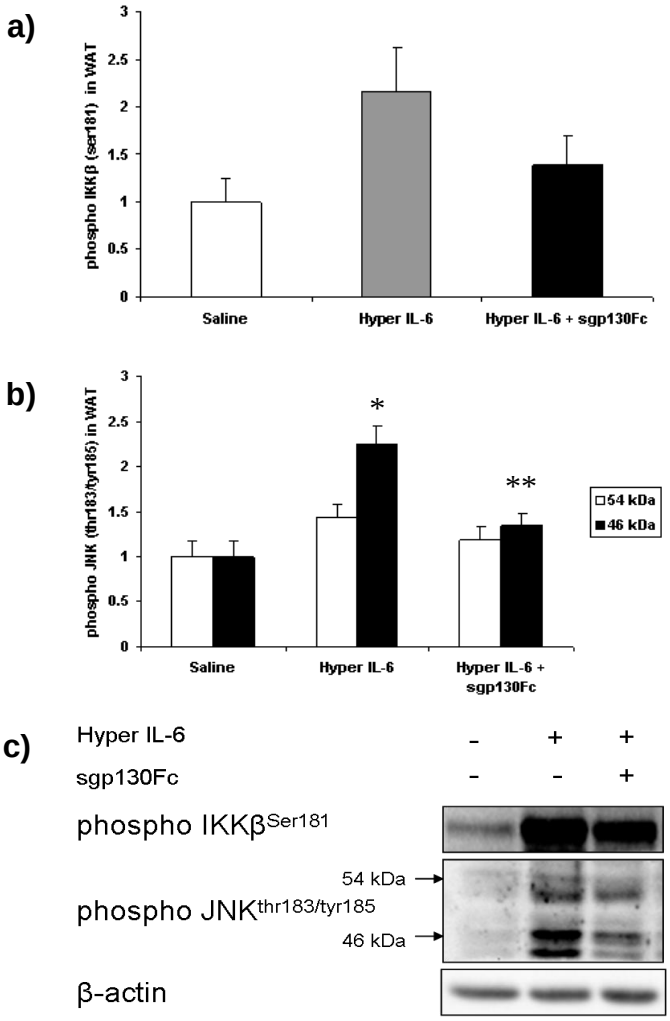


Figure 5.10 Markers of inflammatory signalling are induced in WAT of mice administered Hyper IL-6. a) Quantitation of phospho IKKβ^{ser181} demonstrating a trend of increased phosphorylation in WAT harvested from mice administered 4 µg Hyper IL-6, this had a tendency to be decreased by approximately 25 % when co-administered 40 µg sgp130Fc. b) phospho JNK $^{thr183/tyr185}$ was significantly elevated at the 46 kDa subunit, which was inhibited upon the co-administration of 40 µg sgp130Fc. There was no significant change in the 54 kDa subunit c) Representative blots for the fore mentioned proteins and the loading control β-actin. * denotes Saline vs. Hyper IL-6; $p < 0.001$, ** Hyper IL-6 vs. Hyper IL-6 + sgp130Fc; $p = 0.005$. Data expressed as mean ± SEM, n = 6.

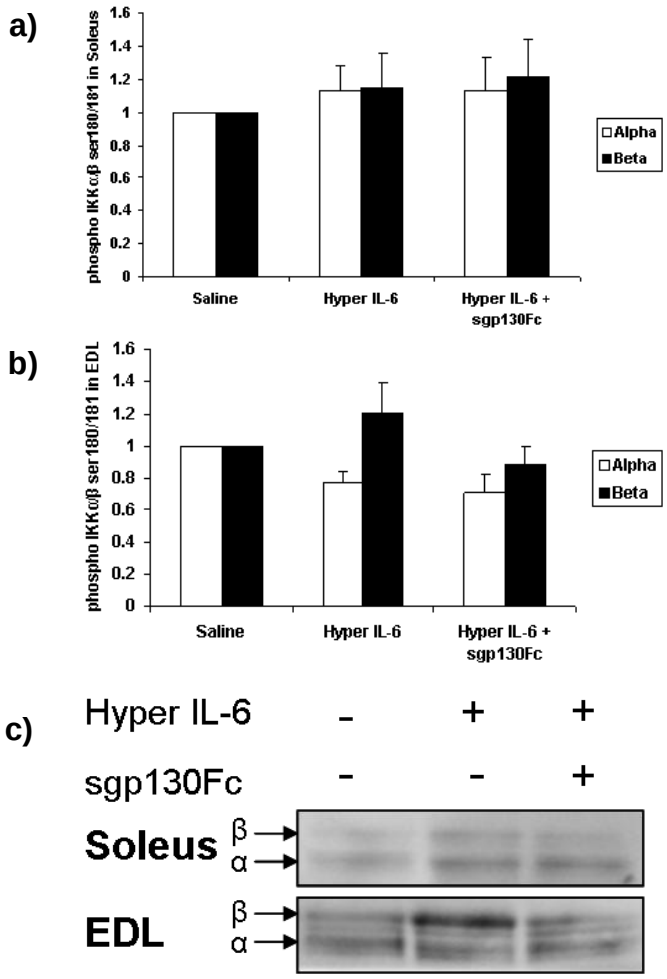


Figure 5.11 Inflammatory signalling was not induced in skeletal muscle upon administration of Hyper IL-6. Quantitation of phospho IKKα/$\beta^{ser180/181}$ in a) *soleus* and b) *EDL* and c) the corresponding representative blots for these muscle types. Data is expressed as mean ± SEM, n = 6.

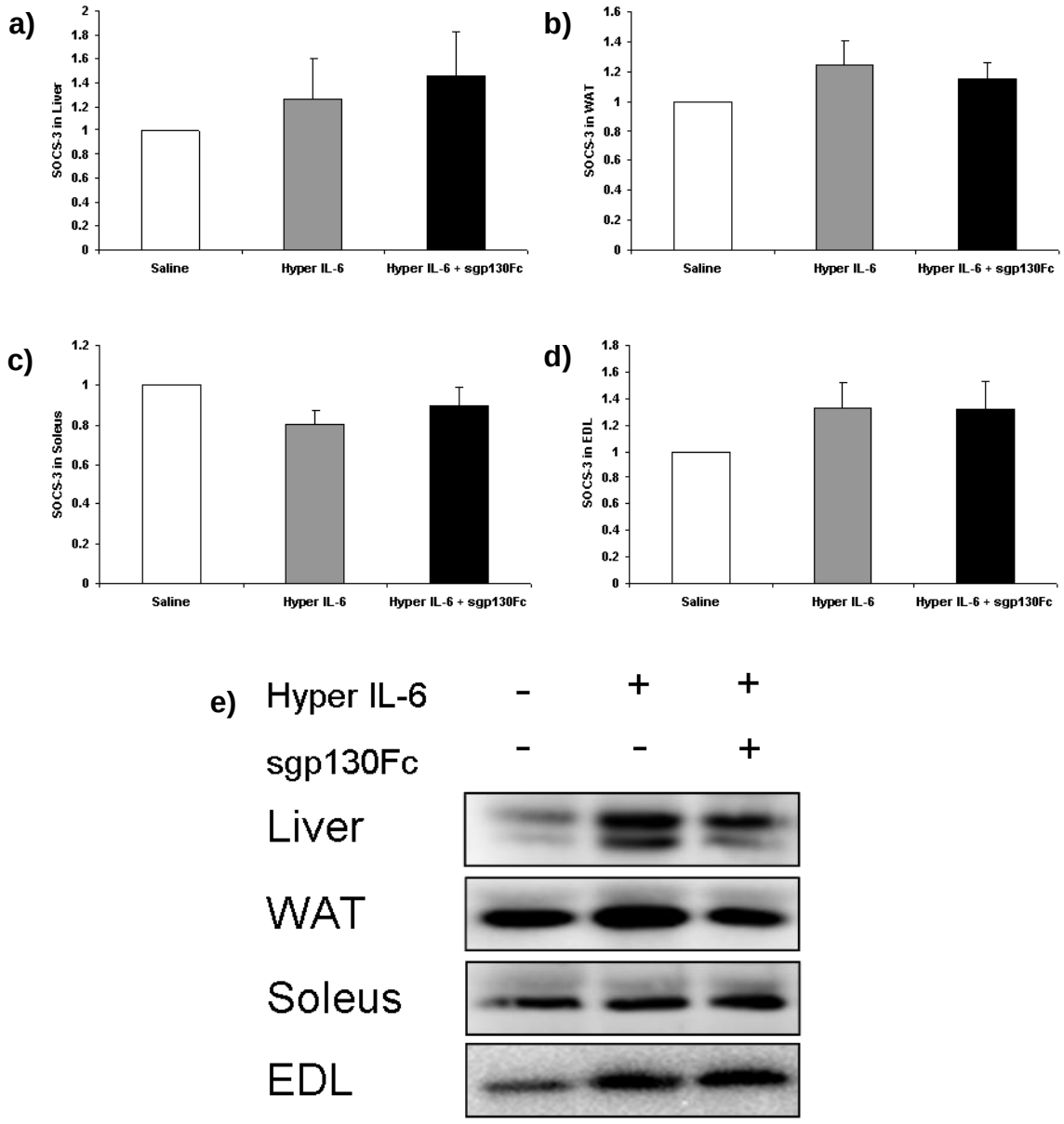


Figure 5.12 SOCS-3 protein expression has a tendency to be induced with acute IL-6/sIL-6R complex mediated signalling events. Expression of SOCS-3, an IL-6 inducible protein, had a tendency to be increased in a) liver, b) white adipose, c) *soleus* and d) *EDL* in mice administered 4 µg Hyper IL-6 ± 40 µg sgp130Fc. SOCS-3 protein remained relatively consistent in c) *Soleus* regardless of treatment group. Note: SOCS-3 is expressed as a doublet in liver. Data expressed as mean ± SEM, n = 6.

5.4 Discussion

The primary aim of this study was to examine the effect of an acute dose of Hyper IL-6 on trans-signalling events in various metabolically active tissues *in vivo* including liver, white adipose tissue, *soleus* and *extensor digitorum longus*. Using Hyper IL-6 to mimic IL-6/sIL-6R activation of gp130 and the subsequent downstream targeted JAK/STAT pathway, it was hypothesised that these trans-signalling events would not only be biologically relevant as previously illustrated in L6 myotubes and CRL1439 hepatocytes *in vitro,* but also in skeletal muscle and adipose tissue as well *in vivo*.

To date there have been no studies that have formally examined the role sIL-6R signalling has on metabolism. To mimic sIL-6R signalling, the designer cytokine Hyper IL-6, a fusion protein of rh-IL-6 and sIL-6R, was used to induce "trans-signalling", i.e. sIL-6R mediated signalling events. However, it is noteworthy that IL-6 trans-signalling is known to cause inflammation within liver and various other tissues as demonstrated within several studies (Miles et al., 1990; Maione et al., 1998; Atreya et al., 2000; Jostock et al., 2001; Nowell et al., 2003; Rabe et al., 2008; Sander et al., 2008). Thus, it was proposed that co-treatment with the inhibitor of Hyper IL-6, sgp130Fc, would prevent inflammatory signalling, particularly within the liver, thereby preventing inflammation within other tissues, whilst maintaining the beneficial effects of this designer cytokine on metabolic cell signalling within skeletal muscle *in vivo*.

To examine the effect that acute IL-6 trans-signalling events have on proteins commonly implicated in exercise induced regulation of metabolism, C57BL6 mice were administered 4 μg of Hyper IL-6 ± 40 μg sgp130Fc via intraperitoneal injection. Forty five minutes post injection, the animals were culled and tissues were promptly excised. The dose and time used in these experiments have previously been used to induce functional changes in downstream targets of JAK/STAT-3 signalling and were thus considered biologically active (Peters et al., 1998).

As the functional role of IL-6/sIL-6R complex signalling in skeletal muscle and WAT remains elusive, the activation of STAT-3 was examined to ensure this phenomenon was biologically relevant within these tissues. Mice were also administered these cytokines in the presence of insulin, however this hormone had little effect on phospho STAT-3^{tyr705}. Subsequently, mice assigned to receive Hyper IL-6 ± sgp130Fc and insulin stimulation prior to harvest were included in the corresponding cytokine treatment group under basal conditions thereby increasing the sample size (to n=12). Clearly, phosphorylation of STAT-3 at the tyr705 residue was increased upon treatment with Hyper IL-6 within liver, an effect that was conserved across skeletal muscle and WAT. The inhibition of Hyper IL-6 induced phospho STAT-3^{tyr705}, using sgp130Fc, occurred in a tissue type specific manner as demonstrated by a reduction of ~60 % in liver compared to that of ~ 40 % in *Soleus* and *EDL*. Notwithstanding, no significant change of this protein was evident in WAT upon co-administration of Hyper IL-6 and sgp130Fc. The varying responses of individual tissues to sgp130Fc may possibly occur as a result of the relative expression of gp130β and the naturally occurring sIL-6R and soluble gp130 within the circulation. Varying signalling responses resulting from limitations of the experimental design can not be excluded. Previous research in NMRI mice has demonstrated that the concentration of Hyper IL-6 within sera peaks at 1 – 3 hours post intraperitoneal administration of the cytokine (Peters et al., 1998) but in culture the Hyper IL-6 activity, as indicated by the phosphorylation of STAT-3, was maximal at 30 min post injection (refer to Chapter 4). The time point of 45 minutes was selected because it fell between the two experimentally determined maxima. As animals were culled at only one time point post administration of the cytokines, it is not inconceivable that signalling events which took place either prior to or post 45 minutes were consequently missed. Moreover, given the cytokines were administered by IP injection, the possibility that the Hyper IL-6 was administered directly into the epididymal fat mass can not be ignored. This may have resulted in saturation of WAT with Hyper IL-6 and as a consequence sgp130Fc unable to prevent trans-signalling events via competitive inhibition, thus explaining the limited inhibition of STAT-3 activation in the WAT of mice administered Hyper IL-6 + sgp130Fc. Further experiments covering a broader span of treatment periods

and/or utilising alternative routes of administration would readily verify whether the experimental constraints impacted the results.

It is well established in the literature that many physiological changes occur upon exercise within many tissues. These include; increased glucose oxidation and endogenous glucose production, decreased insulin release by the pancreas, increased lipolysis and fat oxidation. During exercise, many of the fore mentioned physiological processes are concurrent with an increase in IL-6 release from skeletal muscle and for this reason IL-6 is often described as a myokine (Ostrowski et al., 1998; Hisckock et al., 2004). Research from Febbraio et al. (2004) provided insight into the role of IL-6 within muscle metabolism, demonstrating recombinant human IL-6 infusion into humans increased the rate of contraction induced endogenous production and disposal rate of glucose, without changes in hormones such as glucagon, insulin and epinephrine. This was supported by later research that demonstrated rhIL-6 infusion into healthy men increased Insulin stimulated glucose disposal during a hyperinsulinaemic-euglycemic clamp (Carey et al., 2006). Further evidence of the role IL-6 plays in metabolic regulation is provided in an elegant study by van Hall and colleagues (2003) who noted IL-6 increases fatty acid oxidation and lipolysis. Healthy young men were infused with either a low dose (mean concentration in plasma ~143 pg/mL) or a high dose (mean concentration in plasma ~319 pg/mL) of rhIL-6 for 90 min, at which point an increase in the rate of appearance of endogenous free fatty acid and elevated fatty acid concentration was evident compared to those who received saline. It is important to note, skeletal muscle utilises free fatty acids generated upon the hydrolysis of ester linkages of triglycerides from triglyceride storages to generate ATP through β-oxidation. These studies are suggestive of a positive role for IL-6 in metabolic regulation during exercise and are thus contrary to the popular notion within the literature that this cytokine is a mediator of inflammation. Hence forth the effect of Hyper IL-6 ± sgp130Fc was examined on common signalling pathways that have previously been implicated in these processes and IL-6 action.

Akt activation is an important mediator of many processes including cell cycle progression and survival, glucose metabolism, protein synthesis and Nitric Oxide production (for review refer to Ždychová and Komers, 2005). The trend of basal increases in phospho Akt^{ser473} in most tissues of mice that received Hyper IL-6 was therefore of great interest. The presence of sgp130Fc would appear to have no effect on basal increases in phospho Akt^{ser473} and even potentiated it within *soleus*. No significant effect of these designer cytokines were noted in the insulin stimulated state. This was not anticipated considering the results obtained *in vitro* (Chapter 4) whereby Insulin stimulated glucose uptake was potentiated in L6 myotubes treated with Hyper IL-6 + sgp130Fc. This increase in basal activity of Akt may have many implications on many cellular processes including protein synthesis and proliferation and would certainly be a reflection upon the multi-faceted nature of IL-6. Whether IL-6/sIL-6R complex truly regulates insulin signalling in an acute fashion remains undetermined. To elucidate the role of the IL-6/sIL-6R complex in Insulin signalling regulation it would be necessary to examine upstream mediators of this metabolic pathway such as PI3-Kinase and IRS-1 and downstream targets such as AS160 and GLUT4 translocation.

AMPK is thought to be a metabolic regulator through modulation of its activity through changes in cellular [AMP]/[ATP] ratio. Activation of AMPK leads to the suppression of anaerobic pathways that require the use of ATP and subsequently causes a switch to ATP generating aerobic pathways such as β-oxidation, thereby providing metabolic substrates for cells undergoing metabolic stress (for review refer to Hardie and Sakamoto, 2006). Inhibition of ACC activity occurs by AMPK phosphorylation of ACC at the serine 79 residue. This in turn causes inhibition of malonyl CoA synthesis or increased degradation of this protein. This relieves the allosteric inhibition of the mitochondrial fatty acid transporter, CPT1. CPT-1 activity is increased causing increased uptake of long chain fatty acids and thus fat oxidation (for reviews refer to Ruderman, Saha and Kraegen, 2003 and Steinberg and Jørgensen, 2007). It is well established that exercise increases AMPK activity. In rodents, it has previously been shown that a positive correlation exists between contraction intensity induced activation of AMPK and fatty acid uptake and

oxidation (Raney and Turcotte, 2006). Furthermore it has been demonstrated that during exercise, AMPK activity is diminished in skeletal muscle of IL-6 KO mice when compared to that of WT (Kelly et al., 2004) and increases in IL-6 within the circulation correlate positively with AMPK activity within human skeletal muscle (MacDonald et al., 2003). This is in accordance with Carey and co-workers (2006) who demonstrated that IL-6 mediated increases in glucose uptake and fatty acid oxidation occurred in an AMPK dependent manner, further insinuating this cytokine's importance in fat metabolism. In agreement with these previous studies, four micrograms of Hyper IL-6 injected into mice resulted in a significant increase in phospho ACC in liver, an effect that was not influenced by co-treatment with 40 µg sgp130Fc. In WAT and *EDL* Hyper IL-6 had a propensity to increase ACC and AMPK phosphorylation, however in *soleus* phospho AMPK^{thr172} was significantly reduced upon Hyper IL-6 treatment and co-treatment with sgp130Fc, ablated this effect. A similar response was seen in *soleus* of rats infused with IL-6 in a chronic or intermittent manner (refer to Chapter 3), whereby protein expression of PGC-1α, a downstream target of AMPK, was reduced.

It is noteworthy that AMPK is a multifaceted kinase that is not only a master regulator of metabolism but also involved in cell cycle progression through the regulation of protein synthesis, de novo fatty acid synthesis, G1 cell cycle arrest and autophagy through the TSC1/2-mTOR pathway and the p53-p21 axis (for review refer to Motoshima et al., 2006). During periods of energy deprivation, AMPK suppresses cell growth and biosynthetic processes through its negative modulation of the rapamycin sensitive mTOR (mTORC1) pathway, a regulator of protein synthesis (Kimura et al., 2003). Although the present study did not examine mTOR pathway activity, it may be of interest to do so in future studies given the potential effect Hyper IL-6 has on AMPK and cell growth and differentiation, particularly within the liver. In the current study, Hyper IL-6 also stimulated basal increases in phospho-Akt^{ser473} *in vivo*. Upon stimulation with growth factors, activation of Akt results in the phosphorylation of TSC2 and subsequent loss of activity of the TSC1/2 complex, thereby preventing Rheb binding to the complex and subsequent inhibition of the mTOR complex. Upon activation of mTOR,

increased protein synthesis occurs through the recruitment of its downstream effectors $p70^{S6K1}$ and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) to its binding partner raptor (Fingar et al., 2002).

Many studies have also implicated AMPK activity in the inhibition of cell cycle progression through the regulation of the tumour suppressor, p53 during periods of glucose depletion (Jones et al., 2005; Okoshi et al., 2008). The fore mentioned studies have implicated AMPK in the phosphorylation and accumulation of p53, activation of the pro-apoptotic protein Bax, preventing progression of cell cycle from G1 to S phase thus impeding cellular proliferation. This is of great importance as it promotes cellular survival and maintains cellular viability during periods of glucose deprivation. Given the ability of Hyper IL-6 to regulate proliferation of progenitor cells and other studies insinuating a role for IL-6 in cellular proliferation and differentiation, it is certainly plausible that AMPK activation in response to Hyper IL-6 as seen in this study, particularly within the liver, would result in inhibition of protein synthesis through the mTOR pathway and G1 cell cycle arrest through accumulation and activation of p53. Any aberrant AMPK or Akt pathway activity in response to prolonged Hyper IL-6 administration may lead to either cellular senescence or excessive proliferation. Future studies aimed at examining protein synthesis and cell cycle arrest in response to Hyper IL-6 would provide further insight into the purpose of this important regulatory kinase.

As previously mentioned in Chapter 3 of this dissertation, PGC-1α is a co-transcription factor whose physiological function occurs in a tissue type dependent manner and that expression is abnormal in many diseased states. PGC-1α is an important player in metabolism through the regulation of OXPHOS genes and thus mitochondrial biogenesis and uncoupling, cellular respiration and fat oxidation. Previous research from Watt et al. (2006) and Al-Kahlili et al. (2006) have implicated IL-6 family cytokines in the regulation of this protein, whereby PGC-1α expression was increased compared to saline control animals in C57BL6 mice on a HFD treated with the IL-6 family member CNTF; this in turn was concomitant with a decrease in fat mass. In the later study PGC-1α mRNA in human skeletal muscle

was increased over an 8 day IL-6 treatment period, an effect that was abrogated when transduced with AMPK α1/2 siRNA. It is well established that PGC-1α mRNA is transiently increased upon endurance exercise training (Goto et al., 2000; Pilegaard, Saltin and Neufer, 2003; Mathai et al., 2008) and has previously been proposed to occur via an AMPK signalling dependent manner in rat epitrochlearis muscle (Terada et al., 2002). Furthermore, endurance training in male Sprague Dawley rats increased LKB1 (upstream of AMPK) activity with concomitant increases in PGC-1α and citrate synthase, an indicator of mitochondrial activity (Taylor et al., 2005). In support of the fore mentioned notion, Lee and colleagues (2006) demonstrated that AMPK activation increases fatty acid oxidation in C2C12 myotubes via a PGC-1α dependent mechanism. Most recently, using point mutations and mass spectrometry, it was established that within skeletal muscle, AMPK directly interacts with PGC-1α via phosphorylating thr177 and ser538 residues (Jäger et al., 2007). As there is an abundance of literature supporting a role for exercise and IL-6 family cytokines in the regulation of PGC-1α mRNA and protein, most likely via an AMPK dependent mechanism, this was explored within tissues from animals that received an acute dose of Hyper IL-6. In the current study design, by administering mice with an acute dose of Hyper IL-6, the role of IL-6/sIL-6R/gp130 mediated signalling in the regulation of this protein was studied independently of other exercise related mechanisms that may influence expression of this protein in various tissues.

There was no difference between groups of PGC-1α abundance in either skeletal muscle type examined (*soleus* and *EDL*, data not shown). The change in PGC-1α protein may be dependent on the particular fibre phenotype of the muscle under investigation. This would certainly be in accordance with previous studies insinuating that PGC-1α expression occurs in a fibre type phenotypic manner within skeletal muscle. Ikeda and colleagues (2006) proposed that increases in PGC-1α occurred in a muscle fibre type specific manner as mice undergoing wheel exercise training over an 8 week period had increases in PGC-1α protein in plantaris muscle but protein expression remained unchanged in *Tibialis Anterior* or *soleus*. It is also possible that the lack of change seen in PGC-1α protein

abundance may be due to the inability of Hyper IL-6 to regulate transcription and translation of PGC-1α within *EDL* and *soleus*. It may also be possible that the acute time point examined in this cohort may be inappropriate for detection of changes of this protein within skeletal muscle. This is feasible as previous studies show an increase in mRNA at 2 hours post exercise and is sustained for at least 6 hours after cessation of exercise (Pilegaard, Saltin and Neufer, 2003). Future experiments examining a detailed time response experiment of PGC-1α mRNA and protein in skeletal muscle in response to Hyper IL-6 treatment would elucidate whether IL-6/sIL-6R complex signalling is a regulator of this important mediator of mitochondrial metabolism.

Metabolic regulation within liver is a complex system of compensatory mechanisms during periods of fasting and feeding and this concept is pertinent to whole body physiology ensuring energy homeostasis is maintained. This is of central importance as aberrant signalling within metabolic tissues such as the liver forms the basis of metabolic disease. In liver, during periods of fasting, PGC-1α is activated through its interaction with FoxO1 and increased pyruvate stimulated deactylation by SIRT-1 (Puigserver et al., 2003, Rodgers et al., 2005). This enables the induction of transcription factors including Hepatocyte Nuclear Factor 4α (HNF4α) and activation of gene transcription for important mediators of gluconeogenesis, including glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase 1 (PEPCK) (Yoon et al., 2001). Moreover, PGC-1α activation also regulates lipoprotein metabolism via activation of HNF4α (Rhee et al., 2006). Further evidence of PGC-1α involvement in hepatic glucose output is provided by Koo and colleagues (2005) who demonstrated that glucagon, a hormone released during periods of fasting, increased CREB in an AMPK dependent manner and led to greater transcription of PGC-1α. It has also recently been discovered that PGC-1α possesses an Akt phosphorylation site at ser570 and when activated causes an attenuation of gluconeogenesis and fatty acid oxidation (Li et al., 2007). This is consistent with a previous study that showed upon insulin stimulation, increases in phospho Akt ser473 occurred in concert with a decrease in PGC-1α, thus in turn leading to suppression of transcriptional activity

of the co-transcription factor (Koo et al., 2005). These studies suggest insulin is a key player in hepatic glucose production via inhibition of PGC-1α and transcriptional control of its downstream gluconeogenic genes.

During exercise, hepatic glucose production is increased, due to a combination of increased circulating IL-6, lactate and pyruvate and raised AMP/ATP ratio. Of great interest is the aberrant regulation of gluconeogenesis within diabetic patients resulting in hyperglycaemia. During the fed state, increases in insulin results in the activation of Akt, the sequestration of FoxO1 from the nucleus and subsequent inhibition of PGC-1α activity, thus preventing gluconeogenesis. However, in insulin resistant states such as that seen in diabetes there is a loss of insulin dependent suppression of gluconeogenesis and increased triglyceride synthesis and accumulation resulting in steatosis within the liver (Matsumoto et al., 2006). Contrary to the above study, in the present research, livers from mice co-administered Hyper IL-6 and sgp130Fc had elevated basal levels of phospho Akt^{ser473} with a simultaneous increase in PGC-1α protein. It may be of interest to determine whether in healthy mice, the elevation of Akt activity in response to Hyper IL-6 occurs as a compensatory mechanism in response to increased PGC-1α induced by AMPK and hence is aimed at preventing hyperglycaemia.

Acute treatment with Hyper IL-6, regardless of the presence of sgp130Fc, had a tendency to increase PGC-1α protein within liver and WAT. It is not unreasonable to suggest that in an acute manner such as that seen during exercise, the increase in PGC-1α may occur as a consequence of transient increases of IL-6 within the circulation, leading to an increase in hepatic glucose output, thereby providing an alternate source of energy for working muscles. Whether the increase in PGC-1α protein is mediated via the membrane bound IL-6 Receptor or the sIL-6R requires further investigation. In this current study, it was evident Hyper IL-6, used to mimic IL-6/sIL-6R signalling, induced increases in PGC-1α protein in the liver which were unable to be attenuated in the presence of its inhibitor sgp130Fc. This implies that the induction of PGC-1α in liver occurs via membrane bound IL-6 receptor signalling. Moreover, IL-6/gp130/JAK/STAT-3 induced PGC-1α in liver may have

broader implications in a chronic scenario whereby persistently elevated IL-6 within the plasma leads to hyperglycaemia through increased PGC-1α abundance and enhanced gluconeogenic gene transcription.

It is known that insulin sensitivity *in vivo* correlates with PGC-1α mRNA and protein expression in adipose tissue and expression of this protein was reduced in adipose tissue from insulin resistant individuals (Hammarstedt et al., 2003). Moreover, the exact consequence of IL-6 signalling within adipose metabolism remains elusive, as it has previously been associated with both insulin sensitivity and insulin resistance in adipose tissue. The results obtained in Figure 5.6 supports IL-6/sIL-6R complex as a promoter of mitochondrial metabolism as treatment with Hyper IL-6 significantly increased PGC-1α protein expression. Contrary to that of liver, it would appear that this affect was entirely mediated by IL-6/sIL-6R/gp130 signalling as in the presence of sgp130Fc, Hyper IL-6 treatment was unable to increase PGC-1α protein. These studies insinuate induction of this protein is mediated via distinct biochemical mechanisms (ie. membrane bound receptor v soluble receptor signalling) in a tissue type dependent manner.

It was noteworthy that Hyper IL-6 induced significant increases in the 46kDa subunit of phospho-JNK1 and had a tendency to increase phospho IKKβ within WAT, these affects were ameliorated upon co-treatment with sgp130Fc. Contrary to this, Hyper IL-6 administered murine liver had a tendency for increased phospho JNK at both sub units regardless of the presence of sgp130Fc. No change in IKK and JNK were noted in either skeletal muscle type, this was not surprising as muscle has to undergo severe assault before inflammation is induced (unpublished observations, Cellular and Molecular Metabolism Laboratory). A recent study by Sabio and colleagues (2008) provided evidence of metabolic cross-talk between WAT and Liver in an IL-6 dependent manner. Ablation of JNK1 in WAT caused suppression of HFD induced insulin resistance and restored insulin sensitivity in the liver of these mice. They also noted that secretion of IL-6 by WAT was increased upon activation of JNK1 in response to free fatty acids and IL-6, in turn causing a concomitant increase in SOCS-3 expression within the liver. It would be

of interest to examine whether IL-6 secretion from adipose tissue occurs in response to Hyper IL-6 treatment in mice, thereby increasing IL-6 within the circulation, possibly leading to increased membrane bound IL-6 signalling within the liver. This would certainly account for the increased abundance of phospho ACC^{ser79} and PGC-1α noted in liver of Hyper IL-6 treated mice, an effect that occurred regardless of the presence of sgp130Fc.

Many studies have suggested that SOCS-3 induction may lead to the pathogenesis of insulin resistance via its ability to inhibit IRS-1 and IRS-2 activation by binding to the Insulin Receptor at Tyr960, the key residue for IRS recognition (Torisu et al., 2007 and Ueki, Kondo and Kahn, 2004). Over-expression of SOCS-3 in the liver of mice leads to inhibition of IRS-1 and IRS-2, in turn leading to a reduction in activity of PI3-kinase and Akt (Ueki, Kondo and Kahn, 2004). Torisu and colleagues found insulin sensitivity, in mice with a hepatocyte specific SOCS-3 deficiency, was improved as, during an euglycaemic - insulinaemic clamp, hepatic glucose production rate was lower in these mice compared with that of wild type mice. In hepatocytes isolated from SOCS-3 deficient mice, phosphorylation of IRS-1 and IRS-2 and the downstream target Akt was enhanced compared to those from WT mice. More over, when hepatocytes from wild type mice were administered insulin or IL-6 for 16 h, an increase in SOCS-3 protein with a simultaneous decrease in phospho IRS1 and Akt was noted. When mice deficient in hepatic SOCS-3 are aged, regardless of whether they consume normal chow or high fat diet (HFD), a metabolic syndrome like phenotype is evident. These mice became obese, had fat deposits in the liver and had elevated blood glucose, insulin, serum triglycerides and total cholesterol levels. Furthermore, in the muscle of these mice, insulin stimulated phosphorylation of Akt was less than that of WT mice. It was also evident these mice had increased serum levels of the acute phase proteins serum amyloid A and C-reactive protein. The authors hypothesised that in mice deficient in hepatic SOCS-3, insulin resistance develops as a consequence of the inability to inhibit STAT-3, this in turn leads to hyper activation of this protein and thus mimics a state of chronic inflammation. This will be discussed further in Chapter 6 of this dissertation.

The consequences of SOCS-3 induction in skeletal muscle remains poorly understood. In 2006, a study by Spagenburg aimed to examine the effects of exercise training on SOCS-3 expression and the potential role of this protein within skeletal muscle. Upon treadmill training for 12 weeks, increased expression of SOCS-3 and IL-6 mRNA occurred within the *Soleus* and Plantaris muscle of female Sprague Dawley rats when compared to that of sedentary untrained animals. This study also emphasises that the IL-6 gene promoter contains a NFκB consensus site thereby increasing IL-6 upon NFκB activation. Using luciferase reporter assays, SOCS-3 overexpression in C2C12 myotubes drives NFκB transcriptional activity thus increasing IL-6. This effect would appear to be NFκB dependent as co-transfection with an NFκB mutant led to failure of SOCS-3 to induce IL-6 expression. Further more, SOCS-3 has been implicated in leptin resistance through its interaction with the leptin receptor at Tyr985 (Bjørbæk et al., 1998) and augmenting leptin activation of AMPK within skeletal muscle of morbidly obese humans (Steinberg et al., 2006).

The present study does not show significant increases in SOCS-3 induced by Hyper IL-6 in skeletal muscle of lean mice, thus SOCS-3 induced leptin resistance may only be of importance in a chronic model of inflammation, not acute stimulation. Although a one hour treatment with Hyper IL-6 significantly increased SOCS-3 in L6 myotubes *in vitro* (Chapter 4), in the present study this designer cytokine had a tendency to induce SOCS-3 in WAT, liver and *EDL*. It is possible that for a significant induction of this protein to occur, longer administration of the designer cytokine may be required. It was also of great interest to note that in response to Hyper IL-6 administration, there was a trend for increased SOCS-3 in the liver and this was in concert with significant elevations in phospho JNK1 in WAT. This supports the notion presented by Sabio and colleagues (2008).

In summary, the findings of this study demonstrate that IL-6 trans-signalling events, using Hyper IL-6 to mimic IL-6/sIL-6R complex signalling, occur in liver, muscle and epididymal WAT *in vivo*, in a tissue type dependent manner. More over, it was

demonstrated that the inhibitor of Hyper IL-6, sgp130Fc was bioactive within these tissues. However, the degree to which inhibition occurred was tissue type dependent. IL-6 trans-signalling events led to the activation of JAK/STAT-3, AMPK and JNK pathways, also in a tissue type dependent manner. The exact mechanism by which the tissue type dependency occurs remains unknown and requires further investigation. Due to the findings of this study using Hyper IL-6 to mimic IL-6/sIL-6R complex signalling, it is proposed that trans-signalling may be considered beneficial in an acute manner. For example, during exercise, IL-6 is released in a transient manner and acts as a mediator of metabolic regulation via signalling pathways involved in glucose oxidation and disposal and fat oxidation. However, contrary to the transient release seen during exercise, states of disease are often associated with chronically elevated levels of IL-6 and and/or sIL-6R within the circulation (refer to Chapter 1). This may lead to aberrant pathway activation and inflammation within many tissues. As this paradox exists, the next chapter explores the effect of prolonged Hyper IL-6 administration on inflammation and whole body energy metabolism *in vivo*.

CHAPTER SIX

Prolonged Hyper IL-6 treatment perturbs insulin sensitivity and induces liver injury in mice on a high fat diet

6.1 Introduction

IL-6 signalling mediated via the sIL-6R leads to the activation of STAT-3, ERK and PI3-kinase/Akt pathway activation (Weigel et al., 2008; Leu et al., 2003 and Heinrich et al., 2003). These pathways are mediators of many important cellular processes including cell survival and proliferation, metabolism and inflammation. The role of IL-6 in health and disease remains poorly understood, let alone that of signalling via the soluble IL-6 Receptor. This cytokine is often described as pro-inflammatory, yet it also modulates many metabolic processes including fat oxidation and glucose utilisation within metabolically active tissues such as adipose, skeletal muscle and liver (Pedersen and Febbraio, 2008). Many studies have focussed on prolonged IL-6 trans-signalling and the effect this phenomena has on inflammation (Miles et al., 1990; Maione et al., 1998; Atreya et al., 2000; Jostock et al., 2001; Nowell et al., 2003 and Sander et al., 2008). In contrast, there is little evidence within the literature implicating prolonged IL-6 trans-signalling in the regulation of metabolism. It is well established that transient IL-6 release leads to increased fat oxidation, glucose utilisation, lipolysis and suppressed hepatic glucose output (Stouthard, Elferink and Sauerwein, 1996; van Hall et al., 2003; Weigert et al. 2005; Carey et al., 2006; Pedersen and Febbraio, 2008). Contrary to the fore mentioned beneficial functions, there is also mounting evidence that this cytokine is chronically elevated during many states of inflammation, including metabolic syndrome and diabetes (Targher et al., 2001; Kelley et al., 2003; Pradhan et al., 2003). Subsequently, aberrant signalling pathway activation occurs due to chronically elevated IL-6 within the circulation and the inherent consequences are often dire. Thus the primary aim of this study was to examine the effects of prolonged IL-6 trans-signalling on metabolism *in vivo*.

IL-6 is known to be a key player in the development of such diseases as hepatocellular carcinoma, Kaposi's sarcoma, inflammatory bowel diseases and arthritis to name a few (Miles et al., 1990; Maione et al., 1998; Atreya et al., 2000; Jostock et al., 2001; Nowell et al., 2003 and Sander et al., 2008). Furthermore, it has been illustrated that IL-6/sIL-6R signalling is efficacious at inducing experimental models of inflammatory bowel disease, arthritis and hyperplasic

nodules of the liver (Atreya et al., 2000; Nowell et al., 2003; Maione et al., 1998). These studies surmise that sIL-6R targeted signalling is not a suitable therapeutic candidate treatment in metabolic disorders due to the highly likely odds of the patient developing undesirable side effects. Specifically, these unwanted attributes would make the designer cytokine an unsuitable stand-alone agent for the therapeutic treatment of metabolic disorders. As this is the case, it was proposed that ill effects, as a consequence of prolonged treatment with this cytokine, could be abrogated by co-treating with sgp130Fc. It was hypothesised that the beneficial effects associated with IL-6 signalling through the membrane bound IL-6 receptor such as fat oxidation, increased glucose clearance and insulin sensitivity would be maintained whilst counteracting the detrimental inflammatory processes that are associated with prolonged Hyper IL-6 treatment.

6.2 Materials and Methods

Animals - Male C57BL6 mice of 8 weeks of age weighing approximately 27 grams were housed in the AMREP precinct animal facility in a 12:12 hour light:dark cycle. Animals were fed a high caloric diet (approx 60 % of calories from fat) and water *ad libitum* for 6 weeks. All animal experiments were performed as approved by the Animal Ethics Committee of AMREP (ethics application approval number E/0573/2007/B). At 14 weeks of age, animals were randomly assigned to treatment groups and administered Saline (referred to control here on in), 4 µg Hyper IL-6 or 4 µg Hyper IL-6 + 40 µg sgp130Fc by intraperitoneal injection using a 25 gauge needle every alternate day for 16 days (Figure 6.1). Food intake and body mass was monitored throughout treatment period.

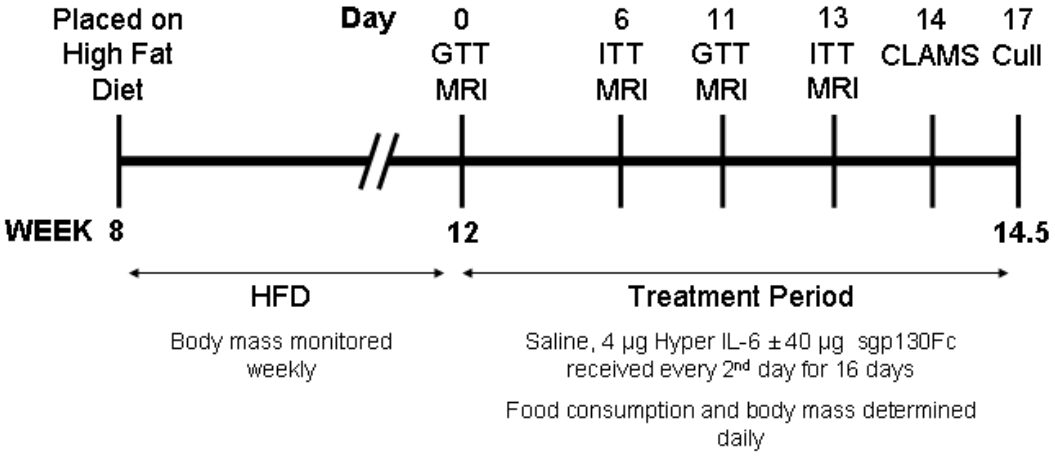


Figure 6.1 Schematic of the experimental design. HFD: High Fat Diet; GTT: Glucose Tolerance Test; ITT: Insulin Tolerance Test.

Twenty four hours post the last injection of the treatment period, mice were food restricted for 4 h, after which they were anaesthetised with 60-80 mg/kg sodium pentobarbital. Upon loss of pedal and blink reflex, basal state tissue was promptly excised. Mice were then administered 1.5 U/kg Insulin (Actrapid®, Novo Nordisk, Bagsraerd, Denmark) via abdominal aortic injection. Three minutes post insulin administration tissues were excised from the contralateral side (referred to as insulin stimulated from here on in). A small amount of liver and gastrocnemius

were collected for histological sectioning. Blood was collected from the heart of the mice and placed in a centrifuge tube containing heparin for further analyses by ELISA. After excision, all tissues were snap frozen in liquid nitrogen and animals were euthanased upon removal of the heart.

^{1}H Magnetic Resonance Imaging – On the day of insulin sensitivity and glucose tolerance testing, lean and fat mass of each individual mouse was determined by MRI. Mice were placed within a tube within the ^{1}H Magnetic Resonance Imaging Unit (Columbus Instruments, Columbus, OH, USA) and a digital output was obtained. The volume of insulin and glucose required for GTT and ITT for each individual animal were calculated relative to its corresponding lean body mass.

Insulin Sensitivity and Glucose Tolerance Tests – At the conclusion of week 4 of HFD and day 11 of the treatment period, whole body glucose tolerance was examined. Insulin sensitivity was determined on days 6 and 13 of the treatment period. Prior to testing, mice were fasted for 6 h. Using a hand held glucometer (Accu-check®, Roche Diagnostics, Mannheim, Germany), basal (*t = 0*) blood glucose concentration were obtained via tail cut. For Glucose tolerance testing, following determination of basal glucose levels the mice were administered 1 g kg^{-1} glucose diluted in saline via an injection into the intraperitoneal (IP) space. Glucose concentration was determined in blood obtained at 15, 30, 45, 60, 90 and 120 min post glucose injection. For insulin sensitivity testing, after basal (*t = 0*) blood glucose concentrations were determined, 0.75 U kg^{-1} insulin (recombinant human insulin, Actrapid®, Novo Nordisk, Bagsraerd, Denmark) was administered IP. As for the GTT, blood glucose concentrations were determined at 15, 30, 45, 60, 90 and 120 min.

Comprehensive Laboratory Animal Monitoring System (CLAMS) – Before initiation of, and at day 13 of treatment, metabolic parameters such as VO_2, RER, food intake, heat output and individual activity, were monitored over a 24 h period by CLAMS (Columbus Instruments, Columbus, OH, USA). Briefly, mice were placed in individual chambers containing sensors for O_2 and CO_2 interfaced with a

computer thereby providing a digital output for parameters of interest. Ambulatory movement of each mouse was monitored through the use of infra red beams on the x, y and z planes. Upon a break of the infra red beam by animal activity a movement event was recorded by the system. Feeding was monitored over this 24 h period by recording the difference in weight of the centre feeder between time points.

Enzyme Linked Immunosorbent Assay (ELISA) analyses of Sera- On the day of harvest, the blood samples taken from each animal for each treatment group were spun in a centrifuge at 3000 g for 10 min, after which the sera containing supernatant was placed in a fresh centrifuge tube and stored at -80 °C until required for ELISA. The concentration of C-reactive protein and Haptoglobin, markers of IL-6 induced acute phase inflammation, were determined using commercially available ELISA kits (Immunology Consultants Laboratory, Inc., Newberg, OR, USA). Briefly, samples were diluted in diluent (for Haptoglobin assay; 1:10000; C - reactive protein; 1:20) and 100 µl of each diluted sample and standards were transferred onto a 96 well plate pre coated with either anti- CRP or anti-haptoglobin antibody. After incubation at room temperature for 15 min, the wells were washed four times using wash solution to remove unbound protein followed by the addition of 100 µl of Enzyme Antibody Conjugate to each well. The plate was incubated for a further 15 min at room temperature followed by thorough washing. One hundred micro litres of TMB Substrate was added to each well, incubated for 10 min and the reaction was stopped upon the addition of 100 µl of the provided stop solution to each well. The absorbance of the reaction was determined by spectrophotometry at 450 nm. The concentration of the sera samples were then interpolated using the standard curve followed by correction for the dilution factor used in the assay.

The concentration of AST and ALT in the sera from each mouse were analysed by the Pathology unit of The Alfred Hospital, Melbourne, Australia.

Histology – Liver lipid content was visualised using Oil Red O staining. Livers mounted in OCT Compound (Tissue Tek, Sakura Finetek, Torrance, CA, USA) were sectioned using a microtome and placed on a glass microscope slide. Slides were then subjected to staining for 15 min using a working solution of 1.5:1; Oil Red O Stock solution (0.5 % (w/v) Oil Red O in dH_2O)-dH_2O. Slides were then rinsed in dH_2O for 15 minutes, mounted in glycerine jelly and allowed to air dry. After which time, images of random fields of view for each section were obtained using a digital SLR camera interfaced with an Olympus image pro microscope. The lipid droplet sizes of 10 random droplets, from 3 representative animals were determined by counting the number of pixels within a square field drawn surrounding the droplet using Adobe Image Ready. Images used were all of the same magnification. The pixel counts for each section for the individual treatment groups were then averaged providing a representative droplet size.

Architecture of the livers harvested from the animals was determined by Haemotoxylin and Eosin (H & E) Staining. After excision, a small amount of liver was removed and placed in Carnoy's Fixative (60 % (v/v) Ethanol, 30 % (v/v) Chloroform and 10 % (v/v) Glacial Acid). Liver samples were then stored in 50 % Ethanol until embedded in paraffin. Following embedding, the livers were sectioned by microtome and placed on a glass microscope slide. Slides were de-waxed by immersion in Xylene for 5 min twice followed by 100 % Ethanol for 5 min, twice and finally 95 % Ethanol for 2 min. Residual xylene and ethanol was removed from the slide by rinsing in dH_2O for 5 min. Tissue sections were then stained by immersing in Haemotoxylin for 2 min, washed for 3 min, followed by staining with Eosin for 30 s. Excess stain was removed by washing thoroughly for 5 min with dH_2O. Sections were then subjected to a dehydration process involving 2 minute immersions in 95 % Ethanol; 100 % Ethanol twice and Xylene twice. Sections were then mounted using DEPEX and allowed to air dry. After which, images of random fields of view for each section were obtained using a digital SLR camera interfaced with an Olympus image pro microscope.

Western Blotting – Endogenous levels of the proteins of interest were determined by western blotting as described in Chapter 3 of this dissertation. Whilst in a pre-chilled ceramic bowl, approximately 50 mg of liver, *soleus*, *EDL* or white adipose tissue were cut from the excised tissues by scalpel blade. Samples were then placed in lysis buffer and ground using a plastic handheld homogeniser tip. The subsequent lysates were then centrifuged at 13000 g for 60 min at 4°C. The supernatant was placed in a fresh microfuge tube and the protein concentration was determined by BCA assay. For each sample, 40 µg of protein was solubilised in Laemmelli's Buffer.

After the constitutive proteins of each sample were separated by electrophoresis, the proteins were transferred to nitrocellulose membrane by semi dry transfer. Membranes were incubated overnight in antibodies for either phospho Akt ser473, phospho Akt thr308, phospho ACC ser79, phospho AMPK thr172, TNFα, β-actin (Cell Signaling Technology, Beverly, CA, USA) or SOCS-3 (Abcam, Cambridge, UK). The relative expression of the proteins of interest was determined by antibody binding and enhanced chemiluminescence. To ensure equal amounts of protein had been analysed for each sample, β-actin protein was evaluated for each sample.

Statistics - Statistics were computed using the statistical analyses software package Sigma Stat to analyse significant differences using a either a one way or two way ANOVA. Multiple comparisons were made using the Student Newman Kuehl's or Tukey's post hoc test to locate significant differences between treatments. Significant differences for all experiments were accepted at $p < 0.05$. All data are expressed as mean fold change of protein expression compared with the expression of the protein of interest in tissues harvested from saline treated mice (Saline Control =1).

6.3 Results

Prolonged Hyper IL-6 administration has no effect on food intake or body mass – C57BL6 mice whole body mass was monitored throughout the experimental period, there was no difference in over all body mass between mice of randomly assigned groups, similar amounts of weight were gained over the 6 weeks of high fat feeding (data not shown). To ensure that any changes noted in body mass over the treatment period were not due to differences between groups in habitual feeding, food intake was monitored (Figure 6.2a). There was no significant difference in food intake between mice that received saline injections and those administered Hyper IL-6 ± sgp130Fc over the 16 day cytokine administration period (Figure 6.2a). A similar loss of total body mass was noted in mice administered saline and Hyper IL-6 + sgp130Fc (Figure 6.2b). The percentage of lean body mass to total body mass (Figure 6.2c) remained unchanged in all groups over the treatment period. At the end of the treatment period, all mice had a lower fat mass to total mass ratio then at the beginning of the treatment (Figure 6.2d). Moreover the loss of fat mass in mice administered Hyper IL-6 alone had a tendency to be greater than that of other treatment groups (25 % vs 10 % respectively), however, upon analysis none of the trends were statistically significant.

Prolonged Hyper IL-6 treatment, regardless of the presence of sgp130Fc, has no effect on glucose tolerance but impedes insulin sensitivity – After 6 weeks of high fat feeding, glucose tolerance was examined in all mice (Figure 6.3a). All animals displayed signs of developing an insulin resistant phenotype as demonstrated by high basal levels of blood glucose of approximately 10 $mmol \cdot l^{-1}$ compared with blood glucose levels typically seen in age matched chow fed C57BL6 mice (unpublished observations, Cellular and Molecular Metabolism Laboratory). Glucose tolerance remained unchanged in all experimental groups after 11 days of cytokine administration (Figure 6.3b and Figure 6.3c). Insulin Sensitivity was examined at day 6 and 13 of treatment period. There was no significant difference in insulin sensitivity between groups after 6 days of treatment (Figure 6.4a).

However, by day 13 (Figure 6.4b) mice that received Hyper IL-6 ± sgp130Fc had impaired insulin sensitivity when compared with control animals (Figure 6.4c, Saline vs Hyper IL-6; $p = 0.021$; Saline vs Hyper IL-6; $p = 0.008$).

Prolonged Hyper IL-6 administration has no effect on VO_2, RER, food intake and activity as determined by CLAMS – Hyper IL-6 had no effect on the oxidative capacity of animals as determined by measuring VO_2 (Figure 6.5a). The respiratory exchange rate also remained unchanged (Figure 6.5b) and there was no significant difference in food intake (Figure 6.5c) between treatment groups over a 24 h period. Activity of mice had a tendency to be higher in those animals administered Hyper IL-6 alone (Figure 6.5d), however this was not of statistical significance (Saline vs Hyper IL-6 $p = 0.078$).

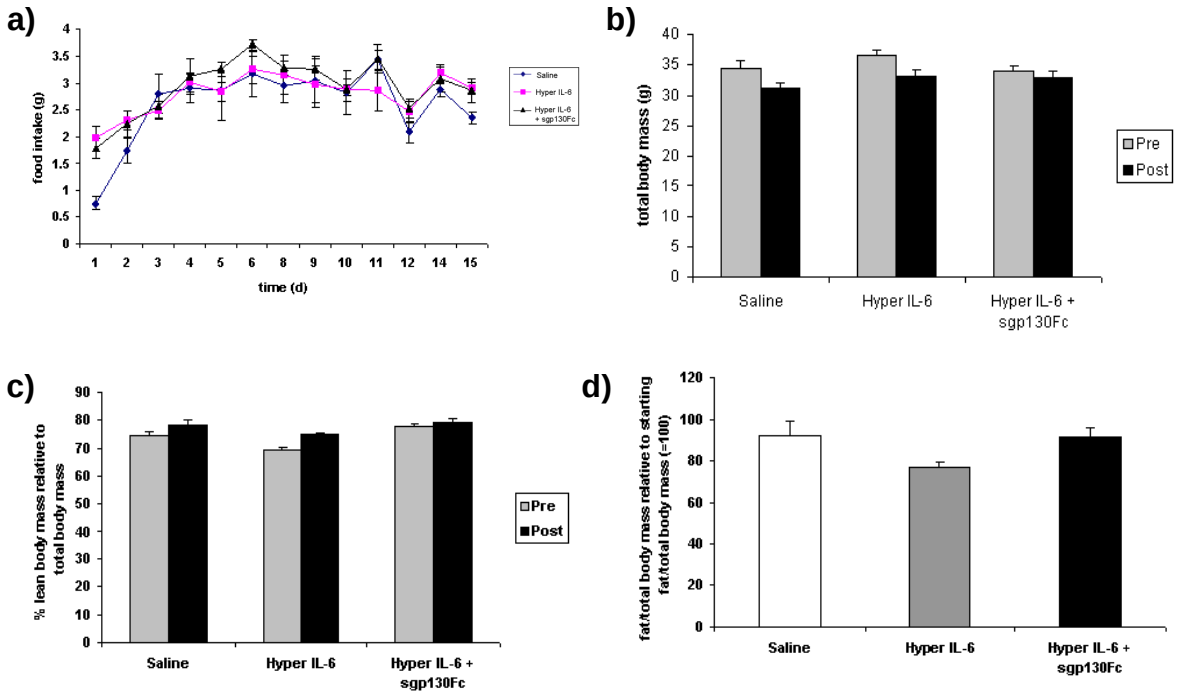


Figure 6.2 Prolonged Hyper IL-6 treatment has no effect on food intake or lean body mass but decreases fat mass relative to whole body weight. a) Food intake remained similar between mice of each treatment group pre and post treatment. b) Total body mass before initiation of treatment and at the end of the treatment period. c) Lean body mass as a percentage of total body mass pre-initiation and post treatment. d) Amount of fat mass lost over entire treatment period as indicated by fat mass/total body mass relative to starting fat mass/total body mass. Data expressed as mean ± SEM, n = 6.

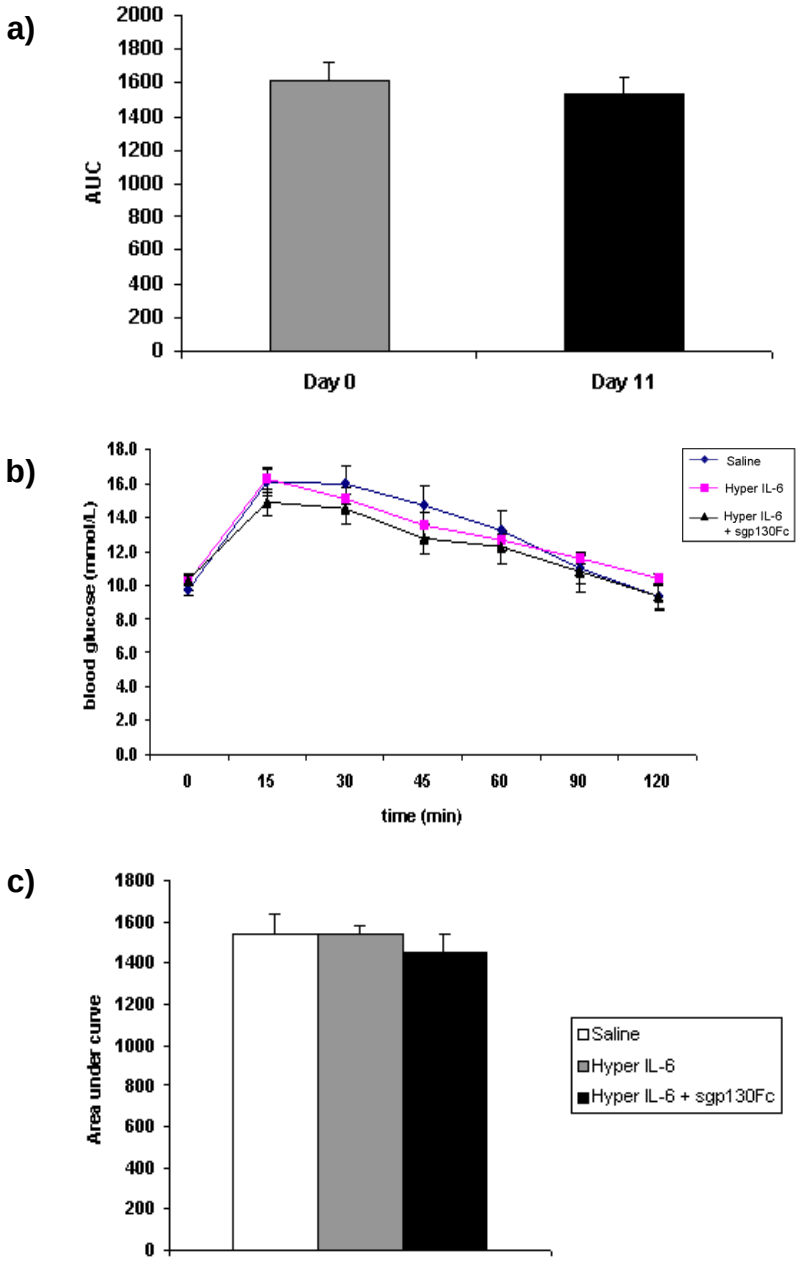


Figure 6.3 Hyper IL-6, regardless of the presence of sgp130Fc, has no effect on glucose tolerance. a) Area under curve for Day 0 and Day 11 for the Saline treatment group only, demonstrating no significant difference in glucose tolerance with time in response to Saline treatment. Glucose Tolerance Test of C57Bl/6 mice b) day 11 of treatment period. c) The area under the curve for each treatment group calculated for day 11 GTT. Data expressed as mean ± SEM, n = 6.

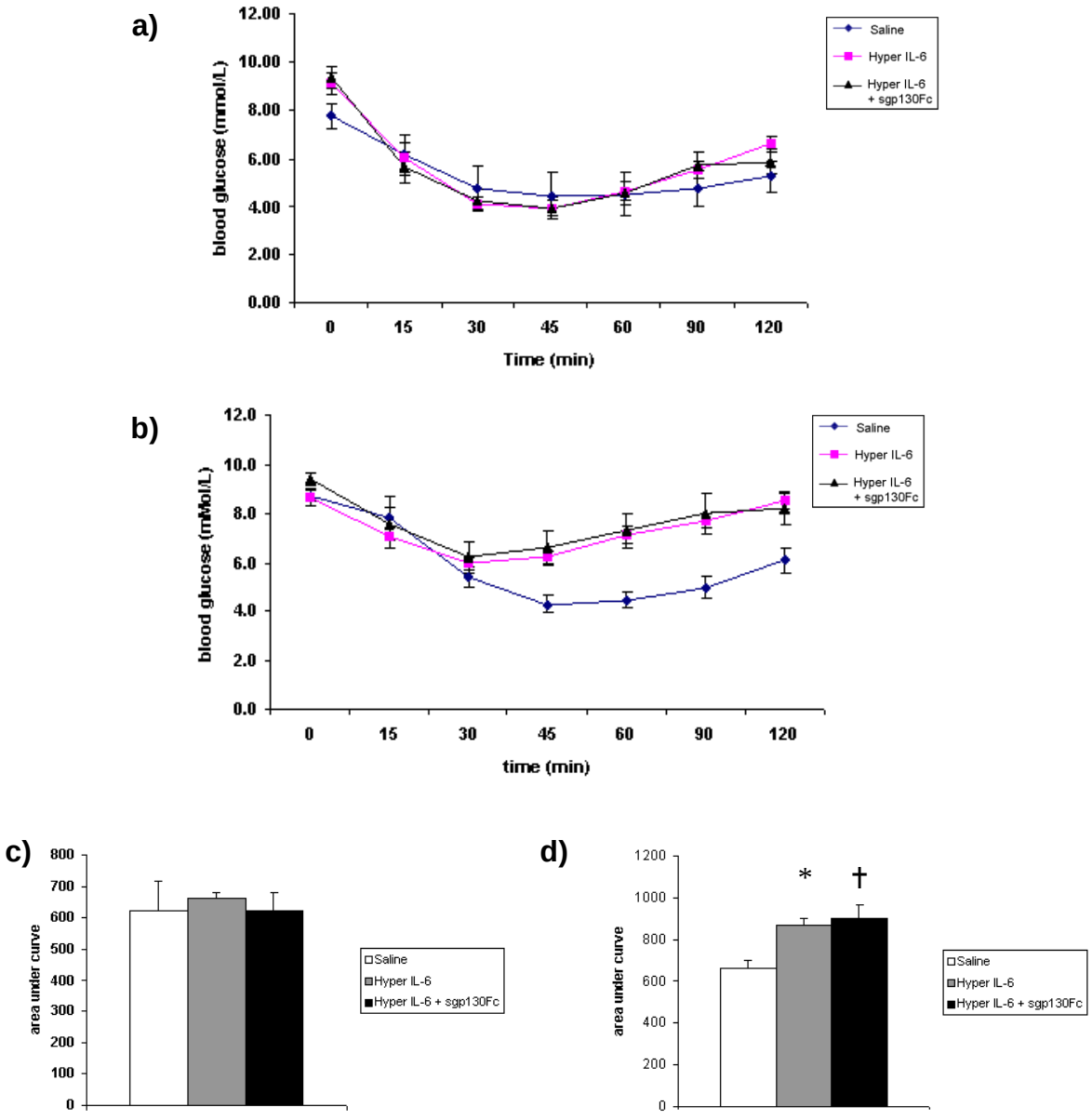


Figure 6.4 Continued Hyper IL-6 treatment decreases insulin sensitivity with time as determined by ITT. Concentration of Blood glucose over time as determined by an insulin tolerance test on a) day 6 and b) day 13 of Hyper IL-6 ± sgp130Fc treatment. Area under curve for Insulin tolerance test performed on c) day 6 and d) day 13 of the treatment period. * Saline vs Hyper IL-6; p = 0.021, † Saline vs Hyper IL-6 + sgp130Fc; p = 0.008. Data expressed as mean ± SEM, n = 6.

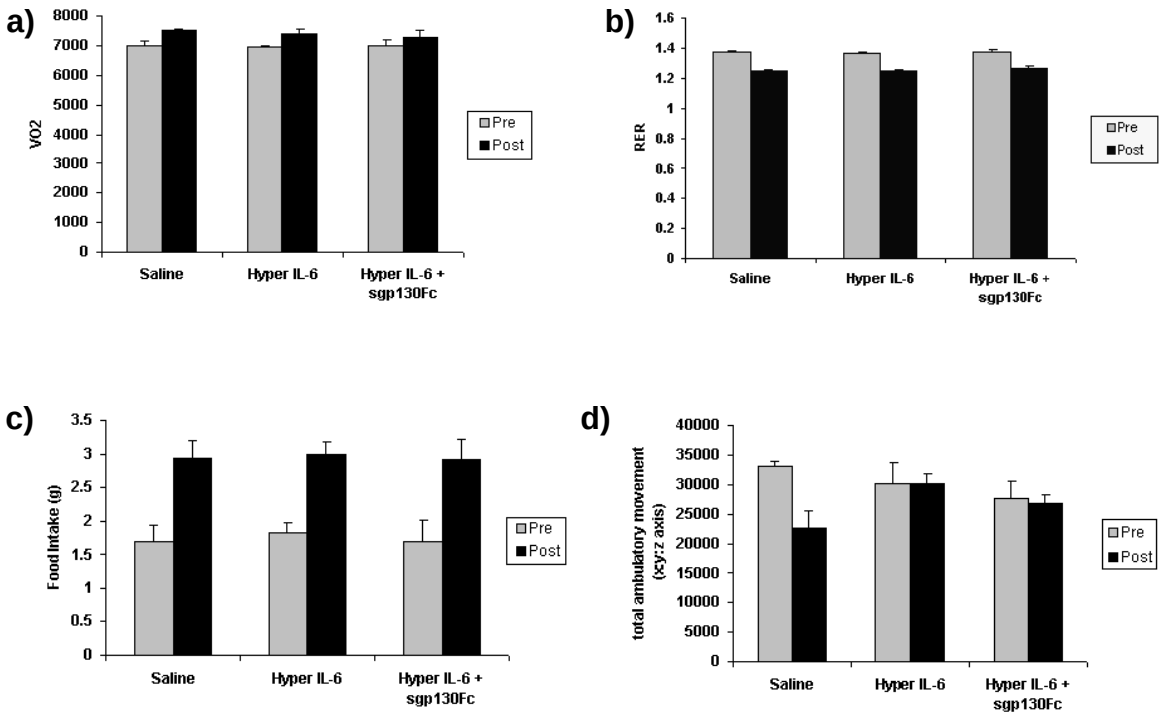


Figure 6.5 Hyper IL-6 treatment has little or no effect on metabolic parameters as determined by CLAMS. Analysis of a) VO_2, b) RER, c) food intake and d) ambulatory movement as determined using the Comprehensive Laboratory Animal Monitoring System. Data expressed as mean ± SEM, n = 3 - 4.

Mice administered Hyper IL-6 only have a higher circulating concentration of ALT and AST, an indication of hepatocellular damage – Circulating levels of AST and ALT, indicators of hepatocellular damage, were determined by ELISA. Animals administered Hyper IL-6 in isolation had a tendency to have higher serum levels of AST (Figure 6.6a) than control animals (Hyper IL-6; 552.3 U/l compared with Saline; 177.6 U/l), an effect that was abrogated upon co-treatment with sgp130Fc (267.3 U/l). Furthermore, mice that received Hyper IL-6 had greater serum concentrations of ALT than control mice or those co-administered sgp130Fc (37.2, 310.5, 42.3 U/l respectively, Figure 6.6b; Saline vs Hyper IL-6). Due to technical difficulties an insufficient amount of sera was collected at time of harvest to perform all necessary analyses. As a consequence many samples were pooled thus limiting group numbers (n=3-5) for analysis of AST and ALT.

Acute phase proteins, C-reactive protein and Haptoglobin, were not induced with prolonged Hyper IL-6 treatment – As previous studies have implicated Hyper IL-6 in acute phase inflammatory protein induction, C-reactive protein and Haptoglobin were examined by ELISA. There was no significant difference in concentration of C-reactive protein between treatment groups (Figure 6.6c). Surprisingly, although Hyper IL-6 had a tendency to increase circulating Haptoglobin concentrations, it was only significantly induced upon co - stimulation with sgp130Fc (Figure 6.6d; Saline vs Hyper IL-6 + sgp130Fc; $p = 0.035$, Hyper IL-6 vs Hyper IL-6 + sgp130Fc; $p = 0.083$).

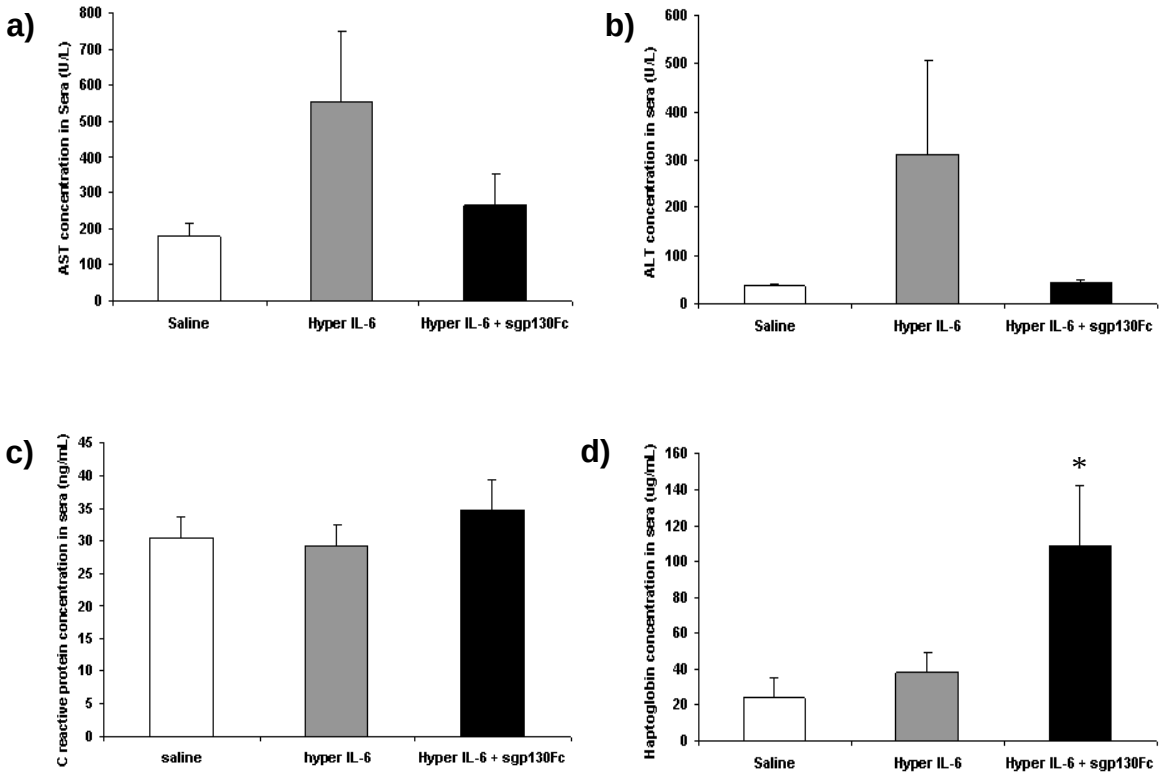


Figure 6.6 Hyper IL-6 administration had little effect on acute phase protein production but a tendency to induce chronic liver injury. Sera concentrations of the markers of hepatocellular damage a) AST, and b) ALT as determined by ELISA. Prolonged Hyper IL-6 treatment did not increase sera concentrations of c) C-Reactive protein and, in the case of d) Haptoglobin, only in the presence of sgp130Fc as determined by ELISA. * Saline vs Hyper IL-6 + sgp130Fc; p = 0.035. Data expressed as mean + SEM. n = 3 - 5 for AST and ALT analysis and n = 6 for C-reactive protein and Haptoglobin analysis.

Saline and co- treated mice on a HFD develop macrovesicular steatosis where as mice administered Hyper IL-6 had greater progenitor cell infiltration plus smaller & uniform lipid droplets – Oil Red O staining was used to examine the extent of steatosis in mice of all treatment groups (Figure 6.7a). Mice in the control and Hyper IL-6 + sgp130Fc treatment groups demonstrated a mixed phenotype steatosis as annotated by the deposition of small and large lipid droplets. By contrast, a difference in lipid droplet size was evident in Hyper IL-6 mice which had an average pixel count of ~400 compared with control and Hyper IL-6 + sgp130Fc treated mice which averaged 550 – 600 pixels in size (Figure 6.7a; saline vs Hyper IL-6; $p = 0.002$; Hyper IL-6 vs Hyper IL-6 + sgp130Fc; $p = 0.035$).

Haemotoxylin and Eosin staining was used to examine morphology of the liver sections obtained. Saline treated mice showed demarcated lobules around central veins, an effect not seen in mice administered Hyper IL-6 only, which maintained classic radial lobule organisation (Figure 6.7b). In mice co-administered sgp130Fc the integrity of the lobules were maintained, however, to a lesser degree than those administered Hyper IL-6 alone. Control mice on the high fat diet showed a steatotic phenotype as illustrated by the increased lipid deposition as determined by oil red o staining, lobular inflammation, hepatocyte ballooning and cytoplasmic reticulation by small aggregates of steatosis in the parenchyma of acinar zone 3 and large vacuole spaces (Figure 6.7a and 6.7c respectively). Mice administered Hyper IL-6 had greater progenitor cell infiltration and larger numbers of fibroblastic stellate cells in the space of disse than either of the other experimental treatment groups. This is an indication that Hyper IL-6 treated mice were undergoing regeneration after liver injury (Figure 6.7c). Hyper IL-6 + sgp130Fc mice appeared to display morphological symptoms of liver disease intermediate to that of mice of the control and Hyper IL-6 experimental treatment groups.

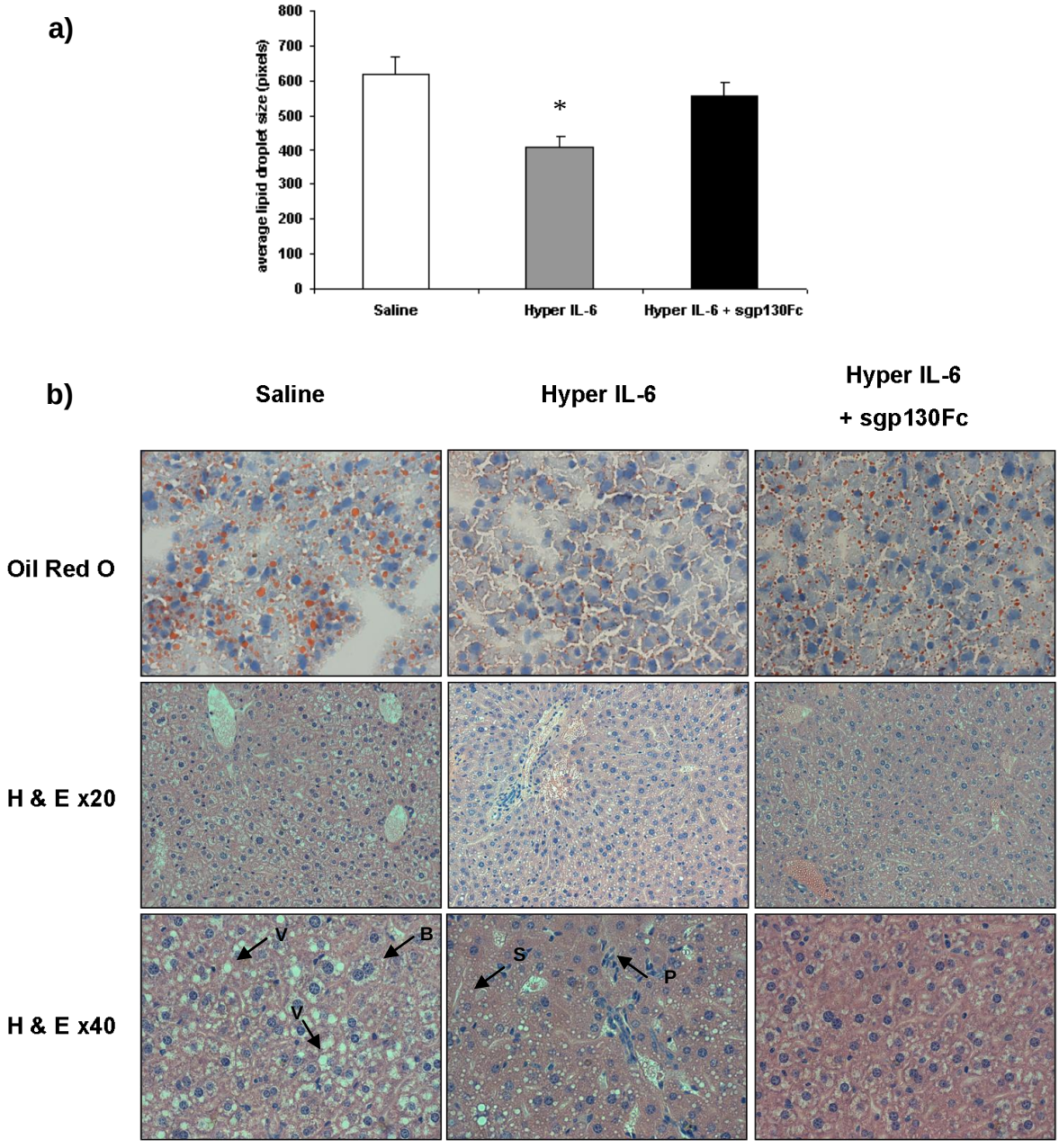


Figure 6.7 Livers from Saline treated C57Bl6 mice on a HFD have disrupted morphology and macro-steatosis whereas Hyper IL-6 treated livers have infiltration of progenitor cells indicating regeneration - a) Quantitation of average number of pixels (size of) for lipid droplets as stained by Oil Red O. Representative histological sections for treatment groups stained with b) Oil Red O, Haemotoxylin and Eosin at magnification x20 and x40. Significant difference * saline vs Hyper IL-6; p = 0.002; Hyper IL-6 vs Hyper IL-6 + sgp130Fc; p = 0.035 V; Vacuoles, B; Hepatocellular Cytoplasmic Ballooning, S; Stellate Cells, P; Progenitor Cells

Prolonged Hyper IL-6 administration modulates phospho Akt^{ser473} and Akt^{thr308} in a tissue type dependent manner – Prolonged Hyper IL-6 administration had a tendency to attenuate basal phosphorylation of Akt at the serine 473 residue in the liver (Figure 6.8a), however in the presence of sgp130Fc this effect was abrogated and thus comparative to the basal levels of control animals. In *soleus* muscle (Figure 6.8b), upon insulin stimulation, expression of phospho Akt^{ser473} was increased by 60 % in Hyper IL-6 treated mice (p=0.01) and control animals (p=0.043), however upon statistical analysis there was no significant change between the basal and insulin stimulated state in co-treated mice (p=0.072). There was no significant difference in basal expression between the treatment groups. In the WAT of Hyper IL-6 and Hyper IL-6 + sgp130Fc treated mice basal expression of phospho Akt^{ser473} had a tendency to be approximately 50 % greater than that of control mice (Figure 6.8c) although upon analysis this was not statistically significant. There was an increase of approximately 2 fold in phospho Akt ^{ser473} under insulin stimulated conditions in all treatment groups.

In liver, there was no significant difference in phospho Akt^{thr308} between treatment groups under basal or insulin stimulated conditions (Figure 6.9a). By contrast, in *soleus* (Figure 6.9b), insulin stimulation caused an increase of approximately 2.5 - 3 fold in the abundance of phospho Akt^{thr308} when compared to that of basal state (Saline vs Saline + Insulin; p = 0.001, Hyper IL-6 vs Hyper IL-6 + Insulin; p = 0.016 and Hyper IL-6 + sgp130Fc vs Hyper IL-6 + sgp130Fc + Insulin; p = 0.019). However, there was no statistically significant difference between the treatment groups.

Expression of phospho AMPK^{thr172} remains similar between treatment groups however Hyper IL-6 + sgp130Fc decreased phospho ACC^{ser79} abundance in liver – Hyper IL-6 administration had no effect on the activation of AMPK (Figure 6.10a) within the liver. The abundance of phospho ACC^{ser79} was reduced by approximately 70 % upon co - treatment with Hyper IL-6 and sgp130Fc (Figure 6.10b; Saline vs Hyper IL-6 + sgp130Fc; p = 0.004, Hyper IL-6 vs Hyper IL-6 + sgp130Fc; p = 0.007).

Prolonged Hyper IL-6 administration does not induce increases in membrane bound TNFα or SOCS-3 protein – There was no significant difference in pro TNFα (Figures 6.11a and 6.11b) nor SOCS-3 (Figure 6.11a) in any of the tissues examined. Although a slight increase in proTNFα in the *soleus* (Figure 6.11a) of mice administered Hyper IL-6 was evident.

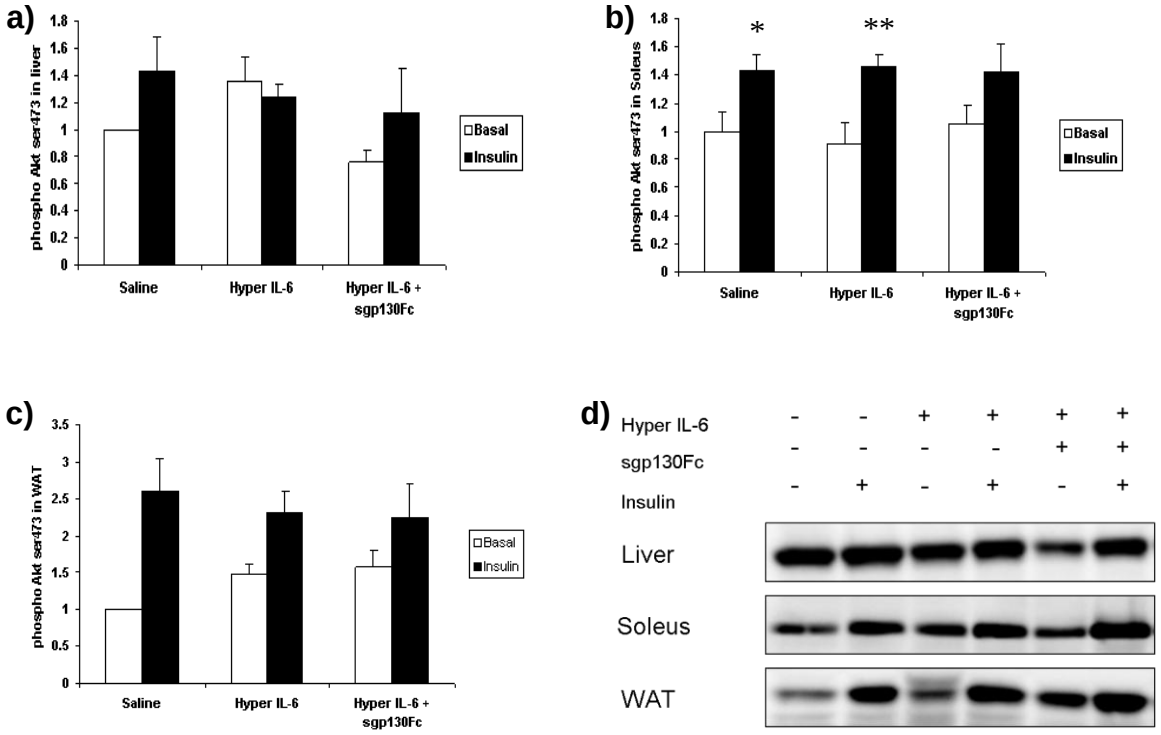


Figure 6.8 High basal expression of phospho Akt^{ser473} is evident in liver of mice regardless of compound administered, an affect that is not conserved in all tissue types. Furthermore, insulin stimulation does not result in a significant increase in phosphorylation of Akt at the ser473 residue in liver of mice treated with saline or Hyper IL-6 ± sgp130Fc - a) Quantitation of phospho Akt^{ser473} under basal and insulin stimulated conditions in a) liver, b) *soleus* and c) WAT. d) Representative immunoblots of phospho Akt^{ser473} in the fore mentioned tissues. For *soleus* the difference in expression; * Saline vs Saline + Insulin; p = 0.043, ** Hyper IL-6 vs Hyper IL+ Insulin; p = 0.010. Data expressed as fold change from saline control =1, mean ± SEM, n = 6.

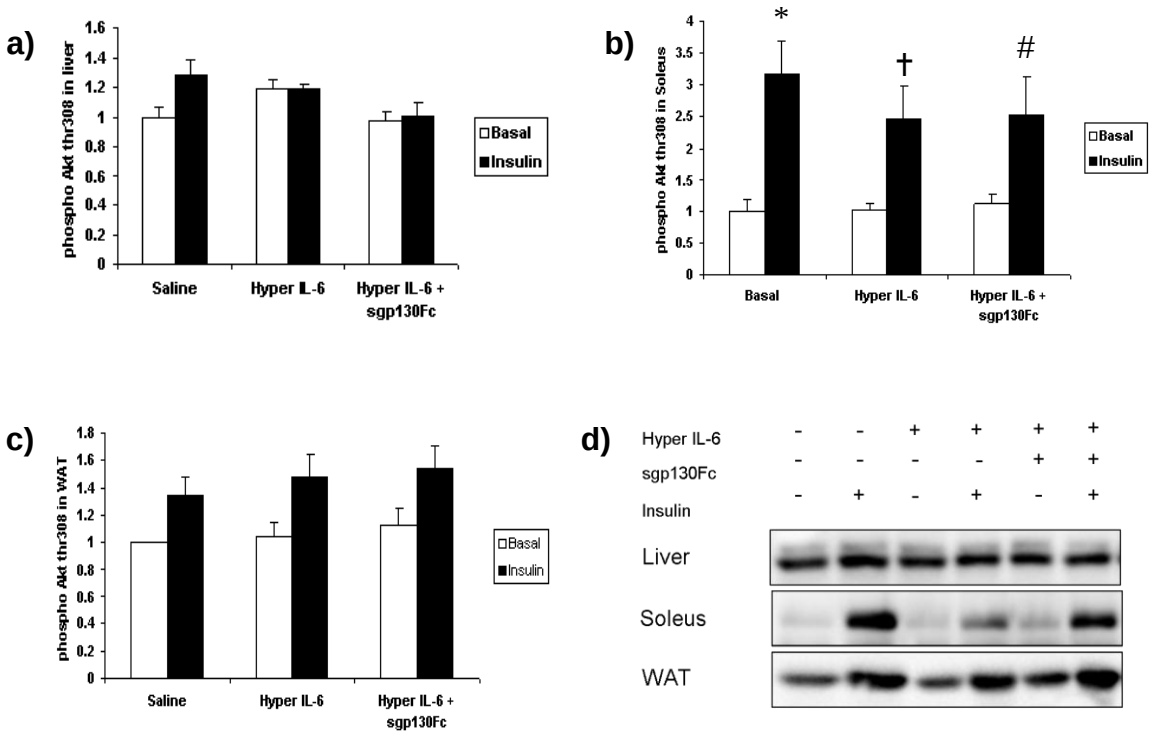


Figure 6.9 Prolonged hyper IL-6 treatment has little effect on phospho Akt^{thr308} protein abundance in liver, *Soleus* or WAT. Densitometry quantitation of protein abundance of phospho Akt^{thr308} as determined by western blotting in a) liver, b) *soleus* and c) WAT. Representative immunoblots for phospho Akt^{thr308} in the fore mentioned tissues. Difference in protein expression of phospho Akt^{thr308} within *soleus*; * Saline vs Saline + Insulin; p = 0.001, † Hyper IL-6 vs Hyper IL-6 + Insulin; p = 0.016, # Hyper IL-6 + sgp130Fc vs Hyper IL-6 + sgp130Fc + Insulin; p = 0.019. Data calculated as fold change from saline control animals under basal conditions (=1). Data expressed as mean ± SEM; n = 6.

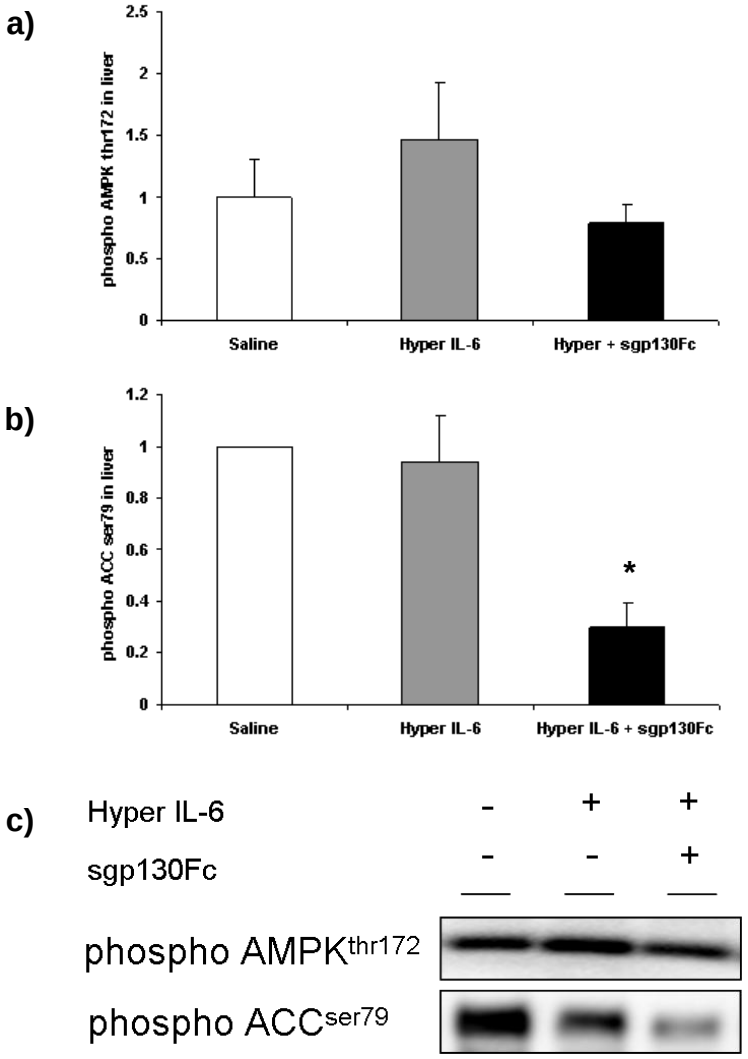


Figure 6.10 Hyper IL-6 has no effect on the activation of AMPK but in the presence of sgp130Fc reduces phosphorylation of ACC in liver. Quantitation of abundance of phospho a) $AMPK^{thr172}$ and b) ACC^{ser79} as determined by western blotting within the liver of Hyper IL-6 ± sgp130Fc treated mice on a HFD. c) Representative immunoblots of phospho $AMPK^{thr172}$ and ACC^{ser79}. * Difference between Saline vs Hyper IL-6; p = 0.004, Hyper IL-6 vs Hyper IL-6 + sgp130Fc; p = 0.007. Data expressed as mean + SEM fold change relative to Saline control;n = 6.

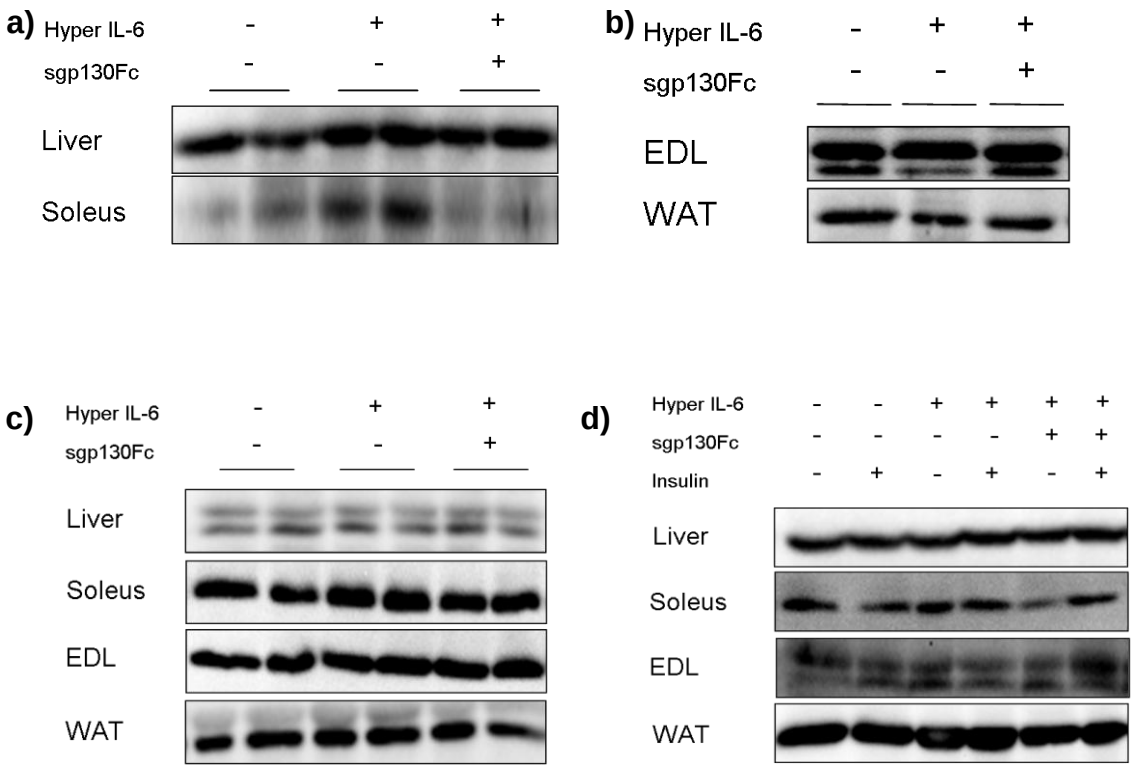


Figure 6.11 Prolonged Hyper IL-6 treatment does not significantly alter protein abundance of pro TNFα or SOCS-3 in tissues of interest in mice on a HFD. Representative immunoblots of pro TNFα in a) Liver and *soleus*, b) *EDL* and WAT, c) SOCS-3 protein in Liver, *Soleus, EDL* and WAT and d) β-actin in fore mentioned tissues.

6.4 Discussion

It is well established IL-6 signalling events are able to modulate metabolism in an acute manner. This occurs through the activation of cellular signalling pathways commonly associated with physiological processes such as fat oxidation and glucose utilisation both *in vitro* and *in vivo*. Studies within this dissertation demonstrate acute Hyper IL-6 treatment is capable of regulating glucose uptake and fat oxidation in myotubes *in vitro* and signalling pathways implicated in metabolic processes and inflammation in various tissues *in vivo.* Importantly, these studies also demonstrate inhibition of Hyper IL-6 using sgp130Fc, occurs in a tissue type dependent manner. The prolonged effect of Hyper IL-6 on metabolism has yet to be examined. However, it is imperative to note prolonged IL-6/sIL-6R treatment is known to cause inflammation (Maione et al., 1998; Atreya et al., 2000; Jostock et al., 2001; Nowell et al., 2003), thereby making it an unsuitable candidate as a stand alone therapeutic agent for the treatment of metabolic disorders such as obesity and diabetes. As this paradigm exists, the main thrust of this study was to examine the effect of prolonged co-treatment with Hyper IL-6 and its competitive inhibitor sgp130Fc on compromised metabolism and inflammation *in vivo*.

To examine the fore mentioned hypotheses, C57Bl6 mice were placed on a HFD, whereby a high percentage of caloric intake originates from dietary trans fats. This ensured mice displayed signs of metabolic syndrome, such as increased fat mass, glucose intolerance and insulin resistance. After 6 weeks of HFD, mice were randomly assigned to a treatment group and administered via IP injection saline, Hyper IL-6 or Hyper IL-6 + sgp130Fc every alternate day for 16 days. Cytokine was administered every alternate day as Hyper IL-6 remains bioactive for 48 h inclusive (Peters et al., 1998). As discussed in Chapter 5 of this dissertation, 40 µg of sgp130Fc was used to ensure that there was a molar excess of the inhibitor in the presence of Hyper IL-6.

To determine if Hyper IL-6 had detrimental side effects many parameters were monitored through out the entire experimental period, including fat mass and lean body mass and food intake. Monitoring body mass was of particular importance to

determine if the designer cytokine possessed anti-obesigenic properties. The food intake of each mouse was monitored daily to ensure that any perturbation in body mass was due to the cytokine affecting whole body metabolism and not due to a change in habitual feeding associated with treatment. It was noted that feeding patterns remained similar between all treatment groups. Despite this, mice administered Hyper IL-6 had a tendency towards greater loss of fat mass over the treatment period than those mice assigned to receive saline or co-treatment with sgp130Fc. The fat mass loss could not be attributed to improved glucose tolerance or improved insulin sensitivity, as mice that received prolonged treatment with Hyper IL-6 displayed evidence of compromised insulin sensitivity and glucose tolerance.

In the current study, mice that received prolonged Hyper IL-6 treatment had little change in lean body mass. This was surprising, given elevated IL-6 as a result of aberrant secretion from tumours such as breast and prostate carcinoma, correlates positively with loss of lean body mass in humans (Evans et al., 2008). Subsequently, the presence of IL-6 and CRP are often used in the diagnosis of cancer induced cachexia (Evans et al., 2008). An additional factor may be required for the onset of cancer induced cachexia, one such candidate may be the protein proteolysis inducing factor (Hussey et al., 2000). Hyper IL-6 may not induce the expression of the necessary co-factor for cachexia to occur, thus explaining why there is little change in lean body mass over time with treatment.

The tendency towards greater fat mass loss could not be attributed to increased activity or improved oxidative capacity, as these remained similar across the three treatment groups. Prolonged Hyper IL-6 administration within these animals, even in the presence of sgp130Fc, had comparatively similar VO_2, RER, ambulation and heat production (unpublished observation). It is noteworthy, a symptomatic hallmark of chronic inflammatory bowel disease is weight loss, attributed to the loss of protein, water and most notably, fat stores (Powell-Tuck, 1986). As Hyper IL-6 has previously been used to induce experimental models of colitis it is feasible to suggest that in the current cohort mice suffered from cytokine induced chronic

inflammation of the gastrointestinal tract, inadvertently accounting for the trend of reduced fat mass (Figure 6.2). In future experiments, it may be of interest to observe if animals treated with Hyper IL-6 are indeed suffering from IBD by examining stool consistency and blood content, rectal health and histological examination of the colon and determining cell population subsets within the tissue through the use of immunohistochemistry.

IL-6 induces and modulates the production of acute phase proteins by hepatocytes (Stoyan et al., 1993; Weiergräber et al., 1995; Lienenlüke and Christ, 2007; Rabe et al., 2008). In the current study, upon prolonged stimulation with Hyper IL-6 alone, the sera concentration of the acute phase proteins CRP and Haptoglobin remained similar to that of control mice. Surprisingly, mice that were co-administered Hyper IL-6 and sgp130Fc had no change in CRP yet significantly increased levels of Haptoglobin. This suggests that, unlike their experimental counterparts, co-treated mice were in a state of acute phase inflammation.

Raised sera concentrations of the amino transferases aspartate amino transferase (AST) and alanine amino transferase (ALT), is a consequence of increased proteolysis and is indicative of chronic liver assault (Bugianesi et al., 2005; Yin et al., 2008). In the present cohort, a trend of elevated sera concentrations of AST and ALT from Hyper IL-6 treated mice was evident when compared with controls. It is important to note however, this result was not considered statistically significant, likely due to a lack of power as there was a limited volume of serum obtained for analysis. To obtain enough volume for analysis, some samples were pooled. AST and ALT results combined with the minimal change in levels of acute phase proteins in saline control and Hyper IL-6 treated mice are suggestive of chronic liver injury, providing support to the notion that Hyper IL-6 is a hepatocellular cytotoxic agent. Further more, it is evident from this data set that sgp130Fc was able to impair the onset of chronic liver disease induced by Hyper IL-6, as co-administration did not augment circulating amino transferases. Importantly, although not as deleterious to the liver as prolonged Hyper IL-6 administration,

prolonged exposure to the designer cytokine and its inhibitor sgp130Fc is still able to induce an acute phase inflammatory response.

Histological staining was utilised to examine the general health of the liver within these animals. It is well established that chronic injury of the liver results in inflammation and increased cytokine production such as TNFα, IFNγ and IL-6, which in turn causes proliferation of progenitor cells (Knight et al., 2005). Progenitor cells are stem cells that differentiate into hepatocytes, replacing injured parenchyma. Thus progenitor cell infiltration is often considered indicative of severity of liver disease (Lowes et al., 1999). In the current study, using Haemotoxylin and Eosin staining, further evidence of chronic liver injury was provided by increased progenitor cell infiltration in livers from Hyper IL-6 treated mice, an anomaly not seen in saline treated nor Hyper IL-6 + sgp130Fc co-treated mice. This is in accordance with previous studies which demonstrate progenitor cell infiltrate and liver regeneration is induced by IL-6/Hyper IL-6 signalling (Yeoh et al., 2007 and Galun et al., 2000) It is unlikely that the increased infiltration of progenitor cells was induced by TNFα, as there was no increase in the pro form of this cytokine in the liver of Hyper IL-6 treated mice compared to that of animals assigned to the control group.

Furthermore, there was no significant change in proTNFα in *EDL* or WAT but there was a strong trend for elevated proTNFα in *soleus* with Hyper IL-6 treatment, an effect that was abrogated in the presence of sgp130Fc (Figure 6.11). The current data suggest Hyper IL-6 has differential effects on proTNFα expression according to tissue type. This is in accordance with Yuen et al. (2009), who demonstrate cell specific effects occur in response to IL-6 with elevated production of TNFα in human aortic endothelial cells but not within mixed hindlimb skeletal muscle.

Insulin resistance is associated with increased hepatic glucose and lipid production, leading to intrahepatic accumulation of fat due to perturbed fat oxidation, a condition known as Steatosis. Using Oil red O staining of liver sections, macro-vesicular steatosis, characterised by accumulation of mixed small

and large lipid droplets (Brunt, 2007), was evident in saline control and Hyper IL-6 and sgp130Fc co-administered mice. By contrast, mice administered Hyper IL-6 had micro-steatosis as demonstrated by small uniform lipid droplets. Microsteatosis is considered to be a manifestation of chronic liver injury (Brunt, 2007). Steatotic classification suggests that in saline control and co-treated mice, steatosis is a manifestation of fatty acid influx and triglyceride accumulation (Reddy and Rao, 2007). By comparison, in Hyper IL-6 treated mice steatosis is likely due to localised hepatocellular damage causing inherent mitochondrial and peroxisomal β-oxidation deficiencies (Reddy and Rao, 2007). In liver of mice treated with Hyper IL-6, there is also evidence of an increased stellate cell population, indicative of liver regeneration and increased fibrosis, an effect not seen in saline control and Hyper IL-6 + sgp130Fc co-treated mice. However, to confirm that there is indeed increased fibrosis it would be necessary to examine sections using specific histochemical stains.

As Oil Red O staining of liver sections demonstrated a perturbation in fatty acid metabolism existed, AMPK pathway signalling was examined. There was little change in protein abundance of phospho AMPK^{thr172}. Surprisingly, a significant reduction in phospho ACC^{ser79} abundance in livers of mice co-administered Hyper IL-6 and sgp130Fc compared with control mice was evident. As to why this occured remains unknown and requires further investigation. Conversely, this reduction was not seen in mice administered Hyper IL-6 alone who displayed similar levels of phospho ACC ^{ser79} abundance compared with control mice. This suggests that fat oxidation potential is comparative between the Hyper IL-6 treated and control mice.

Hyper IL-6 treated mice display signs of insulin resistance as indicated by an impaired response to insulin during an insulin tolerance test. Co-treating with sgp130Fc was unable to rescue Hyper IL-6 induced insulin resistance. This is in accordance with other studies that suggest IL-6 signalling leads to insulin resistance (Senn et al., 2002 and Kanemaki et al., 1998). The data obtained in the current study suggest Hyper IL-6 promotes the insulin resistant phenotype typically

seen within mice fed a HFD. Furthermore, chronically elevated IL-6/sIL-6R complex within the circulation of C57BL6 mice may exacerbate any insulin resistant phenotype associated with pre existing diet induced metabolic syndrome.

In support of the insulin resistant phenotype, in liver the of Hyper IL-6 treated mice, Akt activation at both residues seemed equivocally regulated. Hyper IL-6 had a tendency to increase basal expression of phospho Akt^{ser473} but, upon insulin stimulation was not further potentiated. Basal abundance of phospho Akt^{ser473} was elevated compared to saline control in *soleus* and adipose. However, converse to that of liver signalling appeared to remain responsive to insulin with a 50 % increase in this protein evident (Figures 6.8 and 6.9). In all tissues, activation of Akt at the Thr308 residue closely reflected that of phosphorylation at the Ser473 residues. These signalling data suggest that the insulin resistance seen in all mice treated with Hyper IL-6 may be attributed to impaired liver function rather than metabolic perturbations of skeletal muscle.

SOCS-3 protein expression is induced by IL-6 activation of STAT-3 and inhibits the activity of Janus kinases. It is important to note in the current study there was no change in SOCS-3 protein with any of the treatments. This may be a reflection of a) the relative short half life, 1-2 hours, of SOCS-3 (Siewart et al., 1999) or b) increased proteosomal degradation of the protein. JAK induced phosphorylation of SOCS-3 at the SOCS box, prevents the protein from forming a complex with Elongin C, thereby decreasing stability and accelerating proteosomal degradation of the protein (Haan et al., 2003). It is plausible to suggest Hyper IL-6 results in chronic activation of JAK/STAT-3 and subsequently increased binding of JAK to SOCS-3, leading to instability and accelerated proteosomal degradation of this protein.

In summary, in line with the hypothesis, this current study provides further experimental evidence that sgp130Fc is able to prevent severe injury and chronic inflammation of the liver induced by prolonged Hyper IL-6 treatment. Mice co-administered Hyper IL-6 and sgp130Fc show little sign of chronic liver injury *per se*.

However, it is apparent these mice suffered from acute inflammation with elevated levels of the IL-6 inducible acute phase protein Haptoglobin and hepatocyte degeneration noted. Mice administered Hyper IL-6 lost fat mass over the treatment period but also demonstrated overt insulin resistance. sgp130Fc was unable to ameliorate insulin resistance associated with prolonged Hyper IL-6 treatment.

CHAPTER SEVEN

Conclusions and Future Directions

7.1 Conclusions and Future Directions

The primary aim of this dissertation was to elucidate whether IL-6 signalling mediated via the agonistic sIL-6R (IL-6 trans-signalling) can be considered biologically relevant in the regulation of common metabolic processes such as mitochondrial oxidative phosphorylation, fatty acid oxidation, glucose utilisation and insulin sensitivity within skeletal muscle, adipose and liver.

Chapter Three sought to examine IL-6 trans-signalling, using the designer cytokine Hyper IL-6, in the regulation of transcription and translation of PGC-1α, an important mediator of mitochondrial function through its ability to modulate OXPHOS gene expression, mitochondrial biogenesis and adaptive thermogenesis. *In vitro*, Hyper IL-6 was not a transcriptional or translational regulator of PGC-1α as evidenced by little change in mRNA or protein expression of PGC-1α in response to the designer cytokine within the cell types examined. Furthermore, prolonged treatment with IL-6 had little effect on mRNA or protein expression of PGC-1α *in vivo*. It is important to note, however, that there was limited tissue available for analysis of PGC-1α expression and as a consequence, this aspect of the study was only examined in *Tibialis Anterior* muscle. This does not discount the possibility that IL-6 regulates expression of PGC-1α and other mitochondrial genes in muscle types other than *Tibialis Anterior* and requires further investigation.

An abundance of studies examining IL-6 signalling mediated via the sIL-6R in many tissues including synovial fluid, the bowel and liver is evident within the literature. Conversely, there is little evidence of this phenomenon occurring in metabolically active tissues such as skeletal muscle and adipose tissue. This may be considered as surprising given the prominent role acute release of IL-6 plays in the regulation of metabolism within such tissues as adipose and skeletal muscle during exercise. Consequently this formed the basis of Chapter 4, whereby acute IL-6 trans-signalling events were examined in L6 myotubes and CRL1439 hepatocytes *in vitro*. Using Hyper IL-6 to mimic IL-6/sIL-6R/gp130 activation, the signalling pathways activated and the biological consequences of acute IL-6 trans-signalling in L6 myotubes *in vitro* were explored. This study illustrated L6

myotubes are responsive to IL-6 trans-signalling events as indicated by the phosphorylation of such proteins as STAT-3 and Akt. Moreover, IL-6/sIL-6R complex signalling was adept at regulating glucose uptake and fat oxidation in L6 myotubes *in vitro*. Although it was apparent Hyper IL-6 increased glucose uptake, the direct mechanism by which this occurred requires further experimentation.

A comparison between IL-6 and Hyper IL-6 induced cell signalling in L6 myotubes and CRL1439s provides further support to the notion IL-6/sIL-6R occurs in a cell type dependent manner. The degree to which STAT-3 and Akt were phosphorylated in response to Hyper IL-6 was variable between the two cell types examined. The diverse effects of Hyper IL-6 is likely to be a reflection of its bioactivity within individual cell types and is most likely due to the relative expression of membrane bound IL-6Rα and gp130β within these cells. Not withstanding the naturally occurring competitive inhibitor of sIL-6R signalling, sgp130 also had a tissue specific effect, with partial inhibition of phospho STAT-3 evident in CRL1439 but not L6 myotubes. This too, is likely due to the relative abundance of the membrane IL-6R and gp130.

As Chapter 4 established L6 myotubes were responsive to IL-6 trans-signalling, and that Hyper IL-6 was proficient at regulating glucose uptake and fat oxidation *in vitro,* it was of utmost importance to determine if this scenario translated to an *in vivo* model. Chapter 5 examined components of various cell signalling pathways involved in inflammation, metabolism and cell cycle, in response to an acute Hyper IL-6 treatment within skeletal muscle, adipose tissue and liver, *in vivo*. It was evident that activation of proteins involved in the fore mentioned processes occurred in a tissue type dependent manner as evident by the relative phosphorylation of STAT-3, AMPK and JNK within liver, muscle and WAT.

Due to observations made by previous investigators and those of the current cohort, it was proposed IL-6 trans-signalling may be beneficial in an acute manner. For example, during exercise, IL-6 is released into the circulation in a transient manner and acts as a mediator of metabolic regulation via signalling pathways

involved in glucose oxidation and disposal and fat oxidation. However, it is also well established that states of disease are often associated with chronically elevated levels of IL-6 and and/or sIL-6R within the circulation. This may lead to aberrant pathway activation and inflammation within many tissues. As this paradox exists, Chapter 6 explored prolonged Hyper IL-6 administration on inflammation and whole body energy metabolism *in vivo*.

Mice administered Hyper IL-6 over a prolonged period lost fat mass over the treatment period, had overt insulin resistance and severe liver injury. With the ideal of preventing any associated inflammatory signalling whilst maintaining any beneficial effect Hyper IL-6 treatment had on metabolism, mice were co-treated with Hyper IL-6 and sgp130Fc. Although sgp130Fc prevented severe injury and chronic inflammation of the liver induced by Hyper IL-6, it was apparent these mice suffered from acute inflammation with elevated levels of Haptoglobin and hepatocyte degeneration evident. Surprisingly, sgp130Fc co-treatment was unable to ameliorate insulin resistance associated with prolonged Hyper IL-6 treatment. It is plausible that the insulin resistance associated with Hyper IL-6 treatment, regardless of the presence of sgp130, is due to the enhanced bioactivity and delayed clearance of the designer cytokine.

Although Hyper IL-6 was a useful tool in the examination of IL-6/sIL-6R signalling within various tissues it remains a poor candidate as a therapeutic tool in the war against obesity. This is primarily due to its enhanced bioactivity as a consequence of high binding affinity for the gp130 receptor and slow internalisation resulting in hyper activation of many pro-inflammatory pathways. As a consequence of the enhanced bioactivity, co-treating with sgp130Fc may have not been adequate to prevent inflammation induced by this designer cytokine. This, however, does not discount the potential of a gp130 receptor/ligand like drug design as an anti-obesity agent. Studies aimed at examining the effects of IL-6 treatment within transgenic mice over expressing sgp130Fc, with the ideal of determining if it is possible to prevent “pro-inflammatory” signalling associated with gp130 receptor/ligand activity whilst maintaining beneficial metabolic signalling are planned for the near future.

Similar studies to those performed in this dissertation utilising recombinant IL-6, rather than the potent and highly bioactive Hyper IL-6, in the presence of sgp130Fc may provide insight into the mechanism behind the beneficial effects associated with membrane bound IL-6R signalling. Not withstanding, many IL-6 family cytokines utilise gp130 to mediate cellular functions and hence potentially modulate metabolism and inflammation. Through understanding how gp130 ligands elicit cellular effects, it may be possible to design a cytokine/ therapeutic agent that utilises specific components of individual IL-6 family members thereby selectively targetting components of gp130 mediated signalling. This would ultimately ensure beneficial effects on metabolism are maintained whilst avoiding those that may contribute to the pathogenesis of inflammation.

Collectively, these data demonstrate in skeletal muscle Hyper IL-6 induced trans-signalling, regardless of the presence of sgp130Fc remains intact, contrary to that of hepatocytes *in vitro*. In C57BL6, acute administration of this designer cytokine by IP injection is able to regulate signalling pathways associated with metabolism and inflammation, an effect that sgp130Fc co-administration inhibited in a tissue type dependent manner. Although prolonged Hyper IL-6 treatment in mice on a HFD led to a propensity towards fat mass loss, it also caused severe liver injury and insulin resistance, an affect that was unable to be prevented by co-treatment with sgp130Fc. As a consequence Hyper IL-6 remains a poor candidate as an anti-obesity drug.

REFERENCES

Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, Yan Z. 2005. Exercise stimulates pgc-1α transcription in skeletal muscle through activation of the p38 MAPK pathway. J Biol Chem. 280:19587-19593

Al-Khalili L, Bouzakri K, Glund S, Lönnqvist F, Koistinen HA and Krook A. 2006. Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. Mol Endocrinol. 20:3364-3375

Andersson LC, Gahmberg CG, Kimura AK and Wigzell H. 1978. Activated human T lymphocytes display new surface glycoproteins. Proc Natl Acad Sci. 75:3455-3458

Antonescu CN, Huang C, Niu W, Liu Z, Eyers PA, Heidenreich KA, Bilan PJ and Klip A. 2005. Reduction of insulin stimulated glucose uptake in L6 myotubes by the protein kinase inhibitor SB203580 is independent of p38 MAPK activity. Endocrinology. 146:3773-3781

Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, Schütz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schürmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S and Neurath MF. 2000. Blockade of Interleukin 6 trans signalling suppresses T-Cell resistance against apoptosis in chronic intestinal inflammation: Evidence in Crohn disease and experimental colitis *in vivo*. Nat Med. 6: 583-588

Auernhammer CJ, Bousquet C and Melmed S. 1999. Autoregulation of pituitary corticotroph SOCS-3 expression: Characterisation of the murine SOCS-3 promoter. Proc Natl Acad Sci USA. 96:6964-6969

Australian Government: Department of Health and Ageing. Health Priorities – Diabetes: Diabetes Data and Trends. 2008. Accessed online at http://www.health.gov.au/internet/main/publishing.nsf/content/pq-diabetes-stats. last updated: May 2008

Baeza-Raja B and Muňoz-Cánoves. 2004. p38 MAPK-induced nuclear factor-κB activity is required for skeletal muscle differentiation: role of interleukin-6. Mol Biol Cell. 15:2013-2026

Bazan JF. 1990. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA. 87:6934-6938

Bjørbæk C, Elmquist J, Frantz JD, Shoelson SE and Flier JS. 1998. Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell. 1:619-625

Black S, Kushner I and Samols D. 2004. C-reactive protein. J Biol Chem. 279:48487-48490

Boulanger MJ, Chow D, Brevnova EE and Garcia KC. 2003. Hexameric structure and assembly of the Interleukin-6/IL-6 α-receptor/gp130 Complex. Science. 300: 2101-2104

Briso EM, Dienz O and Rincon M. 2008. Cutting Edge: Soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T Cells. J Immunol 180: 7102-7106

Brunt EM. 2007. Pathology of fatty liver disease. Modern Pathol. 20:S40-S48

Bugianesi E, McCullough AJ and Marchesini G. 2005. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatology. 42:987-1000

Carey, AL and Febbraio, MA. 2004. Interleukin 6 and insulin sensitivity: friend or foe? Diabetologia. 47: 1135-1142

Carey AL, Steinberg GR, Macaulay SL, Holmes AG, Watt MJ, Kemp BE, Pedersen BK and Febbraio MA. 2006. The insulin sensitizing effect of acute Interleukin-6 treatment is mediated via activation of AMP-kinase. Diabetes. 55:2688-2697

Chalaris A, Rabe B, Paliga K, Lange H, Laskay T, Fielding CA, Jones SA, Rose-John S and Scheller S. 2007. Apoptosis is a natural stimulus of IL6R shedding and contributes to the pro-inflammatory trans-signalling function of neutrophils. Blood. 110:1748-1755

Chen SL, Loffler KA, Chen D, Stallcup MR and Muscat GEO. 2002. The co-activator associated Arginine Methyltransferase is necessary for muscle differentiation. J Biol Chem. 277:4324-4333

Chow D, He X, Snow AL, Rose-John S and Garcia KC. 2001. Structure of the extracellular gp130 cytokine receptor signaling complex. Science. 291:2150-2155

Content J, De Wit L, Pierard D, Derynck R, De Clercq and Fiers W. 1982. Secretory proteins induced in human fibroblasts under conditions used for the production of interferon β. Proc Natl Acad Sci USA. 79:2768-2772

Dalmon J, Laurent M and Courtois G. 1993. The human beta fibrinogen promoter contains a hepatocyte nuclear factor 1-dependent interleukin-6-responsive element. Mol Cell Biol. 13:1183-93.

De Filippis E, Alvarez G, Berria R, Cusi K, Everman S, Meyer C and Mandarino LJ. 2008. Insulin resistant muscle is exercise resistant: evidence for reduced response of nuclear encoded mitochondrial genes to exercise. Am J Physiol Endocrinol Metab. 294:E607-E614

Després J and Lemieux I. 2006. Abdominal obesity and metabolic syndrome. Nature. 444:881-887

Diamant M, Rienecka K, Mechtib N, Zhang X, Svensona M, Bendtzena K and Klein B. 1997. Cloning and expression of an alternatively spliced mRNA encoding a soluble form of the human Interleukin-6 signal transducer gp130. FEBS Lett. 412: 379-384

Ettinger MP, Littlejohn TW, Schwartz SL, Weiss SR, McIlwain HH, Heymsfield SB, Bray GA, Roberts WG, Heyman ER, Stambler N, Heshka S, Vicary C and Guler H. 2003. Recombinant variant of ciliary neurotrophic factor for weight loss in obese adults. JAMA 289: 1826-1832

Evans WJ, Morley JE, Argilés J, Bales C, Baracos V, Guttridge D, Jatoi A, Kalantar-Zadeh K, Lochs H, Mantovani G, Marks D, Mitch WE, Muscaritoli M, Najand A, Ponikowski P, Fanelli FR, Schambelan M, Schols A, Schuster M, Thomas D, Wolfe R and Anker SD. 2008. Cachexia: a new definition. Clin Nutr. 27:793-799

Febbraio MA. 2007. gp130 receptor ligands as potential therapeutic targets for obesity. 117:841-849

Febbraio MA, Hiscock N, Sacchetti M, Fischer CP and Pedersen BK. 2004. Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. Diabetes. 53:1643-1648

Fielding CA, McLoughlin RM, McLeod L, Colmont CS, Najdovska M, Grail D, Ernst M, Jones SA, Topley N and Jenkins BJ. 2008. IL-6 regulates neutrophil trafficking during acute inflammation via STAT-3. J Immunol. 181:2189-2195

Fingar DC, Salama S, Tsou C, Harlow E and Blenis J. 2002. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. 16:1472-1487

Fischer M, Goldschmitt J, Peschel C, Brakenhoff JPG, Kallen K, Wollmer A, Grötzinger J and Rose-John S. 1997. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. Nat Biotech. 15: 142-145

Gabay C. 2006. Interleukin-6 and Chronic Inflammation. Arthritis Research and Therapy 8(suppl 2):S3-S11

Galun E, Zeira E, Pappo O, Peters M and Rose-John S. 2000. Liver regeneration induced by a designer human IL-6/sIL-6R fusion protein reverses severe hepatocellular injury. FASEB J. 14:1979-1987

Garnier A, Fortin D, Zoll J, N'Guessan B, Mettauer B, Lampert E, Veksler V, Ventura-Clapier R. 2005. Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. FASEB J. 19:43-52

Gauldie J, Richards C, Harnish D, Lansdorp P and Bauman H. 1987. Interferon β_2/B Cell Stimulatory Factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA. 84:7251-7255

Geiger PC, Wright DC, Han D, and Holloszy JO. 2005. Activation of p38 MAP kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab. 288: E782-E788

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z and Puigserver P. 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/ PGC-1α. EMBO J. 26:1913-1923

Graeve L, Korolenko TA, Hemmann U, Weiergräber O, Dittrich E, Heinrich PC. 1996. A complex of the soluble Interleukin-6 receptor and Interleukin-6 is internalized via the signal transducer gp130. FEBS Lett. 399: 131-134

Gerhatz C, Heesel B, Sasse J, Hemmann U, Landgraf C, Schneider-Mergener J, Horn F, Heinrich PC and Graeve L. 1996. Differential activation of acute phase response factor/ STAT3 and STAT1 via the cytoplasmic domain of the Interleukin-6 Signal Transducer gp130. J Biol Chem. 271:12991-12998

Gerhartz-Hines Z, Rodgers JT, Bare O, Levin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z and Puigserver P. 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1α. EMBO J. 26:1913-1923

Goto M, Terada S, Kato M, Katoh M, Yokozeki T, Tabata I and Shimokawa T. 2000. cDNA cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. Biochem Biophys Res Commun. 274:350-354

Haan S, Ferguson P, Sommers U, Hiremath M, McVicar DW, Heinrich PC, Johnston JA and Cacalano NA. 2003. Tyrosine phosphorylation disrupts elongin interaction and accelerates SOCS-3 degradation. J Biol Chem. 278:31972-31979

Haegeman G, Content J, Volckaert G, Derynck R, Tavernier J and Fiers W. 1986. Structural analysis of the sequence coding for an inducible 26-kDa protein in human fibroblasts. Eur J Biochem. 159: 625-632

Hammarstedt A, Jansson PA, Wesslau C, Yang X and Smith U. 2003. Reduced expression of PGC-1 and insulin signalling molecules in adipose tissue is associated with insulin resistance. Biochem Biophys Res Commun. 301:578-582

Handschin C and Spiegelman BM. 2006. Peroxisome proliferators-activated receptor γ coactivator 1 coactivators, energy homeostasis and metabolism. Endocrin Rev. 27:728-735

Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, LeBrasseur NK, Yan Z and Spiegelman BM. 2007. Skeletal muscle fiber-type switching, exercise intolerance,

and myopathy in PGC-1α muscle-specific knock out animals. J Biol Chem. 282: 30014-30021

Hardie DG and Sakamoto K. 2006. AMPK: a key sensor of fuel and energy status in skeletal muscle. Physiol. 21:48-60

Hattori M, Abraham LJ, Northemann W and Fey GH. 1990. Acute phase reaction induces a specific complex between hepatic nuclear proteins and the interleukin-6 response element of the rat α_2-macroglobulin gene. Proc Natl Acad Sci USA. 87:2364-2368

Heinrich PC, Behrmann I, Müller-Newen G, Schaper F and Graeve L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J. 334: 297-314

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G and Schaper F. 2003. Principles of Interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J. 374:1-20

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, Tsunasawa S, Sakiyama F, Matsu H, Takahara Y, Taniguchi T and Kishimoto T. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature. 324:73-76

Hirano T, Ishihara K and Hibi M. 2000. Roles of STAT-3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family cytokine of receptors. Oncogene. 19:2548-2556

Hiscock N, Chan MH, Bisucci T, Darby IA and Febbraio MA. 2004. Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fibre type specificity. FASEB J. 18: 992-4

Holmes AG, Mesa JL, Neill BA, Chung J, Carey AL, Steinberg GR, Kemp BE, Southgate RJ, Lancaster GI, Bruce CR, Watt MJ and Febbraio MA. 2008. Prolonged interleukin-6 administration enhances glucose tolerance and increases skeletal muscle PPARα and UCP2 expression in rats. J Endocrinol. 198:367-374

Hotamisligil GS. 2006. Inflammation and metabolic disorders. Nature. 444:860-867

Houten SM, Auwerx J. 2004. PGC-1α: Turbocharging Mitochondria. Cell. 119:5-7

Hussey HJ, Todorov PT, Field WN, Inagaki N, Tanaka Y, Ishitsuka H and Tisdale MJ. 2000. Effect of a fluorinated pyrimidine on cachexia and tumour growth in murine cachexia models: relationship with a proteolysis inducing factor. Br J Cancer. 83:56-62

Ikeda S, Kawamoto H, Kasaoka K, Hitomi Y, Kizaki T, Sankai Y, Ohno H, Haga S and Takemasa T. 2006. Muscle type-specific response of PGC-1α and oxidative enzymes during voluntary wheel running in mouse skeletal muscle. Acta Physiol. 188:217-223

Islam O, Gong X, Rose-John S and Heese K. 2009. Interleukin-6 and neural stem cells: more than gliogenesis. Mol Biol Cell. 20: 188-199

Jäger S, Handschin C, St-Pierre J and Spiegelman BM. 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. Proc Natl Acad Sci. 104:12017-12022

Jenkins BJ, Grail D, Nheu T, Najdovska M, Wang B, Waring P, Inglese M, McLoughlin RM, Jones SA, Topley N, Baumann H, Judd LM, Giraud AS, Boussioutas A, Zhu H and Ernst M. 2005. Hyperactivation of Stat3 in gp130 mutant mice promotes gastric hyperproliferation and desensitizes TGF-β Signalling. Nat Med. 11: 845-852

Jenkins BJ, Roberts AW, Najdovska M, Grail D and Ernst M. 2005. The threshold of gp130-dependent STAT3 signalling is critical for normal regulation of hematopoiesis. Blood. 105:3512-3520

Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ and Thompson CB. 2005. AMP-activated protein kinase induces p53-dependent metabolic checkpoint. Mol Cell. 18:283-293

Jones SA. 2005. Directing Transition from Innate to Acquired Immunity: Defining a role for IL-6. J Immunol 175: 3463-3468

Jostock T, Müllberg J, Özbek S, Atreya R, Blinn G, Voltz N, Fischer M, Neurath MF and Rose-John S. 2001. Soluble gp130 is the natural inhibitor of soluble Interleukin-6 receptor transsignalling responses. Eur J Biochem. 268: 160-167

Kahn SE, Hull RL and Utzschneider KM. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 444:840-846

Kami K, Morikawa Y, Sekimoto M and Senba E. 2000. Gene expression of receptors for IL-6, LIF, and CNTF in regenerating skeletal muscles. J Histochem Cytochem. 48:1203-1213

Kanemaki T, Kitade H, Kaibori M, Sakitani K, Hiramatsu Y, Kamiyama Y, Ito S and Okumura T. 1998 Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. Hepatology. 27:1296-303.

Keller C, Keller P, Marshal S and Pedersen BK. 2003. IL-6 gene expression in human adipose tissue in response to exercise: effect of carbohydrate ingestion. J Physiol. 550:927-931

Kelley DE, McKolanis TM, Hegazi RAF, Kuller LH and Kalhan SC. 2003. Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids and insulin resistance. Am J Physiol Endocrinol Metab. 285:E906-E916

Kelly M, Keller C, Avilucea PR, Keller P, Luo Z, Xiang X, Giralt M, Hidalgo J, Saha AK, Pedersen BK and Ruderman NB. 2004. AMPK activity is diminished in tissues of IL-6 knock out mice: the effect of exercise. Biochem Biophys Res Commun. 320:449-454

Kimura N, Tokunaga C, Dalal S, Richardson C, Yoshino K, Hara K, Kemp BE, Witters LA, Mimura O and Yonezawa. 2003. A possible link between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. Genes Cells. 9:65-79

Knight B, Matthews VB, Akhurst B, Croager EJ, Klinken E, Abraham LJ, Olynyk JK and Yeoh G. 2005. Liver inflammation and cytokine prodution, but not acute phase protein synthesis, accompany the adult progenitor (oval) cell response to liver injury. Immunol Cell Biol. 83:364-374

Koo S, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H and Montminy M. 2005. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature. 437:1109-1114

Kordula T, Rydel RE, Brigham EF, Horn F, Heinrich PC and Travis J. 1998. Oncostatin M and the Interleukin-6 and Soluble Interleukin-6 receptor complex regulate α_1-antichymotrypsin expression in human cortical astrocytes. J Biol Chem. 273:4112-4118

Körmöczi GF, Säemann MD, Buchta C, Peck-Radosavljevic M, Mayr WR, Scwartz DWM, Dunkler D, Spitzauer S and Panzer S. 2006. Influence of clinical factors on the haemolysis marker haptoglobin. Eur J Clin Invest. 36:202-209

Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirschman MF and Goodyear L. 2006. AS160 regulates insulin and contraction stimulated glucose uptake in mouse skeletal muscle. J Biol Chem. 281: 31478-31485

Krebs DL and Hilton DJ. 2001. SOCS proteins: negative regulators of cytokine signalling. Stem Cells. 19: 378-387

Lahn M, Kunzmann R, Köhler G, Iklé DN, Hentrich I, Jesuiter H, Kilmburg P, Veelken H, Mackensen A, Rosenthal F and Lindemann A. 1997. Comparison of cytogenetics, cytokine secretion, and oncogene expression in primary cultures of renal carcinoma cells. Oncology. 54: 429-437

Larance M, Ramm G, Stöckli J, van Dam EM, Winata S, Wasinger V, Simpson F, Graham M, Junutula JR, Guilhaus M and James DE. 2005. Characeterisation of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. J Biol Chem. 280:37803-37813

Lee WJ, Kim M, Park H, Kim HS, Jeon MJ, Oh KS, Koh EH, Won JC, Kim M, Oh GT, Yoon M, Lee K and Park J. 2006. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1. Biochem Biophys Res Commun. 340:291-295

Lee YM, Lee JO, Jung JH, Kim JH, Park SH, Park JM, Kim EK, Suh PG and Kim HS. 2008. Retinoic acid leads to cytoskeletal rearrangement through AMPK-Rac1 and stimulates glucose uptake through AMPK-p38 MAPK in skeletal muscle. J Biol Chem. 283:33969-33974

Lemieux K, Konrad D, Klip A and Marette A. 2003. The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules but stimulates glucose uptake and p38 mitogen-activated protein kinase α and β in skeletal muscle. FASEB J. 17:1658-1665

Lerin C, Rodgers JT, Kalume DE, Kim SH, Pandey A, and Puigserver P. 2006. GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. Cell Metab. *3*: 429-438

Leu C, Wong F, Chang C, Huang S and Hu C. 2003. Interleukin-6 acts as an antiapoptotic factor in human esophagael carcinoma cells through the activation of both STAT3 and mitogen activated protein kinase pathways. Oncogene. 22:7809-7818

Li X, Monks B, Ge Q and Birnbaum MJ. 2007. Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1α transcription co-activator. Nature. 447:1012-1016

Li Y, Li J, Zhu J, Sun B, Branca M, Tang Y, Foster W, Xiao X and Huard J. 2007. Decorin gene transfer promotes muscle cell differentiation and regeneration. Mol Ther. 15: 1616-1622

Lienenlüke B and Christ B. 2007. Impact of interleukin-6 on the glucose metabolic capacity in rat liver. Histochem Cell Biol. 128:371-377

Lin J, Wu H, Tarr PT, Zhang C, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson E, Lowell BB, Bassel-Duby R and Spiegelman BM. 2002. Transcriptional co-activator PGC-1α drives the formation of slow twitch muscle fibres. Nature. 418: 797-801

Liu Z and Fuller GM. 1995. Detection of a novel transcription factor for the A alpha fibrinogen gene in response to interleukin-6. J Biol Chem. 1995 Mar 31;270(13):7580-6.

Lowes KN, Brennan BA, Yeoh GC and Olynyk JK. 1999. Oval cell numbers in human chronic liver disease are directly related to disease severity. Am J Pathol. 154:537-541

Lust JA, Donovan KA, Kline MP, Greipp PR, Kyle RA and Maihle NJ. 1992. Isolation of an mRNA encoding a soluble form of the human Interleukin-6 receptor. Cytokine. 4:96-100

Lütticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC and Horn F. 1994. Association of transcription factor APRF and protein kinase Jak1 with the Interleukin-6 signal transducer gp130. Science. 263:89-92

MacDonald C, Wojtaszewski JF, Pedersen BK, Kiens B and Richter EA. 2003. Interleukin-6 release from human skeletal muscle during exercise: relation to AMPK activity. J Appl Physiol. 95:2273-2277

Maione D, Di Carlo E, Li W, Musiani P, Modesti A, Peters M, Rose-John S, Della Rocca C, Tripodi M, Lazzaro D, Taub R, Savino R and Ciliberto G. 1998. Coexpression of IL-6 and soluble IL-6R causes nodular regenerative hyperplasia and adenomas of the liver. EMBO J. 17: 5588-5597

Mathai AS, Bonen A, Benton CR, Robinson DL, Graham TE. 2008. Rapid exercise-induced changes in PGC-1α mRNA and protein in human skeletal muscle. J Appl Physiol. 105:1098-1105

Matsumoto M, Han S, Kitamura T and Accili D. 2006. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest. 116:2464-2472

Matthews V, Schuster B, Schütze S, Bussmeyer I, Ludwig A, Hundhausen C, Sadowski T, Saftig P, Hartmann D, Kallen K and Rose-John S. 2003. Cellular cholesterol depletion triggers shedding of the human Interleukin-6 receptor by ADAM10 and ADAM17 (TACE). J Biol Chem. 278:38829-38839

May LT, Santhanam U, Tatter SB, Ghrayeb J and Sehgal PB. 1989. Multiple forms of human Interleukin-6 – phosphoglycoproteins secreted by many different tissues. Annals NY Acad Sci. 557: 114-119

Miles SA, Rezai AR, Salazar-González JF, Vander Meyden M, Stevens RH, Logan DM, Mitsuyasu RT, Taga T, Hirano T and Kishimoto T. 1990. AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc Natl Acad Sci U S A. 87:4068-72

Mock BA, Nordan RP, Justice MJ, Kozak C, Jenkins NA, Copeland NG, Clark SC, Wong GG and Rudikoff S. 1989. The murine IL-6 gene maps to the proximal region of chromosome 5. J Immunol. 142:1372-1376

Montero-Julian FA, Brailly H, Sautès C, Joyeux I, Dorval T, Mosseri V, Yasukawa K, Wijdenes J, Adler A, Gorin I, Fridman WH and Tartour E. 1997. Characterization of soluble gp130 released by melanoma cell lines: a polyvalent antagonist of cytokines from the Interleukin 6 family. Clin Can Res. 3:1443-1451

Mootha VK, Lindgren CM, Eriksson K, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC. 2003. PGC-1α-responsive genes involved in oxidative phosphorylation are co-ordinately downregulated in human diabetes Nature Genetics. 34: 267-273

Motoshima H, Goldstein BJ, Igata M and Araki E. 2006. AMPK and cell proliferation – AMPK as a therapeutic target for atherosclerosis and cancer. J Physiol. 574:63-71

Müllberg J, Dittrich E, Graeve L, Gerhartz C, Yasukawa K, Taga T, Kishimoto T, Heinrich P and Rose-John S. 1993. Differential shedding of the two subunits of the interleukin-6 receptor. FEBS Lett. 332: 174-178

Nandurkar HH, Hilton DJ, Nathan P, Willson T, Nicola N and Begley CG. 1996. The human IL-11 receptor requires gp130 for signaling: demonstration by molecular cloning of the receptor. Oncogene. 12:585-593

Narazaki M, Yasukawa K, Saito T, Oshugi Y, Fukui H, Koishihara Y, Yancopoulos GD, Taga T and Kishimoto T. 1993. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. Blood. 82:1120-1126

Naugler WE, Sakurai T, Kim S, Maeda S, Kim KH, Elsharkawy AM and Karin M. 2007. Gender disparity in Liver Cancer due to sex differences in MyD88-dependent IL-6 production. Science. 317:121-124

Niesler CU, Myburgh KH and Moore F. 2007. The changing AMPK expression in differentiating mouse skeletal mouse myoblast cells helps confer increasing resistance to apoptosis. Exp Physiol. 92:207-217

Niu W, Huang C, Nawaz Z, Levy M, Somwar R, Li D, Bilan PJ and Klip A. 2003. Maturation of the regulation of GLUT4 activity by p38 MAPK during L6 myogenesis. J Biol Chem. 278:17953-17962

Northemann W, Braciak TA, Hattori M, Lee F and Fey GH. 1989. Structure of the rat interleukin-6 gene and its expression in macrophage derived cells. J Biol Chem. 264:16072-16082

Nowell MA, Richards PJ, Horiuchi S, Yamamoto N, Rose-John S, Topley N, Williams AS and Jones SA. 2003. Soluble IL-6 Receptor governs IL-6 activity in experimental arthritis: Blockade of arthritis severity by soluble glycoprotein 130. J Immunol. 171: 3202-3209

Nowell MA, Williams AS, Carty SA, Scheller J, Hayes AJ, Jones GW, Richards PJ, Slinn S, Ernst M, Jenkins BJ, Topley N, Rose-John S and Jones SA. 2009. Therapeutic targeting of IL-6 trans signaling counteracts STAT3 control of experimental inflammatory arthritis. J Immunol. 182:613-622

Oliviero S and Cortese R. 1989. The human haptoglobin gene promoter: interleukin-6-responsive elements interact with a DNA-binding protein induced by interleukin-6. EMBO J. 8: 1145-1151

Okada M, Sakaguchi N, Yoshimura N, Hara H, Shimizu K, Yoshida N, Yoshizaki Z, Kishimoto S, Yamamura Y and Kishimoto T. 1983. B Cell growth factors and B Cell differentiation factor from human T Hybridomas. J Exp Med. 157: 583-590

Okoshi R, Ozaki T, Yamamoto H, Ando K, Koida N, Ono S, Koda T, Kamijo T, Nakagawara A, Kizaki H. 2008. Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. J Biol Chem. 283:3979-3987

Ostrowski K, Rohde T, Zacho M, Asp S and Pedersen BK. 1998. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. J Phsiol. 508:949-953

Özbek S, Peters M, Breuhahn K, Mann A, Blessing M, Fischer M, Schirmacher P, Mackiewicz A and Rose-John S. 2001. The designer cytokine hyper IL-6 mediates growth inhibition and GM-CSF-dependent rejection of B16 melanoma cells. Oncogene. 20: 972-979

Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyakazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn RC, Mandarino LJ. 2003. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci USA. 100: 8466-8471

Pedersen BK and Febbraio MA. 2008. Muscle as an endocrine organ: focus on muscle derived interleukin-6. Physiol Rev. 88: 1379-1406

Peters M, Jacobs S, Ehlers M, Vollmer P, Müllberg J, Wolf E, Brem G, Meyer zum Büschenfelde K and Rose-John S. 1996. The function of the soluble Interleukin-6 (IL-6) Receptor in vivo: Sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of plasma half-life of IL-6. J Exp Med. 183: 1399-1406

Peters M, Schirmarcher P, Goldschmitt J, Odenthal M, Peschel C, Fattori E, Ciliberto G, Dienes H, Meyer zum Büschenfelde K and Rose-John S. 1997. Extramedullary Expansion of Hematopoietic Progenitor Cells in Interleukin (IL)-6-sIL-6R Double Transgenic Mice. J Exp Med. 185: 755-766

Peters M, Odenthal M, Schirmacher P, Blessing M, Fattori E, Ciliberto G, Meyer zum Büschenfelde K and Rose-John S. 1997. Soluble IL-6 receptor leads to a paracrine modulation of the IL-6 hepatic acute phase response in double transgenic mice. J Immunol. 159: 1474-1481

Peters M, Blinn G, Solem F, Fischer M, Meyer zum Büschenfelde K and Rose-John S. 1998. In vivo and in vitro activities of the gp-130 stimulating designer cytokine Hyper IL-6. J Immunol. 161: 3575-3581

Pilegaard H, Saltin B, Neufer PD. 2003. Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle. J Physiol 546: 851-858

Poupart P, Vandenabeele P, Cayphas S, Van Snick J, Haegeman G, Kruys V, Fiers W and Content J. 1987. B Cell growth modulating and differentiating activity of recombinant human 26-kd protein (BSF-2, HuIFN-β_2, HPGF). EMBO J. 6: 1219-1224

Powell-Tuck J. 1986. Protein metabolism in inflammatory bowel disease. Gut. 27:67-71

Pradhan AD, Manson JE, Rifai N, Buring JE and Ridker PM. 2001. C-reactive protein, interleukin-6, and risk of developing type 2 Diabetes Mellitus. JAMA. 286:327-334

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang C, Krauss S, Mootha VK, Lowell BB, Spiegelman, BM. 2001. Cytokine stimulation of energy expenditure through p38 MAP Kinase Activation of PPARγ Coactivator-1. Mol. Cell. 8:971-982

Puigserver P, Spiegelman BM. 2003. Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α): Transcriptional Coactivator and Metabolic Regulator. Endocrine Reviews. 24: 78-90

Pullen AH. 1977. The distribution and relative sizes of three histochemical fibre types in the rat *tibialis anterior* muscle. J Anat. 123:1-19

Rabe B, Chalaris A, May U, Waetzig GH, Seegert D, Williams AS, Jones SA, Rose-John S and Scheller J. 2008. Transgenic Blockade of Interleukin-6 trans-sginalling abrogates inflammation. Blood. 111:1021-1028

Rakemann T, Niehof M, Kubicka S, Fischer M, Mann MP, Rose-John S and Trautwein C. 1999. The designer cytokine Hyper Interleukin-6 is a potent activator of STAT-3 dependent gene transcription in vivo and in vitro. J Biol Chem. 274: 1257-1266

Raney MA and Turcotte LP. 2006. Regulation of contraction-induced FA uptake and oxidation by AMPK and ERK1/2 is intensity dependent in rodent muscle. Am J Physiol Endocrinol Metab. 291:E1220-E1227

Reddy JK and Rao MS. 2006. Lipid metabolism and liver inflammation. Fatty Liver disease and fatty acid oxidation. Am J Physiol Gastrointest liver physiol. 290:G852-G858.

Rhee J, Ge H, Yang W, Fan M, Handschin C, Cooper M, Lin J, Li C and Spiegelman BM. 2006. Partnership of PGC-1α and HNF4α in the regulation of lipoprotein metabolism. J Biol Chem. 281:14683-14690

Richards PJ, Nowell MA, Horiuchi S, McLoughlin RM, Fielding CA, Grau S, Yamamoto N, Ehrmann M, Rose-John S, Williams AS, Topley N and Jones SA. 2006. Functional characterisation of a soluble gp130 isoform and its therapeutic capacity in an experimental model of inflammatory arthritis. Arthritis and Rheumatism. 54: 1662-1672

Ritchie DG and Fuller GM. 1983. Hepatocyte stimulating factor: a monocyte derived acute phase regulatory protein. Ann N Y Acad Sci. 408:490-502

Rodgers JT, Lerin C, Haas W, Gygl SP, Spiegelman BM and Puigserver P. 2005. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. Nature. 434:113-118

Rose-John S. 2003. Interleukin-6 biology is co-ordinated by membrane bound and soluble receptors. Acta Biochim Pol. 50:604-611

Rose-John S, Scheller J, Elson G and Jones SA. 2006. Interleukin-6 biology is co-ordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol. 80:227-236

Ruderman NB, Saha AK and Kraegen EW. 2003. Minireview: malonyl CoA, AMP-activated protein kinase and adiposity. Endocrinology. 144:5166-5171

Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK and Davis RJ. 2008. A stress signalling pathway in adipose tissue regulates hepatic insulin resistance. Science. 322:1539-1543

Sander LE, Obermeier F, Dierssen U, Kroy DC, Singh AK, Seidler U, Streetz KL, Lutz HH, Müller W, Tacke F and Trautwein C. 2008. Gp130 Signalling promotes development of acute experimental colitis by facilitating early neutrophil/macrophage recruitment and activation. J Immunol. 181:3586-3594

Sano H, Eguez L, Teruel MN, Fukuda M, Chuang TD, Chavez JA, Lienhard GE and McGraw TE. 2007. Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. Cell Metab. 5: 293-303

Santhanam U, Ghrayeb J, Sehgal PB and May LT. 1989. Post translational modifications of human interleukin-6. Arch Biochem Biophys. 274: 161-170

Schwabe M, Zhao J and Kung H. 1994. Differential expression and ligand-induced modulation of the human interleukin-6 receptor on interleukin-6 responsive cells. J Biol Chem. 269:7201-7209

Semple RK, Crowley VC, Sewter CP, Laudes M, Christodoulides C, Considine RV, Vidal-Puig A, O'Rahilly S. 2003. Expression of the thermogenic nuclear hormone receptor coactivator PGC-1α is reduced in the adipose tissue of morbidly obese subjects. Int. J. Obesity: 1-4

Senn, JJ., Klover, PJ, Nowak, IA and Mooney, RA. 2002. Interleukin-6 induces cellular insulin resistance in Hepatocytes. Diabetes 51: 3391-3399

Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M and Muňoz-Cánoves P. 2008. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab. 7:33-44

Siewart E, Müller-Esterl W, Starr R, Heinrich PC and Schaper F. 1999. Different protein turnover of interleukin-6-type cytokine signalling components. Eur J Biochem. 265:251-257

Somers W, Stahl M and Seehra JS. 1997. 1.9 Å crystal structure of Interleukin 6: Implications for a novel mode of receptor dimerization and signalling. EMBO J. 16:989-997

Spagenburg EE, Brown DA, Johnson MS and Moore RL. 2006. Exercise increases SOCS-3 expression in rat skeletal muscle: potential relationship to IL-6 expression. J Physiol. 572:839-848

Steinberg GR and Jœrgensen SB. 2007. The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. Mini Rev Med Chem. 7:519-526

Steinberg GR, McAinch AJ, Chen MB, O'Brien PE, Dixon JB, Cameron-Smith D and Kemp BE. 2006. The suppressor of cytokine signalling 3 inhibits leptin activation of AMP-Kinase in cultured skeletal muscle of obese humans. J Clin Endocrinol. 91:3592-3597

Stouthard JML, Oude Elferink RPJ and Sauerwein HP. 1996. Interleukin-6 enhances glucose transport in 3T3-L1 adipocytes. Biochem Biophys Res Commun. 220:241-245

Stoyan T, Michaelis U, Schooltink H, Van Dam M, Rudolph R, Heinrich PC and Rose-John S. 1993. Recombinant soluble human interleukin-6 receptor:

expression in Escherichia Coli, renaturation and purification. Eur J Biochem. 216:239-245

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN and Yancopoulos GD. 1994. Association and activation of Jak-Tyk Kinases by CNTF-LIF-OSM-IL-6β receptor components. Science. 263: 92-95

Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Yancopoulos GD. 1995. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science. 267:1349-1353

Steinberg GR and Jørgensen SB. 2007. The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. Mini reviews Med Chem. 7:521-528

Streetz KL, Luedde T, Manns MP and Trautwein C. 2000. Interleukin-6 and liver regeneration. Gut. 47:309-312

Strick-Marchand H, Masse GX, Weiss MC and Di Santo JP. 2008. Lymphocytes support oval cell-dependent liver regeneration. J Immunol. 181:2764-2771

Suliman HB, Carraway MS, Welty-Wolf KE, Whorton AR and Piantadosi CA. 2003. Lipopolysaccharide stimulates mitochondrial biogenesis via activation of nuclear respiratory factor-1. J Biol Chem. 278:41510-41518

Sweeney G, Somwar R, Ramlal T, Volchuk A, Ueyama A and Klip A. 1999. An inhibitor of p38 mitogen activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes,

Tanabe O, Akira S, Kamiya T, Wong GG, Hirano T and Kishimoto T. 1988. Genomic structure of the murine IL-6 gene: high degree conservation of potential regulatory sequences between mouse and human. J Immunol. 141:3875-3881

Tanaka M, Kishimura M, Ozaki S, Osakada F, Hashimoto H, Okubo M, Murakami M and Nakao K. 2000. Cloning of novel soluble gp130 and detection of its neutralizing autoantibodies in rheumatoid arthritis. J Clin Invest. 106: 137-144

Tang Y, Kitisin K, Jogunoori W, Li C, Deng C, Mueller SC, Ressom HW, Rashid A, He AR, Mendelson JS, Jessup JM, Shetty K, Zasloff M, Mishra B, Reddy EP, Johnson L and Mishra L. 2008. Progenitor/stem cells give rise to liver cancer due to aberrant TGF-β and IL-6 Signalling. Proc Natl Acad Sci USA. 105:2445-2450

Targher G, Zenari L, Bertolini L, Muggeo M, Zoppini G. 2001. Elevated levels of interleukin-6 in young adults with type I diabetes without clinical evidence of microvascular and macrovascular complications. Diabetes Care. 24:956-957

Tasić D, Dimov D, Gligorijević J, Veličković L, Katić K, Krstić M and Dimov I. 2003. Muscle fibre types and fibre morphometry in the tibialis posterior and anterior of the rat: a comparative study. Med Biol. 10:16-21

Taylor EB, Lamb JD, Hurst RW, Chesser DG, Elligson WJ, Greenwood LJ, Porter BB, Herway ST and Wander WW. 2005. Endurance training increases skeletal muscle LKB1 and PGC-1α protein abundance effects of time and intensity. Am J Physiol Endocrinol Metab. 289:E960-E968

Tenhumberg S, Waetzig GH, Chalaris A, Rabe B, Seegert D, Scheller J, Rose-John S and Grötzinger. 2008. Structure optimisation of the Interleukin-6 trans-signalling antagonist sgp130. J Biol Chem. 283: 27200-27207

Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T andTabata I. 2002. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 296: 350-354

Teyssier C, Ma H, Emter R, Kralli A and Stallcup M. 2005. Activation of nuclear receptor co-activator PGC-1α by arginine methylation. Genes Dev. 19:1466-1473

Torisu T, Sato N, Yoshiga D, Kobayashi T, Yoshioka T, Mori H, Iida M and Yoshimura A. 2007. The dual function of hepatic SOCS-3 in insulin resistance *in vivo*. Genes Cells. 12:143-154

Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jørgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR and Wojtaszewski JFP. 2006. AMPK Mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. Diabetes 55:2051-2058

Ueki K, Kondo T and Kahn CR. 2004. Suppressor of cytokine signalling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol. 24:5434-5446

Van Damme J, Cayphas S, Van Snick J, Conings R, Put W, Lenaerts J, Simpson RJ and Billiau A. 1987. Purification and characterization of human fibroblast-derived hybridoma growth factor identical to T-cell-derived B-cell stimulatory factor-2 (interleukin-6). Eur J Biochem. 168: 543-550

Van Damme J, Opdenakker G, Simpson RJ, Rubira MR, Cayphas S, Vink A, Billiau A and Van Snick J. 1987. Identification of the human 26-kD protein, Interferon β_2 (IFN- β_2), as a B Cell hybridoma/plasmacytoma growth factor induced by Interleukin 1 and Tumor Necrosis Factor. J Exp Med. 165: 914-919

van Hall G, Steensberg A, Sacchetti M, Fischer C, Keller C, Schjerling P, Hiscock N, Møller K, Saltin B, Febbraio MA and Pedersen BK. 2003. Interleukin-6 stimulates lipolysis and fat oxidation in humans. J Clin Endocrinol Metab. 88:3005-3010

Ventura-Clapier R, Garnier A and Veksler V. 2008. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1α. Cardiovasc Res. 79:208-217

Vollmer P, Walev I, Rose-John S and Bhakdi S. 1996. Novel pathogenic mechanism of microbial metalloproteinase: liberation of membrane anchored molecules in biologically active form exemplified by studies with the human Interleukin-6 receptor. Inf Immun. 64:3646-3651

Walev I, Vollmer P, Palmer M, Bhakdi S and Rose-John S. 1996. Pore forming toxins trigger shedding of receptors for interleukin 6 and lipopolysaccharide. Proc Natl Acad Sci USA. 93:7882-7887

Wang Y and Fuller GM. 1994. Phosphorylation and internalization of gp130 occur after IL-6 activation of Jak2 Kinase in hepatocytes. Mol Biol Cell. 5: 819-828

Ward LD, Howlett GJ, Discolo G, Yasukawa K, Hammacher A, Moritz RL and Simpson RJ. 1994. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor and gp130. J Biol Chem. 16:23286-23289

Watt MJ, Dzamko N, Thomas WG, Rose-John S, Ernst M, Carling D, Kemp BE, Febbraio MA and Steinberg GR. 2006. CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK Nat Med 12:541-548

Wegenka UM, Buschmann J, Lütticken C, Heinrich PC and Horn F. 1993. Acute-phase response factor, a nuclear factor binding to acute-phase response elements,

is rapidly activated by Interleukin-6 at the post-translational level. Mol Cell Biol. 13: 276-288

Wegiel B, Bjartell A, Culig Z and Persson JL. 2008. Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. Int J Cancer. 122:1521-1529

Weiergräber O, Hemmann U, Küster A, Müller-Newen G, Schneider J, Rose-John S, Kurschat P, Brakenhoff JPJ, Hart MHL, Stabel S and Heinrich PC. 1995. Soluble human interleukin-6 receptor: Expression in insect cells, purification and characterisation. Eur J Biochem. 234:661-669

Weigert C, Hennige AM, Brodbeck K, Häring HU and Schleicher ED. 2005. Interleukin-6 acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser473 of Akt. Am J Physiol Endocrinol Metab 289: E251-7

Weissenbach J, Chernajovsky Y, Zeevi M, Shulman L, Soreq H, Nir U, Wallach D, Perricaudet M, Tiollais P and Revel M. 1980. Two interferon mRNAs in human fibroblasts: *in vitro* translation and *Escherichia coli* cloning studies. Proc Natl Acad Sci. 77:7152-7156

von Haehling S, Genth-Zotz S, Anker SD, Volk HD. 2002. Cachexia: a therapeutic approach beyond cytokine antagonism. Int J Cardiol. 85: 173-183

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic co-activator PGC-1. Cell. 98:115-124

Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T and Kishimoto T. 1988. Cloning and expression of the human Interleukin-6 (BSF-2/IFNβ 2) receptor. Science. 241:825-828

Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S and Kishimoto T. 1987. Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. EMBO J. 6: 2939-2945

Yasumoto K, Mukaida N, Harada A, Kuno K, Akiyama M, Nakashima E, Fujioka N, Mai M, Kasahara T, Fujimoto-Ouchi K, Mori K, Tanaka Y and Matsushima K. 1995. Molecular analysis of the cytokine network involved in cachexia in colon 26 adenocarcinoma bearing mice. Cancer Res. 55:921-927

Yawata H, Yasukawa K, Natsuka S, Murakami M, Yamasaki K, Hibi M, Taga T and Kishimoto T. 1993. Structure-Function analysis of human IL-6 receptor: dissociation of amino acid residues for IL-6-binding and for IL-6 signal transduction through gp130. EMBO J. 12:1705-1712

Yeoh GCT, Ernst M, Rose-John S, Akhurst B, Payne C, Long S, Alexander W, Croker B, Grail D and Matthews VB. 2007. Opposing roles of gp130-mediated STAT-3 and ERK-1/2 signalling in liver progenitor cell migration and proliferation. Hepatology. 45:486-494

Yin XM, Ding W and Gao W. 2008. Autophagy in the liver. Hepatology. 47:1773-1785

Yki-Järvinen H, Young AA, Lamkin C, and Foley JE. 1987. Kinetics of glucose disposal in whole body and across the forearm in man. J Clin Invest. 79: 1713-1719

Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB and Spiegelman BM. 2001. Control of

hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature. 413: 131-138

Yuen DYC, Dwyer RM, Matthews VB, Zhang L, Drew BG, Neill B, Kingwell BA, Clark MG, Rattigan S and Febbraio MA. 2009. IL-6 attenuates insulin-mediated increases in endothelial cell signalling but augments skeletal muscle insulin action via differential effects on Tumor Necrosis Factor α expression. Diabetes 58: 1086-1095

Ždychová J and Komers R. 2005. Emerging role of Akt Kinase/Protein Kinase B signalling in pathophysiology of diabetes and its complications. Physiol Res. 54:1-16

Zhang D, Sun M, Samols D and Kushner I. 1996. STAT3 participates in transcriptional activation of the C-reactive protein gene by Interleukin-6. J Biol Chem. 271:9503-9509

Zhang X, Blenis J, Li H, Schindler C and Chen-Kiang S. 1995. Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science. 267:1990-1994

Zhang Z, Fuentes NL and Fuller GM. 1995. Characterization of the IL-6 responsive elements in the gamma fibrinogen gene promoter. J Biol Chem. 270:24287-91.

Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum M, Shulman GI. 2002. AMP Kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc Natl Acad Sci USA 99:15983-15987