ROLE OF INTERLEUKIN-6 IN STATES OF METABOLIC HEALTH AND DISEASE

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

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DECLARATION

I, the candidate, Anna Greer Holmes, certify that:

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

b) the work has not been submitted previously, in whole or in part, to qualify for any other academic award;

c) the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program.

Signature of the candidate

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<tbody>
<tr>
<td>ADR</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>Akt</td>
<td>Acute transforming retrovirus thymoma</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-dependent protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Chow</td>
<td>Standard chow diet</td>
</tr>
<tr>
<td>Con</td>
<td>Control</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EDL</td>
<td><em>Extensor digitorum longus</em> muscle</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>GDR</td>
<td>Glucose disposal rate</td>
</tr>
<tr>
<td>GIR</td>
<td>Glucose infusion rate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter protein</td>
</tr>
<tr>
<td>HF</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HGP</td>
<td>Hepatic glucose production</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostatic model assessment</td>
</tr>
<tr>
<td>IkBa</td>
<td>Inhibitor of kappa B kinase alpha</td>
</tr>
<tr>
<td>IkKB</td>
<td>Inhibitor of kappa kinase beta</td>
</tr>
<tr>
<td>IkK</td>
<td>Inhibitor of kappa B kinase</td>
</tr>
<tr>
<td>IONO</td>
<td>Ionomycin</td>
</tr>
<tr>
<td><em>i.p.</em></td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-6&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Interleukin-6 knockout mice</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus-activated protein kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LCACoA</td>
<td>Long-chain acyl-coenzyme A</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified free fatty acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-beta</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferators-activated receptors</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>rhIL-6</td>
<td>Recombinant human IL-6</td>
</tr>
<tr>
<td>rmIL-6</td>
<td>Recombinant murine IL-6</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sIL-6R&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Soluble interleukin-6 receptor</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressors of cytokine signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VO$_2$</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>VO$<em>2$$</em>{\max}$</td>
<td>Maximal oxygen consumption</td>
</tr>
<tr>
<td>VO$<em>2$$</em>{\text{peak}}$</td>
<td>Peak oxygen consumption</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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</table>
ABSTRACT

Obesity and type 2 diabetes are the most prevalent metabolic diseases affecting over 50% of people in the western world. Although the pathogenesis of type 2 diabetes is not fully understood, growing evidence links this disease to a state of chronic inflammation, which occurs in metabolically active tissue such as the liver, adipose tissue and skeletal muscle and results in the secretion of inflammatory cytokines, of which interleukin-6 (IL-6) is one. It is generally accepted that elevations in the plasma and/or tissue of this family of cytokines have a negative effect on whole body glucose homeostasis. While there is compelling evidence for the negative effects of resistin and TNF-α on insulin sensitivity, the role of IL-6 in the etiology of insulin resistance is not fully understood. The notion of negative effects of IL-6 in metabolic processes is further confounded by the marked elevations of IL-6 which occur in conjunction with the beneficial activity of exercise. We firstly sought to examine the effect of the lipolytic hormone adrenaline on IL-6 expression and release in order to establish whether IL-6 acts independently of adrenaline in the regulation of fat metabolism. Reporting the absence of an effect of adrenaline on IL-6, we then investigated the role of IL-6 on metabolic processes in humans at rest and during exercise in circumstances where lipolysis was inhibited. Marked increases in IL-6 circulating protein and tissue gene expression were observed with exercise and further so with fatty acid suppression. In a mouse model of IL-6 depletion marked insulin sensitivity was observed, which was reversed with IL-6 treatment. In a mouse model with normal endogenous IL-6 levels IL-6 treatment also impaired glucose tolerance. Contrastingly, in a rat model both chronic and acute IL-6 treatment improved glucose tolerance.
In summary, studies from this thesis suggest that, rather than being causally related to insulin resistance, the cytokine IL-6 increases lipolysis, fat oxidation, and glucose metabolism in insulin sensitive tissues in humans. This does not appear to be the case in the mouse, where contrasting actions are observed, perhaps due to differences in the reliance of various parameters for metabolic processes between the species.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW
1.1 General introduction

Interleukin-6 (IL-6) was originally identified and reported by several groups, thus receiving a variety of different identifying names reflecting its multifunctional characteristics (Kamimura et al., 2003). The protein was originally sequenced 25 years ago (Weissenbach et al., 1980), and it was cloned and structurally characterized almost 20 years ago by Hirano (Hirano et al., 1986), and Yasukawa and colleagues (Yasukawa et al., 1987). The diverse repertoire of IL-6 expression includes T and B cells, carcinoma cells, endothelial cells, macrophages, mast cells, dendritic cells, islet β cells, and more recently has been found to be expressed in and released from adipose tissue (Mohamed-Ali et al., 1997; Fasshauer et al., 2004), skeletal (Chan et al., 2004a) and smooth (Zampetaki et al., 2005) muscle cells (Chan et al., 2004a; Zampetaki et al., 2005). Many immunomodulatory functions have been ascribed to IL-6, as reviewed by Hirano and colleagues (Hirano et al., 1990). However, more recently IL-6 has been shown to act on a number of tissues, whose principal roles are not of an inflammatory nature (Febbraio & Pedersen, 2002). The recent findings that IL-6 is both a prominent “adipokine” (Lazar, 2005) and “myokine” (Febbraio & Pedersen, 2005) has lead to much interest in the role of this cytokine on metabolic processes during health and disease.

1.2 Induction of interleukin-6 and subsequent signal transduction

IL-6 production is stimulated in response to a variety of stimuli. The signalling events leading to IL-6 production due to pathological stressors such as reactive oxygen species (Frost et al., 2003) and the inflammatory cytokines IL-1 and TNFα have been well established (Akira et al., 1993). Pathogens such as lipopolysaccharide also potently induce IL-6 production by mounting an innate immune response through toll-like receptors in skeletal
muscle and immune cells (Frost et al., 2005). Additionally, it has been known for some time
know that IL-6 is produced from contracting skeletal muscle in the absence of inflammation
(Febbraio & Pedersen, 2005). The differential factors regulating contraction-induced versus
inflammation-induced IL-6 production have not been fully described. Factors increasing
concomitantly with exercise have been implicated in IL-6 production independently of
exercise.

The calcium ionophore, ionomycin, potently increases IL-6 transcription in muscle cell
levels increase dramatically and could thus stimulate contraction-induced IL-6 production.
Prolonged contractile activity is characterised by low-amplitude and sustained elevations in
intracellular Ca^{2+}, leading to increased activity of calcineurin, a ubiquitous Ca^{2+}–calmodulin-
dependent protein phosphatase known to be a key mediator of Ca^{2+} signalling in muscle cells,
and contraction-induced IL-6 transcription has recently been associated with enhanced
calcineurin activity (Banzet et al., 2005). Free fatty acids also induce IL-6 production in
adipose and muscle cell culture (Weigert et al., 2004; Ajuwon & Spurlock, 2005; Jove et al.,
2005). The greater free fatty acid levels in exercise make a likely candidate for contraction-
induced IL-6 production. Furthermore, there is evidence to suggest that adrenergic regulation
may be important in regulating IL-6 as administration of adrenaline increases plasma IL-6 in
both rats (DeRijk et al., 1994) and humans (Sondergaard et al., 2000). During exercise the
increase in plasma adrenaline is marked and correlates with increases in IL-6 (Papanicolaou et
al., 1996) leading to the hypothesis that the increase in circulating adrenaline could mediate
IL-6 production (Nehlsen-Cannarella et al., 1997; Nieman et al., 1998; Nieman et al., 2003).
Mitogen-activated protein kinases (MAPK) (Luo et al., 2003) and more specifically the
nuclear phosphorylation of p38 MAPK (Chan et al., 2004b) also appears to regulate IL-6 gene
transcription in exercising (Chan et al., 2004b) and non-exercising states (Luo et al., 2003;
Chan et al., 2004b). The complexity of the interaction of these and likely many more factors signalling IL-6 production remain to be revealed.

In a target cell, IL-6 can simultaneously elicit signals through its receptor complex, IL-6Rα and gp130, which can be functionally distinct or sometimes contradictory. To explain how IL-6 is capable of generating pro-inflammatory or anti-inflammatory effects contingent on the in vivo environmental circumstance Hirano and colleagues (Hirano et al., 1997; Hirano, 1999) eloquently applied the concept of the ‘signal orchestration model’ whereby the ‘final physiological output can be thought of as a consequence of the orchestration of the diverse signalling pathways generated by a given ligand’. IL-6 signal transduction occurs when IL-6 binds with either its membrane bound receptor α subunit (IL-6Rα) (Taga et al., 1989) or a cleaved soluble version of the membrane-bound receptor (sIL-6Rα) (Mullberg et al., 1993; Mullberg et al., 1994). Although IL-6 can bind to IL-6Rα, gp130 is required for formation of the high-affinity receptor and generation of signal transduction (Yamasaki et al., 1988; Taga et al., 1989; Hibi et al., 1990). IL-6 is able to function in a wide variety of systems (eg immune system, acute-phase reaction, nervous and endocrine systems, cancer, bone metabolism, hematopoiesis) and many human diseases in the body due to the widespread distribution of gp130 and shedding of the soluble form of the IL-6Rα (Kamimura et al., 2003). For example, cells that have either no or very low expression of IL-6Rα, but express gp130, such as skeletal muscle (Helgren et al., 1994; Kami et al., 2000; Zhang et al., 2000) are potentially responsive to cytokine stimulation as facilitated by the presence of sIL-6Rα (Ishihara & Hirano, 2002; Jones & Rose-John, 2002; Ernst & Jenkins, 2004). Regulation of gp130 signalling is considerably complex since the pleiotropic family of IL-6-related cytokines (IL-11, LIF, OsM, CT-1, CNTF, CLC) share the use of gp130 as a common receptor subunit and thus, gp130 signalling regulates a multitude of aspects involved in cellular and tissue homeostasis. IL-6 and IL-11 bind specific α-receptors and induce gp130
homodimerisation, LIF and OsM directly induce gp130/LIF heterodimerisation, whereas CNTF binds to an α-receptor inducing heterodimerisation of the gp130 and LIF receptors, which trigger intracellular signalling cascades. Formation of receptor complexes and signalling are not specifically limited to the interaction between a cytokine and its α-receptor alone as a large variety of receptor complexes are possible. To highlight this, the IL-6R can serve as a substitute α-receptor for CNTF, enabling functional assembly of the receptor complex (Schuster et al., 2003). Ultimately, the IL-6 family of cytokines activate a limited number of intracellular signalling cascades such as JAK/STAT and Ras/MAPK. Tissue-specific responsiveness is limited at the extracellular surface by restricted expression profiles of α-receptors and regulated local and rapid cytokine production.

Following IL-6/IL-6Rα or IL-11/IL-11Rα binding, gp130 homodimerizes (Murakami et al., 1993) and a hexameric signalling-competent complex with a stoichiometry of 2:2:2 (Ward et al., 1994) is formed (picture). Binding of all other cytokines of the IL-6-related family induces heterodimerization of gp130 with a structurally and functionally similar β subunit, LIFRβ or OsMRβ. Membrane association of α subunits is not required for signal initiation. Therefore, soluble α subunits can be synthesised locally and bind to ligands, which facilitate β subunit dimerization and generate cellular responses. Where this occurs in the absence of membrane-associated α subunits it is referred to as receptor conversion or trans-signalling (Ernst & Jenkins, 2004). The manner in which these interactions and signals ultimately elicit specificity in their response is complex and is reviewed in detail by Ernst & Jenkins (Ernst & Jenkins, 2004).

Specific regions of the IL-6 protein have been revealed as important sites for receptor binding and signal transduction (Kamimura et al., 2003), and cellular effects are induced through signalling via the JAK/STAT pathway. In particular, activation involves members of the
Janus-activated protein kinases (JAK), JAK1, JAK2 and TYK2, which then phosphorylate and activate the signal transducer and activator of transcription (STAT)-1 and -3 (Heinrich et al., 1998). Cytokines, which signal through STAT induce transcription of a family of proteins known as the suppressors of cytokine signalling (SOCS) (Starr et al., 1997). Several STAT1/3 consensus binding sites are located on the distal portion of all β subunits. Ligand-induced β subunit homo- or heterodimerization and tyrosine phosphorylation by constitutively associated JAK family kinases is essential for subsequent activation of STAT1/3 and SHP2/ERK mitogen-activate protein kinases (MAPK) intracellular signalling cascades. JAKs tyrosine phosphorylate receptor-bound SHP2 causing Ras/ERK and Akt pathway activation. JAKs also phosphorylate receptor-bound STAT1/3, which form homo or heterodimers then translocate to the nucleus to activate target genes. Negative regulation of gp130 complex signalling may involve proteosomal degradation of ligand-bound receptor complex components due to STAT3-mediated induction of SOCS1/3 or attenuation of signalling by tyrosine phosphorylated SHP2 via inactivation of JAKs. Additionally, nuclear translocation and target gene activation can be inhibited by protein inhibitors of activated STAT proteins, which sequester STAT and thus prevent dimerization. More detailed regulation of these pathways are reviewed by Ernst & Jenkins (Ernst & Jenkins, 2004). The IL-6 family of cytokines are thought to elicit their divergent cellular responses by way of different thresholds of activation of STAT1/3 and SHP2. Such mechanisms utilise multi-site phosphorylation (initiating signal diversity and altering strength and duration of signalling) and multi-faceted negative regulatory processes (restricting target gene activation) (Ernst & Jenkins, 2004), the details of which are beyond the scope of this literature review.

Downstream signalling pathways generated by IL-6/IL-6R/gp130 and other gp130-associated complexes are diverse and elicit respective biological effects by activating different sets of genes. Many immunological roles for downstream targets of cytokine receptors have been
well described. However, other functions are beginning to emerge, whereby roles in the control of metabolism are becoming evident. Primary downstream effectors of IL-6 signalling such as MAPKs (p38, p42/44, c-jun N-terminal kinase, JNK), inhibitor of kappa B kinase (IκK) (Marette, 2002) and AMP-activated protein kinase (AMPK) (Carey et al in review after revision; Kelly et al., 2004) are known to activate processes associated with glucose uptake and fat oxidation.
Figure 1.1  Schematic representation of the association of IL-6 in the formation of active receptor complexes and known downstream targets of gp130-dependent signalling cascades.
1.3 Interleukin-6 in states of health

IL-6 is produced by a large and diverse population of cells, thus, it is difficult to elucidate the relative contribution of each cell type to the relatively low circulating levels (< 4 pg.ml\textsuperscript{-1}) observed in the basal state of healthy individuals (Kado et al., 1999; Bastard et al., 2000; Pickup et al., 2000b; Starkie et al., 2001b; Vozarova et al., 2001). The rapid clearance rate of circulating IL-6 levels, whether induced endogenously (Steensberg et al., 2001a) or exogenously through infusion of high physiological doses (Steensberg et al., 2003b; van Hall et al., 2003), makes it difficult to isolate the in vivo cellular/tissue sources. It has been demonstrated that in healthy humans at rest the brain releases detectable amounts of IL-6 (Nybo et al., 2002), while the hepatosplanchnic viscera has a small uptake of IL-6 (Febbraio et al., 2003a). Human subcutaneous adipose tissue both expresses and releases IL-6 in vivo under non-inflammatory healthy resting conditions. This release has been estimated to contribute ~30% of the circulating IL-6 levels at rest (Mohamed-Ali et al., 1997; Lyngso et al., 2002a) and is exacerbated after exercise (Lyngso et al., 2002a; Keller et al., 2003a). Skeletal muscle cells express IL-6 mRNA and protein in the basal state (Hiscock et al., 2004), however, their contribution to circulating levels at rest appears to be negligible (Febbraio et al., 2003c). Of note, IL-6 has also been shown to stimulate its own expression in skeletal muscle (Keller et al., 2003b) and adipocytes (Fasshauer et al., 2004).

The detection of increased systemic levels of IL-6 after physical exercise is a remarkably consistent finding. It has been repeatedly demonstrated that during muscular exercise the plasma concentration of IL-6 increases (Drenth et al., 1995; Papanicolaou et al., 1996; Ostrowski et al., 2000; Starkie et al., 2000; Steensberg et al., 2000; Keller et al., 2001; Mazzeo et al., 2001; Starkie et al., 2001a; Steensberg et al., 2001b; Langberg et al., 2002; Lyngso et al., 2002a; Steensberg et al., 2002; Suzuki et al., 2002; Febbraio et al., 2003a;
Febbraio et al., 2003b; Febbraio et al., 2003c; Helge et al., 2003; Hiscock et al., 2003; Keller et al., 2003a; MacDonald et al., 2003; Nieman et al., 2003; Lundby & Steensberg, 2004) in some circumstances more than 100 fold (Starkie et al., 2000; Suzuki et al., 2002). IL-6 is produced in and released from contracting skeletal muscle during exercise over and above basal levels (Febbraio & Pedersen, 2002) and is hence referred to as a “myokine” (Febbraio & Pedersen, 2005). Numerous studies have shown increases in skeletal muscle IL-6 mRNA in both humans (Steensberg et al., 2000; Keller et al., 2001; Starkie et al., 2001a; Steensberg et al., 2001a; Nieman et al., 2003; Chan et al., 2004a; Fischer et al., 2004) and rodents (Jonsdottir et al., 2000), as well as net release of IL-6 from the contracting limb (Steensberg et al., 2000; Steensberg et al., 2001a; Steensberg et al., 2002; Febbraio et al., 2003b; Keller et al., 2003b; Lancaster et al., 2003; Nieman et al., 2003; Chan et al., 2004a; Fischer et al., 2004) and adipose tissue (Lyngso et al., 2002a), brain (Nybo et al., 2002), and peri-tendon (Langberg et al., 2002) have also been identified as tissues, which increase gene transcription and or protein release of IL-6 following exercise, thus contributing to elevated circulating levels.

The appearance of IL-6 into the circulation after exercise is subject to exercise intensity (Ostrowski et al., 2000; Lundby & Steensberg, 2004), duration (Nybo et al., 2002) and mode (Nielsen et al., 1996; Nieman et al., 1998; Starkie et al., 2001a). Additionally, the energy status of, or supply to, the working muscle is another factor. Carbohydrate feeding during exercise consistently attenuates the increases in plasma IL-6 (Nehlsen-Cannarella et al., 1997; Starkie et al., 2001a; Febbraio et al., 2003b; Keller et al., 2003a; Nieman et al., 2003). Consistent with the effect of different modes of exercise, IL-6 gene expression in the contracting skeletal muscle has been shown to be attenuated (Nieman et al., 2003) or unaffected (Starkie et al., 2001a) by carbohydrate ingestion. Moreover, this release is
potentiated by low-intramuscular glycogen stores (Keller et al., 2001; Steensberg et al., 2001a; Helge et al., 2003; MacDonald et al., 2003; Chan et al., 2004a). The effects of exercise on IL-6 levels have been associated with concomitant increases in glucose uptake (Febbraio et al., 2003c; Helge et al., 2003). High and low doses of IL-6 infused into healthy humans (Steensberg et al., 2003c) and rats (Rotter Sopasakis et al., 2004) under resting conditions have been shown to have no effect on whole-body glucose disposal (Steensberg et al., 2003c; Rotter Sopasakis et al., 2004), endogenous glucose production, or leg-glucose uptake (Steensberg et al., 2003c). When a low-intensity exercise bout was supplemented with IL-6 administration, to achieve circulating levels observed in a high-intensity exercise bout, the rate of glucose appearance and the rate of whole body glucose disposal, and thus the metabolic clearance rate of glucose, were increased above levels achieved with exercise alone (Febbraio et al., 2004). Stouthard and colleagues (Stouthard et al., 1995) infused human subjects with a high dose of recombinant human IL-6 (rhIL-6) and reported that it increased whole body glucose disposal and subsequent oxidation. However, administration of physiological doses of IL-6, corresponding to plasma concentrations obtained during prolonged exercise, did not affect glucose metabolism (Steensberg et al., 2003c). Thus, it is likely that IL-6 is linked to regulation of glucose homeostasis during exercise and or that it acts as a carbohydrate sensor, while in the absence of contraction-induced stimuli, physiological doses of IL-6 alone are not sufficient to induce hepatic glucose out-put or increase glucose disposal.

Petersen and colleagues, and van Hall and colleagues demonstrated that IL-6 infusion into healthy (van Hall et al., 2003) and type 2 diabetic (Petersen et al., 2005a) patients elevated circulating fatty acid (FA) levels and increased FA turnover, without reducing glucose turnover, causing adverse side effects, or causing hypertriacylglyceridemia, and that these effects of IL-6 were acting independently to changes in circulating hormones. *In vitro*
investigations show that IL-6 increases lipolysis in adipocytes originating from human breast (Path et al., 2001), subcutaneous (Petersen et al., 2005a) and omental (Trujillo et al., 2004) depots, without the presence of hormones (Trujillo et al., 2004) and without altering basal or insulin-stimulated glucose transport (Path et al., 2001). Pulse-chase studies on excised rats muscles incubated in the presence of IL-6 display increased exogenous and endogenous FA oxidation without effect on FA uptake or incorporation of FA into endogenous lipid pools. Furthermore, IL-6 attenuated insulin's suppressive effect on FA oxidation, increasing exogenous FA oxidation (Bruce & Dyck, 2004). It is also reported that AMPK activity is elevated skeletal muscle and adipocytes when incubated in the presence of IL-6 (Kelly et al., 2004). Consistent with this, FA and triacylglycerol concentrations increased in a dose-dependent manner when rats were infused with IL-6 (Nonogaki et al., 1995). The temporal profile of post-exercise IL-6 output from abdominal subcutaneous adipose tissue of healthy subjects closely resembles that of glycerol and FFA (Lyngso et al., 2002b). Of note, however, the IL-6 release begins approximately 30 min before glycerol and fatty acid outputs begin to increase, suggesting that this IL-6 production post-exercise may influence lipolysis and fatty acid mobilisation rate from this lipid depot (Lyngso et al., 2002a). When contracting skeletal muscle is confronted with increasing concentrations of fatty acids originating from other tissues, energy utilization should be favored in the muscle, while other organs act as energy suppliers. Thus, IL-6 released from the contracting muscle and adipose tissue may trigger energy supply through paracrine and autocrine lipolytic actions on adipose tissue. In general, the weight of evidence suggests that IL-6 may be a contributing factor to the health benefits associated with exercise; improved fatty acid metabolism, glucose tolerance, and insulin sensitivity. Despite recent literature indicating such beneficial effects, IL-6 is commonly attributed a causative role in metabolic disease, largely because small elevations in circulating IL-6 are often seen in obese and insulin resistant patient populations.
1.4 Interleukin-6 in states of metabolic disease

Circulating IL-6 is strongly associated with obesity and is often used as a predictor of the development of type 2 diabetes (Kern et al., 2001). Substantial evidence also indicates that obesity is accompanied by a low-level inflammatory state. The release of cytokines by adipose tissue ("adipokines") contributes to the inflammatory state observed in obesity. Monocytes infiltrate adipose tissue in proportion to adiposity and can be a source of adipose tissue-derived inflammatory cytokines (Weisberg et al., 2003). Supporting the connection between obesity, inflammation, and insulin resistance, IL-6 and TNFα directly cause insulin resistance in model systems (Hotamisligil et al., 1994; Emanuelli et al., 2001; Senn et al., 2002; Rotter et al., 2003; Senn et al., 2003). A range of investigators conclude that IL-6 decreases (Bastard et al., 2002; Senn et al., 2002; Klover et al., 2003; Lagathu et al., 2003; Rotter et al., 2003), increases (Stouthard et al., 1995; Stouthard et al., 1996; Wallenius et al., 2002b; Carey et al. in review after revision), or has no effect (Steensberg et al., 2003c; Petersen et al., 2005a) on insulin sensitivity. In addition, the measurement of IL-6 levels and gene polymorphisms in relation to insulin sensitivity is equivocal (Makino et al., 1998; Bastard et al., 2002). Thus, the cause or effect relationship between IL-6 and insulin resistance and the development of type 2 diabetes has not been completely resolved.

While IL-6 leads to insulin resistance in rodent liver tissue, its role in skeletal muscle and adipose tissue is unclear. Liver cells treated with IL-6 demonstrate reduced insulin-stimulated glycogen synthase activity, blunted glycogen phosphorylase, decreased glycogen synthesis, and increased glycogen breakdown (Kanemaki et al., 1998). Insulin receptor signalling is also impaired in IL-6 treated liver through inhibition of receptor autophosphorylation, tyrosine phosphorylation of IRS-1 and subsequent activation of PI3-K and Akt through stimulation of the insulin receptor inhibitor, SOCS3 (Senn et al., 2002; Klover et al., 2003).
Muscle tissue insulin action is inhibited in vivo by IL-6 (Kim et al., 2004a). When mice (Kim et al., 2004b) and differentiated myotubes (Rieuxset et al., 2004) were acutely treated with IL-6 before or during insulin stimulation, glucose disposal and skeletal muscle insulin signalling were impaired. The opposite has also been reported by Carey and colleagues, whereby the effect of IL-6 treatment on insulin signalling was additive to that of insulin (unpublished observations). Chronic IL-6 administration in mice, eliciting levels similar to those found in obese individuals, induced hepatic insulin resistance without impairing skeletal muscle insulin signalling; suggesting a tissue-specific effect of IL-6 on insulin action in vivo (Klover et al., 2003). However, the reverse was found in vitro by Weigert and colleagues, where IL-6 treatment of human muscle cells induced Akt activation, glycogen synthase kinase-3 phosphorylation, and glycogen synthesis. Moreover, these effects were observed in skeletal muscle but not liver tissue (Weigert et al., 2005). Data of IL-6 treatment in vitro in adipocytes is also conflicting with Rotter and colleagues reporting impaired insulin signalling (Rotter et al., 2003) and Stouthard and colleagues reporting positive effects additive to those of insulin (Stouthard et al., 1996). IL-6 infusion during a hyperinsulinemic-euglycemic clamp in rodents either decreases (Kim et al., 2004a) or has no effect (Rotter Sopasakis et al., 2004) on whole body glucose disposal, and recently, Febbraio and colleagues conducted a 4-h IL-6 infusion in healthy human subjects, which improved insulin sensitivity and did not impair insulin’s ability to suppress hepatic glucose production (Carey et al in review after revision).

Increasing severity of insulin resistance has been associated with intramuscular triacylglyceride accumulation (Phillips et al., 1996; Pan et al., 1997; Krssak et al., 1999), increased abdominal adiposity, increased intramuscular long-chain acyl-CoA content (Oakes et al., 1997a; Oakes et al., 1997b), and alterations in membrane phospholipid fatty acid composition (Storlien et al., 1991). Furthermore, lipid-activated signalling pathways interfere
with insulin signal transduction in skeletal muscle to cause insulin resistance (Boden, 1997; Dresner et al., 1999; Griffin et al., 1999). Epidemiological evidence suggests that it is the ingestion of saturated rather than unsaturated fat that is closely linked to insulin resistance and hyperinsulinemia (Dobbins et al., 2002). Specific lipids with distinct effects in different cell types can influence insulin signalling at different sites. Moreover, aberrant deposition of lipid substrates in skeletal muscle and liver tissue as a result of lipid oversupply contributes to the development of insulin resistance (Krebs & Roden, 2005). Adipose tissue is no longer regarded simply as an energy store. It is now well recognised that adipose tissue is an active endocrine organ. Adipose tissue itself is responsive to central and peripheral metabolic signals and is capable of integrating such signals and responding by regulating the secretion of multiple adipose-specific or enriched adipokines, which are implicated in the regulation of energy homeostasis, innate immune system, and lipid metabolism. The systemic effects of impaired insulin sensitivity associated with obesity may be a reflection of the lipotoxic effects of fatty acids (Lelliott & Vidal-Puig, 2004) as well as an imbalance of adipokines (Fantuzzi, 2005) (Figure 1.2).
Figure 1.2 Schematic representation of the major intracellular defects associated with insulin resistance which coincide with IL-6 production,
Elevated circulating levels of IL-6 are consistently reported to be implicated in causing insulin resistance in obese and type 2 diabetic patients (Bastard et al., 2000; Kern et al., 2001; Bastard et al., 2002). It is plausible that IL-6 is elevated in this patient population as a consequence of increased fat mass, rather than being associated with insulin resistance per se. Consolidating this notion are a few studies, which have dissected IL-6 levels from insulin resistance per se. Levels of IL-6 in obese non-diabetic subjects were shown to correlate with percentage body fat and not insulin sensitivity once corrected for body fat levels (Vozarova et al., 2001). Carey and colleagues have also reported that elevated plasma concentrations of IL-6 observed in patients with type 2 diabetes and age-matched control subjects are not related to insulin sensitivity, but are rather an index of adiposity (Carey et al., 2004). In vitro investigations mentioned in the previous section further support the role of IL-6 in lipid metabolism; decreasing intramyocellular fatty acid accumulation. However, this needs to be further investigated in vivo in the context of metabolic disease.

Chronic inflammation in metabolically active tissues such as skeletal muscle, liver and adipose tissue, characterised by abnormal production of pro-inflammatory cytokines and acute-phase reactants by adipocytes and macrophages, is tightly associated with obesity (Sethi & Hotamisligil, 1999). This observation is consistent with the long held notion that type 2 diabetes is a chronic low-grade inflammatory disease (Pickup et al., 1997; Pickup & Crook, 1998). However, whilst obesity is a major predictor of fulminate type 2 diabetes, there are multiple risk factors and obesity alone does not necessarily confer diagnosis, as impaired glucose tolerance and insulin sensitivity must also be present. The literature clearly demonstrates that lipids are involved in the induction of insulin resistance, and that adipocytes are intricately involved in the control of insulin responsiveness at the whole body level. Adipocytes express and release a number of adipokines (e.g. adiponectin, leptin, resistin, IL-6, TNF-α) that have both positive and negative effects on peripheral and central tissues (for
review see Rajala & Scherer, 2003). The adipocyte is a likely candidate for the production and release of acute phase reactants as many general transcription factors for the acute-phase reactant response are expressed abundantly in the adipocyte (Trayhurn & Wood, 2004) and cytokines that are considered to be acute-phase inflammatory markers are correlated with markers of the metabolic syndrome (Pickup et al., 1997; Pickup & Crook, 1998; Pickup et al., 2000a; Pickup et al., 2000b; Rajala & Scherer, 2003).

The pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) production within and release from adipose tissue is associated with obesity. Weisberg and colleagues have shown more specifically, that macrophages within the adipose tissue, rather than adipocytes per se, that are responsible for almost all adipose tissue TNF-α production (Weisberg et al., 2003). TNF-α promotes insulin resistance in several insulin-responding cells and tissues, inhibits tyrosine phosphorylation of IRS-1 and results in a state that is suggestive of type 2 diabetes and obesity (Hotamisligil et al., 1993). Shulman has shown that an increase in intracellular FA also elicited this phenomenon (Shulman, 2000). Furthermore, neutralization of TNF-α improves insulin resistance (Hotamisligil et al., 1993). In addition to the inhibition of IRS-1 tyrosine phosphorylation, TNF-α also stimulates IRS-1 serine phosphorylation, which in contrast to tyrosine phosphorylation, blunts insulin signalling (Hotamisligil et al., 1996; Kanety et al., 1996; Aguirre et al., 2000). JNK, which serves as a sensing juncture for cellular stress and inflammatory status, also phosphorylates IRS-1 serine residues (Hiratani et al., 2005). JNK activity is elevated in the tissues of several obesity models, including high-fat diet-induced obesity and genetic leptin deficiency (Hirosumi et al., 2002), and its ablation in an animal model improves insulin sensitivity and prevents obesity (Hirosumi et al., 2002). There is strong evidence to suggest that the disruption of insulin signalling caused by cytokines that are considered to be acute-phase inflammatory markers (TNF-α, IL-6) is mediated by activation of inhibitor of kappa B kinase β (IkKβ), which removes the inhibitor
of kappa B (IkB) protein from its inhibitory site on nuclear factor kappa B (NF-κB), the major inflammatory-mediating transcription factor (Yin et al., 1998; Yuan et al., 2001). Moreover, the fatty acid-induced insulin resistance previously discussed, which is mediated through LCACoA and DAG activating JNK and the PKC–theta isozymes, consequently activates IkKβ (Perseghin et al., 2003). Since IL-6 is often elevated in type 2 diabetes and its production is stimulated by TNF-α (Neta et al., 1992), it was hypothesised that IL-6 might contribute to the development of type 2 diabetes (Pickup et al., 1997; Pradhan et al., 2001). However, the etiology of IL-6 on these parameters in obesity and insulin resistance are limited and this area warrants further investigation.

Recently, a mouse model of IL-6 deficiency (IL-6-/-) demonstrated increased fat mass, mature-onset obesity, leptin resistance and glucose intolerance; characteristics which were attenuated with subsequent IL-6 treatment (Wallenius et al., 2002b). Chronic intracerebroventricular IL-6 treatment in rats also decreased body weight and fat mass by decreasing food intake and stimulating energy expenditure at the level of the central nervous system (Wallenius et al., 2002a; Jansson et al., 2003). Young, pre-obese IL-6-/- mice were reported to have diminished AMPK phosphorylation in muscle and adipose tissue, which, given the role of AMPK activation in metabolic regulation, may contribute to the developing phenotype (Kelly et al., 2004). In contrast to these studies, Di Gregorio and colleagues reported that IL-6-/- mice did not demonstrate obesity, fasting hyperglycemia or abnormal lipid metabolism (Di Gregorio et al., 2004). The mechanisms perturbing glucose tolerance, insulin sensitivity and energy regulation in the IL-6-/- mouse remain to be determined.

In summary, the role of IL-6 in the induction of insulin resistance, particularly in insulin sensitive tissues is ambiguous. The direct metabolic effects of IL-6 in the regulation of glucose and fatty acid metabolism in skeletal muscle and adipose tissue remain to be clarified.
This thesis sought to investigate the biological role of IL-6 in metabolic states of health and disease.
CHAPTER TWO

AIMS OF THE THESIS
2.1 Aims of the thesis

The primary aim of this thesis was to determine the role of IL-6 in the regulation of metabolism, particularly with respect to its role in a) exercise metabolism and b) insulin sensitivity. Such investigation encompassed the examination of in vivo and in vitro (human and animal) models, as well as the execution of a number of analytical techniques.

The expression and release of IL-6 from contracting skeletal muscle was known to be altered in metabolically perturbed states. Affected by exercise mode, intensity, and duration, as well as glycogen state, the hypothesis within the literature was that IL-6 was released from skeletal muscle during exercise to act in a "hormone-like" manner to increase metabolic substrate supply to the muscle. As adrenaline levels are also altered by these variables and are known to regulate lipolysis, the first study (Chapter 3) investigated whether adrenaline per se induced IL-6 protein production and or release from skeletal muscle. After establishing that adrenaline was not stimulating IL-6 production, the second study (Chapter 4) investigated the affect of the suppression of lipolysis on IL-6 levels during exercise.

The ablation of IL-6 within a whole body system was then investigated in a genetically modified mouse model. The third study (Chapter 5) endeavoured to characterise the regulation of fatty acid metabolism and insulin signalling in the IL-6 knockout mouse in the basal state as well as in a state of diet-induced insulin resistance. These parameters were then investigated following chronic short-term IL-6 treatment (Chapter 6).

Acute elevations in IL-6 levels with exercise would appear to be associated with beneficial effects on metabolism and insulin sensitivity, yet chronically elevated IL-6 levels have been associated with negative effects in conditions of obesity and insulin resistance. Thus, the final
study (Chapter 7) investigated whether the mode of IL-6 administration had disparate effects on these parameters.
CHAPTER THREE

IONOMYCIN, BUT NOT PHYSIOLOGIC DOSES OF
ADRENALINE, STIMULATES SKELETAL MUSCLE

INTERLEUKIN-6 mRNA EXPRESSION AND PROTEIN RELEASE
3.1 Introduction

Interleukin-6 (IL-6) is a multifunctional immune-modulating cytokine that is elevated in stressed states and in patient populations. Expression of IL-6 is putatively associated with insulin resistance, visceral obesity, atherosclerosis, and glucocorticoid receptor dysfunction (for review see (Febbraio & Pedersen, 2002). It is not surprising that IL-6 is associated with obesity-related disorders as it is released from adipose tissue (Mohamed-Ali et al., 1997) and positively correlates with body fat levels (Vozarova et al., 2001; Carey et al., 2004). Recent evidence suggests, however, that IL-6 is produced by contracting skeletal muscle (Febbraio & Pedersen, 2002; Pedersen et al., 2003), but the precise mechanism(s) that regulate IL-6 production by skeletal muscle are unclear. Of note, skeletal muscle IL-6 mRNA expression is not elevated in insulin-resistant humans, nor is it activated by insulin in these patients (Carey et al., 2004), suggesting that factors associated with physical exercise and/or muscle contraction regulate IL-6 transcription in muscle (Febbraio, 2003).

It is well known that during exercise the increase in plasma adrenaline is marked, and it has often been hypothesized that the increase in circulating adrenaline could mediate, in part, the IL-6 response (Nehlsen-Cannarella et al., 1997; Nieman et al., 1998; Nieman et al., 2003). Indeed, there is evidence to suggest that adrenergic regulation may be important in regulating IL-6. Administration of adrenaline increases plasma IL-6 in both rats (DeRijk et al., 1994) and humans (Sondergaard et al., 2000). Furthermore, plasma adrenaline concentrations correlate with plasma IL-6 (Papanicolaou et al., 1996), while the release of IL-6 from skeletal muscle has been shown to be positively correlated to exercise intensity and arterial adrenaline concentration (Helge et al., 2003) during exercise in humans. However, these studies do not provide evidence that adrenaline increases IL-6 production in skeletal muscle, because they
either did not identify the source of the systemic increase when either animals or humans were infused at rest, or the present associative data collected during exercise.

In contrast, during exercise, plasma IL-6 increased 30-fold, however, an adrenaline infusion mimicking closely the exercise-induced increase in plasma adrenaline increased plasma IL-6 only 4-fold (Steensberg et al., 2001b), suggesting that during exercise the role of plasma adrenaline on the IL-6 response is relatively minor. In addition, during 2 legged knee extensor exercise, both the IL-6 gene transcription and protein release was augmented in a leg previously depleted of glycogen, even though both legs were subjected to the same level of circulating adrenaline (Steensberg et al., 2001a), also suggesting that factors other than adrenaline play a more important role in IL-6 production during contraction. The direct effect of adrenaline on IL-6 gene expression and protein release from skeletal muscle has not been experimentally investigated, and this was the primary aim of the present study. Using an in vitro rat skeletal muscle incubation model, we hypothesized that physiologic adrenaline would neither result in an increase in IL-6 mRNA nor protein release from these muscles.

The rapid increase in IL-6 nuclear transcription rate seen within the first 30 min of exercise has been proposed to be attributable to cytosolic calcium (Ca^{2+}) levels (Keller et al., 2001). The role of intracellular Ca^{2+} ([Ca^{2+}]_i) in the control of a diverse range of cellular functions, including gene expression and proliferation, is well documented (Berridge, 1993; Ghosh & Greenberg, 1995) and the [Ca^{2+}]_i concentration has been implicated in signalling cascades as a potent signalling factor for IL-6 transcription (Febbraio & Pedersen, 2002; Febbraio, 2003). Indeed, it has been demonstrated that the calcium ionophore, ionomycin, can increase IL-6 gene expression in cultured human primary muscle cells after a 6-hour incubation period, and further so by 48 hours (Keller, 2002). The effect of Ca^{2+} stimulation on IL-6 expression has only been investigated under such long-term circumstances, and whether this response has a
more rapid effect, or whether ionomycin treatment results in IL-6 protein release has not been investigated. This was the secondary aim of this study, and we hypothesized that ionomycin administration would result in IL-6 gene transcription in, and protein release from, skeletal muscle.
3.2  Methods

Animals and muscle preparation. Male Sprague-Dawley rats of 4 weeks of age and 85 ± 3 g body weight were purchased from Monash Medical Centre (Clayton, Victoria, Australia). The animals were housed in the RMIT University animal facility in a controlled environment with a 12:12 hour light:dark cycle and fed rat chow and water ad libitum. All experimental procedures were approved by the Animal Experimentation Ethics Committee of RMIT University (AEEC # 0313). Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg·100g⁻¹ body weight, Nembutal; Rhone Merieux, Queensland, Australia), and the soleus muscle from both hind limbs were excised from tendon to tendon. Care was taken to avoid damage to the muscle during surgery, and animals were immediately euthanized with an overdose of Nembutal.

Muscle metabolite experiments. Viability of our soleus muscle preparation was established in a preliminary experiment. Paired muscles were isolated as described. To determine the viability of the muscle over the incubation period, 1 muscle was rapidly frozen, whereas the contralateral muscle was incubated for 60 min in gassed Krebs buffer (see below for details), then snap-frozen in liquid nitrogen. Muscles were freeze-dried and powdered, and non-muscle contaminants were removed. The freeze-dried muscle was extracted in 0.5 mol/L HClO₄ (1 mmol/L EDTA) and neutralized with 2.2 mol/L KHCO₃. Adenosine triphosphate (ATP), phosphocreatine (PCr), creatine, and lactate were subsequently determined by spectrophotometric assays (Harris et al., 1974; Passonneau & Lauderdale, 1974).

Experimental protocol. Each intact muscle was preincubated in a 20-mL vial containing 3 mL pregassed (95 % O₂, 5 % CO₂) Krebs buffer (8 mmol/L glucose) for 10 min at 30 °C. After preincubation, either Krebs buffer (CON, n = 5), 10 µmol/L ionomycin (IONO, n = 5),
or adrenaline at concentrations of 1,000 (ADR 1,000, n = 8), 100 (ADR 100, n = 7), and 10 nmol/L (ADR 10, n = 9) was added to the appropriate vial and incubated for a further 40 min. All adrenaline stock solutions were prepared in Krebs buffer with 2 mg/mL ascorbic acid to prevent oxidation. A 3 mmol/L stock solution of adrenaline bitartrate (AstraZeneca, North Ryde, NSW, Australia) was diluted to 3 µmol/L daily such that the appropriate volume could be added to the bath to achieve concentrations of 10, 100, or 1,000 nmol/L ADR in a final bath volume of 3.0 mL. The total volume added for all conditions was 100 µL. At the end of the incubation, muscles were snap-frozen in liquid nitrogen and stored at –80°C for later analysis. A 1 mL aliquot of incubation media was also removed and stored at –80°C for later analysis.

Determination of IL-6 gene expression and protein release. The soleus muscle was extracted for total RNA using the acid guanidinium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi (Chomczynski & Sacchi, 1987), modified as previously described (Febbraio & Koukoulas, 2000). Samples were reverse transcribed using Taqman Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) at a final concentration of 10 ng/µl. PCR reactions were completed with individual reactions of 5 µg total RNA, 1 x Bio-Rad iQ Supermix ((Bio-Rad, Hercules, CA, USA), 200 nM Taqman fluorescent probe (Applied Biosystems) and 900 nM PCR primer (Sigma Genosys, Castle Hill, NSW, Australia) in a total volume of 25 µl using a Bio-Rad iCycler (Bio-Rad). Primer and Taqman probe were designed using Primer Express software (Applied Biosystems) to appropriate specifications from the rat IL-6 mRNA sequence (GenBank/EMBL Accession No. M26744). An 81-bp IL-6 gene fragment was amplified using the forward primer 5´-ATATGTTTCTCAGGGAGATCTTGAA-3´ and reverse primer 5´-GTGCATCATCGCTGTTCATA-3´ (Sigma Geno-sys, Castle Hill, NSW, Australia). Probes were designed with the FAM (6-carboxyfluorescein) reporter dye on the 5´ end and
TAMRA (6-carboxy-tetramethylrhodamine) quencher dye to the 3’ end. 18S mRNA was also amplified, and the TaqMan probes and primers for this gene were supplied in a control reagent kit (Applied Biosystems). Gene expression was quantified with a multiplex comparative critical threshold (C_T) method (Bio-Rad iCycler IQTM, Hercules, CA). The cycle number at which the cDNA amplification is first detected is reflected by the C_T value. For each sample, a change (Δ) in C_T value was obtained by subtracting 18S C_T from IL-6 C_T. A ΔΔC_T value was obtained by subtracting the intervention value from the control value. The expression of rodent IL-6 was then evaluated by 2−ΔΔCT. The IL-6 protein released from the muscle into the incubation media was quantified by enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, MN).

Statistical analysis. Comparisons between means were made using the paired t test. A P value of less than 0.05 defined statistical significance. All values are presented as mean ± SEM.
3.3 Results

Isolated muscles were viable over the 60 min incubation period as indicated by the maintenance of ATP and total creatine within the muscle (rapid freeze: 15.9 ± 0.7 and 82.2 ± 5.6; postincubation: 18.4 ± 3.0 and 90.6 ± 3.9 mmol·kg⁻¹·dm, respectively). IL-6 mRNA was increased by IONO and pharmacologic levels of ADR (1,000 nmol/L; \( P < 0.05 \)) compared with CON. Lower levels of ADR (10 and 100 nmol/L) were without effect on IL-6 mRNA (Fig 3.1, upper). IL-6 protein increased \((P < 0.05)\) in the incubation media with IONO compared with CON. Despite the observed increase in IL-6 mRNA with pharmacologic ADR, there was no measurable increase in IL-6 protein in the incubation media with ADR at all concentrations (Fig 3.1 lower).
Figure 3.1  IL-6 mRNA expression (upper) in and release (lower) from rat soleus muscle incubated for 60 min in saline (control, n = 5), 10 µmol/L ionomycin (IONO, n = 5), 1,000 nmol/L adrenaline (ADR 1,000, n = 8), 100 nmol/L adrenaline (ADR 100, n = 7), 10 nmol/L adrenaline (ADR 10, n = 9). *Indicates difference ($P < 0.05$) from control. Data are expressed as mean ± SEM.
3.4 Discussion

This is the first study to investigate the direct effect of adrenaline on IL-6 gene expression within, and protein release from, skeletal muscle. In the present study, IL-6 mRNA was not altered by short-term exposure to high physiologic and supra-physiologic adrenaline concentrations, whereas adrenaline, at a concentration of more than 1,000 times that occurring physiologically, while increasing IL-6 mRNA, did not increase IL-6 protein release. These data indicate that adrenaline is unlikely to be the stimulus for the exercise-induced increase in IL-6 gene expression and does not increase IL-6 protein release from skeletal muscle.

Helge and colleagues (Helge et al., 2003) correlated plasma IL-6, released from skeletal muscle during exercise, with arterial adrenaline concentrations, and Nieman and colleagues (Nieman et al., 2003) suggested that during a prolonged exercise bout differences in IL-6 gene expression were due to differences in blood adrenaline levels. Previous studies have investigated the effect of adrenaline infusion on IL-6 levels. DeRijk and colleagues (DeRijk et al., 1994) infused adrenaline to physiologic concentrations and observed an increase in plasma IL-6, and in the face of a 24-fold increase in adrenaline concentration Søndergaard and colleagues (Søndergaard et al., 2000) saw a 2-fold to 3-fold increase in plasma IL-6. These studies have reported measurements of circulating factors and have not isolated the impact of adrenaline on skeletal muscle IL-6 per se. Based on our present data it appears, therefore, that in the studies mentioned above, the source of the IL-6 was not the skeletal muscle. It must be noted, however, that in the present study we investigated the effects of adrenaline per se on the IL-6 response in muscle and did not excise muscle from animals following contraction. Thus, we cannot rule out the possibility that following exercise the relationship between adrenaline and IL-6 may differ, because the high-energy phosphagen
content of contracting compared with non-contracting muscle may differ markedly, and this may affect the response to adrenaline.

It is well known that intracellular calcium ([Ca^{2+}]_i) controls a diverse range of cellular functions including gene expression and proliferation, and the literature suggests that [Ca^{2+}]_i is a potent signalling factor for IL-6 transcription (Keller et al., 2002). Our finding that the Ca^{2+} ionophore, ionomycin, increased the IL-6 gene expression is consistent with previous findings (Keller et al., 2002). Keller and colleagues incubated isolated human muscle cells with ionomycin and reported progressive increases in IL-6 mRNA from 6 to 48 hours of incubation. In the present study, we extended on their findings and demonstrated increased IL-6 gene expression within 1 hour. Moreover, these data demonstrate that Ca^{2+} increases IL-6 protein release from skeletal muscle. The present finding showing that the Ca^{2+} ionophore ionomycin increases IL-6 protein release from skeletal muscle is consistent with marked increases in IL-6 protein release seen within 30 min of exercise (Febbraio et al., 2003c), which coincides with a period when there is a rapid increase in cytosolic Ca^{2+} levels due to mechanical load (Obici & Rossetti, 2003). Ca^{2+} signals are known to propagate to the nucleus where they activate or inhibit the function of various transcription factors, and hence, influence gene transcription. Some of the signals downstream of Ca^{2+} have been identified as activators of cytokine gene transcription (Febbraio & Pedersen, 2002), but the degree to which IL-6 is activated in skeletal muscle by such signalling pathways is not known.

It is noteworthy, that while IONO increased both IL-6 mRNA and protein release, ADR 1,000, did not result in protein release (Figure 3.1, lower), despite the fact that the mRNA increase was marked (Figure 3.1 upper). It is difficult to interpret these results, however, the dose of adrenaline in these circumstances was pharmacologic, and the stimulus for IL-6 mRNA transcription may have been different compared with IONO, resulting in
posttranscriptional modification. Alternatively, the release of IL-6 may have been impaired under these circumstances.

In conclusion, we have demonstrated for the first time that calcium stimulates IL-6 gene expression within, and protein release from skeletal muscle, but that physiologic concentrations of adrenaline do not stimulate IL-6 gene expression within or protein release from rat soleus muscle in vitro.
CHAPTER FOUR

SUPPRESSING LIPOLYSIS INCREASES INTERLEUKIN-6 AT
REST AND DURING PROLONGED MODERATE-INTENSITY
EXERCISE IN HUMANS
4.1 Introduction

Interleukin (IL)-6 is released from adipose tissue under non-inflammatory conditions, contributing ~30% of the circulating IL-6 levels at rest (Mohamed-Ali et al., 1997), which is exacerbated after exercise (Keller et al., 2003a). Recent work has demonstrated that IL-6 is released from skeletal muscle during exercise (for review, (Febbraio & Pedersen, 2002). Specifically, the net leg release of IL-6 can account for the majority of the marked elevation of systemic IL-6 observed during exercise (Steensberg et al., 2000). Moreover, this release is potentiated by low-intramuscular glycogen stores (Steensberg et al., 2001a) and is markedly attenuated when subjects are fed glucose throughout exercise (Febbraio et al., 2003b). In circumstances in which muscle glycogen is low, the reliance on fatty acids as a fuel source is altered, and, therefore, these studies may suggest that one metabolic role of muscle-derived IL-6 is to act on adipose tissue to increase lipolysis, thereby providing an extracellular substrate. Two recent studies have demonstrated that recombinant human IL-6 infusion enhances lipolysis. Van Hall and colleagues (van Hall et al., 2003) demonstrated that IL-6 infusion into healthy subjects increased adipose tissue lipolysis and increased fat oxidation in the absence of changes in other lipolytic hormones. In addition, Lyngso and colleagues (Lyngso et al., 2002b) showed that IL-6 infusion gave rise to an increase in net glycerol release in subcutaneous adipose tissue, leading these authors to conclude that IL-6 elicits lipolytic effects in human adipose tissue in vivo. Further research from transgenic mice studies also suggests that IL-6 is a lipolytic cytokine (Wallenius et al., 2002b).

The exercise-induced increase of IL-6 has been hypothesized to have an energy-sensitizing role, sensing metabolically demanding situations, whereby it is released from contracting skeletal muscle to act in a hormone-like manner, mobilizing extracellular substrates, and/or augmenting substrate delivery during exercise (Pedersen et al., 2001; Febbraio & Pedersen,
2002). To further investigate this potential role of IL-6 in relation to fat metabolism, in the present study, we tested the hypothesis that acute suppression of plasma free fatty acid (FFA) levels would augment IL-6 mRNA expression in muscle and adipose tissue and increase the plasma concentration of IL-6 in the circulation at rest and during prolonged moderate-intensity exercise.
4.2 Methods

Resting Experiments

Subject characteristics. Five healthy, young men (28.8 ± 2.7 yr, 185.0 ± 3.7 cm, 76.3 ± 2.1 kg, means ± SEM) volunteered to participate in this study, which was approved by the RMIT Human Research Ethics Committee. All subjects were informed of the experimental protocol, and the possible associated risks of the study were explained to subjects, both orally and in writing, before written, informed consent was obtained. No subject was taking any medication or had any confounding medical history.

Experimental protocol. Subjects reported to the laboratory in the morning after a 10- to 12-h overnight fast and rested quietly. An indwelling cannula was inserted into an antecubital vein for blood sampling and was kept patent by periodic saline infusion. A resting blood sample was taken for analysis of FFA, IL-6, adrenaline, and growth hormone (GH) (as described below). Subjects initially ingested a 10 mg/kg dose of nicotinic acid (NA; 250 mg tablets; Aspen Pharmacare) (time = 0 min), followed by three subsequent doses of 5 mg/kg at 30 min intervals. Additional blood samples were obtained at 60 and 120 min. Muscle and adipose tissue were not sampled in this resting component of the study. All subjects experienced the expected side effects of NA ingestion, which included flushing (a reddening of the skin due to peripheral vasodilation over most of the body), a sensation of heat, and a tingling sensation, which started ~15–20 min after the first dose. No subjects experienced stomach or gastrointestinal upset.

Exercise Experiments

Subject characteristics. Seven active men (24.3 ± 2.9 yr, 185.2 ± 2.2 cm, 80.4 ± 3.4 kg, means ± SE) volunteered to participate in this study, which was approved by the RMIT
Human Research Ethics Committee, in accordance with the Declaration of Helsinki. All subjects were informed of the experimental protocol, and the possible associated risks of the study were explained to subjects both orally and in writing before written, informed consent was obtained. No subject was taking any medication or had any confounding medical history.

Pre-experimental procedures. Subjects visited the laboratory on four occasions. On the first, they performed a continuous and incremental workload test to exhaustion on an electrically braked cycle ergometer (LODE Instrument, Groningen, The Netherlands) to determine their peak pulmonary oxygen uptake ($\dot{V}O_2$ peak), which averaged 4.3 ± 0.3 L/min. Subjects returned to the laboratory 1 week later to complete a familiarization trial that consisted of cycling at 60% $\dot{V}O_2$ peak for 120 min with NA ingestion (described subsequently). Tissue sampling did not occur during this trial, but subjects' response to NA was monitored. Expired pulmonary gases (Quark b2, COSMED, Rome, Italy) were collected and analyzed online at 30-min intervals. The purposes of the practice ride were to familiarize the subject with the protocol, confirm the subject's tolerance to oral NA supplementation, and confirm the exercise power output of ~60% $\dot{V}O_2$ peak. The absolute power output for the trials was 156 ± 5 W.

Experimental protocol. Each subject performed two experimental trials, separated by at least 7 days. For the day preceding each trial, subjects were provided with a food parcel [14 MJ, 80% carbohydrates (CHO)] and were required to abstain from exercise, caffeine, and alcohol. Subjects were also instructed to consume 5 ml/kg of tap water on waking to ensure euhydration. Subjects reported to the laboratory in the morning after a 10- to 12-h overnight fast and rested quietly. An indwelling cannula was inserted into an antecubital vein, as described above. Three incisions were made through the skin and fascia over the vastus lateralis muscle of one leg, and one incision was made in the ventrolateral abdominal wall, ~10 cm lateral of the navel under local anaesthesia (2% lidocaine), for subsequent percutaneous
muscle and adipose tissue biopsy sampling, respectively. Trials were randomized, with subjects consuming either NA (described below) or nothing (Con) during the 60 min before exercise. Due to the mild side effects of NA (described above), we could not disguise the treatment, and, therefore, it was of no benefit to consume a placebo capsule during Con. Blood samples were obtained immediately before NA supplementation (−60 min), immediately before the start of exercise (0 min), and at 30, 60, 90, 120, 150, and 180 min during the exercise protocol. Immediately before exercise, resting muscle and adipose tissue biopsies were obtained while subjects rested on a bed. Samples were immediately frozen in liquid nitrogen and stored until analysis. Subjects subsequently moved to the cycle ergometer and, after a 5-min warm-up at 100 W, commenced cycling at the predetermined workload. Subjects ingested 100 ml of water every 60 min to ensure hydration. Additional muscle and adipose tissue samples were obtained at 90 and 180 min of exercise while subjects remained on the cycle ergometer. The elapsed time between cessation of exercise for biopsy sampling and resumption of exercise was < 30-sec. Expired pulmonary gases were collected at 30-min intervals for the measurement of expired fractions of O₂, CO₂, and ventilation. All trials were performed at an ambient temperature of 19 – 21°C. During the NA trial, subjects ingested a total of 45 mg/kg NA over eight individual doses: 10 mg/kg 60 min before exercise, 5 mg/kg 30 min before (−30) and immediately before (0 min) exercise, and at 30-min intervals throughout exercise.

Fat and carbohydrate oxidation. Whole body CHO and fat oxidation rates were calculated from each period of gas collection and estimated by using stoichiometric equations (Peronnet & Massicotte, 1991).

Blood metabolites and hormonal analyses. Venous blood was placed into precooled tubes containing EDTA for IL-6, growth hormone (GH), and cortisol analysis. IL-6 was analysed
by commercially available enzyme-linked immunosorbent assay (Qunatikine high sensitivity hIL-6 ELISA; R&D Systems Europe, Oxon, UK), and GH and cortisol were analysed by radioimmunoassay (Coat-a-Count, Diagnostic Products). Five milliliters of whole blood were placed into pre-cooled tubes containing lithium heparin for FFA, insulin, glucose, and lactate analysis. FFA was analysed by an enzymatic colorimetric method (NEFA C, Wako Chemicals), and insulin was analysed by radioimmunoassay (Coat-a-Count, Diagnostic Products). Glucose and lactate were immediately analysed by an automated method (Yellow Springs Instruments 2300 STAT). Samples for adrenaline determination were treated with EGTA/GSH and later analysed by radioimmunoassay (LDN, Nordhorn, Germany). All tubes were immediately spun at 1,300 rpm at 4°C for 3 min, and plasma was recovered and stored at –80°C for the respective analysis.

Muscle glycogen content. Muscle samples were freeze-dried and then powdered so as to be able to separate and remove all non-muscle tissue such as connective tissue and fat. Samples were extracted in 250 µl of 2 M HCl at 100 °C for 2 h and then neutralized with 750 µl of 0.667 M NaOH. The extract was then analysed for glucose, with the absorbance at 340 nm determined before and after addition of 1 µg of hexokinase (Passonneau & Lauderdale, 1974). Glycogen content was expressed as micromoles of glucosyl units per milligram of dry tissue.

Muscle and adipose tissue IL-6 mRNA analyses. Muscle and adipose tissue samples were extracted for total RNA by using the acid guanidium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi (Chomczynski & Sacchi, 1987), modified as previously described (Febbraio & Koukoulas, 2000). IL-6 mRNA expression was determined on these samples by real-time PCR, as previously described (Starkie et al., 2001a). IL-6 probe and primers were designed from the human IL-6 gene sequences (GenBank/EBML accession nos. M54894 and M38669). An 81-base pair IL-6 fragment was amplified by using
the forward primer 5'-GGTACATCCTCGACGGCATCT-3' and reverse primer 5'-
GTGCTCTTTGCTGCTTTCAC-3' (Sigma Geno-sys, Castle Hill, NSW, Australia). A
TaqMan fluorescent probe, 5'-FAM (6-carboxyfluorescein)-
TGTTACTCTTTGTTACATGTCTCCTTTTCAGGGCT-3' TAMRA (6-carboxy-
tetramethylrhodamine) (Applied Biosystems), was included with the primers in each reaction.
18S mRNA was also amplified, and the TaqMan probes and primers for this gene were
supplied in a control reagent kit (Applied Biosystems). Gene expression was quantified with a
multiplex comparative critical threshold (C_T) method (Bio-Rad i Cycler IQTM, Hercules,
CA). The cycle number at which the cDNA amplification is first detected is reflected by the
C_T value. For each sample, a change (Δ) in C_T value was obtained by subtracting 18S C_T
from IL-6 C_T with the resting value as the control. Resting values for each subject were
subtracted from the exercise samples for each subject to derive a Δ–ΔC_T value. The
expression of human IL-6 was then evaluated by 2–Δ–ΔCT.

Statistical analysis. A one-way ANOVA with repeated measures (time) was performed on the
resting data. Statistical analysis for the exercise study was performed by two-way ANOVA
with repeated measures (time x trial), and specific differences were identified by using a
Student-Newman-Keuls post hoc procedure. Statistical significance was set at P≤0.05. Data
are expressed as means ± SEM.
4.3 Results

Resting Experiments

Plasma FFAs, adrenaline, and IL-6 response. The plasma FFA concentration was reduced over the 120 min of NA ingestion (0 min: 0.26 ± 0.5 mM; 120 min: 0.09 ± 0.02 mM; \( P < 0.002 \)), whereas the plasma IL-6 concomitantly increased approximately eightfold (0 min: 0.75 ± 0.18 pg/ml; 120 min: 6.05 ± 0.89 pg/ml; \( P < 0.001 \)) (Figure 4.1). Small increases were observed for plasma adrenaline (0 min: 0.22 ± 0.01 nM; 120 min: 0.33 ± 0.02 nM; \( P < 0.05 \)) and GH (0 min: 0.39 ± 0.04 µg / mL; 60 min: 0.64 ± 0.04 µg / mL; \( P < 0.05 \)).

Figure 4.1 Plasma free fatty acid (FFA; A) and IL-6 (B) concentration before (0 min) and after (60 and 120 min) nicotinic acid ingestion. Values are means ± SE (n = 5). *Different from rest, \( P < 0.05 \).
Table 4.1  Whole body carbohydrate and fat oxidation during 180-min cycling exercise at 62 ± 5% \( \dot{V}O_{2\text{peak}} \) with or without nicotinic acid ingestion.

<table>
<thead>
<tr>
<th>Exercise Trial</th>
<th>Time, min</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fat oxidation, kJ/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Con</td>
<td>13.1 ± 1.7</td>
<td>14.9 ± 2.2</td>
<td>16.3 ± 3.3</td>
<td>22.1 ± 2.1</td>
<td>26.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>10.1 ± 1.8</td>
<td>13.8 ± 1.4</td>
<td>12.8 ± 2.3</td>
<td>18.6 ± 5.6</td>
<td>16.3 ± 8.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbohydrate oxidation, kJ/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Con</td>
<td>40.3 ± 2.4</td>
<td>37.4 ± 2.6</td>
<td>37.4 ± 2.2</td>
<td>32.6 ± 2.3</td>
<td>33.3 ± 4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
<td>41.7 ± 2.0</td>
<td>39.3 ± 1.3</td>
<td>42.6 ± 2.9</td>
<td>40.3 ± 5.8</td>
<td>44.1 ± 7.6</td>
</tr>
</tbody>
</table>

Values are means ± SEM; \( n = 7 \). \( \dot{V}O_{2\text{peak}} \), peak oxygen uptake; Con, control; NA, nicotinic acid.

* Significant difference from 0 min (\( P < 0.05 \)).
† Significant difference from Con (\( P < 0.05 \)).

Exercise Experiments

Respiratory responses during exercise. In both trials, oxygen uptake was initially 57 ± 3% of maximal oxygen uptake and increased (\( P < 0.05 \)) to 67 ± 5% of \( \dot{V}O_{2\text{peak}} \) during the last 60 min of exercise. The perceived rate of exertion and ventilation also increased (\( P < 0.05 \)) late in exercise (data not shown) and were not different between trials. Whole body CHO oxidation was lower (\( P < 0.05 \)) and fat oxidation higher (\( P < 0.05 \)) throughout exercise in Con compared with NA (Table 4.1).
**Plasma hormone metabolite and IL-6 responses.** Plasma FFA concentrations were not different when comparing trials before drug administration. However, consistent with our basal experiment, plasma FFA concentration was reduced ($P < 0.05$) after 60 min of rest (0 min) in NA compared with Con. In Con, plasma FFA increased ($P < 0.05$) during exercise, but this was completely suppressed ($P < 0.05$) by NA ingestion, such that values throughout exercise were lower ($P < 0.05$) in NA compared with Con (Figure 4.2 A). Plasma IL-6 was not different at rest, and there was a tendency ($P = 0.09$) for it to increase at rest after NA treatment. Although plasma IL-6 concentration increased ($P < 0.001$) throughout exercise in both trials, the increase was augmented ($P < 0.05$) throughout exercise in NA (Figure 4.2 B).

Plasma glucose concentration was elevated ($P < 0.05$) at 0 min in NA compared with Con, decreased ($P < 0.05$) in both trials late in exercise, and was lower ($P < 0.05$) in NA at 180 min compared with Con (Table 4.2). Plasma lactate was increased ($P < 0.05$) from resting levels late in exercise, and there was no difference between trials (Table 4.2). Plasma insulin concentration decreased ($P < 0.05$) during exercise in both trials, but there were no differences when comparing trials (Figure 4.3 A). Both plasma adrenaline (Figure 4.3 B) and GH (Figure 4.3 C) increased ($P < 0.05$) during exercise in both trials. However, these increases were exacerbated ($P < 0.05$) in NA compared with Con after 180 min. Plasma cortisol was not significantly affected by exercise in either trial. However, after 180 min, plasma cortisol was higher ($P < 0.05$) in NA compared with Con (Figure 4.3 D).
Figure 4.2 Plasma FFA (A) and IL-6 (B) concentration before (−60, −30, 0 min) and during (subsequent time points) 180-min cycling exercise at 62 ± 5% peak pulmonary oxygen uptake ($\dot{V}O_2$ peak) with (●) or without (○) nicotinic acid ingestion. Values are means ± SEM ($n = 7$).

*Different from control (Con), $P < 0.05$. 
Table 4.2  Plasma glucose and lactate concentration during 180-min cycling exercise at 62 ± 5% \( \dot{V}O_{2\text{peak}} \) with or without nicotinic acid ingestion

<table>
<thead>
<tr>
<th>Exercise Trial</th>
<th>-60</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>4.7 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>4.5 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>5.4 ± 0.1</td>
<td>6.0 ± 0.1†</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>4.6 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>3.8 ± 0.1*†</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>1.3 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td>2.0 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>2.5 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>1.1 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>2.6 ± 0.4</td>
<td>2.1 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>2.7 ± 0.3</td>
<td>3.0 ± 0.3*</td>
</tr>
</tbody>
</table>

Values are means ± SEM; \( n = 7 \).

* Significant difference from 0 min (\( P < 0.05 \)).
† Significant difference from Con (\( P < 0.05 \)).
Figure 4.3  Plasma insulin (A), adrenaline (B), growth hormone (C), and cortisol (D) before (0 min) and during (90 and 180 min) 180-min cycling exercise at 62 ± 5% \( \dot{V}O_2 \text{peak} \) with (■) or without (□) nicotinic acid ingestion. Values are means ± SE (n = 7). *Different from Con (\( P < 0.05 \)).
Figure 4.4  Muscle glycogen concentration before (0 min) and during (90 and 180 min) 180-min cycling exercise at 62 ± 5% $\dot{V}O_2$ peak with (solid bars) or without (open bars) nicotinic acid ingestion. Values are means ± SEM ($n = 7$). dm, dry tissue mass.

Intramuscular glycogen and IL-6 gene expression in muscle and adipose tissue.

Intramuscular glycogen content was not different when comparing the exercise trials at rest. Whereas intramuscular glycogen decreased ($P < 0.05$) during exercise in both trials, there were no significant differences either at 90 or 180 min when comparing NA with Con (Figure 4.4).

Skeletal muscle IL-6 mRNA increased ($P < 0.01$) in both trials throughout exercise. In the NA trial, skeletal muscle IL-6 mRNA was elevated two- to threefold above Con levels at 180 min, but this did not reach statistical significance ($P = 0.10$) (Figure 4.5). Adipose tissue IL-6 mRNA increased ($P < 0.001$) in both trials throughout exercise and was approximately sixfold higher ($P < 0.01$) in NA compared with Con at 180 min (Figure 4.5). Biopsy samples were
not obtained in the resting NA trial. However, comparison of the resting samples from Con in the exercise trial with the resting NA exercise trial samples (1 h after NA ingestion) revealed no differences in the parameters measured.

**Figure 4.5** IL-6 mRNA expression in skeletal muscle (A) and adipose tissue (B) before (0 min) and during (90 and 180 min) 180-min cycling exercise at 62 ± 5% \(\dot{V}\text{O}_2\text{peak} \) with (solid bars) or without (open bars) nicotinic acid ingestion. Values are means ± SE (\( n = 6 \)).

*Different from Con, \( P < 0.05 \).
4.4 Discussion

These results demonstrate that circulating IL-6 is elevated in response to acute suppression of FFAs, both at rest and during prolonged moderate-intensity exercise. Furthermore, a reduction in plasma FFA availability induced marked increases in adipose tissue IL-6 mRNA. We interpret these data to suggest that IL-6 may be released from metabolically active tissues in response to reduced FFA in the circulation.

Previous studies have demonstrated strong associations between IL-6 and lipolytic processes. Infusion of IL-6 into rats elicited a dose-dependent increase in serum FFA levels (Nonogaki et al., 1995), and elevated plasma FFA were reported in cancer patients receiving pharmacological recombinant IL-6 infusion (Stouthard et al., 1995); however, a direct lipolytic effect of IL-6 could not be ascribed because adrenaline, a lipolytic hormone, was concomitantly elevated. Wallenius et al. (Wallenius et al., 2002b) demonstrated that IL-6–/– mice have an increased energy intake and develop mature onset obesity, displaying a rapid increase in body fat compared with control mice. Importantly, fat mass was decreased in IL-6+/– mice treated with IL-6, without reducing food intake, an effect that was not observed in wild-type mice. Whereas these studies infer a lipolytic role for IL-6, a recent study using tracer isotope methodology clearly demonstrated increased adipose tissue lipolysis and increased fat oxidation during IL-6 infusion in the absence of changes in anti-lipolytic (insulin) and lipolytic hormones (adrenaline, or cortisol) (van Hall et al., 2003). Hence, one biological role of IL-6 may be to increase lipolysis, albeit a relatively modest effect compared with the potent effects of insulin and adrenaline on lipolysis.

IL-6 is secreted by adipose tissue in resting conditions (Mohamed-Ali et al., 1997; Orban et al., 1999), accounting for ~30% of the circulating IL-6 (Mohamed-Ali et al., 1997), and IL-6
mRNA expression is increased in adipose tissue during (Keller et al., 2003b) and following (Keller et al., 2001) exercise. Consistent with previous studies (Keller et al., 2003b), we demonstrated a marked (~50-fold) increased in adipose tissue IL-6 mRNA expression during exercise in Con. However, this increase was markedly augmented in NA (Figure 4.2). There are two possibilities to explain this observation. First, IL-6 infusion has been demonstrated to increase adipose tissue IL-6 mRNA (Keller et al., 2001). It is possible, therefore, that NA may have increased IL-6 release from other tissues with the subsequent increase in circulating IL-6 acting on adipocytes in this manner. Second, the adipocytes may have increased IL-6 gene expression as a direct effect of NA and/or secondary to NA-induced reduced lipolysis. From our data, we are unable to determine which of these possibilities is likely to have occurred.

In the present study, we were unable to determine the source of the increase in circulating IL-6 with NA, because we did not measure arteriovenous differences across adipose tissue, contracting skeletal muscle, or other potential IL-6-releasing tissues. In normal circumstances, the net release of IL-6 from the contracting skeletal muscle can account for the majority of the circulating plasma IL-6 (Steensberg et al., 2000). IL-6 protein is produced neither by monocytes (Starkie et al., 2001b) nor hepatosplanchnic viscera (Febbraio et al., 2003a), but a small amount of IL-6 is released from the peritendon (Langberg et al., 2002) and brain (Nybo et al., 2002). Of note, during a standard exercise bout (1-h two-legged bicycle exercise at 60% oxygen uptake), the adipose tissue does not release IL-6 (Lyngso et al., 2002a). However, given the profound effects of NA on IL-6 mRNA in adipose tissue, we cannot rule out the possibility that the adipose tissue contributed to the approximately two-fold increase in circulating IL-6 during exercise. Notwithstanding this, the fact that IL-6 mRNA tended to be higher in NA and that the contracting limb is the major source of the contraction-induced increase in plasma IL-6, our data suggest that the increase in plasma IL-6...
with NA was due to a increase in net leg release of IL-6 during NA. Further investigation using arteriovenous balance methodology is required to determine the IL-6 release across the working leg and adipose tissue with NA at rest and during exercise.

Although NA had no effect on insulin, it increased adrenaline and GH during both the resting and exercise experiments, whereas cortisol was increased during exercise. One cannot categorically rule out the possibility, therefore, that the effects of NA on the hormonal milieu mediated the changes in IL-6. It has recently been suggested (Jensen, 2003) that the lipolytic action of IL-6 during human in vivo studies (Lyngso et al., 2002a; van Hall et al., 2003) may be mediated by increases in GH. This is entirely plausible because IL-6 often (Swolin & Ohlsson, 1996; Tseng et al., 1997; Uronen-Hansson et al., 2003), but not always (Sesmilo et al., 2000; Adamopoulos et al., 2002), results in increases in GH production, and it is well known that GH per se can induce lipolysis (Fain & Bahouth, 2000; Nam & Marcus, 2000; Sesmilo et al., 2000). In recent studies, we have incubated 3T3 L1 adipocytes with IL-6 in the presence or absence of lipolytic hormones, including GH, and measured glycerol release as a marker of lipolysis. Rather than augment the IL-6-induced lipolysis, GH tended to attenuate this response (Petersen et al., 2005a). Therefore, although we cannot rule out the possibility that IL-6 is not directly associated with lipolytic processes, these preliminary studies would argue against such a hypothesis.

Adrenaline has been suggested to be a hormone-mediating IL-6 production (Helge et al., 2003) and actively increases IL-6 production when administered at supra-physiological and pharmacological concentrations (DeRijk et al., 1994; Vicennati et al., 2002; Frost et al., 2004). However, results pertaining from physiological situations are more applicable in deciphering the results of the present study. A number of physiological studies suggest that adrenaline is unlikely to mediate the increased IL-6 mRNA and protein release. An
adrenaline infusion in resting humans, which closely mimicked the exercise-induced increase in plasma adrenaline, increased plasma IL-6 only 4-fold, in contrast to the 30-fold increase observed during exercise (Starkie et al., 2001b). In agreement, the previous study (Chapter 3) showed that physiological to pharmacological doses (10–1,000 nM) of adrenaline did not stimulated either IL-6 mRNA expression in, or protein release from, isolated rodent skeletal muscle. As such, it is possible that the small elevation in plasma adrenaline induced by NA may have partly influenced the plasma levels of IL-6 during exercise. It is unlikely that the small elevation in cortisol is responsible for the increase in plasma IL-6 seen with NA because cortisol has been demonstrated to down regulate plasma IL-6 in humans (Kunz-Ebrecht et al., 2003). Therefore, the observed changes in IL-6 are unlikely to be due to the changes observed in the hormonal milieu.

The direct effect of NA on IL-6 production in the present study is not known. NA has, however, been shown to decrease IL-6 by 95 % in whole blood in response to endotoxin (Ungerstedt et al., 2003). The increase in adrenaline in resting and exercising humans due to NA is ~30–40 % (Hawley et al., 2000) and is thus not likely to play a large part in the increases observed in the present study. The vasodilatory effect of NA on peripheral blood vessels could potentially dilute circulating IL-6 levels rather than increase them, as IL-6 is not known to be induced by vasodilation, rather it itself induces vasodilation (Minghini et al., 1998). Hence, it is unlikely that NA had a pronounced effect on the present measurements other than FFA.

Both IL-6 mRNA expression in (Steensberg et al., 2001a; Keller et al., 2003a), and protein release from (Steensberg et al., 2001a), skeletal muscle are augmented by reduced glycogen availability. In the present study, CHO oxidation was augmented in NA compared with Con (Table 4.1), consistent with previous findings whereby NA administration increased the rate
of muscle glycogen utilization and whole body CHO oxidation during prolonged exercise (Carlson et al., 1963; Bergstrom et al., 1969; Gollnick & Hermansen, 1973). Despite this, no statistical differences were observed in intramuscular glycogen levels when comparing trials (Figure 4.4), and despite the tendency for a difference at 180 min, the glycogen levels at 90 min of exercise were almost identical, whereas the plasma IL-6 levels were much greater in NA at this time point, suggesting that glucose uptake and oxidation were elevated during the NA trial. We have reported that muscle glycogen depletion increases IL-6 levels (Steensberg et al., 2001a); however, it appears that our present results were not solely mediated by glycogen availability. Our laboratory's previous findings that an increase in glucose uptake and oxidation attenuate the increase in IL-6 release during exercise (Febbraio et al., 2003b) may appear contradictory to the present results; however, glucose uptake and oxidation were increased due to CHO ingestion such that the energy demand of exercise was met. In the present study, however, energy demand was high, supporting the hypothesis that IL-6 increase is associated with increased metabolic demand. To ascertain whether the increase in IL-6 is due to the lowering of plasma FFA per se or can be attributed to NA ingestion, further investigation is required whereby NA is administered in the presence of a maintained plasma FFA concentration such as obtained through an intralipid infusion.

In conclusion, this study demonstrates that circulating IL-6 is markedly elevated at rest and during prolonged moderate-intensity exercise when lipolysis is suppressed. Significant elevation of IL-6 gene expression in adipose tissue during exercise and further with plasma FFA suppression suggests that IL-6 may be released from active tissue beds such as adipose tissue and/or skeletal muscle during exercise and under metabolic challenge to mobilize fat in an "endocrine-like" manner, and such a hypothesis warrants further investigation.
CHAPTER FIVE

ROLE OF INTERLEUKIN-6 DEFICIENCY ON DIET INDUCED
INSULIN RESISTANCE IN MICE
5.1 Introduction

The IL-6 knockout (IL-6-/-) mouse was generated more than 10 years ago by Kopf and colleagues (Kopf et al., 1994) as a way in which to circumvent a number of potential problems associated with the use of anti-IL-6 antibodies and provide a unique opportunity to study the function of IL-6 in the authentic absence of endogenous IL-6. IL-6-/- mice have been shown to have normal development of organ systems and normal expression of functional cell surface markers on immune cell types (Kopf et al., 1994) and have been used as an experimental model on numerous occasions with regards to immunological parameters. However, only a few recent studies have investigated this mouse in the context of metabolism. Of note, the over-expression of IL-6 results in growth retardation (De Benedetti et al., 2001; Lieskovska et al., 2002, 2003) and fatal renal failure in early life (Katsume et al., 1997; De Benedetti et al., 2002; Katsume et al., 2002).

Wallenius and colleagues reported that IL-6-/- mice spontaneously develop mature-onset obesity (Wallenius et al., 2002b). These mice had significantly increased body fat compared with controls, elevated basal glucose levels, impaired glucose tolerance and elevated leptin. Interestingly, subsequent IL-6 treatment for 18-days significantly decreased body weight and leptin levels in the IL-6-/- mice, with no effect on control mice. However, no mechanistic investigation was pursued in an attempt to explain this phenotype. In direct contrast, Di Gregorio and colleagues (Di Gregorio et al., 2004) did not observe any differences in body mass, total body fat, glucose tolerance or insulin sensitivity between wild type and IL-6-/- mice at the same age as investigated by Wallenius (Wallenius et al., 2002b). In addition, leptin levels were similar in IL-6/- and WT, in contrast to previously reported three-fold elevations in IL-6-/- mice (Wallenius et al., 2002b). Therefore, Di Gregorio and colleagues
concluded that IL-6$^{-/-}$ mice do not develop a phenotype indicative of mature-onset obesity or diabetes, but neither did they observe improved insulin sensitivity (Di Gregorio et al., 2004).

The stark discrepancies between these two investigations at the phenotypic level are yet to be reconciled. As evidenced by the literature, and discussed in the introduction, the controversial role, or perhaps multi-faceted roles of IL-6 in insulin resistance and glucose and fatty acid metabolism remain unclear. Thus, the aim of this study was to further investigate the IL-6$^{-/-}$ mouse, in particular, to characterise the molecular regulation of insulin signalling and fat metabolism. The hypothesis was that IL-6 deficiency would cause perturbed regulation of both fat metabolism and insulin signalling.
5.2 Methods

Animals. Eight-week old male C57BL/6 mice (wild type: WT) were purchased from Monash Medical Centre (Clayton, Victoria, Australia). Eight-week old male IL-6 knockout mice were purchased from MouseWorks (Clayton, Victoria, Australia). WT mice were used as a control as we were informed by the supplier of the IL-6 knockout mice that they were on a pure C57BL/6 background backcrossed at least 8 generations. Mice were housed under controlled temperature (21 °C) and lighting (12 hours of light, 0700–1900 hours; 12 hours of dark, 1900–0700 hours) with free access to water and standard mouse chow until 10 weeks of age. All experimental procedures were approved by the Animal Experimentation Ethics Committee of RMIT University (AEEC # 0419). When mice were 10 weeks of age, the experimental protocol commenced (week 0). Mice were put onto either a standard mouse chow (3 % fat, 77 % carbohydrate, 20 % protein) or a high-fat diet (35.5 % fat, 35.7 % carbohydrate, 20 % protein; Diet F4837; Bio-Serv, Frenchtown, NJ; % by weight), both of which were available ad libitum, for 16 weeks. This model of dietary-induced insulin resistance achieved by the high fat diet creates both fasting hyperglycemia and hyperinsulinemia, a reasonable model for the human insulin resistance (Surwit et al., 1988; Burcelin et al., 2002). Daily food consumption was monitored for a 4-week period (weeks 6–10) and body mass was recorded weekly. Daily caloric intake was calculated by multiplying the consumption of the diet by its caloric density provided by the manufacturer (chow: 4.15; HF: 5.43 kcal/g).

IL-6 knockout verification. To ensure that our mice were, in fact IL-6 knockout, WT and IL-6-/- mice were stimulated intraperitoneally with a sublethal dose of lipopolysaccharide (LPS; 1.0 µg/g bw), a potent inducer of IL-6 production (Kondo et al., 2001; Frost et al., 2003). After two hours, mice were anaesthetised with sodium pentobarbital (60 mg/kg; Sigma, St. Louis, Missouri, USA) and tissues (heart, spleen, liver, muscle, and plasma) were collected.
for determination of IL-6 gene expression and protein by RT-PCR and western blot, respectively. Tissues were extracted for total RNA and IL-6 gene expression was quantified and analysed as previously described (Chapter 3). Western blot analysis for IL-6 protein was performed as described below.

**Glucose and insulin tolerance tests.** Glucose and insulin tolerance tests were performed on both strains of mice at week 8 and glucose tolerance tests were performed again at week 14. For glucose and insulin tolerance tests, mice were fasted for 6 h (0600–1200). Fasting protocols for glucose and insulin tolerance tests and hyperinsulinemic-euglycemic clamps were the same for consistency and comparative reasons. Six-hour fasting was chosen rather than overnight fasting for several reasons; i) mice are nocturnal feeders and overnight fasting would thus commence when they are in a low-consumption mode; ii) overnight fasting significantly decreases fat and muscle tissue and thus body weight (approximately 16%), decreases hepatic glycogen content, plasma glucose and insulin; iii) overnight fasting increases insulin sensitivity and makes maintenance of euglycaemia more difficult (Feuger et al. 2004; Ayala et al. 2006).

An initial blood sample ($t = 0$) was taken from the tail for measurement of fasting glucose. For glucose tolerance tests dextrose (1 mg/g body mass) was administered intraperitoneally. Blood samples for glucose analysis were collected from the tail at 10, 20, 30, 45, 60, 90, and 120 min following injection. Blood glucose was measured from whole blood immediately using a HemoCue Glucose 201+ Analyser (HemoCue, Ängelholm, Sweden). Blood for insulin measurement was taken at 0, 15, or 60 min (one measure per mouse) and treated with EDTA. For insulin tolerance tests, insulin (recombinant human insulin; Actrapid®; 0.85 IU/kg body weight) was administered intraperitoneally and blood samples for glucose analysis were taken at 15, 30, 45, 60, 90, and 120 min after injection.
Surgical Procedures. Approximately 2 weeks after the week-14 tolerance test, each mouse was implanted with 2 catheters (Micro-Renathane MRE 025, 0.025-inch outer diameter, 0.012-inch inner diameter; Braintree Scientific Inc., Braintree, Massachusetts, USA) into the right jugular vein. The anaesthetic cocktail consisted of ketamine hydrochloride (80 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa, USA), acepromazine maleate (0.5 mg/kg; Butler Co., Columbus, Ohio, USA), and xylazine (1.6 mg/kg; Butler Co.) given intraperitoneally. Catheters were tunnelled subcutaneously, exteriorized at the back of the neck, and filled with heparinized saline and sealed with stainless steel plugs. Animals were each given approximately 1 mL of saline subcutaneously and were maintained on a heating pad for a 24-hour recovery period.

Hyperinsulinemic-Euglycemic Clamp. After 16 weeks on the high fat diet (approximately 6 months of age) and four days after surgery, glucose turnover was measured in either the basal state or during a hyperinsulinemic-euglycemic clamp (the latter is a measure of insulin sensitivity). Mice were fasted for 6 hours and then placed in a restrainer to which they had been conditioned on a daily basis for the previous 2 weeks. Exteriorised catheters were exposed and plugs removed. Micro-Renathane tubing was connected to the catheter lead, gently flushed with heparinized saline to confirm patency, and linked up to infusion syringes. Following basal blood glucose sampling the hyperinsulinemic-euglycemic clamp was performed by infusing recombinant human insulin (Novolin R; Novo Nordisk Pharmaceutical Industries Inc., Clayton, North Carolina, USA) at 6 mU/kg/min into the jugular cannula. The insulin infusate comprised the relevant insulin volume, 100 µL/mL, 8% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA) and was diluted to a final volume with saline (0.9 % NaCl). Blood samples (5 µL) were drawn at 10 min intervals for the immediate determination of blood glucose using a HemoCue Glucose 201+ Analyser. Based on these
values, 50% dextrose (Abbott Laboratories, Chicago, Illinois, USA) was variably infused into the other jugular catheter to maintain the plasma glucose concentration at approximately 7.8 ± 0.2 mmol. Steady state (stable plasma glucose concentration and exogenous glucose infusion rate for a minimum of 30 min) was generally achieved within 90–120 min. Each animal was then promptly anaesthetised (ketamine hydrochloride; 80 mg/kg, acepromazine maleate; 0.5 mg/kg, and xylazine; 1.6 mg/kg). Soleus muscles from basal clamp animals were carefully excised tendon to tendon for fatty acid oxidation (see below). Blood was collected from the inferior vena cava for insulin analysis. Tissues (liver, skeletal muscle, brown and white adipose, heart, and spleen) were harvested and rapidly frozen in liquid nitrogen and stored at –80 °C for subsequent analysis.

**Plasma Analysis.** Plasma insulin was measured by ELISA (#EZRMI-13K, Linco Research, Missouri, USA). Free fatty acid content (FFA) was measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA C kit; Wako Chemicals, Richmond, VA).

**Analysis of muscle and liver lipid species.** Intramuscular triacylglycerol (IMTG) content was analysed as previously described (Frayn & Maycock, 1980b). Lipid was extracted from liver and muscle tissue by Folch extraction (Folch et al., 1957). Wet tissue was homogenised in 2.5 ml chloroform:methanol (2:1) plus 0.5 ml chloroform, then gently mixed with 1.5 ml 4 mM MgCl₂ and centrifuged at 100 g for 30 min. The upper aqueous phase was carefully aspirated then 1.8 ml of the lower organic phase was transferred to another vial and evaporated under nitrogen at 37 °C. Triacylglycerol was saponified to glycerol and FFA in 250 µl warm ethanoic KOH (5 parts 80 % KOH : 95 parts absolute ethanol), incubated at 60 °C for 60 min. 0.5 ml of 0.15 M MgSO₄ was added and mixed thoroughly to neutralise the solution, which was then centrifuged at 10 000g for 2 min to pack down the precipitate. The supernatant was collected and stored at –80 °C for fluorometric analysis of glycerol. The
extract was then analyzed for glycerol, with the absorbance at 340 nm determined before and after addition of glycerol kinase and glycerol-3-phosphate dehydrogenase. Glycerol content was expressed as nanomoles per milligram of tissue (wet weight). Diacylglycerol (DAG) and ceramide were extracted and quantified according to the methods described by Preiss and colleagues (Preiss et al., 1987). Briefly, lipids were extracted from liver and mixed gastrocnemius muscle by homogenising tissue in 1.9 mL chloroform/methanol/PBS and 0.2 % sodium dodecyl sulfate (1:2:0.8), adding 500 µl chloroform and thoroughly mixing. The organic and solvent phases were separated by adding 500 µl 1 % perchloric acid, mixed and centrifuged at 2000 rpm for 2 min. Without disturbing the tissue/chloroform interface, the upper layer was aspirated off, and then 450 µl of the lower chloroform phase was collected and dried under nitrogen in a 37 °C water bath. The samples were then reconstituted in 400 µl chloroform/methanol (2:1) and mixed. Samples were again dried under nitrogen (37°C). The reaction was primed by adding 20 µl octyglucoside/cardiolipin to samples, mixed thoroughly, and allowed to stand for 10-15 min (RT). A reaction mixture containing diacylglycerol kinase and [γ-32P]ATP (15 µCi/µmol cold ATP) was added to extracts, gently mixed and incubated for 2-h (RT). The reaction was stopped using 2 ml chloroform/methanol (2:1) and mixed thoroughly. The phase was broken by addition of 500 µl 1 % perchloric acid and thoroughly mixing. After centrifugation (5 min, 1000 rpm) the upper phase was aspirated off and a total of 830 µl of the lower phase recovered and dried under nitrogen (37°C). Samples were mixed with 30 µl chloroform/methanol (2:1) and spotted onto thin layer chromatography plates, developed in chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10) to two thirds of the total plate length. Bands corresponding to DAG and ceramide were identified against standards after phosphoimaging, dried, scraped from the thin layer chromatography plate, and counted in a liquid scintillation analyser (Tri-Carb 2500TR, Packard, Canberra, Australia).
Fatty acid oxidation in soleus muscle:

Pre-incubation and Pre-experimental labelling of the intramuscular lipid pools. Soleus muscles were incubated in 2 ml of base buffer for 30 min to permit equilibration. Briefly, muscle strips were removed from the pre-incubation buffer and transferred to a second vial with incubation medium containing 0.5 μCi/ml of [1-14C] palmitate (Amersham Life Science) for 90 min. Palmitate oxidation and esterification were monitored by the production of 14CO2 and incorporation of [1-14C] palmitate into endogenous lipids.

Extraction of Muscle Lipids. Muscles were placed in 13 ml plastic centrifuge tubes containing 5.0 ml of ice-cold 1:1 chloroform methanol (vol/vol) and homogenised using a polytron (Brinkman Instruments, Mississauga, ON, Canada). Following homogenisation, samples were centrifuged at 2000 g (4°C) for 10 min. The supernatant was removed with a glass Pasteur pipette and transferred to a clean centrifuge tube. Distilled water (2.0 ml) was added, samples were shaken for 10 min, and centrifuged as before to separate the aqueous and lipophilic phases. One ml of the aqueous phase was quantified by liquid scintillation counting to determine the amount of 14C labelled oxidative intermediates resulting from isotopic fixation. This represented a 2-fold correction factor for exogenous 14C-palmitate oxidation. Gaseous 14CO2 produced from the exogenous oxidation of [1-14C] palmitate during the incubation was measured by transferring 1.0 ml of the incubation medium to a 20 ml glass scintillation vial containing 1.0 ml of 1 M H2SO4 and a 0.5 ml Fisher microcentrifuge tube containing 1 M benzethonium hydroxide. Liberated 14CO2 was trapped in the benzethonium hydroxide over 60 min, and the microcentrifuge tube containing trapped 14CO2 was placed in a scintillation vial and counted. Total palmitate oxidation was calculated as oxidative intermediates plus 14CO2 produced.
Western blot analysis of tissue proteins. Tissues were homogenized (Polytron; Brinkman Instruments, New York, NY, USA) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 0.5 % Triton X-100, 10 % glycerol (v/v), 3 µl/ml protease inhibitor cocktail, 100 µg/ml phenylmethylsulfonyl fluoride and 3 µl/ml phosphates inhibitor cocktail. Homogenates were centrifuged and the supernatant was removed. An aliquot was used to determine protein concentration (Pierce, Rockford, IL, USA). Subsequent aliquots of 100 ug protein were solubilized in 4 x Laemmli sample buffer and boiled for 5 min then rapidly frozen in liquid nitrogen and stored at –80 °C. Tissue lysates were resolved by SDS-PAGE on 6-16 % polyacrylamide gels (120 V for 30 min, then 140 V for approx 60 min), transferred to a nitrocellulose membrane (120 min; 0.06 A/membrane), washed twice in TBST, blocked with 5% bovine serum albumin in TBS (120 min at room temperature), washed thrice in TBST, and then immunoblotted with the relevant antibody (overnight at 4°C). Antibodies used are as follows: anti-IRS-1, anti-IRS-2, anti-phospho p85-IRS-1-PI3K, anti-phospho(Ser79)-ACC (Upstate Cell Signalling Solutions, Lake Placid, NY), anti-phospho (Tyr805)-IRS-1 (Oncogene Research Products, Boston, MA), anti-phospho (Ser473)-Akt, anti-Akt, anti-phospho-AMPK-Thr172, anti-AMPK, anti-phospho-p38 MAP kinase (MAPK) (Cell Signalling Technology, Beverly, MA), anti-UCP3 (Alpha Diagnostics, San Antonio, TX), anti-SOCS3, anti-TNFα, anti-phospho (Ser32)-IκBα, anti-IκBα (Santa Cruz Biotechnology, CA), anti-phosphoPGC1α or anti-PGC1α antibody (Chemicon International, Boronia, Victoria, Australia). The next morning membranes were washed thoroughly in TBST (6 x 5 min) then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000–1:4000; Amersham Biosciences, Castle Hill, NSW, Australia), then washed thoroughly again (3 x 5 min, 3 x 10 min). The immunoreactive proteins were then detected with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, England) on a Chemidoc XRS imaging system (BIO-RAD, Hercules, USA). Proteins were quantified by densitometry (Quantity One 1-D Analysis Software). Membranes were then
stripped (stripping buffer details; 5 min RT) of primary and secondary antibodies, washed, re-blocked, and then probed with the corresponding total primary antibody. All values reported are expressed relative to basal WT chow.

**AMPK α1 and α2 activity.** Lysates from muscle, liver and WAT were incubated with AMPK α1 and AMPK α2 antibody-bound protein A agarose beads for 2-h, immuno-complexes were washed and AMPK activity was determined in the presence of 200 µM AMP (Chen et al., 2000).

**Statistical analysis.** Data was analyzed using a one way or two-way analysis of variance (ANOVA) with repeated measures (SPSS), with significance accepted with a $P$ value of $< 0.05$. If analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. Data are presented as means ± SEM.
5.3 Results

*IL-6 knockout verification.* Western blot analysis of IL-6 in spleen and muscle confirmed the deficit of IL-6 protein in IL-6−/− mice (Fig 5.1). Results from RT-PCR also confirmed the absence of IL-6 in IL-6−/− mice as gene amplification was detected in WT and not IL-6−/− mice. On obtaining results that appeared contrary to the published literature (Wallenius *et al.* 2002) we decided to confirm that our WT mice were indeed the appropriate controls, by genetically testing the strains. Despite being told by the supplier of the IL-6 knockout litter that the mice were on a pure C57BL/6 background, genetic tests showed that the IL-6 knockout mice were on a mixed (C57BL/6 x 129J) background. This makes the comparison of our knockout mice with our WT mice difficult to interpret.

*Body and Tissue Mass.* Body mass of mice commencing the experimental protocol was not different between the strains (Table 5.1). All mice increased body mass over the experimental timeframe in accordance with the expected growth rate. During the 16-weeks of HF diet WT, but not IL-6−/− mice increased body mass compared with chow-fed mice (*P* < 0.05). At the end of the experiment IL-6−/− mice on both diets had a lower body mass than WT mice (*P* < 0.05; Table 5.1). Various tissue masses of the groups are reported in Table 5.1. Of note, muscle mass was not significantly different between strains. White adipose depots were smaller in IL-6−/− mice (*P* < 0.05) and elevated by HF in both strains (*P* < 0.05). The spleen and kidneys of IL-6−/− mice were heavier than those of WT mice (*P* < 0.05).

*Food intake.* As demonstrated in Table 5.1, WT mice on HF diet maintained the same daily energy intake as WT Chow mice. IL-6−/− Chow mice had a greater daily energy intake then any other group of mice (*P* < 0.05), and IL-6−/− HF mice had a lower caloric intake than other groups (*P* < 0.05), which was accounted for by their lower body mass.
Glucose and insulin tolerance tests. At week-8 fasting basal glucose was elevated in HF, consistent with induction of insulin resistance ($P < 0.05$). In IL-6$^{-/-}$ fasting basal glucose levels were lower than that of WT on respective diets ($P < 0.05$; Table 5.2). After 14 weeks of the dietary protocol the fasting basal glucose concentrations in all groups had increased slightly, consistent with the aging of the animal, but the pattern of lower levels in IL-6$^{-/-}$ and elevated levels with HF was maintained ($P < 0.05$).

Insulin tolerance tests performed at week-8 showed that IL-6$^{-/-}$ were much more sensitive to the insulin stimulus than WT (Figure 5.2). Relative to WT chow the insulin bolus caused a greater drop in IL-6$^{-/-}$ chow blood glucose levels. This observation also held true when values were expressed as a proportion of initial glucose levels, as IL-6$^{-/-}$ chow had lower basal glucose levels than WT ($P < 0.05$). The recovery of glucose levels was not rapid in IL-6$^{-/-}$ chow because glucose levels were reduced to such low levels. The insulin dose used was chosen because it is commonly used in the literature and preliminary studies showed that this dose elicited an approximately 50 % reduction in blood glucose in WT. However, given the low glucose levels of IL-6$^{-/-}$, a lower insulin dose should have been used, in which case we might have seen a restoration of glucose levels. The insulin tolerance test did show that in WT the HF elicited an impaired ability to restore blood glucose. Expressing glucose values as a percentage of basal glucose, the area under the curve for WT chow was 64 ± 5 % where as that for WT HF was 55 ± 3 %, that of IL-6$^{-/-}$ HF was 47 ± 3 %, but how this relates to IL-6$^{-/-}$ chow cannot be established through this line of data. It was deemed unnecessary to perform ITT at week-14, as the hyperinsulinemic-euglycemic clamp at week-16 was a more rigorous measure of insulin sensitivity.
The glucose bolus administered for the glucose tolerance test increased WT and IL-6⁻/⁻ chow and HF glucose levels to a similar extent above baseline (175 ± 9 %; 171 ± 6 % respectively, $P < 0.05$). Figure 5.3 shows the glucose concentrations during GTT at week-8. This test shows that IL-6⁻/⁻ chow are more glucose tolerant than WT chow. After 8 weeks the HF diet had impaired glucose tolerance in WT and even more so in IL-6⁻/⁻ as indicated by the elevated curve ($P < 0.05$). The characteristic of lower glucose in IL-6⁻/⁻ and the effect of HF persisted at week-14 ($P < 0.05$; Figure 5.3). Interestingly, by week-14 IL-6⁻/⁻ HF had rectified their glucose tolerance to a large extent and their presiding impairment at this time was similar to that seen in WT. When these glucose curves are expressed as the area under the curve (AUC), i.e. the amount of glucose in circulation during the 120 min, the status of glucose tolerance becomes more apparent. AUC for WT HF was higher than WT chow ($P < 0.05$). This was also the case in IL-6⁻/⁻ ($P < 0.05$). The lower basal glucose in IL-6⁻/⁻ could account for the AUC for IL-6⁻/⁻ being lower than WT. In fact, when AUC was calculated relative to baseline glucose levels, AUC for IL-6⁻/⁻ chow and HF tended to be lower than WT, although did not reach significance (169±6; 170±7; 181±5; 192±6% respectively).

**Hyperinsulinemic-euglycemic clamp.** The week-14 fasting glucose measures of all animals were averaged and the resulting value, 7.8 ± 0.2 mmol, was deemed to be the glucose concentration which all mice would be clamped at when maintaining euglycemia. There were no differences between groups regarding the level of euglycemia; all animals were clamped at 7.7 ± 0.2 mmol (Figure 5.4). The glucose infusion rate for WT chow was 62.5 ± 8.2 mg/kg/min. This value means that for the 6 mU/kg of insulin being infused, approximately 62.5 mg/kg of glucose was required to maintain the blood glucose at approximately 7.7 mmol. This value was lower in WT HF (47.4 ± 9.6 mg/kg/min: $P < 0.05$) meaning that there was a lesser degree of insulin-stimulated glucose disposal and thus WT HF were less insulin sensitive than WT chow. IL-6⁻/⁻ chow were more insulin sensitive than WT chow, with a
GIR of 93.4 ± 9.4 mg/kg/min \((P < 0.05)\). Insulin sensitivity of IL-6\(^{-/}\) was impaired by HF (GIR 62.4 ± 3.4 mg/kg/min), however this was comparable to WT chow GIR (Figure 5.4).

**Plasma measures.** FFA were lower in HF groups of both strains and lower in IL-6\(^{-/}\) than WT when compared with WT chow \((P < 0.05; \text{Table 5.2})\). This was an unexpected finding, but likely due to metabolic flexibility allowing a shift to greater fat metabolism when confronted with high fat consumption. Basally, plasma insulin was lower in IL-6\(^{-/}\) chow than WT chow \((P < 0.05)\). HF increased insulin in WT and IL-6\(^{-/}\). When insulin measures from ITT were calculated as AUC, there were no differences between the strains, but higher values in HF groups \((P < 0.05; \text{Table 5.2})\).

**Homeostasis model assessment of insulin resistance (HOMA-IR):** Insulin resistance was estimated by HOMA-IR and was defined as fasting plasma insulin \((\mu\text{U/mL})\) x fasting plasma glucose \((\text{mmol/l})/22.5\) (Matthews et al., 1985). The HOMA method has been shown to be a useful measure for assessing insulin resistance across a wide range of glucose levels in the human population (McAuley et al., 2001). HOMA-IR indicated that HF impaired insulin sensitivity in both strains \((P < 0.05; \text{Table 5.2})\).

**Triacylglycerol content of gastrocnemius muscle and liver.** Levels of TAG in gastrocnemius muscle were lower in IL-6\(^{-/}\) chow than WT chow \((P < 0.05)\). HF increased TAG content in both strains \((P < 0.05)\), with similar levels observed in WT and IL-6\(^{-/}\) HF (Figure 5.5). Levels of TAG in liver tissue were lower in IL-6\(^{-/}\) than WT \((P < 0.05)\). HF increased TAG content in both strains \((P < 0.05)\), but levels in IL-6\(^{-/}\) HF were much lower than WT HF \((P < 0.05)\) and were similar to WT chow (Figure 5.5).
Diacylglycerol and ceramide content of gastrocnemius muscle and liver. Gastrocnemius muscle DAG content was not different between WT and IL-6/−/− chow, but was elevated by HF (P < 0.05), although to a lower extent in WT HF than IL-6/−/− HF tissue (P < 0.05; Figure 5.5). Liver DAG content was lower in IL-6/−/− chow than WT chow (P < 0.05), and was neither affected by HF nor different between strains on HF. Gastrocnemius muscle ceramide content was elevated in IL-6/−/− chow (P < 0.05), but not significantly altered by HF in either strain (Figure 5.5). Liver ceramide content was elevated in IL-6/−/− compared with WT on both diets (P < 0.05).

Soleus fatty acid oxidation. Oxidation of palmitate in soleus muscles under basal conditions was higher in IL-6/−/− than WT in both diet groups (P < 0.05) and HF decreased palmitate oxidation in both strains (P < 0.05; Figure 5.6).

Insulin signalling proteins in liver and skeletal muscle. Total IRS-1 and -2 protein in liver were increased in IL-6/−/− chow (P < 0.05) but not other groups compared with WT chow. No differences were seen in total IRS-1 or -2 protein in gastrocnemius muscle (Figure 5.7). IRS-1-associated PI3K regulatory subunit, p85, was not detectably different between groups but was increased with insulin stimulation (Figure 5.8). No differences were seen in IRS-1 Tyr895 phosphorylation (Figure 5.8).

Akt in liver and skeletal muscle. HF in WT did not affect total Akt in liver, and IL-6/−/− HF was not different to WT. However, total Akt was higher in IL-6/−/− chow compared with WT chow and IL-6/−/− HF (P < 0.05). Basal Ser473 phosphorylation (pSer473) of Akt was lowered by HF in WT (P < 0.05) but not IL-6/−/−, and was lower in IL-6/−/− chow than WT (P < 0.05). Insulin stimulation increased pSer473 in all groups to a similar level (P < 0.05; Figure 5.9).
**Peroxisome proliferator activated receptor gamma coactivator 1 (PGC1α) in liver and skeletal muscle.** PGC1α, a regulator of transcription enzymes necessary for substrates oxidation was measured in muscle and liver tissue. *Gastrocnemius* muscle PGC1α was not different between strains or diets, however there was a trend to increase levels in IL-6−/− HF (*P* = 0.09). Liver PGC1α was decreased in both strains with HF compared with WT chow (Figure 5.10).

**AMP-activated protein kinase (AMPK) in liver, skeletal muscle and adipose tissue.** AMPK is a known cellular energy sensor, the phosphorylation of which, at Thr^{172} (pAMPK), was investigated as a measure of its activation status. Total AMPK protein was not different between the strains or diets in WAT, liver, *gastrocnemius* or *quadriceps* tissues. Basal phosphorylation of AMPK at Thr^{172} was elevated in WAT (*P* < 0.05), decreased in liver (*P* < 0.05), and not different in *gastrocnemius* muscle of IL-6−/− chow compared with WT chow. HF decreased AMPK Thr^{172} phosphorylation in liver of both strains (*P* < 0.05; Figure 5.11).

**AMPK α1 and α2 activity in liver, skeletal muscle and adipose tissue.** WAT AMPK α1 activity was higher in IL-6−/− mice than WT mice (*P* < 0.05). Liver AMPK α1 activity tended to be lower in IL-6−/− mice than WT mice (borderline significance *P* = 0.06). No differences were seen between the strains in liver α2, quad α1 or α2 activity (Figure 5.12).

**Acetyl-CoA carboxylase (ACC) in liver, skeletal muscle and adipose tissue.** Phosphorylation, and thus inhibition, of ACC, to allow fatty acid oxidation, was increased in WAT (*P* < 0.05) and tended to be decreased in liver and *quadriceps* muscle of IL-6−/− mice compared with WT mice (borderline significance *P* = 0.08, *P* = 0.06, respectively; Figure 5.13).
Uncoupling protein 3 (UCP3) in adipose tissue. WAT UCP3 was increased in IL-6^{-/-} HF (P < 0.05) but not WT HF compared with WT chow in the basal state (Figure 5.14).

Suppressor of cytokine signalling 3 (SOCS3) in liver and skeletal muscle. HF did not alter SOCS3 protein in liver tissue or gastrocnemius muscle. No differences were seen between the strains in liver tissue, but SOCS3 was ~10% lower in basal IL-6^{-/-} gastrocnemius muscle compared with WT (P = 0.08; Figure 5.15).

Tumour necrosis factor alpha (TNFα) in liver. Basally, TNFα was elevated in liver of all groups compared with WT chow (P < 0.05) but was not different between the strains on HF (Figure 5.16).

Inhibitor of kappa B alpha (IκBα) in liver. Total IκBα protein in liver was not different between strains or diets. pSer^{32} IκBα was increased basally in chow and HF IL-6^{-/-} compared with WT chow and HF (P < 0.05; Figure 5.17).
Table 5.1 Food intake and tissue mass of WT and IL-6<sup>-/-</sup> mice on chow and HF diets.

Tissue mass is reported in mg from 26-week old mice. All data are expressed as mean ± SEM (n = 12-16). # P < 0.05 vs. WT; § P < 0.05 vs. Chow.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>IL-6&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chow</td>
<td>HF</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>26 ± 0</td>
<td>26 ± 0</td>
</tr>
<tr>
<td>Food intake (kcal/d)</td>
<td>18.2 ± 0.4</td>
<td>17.3 ± 0.8</td>
</tr>
<tr>
<td>Body Mass 10wo (g)</td>
<td>27.6 ± 0.4</td>
<td>-</td>
</tr>
<tr>
<td>Body Mass 26wo (g)</td>
<td>33.7 ± 0.3</td>
<td>36.5 ± 0.9&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart</td>
<td>157.1 ± 7.4</td>
<td>143.5 ± 5.8&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spleen</td>
<td>101.2 ± 5.3</td>
<td>100.5 ± 7.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>204.0 ± 3.4</td>
<td>-</td>
</tr>
<tr>
<td>Soleus</td>
<td>16.1 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td>EDL</td>
<td>9.3 ± 0.4</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Tibialis Anterior</td>
<td>32.5 ± 1.3</td>
<td>32.1 ± 1.1</td>
</tr>
<tr>
<td>Quadriceps Muscle</td>
<td>129.2 ± 4.1</td>
<td>124.8 ± 5.2</td>
</tr>
<tr>
<td>BAT</td>
<td>62.0 ± 5.2</td>
<td>59.8 ± 4.9</td>
</tr>
<tr>
<td>Infrarenal adipose pad</td>
<td>6.8 ± 0.9</td>
<td>18.9 ± 2.8&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymal adipose pad</td>
<td>32.8 ± 4.9</td>
<td>39.8 ± 5.6&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 5.2 Basal plasma measures from 6-h fasted mice. All data are expressed as mean ± SEM (n = 12-16). #: P < 0.05 vs. WT; § P < 0.05 vs. Chow.

<table>
<thead>
<tr>
<th>Glucose (mmol)</th>
<th>Wild Type</th>
<th></th>
<th></th>
<th>IL-6−/− KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chow</td>
<td>HF</td>
<td>Chow</td>
<td>HF</td>
</tr>
<tr>
<td>Week 0</td>
<td>7.8 ± 0.4</td>
<td>-</td>
<td>5.7 ± 0.4#</td>
<td>-</td>
</tr>
<tr>
<td>Week 8</td>
<td>8.1 ± 0.4</td>
<td>9.7 ± 0.3§</td>
<td>6.1 ± 0.3#</td>
<td>7.4 ± 0.4#§</td>
</tr>
<tr>
<td>Week 14</td>
<td>9.1 ± 0.3</td>
<td>10.4 ± 0.3§</td>
<td>6.9 ± 0.3#</td>
<td>7.8 ± 0.4#§</td>
</tr>
<tr>
<td>FFA (mmol)</td>
<td>0.68 ± 0.09</td>
<td>0.39 ± 0.03§</td>
<td>0.52 ± 0.04#</td>
<td>0.35 ± 0.04§</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.55 ± 0.07</td>
<td>0.95 ± 0.16§</td>
<td>0.47 ± 0.07#</td>
<td>0.65 ± 0.08#§</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.2 ± 0.7</td>
<td>6.9 ± 0.9</td>
<td>3.7 ± 0.5</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>AUC (GTT insulin)</td>
<td>41.0 ± 3.5</td>
<td>60.5 ± 4.6§</td>
<td>41.7 ± 2.7</td>
<td>56.1 ± 4.7§</td>
</tr>
</tbody>
</table>
Figure 5.1 IL-6 protein in LPS-stimulated spleen and muscle tissue of WT (solid bars) and IL-6$^{-/-}$ (open bars) mice. Representative blots are shown. All data are expressed relative WT as mean ± SEM ($n = 6$). # P < 0.05 vs. WT.
Figure 5.2 Glucose measures during insulin tolerance tests (0.85 mU/g bm) performed at week-8 in WT chow (▬■▬) and HF (---■---), and IL-6<sup>−/−</sup> chow (▬◊▬) and HF (---◊---) mice. Glucose data are expressed as area under the curve (AUC) for 120-min insulin tolerance test in WT (solid bars) and IL-6<sup>−/−</sup> (open bars) chow and HF mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
**Figure 5.3** Glucose measures during glucose tolerance tests (1 mg/g bm) performed at week-8 (left) and week-14 (right) in WT chow (▬■▬) and HF (---■---), and IL-6^/-^ chow (▬◊▬) and HF (---◊---) mice. Glucose data expressed as area under the curve (AUC) for 120-min glucose tolerance test in WT (solid bars) and IL-6^-/-^ (open bars) chow and HF mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.4  Plasma glucose of mice during steady state of the hyperinsulinemic-euglycemic clamp (upper). Glucose infusion rate (GIR) at euglycemia with 6 mU/kg/min insulin infusion in WT (solid bars) and IL-6−/− (open bars) chow and HF mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.5  Triacylglycerol (upper), diacylglycerol (middle), and ceramide (lower) content in skeletal muscle (left) and liver tissue (right) of WT (solid bars) and IL-6\(^{+/−}\) (open bars) chow and HF mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.6 Palmitate oxidation in *soleus* muscles of WT (solid bars) and IL-6<sup>-/-</sup> (open bars) chow and HF mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.7 Basal *gastrocnemius* muscle (left) and liver tissue (right) IRS1 (upper) and IRS2 (lower) protein of WT (solid bars) and IL-6⁻/⁻ (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM ($n = 6$). # $P < 0.05$ vs. WT; § $P < 0.05$ vs. chow.
Figure 5.8  Basal and insulin-stimulated liver tissue IRS1 Tyr 895 phosphorylation (upper) and IRS1-associated PI3K p85 protein (lower) of WT (solid bars) and IL-6−/− (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.9  Akt Ser\textsuperscript{473} phosphorylation (upper), total protein (middle), and phosphorylation relative to total (lower) in liver tissue of WT (solid bars) and IL-6\textsuperscript{-/-} (open bars) chow and HF mice under basal and insulin-stimulated conditions as indicated. Representative blots are shown. All data are expressed relative to basal WT chow as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.10  PGC1α protein in *gastrocnemius* muscle (upper) and liver tissue (lower) of WT (solid bars) and IL-6−/− (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM (*n* = 6). # *P* < 0.05 vs. WT; § *P* < 0.05 vs. chow.
Figure 5.11  AMPK Thr^{172} phosphorylation relative to total AMPK protein in *gastrocnemius* muscle (upper), liver (middle), and white adipose tissue of WT (solid bars) and IL-6^{-/-} (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM (*n* = 6). # *P* < 0.05 vs. WT; § *P* < 0.05 vs. chow.
Figure 5.12 AMPK α1 and α2 subunit activity in liver tissue (upper), quadriceps muscle (middle), and epididymal white adipose tissue (lower) of chow-fed WT (solid bars) and IL-6−/− (open bars) mice. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. WT.
Figure 5.13 ACCβ phosphorylation in quadriceps muscle, liver, and epididymal white adipose tissue (WAT) of chow-fed WT (solid bars) and IL-6⁻/⁻ (open bars) mice. Representative blots are shown. All data are expressed as a proportion of total protein relative to WT and reported as mean ± SEM ($n = 6$). # $P < 0.05$ vs. WT.
**Figure 5.14** UCP3 in epididymal white adipose tissue of chow-fed WT (solid bars) and IL-6⁻/⁻ (open bars) mice. Representative blots are shown. All data are expressed relative to WT and reported as mean ± SEM ($n = 6$). # $P < 0.05$ vs. WT.
Figure 5.15 SOCS3 protein in liver tissue of WT (solid bars) and IL-6−/− (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
Figure 5.16  TNFα protein in liver tissue of WT (solid bars) and IL-6\(^{-/-}\) (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM \((n = 6)\). \# \(P < 0.05\) vs. WT; § \(P < 0.05\) vs. chow.
Figure 5.17  IκBα Ser^{32} phosphorylation relative to total IκBα protein in basal (left) and insulin stimulated (right) liver tissue of WT (solid bars) and IL-6^{-/} (open bars) chow and HF mice. Representative blots are shown. All data are expressed relative to WT chow as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. chow.
5.4 Discussion

There is conflicting literature regarding the effect of IL-6 knockout on metabolism (Wallenius et al., 2002b; Di Gregorio et al., 2004). In the present study, we did not detect the error in the genetic background of the animals, until we started the analyses. Therefore, the fact that our mice were on different genetic backgrounds makes comparisons very difficult to interpret. In general, however, data from this study demonstrate that IL-6^-/- mice display low basal glucose, rapid glucose elimination, and a high rate of insulin-stimulated glucose utilisation compared with WT. Thus, they are markedly glucose tolerant and insulin sensitive, which is likely to be the consequence of improved fat metabolism. The investigation also confirms the development of glucose intolerance and insulin resistance in high fat-fed mice.

Despite the fact that comparisons are difficult to make when comparing WT with IL-6^-/- mice, when one carefully examines the data within rather than between groups, our data suggest two major findings. The first is that the genetic background of the mice is very important when studying metabolic parameters. The second is that, at least in mice that are on a mixed (C57BL/6 x 129J) background, IL-6 knockout prevents some of the deleterious effects of diet induced obesity. Wallenius and colleagues (Wallenius et al., 2002b) reported that IL-6 knockout resulted in greater weight gain as the mice aged. In our study, this was not the case. As can be seen in Table 5.1, there was no difference when comparing strains at 10 or 26 weeks of age. Moreover, at 26 weeks the IL-6^-/- mice were resistant to weight gain on the HF diet. This suggests that when the IL-6 knockout is made in a mouse on a mixed background, it is protected from the deleterious effects of a HF diet. It is reasonable to expect that the sensitivity of determining food intake was similar for all groups, thus the lower body mass and energy intake of IL-6^-/- Hfmice may account for some of the observations but cannot be quantitatively assessed. A mechanism for decreased food intake warrants investigation.
The HF diet was effective in reducing glucose tolerance and inducing insulin resistance. This was reflected by increased basal blood glucose, increased AUC during GTT, and reduced GIR during the clamp in HF-fed animals. Moreover, relative HF-induced impairments of many measured parameters were similar in each strain. Of note, the GTT area under the curve (Figure 5.3), GIR during the clamp (Figure 5.4) and the rates of fatty acid oxidation in muscle (Figure 5.6) were all similarly affected when animals were placed on a HF diet. This may suggest that the effect of genetic background was quantitatively more important than deletion of IL-6, as the mice responded similarly when subjected to the diet.

Targeted mutagenesis in mice is a powerful tool for the analysis of gene function and human disease. The recognition that different inbred strains harboured different susceptibilities for the development of obesity and diabetes came about as early as the 1970s. It was observed that the spontaneous mutation of the leptin gene (ob/ob) or the leptin receptor gene (db/db) brought about disparate phenotypes depending whether it occurred in the C57BL/KsJ or C57BL/6 strain (Hummel et al., 1972; Coleman & Hummel, 1973). It is becoming increasingly popular to manipulate candidate genes in mouse models in order to assess the impact of a particular genetic defect in the context of glucose metabolism and the development of type 2 diabetes (Valet et al., 2002; Kahn, 2003; Andrikopoulos et al., 2005). However, as more investigations have been reported, the clearer it has become, that the same genetic alteration, when present on different genetic backgrounds, results in profoundly different phenotypes.

The IL-6−/− mice studied by Wallenius, Di Gregorio, Faldt, and Kelly and colleagues were reported as being backcrossed with C57BL/6, C57BL/6J-Il6, C57BL/6, and C57black mice
respectively (Wallenius et al., 2002b; Di Gregorio et al., 2004; Faldt et al., 2004; Kelly et al., 2004). The mice used in this study were thought to have a C57BL/6 genetic background. However, only after completion of the study, was it discovered that the IL-6−/− mice used were in fact derived from interbreeding C57BL x 129 F1 animals to obtain mice homozygous for the IL-6 allele, not from the C75BL/6 strain, as believed. This fact makes one dubious of making any comparison between the results of the studies, specifically those utilizing different strains. Primarily, the WT data cannot be considered appropriate controls with which to compare the IL-6−/− data. Andrikopoulos and colleagues recently investigated the differential effect of C57BL/6 and 129 mouse strains on high fat diet on glucose-induced insulin release (Andrikopoulos et al., 2005). This study helps us to resolve what of our findings may be the consequence of differences in background strain. According to Andrikopoulos, compared with C57BL/6, 129 mice have greater body mass and epididymal fat pad mass on a chow diet; the same weight gain and fat mass on HF; higher basal plasma glucose; decreased insulin levels; are glucose intolerant; and secrete less insulin in response to glucose. This phenotype is in contrast to, and thus does not appear to account for, the marked insulin sensitivity observed in IL-6−/− mice investigated here. However, this comment is speculative. Further to this, Simpson and colleagues report that within the 129 strain substrains exist, and that deliberate and accidental outcrossing of substrains leads to extensive genetic variability (Simpson et al., 1997). Purely acquiring a C57BL x 129 mouse line would not be considered an acceptable control. Stringent breeding criteria and thorough inbreeding are mandatory in order to achieve appropriate littermates, homozygous for the IL-6 allele, to serve as true control animals. This study highlights the fact that investigators need to be very cautious, firstly when selecting animal lineage on which to conduct research, and secondly when evaluating results, both self-generated and published by others. It is very clear from this investigation that stark differential effects arise from the same genetic mutation, which is presumably primarily the result of a difference in background strain and may also be
attributed to differences in rearing and environment. The state of IL-6 deficiency in mice has now been shown to cause profound insulin resistance and obesity (Wallenius et al., 2002b), no obvious phenotype (Di Gregorio et al., 2004), and profound insulin sensitivity and fat metabolism (current study). Such results are likely to be just as varied within and between other species when studied under varying parameters.

Taking into consideration possible variation due to genetic background it is difficult to make comparisons with investigations in alternative backcrosses. However, upon considering the differential phenotypes of the C57BL/6 and 129 mice, it is difficult to believe that such is the root of the disparate phenotype of mice observed in this study. The absence of a phenotype described by Di Gregorio and colleagues (Di Gregorio et al., 2004) compared with that by Wallenius and colleagues (Wallenius et al., 2002b) can plausibly be attributed to slight differences in genetic background. However, that our results are in stark contrast to Wallenius and colleagues is of concern. While the literature is at times contradictory with respect to the actions of IL-6 on insulin signalling and metabolic regulation, there is much support for our findings. Of note, Klover and colleagues used antibodies against IL-6 to deplete IL-6 levels in obese insulin resistant mice and found that IL-6 depletion significantly improved insulin signalling (Klover et al., 2003). Cai and colleagues also demonstrated improved insulin sensitivity in mice by systemic neutralization of IL-6 (Cai et al., 2005). Further, Kim and colleagues treated mice with IL-6 for 5-days and found this to drastically reduce skeletal muscle glucose uptake and insulin signalling (Kim et al., 2004a). Thus we conclude that the IL-6 deficient (C57BL x 129) mice are markedly insulin sensitivity. In conjunction with the literature and the previous findings in this thesis we believe that this phenotype may be due to improved metabolism of lipid. However, the limitations of this study mean that this phenotype can not be attributed to the IL-6 deletion alone as HF-induced
impairments were comparable between IL-6⁻/⁻ and WT strains and decreased food intake in IL-6⁻/⁻ HF warrants further investigation. Further stringent investigations are required. Firstly, to fully characterise strain-specific metabolic regulation, and secondly, to tease apart the many confounding processes associated with IL-6 and its aetiology in insulin sensitivity and metabolism.
CHAPTER SIX

INTERLEUKIN-6 TREATMENT IMPAIRS GLUCOSE TOLERANCE
AND AMPK ACTIVATION IN IL-6\(^{-/-}\) AND WT MICE
In the previous chapter an attempt was made to elucidate the role of IL-6 in metabolic processes using a “loss of function” approach, that being the study of mice with IL-6 deletion. As discussed, we discovered that our IL-6/−/− mice were on a different genetic background compared with our WT mice, making it very difficult to determine whether the positive phenotype observed in our IL-6/−/− mice was due to the deletion of IL-6 or due to the specific genetic background of the mice. In order to answer this question, we next decided to adopt a “gain of function” approach, that being chronic treatment of the WT and IL-6−/− mice with recombinant mouse IL-6. We rationalised that should the responses to rmIL-6 treatment be similar when comparing the WT and IL-6−/− mice, then the results seen in the previous chapter would be more likely due to the genetic deletion of IL-6.

It is noteworthy that the majority of the studies reported in the literature that have treated mice with IL-6 have reported a negative phenotype with respect to insulin action. IL-6 treatment in mice during an hyperinsulinemic-eyglycemic clamp results in insulin resistance as evidenced by reduced GIR, whole body glucose turnover, skeletal muscle glucose uptake, IRS1-associated PI3K (muscle), IRS2-associated PI3K (liver), glycolysis (whole body and skeletal muscle), and liver fatty acylCoA, and increased muscle fatty acylCoA and hepatic glucose production (Kim et al., 2004a). In addition, two studies from Robert Mooney’s group in Rochester support the hypothesis that IL-6 leads to insulin resistance. In their first study, chronic IL-6 treatment (5-day) lead to the development of insulin resistance in liver tissue and not skeletal muscle (Klover et al., 2003). The chronic IL-6 exposure in vivo inhibited the ability of insulin to signal through its receptor and to phosphorylate two major metabolic substrates, IRS1 and IRS2. In their second study, they reported that, in diet-induced obese (ob/ob) mice, depletion of IL-6 using neutralizing antibodies improved insulin responsiveness
in 2-h insulin tolerance tests (Klover et al., 2005). From these studies, it appears that IL-6 promotes insulin resistance and, in this context, our results presented in the previous chapter would tend to suggest that the results were attributable to IL-6 deletion and not genetic background.

It must be noted, however, that not all studies demonstrate that IL-6 treatment leads to insulin resistance. We have previously reported that acutely treating type 2 diabetic human patients with IL-6 normalises insulin sensitivity as measured by the HOMA-IR (Petersen et al., 2005a). Moreover, in unpublished work from our laboratory, humans infused acutely with physiological levels of IL-6 during an hyperinsulinemic-euglycemic clamp displayed improved peripheral insulin sensitivity (Carey et al in review after revision). Finally, as reported in a subsequent chapter of this thesis (Chapter 7), treating rats with IL-6 for 14 days markedly improved glucose tolerance.

The aim of this study, therefore, was to determine whether the differences observed between WT and IL-6−/− mice in the previous study were the consequence of a direct effect of the absence of IL-6, or due to differences in the genetic background of the mice. Accordingly, we repeated the study design of the previous chapter (placing WT and IL-6−/− mice on a HF diet), with or without rmIL-6 treatment for 14 days. The hypothesis was that IL-6 treatment would alter the phenotype of IL-6−/− mice to be more like that of WT mice; i.e. impair glucose tolerance, insulin sensitivity and regulation of fat metabolism.
6.1 Methods

Animals and IL-6 treatment. The description of the mice was identical to that reported in the previous chapter (Chapter 5).

After 8 weeks on the diet, which was sufficient to induce insulin resistance, fasting hyperglycemia and hyperinsulinemia, as demonstrated in Chapter 5, mice were treated with recombinant murine (rm) IL-6 or vehicle. Alzet mini osmotic pumps (model no. 1002; Durect, Cupertino, CA), with a 101 ± 2 µl capacity and an infusion rate of 0.21 ± 0.01 µl/h were used. Pumps were filled to capacity with 4 µg/ml rmIL-6 diluted in carrier (0.9% NaCl and 0.1% BSA) resulting in a daily delivery of approx 201 ng rmIL-6. Pumps were prepared as per the directions supplied and incubated at 37°C overnight to ensure steady state pumping was achieved by the time of implantation. Mice were anaesthetised with a cocktail consisting ketamine hydrochloride (80 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa, USA), acepromazine maleate (0.5 mg/kg; Butler Co., Columbus, Ohio, USA), and xylazine (1.6 mg/kg; Butler Co.) given intraperitoneally. A small incision was made in the skin on the back of the neck and pumps were implanted in a dorsal orientation into the intrascapular subcutaneous space. Incisions were closed with interrupted surgical silk sutures. Control (saline-treated) animals received pumps containing vehicle carrier (0.9% NaCl and 0.1% BSA) only (n = 6).

Food consumption was measured daily from 2 weeks prior to treatment through until the end of the study. Daily caloric intake was calculated by multiplying the consumption of the diet by its caloric density provided by the manufacturer (chow: 4.15; HF: 5.43 kcal/g). Body mass was recorded weekly.
**Glucose tolerance tests, insulin stimulation and tissue collection.** Glucose tolerance tests were performed as per the previous chapter. Two days after the GTT, mice were again fasted for 6 h (0600–1200 hours). Blood was obtained from the tail for measurement of basal insulin (EDTA treatment) and FFA (EGTA/GSH treatment). Mice were injected *i.p.* with 1.5U/kg insulin. Mice were then anaesthetised and tissues (liver, *quadriceps*, *gastrocnemius*, *EDL*, *soleus*, WAT, BAT, spleen) were collected 15 min after insulin injection. All samples were rapidly frozen in liquid nitrogen and stored at –80°C.

**Plasma Measures.** Plasma insulin was measured by ELISA (#EZRMI-13K, Linco Research, Missouri, USA). FFA was measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA C kit; Wako Chemicals, Richmond, VA).

**Tissue analysis.** All analytic methods were identical to those reported in Chapter 5.

**Statistical analysis.** Data was analyzed using a one way or two-way analysis of variance (ANOVA) with repeated measures (SPSS), with significance accepted with a *P* value of < 0.05. If analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. Data are presented as means ± SEM.
6.2 Results

Body Mass. At 10 weeks of age body mass was not different between strains (WT: 28.3 ± 0.5; IL-6⁻/⁻: 27.7 ± 0.4 g; Table 6.1). After 10 weeks WT HF were heavier than WT chow (WT chow: 34.6 ± 1.4; WT HF: 39.9 ± 1.8g), but IL-6⁻/⁻ chow and HF had similar body mass and were lighter than WT HF (IL-6⁻/⁻ chow: 30.6 ± 0.8; IL-6⁻/⁻ HF: 29.4 ± 1.1; P < 0.05). By this age, IL-6⁻/⁻ chow mice also exhibited a lower body mass than WT chow mice (P < 0.05). IL-6⁻/⁻ mice did not display the weight gain, which WT HF did (P < 0.05). While surgery had an initial effect on body mass, mice returned to their pre-surgery weight within 3-4 days. Neither saline- nor IL-6-treatment had deleterious effects on tissue mass (heart, spleen, kidney, BAT, WAT, soleus, EDL, tibialis anterior, quadriceps muscle) or body mass of mice.

Food Intake. Food intake is expressed as energy intake per day (kcal/day) and reported in Table 6.1. IL-6⁻/⁻ chow had a greater caloric intake than WT chow (P < 0.05), as reported in Chapter 5. WT HF had a similar food intake to WT chow, whilst IL-6⁻/⁻ HF ate less than either strain on chow (P < 0.05). Neither saline- nor IL-6-treatment altered food consumption in any group.
Table 6.1  Food intake and body mass of WT and IL-6$^{-/-}$ mice on chow and HF diets two weeks pre- and post-saline or IL-6-treatment. All data are expressed as mean ± SEM ($n = 6$). # $P < 0.05$ vs. WT; § $P < 0.05$ vs. Chow; † $P < 0.05$ vs. saline.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th></th>
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<tr>
<td></td>
<td>Chow</td>
<td>HF</td>
<td>Chow</td>
<td>HF</td>
</tr>
<tr>
<td>Body Mass</td>
<td></td>
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<tr>
<td>Pre:</td>
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<td>39.8 ± 1.8</td>
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<td>§ 32.4 ± 0.8</td>
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<td>17.4 ± 0.7</td>
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<td>17.9 ± 0.2</td>
<td>15.1 ± 0.7</td>
<td>§ 19.2 ± 0.5</td>
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Plasma Measures

Basal glucose levels and response to glucose tolerance test. As seen in Chapter 5, basal fasting plasma glucose levels were elevated by HF in both WT and IL-6$^{-/-}$ mice ($P < 0.05$; Table 6.2) and glucose levels were lower in IL-6$^{-/-}$ chow and HF mice compared with WT mice ($P < 0.05$; Table 6.2). Saline treatment did not alter glucose concentrations. IL-6 treatment increased basal glucose levels in WT and IL-6$^{-/-}$, chow and HF groups ($P < 0.05$; Table 6.2). Glucose responsiveness was assessed by measuring plasma glucose over 120 min following injection of a glucose bolus and then calculating the area under the curve generated by these measures as shown in Figure 6.1. Saline treated mice exhibited a similar glucose elimination rate as reported in the previous study. HF mice had impaired glucose tolerance,
as indicated by the larger area under the curve, compared with chow mice, and chow and HF IL-6−/− mice displayed greater glucose tolerance than WT. IL-6-treatment impaired glucose tolerance in all groups (P < 0.05; Figure 6.1).

**Basal insulin levels and response to glucose tolerance test.** When treated with saline, insulin levels were greater with HF than chow in both strains (P < 0.05; Table 6.2). IL-6−/− chow and HF displayed lower insulin levels than WT. With IL-6-treatment there was an increase in insulin levels in WT chow (P < 0.05) and tendency for increased insulin levels in IL-6−/− groups, but not WT HF (Table 6.2). AUC during the GTT for insulin measures was higher in HF (P < 0.05), lower in IL-6−/− (P < 0.05), and not affected by IL-6-treatment (Table 6.2).

**Free fatty acid.** Fasting plasma FFA was reduced approximately 30% by the HF diet in both mouse strains (P < 0.05, Table 6.2). FFA was lower in IL-6−/− chow than WT chow (P < 0.05), but not different between the strains on HF. IL-6 treatment did not alter FFA in WT or HF groups, whereas increased FFA was seen in IL-6-treated IL-6−/− chow (P < 0.05).
Table 6.2 Basal plasma measures from 6-h fasted mice and AUC for insulin measures during GTT. All data are expressed as mean ± SEM (n = 12-16). # P < 0.05 vs. WT; § P < 0.05 vs. Chow; † P < 0.05 vs. saline.

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<td></td>
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<td>HF</td>
<td>Chow</td>
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<td>7.0 ± 0.3 #</td>
<td>7.5 ± 0.3 #§</td>
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<td>8.4 ± 0.2 # †</td>
<td>8.7 ± 0.3 # †</td>
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<td>FFA (mmol)</td>
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<td>0.52 ± 0.04 #</td>
<td>0.35 ± 0.04 §</td>
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<td>0.33 ± 0.03§</td>
<td>0.65 ± 0.05 †</td>
<td>0.37 ± 0.03 §</td>
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<tr>
<td>Insulin (ng/ml)</td>
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<td></td>
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<tr>
<td>Post: Saline</td>
<td>0.49 ± 0.04</td>
<td>0.64 ± 0.05</td>
<td>0.41 ± 0.04 #</td>
<td>0.55 ± 0.04</td>
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<td>0.50 ± 0.07</td>
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<td>AUC (GTT insulin)</td>
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<tr>
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</tr>
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<td>63.3 ± 7.5</td>
<td>37.2 ± 5.7</td>
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Diacylglycerol and ceramide content in liver tissue. As reported in Chapter 5 and shown in Figure 6.2, liver DAG content was lower in IL-6<sup>−/−</sup> chow than WT chow (P < 0.05), and was not affected by HF. Liver ceramide content was elevated in IL-6<sup>−/−</sup> compared with WT (P < 0.05). Importantly, IL-6-treatment increased both liver ceramide and DAG content in WT HF (P < 0.05) with no affect on any other treatment group (Figure 6.2).

AMPK in skeletal muscle and liver tissue. Phosphorylation of AMPK at Thr<sup>172</sup> (pAMPK) was investigated as a measure of the activation status of AMPK. Total AMPK protein was
measured and was not different between groups in either tissue. Data is presented as pAMPK / total AMPK. IL-6 treatment decreased pAMPK in all groups in both skeletal muscle and liver tissue ($P < 0.05$) except for skeletal muscle of IL-6$^-/-$ HF (Figure 6.3).

*Akt in skeletal muscle and liver tissue.* IL-6 treatment increased liver Akt Ser$^{473}$ phosphorylation in all groups ($P < 0.05$) but did not affect *gastrocnemius* muscle Akt phosphorylation (Figure 6.4).

*PGC1α in skeletal muscle and liver tissue.* IL-6-treatment increased *gastrocnemius* muscle PGC1α in WT chow ($P = 0.03$) and there was a tendency for increased levels in IL-6$^-/-$ ($P = 0.1$ chow, 0.17 HF) compared with saline-treatment. IL-6-treatment increased PGC1α in liver tissue in all groups ($P < 0.05$; borderline significance in IL-6$^-/-$ chow $P = 0.07$; Figure 6.5).
Figure 6.1 Glucose tolerance test blood glucose (upper), area under the curve (middle) and plasma insulin (lower) for saline-treated (solid bars) and IL-6-treated (striped bars) WT (black bars) and IL-6⁻/⁻ (white bars) mice. All data are expressed as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. Chow; † P < 0.05 vs. saline.
Figure 6.2  Diacylglycerol (upper) and ceramide (lower) content in liver tissue of saline-treated (solid bars) and IL-6-treated (striped bars) WT (solid black bars) and IL-6^{-/-} (solid white bars) mice. All data are expressed as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. Chow; † P < 0.05 vs. saline.
Figure 6.3  AMPK phosphorylation relative to total AMPK protein in *gastrocnemius* muscle (upper) and liver tissue (lower) of saline-treated (solid bars) and IL-6-treated (striped bars) WT (solid black bars) and IL-6−/− (solid white bars) mice. All data are expressed relative to saline-treated WT chow as mean ± SEM (n = 6). # P < 0.05 vs. WT; § P < 0.05 vs. Chow; † P < 0.05 vs. saline.
Figure 6.4  Akt Ser<sup>473</sup> phosphorylation in *gastrocnemius* muscle (upper) and liver tissue (lower) of saline-treated (solid bars) and IL-6-treated (striped bars) WT (solid black bars) and IL-6<sup>−/−</sup> (solid white bars) mice. All data are expressed relative to saline-treated WT chow as mean ± SEM (*n* = 6). # *P* < 0.05 vs. WT; § *P* < 0.05 vs. Chow; † *P* < 0.05 vs. saline.
Figure 6.4 PGC1α protein in *gastrocnemius* muscle (upper) and liver tissue (lower) of saline-treated (solid bars) and IL-6-treated (striped bars) WT (solid black bars) and IL-6−/− (solid white bars) mice. All data are expressed relative to saline-treated WT chow as mean ± SEM (*n* = 6). *# P < 0.05 vs. WT; § P < 0.05 vs. Chow; † P < 0.05 vs. saline.*
6.3 **Discussion**

The main finding of this study is that chronic IL-6 treatment impairs glucose tolerance and AMPK activity in both IL-6\(^{-/-}\) and WT mice. Of note, when treated with rmIL-6, mice on a standard chow diet had elevated basal glucose levels while basal insulin levels tended to increase with this treatment. Moreover, in all groups of mice tested, IL-6 treatment induced glucose intolerance. Importantly, with respect to both basal glycemia and glucose tolerance, the response to IL-6 treatment was similar in both WT and IL-6\(^{-/-}\) mice, despite the fact that the mice were of different genetic background. In addition, the results from this study, which show that “gain of function” with respect to IL-6 produced a negative phenotype, are consistent with the results from the previous chapter, which showed that “loss of function” produced a positive phenotype. These current findings support previous experiments in mice that showed that IL-6 induced insulin resistance (Klover *et al.*, 2003; Kim *et al.*, 2004b; Klover *et al.*, 2005). Hence, based on these series of experiments it appears that IL-6 induces glucose intolerance in mice.

As discussed in the introduction of this chapter, the studies in mice appear to be in conflict with studies conducted in humans (Petersen *et al.*, 2005a) or in rats (Rotter Sopasakis *et al.*, 2004; Chapter 7 of this thesis). The data presented here are supported by an number of *in vivo* studies where IL-6 is seen to impair insulin sensitivity (Klover *et al.*, 2003; Kim *et al.*, 2004b; Cai *et al.*, 2005). Klover and colleagues demonstrated defects in downstream actions of insulin in mice chronically treated with IL-6. These mice presented with abnormal insulin tolerance test, increased postprandial serum glucose levels, and reduced postprandial induction of glucokinase (Klover *et al.*, 2003). Kim and colleagues showed that IL-6 treatment lowered GIR, whole body glucose turnover, skeletal muscle glucose uptake, muscle
and liver insulin signalling, and increased hepatic glucose production (Kim et al., 2004a). Cai and colleagues reported that mice presenting with profound hepatic, and moderate systemic insulin resistance exhibited elevated IL-6 production (Cai et al., 2005). When IL-6 was systemically neutralised the insulin resistant phenotype was reversed (Cai et al., 2005). Together, these data demonstrate that chronic exposure to circulating levels of IL-6 that are within the range attainable in obesity and type 2 diabetes are able to recapitulate several of the characteristic metabolic defects of obesity-mediated insulin resistance and diabetes. It is difficult to determine why there should be such species differences, but it is important to note that while insulin treatment results in 100% suppression of hepatic glucose production in humans (Petersen et al., 2000) and rats (Watt et al., 2006), hepatic glucose output is only suppressed ~70% in mice (He et al., 2003; Hevener et al., 2003). This may be an important consideration when examining the effect of IL-6 on metabolic processes in various species.

Investigation into accumulation of fatty acid species in liver tissue after IL-6 treatment did not account for impaired glucose tolerance as DAG and ceramide levels were only elevated in WT HF, despite the development of glucose intolerance in all groups. Further investigation into the lipid profile of other tissues is warranted, as tissue-specific differences are likely. IL-6 treatment decreased phosphorylation of AMPK in liver in all groups, with a less prominent phenotype observed in gastrocnemius muscle. Such a profound affect on AMPK activity would more than likely decrease fatty acid oxidation and glycolysis to contribute to glucose intolerance. Fatty acid metabolism and oxidation could be investigated in specific tissues to verify this. The finding of decreased AMPK activity is consistent with impaired glucose tolerance; however, it is at odds with the knowledge that IL-6 is released from skeletal muscle in large amounts during and after sustained physical activity (Febbraio & Pedersen 2002) and that AMPK activity is increased several fold following exercise (Vavvas et al., 1997). Kelly and colleagues incubated isolated rat skeletal muscles in the presence of IL-6 and observed
increases in AMPK phosphorylation (Kelly et al., 2004). The same investigation reported diminished AMPK activity in IL-6 knockout mice at rest and following exercise. It is difficult to resolve the discrepancy between the results of Kelly and colleagues and those of the current study as experimental conditions and data presented do not allow for comparison. Furthermore, in our hands, in vivo IL-6 treatment in rats does not alter AMPK activity (Chapter 7).

In conflict with decreased AMPK activity in the current study, IL-6 treatment increased phosphorylation of the transcription factor, PGC1α, which is a key regulator of metabolic processes, such as oxidative phosphorylation, liver gluconeogenesis, and mitochondrial biogenesis (Spiegelman et al., 2000; Scarpulla, 2002; Puigserver & Spiegelman, 2003). Increased activation of PGC1α in liver and muscle tissue is not consistent with the observed glucose intolerance in IL-6 treated mice and, as such, this result was unexpected. Likewise, increased phosphorylation of Akt in liver with IL-6 treatment was unexpected. However, chronic activation of Akt may contribute to insulin resistance in insulin-sensitive tissues as hypothesised by Carey and Febbraio (Carey & Febbraio, 2004).

In conclusion, we demonstrate that IL-6 treatment impairs glucose tolerance. Furthermore, this investigation supports the findings of the previous study that the deficit of IL-6 improves glucose tolerance and insulin sensitivity. That IL-6 treatment impaired glucose tolerance in both strains of mice enables us to further ascribe the improved insulin sensitivity in IL-6−/− mice to IL-6 deficiency per se rather than differences in background.
CHAPTER SEVEN

PROLONGED IL-6 TREATMENT IMPROVES GLUCOSE TOLERANCE BUT NOT INSULIN SENSITIVITY IN RATS
7.1 Introduction

The results presented in first two experimental chapters of this thesis, demonstrated that IL-6 is produced by skeletal muscle in response to calcium (Chapter 3), and that the exercise-induced production and release of IL-6 is exacerbated with suppression of lipolysis (Chapter 4). Over 5 years ago, Steensberg and colleagues first reported that IL-6 is produced from contracting muscle (Steensberg et al., 2000) and since that time several papers ((Keller et al., 2001; Starkie et al., 2001a; Steensberg et al., 2001a; Hiscock et al., 2003; Febbraio et al., 2004; Hiscock et al., 2004; Tomiya et al., 2004; Keller et al., 2005a; Keller et al., 2005b) have verified that IL-6 is produced and released by skeletal muscle during exercise. It is important to note, that during exercise muscle glucose uptake is markedly increased compared with rest. These exercise studies, coupled with studies conducted in a muscle cell culture model where IL-6 was acutely administered (Weigert et al., 2006), suggest that during contraction, IL-6 may be produced to activate signalling pathways to increase glucose uptake.

In contrast with the acute cell culture model (Weigert et al., 2005), experiments reported in Chapter 6 of this thesis, along with those reported by Mooney and co-workers (Senn et al., 2002; Klover et al., 2003; Senn et al., 2003), clearly demonstrate that chronic IL-6 administration in the mouse impairs insulin sensitivity. Why would this apparent paradox exist? Careful examination of the literature generally suggests that acute IL-6 treatment in vivo results in positive effects with respect to insulin sensitivity, whereas chronic IL-6 treatment has the opposite effect. Highlighting this notion is the fact that athletes, who experience frequent saccadic increases in IL-6 following training, are profoundly insulin sensitive. Furthermore, that IL-6 is rapidly cleared following infusion (Steensberg et al., 2003a; van Hall et al., 2003) or exercise (Steensberg et al., 2001a), suggests that chronic elevation may be undesirable. In a recent review, Carey and Febbraio (2004) put forward the
hypothesis chronic elevations in IL-6, such as in obesity and type 2 diabetes, may impair insulin sensitivity in insulin-responsive cells by serine phosphorylation of IRS1, which would down-regulate PI3K via activation of the mammalian target of rapamycin (mTOR), which is activated by Akt (Takano et al., 2001; Tremblay & Marette, 2001; Gual et al., 2003). IL-6 may also inhibit tyrosine phosphorylation of IRS1 by activating suppressors of cytokine signalling (SOCS1/3) via the Jak/STAT pathway. The acute elevation of IL-6 however may not be sufficient for the activation of mTOR as the short stimulation may not cause chronic Akt activation (Figure 7.1). In this study we sought to test the hypothesis proposed by Carey & Febbraio (2004). Our aim was to exogenously administer IL-6 to rats eliciting either chronic or acute elevations in systemic IL-6 and investigate the subsequent effect on whole body glucose tolerance and insulin sensitivity. We chose the rat as a model for study for 2 reasons. Firstly, since the rat is ~10 times greater in mass compared with a mouse we could comprehensively examine metabolites and signalling molecules in many tissues in the single animal. Secondly, and more importantly, under conditions of insulin stimulation the rat behaves more like the human than the mouse. Specifically, during a hyperinsulinemic euglycemic clamp both rats (Watt et al., 2006) and humans (Petersen et al., 2005b) display 100% suppression, whereas mice only display ~50-70 % suppression (Hevener et al., 2003) of hepatic glucose output. We hypothesised that the mode of IL-6 treatment would have divergent effects on these parameters in an in vivo setting.
Figure 7.1 Hypothesised role for IL-6 in the aetiology of insulin sensitivity within insulin responsive cells (From Carey & Febbraio, 2004. "Interleukin-6 and insulin sensitivity: friend or foe?" Diabetologia 47: 1135-1142).
7.2 **Methods**

**Animals.** Male Wistar rats weighing approximately 220 g were purchased from Monash Animal Facility (Clayton, Victoria, Australia). The animals were housed in the RMIT University animal facility in a controlled environment with a 12:12 hour light:dark cycle and fed rat chow and water *ad libitum*. All experimental procedures were approved by the Animal Ethics Committee of RMIT University (AEC #0407). Animals were randomly assigned to receive IL-6 or saline vehicle in a chronic or phasic manner, with 12 animals in each group.

**IL-6 treatment: Chronic.** For experiments examining chronic IL-6 exposure, osmotic pumps (Alzet 2ML2; Durect, Cupertino, CA) with a 14-day pumping capacity and an infusion rate of 4.5 µl/h were used. Pumps were filled to capacity with 22.5 µg/ml rhIL-6 diluted in carrier (0.9% NaCl and 0.1% BSA) resulting in a daily delivery of 2400 ng rhIL-6. Following induction of general anaesthesia with sodium pentobarbital (6 mg.100 g⁻¹ bw; Sigma), pumps were implanted into the intrascapular subcutaneous space. Incisions were closed with interrupted absorbable sutures. Control animals received pumps containing the carrier alone (herein referred to as Control).

**IL-6 treatment: Phasic.** Phasic IL-6 treatment was achieved by injecting rats with 2400 ng rhIL-6 into the i.p. space twice daily. 1200 ng rhIL-6 diluted in carrier (0.9 % NaCl and 0.1 % BSA) was injected at 0700 and 1900 daily for 14-days. Control animals received saline injections of the same volume at the same time.

**Insulin and glucose tolerance tests.** Rats were fasted for 12 h overnight and tested at 0700 on day 14. Basal blood samples (*t = 0*) were taken from the tail of restrained rats for blood glucose and plasma insulin measures. For glucose tolerance tests 1 g. kg⁻¹ bw glucose was
injected intraperitoneally. Blood samples for measurement of glucose and plasma insulin levels were obtained from the tail at 15, 30, 60, and 120 min. For insulin tolerance tests 0.75 U. kg\(^{-1}\) bw insulin (recombinant human insulin; Actrapid®) was injected intraperitoneally. Blood samples for glucose measurement were obtained from the tail at 15, 30, 60, and 120 min.

**Tissue collection.** Approximately 24-hours after tolerance testing rats were anaesthetised (6 mg.100 g\(^{-1}\) bw sodium pentobarbital; Sigma, Steinheim, Germany), and injected with insulin (150 mU.g\(^{-1}\)) or saline vehicle intraperitoneally. After approximately 10 min *soleus*, *gastrocnemius*, liver and adipose tissues were excised and rapidly frozen in liquid nitrogen for storage at –80°C.

**Plasma Analysis.** Glucose measures were assessed immediately at the time of blood collection using a HemoCue Glucose 201+ Analyser (HemoCue, Ängelholm, Sweden). Free fatty acid content (FFA) was measured spectrophotometrically by an enzymatic colorimetric assay (Wako NEFA C kit; Wako Chemicals, Vancouver, USA).

**Analysis of muscle and liver triacylglycerol.** Analytic methods were identical to those reported in Chapter 5.

**Tissue protein extraction, immunoblotting and western blot analysis.** Analytic methods were identical to those reported in Chapter 5. All values reported are expressed relative to control.

**AMPK Activity.** Analytic methods were identical to those reported in Chapter 5.

**Statistical Analyses.** Data was analyzed using a one way or two-way analysis of variance
(ANOVA) with repeated measures (SPSS), with significance accepted with a $P$ value of < 0.05. If analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. Data are presented as means ± SEM.
7.3 **Results**

No differences in any measures were observed between phasic saline- and chronic saline-treated groups. Hence, data from these two groups were pooled and are henceforth referred to as control (Con).

*IL-6 enhances whole body glucose tolerance irrespective of mode of delivery.* Fasting basal blood glucose was not different between any of the groups. The glucose bolus administered for the glucose tolerance test (GTT) elicited an ~ 2.4-fold increase in blood glucose concentration at 15 min in Con rats \( (P < 0.05) \). The glucose concentration in both groups of IL-6-treated rats was elevated ~ 1.8-fold over basal levels 15 min after administration of the glucose bolus \( (P < 0.05) \). This increase was lower in IL-6-treated rats than that of Con rats \( (P < 0.05) \). During the GTT, glucose was consistently lower in both phasic and chronic IL-6-treated rats than in Con. When glucose measures were expressed as area under the curve (AUC) for the duration of the GTT, AUC for both IL-6-treated groups was ~ 30 % lower than Con \( (P < 0.05) \) and this was not different between phasic or chronic IL-6 treatment. At the 120 min time point glucose of all rats was ~ 10 % above fasting basal levels (Figure 7.2).

*IL-6 does not effect whole body insulin tolerance, insulin signalling or lipid metabolite accumulation in muscle and liver irrespective of mode of delivery.* The insulin bolus administered decreased blood glucose at all time points in all groups relative to fasting basal values \( (P < 0.05) \). Although there was a slight tendency for glucose to be lower at 30 min when comparing phasic IL-6 with Con, the results were not significant differences in blood glucose were observed between the groups at any time point during the ITT (Figure 7.3). Consistent with this finding, although insulin stimulation increased phosphorylation of Akt
(Ser^{473}) in muscle and liver tissue ($P < 0.05$), no differences were observed when comparing the groups (Figure 7.4). Since IL-6 has been known to increase lipolysis and lipid oxidation (van Hall et al., 2003; Petersen et al., 2005a), we next sought to determine if IL-6 treatment influenced triglyceride content in skeletal muscle (soleus) or liver tissue, but this was not the case (Figure 7.5).

*IL-6 treatment does not affect AMPK signalling in skeletal muscle or liver, but decreases AMPK in adipose tissue.* Since acute IL-6 treatment activates AMPK (Kelly et al., 2004) we next sought to determine the effect of chronic IL-6 treatment on AMPK and downstream signalling. IL-6 treatment did not alter activity of the AMPK subunits, $\alpha_1$ and $\alpha_2$, in EDL muscle or liver tissue, nor did effect phosphorylation of ACC in liver. There was, however, a small but significant decrease in p-ACC in EDL with chronic, but not phasic IL-6 treatment. Chronic IL-6 treatment decreased AMPK $\alpha_1$ activity in WAT compared with Con ($P < 0.05$) but no differences were seen between the IL-6 treated groups or between Con and IL-6 Phasic (Figure 7.6). However, this effect was not matched by a reduction in p-ACC with chronic treatment (Figure 7.7).

*IL-6 treatment increases PPAR alpha and UCP2 protein content in skeletal muscle tissue.* IL-6 is a potent lipolytic factor (van Hall et al., 2003; Petersen et al., 2005a) and has been shown to enhance whole body VO2 in rats (Wallenius et al., 2002a). Since fatty acids can enhance UCP levels in myocardium via a PPAR dependent mechanism, we next sought to determine whether IL-6 increased PPAR and UCP isoforms. Phasic IL-6 treatment increased PPAR$\alpha$ content in tibialis anterior muscle compared with Con ($P < 0.05$) but no differences were seen between other groups or the PPAR $\gamma$ and $\delta$ isoforms (Figure 7.8). Both phasic and chronic IL-6 treatment increased UCP2 content in tibialis anterior muscle compared with Con ($P < 0.05$) but no differences were seen in UCP3 content (Figure 7.9).
IL-6 treatment does not affect GLUT4, STAT or mTOR signalling or markers of inflammation. No differences were seen in skeletal muscle GLUT4, SOCS3, STAT3 phosphorylation (Tyr705), NFκB, IKβ-α phosphorylation (Ser32/36) mTOR phosphorylation (Ser2448), or p70 S6K (Figure 7.10). No differences were seen in liver tissue SOCS3 or IKβ-α phosphorylation (Ser32/36) (Figure 7.11).
Figure 7.2 Glucose measures (upper) and area under the curve (lower) for glucose tolerance tests (1 g.kg bw⁻¹) of saline-treated control (■ ■ ■, black bars), phasic IL-6-treated (---◊---, striped bars), and chronic IL-6-treated (◊■◊, open bars) rats. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs.
Figure 7.3 Glucose measures for insulin tolerance tests (0.75 U.kg bw\(^{-1}\)) of saline-treated control (■■■), phasic IL-6-treated (---◊---), and chronic IL-6-treated (■◊■) rats. All data are expressed as mean ± SEM (n = 12).
Figure 7.4 Akt protein content and Ser\textsuperscript{473} phosphorylation of basal (B) or insulin-stimulated (I) tibialis anterior muscle (upper) and liver tissue (lower) of saline-treated control (solid black bars, S), phasic IL-6-treated (striped bars, P), and chronic IL-6-treated (open bars, C) rats. Representative blots are shown. All data are expressed as mean ± SEM (n = 6). * P < 0.05 vs. basal.
Figure 7.5  Triacylglycerol content of soleus muscle (left) and liver tissue (right) of saline-treated control (solid black bars), phasic IL-6-treated (striped bars), and chronic IL-6-treated (open bars) rats. All data are expressed as mean ± SEM (n = 12).
Figure 7.6  AMPK α1 and α2 subunit activity in EDL muscle (upper), liver (middle), and adipose tissue (lower) of saline-treated control (solid black bars), phasic IL-6-treated (striped bars), and chronic IL-6-treated (open bars) rats. All data are expressed as mean ± SEM (n = 12). # P < 0.05 vs. Con.
Figure 7.7  ACC phosphorylation of EDL muscle (upper), liver tissue (middle), and white adipose tissue (lower) of saline-treated control (solid black bars), phasic IL-6-treated (striped bars), and chronic IL-6-treated (open bars) rats. All data are expressed as mean ± SEM (n = 6).

# P < 0.05 vs. Con.
Figure 7.8  PPAR isoforms in *tibialis anterior* muscle tissue of saline-treated control (solid black bars), phasic IL-6-treated (striped bars), and chronic IL-6-treated (open bars) rats. All data are expressed as mean ± SEM (*n = 6*). # *P < 0.05* vs. Con.
Figure 7.9  Uncoupling protein 2 and 3 content in *tibialis anterior* muscle tissue of saline-treated control (solid black bars), phasic IL-6-treated (striped bars), and chronic IL-6-treated (open bars) rats. All data are expressed as mean ± SEM (*n* = 6). # *P* < 0.05 vs. Con.
Figure 7.10 Representative blots of GLUT4, SOCS3, STAT3 (Tyr^{705}), NFκB, IκBα (Ser^{32/36}), mTOR (Ser^{2448}), and p70 S6K protein content in basal and insulin stimulated *tibialis anterior* muscle tissue of control, phasic IL-6-treated, and chronic IL-6-treated rats. No differences were observed between the groups.

Figure 7.11 Representative blots of SOCS3, p-IκBα (Ser^{32/36}), and IκBα protein content in basal and insulin stimulated liver tissue of control, phasic IL-6-treated, and chronic IL-6-treated rats. No differences were observed between the groups.
A common perception is that chronically elevated levels of IL-6 in obesity and disease states promotes insulin resistance (Lazar, 2005). Yet in light of the positive effects of exercise on insulin sensitivity, it is also believed that the acute exercise-induced increases in IL-6 have a beneficial effect on metabolism and insulin sensitivity (Febbraio & Pedersen, 2005). The results from this study show that rhIL-6 treatment does not impair glucose tolerance or insulin signalling in rats at the peripheral level, but rather, improves insulin-stimulated glucose disposal during a glucose tolerance test. Furthermore, chronic and phasic elevations in exogenous IL-6 do not exhibit divergent effects.

According to the insulin tolerance test data, neither phasic nor chronic IL-6 treatment impaired insulin sensitivity in peripheral tissues, which was corroborated by normal insulin-stimulated Akt phosphorylation in skeletal muscle and liver tissue. Likewise, IL-6 treatment did not affect proteins associated with inflammation and insulin resistance in muscle tissue as we hypothesized would occur with chronic IL-6 treatment. Consistent with this finding, rhIL-6 infusion during a hyperinsulinemic-eyglycemic clamp in rats has recently been shown to have no effect on whole body glucose disposal or insulin sensitivity (Rotter Sopasakis et al., 2004). Other investigators report contradictory findings, albeit in a different species. Klover and colleagues demonstrated defects in downstream actions of insulin in mice chronically treated with IL-6 (Klover et al., 2003). These included abnormal response to an insulin tolerance test, increased postprandial serum glucose levels, and reduced postprandial induction of glucokinase. These data demonstrated that chronic exposure to circulating levels of IL-6 induced hepatic insulin resistance without impairing skeletal muscle insulin signalling (Klover et al., 2003). Accounting for body weight, this dose was approximately 2-fold greater than used in the current study. Another study, by Kim and colleagues, reported that
mice acutely treated with recombinant murine IL-6 before or during an hyperinsulinemic-euglycemic clamp experienced impaired glucose disposal and insulin signalling (Kim et al., 2004a). Impairments were observed in insulin-stimulated IRS1- and IRS2-associated PI3K activation in skeletal muscle and liver, respectively, with skeletal muscle showing greater degree of impairment than liver tissue (Kim et al., 2004a); contrary to the findings of Klover and colleagues (Klover et al., 2003). The dose of IL-6 administered in the above mouse study (0.5 mg/h/mouse) far exceeded that employed in the current study (100 ng/h/rat) and thus raises concerns over the physiological relevance of such results. Even so, in the previously mentioned mouse study insulin-stimulated Akt activity was not affected in either tissue type (Kim et al., 2004a). It is difficult to ascertain what comparisons can be made between studies utilising different species. During hyperinsulinemic-euglycemic clamps various mouse strains exhibit glucose infusion rates often 5-10-fold greater than those of various rat strains, meaning that mice have far greater rates of insulin-stimulated glucose uptake. Thus mice are much more insulin sensitive than rats and are likely to have alternate sensitivity to and regulation of such interventions, which may account for the detrimental results observed in mice but not in rats. Furthermore, in support of our findings Carey and colleagues recently conducted a 4-h IL-6 infusion in healthy human subjects, which improved whole body glucose disposal, oxygen consumption, glucose oxidation, and insulin sensitivity and did not impair insulin’s ability to suppress hepatic glucose production (Carey et al., in review after revision).

The finding of improved glucose tolerance in IL-6-treated rats during the first 60 min of the GTT was a consistent finding across all animals. However, without further investigation into regulation of glucose metabolism at the level of the liver, we cannot rule out this being a transient effect, with questionable physiological relevance. IL-6-treatment may have affected food-seeking behaviour, thus increasing activity and energy expenditure, which could account
for the observed improvement in glucose tolerance, however, food intake and activity patterns of rats were not monitored. We observed increases in both PPARα and UCP2 protein expression in skeletal muscle of IL-6 treated rats. The physiological relevance of these increases may indeed be marginal, however both of these proteins play integral roles in the regulation of metabolism, particularly that of fatty acid oxidation. IL-6 may be enhancing fat oxidation by way of PPARα. Alternatively, the increase in PPARα may be a negative feedback response to the circulating IL-6 levels as PPARα has previously been shown to inhibit activation of IL-6 (Chinetti et al., 2000; Gervois et al., 2004). The effect of IL-6 on UCP expression has not been studied and requires investigation. Previous reports demonstrated increased VO₂ following rhIL-6 treatment into rats (Wallenius et al., 2002a) and humans (Carey et al., in review after revision), which increased insulin-stimulated glucose disposal and appeared to be due to enhanced oxidative glucose metabolism. As such, we suggest that IL-6 may be augmenting cellular respiration leading to an increase in glucose oxidation by increasing mitochondrial uncoupling.

It should be noted as an element of concern that the pharmacological effect of a given cytokine may differ from its biological effect. Issues arising from exogenous administration of compounds have been previously highlighted. For example, the use of anti-IL-6 antibodies to abrogate circulating IL-6 levels has been suggested to enhance IL-6 release and has been shown to in fact have the opposite effect whereby it increased the bioactivity of circulating IL-6 (Heremans et al., 1992). The low homology between human and rat IL-6 (~58 %) is of concern and may account for the absence of a phenotype developed in IL-6-treated rats. rhIL-6 has previously been reported to elicit effects in rodent brain tissue (Schobitz et al., 1995; van der Meer et al., 1996), hepatocytes (Schieferdecker et al., 2000; Moran et al., 2005) and skeletal muscle (Baudry et al., 1996), with similar effects seen with rhIL-6 and rrIL-6 (Schieferdecker et al., 2000). Other studies have reported that exogenous rhIL-6 treatment in
rodents is efficacious providing it is supplemented with soluble recombinant human IL-6Ra (Marz et al., 1997; Marz et al., 1999; Thier et al., 1999; Galun et al., 2000). A previous study treating rats systemically with rhIL-6 has also reported a null effect (Rotter Sopasakis et al., 2004), yet rats treated intracerebroventricularly with rodent IL-6 exhibit profound results (Wallenius et al., 2002a). The homology between human and mouse IL-6 is even lower; at ~42%. Given that rhIL-6 of similar concentrations to that used in the current study has been administered to mice and has been observed to have detrimental outcomes on insulin signalling (Klover et al., 2003), it is unlikely that rhIL-6 used in this study was without biological function in the rats. The divergent outcomes in response to IL-6 may be species-specific. This notion is highlighted by the fact that the results of this study are in contrast to that of the previous study (Chapter 6) whereby; administration of equivalent doses of IL-6 (kg bw\(^{-1}\)) elicited quite different results in mice to that in rats in the current study. Admittedly, mice were treated with rmIL-6 and rats, rhIL-6. An investigative comparison of human, rodent and murine IL-6 bioactivity and elimination within and across species is warranted.

The observation of elevated IL-6 levels in the circulation and adipose tissue of type 2 diabetic patients has been instrumental in the notion of IL-6 as a cause of insulin resistance (Carey & Febbraio, 2004). A substantial portion of circulating IL-6 is released from adipose tissue at rest (Mohamed-Ali et al., 1997), and increased circulating IL-6 levels in type 2 diabetic patients have been shown to be related to fat mass and not insulin sensitivity (Carey et al., 2004). Furthermore, IL-6 production and release from contracting skeletal muscle positively correlates with muscle glucose uptake during contraction (Steensberg et al., 2001a). This study found that exogenous rhIL-6 did not effect insulin sensitivity but did somewhat improve insulin-stimulated glucose disposal. Thus we propose that exogenous IL-6 administration does not cause insulin resistance as the augmented secretion of IL-6 in obesity and insulin resistance is a response to the obese insulin resistant state, rather than a cause of insulin
resistance. Further investigation needs to be conducted into clarifying the effect of IL-6 on metabolic parameters in various species and tissue types and delineating efficacious doses of IL-6 in both chronic and acute conditions.
CHAPTER EIGHT

SUMMARY AND CONCLUSIONS
8.1 Summary and Conclusions

The primary aim of this thesis was to investigate the role of interleukin-6 in insulin sensitivity and fat metabolism in states of metabolic health and disease.

The first study (Chapter 3) found that skeletal muscle IL-6 mRNA and protein were up-regulated in vitro in a calcium-dependent manner, specifically, in response to the calcium ionophore, ionomycin, but not in response to high physiological concentrations of adrenaline. In the literature, IL-6 production during exercise is attributed with having a role in the regulation of lipolysis, and during exercise, plasma adrenaline and IL-6 levels are closely correlated. Adrenaline is known to have a profound effect on fuel metabolism and thus the concept that adrenaline might cause the increase in IL-6 production associated with exercise had previously been raised. However, this was the first study to examine the effect of adrenaline per se on IL-6 production within and from skeletal muscle and it indicated that in physiological situations adrenaline per se does not induce IL-6. The second study (Chapter 4) verified the exercise-induced increase in circulating IL-6 and demonstrated that both the contracting skeletal muscle and adipose tissue contribute to this by increasing IL-6 gene expression within the tissue. This study further investigated the role of IL-6 in the regulation of fat metabolism by preventing the utilisation of fatty acids as a metabolic substrate in both the resting and exercising state in healthy human subjects. The most important findings of this study (Chapter 4) were firstly, that when fatty acid release was inhibited IL-6 levels increased in both the resting and exercising conditions, and secondly, that under these conditions the increase in IL-6 mRNA within adipose tissue was marked. These studies are important as they add weight to the body of literature which indicates that IL-6 per se is produced in and released from metabolically sensitive tissues in metabolically demanding circumstances and plays a part in the regulation of fat metabolism in humans (Figure 8.1).
This study would benefit from extending the investigation to include the infusion of lipid concomitantly with NA ingestion and exercise. Furthermore, given the apparent species-specific differences, these investigations in rodents are warranted.

![Figure 8.1 Schematic presentation of the biological effect of muscle derived IL-6 on adipose tissue. (Adapted from Febbraio & Pedersen FASEB J. 16: 1335-1347, 2002)](image)

Having concluded from the first two studies that IL-6 was likely involved in the regulation of energy utilisation in humans, and the previous investigation reporting IL-6 knockout mice to be obese and insulin resistant, the third study (Chapter 5) aimed to investigate the mechanisms underlying the insulin resistance and altered metabolic state of mice deficient in IL-6. The IL-6<sup>−/−</sup> mice we investigated in this study were markedly insulin sensitive, but were
similarly affected by a high-fat diet as WT mice were. It is however, important to be mindful that this study alone does not add to the literature in a particular regard, but serves as an important warning for researchers to be careful in the planning of their experiments so as to ensure appropriate controls are conducted. The issues raised in the discussion (Chapter 5) make it very difficult to interpret the data from this study as to the effect of the absence of IL-6 within the system. The importance of the strain background and health status of the animal can not be understated. In future, when employing genetically modified models researchers need to be especially diligent in ensuring the purity of the breeding line they are using and fully characterising the strain both with and without the genetic modification. Additionally, the health of animals needs to be thoroughly assessed regularly and rigorous procedures adhered to within the breeding facility in order to ensure no diseases compromise the colony’s health.

By adopting a “gain of function” approach and treating mice with IL-6 we attempted to validate the results from the “loss of function” study in Chapter 5. An investigation into the mechanisms of food regulation would be of interest in these groups of mice as underlying differences at this level were not addressed and may provide a better understanding of the aetiology. Whilst the absence of diet-induced differences between the strains in Chapter 5 indicated that the background, rather than the IL-6 deficiency, was the likely cause of differences in insulin sensitivity, the IL-6 treatment indicated to the contrary. In all groups of mice tested, IL-6 treatment induced glucose intolerance. Importantly, with respect to both basal glycemia and glucose tolerance, the response to IL-6 treatment was similar in both WT and IL-6⁻/⁻ mice, despite the fact that the mice were of different genetic background. The phenotype of the IL-6⁻/⁻ mice appeared to be mediated by improved fat metabolism as relevant measures were increased basally and impaired by IL-6 treatment. These analyses were not
conducted as thoroughly as is perhaps warranted following IL-6 treatment but this is an avenue for future investigation.

These observations of negative effects of IL-6, quite disparate to those made in humans, are potentially due to the fact that the two species regulate metabolic parameters differently, rather than being a manifestation of genetic manipulation \textit{per se}, as indicated by the reversal of the phenotype with IL-6 treatment. Such species-specific differences are particularly apparent when performing a hyperinsulinemic-euglycemic clamp. A maximal insulin dose suppresses glucose production by \~50-70\% in healthy mice, compared with \~100\% suppression in humans, indicative of the increased hepatic sensitivity to insulin in humans, yet mice display \~10-fold greater rates of insulin-stimulated glucose uptake than humans indicating greater insulin sensitivity at a whole body level.

We then went on to investigate the effect of exogenous IL-6 in rats (Chapter 7). This study was to serve two purposes; firstly, to enable us to make another species comparison of the effect of IL-6, secondly, and most importantly, to determine whether discrepancies in the literature concluding positive, null, and negative effects of IL-6 on insulin sensitivity could be attributed to the mode of administration being either acute of chronic. Analysis revealed no differences due to the mode of IL-6 treatment. Although the dose of IL-6 in this study was comparable to that in the mouse study (Chapter 6) it did not impair insulin sensitivity in the rats, and actually improved glucose tolerance. Again, these results are quite different to those in mice following IL-6 treatment (Figure 8.2). These results further support the notion that there are pertinent species-specific differences in metabolic regulation and response to IL-6 which require further investigation.
In conclusion, this thesis suggests that the factor which contributes most pertinently to the controversy in the literature with respect to IL-6 having positive and negative effects on insulin sensitivity is the species studied. On the whole, it would appear that IL-6 has beneficial metabolic effects in humans, particularly with respect to substrate utilisation and unfavourable effects in mice regarding insulin sensitivity. Ongoing investigations will hopefully tease out the differences in metabolic regulation between the rodent species commonly studied and that of humans. By being aware of the similarities and differences between species this knowledge will in turn assist future experimenters direct their investigations in an appropriate manner so as to ultimately resolve the components important in insulin sensitivity and other metabolic processes in order to treat the likes of obesity and type 2 diabetes.
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