INVESTIGATION OF THE INTRACELLULAR SIGNALLING PATHWAY
FOR INTERLEUKIN-6 GENE EXPRESSION IN SKELETAL MUSCLE

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

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Data contained within this thesis form the basis of the following publications:


DECLARATION

I, the candidate, Ming-Hang Stanley Chan, certify that:

a) Data from chapter three was obtained in conjunction with Dr. Natalie Hiscock, a post-doctoral scientist in our laboratory.

b) Except where due acknowledgement has been made, the work is that of the candidate alone while enrolled as a student at the RMIT University;

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

d) 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.

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α-MEM Alpha minimum essential medium
AMP Adenosine mono-phosphate
AMPK Adenosine mono-phosphate-activated protein kinase
ANOVA Analysis of variance
AP-1 Activator protein-1
ATCC American type culture collection
BMNCs Blood mononuclear cells
bp Base pair
C2C12 Murine skeletal muscle cell line
Ca²⁺ Calcium
cAMP Cyclic adenosine-3',5'-monophosphate
cDNA Complimentary deoxyribonucleic acid
CK Creatine kinase
CNTF Ciliary neurotropic factor
Con Control
CREB cAMP response element binding protein
CT Critical threshold
CT-1 Cardiotrophin 1
DMEM Dulbecco's modified eagle's medium
DNA Deoxyribonucleic acid
FBS Foetal bovine serum
FFAs Free fatty acids
gp130 Glycoprotein 130
GRE Glucocorticoid receptor response element
HLH Helix-loop-helix
IFN-β2 Interferon-β2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappa B kinase</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-11</td>
<td>Interleukin-11</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IONO</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor associated kinase</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo-bases</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-dalton</td>
</tr>
<tr>
<td>L6</td>
<td>Rat skeletal muscle cell line</td>
</tr>
<tr>
<td>LCHO</td>
<td>Low carbohydrate</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>Lo Gly</td>
<td>Low glycogen trial</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein α</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>Macrophage inflammatory protein β</td>
</tr>
<tr>
<td>MJ</td>
<td>Milli-Joules</td>
</tr>
<tr>
<td>MRE</td>
<td>Multiple response element</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary-response protein 88</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-IL-6</td>
<td>Nuclear factor interleukin-6</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor of kappa-B cells</td>
</tr>
</tbody>
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OSM  Oncostatin M
PAS  Periodic Acid-Schiff Staining
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PFA  Paraformaldehyde
PKC  Protein kinase C
PMSF  Phenylmethylsulfonyl fluoride
RAW264.7  Murine macrophage cell line
rhIL-6  Recombinant human IL-6
RNA  Ribonucleic acid
ROS  Reactive oxygen species
RT-PCR  Reverse transcription-PCR
SDS-PAGE  Sodium dodecylsulfate – polyacrylamide gel electrophoresis
SE  Standard errors of mean
siRNA  Small interfering RNA
sTNF-R  Soluble tumour necrosis factor receptors
TLR  Toll-like receptor
TNF-R  Tumour necrosis factor receptors
TNF-α  Tumour necrosis factor α
TRAF  Tumour necrosis factor α receptor associated factor
VO2 peak  Peak pulmonary oxygen uptake
It has been recently demonstrated that the cytokine interleukin (IL)-6 is unique among the so called “inflammatory cytokines” in that it can be rapidly produced by and released from contracting skeletal muscle into the circulation in the absence of muscle damage or markers of inflammation. Moreover, previous studies have also indicated that the muscle-derived IL-6 may play an important role in insulin sensitivity and fat metabolism, which may help to explain some of the beneficial effects associated with exercise. Despite the fact that the magnitude of the contraction mediated IL-6 gene expression is often dependent on the mode, intensity and duration of the exercise bout performed, the signalling events that mediate these processes are poorly understood. Accordingly, the central aim of this thesis of work was to identify the signalling pathways for IL-6 gene transcription in contracting skeletal muscle. We firstly sought to examine the cellular origin and distribution of IL-6 within the mass of skeletal muscle in response to exercise. Confirming that skeletal myocytes are a source of the exercise-induced elevation in IL-6 in human skeletal muscle by demonstrating that IL-6 mRNA increases intramyocelluarly using in situ hybridisation, we next investigated the pattern of cytokine gene expression in response to contraction. Our data revealed that cytokines are not ubiquitously expressed in human skeletal muscle, and the expression of IL-6 and -8 are both influenced by glycogen availability within the contracting muscle. Moreover, a reduction in intramuscular glycogen also led to a marked phosphorylation of the nuclear p38 MAPK,
which was then proven to have a critical role in the mRNA induction of IL-6. Importantly, the transcriptional activation of IL-6 in skeletal muscle appeared to be regulated by a NFκB-independent mechanism, and that NFAT inhibition may have a sensitizing effect on the skeletal muscle to Ca^{2+} stimulation.

In summary, studies from this thesis suggest that contracting skeletal muscle cells actively produces IL-6 via a mechanism that is atypical from those seen during inflammation. It is clear from this series of studies that IL-6 is produced in muscle cells by a unique pathway that involves changes in metabolic and contractile processes, but not inflammatory signalling cascades.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW
1.1 General introduction

It has been well demonstrated that physical exercise induces considerable changes in physiological homeostasis (Pedersen and Hoffman-Goetz, 2000). The discovery that cytokines are markedly increased in the circulation during exercise has lead to a great deal of attention within the scientific community in the recent years. Early studies on the systemic appearance of cytokines mainly came from various models of sepsis and inflammation. In these models, the local production of pro-inflammatory cytokines namely, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Dinarello, 1992) result in systemic appearance of anti-inflammatory cytokines and cytokine inhibitors like interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1ra) and soluble tumour necrosis factor receptor (sTNF-R) (Akira et al., 1993). Among these molecules, IL-6 is unique in that it has been reported to have both pro- and anti-inflammatory properties and its functional role appears to be context specific (Taaffe et al., 2000; Tilg et al., 1997).

The first study to establish a link between exercise and cytokine response was performed by Cannon et al. in 1983, in which they saw an elevated rectal temperature in rats that resembles an infection, following injection of post-exercise plasma from human subjects. Since then, various studies have consistently reported the elevation of circulating cytokine levels, particularly IL-6, in response to different forms of exercise (Bruunsgaard et al., 1997; Cannon et al., 1986; Castell et al., 1997; Drenth et al.,
Following the onset of exercise, IL-6 is the first cytokine to appear in circulation with a magnitude of greater than 100-fold has been observed during exercise (Febbraio and Pedersen, 2002; Fischer, 2006; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001). This increase is then accompanied by the emergence of cytokine inhibitors IL-1 receptor antagonist (IL-1ra), soluble tumour necrosis factor receptor (sTNF-R) and the anti-inflammatory cytokine, interleukin-10 (IL-10) (Ostrowski et al., 1998a; Ostrowski et al., 1999; Ostrowski et al., 1998b), while the classic pro-inflammatory cytokines TNF-α and IL-1β remained either unaffected with exercise (Petersen and Pedersen, 2005) or exhibits relatively small, delayed increments (Suzuki et al., 2002). In addition, plasma interferon (IFN)-α and –γ do not appear to elevate in response to exercise, while endurance exercise seemed to induce a systemic release of granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein 1 (MCP-1), and chemokines like interleukin (IL)-8, macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta, but suppresses IL-2 (Ostrowski et al., 2001; Suzuki et al., 2002; Suzuki et al., 2000). Although, exercise may affect the plasma concentration of other cytokines, the increase of IL-6 is by far the most marked and its appearance precedes that of the other cytokines (figure 1.1).
Figure 1.1 The plasma cytokine response to sepsis (A) and exercise (B) (Petersen and Pedersen, 2005). In sepsis, the pattern of cytokine response mainly consisted of TNF-α, IL-1β, IL-6, IL-1ra, TNF-R, and IL-10. In contrast, exercise results in a marked elevation of IL-6, in the absence (or minimal expression) of the pro-inflammatory cytokines namely TNF-α and IL-1.
1.2 Background of IL-6

The cytokine IL-6, was first discovered and named interferon-β₂ (IFN- β₂) in 1980 by Weissenbach and colleagues (Weissenbach et al., 1980) during an effort to clone and characterize the interferon-β gene in human fibroblasts. The cytokine was subsequently named B-cell stimulatory factor-2 (Hirano et al., 1985), B cell differentiation factor, T cell-replacing factor, 26-kDa protein (Content et al., 1982; Haegeman et al., 1986), hybridoma growth factor (Brakennhoff et al., 1987a; Van Snick et al., 1986), interleukin hybridoma plasmacytoma factor 1, plasmacytoma growth factor (Nordan et al., 1987), hepatocyte-stimulating factor (Gauldie et al., 1987), macrophage granulocyte-inducing factor 2, cytotoxic T cell differentiation factor (Takai et al., 1988) and thrombopoietin due to its biological functions. In 1989, when these variously named proteins were found to be identical on the basis of their amino acid and/or nucleotide sequences, the name IL-6 was settled upon (Akira et al., 1993; Song and Kellum, 2005). IL-6 is a member of a cytokine family that consists of leukemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF), IL-11, oncostatin M (OSM) and cardiotrophin 1 (CT-1). Members of this cytokine family contain four antiparallel α-helices termed A, B, C and D that are connected by two long, and one short, loops (Figure 1.2) (Heinrich et al., 2003; Song and Kellum, 2005). Although each of the IL-6 type cytokine is recognized by a specific receptor complex, they all share a common subunit, glycoprotein-130 (gp-130) for signal transduction (Figure 1.3) (Gadient and Patterson, 1999; Hibi et al., 1996; Hirano et al., 1994).
In humans, the gene for IL-6 is located on chromosome 7p21 of approximately 5 kilobase (kb) in size (Sehgal et al., 1986), while it is 7 kb on chromosome 5 of the mouse genome (Mock et al., 1989). They both consist of five exons (Figure 1.4) and display a high degree of homology in the protein coding regions, as well as the 3’ and 5’ untranslated regions (Tanabe et al., 1988; Yasukawa et al., 1987; Zilberstein et al., 1986). IL-6 is synthesized as a precursor of 212 amino acids, in which the hydrophobic signal sequence at the N-terminal is cleaved during post-translational processing to obtain the mature IL-6 peptide containing 184 amino acids (Hirano et al., 1985; Nordan et al., 1987; Simpson et al., 1988a; Van Damme et al., 1987; Van Snick et al., 1986). Of interest, in concert with the full-length peptide, a truncated form of IL-6 lacking the signal-transducing domain (encoded by exon II) due to alternative splicing has also been reported in peripheral blood mononuclear cells (Kestler et al., 1995). Depending on the cellular origin and the amount of post-translational modifications including N- and O-linked glycosylations, and phosphorylations that occurs at multiple serine residues, the molecular mass of the mature IL-6 peptide may vary between 21-30 kDa (Cayphas et al., 1987; Hirano et al., 1985; Hirano et al., 1986; Van Damme et al., 1987; Van Damme et al., 1988). However, these modifications appeared to have minimal effect on the biological activity of IL-6, since recombinant IL-6 synthesized in prokaryotes is functionally indistinguishable from the natural form (Brakenhoff et al., 1987b; Geiger et al., 1988; Simpson et al., 1997; Simpson et al., 1988b; Van Damme et al., 1987).
**Figure 1.2** Structures of IL-6 and OSM (Heinrich et al., 2003). The four helices A, B, C and D are highlighted in different colours; circles indicate the three receptor-binding domains.
Figure 1.3  Members of the IL-6-type cytokine family and their respective receptors (Heinrich et al., 2003). Each of the IL-6-type cytokine is recognized by a different ligand-binding subunit, but they all require the transmembrane gp130 subunit for signal transduction.
Figure 1.4 Structural comparison of the murine and human IL-6 genes (Tanabe et al., 1988). The five exons are represented by open (coding) and closed (non-coding) boxes. Numbers above/below the two genes indicate nucleotide base pair (bp) in each coding region of the exon and intron, respectively. Sequence homologies between human and murine in the coding region of each exon are indicated at the bottom of the diagram.
In healthy individuals, the resting plasma concentration of IL-6 is usually around 1 pg•ml\(^{-1}\) or less (Bruunsgaard et al., 1997; Ostrowski et al., 1998a). The circulating concentration of this cytokine may peak to 100 pg•ml\(^{-1}\), in response to exercise (Fischer, 2006), or a much higher concentration (10 000 pg•ml\(^{-1}\)) has been observed in the case of severe systemic infections (Friedland et al., 1992). In contrast, chronic low-level increases in plasma IL-6 of 10 pg•ml\(^{-1}\) or less has been demonstrated in obesity (Bastard et al., 2000), sedentary individuals (Fischer et al., 2006; Panagiotakos et al., 2005), insulin resistance (Bruun et al., 2003), type 2 diabetes (Kado et al., 1999) and cardiovascular disease (Fisman et al., 2006), and may be used as a predictor of mortality (Bruunsgaard, 2002).

### 1.3 Kinetics of the IL-6 response to exercise

It has been well established that the plasma level of IL-6 may increase over 100-fold in response to strenuous exercise (Starkie et al., 2001b). While this may represent an atypical and exaggerated response to exhaustive exercise such as marathon running (Fischer, 2006), the kinetics of IL-6 appearing in the circulation seems to differ based on three major factors: the mode, intensity and duration of exercise performed. For instance, Nielsen et al. (1996) demonstrated a twofold increase in the plasma concentration of IL-6 after 6 minutes of maximal rowing exercise. However, in running, the increase of plasma IL-6 was not seen until 30 minutes after the onset of exercise, which was peaked after 2.5 hours of exercise (Ostrowski et al., 1998a). It is noteworthy that the exercise-
induced increase in plasma IL-6 is not linear throughout the course of exercise, but rather is somewhat exponential as exercise progresses (Fischer et al., 2004a; Ostrowski et al., 1998a; Steensberg et al., 2000b).

It is clear that the kinetics of the IL-6 response to exercise of concentric nature differs from that of eccentric nature, the latter of which is often associated with notable muscle damage (Bruunsgaard et al., 1997; Ostrowski et al., 1998b; Rohde et al., 1997). In concentric contractions, the increase of IL-6 in circulation has been demonstrated to be closely related to the duration of exercise, and the appearance of IL-6 in the plasma exhibits a logarithmic relationship to the duration of muscular activity (Ostrowski et al., 1998a). The maximal level of IL-6 for this type of contraction is usually observed either immediately post exercise or shortly thereafter, followed by a rapid decline towards pre-exercise level within a few hours (Fischer et al., 2004a; Ostrowski et al., 1998a). Conversely, the IL-6 level in response to “muscle damaging” (eccentric) exercise is of somewhat a lower magnitude, in which the peak level is often seen some time after the cessation of contraction and the plasma level may remain elevated for several days (Pedersen et al., 2001; Toft et al., 2002).

1.4 The cellular origin of the exercise-induced IL-6

In mammals, IL-6 is mainly produced by various cells of the immune system including activated monocytes/macrophages (Aarden et al., 1987;
Van Snick et al., 1986), fibroblasts (Weissenbach et al., 1980) and vascular endothelial cells (Corbel and Melchers, 1984). Moreover, other cells including T-cells (Hirano et al., 1985; Van Snick et al., 1986), keratinocytes (Baumann et al., 1984), osteoblasts, B-cells, neutrophils, eosinophils, mast cells, smooth muscle cells (Akira et al., 1993), folliculostellate cells in the anterior pituitary (Vankelecom et al., 1989), adipocytes (Arner, 2005; Trayhurn and Wood, 2004) and skeletal muscle cells (Nagaraju et al., 1998) are also known to produce IL-6 under various conditions. Given that exercise primarily involves muscular contraction from the various body parts, skeletal muscle was proposed to be a potential candidate for the exercise-induced increase in plasma IL-6 (Febbraio and Pedersen, 2002). In 2000, Steensberg et al. (2000a) used a one legged knee extending model and showed a steady increase in systemic IL-6 concentration over the course of experiment. Intriguingly, when they compared the net IL-6 release from the two legs, IL-6 was observed to be significantly released from the contracting, but not the resting limb. Moreover, the kinetics of IL-6 release from the exercising leg closely matched the systemic concentration suggesting the contracting muscles are accountable for the systemic appearance of IL-6.

In resting human skeletal muscle, the level of IL-6 mRNA appeared to be minimal, with a basal expression of the IL-6 protein predominantly found in the slow-twitch (type I) fibres (Plomgaard et al., 2005). Exercise rapidly increases the nuclear transcriptional rate of IL-6 within the contracting muscles, with a 10- to 20-fold increase in mRNA content observed
following 30 minutes of “non-damaging” exercise, which may attain its peak (up to 100-fold) immediately post-exercise (Keller et al., 2001a), while the protein of IL-6 have also been observed to be expressed uniformly across post-exercise muscle fibres (Penkowa et al., 2003). Together with the finding that exercise do not induce the IL-6 mRNA (Moldoveanu et al., 2000) or protein expression (Starkie et al., 2000a; Starkie et al., 2001a) in monocytes, which is often seen during sepsis (Pedersen and Hoffman-Goetz, 2000), these data seemed to suggest that skeletal myocytes may be responsible for making IL-6 during exercise.

1.5 Regulation of the exercise-induced IL-6 response

It is generally understood that the contraction induced IL-6 gene expression is related to the mode, intensity and duration of the exercise, and one’s endurance capacity (Febbraio and Koukoulas, 2000; Fischer, 2006; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001). In the past, this response was hypothesized to be derived from the immune cells, based on the common belief that the exercise-induced IL-6 increase in the plasma is a consequence of an immune response from the injured working muscle (Nehlsen-Cannarella et al., 1997a). Recent studies, however, revealed that the cytokine response seen during exercise differs greatly from that of pathophysiologic nature (Figure 1.1) (Febbraio and Pedersen, 2002; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2001; Suzuki et al., 2002). The fact that exercise induces IL-6 increase in the circulation without observable markers of inflammation (TNF-α and IL-
1β) or tissue injuries (Ostrowski et al., 1998a; Ostrowski et al., 1999) is linking IL-6 to biological processes other than inflammation. In most cases, exercise does not increase the circulating concentration of TNF-α and IL-1β which are the classic markers of inflammation (Febbraio and Pedersen, 2002; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 2004b; Suzuki et al., 2002), but elevates the systemic concentration of anti-inflammatory cytokines and cytokine inhibitors like IL-10, sTNF-R and IL-1ra (Ostrowski et al., 1999; Ostrowski et al., 2000; Pedersen et al., 2004b). IL-6 appears to directly suppress the production of TNF-α both in vitro (Schindler et al., 1990) and in vivo (Matthys et al., 1995; Mizuhara et al., 1994) and IL-1β (Petersen and Pedersen, 2005; Rehman et al., 1997). IL-6 also stimulates the production of IL-1ra and IL-10 which have been found to have negative effects on the production of IL-1α, IL-1β and TNF-α, as well as various chemokines (Moore et al., 1993; Pretolani, 1999; Steensberg et al., 2003a). Furthermore, it has been demonstrated that exercise could negate the endotoxin-induced inflammatory response in humans (Starkie et al., 2003) and TNF-α overexpression in mice (Keller et al., 2004). Even in the context of eccentric exercise whereby the exaggerated muscular damage is represented by 1000-fold increase in the post-exercise plasma creatine kinase (CK) level, the concentration of circulating IL-6 was only elevated by 4-fold (Pedersen et al., 2001) compared to 30-fold during concentric exercise, (Steensberg et al., 2001c) suggesting muscle damage is not required for the regulation of IL-6 during exercise.
Ostrowski et al. (2000) has demonstrated a positive correlation between the magnitude of IL-6 response and exercise intensity which is a determining factor controlling the mass of muscle being recruited for contractile activity. Given that skeletal muscle is a major source of the circulating IL-6 seen during exercise (Fischer et al., 2004a; Steensberg et al., 2000b), it is therefore understandable why the most substantial increase of IL-6 is often observed in response to strenuous exercise that involves contraction of multiple large muscle groups, such as running (Nieman et al., 2005; Ostrowski et al., 1999; Suzuki et al., 2000). Of note, the effects of exercise intensity should always be considered together with duration of the exercise, since exercise of high intensity is often associated with a shorter duration and there appeared to be an inverse relationship existing between the two. However, exercise duration alone is believed to be an important factor that is accountable for over 50% of the variation in post–exercise plasma IL-6 level (Fischer, 2006). Furthermore, regular exercise training have also been reported to lower the basal levels of plasma IL-6 (Cesari et al., 2004; Colbert et al., 2004; Panagiotakos et al., 2005), and markedly reduces the contraction-induced IL-6 mRNA expression in human skeletal muscle (Fischer et al., 2004b). Although, the exact mechanism awaits further investigation, it is possible that this phenomenon could be due to muscle adaptation to exercise training, since trained skeletal muscles have a higher pre-exercise glycogen content and substrate turn over rate (Schantz et al., 1983), as a result, they are more fatigue resistant and rely less on the extracellular substrates during exercise (Phillips et al., 1996).
1.6 **Mechanisms for IL-6 production in skeletal muscle**

Although, the aforementioned factors may serve as extracellular signals to modulate the IL-6 response (Fischer, 2006), the fact that IL-6 is made and secreted only by the contracting muscles, and not the resting muscles that were subjected to the same blood supply (Ji et al., 2004; Steensberg et al., 2000b), indicates that the exercise induced IL-6 response is regulated by mechanisms other than those of endocrine origin. Indeed, a rapid increase in the nuclear transcriptional rate of IL-6 has been demonstrated in isolated muscle nuclei, following the onset of exercise (Keller et al., 2001b), and that the plasma IL-6 has also been observed to have a relatively short half-life (Montero-Julian et al., 1995; van Hall et al., 2003) suggesting transcriptional activation may play an important role in regulating the expression of IL-6 during exercise.

The transcription regulatory region, also known as the promoter region, of IL-6 is located at the 5’ flanking region immediately upstream of the first coding exon. This promoter region contains cis-acting response elements that are important in dictating gene expression upon binding with transcription factors resulting from signalling pathway activation (Figure 1.5). The importance of this region was highlighted, when the first 300 bp sequence of the human promoter was found to share greater than 80% homology with that of the mouse (Tanabe et al., 1988) suggesting its role in this region is of evolutionary importance. Using a site-directed mutagenesis approach, Dendorfer et al. (1994a) reported the mapping of potential transcription factor docking sites within the IL-6 promoter.
Figure 1.5 Schematic presentation of the IL-6 promoter showing the putative cis-regulatory elements of the human IL-6 promoter (Dendorfer et al., 1994b). The approximate locations of each response element, relative to the major transcription start site (+ 1), are indicated at the bottom of the diagram. Abbreviations: HLH, helix-loop-helix.
These included response elements for glucocorticoid receptor (GRE), activator protein-1 (AP-1), Ets family of transcription factors, GATA proteins, NFκB and a multiple response element (MRE) which comprised of elements for nuclear factor interleukin-6 (NF-IL-6) and cAMP response element binding protein (CREB). Of interest, both AP-1 and GATA proteins are known transcription partners of NFAT, their synergistic dimerization has been shown to enhance the transcriptional activity of NFAT on a variety of target genes (Im and Rao, 2004; Macian et al., 2001). Moreover, analysis of muscle biopsies from human subjects that had undergone one hour of cycling exercise demonstrated an increased nuclear abundance of both total and phosphorylated p38 MAPK, but the nuclear localization of NFAT was unaffected (McGee and Hargreaves, 2004). Of note, p38 MAPK is a known regulator of ATF-2 and Elk-1, in which ATF-2 is a subunit of the AP-1 heterodimer (Jun:ATF) (Derijard et al., 1995; Raingeaud et al., 1995; van Dam and Castellazzi, 2001), while Elk1 is a member of the Ets superfamily of transcription factors (Whitmarsh et al., 1995; Yordy and Muise-Helmericks, 2000). More importantly, in cultured murine muscle cells, p38 MAPK has been observed to induce the activity of NFκB and IL-6 gene expression by simultaneously reducing inhibitor of kappa B-alpha (IκB-α) levels and enhancing the DNA binding activity of NFκB, as well as potentiating the transactivating activity of the p65 subunit (Baeza-Raja and Munoz-Canoves, 2004). It is important to note that the activity of p38 MAPK is often increased in skeletal muscle following contraction (Boppart et al., 2000b; Goodyear et al., 1996b), and that the activation of p38 MAPK has been associated with
depleted intramuscular glycogen content and disrupted Ca\(^{2+}\) homeostasis resulting from prolonged exercise (Febbraio and Pedersen, 2002). Therefore, even though the IL-6 promoter does not appear to contain consensus response sequences for p38 MAPK, it is this kinase that is involved in the regulation of IL-6 expression through the transactivation of other transcription factors. In addition, p38 MAPK has also been associated with post-transcriptional mRNA stabilization of IL-6 via the action of its downstream target, p38 MAP kinase-activated protein kinase (MAPKAP K2 or MK2) (Patil et al., 2004; Wang et al., 1999; Winzen et al., 1999).

Although, the activity of NFκB can be regulated by the activation of p38 MAPK, it should be brought to attention that NFκB per se is also a renowned transcriptional regulator of IL-6 (Liang et al., 2004a). NFκB is a redox- (reduction-oxidation) and oxygen-sensitive transcription factor that is known to be activated upon exposure to reactive oxygen species (ROS) (Haddad, 2002; Schreck et al., 1991). Increased ROS formation has been observed in the contracting muscles of both animal (Davies et al., 1982; Jackson et al., 1985; Sandstrom et al., 2006) and human models (Jackson, 2005; Smith and Reid, 2006) during exercise. In cultured C2C12 myotubes, Kosmidou et al. (2002) demonstrated that ROS stimulated the production of IL-6 in a manner that involves transcriptional activation of the IL-6 gene through an NFκB/AP-1-dependent mechanism. Supplementation of different antioxidants have been shown to associate with a reduced expression of IL-6 both \textit{in vivo} (Thompson et al., 2001;
Vassilakopoulos et al., 2003) and in vitro (Kosmidou et al., 2002). Inhibition of NFκB activation via oral administration of a non-steroidal anti-inflammatory drug, indomethacin, ablated the exercise-induced increase of plasma IL-6 in humans (Rhind et al., 2002). However, the activation of NFκB through muscle-specific transgenic expression of its upstream kinase, the IkappaB kinase beta (IkKB) in mice did not increase the transcription of IL-6 in skeletal muscle (Cai et al., 2004), suggesting NFκB may not be solely responsible for the transcription of IL-6 gene in contracting muscle.

It has been well established that mechanical load during contractile activity is a potent stimulus for Ca$^{2+}$ release from the sarcoplasmic reticulum (Olson and Williams, 2000a). While elevated Ca$^{2+}$ levels have been shown to activate nuclear factor of activated T-cells (NFAT) (Dolmetsch et al., 1998; Im and Rao, 2004; Olson and Williams, 2000a) and nuclear factor kappa B (NFκB) (Dolmetsch et al., 1998; Kubis et al., 2003) in vitro, incubation of human muscle cells in culture or ex-vivo with a calcium ionophore also results in an increased expression of IL-6 (Holmes et al., 2004; Keller et al., 2002a). Moreover, analysis of muscle biopsies from human subjects that had undergone one hour of cycling exercise demonstrated an increased nuclear abundance of both total and phosphorylated p38 MAPK, but unaltered nuclear localization of NFAT (McGee and Hargreaves, 2004), while the mRNA expression of calcineurin A, a serine/threonine phosphatase that is sensitive to elevated intracellular Ca$^{2+}$, was increased in response to knee extensor exercise.
NFAT is a transcription factor whose overall activity depends on the balance between dephosphorylation (activation) by calcineurin, and re-phosphorylation (de-activation) by NFAT kinases like the glycogen synthase kinase (GSK)-3 (Fiedler and Wollert, 2004). Therefore, it is possible that the lack of nuclear abundance of NFAT observed after exercise (McGee and Hargreaves, 2004), may be due to a rapid rephosphorylation of NFAT which resulted in a subtle nuclear localization. This notion is supported by a more recent study that observed nuclear localization of NFAT following a 20 minute stimulation with ionomycin, a potent calcium ionophore (Noguchi et al., 2004a). Of interest, it is known that muscle cell expresses high levels of NFAT (Olson and Williams, 2000a), and its activity is markedly upregulated in response to a low sustained elevation of cytosolic Ca\textsuperscript{2+} levels (Dolmetsch et al., 1998; Febbraio and Pedersen, 2002). A low sustained elevation of intracellular Ca\textsuperscript{2+} is also a characteristic of prolonged muscular contraction (Konishi, 1998) suggesting NFAT signalling may be responsible for the contraction-mediated IL-6 synthesis.

Although NFAT, in itself, can lead to cytokine gene transcription, in the nucleus, NFAT has been shown to interact with the transcription factor AP-1 mediating the transcription of cytokine genes (Crabtree, 1999; Rao et al., 1997). It is possible that large intracellular Ca\textsuperscript{2+} transients seen during maximal contraction may also activate the transcription of IL-6 via NFkB and c-jun N-terminal kinase (JNK)–dependent mechanism (Dolmetsch et al., 1998; Febbraio and Pedersen, 2002). The activation of JNK is
associated with IL-6 gene transcription in monocytes via the action of NFκB (Tuyt et al., 1999) and muscle contraction markedly increases JNK activation (Boppart et al., 2000b), whereby the downstream substrates of JNK are known to mediate transcription of cytokine genes via AP-1 dimerization (Hibi et al., 1993). Taken together, although the involvement of these signalling pathways in the activation of IL-6 in skeletal muscle is yet to be determined, it is possible that during more intense muscular activity the serial activation of these pathways may give rise to the pronounced IL-6 response observed in the plasma.

Besides the aforementioned mechanisms, intramuscular glycogen content has also been implicated in the contraction-induced increase in IL-6 gene transcription. In humans, a reduced intramuscular glycogen content has been shown to exacerbate the contraction-mediated increase in IL-6 gene transcription (Keller et al., 2001a; Steensberg et al., 2001a), and therefore its mRNA expression (Gleeson and Bishop, 2000; Steensberg et al., 2001b) and protein release (Steensberg et al., 2001b). Moreover, IL-6 mRNA abundance (Febbraio et al., 2003b; Starkie et al., 2000b) and net contracting limb IL-6 protein release (Febbraio et al., 2003b) is reversed by the carbohydrate supplementation. Although, the signalling mechanism by which muscle glycogen enhances the transcription of IL-6 is yet to be determined, depletion of intramuscular glycogen content has been associated with a limited energy availability for the working muscles, which could have profound effects on a variety of cellular processes including gene transcription (Hargreaves, 2004). p38 MAPK is a stress-
activated protein kinase (Ben-Levy et al., 1998) which is often activated in skeletal muscle during contraction (Boppart et al., 2000a; Goodyear et al., 1996a; Williamson et al., 2003a), and low intramuscular glycogen content disrupts the uptake and release of calcium by the sarcoplasmic reticulum in both animals (Byrd et al., 1989; Chin and Allen, 1996; Stephenson et al., 1999) and humans (Booth et al., 1997). Taken together, these findings suggest that the reduced glycogen content during prolonged exercise may potentiate the transcription of IL-6 in the working muscles via a pathway cross-talk between Ca\(^{2+}\) and p38 MAPK (Febbraio and Pedersen, 2002; Febbraio and Pedersen, 2005) (Figure 1.6).
**Figure 1.6** Schematic presentation of the proposed signalling cascades and biological effects of the muscle-derived IL-6. Altered Ca\(^{2+}\) homeostasis, depleted glycogen stores and increased formation of ROS are all capable of inducing the expression of IL-6 via activation of the stated signalling pathways. Once synthesized, IL-6 may act locally within the contracting skeletal muscle in an auto-/para-crine manner or be secreted into the circulation exerting a myriad of systemic effects.
1.7 Biological roles of muscle-derived IL-6

As previously mentioned, one of the biological functions of muscle-derived IL-6 is to down-regulate the TNF-α and IL-1 induced inflammation (Figure 1.6). This is thought to be one way physical activity may provide beneficial effects against chronic medical disorders associated with low-grade inflammation such as atherosclerosis and Type 2 diabetes, as well as various forms of cancers (Blair et al., 2001; Petersen and Pedersen, 2005). Recent studies have suggested that muscle derived-IL-6 may act as a hormone and play an important role in energy metabolism (Febbraio and Pedersen, 2002; Pedersen et al., 2004a; Pedersen et al., 2001; Petersen and Pedersen, 2005). It is known that physical exercise rapidly increases glucose uptake and utilization by the working skeletal muscles. This results in a sudden fall of the blood glucose level (hypoglycaemia) which then triggers the production and release of glucose from the liver, in order to maintain blood glucose homeostasis (euglycaemia) (Kjaer, 2001). Despite intensive research into the possible pathways that may be involved in this process, very little is known regarding the regulation of hepatic glucose production during exercise. It has been demonstrated that exercise alters the circulating levels of various endocrine hormones including insulin and glucagon (Kjaer, 2001), cortisol (Cryer, 1993), adrenaline (Howlett et al., 1999), as well as adrenergic neural stimulation (Sigal et al., 2000; Sigal et al., 1994). However, none of these factors seem to fully account for the rise in hepatic glucose production during exercise (Pedersen et al., 2001), which raised a possibility that other factor(s) such as IL-6, released from the working muscle cells may
contribute to the increase in hepatic glucose production (Howlett et al., 1999).

IL-6 has been shown to have a marked influence on hepatic glucose metabolism by the inhibition of glycogen synthase and activation of glycogen phosphorylase, which are the rate-limiting enzymes in glycogenolysis (Kanemaki et al., 1998). Moreover, infusion of human subjects with recombinant human IL-6 (rhIL-6) also resulted in an increase in the hepatic glucose production (Stouthard et al., 1995), as well as the fasting blood glucose level in a dose-dependent manner (Tsigos et al., 1997). However, these data were collected from patients with renal cell carcinoma (Stouthard et al., 1995) or involved pathophysiological infusions of IL-6 (Tsigos et al., 1997). In subsequent studies that infused physiological levels of IL-6 at rest, no increase in hepatic glucose production was observed (Steensberg et al., 2003b). However, in 2004 Febbraio et al. (2004) demonstrated that IL-6 indeed contributed to the exercise-induced increase in hepatic glucose production, demonstrating that IL-6 indeed signalled from muscle to liver during exercise. This study identified skeletal muscle as an endocrine organ and IL-6 as the first “myokine”. Since, these data were in contrast to those data obtained by this group at rest (Steensberg et al. 2003), these authors concluded that IL-6 requires a “co-factor” produced during exercise to increase liver glucose output.
Along with the observation on the hepatic glucose output, Stouthard et al. (1995) also reported an elevated level of circulating free fatty acids (FFAs) during rhIL-6 infusion in their patient population. IL-6 infusion into rats have also been found to elevate the serum levels of triglyceride and FFAs resulting from an increased hepatic triglyceride secretion and lipolysis, respectively (Nonogaki et al., 1995). In more recent studies, IL-6 infusion into healthy humans (van Hall et al., 2003) and patients with type 2 diabetes (Watt et al., 2005) has been shown to result in an increase in lipolysis, fatty acid uptake and whole body fatty acid oxidation. Interestingly, a study by Wallenius et al. (2002) demonstrated that IL-6 deficient mice developed mature-onset obesity that is reversible by exogenous supplementation suggesting that IL-6 is a powerful lipolytic factor, and raise a possibility that the elevated circulating concentration of FFA during exercise may be partly mediated by the muscle derived IL-6 (Figure 1.6).

IL-6 has also been shown to improve glucose absorption in the gut (Hardin et al., 2000), and the administration of IL-6 also appeared to have a positive effect on glucose uptake by insulin sensitive tissues (Figure 1.6) (Stouthard et al., 1996). While a polymorphism of the IL-6 gene has been linked to an altered insulin sensitivity in humans (Fernandez-Real et al., 2000), mice with transgenic disruption of the IL-6 gene developed impaired glucose tolerance which was reverted by exogenous supplementation of IL-6 (Wallenius et al., 2002). Taken together, the muscle-derived IL-6 appears to act in an endocrine hormone-like manner,
maintaining energy homeostasis by mobilising substrate uptake and release in different tissues.

Besides the endocrine effect of IL-6, this cytokine has also been suggested to be involved in local processes occurring within the producing muscle (Figure 1.6). Exogenous IL-6 has been shown to have proliferative effects on both human myoblast and satellite cells, and that the proliferation of satellite cells is regulated by the endogenous production of IL-6 in an autocrine manner (Austin et al., 1992; Austin and Burgess, 1991; Cantini et al., 1995). Together with the finding that IL-6 treatment of myoblasts enhances their differentiation in culture (Okazaki et al., 1996) has led to suggestion that the locally produced IL-6 may be implicated in muscle hypertrophy seen in resistance exercise (Vierck et al., 2000).

In summary, the literature demonstrates that contracting skeletal muscles actively produce and release IL-6 into the circulation independent of inflammation. It appears that the elevated Ca^{2+} levels seen during muscle contraction may play a regulatory role in this process, however the exact intracellular mechanism is yet to be characterized, and it is likely to involve pathway crosstalk. This thesis sought to investigate the intracellular signalling pathway for interleukin-6 gene transcription in skeletal muscle.
CHAPTER TWO

AIMS OF THE THESIS
2.1 Aims of the thesis

The primary aim of this thesis was to determine the intracellular regulatory mechanism mediating the expression of the IL-6 gene in skeletal muscle. Such investigation encompassed the study of in vivo and in vitro (human and cell culture) models utilizing a variety of well-established analytical techniques.

It is known that acute skeletal muscle contraction elicits a rapid elevation of IL-6 in the plasma through a rapid rate of gene transcription, translation and release of the IL-6 protein from skeletal muscles. However, the cellular source of IL-6 within skeletal muscle tissue during contraction has yet to be clearly elucidated. The first study (Chapter 3) investigated whether skeletal myocytes per se is a source of the contraction-induced IL-6. After confirming the cellular origin of the contraction-induced IL-6, the second study (Chapter 4) examined the effect of intramuscular glycogen levels on the expression of cytokine genes in skeletal muscle.

The intracellular signalling events in relation to altered glycogen levels and contraction were then investigated in muscle biopsy samples of post-exercise human subjects. The third study (Chapter 5) aimed to characterize the regulatory mechanisms involved in the regulation of IL-6 during exercise in both normal and glycogen-depleted states. Given that the literature has suggested the possible involvement of both elevated
Ca^{2+} levels and activation of p38 MAPK in the regulation of IL-6 synthesis, the following study (Chapter 6) examined the effect of these factors on the expression of IL-6 in cultured skeletal muscle (myotubes).

It has been demonstrated that the expression of IL-6 in muscle may also required the activation of intermediate molecules that are involved in the classic pro-inflammatory pathways, particularly those of the NFκB pathway. However, according to our findings (Chapter 5) and the literature, conflicting evidence seems to exist concerning the role of this pathway during muscle contraction. Therefore, the final study (Chapter 7) investigated the expression pattern of IL-6 and other closely associated cytokines in response to different stimuli in cultured skeletal myotubes, with particular focus on the role of NFκB in the regulation of IL-6 gene transcription.
CHAPTER THREE

SKELETAL MYOCYTES ARE A SOURCE OF INTERLEUKIN-6 mRNA EXPRESSION AND PROTEIN RELEASE DURING CONTRACTION
3.1 Introduction

It is now well established that acute skeletal muscle contraction results in a rapid elevation of the plasma level of the cytokine, interleukin (IL)-6 (Febbraio and Pedersen, 2002). This increase can be attributed to a rapid rate of gene transcription within skeletal muscle biopsy specimens (Keller et al., 2001b) and translation of IL-6 protein that is released from the contracting skeletal muscle (Steensberg et al., 2000b).

The cellular source of IL-6 within skeletal muscle tissue during contraction has yet to be clearly elucidated. Given that skeletal muscle contains cell types other than myocytes, including smooth muscle cells, fibroblasts, endothelial cells, and macrophages, that are also known to produce IL-6 (Cicco et al., 1990; De Rossi et al., 2000; Klouche et al., 1999; Podor et al., 1989). Therefore, it is possible that the elevation in IL-6 gene expression in biopsy tissue and IL-6 protein release could be originated from those cells. In accordance with this hypothesis, Malm et al. (2000) detected IL-6 protein in both muscle and non-muscle cells using immunohistochemical staining of muscle biopsies taken before and after acute contraction, while the group was unable to show any change in the amount of IL-6 protein as a result of muscle contraction. Recently, however, Penkowa et al. (2003) reported a qualitative elevation in IL-6 protein measured in muscle cells within human muscle biopsy sections by immunohistochemistry. In that study, IL-6 was uniformly expressed throughout each fibre, and the authors also stated that IL-6 expression
was not different between fibre types. These findings lend support to the hypothesis that IL-6 is produced in, and released in, muscle tissue but does not necessarily confirm that myocytes are a source of IL-6 production, because the authors could not rule out the possibility that cells other than myocytes produced the protein, which then trafficked into the muscle cells to perform important functions such as the initiation of a stress protein response (Febbraio et al., 2002) and energy metabolism (Pedersen et al., 2004a). The aim of the present study is to determine whether muscle cells *per se* are a source of the exercise-induced elevation in IL-6 in human skeletal muscle and to characterize the distribution of IL-6 within and between fibres. An analysis of both IL-6 protein and mRNA expression within sectioned muscle biopsy tissue, collected before and after contraction, and further characterization of the fibre type and glycogen content of each fibre was performed. By simultaneously examining the cellular localization of the IL-6 mRNA and protein within fibre cross sections, this study would provide further evidence on whether muscle cells are indeed a source of IL-6 during contraction. The current hypothesis is that IL-6 mRNA and protein would be increased within myocytes following exercise and that this would correspond with glycogen-depleted fibres.
3.2 Materials and Methods

Subjects and experimental protocol. Seven healthy, active males volunteered to participate in this investigation. Subjects signed a written consent form after being informed of all procedures and potential risks of the study, which was approved by the appropriate Ethics Committee. Subjects performed 120 min of continuous cycle ergometry at a power output equivalent to ~55% of their individual peak oxygen consumption (VO$_2$ peak). The rationale for the exercise bout chosen in this study was based on previous data (Febbraio et al., 2004) which demonstrated that this mode of exercise would result in appearance of IL-6 in the plasma without observable markers of muscle tissue damage or inflammation. Muscle biopsies were obtained from vastus lateralis muscle before (PRE) and after (POST) exercise using the percutaneous needle method with suction under local anesthesia. Immediately following each biopsy, muscle tissue was removed from the needle using sterile tweezers and separated into two sections: one of these was immediately frozen in liquid nitrogen, and one was mounted in TissueTek (Fronine, Riverstone, Australia), frozen in isopentane (BDH Laboratory Supplies, Poole, UK) over liquid nitrogen, and stored at −80°C until later analysis.

Histological preparation. Multiple 10 µm sections were cut from mounted muscle biopsy samples in a cryostat at −20°C. Sections were fixed onto gelatin-coated microscope slides by immersion in 5% paraformaldehyde (PFA) for 5 min and 70% ethanol for 5 min, air dried, and stored at −80°C.
until required for analysis. For fibre type analysis, three sections were placed on uncoated microscope coverslips and stored at 4°C until required.

**Immunohistochemical staining for human IL-6 protein.** IL-6 protein was detected in muscle fibre cross-sections. In brief, sections were washed in 5% Triton X (Sigma-Aldrich, Castle Hill, Australia) for 10 min and then washed twice with 0.02 M phosphate-buffered saline (PBS) and allowed to dry. Sections were then blocked overnight with 3% sheep serum (Silenus Laboratories, Melbourne, Australia). The next day, sections were washed three times in PBS and incubated overnight with a rabbit polyclonal IL-6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:20 dilution in 1% sheep serum at 4°C. Negative slides were prepared using 1% sheep serum in the absence of the primary antibody. The following day, sections were washed three times with PBS and labeled with a fluorescein conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) (1:50 dilution in 1% sheep serum) for 2 h at room temperature in the dark. After three washes in PBS, sections were air dried and mounted in Fluoroguard (Bio-Rad Laboratories, Hercules, CA). Coverslips were sealed with clear nail polish and allowed to dry. The IHC methodology described above was optimized before sample analysis to provide clear fluorescence with minimal nonspecific binding (background). Several concentrations of primary antibody were detected by the secondary antibodies fluorescein, Texas Red, and biotinylated streptavidin-peroxidase complex. We found that detection with Texas Red and biotinylated streptavidin-peroxidase
showed consistently high background, suggesting high levels of nonspecific binding. Thus, we chose to analyze our samples using fluorescein.

**Detection and quantitation of IL-6 protein.** IL-6 protein was visualized using an Olympus BX60 (Olympus Optical Company, Hamburg, Germany) fluorescent microscope, and multiple images were captured by a Sensicam Imaging camera controlled by Camware software (version 1.21, PCO Imaging, Kelheim, Germany). For both positively and negatively stained slides, three images were captured on each of three sections. Thus, nine sections were captured for each subject at each time point. Fluorescence intensity was quantitated using Metamorph software (version 4.6, Universal Imaging Corporation, Downingtown, PA). Mean intensity was measured on each image, and total mean fluorescence was calculated for each sample. To exclude background fluorescence, we subtracted quantitated data for the negative slides from the data from positive slides, and mean fluorescence was compared between PRE and POST samples.

**Comparison of IL-6 fluorescence between glycogen-rich and glycogen-poor fibres.** Muscle fibre glycogen content was qualitatively assessed on sectioned biopsy tissue using Periodic Acid-Schiff Staining (PAS) as described previously (Febbraio et al., 1994). This assay is used to visualize glycols by producing a magenta stain that is proportional to the amount of glycogen in each cross-sectioned muscle fibre. As expected, glycogen content was uniformly rich in samples collected before exercise.
and, therefore, these were not classified. In post-exercise samples, however, each fibre was classified as being rich, moderate, or poor in glycogen content using a visual scale. To examine whether individual fibre glycogen content was associated with IL-6 fluorescence following exercise, three glycogen-poor and three glycogen-rich fibres from each section were outlined and quantitated. These fibres were matched to their identical fibres in the IL-6 IHC-stained sections, and these fibres were quantitated using Metamorph software. In essence, we compared IL-6 protein between glycogen-rich and glycogen-poor fibres.

**Fibre typing.** Muscle fibre type was determined in 10 µm sections by one of two methods: staining for myofibrillar ATPase activity after reincubation with alkaline or acidic buffers, as described elsewhere (Mabuchi and Sreter, 1980), which stains type 2 muscle fibres a dark color, or by IHC, essentially as described above for IL-6 protein, except that the primary antibody used was a mouse anti-slow muscle myosin monoclonal antibody (which detects type 1 fibres) (Chemicon International, Temecula, CA) diluted 1:500 in 1% sheep serum, and the secondary antibody used was a fluorescein conjugated anti-mouse (Santa Cruz Biotechnology) diluted 1:50 in 1% sheep serum. Sections were washed, air dried, mounted, and sealed as described above. We initially used the myofibrillar ATPase activity when performing comparisons with IL-6 protein. However, we found that this method resulted in shrinkage of the fibres (Figure 3.1), making it more difficult to match the sections with the IL-6 protein and PAS cross-sections. Hence, when subsequently performing the *in situ*
hybridization analyses, we used the IHC method (Figure 3.4). We suggest, therefore, that this method is superior because it does not affect the size of the muscle fibres.

*In situ hybridization.* The IL-6 probe used was derived from a 1.23 kb human sequence. The antisense probe for human IL-6 was linearized with EcoR1 and labeled using T7 polymerase with 33P-UTP for 2 h at 37°C. The probe was hydrolyzed by exposure to alkaline pH for 50 min to produce a probe with length of ~0.15 kb. The labelled probe was counted on a scintillation counter and diluted in hybridization buffer to a final concentration of $40 \times 10^6$ dpm•ml$^{-1}$. *In situ* hybridization was carried out on 10 µm frozen sections of tissue from each of the pre- and post-exercise biopsies. Sections were fixed by immersion in buffered 4% paraformaldehyde, washed in PBS, and then stored at −20°C after dehydration in 70% ethanol. Metastatic breast tissue sections were prepared in an identical manner to act as positive controls. Pretreatment of sections before hybridization was carried out by microwaving in antigen retrieval solution (Citra Plus, BioGenex, San Ramon, CA), followed by incubation in pronase E (Sigma, St. Louis, MO) at a concentration of 125 µg•ml$^{-1}$ for 10 min at 37°C. Hybridization was carried out overnight in hybridization buffer containing 50% formamide at 60°C. Posthybridization washes were performed in 50% formamide in 2× SSC at 55°C. In addition, unbound probe was removed by incubation of sections in RNase A (Sigma, St Louis, MO) at a concentration of 125 µg•ml$^{-1}$ at 37°C for 1 h. Positive signals were detected using Amersham (Little Chalfont
Buckinghamshire, UK) LM-1 emulsion and development in Kodak (Rochester, NY) Dektol developer, then fixation in Ilford Hypam (Cheshire, UK) rapid fixer. Slides were lightly counterstained with hematoxylin and viewed using a dark-field condenser.

Measurement of IL-6 mRNA in muscle biopsies by RT-PCR. Total RNA was extracted using the acid phenol method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) and reverse transcribed using Taqman Reverse Transcription Reagents, using random hexamer primers, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). IL-6 mRNA was detected using real-time PCR as described previously (Starkie et al., 2001b). For each sample, the IL-6 mRNA content was normalized to the housekeeping gene 18S (given a $\Delta C_T$ value). All samples from a given subject was expressed as fold changes relative to the PRE value, which was set to 1, using the $\Delta\Delta C_T$ method (Applied Biosystems).

Statistical analysis. Paired t-tests were used to detect the effect of contraction on quantitated total IL-6 protein (PRE vs. POST), and IL-6 protein in HIGH and LOW glycogen fibres. $P < 0.05$ was considered to indicate statistical significance. Data are expressed as mean ± SE. Statistical analyses were performed using SigmaStat version 2.03 (SPSS, Inc., Chicago, IL).
3.3 Results

*IL-6 protein is elevated after exercise in skeletal muscle fibres.* IL-6 protein was elevated in muscle biopsy cross-sectional tissue from PRE to POST contraction (P < 0.05) that could clearly be observed both within and between muscle fibres (Figure 3.1 and 3.2). At PRE, IL-6 protein was uniformly expressed across muscle fibres at low levels (Figure 3.1). At POST, however, IL-6 protein was expressed in a fibre-specific manner. The distribution of IL-6 protein was then examined by quantitatively comparing muscle fibres with LOW glycogen content (type 1 fibres) and fibres with HIGH glycogen content (type 2 fibres) at POST. This analysis revealed that IL-6 protein was expressed predominantly in type 2 muscle fibres with HIGH glycogen content (P < 0.05) (Figure 3.3).

*IL-6 mRNA is elevated after exercise in skeletal muscle fibres.* IL-6 mRNA, as measured by real-time RT-PCR, was increased (P < 0.05) 18 ± 8-fold when comparing PRE with POST. As can be seen in Figure 3.4, this coincided with an increase in IL-6 mRNA, as measured by *in situ* hybridization, within skeletal muscle fibres comparing PRE with POST contraction. At PRE, IL-6 mRNA was expressed peripherally at low levels around all fibres, and there was a virtual absence of IL-6 in the center of the myocytes, suggesting that, at rest, IL-6 in skeletal muscle tissue is likely to be located in cell types other than myocytes (Cicco et al., 1990). However, at POST, IL-6 mRNA was selectively expressed throughout some, but not all, muscle fibres (Figure 3.4). Further examination of these
fibres showed that type 2 fibres were expressing IL-6 mRNA at a much greater level than type 1 fibres and that these fibres also had greater glycogen content (Figure 3.4)
Figure 3.1  IL-6 protein (left), glycogen content (middle), and fibre type (type 2 fibres dark, right) in muscle biopsy sections before (PRE) and after (POST) 120 min continuous recumbent cycle ergometry. Yellow circle in POST images indicates the same muscle fibre.
**Figure 3.2** IL-6 protein before (PRE) and after (POST) 120 min continuous recumbent cycle ergometry. #Difference between PRE and POST (P < 0.05). Values are expressed as mean ± SE.
Figure 3.3  IL-6 protein in type 1 (low glycogen content) and type 2 (high glycogen content) fibres after (POST) 120 min continuous recumbent cycle ergometry. *Difference between PRE and POST (P < 0.05). Values are expressed as mean ± SE.
**Figure 3.4** IL-6 mRNA (left), glycogen content (middle), and fibre type (type 1 fibres light, right) in muscle biopsy sections before (PRE) and after (POST) 120 min continuous recumbent cycle ergometry. Human metastatic breast tissue was used as a positive control (bottom). Yellow circle in the POST images indicates the same muscle fibre.
3.4 Discussion

This is the first study to identify that IL-6 is produced within muscle cells during muscular contraction. In a recent study by Penkowa et al. (2003), IL-6 protein was detected within muscle fibres and shown to increase following acute contraction. Although this study showed that IL-6 protein was indeed increased in muscle cells, the authors did not measure the cellular localization of IL-6 mRNA. Therefore, while providing a good indication that myocytes were a source of IL-6 during contraction, this previous study could not rule out the possibility that IL-6 may have been produced by other cell types before being trafficked into the muscle cell to perform intracellular functions. By using in situ hybridization to determine the cellular localization of IL-6, coupled with the quantitatively analyzed IL-6 protein within muscle fibres using IHC detection, our data provide the first solid evidence that human skeletal myocytes indeed produce IL-6 in response to contraction.

Our IL-6 protein data differ somewhat from that reported by Penkowa et al. (2003), who showed that IL-6 protein was distributed homogenously across each muscle fibre and did not appear to vary between fibre types. In contrast, the distribution of IL-6 protein after contraction in the current study could clearly be detected in predominantly type 2 fibres. It is difficult to identify the cause of this disparity between studies, given that both studies used a prolonged concentric model of contraction and the sampling methodologies of each study were similar. However, as reported
in our methods, our optimization for IHC not only included fluorescein as a secondary antibody, but also biotinylated streptavidin-peroxidase and Texas Red tagged secondary antibodies. We found that these two latter antibodies were less effective than fluorescein in eliminating background expression, and it is possible that the difference when comparing the results from the study of Penkowa et al. with the present study was the choice of the secondary antibody. Irrespective of why our data differ with those of Penkowa et al., we are confident that our protein data are an accurate representation of the fibre distribution of IL-6 because they are very consistent with our in situ hybridization data.

Our data reporting that IL-6 protein and mRNA expression was located in predominantly type 2 fibres with high glycogen content after contraction is an important novel finding. This finding was very surprising to us, and contrary to our hypothesis, which was based on the consistent finding from previous studies that IL-6 mRNA expression and protein release is exacerbated with glycogen depletion (Keller et al., 2001b; Steensberg et al., 2001b). However, it is well known that during prolonged steady-state cycling, muscle glycogenolysis occurs predominantly in type 1 fibres, and glycogenolysis in the type 2 fibres only occurs when type 1 fibres are glycogen depleted (Gollnick et al., 1973). Given our data that IL-6 protein and mRNA are present in predominantly type 2 fibres, we propose that an augmented increase in IL-6 mRNA and protein release is evident with low pre-exercise glycogen content because type 1 fibres become glycogen depleted earlier, resulting in the earlier recruitment of type 2 fibres.
In conclusion, our data clearly demonstrate that human skeletal myocytes 
*per se* are a source of contraction induced IL-6 and that was IL-6 is 
predominantly located in type 2 muscle fibres.
CHAPTER FOUR

CYTOKINE GENE EXPRESSION IN HUMAN SKELETAL MUSCLE DURING CONCENTRIC CONTRACTION: EVIDENCE THAT IL-8, LIKE IL-6, IS INFLUENCED BY GLYCOGEN AVAILABILITY
4.1 Introduction

Cytokines are pleiotropic proteins produced by virtually every nucleated cell in the body (Tilg, 2001). However, in most tissues including skeletal muscle, the constitutive expression of these cytokines is either minimal or absent (Frost et al., 2002). Rather, the expression of cytokines is induced by a myriad of physiological, pathological or chemical stimuli. Recently, Frost et al. (2002) demonstrated that lipopolysaccharide (LPS) induced the gene expression of a number of cytokines, namely interleukin (IL)-6, tumour necrosis factor (TNF)-α, IL-12, IL-1α, IL-1ra and TNF-β in mouse skeletal muscle. In addition, it was also demonstrated that 3 hours of running exercise, which would result in some tissue damage, induced the mRNA expression of IL-1β, IL-6, IL-8, IL-10 and TNF-α in human skeletal muscle (Nieman et al., 2003). These results were not surprising, because LPS is a potent stimulus for cytokine production in a variety of cell types including blood mononuclear cells, whereas tissue damage will result in neutrophil and macrophage infiltration into skeletal muscle (McLoughlin et al., 2003) which are known to produce a variety cytokines (Akira et al., 1993). Work from our group has focused on the effect of “non-damaging” muscle contraction on the induction of cytokines within skeletal muscle. It has been demonstrated that muscle contraction, in the absence of markers of muscle damage, rapidly increases IL-6 mRNA expression in skeletal muscle biopsy samples (Starkie et al., 2001b; Steensberg et al., 2001b; Steensberg et al., 2002; Steensberg et al., 2000b), whereas the intramuscular nuclear transcriptional activity of IL-6 is rapidly increased with the onset of such exercise (Keller et al., 2001b). Of note, however, is
the observation that unlike LPS, this type of muscle contraction does not increase IL-6 mRNA (Ullum et al., 1994) or protein (Starkie et al., 2000b; Starkie et al., 2001c) expression in blood mononuclear cells. This has lead us to hypothesize that the contraction induced IL-6 expression in skeletal muscle is not a consequence of trauma or damage, but rather is a specific biochemical phenomenon to allow the muscle to release this cytokine to mobilize substrate from fuel depots within the body to facilitate energy metabolism (Febbraio and Pedersen, 2002). To this end, IL-6 protein is released from skeletal muscle during prolonged knee extensor or bicycle exercise (Steensberg et al., 2001b; Steensberg et al., 2002; Steensberg et al., 2000b) and its release is further elevated when intramuscular glycogen stores are low (Steensberg et al., 2001b) or attenuated when glucose availability is increased (Febbraio et al., 2003b). In addition, it appears that IL-6 acts in a “hormone like” manner to increase lipolysis and fat oxidation (van Hall et al., 2003).

In contrast to the observations of contraction-induced skeletal muscle release of IL-6 and the data obtained after 3 hours of treadmill running (Nieman et al., 2003), concentric muscle contractions do not increase TNF-α gene expression in contracting skeletal muscle (Steensberg et al., 2002). In addition, chronic muscular activity down-regulates TNF-α gene expression in the skeletal muscles of elderly humans (Greiwe et al., 2001). The failure for such muscular contraction to induce TNF-α gene expression in skeletal muscle is not surprising given the marked increase in glucose uptake during exercise and the well described negative effect of
TNF-α on glucose disposal (Febbraio and Pedersen, 2002). Whether muscle contractions *per se* increase the expression of other cytokines within skeletal muscle or whether carbohydrate availability mediates such expression is not known and this was the aim of the present study. We chose to study humans during short-term cycling exercise to minimize any possibility of muscle damage inducing a local inflammatory response. We hypothesised that muscle contraction would not result in the ubiquitous induction of cytokine genes. Rather, we hypothesized that IL-6 would be the only cytokine markedly increasing its mRNA expression in response to contraction or glycogen availability, because of its specific “endocrine-like” role that has been identified.
4.2 Materials and Methods

Subjects and experimental protocol. Eight active, but not specifically trained, males (24 ± 2 yr, 77 ± 3kg, and 181 ± 2 cm) participated in the study, which was approved by the Royal Melbourne Institute of Technology (RMIT) University Human Ethics Committee. Written informed consent was obtained from all human subjects. Each visited the laboratory and performed an incremental exercise test on a cycle ergometer (Lode, Excalibur, Groningen, The Netherlands) until they reached volitional exhaustion. The peak pulmonary oxygen uptake (VO$_2$peak) averaged 49.0 ± 3.2 ml•kg$^{-1}$•min$^{-1}$. Expired pulmonary gases (Quark b$^2$, COSMED, Rome, Italy) were collected and analyzed online to compute VO$_2$peak.

The subjects subsequently visited the laboratory for two experimental trials separated by at least 1 week and conducted in random and counterbalanced order. During these trials they attended the laboratory between 1600 and 1700 to perform glycogen-depleting exercise and returned the following morning at ~0800 to perform the experimental trial. The glycogen-depleting exercise involved two exercise bouts consisting of continuous cycling at 70% VO$_2$peak for 20 minutes followed by 20 minutes intermittent exercise. The intermittent exercise consisted of 2 min cycling at 90% VO$_2$peak followed by 2 min at 50% VO$_2$peak. Subjects then performed ~20 minutes arm-cranking exercise (~50 W) to deplete glycogen of the upper arms. After resting for 5 minutes, the aforementioned cycling protocol was repeated until subjects reached
volitional exhaustion. Five all-out sprints were then performed for 30 seconds with a 2 minutes recovery period. The duration of the glycogen-depleting exercise was ~2.5 hours (average of 92 ± 3 minutes cycling exercise). Water intake was permitted throughout all trials, and no adverse events were reported by subjects. Subjects were provided with an overnight food parcel. On one occasion, the 6.2 MJ diet consisted of 79% carbohydrate, 3% fat, and 18% protein (denoted herein as Con); whereas, on the other visit, the 6.4 MJ diet consisted of 4% carbohydrate, 59% fat, and 37% protein (denoted herein as Lo Gly). Subjects were instructed to consume all food before 2200 and were permitted to consume water thereafter. Subjects arrived at the laboratory the following morning after an overnight fast. They voided, lay supine on a bed, and a Teflon catheter was inserted into an antecubital vein for blood sampling. One leg was prepared for subsequent needle biopsy by making two incisions through the skin and fascia of the vastus lateralis under local anesthesia. Immediately before exercise, a muscle sample was obtained by needle biopsy. The leg was bandaged and the subject moved to the cycle ergometer and commenced cycling at ~70% VO₂peak. Venous blood samples were obtained at 20 minutes intervals during exercise. In addition, expired pulmonary gases (Quark b², COSMED) were collected and analyzed online at 20 minutes intervals during exercise. A second muscle sample was obtained from the second incision immediately after the cessation of exercise (at 60 minutes) with the subject on the cycle ergometer. Muscle samples were obtained from the contralateral leg in the subsequent trial.
Muscle Tissue Analyses

Muscle glycogen. A portion of muscle (~10 mg) was freeze-dried, dissected free of any blood and connective tissue, powdered, and analyzed for glycogen as previously described (Febbraio et al., 2003b).

Measurement of mRNA expression. A portion of muscle (~30 mg) was extracted for total RNA using a modification of the acid guanidium thiocyanate-phenol chloroform extraction method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) described elsewhere (Febbraio and Koukoulas, 2000). The total RNA was quantified two to three times before 1 ng of each total RNA sample was reverse transcribed in a 10 µl reaction containing 1 X TaqMan RT buffer, 5.5 mM MgCl₂, 500 mM each 2’-deoxynucleoside 5’-triphosphate, 2.5 mM random hexamers, 0.4 U·ml⁻¹ RNase inhibitor, 1.25 U·ml⁻¹ Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) and made up to volume with milli-Q H₂O (0.05% DEPC treated). Control samples were also analyzed where all the above reagents are added to RNA samples except the Multiscribe Reverse Transcriptase. The reverse transcription reactions were performed using a GeneAmp PCR system 2400 (Perkin, Elmer, Wellesley, MA) with conditions at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. EDTA (2 ml 0.5 M; pH 8.0) was added to each sample and stored at -20°C until further analysis.
Real-time PCR was employed to quantitate human IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, the p35 and p40 subunits of IL-12 (IL-12p35, IL-12 p40), IL-15, interferon (IFN)-γ, and TNF-α. All cytokines except IL-6 were analyzed using the Taqman Human Cytokine Gene Expression Plate (Applied Biosystems, Foster City, CA). Human probe and primers for IL-6 were designed (Primer Express version 1.0 Applied Biosystems) from the human gene sequence accessed from GenBank/EMBL as previously described (Starkie et al., 2001b). For all genes, a TaqMan probe was labeled with the fluorescent tags FAM (6-carboxyfluorescein) at the 5’ end and TAMRA (6-carboxy-tetramethylrhodamine) at the 3’ end and ribosomal 18S mRNA was also amplified as our reference gene. We quantitated gene expression using a multiplex comparative critical threshold (C_T) method (Bio-Rad i Cycler IQ, Hercules, CA).

PCR reactions were carried out in duplicate in 50 µl reactions of TaqMan universal PCR master mix (1 X), 50 nM TaqMan 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, and probes and primers at specific concentrations. Twenty nanograms of cDNA were amplified using the following conditions: 50°C for 2 min, 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Of note, in previous experiments where we have followed these procedures, we compared our gene expression data obtained from real-time PCR methodology with that obtained using a Northern blot and have demonstrated comparable results (Steensberg et al., 2001b). For each sample, 18S C_T values were subtracted from the gene of interest C_T values
to derive a $\Delta C_T$ value. The resting value for each subject during each trial was then subtracted from the exercise samples for each subject to derive a $\Delta\Delta C_T$ value. The expression of the genes of interest relative to the resting samples was then evaluated using the expression $2^{-\Delta\Delta C_T}$. The coefficient of variation, determined on the $C_T$ value of the 18S samples, was less than 1%.

**Blood analyses.** Blood for plasma glucose analysis was collected into a tube containing fluoride EDTA, mixed, and spun in a centrifuge at 2200 g for 8 minutes at 0°C. The plasma was later analyzed for glucose using an automated glucose/lactate analyzer (YSI 2300 STAT PLUS, Yellow Springs, OH). Blood samples for plasma IL-6 and IL-8 concentration were drawn into glass tubes containing EDTA. The tubes were spun immediately at 2,200 g for 15 minutes at 4°C and the plasma was stored at -80°C until analyses were performed. ELISA kits (R&D Systems, Minneapolis, MN), were used to quantify plasma IL-6 and IL-8, which were quantified using chemiluminescence. Plasma creatine kinase (CK) was determined by automated enzyme reactions using the International Federation of Clinical Chemistry recommended method (automated analyses for AU5000, Olympus, Tokyo, Japan).

**Histological determination of muscle fibre cross sections.** In the present study we did not freeze clamp muscle in isopentane for histological determination. To provide further evidence that bicycle exercise does not induce muscle damage, we collected muscle samples prepared for
histochemical determination from a separate cohort of subjects who exercised for a similar duration and intensity on a bicycle ergometer. It is standard practice in our laboratory to perform the glycogen depletion protocol before experimentation to minimize any differences in pre-exercise glycogen content between subjects. Consequently, the subjects in this cohort also performed the same glycogen depletion protocol before being provided with the identical diet that was given to subjects in Con. Muscle samples obtained before and after exercise were rapidly frozen in isopentane, subsequently sectioned frozen in a cryostat (10 µm sections) and stained with hematoxylin and eosin.

Statistical analysis. Analysis of the IL-6 and IL-8 mRNA measures revealed that the data were not normally distributed. To ensure homogeneity of the data, data were log transformed before statistical analysis. All other parameters were normally distributed. A two-way (trial X time) ANOVA with repeated measures on the time factor was used to compute the statistics (Statistica, Tulsa, OK), 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 ± SE.
4.3 Results

Workload and pulmonary measures. Subjects cycled at an average of 174 ± 12 W during the experimental trials. Although mean VO\textsubscript{2}\text{peak} did not differ when comparing trials (34.7 ± 2.2 versus 34.2 ± 2.2 ml\textbullet kg\textsuperscript{-1}\textbullet min\textsuperscript{-1}) for Con and Lo Gly, respectively), respiratory exchange ratio was higher (P < 0.05) in Con (0.90 ± 0.01) compared with Lo Gly (0.81 ± 0.01).

mRNA abundance of cytokines. The pattern of cytokine gene expression is presented in Table 4.1. Of the 13 cytokines analyzed, 5 were detected in the muscle biopsy samples. These were IL-1β, IL-6, IL-8, IL-15, and TNF-α. Only IL-6 and IL-8 were induced (P < 0.05) by contraction. In addition, the contraction-induced expression of these cytokines was augmented (P < 0.05) during Lo Gly (Figure 4.1).

Muscle glycogen and plasma glucose. Resting muscle glycogen was higher (P < 0.05) in Con compared with Lo Gly (375 ± 35 versus 163 ± 27 mmol glycosyl units\textbullet kg\textsuperscript{-1} dry mass for Con and Lo Gly, respectively) and was reduced (P < 0.05) to 102 ± 32 versus 17 ± 5 mmol glycosyl units\textbullet kg\textsuperscript{-1} dry mass for Con and Lo Gly, respectively (Figure 4.2). Plasma glucose was not different when comparing trials at rest, and whereas plasma glucose concentration was maintained in Con, it fell (P < 0.05) in Lo Gly such that the values at 40 and 60 minutes were lower (P < 0.05) in Lo Gly compared with Con (Figure 4.2).
\textit{Plasma cytokines and creatine kinase}. Plasma IL-6 was not different at rest when comparing trials. Plasma IL-6 increased (P < 0.05) after 40 minutes in both trials, but the increase was greater at 40 and 60 minutes in Lo Gly such that the concentrations at these times were higher (P < 0.05) compared with Con (Figure 4.3). In contrast, IL-8 was not different at rest or at any point during exercise when comparing Con with Lo Gly, and exercise per se did not increase plasma IL-8 in either trial (Figure 4.3). Plasma creatine kinase (CK) was not different at rest when comparing trials and was not increased when comparing values at 60 minutes with those at rest (Table 4.2).

\textit{Histological determination of muscle fibre cross sections}. We were unable to detect any differences in the general histological architecture of the muscle cross sections before and after exercise. Pre- and post-exercise cross sections from a single subject are displayed in Figure 4.4.
Table 4.1 Pattern of cytokine expression in skeletal muscle. The expression of interleukin (IL)1-β, IL-6, IL-8, IL-15, and tumour necrosis factor (TNF)-α were detected in the pre-exercise muscle biopsy samples. Of these only IL-6 and IL-8 mRNA were contraction inducible, and their increase were exacerbated in the presence of low intramuscular glycogen.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Basal Expression</th>
<th>Concentric Contraction Inducible?</th>
<th>Influenced by Glycogen Content?</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>IL-2</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<td>IL-4</td>
<td>No</td>
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<td>IL-5</td>
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<tr>
<td>IL-6</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>IL-8</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>IL-10</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>IL-12p40</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>IL-15</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>IFN-γ</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 4.1  Contraction-induced increase in interleukin (IL)-6 (A) and IL-8 (B) mRNA during exercise with normal (Con) or low (Lo Gly) pre-exercise intramuscular glycogen content. Values are expressed as a fold change from rest with rest equal to 1 arbitrary unit (means ± SE). * Difference (P < 0.05) compared with Con; n = 8.
Figure 4.2 Muscle glycogen (A) and plasma glucose (B) concentrations during exercise with normal (■) or low (□) pre-exercise intramuscular glycogen.* Difference (P < 0.05) compared with 0 min. Values are expressed as mean ± SE; n = 8.
Figure 4.3  Plasma IL-6 (A) and IL-8 (B) concentrations during exercise with normal (■) or low (□) pre-exercise intramuscular glycogen content.

* Difference (P < 0.05) compared with Con. Values are expressed as mean ± SE; n = 8.
Table 4.2  Plasma creatine kinase concentrations before (0 minute) and after (60 minutes) 1 hour of cycling exercise with either Con or Lo Gly intramuscular glycogen availability. Values expressed in U•L\(^{-1}\) as means ± SE (n = 8). Con, normal; Lo Gly, reduced.

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Lo Gly</th>
</tr>
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<tbody>
<tr>
<td>0 min</td>
<td>343 ± 60</td>
<td>291 ± 88</td>
</tr>
<tr>
<td>60 min</td>
<td>353 ± 69</td>
<td>294 ± 84</td>
</tr>
</tbody>
</table>
Figure 4.4 Muscle cross section from the vastus lateralis muscle taken from an individual before (A) and after (B) cycling exercise performed at 70% peak pulmonary oxygen uptake (VO₂peak) for 60 minutes. Greyscale images were stained for hematoxylin and eosin.
4.4 Discussion

The results from this study demonstrate that cytokines are not ubiquitously expressed in skeletal muscle and the mRNA abundance of IL-6 and IL-8 alone appear to be increased by the type of exercise used in this study. Because the mode of exercise used in the present study did not induce observable markers of muscle tissue damage or inflammation and because the contraction-induced increase in IL-8 and IL-6 was potentiated when glycogen availability was reduced, our data suggest that the mRNA abundance for these two cytokines is influenced by metabolic processes rather than inflammation secondary to muscle damage.

In the present study we only measured the mRNA abundance and not the rate of gene transcription. Therefore, our measures may reflect an increase in gene transcription and/or increases in mRNA stability or, indeed, a combination of both. Although we have no evidence regarding the contraction induced rate of transcription for IL-8, however, Keller et al. (2001b) demonstrated that the rate of IL-6 gene transcription closely matches the mRNA abundance during exercise with regardless of the pre-exercise glycogen levels. In this respect we are confident, at least for our IL-6 measures, that our results reflect the rate of gene transcription. It is well known that skeletal muscle not only contains myocytes, but also smooth muscle cells, fibroblasts, endothelial cells, and macrophages, and these cells are also known to produce IL-6 (Cicco et al., 1990; De Rossi et al., 2000; Klouche et al., 1999; Podor et al., 1989). Because we only obtained muscle biopsy samples, it was possible that the elevations seen
in IL-6 and IL-8 mRNA expression may have been due to an increased mRNA expression in cells other than skeletal myocytes. We have not examined mRNA localization of IL-8 in muscle cells; however, our in situ hybridization data, presented in the previous chapter, have clearly demonstrated that the mRNA abundance of IL-6 increases within the skeletal muscle cells after contraction, and the quantification of mRNA abundance using this method matches the rate seen using RT-PCR in muscle biopsy homogenates.

The results from the present study differ somewhat with those that have stimulated muscle with LPS (Frost et al., 2002) or muscle contraction with a large eccentric component (Nieman et al., 2003). In particular, whereby these two stimuli resulted in a marked increase in the mRNA abundance of TNF-α and IL-1β, our data showed no such effect, although both TNF-α and IL-1β were basally expressed in our tissue samples. The current findings are consistent with the previous observations in humans during knee extensor exercise (Steensberg et al., 2002), which has also been shown to result in minimal muscle damage (Steensberg et al., 2000b). It is well known that TNF-α is expressed in response to tissue damage and inflammation (Broussard et al., 2003). In addition, Fielding et al. (1993) demonstrated that ultrastructural damage to skeletal muscle is associated with neutrophil infiltration and IL-1β accumulation. In the previous study reported by Nieman et al. (Nieman et al., 2003), the exercise-induced increase in TNF-α and IL-1β was not affected by carbohydrate availability,
providing further evidence that the observed increases were probably due to inflammatory and not metabolic processes.

Although our data cannot categorically rule out the presence of some muscle damage, the evidence strongly argues against such an occurrence. Firstly, as discussed, we saw no evidence that either TNF-α and IL-1β were increased by contraction, and these cytokines are often, if not always, elevated when muscle damage occurs (Fielding et al., 1993; Frost et al., 2002; Nieman et al., 2003). Secondly, although the resting plasma creatine kinase (CK) levels were in the high range of normal, there was no indication that CK rose during 60 minutes of exercise. This is in contrast with previous studies where subjects ran (Walsh et al., 2001) or performed eccentric cycling (Toft et al., 2002) for 60 minutes. In these studies, where exercise involved an eccentric component, a marked increase in plasma CK was observed after 60 minutes. Thirdly, our previous data have demonstrated that this mode of exercise results in intramyocellular increases in both IL-6 mRNA and protein but does not increase IL-6 mRNA in the area surrounding the fibres (Chapter 3). Finally, despite our histological data are from a separate cohort of subjects, these subjects nonetheless underwent a similar exercise regimen, including exercise the previous day. The histology did not provide any evidence of ultrastructural damage. Our data, therefore, suggest that there is no observable muscle damage during short-term (60 minutes) continuous exercise.
Our observations that IL-6 increased with non-contracting skeletal muscle and that the increase was augmented when intramuscular glycogen levels were low are entirely consistent with previous studies that have examined this relationship (Keller et al., 2001b; Steensberg et al., 2001b). Although in the present study we did not sample for leg IL-6 release, the fact that leg IL-6 release can account for much of the plasma IL-6 accumulation (Steensberg et al., 2000b), suggests that our plasma IL-6 data suggest that, like previous studies (Steensberg et al., 2001b), circulating IL-6 appears to be related to glycogen availability. Recently, however, using \textit{in situ} hybridization and immunohistochemical techniques, we identified that IL-6 is produced almost exclusively by type 2 muscle fibres. Given that type 1 fibres are recruited during prolonged contraction, we showed that IL-6 was inversely related to glycogen content within muscle fibres (Chapter 3). Although these recent results seem somewhat contradictory to the present and previous findings (Keller et al., 2001b; Steensberg et al., 2001b), there is a viable explanation for the apparent anomaly. In the present and previous studies (Keller et al., 2001b; Steensberg et al., 2001b), muscle glycogen levels were analysed from mixed fibre muscle biopsies and patterns of glycogen depletion within specific fibre types were not obtained. It is likely, however, that as type 1 fibres became depleted of muscle glycogen, type 2 fibres were subsequently recruited, producing and then releasing IL-6.

A key finding from this study was that like IL-6, IL-8 mRNA is induced by concentric muscle contraction and that its induction is markedly
exacerbated by low intramuscular glycogen stores. It must be noted, however, that where the increase of IL-6 was ~150-fold in Lo Gly, the increase in IL-8 was ~40-fold (Figure 4.1). In addition, whereas IL-6 increased in the circulation in both trials, but to a greater extent in Lo Gly, no such increase in IL-8 was observed. Hence, there appear to be differences in the biological profile of contraction-induced increases in these two cytokines. IL-8 has been shown to increase in skeletal muscle, but is blunted by carbohydrate ingestion throughout exercise (Nieman et al., 2003). However, the biological significance of this increase was not discussed in this previous paper (Nieman et al., 2003) and little is known regarding the significance of the increase in skeletal muscle IL-8 mRNA. IL-8 was identified over a decade ago (Baggiolini and Clark-Lewis, 1992) and is classed as one of 40 chemotactic peptides ranging from 8 to 14 kDa. In this respect, the biological action and size of IL-8 are quite different compared with IL-6. In recent years, biological roles have been identified for muscle-derived IL-6, both as an intramyocellular signal for heat shock proteins (Febbraio et al., 2002) and as an endocrine-like molecule to be released from the muscle to modulate metabolic processes such as lipolysis (Febbraio and Pedersen, 2002; van Hall et al., 2003). In the current study, we saw no evidence that IL-8 was released into the circulation, so in this respect, it is unlikely to have significant circulatory actions. Whereas it is possible that IL-8 has some biological role to play in the etiology of muscle metabolism, it is also possible that its induction is simply a consequence of a disruption to myocellular homeostasis. In most cell types IL-8 is markedly induced via the mitogen activated protein
kinase (MAPK) pathways. In particular, p38 appears to be a potent upstream signalling molecule for IL-8 in monocytes, neutrophils, and smooth muscle cells (Hoffmann et al., 2002).

In summary, our data demonstrate that cytokines are not ubiquitously expressed in skeletal muscle and that IL-8, like IL-6, is influenced by the glycogen availability within the contracting muscle. The biological role of IL-8 during exercise is yet to be elucidated.
CHAPTER FIVE

ALTERING DIETARY NUTRIENT INTAKE THAT REDUCES GLYCOGEN CONTENT LEADS TO PHOSPHORYLATION OF NUCLEAR p38 MAPK IN HUMAN SKELETAL MUSCLE
5.1 Introduction

As discussed, interleukin (IL-6) is a pleiotropic cytokine produced by a variety of tissues during inflammation arising from a variety of pathophysiological conditions (Pinsky et al., 1993; Strassmann et al., 1992; Ullum et al., 1996). As discussed in Chapter 4, skeletal muscle cells are also capable of producing IL-6 in response to various chemical stimuli such as incubation with lipopolysaccharide (LPS) (Frost et al., 2002; Frost et al., 2003), reactive oxygen species (ROS) (Kosmidou et al., 2002) and inflammatory cytokines (Frost et al., 2003; Luo et al., 2003). In these circumstances, the upstream signalling events that lead to the induction of IL-6 have been well categorized. Both LPS and inflammatory cytokines such as IL-1β and TNF-α can activate IL-6 via the mitogen-activated protein kinases (MAPK) c-jun terminal kinase (JNK) (Frost et al., 2003), p38 MAPK (Luo et al., 2003), and the transcription factors nuclear factor kappa-β (NFκβ) and activator protein-1 (AP1) (Kosmidou et al., 2002). These signalling events that lead to IL-6 production in cultured skeletal muscle cells are consistent with experiments conducted in cardiac myocytes (Craig et al., 2000) and monocytes (Tuyt et al., 1999). However, no studies have examined upstream IL-6 signalling in skeletal muscle in vivo.

As discussed in Chapter 1, human skeletal muscle appears unique in that it can produce IL-6 during contraction in the absence of observable markers of inflammation (Febbraio and Pedersen, 2002), linking IL-6 to
metabolism rather than inflammation. Penkowa and colleagues (2003) have used immunohistochemical techniques to demonstrate that the contraction-induced increase in IL-6 protein formation occurs within the muscle cells, a finding that was confirmed by measuring immunohistochemical detection of IL-6 protein and in situ hybridization determination of IL-6 mRNA by our group (Chapter 3). In both of these previous experiments, the mode of contraction was prolonged and concentric, and markers of damage were absent. During this type of exercise, the rate of IL-6 gene transcription within human skeletal muscle is rapid (Keller et al., 2001b) and it has been previously hypothesized that this is due to calcium (Ca^{2+}) being released from the lateral sacs of the sarcoplasmic reticulum to activate IL-6 through activation of nuclear factor of activated T cells (NFAT) (Febbraio and Pedersen, 2002). Previous studies in B lymphocytes have demonstrated that the activation of NFAT was induced by a low sustained [Ca^{2+}]i (Dolmetsch et al., 1998), which is characteristic of prolonged skeletal muscle contractile activity. NFAT is present in ~10-fold higher concentrations in neuronal and muscle cells when compared with other cell types (Olson and Williams, 2000b). When activated, calcineurin binds to and dephosphorylates NFAT, allowing it to translocate to the nucleus where it associates with other transcription factors (Olson and Williams, 2000b).

Although muscle contraction induces IL-6 gene transcription in skeletal muscle, it is also clear that this effect is exacerbated in the presence of low intramuscular glycogen stores as we showed in Chapter 4 and others
have shown previously (Keller et al., 2001b; Steensberg et al., 2001b). Despite these observations, the signalling events that mediate these processes are not well understood. It is, however, well known that p38 MAPK often (Boppart et al., 2000b; Goodyear et al., 1996b) but not always (Williamson et al., 2003b) increases in skeletal muscle during contraction. To date, no studies have determined whether glycogen availability influences the expression of p38 MAPK in contracting skeletal muscle. However, since p38 MAPK is a stress-activated protein kinase and low glycogen decreases the energy availability in contracting muscle, this hypothesis is plausible.

Based on the previous studies in skeletal muscle cell culture experiments and other cell/tissue types, a model was recently proposed which hypothesized that contraction could activate IL-6 transcription through activation of NFAT, and that low glycogen would activate p38 MAPK leading to a potentiation of IL-6 transcription and protein production (Febbraio and Pedersen, 2002). In the present study, we tested the hypothesis that the transcription of IL-6 during predominantly concentric exercise was activated by NFAT, but enhanced with low glycogen due to increased nuclear localization and phosphorylation of p38 MAPK. To do this, we isolated nuclear fractions of muscle samples before and after exercise and probed them for total protein or phosphorylated proteins using Western blot analyses.
5.2 Materials and Methods

Human Experiments

The present experiments were performed on muscle samples obtained from our human study described in Chapter 4. Con and LCHO denote control and low carbohydrate trials, respectively.

Muscle tissue analyses

Measurement of mRNA expression. The measurement of IL-6 mRNA was as described in Chapter 4.

Extraction of nuclear proteins. Nuclear proteins were extracted from the muscle samples using a technique described previously (McGee et al., 2003). Briefly 30 mg of the muscle was homogenized in 500 mL of prechilled Buffer A (250 mM, sucrose, 10mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 ml per 30 mg tissue protease inhibitor cocktail). The homogenate was spun at 500 g for 5 minutes at 4°C. The supernatant containing cytosolic materials (crude fraction) was removed and stored at -80°C until required. Then 500 ml of prechilled Buffer B (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM MgCl2, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM DTT, 1 mM PMSF, 2 ml per 30 mg tissue protease inhibitor cocktail) was added to resuspend the remaining pellet. After 10 minutes incubation on ice with occasional mixing, the pellet mixture was then spun for 5 minutes at 3000 g at 4°C.
The supernatant representing the nuclear fraction was extracted and stored at -80°C until required. Fraction purity was assessed by Western blotting on Histone H1 as described previously (McGee et al., 2003).

**Western blotting.** SDS-PAGE was used to separate and identify proteins from the two fractions (nuclear and cytosolic). Thirty micrograms of protein from each sample was transferred by electrophoresis on 10% SDS polyacrylamide gel at 130 V. The separated proteins were then transferred using a semidry transfer on to a nitrocellulose membrane and incubated in blocking buffer [5% skim milk powder in Tris-buffered saline with 0.25% Tween (TBST)] for 1 h. After an overnight exposure to primary antibodies against total and phosphorylated p38 MAPK, total and phosphorylated JNK, NFκB (Cell Signalling Technology, Inc., Beverly, MA), or NFAT (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (1 in 1000 in blocking buffer) at 4°C, the membrane was then subjected to anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1 in 10,000 in blocking buffer) for 1 h. After 30 minutes of washing, antibody binding was detected by enhanced chemiluminescence substrate (Pierce SuperSignal chemiluminescent; Pierce, Rockford, IL) and a Kodak Image Station 440CF (NEN Life Science Product, Boston, MA). Band intensities (arbitrary units) were measured by Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

**Statistical analysis.** Statistical analysis was carried out by Two-way (trial x time) ANOVA with repeated measures on the time factor. If analyses
revealed a significant interaction, a Student Newman-Keuls test was used to locate specific differences. Significance was accepted as $P < 0.05$. Data are means ± SE. All statistics were completed using Statistica software for windows (StatSoft, version 5.1, 1997, Statistica, Tulsa, OK).
5.3 Results

Muscle Glycogen. Resting muscle glycogen was higher (P < 0.05) in Con compared with LCHO (375 ± 35 versus 163 ± 27 mmol glycosyl units•kg⁻¹ dry mass for Con and LCHO, respectively) and was reduced (P < 0.05) to 102 ± 32 vs. 17 ± 5 mmol glycosyl units•kg⁻¹ dry mass for Con and LCHO, respectively.

Nuclear Abundance of Signalling Proteins. The nuclear abundance of total JNK was neither affected by glycogen nor contraction. Exercise increased (P < 0.05) phosphorylation of JNK in the nucleus compared with basal levels, but the degree of increase was not different when comparing trials (Figure 5.1). The nuclear abundance of NFκB and NFAT was neither affected by glycogen availability or contraction (Figure 5.2).

No difference was observed in nuclear total p38 MAPK either before or after exercise in either trial. However, the phosphorylated form of p38 MAPK was higher (P < 0.05) in LCHO compared with Con both before and after exercise. Despite this, the cytosolic abundance of phosphorylated p38 MAPK was not different between trials, although it tended (P = 0.09) to be higher in LCHO compared with Con after exercise (Figure 5.3).

Exercise resulted in an increase (P < 0.05) in IL-6 mRNA in both CON and LCHO. However, the exercise-induced increase was markedly exacerbated (P < 0.05) in LCHO (Figure 5.4). When the pre-exercise nuclear
abundance of phosphorylated p38 MAPK was plotted against the exercise-induced increase in IL-6 mRNA, a highly significant correlation ($r = 0.96; P < 0.01$) was observed (Figure 5.4).
Figure 5.1  Nuclear abundance of total (A) and phosphorylated (B) c-jun amino terminal kinase isoforms 1 and 2 (JNK 1/2) before (basal) and after (contraction) 60 minutes of concentric exercise with normal (CON; filled bars) or low (LCHO; open bars) pre-exercise muscle glycogen content. † Difference (P < 0.05) from basal (main time effect). Values are means ± SE (n = 8).
Figure 5.2  Nuclear abundance of nuclear factor kappa B (NFκB; A) and nuclear factor of activated T cells (NFAT; B) before (basal) and after (contraction) 60 minutes of concentric exercise with normal (CON; filled bars) or low (LCHO; open bars) pre-exercise muscle glycogen content. Values are means ± SE (n = 8).
Figure 5.3 Nuclear abundance of total (A) and phosphorylated (B) p38 mitogen activated protein kinase (p38 MAPK) and cytosolic abundance of phosphorylated p38 MAPK (C) before (basal) and after (contraction) 60 minutes of concentric exercise with normal (CON; filled bars) or low (LCHO; open bars) pre-exercise muscle glycogen content. * Difference (P < 0.05) in LCHO compared with CON. Values are means ± SE (n = 8).
Figure 5.4 Exercise induced fold change in interleukin-6 (IL-6) mRNA with normal (CON; filled bars) or low (LCHO; open bars) pre-exercise muscle glycogen content (A) and the relationship between pre-exercise p38 MAPK and the exercise induced fold change in IL-6 (B) mRNA. * Difference (P < 0.05) in LCHO compared with CON.
5.4 Discussion

The results from the present study suggest that low intramuscular glycogen is associated with the phosphorylation of the nuclear p38 MAPK. Since the phosphorylation of p38 in the nuclear fraction prior to exercise was associated with the exercise-induced fold increase in IL-6 mRNA, it appeared that one role of phosphorylation of p38 MAPK within the nucleus was to act as an upstream target for IL-6 in contracting human skeletal muscle.

From our results, it appears that a diet low in carbohydrate that results in low intramuscular glycogen per se does not lead to the nuclear translocation of p38 MAPK, but rather phosphorylates p38 MAPK that resides in the nucleus (Figure 5.3). Subjects performed the same pre-trial exercise regime, so the increase in pre-exercise nuclear p38 MAPK phosphorylation observed in LCHO cannot be attributed to prior contractile activity. However, we cannot rule out the possibility that a high fat diet and not low glycogen per se was responsible for this observation because subjects ingested a high fat diet in the intervening period between the glycogen depleting exercise and the experimental trial in LCHO. Indeed, chronic exposure of human skeletal muscle cells to palmitate, a saturated fatty acid, increases the activation of p38 MAPK (Aas et al., 2004); whereas treatment with the unsaturated fatty acid, oleate, promotes a reduction in phosphorylation of this kinase and hence its activity (Aas et al., 2006). At the present time, there are no data that have examined the direct effect of low glycogen on p38 MAPK phosphorylation in skeletal
muscle, so we are unable to shed further light on whether our observation of nuclear p38 MAPK phosphorylation was due to low glycogen *per se*. Of note, however, Polekhina *et al.* (2003) have demonstrated that the AMP-activated protein kinase (AMPK) β subunit contains a functional glycogen binding domain. In addition, when glycogen binds to this domain, the activity of AMPK and its upstream kinase AMPK-kinase is down-regulated (Steinberg *et al.*, 2006). Although highly speculative, it is possible that kinases upstream of p38 MAPK, such as MKK3 and MKK6, may also have a functional binding domain that inhibits phosphorylation when bound to glycogen, although it must be noted that p38 MAPK phosphorylation in the cytosolic fraction was unaffected by glycogen content before exercise. Alternatively, the up-regulation of p38 MAPK in the presence of low glycogen may be a direct effect of AMPK activity as it has been demonstrated that the AMPK activator AICAR increases p38 MAPK phosphorylation in skeletal muscle (Lemieux *et al.*, 2003).

It is well established that when muscle cells are incubated with inflammatory agents such as LPS (Frost *et al.*, 2002; Frost *et al.*, 2003), reactive oxygen species (Kosmidou *et al.*, 2002) IL-1β and TNF-α (Frost *et al.*, 2003; Luo *et al.*, 2003), IL-6 can be activated by JNK (Frost *et al.*, 2003), NFκB, and AP-1 (Kosmidou *et al.*, 2002), which associates with NFAT to activate IL-6. Since it has been shown that the calcium ionophore, ionomycin, can induce IL-6 mRNA in human cultured muscle cells (Keller *et al.*, 2002b; Keller *et al.*, 2006), we hypothesized that NFAT would be activated by cytosolic Ca$^{2+}$ accumulation upon contraction and
that this would lead to IL-6 gene transcription. From our results, this does not appear to be the case. Although the nuclear abundance of NFAT appeared to be higher in LCHO prior to exercise, this was largely due to the results obtained from one subject. Moreover, there was a 20-fold increase in the mRNA abundance of IL-6 in CON, but in these circumstances no increase in nuclear NFAT was observed. Therefore, while we cannot rule out the possibility that the rapid IL-6 gene transcription seen at the onset of contraction is mediated by NFAT secondary to increased cytosolic Ca\textsuperscript{2+} accumulation, our results are not consistent with this hypothesis. Likewise, there was no indication that NFkB mediated the increase in IL-6 mRNA during contraction. It is well known that NFkB is involved in the regulation of a large number of genes that control various inflammatory responses including IL-6 (Li et al., 2000b), while in skeletal muscle cell culture, NFkB induced IL-6 transcription appears most sensitive to incubation with LPS (Kosmidou et al., 2002). As discussed, however, the present study involved 60 minutes of concentric exercise that does not result in inflammation. Therefore, we were not surprised that the nuclear abundance of NFkB was not increased. In addition, as discussed previously, Cai et al (2004) showed in a mouse model with constitutive IKK expressed in skeletal muscle, IL-6 is not elevated, arguing against the NFkB pathway activating IL-6 in this tissue. However, it must be pointed out that, in this study, we measured nuclear abundance of NFkB and not the activity level of this protein by mobility shift analysis or phosphorylation of IxB. Therefore, our data cannot rule out the possibility that nuclear NFkB was activated in the nucleus by contraction.
In our experiment, nuclear JNK 1/2 phosphorylation was increased by contraction in both CON and LCHO (Figure 5.1), a result consistent with previous experiments that measured JNK activity in crude muscle homogenates from humans subjected to 60 minutes of cycling exercise (Aronson et al., 1998). Although the exercise induced increase in nuclear JNK 1/2 phosphorylation was similar when comparing the two trials, it is possible that JNK phosphorylation at the onset of exercise is an upstream signal for IL-6 gene transcription during contraction of this type. As discussed, Frost et al. (2003) has demonstrated that IL-6 gene expression is a downstream target of JNK in C2C12 myoblasts subjected to LPS. Clearly the hypothesis that JNK may mediate IL-6 gene transcription in contracting skeletal muscle is plausible and warrants further examination.

The most important finding of this study was that the increase in phosphorylation of p38 MAPK in the nucleus due to low glycogen was associated with the magnitude of the increase in IL-6 mRNA (Figure 5.4). We acknowledge that the subject number is relatively low, but the tightness of this relationship suggests that phosphorylation of p38 MAPK at the nucleus may result in regulation of co-repressors or -activators binding to the 5’ flanking region of the IL-6 gene. This result stimulated us to examine this process further (see Chapter 6).

In conclusion, we have shown that dietary manipulation that resulted in low intramuscular glycogen was associated with the phosphorylation of the nuclear p38 MAPK. In addition, the phosphorylation of p38 MAPK in
the nuclear fraction, before exercise was tightly related to the exercise-induced fold increase in IL-6 mRNA.
CHAPTER SIX

THE CALCIUM IONOPHORE, A23187 STIMULATES IL-6 GENE EXPRESSION IN CULTURED MYOTUBES, A RESPONSE THAT IS ABOLISHED WITH p38 MAPK INHIBITION
6.1 Introduction

In accordance with the literature, our previous findings described in detail in Chapters 3 & 4, have clearly shown that the production of IL-6 during “non-damaging” exercise is not related to inflammation or other cytokines, but rather is related to the intramuscular glycogen levels and the mode of contraction. Like other tissues, the constitutive expression of cytokines within skeletal muscle are usually minimal or absent at a resting state (Frost et al., 2002). Exercise induces a rapid increase of the nuclear transcriptional rate of IL-6 (Keller et al., 2001b), and its release into the circulation (Febbraio and Pedersen, 2002). Once in the circulation, IL-6 has been reported to have a relative short half-life (Castell et al., 1988; Suzuki et al., 2000) and exhibit a rapid plasma clearance by the kidneys and the liver (Febbraio et al., 2003a; Montero-Julian et al., 1995), suggesting this cytokine may be largely regulated at the level of expression.

Muscular contraction is a potent stimulus for Ca\(^{2+}\) release from the lateral sacs of the sarcoplasmic reticulum (Olson and Williams, 2000b), and elevated levels of Ca\(^{2+}\) have been shown to implicate in the transcription of IL-6 (Holmes et al., 2004; Keller et al., 2002b). In addition, contractile activity is known to result in phosphorylation and activation of the MAPK, p38 (Dentel et al., 2005). In the previous Chapter, we reported that the phosphorylation of p38 MAPK in the nucleus closely correlated with the expression of IL-6. Therefore, we hypothesized that the elevated levels of
Ca^{2+} might be serving as an upstream signal to activate the p38 MAPK, which then upregulates the expression of IL-6 in skeletal muscle. To test the hypothesis, we moved to a tissue culture experimental model, where we could carefully control the experimental environment. Accordingly, L6 myotubes were stimulated with the calcium ionophore, A23187, in the presence of the p38 MAPK inhibitor, samples collected at the end of the experiment were subjected to Western blotting and RT-PCR analyses.
6.2 Materials and Methods

Cell culture experiments. L6 myoblasts were maintained at 37°C on 100 mm collagen-coated plastic dishes in 5% CO₂/95% O₂ humidified air in α-MEM + 10% foetal bovine serum (FBS) culture media + 1% penicillin-Streptomycin (stock concentration: 10 000 units.ml⁻¹ penicillin G, 10 000 µg.ml⁻¹ Streptomycin). Differentiation was induced by switching to medium containing 2% FBS when the myoblasts were ~90% confluent. Experimental treatments commenced after 7 days, by which time nearly all of the myoblasts had fused to form myotubes. The evening before experiments, cells were serum-starved in α-MEM +0.1% FBS. Experiments were performed the following morning in α-MEM media + 0.1% FBS containing 4% fatty acid free bovine serum albumin, the cells were treated with different doses of the calcium ionophore, A23187 for 6 hours as recommended (Keller et al., 2002b), the optimal treating dosage was determined by analysing the extracted RNA using RT-PCR with rodent specific probe/primers as described previously (Carey et al., 2003).

In successive experiments, the overnight serum-starved myotubes were treated in 500 nmol.L⁻¹ A23187, with or without the addition of 10 µmol.L⁻¹ of the p38 MAPK inhibitor, SB203580, employing the appropriate vehicles as controls. After 6 hours, cells were harvested. In one experiment (n = 6 plates for each treatment), the nuclear fractions of lysed cells were isolated and subsequently analysed for total and phosphorylated form of p38 MAPK as described in the previous chapter.
(chapter 5). In a second experiment (n = 5-6 plates for each treatment), cells were lysed and extracted for RNA, and analysed using RT-PCR as described above.

*Statistics.* Statistical analysis was carried out by Student t-test followed by Bonferroni correction. Significance was accepted as \( P < 0.02 \). Data are expressed as means ± SE. All statistics were completed using Prism 4 for windows (version 4.01, 2004, GraphPad Software Inc).
6.3 Results

The calcium ionophore, A23187 induces the expression of IL-6 mRNA in L6 myotubes following a dose-dependent manner, with significant induction (P < 0.05) observed starting at a dosage of 500 nmol•L\(^{-1}\) (Figure 6.1).

Treatment of L6 myotubes with the A23187, and/or the pyridinylimidazole p38 MAPK inhibitor SB203580 did not affect nuclear p38 MAPK. However, treating cells with ionomycin increased (P < 0.05) phosphorylation of nuclear p38 MAPK while addition of SB203580 attenuated (P < 0.05) this response (Figure 6.2). As with previous experiments (Keller et al., 2002b), ionomycin treatment increased (P < 0.05) IL-6 mRNA. However, the addition of the SB203580 to the A23187 blunted (P < 0.05) this response (Figure 6.2).
Figure 6.1 The calcium ionophore, A23187 induced fold-increase of interleukin-6 (IL-6) mRNA expression in cultured L6 myotubes. Fold change in with normal. * Difference (P < 0.02) in treatment dosages compared with control. Values are expressed as means ± SE (n = 3).
Figure 6.2  Ratio between phosphorylated and total p38 MAPK (A) and IL-6 mRNA (B) in cultured L6 myotubes treated with the calcium ionophore, A23187 in the presence or absence of the pyridinylimidazole p38 MAPK inhibitor SB203580. * Difference (P < 0.05) compared with - A23187; - SB203580 (vehicle control). † Difference (P < 0.05) compared with + A23187; + SB203580. Values are expressed as means ± SE (n = 6).
6.4 Discussion

The present study demonstrated that pharmacological inhibition of p38 MAPK phosphorylation results in a complete ablation of the IL-6 response in stimulated skeletal myotubes. We performed tissue culture experiment, in order to further examine the strong association of p38 MAPK phosphorylation and the level of IL-6 gene expression seen in the previous chapter (Chapter 5).

Treatment of skeletal myotubes with calcium ionophore has been previously shown to increase IL-6 mRNA expression (Keller et al., 2002b), and since muscle contraction results in a rapid and marked increase in intracellular Ca\textsuperscript{2+}, which is a powerful stimulus for the phosphorylation of p38 MAPK, we hypothesized that A23187 would also induce phosphorylation of p38 MAPK in L6 myotubes.

Treatment of L6 myotubes with A23187 dose-dependently increased the mRNA abundance of IL-6, therefore its rate of transcription (Keller et al., 2001b) with an effective dosage starting at 500 nmol•L\textsuperscript{-1}. While the same treatment had no effect on total nuclear p38 MAPK, A23187 phosphorylated nuclear p38 MAPK and resulted in a marked increase in IL-6 mRNA. Importantly, inhibition of p38 MAPK in the nucleus using SB203580, reduced the phosphorylation of nuclear p38 MAPK, and totally ablated the increase in IL-6 mRNA. Although our in vitro data cannot provide definitive proof that the increase in p38 MAPK phosphorylation in the nucleus and the subsequent increase in IL-6 mRNA seen in vivo were
due to dietary manipulation that resulted in low intramuscular glycogen. Indeed, our tissue culture experiments have no evidence for the role of glycogen in this process. In addition, the factors that affect gene transcription in vitro cannot be totally representative of what occurs in vivo due to the complexity of the in vivo model.

Notwithstanding these limitations, our in vitro data provide evidence that the relationship between phosphorylation of nuclear p38 MAPK and the increase in IL-6 mRNA observed in Chapter 5, was not merely associative but that phosphorylation of p38 MAPK participated in the induction of IL-6 mRNA. Although other MAPK signalling molecules such as JNK have been shown to be capable of increasing IL-6 mRNA (Frost et al., 2003), we are confident that our inhibitor (SB203580) was specific for p38 MAPK, since this compound has been shown to prevent p38 phosphorylation and no other protein kinase at the concentration used in this experiment (Davies et al., 2000). The control of IL-6 promoter activity is very complex and not fully categorized, despite it being the subject of numerous studies in various cell types including monocytes, endothelial cells, fibroblasts, HeLa cells, and lymphocytes (Keller et al., 1996). To our knowledge, no studies have examined the transcription factors that may bind to the IL-6 promoter region in muscle cells to induce expression of this gene, but based on our results, we suggest that candidate transcription factors may be mediated by p38 MAPK. It is important to note, that the β-isoform of p38 MAPK contains an LPS kinase domain (Shi and Gaestel, 2002), and therefore, while our antibody detected both α and β-isoforms, it is more
likely that the β-isoform of p38 MAPK would be the isoform that may lead to the activation of IL-6.

In summary, along with our previous finding that the phosphorylation of nuclear p38 MAPK was associated with low intramuscular glycogen level and IL-6 gene expression, the current study provides good evidence that phosphorylation of p38 MAPK in the nucleus may participate in the regulation of corepressors or coactivators binding to the promotor region of the IL-6 gene in skeletal muscle cells.
CHAPTER SEVEN

A UNIQUE SIGNALLING PATHWAY FOR IL-6 GENE
TRANSCRIPTION IN SKELETAL MUSCLE THAT IS
ACTIVATED BY CALCIUM BUT NOT
LIPOPOLYSACCHARIDE
7.1 Introduction

The previous chapters have clearly showed that skeletal muscle cells are capable of synthesizing IL-6 in the absence of observable inflammation. In lipopolysaccharide (LPS)-stimulated macrophagic cells, it is clear that the expression of IL-6 is regulated via the Toll-like receptor (TLR)/NFκB pathway (de Waal Malefyt et al., 1991; Kreutz et al., 1997; Randow et al., 1995). In these cells, binding of LPS to the Toll-like receptor (TLR)-4 recruits myeloid differentiation primary-response protein 88 (MyD88) to its cytoplasmic domain. By acting as an adaptor molecule, MyD88 triggers a cascade of intracellular signalling consisting of IL-1 receptor-associated kinase (IRAK)-1, TNF-α receptor-associated factor (TRAF)-6, leading to the activation of the IkappaB kinase (IKK)-nuclear factor of κB (NFκB) pathway. NFκB is a transcription factor that usually resides in the cytosol under resting conditions, where its activity is highly restricted by the association with IkappaB (IκB), the inhibitory subunit of NFκB. Activation of IKK phosphorylates this inhibitory subunit at specific serine sites, targeting it for ubiquitination and hence subsequent proteasomal degradation, resulting in the activation of NFκB. The activated NFκB is then able to translocate into the nucleus, and exert its transcriptional effects on a variety of immunogenic genes including IL-1β, IL-6 and TNF-α resulting in the induction of the classic inflammatory response (Figure 7.1) (Akira, 2003; Akira and Takeda, 2004; Beutler, 2000; Guha et al., 2001; Kreutz et al., 1997; Li et al., 2000a; Liang et al., 2004a; Nam, 2006).
In contrast, the role of NFκB in the regulation of IL-6 gene expression in skeletal muscle cells remains largely controversial. Several studies have indicated that skeletal muscle cells may synthesize IL-6 via an NFκB-dependent mechanism (Baeza-Raja and Munoz-Canoves, 2004; Kosmidou et al., 2002; Liang et al., 2004b; Rhind et al., 2002). However, constitutive activation of NFκB did not appear to alter the transcription of IL-6 in skeletal muscle (Cai et al., 2004), nor the nuclear localization of NFκB seemed to be affected after one hour of concentric exercise, as reported in previous chapter. Although, we did not obtain data regarding the activity of nuclear NFκB in response to contraction due to our study limitations, nonetheless, these findings indicated that a divergent signalling pathway might exist for IL-6 gene transcription in skeletal muscle. The primary objective of the present study was to unravel the molecular mechanism for the regulation of IL-6 within skeletal myotubes, particularly the functional role of NFκB in this process. To achieve this, two well-characterized murine cell-lines: C2C12 myotubes and RAW 264.7 macrophages were chosen for the study, in which the LPS-stimulated macrophages were serving as a positive reference for NFκB activation.

Despite the inherent limitations, in the present study we used Ca^{2+} release as a proxy for contraction. At the time of submitting this thesis, work in our laboratory has established a technique for contracting muscle cells in culture and we have been able to show similarities between contracting cells in culture and using calcium ionophores on many signalling parameters (data not shown). In the current study, we proposed that
skeletal muscle cells are able to synthesize IL-6 in response to increased cytosolic Ca\(^{2+}\) independent of NFκB activation. This hypothesis was based on the data presented in Chapter 6, where we observed that the Ca\(^{2+}\) ionophore A23187 increased the IL-6 mRNA abundance, but that this was blocked by inhibition of p38 MAPK. As discussed, p38 MAPK is not a transcription factor but is involved in the signal transduction of calcineurin/NFAT (Fiedler and Wollert, 2004), which is known to modulate cytokine gene expression (Dolmetsch et al., 1998; Fisher et al., 2006; Im and Rao, 2004; Olson and Williams, 2000a). Of note, in a recent study, inhibition of the NFAT upstream regulator, calcineurin impaired the Ca\(^{2+}\)-induced IL-6 response in cultured skeletal myotubes (Keller et al., 2006). Therefore, the second aim of the study was to examine whether NFAT may be responsible for the transcription of IL-6 in skeletal myotubes. Based on the literature and data collected in previous chapters from this thesis, we proposed a signal transduction hypothesis summarised in Figure 7.1 where LPS stimulated macrophages activate inflammatory cytokines via NFκB in the classical manner, but that Ca\(^{2+}\), signalling through p38 MAPK and calcineurin acts through NFAT to stimulate IL-6 gene transcription.
Figure 7.1 The proposed cytokine signalling pathways for macrophage (left) and skeletal muscle (right). CD14 is a membrane-associated glycosylphosphatidylinositol-linked protein expressed at the surface of macrophages, which acts as a co-receptor in concert with TLR 4 for the recognition of bacterial lipopolysaccharide.
7.2 Materials and Methods

Cell culture. C2C12 cell line was kindly provided by Dr. Gregory Steinberg (St. Vincent Medical Research Institute, Melbourne), RAW 264.7 cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The C2C12 myoblasts were grown in α-MEM + 10% foetal bovine serum (FBS) + 1% penicillin-Streptomycin (stock concentration: 10 000 units•ml⁻¹ penicillin G, 10 000 µg•ml⁻¹ Streptomycin). The RAW 264.7 cells were maintained in DMEM low modified (SAFC Biosciences Pty. Ltd) supplemented with 10% FBS + 1% penicillin-Streptomycin. The cell lines were maintained at 37°C in a humidified incubator supplemented with 5% CO₂.

Differentiation of the myoblasts was induced by switching to medium containing 2% FBS when the myoblasts were ~90% confluent. Experimental treatments commenced after 7 days, by which time nearly all of the myoblasts had fused to form myotubes. The evening before experiments, cells were serum-starved in α-MEM + 0.1% FBS. Experiments were performed the following morning in α-MEM media + 0.1% FBS, the cells were treated with either 1 µM of the calcium ionophore, A23187 (Sigma) or 100 ng•ml⁻¹ of lipopolysaccharide (LPS) (E. coli O26:B6; Sigma), or combination of the two as previously optimized. RNA was isolated using RNeasy® Mini Kit (Qiagen, Australia) according to the Manufacturer’s protocol, quantified and reverse-transcribed, before subjected to real time-PCR analysis for gene expression as described in
previous chapters (Chapter 4–6). All cytokines were analysed using Assays-on-Demand™ primer and probe mix (Applied Biosystems, USA).

**Western Blotting.** The cells were washed twice in ice-cold PBS before lysing in ice-cold buffer (50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na$_3$VO$_4$, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (v:v), 100 µg•ml$^{-1}$ phenylmethylsulfonyl fluoride, 5 µl•ml$^{-1}$ protease- and phosphatase inhibitor cocktail) and centrifuged at 4°C at maximum speed for 30 minutes, the protein containing supernatants were transferred to fresh microfuge tubes and kept frozen at −80°C until analysis. Analyses of protein were then carried out using SDS-PAGE with equal amount of protein loading in each well as described in previous chapters, and antibody-binding was detected using ChemiDoc™ XRS System (Bio-Rad Laboratory Inc., Hercules, CA).

**NFκB inhibition.** The overnight serum-starved cells were pre-incubated in medium containing various concentrations of parthenolide (Sigma) for one hour. The cells were washed before stimulated with 100 ng•ml$^{-1}$ LPS. The stimulated cells were then harvested three hours later, and isolated proteins were subjected to Western blotting analysis. In successive experiments, the C2C12 myotubes were pre-incubated in 40 µM for one hour before stimulation with either A23187 or LPS for 3 hours and analysed in the same manner as described above.
**NFkB silencing.** Two siRNA sequences of murine NFκB were tested for their capacity to reduce the expression of NFκB protein in C2C12 myotubes. Gene silencing was achieved with equal efficacy with the following pairs of 21-bp oligonucleotide sequences (accession number NM_008689, siRNA ID 155566 (seq 1) and siRNA ID 155568 (seq 2); Ambion, Austin, TX, USA). The oligonucleotide pairs were annealed and reconstituted in the supplied RNase-free water. A negative control siRNA was also employed to account for off-target effects. Cells were seeded onto 6-well plates one day before transfection. On the day of transfection, the cell culture medium was replaced to a serum- and antibiotic-free α-MEM media and 75 pmol of the respective siRNA was added with Lipofectamine 2000™ (Invitrogen, Mount Waverley, VIC, Australia) to give a final concentration of 45 nmol•L⁻¹. The transfection medium was removed after 16–20 hours, and cells were washed with phosphate-buffered saline (PBS) and differentiated as described.

**NFAT inhibition.** The serum-starved C2C12 myotubes were pre-incubated in medium containing 1 μM of 11R-VIVIT (Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) for one hour as reported (Noguchi et al., 2004b). After washing with 1 X PBS, the cells were stimulated with 1μM of ionomycin (Sigma) or 100 ng•ml⁻¹ LPS for three hours before RNA were extracted, and analysed using RT-PCR described above.

**Statistics.** Statistical analysis was carried out by student t-test coupled with Bonferroni correction and One-way ANOVA. If ANOVA revealed a
significant interaction, Student Newman-Keuls test was then used to
locate specific differences. Significance was accepted as P < 0.05. Data
are expressed as means ± SE. All statistics were completed using Prism 4
for windows (version 4.01, 2004, GraphPad Software Inc).
7.3 Results

Cytokine response in macrophage and muscle cells. Immunoblotting against the macrophage-specific antigen revealed that the presence of F4/80 positive cells in our C2C12 cell line is minimal (Figure 7.2). In the macrophage, the mRNA expression of IL-6, IL-1β and TNF-α increased dramatically (p < 0.05) in response to LPS treatment, but not to the calcium ionophore, A23187, while treatment with A23187 + LPS lowered the fold induction of IL-6 and IL-1β (p < 0.05), but not the expression of TNF-α (Figure 7.3). In skeletal myotubes, A23187 induced a significant increase in IL-6 mRNA (p < 0.05), but not TNF-α, while IL-1β was only detected at basal levels. LPS treatment lead to a moderate increase of IL-6 mRNA expression (p < 0.05), that is further augmented in the presence of A23187 (p < 0.05) (Figure 7.4).

Kinetics of the cytokine responses. In the macrophage, incubation with LPS induced a marked expression of IL-6 and IL-1β (p < 0.01) observed at three hours, followed by a significant decline thereafter, while the TNF-α response is much more rapid, with significant increase detected as early as one hour (p < 0.01), reaching its peak at three hours before the decline at five hours (Figure 7.5). A23187 induced a rapid and marked expression of the IL-6 mRNA in the skeletal myotubes that was sustained over five hours (p < 0.05), while LPS treatment elicited an observable response of TNF-α and IL-1β at five hours (p < 0.05) (Figure 7.6).
**NFκB inhibition and the cytokine responses.** LPS stimulation lead to a marked phosphorylation of IkBa in the macrophage but not the skeletal myotubes (Figure 7.7). Time course experiment revealed that the phosphorylation of IkBa and IKK were potentiated in macrophage after 1-hour incubation with LPS but not in skeletal myotubes, while A23187 treatment did not appear to affect the phosphorylation of neither IkBa nor IKK when compared to control in all of the tested time points (Figure 7.8). Pharmacological inhibition of NFκB dose-dependently reduced the LPS-induced phosphorylation of IkBa in both macrophage and skeletal myotubes, but no significant difference was observed in the A23187 stimulated skeletal myotubes (Figure 7.9). Parthenolide administration resulted in total and partial inhibition (p < 0.01) of the cytokine gene expression in a RAW 264.7 cell and C2C12 myotubes, respectively (Figure 7.10).

**NFκB-slicencing and the cytokine responses.** Transfection of myotubes with silencing sequence against NFκB resulted in marked reduction (greater than 50%) in protein expression of each subunits, however, no significant difference in IL-6 expression was observed between the wild-type and the NFκB-silenced myotubes in response to A23187 stimulation, nor the mRNA expression of TNF-α was affected by the silencing, while IL-1β was only detected at basal levels (Figure 7.11).

**NFAT inhibition and IL-6 response.** Ionomycin is another commonly used calcium ionophore that has been reported to be more effective than
A23187 (Liu and Hermann, 1978). Ionomycin induced a significant increase in IL-6 mRNA expression in the skeletal myotubes at both 1- and 3-hours, the NFAT inhibitor, 11R-VIVIT dramatically potentiated the ionomycin-induced IL-6 expression (Figure 7.12).
Figure 7.2 Immunoblotting of the F4/80 antigen in C2C12 and RAW264.7. F4/80 is a macrophage-specific antigen that is used to check for macrophage contamination in the C2C12 cell line.
Figure 7.3 Cytokine gene expressions of IL-1β and IL-6 (A), and TNF-α (B) in murine macrophage cell. RAW264.7 cells were exposed to the calcium ionophore, A23187, LPS or the combination of both for 3 hours, total RNA was isolated for real-time PCR analysis. * Difference (P < 0.02) compared with vehicle and control; † Difference (P < 0.05) compared with LPS treatment. Values are expressed as means ± SE (n = 3).
Figure 7.4  Cytokine gene expressions of IL-1β and IL-6 (A), and TNF-α (B) in murine skeletal muscle cell. C2C12 myotubes were exposed to the calcium ionophore, A23187, LPS or the combination of both for 3 hours, total RNA was isolated for real-time PCR analysis. * Difference (P < 0.05) compared with vehicle and control; † Difference (P < 0.05) compared with A23187 and LPS treatments; ‡ Difference (P < 0.05) compared with LPS treatment. Values are expressed as means ± SE (n = 6).
Figure 7.5 Kinetic of cytokine response of IL-6 (A), TNF-α (B) and IL-1β (C) in macrophages. RAW264.7 cells were exposed to LPS, total RNA was isolated at various time points for real-time PCR analysis. * Difference (P < 0.01) compared with control of the respective time points; † Difference (P < 0.05) compared with 1 and 5 hours of LPS treatment; ‡ Difference (P < 0.05) compared with 1 hour LPS treatment. § Difference (P < 0.05) compared with 1-hour LPS treatment. Values are expressed as means ± SE (n = 6).
Figure 7.6  Kinetic of cytokine response of IL-6 (A), TNF-α (B) and IL-1β (C) in skeletal muscle cell. C2C12 myotubes were exposed to either A23187 or LPS, total RNA was isolated at various time points for real-time PCR analysis. * Difference (P < 0.05) compared with control and vehicle of the respective time points; # Difference (P < 0.05) compared with LPS; † Difference (P < 0.05) compared with 5 hours of A23187 treatment; ‡ Difference (P < 0.05) compared with the same treatment of other time points. Values are expressed as means ± SE (n = 6).
**Figure 7.7** Immunoblotting of the phosphorylated-\(\mathrm{I}\kappa\mathrm{B}a\) (serine 32) in C2C12 and RAW264.7. Wild type C2C12 myotube and RAW264.7 macrophage were stimulated with LPS for 3 hours before whole cell lysates were collected for analysis. The phosphorylation of \(\mathrm{I}\kappa\mathrm{B}a\) is a proxy measure of \(\mathrm{NF}_{\kappa}\mathrm{B}\) activation, the fold difference is expressed relative to the C2C12 (\(n = 3\)). * Difference (\(P < 0.05\)) compared with C2C12.
Figure 7.8 Immunoblotting of the phosphorylated-IκBα (serine 32) and -IKK α/β (serine 180/181) in RAW264.7 and C2C12 myotubes. The cells were incubated with either A23187 (A) or LPS, whole cell lysates were collected at various time points (n = 4). IKK is the upstream kinase of IκBα, their phosphorylations are indicative of NFκB activation.
**Figure 7.9** Immunoblotting of the phosphorylated-IκBa (serine 32) in RAW264.7 and C2C12 myotubes. The cells were pre-incubated in various concentrations of the NFκB inhibitor, parthenolide, for 1 hour prior to LPS stimulation, whole cell lysates were collected after 3 hours (A) (n = 3). C2C12 myotubes were pre-incubated in 50 µM of parthenolide for 1 hour before stimulation with either A23187 for 3 hours (B) (n = 3). The phosphorylation of IκBa is serving as a proxy measure of NFκB activation.
Figure 7.10 Pro-inflammatory cytokine response in RAW 264.7 (A) and C2C12 (B) cells. The cells were pre-incubated in the presence of the NFκB inhibitor, parthenolide for 1 hour before the addition of LPS or A23187, total RNA was isolated after 3 hours and analysed using real-time PCR. The level of gene expression is shown as mRNA fold change relative to control (n = 3). * Difference (P < 0.02) compared with control; † Difference (P < 0.02) compared pather nolode control; # Difference (P < 0.01) compared with the same treatment in the absence of parthenolide.
**Figure 7.11** The effect of NFκB-silencing on the expression of NFκB (A) and cytokine gene expressions (B) in skeletal muscle cells. The transfected C2C12 myotubes were exposed to either A23187 or LPS for 3 hours, total RNA was isolated and analysed using real-time PCR. The level of gene expression is shown as mRNA fold change relative to control (n = 3). * Difference (P < 0.01) compared with scramble sequence; † Difference (P < 0.01) compared with siRNA 1; # Difference (P < 0.02) compared with control and vehicle; § Difference (P < 0.01) compared LPS treatment. § Difference (P < 0.01) compared with A23187.
Figure 7.12 The effect of NFAT inhibition on the expression of IL-6 in skeletal muscle cells. The C2C12 myotubes were pre-incubated with the NFAT inhibitor, 11R-VIVIT, for 1 hour prior to addition of either ionomycin or LPS for 1 (A) and 3 hours (B), total RNA was isolated for real-time PCR analysis. The level of gene expression is shown as mRNA fold change relative to control (n = 3). * Difference (P < 0.02) compared with control; † Difference (P < 0.01) compared with LPS treatment; ‡ Difference (P < 0.001) compared with vehicle IONO treatment.
7.4 Discussion

The present study demonstrated that the expression of IL-6, but not TNF-\(\alpha\) or IL-1\(\beta\) is inducible by elevation of intracellular Ca\(^{2+}\) levels in skeletal muscle cells, and that the expression of IL-6 appeared to be regulated via mechanisms other than those of the classic inflammatory pathway. In accordance with the previous findings (Holmes et al., 2004; Keller et al., 2002b; Keller et al., 2006), treatment of murine myotubes with calcium ionophores (A23187 and ionomycin) markedly increased the IL-6 gene expression, confirming C2C12 myotubes (mouse) like those of human and rat (L6) origin can synthesize IL-6 in response to Ca\(^{2+}\) transients, and that calcium ionophores can be used as an *in vitro* proxy for contraction for IL-6 expression. Importantly, the calcium ionophore induced IL-6 gene expression, occurred in the absence of activation of the NF\(\kappa\)B signalling cascade, identifying a novel NF\(\kappa\)B independent signal transduction cascade in skeletal muscle cells.

From our results, it appears that the cytokine response observed in skeletal muscle cells differs greatly from that observed in the macrophage. First of all, the calcium ionophore, A23187 induced a dramatic IL-6 response in the skeletal myotubes, but not in macrophages. In contrast, the LPS-stimulated macrophage showed a robust increase in IL-6 and IL-1\(\beta\) expression, while the same treatment in skeletal myotubes only resulted in a moderate increase in IL-6, while TNF-\(\alpha\) and IL-1\(\beta\) remained at basal levels. Moreover, when applied together, A23187 appeared to antagonize the effect of LPS on IL-6 and IL-1\(\beta\) expression in
macrophages, but greatly potentiated the IL-6 and TNF-α response to LPS in myotubes. So far, there are no studies that have examined the direct effect of elevated intracellular Ca^{2+} level on the LPS-mediated cytokines expression in muscle, therefore, we are unable to shed further light on this observation. However, Chen and Lin-Shiau et al. (2000) have demonstrated that thapsigargin, a compound that increases intracellular Ca^{2+} concentrations via inhibition of the ER Ca^{2+} -ATPase resulting in depletion of the endoplasmic reticulum Ca^{2+} stores, profoundly potentiated the LPS-mediated TNF-α production in RAW 264.7, but not the calcium ionophores, A23187 or ionomycin providing evidence that factors other than those of altered cytosolic Ca^{2+} content may be contributing to our observation.

Secondly, our time-course study revealed that, although of a much lower magnitude when compared with the LPS-stimulated macrophage, the kinetics of the IL-6 response in the myotubes occurred at a much faster rate with the highest increase detected as early as one hour which remained elevated after three hours of treatment before a decline was observed. Of note, at one and three hours where the most significant IL-6 increase was observed, the mRNA expression of TNF-α and IL-1β were only detected at basal levels, even at time point whereby A23187 induced a significant TNF-α expression, IL-6 was only increased by < 100-fold indicating that the myotubes are producing IL-6 via a TNF-independent pathway. In contrast, the peak increase of IL-6 mRNA in the macrophages was not observed until after three hours of treatment, which was
accompanied by the peak expression of TNF-α and IL-1β, and that the three cytokines exhibited a similar kinetic over the course of the experiment. Given that both TNF-α and IL-1β are potent inducers of IL-6 (Akira et al., 1993), it is possible that the delayed IL-6 peak observed in the macrophages may represent a TNF-α and IL-1β-dependent mechanism.

In monocytes and macrophages, it has been demonstrated that LPS stimulates the production of pro-inflammatory cytokines via activation of the NFκB pathway (Akira, 2003; Akira and Takeda, 2004; Beutler, 2000; Guha et al., 2001; Kreutz et al., 1997; Li et al., 2000a; Liang et al., 2004a; Nam, 2006), and that once produced, both TNF-α and IL-1-β have also been shown to activate the classical NFκB pathway (Nishikori, 2005) and positively regulate their own expressions (Brinkman et al., 1999; Ilyin et al., 2000). In the present study, LPS stimulation of the RAW 264.7 cells resulted in a marked phosphorylation of IκBα and IKK (Figure 7.8 & 7.9). However, when C2C12 myotubes were stimulated with LPS, the phosphorylation of IκBα and IKK was much less marked (Figure 7.8 & 7.9). These data suggested that in macrophages the LPS-induced expression of IL-6 involves NFκB activation, while the activation of NFκB in skeletal myotubes seemed much less sensitive to LPS stimulation. Of equal importance was the observation that stimulation of C2C12 cells with A23187 had absolutely no effect on activation of the NFκB signalling cascade and yet such stimulation increased IL-6 mRNA expression nearly
1000 fold (Figure 7.4). Together, these observations suggest that the Ca$^{2+}$ mediated IL-6 gene transcription is NFkB independent.

As a collary to simply examining the effect of Ca$^{2+}$ on NFkB and IL-6 mRNA expression, we also used strategies to inhibit NFkB signalling in skeletal muscle. Accordingly, a pharmacological inhibitor of NFkB, parthenolide was used. Parthenolide mediates the inhibition of NFkB by preventing the activation of IKK without affecting the activation of JNK and p38 MAPK (Hehner et al., 1999; Nam, 2006). Pre-treatment of RAW 264.7 cells with parthenolide blunted the LPS-induced NFkB activation in a dose-dependent manner, with a noticeable reduction observed starting at 25 µM (Figure 7.9). Accordingly, LPS induced activation of cytokine mRNA expression was completely blunted by parthenolide treatment (Figure 7.10). A23187 had no effect on NFkB activation (Figure 7.8 and 7.9), while parthenolide had no effect on this pathway either (Figure 7.10). It was very surprising, therefore, that treatment of myotubes with parthenolide blunted the A23187-induced IL-6 mRNA response. From these data we can only conclude that parthenolide is not a specific inhibitor of NFkB activation and that it must be working on other signal transduction pathways.

Given these problems with the parthenolide experiments, it was necessary to try and specifically inhibit NFkB. Accordingly, we performed RNA silencing experiments. Transient transfection of C2C12 myotubes with small interfering RNA (siRNA) reduced the basal expression of the NFkB
protein by over 50% (Figure 7.11). Importantly, when stimulated, the A23187-induced IL-6 mRNA response in C2C12 myotubes was not different when comparing siRNA cells targeted to NFκB with cells transfected with the scramble sequence (negative control). While we acknowledge that our data cannot rule out the possibility that the residual expression of NFκB may be sufficient to maintain the cytokine response in the stimulated myotubes, they provide further evidence supporting the notion that skeletal muscle may utilise a different signalling mechanism for IL-6 gene transcription in muscle, and that the full expression of NFκB is not essential to initiate the transcription of IL-6 in response to Ca2+.

Given that our data suggested that NFκB was not involved in Ca2+-induced IL-6 mRNA expression, we next examined whether NFAT was involved as shown in Figure 7.1. Accordingly, we treated cells with the NFAT inhibitor peptide, 11R-VIVIT. Treatment of cells with LPS in the presence or absence of 11R-VIVIT had no effect on IL-6 mRNA expression. In contrast, and contrary to our hypothesis, 11R-VIVIT enhanced, rather than repressed the Ca2+-induced IL-6 mRNA expression in a time-dependent manner. Given that NFAT is a transcription factor that is known to play a pivotal role in the activation of cytokine gene expression, including IL-6 (Abbott et al., 2000; Rao et al., 1997), and that inhibition of NFAT (Nilsson et al., 2006) or its upstream regulator, calcineurin (Banzet et al., 2007), has been shown to significantly reduce the transcription of the IL-6 gene, this result was most surprising. The precise reason for this observation is currently unknown, but it is possible that our observation
may be attributed to the specificity of our inhibitor. Therefore, alternate approaches for inhibition in a well-characterized human muscle cell-line may be necessary to determine the role of NFAT in the Ca\textsuperscript{2+} induced increase in IL-6 mRNA expression.

In summary, we have shown that the expression of IL-6 is differentially regulated in macrophages and skeletal myotubes. In skeletal myotubes, IL-6 may be activated by acute elevation in intracellular Ca\textsuperscript{2+} levels via an NFκB-independent mechanism, and that the IL-6 response precedes the appearance of TNF-α. Blockage of the NFAT pathway did not attenuate the IL-6 response to Ca\textsuperscript{2+} stimulation. Although the molecular mechanism for IL-6 gene activation in contracting skeletal muscle, particularly the role of NFAT, warrants further investigation, the current study identifies a divergent signalling pathway may exist for IL-6 gene transcription in skeletal muscle.
CHAPTER EIGHT

SUMMARY AND CONCLUSIONS
8.1 Summary and conclusions

The primary aim of this thesis was to investigate the intracellular mechanism for IL-6 gene transcription in skeletal muscle.

The first study (Chapter 3) confirmed that human skeletal myocytes per se are a source of the contraction induced IL-6, and that the mRNA and protein expression of IL-6 was predominantly located in type 2 muscle fibres with high glycogen content. Although, this finding is contrary to previous findings that IL-6 mRNA expression and protein release are exacerbated with glycogen depletion, it is possible that during prolonged exercise as the type 1 fibres are becoming glycogen-depleted, the type 2 fibres may be subsequently recruited. Nevertheless, this was the first study to identify that IL-6 is produced within muscle cells during muscular contraction because we measured the mRNA levels by in situ hybridisation.

Given that the expression of IL-6 is known to be regulated by other cytokines, particularly those that are implicated in the inflammatory processes, the second study (Chapter 4) aimed to determine whether muscle contractions per se increase the expression of other cytokines within skeletal muscle, as well as the implication of carbohydrate availability in such a process. The second study (Chapter 4) demonstrated that cytokines are not ubiquitously expressed in human skeletal muscle, and like IL-6, the expression of IL-8 is also influenced by glycogen
availability within the contracting muscle, which provided further evidence that the transcription of IL-6 is related to metabolic processes rather than inflammation secondary to muscle damage.

Having concluded from the first two studies that contraction induces the expression of IL-6 in skeletal muscle cells that is influenced by the availability of intramuscular glycogen levels, and that previous investigations have also consistently reported the activation of p38 MAPK in skeletal muscle during contraction, the third study (Chapters 5 & 6) sought to investigate the effect of reduced intramuscular glycogen levels on the activation of p38 MAPK, and subsequently the expression of IL-6, in response to “non-damaging” muscular exercise. From our results, it appeared that a reduction in intramuscular glycogen per se does not lead to the nuclear translocation of p38 MAPK, but rather phosphorylates nuclear p38 MAPK. Moreover in the human study, the phosphorylation of p38 MAPK in the nucleus appeared to be closely associated with the contraction-induced mRNA expression of IL-6. To determine whether this relationship was due to a casual or random association, it was necessary to perform tissue culture experiments (Chapter 6). We found that an elevation in intracellular Ca\(^{2+}\) phosphorylated the nuclear p38 MAPK which resulted in a marked increase in IL-6 mRNA expression. More importantly, pharmacological suppression of p38 mediated a total ablation of this response providing evidence that the relationship between phosphorylation of nuclear p38 MAPK and the increase in IL-6 mRNA was
not merely associative but that phosphorylation of p38 MAPK participated in the induction of IL-6 mRNA.

Although, we have demonstrated that skeletal muscle cells express IL-6 in a p38 MAPK dependent manner, several studies have indicated that the cytokine transcription factor, NFκB may also be involved in the activation of IL-6 by acting in concert with p38 MAPK. In the next study (Chapter 7), we aimed to unravel the underlying mechanism for expression of IL-6 within skeletal myotubes, with particular focus on the implication of NFκB in this process.

Our data revealed that the pattern of cytokine response in skeletal muscle cells differs greatly from that in the macrophage. It appears that skeletal muscle cells are able to attain a maximal expression of IL-6 without TNF-α and IL-1β, which are the primary inducers of IL-6 during systemic inflammation, and that the activation of IL-6 in skeletal muscle seems to be regulated by an NFκB-independent mechanism (Figure 8.1). Given that NFκB plays a pivotal role in the activation of cytokine gene expressions in various immune cells, particularly macrophage cells, in response to pathological challenges, our observation further supports the notion that the expression IL-6 in muscle is not pathophysiological. Importantly, inhibition of the Ca^{2+} sensitive transcription factor, NFAT, not only failed to attenuate the effect of Ca^{2+}, but surprisingly it potentiated the expression of IL-6 in the stimulated myotubes. Although, the exact reason for this observation is unknown at the present time, however, the role of NFAT in
this process is warrant for further investigations. The use of pharmacological agents to stimulate or inhibit signalling pathways in vitro is a useful and relatively basic technique to delineate biochemical signalling pathways. However, it is worth bearing in mind the limitations of this approach such as off-target effects that some of these compounds might have when planning for future experiments.

In conclusion, the thesis demonstrated that the expression of IL-6 in skeletal muscle is regulated by a network of signalling cascades that is likely to involve crosstalk between the Ca\textsuperscript{2+} and glycogen/p38 MAPK pathways (Figure 8.1). Ongoing research is necessary to gain further insight into the molecular mechanism in which IL-6 is regulated in the contracting muscles. Understanding the regulation mechanism of this process not only would help explain some of the beneficial effects of physical exercise on health, it may also lead to IL-6 being used as a potential therapeutic aid in the treatment of metabolic diseases like type 2 diabetes.
**Figure 8.1** Summary of cytokine expression in macrophage and skeletal muscle.
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