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THE ROLES OF IL-6 IN THE REGULATION OF GLUCOSE HOMEOSTASIS

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

JENNY ELIZABETH GUSBA

In partial fulfilment of requirements

For the degree of

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ABSTRACT

THE ROLES OF CYTOKINES IN CARBOHYDRATE METABOLISM IN SKELETAL MUSCLE

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University of Guelph, 2008

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Dr. T. E. Graham

This thesis examined the roles of IL-6 in the regulation of glucose homeostasis with a specific focus on skeletal muscle. Study 1 sought to determine whether muscle glycogen content is a stimulus for the production of IL-6, examining the period during and after exercise. The relationship between IL-6 and muscle glycogen content was measured during similar bouts of exhaustive exercise on two occasions. On one occasion, subjects received carbohydrate during recovery to facilitate rates of glycogen resynthesis. During exercise, subjects performed similar bouts of exercise, such that differences in an individual's glycogen levels between trials could be compared to differences in IL-6. No correlation was detected between the net change in glycogen content and plasma IL-6 or IL-6 mRNA from rest to exhaustion. Moreover, there was no correlation between the difference within subjects at exhaustion in IL-6 and glycogen. During recovery, although carbohydrate intake significantly increased glycogen resynthesis, there was no change in post-exercise IL-6 mRNA level or plasma IL-6 concentration. Therefore, glycogen was decidedly not the sole regulator of IL-6 production in skeletal muscle. Study 2 examined the direct effect of IL-6 and TNF- α on glucose transport and the phosphorylation of key signalling proteins in rodent muscle under basal, insulin-stimulated, and contraction-mediated conditions. Under basal conditions, IL-6 increased glucose transport in

association with an increase in AMPK and AS160 phosphorylation, but IL-6 decreased insulin-stimulated glucose transport via a reduction in phosphorylation of CaMKII and AS160. A novel finding was the direct involvement of IL-6 in contraction-mediated glucose transport; increasing glucose transport via phosphorylation of CaMKII and AS160. This research suggests that the activation of CaMKII is involved in the actions of IL-6 under insulin-stimulated and contraction-mediated conditions. Furthermore, it also suggests that AS160 may be a point of convergence for multiple signalling pathways. Finally, TNF-alpha's actions mimicked that of IL-6, with the exception of during contraction where TNF-alpha had no significant effect on glucose transport and attenuated the effects of IL-6.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
LIST OF TABLES.....	iv
LIST OF FIGURES.....	v
LIST OF ABBREVIATIONS.....	vii
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: REVIEW OF LITERATURE	
2.1 Glucose homeostasis.....	7
2.1.1 Skeletal muscle is a key tissue	7
2.2 Cytokines as regulators of muscle metabolism.....	8
2.2.1 IL-6 as a key cytokine.....	9
2.2.2 Skeletal muscle as a source of IL-6.....	11
2.2.3 Skeletal muscle as a target for IL-6.....	17
2.2.4 Regulation of glucose transport in skeletal muscle	18
2.2.5 IL-6 and resting glucose transport.....	26
2.2.6 Molecular mechanisms of IL-6 in resting skeletal muscle	28
2.2.7 IL-6 and glucose transport during exercise.....	34
2.3 Are there other cytokines acting with or against IL-6?.....	35
2.3.1 TNF-alpha as another important modulator of glucose homeostasis.....	35
2.3.2 Skeletal muscle is not an important source of TNF-alpha.....	36
2.3.3 The effect of TNF-alpha on glucose transport.....	36
2.3.4 Molecular mechanism of TNF-alpha in resting skeletal muscle.....	40
2.3.5 TNF-alpha is not involved in glucose transport during muscle contraction.....	43
2.3.6 Is there a relationship between TNF-alpha and IL-6?.....	44
CHAPTER 3: PURPOSE OF RESEARCH	46
CHAPTER 4: STUDY 1- Interleukin-6 and its mRNA responses in exercise and recovery: relationship to muscle glycogen.....	49
Abstract.....	50
Introduction.....	51
Methods.....	52
Results.....	58
Discussion.....	64
CHAPTER 5: STUDY 2- Effect of Interleukin-6 and Tumour Necrosis Factor Alpha on basal, insulin-stimulated, and contraction-induced glucose transport in skeletal muscle..	70
Abstract.....	71
Introduction.....	72
Methods.....	74
Results.....	78
Discussion.....	84
CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	92
REFERENCES.....	121
APPENDICES.....	148

LIST OF TABLES

Chapter 2: Review of Literature

Table 1: Studies examining the effect of IL-6 on glucose homeostasis and glucose transport in resting skeletal muscle	27
Table 2: Studies examining the molecular mechanisms of IL-6 in glucose metabolism in resting skeletal muscle.....	29
Table 3: Effect of TNF-alpha on glucose homeostasis and glucose transport in skeletal muscle.....	37
Table 4: Studies examining the molecular mechanisms of IL-6 in glucose metabolism in resting skeletal muscle.....	40

Chapter 4: Study 1- Interleukin-6 and its mRNA responses in exercise and recovery: relationship to muscle glycogen

Table 5: Blood glucose, insulin, and free fatty acids.....	58
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Chapter 5: Study 2 - Effect of Interleukin-6 and Tumour Necrosis Factor Alpha on basal, insulin-stimulated, and contraction-induced glucose transport in skeletal muscle

Table 6: Glucose transport in soleus muscle strips.....	78
Table 7: The effect of IL-6, TNF-alpha, and IL-6 + TNF-alpha on PI3-kinase and Akt (ser ⁴⁷³ and thr ³⁰⁸) in rat soleus muscle strips.....	82

LIST OF FIGURES

Chapter 2: Review of Literature

Figure 1: Figure 1. Molecular mechanisms associated with insulin signalling.....	20
Figure 2. Potential contraction-mediated signalling pathways in skeletal muscle leading to GLUT4 translocation.....	22
Figure 3. Suggested points of interaction between IL-6 and intermediates involved in glucose transport in skeletal muscle.....	33

Chapter 4: Study 1: Interleukin-6 and its mRNA responses in exercise and recovery: relationship to muscle glycogen

Figure 4: Skeletal muscle glycogen following ~2 h of exhaustive exercise and 5h of recovery.....	59
Figure 5: Plasma IL-6 following ~2 h of exhaustive exercise and 5 h of recovery.....	61
Figure 6a: Correlational analysis between glycogen depletion (change from rest to exhaustion) and rise in interleukin-6 in both trials.....	62
Figure 6b: Correlational analysis between the difference in IL-6 in the 2 tests and that for glycogen at exhaustion within subjects.....	63
Figure 7: IL-6 mRNA following ~2h of exhaustive exercise and 5h of recovery	64
Figure 8: Correlational analysis between the change in glycogen and interleukin-6 mRNA from rest to exhaustion	67

Chapter 5: Study 2 - Effect of Interleukin-6 and Tumour Necrosis Factor-Alpha on basal, insulin-stimulated, and contraction-induced glucose transport in skeletal muscle

Figure 9: Phosphorylation of AMPK (Thr172) by IL-6, TNF- α , and IL-6 + TNF- α in the absence insulin.....	79
Figure 10: Phosphorylation of AS160 by IL-6, TNF-alpha, and IL-6 TNF- α in the absence of insulin.....	79
Figure 11: Phosphorylation of CaMKII by IL-6, TNF-alpha, and IL-6 TNF- α in the presence of insulin.....	80
Figure 12: Phosphorylation of AS160 by IL-6, TNF-alpha, and IL-6 TNF- α in the presence of insulin	81
Figure 13: Phosphorylation of CaMKII A) at rest versus during contraction; B) during contraction in the presence of IL-6, TNF- α , and IL-6 + TNF- α	83
Figure 14: Phosphorylation of AS160 A) at rest versus during contraction; B) during contraction in the presence of IL-6, TNF- α , and IL-6 + TNF- α	84
Figure 15: Phosphorylation of AMPK at rest versus contraction.....	84

Chapter 6: Discussion and Future Directions

Figure 16: Mechanism by which IL-6 increases basal glucose transport in study 2.....	98
Figure 17: Mechanism by which IL-6 decreases glucose transport in the presence of insulin in study 2.....	99
Figure 18: Interactions between IL-6 signalling pathway and mediators of glucose transport.....	101
Figure 19. Regulation of AMPK phosphorylation by upstream kinases and downstream phosphatases: the potential influence of insulin.....	104
Figure 20. AS160 phosphorylation versus glucose transport.....	105
Figure 21. AMPK phosphorylation versus glucose transport.....	106
Figure 22: IL-6 actions in muscle contraction; role of CaMKII.....	110

LIST OF ABBREVIATIONS

Abbreviation	Definition
ACC	acetyl-CoA carboxylase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	Akt substrate 160
Ca ²⁺	Calcium ion
CaMKII	calcium/calmodulin dependent protein kinase II
cGMP	cyclic guanosine monophosphate
CRP	C-reactive protein
ERK1/2	extracellular signal-regulated kinase ½
GIR	glucose infusion rate
GLUT 4	glucose transporter 4
GSV	GLUT storage vesicles
IL-6	interleukin-6
IR	insulin receptor
IRS-1	insulin receptor substrate-1
JAK	janus kinase
JNK	c-Jun NH2-terminal kinase
MAPK	mitogen activated protein kinase
mRNA	messenger RNA
NF-κB	nuclear factor kappa-B
PDK	phosphoinoside-dependent kinase
PIP3	phosphatidylinositol (3,4,5)P3
PIP2	phosphatidylinositol (4,5)P2
PI3-kinase	phosphinositol-3-kinase
PKB/Akt	protein kinase B/Akt
PKC	protein kinase C
Ra	rate of appearance
Rd	rate of disappearance
TNF-α	tumor necrosis factor-alpha
SR	sarcoplasmic reticulum
STAT	signal transducer and activator of transcription

CHAPTER 1: INTRODUCTION

Understanding the regulation of glucose homeostasis is a great challenge in metabolic physiology and is also important clinically, particularly in diabetes. Skeletal muscle is responsible for nearly 80% of insulin-stimulated glucose disposal and thus, is a key component in the maintenance of homeostasis. An additional layer of complexity regarding skeletal muscle is that its regulation is markedly different depending on its metabolic status (e.g., rest versus exercise). In type 2 diabetes, there is a reduced response to insulin in skeletal muscle, but exercise-induced glucose transport tends to remain unaltered. Therefore, from a therapeutic perspective, there is large research focus on identifying the molecular mechanisms responsible for insulin- and contraction-mediated glucose transport.

As research progresses in this area, it has become clear that although insulin and aspects of muscle contraction are critical to regulating skeletal muscle glucose transport and metabolism, there are other factors involved, including the cytokines interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-alpha). The nature of their involvement may depend on the metabolic status of the muscle.

Both IL-6 and TNF-alpha have been regarded in some cases as “negative” effectors of insulin sensitivity and glucose metabolism. From a biological perspective, although these factors may be considered “negative” in their actions, it is possible that they are an important part of a compensatory response and may not actually be negative or harmful. For example, in diabetes, if the muscle did not become insulin resistant and excessive amounts of glucose were allowed to enter the muscle cell, the metabolic consequences could be far more severe than currently observed with increasing concentrations of glucose in the blood. Therefore, although IL-6 and TNF-alpha will be

referred to as “positive” or “negative” effectors, it should be clear that this terminology reflects an enhancement or reduction in normal metabolism and does not suggest that they are detrimental.

IL-6 is a pleiotrophic cytokine that in the last two decades has been found to be involved in various aspects of fat and carbohydrate metabolism throughout the body. From the perspective of glucose, the nature of its regulatory influence has been widely debated, as detailed in recent point: counterpoint papers (139; 156; 157). It has been proposed to be a negative effector because elevated circulating concentrations are observed with disease states such as diabetes and obesity (137; 169). Furthermore, IL-6 infusion has been shown to induce insulin resistance in rodents (114).

On the other hand, those who suggest that IL-6 is a positive effector provide evidence using *in vivo* data from humans that suggests that with IL-6 infusion, insulin sensitivity is not disrupted (33; 211). Furthermore, insulin resistance is observed when IL-6 is pharmacologically blocked (43; 145) and in IL-6 knockout mice (224). These studies indicate that IL-6 is required for insulin sensitizing purposes. In addition, the fact that IL-6 is released in copious amounts from skeletal muscle during exercise (40; 110; 202; 204), suggests that if IL-6 was a negative effector, it would be counter-intuitive for IL-6 to be released during exercise. Biological changes are logically induced to protect tissues, enhance their function, etc; therefore, possible reasons for these conflicting discussions on the nature of IL-6's actions in the body may be that: 1) IL-6 could have different effects on different tissues, 2) its effects may differ under chronic versus acute situations, 3) there may be differences between species in their response to IL-6, 4) varying concentrations of IL-6 may have different effects on metabolism (i.e. dose-

response), and 5) IL-6 could be acting in concert with other factors, such as TNF-alpha, which could alter its impact in a whole body model.

At rest, the effect of IL-6 on glucose homeostasis has provided conflicting findings, particularly at the level of skeletal muscle. Recent findings suggest that IL-6 increases glucose transport in the absence of insulin (9; 33; 73; 75). However, examinations of insulin-stimulated glucose transport have generated mixed findings. Two studies found no change in insulin-stimulated glucose transport (73; 75), whereas other studies observed approximately 4-fold increase in insulin-stimulated transport (9; 33). Clearly further study is required to examine the exact nature of IL-6's involvement in the regulation of skeletal muscle glucose metabolism, particularly in the presence of insulin.

During exercise, the maintenance of glucose homeostasis involves precise communication between the liver and skeletal muscle. It has been suggested that IL-6 produced and released by skeletal muscle may serve as a communication molecule providing feedback and feedforward signals to coordinate carbohydrate metabolism. The stimuli for production of IL-6 by active skeletal muscle remain elusive, but one suggested candidate is declining concentrations of glycogen in the muscle (202). There are many studies that have evaluated the relationship between IL-6 and glycogen and have shown that when glycogen levels are low prior to exercise, IL-6 release is exaggerated (37; 109; 202). These studies are correlative and thus, do not prove causation. However, more recently, Hiscock et al (2004) suggested that a declining concentration of glycogen in the active muscle was not directly involved in IL-6 production. They found that IL-6 mRNA was concentrated in type II fibres, with high residual glycogen concentrations following 120 minutes (min) of exercise at 55% VO_2 max (92). In addition, studies have not

considered the relationship between IL-6, post exercise nutrition, and glycogen restoration. Therefore, further study is required to examine the nature of the relationship between IL-6 and glycogen content both during and after exercise as well as the role of calcium in the regulation of IL-6 mRNA expression.

Regardless of the stimuli causing IL-6 release during exercise, it is possible that once it is released by the skeletal muscle it is capable of acting in a paracrine fashion on the muscle to initiate metabolic actions (e.g., glucose transport). One study has examined this concept and found that low intensity exercise combined with IL-6 infusion in young men resulted in a greater rate of disappearance (Rd) than low intensity exercise alone (59). However, the direct effect of increased local concentrations of IL-6 on glucose transport during muscle contraction has not been examined.

Metabolic signals do not act in isolation; consequently, IL-6 may interact with other cytokines including TNF- α . It is generally accepted that TNF- α is a negative effector of metabolism, implicated in a variety of disease states including type 2 diabetes. Infusion of TNF- α in humans (171) and rodents (125; 128; 134; 191) resulted in insulin resistance at the whole body level, regardless of infusion duration. Similarly, hindlimb infusion of TNF- α in rodents caused a reduction in insulin-stimulated glucose transport (241; 244). Although whole body and hindlimb models consistently show a negative effect of TNF- α on glucose transport, experiments using cell culture and isolated muscle strips have generated mixed findings.

The mechanism by which TNF- α could be exerting its effects is unclear and it is only beginning to receive significant attention. From the existing literature it appears that TNF- α may be altering insulin signalling by modulating signalling intermediates such as insulin receptor substrate-1 (IRS-1) phosphorylation, its subsequent interaction

with phosphoinositol-3-kinase (PI3-kinase) (50; 238) or via increases in mitogen activated protein kinases (MAPKs) (48; 77; 239).

In contrast to IL-6, TNF-alpha release from skeletal muscle (62; 204) or elevations in the systemic circulation (149; 150; 200) have not been consistently observed during exercise. However, TNF-alpha is present in elevated concentrations in some disease conditions (e.g. obesity, diabetes) and, therefore, it is conceivable that these chronically elevated levels of TNF-alpha may influence normal glucose metabolism in skeletal muscle during exercise. Only one study has examined the effect of TNF-alpha on muscle during contraction. Zhang et al. (2003) infused TNF-alpha into an electrically stimulated rodent hindlimb for 1 hour, and although they found that TNF-alpha caused insulin resistance in the resting hindlimb, no significant effect on glucose transport during muscle contraction was detected (244).

Both TNF-alpha and IL-6 have been proposed to disrupt normal glucose metabolism and homeostasis. Therefore, it is possible that these two cytokines interact; however, it is not surprising that very little is known about any interaction as there is much uncertainty about their individual actions.

A number of studies have shown that TNF-alpha stimulates IL-6 production in adipose tissue (167), smooth muscle (26), and skeletal muscle (11; 40). Furthermore, plasma IL-6 has been shown to be decreased with anti-TNF-alpha therapy (187). Because TNF-alpha may stimulate IL-6 production, it has been argued that any increase in IL-6 production may only reflect increases in TNF-alpha and may not be as biologically relevant as elevations in TNF-alpha (167). On the other hand, IL-6 has been shown to suppress TNF-alpha production (167), which may indicate a feedback/feedforward interaction between the two cytokines. It is also possible that other cytokines or factors

interact with IL-6 and TNF-alpha to influence their production and/or actions. Clearly there are many gaps in the existing body of research and further study will help to answer these and other questions related to IL-6 and TNF-alpha.

Therefore, this thesis intended to: 1) to determine the relationship between IL-6 and muscle glycogen, particularly during the post exercise period, 2) to determine the direct effect of IL-6 and TNF-alpha and the combination of IL-6 and TNF-alpha on basal and insulin-stimulated glucose transport and the phosphorylation of key signalling intermediates, and 3) to determine the direct effect of IL-6, TNF-alpha and their combination on glucose transport and proposed molecular mechanisms in contracting muscle.

CHAPTER 2. LITERATURE REVIEW

2.1 Glucose Homeostasis

Maintenance of glucose homeostasis is a highly regulated, complex process. Essential to this process is the communication between many tissues within the body particularly the gut, liver, adipose tissue and skeletal muscle. Hormones and other factors are produced from these tissues which provide feedforward and feedback signals that influence not only each other, but tissues such as the pancreas and brain (reviewed by Herman and Khan (2006) (90). For instance, recently it has been identified that the gut releases incretins in response to carbohydrate ingestion (177; 179). Incretins have been shown to act on the pancreas, for example, to provide a feedforward signal to promote insulin release and may account for nearly 50 percent of insulin production (223). Although hormones such as epinephrine, norepinephrine (193), growth hormone (102), and incretins (164) are involved in the regulation of glucose homeostasis, the critical regulator is unquestionably insulin. It is increasingly clear, however, that other factors such as adenosine and cytokines (e.g., interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-alpha)) may play an important role. Cytokines, in particular, may be important in pathological situations, such as insulin resistance where their actions may oppose those of insulin.

2.1.1 Skeletal muscle is a key tissue

As discussed above, there are a number of tissues involved in the maintenance of glucose homeostasis; however, skeletal muscle may be the most important in this process. Skeletal muscle is considered a major site of regulation of glucose metabolism and plays a critical role when glucose homeostasis is challenged acutely (e.g. exercise, carbohydrate ingestion) or chronically (e.g. diabetes). The mechanisms involved in the maintenance of

glucose homeostasis by skeletal muscle vary depending whether the muscle is at rest or contracting. As research progresses in this area, it is clear that although insulin and muscle glycogen concentrations are critical to regulating skeletal muscle glucose transport and metabolism, there are other factors involved, including the cytokines IL-6 and TNF-alpha. The nature of their involvement may depend on whether insulin is present or the muscle is contracting.

2.2 Cytokines as regulators of muscle metabolism

Cytokines were once defined as “regulatory proteins secreted by white blood cells and a variety of other types of cells in the body; the pleiotropic actions of cytokines include a numerous effects on cells of the immune system and modulation of inflammatory responses” (1). However, this definition no longer encompasses all of the known actions of cytokines. It is recognized that they play a larger role in regulating cellular responses. In this manner, cytokines appear to act much like hormones; however, there are some important differences. While cytokines can also fulfill roles such as maintaining metabolic homeostasis and responding to changes in nutritional status, they differ from classical hormones as each cytokine is produced by numerous cell types. Cytokines have been classified into groups by the cell type from which they were produced (i.e., adipose tissue derived cytokines can be referred to as adipokines) (1).

Cytokines have been shown to exert their metabolic regulatory effects on many tissues within the body including the liver, adipose, and muscle tissues. In this respect, their ‘normal’ physiological roles are thought to be communication molecules between these tissues (60; 155). However, little is known in this regard, in part because they have been largely studied in pathological states such as diabetes, cardiovascular disease, obesity, and sepsis rather than in healthy states.

As a result, the effects of cytokines such as IL-6 and TNF-alpha are often interpreted as “negative”. As described in the introduction, the use of the word “negative” in this thesis does not necessarily mean that the actions of the cytokine are harmful. From a biological perspective, it is possible that they could be acting in a manner that alters normal metabolism so as to defend the tissues from more serious metabolic dysregulation and consequences.

Recent research in this area identifies IL-6 and TNF-alpha as important cytokines, but undoubtedly a number of other cytokines are playing diverse and vital roles in metabolic control. In light of recent work examining the regulation of carbohydrate metabolism attributed to IL-6 and TNF-alpha, this thesis is focusing primarily on IL-6 and secondarily on TNF-alpha. Furthermore, because of the key role of skeletal muscle in glucose homeostasis, it is with regard to this tissue that these cytokines will be examined.

2.2.1. IL-6 as a key cytokine

Interleukin-6 (IL-6) is a glycoprotein that has a molecular mass of 21-28 kDA. It is found in 4 helices (198) and has a half-life of approximately 3h in rats (36). The plasma half-life of IL-6 can be extended if bound to the circulating soluble IL-6 receptor (166). Interleukin-6 acts via a complex signalling pathway consisting of an IL-6 specific receptor and a subunit (gp130) that initiates the signalling pathway (86).

Interleukin-6 is produced by a number of cells including stimulated monocytes/macrophages, fibroblasts, vascular endothelial cells, keratinocytes, osteoblasts, T-cells, B-cells, neutrophils, eosinophils, adipose tissue, mast cells, smooth muscle cells (8) and skeletal muscle cells (206). However, the regulation of IL-6 production is still unclear.

Under resting conditions, adipose tissue (both visceral and subcutaneous) is largely responsible for maintaining systemic concentrations of IL-6 (138). Although circulating levels of IL-6 exhibit a circadian rhythm (222), typical, resting levels are approximately 1 pg/ml in young, healthy adults (162). Elevated levels of IL-6 have been associated with insulin resistance (137; 169), osteoporosis (58; 237), and cardiovascular disease (104; 225). Circulating concentrations in these disease states have been shown to vary, but in elderly individuals with diabetes, Pedersen et al. (2003) observed IL-6 levels of 3.0 pg/ml (162). A much more dramatic increase in plasma IL-6 has been noted in certain types of cancer, with levels often greater than 10 pg/ml (23; 218). In one particular investigation, cancer patients with metastatic renal cell carcinoma and serum IL-6 concentrations greater than 300 pg/ml had a reduced life expectancy and did not respond to the treatment in the study (23). In extended bouts of exhaustive exercise, large, transient increases in IL-6 concentrations can approach 100 pg/ml (149; 152). The usefulness of plasma IL-6 as a diagnostic tool for diseases such as insulin resistance is unlikely given that elevated levels of IL-6 are not consistently noted in individuals with diabetes (32). Although efforts have been made to determine the effect of increases of these cytokines on glucose homeostasis, this area of research is very limited and controversial and will be discussed further in subsequent sections of this literature review.

Major targets of circulating IL-6, under resting conditions, are the liver (129; 155), endothelium (226), skeletal muscle (75; 114), and adipose tissue (129; 155; 161). Interleukin-6 has been shown to act on the liver and the endothelial and smooth muscle cells to produce factors that increase the risk of diseases such as cardiovascular disease and diabetes. In the liver, IL-6 has been shown to induce C-reactive protein production

(71). Sattar et al (2003) reported that elevated levels of C-reactive protein increased the risk of cardiovascular disease by 3.7-fold and type 2 diabetes by 24.5-fold (196).

Adipose tissue, the primary source of IL-6 at rest, is also one of its primary targets. Interleukin-6 may act in an autocrine or paracrine fashion or IL-6 from other sources, may be directed toward adipose (129). The effects of IL-6 on normal adipose tissue metabolism are somewhat controversial, however, it has been suggested that it may interfere with insulin signalling pathways (189).

Skeletal muscle has been consistently shown to be an important source of IL-6 during exercise (92; 101; 204) and may also be another important target of IL-6 (75; 110; 111). While attention has been given to IL-6 as a potential mediator of insulin resistance in skeletal muscle (114), this is highly debated. Subsequent sections of this chapter will address skeletal muscle as a source and target for IL-6 as this is the foundation of this thesis.

2.2.2. Skeletal muscle as a source of IL-6

The production of IL-6 by skeletal muscle, as reflected by increasing mRNA levels, is not significant at rest, but is very large at the end of prolonged, exhaustive exercise (109; 149; 152; 163; 204). Interleukin-6 is not only produced by skeletal muscle during exercise (37; 152; 163; 204), but is also released in large quantities, as shown by arterial-femoral venous differences for an exercising leg (202; 204). This release is not due to muscle damage incurred during exercise, as initially speculated (30; 149; 153). There is some debate regarding which fibre types are primarily responsible for producing IL-6 during exercise. Penkowa et al. (2003), using immunohistochemical techniques, showed that IL-6 was produced equally in type I and II muscle fibres (163), whereas Hiscock et al. (2004) found that type II fibres were the primary producers of IL-6 (92).

More recently, Banzet et al. (2005) observed an increase in IL-6 gene transcription primarily in myofibres recruited early in exercise (type I and IIa) (16). While there is convincing evidence that active muscle cells produce large quantities of IL-6, clearly a consensus has not been established regarding the fibre type responsible for IL-6 production.

Interleukin-6 released from skeletal muscle results in a circulating concentration that may be up to 100-fold greater than the concentration observed at rest (178). The magnitude of the release of IL-6 from skeletal muscle is dependent on exercise mode (144), intensity (88; 153), and duration (152); the longer the exercise bout and/or the higher the intensity, the greater the release of IL-6 into the circulation (153).

There are very few investigations that have continued to measure IL-6 production or release from the muscle during recovery from exercise. Penkowa et al (2003) found that in muscle IL-6 mRNA peaked immediately following exercise and protein expression was at its highest level 3 h post-exercise. However, plasma levels of IL-6 peaked at the end of exercise (163). Similarly, Ostrowski et al. (1998) noted an elevation in plasma levels of IL-6 in the circulation after the cessation of exercise, but they observed a dramatic decline immediately following exercise (149).

When released into the systemic circulation, IL-6 has been suggested to function as a metabolic communication molecule (158; 160). Pedersen et al. (2003) proposed that IL-6 released from contracting muscle acts to communicate between the liver and skeletal muscle to coordinate carbohydrate metabolism, especially as the energy demands of exercise increases (158). These tissues, as discussed above, are particularly important for maintaining glucose homeostasis. For optimal regulation of glucose metabolism, integration is required between the tissues with feedforward and feedback signals to

control glucose production, storage, and utilization. It is possible that IL-6 could be a key signal, acting in both a feedforward and feedback manner.

In this role, when skeletal muscle requires glucose from sources outside the muscle cell, IL-6 is released into the circulation and is thought to stimulate glycogenolysis and gluconeogenesis in the liver (220), to potentially direct glucose from the circulation to the muscle (59; 159). Studies examining the role of dietary carbohydrate in dynamics of IL-6 action have also provided support for a role of IL-6 in management of substrate utilization (61; 199). For example, the availability of dietary carbohydrate would lessen the requirement for increased hepatic glycogenolysis and the production by the muscle may be lessened as its role as a communication molecule is not required (61). Although this scenario is highly speculative, carbohydrate ingestion during exercise has been shown to attenuate increases in circulating IL-6 observed with exhaustive exercise (61; 199). Clearly IL-6 production is responsive to exogenous carbohydrate, but the mechanism responsible for adjusting IL-6 production is unclear.

A number of other stimuli have been suggested to cause the synthesis and release of IL-6 from skeletal muscle during exercise. Possible candidates include changing calcium concentrations or proteins activated by calcium that would be observed during muscle contractions (15; 16), changes in plasma glucose concentration (22; 61; 199), and declining muscle glycogen concentration (37; 109; 202). Furthermore, metabolic proteins within the muscle such as MAPKs are proposed to mediate the transcription of IL-6. These signalling intermediates have been shown to be involved in IL-6 production following exposure to other cytokines, lipopolysaccharides, or epinephrine (67; 68). This area of research is in its infancy; and while these factors may all play important roles in

the production and release of IL-6 from skeletal muscle, the relative contribution of each factor remains unclear.

The calcium released from the sarcoplasmic reticulum during muscle contraction has been suggested to directly or indirectly stimulate IL-6 mRNA production (60; 94). Evidence for a direct role of muscle contraction on IL-6 production was observed when rats were subjected to electrically stimulated contractions in one limb and the exercising limb released IL-6, but the resting limb did not (101). Steensberg et al (2000) observed a similar release in humans during one-legged knee extensor exercise (206). Therefore, a local factor is acting during muscle contraction to stimulate IL-6 production.

Calcium itself may trigger the synthesis of IL-6 in some unknown manner; however, it is speculated that the relationship between this stimulus and IL-6 may be due to the increased activity of calcineurin, a key mediator of calcium signalling in muscle cells (15; 16). Pharmacological inhibition of calcineurin blunts the increase in IL-6 mRNA levels typically noted with exercise in rodents. Alternatively, other proteins that are activated by calcium (calcium calmodulin-dependent protein kinase (CaMK), protein kinase C (PKC)) may be involved in stimulating the production of IL-6. Therefore, there are a number of calcium-induced proteins to consider and further work should focus on these proteins as potential mediators of exercise-induced IL-6 synthesis in skeletal muscle.

During exercise, as glycogen concentrations within the muscle are reduced, there is a greater reliance on blood glucose. Therefore, it has been proposed that IL-6 production and release is stimulated by challenges to blood glucose homeostasis as the release of IL-6 is greater with increased duration of the exercise bout (152). It is likely

that this relationship between IL-6 and blood glucose concentrations is further evidence for an overall role for IL-6 as a coordinator of substrate metabolism throughout the body.

Declining levels of muscle glycogen are associated with many changes in cellular function including gene expression (170) and the regulation of glucose transport (177; 179). Reduced concentrations of glycogen in skeletal muscle have been correlated to IL-6 gene transcription and proposed to have a causal relationship (92; 202). Steensberg et al (2001) suggested that although muscle contraction may initiate the production of IL-6, this effect was intensified by low muscle glycogen (202). Glycogen has been proposed as a regulator of the production of a number of metabolic proteins (i.e., glucose transporter-4 (GLUT 4), phosphoinoside-dependent kinase-4 (PDK 4), etc.) (170). Thus, it is conceivable that it also plays a role in the regulation of cytokine production and signalling. As exercise progresses and glycogen levels fall, IL-6 release from the muscle has been inversely correlated with falling muscle glycogen concentrations (202). Some studies have also examined the effect of glycogen depletion prior to the exercise bout (22; 202). One study found that initiating exercise with a reduced glycogen concentration enhanced IL-6 release from the muscle (22). Similarly, another investigation reduced glycogen in one leg prior to exercise and then exercised both legs (cycling) until exhaustion. In the "low glycogen" leg, IL-6 release occurred earlier than in the leg with normal glycogen. Moreover, at the end of the exercise bout, regardless of pre-exercise glycogen concentration, IL-6 mRNA was highest in the leg with the lowest glycogen content (202). Despite the growing body of evidence that glycogen levels are related to IL-6 production, Hiscock et al (2004) found low glycogen concentrations were not directly associated with IL-6 production as the IL-6 mRNA was concentrated in type II fibres, with high residual glycogen concentrations (92). This study challenges existing

investigations that have demonstrated a correlation between IL-6 and glycogen content, suggesting that further study is warranted.

As stated earlier, recovery from exercise is characterized by a dramatic resynthesis of muscle glycogen (35; 214) and a corresponding decline in circulating IL-6 concentrations (149). Studies to date have investigated glycogen breakdown and its relationship with increasing levels or production of IL-6, but have not considered the period of resynthesis of glycogen following a bout of exercise and IL-6 decline.

Interleukin-6 may also be involved in early post exercise (insulin-independent) events related to glycogen resynthesis, but this concept has not been examined to date.

Furthermore, since the ingestion of carbohydrate during exercise attenuates IL-6 release (61; 199), it is conceivable that provision of carbohydrate in the recovery period may also affect the production and release of IL-6 from skeletal muscle during the initial recovery period. Furthermore, intake of carbohydrate immediately post-exercise and for up to 5 hours post-exercise has been shown to dramatically increase glycogen synthesis rates (3; 35; 55; 243) and, therefore, this could also influence IL-6 production early in recovery.

From the above discussion, it is apparent that IL-6 may serve as a sensor of substrate availability in skeletal muscle. Despite the potential metabolic function of IL-6, these relationships are very speculative. If IL-6 production and low glycogen levels are in fact directly related, then altering the rate of glycogen repletion through provision of carbohydrate during recovery could also affect IL-6 levels in the circulation (i.e. a more dramatic decrease in production of IL-6 upon cessation of exercise due to a more rapidly decreasing stimulus). In addition, it is possible that IL-6 acts in a paracrine fashion such that IL-6 could alter glucose transport.

2.2.3. Skeletal muscle as a target for IL-6

Skeletal muscle as a target for IL-6 has been introduced above. The presence of IL-6 receptors on the surface of the muscle suggests that IL-6 is able to exert an influence on the metabolic actions of the muscle. Furthermore, expression of receptors and protein itself was enhanced by muscle contraction indicating that IL-6 release during exercise may be able to have a local effect on the muscle itself (108). There is also evidence of large interstitial increases in IL-6 with even low intensity arm exercise. Rosendal et al. (2005) found that the local concentrations of IL-6 during low force exercise was ~1246 pg/ml (186), compared to typical circulating levels during high intensity exercise that range from 10-80pg/ml. Therefore, ample IL-6 would be located in the vicinity of the muscle and receptors present on the muscle membrane. As a result it is plausible that IL-6 exerts local effects on the muscle to promote glucose transport, storage and oxidation. The consequences of skeletal muscle being targeted by IL-6 are unclear, although there has been a significant amount of recent research focused on answering this question.

The effects of IL-6 on skeletal muscle glucose metabolism and insulin sensitivity are highly controversial. In particular, the effect of IL-6 on glucose transport and insulin signalling intermediates remains unclear. This highly controversial issue was recently discussed in point-counterpoint papers in which the authors detail their opposing opinions on the nature of the relationship between IL-6 and carbohydrate metabolism (139; 156; 157). Various points included in this series of papers will be addressed in subsequent discussions. To examine further this controversial topic, a general understanding of glucose transport and related molecular mechanisms under basal, insulin-stimulated, and contraction-mediated conditions are necessary.

2.2.4. Regulation of glucose transport in skeletal muscle

Glucose transport into skeletal muscle is a tightly regulated process that is controlled through the delivery of glucose to the muscle, transport of glucose across the plasma membrane, and the flux of glucose through intracellular metabolism (i.e., conversion to glucose-6-phosphate) (168; 185). Which site of regulation is most limiting depends, in part, on the muscle fibre type (81; 168). Furthermore, the rate limiting step may depend on the signals altering the metabolism of the muscle. In the basal state, transport of glucose across the membrane is considered rate limiting (168), but in conditions such as hyperinsulinemia (25; 80) or exercise (80; 117) transport is greatly increased such that delivery of glucose to muscle or glucose phosphorylation within muscle become more significant points of control. This thesis will focus on the transport of glucose across the membrane and the role that factors such as IL-6 may play in mediating or inhibiting mechanisms associated with movement of glucose into the muscle cell.

Glucose transporters in skeletal muscle.

Glucose is able to enter into skeletal muscle via glucose transporters (GLUT). Different isoforms exist and their presence varies depending on the tissue in which they are expressed. The dominant glucose transporter isoforms found in skeletal muscle are GLUT-1 and GLUT-4 (72; 132), although GLUT-4 is in more abundant quantities than GLUT-1. GLUT-1 is localized to the plasma membrane for basal glucose transport (132), but GLUT-4 has been shown to be the primary glucose transporter in response to insulin and in muscle contraction (192; 245). GLUT-4 is found in glucose transporter storage vesicles (GSVs), which are thought to protect GLUT4 from degradation (98). These GSVs may exist in distinct pools for contraction and insulin-stimulated glucose transport

(27; 54). The GSVs are thought to move along a cytoskeletal network towards the plasma membrane (148), although the requirement for an intact cytoskeletal network is debated by some (7). More recent evidence suggests that once a GSV translocates to the plasma membrane, it fuses to the plasma membrane, and allows GLUT4 to attach to the surface of the membrane (66). It has recently been shown that in addition to the requirement of translocation of GLUT4 to the plasma membrane increases in glucose transport are also dependent on amplification in intrinsic activity of the transporters (10; 172). On the other hand, exercise has been shown to increase the number of glucose transporters on the cell surface, but not their affinity for glucose (173).

Molecular mechanisms leading to GLUT-4 translocation

The effects of insulin and contraction on glucose transport are additive (89). Furthermore, there is evidence that they act via independent signalling pathways. For example, using wortmanin, a phosphoinositol-3-kinase (PI3-kinase), inhibitor, insulin-stimulated glucose transport was blocked, but contraction-mediated transport of glucose was unaffected. Therefore, contraction-mediated glucose transport occurs via a PI3-kinase independent mechanism (127).

Molecular aspects of insulin signalling.

Insulin initiates its action by binding to its receptor on the cell surface (Figure 1) (38). Following interaction with the insulin receptor, insulin receptor (IR) tyrosine kinase is activated, which in turn phosphorylates insulin receptor substrate proteins (IRS) (38). The IRS proteins then interact with PI3-kinase, a key signalling intermediate that is thought to be essential to mediating insulin's actions (38). Activation of PI3-kinase, in turn, leads to the formation of phosphorylated inositol-containing lipids (e.g.,

phosphatidylinositol (3,4,5) P_3 (also known as PIP $_3$) (221). PIP $_3$ activates phosphoinositide-dependent kinase 1 (PDK1), which activate protein kinase B (PKB)/Akt (referred to from this point forward as Akt) (194; 221).

Figure 1. Molecular mechanisms associated with insulin signalling. A '?' indicates a proposed interaction between proteins. IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3-kinase, phosphoinositide-3-kinase; PIP $_3$, phosphatidylinositol (3,4,5) P_3 ; PDK-1/2, phosphoinositide-dependent kinase 1; PKB/Akt, protein kinase-B/Akt; AS160, Akt substrate 160; GLUT4, glucose transporter 4; CaMKII, calcium calmodulin kinase-2; AMPK, AMP-activated protein kinase; Ca $^{2+}$, calcium

Downstream of this step, the mechanisms of the pathway become increasingly unclear. Akt substrate 160 (AS160), a protein involved in GLUT4 exocytosis in the basal state (57; 126), is thought to be bound to the GSVs. Activation of Akt2 is suggested

to phosphorylate AS160, which causes it to become unbound from the GSVs, which are then free to move towards the plasma membrane (126). The GSVs are then translocated along the cytoskeletal network by a series of Rab proteins to the cell surface. A recent study by Thong et al (2007) showed that AS160 may be a point of convergence for a number of intermediates other than Akt, including AMP-activated protein kinase (AMPK) and novel and conventional PKCs (215).

While the mechanism of insulin-stimulated glucose transport has been shown to be PI3-kinase-dependent, it has been more recently shown that calcium ions (Ca^{2+}) may also be involved (34; 234). Reactions activated by Ca^{2+} ions, leading to translocation of glucose transporters are a suggested pathway, which may include proteins such as CaMKs (234). CaMKII, in particular, has been found to be expressed in skeletal muscle (182; 183) and has been recognized as a possible candidate to be involved in insulin-stimulated glucose transport (234). It has also been proposed that this kinase could be activated by intermediates in the insulin-signalling pathway. Nevertheless, the involvement of a calcium-dependent mechanism in insulin-stimulated glucose transport remains controversial (234).

Although proximal aspects of the insulin signalling pathway in skeletal muscle have been well studied, the more distal aspects of the pathway and associated proteins require further investigation. Furthermore, the details related to the regulation of these proteins may be important in the treatment of diseases such as type 2 diabetes. For example activating AS160 by phosphorylation is accomplished through the insulin signalling pathway, which is reduced by ~39% in skeletal muscle of type 2 diabetics. However, a reduced phosphorylation of AS160 is likely not solely responsible for the large (~90%) reduction in GLUT4 translocation observed in diabetes (105).

Molecular aspects of contraction signalling

The mechanism by which contraction mediates its effects on glucose transport is not as well defined as insulin. It is known that the contraction-mediated signalling pathway leading to GLUT4 translocation is PI3-kinase independent, and it is likely that it is regulated by two separate mechanisms, the metabolic status of the cell (i.e. energy and substrate levels) and intramyocellular calcium concentration (178). Proposed mechanisms may vary depending on the fibre type of the muscle examined (235). Figure 2 shows the potential signalling pathways that may be involved in contraction mediated glucose transporter trafficking.

Figure 2. Potential contraction-mediated signalling pathways in skeletal muscle leading to GLUT4 translocation. Solid lines represent metabolic pathways, dashed lines represent pathways leading to unknown effectors. AMP, adenosine monophosphate; Ca, calcium; cPKC, conventional PKC; AMPK, AMP-activated protein kinase; CaMKII, Calcium calmodulin kinase-2; AS160, Akt substrate 160; GLUT, glucose transporters.

AMPK has been suggested to be a key monitor of metabolic status and a regulator of glucose transport during exercise (142; 143). It is activated *in vivo* in response to exercise in rat (65), electrically stimulated muscle contractions in situ, and contractions of isolated muscles *in vitro* (85; 197). It is regulated allosterically by increases in cellular AMP:ATP ratio and covalently through phosphorylation of the Thr¹⁷² residue within the α -catalytic subunit (4). AMPK can also be activated by hormones such as leptin (135), IL-6 (9; 33; 73; 75), adiponectin (240), and ciliary neurotrophic factor (227) or suppressed by hormones such as resistin (195). Finally, AMPK is activated by 5 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) in resting muscle. AICAR, when taken up by skeletal muscle, is phosphorylated to form ZMP (AICAR monophosphate), which mimics both the allosteric activation and promotes phosphorylation of the upstream kinase AMPK kinase.

Evidence of the involvement of AMPK in contraction-mediated glucose transport was provided by Mu et al (140), who used a dominant inhibitory mutant of AMPK in mouse muscle and found a 30-40% decrease in glucose transport. This finding suggests that AMPK is involved, but it unlikely the only mediator (236).

In rodents, the connection between AMPK activation and contraction-mediated glucose transport appears to be unique to fast twitch muscle, as suggested by a study demonstrating a dissociation between AMPK activation and glucose transport in rat soleus muscle *in vitro* (51). Similarly, more recent research showed that AICAR did not increase glucose transport in rat soleus muscle (235). Therefore, it is suggested that a calcium-induced mechanism serves a primary role in regulation of glucose transport in contracting soleus muscle (235).

Calcium concentrations in the cytoplasm are only elevated for a very short time after each contraction, but glucose transport may persist for an extended period after the cessation of contraction. Therefore, it is likely via the activation of calcium-mediated proteins, including CaMK and some isoforms of PKC that increased intracellular calcium concentrations mediate glucose transport in skeletal muscle.

CaMK is activated by an elevation in intracellular Ca^{2+} levels, subsequent binding of Ca^{2+} to calmodulin, and allosteric activation of CaMKII by Ca^{2+} –calmodulin binding (97). The isoform best characterized and known to be present in skeletal muscle is CaMKII (31; 41; 183; 184). It has been shown that CaMKII is activated during exercise. In situ contractions of rat gastrocnemius muscle resulted in a rapid increase in CaMKII phosphorylation at Thr²⁸⁷ (peaking at 1-3 min and reversed after 30min of contraction (182). The time course of CaMKII activation has been examined in human skeletal muscle (184) and fast twitch rodent skeletal muscle (182), but not slow twitch muscle.

The role of CaMK in glucose transport in skeletal muscle has been examined using inhibitors specific to CaMKs. In rodent epitrochlearis muscle, a fast twitch muscle, there was a 50% reduction in glucose transport with inhibition of CaMK. Therefore, the authors concluded that both AMPK activation and a rise in cytosolic calcium, thus activating CaMKs were responsible for mediating glucose transport during contraction (236). However, in soleus muscle, glucose transport was completely prevented by the SR calcium channel blocker dantrolene a CaMK-specific inhibitor. Therefore, in slow twitch muscle, rising cytosolic calcium appears to be the sole mechanism responsible for the activation of factors responsible for increases in glucose transport during contraction (235).

Another calcium-mediated factor that may be implicated in contraction-mediated glucose transport is PKC and its various isoforms (165). For example, it has been proposed that the PKC- ζ/λ isoforms can mediate both insulin- and exercise induced glucose transport through independent signalling pathways (39). Perrini et al. (2004) observed a corresponding increase in phosphorylation and abundance of these isoforms of PKC during 30 and 60 min of exercise (165). It is apparent, however, that further research is required to elucidate the roles of the various isoforms of PKC in glucose transport in muscle.

As suggested earlier, activation of Akt, PKC, and AMPK may lead to the phosphorylation of AS160 (215). The proposed role of AS160 in insulin signalling, as a result of Akt activation, was described above and its resultant actions may be similar when activated by PKC, AMPK (215), or CaMKII (120). Studies have shown that phosphorylation of AS160 occurs following both insulin and contraction in skeletal muscle (29; 120; 121). Using mutant constructs of AS160, Kramer et al (2006) found a serious impairment of both contraction- and insulin-stimulated glucose transport (121).

Nitric oxide and MAPKs have also been proposed to act as mediators of glucose transport during exercise. The mechanism of action of nitric oxide is suggested to be distinct from the mechanisms proposed above and may involve intracellular calcium, AMPK, or increases in reactive oxygen species. Nitric oxide may be exerting its effects by altering cyclic guanosine monophosphate (cGMP) levels (242), and subsequent promotion of glucose transport potentially via a cGMP-dependent protein kinase mechanism (242).

Early research on the MAPK family of proteins (extracellular signal-regulated kinases (ERKs), p38 MAPK, and JNK) suggested that there was a role for p38 MAPK in

glucose transport during muscle contraction. These proteins are stimulated by exercise as well as by factors such as cytokines, growth factors and cellular stress (119). However, the role of these MAPK proteins in regulation of glucose transport has been questioned as inhibitors of MAPK/ERK kinases (83; 232) and JNK (231) have not affected glucose transport. The use of dominant negative p38 mutants did not affect glucose transport (12) and overexpression of p38 γ actually decreased expression of GLUT4 transporters and corresponding glucose transport (93). Therefore, the involvement of any of the MAPK family of proteins in glucose transport in skeletal muscle is unlikely.

2.2.5. IL-6 and resting glucose transport

Many different models have been employed to determine the effects of IL-6 in skeletal muscle on glucose transport and molecular signalling mechanisms, ranging from biopsies of humans following infusion of IL-6, to cell culture. The resulting variability of findings may be due to the comparison of results using different models, different concentrations of cytokines utilized, and varying duration of exposure to the cytokines (see Table 1). As such, a consensus does not exist concerning the effects of IL-6 on glucose transport into skeletal muscle.

Acute infusion of IL-6 in humans and rodents has provided conflicting effects on insulin sensitivity. For example, in resting humans, Tsigos et al (1997) found that a subcutaneous injection of IL-6 caused an elevation in blood glucose levels, with increasing doses of IL-6. The authors suggested that this finding might be due to reduced insulin sensitivity in skeletal muscle or increased hepatic glucose production, although only plasma glucose was measured (217). Conversely, in cancer patients, an increase in insulin sensitivity was noted after IL-6 infusion due to an increase in glucose clearance despite elevations in HGP. The authors were confident that, although this finding may be unique because of the patient population utilized, the presence of cancer did not influence their results (211).

Steensberg et al. (2003), on the other hand, did not detect alterations in insulin sensitivity and no change in HGP with 3h of IL-6 infusion. They also observed no change in either whole-body glucose disposal or net-leg glucose transport (203).

Table 1. Studies examining the effect of IL-6 on glucose homeostasis and glucose transport in resting skeletal muscle.

Authors/Species Tissues	[IL-6]	Duration	Findings
<i>In vitro</i>			
Geiger et al. (2007) (73); Rodent Epitrochlearis/ Soleus	4, 10, 120 ng/ml	30min	Basal & Ins: ↑ 2- & 1.5-fold epitrochlearis; n/c soleus
Glund et (2007) (75) Human <i>Vastus lateralis</i>	120 ng/ml	15,30, 80min	Basal: ↑ 1.3-fold Ins: n/c
Al Khalili et al.(2006) (9) Human Cell culture (<i>rectus abdominus</i>)	5,25,100 ng/ml	20min, 3h, 8d	Basal ↑ 1.2-fold (3h/8d) Ins: ↑ 1.7-fold (8d)
Carey et al.(2006) (33) Human, L6 myotubes	1-100 ng/ml	120min	Basal: ↑ 2-fold Ins: ↑ 4-fold
<i>In vivo</i>			
Carey et al.(2006) (33) Human Whole body	Infusion pl [100-140 pg/ml]	4h	GIR: ↑15%
Stouthard et al. (1995) (211) Human, cancer patients Whole body	Infusion pl [175-7584 pg/ml]	3h	Ra: ↑ 20% clearance: ↑
Tsigos et al.(1997) (217) Human Whole body	sc injection pl [8,22,65,290, 4050 pg/ml]	0-4h	Pl glu: ↑ (1,3,10mg/kg/bw)
Krogh-Madsen (2006) (122) Human Whole body	Infusion pl [55 pg/ml]	3h	Ra: n/c Rd: n/c
Steensberg et al.(2003) (203) Human Leg	Infusion pl [143, 319 pg/ml]	3h	Ra:n/c; Rd:n/c Leg: n/c
Kim et al.(2004) (114) Rodent Muscle	Infusion 0.5 µg/h; Clamp	2h	GIR: ↓25% Ins: ↓glu turnover
Rotter-Sopasakis et al. (2004) (190) Rodent	Infusion pl [~0.85 nmol/L]	120min	GIR: n/c
Other			
Jové, 2005 (103) Mouse C2C12 myoblasts	palmitate-induced IL-6; pl [1000 pg/ml]	16h	Ins: ↓34%

pl – plasma; Ins – insulin; Ra - rate of appearance; Rd - rate of disappearance;
GIR - glucose infusion rate; Glu – glucose; min – minutes; h – hour; n/c – no change; ↑-
increase; ↓- decrease.

There have been very few studies examining the direct effects of IL-6 on glucose transport into skeletal muscle. These existing studies have only considered the effect of IL-6 on glucose transport under basal or insulin-stimulated conditions at rest, rather than during muscle contraction.

Generally, these studies have shown that IL-6 enhances basal (9; 33; 73; 75) and insulin-stimulated glucose transport in skeletal muscle (9; 33; 73). However, Geiger et al. (2007) observed enhanced glucose transport in soleus and epitrochlearis muscle strips only when exposed to supraphysiological levels of IL-6 (120 ng/ml) (73). Meanwhile, an earlier study found that lower concentrations (10 ng/ml) resulted in a 2- and 4-fold increase in basal and insulin stimulated glucose transport, respectively, in L6 myotubes (33). Glund et al. (2007), utilizing human *vastus lateralis* muscle strips found that IL-6 increased basal glucose transport 1.3-fold. However, they did not observe an increase in insulin-stimulated glucose transport with IL-6 exposure (75).

It appears that the few studies examining the direct effect of IL-6 on skeletal muscle in an isolated model have observed an increase in basal and insulin-stimulated glucose transport with IL-6 present. However, *in vivo* measures of glucose homeostasis with IL-6 infusion are less consistent.

2.2.6. Molecular mechanisms of IL-6 in resting skeletal muscle

The research investigating the molecular mechanisms by which IL-6 could exert its effects on skeletal muscle glucose transport does not provide a clear consensus. Once IL-6 is bound to its receptor, it induces a series of signalling events including the activation of the Janus Kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway (86). It is unclear how the signalling intermediates of IL-6 interact with other molecular proteins in muscle, however, the following review will describe a number

of proteins related to glucose transport in muscle that have been shown to be affected by IL-6 exposure. Table 2 summarizes the studies examining the potential metabolic mechanisms of IL-6.

Table 2. Studies examining the molecular mechanisms of IL-6 in glucose metabolism in resting skeletal muscle.

Protein	Authors Species, Tissue	[IL-6]	Duration	Findings
IR	Klover, 2003 (118) Rodent infusion	16µg/ml	5d	Ins: n/c
	Rotter-Sopaskis, 2004 (190) Rodent cell culture	infusion pI[~0.85nmol/L]	120min	Ins: n/c
	Rieusset, 2004 (180) Human cell culture	20ng/ml	90min	Ins: n/c
IRS 1/2	Weigert, 2006 (229) rodent injection	2.5ug/kg	5min	Basal: ↑ (Ser318)
	Rotter-Sopaskis, 2004 (190) Rodent infusion	infusion pI[~0.85nmol/L]	120min	Ins: n/c
	Weigert, 2006 (229) Mouse, C2C12 myotubes		10min	
	Human myotubes	20ng/ml	90min	Basal: ↑ serP; n/c tyrP
	Al-Khalili, 2006 (9) Human cell culture	25ng/ml	20min, 3h	Basal: n/c; Ins: n/c
	Carey, 2006 (33) Human L6 myotubes	100ng/ml	10min	Basal: n/c; Ins: n/c
IRS-1 assoc w PI3- kinase	Kim, 2004 (114) Rodent infusion	0.5µg/h	2h	Ins: ↓
	Rieusset, 2004 (180) Human cell culture	20ng/ml	90min	Ins: ↓
	Glund 2007 (75) Human strips	120ng/ml	15,30,80min	Basal: n/c; Ins: n/c
	Carey, 2006 (33) Human L6 myotubes	100ng/ml	10min	Basal: n/c; Ins: n/c
PI3- kinase	Al-Khalili, 2006 (9) Human myotubes, myoblasts	25ng/ml	3h, 8d	Basal: ↑; Ins: n/c
Akt	Kim, 2004 (114) Rodent infusion	0.5µg/h	2h	Ins: n/c
	Weigert, 2005 (228) Mouse, njection/intraperitoneal Infusion	50ng 10 and 30ng/ml	5min/15- 60min	Basal: ↑ (Ser473); n/c (Thr308); Ins: ↑ (Ser473/Thr308)
	Human myotubes		20-180min	
	Rieusset, 2004 (180) Human cell culture	20ng/ml	90min	Ins: ↓
	Al-Khalili, 2006 (9) Human cell culture	25ng/ml	20min, 3h	Basal: ↑ (Ser473/Thr308) Ins: n/c
	Geiger, 2007 (73) Rodent strips	4, 10, 120ng/ml	30min	Basal: n/c; Ins: n/c (Thr308)
	Glund, 2007 (75) Human strips	120ng/ml	15,30,80min	Basal: n/c; Ins: n/c (Ser473/Thr308)
	Carey, 2006 (33) Human L6 myotubes	100ng/ml	120min	Basal: n/c; Ins: ↑

Protein	Authors Species, Tissue	[IL-6]	Duration	Findings
AS160	Al-Khalili, 2006 (9) Human cell culture	25ng/ml	3h	Basal: n/c; Ins: n/c
	Glund, 2007 (75) Human strips	120ng/ml	30min	Basal: n/c; Ins:n/c
AMPK	Al-Khalili, 2006 (9) Human cell culture	25ng/ml	3h	Basal: ↑ (3.8 fold) Ins: n/c
	Carey, 2006 (33) Human L6 myotubes IL-6-/- mice Gastrocnemius	1-1000ng/ml No incubations	10-120min	Basal: ↑ (2-fold) ↓pAMPK-α2; n/c AMPK-α1
	Geiger, 2007 (73) Rodent strips	4, 10, 120ng/ml	30min	Basal: ↑ in epitrochlearis; n/c in soleus
	Glund, 2007 (75) Human strips	120ng/ml	15,30,80min	Basal: ↑ (2-fold); Ins: n/c
ACC	Carey, 2006 (33) Human L6 myotubes IL-6-/- mice Gastrocnemius	100ng/ml no incubations	10-120min	Basal: ↑ ↓pACC
MAPKs	Al-Khalili, 2006 (9) Human cell culture	25ng/ml	3h	Basal: ↑ERK ½; Ins: n/c
	Glund, 2007 (75) Human strips	120ng/ml	15,30,80min	Basal: ↑; Ins: ↑ (p38MAPK)
PKC	Kim, 2004 (114) Rodent infusion	0.5µg/h	2h	Ins: n/c (PKC λ/γ)

IR – insulin receptor; IRS-1 – insulin receptor substrate-1; IRS-1 assoc w PI3-kinase – insulin receptor substrate associated with phosphoinositide-3 kinase; PI3-kinase – phosphoinositide-3 kinase; AS160 – Akt substrate 160; AMPK – adenosine monophosphate kinase (AMP) – activated protein kinase; ACC – acetyl-CoA-carboxylase; MAPKs – mitogen activated protein kinases; ERK – extracellular signal-regulated kinase; PKC – protein kinase C; h – hour; min- minute; ↑ - increase; ↓ - decrease; n/c – no change; ser – serine; tyr – tyrosine; Ins - insulin

One proposed mediator of the effects of IL-6 is AMPK (73; 75; 130). The proposed role of AMPK in glucose transport was described in detail earlier. Recent studies have examined the possibility that IL-6 may exert its effects via AMPK activation under basal and insulin-stimulated conditions (73; 75) and during contraction (112; 130). Under resting conditions, there is some evidence to support the involvement of AMPK in IL-6-mediated metabolic effects. In studies examining IL-6 knockout mice, extensor digitorum longus (EDL) muscle strips from the null mice demonstrated only 40-90% of normal AMPK activity and acetyl Co-A carboxylase (ACC) phosphorylation. Incubation of the muscle from the IL-6^{-/-} mice with IL-6 increased AMPK activity by 1.5-3-fold

(112). Similar results were noted in gastrocnemius muscle strips also from IL-6^{-/-} mice (33). Other investigations utilizing L6 myotubes (33) and muscle strips from human vastus lateralis (75) have found that IL-6 exposure resulted in an elevation in AMPK and ACC phosphorylation within 10 min and lasted 120 min. Furthermore, IL-6 did not affect basal or insulin-stimulated glucose transport in myotubes transfected with AMPK dominant/negative construct (33).

Very few studies have investigated the interaction between IL-6 and AMPK during exercise. IL-6 release has been correlated with AMPK activity during exercise, but correlations do not provide proof of a direct interaction (130). Kelly et al. (2004) found that in IL-6 knockout mice, AMPK activity was reduced compared to control animals with exercise (112). However, the direct effect of IL-6 on AMPK activity during contraction has not been examined.

Studies examining the effect of IL-6 on insulin signalling proteins have generated a wide spectrum of findings. This variability in findings may be due to the many different models employed or the duration of exposure to IL-6. However, the body of research is still quite small and proteins in the distal aspect of the signalling pathway remain elusive and further research is required.

Studies examining acute introduction of IL-6 in rodents via injection or infusion have also provided varied findings. Two investigations examining IL-6 injection observed an increase in insulin sensitivity (228; 229). The increase in insulin sensitivity could be attributed to the IL-6-induced increase in serine phosphorylation of IRS-1 (229) and increased phosphorylation of the serine residue of Akt observed in these studies (228). On the other hand, another investigation found that 120 min of IL-6 infusion resulted in no change in protein levels of IRS-1, or IR phosphorylation (190). Finally, Kim et al (2004) found that IL-6

infusion during a euglycemic hyperinsulinemic clamp led to a reduction in IRS-1 associated PI3-kinase activity (114). These varied findings may be attributed to the time of IL-6 exposure as the first two studies involved injection of IL-6 and measurements after 5 min (228; 229). The latter experiments were based on more prolonged (120min) exposure to IL-6 (114; 190).

To date, only a single study has examined the effects of chronic infusion of IL-6 in rodents. Five days of IL-6 exposure had no significant effect on insulin receptor signalling or insulin sensitivity (118).

The direct effect of IL-6 on insulin signalling in skeletal muscle has been evaluated through incubation of resting rodent and human muscle cells and muscle strips with IL-6. Rieusset et al. (180) found insulin signalling to be altered after 90 min of IL-6 and insulin incubation. Interleukin-6 prevented both the effect of insulin-induced IRS-1 phosphorylation and the association of p85 α P13K with IRS-1, while also attenuating insulin-induced increase in phosphorylation of PKB/Akt. There was no significant effect of IL-6 on insulin receptor autophosphorylation or the insulin receptor and IRS-1 protein content. On the other hand, two studies found that exposure to IL-6 had an insulin-sensitizing effect on human muscle cells by inducing the phosphorylation of the serine residue of Akt (33; 228); however, without insulin present, IL-6 did not have an effect on Akt ser⁴⁷³, nor did it change protein levels of p85 subunit of PI3-kinase or IRS-1(33). Finally Glund et al. (2007) found no significant effect of IL-6 on IRS-associated PI3-kinase activity, and Akt and AS160 phosphorylation in human *vastus lateralis* muscle strips (75).

Very recently a study noted an increase in GLUT4 translocation with IL-6 exposure and an additive effect was observed when insulin and submaximal

concentrations of IL-6 were combined (33). Therefore, it is possible that regardless of the intermediate activated by IL-6, glucose transporters could be at higher concentrations on the cell surface.

Figure 3. Suggested points of interaction between IL-6 and intermediates involved in glucose transport in skeletal muscle. A solid line represents an established metabolic pathway. A dashed line represents a proposed pathway. A '*' represents the interaction favoured by existing research, and a '?' represents a relationship that is unclear based on the existing evidence.

Although the literature is varied in this area, a likely candidate that may moderate the activities of IL-6 in skeletal muscle is AMPK. The four recent studies examining the direct effect of IL-6 on glucose transport also measured AMPK and found

phosphorylation to be increased with IL-6 exposure. However, Geiger et al (2007) observed an increase in AMPK phosphorylation in rodent epitrochlearis muscle strips, but did not detect an increase in soleus muscle (73). Other possible signalling intermediates that IL-6 may interact with include IRS-1/2 and Akt, although the evidence is fairly mixed, as with other proteins. Clearly further research is required to reveal mediators of IL-6's actions. Figure 3 provides an overview of possible interactions between IL-6 and mediators of glucose transporter translocation.

2.2.7. IL-6 and glucose transport during exercise.

As suggested above, it is possible that IL-6 released from muscle is acting in an autocrine, paracrine, or endocrine fashion (60; 110; 111). The local influence of IL-6 on contraction-mediated glucose transport in muscle has not been directly investigated. However, a single study (59) attempted to isolate the effects of IL-6 released during skeletal muscle contraction on whole body glucose homeostasis. Febbraio et al. (2004) found that low intensity exercise combined with IL-6 infusion resulted in a greater rate of glucose disappearance than low intensity exercise alone, but did not surpass that of high intensity exercise. This study illustrates that while IL-6 appears to be important in the regulation of glucose homeostasis during exercise *in vivo*, other factors related to muscle contraction are also involved, such as changing calcium levels and declining glycogen concentrations (59). Clearly further research is needed to examine the direct effect of locally produced IL-6 on glucose transport during exercise.

Molecular mechanisms of IL-6 in contracting skeletal muscle

During exercise, the mechanism by which IL-6 may exert any effect on skeletal muscle has not been investigated. As discussed above, the signalling pathways leading to GLUT-4 translocation during contraction are somewhat unclear; however, key mediators

have been suggested to be AMPK (236) and calcium-induced proteins such as CaMK (235; 236) and PKCs (39). Therefore, it is possible that any effect of IL-6 during contraction may be via interactions with these proteins.

2.3. Do other cytokines antagonize or agonize the actions of IL-6?

When considering a role for IL-6 in the regulation of glucose homeostasis, other cytokines that may interact to influence any of its effects should be considered. TNF-alpha is a likely candidate and is implicated in a variety of disease states including type 2 diabetes. In order to address any combined effects that IL-6 and TNF-alpha may exert, it is important first to address the effects of TNF-alpha alone on glucose homeostasis and glucose transport in skeletal muscle.

2.3.1. The involvement of TNF-alpha in glucose homeostasis.

Tumour necrosis factor-alpha is a cytokine originally termed for its role in tumour necrosis in septic animals and cancer cachexia (45). The biologically active form of TNF-alpha (17 kDa) is a soluble protein that has undergone cleavage from a 26-kDa cell surface transmembrane protein.

Tumour necrosis factor-alpha is synthesized by many cell types including monocytes/macrophages, lymphocytes, and adipocytes (216). The biological effect of TNF-alpha on target tissues is dependent upon its concentration, the duration of exposure, and the presence of other factors that may mediate its effects (21). Interestingly, TNF-alpha receptors (TNF-R) are found on nearly all cell surfaces, but there is no correlation between the receptor density and the corresponding biological response (63). Similar to IL-6, TNF-Rs exist in both a membrane bound (5) and a soluble form (91; 147).

Tumour necrosis factor-alpha has been implicated in many disease states including insulin resistance (14; 122; 134; 137), obesity (14), cardiovascular disease

(212), sepsis (213), and rheumatoid arthritis (187). Tumour necrosis factor-alpha was the first secretory protein from adipose tissue to be identified as a causal link between obesity and insulin resistance (96). Evidence of this was shown in rodents, where administration of TNF-alpha resulted in insulin resistance (125; 128; 134; 191). Normal circulating concentrations are approximately 1.5 pg/ml, but in a variety of disease states, circulating concentrations of TNF-alpha are elevated (91). In the case of diabetes, however, elevations in circulating concentrations of TNF-alpha are not consistently observed (24; 113). Therefore utilizing higher than normal plasma TNF-alpha levels as a marker for diabetes would not be a reliable method.

2.3.2 Skeletal muscle is not an important source of TNF-alpha

While IL-6 is released in large quantities from skeletal muscle during exercise, the same does not appear to be consistently true for TNF-alpha. There are two findings of modest, but significant increases in plasma TNF-alpha following exhaustive exercise (150; 200). Therefore, it is plausible that TNF-alpha may also be released during exercise, but Febbraio et al. (62) found that, there was no net release of TNF-alpha from exercising muscle. Similarly, it has been found (62) that muscle is not a source of TNF-alpha in the circulation under resting conditions. Once again, findings are mixed on this topic as other studies have not observed increases in systemic TNF-alpha (149; 204) or its mRNA (152) in skeletal muscle.

2.3.3 The effect of TNF-alpha on glucose transport

As suggested earlier, TNF-alpha has been implicated in obesity and insulin resistance because elevations in circulating levels are observed in these disease states (137). A substantial amount of work has examined the effects of TNF-alpha on glucose metabolism and insulin signalling *in vivo* and *in vitro*. Although the evidence that TNF-

alpha causes disruptions to normal carbohydrate metabolism is convincing, there are some mixed findings, worthy of discussion (Table 3).

Table 3. Effect of TNF-alpha on glucose homeostasis and glucose transport in skeletal muscle

Author/Species/Tissue	[TNF-alpha]	Duration	Findings
<i>In vitro (Cell Culture)</i>			
Ciaraldi, 1998 (44) Human, T2D Myotubes	5ng/ml	90min, 24h	Basal: ↑ Ins n/c (90min); ↑ 24h
Grohmann, 2005 (77) Human Myotubes	20ng/ml	24h	Basal: ↑
Rosenzweig, 2002 (188) Mouse Myotubes	1nmol/L	5,15,30min	Ins: ↓ 50% Basal: n/c
Storz, 1999 (210) Mouse Myotubes	50ng/ml	2h	Basal: ↑ (~1.5-fold) Ins: ↑
Begum, 1996 (18) Rodent L6 myotubes	10ng/ml	1-12h	Basal: ↓ 40% Ins: ↓ 50%
Del Aguila, 1999 (50) Rodent C2C12myotubes	10ng/ml	1h	Basal: ↓ 43% Ins: ↓ 30%
Yamaguchi, 2003 (238) Rodent L6 myotubes	20ng/ml	6h	Ins: ↓ 40%
Patiag, 2000 (154) Rodent L6 myotubes	1, 10ng/ml	24h	Basal: n/c Ins: n/c
de Alvaro, 2004 (48) Rodent Myotubes	1nM	24h	Ins: n/c Basal: ↑ (3-fold)
Yamasaki, 1996 (239) Rodent L6 myotubes	23nM	48h	Basal: ↑ (3-fold)
Ranganathan, 1996 (175) Rodent L6 myotubes	5,10, 20ng/ml	4,5,8d	Basal: ↑ (30%) Ins: n/c
<i>In vitro (Muscle Strips)</i>			
Furnsinn, 1997 (69) Rodent Soleus	10, 100, 1000pmol/L	30min, 6h, 24h	Basal: n/c Ins: n/c
Nolte, 1998 (146) Rodent Soleus, Epitrochlearis	6nmol/L	45min, 4h	Basal: n/c Ins: n/c

Author/Species/Tissue	[TNF-alpha]	Duration	Findings
<i>In vivo (Infusion)</i>			
Plomgaard, 2005 (171) Human Whole Body	1000ng/h/m2 (8-fold↑ pl [TNF])	1h	Ga: no ↑ EGP: n/c
Krogh-Madsen, 2006 (122) Human Whole body	1000ng/h/m2	3h	Gd: ↓
Rask-Madsen, 2003 (176) Human Forearm	pl [252ng/L] in perfused arm		Basal: n/c Ins: ↓ 100%
Ling, 1994 (128) Rodent Whole Body	20ug/kg	3h	GIR: ↓ (50%) Ins: n/c HGP: ↑
Lang, 1992 (125) Rodent Whole Body Gastrocnemius	1ug/kg	18h	GIR: ↓ Ins: ↓ (Gastrocnemius)
Steinberg, 2006 (208) Mouse Whole Body, Gastrocnemius	1ug/kg/d	24h	Gd: ↓ ~25% Ins: ↓ 50%
Miles, 1997 (134) Rodent Whole Body	0.7, 0.5ug/kg/h	4-5d	Gd: ↓
Ruan, 2002 (191) Rodent Whole Body	0.27mg/h	24h or 4d	Insulin resistance with insulin tolerance test
<i>In vivo (Infusion)</i>			
Youd, 2000 (241) Rodent Hindlimb Soleus, Plantaris	0.5ug/kg/h	3h	Ins: ↓ 61% hindlimb Ins: ↓ 50% soleus and 68% plantaris Basal: n/c
Zhang, 2003 (244) Rodent Hindlimb	0.5ug/kg/h	3h	Basal: n/c Ins: ↓ (3, 10 mU), n/c 30 mU
<i>In vivo (Electrical Stimulation)</i>			
Zhang, 2003 (244) Rodent Hindlimb	0.5ug/kg/h	2h	Contraction: n/c

Ins = insulin stimulated glucose transport; Gd = glucose rate of disappearance; GIR = glucose infusion rate; Basal = basal glucose transport; HGP = hepatic glucose production; T2D = type 2 diabetes; ↑ - increase; ↓ - decrease; n/c – no change

Muscle insulin resistance is consistently noted with TNF-alpha infusion. Infusion of TNF-alpha in rodents causes insulin resistance and an increase in hepatic glucose

production (125; 128; 134; 191). Similarly, hindlimb infusion of TNF-alpha in rodents resulted in decreased insulin sensitivity as seen through a reduction in insulin-stimulated glucose transport (241; 244). These findings were consistent regardless of infusion duration. Two studies infusing TNF-alpha into humans confirmed that this resulted in insulin resistance in muscle, but Plomgaard et al. could not demonstrate a reduction in hepatic glucose production (171).

Contrary to the infusion studies reported above, the direct effect of TNF-alpha on insulin resistance in cell culture and isolated muscle strips has provided mixed findings. In most studies using rodent cell culture, a reduction in insulin-stimulated glucose transport has been noted (19; 19; 50; 188; 238). However, three studies reported that TNF-alpha had no effect on insulin sensitivity on cell cultures after chronic (24h-8d) exposure (48; 154; 175). Similarly, acute and chronic incubation of isolated rodent muscle strips (soleus and extensor digitorum longus (EDL)) did not result in a change in insulin-stimulated glucose transport (69; 146).

On the other hand, exposing cultured human muscle cells to TNF-alpha for 90min and 24h led to an increase in basal glucose transport (44; 77), whereas after 24h of TNF-alpha exposure there was an increase in insulin-stimulated glucose transport (44). Interestingly, these studies both noted an increase in GLUT1 total protein, but no change in GLUT-4 total protein (44; 77). These contrary findings may reflect a species difference between humans and rodents.

A number of the aforementioned studies also examined the effect TNF-alpha on basal glucose transport in skeletal muscle and the results were inconsistent. For instance, an increase (175; 239), no effect (50; 154), and a reduction (18) in basal transport into

the muscle have been documented. Many investigations expose cells or muscle strips to concentrations at least 10-fold greater than physiological concentrations.

2.3.4. Molecular mechanism of TNF-alpha in resting skeletal muscle

In rodent cell culture, most studies have found a reduction in insulin-stimulated glucose transport as well as a decrease in the activation of proteins that may mediate the translocation of GLUT4 such as MAPK (48; 77; 171; 239), PKCs (188), and other insulin signalling intermediates (18; 19; 48; 50; 188; 238). Table 4 includes a summary of studies examining the molecular mechanisms of TNF-alpha's actions in skeletal muscle glucose transport.

Table 4. Studies examining the molecular mechanisms of TNF-alpha in glucose metabolism in resting skeletal muscle.

Protein	Author/Species/Tissue	[TNF]	Duration	Findings
IR	Nolte, 1998 (146) Rodent, strips	6nmol/L	45min, 4h	Ins: n/c
	Rosenzweig, 2002 (188) Mouse, myotubes	1nmol/L	5,15,30min	Basal: ↑; Ins: ↓
	Storz, 1999 (210) Mouse, myotubes	50ng/ml	2h	Ins: ↓40%
	Yamaguchi, 2003 (238) Rodent, L6 myotubes	20ng/ml	6h	Basal: n/c; Ins: n/c
	de Alvaro, 2004 (48) Rodent, myotubes	1nmol/L	24h	Ins: ↓
IRS 1/2	Plomgaard, 2005 (171) Human Infusion	pl [↑ 8-fold]	1h	Ins:↓; Basal:↑
	Steinberg, 2006 (208) Mouse, infusion	1ng/kg/d	24h	Basal: ↓~40%
	Nolte, 1998 (146) Rodent, strips	6nmol/L	45min, 4h	Ins: n/c
	Rosenzweig, 2002 (188) Mouse, myotubes	1nmol/L	5,15,30min	Basal: ↑; Ins: ↓
	Del Aguila, 1999 (50) Rodent, C2C12myotubes	10ng/ml	1h	Ins:↓; Basal: n/c
	Storz, 1999 (210) Mouse, myotubes	50ng/ml	2h	Ins:↓60%
	Yamaguchi, 2003 (238) Rodent, L6 myotubes	20ng/ml	6h	Basal: n/c; Ins: n/c
	de Alvaro, 2004 (48) Rodent, myotubes	1nmol/L	24h	Ins: ↓
	Yamasaki, 1996 (239) Rodent, myotubes	23nmol/L	48h	Basal: n/c

Protein	Author/Species/Tissue	[TNF]	Duration	Findings
IRS-1 assoc w PI3- kinase	Steinberg, 2006 (208) Mouse, infusion	1ng/kg/d	24h	Basal: ↓~40%
	Nolte, 1998 (146) Rodent, strips	6nmol/L	45min, 4h	Ins: n/c
	Rosenzweig, 2002 (188) Mouse, myotubes	1nmol/L	5,15,30min	Basal: ↑; Ins: ↓
	Del Aguila, 1999 (50) Rodent, C2C12myotubes	10ng/ml	1h	Ins:↓; Basal: n/c
	de Alvaro, 2004 (48) Rodent, myotubes	1nmol/L	24h	Ins: ↓
Akt	Halse, 2001 (79) Human, myoblasts	5ng/ml	5d	Ins: n/c; Basal: n/c
	Steinberg, 2006 (208) Mouse, infusion	1ng/kg/d	24h	Basal: ↓~25%
	Storz, 1999 (210) Mouse, myotubes	50ng/ml	2h	Ins: n/c;
	Yamaguchi, 2003 (238) Rodent, L6 myotubes	20ng/ml	6h	Basal: n/c; Ins: n/c
	de Alvaro, 2004 (48) Rodent, myotubes	1nmol/L	24h	Ins: ↓ (Ser473; Thr308)
AS160	Plomgaard, 2005 (171) Human Infusion	pl [↑ 8-fold]	1h	Basal: ↓
AMPK	Steinberg, 2006 (208) L6 myotubes	10ng/ml	8-16h	Basal:↓ pAMPK and activity
	Steinberg, 2006 (208) Mouse, infusion	1ug/kg/day	24h	Basal:↓ pAMPK and activity
ACC	Steinberg, 2006 (208) L6 myotubes	10ng/ml	8-16h	Basal:↓ ~80%
	Steinberg, 2006 (208) Mouse, infusion	1ug/kg/day	24h	Basal:↓ ~50%
MAPKs	Plomgaard, 2005 (171) Human Infusion	pl [↑ 8-fold]	1h	↑ERK-1/2, JNK
	Grohmann, 2005 (77) Human, myotubes	20ng/ml	24h	Basal: ↑
	Nolte, 1998 (146) Rodent, strips	6nmol/L	45min, 4h	Basal: ↑(70%) pp42, pp44 MAPK,
	Del Aguila, 1999 (50) Rodent, C2C12myotubes	10ng/ml	1h	Ins:↓; Basal: n/c
	Yamaguchi, 2003 (238) Rodent, L6 myotubes	20ng/ml	6h	Ins: n/c; Basal: n/c (ERK 1/2)
	de Alvaro, 2004 (48) Rodent, myotubes	1nmol/L	24h	Basal: ↑
	Yamasaki, 1996 (239) Rodent, myotubes	23nmol/L	48h	Basal: ↑
PKC	Rosenzweig, 2002 (188) Mouse, myotubes	1nmol/L	5,15,30min	Ins: ↑δ α activity;↓ coprecipitation with IR
	Steinberg, 2006 (208) Mouse, infusion	1ng/kg/d	24h	Basal: ↑ (θ,ε isoforms)

IR – insulin receptor; IRS-1 – insulin receptor substrate-1; IRS-1 assoc w PI3-kinase – insulin receptor substrate associated with phosphoinositide-3 kinase; PI3-kinase – phosphoinositide-3 kinase; AS160 – Akt substrate 160; AMPK – adenosine monophosphate kinase (AMP) – activated protein kinase; ACC – acetyl-CoA-carboxylase; MAPKs – mitogen activated protein kinases; ERK – extracellular signal-regulated kinase; PKC – protein kinase C; h – hour; min – minute; ↑ - increase; ↓ - decrease; n/c – no change; ser – serine; tyr – tyrosine; Ins - insulin

Mitogen-activated protein kinases, critical positive and negative modulators of insulin signalling, and glucose transport were also affected by TNF-alpha incubation. A few studies found an increase in MAPK phosphorylation (48; 77; 171; 239). These studies found that increasing MAPK phosphorylation resulted in a reduction in insulin sensitivity. Interestingly, phosphorylation of inhibitory MAPKs, p38, JNK, and ERK were increased with chronic (24h) incubation. Both findings led to a reduction in insulin sensitivity (48).

Rosenzweig et al. (2002) found that acute incubation of myotubes in TNF-alpha caused a reduction in insulin-stimulated tyrosine phosphorylation of the insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and its association with PI3-kinase. Furthermore, TNF-alpha was shown to block the interaction between PKCs and IRS-1 (188). A couple of other studies confirm these findings in rodents, finding a reduction in IRS-1 phosphorylation and its subsequent interaction with PI3-kinase (50; 238). However, two other studies did not observe a change in IRS-1 (146; 239). These latter studies were chronic (48 h) incubations (239) of muscle strips, whereas the former studies were acute incubations (30 min, 1 h, 6 h) using rodent cell culture (50; 188; 238). In addition, following stimulation of human myoblasts (79) and rodent L6 myotubes (238), there was no change in Akt phosphorylation. However, Plomgaard et al (2005) observed impairment in AS160 phosphorylation which is downstream of Akt (171). The evidence of an interaction with insulin signalling intermediates by TNF-alpha likely takes place in more proximal aspects of the signalling cascade, such as IRS-1/2; nevertheless, AS160 is also a promising candidate (238). However, research examining the distal aspects of the insulin signalling pathway, such as Akt, AS160 and other more distal proteins are limited.

Interestingly, Rosenzweig et al. (2002) examined the interaction of PKC α and δ with IR and IRS-1 and the effect of TNF- α exposure in mouse myocytes (188). Tumour necrosis factor- α prevented insulin-induced coprecipitation of PKC α and δ with IR. It seems that although both isoforms are associated with IRS-1, insulin decreased coprecipitation of IRS-1 with PKC α and increased coprecipitation of IRS-1 with PKC δ ; TNF-blocked the effects of insulin on association of both. Further examination of this relationship by overexpressing these PKC isoforms demonstrated that the interaction between TNF- α and IR tyrosine phosphorylation could be due to a disruption in insulin mediated PKC α and δ with upstream signalling intermediates such as IR and IRS-1.

Based on the aforementioned research, TNF- α may be interfering with the insulin signalling pathway by either decreasing IRS-1 phosphorylation or reducing the association between IRS-1 and PI3-kinase. However, whether other signalling proteins are affected by TNF- α is unclear.

The molecular mechanism involved with any interaction between TNF- α and contraction-mediated glucose transport has not been examined.

2.3.5. TNF- α is not involved in glucose transport during muscle contraction.

As discussed above, it is likely that during exercise TNF- α is not released in significant quantities from skeletal muscle nor has it been found to be acutely elevated in the circulation. However, TNF- α is often present in chronically elevated concentrations in conditions like obesity and diabetes. Because TNF- α is in elevated concentrations in disease states that are characterized by insulin resistance, it is important to investigate any possible antagonistic effects of TNF- α on glucose transport in contracting muscle. To date, only Zhang et al. (2003) has explored this possibility. They

infused TNF-alpha into an electrically stimulated hindlimb for 1h and while they found that TNF-alpha caused insulin resistance in the resting hindlimb, there was no significant effect on glucose transport during muscle contraction (244).

2.3.6. Is there a relationship between TNF-alpha and IL-6?

Both TNF-alpha and IL-6 have been proposed to cause disruptions to normal glucose metabolism and homeostasis. As a result, chronic elevations in these cytokines have been implicated in diabetes. However, very little is known about the interaction between IL-6 and TNF-alpha. From the above discussion it is clear that our understanding of the individual actions of these cytokines is limited. So, it is not surprising that there is even less known about their interaction.

A few studies have looked at TNF-alpha induced IL-6 production and have found that TNF-alpha increases the production of IL-6 in many tissues including adipose tissue (167), smooth muscle (26), and skeletal muscle (11; 40). The effect of TNF-alpha infusion on circulating concentrations of IL-6, on the other hand, has generated mixed findings. Some studies have found no increase in plasma IL-6 with TNF-alpha infusion (171; 176), whereas others have found an increase in IL-6 (122). Interestingly, anti-TNF-alpha therapy resulted in a decrease in plasma IL-6 (187). Alternatively, IL-6 may suppress TNF-alpha production (107), but recently Carey et al. (2006) infused IL-6 into human subjects and found no change in circulating TNF-alpha (33). The interaction observed between these two cytokines to date could indicate the presence of feedforward and feedback communication.

It may be possible that the interaction between IL-6 and TNF-alpha is synergistic in nature or the presence of one cytokine may override the effects of the other. Bruce and Dyck (2004) found that the TNF-alpha-induced increase in fatty acid incorporation into

diacylglycerol in skeletal muscle was inhibited by IL-6 (28). However, to my knowledge, it is unknown how IL-6 and TNF- α interact to affect glucose transport.

CHAPTER 3: PURPOSE OF RESEARCH

There has been a great deal of interest in the role of cytokines in metabolic processes with the hopes of providing further understanding of the mechanisms behind the dysregulation of glucose and fatty acid metabolism in disease states. Interleukin-6 and TNF-alpha have been considered to be likely candidates for metabolic regulators, particularly in skeletal muscle metabolism, as they have been implicated in disease states, such as diabetes and cardiovascular disease.

The role of IL-6 in carbohydrate metabolism is highly controversial. It has been well established that during exercise IL-6 is released in significant quantities (40; 110; 202; 204). Exercise bouts that result in IL-6 production are also characterized by glycogen degradation and glucose utilization (205). A number of studies have demonstrated an inverse correlation between glycogen levels and circulating plasma levels of IL-6 (109; 202). However, this has been challenged by Hiscock et al. (92). Furthermore, studies have not carefully examined post exercise events where glycogen resynthesis is taking place and IL-6 levels are declining in the circulation.

The presence of IL-6 receptors on the muscle (111) suggests that when IL-6 is released during exercise, it may be acting locally on the muscle to facilitate glucose transport. The question has been posed (60), if IL-6 is released from an exercising skeletal muscle, then why is it implicated as a biomarker of disease? This is a highly debated question and studies examining the effect of IL-6 on glucose transport and insulin signalling have generated conflicting findings.

Tumour necrosis factor alpha is also a cytokine of metabolic interest, as it is implicated in a variety of diseases. Although there is general agreement that TNF-alpha causes insulin resistance in skeletal muscle, there are some inconsistencies. Furthermore,

there is only one study to date examining the direct effect of elevated TNF-alpha concentrations on glucose metabolism in contracting skeletal muscle (244).

Finally, there is very little research examining the interaction between IL-6 and TNF-alpha. The existing research suggests that these cytokines can influence the actions of each other, but their combined effect on glucose transport remains elusive.

Therefore, this thesis sought to ascertain 1) whether a relationship exists between glycogen and IL-6 during exhaustive exercise and in the period of recovery when dietary carbohydrate is provided (study 1); 2) to examine if there is a direct relationship between IL-6 and TNF-alpha on basal and insulin-mediated skeletal muscle glucose transport and insulin signalling in soleus muscle strips (Study 2), and 3) to determine the direct effect of IL-6 and TNF-alpha on contraction-mediated glucose transport and signalling intermediates in soleus muscle strips (Study 2).

Research Hypotheses:

1. The reduction in muscle glycogen experienced during two similar bouts of prolonged exercise will be related to increases in circulating IL-6 and muscle IL-6 mRNA. Secondly, accelerating the rate of muscle glycogen restoration via CHO feeding early in recovery will result in a more rapid decline in circulating IL-6 and skeletal muscle IL-6 mRNA during recovery.
2. Exposure of resting rodent soleus muscle strips to IL-6 will result in increases in glucose transport; however, exposure of muscle strips to TNF-alpha will decrease glucose transport. The combination of the two cytokines will result in a net negative effect on glucose transport into soleus muscle. Further, similar findings will be observed with or without insulin present.

3. Incubation of contracting soleus muscle strips in IL-6 will result in an increased glucose transport; however, exposure of contracting muscle strips to TNF-alpha will decrease glucose transport. Exposure of the muscle to both IL-6 and TNF-alpha will result in no change in glucose transport.

4. There is limited and conflicting research regarding the mechanism by which IL-6 and TNF-alpha exert their effects in skeletal muscle. As such, I am unable to create a hypothesis but rather pose the research question, "what effect does the acute incubation of soleus muscle strips with both IL-6 and TNF-alpha have on glucose transport and the phosphorylation of relevant signalling proteins".

CHAPTER 4

Interleukin-6 and its mRNA responses in exercise and recovery: relationship to muscle glycogen

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ABSTRACT

Increases in circulating IL-6 during exhaustive exercise has been suggested to be related to declining muscle glycogen. We addressed two hypotheses: a) exhaustive exercise on two occasions will result in similar decreases in glycogen and increases in circulating IL-6 and its muscle mRNA; b) increasing the rate of glycogen restoration via high carbohydrate feeding in recovery will be correlated with more rapid declines in muscle mRNA and circulating IL-6. Ten male subjects (22.6 ± 0.8 y) cycled to exhaustion (65% $\text{VO}_{2\text{max}}$) on two occasions (117.8 ± 2.9 min). Carbohydrate (1 g/kg bw) or water was ingested at exhaustion, 60, 120, 180, and 240 min post-exercise. Muscle biopsies were taken at rest, exhaustion, 30, 60, 120 and 300 min of recovery. Exercise resulted in a 14.5-fold increase ($p < 0.05$) in IL-6 mRNA, 14.4-fold increase ($p < 0.05$) in circulating IL-6, and an 80% decrease ($p < 0.05$) in muscle glycogen from rest. The decline in glycogen was not correlated with the increase in plasma IL-6 or IL-6 mRNA. During recovery, circulating IL-6 and its muscle mRNA decreased similarly in both trials, however, glycogen increased 150% ($p < 0.05$) and 40% in the carbohydrate and water trials, respectively. Therefore, the declining IL-6 mRNA and IL-6 plasma concentrations during recovery were not related to carbohydrate availability or changes in glycogen.

Key Words: cytokine, carbohydrate, exhaustion, gene expression, fatigue

Introduction

Under resting conditions, skeletal muscle does not release large quantities of interleukin-6 (IL-6). However, during prolonged exercise, dramatic increases in circulating levels of IL-6 have been observed (149; 152). The increase in IL-6 is due to increased production and release by active skeletal muscle (92; 186; 206). Qualitative increases in IL-6 protein in skeletal muscle cells have also been detected following exercise (163).

A putative stimulus for the production and release of IL-6 from the active muscle is the declining muscle glycogen concentration (109; 202). These studies showed a strong correlation between declining muscle glycogen levels during exercise and both the increasing IL-6 release and the increase in IL-6 mRNA. Moreover, when pre-exercise levels of glycogen were reduced, release of IL-6 occurred earlier in exercise (202). Furthermore, provision of carbohydrate (CHO) during exercise attenuated the release of IL-6 (199). Interestingly, the increase in IL-6 mRNA typically detected during exercise was maintained, suggesting that carbohydrate availability affects either translation of mRNA or the release of IL-6 from the muscle.

Few studies have examined IL-6 during recovery from exhaustive exercise. Shortly after the cessation of exercise, plasma levels of IL-6 begin to decline, but remain above basal concentration after 4h of recovery (64; 150; 151; 163). Furthermore, immunohistochemical detection of IL-6 expression in skeletal muscle showed that levels remained elevated for at least 9 h following exhaustive cycling exercise (163). Although the decrease in plasma IL-6 post- exercise occurred while glycogen levels begin to rise, the relationship between these two parameters has not been investigated.

Therefore, the following investigation addressed two hypotheses. First, bouts of prolonged exercise of identical intensity and duration that reduces glycogen on two occasions will result in similar increases in circulating IL-6 and muscle IL-6 mRNA. Secondly, accelerating the rate of glycogen restoration via CHO feeding early in recovery will result in a more rapid decline in circulating IL-6 and skeletal muscle IL-6 mRNA during recovery.

Materials and Methods

Subjects.

Ten physically-active, male subjects (22.6 ± 2.8 y; 80.4 ± 2.8 kg; 183.6 ± 2.1 cm (mean $\pm$ SE) who participated in physical activity at least 3 times per week were recruited. None were taking any medications, particularly those known to affect muscle performance. Other exclusion criteria included diabetes, liver and/or kidney dysfunction, orthopaedic conditions, and neurological disorders. The study was approved by the University of Guelph Human Ethics Committee and informed, written consent was obtained from each subject prior to participation in the study.

Preliminary Testing.

Subjects performed a maximal oxygen uptake protocol prior to the start of testing. $\text{VO}_{2\text{max}}$ was determined (4.4 ± 0.2 L/min) with cycle ergometry using a graded workload protocol. The resistance was increased by 50 watts (W) every 2 min until 300 W was reached and then increased by 25 W each minute until voluntary exhaustion. Breath-by-breath expired oxygen (VO_2), carbon dioxide (VCO_2), ventilation (L/min), and calculated $\text{VO}_{2\text{max}}$ (L/min) were recorded using the Sensor Medix Vmax Series Metabolic Measurement System. Two days prior to each of the 2 trials, subjects performed a 1 h bout at 65% of $\text{VO}_{2\text{max}}$ at a pre-determined, constant cadence chosen by the subject.

The subject would exercise at this cadence during subsequent testing. After this exercise bout, subjects were given pre-packaged meals (CHO 55%, fat 30%, protein 15%; 3000 kcal per day) to ingest for the next 36 h, until their arrival in the lab on the day of the trial. Subjects were advised to eat only the food assigned to them and to refrain from caffeine-containing products, alcohol and physical activity for 3 d prior to each experiment.

Experimental Protocol.

Each subject completed 2 trials, separated by at least 1 wk. On the day of the experiment, subjects were fed a standardized breakfast (CHO 55%, fat 30%, protein 15%) 2 h prior to the exercise trial. A catheter was inserted in an antecubital vein for blood sampling and kept patent with a saline drip. After a resting muscle biopsy and blood sample were taken, subjects exercised at 65% of VO₂ max until voluntary exhaustion (117.8 ± 2.9 min) on an electronically braked, Lode cycle ergometer. Subjects consumed water throughout the trial. The total volume of water was recorded in the first trial and repeated during the second trial. Blood samples were taken every 30 min during exercise and measurements of oxygen consumption were taken every 20 min to ensure that the subject was exercising at the desired intensity. At exhaustion, a muscle biopsy was taken. Additional muscle biopsies were taken at 30, 60, 120, and 300 min post exercise. Muscle biopsies were taken from the *vastus lateralis* by percutaneous needle biopsy technique under local anesthesia. Four biopsies were taken from one leg and two from the other (each incision allowed for two biopsies) on the first day of testing. The selection of legs was reversed on the second day of testing. Blood was sampled every 30 min for the first 2 h of recovery and every hour thereafter. In the second trial, subjects were instructed to exercise for the same duration and at the same power output. During recovery, subjects were given equal volumes of either water (placebo (PL)) or 1 g/kg (80.4 ± 8.7 g) CHO

(Gatorlode ®, mixed with water) at exhaustion, 60, 120, 180, and 240 min of recovery.

The order of the CHO and PL trials were randomized and counterbalanced. By the end of 5 h of recovery, subjects had ingested either ~400 g of CHO or an equivalent volume of water.

Analysis.

Blood samples.

Mixed venous blood was analyzed for glucose, insulin, free fatty acids (FFA), and IL-6. Whole blood glucose was analyzed immediately by a glucose oxidase method (YSI 2300 Stat Plus Glucose Analyzer). At each time point, ~5 ml of blood was collected in a non-heparinized tube and allowed to clot at room temperature. Samples were centrifuged for 10 min at 1200 x g and serum was stored at -20°C until analyzed for insulin (Coat-a-count Insulin, Intermedico Diagnostic Products, intra-assay CV was 3.5%) and FFA (NEFA kit, Wako Chemical, intra-assay CV was 1.1%). IL-6 in plasma was determined from blood collected in EDTA tubes. These tubes were kept on ice and spun at 4°C and 1000 x g for 15 min. Coefficient of variation for the IL-6 assay was 7%. The concentration of IL-6 was assessed using a quantitative sandwich ELISA kit (R&D Systems, Inc. Minneapolis, MN). Samples taken late in the exercise bout (90 min, 120 min), exhaustion, and early recovery (30 min) were diluted (4-10x) in order to keep the readings within the assay standard curve (0-10 pg/ml).

All blood assays were performed in duplicate and means of duplicates were evaluated.

Muscle Samples.

Skeletal muscle biopsies were dissected free of fat and any visible connective tissue and frozen in liquid nitrogen in approximately 15 seconds and stored at -80°C for later analysis.

Glycogen.

Samples (~15-20 mg wet weight (ww)) were freeze dried and stored at -80°C. The dried muscle was manually powdered to obtain 2-3 mg dry weight (dw) of sample. Pro- (PG) and macroglycogen (MG) were analyzed enzymatically as previously described (2; 3). Briefly, PCA was used to separate the acid-soluble (macroglycogen fraction) from the acid-insoluble (proglycogen fraction). Hydrochloric acid was then added to each fraction to hydrolyze glycogen to glucose. After 2 h incubation at 100°C, Tris buffer was added to neutralize both fractions. The extract was then analyzed for glucosyl units (20). The sum of PG and MG fractions equals total glycogen and only the total is reported in this paper. The coefficient of variation for this method was 6.0%.

RNA extraction.

RNA was extracted from 15-20 mg of fresh muscle using a modified Chomczynski and Sacchi (1987) method (42). Briefly, 500 µl of TRIZOL reagent was added and tissues were homogenized for 30 s (Powergen 125, Fischer). After incubating samples for 3 min at room temperature, 100 µl of chloroform was added and shaken by hand for 15 s. Following a second room temperature incubation (5 min), samples were centrifuged for 15 min at 12,000 x g at room temperature. The aqueous phase was then removed to a separate tube at 250 µl of isopropanol was added. RNA was precipitated after 30 min incubation on ice and subsequent centrifugation at 12,000 x g at 4°C for 30 min. The pellet was recovered, washed with 500 µl of 75% ethanol and centrifuged for 5 min at 7500 x g at 4°C. Once again the pellet was isolated, dried, and DEPC (diethylpyrocarbonate)-treated water (40 µl at 55°C) added. Finally, the pellet was dissolved after being heated for 5 min at 55°C. The concentration and purity of the RNA

isolation was determined using 2 µl of the extract by spectroscopy. The product was stored at -80°C for later use.

cDNA Synthesis.

Reverse transcription was performed on samples using SuperScript™ II RT (Invitrogen). Simply, RNA was combined with random primers (150 ng) and dNTP (nucleotides) mix (10 mM each) and brought to a total volume of 12 µl with sterile, distilled water. The RNA was denatured at 65°C for 5min, quickly chilled on ice and then briefly centrifuged. Then 5X first-strand buffer (4 µl) and 0.1 M DTT (dithiothreitol) (2 µl) was added, mixed gently, and then incubated at 25°C for 2 min. Finally, 200 units of SuperScript™ II RT (1 µl) was added along with sterile, distilled water (total volume 20 µl) and then incubated at 42°C for 50 min. The reaction was inactivated by heating at 70 °C for 15 min. The cDNA was stored at -20 °C for later use.

RT-PCRs

Quantification of mRNA expression was performed in triplicate by real-time RT-PCR using the ABI PRISM 5700 sequence detection system (Applied Biosystems). PCR reactions were carried out in triplicate using SYBR® Green qPCR SuperMix UDG Kit (Invitrogen). For each reaction, 0.5 µl cDNA (1 µg) was combined with 12.5 µl Supermix, 0.25 µl ROX Passive Reference Dye, 0.5 µl (10 pM) forward and reverse primers (for 18S or IL-6), and 6.75 µl Milli-Q to a final volume of 25 µl. Primers were designed using Premier Biosoft primer program (Netprimer). GenBank sequences were used to design primers for IL-6 (Accession no. M54894) and 18S rRNA (Accession no. M10098.1). IL-6 forward and reverse primers were 5'-TGA AAG CAG CAA AGA GGC ACT-3' and 5'-GCA AGT CTC CTC ATT GAA TCC AG-3', respectively, with an amplicon size of 100-bp. 18S rRNA forward and reverse primers were 5'-GAC TCA

ACA CGG GAA ACC TCA C-3' and 5'-ATC GCT CCA CCA ACT AAG AAC G-3', respectively, with an amplicon size of 114-bp (Lab Services, University of Guelph). No template controls and cDNA were amplified in triplicate using the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each sample, a critical threshold (C_T) was detected that identified the cycle number that gene amplification is first detected. A delta (Δ) C_T value was obtained by subtracting the C_T for 18s from that of IL-6. The resting ΔC_T were subtracted from exercise and recovery samples to determine a $\Delta\Delta C_T$ value. The efficiency was assessed to be 100% (See Appendix D); therefore the expression of IL-6 was assessed based on $2^{-(\Delta\Delta C_T)}$. Dissociation curve analysis confirmed a single product for both the endogenous control and the gene of interest. The coefficient of variation for this method was calculated to be 5.3% using the C_T values.

Data Analysis.

Data was analyzed using a two-way, repeated measures ANOVA using Sigma Stat 3.1 and was reported as mean $\pm$ SEM. Data was log-transformed where necessary. Significance was established at $P < 0.05$. When a significant interaction was identified, a Tukey's post-hoc test was used. A small number of data points were deemed to be outliers. Outliers were data that more or less than 1.5 times the interquartile range (IQR) and were not included in this analysis. For example, 9 of 120 IL-6 mRNA data points met these criteria. Four percent of all data were found to be outliers based on the criteria used. The relationship between IL-6 (either circulating concentration or mRNA) and glycogen, insulin, glucose, and free fatty acids was assessed using Pearson Product Moment correlation and linear regression analysis. Finally, a paired t-test was used to examine differences in area under the curve.

Results

Glucose, Insulin and Free Fatty Acids

Exhaustive exercise repeated on two occasions resulted in similar circulating glucose, insulin and FFA responses (*Table 5*). Serum insulin declined ($p < 0.05$) during exercise while blood glucose levels were maintained ($p < 0.05$).

Table 5. Blood Glucose, Insulin, and Free Fatty Acids¹

	EXERCISE				RECOVERY				
	Baseline	30 min	60 min	90 min	EXH	30 min	60 min	120 min	300 min
<i>Treatment</i> ²	<i>Glucose (mM)</i>								
Placebo	4.3±0.2	3.8±0.2	3.7±0.1	3.5±0.1	3.1±0.2	4.0±0.4	4.1±0.5	4.4±0.4	4.2±0.3
	<i>a,c,f</i>	<i>b,a,c</i>	<i>b,a,c</i>	<i>b,a</i>	<i>b</i>	<i>c,e*</i>	<i>e*</i>	<i>e*</i>	<i>a,e*</i>
CHO	4.0±0.2	3.6±0.1	3.7±0.2	3.4±0.2	3.3±0.2	6.0±0.4	6.9±0.5	6.6±0.5	4.6±0.1
	<i>Insulin (pM)</i>								
Placebo	13.9±2	4.1±0.6	1.7±0.4	1.1±0.1	1.0±0.3	1.4±0.4	1.4±0.3	4.5±3	2.3±0.3
	<i>b,d</i>	<i>A</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a,b*</i>	<i>c,b*</i>	<i>d*</i>	<i>d*</i>
CHO	16.6±3	3.2±0.7	1.8±0.3	1.0±0.2	0.8±0.2	12.6±2	17.3±3	36.5±6	48.7±15
	<i>Free Fatty Acids (μM)</i>								
Placebo	226±45	296±66	576±145	861±152	1163±131	1792±203	1888±199	1869±155	1268±86
	<i>a</i>	<i>a</i>	<i>a,e</i>	<i>b</i>	<i>b</i>	<i>c*</i>	<i>c,b*</i>	<i>b,c,d*</i>	<i>b,c,d,e*</i>
CHO	125±15	270±44	542±90	952±122	1096±101	993±118	602±182	169±52	69±21

¹ mean±SE

² The treatment was not introduced until exhaustion; therefore both trials were similar during baseline, 30, 60, 90 min of exercise

CHO = carbohydrate

* $P < 0.05$ between treatments

Unlike letters represent $P < 0.05$ within a blood measure (FFA, insulin, glucose)

FFA were significantly ($p < 0.05$) elevated by the end of the exercise bout. After the cessation of exercise, ingestion of dietary CHO throughout recovery caused an increase ($p < 0.05$) in serum insulin and blood glucose levels as anticipated. Circulating glucose returned ($p > 0.05$) to levels comparable to placebo by 300 min of recovery. From 30-300 min post-exercise in the placebo trial, glucose and insulin levels were not different ($p > 0.05$) from the pre-exercise values. FFA remained elevated ($p < 0.05$) in the placebo trial, but declined with the ingestion of CHO as anticipated.

Muscle Glycogen

Figure 4. Skeletal muscle glycogen following ~2 h of exhaustive exercise and 5h of recovery. Subjects ingested 1g/kg of carbohydrate (CHO) or an equal volume of water (PL) every 60 min for 300 min of recovery. An * indicates between group differences ($p < 0.05$), + indicates within group differences ($p < 0.05$) vs EXH, 30, 60, 120, 300, § indicates within group differences ($p < 0.05$) vs EXH, 30, 60 min, and † indicates within group differences ($p < 0.05$) vs Exh, 30 min.

Concentrations of muscle glycogen are shown in **Figure 4**. Resting muscle glycogen was similar in both trials (CHO, 450.5 ± 30.4 ; PL, 459.7 ± 27.6 mmol glucosyl units/kg dw). Exercise duration was not significantly ($p < 0.05$) different between the two trials (Trial 1, 118.5 ± 3.5 min; Trial 2, 117.1 ± 4.9 min). However, times to exhaustion are not identical because 3 subjects fatigued prematurely on day 2. These subjects demonstrated similar variability in outcome measures as those who cycled for the same duration in both trials. Muscle glycogen levels at exhaustion were also similar ($p > 0.05$) in both trials (Trial 1, 108.2 ± 19.5 (24% of pre-exercise levels); Trial 2, 73.4 ± 12.7 (16% of pre-exercise levels) mmol glucosyl units/kg dw). Thus, prior to the two treatments, the procedure was successful in achieving very similar metabolic conditions in terms of exercise duration, changes in glycogen, and blood insulin, glucose and FFA.

Following introduction of the treatments in recovery, a significant ($p < 0.05$) increase in total glycogen was noted at 120 and 300 min in the carbohydrate trial. By 300 min, muscle glycogen levels were approximately 59% of resting levels. Alternatively, ingestion of water (placebo) during 300 min did not significantly ($p > 0.05$) increase glycogen levels, which were 22% of rest at 300 min of recovery.

Plasma IL-6

Mixed venous IL-6 concentrations are shown during and after exercise in **Figure 5**. Systemic IL-6 levels increased ($p < 0.05$) 10-15 fold with exercise, reaching a maximal value at exhaustion (CHO, 11.4 ± 1.2 pg/ml; PL, 13.4 ± 2.3 pg/ml; $p = 0.51$) and declined after the cessation of exercise.

Compared to the end of exercise, IL-6 levels were significantly ($p < 0.05$) lower after 60 min of recovery in both groups. Despite the different metabolic conditions, there

was no difference in IL-6 between CHO and PL ingestion during recovery. Similarly, area under the curve analysis failed to show a distinction between CHO and PL (CHO, 2145.5 ± 338.0 pg/ml; PL, 2385.9 ± 378.6 pg/ml, $p=0.48$).

Figure 5. Plasma IL-6 following ~2 h of exhaustive exercise and 5 h of recovery. Subjects ingested 1g/kg of carbohydrate (CHO) or an equal volume of water (PL) every 60 min for 300 min of recovery. An * indicates within group differences ($p<0.05$) vs Rest, E30, + indicates within group differences ($p<0.05$) vs E60, E90, 60, 120, 30 min.

Increasing levels of plasma IL-6 observed throughout exercise (rest, 30, 60, 90, exhaustion) were correlated ($p<0.001$) negatively with glucose ($r=0.50$), insulin ($r=0.40$), and positively with FFA ($r=0.53$). In marked contrast, glycogen degradation with exercise during the two trials was not correlated with increases in IL-6 (trial 1: $r=0.50$, $p=0.14$; trial 2: $r=0.03$; $p=0.92$). When the net change from rest to exhaustion from both trials were combined, the result remained nonsignificant ($p>0.05$) (*Figure 6a*).

Figure 6a. Correlational analysis between glycogen depletion (change from rest to exhaustion) and rise in interleukin-6 in both trials (n=9). The data of the two trials for the same subject are connected with a solid line. The dashed line indicates linear regression ($r^2 = 0.009$; $p=0.7$). Examples of the inconsistencies of a possible IL-6 and glycogen relationship are highlighted in two subjects: (Δ) similar decreases in glycogen, but a large difference in IL-6 and ($\square$) large difference in glycogen, but very similar elevations in IL-6.

Similarly, at exhaustion, a significant ($p>0.05$) correlation was not found when the difference between trials (Trial 1 – Trial 2) for IL-6 and glycogen were compared ($r=-0.38$; $p=0.31$) (*Figure 6b*). Over 5 h of recovery, plasma IL-6 was not correlated ($p>0.05$) with circulating insulin, glucose, or FFA. Likewise, during recovery, changes in glycogen with ingestion of CHO was not correlated with decreases in plasma IL-6 ($r=-0.15$; $p=0.29$). There was also no correlation in these parameters in the PL trial ($r=-0.27$; $p=0.06$).

Figure 6b. Correlational analysis between the difference in IL-6 in the 2 tests and that for glycogen at exhaustion within subjects ($n=9$). One subject was omitted for technical reasons. Each data point represents a subject and was generated by subtracting the glycogen or IL-6 at exhaustion on day 1 from the respective value on day 2. The dashed line indicates linear regression ($r^2 = 0.14$; $p=0.3$).

IL-6 muscle mRNA

Expression of IL-6 in skeletal muscle is shown in **Figure 7**. Four data points at exhaustion are not included due to insufficient sample. Exhaustive exercise resulted in a 14.5-fold increase in IL-6 mRNA ($p<0.05$). After 30 min of recovery, IL-6 mRNA remained elevated, but then declined toward resting levels by 60 min. Increase in IL-6 mRNA during exercise was significantly ($p<0.05$) correlated with corresponding plasma levels of IL-6 during exercise ($r=0.54$) and also in recovery (0.57 ; $p<0.05$).

Figure 7. IL-6 mRNA following ~2h of exhaustive exercise and 5h of recovery (n=10). IL-6 mRNA is expressed as a fold change from rest. A + indicates within group differences versus rest and ‡ indicates within group differences ($p<0.05$) vs 30, 60, 120, 300 min.

In contrast, muscle glycogen and IL-6 mRNA was not correlated over 5 h of recovery when CHO ($r=-0.05$; $p=0.90$) or PL ($r=-0.46$; $p=0.25$) was ingested. IL-6 mRNA decreases were comparable despite differences in glycogen levels throughout recovery. Furthermore, the net change in glycogen from rest to exhaustion was not correlated with the net change in IL-6 mRNA ($r=-0.39$; $p=0.14$) (*Figure 8*).

Discussion

The current investigation examined two hypotheses. The first was that decreases in muscle glycogen concentrations observed during similar bouts of prolonged exercise on two different occasions would be correlated with increases in plasma IL-6 and its

mRNA. The second was that increasing the rate of glycogen repletion with CHO ingestion in recovery will result in a more rapid decline of IL-6 in the circulation.

As expected, prolonged exercise to exhaustion lowered glycogen stores, decreased blood insulin and glucose, and increased serum FFA, and there was no significant difference between trials suggesting that similar metabolic situations were generated. In addition, exercise resulted in a marked rise in plasma IL-6, consistent with past exercise studies (109; 149; 152; 199). There was no significant difference between trials in IL-6 at exhaustion. As glycogen decreased, circulating IL-6 increased and a significant, negative correlation between rest and exhaustion IL-6 and glycogen concentration was noted during exercise on both occasions. While this gross comparison supports the hypothesis that increasing IL-6 during exercise is related to declining muscle glycogen, a more detailed examination of the net change in glycogen and the net change in IL-6 from rest to exhaustion, failed to generate a significant correlation (Figure 3a). Of particular importance is the within subject day-to-day variations in changes in glycogen. When the difference within subjects at exhaustion in IL-6 and glycogen was compared, there was no correlation between the two variables. For example, in one subject, differences in glycogen depletion in the two trials (475.3 mmol glycosyl units/kg dw; 545.1 mmol glycosyl units/kg dw) were associated with modest differences in the increase in IL-6 (15.3 and 13.1 pg/ml, respectively). In another subject who had similar differences in glycogen in the two trials (377.1 mmol glycosyl units/kg dw; 444.7 mmol glycosyl units/kg dw), demonstrated large differences in the increase in IL-6 (15.2 and 25.0 pg/ml, respectively). These data fail to support the hypothesis that decreases in muscle glycogen are related to elevations in IL-6 with exercise. While we do not have a

direct measure of IL-6 release or intramuscular concentration, previous studies (92; 163; 202) demonstrated that blood IL-6 is an accurate reflection of muscle production and release.

IL-6 mRNA increased nearly 15-fold at the end of exercise. The magnitude of increase in IL-6 mRNA is similar to previously observed levels (92; 109; 202). There was no significant correlation between the amount of glycogen breakdown and the fold change of mRNA from rest (Figure 5a). This is consistent with the lack of correlation between glycogen and IL-6 gene expression in muscle and fails to support the hypothesis.

Previous studies (64; 163; 186) have noted that upon cessation of exercise, a decrease in circulating IL-6 was matched by a reduction in IL-6 release from skeletal muscle and thus we interpret the decrease in IL-6 in the present study as a reflection of a decrease in production and release from muscle. As expected from previous studies (3; 17), glycogen resynthesis was markedly increased with provision of 1 g/kg/h of CHO, as were circulating glucose and insulin levels, while FFA levels were blunted. Neither plasma IL-6, nor skeletal muscle IL-6 mRNA, was affected by ingestion of CHO during the 5 h following exercise. There were no significant correlations during recovery between IL-6, IL-6 mRNA and circulating glucose, insulin or FFA levels. Similarly, we did not observe a correlation between plasma IL-6 and glycogen during 5 h of recovery from exercise. Therefore, changes in substrate availability and subsequent CHO storage during recovery from exhaustive exercise do not appear to influence plasma IL-6 levels or gene expression in skeletal muscle.

Figure 8. Correlational analysis between the change in glycogen and interleukin-6 mRNA from rest to exhaustion. The dashed line indicates linear regression ($r = -0.39$; $p = 0.13$; $n = 16$). Two trials for the same subject are connected by a line ($n = 6$). Four subjects were missing a value at exhaustion and thus only have one data point on the graph.

The skeletal muscle biopsies taken would be a mix of type I and type II fibres and it is likely that many of the type II fibres were not active in the exercise bout (76). There is controversy regarding skeletal muscle fibre type and production of IL-6. Penkowa et al. (2003) found IL-6 to be equally distributed in both type I and type II fibres using immunohistochemical procedures (163). On the other hand, Hiscock et al. (2004) found that following an exercise bout, similar to that of the current study, increases in IL-6 protein and mRNA expression were predominantly in type II fibres. The authors were unable to determine why their results varied from that of Penkowa et al. (163), other than some slight methodological differences in immunohistochemical detection (92). If the

latter study is correct, this could explain our lack of relationship between glycogen and IL-6.

A possible limitation of the current study design is the taking of two biopsies from a single incision. Malm et al. (2000) demonstrate that the biopsy technique causes an inflammatory response; however, the inflammatory response commences at 6h of recovery. Furthermore, they do not evaluate the effect of multiple biopsies from the same incision site (131). If a second biopsy from the same site should bear evidence of a local inflammatory reaction, then every second data point should show a large increase in the mRNA for IL-6. Examination of figure 4 (and our examination of the data for individual subjects) shows no sign of such elevations in the data especially that at the 4th and 6th data point.

If alterations in muscle glycogen are not the stimulus for exercise or recovery levels of IL-6, then there must be other factor(s) enhanced by exercise, and then inhibited early in recovery. One possible mediator of IL-6 may be the rapid increase in cytosolic calcium levels that occurs during exercise (60). It is likely that there are a number of regulatory factors for the expression of the IL-6.

IL-6 has also been proposed to coordinate the systemic provision of fuels to the active skeletal muscle during exercise (60; 74). In support of this, in the current study, IL-6 levels increased during exercise as glucose and insulin levels declined and FFA increased. However, in recovery, similar declines in IL-6 in the two trials despite different FFA and glucose levels suggests that IL-6 is not a dominant metabolic signal at this time and that other factors such as insulin are more important.

In conclusion, this study is novel as it used similar bouts of exercise in the same subject to examine the relationship between declining glycogen stores and rises in plasma IL-6 and employed different nutritional strategies to determine if post exercise IL-6 responses are sensitive to fuel availability. Based on the current findings, neither glycogen depletion nor repletion appears to have a direct relationship with IL-6 during and following exhaustive exercise. Further research is required to clarify the mechanism by which IL-6 gene expression is initiated and IL-6 is released from the muscle.

Perspectives

Nutrition immediately following exercise is an important topic in sports medicine: IL-6 has been proposed to be an important metabolic signal (158) to liver and adipose tissue during exercise. While the role(s) of IL-6 released from muscle remain to be clarified, if IL-6 served such a function during the early phase of recovery, then providing CHO exogenously could interfere with these processes. This study shows that ingestion of CHO during recovery does not disrupt the normal IL-6 response. It is unclear from these findings whether IL-6 is a vital metabolic signal during recovery.

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ABSTRACT

Interleukin-6 and TNF- α have been suggested to influence glucose transport in skeletal muscle, but the nature of their actions are controversial. We addressed the hypothesis that IL-6 increases glucose transport in rodent soleus muscle strips with or without insulin present and during muscle contraction. Furthermore, we hypothesized that TNF- α would interfere with the actions of IL-6 when present together in the medium. We observed a ~1.5-fold increase ($P < 0.05$) in basal glucose transport with IL-6 (5 ng ml^{-1}), TNF- α (5 ng ml^{-1}), and IL-6 + TNF- α , and a corresponding increase ($P < 0.05$) in phosphorylation of AMPK and AS160. Insulin-stimulated glucose transport was decreased ($P < 0.05$) in the presence of IL-6 (-21%); TNF- α (-17%); IL-6 + TNF- α (-26%). Similarly, CaMKII and AS160 phosphorylation were decreased ($P < 0.05$). There was no additive effect of IL-6 and TNF- α on glucose transport or phosphorylation of these proteins. Electrical stimulation of soleus muscle strips increased ($P < 0.05$) glucose transport 1.9-fold versus basal. A further 25% increase ($P < 0.05$) in glucose transport was observed with the addition of IL-6 to the incubation medium. IL-6 also increased ($P < 0.05$) CaMKII and AS160 phosphorylation compared to control (contraction with no IL-6). There was no significant effect of TNF- α on contraction-mediated glucose transport; however, when TNF- α was present in the medium with IL-6, it prevented the increase in glucose transport and phosphorylation of CaMKII and AS160 observed with IL-6 alone. Taken together, our data suggest that the actions of IL-6 and TNF- α appear to depend on the signals present (i.e., insulin, contraction) that alter muscle metabolism.

Key Words: Soleus, rodent, exercise, AMPK, CaMKII, AS160, diabetes

Introduction

Interleukin-6 (IL-6) has been suggested to be involved in the regulation of glucose homeostasis. However, whether IL-6 promotes insulin resistance or enhances carbohydrate metabolism has been widely debated (133; 139; 156; 157). Interleukin-6 has been proposed to be a negative “effector” of metabolism because chronically elevated circulating concentrations are observed with disease states such as diabetes and obesity (141; 169; 174). Conversely, some studies have demonstrated increases in insulin sensitivity with IL-6 infusion (33; 211).

Under resting conditions, adipose tissue is primarily responsible for maintaining systemic concentrations of IL-6 (138). However, during exercise, IL-6 is produced by skeletal muscle (37; 149; 152; 204), with circulating IL-6 levels ranging from 10-80 pg ml⁻¹ and interstitial muscle concentrations reaching ~1200 pg ml⁻¹ during low-force exercise (186). As a result, IL-6 released from skeletal muscle has been proposed to act locally in an autocrine and paracrine fashion and systemically as an endocrine factor. Interleukin-6 release from contracting skeletal muscle has been positively correlated with skeletal muscle glucose transport (204). However, the direct effect of IL-6 on glucose transport in contracting muscle has not been examined. Recent investigations have reported a direct increase in basal glucose transport in skeletal muscle with exposure to IL-6 (9; 33; 73; 75); however, the findings under insulin-stimulated conditions are not as consistent. Two studies (9; 33) utilizing cell culture reported that IL-6 increased insulin-stimulated glucose transport. Geiger et al (2007) observed similar findings in rodent epitrochlearis muscle strips, but failed to observe a change in glucose transport in soleus

muscle strips (73). Another study (75) using human vastus lateralis muscle strips did not detect a change in insulin-stimulated glucose transport when IL-6 was present.

A number of proteins involved in translocation of glucose transporters to the cell surface have been evaluated as potential mediators of IL-6's effects on glucose transport in muscle. AMP-activated protein kinase (AMPK) has been identified as a likely candidate (9; 33; 73; 75), but it is possible that other proteins involved in GLUT-4 translocation could also be involved.

If IL-6 is involved in the regulation of glucose homeostasis, it is possible that it does not act independently, but interacts with factors, particularly other cytokines (e.g., TNF- α). Some attention has been given to the TNF- α -induced IL-6 production in adipose tissue (167), smooth muscle (26), and skeletal muscle (11). Furthermore, Bruce and Dyck found that IL-6 inhibited the TNF- α -induced increase in fatty acid incorporation into diacylglycerol in skeletal muscle (28).

Therefore, the present investigation examined the influence of IL-6 on glucose homeostasis, by exploring the direct effect of IL-6 and TNF- α on basal, insulin-stimulated, and contraction-mediated glucose transport in isolated rodent soleus muscle strips. It was hypothesized that IL-6 would increase glucose transport under all conditions and TNF- α would inhibit. It was also hypothesized that the effects of TNF- α would antagonize that of IL-6.

Methods

Ethical approval

All procedures were approved by the Animal Care Committee at the University of Guelph.

Animals and preparation of muscle strips

Female Sprague Dawley rats (Charles River Laboratory, QC, Canada; ~100-140g, n=125) were housed in a controlled environment on a 12h reversed light dark cycle and fed Purina rat chow. Female rats were chosen because of their small size and because of their mild temperament. Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg per 100g body weight). At the end of the dissection procedure, the rats were humanely killed with an intracardiac injection of sodium pentobarbital.

Soleus muscles were split longitudinally into strips prior to incubation with intact tendons using a 27-gauge needle as described previously (207). For the resting conditions (basal and insulin-stimulated), each strip was placed in a 20ml glass vial with 2 ml of pre-gassed Krebs-Henseleit buffer (KHB) + 0.1% albumin + gassed 1.3% bicarbonate warmed to 30 °C. This was the base buffer for all experiments.

Muscle strips designated to be electrically stimulated were dissected with tendons intact, sutured, removed, and suspended on brass hooks in a 20 ml incubation reservoir. The muscle strip was fixed to maintain resting tension, as previously described (56). Twelve millilitres of base buffer as described above was added to the incubation reservoir. The buffer was maintained at 30°C by an exterior circulating water system and was

continuously gassed. A layer of mineral oil was added to the top of the incubation buffer to maintain gassing pressures.

Protocol overview

Due to the limitation of tissue mass available for analysis, one series of experiments was conducted for measurement of glucose transport and a second series to facilitate the measurement of various signalling proteins. In both series, soleus muscle strips were either studied at rest, with or without insulin present, or during 30 min of contractions. In all cases the duration of the experiment was 70 min except for those of resting muscle when insulin was not present. Under this condition, the duration was extended to 90 min as a low rate of glucose transport was anticipated. To investigate the impact of contractions, the muscle was stimulated (150 ms trains composed of 0.5 ms impulses (20-40V, 60Hz frequency) at 20 tetani per minute) for 30 min.

Pre-incubation

The soleus muscle strips were incubated in the base buffer described above (KHB, NaHCO₃ and 0.1% albumin) plus 8mM glucose and 32mM mannitol for 30 min. For basal and insulin-stimulated conditions, after 30 min the muscle strip was then transferred to a second vial for 10 min containing the daily buffer and 32mM mannitol and 4mM pyruvate. A second wash was performed for 10 min after which the strip was moved to the third vial. The second wash was omitted in electrically stimulated muscle strips and rather, contraction was initiated in a buffer containing 8mM glucose and 32mM mannitol. If the protocol required the muscle to be exposed to IL-6 (5ng ml⁻¹) and/or TNF-alpha (5ng ml⁻¹) (Peprotech, Cedarlane Laboratories, Ontario, Canada), the cytokine

was present in the medium throughout the pre-incubation regardless of whether the final situation was exposure to insulin or not or was muscle contractions.

Experimental Incubation

The soleus muscle strip was moved to a final vial containing the experimental buffer (KHB base buffer) plus 8mM 3-O-[^3H] methyl-D-glucose ($0.5\mu\text{Ci ml}^{-1}$), 32mM [^{14}C] mannitol ($0.2\mu\text{Ci ml}^{-1}$). This medium also contained cytokine(s) and/or insulin, according the amounts required by the protocol. For resting muscle, the muscle strip remained in this vial for 20 min in the presence of insulin (60 pmol L^{-1}) and for 40 min when insulin was not present. The same buffer was employed for electrically stimulated muscle strips to measure glucose transport during the last 20 min of contraction.

Glucose transport

In the three experimental conditions, glucose transport (via 3-O-[^3H] methyl-D-glucose) was measured for the last 20 min in the contraction and insulin treatments and for the last 40 min in the resting muscle with no insulin. Upon removal of the strip from the final vial or the contraction reservoir, the muscle strip was dissected free from its tendons, blotted dry, and weighed. The strip was then added to 1M NaOH and heated to 100°C for 10 min. A 200ul sample of muscle digest was added to 5ml of scintillation fluid and left to quench overnight. Samples were counted using standard liquid scintillation counting techniques.

Signalling proteins by western blot analysis

In the three conditions, signalling proteins were measured in the muscle at the end of the protocol. In the condition with insulin present, insulin was added during the last 10 min of the protocol. Muscle strips designated for western blot analysis were blotted dry

and frozen immediately in liquid nitrogen for later analysis. Protein levels of phosphorylated ACC (ser⁷⁹; Cell Signalling), Akt (ser⁴⁷³/thr³⁰⁸; Cell Signalling), AS160 (thr⁶⁴²; BioSource International), AMPK (thr¹⁷²; Upstate), CaMKII (thr²⁸⁶; Cell Signalling), and total PI3-kinase p85 (Upstate) were measured. Frozen muscle samples were homogenized in ice cold homogenization buffer and 50-60 µg of total protein was loaded per sample. Samples were transferred to polyvinylidene fluoride (PVDF) membrane following SDS-PAGE electrophoresis (124). Equal loading was confirmed by nonspecific protein staining with Ponso-S. Blots designated to determine phosphorylated protein levels were blocked in Tris-buffered saline-Tween solution (TBS-T) containing 5% BSA (Sigma) (ACC) or 2.5% BSA (Akt (ser⁴⁷³ and thr³⁰⁸)) or 5% skim milk (Nestle) (PI3-kinase, AMPK, CaMKII). After washing with TBST, blocked membranes were incubated with the corresponding antibody overnight at 4°C or in the case of pACC, 1h at room temperature (RT). Membranes were washed with TBS-T for 30 min (3x10 min intervals) and were subsequently incubated for 1 h at RT with goat anti-rabbit IgG HRP-linked antibody (Santa Cruz). Proteins were visualized and relative densities quantified using the ChemiGenius 2 imaging system (Syngene). All data are expressed relative to basal (no cytokine or insulin present) or insulin-only conditions (no cytokines present).

Data Analysis

Data was analyzed using Sigma Stat 3.1 and was reported as mean ± SEM. Significance was established at $P < 0.05$. A one-way ANOVA and Fischer LSD post hoc determined differences between treatments. In the case where normality testing failed ($P > 0.05$), a one-way ANOVA on ranks and appropriate post-hoc tests were run.

Results

Basal conditions

Under basal conditions (no insulin) a significant ($P<0.05$) increase in glucose transport was observed following 70 min of exposure to IL-6 (+44%), TNF-alpha (+67%), and the combination of IL-6 and TNF-alpha (+44%). There was no evidence of an additive effect of IL-6 and TNF-alpha on basal glucose transport (*Table 6*).

Table 6. Glucose transport in soleus muscle strips ($\mu\text{mol/g/5min}$).

	<i>Control</i>	<i>IL-6</i>	<i>TNF-alpha</i>	<i>IL-6 + TNF-alpha</i>
<u>Resting</u>				
Basal	0.09 ± 0.01^a	0.13 ± 0.01^b	0.15 ± 0.01^b	0.13 ± 0.01^b
Insulin	$0.27 \pm 0.01^{a*}$	$0.21 \pm 0.02^{b*}$	$0.23 \pm 0.01^{b*}$	$0.20 \pm 0.01^{b*}$
Net Insulin ¹	$0.18 \pm 0.02^{a*}$	0.09 ± 0.01^b	0.08 ± 0.01^b	0.08 ± 0.01^b
<u>Contraction</u>	$0.17 \pm 0.02^{a*}$	0.22 ± 0.02^b	0.15 ± 0.01^a	0.14 ± 0.02^a

Values are means $\pm$ SE; n=12

¹Data were calculated by subtracting respective rates of basal transport from the insulin response. Unlike letters, $P<0.05$ within the same condition. * $P<0.05$ vs basal under control conditions

AMPK phosphorylation was increased under basal conditions ($P<0.05$) when exposed to IL-6 (+58%) and IL-6 + TNF-alpha (+60%) to a level similar to that observed with insulin (*Figure 9*). There was also a strong trend ($P=0.068$) for a similar result in the basal condition with TNF-alpha (+53%). In addition, Figure 10 shows that basal levels of AS160 phosphorylation were increased ($p<0.05$) by IL-6 (+56%), TNF-alpha (+49%), and IL-6 + TNF-alpha (+28%), but not to the extent of the insulin-induced phosphorylation of AS160 (+230%). There was no significant effect of these treatments

on PI3-kinase, or the phosphorylation of Akt (*Table 7*), AMPK, ACC, and CaMKII (*Appendix B*) under basal conditions.

Figure 9. Phosphorylation of AMPK (Thr172) by IL-6, TNF-alpha, and IL-6 + TNF-alpha in the absence of insulin. Values are means $\pm$ SE; n=6. *P<0.05 versus basal.

Figure 10. Phosphorylation of AS160 by IL-6, TNF-alpha, and IL-6 TNF-alpha in the absence of insulin. Values are means $\pm$ SE; n=6* P<0.05 v. basal, # P<0.05 vs insulin.

Insulin-stimulated conditions

Insulin stimulation increased glucose transport 3-fold over that of the basal condition. Insulin also increased PI3-kinase (*Table 7*) and the phosphorylation of AMPK (*Figure 9*), AS160 (*Figure 10 and 12*), CaMKII (*Figure 11*), Akt (ser⁴⁷³), and Akt (thr³⁰⁸). In contrast, there was a decrease ($P < 0.05$) in insulin-stimulated glucose transport in the presence of IL-6 (-21%); TNF-alpha (-17%); IL-6 + TNF-alpha (-26%) compared to the insulin-alone condition. Given that the cytokines increased basal glucose transport, the respective rates of basal glucose transport were subtracted from the comparative insulin condition (e.g., (IL-6 + insulin) – (IL-6 with no insulin present)). This calculation resulted in identifying an even greater decrease in transport (IL-6, -44%; TNF-alpha, -32%; and IL-6 + TNF-alpha, -44%). There was no additive effect on insulin-stimulated glucose transport when IL-6 and TNF-alpha were combined.

Figure 11. Phosphorylation of CaMKII by IL-6, TNF-alpha, and IL-6 TNF-alpha in the presence of insulin. Values are means $\pm$ SE; $n=6$, # $P < 0.05$ vs insulin.

Figure 12. Phosphorylation of AS160 by IL-6, TNF-alpha, and IL-6 TNF-alpha in the presence of insulin. Values are means $\pm$ SE; n=6, # $P < 0.05$ vs insulin, * $P < 0.05$ vs basal.

Figure 11 shows the decrease in insulin-stimulated CaMKII phosphorylation observed following incubation with IL-6 (-34%), TNF-alpha (-31%), and IL-6 + TNF-alpha (-31%). In addition, a reduced level of insulin-stimulated phosphorylation of AS160 by IL-6 (-26%), TNF-alpha (-39%), and IL-6 + TNF-alpha (-24%) was also noted, but remained greater ($P < 0.05$) than basal (**Figure 12**). Therefore, the effect of insulin on phosphorylation of AS160 was not completely obliterated and this is compatible with the glucose transport findings as there was still an elevation above basal in the presence of the cytokines. In the case of both CaMKII and AS160, no additive effect of IL-6 and TNF-alpha was detected. The cytokine treatments did not affect AMPK, ACC, Akt (ser⁴⁷³), Akt (thr³⁰⁸) and PI3-kinase with the one exception that TNF-alpha caused a significant reduction in the insulin-stimulated phosphorylation of Akt (thr³⁰⁸) as reported in Table 7.

Table 7. The effect of IL-6, TNF-alpha, and IL-6 + TNF-alpha on PI3-kinase and Akt (ser⁴⁷³ and thr³⁰⁸) in rat soleus muscle strips.

<i>Condition</i>	<i>Cytokine</i>	<i>PI3-kinase</i>	<i>Akt (ser⁴⁷³)</i>	<i>Akt (thr³⁰⁸)</i>
Basal ¹	none	1 ^a	1 ^a	1 ^a
	IL-6	0.89±0.2 ^a	0.98±0.2 ^a	1.02±0.2 ^a
	TNF-alpha	1.04±0.1 ^a	1.0±0.1 ^a	0.85±0.1 ^a
	IL-6 + TNF-alpha	1.13±0.2 ^a	0.94±0.2 ^a	0.84±0.1 ^a
Insulin ¹	None	1.28±0.1 ^b	2.76±0.5 ^b	9.07±2.7 ^b
	IL-6	1.28±0.2 ^b	2.53±0.6 ^b	10.8±1.7 ^b
	TNF-alpha	1.32±0.1 ^b	2.72±0.8 ^b	6.80±1.5 ^c
	IL-6 + TNF-alpha	1.25±0.2 ^b	2.68±0.6 ^b	9.25±1.1 ^b
Contraction ²	None	1	1	1
	IL-6	1.03±0.05	1.06±0.1	0.82±0.1
	TNF-alpha	1.09±0.2	0.92±0.2	0.76±0.1
	IL-6 + TNF-alpha	1.14±0.1	1.02±0.1	0.77±0.2

Unlike letters, P<0.05 within the same column.

¹Fold change versus Basal, no cytokines present

²Fold change versus Contraction, no cytokines present

Muscle contraction

Thirty minutes of electrical stimulation of soleus muscle strips resulted in a 1.9-fold increase in glucose transport compared to basal (rest with no insulin) (Table 6).

Contraction was associated with a 66% increase (P<0.05) in CaMKII (*Figure 13A*) and a 63% increase in AS160 (*Figure 14A*) compared to control (no contraction, no insulin).

There was no significant change in AMPK phosphorylation after 30 min (*Figure 15*).

Figure 13. Phosphorylation of CaMKII A) Basal versus contraction, [§] $P < 0.05$ vs. basal. B) during contraction in the presence of IL-6, TNF- α , and IL-6 + TNF- α , ^{*} $P < 0.05$ versus control. Values are means $\pm$ SE; $n = 6$.

In the presence of IL-6, there was a 2.4-fold increase in glucose transport compared to basal, which was 25% greater ($P < 0.05$) than contraction with no cytokines present. There was no significant effect of TNF- α or IL-6 + TNF- α on contraction-mediated glucose transport (*Table 6*).

IL-6 correspondingly increased contraction-mediated phosphorylation of CaMKII by 51% (*Figure 13B*) and AS160 by 70% (*Figure 14B*). In agreement with no significant change in glucose transport with either TNF- α alone or in combination with IL-6, there was no effect ($P > 0.05$) on the phosphorylation of these proteins. There was also no significant effect of contraction with or without cytokines on PI3-kinase (*Table 2*) or the phosphorylation of AMPK, ACC, (data not shown), and Akt (ser⁴⁷³, thr³⁰⁸).

Figure 14. Phosphorylation of AS160 A) basal versus contraction, [§] P<0.05 vs. basal. B) during contraction in the presence of IL-6, TNF- α , and IL-6 + TNF- α , *P<0.05 versus control. Values are means $\pm$ SE; n=6.

Figure 15. Phosphorylation of AMPK (thr¹⁷²) basal versus contraction.

Discussion

The present investigation used isolated rodent soleus muscle strips to examine the direct effect of IL-6 and TNF- α on glucose transport in skeletal muscle. The effects

of these cytokines were examined at rest, with or without insulin present, and during contraction. To our knowledge this is the first study to provide evidence for a direct role of IL-6 in skeletal muscle glucose transport during contractions. Other important observations included : 1) CaMKII, a calcium-induced protein, was found to be involved in the actions of IL-6 in skeletal muscle under insulin-stimulated and contraction-mediated conditions; 2) activation of AS160 was influenced by IL-6 under all metabolic conditions, which may suggest that AS160 is a point of convergence for different signalling pathways in skeletal muscle; 3) during contractions, TNF-alpha prevented the IL-6-induced increase in glucose transport; and 4) glucose transport generally changed in proportion to the degree of phosphorylation of CaMKII and AS160.

Effect of IL-6 on resting skeletal muscle

IL-6 and TNF-alpha increased basal glucose transport by 60 percent. The magnitude of increase in basal glucose transport by IL-6 is consistent with several recent studies (9; 33; 73; 75). However, although Geiger and coworkers observed an increase in transport in the epitrochlearis muscle of rodents, they also reported no change in glucose transport in soleus muscle strips (73). As discussed below, the protocol employed in the latter study may have accounted for the differences.

The IL-6- and TNF-alpha-induced increase in basal glucose transport in the present study corresponded to an increase in phosphorylation of AMPK and AS160. Previous reports (9; 33; 73; 75) have also proposed that AMPK may facilitate glucose transport via an increase in AS160 phosphorylation (215). While our findings support this theory, two recent investigations (9; 75) were unable to detect a change in AS160

phosphorylation with IL-6 exposure, although they observed an increase in glucose transport.

As expected, insulin markedly increased glucose transport and this was associated with increased PI3-kinase (p85) and phosphorylation of Akt (especially thr³⁰⁸). Increases in the phosphorylation of AMPK, CaMKII, and AS160 were also observed. The increase in AMPK phosphorylation observed during insulin-stimulation was unexpected as it is contrary to previous investigations (52; 84; 85; 120; 136). However, others have found that AMPK responds differently in glycolytic versus oxidative muscle (51, 236) and existing studies have used primarily rat glycolytic muscle preparations, rather than oxidative (e.g., soleus) muscle as a model in which to study the relationship between AMPK phosphorylation and insulin. Furthermore, the studies performed using intact rat muscle, used males animals (52; 84; 85; 120; 136). The current investigation used female rats, which could suggest that both fibre type and sex-differences may exist in the activation of AMPK by insulin. Roepstorff et al. (2006) found that in humans, during exercise, the increase in AMPK Thr172 phosphorylation and AMPK α 2 activity observed in male subjects was not observed in female participants. The authors attributed this difference to a number of factors including sex-differences in fibre type, with females having a greater percentage of type 1 fibres (181). Clearly, further research is warranted.

In contrast, exposure of the soleus muscle strips to IL-6 resulted in a decrease in insulin-mediated glucose transport. These findings are consistent with the association observed between IL-6 and insulin resistance in whole body studies (209). However, two other studies (73; 75) utilizing isolated muscle strips failed to detect a change in insulin-stimulated glucose transport with IL-6 present. The incubation protocols of these

investigations were very different from that in the current study. For example, Geiger et al (2007) incubated rodent soleus muscle strips with an IL-6 concentration of 120 ng ml⁻¹ (i.e. 24 times that in the present study) for 30 min followed by a 3h recovery incubation without IL-6 present when insulin-stimulated glucose transport was measured (73). Two other studies (9; 33) observed an increase in insulin-mediated glucose transport in human cell cultures. One of these studies (9) observed an increase in insulin-stimulated glucose transport following 8 days of exposure to IL-6 that was 5 times the concentration used in our protocol, while the other study (33) incubated myotubes for 120 min with an IL-6 concentration 20 times that of the current study present in the medium. In comparison to the present investigation, the conditions of these previous studies were far more extreme, mimicking pathological states.

The decrease in insulin-stimulated glucose transport caused by IL-6 and TNF- α was associated with decreases in phosphorylation of CaMKII and AS160 to basal levels. Wright et al. (2004) provided evidence that CaMKII is involved in insulin-stimulated glucose transport. Furthermore, using a CaMKII-specific inhibitor they showed a 30% decrease in insulin-mediated glucose transport (234).

In the present investigation, both IL-6 and TNF- α increased basal glucose transport and reduced insulin-stimulated transport with corresponding changes in phosphorylation of the same signalling intermediates. However, there was no additive effect of IL-6 and TNF- α . Therefore, it is likely that IL-6 and TNF- α act via a similar signalling pathway to influence glucose transport in resting muscle.

It is unclear how IL-6 and TNF- α interact with AMPK and CaMKII. The suppressor of cytokine signalling (SOCS) proteins have been suggested to mediate “cross

talk” between IL-6 and signalling intermediates involved in glucose transport. Ueki et al (2004) have shown that SOCS-1 and -3 interact with the insulin receptor (IR) and insulin receptor substrates 1 and 2 (IRS-1/2) (219). However, in the current study, proteins distal to IRS-1, including Akt, was unchanged, which could suggest that SOCS-1 or -3 are not responsible for mediating the cytokine induced changes in phosphorylation AMPK and CaMKII. Alternatively, the SOCS proteins could be acting on proteins other than, or in addition to, the insulin receptor and its substrate (IRS-1/2).

Effect of IL-6 on contracting skeletal muscle

Skeletal muscle has been shown to release IL-6 during contraction, leading to local concentrations of ~2 ng/ml (186) and it has been proposed that IL-6 may act in a paracrine fashion to regulate the metabolism of the muscle. We observed that IL-6 induced an increase in contraction-mediated glucose transport. The IL-6-induced increase in glucose transport was accompanied by an increase in phosphorylation of CaMKII and AS160. Although this is the first study to find that IL-6 can directly increase contraction mediated transport of glucose into the muscle, Febbraio et al. (2004) provided earlier evidence that suggested that IL-6 could be acting in this manner. They found that when low intensity exercise (40% VO₂ max) was combined with IL-6 infusion to reach circulating IL-6 levels comparable to that of high intensity exercise (75% VO₂ max), there was a ~20% greater rate of disappearance (R_d) of glucose than low intensity exercise alone (59).

Previous reports (6; 51; 106) have indicated that the activation of AMPK is not responsible for mediating glucose transport during contraction in slow-twitch rat skeletal muscle. Rather, it appears that calcium-related mechanisms are responsible for glucose

transport during contraction in rodent soleus skeletal muscle (235). Similarly, evidence has been provided to suggest that CaMKII, a calcium-induced protein, is an important mediator of contraction mediated glucose transport, independent of AMPK (184; 230; 235). However, Richter's group have found that CaMKII acts via AMPK, but this pathway only appears to function during low intensity stimulation. Furthermore, CaMKII was only found to act through increasing AMPK activity for the first 2 min of exercise (100). Alternatively, Wright et al (2004) observed that CaMKII acted independently of AMPK with 10 min of high intensity stimulation of skeletal muscle (236). Our findings concur with these investigations as we observed an increase in CaMKII phosphorylation in contracting soleus muscle strips, whereas there was no significant increase in AMPK phosphorylation.

Unlike IL-6, TNF-alpha exposure did not induce an increase in glucose transport in the soleus muscle strips. These findings confirm the work by Zhang et al (2003), who found no significant effect of TNF-alpha on glucose transport in contracting rodent hindlimb (244). However, when IL-6 and TNF-alpha were both present in the incubation medium, the effect of IL-6 was abolished, as was the IL-6-mediated increase in phosphorylation of CaMKII and AS160. The mechanism by which TNF-alpha interfered with the phosphorylation of CaMKII and AS160 remains unknown.

The findings from the current study support the hypothesis that IL-6 can act in a paracrine fashion to upregulate glucose transport in the working muscle. While TNF-alpha interfered with this action, it is important to note that this cytokine does not change with exercise (62; 149; 204) while there is an enormous increase in IL-6 (37; 78; 152; 204). However, elevated concentrations of IL-6 (209) and TNF-alpha (95) are observed

in individuals with diabetes. Therefore, in those who have chronically elevated levels of TNF-alpha, the local effect of IL-6 could be impaired. Such people would still be able to increase glucose transport during exercise via contraction mediated means (phosphorylation of CaMKII and AS160), but might not benefit from an IL-6 effect.

It is paradoxical that IL-6 and TNF-alpha affected glucose transport in a completely different manner in the absence and presence of insulin. However, important biological systems commonly have a number of redundancies, presumably to ensure that the important metabolic events (e.g., glucose transport) take place. While it is important to study stimuli such as a cytokine or insulin in isolation, this never happens *in vivo*.

What happens as a result of a single stimulus is often not seen when these signals are integrated. Our results suggest that at least part of the signalling pathway for the cytokines interacts with that of insulin. When insulin was absent, IL-6 was able to increase phosphorylation of AMPK and AS160 to enhance glucose transport, whereas in the presence of insulin, phosphorylation of CaMKII and AS160 were decreased and there was no significant effect on PI3-kinase. It appears that for resting muscle, the primary stimulus for glucose transport is insulin and that the concentrations of other factors such as the cytokines may provide more detailed feedback regarding metabolic status and may modify the insulin-mediated outcome. Similarly, during exercise, the primary stimulus is contraction and it is possible that cytokines could reflect the metabolic status of the muscle and the body to provide modification of the degree of glucose transport.

Consistently, the extent of phosphorylation of AS160 reflected the resulting change in glucose transport, suggesting its potential importance as a point of regulation. These findings are in agreement with recent evidence that AS160 is a point of convergence for a

number of signalling pathways (120; 215). Future work should focus on elucidating the mechanism by which IL-6 and TNF- α alter the phosphorylation of this protein.

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CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The regulation of carbohydrate homeostasis is a complex process with many redundancies. Galbo (70) and others (115; 116) have shown that not only are there many feedback mechanisms to regulate glucose homeostasis, but 'feed forward' signals are also critical. They hypothesized that changes in hormones responsible for maintaining glucose availability take place too quickly to be primarily accounted for entirely by feedback from alterations in metabolic status (e.g., decreased blood glucose concentration). Therefore, other signals such as stimulation of the motor centers of the brain may provide a feed forward signal to stimulate early growth hormone, catecholamine and insulin responses. These factors, then in turn mobilize extramuscular fuel in exercise (115).

Other systemic signals may also function in this manner to provide advance information of challenges to metabolism, including cytokines such as IL-6. Skeletal muscle, a key tissue involved in glucose homeostasis, has been shown to be responsible for nearly 80% of insulin-stimulated glucose disposal (49), and thus, is a critical tissue for the maintenance of homeostasis. During exercise skeletal muscle is a major consumer of carbohydrate, through the use of its muscle glycogen and by taking up large quantities of glucose from the blood. Integration of muscle metabolic regulation with that of the liver and other tissues are essential to the tight control of blood glucose levels, especially during exercise. For example, in Study 1, there was only a small decrease in blood glucose (0.6 mM/L in 90 min). Given a blood volume of 5 L, this would amount to a decrease of only 3 mmols of glucose, whereas the magnitude of glucose utilized by the

body during such exercise intensity and duration has been reported to be as great as ~2500 mmol (53).

Glycogen is not a key regulator of IL-6 production in skeletal muscle

Traditionally insulin is thought of as the key to carbohydrate metabolism but the management of carbohydrate delivery, transport, storage, and utilization in skeletal muscle has been shown to be determined to a large extent by glycogen levels in the muscle; a muscle with lower glycogen levels is characterized by higher rates of glucose transport than a muscle with much higher stores of glycogen (177). It is likely that glycogen itself does not directly regulate carbohydrate management, but rather modulates signals that can modify glucose supply, transport and utilization (177; 233). Interleukin-6 has been identified to be a possible signal that may be controlled by changing levels of muscle glycogen (109; 202).

During exercise muscle has been shown to release IL-6 in large quantities (202; 204). Given that IL-6 is being produced at a time when metabolic demands are high, IL-6 has been assumed to be biologically important. Its role in metabolism is unclear, but it was suggested to act both systemically and locally in the vicinity of the muscle. A number of studies have shown a correlation between declining glycogen levels in muscle and corresponding increases in systemic concentrations of IL-6 (92; 202). However, the findings from this thesis suggest that IL-6 production is not determined solely by glycogen levels and, therefore, other metabolic signals must be involved in the regulation of carbohydrate metabolism in skeletal muscle.

The findings from study 1 generally confirmed those of previous studies, that declining glycogen was correlated with increases in plasma IL-6 and IL-6 mRNA in

skeletal muscle. However, a closer examination of the net change in glycogen and the net change in IL-6 from rest to exhaustion, failed to generate a significant correlation. When the difference within subjects at exhaustion in IL-6 and glycogen was compared, there was no correlation between the two variables. Furthermore, there was also no significant correlation observed between the amount of glycogen breakdown and the fold change of IL-6 mRNA from rest despite a nearly 15-fold increase in IL-6 mRNA at the end of exercise.

A peak in muscle IL-6 mRNA levels has also been observed immediately following exercise, with protein expression reaching a maximum level 3 hours after exercise (163). However, previous investigations have not examined the relationship between IL-6 and glycogen in the recovery period. Study 1 examined the post-exercise period, characterized by a resynthesis of glycogen and a corresponding decline in circulating IL-6 concentrations and found no correlation between IL-6 and glycogen in this period.

This thesis provides evidence that alterations in muscle glycogen are not the primary stimulus for IL-6 production in muscle, but the metabolic signal is not evident. Presumably it must increase with exercise, and then dissipate early in recovery. As glycogen levels decrease and fatty acid concentrations rise, signalling pathways sensitive to these changes in the muscle's metabolic state (e.g., NF- κ B) could stimulate IL-6 production (119). Another possible factor may be rapid increases in cytosolic calcium levels with exercise, which leads to the activation of a number of factors including calcineurin (60). It is also possible that other calcium-induced factors could trigger the

production of IL-6, including CaMKII. The latter possibility is addressed later in this discussion.

Study 1 has provided support for the proposed (60; 155) role of IL-6 as a systemic communication factor during exercise, potentially as a feed forward signal to the liver and possibly adipose tissue. The ingestion of carbohydrate during exercise has been shown to both reduce the magnitude of glycogen use as well as attenuate IL-6 production by the muscle (61; 144; 199). Starkie et al. showed that carbohydrate ingestion did not affect gene expression in the muscle, but suggested that release of IL-6 from the muscle may be affected (199). Their findings suggested that because there was a large amount of glucose provided to sustain increases in energy requirements during exercise, IL-6 was not required to signal to the liver and other tissues to provide fuel for the active muscle. Study 1 showed a negative correlation between increasing levels of plasma IL-6 observed throughout exercise and systemic glucose and insulin concentrations and a positive association with free fatty acid levels. While it is likely that IL-6 is an important factor in the management of fuel for skeletal muscle, further investigations are required to probe potential signalling mechanisms and relationships with fat metabolism rather than only carbohydrate metabolism.

IL-6 affects glucose transport in resting skeletal muscle

The second study in this thesis (Chapter 5) has provided evidence that IL-6 can increase basal (no insulin present) glucose transport. The observed increase in basal glucose transport by IL-6 (1.6-fold increase) is consistent with recent studies (9; 33; 73; 75), with the exception of Geiger et al (2007), who failed to observe an increase in glucose transport in rodent soleus muscle strips (73).

In the presence of insulin, IL-6 decreased glucose transport by 44 percent. These findings are contrary to the observations of the recently published studies introduced above (9; 33; 73; 75). As noted in Chapter 5, these studies employed a longer duration of exposure to extremely high concentrations of IL-6.

Molecular mechanisms mediating the effects of IL-6 in resting muscle

A number of key proteins involved in GLUT 4 translocation to the cell surface have been evaluated as potential mediators of the effects of IL-6 in muscle. The literature is inconsistent (see Table 2, Chapter 2) probably due to the wide range of concentrations of IL-6 employed, acute versus chronic exposure and various models used. However, AMPK has been identified as a likely candidate (9; 33; 73; 75). Four recent studies examined the direct effect of IL-6 on glucose transport and found that IL-6 increased AMPK phosphorylation in the absence of insulin (9; 33; 73; 75). However, Geiger et al (2007) failed to detect a change in AMPK phosphorylation when studying rodent soleus muscle strips, which coincided with their glucose transport findings (73). Other suggested candidates are Akt (9; 67) or IRS-1/2 (114; 180; 229), but the findings related to these signalling proteins are mixed.

In the present study, IL-6 increased phosphorylation of AMPK and AS160 under basal conditions (*Figure 16*). These data are consistent with existing findings that suggested that activation of AMPK mediates insulin-independent stimulation of glucose transport in resting skeletal muscle (123). Furthermore, the current findings concur with that of the aforementioned research (9; 11; 33; 99) that examined the relationship between IL-6 and AMPK under basal conditions. It has been suggested that AMPK may act to facilitate GLUT-4 translocation via phosphorylation of AS160 (215). However,

existing studies have not observed a change in AS160 phosphorylation (9; 75). The current work supports the theory that AMPK acts via AS160, but does not address the mechanisms for how this AS160 activation occurs. Therefore, further investigation is required to reveal the mechanism by which these two proteins interact.

To my knowledge it is unknown how IL-6 activates AMPK, but there is some evidence that IL-6 activates the MAPK signalling pathway involving p38MAPK and ERK1/2 (86). It is possible that MAPK could be responsible for mediating the IL-6-induced changes in AMPK phosphorylation, but this is speculative. Skeletal muscle exposed to insulin was characterized by an increase in PI3-kinase and the phosphorylation of Akt (ser⁴⁷³), Akt (thr³⁰⁸), AMPK, AS160, and CaMKII (*Figure 17A*). When IL-6 was also present, phosphorylation of CaMKII and AS160 was reduced in association with a decrease in glucose transport (*Figure 17B*). CaMKII phosphorylation was reduced to the level observed under basal conditions. While the role of CaMKII in insulin-stimulated glucose transport is somewhat controversial, Wright et al (2004) provided evidence of the involvement of CaMKII by demonstrating a partial reduction in glucose transport when CaMKII phosphorylation was inhibited (236). To my knowledge, the current thesis contains the first set of experiments that have evaluated the role of CaMKII in the actions of IL-6. In study 2, insulin-stimulated glucose transport was only partially reduced by IL-6. It is possible, based on the findings of the current study and the work by Wright et al (236), that IL-6 did not interfere with signalling intermediates in the proximal aspect of the PI3-kinase pathway (e.g., PI3-kinase, Akt), but rather reduced insulin-stimulated CaMKII phosphorylation. Furthermore, decreased AS160

phosphorylation may be a result of a reduction in CaMKII phosphorylation, rather than due to changes in activation via the PI3-kinase pathway.

Figure 16. Schematic summarizing the possible mechanisms by which IL-6 increases basal glucose transport. The number and size of the arrows depicting glucose transport represents the magnitude compared to basal. The proteins observed to be affected by IL-6 exposure in study 2 are located at the end of the dashed arrows. A '+' indicates an increase in phosphorylation of the protein and '?' indicates a proposed interaction between proteins.

It is unclear how IL-6 may exert its effects on these signalling intermediates. One possible mechanism is “cross-talk” between signalling intermediates activated upon IL-6 binding to its receptor (e.g., JAK/STAT pathway) and the proteins involved with glucose transport (*Figure 18*) (87).

The JAK/STAT pathway is initiated when IL-6 binds to its membrane-bound or soluble receptor. The activation of the signal transducing receptor complex (glycoprotein 130 (gp130)), which leads to the activation of the Janus kinase (JAK) tyrosine kinase family of proteins. They, in turn, initiate the STAT (signal transducers and activators of transcription) transcription factors. STAT phosphorylation results in the transcription of the suppressor of cytokine signalling (SOCS) proteins, which act as a negative feedback component of this pathway (86). The SOCS proteins have been shown to act on the JAKs, thereby inhibiting phosphorylation of the STATs and preventing further signal transduction (201).

It has been suggested that the activation of this signalling pathway can lead to the initiation of other signalling cascades, such as PI3-kinase, potentially via interaction of the JAKs with IRS/PI3-kinase (229). This area of research is in its infancy, so although a number of alternative mechanisms can be proposed, it is important to note that they are speculative and to my knowledge have not been studied to date.

If the SOCS proteins act on the JAKs to prevent activation of the insulin signalling pathway at the point of IRS interaction with PI3-kinase, this could be a possible mechanism by which IL-6 negatively affected insulin signal transduction. SOCS itself has also been shown to directly interact with IR to impair phosphorylation of IRS-

Figure 18. Schematic summarizing the proposed and previously observed interactions between IL-6 signalling pathway and mediators of glucose transport. Interactions observed in past research are indicated by a solid line and the proposed interaction based on the current work is indicated by a dashed line. A '+' indicates an increase in phosphorylation of the protein and '?' indicates a proposed interaction between proteins.

1/2, which provides additional evidence for a role for SOCS in the regulation of insulin signal transduction (219). The phosphorylation of IR or IRS-1/2 was not measured in the current study; however, there was no change in PI3-kinase, which is downstream of IR and IRS-1/2, despite reductions in glucose transport. Therefore, an interaction between SOCS and IRS-1/2 to inhibit signal transduction was not consistent with the current findings. It is also possible that SOCS interacted with proteins in addition to the JAKs or IRS-1/2, potentially downstream of PI3-kinase (e.g., AS160), which caused a corresponding decrease in phosphorylation of AS160 as observed in Study 2. Furthermore, SOCS may have affected CaMKII activation or proteins upstream of

CaMKII, but this has not been studied to date. Finally, it is feasible that other signalling proteins in the IL-6 pathway (e.g., STATs, or other unknown intermediates) interacted with signalling intermediates responsible for glucose transport. In the case of study 2, SOCS proteins were not measured because it is extremely difficult to detect this protein in muscle with current available methods.

While it appears paradoxical that soleus muscle strips incubated in IL-6 and TNF- α altered glucose transport differently depending on whether insulin was present or absent, these findings likely reflect the redundancies found in important biological systems; specifically, the multiple pathways leading to glucose transport in skeletal muscle. The direction of change in glucose transport, regardless of metabolic situation, coincided consistently with alterations in the phosphorylation of regulatory proteins. For example IL-6-induced changes in glucose transport are reflected in changes in phosphorylation of AS160 and, with the exception of the basal conditions, are also reflected in phosphorylation of CaMKII.

Although it is important to examine a stimulus such as IL-6 in isolation, this situation is not representative of a biological system. The metabolic consequence of the individual stimulus appears to be very different when other signals are integrated and the final outcome also depends on the metabolic status of the muscle itself. While it appeared that insulin is a major stimulus for glucose transport in resting muscle, the insulin-mediated response can be altered by other factors such as IL-6 that provide feedback regarding metabolic status. For example, in the absence of insulin, IL-6 increased the phosphorylation of AMPK and AS160 to enhance glucose transport. Alternatively, with insulin present, the phosphorylation of CaMKII and AS160 were

decreased and PI3-kinase was unaffected. Changes in these signalling intermediates may indicate the ability of IL-6 to affect different proteins depending on the signals present to alter muscle metabolism.

In Study 2 an increase in AMPK phosphorylation was detected with insulin exposure. This finding varies from the existing literature where the effect of insulin on AMPK phosphorylation has been directly measured in skeletal muscle (52; 84; 85; 120; 136). As discussed in Study 2, differences could be explained due to the use of soleus muscle and the muscle was obtained from female rats. D'Eon et al. (2005) found that estradiol treatment increased the activation of AMPK in a dose dependent manner. Therefore, it is possible that the presence of estrogen in female skeletal muscle could be responsible for the difference in findings (46). However, for estrogen to be implicated, the effect of estrogen would have to be specific to insulin-stimulated conditions as AMPK phosphorylation was not elevated under all conditions (e.g., contraction). In the intact female animal of reproductive age, with the exception of type 1 diabetes, there would never be a situation where insulin was not present.

The mechanism by which insulin is increasing AMPK phosphorylation is unclear. Given that the AMP/ATP ratio is not changing in these muscle strips over 70 min (see appendix C), it is likely that insulin is activating AMPK via upstream kinases such as LKB-1 and CaMKK. CaMKK, in particular, is a likely candidate because it can be activated by calcium ions, without any change in AMP/ATP (82). Alternatively, because the phosphorylation of the Thr172 site of AMPK is also dependent on the inhibition of dephosphorylation by protein phosphatases (47), insulin may be acting to inhibit phosphatases such as PP2A or C (*Figure 19*).

Figure 19. Regulation of AMPK phosphorylation by upstream kinases and downstream phosphatases: the potential influence of insulin (adapted from Steinberg et al (208)).

IL-6 acts locally to increase glucose transport in contracting skeletal muscle

Systemic IL-6 would appear to be an important metabolic modifier of insulin's effects and muscle may also employ the production of IL-6 to communicate metabolic information to the liver and adipose tissue. In addition, with large local concentrations (~1200 pg/ml) of IL-6 measured in the vicinity of the muscle during muscle contractions (186), IL-6 has been proposed (59; 60) to act in a paracrine fashion to influence muscle glucose transport, storage, and oxidation.

There are a limited number of investigations that have considered the influence of IL-6 on glucose homeostasis during exercise. Febbraio et al. (2004) combined exercise and IL-6 infusion and measured glucose appearance, disappearance, and clearance rate. They found that low intensity exercise combined with IL-6 infusion resulted in a greater rate of glucose appearance and disappearance as well as clearance than low intensity exercise alone (59). In addition, Helge et al (2003) showed that thigh IL-6 release was positively related to thigh glucose transport during knee extension exercise (88). These

results suggest that IL-6 may be capable of directly increasing glucose transport into skeletal muscle.

Figure 20. AS160 phosphorylation versus glucose transport A) All data (n=12) and B) basal and contraction trended and insulin trended alone. Triangle markers represent basal data, square markers are insulin-stimulated conditions, and circle markers represent contraction data points.

The current study is the first that evaluated the direct effect of IL-6 on contracting skeletal muscle. Contractions resulted in an almost 2-fold increase in glucose transport above the basal rate; this is a slightly lower than the 2.5-3-fold increase previously measured (13; 235; 236). These small differences are most likely due to differences in the stimulation protocols. Associated with this enhanced glucose transport were increases in CaMKII and AS160 phosphorylation. Consistently, the extent of phosphorylation of AS160 reflected the resulting change in glucose transport, suggesting its potential importance as a point of regulation (*Figure 20*). While AMPK phosphorylation tended to increase with exercise, the difference was not significant. This lack of a large AMPK activation with exercise may suggest the important role of other factors such as calcium induced proteins (CaMKII) in the regulation of glucose transport during contraction in isolated soleus muscle. As hypothesized, IL-6 further increased

glucose transport, to levels 2.4 fold greater than basal. This increase was also significantly greater than the contraction condition without IL-6 present.

Although there was no change in AMPK phosphorylation during contraction in rodent soleus muscle, a significant relationship between AMPK phosphorylation and glucose transport under basal, insulin-stimulated, and contraction-mediated conditions was observed (*Figure 21A*). However, a closer examination of the data suggests that the overall relationship between AMPK phosphorylation and glucose transport is due to the strong association under basal and insulin-stimulated conditions (*Figure 21B*), whereas there appears to be no relationship between AMPK phosphorylation and glucose transport when the contraction data is isolated (*Figure 21D*). Due to the sample size, these findings need to be viewed as preliminary.

Figure 21. AMPK phosphorylation versus glucose transport. A) All data, $n=12$ ($P<0.05$); B) Basal and insulin-stimulated conditions, $n=8$ ($P<0.05$); C) Basal, $n=4$ ($P=0.14$); D) Contraction, $n=4$ ($P>0.05$). Triangle markers represent basal conditions; square markers indicate insulin-stimulated data points; and the circles represent the contraction conditions.

The observed contraction-mediated activation of CaMKII confirms past research (235; 236), which demonstrated that CaMKII is important in the regulation of glucose transport during muscle contraction, but study 2 was the first to associate IL-6 and the phosphorylation of CaMKII under these conditions. It is also possible that CaMKII acts via AS160 to mediate GLUT 4 translocation (121).

Earlier in this discussion, AS160 was suggested to be a common signalling element that was increased under basal conditions and decreased in the presence of insulin. These changes in its phosphorylation under basal and insulin-stimulated conditions were accompanied by corresponding shifts in the phosphorylation of AMPK and CaMKII, respectively. During contraction, IL-6 was found to increase the phosphorylation of CaMKII and AS160. These data are consistent with others (121; 215), and suggests that AS160 may be a point of convergence for a variety of signalling pathways leading to the translocation of glucose transporters. Figure 20A shows the relationship between glucose transport and AS160 phosphorylation when all treatments (control, IL-6, TNF-alpha, IL-6 + TNF-alpha) under all conditions (basal, insulin-stimulated and contraction) were considered. When the insulin-stimulated data are treated separately (*Figure 20B*), the R^2 increased from 0.17 to 0.77 and a significant ($P<0.05$) relationship between AS160 phosphorylation and glucose transport is observed. Although there are only 4 data points in the insulin-stimulated condition, these data also are tightly aligned ($P=0.07$) but markedly shifted upward. It is unclear why the insulin-stimulated data does not fit the trendline to the same extent of the basal and contraction data when all data are plotted together, however, it would appear that insulin is able to induce a greater glucose uptake for a given change in AS160 phosphorylation. For

example, for a 100% increase in AS160 phosphorylation, the predicted glucose uptake without insulin would be approximately 1.7 as compared to 2.7 $\mu\text{mol/g/5 min}$ with insulin present.

CaMKII may have a greater role than mediating changes in glucose transport; it may also be involved in stimulating IL-6 production in the muscle (**Figure 22B**). Keller et al. (2003) infused IL-6 and observed corresponding increases in IL-6 mRNA in the skeletal muscle, therefore, regulating its own production (110). The mechanism for this IL-6-induced IL-6 production is unknown; however, given that glycogen is not a key factor stimulating its manufacture, calcium-related factors such as CaMKII could be responsible. Therefore, these findings taken together with the work from this thesis suggest that activation of CaMKII could be an important part of a regulatory loop, controlling both the actions and production of IL-6 during contraction (**Figure 22B**).

While the SOCS proteins may be involved in the interaction between IL-6 and these signalling intermediates, it is also possible that IL-6 affected the phosphorylation of CaMKII via an upregulation of the MAPK signalling cascade. However, the involvement of the MAPK family of proteins in glucose transport during contraction has been questioned (93). The period immediately following exercise is metabolically important, characterized by a high level of plasma IL-6 and a replenishment of lost fuel stores (i.e., glycogen). Study 1 provided evidence that plasma IL-6 levels remained more than 5 times greater than resting concentrations 5h post exercise. At that time, circulating insulin concentrations also remained low. It is possible that IL-6 may be involved in the insulin-independent events in the early post exercise, but this concept has not been examined to date. Findings from the second study of this thesis (Chapter 5) demonstrated

that a high concentration of IL-6 in the local milieu of the muscle, combined with low insulin levels (also observed immediately post exercise) may result in an enhanced glucose transport. Therefore, it is conceivable that IL-6 is associated with the insulin-independent mechanism, which helped to promote restoration of glycogen in the early phase of recovery. However, in study 1 when 1 g/kg/h of carbohydrate was provided during recovery, resulting in elevated insulin levels, and FFA levels were blunted, neither plasma IL-6, nor skeletal muscle IL-6 mRNA was affected by ingestion of the carbohydrate. Plasma IL-6 and IL-6 mRNA were not found to be associated with circulating levels of glucose, insulin, or FFA, nor muscle glycogen. These data provided evidence that IL-6 is not necessarily involved in the insulin-independent events post-exercise, as insulin levels were high immediately after consumption of the carbohydrate beverage and IL-6 levels remained elevated.

Similar to the role of insulin in resting muscle, contraction is the fundamental stimulus regulating glucose transport during exercise. It is well known that these two basic stimuli act to promote GLUT4 translocation via different signalling mechanisms.

Figure 22. A. Signalling intermediates involved in muscle contraction and possibly IL-6 production in skeletal muscle. B. Interaction between IL-6 and key signalling intermediates in contraction-mediated glucose transport in skeletal muscle. The size and number of arrows reflects the difference in magnitude of glucose transport compared to basal (see Figure 1). The proteins affected by IL-6 exposure in study 2 are shown by a dashed arrow. An increase in phosphorylation of a protein is indicated by “+” and “?” represents a potential interaction between proteins.

The present study suggests that while IL-6 influences both of these fundamental processes, it does so with two different effects. The sources of IL-6 may also be different. Muscle produces insignificant amounts of IL-6 at rest, so in this state a decrease in insulin-mediated glucose transport would likely be from excessive IL-6 released by adipose tissue. During exercise the large intramuscular production of IL-6 presents the potential for a local paracrine action that could be specific to the active fibre itself. Given the role of skeletal muscle as a critical tissue in carbohydrate storage, it is not surprising that the regulation would have multiple feedback and feedforward systems that reflect the metabolic status of many aspects of the body. In addition, it would be expected that a number of redundancies would be present and that IL-6 could be an important regulatory protein acting via one or more of the systems in place to maintain glucose homeostasis.

TNF-alpha affects glucose transport in resting skeletal muscle

As discussed above, there are many redundancies and feedback mechanisms to monitor and reflect metabolic status and to refine basic signals such as insulin or contraction. Interleukin-6 has been identified as one such mechanism and it is highly likely that the effects are integrated with other factors, particularly other cytokines (e.g., TNF-alpha). Although the primary focus of this thesis was IL-6, TNF-alpha was also evaluated since it has been shown that TNF-alpha can interact with IL-6 at target tissues (28). However, before considering their combined effects, the individual influence of TNF-alpha on glucose homeostasis and glucose transport in skeletal muscle was considered.

Tumour necrosis factor-alpha has been implicated in obesity and insulin resistance as elevations in circulating levels are observed in these disease states (137). While the evidence that TNF-alpha causes disruptions to normal carbohydrate metabolism is convincing, there are inconsistent findings, particularly when the direct effect of TNF-alpha was measured. At the whole body level, infusion of TNF-alpha caused insulin resistance (125; 128; 134; 191). In muscle cell culture and muscle strips, the effect of TNF-alpha insulin-stimulated glucose transport has shown either a reduced (18; 50; 188; 238), unchanged (44; 48; 69; 146; 154; 175), or an increased glucose transport (210). These studies have utilized a variety of TNF-alpha concentrations and durations of incubation.

In the current investigation, TNF-alpha caused a reduction in insulin-stimulated glucose transport, which is in agreement with the general consensus that TNF-alpha results in insulin resistance. TNF-alpha exposure resulted in a reduction in CaMKII and AS160 phosphorylation. A role for CaMKII in the actions of TNF-alpha is a novel finding and is very similar to the actions of IL-6 as reported above. Based on Figure 20B, the TNF-alpha data was found to fit on the resultant trendline when changes in insulin-stimulated AS160 phosphorylation and glucose transport were compared.

Although the literature varies regarding a possible molecular mechanism of TNF-alpha's action, many studies observed a reduction in IRS-1 activation with TNF-alpha exposure in the presence of insulin (48; 50; 171; 188; 210). IRS-1 was not measured as part of this thesis work, however, PI3-kinase and phosphorylation of Akt was unchanged, which suggests that under the conditions of the present investigation any reduction in phosphorylation of proteins found in the PI3-kinase pathway are further downstream

(e.g., AS160). An alternative mechanism may involve signalling intermediates that are insulin sensitive but act independently of PI3-kinase (e.g., CaMKII).

Under basal conditions, an increase in glucose transport was noted. This finding was unexpected as at least in rodent cell culture, acute incubation (<24h) generally caused a decrease in basal glucose transport (18; 50), whereas a longer incubation resulted in no change (154) or increased (48; 175; 239) transport. However, two investigations using a rodent soleus strip model reported no change in basal glucose transport in the presence of TNF-alpha (69; 146). Differences in the concentration of TNF-alpha utilized as well as the duration of incubation may have resulted in the contrary findings to that of the current study.

In the present investigation, basal levels of AMPK and AS160 phosphorylation was increased with TNF-alpha exposure, comparable levels observed with IL-6. The findings related to AMPK are contrary to Steinberg et al. (2006), who showed a reduction in AMPK under basal conditions in L6 myotubes and following TNF-alpha infusion in mice (208). However, the previous study (208) utilizes L6 myotubes, rather than intact muscle as used in the present investigation. Furthermore, cells were incubated for 8 h rather than the acute protocol (70min exposure) used in study 2. The other candidates that have been identified in the existing literature that were not measured in this thesis are the MAPKs. Despite the mixed findings in the existing literature, a few studies have shown increased activation of MAPKs (77; 146; 239), but not all studies demonstrated these results.

In summary, similar to IL-6, TNF-alpha clearly plays a role in glucose transport in skeletal muscle. Furthermore, the nature of its actions appears to depend on the signals present to alter metabolism.

IL-6 and TNF-alpha do not act in a synergistic manner in resting skeletal muscle

The combination of IL-6 and TNF-alpha together in the incubation medium resulted in similar findings as detailed above for IL-6 or TNF-alpha. To my knowledge this is the first study that has combined these cytokines to examine their collective effects on glucose transport and related signalling elements. An additive effect was not detected, nor was there any reduction in the response observed with each cytokine independently. Furthermore, both IL-6 and TNF-alpha initiated similar changes glucose transport as well as in signalling intermediates measured under both basal and insulin-stimulated conditions. Taken together, these data suggest that IL-6 and TNF-alpha do not act via independent pathways in resting skeletal muscle.

TNF-alpha interferes with the actions of IL-6 in contracting skeletal muscle

As described earlier, investigations have routinely observed significant IL-6 production by skeletal muscle during exercise (37; 152; 163; 204) while TNF-alpha is not (204). When electrically stimulated rodent soleus muscle strips were incubated with TNF-alpha, there was no change detected in glucose transport or key signalling intermediates compared with the untreated, contracting muscle strips. The only study, to my knowledge, that has examined the direct effect of TNF-alpha on contracting muscle found that TNF-alpha infused into an electrically stimulated rodent hindlimb for 1h did not alter glucose transport (244). The present results concur with these findings and suggest that TNF-alpha does not influence glucose transport during exercise.

From a teleological perspective, the current findings are logical; TNF-alpha inhibits the actions of insulin in resting muscle; TNF-alpha is not produced by contracting skeletal muscle, nor is it present in elevated concentrations during muscle contraction; therefore, with exercise, there is no increase in TNF-alpha, no muscle release of TNF-alpha, and little insulin present. Therefore, one would not expect there to be an effect of TNF-alpha on contracting muscle.

On the other hand, when the electrically stimulated muscle strips were exposed to IL-6 and TNF-alpha simultaneously, the IL-6-induced increase in glucose transport and phosphorylation of CaMKII and AS160 was abolished. Therefore, it appears that the 'cytokine and contraction pathways' converge at these critical proteins. Interestingly, however, the TNF data fit the resultant trendline when basal and contraction-mediated AS160 phosphorylation and corresponding glucose transport values were plotted (Figure 20B).

These findings may also be clinically significant when TNF-alpha is elevated in disease conditions, as it may have a negative impact both on the effect of insulin on resting muscle and on the ability of IL-6 released from the muscle to facilitate the transport of glucose into active muscle. This would imply that while such a person can increase muscle glucose transport via exercise, the additive effect of IL-6 acting locally would likely be inhibited with the net result being a subnormal glucose transport. However, a reduction in glucose transport in a disease state may not be "bad" but rather protect the muscle from excess glucose uptake.

Summary

The maintenance of glucose homeostasis is a complex process that is regulated by redundant systems. This thesis suggests that IL-6 can act as a systemic and local regulator of glucose metabolism, particularly during muscle contraction.

The findings from this thesis provided insight, for the first time, into the ability of IL-6 to increase glucose transport during contraction. The mechanism by which this was shown to occur was via increased CaMKII and AS160 phosphorylation. Nevertheless, the signaling events leading from IL-6 binding to its receptor and the activation of these proteins remains elusive. Under resting conditions, however, IL-6 and TNF-alpha were found to reduce insulin-stimulated glucose transport. It is possible that the insulin resistance caused by these cytokines in an isolated model is actually part of a system to control the response of muscle to the larger signal of insulin. The mechanism by which these cytokines were found to decrease the actions of insulin was via a reduction in CaMKII and AS160 phosphorylation. In the absence of insulin, IL-6 and TNF-alpha increased glucose transport through an increase in AMPK and AS160.

These experiments provide evidence that AS160 is critical in the actions of IL-6 regardless of the signal responsible for altering metabolism (e.g., insulin, contraction). Furthermore, these findings also support a role for AS160 as a point of convergence for multiple pathways leading to glucose transport.

This thesis also examined the effect of TNF-alpha on the actions of IL-6. Evidence was provided that when contracting muscle is exposed to TNF-alpha and IL-6 in combination, the IL-6-induced increase in glucose transport and phosphorylation of CaMKII and AS160 is attenuated. This finding suggests that TNF-alpha is able to block

the actions of IL-6, but the mechanism remains elusive. It is clear from the above discussions that there is an extensive amount of work required to clarify the relationship between IL-6, TNF-alpha, and glucose homeostasis.

Future Directions

Glucose homeostasis is challenged by acute and chronic alterations to normal metabolism. For instance, ingestion of carbohydrates and exercise are examples of acute challenges and diabetes (i.e., insulin resistance) is an example of a chronic burden to the maintenance of glucose homeostasis. To date, there have been great efforts to elucidate the mechanisms involved in the regulation of glucose homeostasis under different metabolic conditions. Recent work has been focused on identifying the mechanisms responsible for insulin resistance, particularly in skeletal muscle. Of these factors, cytokines, (i.e., IL-6) have been identified as a key component in the regulation of glucose metabolism. However, the results remain highly variable. Therefore, the overall question remains: is IL-6 a key regulator of glucose homeostasis?

Study 1 in this thesis concluded that alternative factors including calcium-induced factors (i.e., calcineurin) are likely involved in the production of IL-6 in skeletal muscle and should be examined further. Initial research indicated that glycogen was somehow responsible for IL-6 expression in muscle and there is very little work examining other possible factors. Therefore, future studies should examine the role of other contraction-mediated factors. One such factor that might be involved is CaMKII. Future work should measure the activation of CaMKII in exercising muscle. Blocking CaMKII and measuring subsequent IL-6 production, subsequent release and IL-6-induced changes to glucose transport may provide more insight into the role of CaMKII in IL-6 actions.

Alternatively, contracting isolated muscle strips could be exposed to IL-6 and IL-6 mRNA and CaMKII could be measured during contraction and correlated.

While Study 2 identified AS160, AMPK, and CaMKII as key signalling intermediates, mediating the effects of IL-6 on skeletal muscle, there are very few investigations examining the cross-talk between the JAK/STAT pathway and signalling intermediates leading to glucose transport in skeletal muscle. Furthermore, these studies have focused on signalling intermediates involved in the PI3-kinase pathway and have identified IRS-1 as a point of interaction. The findings from this thesis suggest that IL-6 interacts with signalling pathways downstream of PI3-kinase. Other pathways activated by IL-6 (i.e., MAPK) should be investigated to determine whether there is any cross-talk with the signalling pathways involving AMPK, CaMKII, and AS160.

Another finding that should be investigated further was the shift the AS160 curve (Figure 5A) by insulin when AS160 phosphorylation was plotted compared to glucose transport. When insulin was present, a greater glucose transport was observed for a given level of AS160 phosphorylation. It is may be that different pools of GLUT4 are recruited with insulin versus basal or contraction and AS160 acts primarily on one pool.

Insulin also increased the phosphorylation of AMPK, which was an unexpected finding. As discussed earlier, fibre type and sex-differences may be responsible for this novel finding. Given that the current study used female rats, future studies could examine the effect of estrogen or other sex hormones on AMPK phosphorylation in the presence of insulin in skeletal muscle. Furthermore, future work could examine upstream kinases such as LKB-1 and CaMKK and downstream protein phosphatases such as PP2A or C to determine how insulin could be altering the phosphorylation of AMPK.

Interleukin-6 also increased CaMKII phosphorylation in contracting muscle. Common to all of these metabolic states, IL-6 also affected the phosphorylation of AS160. There is evidence that AS160 is a point of convergence for a variety of signalling pathways, including AMPK, Akt, and CaMKII. Thong et al (2007) transfected AS160 mutants to determine its contribution to GLUT4 traffic in cells treated with substances that activate Akt, PKC, or AMPK (215). They found that AS160 was involved in all signalling pathways. Furthermore CaMKII has been shown to act via AS160. Future work could apply the same model used by Thong et al (2007) to examine the pathway that includes CaMKII (215).

The interaction between TNF-alpha and IL-6 has not been extensively examined and controversy remains regarding their individual influences on glucose homeostasis. However, it has been proposed that IL-6 may interact with other cytokines, and in particular, TNF-alpha. This study does not provide evidence of an interaction between TNF-alpha and IL-6 under resting conditions in soleus muscle strips, while TNF-alpha interfered with the actions of IL-6 in contracting muscle strips. In the contracting muscle strips, the increase in CaMKII and AS160 observed with IL-6 exposure was not evident when TNF-alpha was also present in the medium. It is unclear how TNF-alpha could be interfering with the actions of IL-6 and subsequent studies should examine this further. Future studies could examine the TNF-alpha and IL-6 signalling pathways to determine any points of convergence or interference. Alternatively, an examination of CaMKII and AS160 phosphorylation residues to determine if TNF-alpha is capable of blocking the ability of IL-6 to interact with the residue may provide further insight.

Finally, this thesis and the above discussions have focused solely on carbohydrate metabolism. However, carbohydrate metabolism does not operate in isolation but rather is intimately integrated with fat metabolism. It is possible that IL-6 may be affecting the signalling intermediates described above through changes in fat metabolism. Future studies should consider the role of fatty acid and ceramide accumulation in skeletal muscle as a potential mechanism for the development of insulin resistance with IL-6 exposure. Specific focus should be placed on changes in muscle fat metabolism and signalling proteins found to affect glucose transport (e.g., CaMKII) in study 2. For instance, an examination of the role of CaMKII and AS160 in IL-6-induced changes in fatty acid transport and storage in muscle strips could determine whether IL-6 is able to alter the phosphorylation of these proteins through changes in fat metabolism.

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APPENDICES

APPENDIX A. Glucose transport - Dose response

Figure 1. Insulin-stimulated glucose transport. Dose response pilot testing (n=3)

Figure 2. Basal glucose transport. Dose response pilot testing (n=3).

APPENDIX B. Summary of protein data (not shown in Chapter 5)

Phosphorylation of AMPK, ACC, and CaMKII in the presence of IL-6, TNF- α , and IL-6 + TNF- α .

<i>Condition</i>	<i>Cytokine</i>	<i>AMPK</i>	<i>ACC</i>	<i>CaMKII</i>
Rest Basal ¹	————	1 ^a	1 ^a	1 ^a
	IL-6	1.6±0.2 ^b	0.9±0.1	1.4±0.2 ^a
	TNF- α	1.5±0.3 ^b	1.0±0.3	0.9±0.2 ^a
	IL-6 + TNF- α	1.6±0.1 ^b	0.9±0.2	1.2±0.3 ^a
	————	2.0±0.2 ^b	1.8±0.3 ^b	1.6±0.2 ^b
Insulin ¹	————	1.7±0.2 ^b	1.3±0.3	0.87±0.1 ^a
	IL-6	2.0±0.1 ^b	1.6±0.7	0.9±0.1 ^a
	TNF- α	1.5±0.1 ^b	0.9±0.2	0.9±0.1 ^a
	IL-6 + TNF- α	1.1±0.05	1.2±0.4	1.5±0.3 ^b
	————	0.9±0.1	1.2±0.3	1.1±0.2
Contraction ²	————	1.1±0.1	1.0±0.4	0.7±0.2
	IL-6	1.1±0.05	1.2±0.4	1.5±0.3 ^b
	TNF- α	0.9±0.1	1.2±0.3	1.1±0.2
	IL-6+TNF- α	1.1±0.1	1.0±0.4	0.7±0.2
	————	1	1	1 ^a

AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; CaMKII, calcium-calmodulin kinase II;

Unlike letters, P<0.05 within the same column (within rest or contraction).

¹ Fold change versus Basal, no cytokines present

² Fold change versus Contraction, no cytokines present

APPENDIX C. Phosphagen Data

Muscle viability (ATP and phosphocreatine) of resting isolated rat soleus muscle after 0 and 70 min of incubation in modified Krebs-Henseleit buffer.

	Time	Sample					Average	SEM
		1	2	3	4	5		
ATP	0 min	19.2	21.7	22.9	21.7	22.1	21.5	0.6
	70 min	20.1	17.6	19.6	19.2	19.3	19.2	0.4
PCr	0 min	41.4	43.6	40.7	45.1	45.9	43.3	0.9
	70 min	38.1	39.9	34.0	27.4	33.3	34.5	1.5

ATP, adenosine triphosphate; PCr, phosphocreatine. Values are means $\pm$ S.E.M., mmol (kg dry mass)⁻¹

APPENDIX D – mRNA – Efficiency Calculation for IL-6 and 18s rRNA.

Efficiency was calculated by plotting the concentration of a serial dilution of cDNA and the corresponding Ct value and the equation $E = 10^{-1/\text{slope}}$ used.

Gene	Slope	$E=10^{-1/\text{slope}}$	Efficiency
18S	-3.48	1.93507253	0.9350725
IL-6	-3.37	1.978718954	0.978719

The difference between slopes was 0.11, therefore, the genes were deemed to be of equal efficiencies.