Regulation of Insulin Signalling by Exercise in Skeletal Muscle

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CANDIDATE DECLARATION

I am the author of the thesis

entitled

Regulation of insulin signalling by exercise in skeletal muscle

Submitted for the degree

of

Doctor of Philosophy

Is the result of my own research, except where otherwise acknowledged, and that this thesis in whole or in part has not been submitted for an award, including a higher degree, to any other university or institution.

Full Name: Glenn Wadley

Signed: ____________________________  Date: ________________
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DEFINITION OF TERMS

The symbols and definition of terms used in this thesis are:

- **BMI**: body mass index (kg.m$^{-2}$)
- **BSA**: bovine serum albumin
- **CAP**: c-Cbl associating protein
- **CHO**: carbohydrate
- **DMEM**: Dulbecco's modified Eagle’s medium
- **EGF**: extracellular growth factor
- **ELISA**: enzyme-linked immunosorbent assay
- **g**: relative centrifugal force
- **GIR**: glucose infusion rate
- **GLUT4**: glucose transporter isoform 4
- **HOMA**: homeostatic model of assessment
- **HRP**: horse radish peroxidase
- **IDDM**: insulin dependent diabetes mellitus
- **IgG**: immunoglobulin G
- **IR**: Insulin receptor
- **IRS**: Insulin receptor substrate
- **LAR**: leukocyte antigen-related PTPase
- **LRP**: leukocyte common antigen-related PTPase
- **MAPK**: mitogen activated protein kinase
- **min**: minute
- **mRNA**: messenger ribonucleic acid
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<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>$p$</td>
<td>level of probability</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>tensin homolog deleted on chromosome ten</td>
</tr>
<tr>
<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTPase</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>pY</td>
<td>phosphotyrosine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHIP2</td>
<td>Src Homology 2 domain containing inositol 5-phosphatase 2</td>
</tr>
<tr>
<td>SHPTP2</td>
<td>Src Homology (SH) PTPase 2</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>VO$_2$</td>
<td>rate of oxygen uptake</td>
</tr>
<tr>
<td>VO$_2$ peak</td>
<td>peak rate of oxygen uptake per minute</td>
</tr>
<tr>
<td>VCO$_2$</td>
<td>rate of carbon dioxide production</td>
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ABSTRACT

Regulation of insulin signalling by exercise in skeletal muscle

Regular physical activity improves insulin action and is an effective therapy for the treatment and prevention of type 2 diabetes. However, little is known of the mechanisms by which exercise improves insulin action in muscle. These studies investigate the actions of a single bout of exercise and short-term endurance training on insulin signalling. Twenty-four hours following the completion of a single bout of endurance exercise insulin action improved, although greater enhancement of insulin action was demonstrated following the completion of endurance training, implying that cumulative bouts of exercise substantially increase insulin action above that seen from the residual effects of an acute bout of prior exercise. No alteration in the abundance and phosphorylation of proximal members of the insulin-signalling cascade in skeletal muscle, including the insulin receptor and IRS-1 were found. A major finding however, was the significant increase in the serine phosphorylation of a known downstream signalling protein, Akt (1.5 fold, p<0.05) following an acute bout of exercise and exercise training. This was matched by the observed increase in protein abundance of SHPTP2 (1.6 fold, p<0.05) a protein tyrosine phosphatase, in the cytosolic fraction of skeletal muscle following endurance exercise. These data suggest a small positive role for SHPTP2 on insulin stimulated glucose transport consistent with transgenic mice models. Further studies were aimed at examining the gene expression following a single bout of either resistance or endurance exercise. There were significant transient increases in IRS-2 mRNA concentration in the few hours following a single bout of both endurance and resistance exercise. IRS-2 protein abundance was also observed to significantly increase 24-hours following a single bout of endurance exercise indicating transcriptional regulation of IRS-2 following muscular contraction. One final component of this PhD project was to examine a second novel insulin-signalling pathway via c-Cbl tyrosine phosphorylation that has recently been shown to be essential for insulin stimulated glucose uptake in adipocytes. No evidence was found for the tyrosine phosphorylation of c-Cbl in the skeletal muscle of Zucker rats despite demonstrating significant phosphorylation of the insulin receptor and Akt by insulin treatment and successfully immunoprecipitating c-Cbl protein. Surprisingly, there was a small but significant increase in c-Cbl protein expression following insulin-stimulation, however c-Cbl tyrosine phosphorylation does not appear to be associated with insulin or exercise-mediated glucose transport in skeletal muscle.
CHAPTER ONE
REVIEW OF LITERATURE

1.1 Introduction

Currently around 1 million Australians and 124 million people worldwide have diabetes mellitus, and this figure is expected to increase to approximately 300 million worldwide by the year 2025 (Amos et al., 1997; Dunstan et al., 2001). Diabetes mellitus is a collection of disorders characterised by high glucose levels in the blood and body tissues. The two major forms of diabetes mellitus are insulin dependent (IDDM or type 1 diabetes) and non insulin dependent diabetes mellitus (NIDDM or type 2 diabetes). Type 1 diabetes is caused by the destruction of the insulin secretory capacity of the pancreatic beta cells by an autoimmune response and affects approximately 15% of all cases worldwide (Alberti et al., 1997). Type 2 diabetes is by far the more prevalent form of the disease, accounting for up to 90% of cases in Western countries (Alberti et al., 1997) and is characterised by defects in both insulin secretion and insulin resistance of peripheral target tissues such as skeletal muscle (DeFronzo et al., 1985). The burgeoning epidemic of type 2 diabetes and obesity will be among the most significant public health issues facing industrialised nations within the next decade. The development of insulin resistance is a particularly crucial and an early event in the aetiology of syndrome X, a cluster of overlapping conditions, which include obesity, atherosclerosis, type 2 diabetes and hypertension (Reaven, 1994; Weyer et al., 1999). The most important site of insulin resistance in type 2 diabetics has been found to be skeletal muscle; which can account for approximately 80 – 90% of insulin stimulated
glucose uptake (DeFronzo et al., 1985). Therefore, the intracellular defects that contribute to insulin resistance in skeletal muscle will be a focus of this discussion.

There is considerable evidence suggesting physical activity is an effective therapy for the treatment and prevention of type 2 diabetes, however little is known of the intracellular mechanisms by which physical activity exerts such a positive influence on skeletal muscle insulin action. Considerable recent gains have been made in determining the cellular basis of the actions of insulin and intracellular sites of insulin resistance, providing new clues in the hunt to identify the mechanisms by which regular physical activity is able to exert a positive benefit on insulin sensitivity in humans. This review will provide an overview of the current literature that has examined how insulin and exercise regulate glucose uptake in skeletal muscle, identifying what is known and hypothesised regarding the interactions between these pathways. There are significant benefits in determining the intracellular pathways through which physical activity improves insulin action as this knowledge may provide a basis for the development of more efficacious therapies in the treatment and prevention of type 2 diabetes.

1.2 Physical activity, insulin resistance and type 2 diabetes

Physical inactivity is believed to be causally associated with the development of chronic diseases such as obesity, atherosclerosis, type 2 diabetes and hypertension. Indeed, regular physical activity is inversely correlated with type 2 diabetes (Helmrich et al., 1991; Manson et al., 1992; Manson et al., 1991) coronary heart disease (Manson et al., 1999), and importantly total mortality (Powell & Blair, 1994), a relationship which has been shown to be independent of body fat mass (Lee et al., 1999). A large proportion of
Australians do not currently undertake sufficient physical activity to obtain some health benefits. Approximately half of all Australians over 25 years of age accumulate less than the recommended minimum of 150 minutes of physical activity per week (including walking, moderate and vigorous activity), which also coincides with 24% of Australians over the age of 25 having some form of impaired glucose metabolism (Dunstan et al., 2001). Vigorous exercise at least once per week has been found to significantly reduce the risk of developing type 2 diabetes (Manson et al., 1991) whilst increased frequency of vigorous physical activity per week progressively decreases the risk of developing type 2 diabetes (Manson et al., 1992). It is not only vigorous exercise that has such a profound therapeutic benefit, since at least 180 minutes of brisk walking per week is able to reduce the risk of type 2 diabetes and coronary heart disease to a similar extent as regular vigorous exercise (Hu et al., 1999; Manson et al., 1999). Furthermore, long-term interventions (3-6 years), which involve physical activity and diet modification have been shown to reduce the progression to type 2 diabetes in subjects already with impaired glucose tolerance (Eriksson & Lindgarde, 1991; Knowler et al., 2002; Tuomilehto et al., 2001). A landmark study (Knowler et al., 2002) recently demonstrated that lifestyle interventions involving both diet and physical activity modification are more effective at preventing type 2 diabetes than treatment with common antihyperglycemic drugs. Over 3000 obese non-diabetics with impaired glucose tolerance underwent long-term treatment involving standard lifestyle recommendations plus metformin, a placebo or an intensive lifestyle intervention program involving 150 minutes of brisk walking per week, individualised dietary education promoting weight reduction and a healthy low calorie, low fat diet. After 3 years the incidence of diabetes was 58% lower in the lifestyle intervention group and only 31% lower in the metformin group when compared to the placebo group. These
studies demonstrate the powerful effect that a healthy lifestyle has on the prevention of metabolic disorders. However they do not address whether physical activity independently of diet modification provides protection against type 2 diabetes.

There does however appear to be a distinct therapeutic benefit of physical activity alone in the prevention of type 2 diabetes. A study into a cohort of the Amish population of America revealed that despite similar rates of obesity when compared to the general Caucasian population in America, there was a significantly lower prevalence of type 2 diabetes (Hsueh et al., 2000). The Amish population of America are a cultural group that forgo many of the labour saving devices of the typical American population and are therefore thought to be significantly more physically active (Hsueh et al., 2000), which supports the independent benefits of physical activity in diabetes prevention. More direct evidence also demonstrates the powerful effect of physical activity on diabetes prevention. In one of the most definitive studies so far, almost 600 Chinese citizens with impaired glucose tolerance underwent a randomised clinical control trial over a six-year period. Regular physical activity was found to be significantly more effective in preventing type 2 diabetes than diet modification with no further benefit when diet and exercise were combined (Pan et al., 1997). Therefore, the reasons why physical activity is effective in reducing the prevalence and development of type 2 diabetes are probably twofold.

Firstly, regular physical activity is protective against excessive weight gain (Paffenbarger et al., 1986), with adiposity strongly associated with insulin resistance and diabetes development obesity (Goodyear et al., 1995a; Kelley et al., 1999). High fat feeding in rats induces insulin resistance (Kim et al., 2000a), while weight loss in
humans via a low calorie diet improves insulin sensitivity (Franssila Kallunki et al., 1992) demonstrating the clear link between body fatness and insulin action.

Secondly, independent of body fatness physical activity improves skeletal muscle insulin sensitivity. The sensitivity of tissues such as muscle and fat to exogenous insulin is commonly measured in research by a euglycemic, hyperinsulinemic clamp. A primed, constant infusion of insulin is used to raise and maintain plasma insulin levels. The negative feedback principle is used to hold plasma glucose concentration at basal levels via a variable glucose infusion (DeFronzo et al., 1979). Under steady-state euglycemia, the glucose infusion rate is equal to the glucose uptake by all insulin sensitive tissues in the body, primarily skeletal muscle and fat (DeFronzo et al., 1985). The euglycemic, hyperinsulinemic clamp is the recognised gold standard for measuring whole-body insulin sensitivity (DeFronzo et al., 1979). In cross-sectional studies, those who are physically active have greater insulin sensitivity than sedentary individuals (Ebeling et al., 1993; Kirwan et al., 2000; Takala et al., 1999). Indeed, a single bout of exercise provides sufficient stimuli to improve insulin sensitivity in humans (Mikines et al., 1988; Thorell et al., 1999; Wojtaszewski et al., 1997) and rodents (Hansen et al., 1998). Whilst endurance training in humans (1-12 weeks, 30-60 min/day) has also been shown in many studies to improve insulin sensitivity (DeFronzo et al., 1987; Dela et al., 1994; Houmard et al., 1999; Hughes et al., 1993) with even one week of endurance training being sufficient to improve glucose tolerance in humans with mild type 2 diabetes (Rogers et al., 1988). Recently, a 6-month walking program involving moderate or vigorous walking for 30 minutes per day, 3-7 days per week in older, mildly overweight non-diabetics was sufficient to improve insulin sensitivity despite no changes in bodyweight (Duncan et al., 2003). Thus, there is considerable evidence that physical
training and more specifically, endurance training has a powerful impact on insulin action in human skeletal muscle. Yet it remains to be fully elucidated how regular muscle contraction is able to elicit such a profound effect on the intracellular actions of insulin. An obvious starting point when searching for the underlying mechanisms of insulin resistance is to discover where differences lie along the pathway of insulin signalling to glucose transport and glycogen synthesis in type 2 diabetics compared with subjects displaying normal glucose tolerance. Downstream of insulin signalling, defects in the skeletal muscle of type 2 diabetics are apparent, including reduced hexokinase II and glycogen synthase activity (Cusi et al., 2000; Kruszynska et al., 1998). Type 2 diabetics also have a downregulation of a number of steps involved in insulin-signalling and glucose transport (Cusi et al., 2000). To understand this further it is important to examine firstly the pathway of insulin action, how this pathway is dysregulated in insulin resistant states and then identify how exercise might interact with the insulin-signalling cascade to enhance insulin action.

1.3 Pathway of insulin signalling

The binding of insulin to its receptor initiates a complex cascade of signalling events, which ultimately leads to the translocation of the GLUT4 glucose transporter to the plasma membrane. The key proteins involved in insulin signalling and their interaction, resulting in GLUT4 translocation, is not fully understood. However, several steps have been well elucidated and are known to be crucial in this pathway (Figure 1.1).

The insulin receptor is a transmembrane protein comprising two alpha subunits, each linked to a beta subunit and each other by disulfide bonds. The two alpha subunits are
extracellular and contain the insulin binding sites, whilst the two beta subunits are transmembrane with the intracellular portion containing the insulin regulated tyrosine protein kinase (for reviews, see Kellerer et al., 1999; White & Kahn, 1994). Binding of insulin to the alpha subunits activates the tyrosine kinase of the beta subunits, enabling autophosphorylation of the insulin receptor (IR) via tyrosine phosphorylation. This autophosphorylation of the IR in turn enables phosphorylation of a family of multi-site docking proteins called insulin receptor substrates (IRS), each containing numerous tyrosine and serine phosphorylation sites including at least 8 tyrosine sites on IRS-1 alone that are phosphorylated by the activated IR. (Lavan et al., 1997a; Lavan et al., 1997b; Sun et al., 1993; Sun et al., 1995). The 4 known members of the IRS family are IRS-1, IRS-2, IRS-3 and IRS-4 (Lavan et al., 1997a; Lavan et al., 1997b; Sun et al., 1993; Sun et al., 1995). Of the 4 IRS proteins, various combinations of genetic knockout mice models have demonstrated a major role for IRS-1 and IRS-2 in insulin signalling, with IRS-1 being a major regulator in muscle and IRS-2 being prominent in liver (Kido et al., 2000). Recent findings also point to a partial insulin-signalling role for IRS-2 in skeletal muscle immediately after exercise (Howlett et al., 2002). The tyrosine phosphorylation of IRS-1 binds a number of proteins containing the Src-homology 2 and 3 domains (SH2 and SH3). These include Src homology protein tyrosine phosphatase 2 (SHPTP2, also called SHP2); a protein tyrosine phosphatase, GRB2; an adaptor molecule that links the RAS pathway and is involved in cell growth and metabolism, Nck; an adaptor protein involved in cell proliferation and the lipid cleavage enzyme phosphatidylinositol 3-kinase (PI 3-kinase) that is a key enzyme required for insulin stimulated glucose transport (Figure 1.1) (Lee et al., 1993; Myers et al., 1992; Skolnik et al., 1993; Sun et al., 1993). In addition to phosphorylating PI 3-kinase the IRS proteins traffic insulin signalling via the mitogenic pathways (for
reviews see (Kellerer et al., 1999; Virkamaki et al., 1999; White & Kahn, 1994). Recent evidence also points to a role in GLUT4 activation for the p38 subgroup of the mitogen activated protein kinase (MAPK) pathways (Somwar et al., 2001; Thong et al., 2003) independently of IRS’s and PI 3-kinase, although the mitogenic pathways are not the focus of this review.

To add further to this complexity, PI 3-kinase is a heterodimeric enzyme composed of a p85 regulatory subunit and a p110 catalytic subunit. The p85 regulatory subunit resides predominantly in the cytosol in the basal state. Following insulin stimulation, the p85 subunit translocates from the cytosol to the intracellular membrane whereby it associates the activated IRS proteins and is activated (Clark et al., 1998; Inoue et al., 1998). Currently, five isoforms of the regulatory subunits have been identified, two 85 kDa proteins (p85α, p85β), two 55 kDa proteins (p55α, p55γ) and a 50 kDa protein (p50α) (Inukai et al., 1997; Shepherd et al., 1997). The isoforms have tissue-specific distribution and exhibits different degrees of activation in response to stimulation by insulin (Inukai et al. 1997). Of these isoforms, p85α is ubiquitous and is expressed most abundantly in muscle, fat and liver (Inukai et al., 1997; Shepherd et al., 1997). Chemical inhibition, and more recently genetic manipulation to generate dominant negative mutant p85 cells have demonstrated the key role of PI 3-kinase in the stimulation of GLUT4 translocation by insulin (Cheatham et al., 1994; Lund et al., 1995; Wojtaszewski et al., 1996).
Figure 1.1 Pathway of insulin signalling. Upon insulin binding to its receptor it undergoes autophosphorylation, whereby it catalyses phosphorylation of a number of proteins, including the IRS family that is involved in glucose transport. Insulin receptor autophosphorylation also initiates numerous cellular processes involved in glucose metabolism, glycogen synthesis, cell growth and differentiation, protein synthesis and gene expression. Adapted from (Saltiel & Kahn, 2001). Note: SHP2 is also called SHPTP2 and is known to associate with IRS-1 upon insulin-stimulation and is thought to be involved in glucose transport (Kuhne et al., 1993; Maegawa et al., 1999).
Much of the complexity of this pathway resides beyond PI 3-kinase activation, with many of the steps not yet identified. Little is known of the proteins downstream of PI 3-kinase that are involved in the regulation of GLUT4 translocation. Akt, also known as protein kinase B, is potentially a key protein involved in insulin stimulated GLUT4 translocation (Kim et al., 1999b; Thorell et al., 1999; Wojtaszewski et al., 1999) whilst some atypical isoforms of protein kinase C (aPKC) such as zeta (ζ) and lambda (λ) may also have a role (Standaert et al., 1999).

Akt is a downstream target of PI 3-kinase, that is activated by insulin and has a potential role in glucose transport since overexpression of constitutively active Akt increases GLUT4 translocation and glucose transport in 3T3L1 adipocytes and L6 muscle cells (Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997; Ueki et al., 1998). Recently, some isoforms of Akt (Akt-2 and Akt-3) have shown defective activity in obese, insulin resistant humans compared with lean controls (Brozinick et al., 2003). The precise role of Akt in glucose transport is somewhat unclear since overexpression of a dominant negative mutant Akt in chinese hamster ovary (CHO) cells does not effect insulin stimulated glucose transport (Kitamura et al., 1998) suggesting that it may not play a major role in glucose transport. However, inhibitory mutant models are problematic with chronic mutant overexpression potentially allowing the adaptation of alternate pathways (Hill et al., 1999). Recently, depletion of Akt-1 and/or Akt-2 isoforms in 3T3L1 adipocytes by RNA directed gene silencing suggest a primary role for Akt-2 and a lesser role for Akt-1 in insulin-stimulated glucose transport (Jiang et al., 2003).

Insulin stimulation of Akt has been shown to increase glycogen synthesis in L6 myotubes by inhibiting glycogen synthase kinase-3 (GSK-3), a known inhibitor of glycogen synthase (Cross et al., 1995; Cross et al., 1997; Ueki et al., 1998) whilst in cell
lines lacking GSK-3 such as 3T3L1 adipocytes, overexpression of Akt has no effect of glycogen synthesis (Ueki et al., 1998). Activation of Akt also has been shown to increase protein synthesis in both L6 myotubes and adipocytes (Hajduch et al., 1998; Ueki et al., 1998). The pathway for insulin stimulated protein synthesis via the activation of Akt appears to involve the downstream activation of p70 S6 kinase, since inhibition of Akt reduces activation of p70 S6 kinase in CHO cells and 3T3L1 adipocytes (Kitamura et al., 1998). These findings suggest that not only is Akt a crucial insulin signalling protein that resides downstream of PI 3-kinase, but that it has multiple signalling roles in response to insulin stimulation that appear to involve glucose transport as well as glycogen and protein synthesis.

Other components of the insulin signalling pathway downstream of PI 3-kinase that have been shown to have a role in insulin stimulated glucose transport is the activation of atypical PKC isoforms ζ and λ via activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Grillo et al., 1999; Standaert et al., 1999). The role of these downstream kinases in the insulin stimulated translocation of GLUT4 has still to be fully elucidated and will not be a major focus of this review although they are a promising area of future investigation since atypical PKC activity is associated with impaired insulin-stimulated GLUT4 translocation brought about by high fat feeding and fatty acid infusion in rodents (Kim et al., 2002; Tremblay et al., 2001).

This leaves the perplexing situation in which it has yet to be adequately described how insulin stimulates glucose transport. Clearly such research is a focus of major research endeavours.
1.4 A second pathway for insulin signalling

Recently, a second novel insulin-signalling pathway has been described (Baumann et al., 2000) that has been shown to be essential for insulin stimulated glucose uptake in adipocytes. This pathway involves the recruitment of the protein c-Cbl to the IR by the c-Cbl associating protein (CAP) (Figure 1.1). c-Cbl is a 120-kDa cellular homologue of the transforming v-Cbl oncogene. In 3T3L1 adipocytes c-Cbl is rapidly tyrosine phosphorylated by insulin (Ribon & Saltiel, 1997) and is recruited to the IR by CAP (Ribon et al., 1998). Tyrosine phosphorylation via the IR initiates the dissociation of the CAP-Cbl complex enabling c-Cbl interaction with the caveolar protein flotillin. Flotillin forms a ternary complex with both CAP and c-Cbl directing its localisation to lipid rich regions of the plasma membrane known as lipid rafts. Expression of a non-functional CAP mutant in 3T3-L1 adipocytes prevents insulin stimulated GLUT4 translocation and glucose transport, demonstrating the importance of this pathway (Baumann et al., 2000).

A number of other stimuli are able to tyrosine phosphorylate c-Cbl, including extracellular growth factor (EGF) and interleuken-3 (Anderson et al., 1997; Fukazawa et al., 1996; Marcilla et al., 1995; Meisner et al., 1995; Ribon & Saltiel, 1997). Shear stress also tyrosine phosphorylates c-Cbl in endothelial cells (Miao et al., 2002). However, studies are yet to be undertaken to determine the role of this pathway in the primary target for glucose disposal, skeletal muscle. There is evidence that c-Cbl’s role in insulin signalling may be tissue specific. Cell lines such as 3T3L1 fibroblasts do not contain the c-Cbl associating protein, CAP and do not show the same insulin stimulated tyrosine phosphorylation of c-Cbl as 3T3L1 adipocytes (Ribon et al., 1998). In cell lines expressing high levels of functional insulin receptors, such as chinese hamster ovary (CHO) cells overexpressing the human insulin receptor, rat 1 fibroblasts (HIRC) and KRC-7 cells insulin treatment does not induce tyrosine phosphorylation of c-Cbl (Ribon
& Saltiel, 1997). Unfortunately, there has been no investigation into the role of c-Cbl in insulin action in the primary site of glucose disposal, skeletal muscle to confirm if the pathway is a central component of insulin signalling or if it is an ancillary pathway used for insulin signalling in cells lacking sufficient abundance of CAP or insulin receptors.

### 1.5 Exercise and glucose transport

Exercise alone enables GLUT4 translocation to the cell surface and increased GLUT4 glucose transport (Richter et al., 1998; Thorell et al., 1999). The mechanisms for this contraction-mediated activation of GLUT4 remain unknown, although it is now well established that the pathway is independent from the proximal steps in the insulin-signalling cascade. Type 2 diabetics also have normal contraction-stimulated GLUT4 translocation and glucose uptake (Kennedy et al., 1999; Kingwell et al., 2002) so it is unlikely that the contraction-stimulated pathway for GLUT4 translocation is directly involved in the increased insulin sensitivity observed following exercise cessation. The independent effects of exercise on GLUT4 translocation and subsequent glucose transport will therefore not be covered in great detail in this review. For recent insights detailing the independent mechanisms by which muscle contraction alone stimulates glucose transport see reviews (Cortright & Dohm, 1997; Hayashi et al., 1997; Richter et al., 2001; Ryder et al., 2001). Although contraction and insulin seem to stimulate GLUT4 translocation and glucose uptake via independent pathways, it has been mentioned earlier that following exercise cessation there is a substantial increase in insulin action above that exhibited by contraction or insulin alone and can last up to 48 hours following a single exercise bout (Mikines et al., 1988). Acute exercise and exercise training increases hexokinase II and glycogen synthase activity along with insulin sensitivity in normal glucose tolerant rodents (Kim et al., 2000a; O'Doherty et
al., 1993) and also increases glycogen synthase (GS) activity in type 2 diabetic humans
(Cusi et al., 2000). Similar improvements have also been found at a number of steps
involved in glucose transport (Cusi et al., 2000; Houmard et al., 1999). However
nuclear magnetic resonance (NMR) combined with microdialysis measurements of
skeletal muscle found no association between reduced glycogen synthesis and
intracellular glucose accumulation in type 2 diabetics (Cline et al., 1999). This would
suggest that glucose transport is a major rate-limiting step for insulin-stimulated glucose
metabolism in type 2 diabetic muscle. Recent exercise training and high fat diet studies
in rodents would support this view (Kim et al., 2000a).

1.6 Defective insulin signalling in insulin resistant states
Critical to the impairment in insulin action observed in human diseases of insulin
resistance, including obesity and type 2 diabetes, is attenuated activation of the tyrosine
phosphorylation of the key insulin-signalling proteins. Reduced insulin stimulated
tyrosine phosphorylation of IR, IRS-1 and IRS-1 associated PI 3-kinase activity is
evident in human obesity (Cusi et al., 2000), demonstrating reduced activation of the
initial steps of insulin action. In addition, morbid obesity (BMI > 40 kg/m^2) is also
accompanied by a reduction in the protein content of IR, IRS-1 and the p85 subunit of
PI 3-kinase in their muscles (Goodyear et al., 1995a). Similar defects are observed in
obese rats (Anai et al., 1998; Folli et al., 1993; Kerouz et al., 1997). Type 2 diabetics
demonstrate comparable suppression of insulin stimulated IRS-1 tyrosine
phosphorylation and lower activation of PI 3-kinase activity by either IRS-1 or IRS-2
(Bjornholm et al., 1997; Cusi et al., 2000; Kim et al., 1999b; Krook et al., 2000).
Downstream of PI 3-kinase, there is some controversy regarding the defective activation of Akt by insulin in muscle that is insulin resistant. Some studies have shown that obese subjects and type 2 diabetics have normal activation of Akt isoforms (Kim et al., 1999b), whilst recently Brozinick et al. (Brozinick et al., 2003) has shown defective activity of Akt-2 and Akt-3 isoforms but not Akt-1 in morbidly obese subjects. Interestingly, there was an impairment of Akt-1 serine (473) phosphorylation in obese subjects despite normal activation of Akt-1 kinase activity suggesting other sites of regulation for Akt-1 apart from phosphorylation on serine site 473 such as the threonine 308 site (Brozinick et al., 2003). The discrepancies between studies could be due to a number of factors such as different subject populations or muscle analysis although the differences are probably due to antibody specificity. Kim et al. (Kim et al., 1999b) found no defective activation of Akt immunoprecipitated with an antibody that recognises both Akt-1 and Akt-2 isoforms. If Akt-1 were normal in type 2 diabetics then this would mask any defective regulation of Akt-2 using an antibody that recognises both isoforms. Clearly, more investigation into the functional regulation of proteins distal to PI 3-kinase in insulin resistant states is required. However the pattern emerges that in insulin resistant states, a major defect in insulin signalling resides in an impaired capacity to generate a tyrosine phosphorylation signal from the IR through to PI 3-kinase. Further downstream in the insulin signalling pathway it is unlikely that GLUT4 protein abundance is primarily involved in the development of insulin resistance since type 2 diabetics have normal levels of GLUT4 protein (Andersen et al., 1993; Kennedy et al., 1999).
1.7 Interactions between exercise and the insulin signalling pathway

It was mentioned earlier that a remarkable effect of exercise is that enhanced insulin sensitivity is observed for up to 48 hours following the cessation of a single exercise bout (Mikines et al., 1988). There is some evidence that during the later stages of this period enhanced insulin sensitivity may be due to an upregulation of the insulin-signalling cascade. In the hours following a single bout of endurance exercise, improved insulin sensitivity is not due to an upregulation of the proximal steps in the insulin-signalling cascade. Indeed there is no increase in insulin binding and activation of the IR despite blood flow potentially increasing the amount of available insulin to the contracted muscle (Bonen et al., 1985; Treadway et al., 1989). There is no increase in the insulin-stimulated tyrosine phosphorylation of IRS-1, or its associated activation of PI 3-kinase despite significant increases in insulin sensitivity (Goodyear et al., 1995b; Wojtaszewski et al., 1997). Prior exercise alone also reduces basal IR tyrosine phosphorylation without significantly activating IRS-1 or its association with the p85 subunit of PI 3-kinase (Koval et al., 1999). However it appears that following exercise and insulin stimulation, IR/IRS-1 independent pathways can activate PI 3-kinase. Studies in rodents have demonstrated an insulin-stimulated increase in phosphotyrosine associated PI 3-kinase activity following acute exercise (Zhou & Dohm, 1997) with part of the increase in phosphotyrosine associated PI 3-kinase activity attributed to IRS-2 associated PI 3-kinase activity (Howlett et al., 2002). Also, recent work using IR knockout mice suggest that an as yet unidentified tyrosine phosphoprotein is binding and activating PI 3-kinase following exercise (Wojtaszewski et al., 1999).
Also, there is evidence that in the few hours following exercise, a serum factor, most likely a protein, may be responsible for enhanced insulin sensitivity although little research has followed up on this theory (Gao et al., 1994). Alternative mechanisms regulating tyrosine phosphoproteins may also be involved in the enhancement of insulin signalling and these will be discussed at a later stage.

There is also some evidence that some of the downstream insulin signalling proteins may be activated by contraction. Although the activation of Akt by insulin is well known, its activation by contraction is somewhat controversial in skeletal muscle (Brozinick Jr. & Birnbaum, 1998; Sherwood et al., 1999; Turinsky & Damrau-Abney, 1999; Whitehead et al., 2000). Total Akt kinase activity does not increase in response to contraction (Brozinick Jr. & Birnbaum, 1998; Sherwood et al., 1999) although a small increase in Akt-1 kinase activity and serine (473) phosphorylation has been observed following electrical stimulation in rat muscle (Turinsky & Damrau-Abney, 1999; Whitehead et al., 2000). The serine (473) and threonine (308) phosphorylation of all three isoforms (Akt-1 > Akt-2 > Akt-3) by contraction has recently been confirmed (Sakamoto et al., 2002) however this effect is relatively minor and transient compared to the insulin stimulated activation of Akt (Sakamoto et al., 2002; Turinsky & Damrau-Abney, 1999; Whitehead et al., 2000) since maximal activation occurs by 3 minutes and then returns to basal levels by 15 minutes of contraction. In humans, pAkt (Ser473) is increased immediately following exercise (Thorell et al., 1999) although pAkt (Ser473) is not elevated in humans 4-hours after the cessation of exercise (Wojtaszewski et al., 2000). However, it may play a role in enhanced insulin-stimulated glucose uptake in the longer term (24–48 hour period) following exercise cessation. Rodent studies point towards a role for an additive effect of exercise on insulin-stimulated Akt
phosphorylation since it has been shown to be increased in the 16-48 hour period following 5 days and 6 weeks of exercise training in lean, non-diabetic rats (Chibalin et al., 2000; Luciano et al., 2002) with a trend for increased pAkt (Ser473) following one day of exercise (Chibalin et al., 2000). Clearly, more work needs to be done to investigate the potential effect of muscular contraction on Akt activation although it is a potential candidate for the regulation of insulin-stimulated glucose uptake following exercise.

A single bout of exercise and exercise training also enhances GLUT4 gene expression, protein concentration and activation (Cox et al., 1999; Goodyear et al., 1990; Kraniou et al., 2000). Furthermore, overexpression of GLUT4 in transgenic mice improves basal and insulin-stimulated glucose transport (Brozinick et al., 2001; Brozinick et al., 1997) so it is possible that increases in GLUT4 content following exercise could be at least be partly responsible for improvements in insulin sensitivity.

Exactly, how exercise is then able to increase insulin action remains unclear. Based on available data 2 mechanisms can be proposed.

- An acute effect, which lasts for a few hours following exercise cessation and appears to act independently of the early steps in the insulin-signalling cascade.

- And a second chronic response that can last for up to 48 hours following a single exercise bout (Mikines et al., 1988) and may be related to improvements in insulin-signalling transduction or increased protein abundance that may account for greater activation (Chibalin et al., 2000; Cusi et al., 2000). It is this longer
lasting effect of a single bout of exercise or endurance training on insulin action and insulin signalling transduction and the potential regulatory mechanisms that will be a major focus of this thesis. Therefore, unless otherwise stated, the chronic response of insulin action and/or insulin signalling to either a single (acute) bout of exercise or endurance training in the period > 15 hours post exercise will form the basis of the proceeding discussion.

1.7.1 Insulin signalling and acute exercise
One of the remarkable effects of a single exercise bout at a moderate intensity is its ability to exert improvements on insulin sensitivity for up to 48 hours following exercise (Mikines et al., 1988). Investigations into the mechanisms of how insulin sensitivity is increased 24-48 hours after a single exercise bout is an area of considerable interest. Along with the improvements in insulin sensitivity there are also improvements in a number of the early signalling events following insulin stimulation including IRS-1 and IRS-2 associated PI 3-kinase activity in lean, non-diabetic rodents examined 16 hours following exercise (Chibalin et al., 2000). There are few comparative results in humans, particularly in subjects with normal glucose tolerance. In obese non-diabetics, insulin sensitivity was improved 24 hours following a single exercise bout and this was associated with improvements in IR tyrosine phosphorylation and the association of the p85 subunit of PI 3-kinase with IRS-1 (Cusi et al., 2000). When the same experiment was performed in type 2 diabetics, there was no change in insulin sensitivity despite improvements in IR and IRS-1 tyrosine phosphorylation. There was also no improvement in the p85 association with IRS-1 (Cusi et al., 2000) in type 2 diabetics, suggesting that this may be a key rate-limiting step in insulin action. Clearly, further work needs to clarify the differences in insulin action and the signalling...
cascade between normal and insulin resistant subjects. Exercise however, provides a powerful intervention in which to understand the mechanisms behind the intracellular defects in those individuals with impaired glucose tolerance.

It is possible that acute exercise may be exerting changes in the protein abundance of some of the proteins involved in insulin signalling. Most of the primary signalling proteins such as IR and IRS-1 do not change in response to a single bout of exercise although IRS-2 protein abundance and tyrosine phosphorylation significantly increase in the 24 hours following acute exercise (Chibalgin et al., 2000). IRS-2 may exert a major role in mediating insulin action in liver and in the development and maintenance of pancreatic β-cell mass, yet its precise role in skeletal muscle is uncertain (Kido et al., 2000; Withers et al., 1999). In isolated muscle and fat cells, IRS-2 has been shown to have a similar role to IRS-1 in insulin-stimulated glucose transport (Miele et al., 1999; Zhou & Dohm, 1997) and may act as an alternative pathway of insulin action. Although IRS-2 has been shown not to play a major role in exercise or insulin stimulated glucose transport (Higaki et al., 1999), insulin stimulated IRS-2 tyrosine phosphorylation and associated PI 3-kinase activity are elevated immediately post exercise and can account for some of the improvements in insulin stimulated glucose uptake (Howlett et al., 2002) immediately post exercise, however much less is known of its role in the 12-24 hours period after the cessation of acute exercise. Even less is known of the transcriptional regulation of IRS-2 following exercise. If the protein abundance of IRS-2 is altered 24 hours following acute exercise, increases in IRS-2 gene expression may be initiated via contraction, thus upregulating protein expression, although this has not been investigated. It is also unknown if similar transient increases in IRS-2 protein expression are observed in humans following acute exercise.
1.7.2 Insulin signalling and exercise training

Exercise training is typically used to describe bouts of exercise performed on a regular basis. Previous studies examining the effect of exercise training on insulin action have typically used bouts of moderate endurance exercise on most days of the week for a minimum of 5 days up to several weeks (Chibalin et al., 2000; Houmard et al., 1999; Luciano et al., 2002; Tanner et al., 2002). Recent human studies have found significant improvements in whole-body insulin sensitivity following short-term endurance training involving several consecutive days of cycling for 60 minutes per day at an intensity of around 75% $\dot{VO}_2$ peak (Houmard et al., 1999; Tanner et al., 2002; Youngren et al., 2001). However, the mechanisms which insulin enhances signalling following exercise training remain unclear. Principally, the insulin-signalling pathway could be regulated by several means. Exercise training could be mediating improvements in the abundance of key proteins such as IR, IRS-1, and IRS-2 or increased protein abundance of protein kinases such as Akt or specific components in the PI 3-kinase complex. Although skeletal muscle IR protein content in rats is significantly elevated following both a single exercise bout and exercise training (Chibalin et al., 2000) a similar effect is not apparent in humans (Cusi et al., 2000; Youngren et al., 2001). Indeed, IRS-1 protein abundance has been shown to be decreased following short term training in rats, whilst IRS-2 protein content increases after one bout of exercise and returns to sedentary levels following training (Chibalin et al., 2000). Longer-term training (6-7 weeks) in rodents shows no change in protein abundance of any of the major signalling proteins (Christ et al., 2002; Luciano et al., 2002). Therefore transcriptional control of these insulin-signalling proteins does not appear to be a central component of the adaptive response to exercise training. This does not imply that the gene expression of these
proteins cannot change in response to exercise training. The mRNAs of IR, IRS-1 and PI 3-kinase increase in response to 9 weeks of endurance training in rats (Kim et al., 1995; Kim et al., 1999a) however these changes probably occur subsequent to exercise induced improvements in insulin sensitivity. The findings discussed above would therefore suggest that changes in the protein abundance of the various insulin signalling proteins do not appear to be the main mechanisms by which endurance training exerts its influence on insulin sensitivity.

In humans and rats it appears that insulin signal transduction is a major factor, although probably not the only factor, contributing to increased insulin sensitivity following training. In the insulin resistant state, exercise training induced improvements in insulin sensitivity may not be mediated by changes in the proximal components of the insulin signalling pathway such as the IR, IRS-1, PI 3-kinase or Akt (Christ et al., 2002). In obese rats, several weeks of endurance training improves insulin sensitivity despite no changes in IR, IRS-1 tyrosine or Akt serine phosphorylation (Christ et al., 2002). Moderately obese middle-aged men were observed following short term training to have improved insulin sensitivity without any improvement in PI 3-kinase, also suggesting that enhanced insulin action may occur downstream of PI 3-kinase (Tanner et al., 2002). In support of this finding is the recent investigation that 5 months of exercise training in patients with chronic heart failure did not improve insulin signalling at the level of IRS-1, PI 3-kinase or Akt despite improvements in insulin sensitivity (Kemppainen et al., 2003). This would imply that in the insulin resistant state, exercise-induced improvements in insulin sensitivity are either occurring at more distal and as yet unknown steps in the insulin-signalling cascade or via a mechanism independent from insulin signalling.
In the healthy state with normal glucose tolerance, the changes in insulin signal transduction following training are not clearly defined or understood even for the proximal components of the cascade. Endurance training for six weeks in lean, healthy rodents upregulates numerous steps in the insulin-signalling cascade (Luciano et al., 2002) and IRS-1 associated PI 3-kinase activity is significantly higher in trained subjects compared with sedentary subjects (Kirwan et al., 2000). However with such a long time course, most of these changes could be occurring secondary to improvements in insulin sensitivity. Short-term endurance training of between 5-7 days does however significantly improve insulin stimulated IR function and PI 3-kinase activity in both humans and rodents (Chibalin et al., 2000; Houmard et al., 1999; Youngren et al., 2001). However in humans, IR autophosphorylation was measured in vitro (Youngren et al., 2001) in relatively high insulin concentrations and it is unknown if it is improved in vivo at normal physiological levels of insulin. It is also unknown what intracellular mechanisms are regulating this improvement in insulin signalling and this will be covered in some detail at a later stage. Even though in the insulin resistant or diabetic state exercise training may be mediating improvements in the insulin-signalling cascade distal to PI 3-kinase, it is still important to understand how exercise training in the healthy, glucose-tolerant state regulates the insulin-signalling pathway. Not only does this further our understanding of the overall regulation of the insulin signalling cascade, but as discussed above, physically active people not only have enhanced insulin sensitivity compared to sedentary subjects with normal glucose tolerance, they also have an upregulation of numerous steps in the insulin signalling cascade, that may ultimately provide protection from metabolic diseases such as type 2 diabetes.
1.7.3 Insulin sensitivity, insulin signalling and resistance training

Recently, insulin action has shown to be improved by resistance exercise (Dunstan et al., 2002; Ishii et al., 1998; Miller et al., 1994; Poehlman et al., 2000). The mechanisms to account for improvements in insulin action via resistance exercise remain unknown. Some cross sectional and longitudinal studies suggest resistance training mediates improvements in insulin action via increases in lean muscle mass alone without improving insulin sensitivity per unit of skeletal muscle mass (Poehlman et al., 2000; Takala et al., 1999). Other resistance training studies have shown insulin action to be improved independently of changes in lean muscle mass or body composition (Dunstan et al., 2002; Ishii et al., 1998). Muscle overload in rodents has been shown to increase glucose uptake, protein synthesis and IRS-1 and p85 associated PI 3-kinase activity independently of insulin (Carlson et al., 2001; Hernandez et al., 2000) suggesting multiple and divergent roles for these proteins. However the effects of resistance exercise on any aspect of the insulin-signalling pathway have yet to be studied in humans.

1.7.4 Unresolved issues regarding improved insulin signalling and sensitivity via exercise

Even though short-term endurance training in humans has also been shown in many studies to improve insulin sensitivity (DeFronzo et al., 1987; Dela et al., 1994; Houmard et al., 1999; Youngren et al., 2001) one constant criticism is that the effect is primarily due to the residual effects of the last bout of exercise and not the result of cumulative bouts of exercise. Indeed, early studies using an oral glucose tolerance test (OGTT) to measure insulin action have found that a large portion of the improvement in insulin sensitivity following exercise could be attributed to the last bout (Heath et al.,
A single bout of exercise is sufficient to improve insulin sensitivity in humans (Mikines et al., 1988; Thorell et al., 1999; Wojtaszewski et al., 1997) and rodents (Hansen et al., 1998) for up to 48 hours (Mikines et al., 1988) and may therefore be solely responsible for the improvements in insulin action. There is some data in rodents and insulin resistant humans to suggest that this is not the case and that there is likely an additive effect of short-term training on insulin sensitivity. In humans with impaired glucose tolerance, a single bout of exercise does not increase glucose tolerance as measured by an OGTT although following 7 days of exercise-training glucose tolerance is increased by approximately 30% (Rogers et al., 1988). In rats measured 16 hours following cessation of exercise, a single bout of exercise increased insulin-stimulated glucose transport by 30%, while 5 days of exercise training increased glucose transport by 50% (Chibalin et al., 2000) suggesting that cumulative bouts of exercise have an additive effect above that of a single exercise bout, however this effect has not been fully investigated in humans, particularly those with normal glucose tolerance.

Importantly, the development of type 2 diabetes, from impaired glucose tolerance, is associated with decreased insulin signalling transduction. The findings that endurance training increases insulin stimulated IR function and PI 3-kinase activity (Chibalin et al., 2000; Houmard et al., 1999; Youngren et al., 2001) provides the possibility that the major beneficial action of exercise training is to exert a positive influence on insulin signalling via improvements in signal transduction that may ultimately prevent skeletal muscle from becoming insulin resistant.

Further, it remains unresolved if acute endurance exercise has an impact on the transcriptional regulation and abundance of proteins such as IRS-2, particularly in
humans. Also, the impact of acute endurance exercise and exercise training on the insulin-stimulated activation of key proteins has yet to be fully resolved, particularly in humans while even less is known regarding the impact of resistance exercise on insulin signalling. Additionally, little is known of the molecular mechanisms that may be mediating the improvements in the activation of these signalling proteins.

**1.8 Protein tyrosine phosphatases as regulators of insulin signalling**

Significant interest is currently being focussed on the regulation of the insulin-signalling cascade by protein tyrosine phosphatases (PTPases). This diverse family of enzymes have a common conserved catalytic domain made up of around 250 amino acids with a cysteine residue that is essential for catalysing the hydrolysis of phosphotyrosine residues (for reviews see (Goldstein et al., 1998; Ostman & Bohmer, 2001) whilst the rest of the protein differs greatly among this group of enzymes. PTPases act to either dampen or amplify the insulin signal by removing the activated phosphate group from tyrosine amino acids (Elchebly et al., 1999) and may therefore be key regulators of the insulin-signalling cascade. The subcellular localisation of PTPases may also be important since readily abundant PTPases in skeletal muscle and adipocytes each display characteristic subcellular distribution between the cytosol and particulate fractions (Table 1.1) suggesting preferential sites of action towards the insulin receptor and insulin receptor substrates depending upon subcellular localisation (Figure 1.2).

Immunoblot analysis has confirmed the expression of 4 major PTPases in skeletal muscle, leukocyte antigen-related (LAR), leukocyte common antigen related (LRP),
protein tyrosine phosphatase 1B (PTP1B) and Src Homology (SH) SHPTP2 (Ahmad et al., 1997a; Ahmad & Goldstein, 1995) although LRP expression appears to be expressed at relatively lower levels than the other three PTPases (Ahmad & Goldstein, 1995).

The transmembrane enzyme, LAR has been implicated as a negative regulator of insulin signalling. Reduction of LAR protein abundance via antisense RNA expression in rat hepatoma cells increased IR signalling (Kulas et al., 1995). Also, elevated LAR expression accounts for enhanced PTPase activity in the adipose tissue of obese humans (Ahmad et al., 1995a) suggesting a role for this PTPase in the regulation of insulin signalling. The regulation of insulin signalling by LAR is thought to be primarily via its association with the insulin receptor (Ahmad & Goldstein, 1997). However, conflicting findings have cast some doubt onto the role of LAR in the regulation of insulin signalling. Unexpectedly, LAR knockout mice display significant insulin resistance compared to their wild type littermates and post insulin receptor defects involving PI 3-kinase (Ren et al., 1998) in the liver. In transgenic mice that specifically overexpress LAR in skeletal muscle, insulin stimulated glucose uptake is suppressed as expected but the primary sites of action for LAR, namely the IR and IRS-1 were unaltered (Zabolotny et al., 2001). Rather, the effects of LAR overexpression appear to be directed towards downstream targets such as IRS-2 and PI 3-kinase (Zabolotny et al., 2001). The exact role of LAR on insulin signalling remains unclear however it may not reside at the proximal sites of the insulin-signalling cascade as previously thought.
Table 1.1. Classification of Major Protein Tyrosine Phosphatases (PTPases) implicated in the regulation of insulin signalling in skeletal muscle.

<table>
<thead>
<tr>
<th>Name</th>
<th>Other Names</th>
<th>Cellular Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAR</td>
<td></td>
<td>Particulate fraction</td>
</tr>
<tr>
<td>LRP</td>
<td>RPTP-α</td>
<td>Particulate fraction</td>
</tr>
<tr>
<td>PTP1B</td>
<td></td>
<td>Cytosol &amp; Particulate</td>
</tr>
<tr>
<td>SHPTP2</td>
<td>Syp, SHP-2, PTP1D, SHPTP3 &amp; PTP2C</td>
<td>Cytosol &amp; Particulate</td>
</tr>
</tbody>
</table>

(Ahmad & Goldstein, 1995; Calera et al., 2000; Maegawa et al., 1999)

Figure 1.2. Potential regulation of the insulin-signalling pathway.
PTP1B was the first PTPase to be isolated in homogenous form in 1988 from human placenta (Tonks et al., 1988). PTP1B is distributed between the cytosol and particulate fractions in skeletal muscle (Ahmad & Goldstein, 1995) and has been shown in vitro to negatively regulate insulin signalling via dephosphorylation of the IR and IRS-1 (Ahmad et al., 1995b; Calera et al., 2000; Goldstein et al., 2000; Kenner et al., 1996). Perhaps the strongest evidence for the role of PTPases in insulin signalling comes from mice lacking a PTPase gene. Elchebly et al. (1999) generated PTP1B null mice by targeted disruption of exons 5 and 6 (PTP1B Ex5/6−/−). These mice demonstrated enhanced insulin sensitivity and increased phosphorylation of IR and IRS-1 in muscle and liver following insulin infusion. Interestingly, these mice were resistant to weight gain when fed a high fat diet. Klaman et al. (2000) has also generated PTP1B deficient mice however they targeted exon 1 (PTP1B Ex1−/−) and also found increased insulin sensitivity. PTP1B Ex1−/− mice were also shown to have low body fat stores, which is largely due to an increase in energy expenditure via increases in basal metabolic rate (Klaman et al., 2000). The relationship between enhanced insulin sensitivity and increased energy expenditure in PTP1B deficient mice is unclear, however these findings do point towards an important role for PTP1B in the regulation of insulin sensitivity. These effects also appear to be tissue specific since overexpression of PTP1B in adipose cells results in no change in insulin stimulated glucose transport despite significant reductions in signal transduction through IR, IRS-1 and PI 3-kinase (Venable et al., 2000). Inhibition of PTP1B activity or disruption of PTP1B protein abundance in vivo also has a profound effect on insulin signalling. Treatment of obese Zucker rats with a known inhibitor of PTP1B activity, Bis(maltolato)oxovanadium(IV), not only reduced PTP1B activity in vivo but reduced plasma insulin levels (Mohammad et al., 2002). Reduction of PTP1B protein abundance in liver and fat by antisense
oligonucleotide treatment in mice has not only been shown to improve insulin sensitivity but improve insulin stimulated phosphorylation of the IR, IRS-1, IRS-2 and Akt-1 (Gum et al., 2003; Zinker et al., 2002). Not surprisingly, research is now underway into specific inhibitors of PTP1B (Iversen et al., 2000) due to its therapeutic potential in patients with insulin resistance.

Following insulin stimulation, SHPTP2 associates with IRS-1 (Kuhne et al., 1993) and unlike PTP1B, the major site of SHPTP2 activity appears to reside in the cytosolic fraction (Ahmad & Goldstein, 1995), implicating SHPTP2 as a potential candidate to dephosphorylate IRS-1. However, unlike most other PTPases in skeletal muscle, SHPTP2 has been implicated to have a small role as a positive regulator of insulin signalling. In rat fibroblasts there is an increase in insulin stimulated binding of IRS-1 with SHPTP2, IRS-1 tyrosine phosphorylation and PI 3-kinase activity when SHPTP2 is overexpressed, with the opposite observed when SHPTP2 is inhibited (Ugi et al., 1996). Inhibiting SHPTP2 by transfection of a dominant negative mutant in rat adipocytes also reduces insulin-stimulated translocation of GLUT4 (Chen et al., 1997). In vivo studies also implicate SHPTP2 as a positive regulator of insulin signalling. Transgenic mice have been generated to express a dominant negative mutant that lacks a PTPase domain thereby inhibiting SHPTP2 (Maegawa et al., 1999). These transgenic mice exhibit impaired insulin stimulated glucose uptake along with impaired activation of IRS-1, PI 3-kinase and Akt-1 in skeletal muscle. It is unclear how SHPTP2 may positively influence insulin signalling. Ugi et al., (Ugi et al., 1996) postulated that SHPTP2 may be increasing tyrosine phosphorylation of IRS-1 by either inhibiting an as yet unknown PTPase(s) or by activating other tyrosine kinases. SHPTP2 is clearly a
candidate involved in the regulation of insulin signalling but as yet it’s precise role and relative contribution to insulin signalling is yet to be determined.

There is some evidence to suggest that PTPases are negative regulators of insulin signalling in humans. PTPase activity is significantly increased in skeletal muscle and adipose tissue of obese subjects compared to lean subjects (Ahmad et al., 1997a; Ahmad et al., 1995a). Furthermore, enhanced insulin sensitivity following weight loss in obese subjects correlates with a reduction in PTPase abundance (Ahmad et al., 1997b). However, PTPase activity is lower in obese type 2 diabetics suggesting that different regulatory mechanisms may be operating in the diseased state (Ahmad et al., 1997a). Unfortunately, no studies to date have examined the impact of exercise on PTPase abundance or subcellular distribution let alone activity. If PTPase abundance, subcellular distribution or activity were decreased in response to exercise then it is tempting to speculate that insulin sensitivity might increase due to the amplified tyrosine phosphorylation on a number of key proteins, including PI 3-kinase.

There are other potential regulators of the insulin-signalling cascade that will not be the focus of this review but are nevertheless worthy of consideration. Briefly, these include the lipid phosphatases Src homology 2 domain containing inositol 5-phosphatase 2 (SHIP2) and tensin homolog deleted on chromosome ten (PTEN) as potential modulators of the proximal and distal actions of PI 3-kinase (for reviews see (Jiang & Zhang, 2002). SHIP2 is thought to be a negative regulator of insulin signalling via its ability to dephosphorylate PI(3,4,5)P3 which is a product of PI 3-kinase and a known activator of Akt (Jiang & Zhang, 2002). Mice deficient in the SHIP2 gene have increased glucose tolerance and insulin sensitivity along with increased glycogen
synthesis (Clement et al., 2001). PTEN is also a potential regulator of insulin signalling since it dephosphorylates PI(3,4,5)P$_3$ and PI(3,4)P$_2$ and may also be involved in the modulation of Akt activity by PI 3-kinase (Jiang & Zhang, 2002). Inhibition of PTEN via an antisense oligonucleotide decreases PTEN protein expression in liver and fat of mice, thereby increasing insulin sensitivity and insulin-stimulated Akt activity (Butler et al., 2002). These findings clearly implicate SHIP2 and PTEN as potential regulators of the insulin-signalling pathway and therapeutic targets for the treatment of insulin resistance although there is currently no data linking them to the exercise-induced improvements in insulin sensitivity.

1.9 Summary

Based on the literature reviewed there is a clear need for research to examine the physiological regulation of the insulin-signalling cascade by exercise in human subjects. An effective intervention that would provide valuable insights into the regulation of the insulin-signalling pathway involves both acute exercise and exercise training. There is currently little known of the cellular adaptations to endurance exercise, which enables greater insulin sensitivity. Indeed, it is currently unknown if insulin sensitivity is unregulated solely by the residual effects of acute exercise or if exercise training further improves insulin sensitivity. The regulation of the proximal aspects of the insulin signalling cascade including tyrosine phosphorylation of the IR and IRS-1 in response to a physiological in vivo insulin stimulus following both acute exercise and exercise training still requires clarification even in healthy, glucose tolerant humans. Less is known of the impact of exercise on more distal components of the insulin-signalling cascade, such as Akt. Few studies have examined the transcriptional regulation of key
insulin signalling proteins such as IRS-2 in skeletal muscle by exercise. There is also now some evidence in humans and rodents suggesting that resistance exercise can also improve the intrinsic capacity of skeletal muscle to respond to insulin independent of changes in lean muscle mass, although even less is known of the molecular mechanisms that may be regulating this when compared to endurance exercise. The emergence of PTPases as regulators of insulin signalling provides potential mechanisms through which muscle contraction may improve insulin-signalling transduction. Furthermore, in vitro studies have provided an alternative or adjunct pathway by which insulin may stimulate GLUT4 translocation and glucose uptake. No studies to date have examined if this pathway is activated by insulin or contraction in vivo, let alone in skeletal muscle, which is the primary site for glucose uptake in mammals. Understanding the physiological significance of these pathways in vivo and responsiveness to exercise will have a profound impact upon the delivery of diabetic care and management as well as providing insights into the development of safer therapies for the treatment and prevention of this and other metabolic diseases.

1.10 Aims

The aims of this thesis are:

1. To determine the impact of acute endurance exercise and endurance training on the mRNA levels of the key insulin signalling proteins IR, IRS-1, IRS-2 and the p85α subunit of PI 3-kinase in human skeletal muscle.

2. To determine the impact of acute resistance exercise on the mRNA levels of key insulin signalling proteins in human skeletal muscle.
3. To determine if short-term endurance training results in significantly higher levels of whole body insulin sensitivity compared to a single bout of endurance exercise in sedentary males.

4. To determine if a single bout of acute exercise and short-term endurance training results in increased protein abundance and/or insulin-stimulated phosphorylation of IR, IRS-1 and Akt in sedentary humans.

5. To examine in detail whether acute endurance exercise or short-term endurance training alters the abundance or subcellular distribution of PTPases such as SHPTP2 and PTP1B in human skeletal muscle and to see if this coincides with improvements in tyrosine phosphorylation of IR, IRS-1 or serine phosphorylation of Akt.

6. To determine if acute endurance exercise or training alters IRS-2 protein expression in human muscle.

7. To determine if insulin stimulation or acute exercise activates the novel insulin signalling pathway involving tyrosine phosphorylation of c-Cbl in skeletal muscle of rodents.

8. To determine if there are any differences in c-Cbl protein expression or tyrosine phosphorylation in the muscle of lean and insulin resistant rodents.
CHAPTER TWO
DIFFERENTIAL EFFECTS OF EXERCISE ON INSULIN SIGNALLING GENE EXPRESSION IN HUMAN SKELETAL MUSCLE

2.1 Introduction

Despite the epidemiological evidence correlating regular physical activity with improvements in insulin action (Helmrich et al., 1991; Manson et al., 1992; Manson et al., 1991) there is a paucity of consistent data examining the cellular mechanisms of action, particularly in humans. In recent years, it has become apparent that transcriptional regulation of gene expression is an integral component of skeletal muscle adaptation to exercise (Goldspink, 1998; Neufer et al., 1998; Puntschart et al., 1998). Although the mechanisms by which insulin signalling is enhanced following exercise remain unclear, the gene expression of key proteins involved in the insulin-signalling pathway could be mediated by endurance training and may account for some of the improvements in insulin signalling following chronic exercise.

The mRNAs of insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI 3-kinase) increase in response to endurance training in rats (Kim et al., 1995; Kim et al., 1999a), while skeletal muscle IR protein content is significantly elevated following both a single exercise bout and exercise training in rats (Chibalin et al., 2000). In humans, PI 3-kinase activity during insulin infusion is increased following endurance training (Houmard et al., 1999), although mRNA abundance was not reported. In addition to IRS-1, skeletal muscle contains an
alternative substrate of the IR, insulin receptor substrate-2 (IRS-2). The response of IRS-2 appears to differ from IRS-1, with IRS-2 protein content increasing after a single bout of exercise, although this increase is abolished following several bouts of endurance exercise (Chibalin et al., 2000). The regulation of IRS-2 gene expression in human skeletal muscle by exercise has yet to be described.

In this study, it was hypothesised that a mechanism contributing to the enhancement of insulin action following endurance training in human subjects is upregulated gene expression of key members of the insulin-signalling cascade. To examine this hypothesis, the impact of a single bout of exercise and short term endurance training on the mRNA levels of IR, IRS-1, IRS-2 and the p85α subunit PI 3-kinase in untrained human subjects was determined.

2.2 Materials and Methods

2.2.1 Experimental protocol

Seven healthy subjects volunteered to be involved in the study. Four were female and three were male. The age, $\dot{V}O_2$ peak and BMI (mean ± SE) of the subjects prior to the study were 28.9 ± 3.1 years, 37.1 ± 2.7 ml.kg$^{-1}$.min$^{-1}$ and 22.6 ± 1.4 kg.m$^{-2}$, respectively. Body mass did not change with the 9 days of exercise training (67.7 ± 5.4 vs. 67.2 ± 5.3 kg). The study was approved by the Deakin University Human Research Ethics Committee and subjects gave their written consent to participate in this study after all procedures and the possible risks of participation were explained. Prior to testing, all subjects performed an incremental cycling test to exhaustion for the
determination of peak pulmonary oxygen uptake (\(\dot{V}O_2\) peak). All cycling tests were performed on a cycle ergometer (Quinton Excalibur, Groningen, The Netherlands). \(\dot{V}O_2\) was measured using indirect calorimetry (Gould metabolic systems, Ohio, USA).

All subjects reported to the laboratory in the morning after an overnight fast (10-12 hours). For the 24 hours preceding all test sessions, subjects abstained from alcohol, tobacco, caffeine and exercise. Muscle samples were obtained prior to, immediately after and 3 hr after exercise (from the vastus lateralis) on the first and last days of a 9-day training program using the percutaneous needle biopsy technique. Excised muscle tissue from the biopsy was immediately frozen in liquid nitrogen for subsequent analysis. Exercise was 60 min on a cycle ergometer at 63 ± 2 % \(\dot{V}O_2\) peak, which was repeated for nine consecutive days.

2.2.2 Analytical methods

Total RNA was isolated using FastRNA™ Kit-Green (BIO 101, Vista CA) (Dana et al., 1995). RNA was reverse transcribed to synthesise first strand cDNA using AMV Reverse Transcriptase (Promega, Madison, WI). Briefly, the RNA was added to a mixture containing a final concentration of 5 mM MgCl, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1% Triton® X-100, 1 mM of each dNTP, 20 U Recombinant Rnasin Ribonuclease Inhibitor (40 U/\(\mu\)l) and 0.5 \(\mu\)g Oligo (dT)\(_{15}\). Primers were designed using the Primer Express™ software package version 1.0 (Perkin–Elmer, Norwalk, USA) from gene sequences obtained from GeneBank (\(\beta\)-actin: X00351, IR: M10051, IRS-1: NM_005544, IRS-2: AF073310, PI 3-kinase p85\(\alpha\) subunit: M61906). The primer sequences were validated using BLAST (Altschul et al., 1990) to ensure each primer
was homologous with the desired mRNA of human skeletal muscle. The primer sequences are shown in Table 2.1. Real-time PCR was used to quantify mRNA expression and has been described in detail previously (Gerber et al., 1997). This technique has been modified to include SYBR® Green chemistry rather than the oligonucleotide probe used elsewhere (Gerber et al., 1997; Xin et al., 1999). Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR® Green to double stranded DNA (Perkin-Elmer, Norwalk, USA). mRNA levels were quantitated using the threshold cycle (C_T) value, which is the cycle at which the fluorescence emission increases above a threshold level (10 times the standard deviation of the background). Therefore, C_T values are calculated at the initiation of the logarithmic phase of PCR amplification and provide accurate measurement of starting cDNA concentrations (Gerber et al., 1997). Real-time PCR was performed in triplicate using the ABI PRISM® 5700 sequence detection system (Perkin-Elmer, Norwalk, USA). A real-time PCR mix of 2X SYBR® Green Universal PCR Master Mix (Perkin-Elmer, Norwalk, USA); forward and reverse primer (2 µM) and cDNA was run for 40 cycles of PCR in a volume of 25 µl. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-actin mRNA was also quantified, and results were normalised to these values. β-actin mRNA levels have been reported not to change in response to 3, 6 and 12 weeks of training in rat skeletal muscle (Murakami et al., 1994) and therefore considered adequate as an internal control for this study.

Samples were analysed using two-way ANOVA with repeated measures. Post Hoc analysis was performed to determine differences between groups using Newman-Keuls test, where appropriate.
**Table 2.1: Gene primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>GAC AGG ATG CAG AAG GAG ATT ACT</td>
<td>TGA TCC ACA TCT GCT GGA AGG T</td>
</tr>
<tr>
<td>IR</td>
<td>GCC ACC AAT ACG TCA TTC ACA A</td>
<td>GTT GCT GGA ATT CAT CGT GTA C</td>
</tr>
<tr>
<td>IRS-1</td>
<td>CCA CTC GGA AAA CTT CTT CTT CAT</td>
<td>AGA GTC ATC CAC CTG CAT CCA</td>
</tr>
<tr>
<td>IRS-2</td>
<td>ACG CCA GCA TTG ACT TCT TGT</td>
<td>TGA CAT GTG ACA TCC TGG TGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAA</td>
</tr>
<tr>
<td>p85α</td>
<td>GGA AGC AGC AAC CGA AAC AA</td>
<td>TTC GCC GTC CAC CAC TAC A</td>
</tr>
</tbody>
</table>

IR: insulin receptor; IRS-1: insulin receptor substrate-1; IRS-2: insulin receptor substrate-2; p85α: p85α subunit of phosphatidylinositol 3-kinase.
2.3 Results

The impact of acute exercise and exercise training was determined relative to the abundance of β-actin, a commonly used housekeeping gene. To confirm that the expression of β-actin is not modified in human muscle following exercise training, absolute gene expression of β-actin is shown in Figure 2.1. Fluorescent detection by real-time PCR demonstrated no significant training effect on the expression of β-actin. A small reduction in C<sub>T</sub> was evident immediately following the completion of the first exercise bout, suggesting increased mRNA recovery or cDNA synthesis (reverse transcriptase) efficiency at this time-point. Subsequent analysis of gene expression is normalised against β-actin (housekeeping gene), to account for the variability in cDNA abundance between samples.

The gene expression of IR (Figure 2.2A) and IRS-1 (Figure 2.2B) were not significantly altered either immediately post-exercise or 3 hours following a single bout of exercise. Furthermore, there was no significant change in the mRNA expression of these genes following 9 days of endurance training. The gene expression of IRS-2 was found to be 11 ± 4 fold higher (p< 0.01) 3 hours after exercise compared with basal levels, although this effect was reduced to 4 ± 1 fold (p< 0.05) after exercise training (Figure 2.2C). Similarly, the mRNA levels of PI 3-kinase were also higher (p<0.05) 3 hours following exercise in the untrained state but no changes in expression were found after exercise in the trained state (Figure 2.2D).
Figure 2.1. Effect of a single bout of exercise and short-term endurance training on the gene expression of β-actin. Bars on graph represent means ± SE of 7 subjects. Closed bars: untrained; Hatched bars: trained; Pre: immediately before, Post: immediately after, and 3hr Post: 3 hours after a single exercise bout. Note that Critical Threshold (C_T) represents the PCR cycle number at which the fluorescence emission increases above a threshold level. Therefore, a lower C_T value represents a higher initial mRNA concentration. * p<0.05 vs. trained values.
Figure 2.2. Effect of a single bout of exercise and short-term endurance training on the gene expression of A: the insulin receptor (IR), B: insulin receptor substrate-1 (IRS-1), C: insulin receptor substrate-2 (IRS-2) and D: p85α subunit of phosphatidylinositol 3-kinase (p85α PI 3-kinase). Bars on graph represent means ± SE of 7 subjects. Closed bars: untrained; Hatched bars: trained; Pre: immediately before, Post: immediately after, and 3hr Post: 3 hours after a single exercise bout. # p< 0.01 vs. all other values, * p< 0.05 vs. trained values, + p< 0.05 vs. Post untrained values.
2.4 Discussion

The increased sensitivity of insulin-mediated glucose uptake following exercise persists for up to 48 hours, during which time the acute effects of glucose on GLUT4 translocation have been reversed and muscle glycogen concentrations are largely restored (Goodyear et al., 1990; Mikines et al., 1988). The results from this study and others have shown that a single bout of exercise is capable of eliciting increased gene expression of key components of the glucose disposal pathway, including GLUT4 and hexokinase II (Krainiou et al., 2000; Neufer & Dohm, 1993). Recent data have also shown increased levels of GLUT4 protein 22 hours following a single bout in humans (Greiwe et al., 2000). In addition to the upregulated glucose transport capacity, enhanced transduction of the insulin signal cascade is evident in humans and rodents (Chibalin et al., 2000; Houmard et al., 1999), however the impact of exercise on the gene transcription of critical components of this pathway is unknown. The present study examined whether a single bout of exercise and endurance training alters the gene expression of the key insulin-signalling proteins in human subjects. The results of this study demonstrated that gene expression of IRS-2, and to a lesser extent, PI 3-kinase, is increased by a single bout of exercise in untrained individuals. However, other key insulin-signalling intermediates, including IR and IRS-1 were not regulated either by a single exercise bout or by short-term training in humans.

The present study found significantly increased IRS-2 gene expression in the hours following a single bout of exercise, but not immediately following exercise in humans. This finding is in agreement with an increase in IRS-2 protein levels and insulin stimulated IRS-2 associated PI 3-kinase activity measured 16 hours following a single
exercise bout (Chibalin et al., 2000) in rat skeletal muscle. IRS-2 may exert a major role in mediating insulin action in liver and in the development and maintenance of pancreatic β-cell mass, yet its precise role in skeletal muscle is uncertain (Kido et al., 2000; Withers et al., 1999). In isolated muscle and fat cells, IRS-2 has been shown to have a similar role to IRS-1 in insulin-stimulated glucose transport (Miele et al., 1999; Zhou et al., 1997) and may act as an alternative pathway of insulin action. However, IRS-2 is not involved in glucose transport immediately following either a single exercise bout or short-term training (Chibalin et al., 2000; Higaki et al., 1999). A complex interplay between IRS-1 and IRS-2 is evident in the regulation of insulin action in skeletal muscle, with alterations in the balance of these proteins impacting on insulin action (Withers et al., 1999). Therefore, it is tempting to speculate of an early adaptive role of IRS-2 in the exercise-induced increases in insulin signalling in the hours following exercise. This role for IRS-2 might then become redundant following the more slowly initiated adaptive responses of other members of the insulin signalling intermediates, such as IRS-1. In the current study no changes in IRS-1 gene expression, either acutely or following training were observed. However, insulin-stimulated function of IRS-1 has been observed to increase by 5 days of training (Chibalin et al., 2000) in rats. As tempting as it is to speculate on the role of IRS-2 to insulin signalling in the hours following exercise, further evidence is required to establish its contribution.

It was somewhat surprising that there were no observable changes in IR and IRS-1 following endurance training given the enhanced gene expression found previously in rodents following endurance training (Kim et al., 1995; Kim et al., 1999a). This may reflect species differences, the composition of fiber-types analysed and/ or differences in training protocol. The present study used a relatively short (9 days and 60 min per
day) training period, whereas the rodents were trained for 90 min per day over a 9-week period. A further limitation of the present study was that subjects were relatively young and lean, without a family history of type 2 diabetes. Thus, any potential changes in insulin action may be small, such that large changes in the gene expression of IR and IRS-1 might not be expected. However, similar studies have demonstrated enhanced insulin-sensitivity following only a single bout of exercise (Wojtaszewski et al., 1997) and in training programs utilizing relatively short-term programs such as the one used in the present study (Houmard et al., 1999). Thus, it can be concluded that it is unlikely that enhanced gene expression of IR and IRS-1 are central components of the adaptive response to exercise. Exercise-induced improvements in translational control such as RNA stability, protein processing transport and protein stability, as identified previously following exercise training (Welle et al., 1999) may exert a greater influence on IR and IRS-1 protein abundance and activity.

In the present study it is also possible that any significant changes in gene expression occurred outside or between the sampling periods. There are few data on induction of increased gene expression following an exercise stimulus, or the half-lives of many mRNA species in humans (Neufer, 1999). Therefore, in the current study, timing of the biopsy was aimed to maximize the likelihood of identifying altered gene expression in the target genes. A spectrum of responses in human skeletal muscle has been reported previously, with increased mRNA abundance demonstrated within minutes of exercise initiation (Puntschart et al., 1998), immediately following the completion (Gustafsson et al., 1999; Kraniou et al., 2000) and for up to eight hours post-exercise (Seip et al., 1997). Indeed significantly increased gene expression of IR, IRS-1 and PI 3-kinase have been shown in rodents 48 hours following the termination of a 9-week training program.
(Kim et al., 1995; Kim et al., 1999a). In the present study, there were no changes in mRNA levels for IR and IRS-1 either immediately post-exercise or when measured again 3 hours post-exercise. Furthermore there was no evidence of a training effect on the gene expression of IR and IRS-1 as the second pre-exercise biopsy sample was obtained 24 hours following the last training session. These results would therefore suggest that significantly altered gene expression to moderate exercise of the IR and IRS-1 genes is unlikely.

### 2.5 Conclusion

In summary, skeletal muscle IRS-2 gene expression is significantly increased in the few hours following a single bout of exercise, but following training this effect is diminished in humans. Similarly, the mRNA levels of PI 3-kinase appear to be increased in the hours following a single bout of exercise, however this effect was not observed again following short-term training. A single bout of exercise or short-term endurance training does not increase the gene expression of either the IR and IRS-1 genes. Therefore, in untrained human subjects undertaking moderate exercise training there is little evidence of sustained and substantial alterations in the gene expression of key members of the insulin-signalling pathway. Further studies are required to elucidate the mechanisms regulating the increased activity of the insulin-signalling cascade following exercise.
CHAPTER THREE

IRS-2 GENE EXPRESSION IS UPREGULATED BY RESISTANCE EXERCISE IN HUMAN SKELETAL MUSCLE

3.1 Introduction

As discussed in Chapter 1, insulin action is improved by resistance exercise (Dunstan et al., 2002; Ishii et al., 1998; Miller et al., 1994; Poehlman et al., 2000). There is a small amount of evidence in rodents to suggest that resistance exercise is able to increase some of the components of the insulin signalling pathway such as IRS-1 and p85 associated PI 3-kinase activity independently of insulin (Carlson et al., 2001; Hernandez et al., 2000) along with increased glucose uptake and protein synthesis. However, the mechanisms to account for improvements in insulin action via resistance exercise remain unknown. The results obtained in Chapter 2 of this thesis show that endurance exercise has a marked yet temporary impact on the transcriptional activation of the genes encoding major insulin-signalling proteins including IRS-2 and PI 3-kinase whilst the gene expression of other key proteins in this pathway remain unaffected. The effects of resistance exercise on any aspect of the insulin-signalling pathway have yet to be studied in humans. Therefore, this study investigated the impact of a single bout of resistance exercise on the mRNA concentration of key insulin signalling proteins that are differentially regulated following endurance exercise.
3.2 Materials and Methods

3.2.1 Experimental protocol

Ten healthy but untrained male subjects volunteered to be involved in the study. The age, weight, height and BMI (mean ± SE) of the subjects prior to the study were 27.2 ± 1.7 years, 78.4 ± 2.7 kg, 179.5 ± 1.6 cm and 24.2 ± 0.9 kg.m$^{-2}$, respectively. The Deakin University Human Research Ethics Committee approved the study and subjects gave their written consent to participate in this study after all procedures and the possible risks of participation were explained.

One week prior to the experiment, subjects underwent a familiarisation trial on the Cybex dynamometer (Cybex International Inc. UK). During this session, seat and leg positions were recorded and subjects performed 3 sets of 5 maximal isokinetic concentric and eccentric muscle contractions at an angular velocity of 60 deg.sec$^{-1}$.

For the 24 hours preceding the trial, subjects abstained from alcohol, tobacco, caffeine and exercise and consumed a standardised diet consisting of 66% CHO, 20% fat and 14% protein. Subjects were permitted to consume water ad libitum. On the day of the trial, subjects reported to the laboratory in the morning after an overnight fast (10-12 hours). Muscle samples were obtained from the vastus lateralis using the percutaneous needle biopsy technique prior to, 30 min, 4 hours and 24 hours after the conclusion of the exercise bout. After the muscle biopsy at 30 min, subjects were given a small breakfast consisting of 2 slices of toasted white bread with a small amount of butter, jam and 250ml of orange juice. Excised muscle tissue from the biopsy was immediately frozen in liquid nitrogen for subsequent analysis. The exercise bout consisted of 3 sets
of 12 repetitions of maximal isokinetic thigh exercise on the Cybex Dynamometer at an angular velocity of 60 deg.sec\(^{-1}\) with each set being separated by 2 min. This involved both concentric and eccentric muscle contractions of the knee extensors. Concentric and eccentric muscle contractions combined have been shown to produce the largest alterations in myofibrillar disruption (Dudley et al., 1991; Gibala et al., 2000). During each trial, peak and average torque were measured and recorded.

3.2.2 Analytical methods

Total RNA was isolated and reverse transcribed as described in Chapter 2. Primers were identical to those used in Chapter 2 and the primer sequences are shown in Table 3.1. Real time PCR was used to quantify mRNA expression and has been described in detail previously (Chapter 2) and cDNA was run for 40 cycles of PCR in a volume of 20 µl. To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-actin mRNA was also quantified, and results were normalised to these values. The previous study (Chapter 2) in this thesis found that β-actin mRNA levels did not change in response to endurance exercise in humans and was therefore considered as an adequate internal control for this study.

Samples were analysed using repeated measures ANOVA. Post Hoc analysis was performed to determine differences between groups using Newman-Keuls test, where appropriate.
### Table 3.1: Gene primer sequences

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</tr>
<tr>
<td>IRS-1</td>
<td>CCA CTC GGA AAA CTT CTT CTT CAT</td>
<td>AGA GTC ATC CAC CTG CAT CCA</td>
</tr>
<tr>
<td>IRS-2</td>
<td>ACG CCA GCA TTG ACT TCT TGT</td>
<td>TGA CAT GTG ACA TCC TGG TGA TAA</td>
</tr>
<tr>
<td>p85α</td>
<td>GGA AGC AGC AAC CGA AAC AA</td>
<td>TTC GCC GTC CAC CAC TAC A</td>
</tr>
</tbody>
</table>

IRS-1: insulin receptor substrate-1; IRS-2: insulin receptor substrate-2; p85α: p85α subunit of phosphatidylinositol 3-kinase.
3.3 Results

The peak torque (mean ± SE) recorded for each subject during the trials was 229.6 ± 10.0 and 273.4 ± 9.5 N.m⁻¹ for the concentric and eccentric contractions, respectively. The resistance exercise was performed in 3 sets, and the work intensity increased as each set progressed and as the subjects became more familiar with the protocol (Table 3.2). It was also observed that the subjects fatigued rapidly towards the latter stages of the last exercise set.

The impact of resistance exercise was determined relative to the abundance of β-actin, a commonly used housekeeping gene. To confirm that the expression of β-actin is not modified in human muscle following resistance exercise, absolute gene expression of β-actin is shown in Figure 3.1. Fluorescent detection by real-time PCR demonstrated no significant exercise effect on the expression of β-actin. Subsequent analysis of gene expression is normalised against β-actin (housekeeping gene), to account for the variability in cDNA abundance between samples.

Thirty minutes following resistance exercise mRNA concentration of IRS-1 was decreased approximately 2.3 ± 0.6 fold (Figure 3.2A, not significant) and was then increased 3.4 ± 1.1 fold (Figure 3.2A, p<0.05) 3.5 hours later. IRS-2 gene expression increased 3.4 ± 1.2 fold (Figure 3.2B, p<0.05) 4 hours following resistance exercise and was restored to basal levels by 24 hours (Figure 3.2B, p<0.05). There was no change in the mRNA concentration of the p85α subunit of PI 3-kinase (Figure 3.2C).
Table 3.2. Average torque during each repetition of isokinetic thigh exercise.

<table>
<thead>
<tr>
<th></th>
<th>Average Torque (N.m(^{-1}))</th>
<th>Average Torque (N.m(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentric</td>
<td>Eccentric</td>
</tr>
<tr>
<td>Set 1</td>
<td>153.7 ± 9.9</td>
<td>147.3 ± 17.9</td>
</tr>
<tr>
<td>Set 2</td>
<td>188.8 ± 11.5</td>
<td>205.5 ± 18.6</td>
</tr>
<tr>
<td>Set 3</td>
<td>192.7 ± 12.1</td>
<td>215.1 ± 17.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 for all sets

Figure 3.1. Effect of a single bout of resistance training on the gene expression of β-actin.

Bars on graph represent means ± SE of 8 subjects. 0: immediately before exercise, 30min, 4hrs and 24hrs: 30 min, 4 hours and 24 hours following a single bout of resistance exercise, respectively. Note that Critical Threshold (C\(_T\)) represents the PCR cycle number at which the fluorescence emission increases above a threshold level. Therefore, a lower C\(_T\) value represents a higher initial mRNA concentration.
Figure 3.2. Effect of a single bout of resistance exercise on the mRNA concentration of A: insulin receptor substrate-1 (IRS-1), B: insulin receptor substrate-2 (IRS-2). And C: p85α subunit of PI 3-kinase. Bars on graph represent means ± SE of 8 subjects. 0, 30min, 4hrs and 24hrs: immediately before, 30 min, 4 hours and 24 hours following resistance exercise, respectively. +p<0.05 vs. 4hrs, * p<0.05 vs. 0hrs.
3.4 Discussion

There is a considerable amount of data now on the impact of endurance exercise on insulin sensitivity and the molecular components of the insulin-signalling pathway. Resistance exercise has also been shown to improve insulin action although conflicting findings have yet to establish if this improvement is due to an enhanced ability of skeletal muscle to respond to insulin or if this is simply due to increases in lean muscle mass (Dunstan et al., 2002; Ishii et al., 1998; Poehlman et al., 2000; Takala et al., 1999). Furthermore, there has been no investigation into the impact of resistance exercise on any molecular aspect of the insulin-signalling pathway in humans.

This study has for the first time shown that a single bout of resistance exercise is sufficient to significantly alter the gene expression of IRS-1 and IRS-2 in the skeletal muscle of humans. The overall response of IRS-1 suggests that the mRNA concentration tends to decrease 30 min following resistance exercise whereupon it is restored to pre exercise levels over the following 3.5 hours. IRS-2 on the other hand, significantly increases in the few hours following resistance exercise and is restored to basal levels within 24 hours.

Only one study has investigated mRNA concentration of select genes following resistance exercise in humans (Willoughby & Nelson, 2002). They found a small but significant upregulation of the gene expression for myosin heavy chain (MHC) isoform Type IIx, and myogenic regulatory factors (MRF) Myo-D and myogenin immediately after heavy resistance exercise. This effect was also found to be significantly greater 6 hours post exercise with MHC isoforms Type I and IIa also elevated. An upregulation
of genes involved in muscle phenotype and the enlargement or repair of the myofibre is therefore not surprising following heavy resistance exercise.

The major finding of this study was that resistance exercise significantly increases IRS-2 gene expression in the few hours post exercise in humans. A similar response has been found for this gene in the few hours following endurance exercise (Chapter 2) although it appears endurance training resulted in a much more marked response (11 ± 4 fold increase, \( p<0.01 \)) (Chapter 2) compared to the 3 ± 1 fold increase following resistance exercise in the present study. It is however difficult to compare the magnitude of the two results since although the two studies used healthy untrained volunteers, the endurance study involved both men and women and the time course for the sampling of muscle differed. What is clear however is that intense muscular contraction using very different exercise modalities is sufficient to upregulate IRS-2 mRNA concentration for several hours following exercise. This may point towards a role for IRS-2 in muscle fibre growth in addition to a small role on insulin action. The physiological role/s for IRS-2 remain unclear although gene knockout studies have shown it to be important in insulin stimulated PI 3-kinase activity (Howlett et al., 2002) in skeletal muscle and may act as an alternative pathway for insulin action when IRS-1 function is compromised (Kido et al., 2000; Rondinone et al., 1997; Withers et al., 1999). IRS-2 has also been shown to be particularly important in the liver where it plays a major role in insulin action and is critical to the maintenance of \( \beta \)-cell mass in the pancreas (Kubota et al., 2000; Withers et al., 1999).

The present study is limited in its findings as subjects were fed a small meal approximately 35 minutes after exercise, which may have had an effect on the gene
expression of IRS-2. This confounding factor cannot be discounted, but it is unlikely to be a major factor since similar transient increases were observed following endurance exercise despite no food being consumed. There were also no observable changes in the gene expression of IRS-1 or the \( p85_\alpha \) sub unit of PI 3-kinase compared to basal levels during this period. Also, IRS-2 gene expression was not increased above basal levels in the longer term suggesting that the increase in IRS-2 gene expression in the few hours following exercise is largely attributable to the preceding muscular contraction.

Tyrosine phosphorylation of IRS-1 is known to play a major role in insulin stimulated mitogenesis and glucose uptake (for reviews see (Kellerer et al., 1999; Virkamaki et al., 1999; White & Kahn, 1994). Also, IRS-1 and \( p85 \) associated PI 3-kinase activity have been shown to be significantly elevated for 6 – 24 hours in rats who underwent muscle overload by either short term resistance training or surgical ablation, which coincided with enhanced glucose uptake and protein synthesis (Carlson et al., 2001; Hernandez et al., 2000). Unfortunately, IRS-2 tyrosine phosphorylation and any associated activation of PI 3-kinase following such types of intense muscular contraction have yet to be investigated. A constitutively active form of the Ras proto-oncogene that selectively stimulates the PI 3-kinase pathway was found to induce muscle fibre growth in regenerating denervated mammalian muscle (Murgia et al., 2000). The activation of PI 3-kinase by insulin receptor substrates following muscular contraction may therefore play a role in mediating protein synthesis independently from insulin mediated glucose uptake.

Endurance exercise was observed in the previous chapter not to have any effect on the mRNA concentration of IRS-1 in untrained humans. It is therefore surprising to observe
a trend for IRS-1 gene expression to decrease in the short period following resistance exercise. A physiological explanation for the tendency of IRS-1 mRNA concentration to decrease post exercise could be postulated that since resistance exercise results in a large degree of cellular disruption and damage, perhaps there is little need or ability to resynthesise any proteins that are already in abundance at that particular time, although there is no evidence to support this.

### 3.5 Conclusion

In summary, this is the first study to demonstrate altered mRNA concentration of key insulin signalling proteins in skeletal muscle of humans following high intensity resistance exercise. There was a tendency for IRS-1 gene expression to decrease in the short period following resistance exercise, whereas IRS-2 gene expression markedly increased several hours after exercise. The mRNA expression of the p85α subunit of PI 3-kinase was not however altered by resistance exercise. The observation that IRS-2 gene expression is increased following intense muscular contractions following either endurance and resistance exercise points towards roles for the protein not only in insulin action and glucose transport but perhaps in myofibre growth in skeletal muscle independent from insulin.
CHAPTER FOUR
THE EFFECT OF EXERCISE ON POTENTIAL MEDIATORS OF INSULIN SIGNALLING IN HUMAN SKELETAL MUSCLE

4.1 Introduction

As discussed in Chapter 1, exercise improves insulin sensitivity although the effects of exercise on insulin signalling appear time dependent. In the 12-24 hour period following the cessation of exercise the observed improvements in insulin sensitivity may be related to an upregulation of the insulin-signalling cascade. It is this delayed period following exercise cessation that will be the focus of this study rather than the initial few hours following exercise cessation. Understanding the intracellular pathways and mechanisms that are upregulated during sustained periods of enhanced insulin sensitivity provides valuable insights into the development of safer therapies for the prevention and treatment of metabolic diseases such as diabetes.

Insulin receptor (IR) and IRS-1 tyrosine phosphorylation appear to be upregulated in response to short-term exercise training, although it is unclear if these effects are apparent under physiological insulin levels in vivo. It is also yet to be resolved if any improvements in insulin signalling during this period are due to increased protein abundance of key insulin signalling proteins. Furthermore, there is little data on the upregulation of distal parts of the insulin-signalling cascade such as Akt in the 12-24 hours following exercise. Even though short-term endurance training in humans has been shown in many studies to improve insulin sensitivity (DeFronzo et al., 1987; Dela
et al., 1994; Houmard et al., 1999; Youngren et al., 2001) one constant criticism is that the effect is primarily due to the residual effects of the last bout of exercise and not the result of cumulative bouts of exercise. There are some data in rodents and insulin resistant humans (Chibalin et al., 2000; Rogers et al., 1988) to suggest that cumulative bouts of exercise further increase insulin sensitivity when compared to an acute bout of exercise although this effect has not been fully investigated in humans, particularly those with normal glucose tolerance. The primary aims of this study were to determine if a single bout of acute exercise and short-term endurance training resulted in increased protein abundance and/or insulin-stimulated phosphorylation of IR, IRS-1 and Akt in sedentary humans. Another aim of this study was to determine if short-term endurance training resulted in significantly higher levels of whole-body insulin sensitivity compared to a single bout of exercise.

It was also discussed in Chapter 1 that significant interest is currently being focussed on the regulation of the insulin-signalling cascade by protein tyrosine phosphatases (PTPases). This diverse family of PTPases are novel mediators of the insulin-signalling cascade although little is known of the effects of exercise on PTPase abundance, particularly in human muscle. The subcellular localisation of PTPases could be important since readily abundant PTPases in skeletal muscle and adipocytes each display characteristic subcellular distribution between the cytosol and particulate fractions (Ahmad & Goldstein, 1995; Calera et al., 2000). Therefore it is likely there are preferential sites of action between the IR and insulin receptor substrates (IRS) depending upon subcellular localisation.
PTP1B is an abundant PTPase in skeletal muscle and PTP1B knockout mice display markedly enhanced insulin sensitivity along with increased phosphorylation of IR and IRS-1 in muscle and liver following insulin infusion (Elchebly et al., 1999). Recently, reduction of PTP1B protein in liver and fat have been associated with improved insulin action along with increased tyrosine phosphorylation of the IR, IRS-1, IRS-2 and serine phosphorylation of pAkt (Ser473) (Gum et al., 2003; Mohammad et al., 2002; Zinker et al., 2002).

Following insulin stimulation, SHPTP2 associates with IRS-1 (Kuhne et al., 1993) and unlike PTP1B, the major site of SHPTP2 activity appears to reside in the cytosolic fraction (Ahmad & Goldstein, 1995), implicating IRS-1 as a potential candidate for dephosphorylation. However, unlike most other PTPases in skeletal muscle, SHPTP2 has actually been implicated as a positive regulator of insulin signalling. As mentioned in Chapter 1, overexpression of SHPTP2 in various cell lines upregulates insulin signalling at multiple sites including IRS-1 tyrosine phosphorylation and PI 3-kinase activity, with the opposite observed when SHPTP2 is inhibited (Chen et al., 1997; Ugi et al., 1996). In vivo studies also implicate SHPTP2 as a positive regulator of insulin signalling since transgenic mice with impaired SHPTP2 have decreased insulin action and impaired activation of IRS-1, PI 3-kinase and Akt in skeletal muscle (Maegawa et al., 1999). SHPTP2 is clearly a candidate involved in the regulation of insulin signalling although a precise role and relative contribution to insulin signalling is yet to be determined. Furthermore, no studies have sought to investigate the actions of exercise training on SHPTP or PTP1B.
Crucial to the analysis of SHPTP2 and PTP1B activity is examination of their subcellular localisation. If abundance of particular PTPases were altered in response to exercise then insulin sensitivity might increase due to the amplified phosphorylation of key signalling proteins. It could be hypothesised that decreases in PTP1B abundance in the particulate and cytosolic fractions by exercise could increase both IR and IRS-1 tyrosine phosphorylation, with concomitant enhanced phosphorylation of distal signalling proteins such as Akt. Furthermore, if SHPTP2 protein abundance were enhanced in the cytosolic fraction following exercise, then increased IRS-1 tyrosine phosphorylation, along with enhanced Akt activation/phosphorylation may be expected. The secondary aim of this study was therefore to determine if exercise altered the abundance or subcellular distribution of PTP1B or SHPTP2 in humans and if this coincided with exercise-induced increases in the tyrosine phosphorylation of IR and IRS-1 or serine phosphorylation of Akt.

4.2 Materials And Methods

4.2.1 Materials

PTP1B mouse monoclonal IgG (BD, Cat. # 610140) and SHPTP2 mouse monoclonal IgG (BD, Cat. # 610621) were from BD Biosciences (San Diego, USA). IRS-1 rabbit polyclonal IgG (UBI, Cat. # 06-248) was purchased from Upstate Biotechnology (New York, USA). Phosphotyrosine (PY99) mouse monoclonal IgG (SC, Cat. # 7020) and IRS-1 (A-19) rabbit polyclonal IgG (SC, Cat. #560) were from Santa Cruz Biotechnology (California, USA). 83.7 IRβ mouse monoclonal IgG was a gift from Prof. Ken Siddle (University of Cambridge). Phospho-Akt (pAkt) Ser473 rabbit polyclonal IgG (Cat. # 9271) and Akt rabbit polyclonal IgG (Cat. # 9272) were from
Cell Signaling Technology (New England BioLabs, Hartsfordshire, England). Affinity purified peroxidase labelled anti-mouse IgG and anti-rabbit IgG were purchased from Silenus (Victoria, Australia). All other reagents were analytical grade (Sigma, NSW, Australia).

4.2.2 Subjects

A group of 8 untrained, but healthy males volunteered to be involved in this study. The experimental protocol and consent form was approved by the Deakin University Human Research Ethics Committee prior to the commencement of the study. All subjects gave their written consent to participate in this study after all procedures and the possible risks of participation were explained. Subject characteristics were (means ± SE) age, 24 ± 1yrs; height, 180 ± 3cm; mass, 82.3 ± 4.3kg; and body mass index, 25.6 ± 1.5kg·m$^{-2}$.

4.2.3 Experimental protocol

Subjects visited the Exercise Physiology Laboratories at Deakin University for determination of $\dot{V}O_2$ peak and then on ten further occasions. Eight subjects underwent 3 euglycemic-hyperinsulinemic clamps on separate days, each following an overnight fast (Table 4.1). Subjects were requested to refrain from any moderate or strenuous activity or exercise for at least 48 hours prior to the first euglycemic clamp (day 1). Several days later, each subject cycled at 75% $\dot{V}O_2$ peak for 60 min (day 7). Twenty-four hours after finishing the acute bout of exercise (day 8) each subject underwent a second euglycemic clamp procedure. Each subject then cycled for 60 min each day at 75% $\dot{V}O_2$ peak for the following 7 days. During each exercise session, $\dot{V}O_2$ was
monitored and the workload was increased accordingly to maintain a relative exercise intensity of 75% \( \dot{V}O_2 \) peak. Twenty-four hours following the last exercise bout, each subject underwent a third euglycemic clamp procedure (Table 4.1).

In the 24-hour period prior to each euglycemic clamp subjects avoided the consumption of tobacco, alcohol and caffeine and were told to avoid any physical activity other than that prescribed by the study. Furthermore, the day prior to each euglycemic clamp, each subject ate a standardised diet consisting of approximately 14,500KJ with 76% of energy derived from carbohydrate, 12% from protein and 12% from fat.

All cycling tests were performed on a Quinton Excalibur cycle ergometer (Groningen, The Netherlands). The seat height and handlebar position on the cycle ergometer were recorded on the first cycle session and replicated on subsequent visits. During each cycling test, a large domestic fan circulated air onto the subject’s head and chest in order to aid body temperature regulation. \( \dot{V}O_2 \) was measured on line and calculated every 30 seconds using Vista/Turbofit software, version 4.045 (Vacumetrics Inc. California, USA). Ventilation was measured by a turbine flow transducer (KL Engineering Co. California, USA), which was calibrated using a 3.0 L syringe. Expired CO2 and O2 were measured with infrared carbon dioxide and zirconia cell oxygen analysers, respectively (AEI Technologies Inc. Pittsburgh, USA) which were calibrated to 0.01% using alpha rated standard gases (Linde Gases Pty Ltd, Villawood, NSW, Australia). Heart rates were measured continuously using a Polar heart rate monitor (Polar Electro, Finland).
Table 4.1. Summary of the testing sessions.

<table>
<thead>
<tr>
<th>Day</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Euglycemic, hyperinsulinemic clamp 1</td>
</tr>
<tr>
<td>Day 7</td>
<td>Exercise, 75% VO₂ peak</td>
</tr>
<tr>
<td>Day 8</td>
<td>Euglycemic, hyperinsulinemic clamp 2</td>
</tr>
<tr>
<td>Days 8-14</td>
<td>Exercise, 75% VO₂ peak</td>
</tr>
<tr>
<td>Day 15</td>
<td>Euglycemic, hyperinsulinemic clamp 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsies</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

↑ indicates an increase.
Each subject performed an incremental cycling test until volitional exhaustion and the results were used to later estimate submaximal exercise workloads from the linear relationship between oxygen uptake and power output. $\dot{VO}_2\text{peak}$ was deemed as the highest $\dot{VO}_2\text{(L-min}^{-1})$ value during a 60 second period.

4.2.4 Euglycemic hyperinsulinemic clamp

Following an overnight fast, a dose of 1.2 g KCl (Novartis Pharmaceuticals, North Ryde, NSW, Australia) was administered orally to prevent a decrease in plasma potassium concentration during the clamp. A 22 gauge polyurethane catheter was inserted into an antecubital vein in the left arm for infusion of glucose and into the right arm for infusion of insulin. A third polyurethane catheter was inserted retrograde into a dorsal vein of the right hand and the hand was wrapped in an electric heating pad and warmed (approximately 50°C) for sampling of arterialised blood. Five millilitres of the subject’s blood and 95 ml of 0.9% (w/v) saline were then mixed with 20U of human insulin (Actrapid, Novo Nordisk, Denmark) to create the insulin infusate. A one-step euglycemic-hyperinsulinemic clamp was initiated by an intravenous bolus injection given over 1-min (9mU.kg$^{-1}$) followed by a 120-min constant infusion of insulin (40 mU.m$^{-2}$.min$^{-1}$). Arterialised venous blood samples obtained immediately before and at 5 min intervals during the infusion were analysed for glucose concentration using a glucose analyser (EML105, Radiometer Pacific, Melbourne, Australia). Blood glucose levels were clamped at 4.83 ± 0.02 mM for the final 30 min of hyperinsulinemia in each clamp by the use of a variable infusion of 25% glucose. Arterialised venous blood samples were also obtained immediately before and at 10 min intervals during the infusion for the determination of plasma insulin concentrations. These blood samples
were spun in a centrifuge at 13,000 rpm for 3 min and the plasma was frozen at -20°C for later analysis. Glucose infusion rate (GIR) was calculated as the average from the final 30 min of the clamp.

In addition, homeostasis model assessment (HOMA) measures of insulin action were calculated from fasting glucose and insulin values obtained prior to the initiation of insulin infusion. HOMA = fasting plasma insulin (µU/ml) × fasting plasma glucose (mmol.l⁻¹) ÷ 22.5 (Matthews et al., 1985). HOMA values provide a determination of the effectiveness of fasting insulin levels to regulate blood glucose levels (Matthews et al., 1985). HOMA has been used in numerous studies as a measure of insulin action and has been found to correlate highly with whole-body insulin sensitivity as measured by the euglycemic, hyperinsulinemic clamp (Katsuki et al., 2001; Matthews et al., 1985).

4.2.5 Blood biochemistry

Plasma insulin concentrations were determined in duplicate by radioimmunoassay using a commercially available kit (Phadaseph, insulin RIA, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden).

4.2.6 Muscle biopsy procedure

Percutaneous muscle samples (137 ± 6 mg) were obtained under local anaesthesia (Xylocaine, 1% plain) from the vastus lateralis of each subject by using the needle biopsy technique (Bergström, 1962) modified to include suction. Biopsies were taken 5 min prior to and 120 min following the initiation of the insulin infusion on the same leg with the second biopsy being at least 5 cm proximal to the first. Muscle samples were
frozen in the needles within 5 seconds in liquid nitrogen and stored in vented cryotubes. These samples were stored in liquid nitrogen for later analysis.

4.2.7 Preparation of whole muscle lysates

Frozen muscle (10µl of buffer per mg of muscle) was homogenised using a polytron at maximum speed for 30-seconds in freshly prepared ice-cold Buffer A, (50mM HEPES at pH 7.6 containing 150mM NaCl, 20mM Na₃P₂O₇, 20mM β-glycerophosphate, 10mM NaF, 2mM EDTA, 1% v/v Nonidet P-40, 10% v/v glycerol, 1mM MgCl₂, 1mM CaCl₂, 2mM Na₃VO₄, 2mM PMSF and 5µl.ml⁻¹ Protease Inhibitor Cocktail (P8340, Sigma)). Tissue lysates were incubated on ice for 20 min and then spun at 10,000 × g for 20 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, NSW, Australia) with BSA as the standard. The supernatants were stored at –80°C until analysis.

4.2.8 Preparation of fractionated muscle lysates

Frozen muscle (10µl of buffer per mg of muscle) was homogenised using a polytron at maximum speed for 30-seconds in freshly prepared ice-cold Buffer B, (50mM HEPES at pH 7.4 containing 1mM DTT, 4mM EDTA, 2.5 mM Benzamidine, 2mM PMSF, 5µl.ml⁻¹ Protease Inhibitor Cocktail (P8340, Sigma)). The crude homogenate was spun at 350,000 × g for 30 min at 4°C and the resulting supernatant was taken as the cytosol fraction. Proteins in the particulate fraction were then solublised by treating the pellet with ice cold Buffer B containing 1% v/v Triton X-100 and 0.2 M NaCl, incubating for 45min at 4°C, then spinning at 150,000 × g for 60 min at 4°C. The resulting supernatant was taken as the particulate fraction. Protein concentration was determined using a
bicinchoninic (BCA) protein assay (Pierce, IL, USA) with BSA as the standard. The supernatants were stored at −80°C until analysis.

4.2.9 Immunoprecipitations and immunoblotting

For immunoprecipitation, 2µg of anti-IRβ monoclonal antibody (Upstate Biotechnology) and 3µg of anti-IRS-1 (A-19) polyclonal antibody (Santa Cruz) was coupled to 30 µl (50% w/v) Protein A-sepharose beads (Zymed Laboratories, CA, USA) for 20 min at room temperature and then washed once in PBS. Tissue lysates (350µg protein) were incubated with the antibody coupled beads overnight at 4°C with rotation. Immunoprecipitated proteins were then washed once in PBS containing 0.5M NaCl and 0.2% v/v Triton X-100 and twice in PBS containing 0.2% v/v Triton X-100. Immunoprecipitated proteins or equal amounts of protein for determination of pAkt (Ser473) (75µg), PTP1B (10µg), SHPTP2 (10µg), IRS-1 (45µg), and Akt protein abundance (75µg) were suspended in Laemmli sample buffer. Bound proteins were separated by SDS-PAGE and electrotransfer of proteins from the gel to nitrocellulose membranes in towbin transfer buffer (25mM Tris, pH 8.3, 192mM glycine, and 20% v/v methanol) was performed for 100min at 100V (constant). Blots were probed with anti-pAkt (Ser473) rabbit polyclonal (1:1000), anti-PTP1B monoclonal mouse (1:1000), anti-IRS-1 rabbit polyclonal (UBI, 1:1000), anti-Akt rabbit polyclonal (1:1000) or anti-phosphotyrosine PY99 monoclonal mouse (1:1000) antibodies. IRβ immunoprecipitated phosphotyrosine and PTP1B blots were stripped using Restore™ (Pierce, IL, USA), and reprobed with either anti-IRβ monoclonal mouse antibody (1:1000) or anti-SHPTP2 monoclonal mouse (1:1000), respectively, to determine IRβ and SHPTP2 protein abundance. Binding was viewed by enhanced chemiluminescence (Pierce, IL, USA) and
quantified with Kodak 1D version 3.5 densitometry software (Eastman Kodak Co., CT, USA).

4.2.10 IR content and autophosphorylation by ELISA

The IR content and the level of autophosphorylation in skeletal muscle extracts was also quantified by an IR antibody “trap” activation assay using time resolved fluorescence and Europium conjugated reagents. Microtiter 96-well plates were coated with 50µl of 83.7 anti-insulin receptor antibody (a gift from Prof. Ken Siddle, University of Cambridge) (215 µg/ml in 50mM NaHCO₃ buffer, pH 9.6) for 16 hours at 4°C. Antibody was removed from the plate wells and the plate was blocked with 75µl of 0.5% ovalbumin in 1×TBS for 2 hours at room temperature. After removal of blocking solution the plates were washed three times with 1×TBST. Aqueous samples (50ul) of skeletal muscle extracts containing 100µg of protein were applied to wells in triplicate and allowed to bind overnight at 4°C. After washing three times (1×TBST), 50 µl of either Europium-labelled human recombinant insulin (1:4000, a gift from Peter Hoyne, CSIRO, Health Sciences and Nutrition, Melbourne) or anti-phosphotyrosine (1:10000, pY100 Perkin Elmer) antibody in binding buffer consisting of 100mM Hepes at pH 8.0 containing 100mM NaCl, 0.05%Tween 20 and 2µM DTPA was added to each well and allowed to incubate overnight at 4°C. After washing three times (1×TBST), 100µl of europium enhancement solution was added and the wells were counted by a Wallac Victor fluorometer (Perkin Elmer).
4.2.11 Statistical analysis

Standards were included in all immunoblots and interassay variation was accounted for by normalising data to control samples. Data are presented as mean ± SEM.

Differences were determined using either one or two-way analysis of variance (ANOVA) with Newman-Keuls post-hoc analysis, where appropriate. Significance was accepted when \( p < 0.05 \).

4.3 Results

4.3.1 Subjects

Body mass did not change with exercise training (82.3 ± 4.3 vs. 82.4 ± 4.4 kg). The cardiovascular fitness (as measured by \( \dot{V}O_2 \text{peak} \)) prior to the study and the exercise characteristics for the duration of the study are presented in Table 4.2.

There was a measurable increase in cardiovascular fitness following the short-term training program. Exercise heart rate during the final training session was significantly lower (159 ± 3 beats-min\(^{-1}\)) when compared to the first session (166 ± 3 beats-min\(^{-1}\); \( p < 0.05 \)) despite exercising \( \dot{V}O_2 \) remaining the same (2.67 ± 0.17 L-min\(^{-1}\) vs. 2.62 ± 0.16 L-min\(^{-1}\); NS) between the first and final sessions, respectively.

4.3.2 Euglycemic Hyperinsulinemic clamp

The euglycemic, hyperinsulinemic clamp is the recognised gold standard for measuring whole-body insulin sensitivity (DeFronzo et al., 1979). This study performed a 2 hour
clamp procedure on 8 sedentary subjects as outlined in Table 4.1. This procedure allows the present study to measure changes to whole body insulin-sensitivity brought about 24 hours following a single bout of endurance exercise and short-term endurance training. Percutaneous muscle biopsies taken before and during the clamp procedures provided this study with skeletal muscle that was stimulated with or without high physiological doses of insulin. The muscle samples obtained from this study were then measured to examine if changes in whole-body insulin sensitivity via exercise are also matched by alterations in protein abundance and phosphorylation of key insulin-signalling proteins and the abundance of putative mediators of insulin signalling such as PTPases.

Plasma glucose concentration was not significantly different at any time point after 30 min of insulin infusion and was clamped at 4.83 ± 0.02 mM for the final 30 min of each clamp. There was also no significant (NS) difference in plasma glucose concentration during the final 30 min of each clamp between the three clamp conditions (sedentary vs. acute exercise vs. endurance training; NS). Plasma insulin concentration reached a steady state of 421.5 ± 15.2 pmol/l by 20 min of infusion in the sedentary condition. Although insulin infusion rates remained the same during each of the clamps, there was an average 9% decrease ($p<0.05$, Figure 4.1) in plasma insulin concentration from 90-120 min following 8 days of endurance training, possibly reflecting an increase in insulin clearance.
Table 4.2. $\dot{V}O_2$ peak prior to the study and exercise characteristics during the exercise-training period (n = 8 subjects).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Means ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\dot{V}O_2$ peak (mL·kg(^{-1})·min(^{-1}))</td>
<td>42.2 ± 1.6</td>
</tr>
<tr>
<td>Training $\dot{V}O_2$ (%peak)</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>Training heart rate (beats·min(^{-1}))</td>
<td>161 ± 3</td>
</tr>
<tr>
<td>Training heart rate (%max)</td>
<td>83 ± 2</td>
</tr>
</tbody>
</table>

Training $\dot{V}O_2$; average $\dot{V}O_2$ for the eight days of endurance training, expressed as a percentage of $\dot{V}O_2$ peak.

Figure 4.1. Average plasma insulin concentration for 90-120 min during the euglycemic, hyperinsulinemic clamps. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Bars on graph represent means ± SE of 8 subjects. *p<0.05 vs. other variables.
Table 4.3 Fasting plasma characteristics prior to the 3 euglycemic, hyperinsulinemic clamps

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Acute</th>
<th>Training</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting plasma glucose (pmol/l)</td>
<td>4.86 ± 0.23</td>
<td>4.75 ± 0.12</td>
<td>4.84 ± 0.13</td>
</tr>
<tr>
<td>Fasting plasma insulin (mmol/l)</td>
<td>79.4 ± 8.5</td>
<td>59.2 ± 8.2**</td>
<td>49.1 ± 6.0+</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.75 ± 0.41</td>
<td>2.08 ± 0.29*</td>
<td>1.77 ± 0.23**</td>
</tr>
</tbody>
</table>

HOMA, homeostasis model assessment; Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Bars on graph represent means ± SE of 8 subjects. *p<0.05 vs. sedentary; **p<0.01 vs. sedentary; +p<0.001 vs. sedentary.

Figure 4.2. Glucose infusion rate during the euglycemic, hyperinsulinemic clamps.

Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Bars on graph represent means ± SE of 8 subjects. *p<0.05 vs. other variables.
Fasting plasma glucose was not altered by a single bout of exercise or endurance training (Table 4.3; NS). However insulin action was significantly improved following an acute bout of endurance exercise and was further improved following short-term training. Firstly, fasting plasma insulin following a single bout of exercise significantly decreased 21% \( (p<0.01, \text{Table 4.3}) \) and was reduced 34% \( (p<0.001, \text{Table 4.3}) \) following endurance training. Secondly, as assessed via HOMA values at rest, HOMA values were significantly reduced 24% \( (p<0.05, \text{Table 4.3}) \) following acute exercise and 36% following training \( (p<0.01, \text{Table 4.3}) \). GIR during the final 30 min of the clamp was not significantly increased following an acute bout of exercise, however there was a significant 26% \( (p<0.05, \text{Figure 4.2}) \) increase following 8 days of endurance training (5.8 ± 1.1 vs. 6.2 ± 1.0 vs. 7.3 ± 1.0 mg.kg\(^{-1}\).min\(^{-1}\), respectively, Figure 4.2), indicating an improvement in insulin sensitivity that is in agreement with other short term training studies (Houmard et al., 1999; Tanner et al., 2002; Youngren et al., 2001).

4.3.3 Insulin receptor measured by immunoprecipitation

Figure 4.3 and 4.4 shows IR protein expression and tyrosine phosphorylation for the 3-euglycemic, hyperinsulinemic clamps performed for each of the subjects, respectively. There was no significant difference in IR protein abundance or tyrosine phosphorylation following endurance training. Insulin induced a 2.9 ± 0.7 fold increase in IR tyrosine phosphorylation in the sedentary state, a 2.2 ± 0.4 fold increase following acute exercise and a 2.2 ± 0.9 fold increase following endurance training (NS).
4.3.5 IR content and autophosphorylation by ELISA

There was also no significant difference in IR protein abundance following insulin infusion, acute exercise or endurance training for the 3-euglycemic, hyperinsulinemic clamps performed for each of the subjects (Figure 4.5) measured by ELISA. Insulin significantly increased IR tyrosine phosphorylation by approximately 2.1 fold ($p<0.0001$, Figure 4.6) similar to the immunoprecipitation approach. Also, acute exercise and endurance training were not found to alter IR tyrosine phosphorylation similar to the immunoprecipitation approach. It is evident from the standard errors observed in Figure 4.5 and 4.6 compared to the size of the means that IR protein abundance and tyrosine phosphorylation as measured by the antibody “trap” assay were more quantitative and less variable than the method of immunoprecipitation followed by Western blotting. Nevertheless, both methods provided essentially the same finding. Insulin-stimulation induced an approximately 2-fold increase in IR tyrosine phosphorylation, but neither IR protein abundance or tyrosine phosphorylation is altered by acute exercise or training.
Figure 4.3. Insulin receptor protein expression during the euglycemic, hyperinsulinemic clamps as measured by immunoprecipitation. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

Figure 4.4. Insulin receptor tyrosine phosphorylation during the euglycemic, hyperinsulinemic clamps as measured by immunoprecipitation. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values,
closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects. *\( p<0.05 \) vs. basal in the sedentary condition.

Figure 4.5. Insulin receptor number during the euglycemic, hyperinsulinemic clamps as measured by ELISA. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

Figure 4.6. Insulin receptor tyrosine phosphorylation relative to receptor number during the euglycemic, hyperinsulinemic clamps as measured by ELISA. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects. *\( p<0.0001 \) basal vs. insulin-stimulated values.
4.3.5 IRS-1

IRS-1 protein expression was not altered by insulin infusion, acute exercise or endurance training (Figure 4.7). Insulin increased IRS-1 tyrosine phosphorylation approximately 2.9 fold ($p<0.05$, Figure 4.8). Due to technical difficulties, only 7 subjects were analysed for IRS-1 tyrosine phosphorylation. However neither acute exercise nor endurance training was found to alter the insulin stimulated tyrosine phosphorylation of IRS-1.

4.3.6 Akt

There was no significant effect of insulin or exercise on total Akt protein abundance (Figure 4.9). Insulin induced an approximate 3.8 fold increase in pAkt (Ser473) ($p<0.0001$, Figure 4.10). Acute exercise and exercise training were found to increase overall pAkt (Ser473) by approximately 1.5 fold ($p<0.05$, Figure 4.10). However ANOVA showed no additive effect of exercise on insulin-stimulated pAkt (Ser473), nor were basal levels of pAkt (Ser473) found to be significantly increased following exercise.

4.3.7 SHPTP2 and PTP1B

SHPTP2 protein abundance in the cytosolic fraction was increased approximately 1.6 fold following acute exercise and endurance training ($p<0.05$, Figure 4.11) when compared to the sedentary condition. SHPTP2 protein abundance in the particulate fraction was not altered by exercise (Figure 4.12). There were no significant changes in PTP1B protein abundance in either the cytosol or particulate fractions (Figures 4.13 and 4.14, respectively).
Figure 4.7. IRS-1 protein abundance during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-IRS-1 antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

Figure 4.8. IRS-1 tyrosine phosphorylation relative to protein abundance during the euglycemic, hyperinsulinemic clamps. Skeletal muscle extracts were immunoprecipitated with anti-IRS-1 antibody and blots were probed for anti-phosphotyrosine (pY99) antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open
bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 7 subjects. *p<0.05 basal vs. insulin-stimulated values.

Figure 4.9. Akt protein abundance during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-Akt antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

Figure 4.10. pAkt (Ser473) during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-pAkt (Ser473) antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.
graph represent means ± SE of 8 subjects. *$p<0.0001$ basal vs. insulin stimulation, #$p<0.05$ vs. sedentary condition.

**Figure 4.11. SHPTP2 protein abundance in the cytosolic fractions during the euglycemic, hyperinsulinemic clamps.** Protein abundance is determined following western blotting of fractionated skeletal muscle extracts with anti-SHPTP2 antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects. #$p<0.05$ vs. sedentary condition.

**Figure 4.12. SHPTP2 protein abundance in the particulate fraction during the euglycemic, hyperinsulinemic clamps.** Protein abundance is determined following western blotting of fractionated skeletal muscle extracts with anti-SHPTP2 antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min
of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

**Figure 4.13.** PTP1B protein abundance in the cytosolic fraction during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of fractionated skeletal muscle extracts with anti-PTP1B antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent Means ± SE of 8 subjects.

**Figure 4.14.** PTP1B protein abundance in the particulate fraction during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of fractionated skeletal muscle extracts with anti-SHPTP2 antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent Means ± SE of 8 subjects.
of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent Means ± SE of 8 subjects.
4.4 Discussion

The present study observed significant increases in the insulin action of sedentary individuals following an acute bout of exercise and exercise training. Although the volunteers in the present study were healthy non-diabetics, it is still important to understand how exercise training in the healthy, glucose-tolerant state regulates the insulin-signalling pathway. As discussed in Chapter 1, physically active people have enhanced insulin sensitivity compared to sedentary subjects with normal glucose tolerance (Duncan et al., 2003; Kirwan et al., 2000). Furthermore, physically active people have an upregulation of steps in the insulin-signalling cascade that may ultimately provide protection from metabolic diseases such as type 2 diabetes (Kirwan et al., 2000). In the present study, fasting plasma insulin and HOMA values, used as measures of whole-body insulin action, significantly decreased following acute exercise and further decreased following short-term training. Whilst the GIR measured during a euglycemic, hyperinsulinemic clamp is the gold standard for measuring insulin sensitivity; HOMA is a useful measure of insulin action since it has been used in a number of exercise studies as an index to measure improvements in insulin sensitivity (Katsuki et al., 2001; Kinoshita et al., 2002; Youngren et al., 2001) and correlates well with insulin sensitivity (Katsuki et al., 2001; Matthews et al., 1985). GIR during hyperinsulinemic, euglycemic clamps did not increase after an acute bout of exercise, however a significant 26% increase was observed following 8 days of endurance training. These changes occurred despite no significant change in body mass and therefore any improvement in insulin action could largely be attributed to endurance exercise rather than any associated alterations in body composition. These improvements in insulin action are consistent with similar studies using short-term
endurance exercise (Houmard et al., 1999; Tanner et al., 2002; Youngren et al., 2001). Rodents show further increases in insulin action following 5 days of training compared to a single day (Chibalin et al., 2000) and there is a similar improvement in glucose intolerant humans using an oral glucose tolerance test (Rogers et al., 1988). However this is the first study to show in humans with normal glucose tolerance that insulin action is further enhanced following cumulative bouts of exercise compared to a singlebout of exercise. Also, the significant increase in GIR following short-term training could potentially be higher since there was an observed decrease in plasma insulin levels during the euglycemic clamp following short-term training, despite infusion rates remaining the same for each subject.

The lack of statistical significance for GIR following acute exercise when compared to previous studies could be explained by differences in methodology. Mikines et al., (Mikines et al., 1988) used a 4-step euglycemic, hyperinsulinemic clamp following an acute bout of exercise which allowed them to measure insulin sensitivity and insulin responsiveness quite accurately compared to the one-step clamp procedure used in the present study. Other studies to show improvements in insulin sensitivity following a single bout of exercise have employed the one-step clamp procedure within a few hours of the cessation of exercise (Thorell et al., 1999; Wojtaszewski et al., 1997) or have used subjects with impaired glucose tolerance (Cusi et al., 2000).

The alterations in insulin sensitivity detected by HOMA are largely due to reduced circulating insulin concentrations prior to the euglycemic clamp. Furthermore, it was also observed in the present study that plasma insulin concentrations during the clamp were significantly reduced following training despite insulin infusion rates remaining
constant. This could be put down to an increase in insulin clearance with exercise. The internalisation of the IR to the endosomal compartments of the cell following insulin binding and the subsequent dissociation of insulin from the IR and recycling back to the plasma membrane is an important mechanism for insulin clearance (Authier et al., 1994; Burgess et al., 1992; Khan et al., 1989) although the proportional contribution of the liver and muscle to insulin clearance are unknown. Endurance training has been shown to increase hepatic insulin clearance in rodents (Wirth et al., 1982) although relatively little is known of the effects of exercise on insulin clearance, particularly in humans. A single bout of exercise has been observed to increase whole body insulin clearance immediately or up to 18 hours following the cessation of exercise when plasma insulin concentrations are within the range observed in the present study (Brambrink et al., 1997; Mikines et al., 1989; Thorell et al., 1999). When supraphysiological levels of insulin are infused the exercise induced improvement in insulin clearance are not observed (Mikines et al., 1988; Mikines et al., 1989) suggesting that the maximum clearing capacity of insulin is not altered by exercise. None of the recent studies to examine the effects of short-term training on insulin sensitivity and signalling have observed a similar reduction in plasma insulin following training although much higher levels of insulin stimulation (2-3 fold higher) (Houmard et al., 1999; Tanner et al., 2002; Youngren et al., 2001) were utilised compared to the present study, and may therefore account for the lack of exercise effect on insulin clearance.

The results presented in this chapter are consistent with previous studies that demonstrate insulin induces a significant increase in the tyrosine phosphorylation of the IR and IRS-1. However, interestingly exercise did not alter the insulin-stimulated increase in either IR or IRS-1 tyrosine phosphorylation or protein expression despite
improvements in whole-body insulin action. A significant increase in *in vitro* IR autophosphorylation has previously been reported in human muscle following a similar short-term endurance-training program (Youngren et al., 2001) when compared to the present study. However the increases observed by Youngren et al., (Youngren et al., 2001) were only observed when *in vitro* insulin concentrations were at least 960 pmol/l; approximately 2.5 fold higher than the *in vivo* levels observed in the present study, suggesting that IR function may only be increased following training at supraphysiological insulin levels. The present study has shown using two different approaches to measure IR tyrosine phosphorylation that acute exercise and endurance training do not increase insulin-stimulated IR tyrosine phosphorylation, despite observed improvements in insulin action. Few short-term training studies in humans have measured IRS-1 tyrosine phosphorylation. IRS-1 associated PI 3-kinase activity has been shown to be elevated in trained vs. untrained males (Kirwan et al., 2000) and has also been elevated following 7 days of training in young males (Houmard et al., 1999) although these studies also used supraphysiological doses of insulin that resulted in plasma insulin levels approximately 3 times higher than those used in the present study (Houmard et al., 1999; Tanner et al., 2002) making comparisons difficult. These findings suggest that proximal steps in the insulin-signalling cascade are not the primary mechanisms for exercise-induced improvements in insulin signalling in human skeletal muscle. The effects of exercise on glucose transport are more likely mediated on more distal and perhaps as yet unknown signalling proteins.

The major finding of this study was that 24-hours following acute exercise and endurance training; overall pAkt (Ser473) was significantly increased. This is a surprising finding since prior exercise alone has not yet been reported to have any long-
term (12-24 hours) effect on pAkt (Ser473) in humans. Previous studies have shown that muscular contraction is associated with increased serine (473) and threonine (308) phosphorylation of Akt (Sakamoto et al., 2002; Sherwood et al., 1999; Turinsky & Damrau-Abney, 1999) with maximal activation of Akt by 3 min of contraction, then returning to basal levels by 15 min (Sakamoto et al., 2002). In humans, pAkt (Ser473) is increased immediately following exercise (Thorell et al., 1999) although a separate study showed pAkt (Ser473) is not elevated in humans 4-hours after the cessation of exercise (Wojtaszewski et al., 2000) so it is unusual that pAkt (Ser473) was upregulated 24 hours following the cessation of exercise in the present study. A short-term training study in middle-aged men found no change in insulin-stimulated Akt activity when measured 17-hours following the last bout of exercise, although only 4 subjects were analysed so the results must be viewed with caution (Tanner et al., 2002). Insulin-stimulated pAkt (Ser473) has been shown to be increased following 5 days and 6 weeks of training in lean, non-diabetic rats (Chibalin et al., 2000; Luciano et al., 2002) with a trend for increased pAkt (Ser473) following one day of exercise (Chibalin et al., 2000). These findings of increased insulin-stimulated pAkt (Ser473) in rodents 16-48 hours after the last bout of exercise training are slightly different to the observations in the present study where overall pAkt (Ser473) was found to be increased following exercise without an additive effect of exercise on insulin-stimulated pAkt (Ser473). However, the findings from these rodent studies and the present study suggest that in the 16-48 hour period following the cessation of exercise there is a significant effect of exercise on pAkt (Ser473). There are currently few clues as to the precise mechanism/s whereby exercise may be acting upon Akt. Growth hormone, intracellular calcium and cAMP all activate Akt \textit{in vitro} (Sable et al., 1997; Sakaue et al., 1997; Yano et al., 1998) and may account for the increased pAkt (Ser473) observed following exercise in the present
Despite exercise and insulin increasing pAkt (Ser473), there was no interaction between insulin and exercise, suggesting that the effects are not additive. This would imply that exercise might be increasing signalling through Akt for purposes other than, or in addition to insulin-stimulated glucose uptake. Akt seems to have multiple signalling roles, including insulin-stimulated glucose transport, glycogen synthesis and mitogenic signalling although its exact contributions are still controversial (Kitamura et al., 1998). Overexpression of Akt in L6 myotubes increases glucose uptake, glycogen and protein synthesis (Ueki et al., 1998). Also, PI 3-kinase inhibitors not only block insulin-stimulated glucose uptake but Akt activity in isolated rodent muscle (Brozinick Jr. & Birnbaum, 1998; Whitehead et al., 2000) demonstrating a key role for Akt on glucose uptake. Insulin stimulation of Akt has been shown to increase glycogen synthesis in L6
myotubes by inhibiting glycogen synthase kinase-3 (GSK-3), a known inhibitor of glycogen synthase (Cross et al., 1995; Cross et al., 1997; Ueki et al., 1998) whilst in cell lines lacking GSK-3 such as 3T3L1 adipocytes, overexpression of Akt has no effect of glycogen synthesis (Ueki et al., 1998). The pathway for insulin stimulated protein synthesis via the activation of Akt also appears to involve the downstream activation of p70 S6 kinase, since inhibition of Akt reduces activation of p70 S6 kinase in CHO cells and 3T3L1 adipocytes (Kitamura et al., 1998). Also, in isolated rodent muscle, PI 3-kinase inhibitors not only impair insulin-stimulated Akt kinase activity but also p70 S6 kinase activity (Brozinick Jr. & Birnbaum, 1998) further supporting the involvement of Akt in insulin-stimulated protein synthesis. There is some evidence to suggest that the different isoforms of Akt have preferential roles towards the regulation of glucose uptake, glycogen synthesis and mitogenesis. Akt-1 knockout mice have normal insulin sensitivity but are much smaller than their wild type littermates (Cho et al., 2001b), whilst Akt-2 deficient mice have normal growth development but are insulin resistant (Cho et al., 2001a). Akt-2 inhibition in 3T3L1 adipocytes also inhibits insulin-stimulated GLUT4 translocation (Hill et al., 1999). Recently, depletion of Akt-1 and/or Akt-2 isoforms in 3T3L1 adipocytes by RNA directed gene silencing suggest a primary role for Akt-2 and a lesser role for Akt-1 in insulin-stimulated glucose transport (Jiang et al., 2003). Similar preferential roles for Akt-1 and Akt-2 were found for glycogen synthesis (Jiang et al., 2003). Whilst further work is required to elucidate the exact roles of the different Akt isoforms, it appears that Akt is a key regulatory protein involved in insulin-stimulated glucose transport, glycogen synthesis and mitogenesis. However, without downstream measures of the mitogenic or glycogen synthesis pathways such as phosphorylated MAP kinase, p70 S6 kinase or glycogen synthase activity there is no direct evidence for this in the present study. Due to a lack of
available sample, time and financial constraints these variables were not measured in the present study. Nevertheless, this study has successfully shown for the first time that there is a small increase in key component of insulin signalling in response to acute exercise and short-term training that is distal to PI 3-kinase in human skeletal muscle.

Another major finding of this study was that exercise selectively increases the protein expression of SHPTP2 in human skeletal muscle. The finding that SHPTP2 protein abundance was selectively increased in the cytosolic fraction suggests this to be a major site for its intracellular action. In support of this SHPTP2 has been implicated in rat skeletal muscle to account for a large majority of cytosolic PTPase activity (Ahmad & Goldstein, 1995). However, increased cytosolic levels of SHPTP2 are not always associated with states of improved insulin sensitivity. Both SHPTP2 and PTP1B are increased in obese subjects and decreased in obese type 2 diabetics compared to lean controls. These findings suggest a dysregulation of PTPases in the insulin resistant and diabetic states (Ahmad et al., 1997a). Nevertheless, SHPTP2 has been implicated to play a role in the positive regulation of insulin-stimulated glucose transport, glycogen synthesis and mitogenic signalling (Chapter 1). Over expression of SHPTP2 in rat fibroblasts increases the insulin stimulated binding of IRS-1 with SHPTP2, IRS-1 tyrosine phosphorylation, and the associated PI 3-kinase activity whilst inhibition of SHPTP2 has the opposite effect (Ugi et al., 1996). Transgenic mice with impaired SHPTP2 not only display reduced insulin-stimulated glucose transport, but glycogen synthase activity and MAP kinase phosphorylation are also impaired, suggesting multiple regulatory roles for SHPTP2 in skeletal muscle (Maegawa et al., 1999). Both overexpression and inhibition of SHPTP2 in rat fibroblasts also supports the multiple actions of SHPTP2 on insulin signalling (Ugi et al., 1996).
How exactly a PTPase such as SHPTP2 is able to positively influence insulin signalling is unclear. It has been postulated that SHPTP2 amplifies tyrosine phosphorylation of IRS-1 by either inhibiting an as yet undefined PTPase(s) (Ugi et al., 1996) or by the activation of tyrosine kinases. As mentioned in Chapter 1, one of the main regulatory mechanisms for SHPTP2 on insulin signalling involves increased IRS-1 tyrosine phosphorylation (Maegawa et al., 1999; Ugi et al., 1996). However, the present study observed no changes in IRS-1 tyrosine phosphorylation following acute exercise and exercise training. Although it appears that increased SHPTP2 protein abundance is not mediating IRS-1 tyrosine phosphorylation in the present study it is possible that enhanced cytosolic SHPTP2 protein expression indirectly increases pAkt (Ser473). An indirect link between increased SHPTP2 abundance and increased Akt activity has been demonstrated in the past. Overexpression of SHPTP2 is known to increase the activity of the tyrosine kinase, Src (Walter et al., 1999) via the binding to its SH3 domain. The association of Src through its SH3 domain with the C-terminal regulatory region of Akt has been shown to be necessary for the tyrosine phosphorylation and activation of Akt (Jiang & Qiu, 2003). Src association with Akt is also required for its extracellular growth factor (EGF) induced activation (Jiang & Qiu, 2003), although the association of SHPTP2 with Src on the SH3 domain and the subsequent activation of Akt following insulin stimulation are unknown. Although highly speculative, the observation in the current study that SHPTP2 cytosolic protein abundance is associated with increased pAkt (Ser473) following both acute exercise and short-term training, combined with the previous findings of others, indirectly linking SHPTP2 and Akt via the tyrosine kinase, Src, is sufficient to warrant further investigation into this poorly described aspect of insulin signalling.
The other significant finding of the present study was that PTP1B protein abundance or subcellular distribution is unaltered by exercise. There is evidence for the inactivation of PTP1B by insulin and reactive oxygen species such as hydrogen peroxide (Mahadev et al., 2001; Tao et al., 2001) without alteration in protein expression. It is however unlikely that alterations in PTP1B activity may be regulating any exercise-induced improvements in insulin sensitivity since the IR and IRS-1, which are substrates of PTP1B, were not altered by exercise. The vast majority of studies investigating the role of PTPases in the regulation of insulin signalling have focussed on PTP1B. This is not surprising since PTP1B null mice display enhanced insulin sensitivity and improved insulin stimulated IR and IRS-1 tyrosine phosphorylation (Elchebly et al., 1999). Similar results have been found when PTP1B is disrupted by either antisense oligonucleotide or specific inhibitor treatment (Mohammad et al., 2002; Zinker et al., 2002). Weight loss in humans is also associated with reduction of PTP1B in adipose tissue (Ahmad et al., 1997b). Clearly, PTP1B has an important role to play in insulin signalling and therapeutic benefits may be gained for type 2 diabetics via the development of specific PTP1B inhibitors (Iversen et al., 2000; Mohammad et al., 2002). However, it would appear based on the findings of this study that exercise-induced improvements in insulin action are not mediated by changes in PTP1B protein abundance or subcellular localisation.

4.5 Conclusions

In summary, this study has found that 24 hours following a single bout of exercise there was improved whole-body insulin action in sedentary humans. Larger improvements in
insulin action were found 24 hours following the completion of endurance training, which implies that increased insulin action is not only due to the residual effects of the prior bout of exercise, but that cumulative bouts of exercise substantially increase this response. This study also found that there was no effect of exercise on the upstream components of the insulin-signalling pathway such as the IR or IRS-1 protein expression or tyrosine phosphorylation, suggesting that increased insulin sensitivity following exercise is not mediated by changes in the proximal section of the insulin signalling pathway. A major finding of this study was that there was an observed increase in the serine phosphorylation of a known downstream signalling protein, Akt, in human skeletal muscle following an acute bout of exercise and exercise training.

Finally, the present study also found increased abundance of SHPTP2 protein in the cytosolic fraction of skeletal muscle following both acute exercise and short-term training. Previous studies have shown a possible link between SHPTP2 and Akt, so it is possible, although highly speculative, that the upregulation of both SHPTP2 and pAkt (Ser473) following exercise in the present study is directly related, although further research is required to establish this link. It is also possible that the increase in pAkt (Ser473) and/or cytosolic SHPTP2 by exercise could be responsible for increased mitogenic signalling and/or glycogen synthesis and future research should examine these pathways. Also, PTP1B protein expression and cellular distribution was unaltered by exercise, as were its targets for protein tyrosine dephosphorylation, IR and IRS-1, suggesting that PTP1B is not involved in any improvements in insulin stimulated glucose uptake by exercise.
CHAPTER FIVE

EFFECTS OF EXERCISE ON PROTEIN ABUNDANCE OF
IRS-2 AND THE p85α SUB UNIT PI 3-KINASE

5.1 Introduction

In Chapter 2 it was observed that a single bout of endurance exercise is sufficient to increase the mRNA concentration of insulin signalling proteins such as IRS-2 and the p85α sub unit of PI 3-kinase in humans, whilst mRNA concentration of other key insulin signalling proteins such as IR and IRS-1 are unchanged by acute exercise. IRS-2 mRNA concentration is also upregulated following a single bout of resistance exercise, demonstrating the acute transcriptional activation of IRS-2 by intense muscle contraction (Chapter 3). Improvement in IRS-2 protein abundance and insulin stimulated PI 3-kinase activity in rodents has further been shown following an acute bout of exercise pointing to a specialised role for IRS-2 in insulin stimulated glucose transport and/or mitogenesis (Chibalin et al., 2000; Howlett et al., 2002). It remains unclear if this transient, transcriptional activation of IRS-2 and the p85α sub unit of PI 3-kinase gene expression following a single bout of endurance exercise in humans results in an increase in protein abundance. It was therefore decided that subsequent to the results obtained from Chapter 4 further analysis was required to determine if exercise increased the protein abundance of these insulin-signalling components. Therefore, the aim of this study was to determine if acute exercise is sufficient to increase IRS-2 and p85α PI 3-kinase protein expression in humans skeletal muscle.
5.2 Materials And Methods

5.2.1 Materials

PI 3-kinase p85α rabbit polyclonal IgG (UBI, Cat. # 06-195) was purchased from Upstate Biotechnology (New York, USA). IRS-2 (A-19) goat polyclonal (SC, Cat. #1556) was from Santa Cruz Biotechnology (California, USA). Affinity purified peroxidase labelled anti-mouse IgG and anti-rabbit IgG were purchased from Silenus (Victoria, Australia). All other reagents were analytical grade (Sigma, NSW, Australia).

5.2.2 Subjects

This study forms part of the human intervention that was covered in the previous chapter. The subjects recruited, and the methods for exercise training and the euglycemic hyperinsulinemic clamp procedure are the same as per the previous chapter. Briefly, A group of 8 untrained, but healthy males volunteered to be involved in this study. Subject characteristics were as described in the previous chapter.

5.2.3 Experimental protocol

The subjects underwent 3 euglycemic-hyperinsulinemic clamps on separate days, each following an overnight fast and performed acute exercise and endurance training as per the previous chapter.

5.2.4 Immunoprecipitations and immunoblotting

Equal amounts of protein for determination of IRS-2 (75µg) and PI 3-kinase p85 (60µg) were solubilised in Laemmlli sample buffer. Bound proteins were separated by SDS-
PAGE and electrotransfer of proteins from the gel to nitrocellulose membranes (25mM Tris, pH 8.3, 192mM glycine, and 20% v/v methanol) was performed for 100 min at 100V (constant). Blots were probed with anti-IRS-2 goat polyclonal (1:200) or anti-p85 PI 3-kinase rabbit polyclonal (1:1000) antibodies. Binding was viewed by enhanced chemiluminescence (Pierce, IL, USA) and quantified with Kodak 1D version 3.5 software (Eastman Kodak Co., CT, USA).

5.2.5 Statistical analysis

Standards were included in all immunoblots and interassay variation was accounted for by normalising data to control samples. Data are presented as mean ± SEM.

Differences were determined using 2-way analysis of variance (ANOVA) with Newman-Keuls post-hoc analysis, where appropriate. Significance was accepted when $p<0.05$.

5.3 Results

The subject and exercise characteristics are presented in the previous chapter (Table 4.2). Also, refer to the previous chapter for the results of the euglycemic, hyperinsulinemic clamps (Table 4.3 and Figure 4.1 and 4.2).

5.3.1 IRS-2 and p85α PI 3-kinase

The protein expression of the p85α catalytic subunit of PI 3-kinase was not altered by exercise (Figure 5.1). IRS-2 protein expression increased approximately 1.5 fold
following insulin stimulation in the sedentary condition ($p<0.05$, Figure 5.2). There was also a 1.9 fold increase in IRS-2 protein abundance following acute exercise ($p<0.05$, Figure 5.2), which decreased back to sedentary levels following endurance training ($p<0.05$, Figure 5.2).
Figure 5.1. p85α PI 3-kinase protein abundance during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-p85α PI 3-kinase antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects.

Figure 5.2. IRS-2 protein abundance during the euglycemic, hyperinsulinemic clamps. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-IRS-2 antibody. Western blots are representative from one subject. Sedentary: no prior exercise, Acute: 24 hours following 60 min of exercise, Training: 24 hours following 8 days of endurance training. Open bars: basal values, closed bars: insulin stimulated values. Bars on graph represent means ± SE of 8 subjects. *p<0.05 vs. basal value in the sedentary state.
5.4 Discussion

It was discussed in the previous chapter that the present study observed significant increases in the insulin action of sedentary individuals following an acute bout of exercise and further improvements following short-term endurance training that could largely be attributed to exercise rather than any associated alterations in body composition.

We found a small but significant increase in IRS-2 protein expression following prolonged insulin stimulation in the sedentary state. This result was surprising since little is known regarding increases in protein abundance following acute insulin stimulation. Even more surprising was that it was only increased by insulin in the sedentary state and not following acute exercise or endurance training. No studies to our knowledge have investigated the effects of insulin stimulation on IRS-2 protein abundance. Increased IRS-2 protein abundance can account for a significant amount of increased IRS-2 associated PI 3-kinase activity following an acute bout of exercise (Chibalin et al., 2000) although the effects of prolonged insulin stimulation on IRS-2 protein abundance have not been previously measured. It could be postulated that unlike the sedentary condition, no change in insulin-stimulated IRS-2 protein expression was observed following acute exercise since protein abundance was already significantly elevated by the exercise bout, reducing the stimulus for additional protein synthesis. For some as yet unknown reason cumulative bouts of exercise attenuate the transcriptional regulation of IRS-2 compared to acute exercise (Chapter 2) and this may explain the lack of effect for insulin stimulation or endurance training on IRS-2 protein abundance observed in the present study.
The protein expression of IRS-2 also significantly increased by 1.9 fold in the 24 hours following a single bout of endurance exercise and returned to sedentary levels after 8 days of endurance training. Chapter 2 of this thesis reported a significant increase in IRS-2 gene expression several hours following an acute bout of exercise, which would now appear to coincide with increased protein abundance approximately 20 hours later. Furthermore, short-term endurance training diminished the effect of a single bout of exercise on IRS-2 gene expression (Chapter 2), which also coincides with the findings of the present study that IRS-2 protein abundance returned to sedentary levels following training. It is often difficult to correlate mRNA with protein abundance due to the transient nature of mRNA and the longer synthesis and half lives of the proteins. However, it would appear that for IRS-2 at least, large changes in gene expression in the few hours following exercise are associated with significant increases in protein expression up to 20 hours later. It would also suggest that alterations in IRS-2 protein abundance by exercise are mediated by transcriptional factors rather than via translation control. The protein abundance of IRS-2 in rodents (Chibalin et al., 2000) 16 hours following a single bout of exercise also significantly increases with a similar pattern to return to sedentary levels following short term training when compared to the findings of the present study. Chibalin et al. (Chibalin et al., 2000) also found increases in IRS-2 associated PI 3-kinase activity in both the basal and insulin stimulated state, which could largely be attributed to increases in protein abundance. Unfortunately, the measurement of IRS-2 tyrosine phosphorylation requires relatively large volumes of sample and for this reason IRS-2 tyrosine phosphorylation not measured in the present study. Nevertheless, the finding in the present study of increased IRS-2 protein abundance following acute exercise, combined with previous findings in rodents (Chibalin et al., 2000) would suggest an early adaptive role for IRS-2 that is different to
the other insulin receptor substrates with regards to glucose transport and/or mitogenesis. Although IRS-2 has been shown not to play a major role in exercise or insulin stimulated glucose transport (Higaki et al., 1999), insulin stimulated IRS-2 tyrosine phosphorylation and associated PI 3-kinase activity are elevated immediately post exercise and can account for some of the improvements in insulin stimulated glucose uptake (Howlett et al., 2002) immediately post exercise, however much less is known of its role in the 12-24 hours period after the cessation of acute exercise. In the present study, there was only a small improvement in insulin action following acute exercise when compared with exercise training and in light of this it would appear that the observed increases in IRS-2 protein abundance if they are related to any improvements in insulin sensitivity are likely to be small. One other potential role for IRS-2 is via the mitogenic pathway. IRS-2 has a major role in the maintenance of β-cell mass in the pancreas (Withers et al., 1999). Little is known of the role for IRS-2 in the mitogenic pathway, particularly in skeletal muscle although as discussed earlier there is a sustained activation of IRS-2 associated PI 3-kinase activity for up to 16 hours following an acute bout of endurance exercise in rodents independently of insulin that could largely be attributed to increased IRS-2 protein abundance (Chibalin et al., 2000). Selective stimulation of PI 3-kinase independently from insulin has been shown in regenerating rodent muscle to be important for myofiber growth (Murgia et al., 2000). The activation of PI 3-kinase via association with its p85α catalytic subunit and by IRS-1 independent from insulin has been shown following muscle overload in rodents to coincide with increased glucose uptake and protein synthesis (Carlson et al., 2001; Hernandez et al., 2000). It would therefore appear that following intense muscular contractions IRS-2 protein abundance increases, which may activate PI 3-kinase via concomitant increases in the tyrosine phosphorylation of IRS-2. There is some evidence
to suggest that this pathway may play a role in protein synthesis and perhaps following insulin stimulation IRS-2 may also play a minor role in glucose uptake following an acute bout of exercise. This pathway may have an early transitory role in both mitogenesis and insulin stimulated glucose uptake since IRS-2 protein expression and function appear to return to sedentary levels with repeated bouts of intense muscular contractions.

The finding that protein abundance of the p85α sub unit of PI 3-kinase was not increased 24 hours following acute exercise or exercise training suggests that even though acute exercise can stimulate transcriptional activation of the p85α gene, this does not appear to result in improved protein abundance.

5.5 Conclusions

In summary, prolonged insulin stimulation in the sedentary state was found to increase IRS-2 protein abundance and that IRS-2 protein abundance, like its gene expression found previously in Chapter 2, is augmented following acute exercise, but attenuated following cumulative bouts of exercise. In light of these novel findings, future studies investigating if any functional changes occur in IRS-2 tyrosine phosphorylation or related PI 3-kinase activity following a single bout of exercise and exercise training are therefore warranted. This would further support a transient role for IRS-2 in insulin-stimulated glucose uptake and mitogenic signalling in human skeletal muscle.
CHAPTER SIX

THE EFFECT OF INSULIN AND EXERCISE ON c-Cbl PROTEIN ABUNDANCE AND PHOSPHORYLATION IN INSULIN RESISTANT SKELETAL MUSCLE

6.1 Introduction

While the activation of the PI 3-kinase pathway is pivotal in the stimulation of glucose transport, its activation alone is not sufficient to mediate glucose transport. Activation of IRS-1 and/or PI 3-kinase with ligands such as platelet derived growth factor (PDGF), insulin in cell lines with impaired IR function or via integrin engagement on the cell membrane do not result in increased glucose transport (Baumann et al., 2000; Guilherme & Czech, 1998; Krook et al., 1997; Wiese et al., 1995). These studies provide some evidence for another insulin signalling pathway that is required for insulin-stimulated glucose transport, but is independent of PI 3-kinase activation.

It was discussed in Chapter 1 that there has been recent identification of an alternate insulin-signalling pathway for glucose transport involving the recruitment of the protein c-Cbl to the IR by the adapter protein CAP (Baumann et al., 2000). c-Cbl is heavily tyrosine phosphorylated by a variety of kinase signalling pathways (Anderson et al., 1997; Fukazawa et al., 1996; Marcilla et al., 1995; Meisner et al., 1995) and in 3T3-L1 adipocytes c-Cbl is markedly and rapidly tyrosine phosphorylated in response to insulin (Ribon et al., 1998).
Tyrosine phosphorylation of c-Cbl and subsequent localization to the lipid rafts in conjunction with the CAP and flotillin complex may be a necessary insulin-dependent pathway for glucose transport in adipocytes. However, involvement of this pathway in skeletal muscle, the primary tissue of insulin-mediated glucose uptake has yet to be demonstrated. Unlike adipocytes which are responsible for only 10% of insulin stimulated glucose disposal, skeletal muscle accounts for as much as 90% of whole-body glucose uptake (DeFronzo et al., 1985). Furthermore, in insulin resistant states the activation of signalling proteins such as the insulin receptor, IRS-1, PI 3-kinase and some isoforms of Akt are dysregulated (Brozinick et al., 2003; Christ et al., 2002; Cusi et al., 2000; Krook et al., 2000). Therefore, investigation of c-Cbl activation in normal healthy and insulin resistant skeletal muscle is important in order to elucidate the potential role of this novel insulin-signalling cascade.

Exercise is a potent stimuli for the translocation of GLUT4 to the cell surface and the subsequent increase in glucose transport into muscle (Richter et al., 1998; Thorell et al., 1999). The signalling pathways mediating translocation of GLUT4 in response to exercise remain unknown, although it is now well established that the pathway is independent from the insulin-mediated activation of PI 3-kinase (Wojtaszewski et al., 2002). c-Cbl is tyrosine phosphorylated by a wide variety of cell surface receptors including growth factors, cytokines, and lymphocyte antigens (Galisteo et al., 1995; Ribon & Saltiel, 1997; Taher et al., 2002). Additionally, shear stress in endothelial cells, initiates rapid and sustained tyrosine phosphorylation of c-Cbl (Miao et al., 2002). Collectively the results of these studies suggest a possible role for skeletal muscle contractile activity to result in tyrosine phosphorylation of c-Cbl and hence downstream
activation of the flotillin related complex involved in GLUT4 translocation to the cell membrane lipid rafts.

Furthermore, the expression of CAP in skeletal muscle is important since failure of insulin to tyrosine phosphorylate c-Cbl in 3T3L1 fibroblasts has been shown to be associated with a lack of CAP expression in 3T3L1 fibroblasts (Ribon et al., 1998). Skeletal muscle of mice has been previously shown to express CAP mRNA, implying expression of functional CAP protein in skeletal muscle (Ribon et al., 1998). However the expression of CAP protein in skeletal muscle and its abundance relative to other tissues such as 3T3L1 adipocytes is unknown. Therefore, the measurement of CAP expression in skeletal muscle will further elucidate the role of this novel pathway

In the present study the impact of insulin-stimulation and an acute bout of exhaustive swimming exercise on c-Cbl tyrosine phosphorylation in the skeletal muscle of lean and insulin-resistant obese Zucker rats was investigated. Soleus muscle from these animals was harvested for analysis of c-Cbl, IR and Akt phosphorylation and protein abundance following rest, acute exercise or insulin stimulation. A further aim of this study was to briefly determine if CAP protein abundance is much higher in 3T3L1 adipocytes compared to rat skeletal muscle.
6.2 Materials and Methods

6.2.1 Materials

c-Cbl (7G10) mouse monoclonal IgG (UBI, Cat. # 05-440), CAP rabbit polyclonal IgG (UBI, Cat. # 06-994), insulin-receptor (IR$_\beta$) rabbit polyclonal IgG (UBI, Cat. # 06-492) and phosphotyrosine (4G10) mouse monoclonal IgG (UBI, Cat. # 05-321) were purchased from Upstate Biotechnology (NY, USA). CT1 IR$_\beta$ mouse monoclonal IgG was a gift from Prof. Ken Siddle (University of Cambridge). Phospho-Akt (pAkt) Ser473 rabbit polyclonal IgG (Cat. # 9271) and Akt rabbit polyclonal IgG (Cat. # 9272) were from Cell Signaling Technology (New England BioLabs, Hartsfordshire, England). Affinity purified peroxidase labelled anti-mouse IgG and anti-rabbit IgG were purchased from Silenus (Victoria, Australia). All other reagents were analytical grade (Sigma, NSW, Australia).

6.2.2 Animal care and dietary treatment

Female lean (fa/__; n = 18) and obese (fa/fa; n = 18) Zucker rats aged 10-11 wk and weighing ~ 176 and ~296 g, respectively, were obtained from Monash University Animal Services, Victoria, Australia. Animals were housed two per cage in an environmentally controlled laboratory (temperature 22 ± 1°C, relative humidity 50 ± 2%) with a 12:12-h light-dark cycle (light 0700-1900). Animals were fed standard rodent chow (67.5% carbohydrate, 11.7% fat, 20.8% protein; Barastock, Victoria, Australia), given ad libitum access to water; and familiarized to laboratory conditions for 1 wk prior to experimentation. The Animal Experimentation Ethics Committee of RMIT University approved all experimental procedures.
Animals were assigned to one of three subgroups on the basis of 1) whether they remained sedentary control (Con), 2) were exercised (Ex), or 3) were insulin stimulated (Insulin).

6.2.3 Sedentary controls
At 1700 h on the day before the experiment, lean (ZL) animals were restricted to 10 g, and obese (ZO) animals to 12 g, of chow (this amount being ~60% of the animals average daily food consumption from the previous 7 d). ZL rats were assigned to one of three experimental groups: sedentary control (ZL-Con; n = 6), exercised (ZL-Ex; n = 6), and insulin treated (ZL-Insulin; n = 6). ZO rats were also assigned to one of three experimental groups: sedentary control (ZO-Con; n = 6), exercised (ZO-Ex; n = 6), and insulin treated (ZO-Insulin; n = 6).

6.2.4 Exercise
Two groups of rats (ZL-Ex and ZO-Ex) performed a standard exercise regimen in order to deplete their skeletal muscle glycogen stores. Three rats swam together in a steel barrel measuring 60 cm in diameter and filled to a depth of ~60 cm. Water temperature was maintained at 35°C. Prior to the commencement of an experiment, all animals had been familiarized to swimming for 10 min/d for 3 d. The swimming protocol was a modification of the procedure used extensively in previous exercise studies with rats (Bruce et al., 2001; Cartee et al., 1989; Gulve et al., 1990). Rats swam for eight 30 min bouts separated by 5 min rest periods. In the case of the obese animals, a weight equal to ~2.5% of body mass (BM) was attached to the base of the tail after the first 30 min exercise bout in an attempt to compensate for their increased buoyancy. The obese
animals swam with the weight attached for the remaining seven exercise bouts. Weights were chosen so that during the swimming protocol the body angles relative to the surface of the water were similar for both the obese and lean rats (Walberg et al., 1982). Rats were sacrificed immediately after the exercise.

6.2.5 Insulin treatment

Two groups of non-exercised rats (ZL-Insulin and ZO-Insulin) were injected intraperitoneally with insulin (0.15 U.g\(^{-1}\) body weight, Human, Actrapid, Novo Nordisk, Denmark) (Howlett et al., 2002). After 5 min the animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg.kg\(^{-1}\) BM) and sacrificed.

6.2.6 Preparation of rat tissue and blood samples

Approximately 5 min before the due time of death, rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg.kg\(^{-1}\) BM) and soleus muscle was rapidly excised and frozen in liquid N\(_2\). The soleus (84% type I, slow-twitch, oxidative fibers) was deliberately chosen to sample because previous investigations have reported there to be no differences in GLUT4 content between ZL and ZO animals (Brozinick et al., 1992). The exercise protocol used in the present study has been shown in previous studies to significantly reduce muscle glycogen in the soleus, indicating active recruitment of this muscle group during the exercise protocol (Bruce et al., 2001). Furthermore, type I fibers are more insulin sensitive (Kern et al., 1990) and have a higher maximal glucose transport rate than type II fibers (Richter et al., 1988). A blood sample (~1 mL) was obtained via cardiac puncture.
Frozen rat soleus were homogenised using a polytron at maximum speed for 30 sec on ice in 600µl of freshly prepared ice-cold buffer consisting of 50mmol/l HEPES at pH 7.6 containing 150 mmol/l NaCl, 20 mmol/l Na₄P₂O₇, 20 mmol/l β-glycerophosphate, 10 mmol/l NaF, 20 mmol/l EDTA, 1% v/v Nonidet P-40, 10% v/v glycerol, 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 2 mmol/l Na₃VO₄, 2 mmol/l PMSF, 5µl/ml Protease Inhibitor Cocktail (P8340, Sigma). Tissue lysates were incubated on ice for 20 min and then centrifuged at 10,000 × g for 20 min at 4 °C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, NSW, Australia). The supernatants were stored at −80 °C until subsequent analysis.

6.2.7 Cells and culture conditions

Differentiated 3T3-L1 adipocytes were a gift from Dr. Lance Macaulay (CSIRO). Briefly, 3T3L1 fibroblasts were grown and passaged in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 10% (v/v) newborn calf serum at 37°C. Fibroblasts were differentiated 1 to 2 days post-confluence. The differentiation medium contained 10% (v/v) fetal calf serum (FCS), 250nM dexamethasone, 500nM isobutyl methylxanthine and 500nM insulin. After 3 days, the differentiation medium was replaced with post-differentiation medium containing 10% (v/v) FCS and 500nM insulin for a further 2 days. Cells were then fed for another 2 days post-differentiation in DMEM supplemented with 5% (v/v) FCS. Prior to cell lysis, adipocytes were serum starved overnight in DMEM containing 0.5% (v/v) FCS. Adipocytes were then washed twice with ice-cold PBS and incubated for 15 min on ice in lysis buffer containing 50mmol/l Tris-HCl at pH 7.4 containing 150 mmol/l NaCl, 1 mmol/l Na₄P₂O₇, 0.25% sodium deoxycholate (w/v), 1 mmol/l NaF, 1 mmol/l EGTA, 1% Nonidet P-40, 1
mmol/l Na$_3$VO$_4$, 1 mmol/l PMSF, 1µg/ml aprotinin, 1µg/ml leupeptin and 1µg/ml pepstatin. Tissue lysates were then spun at 10,000 × g for 15 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, NSW, Australia). The supernatants were stored at −80°C until analysis. The lysis analysed was from 2 separate 10 cm-diameter dishes.

6.2.8 Immunoprecipitations and immunoblotting

For immunoprecipitation, 1µg of anti-c-Cbl or 2µg of anti-IRβ monoclonal antibody (Upstate Biotechnology) was coupled to 30 µl protein A-sepharose beads (Zymed Laboratories, CA, USA) for 20 min at room temperature and then washed once in PBS. Tissue lysates (375µg protein) were incubated with the antibody coupled beads overnight at 4°C with rotation. Immunoprecipitated proteins were then washed once in PBS containing 0.5 mol/l NaCl and 0.2% v/v Triton X-100 and twice in PBS containing 0.2% v/v Triton X-100. Immunoprecipitated proteins or total lysates for determination of pAkt (Ser473) (75µg), IRβ (45µg), Akt protein abundance (75µg) in rat skeletal muscle and CAP protein abundance in 3T3L1 adipocytes (20µg of total protein per lane) and rat skeletal muscle (75µg of total protein per lane) and were solubilised in Laemmli sample buffer. Bound proteins were separated by SDS-PAGE and electrotransfer of proteins from the gel to nitrocellulose membranes (25 mmol/l Tris, pH 8.3, 192 mmol/l glycine, and 20% v/v methanol) was performed for 100 min at 100V (constant). Blots were probed with anti-c-Cbl monoclonal mouse, anti-CAP polyclonal rabbit, anti-phosphotyrosine 4G10 mouse monoclonal, anti-IRβ CT-1 mouse monoclonal, anti-Akt rabbit polyclonal or anti-pAkt (Ser473) rabbit polyclonal antibodies. Binding was detected with HRP coupled secondary antibodies and by
enhanced chemiluminescence (Pierce, IL, USA). Blots were quantified with Kodak 1D version 3.5 software (Eastman Kodak Co., CT, USA).

6.2.9 Blood biochemistry

Whole blood (~1 mL) was transferred to an EDTA administered tube and was spun in a centrifuge at 12,000 rpm for 3 min. The plasma was analysed for plasma glucose concentration using an automated analyser (Yellow Springs Instruments 2300 Stat Plus Glucose Analyser, Yellow Springs, OH, USA). The remaining plasma was stored at –80°C and was subsequently analysed for plasma insulin concentration by radioimmunoassay using a commercially available kit (Phadeseph, Insulin RIA, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden).

6.2.10 Statistical analyses

Standards were included in all immunoblots and interassay variation was accounted for by normalising data to control samples. Data are presented as mean ± SEM. Analysis of differences between two treatments within a genotype and differences between lean and obese animals was performed using an unpaired \( t \) test. All other differences were determined using a two-way analysis of variance with Newman-Keuls post-hoc analysis, where appropriate. Significance was accepted when \( p<0.05 \).
6.3 Results

6.3.1 Characteristics of lean and obese Zucker rats

As expected, ZO animals were significantly heavier than ZL (296 ± 7g versus 176 ± 3g, \( p < 0.0001 \)). Table 6.1 shows the concentrations of plasma glucose and plasma insulin at rest and following the two treatment interventions. Resting plasma glucose concentrations were similar between ZO and ZL. However, as expected, resting plasma insulin concentrations were 3 to 4-fold higher in ZO compared with ZL rats (\( p < 0.05 \), Table 1). In ZL rats, exercise was associated with a 1.6 and 2.6 fold reduction in plasma glucose and plasma insulin concentrations, respectively (\( p < 0.05 \), Table 6.1). Insulin treatment resulted in supra-physiological plasma insulin levels. Insulin treated rats were 2000-fold and 800-fold higher in ZL and ZO rats, respectively when compared to the control groups (\( p < 0.05 \), Table 6.1). Following insulin treatment, plasma glucose concentrations decreased 2.0 and 1.6 fold in ZL and ZO rats, respectively (\( p < 0.05 \), Table 6.1).
Table 6.1 Plasma glucose and insulin concentrations measured in lean and obese Zucker rats.

<table>
<thead>
<tr>
<th></th>
<th>Glucose, mmol/l</th>
<th>Insulin, pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>Control</td>
<td>8.7 ± 0.1</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>Exercise</td>
<td>5.5 ± 0.9⁺</td>
<td>10.4 ± 0.9⁺</td>
</tr>
<tr>
<td>Insulin</td>
<td>4.4 ± 0.6⁺</td>
<td>7.7 ± 0.5⁺</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 rats in each group. *p<0.05 vs. control group, ⁺p<0.05 vs. lean group in identical conditions.
6.3.2 Insulin receptor protein expression and tyrosine phosphorylation in muscle of Zucker rats

Lean and obese Zucker rats either undertook a single bout of exhaustive swimming exercise, or were treated with a supra-physiological dose of insulin. Immunoprecipitation and immunoblotting were performed on the extracts prepared from skeletal muscle and analysed for protein abundance and tyrosine phosphorylation of the IR and c-Cbl. pAkt (Ser473) and protein abundance of Akt, a kinase phosphorylated following the insulin-stimulated activation of PI 3-kinase was also determined. The results clearly show that insulin treatment markedly increased tyrosine phosphorylation of the IR. ZL-insulin treated rats had a 12-fold higher tyrosine phosphorylation of the IR compared to the ZL-control rats ($p<0.0001$, Figure 6.1). This effect was blunted in obese Zucker rats with IR tyrosine phosphorylation only 3-fold higher for the ZO-insulin treated compared to the ZO-control rats ($p<0.001$, Figure 6.1). This observation may partly be explained by the 40% lower abundance of IR protein in ZO compared to ZL animals ($p<0.0001$, Figure 6.2). IR protein abundance was not altered by either insulin treatment or acute exercise (not significant, Figure 6.2), although there was a trend ($p=0.06$) for ZO animals to have higher IR tyrosine phosphorylation levels than ZL in the control group (Figure 6.1). This finding can probably be explained by the significantly higher basal plasma insulin concentrations of the ZO animals in the two control groups (Table 6.1). Exercise did not increase IR phosphorylation in either lean or obese Zucker rats (Figure 6.1). Indeed, IR tyrosine phosphorylation was unable to be detected following exercise in the ZL rats. Accordingly, a nominal value was assigned to this group relative to the fold changes seen in the ZO animals between the control and exercised groups.
Figure 6.1. Effects of acute exercise and insulin stimulation on insulin receptor (IR) phosphorylation in lean (open bar) and obese (closed bar) Zucker rats. Skeletal muscle extracts were immunoprecipitated with anti-IR<sub>β</sub> antibody and blots were probed for anti-phosphotyrosine (4G10) antibody. Western blots are representative of one rat in each group. Bars on graph represent means ± SEM of 6 rats per group. *<i>p</i>&lt;0.0001 vs. control, #<i>p</i>&lt;0.0001 vs. ZL-insulin.

Figure 6.2. Effects of exercise and insulin on IR<sub>β</sub> protein abundance in lean (open bar) and obese (closed bar) Zucker rats. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-IR<sub>β</sub> antibody. Western blots are representative of one rat in each group. Bars on graph represent means ± SEM of 6 rats per group. *<i>p</i>&lt;0.05 vs. ZL
6.3.3 Akt protein expression and serine (473) phosphorylation in muscle of Zucker rats

ANOVA revealed a main effect for treatment ($p<0.0001$, Figure 6.3), suggesting that exercise increased Akt protein abundance when the genotypes are analysed together. This is probably explained by the 1.7 fold higher protein abundance in the ZL rats following exercise (Figure 6.4) although the lack of interaction between genotype and treatment does not allow this interpretation to be made in statistical terms. Akt protein expression was similar between obese and lean animals (not significant, Figure 6.4). Figure 6.5 shows that insulin treatment significantly increased pAkt (Ser473) in ZL-insulin and ZO-insulin skeletal muscle by 9 and 14 fold respectively, compared to the control groups ($p<0.0001$, Figure 6.5). pAkt (Ser473) was not significantly altered by exercise nor was it different in the control groups between genotypes. However pAkt (Ser473) following insulin treatment was reduced by ~30% in ZO rats compared with lean littermates ($p<0.0001$, Figure 6.5), an effect that cannot be explained by differences in Akt protein expression.
Figure 6.3. Effects of exercise and insulin on Akt protein abundance in lean and obese Zucker rats. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-Akt antibody. Bars on graph represent means ± SEM of 12 rats per intervention (lean and obese rats were combined for each intervention group). *p<0.0001 vs. control.
Figure 6.4. Effects of exercise and insulin on Akt protein abundance in lean (open bar) and obese (closed bar) Zucker rats. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-Akt antibody. Western blots are representative of one rat in each group. Bars on graph represent means ± SEM of 6 rats per group.

Figure 6.5. Insulin treatment but not exercise increases pAkt (Ser473) in lean (open bar) and obese (closed bar) Zucker rats. Protein abundance is determined following western blotting of skeletal muscle extracts with anti-pAkt (Ser473) antibody. Western blots are representative of one rat in each group. Bars on graph represent means ± SEM of 6 rats per group. *p<0.0001 vs. control, #p<0.0001 vs. ZL-insulin.
6.3.4 c-Cbl protein expression and tyrosine phosphorylation in muscle of Zucker rats

Figure 6.6 shows that c-Cbl is an abundant protein in skeletal muscle. However there was no significant difference in c-Cbl protein abundance between genotypes. ANOVA detected a significant treatment effect \( (p<0.05, \text{ Figure 6.7}) \) suggesting that c-Cbl protein abundance increases 1.5-fold in skeletal muscle in response to supraphysiological insulin treatment. However tyrosine phosphorylation of c-Cbl was undetectable (immunoprecipitating Cbl and immunoblotting for tyrosine phosphorylation) between any of the interventions, despite detecting both immunoprecipitated c-Cbl protein and tyrosine phosphorylation of the IR in rodent skeletal muscle (Figure 6.8).
Figure 6.6. No difference in c-Cbl protein abundance between lean (open bar) and obese (closed bar) Zucker rats. Skeletal muscle extracts were immunoprecipitated with anti-c-Cbl antibody and blots were probed for anti-c-Cbl antibody. Western blots are representative of one rat in each group. Bars on graph represent means ± SEM of 6 rats per group.

Figure 6.7. Insulin treatment but not exercise increases c-Cbl protein abundance in Zucker rats. Skeletal muscle extracts were immunoprecipitated with anti-c-Cbl antibody and blots were probed for anti-c-Cbl antibody. Bars on graph represent means ± SEM of 12 rats per intervention (lean and obese rats were combined for each intervention group). *p<0.05 vs. control.
Figure 6.8. c-Cbl is not tyrosine phosphorylated by insulin treatment in rat skeletal muscle. The skeletal muscle extracts from 2 lean Zucker rats (insulin treated) were prepared and immunoprecipitated with anti-insulin receptor (IR) or anti-c-Cbl antibodies. The resulting immunoprecipitates were then subjected to immunoblotting with anti-c-Cbl or anti-phosphotyrosine (anti-pY 4G10 blot) antibodies. The positions of molecular mass markers (in kilo Daltons) are indicated on the left. IP: immunoprecipitation. Insulin +: insulin treated.
6.3.5 CAP protein expression in 3T3L1 adipocytes and skeletal muscle of Zucker rats

For determination of CAP protein expression between rat skeletal muscle and 3T3L1 adipocytes, only one experiment was performed due to time and financial constraints. This provides a qualitative comparison of CAP protein expression between 3T3L1 adipocytes and skeletal muscle of Zucker rats. Figure 6.9 shows the expression of CAP protein from whole cell lysates prepared from 2 separate dishes of 3T3L1 adipocytes and skeletal muscle from 3 lean Zucker rats. The 99kD and 125kD isoforms appear more abundant in 3T3L1 adipocytes compared to rat skeletal muscle.
Figure 6.9. CAP protein expression appears more abundant in 3T3L1 adipocytes compared to rat skeletal muscle. Cell lysates (20 µg of 3T3L1 adipocytes and 75 µg of rat skeletal muscle total protein per well) were solubilised in Laemmli sample buffer and subjected to immunoblotting with anti-CAP antibody. The positions of molecular mass markers (in kilo Daltons) are indicated on the left. The arrows indicate the 2 major isoforms of CAP (125kD and 99kD) in 3T3L1 adipocytes. Two separate dishes of 3T3L1 adipocytes and skeletal muscle from 3 lean Zucker rats.
6.4 Discussion

To determine the role and responsiveness of the c-Cbl pathway, skeletal muscle from normal and obese insulin resistant Zucker rats was examined under resting conditions, after insulin-stimulation and immediately following a single bout of exhaustive swimming exercise. There was no evidence of tyrosine phosphorylation of c-Cbl in the muscle samples analysed under any of the experimental conditions, despite clearly demonstrating insulin-stimulation of its receptor and downstream activation of insulin signalling via Akt phosphorylation. These data suggest that tyrosine phosphorylation of c-Cbl may not be integral to GLUT4 translocation and activation of glucose transport in healthy and insulin resistant skeletal muscle. There was however a very small increase in c-Cbl protein abundance following insulin treatment. Given that the time course of insulin stimulation was relatively short, these results suggest that insulin may cause the translocation of c-Cbl out of a cellular compartment not solubilized by the extraction protocol.

Analysis of the responsiveness of the proximal members of the classical insulin signalling cascade demonstrated that basal tyrosine phosphorylation of the IR was elevated in the obese animals under resting conditions. Insulin stimulation resulted in a significant increase in tyrosine phosphorylation of the IR, and pAkt (Ser473) and these effects were blunted in obese Zucker rats compared with lean littermates. These results are in agreement with previous findings in this animal model (Christ et al., 2002; Kim et al., 2000b; Zhou et al., 1999). A novel finding from the present study was that intraperitoneal insulin administration failed to induce tyrosine phosphorylation of c-Cbl. There is evidence that c-Cbl’s role in insulin signalling may be tissue specific. Cell lines
such as 3T3L1 fibroblasts do not contain the c-Cbl associating protein, CAP and do not show the same insulin stimulated tyrosine phosphorylation of c-Cbl as 3T3L1 adipocytes (Ribon et al., 1998) although CAP abundance in skeletal muscle has not been previously documented. In cell lines expressing high levels of functional insulin receptors, such as chinese hamster ovary (CHO) cells overexpressing the human IR and KRC-7 cells insulin treatment does not induce tyrosine phosphorylation of c-Cbl (Ribon & Saltiel, 1997). This would suggest that in cell lines abundant in functional IR or lacking CAP, the c-Cbl pathway is not a central component for insulin signalling but rather an ancillary pathway. It is however difficult to explain the small, significant increase in c-Cbl protein abundance following a supraphysiological dose of insulin. It is unlikely that 5 min of insulin treatment in the current study was sufficient time for increases to occur via protein synthesis. It is possible that the increase in protein resulted from another storage site within the cell that was not extracted upon cell lysis. The measurement of c-Cbl abundance and tyrosine phosphorylation from the detergent-soluble fraction of the cell was chosen for analysis since it has been shown in 3T3L1 adipocytes that c-Cbl is predominantly in the detergent-soluble fraction under basal conditions and is a major site for its tyrosine phosphorylation following insulin stimulation (Mastick & Saltiel, 1997; Ribon & Saltiel, 1997). Upon insulin stimulation c-Cbl is tyrosine phosphorylated in the detergent-soluble fraction as early as 1 min and for at least 30 min following stimulation (Ribon & Saltiel, 1997). This time course also coincides with partial translocation of c-Cbl into the detergent-insoluble fraction (Baumann et al., 2000; Mastick & Saltiel, 1997). The whole cell lysis method employed in the present study would not extract c-Cbl from the detergent-insoluble fraction of the cell assuming there was any c-Cbl present in this fraction. Small amounts of c-Cbl have been reported in the detergent-insoluble fraction of 3T3L1 adipocytes under basal
conditions (Mastick & Saltiel, 1997) although the subcellular distribution of c-Cbl has not been reported in skeletal muscle. It is possible in skeletal muscle that the small increase measured in c-Cbl protein following insulin treatment could result from release of c-Cbl from the detergent-insoluble fraction following insulin stimulation. Rodent studies examining c-Cbl abundance in the various cellular fractions of adipose tissue and skeletal muscle following insulin treatment may confirm this conjecture.

c-Cbl undergoes activation and tyrosine phosphorylation by a diverse range of cellular stimuli including shear stress (Miao et al., 2002) and a wide array of cytokines and growth factors, including hepatocyte growth factor and epidermal growth factor (Galisteo et al., 1995; Ribon & Saltiel, 1997; Taher et al., 2002). Exercise stimulates the translocation of GLUT4 to the cell surface, increasing glucose transport via a pathway that is independent from the insulin-mediated activation of PI 3-kinase (Richter et al., 1998; Thorell et al., 1999), suggesting a possible role of the alternative c-Cbl pathway. Therefore the present study also examined whether c-Cbl would undergo tyrosine phosphorylation in response to an intense bout of prolonged exercise. The exercise stimuli chosen has been previously shown to result in pronounced reductions in muscle glycogen concentrations (Bruce et al., 2001), demonstrating the energetic demands of this exercise mode. However, c-Cbl tyrosine phosphorylation was unaffected by a single bout of swimming exercise.

An additional finding of the current investigation was that c-Cbl is a readily abundant protein in rodent skeletal muscle. Given the apparent abundance of this protein and the lack of subsequent tyrosine phosphorylation to either supra-maximal insulin-administration or acute, exhaustive exercise the physiological action of c-Cbl in skeletal
muscle remains to be elucidated. Considerable interest is focused on the possible actions of c-Cbl as a negative regulator of growth hormone mediated gene transcription (Goh et al., 2002) or as a negative regulator of non-receptor tyrosine kinases such by enhancement of their ubiquitin-dependent degradation (Ota & Samelson, 1997). Interestingly, despite the many perturbations in cellular homeostasis in the insulin resistant Zucker rat, c-Cbl protein expression was not altered between genotypes.

There was also a significant increase in Akt protein abundance following exercise, which could largely be attributed to a 1.7-fold increase in Akt protein in the lean group. This finding is unusual since there is no published data to support this observation. It is known that pAkt (Ser473 and Thr308) increases transiently during electrical stimulation of short duration in rodent muscle and decreases back to basal levels by 15 min of stimulation (Sakamoto et al., 2002) without any increases in Akt protein abundance. Further research is required to investigate the physiological significance of this finding.

This study also sought to compare the protein abundance of CAP in rodent skeletal muscle with its expression in 3T3L1 adipocytes, since CAP expression is involved in the insulin-stimulated tyrosine phosphorylation of c-Cbl in 3T3L1 adipocytes (Baumann et al., 2000). The major isoforms of CAP in 3T3L1 adipocytes occurs at 99kD and 125kD, whilst other isoforms or spliced variants occur at 75, 53 and 45kD (Ribon et al., 1998). It would appear from this preliminary investigation that the 99 and 125kD isoforms of CAP are much more abundant in 3T3L1 adipocytes compared to rat skeletal muscle, despite approximately 375% more total protein from the skeletal muscle lysates being loaded per well. This particular analysis has obvious limitations since due to time and financial constraints; the results are from one experiment. This
experiment would need to be repeated several times to allow for a thorough statistical examination of the data compared to the qualitative findings presented here. Furthermore, the lysis of the 3T3L1 adipocytes was performed with a slightly different buffer, which may have altered the efficiency of CAP extraction between the two cell types although the total protein assay would have partially controlled for this.

Nevertheless, this is the first reported measurement of CAP protein expression in skeletal muscle, although CAP protein has previously been reported to be abundant in adipose tissue of the same animals (Ribon et al., 1998). Further investigation comparing CAP protein abundance between adipose tissue and skeletal muscle would help to explain the role of the c-Cbl pathway in insulin responsive tissue. It has been shown previously that a lack of CAP protein expression in 3T3L1 fibroblasts is associated with the lack of c-Cbl tyrosine phosphorylation in response to insulin stimulation (Ribon et al., 1998). However the qualitative findings of the present study suggest that CAP may not be an abundant protein in skeletal muscle and this may explain why there is no tyrosine phosphorylation of c-Cbl in this tissue in response to insulin stimulation.

**6.5 Conclusion**

In summary, this is the first study to report that c-Cbl is a readily abundant protein in both healthy and insulin resistant rodent skeletal muscle. The tyrosine phosphorylation of c-Cbl in response to exercise or insulin treatment was undetectable using the protocols employed in the present study despite detecting both immunoprecipitated c-Cbl protein and the tyrosine phosphorylation of the IR in rodent skeletal muscle. Therefore, unlike 3T3L1 adipocytes, 5 minutes of insulin stimulation in rodent skeletal
muscle does not appear to tyrosine phosphorylate c-Cbl. Furthermore, exercise does not seem to have an effect on either c-Cbl protein abundance or tyrosine phosphorylation but there was however a small increase in protein abundance following supraphysiological insulin treatment. Based on the limited findings of the present study, it appears as if CAP protein is much more readily expressed in 3T3L1 adipocytes compared to skeletal muscle. Insufficient abundance of CAP protein in skeletal muscle may explain why insulin stimulation does not tyrosine phosphorylate c-Cbl in this tissue as it has been shown previously by others that CAP protein expression is necessary for the insulin stimulated tyrosine phosphorylation of c-Cbl. Finally, insulin resistant states such as obesity do not appear to result in differential expression of c-Cbl protein in the Zucker rat, which may suggest that it is not a defect associated with insulin resistance in skeletal muscle.
CHAPTER SEVEN

CONCLUSION

7.1 Conclusions

Regular physical activity is an effective therapy for the treatment and prevention of type 2 diabetes. Importantly, physical activity improves insulin sensitivity although there is a paucity of data examining the intracellular mechanisms that account for this improved insulin action that proceed greater muscular contractile activity. Understanding how exercise improves insulin action will not only improve diabetic care and management, but also lead to greater insight into future effective therapies for the prevention and treatment of this and other metabolic diseases. This thesis investigated several aspects involved in the regulation of the insulin-signalling cascade by exercise and sought to clarify the benefits of exercise training on improved insulin action. The thesis also sought to investigate if a novel insulin signalling pathway that has been reported in adipocytes was also involved in the insulin signalling of skeletal muscle.

The residual effect of the last bout of exercise alone has often been attributed to the observed improvements in insulin action following endurance training. A finding of this thesis was that 24 hours following a single bout of endurance exercise insulin action was improved although larger improvements in insulin action were found following the completion of endurance training. This would imply that increased insulin action is not only due to the residual effects of the prior bout of exercise, but that cumulative bouts of exercise provide further enhancement of this response.
Little was previously known of the transcriptional regulation of key insulin signalling proteins by exercise. This thesis found a significant transient increase in IRS-2 mRNA concentration in the few hours following a single bout of both endurance and resistance exercise. While endurance and resistance exercise is not directly comparable, the data from this thesis demonstrates that resistance exercise acts to elicit some alterations in some of the insulin signalling genes. The recent findings of increased insulin action following resistance training (Dunstan et al., 2002) suggest the involvement of as yet unknown mechanisms. The gene expression of the p85α subunit of PI 3-kinase also increased significantly in the few hours following a single bout of endurance but not resistance exercise. This effect was attenuated following several days of endurance training. A single bout of exercise or short-term endurance training also failed to alter the gene expression of either the IR or IRS-1 genes providing little evidence of sustained and substantial alterations in the gene expression of most of the key proteins of the insulin-signalling pathway. Surprisingly, IRS-2 protein abundance was however observed to significantly increase 24 hrs following a single bout of endurance exercise which then decreased back to sedentary levels following short term endurance training indicating transcriptional regulation of this protein via intense muscular contraction. IRS-2 protein abundance was also observed to increase significantly in the sedentary state following prolonged insulin stimulation. The transient nature of this regulation following prolonged insulin stimulation or a single bout of exercise but not cumulative bouts of exercise indicates an early adaptive role for IRS-2 in both insulin stimulated glucose uptake and mitogenesis. Recent work has focussed on the unique actions and roles for IRS-2 in skeletal muscle (Chibalin et al., 2000; Howlett et al., 2002) although the exact physiological function is still to be determined.
This thesis found that there was no effect of a single bout of exercise or short-term endurance training on proximal components of the insulin-signalling cascade such as IR and IRS-1 protein expression or tyrosine phosphorylation. However a major finding of this thesis was that there was an observed increase in the serine phosphorylation of a known downstream signalling protein, Akt, following both an acute bout of exercise and with exercise training. This finding suggests a role for Akt in the upregulation of glucose transport following exercise. However, Akt has been shown by others to have multiple signalling roles, including glycogen synthesis (Cross et al., 1995; Cross et al., 1997; Ueki et al., 1998) and mitogenic signalling (Hajduch et al., 1998; Ueki et al., 1998) and the relative contribution of Akt to all these pathways following exercise cannot be determined from the present study.

Protein tyrosine phosphatases (PTPases) are a diverse family of enzymes that have generated considerable interest as novel targets for the treatment of type 2 diabetes and are currently regarded as key regulators of the insulin-signalling cascade. Readily abundant PTPases in skeletal muscle and adipocytes each display characteristic subcellular distribution between the cytosol and particulate fractions (Ahmad & Goldstein, 1995; Calera et al., 2000) and are therefore likely to have preferential sites of action throughout the insulin-signalling cascade. The major finding of this thesis was that exercise selectively increases the protein expression of SHPTP2 in human skeletal muscle. This is the first study to report an increase in a positive regulator of insulin signalling \textit{in vivo} following exercise in humans. The increase in SHPTP2 protein abundance at a major site of PTPase activity, the cytosolic fraction, in skeletal muscle following exercise was associated with an increase in pAkt (Ser473); an enzyme that is activated downstream of PI 3-kinase by insulin. This finding has important
implications in the search for alternative therapies to improve insulin sensitivity in patients with insulin resistance. Inhibiting SHPTP2 in rat adipocytes and transgenic mice also reduces insulin-stimulated glucose uptake, translocation of GLUT4 and impairs activation of IRS-1, PI 3-kinase and Akt-1 (Chen et al., 1997; Maegawa et al., 1999). If increased SHPTP2 protein abundance is directly involved in insulin-stimulated glucose transport then it provides another target for the pharmacological treatment of type 2 diabetes. And even if SHPTP2 is not directly responsible for improved insulin-stimulated glucose transport following exercise then it at least highlights a poorly described section of the insulin signalling cascade potentially involved in the upregulation of insulin sensitivity by exercise. There was however, no observed improvement in IRS-1 tyrosine phosphorylation following exercise, which is reported to be a major target for the action of SHPTP2 suggesting that it may also be involved in other actions such as mitogenesis and glycogen synthesis (Maegawa et al., 1999; Ugi et al., 1996) although there is no direct evidence for this in the present study. It is possible that SHPTP2 is mediating its effects on insulin-stimulated glucose transport independently of IRS-1. It was discussed in chapter 4 that SHPTP2 could be signalling to downstream components of the cascade, such as Akt, via other tyrosine kinases like Src, although the evidence for this is far from conclusive. The protein expression of another prominent PTPase, PTP1B, was not altered by acute exercise or exercise training in either the particulate or cytosolic fractions, nor was the insulin-stimulated tyrosine phosphorylation of the IR, which is one of its main targets for dephosphorylation. This would suggest that any improvements in insulin action via exercise are not mediated by PTP1B and do not involve alterations in the abundance and tyrosine phosphorylation of proximal members of the insulin signalling such as IR and IRS-1.
A novel insulin-signalling pathway involving c-Cbl tyrosine phosphorylation is essential for insulin stimulated glucose transport in adipocytes although the role of this pathway in skeletal muscle, the primary site for glucose disposal, is unknown. This thesis found no evidence of tyrosine phosphorylation of c-Cbl in the skeletal muscle of Zucker rats by insulin treatment or exercise despite demonstrating significant phosphorylation of the insulin receptor and Akt by insulin treatment and successful immunoprecipitation of c-Cbl protein. There was also no differential expression of c-Cbl protein between lean and obese Zucker rats, suggesting that c-Cbl tyrosine phosphorylation is not associated with insulin or exercise-mediated stimulation of glucose transport in skeletal muscle nor does it participate in insulin resistance in skeletal muscle. Surprisingly, there was a small but significant increase in c-Cbl protein expression following insulin-stimulation. It is possible in skeletal muscle that the small increase in c-Cbl protein observed following insulin treatment could result from release of c-Cbl from the detergent-insoluble fraction following insulin stimulation, as the whole-cell lysis method employed in the present study would not extract c-Cbl from the detergent-insoluble fraction of the cell.

In summary, this thesis evaluated several key aspects of insulin signalling in human skeletal muscle. This work provided new insights into the insulin signalling cascade and response to exercise. Cumulative bouts of exercise were found to increase insulin action above that of a single bout, debunking the commonly held belief that insulin sensitivity is primarily due to the residual effects of a single bout of prior exercise. The thesis found that there is a transient upregulation of IRS-2 gene expression and protein
abundance following acute exercise indicating a role for this protein in insulin-stimulated glucose transport and mitogenesis. There was no observed increase in the protein expression or tyrosine phosphorylation of IR and IRS-1 following acute exercise or short-term training. This would suggest that proximal members of the insulin-signalling cascade such as IR and IRS-1 are not involved in the enhanced insulin-stimulated glucose uptake observed following exercise in human skeletal muscle. A major finding of this thesis was the observed increase in serine phosphorylation of a known downstream signalling protein, Akt, in human skeletal muscle following an acute bout of exercise and exercise training. It is unclear just how exercise is able to increase pAkt (Ser473) however it does suggest that downstream components of the insulin signalling cascade may be involved in enhanced insulin-stimulated glucose uptake. The thesis found that a poorly described section of the insulin signalling cascade involving SHPTP2 in the cytosolic fraction of the cell is increased following exercise. It is unclear if the increased cytosolic SHPTP2 protein abundance is directly related to observed increase in pAkt (Ser473) following exercise although the findings from this thesis warrant further investigation into this area. This thesis also found that a novel insulin-signalling pathway involving c-Cbl tyrosine phosphorylation is not involved with insulin or exercise mediated stimulation of glucose transport in rodent skeletal muscle, although insulin treatment may be involved in c-Cbl translocation.

### 7.2 Future Directions

There are many unresolved issues surrounding the actions of acute and repeated exercise on insulin action. Emerging insights into the control, regulation and downstream signalling components of the insulin-signalling cascade will make this an interesting area of on-going research. The results obtained from the present study
indicate that many unresolved issues remain with respect to some of the aspects of the insulin-signalling cascade and the impact of exercise on insulin signalling.

1. The present investigation observed an increase in SHPTP2 protein abundance and pAkt (Ser473) following acute exercise and exercise training. It is unclear based on the present findings if these changes are directly related. As mentioned earlier, transgenic mouse models involving SHPTP2 inhibition result in decreased Akt activity and insulin-stimulated glucose transport. However a direct link between SHPTP2 and Akt has yet to be established, although as mentioned in Chapter 4 there is some indirect evidence suggesting SHPTP2 activates Akt via the tyrosine kinase Src following EGF stimulation. It is unknown if Src is activated following muscular contraction or if there is an increased association of Src with SHPTP2 following exercise. It is also unknown if this increased association then increases the binding of Src with the regulatory region of Akt, which subsequently increases Akt activation or phosphorylation. Further work would also be required to show the interaction of this pathway following insulin stimulation.

2. The finding that SHPTP2 protein abundance and pAkt (Ser473) are increased by a single bout of endurance exercise and endurance training with concomitant improvements in whole body insulin action is not sufficient to conclude that these intracellular changes are directly involved in improved insulin-stimulated glucose transport. Both SHPTP2 and Akt have been implicated in insulin stimulated glucose transport, glycogen synthesis and mitogenic signalling (Brozinick Jr. & Birnbaum, 1998; Cross et al., 1995; Cross et al., 1997;
Maegawa et al., 1999; Ueki et al., 1998; Ugi et al., 1996). It is possible that upregulation of SHPTP2 protein abundance and pAKT (Ser473) are involved in a combination of these pathways in response to exercise. Further research needs to measure changes in insulin-stimulated glycogen synthase, MAP kinase and p70 S6 kinase activity following acute exercise and short-term training. This would help to establish if an upregulation of these pathways are mediated by increased cytosolic SHPTP2 protein abundance and/or Akt activity/phosphorylation.

3. Two of the predominant PTPases in skeletal muscle were investigated for their role in the regulation of insulin signalling by exercise. There are a number of other PTPases thought to be involved in the regulation of insulin signalling that were not investigated due to time and financial constraints. The PTPase LAR is a highly expressed in skeletal muscle and thought to play a major role in the regulation of the insulin receptor. It is unlikely that LAR is involved in the regulation of insulin signalling by exercise since no changes were observed in the insulin-stimulated tyrosine phosphorylation of the insulin receptor following acute exercise or exercise training. Furthermore, lipid phosphatases such as PTEN and SHIP2 that were briefly mentioned in Chapter 1 are thought to act downstream of IRS-1 and may potentially be involved in the regulation of insulin signalling by exercise.

4. Although the insulin-signalling pathway proximal to PI 3-kinase is well described, the hunt is on worldwide to discover the downstream components of the insulin-signalling cascade that ultimately lead to GLUT4 translocation and
glucose uptake. It has been discussed briefly in Chapter 1 that PDK-1, Akt and the atypical PKC’s (PKCλ and PKCζ) are all thought to be involved in insulin-stimulated GLUT4 translocation. The upregulation of insulin stimulated glucose uptake by exercise could very well occur in the insulin signalling pathway distal to PI 3-kinase as this thesis found in support of the majority of previously published work no evidence for improvements in the proximal section of the pathway involving the insulin receptor or IRS-1. There is good evidence as discussed in Chapter 1, that Akt and atypical PKC activation is downregulated in insulin resistant muscle brought about by fatty acid infusion or high fat feeding (Kim et al., 2002; Tremblay et al., 2001). Future research should examine if exercise induced improvements in insulin stimulated glucose uptake occur via upregulation of these atypical PKC’s. There is also some recent evidence that insulin-stimulated p38 MAPK is upregulated in the few hours following endurance exercise in humans (Thong et al., 2003), suggesting a role for this pathway in the exercise induced improvement of insulin sensitivity.

5. It has been found that in the few hours following resistance and endurance exercise, there is a transient increase in IRS-2 mRNA concentration. The resistance exercise study is limited in its findings as subjects were fed a small meal immediately after exercise, which may have had an effect on the gene expression of IRS-2. Controlling for food intake on IRS-2 gene expression after exercise therefore warrants further investigation. However gene expression of IRS-1 and the p85α subunit of PI 3-kinase did not change during this period and there were no changes in IRS-2 gene expression in the longer term. Also, similar transient increases were observed following endurance exercise despite no food
being consumed. Therefore the results of the present study do justify increasing the scope of the investigation to determine if resistance exercise is able to increase IRS-2 protein content in the 24 hour period post exercise, and if IRS-2 associated PI 3-kinase activity is also improved during this period, which may in part explain small increases in insulin sensitivity observed following resistance exercise. As discussed earlier, IRS-2 potentially plays a role in insulin-stimulated glucose uptake and muscle fibre growth in skeletal muscle (Howlett et al., 2002; Kido et al., 2000; Kubota et al., 2000; Rondinone et al., 1997; Withers et al., 1999) although its exact role in glucose uptake and mitogenesis following acute exercise is still unclear. The finding that IRS-2 gene expression is upregulated following a single bout of endurance and resistance exercise may point towards an early adaptive role for IRS-2 in the combined actions of glucose uptake and mitogenesis following very different types of muscle contraction. Further study investigating IRS-2 protein abundance and basal and insulin-stimulated tyrosine phosphorylation following acute resistance and endurance exercise will help to resolve the physiological role/s of IRS-2 following intense muscular contraction.

6. Although it appears that insulin or exercise do not tyrosine phosphorylate c-Cbl in skeletal muscle there does appear to be tissue specific role for c-Cbl in insulin stimulated glucose transport. A study examining if insulin stimulates tyrosine phosphorylation of c-Cbl in adipose tissue but not skeletal muscle of rodents is required to confirm this. CAP expression appears to be crucial in the tyrosine phosphorylation and subsequent recruitment of c-Cbl to the insulin receptor and translocation to lipid rafts. Preliminary evidence was presented demonstrating
that CAP is expressed more abundantly in 3T3L1 adipocytes compared to skeletal muscle. Therefore the relative abundance of CAP in both skeletal muscle and adipose tissue of rodents would help to explain any tissue specific differences that may exist for this pathway.

7.3 Concluding statement

Many grey and unknown areas exist in the hunt to determine the cellular basis for the beneficial actions of exercise on insulin action. This research area will undoubtedly provide many key insights into the malfunction in insulin resistant states. However, the immutable fact remains that even in young and relatively fit male subjects, as investigated in the current study, exercise is a necessary and central component involved in the optimal regulation of insulin signalling transduction.
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