INSULIN SIGNALLING CAN REGULATE GLUCOSE TRANSPORT IN MUSCLE INDEPENDENTLY OF CHANGES IN TOTAL GLUT-4: EFFECTS OF MUSCLE ACTIVITY AND HIGH-FAT-FEEDING ON THE INSULIN SIGNAL AKT

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Kinesiology

Waterloo, Ontario, Canada, 2000

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Abstract

INSULIN SIGNALLING CAN REGULATE GLUCOSE TRANSPORT IN MUSCLE INDEPENDENTLY OF CHANGES IN TOTAL GLUT-4: EFFECTS OF MUSCLE ACTIVITY AND HIGH-FAT-FEEDING ON THE INSULIN SIGNAL AKT

Physical activity and dietary modification are known to alter insulin-stimulated glucose transport in muscle, in part, by changing the GLUT-4 content of skeletal muscle. The studies in this thesis indicate that total muscle GLUT-4 availability alone does not dictate insulin responsiveness, but that Akt is also involved.

To investigate the impact of varied fatty acid composition of high fat diets on glucose tolerance, rats were provided for 3-4 weeks with either a high safflower oil diet (HF-SAFF), a high mixed oil diet (HF-MIXED) or a low fat diet (LF). Diets differing in fatty acid composition had different effects on whole body glucose disposal, glucose transport into muscle, and glucose transport into adipose tissue. Feeding rats the HF-SAFF diet decreased 2-deoxyglucose (2-DG) uptake in isolated adipocytes compared with HF-MIXED-fed rats and LF-fed rats ($P<0.05$). In contrast, 2-DG uptake by adipocytes from HF-MIXED-fed rats did not differ from LF-fed rats ($P>0.05$). Insulin-stimulated, 3-O-methylglucose (3-O-MG) uptake into oxidative, mixed, and glycolytic muscles was reduced in HF-SAFF ($P<0.02$) and HF-MIXED-fed rats ($P<0.02$), while under basal conditions, 3-O-MG uptake was increased in HF-SAFF and HF-MIXED-fed rats relative to LF-fed rats ($P<0.04$). Glucose tolerance, assessed by an intravenous glucose tolerance test, was reduced in HF-SAFF-fed rats ($P<0.05$), but was unaffected by the HF-MIXED diet ($P>0.05$), compared with the LF diet. These high fat diets, which differ in fatty acid composition, differentially affected skeletal muscle and adipose tissue insulin responsiveness.

Insulin signalling at the level of Akt was examined in muscle made insulin resistant by short-term (24 hours) denervation. Insulin-stimulated glucose transport *in vitro* was reduced by 28% ($P<0.05$) in denervated muscle (DEN). In control muscle (SHAM), insulin increased levels of surface detectable GLUT-4 (i.e. translocated GLUT-4) 1.8-fold ($P<0.05$) while DEN surface GLUT-4 was not increased by insulin ($P>0.05$). Insulin treatment *in vivo* induced a rapid appearance of phospho[SER ^{473}]Akt-1 in SHAM, 3 minutes after insulin injection. In DEN, phospho[SER ^{473}] Akt-1 also appeared at 3 minutes, but SER ^{473} phosphorylated Akt-1 was 36 % lower than in SHAM ($P<0.05$). In addition, total Akt-1protein in DEN was 37% lower than in SHAM ($P<0.05$). Akt-1 kinase activity was lower in DEN at two insulin levels tested; 0.1U insulin / rat (-22%, $P<0.05$) and 1U insulin / rat (-26%, $P<0.01$). These studies indicate Akt-1 is important for increasing surface available GLUT-4. This suggests that the insulin signal Akt is defective in 24 hour denervated rat muscle.

Insulin signalling at the level of Akt was examined in muscle from high-fat-fed rats to determine whether Akt-derived signals might influence glucose transport when insulin resistance is induced by high-fat-feeding. Unlike denervated muscle, in which insulin-induced Akt-1activation was impaired, the alterations in Akt activation observed in muscle from high-fat-fed rats did not explain the insulin resistance induced by high-fat-feeding. Instead, insulin stimulated a greater increase in Akt-1 kinase activity in muscle from HF-SAFF-fed (+61%) and HF-MIXED-fed (+44%) rats than in muscle from LF-fed rats ($P<0.05$). Insulin-induced Akt-2 activation was similar in muscle from all 3 groups of rats

(P>0.05). GLUT-1 levels were found to be 80% higher in muscle of HF-SAFF-fed and HF-MIXED-fed rats than in muscle from LF-fed rats (P<0.05). Basal 2-deoxyglycose glucose (2-DG) transport was also higher in muscle from both groups of high-fat-fed rats (P<0.05). High-fat diets may have affected basal glucose uptake differently since PI3-kinase inhibition lowered glucose uptake well below basal levels in HF-SAFF but not HF-MIXED. These studies suggest that insulin-activatable signals, which could include Akt, help to upregulate basal glucose uptake. This may occur in a variety of ways, including promotion of GLUT-1 biosynthesis.

The final studies determined whether fiber type differences in Akt activation exist in chronically-active skeletal muscles. It is well known that fast twitch glycolytic (FG) muscle has less GLUT-4 and exhibits lower rates of maximal insulin-stimulated glucose transport than slow twitch oxidative (SO) muscle. Chronic low frequency stimulation (CLFS) for 360 minutes / day (7 days) induced a disproportionately higher amount of GLUT-4 protein expression in white tibialis anterior muscle (WTA) than in red tibialis anterior (RTA) muscle. Thus, with 360 minutes / day of CLFS, levels of GLUT-4 in chronically-active RTA and chronically-active WTA were found to be equivalent. However, unlike total GLUT-4, maximal insulin-stimulated 3-O-methylglucose (3-OMG) transport rates were not equalized by 360 minutes / day CLFS. Insulin-stimulated glucose transport was about 2-fold greater in chronically-active RTA muscle than in chronically-active WTA muscle, similar to the normal differences in insulin-stimulated 3-OMG transport rates observed between non-active (control) red and white TA muscles. Insulin-stimulated Akt-1 kinase activity in chronically-active RTA was significantly higher (+92%)

than in control RTA ($P<0.01$). Conversely, insulin-stimulated Akt activation was 37% lower in chronically-active WTA than control WTA ($P<0.01$), possibly suggesting that chronic low frequency stimulation suppressed the normal Akt activation response to insulin in this muscle group. These studies could suggest Akt in the red and white portions of the TA muscle are differentially affected by chronic muscle activity. Akt may represent an important insulin signal that is modulated, in conjunction with an increased quantity of GLUT-4, to control the upregulation of insulin-stimulated glucose transport in chronically-active muscles.

The studies in this thesis support a role for Akt in insulin-stimulated glucose transport. Collectively, the studies in this thesis indicate that Akt contributes to glucose transport activity and has an important role when muscle insulin responsiveness is altered by a change in muscle activity or the consumption of a high-fat diet.

Acknowledgements

I am extremely appreciative of all the help and support I received. Assistance by others in the lab was invaluable. Special thanks to Sylvia Rodriguez, Yoga Arumugam, Rhonda Bell, Don M^{c}Cutcheon and Xioa Xia Han. I am indebted to them for their help with animal care and surgery. Completion of this project in ample time would have been an impossible task without them around.

Thanks to both Joost Luiken and YuXiang Wang for untimely words of wisdom! These were always welcomed and greatly appreciated.

Russ, here's to our time through the PhD program together... cheers!

Rhonda Bell, your enthusiasm for taking trips and making friends was refreshing! Hats off to an engaging scientific discussion on a long road trip from Boston.

A much deserved thank you to Arend Bonen who always directed me with an endpoint in mind. This was most appreciated when I was 'snagged' along the way.

Dedication

This thesis dissertation is dedicated to all the friends and family who knew how to find me, and always did, while I was playing around in my own little world. Thanks for the reality check Mum, Dad, Julie, Eric, Mark, Jeff, Scott, Rick, Shawn, and Jennifer.

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2-DG	2-deoxyglucose
3-OMG	3-O-methylglucose
ATP	adenosine triphosphate
cAMP	cyclic adenosine 3', 5'-monophosphate
DAG	diacylglycerol
DTT	dithiothreitol
g	gram
h	hour
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HF-SAFF	high safflower oil diet
HF-MIXED	high mixed oil diet
HRP	horse radish peroxidase
Hz	Hertz
i.p.	intraperitoneal
IR	insulin receptor
IRS	insulin receptor substrate
IVGTT	intravenous glucose tolerance test
kcal	kilocalories
kg	kilogram
Kg	glucose tolerance

KHB	Krebs Henseleit buffer
LF	low fat
mCi	milliCurie
mg	milligram
$MgCl_2$	magnesium chloride
mL	milliliter
n-3	omega-3
NaCl	sodium chloride
NFDM	non-fat-dry-milk
PDK	phosphatidylinositol-dependent kinase
$PI(3,4,)P_2$	phosphatidylinositol 3,4, diphosphate
PI3-kinase	phosphatidylinositol 3'-kinase
rac (kinase)	related to A and C (kinase)
RIA	radioimmunoassay
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TBS	tris-buffered saline
U	international unit

Chapter 1:

I. INTRODUCTION

Since the discovery of insulin by Banting and Best in 1922 (Banting and Best, 1922) our understanding of how insulin stimulates the uptake of glucose into key target tissues has expanded immensely. For some time, exercise has been known to improve glycemic control by enhancing insulin sensitivity in peripheral tissues such as muscle. In contrast, poor lifestyle-related choices such as physical inactivity or inadequate nutritional habits (i.e. high fat diets) may be detrimental to insulin sensitivity. A plethora of studies show that physical activity and dietary modification can alter insulin sensitivity by changing the concentration of the insulin-regulatable glucose transporter, GLUT-4, in skeletal muscle (Brozinick Jr. et al., 1993; Etgen et al., 1993; Friedman et al., 1990). Yet, recent studies seem to suggest that total muscle GLUT-4 availability alone does not dictate insulin responsiveness, but that an intact and adaptable insulin signalling system may also be vital for altering insulin-stimulated glucose transport activity (Koval et al., 1999).

In a series of studies, the signalling protein Akt was examined under different physiological stresses, since increases in Akt activity generated by insulin stimulation appear to be involved in increasing the quantity of cell surface glucose transporters. Studies in cell culture have inferred that Akt provides signals for glucose transport by inducing the translocation of GLUT-4 glucose transporters (Kohn et al., 1996; Hajduch et al., 1998) and by promoting GLUT-1 biosynthesis (Hajduch et al., 1998). Recent studies in adipose tissue (Tanti et al., 1997; Kupriyanova and Kandor, 1999) have also implicated Akt to be central to insulin-mediated glucose utilization by upregulating surface availability of glucose transporter proteins. Therefore, the focus of this thesis has been to

examine the effects of a) chronically altered muscle activity and b) high-fat diets on insulin-induced Akt activity in skeletal muscle.

In order to place the work in this thesis in perspective a general overview of insulin-regulated glucose transport, glucose transporters and insulin signalling is provided below.

II. GLUCOSE TRANSPORTERS

The model system first showing glucose transporters translocate: adipose cells

Insulin-stimulated glucose transport was demonstrated many years ago *in vitro* in adipocytes (Wardzala et al., 1978), skeletal muscle (Holloszy and Narahara, 1965), and cardiac muscle (Zaninetti et al., 1988). But, much of our understanding behind the mechanisms involved in insulin-stimulated glucose transport originated from the use of isolated white adipocytes from rats. Cushman and Wardzala (1980) demonstrated that the increase in glucose transport after stimulation with insulin is due to an increase in the number of transporter systems accessible to glucose. They showed that the fungal metabolite cytocholasin B will bind to, and inhibit, the activity of a proposed membrane bound protein required for glucose transport. The fractionation and isolation of plasma membranes and intracellular membranes revealed a distribution of labeled-cytocholasin B binding sites between these two membrane pools. Upon insulin stimulation, there was a five-fold increase in the number of glucose transport proteins in the plasma membrane fraction and a proportionate loss of transporters from the intracellular membrane fraction (Cushman and Wardzala, 1980).

At the same time, another research group independently proposed the same hypothesis: namely that insulin increases the number of transporter systems accessible to

glucose in the plasma membrane (Suzuki and Kono, 1980). Suzuki and Kono (1980) developed a method of reconstituting plasma membrane and intracellular membrane vesicles marked with Golgi enzymes for measurements of glucose transport activity. They showed that when isolated adipocytes were stimulated with insulin, before the membrane vesicles were prepared, glucose transport activity increased in plasma membrane-rich vesicles and decreased in the Golgi-rich fraction of vesicles (Suzuki and Kono, 1980).

These experiments, conducted by the research groups of Cushman and Suzuki, were the first to outline the manner by which glucose transporters are made accessible to extracellular glucose in insulin-stimulated cells. These two separate research groups postulated simultaneously, but independently, that insulin increases the translocation of glucose transporters from an intracellular pool to the cell surface.

III. A FAMILY OF GLUCOSE TRANSPORTERS FACILITATE GLUCOSE TRANSPORT

Glucose transport is now known to be regulated by a family of glucose transporter proteins (Bell et al., 1990; Mueckler, 1994) (Table 1). GLUT-1 was the first transporter purified and partially sequenced from human erythrocytes, leading to the isolation of cDNA clones from human HEPG2 cells and rat brain (Mueckler et al., 1985; Birnbaum et al., 1986). Soon after, screening of cDNA libraries for clones that cross-hybridized with GLUT-1 followed, and other glucose transporter isoforms were subsequently discovered. Because of the high degree of similarity among isoforms, a single structure and topology for the glucose transporter protein was proposed based on the GLUT-1 sequence (Mueckler, 1993). It consisted of 12 membrane spanning α-helical segments, an intracellular loop connecting transmembrane sequences 6 and 7 which divide the structure in half, a large exofacial loop, and intracellular NH- and COOH- termini.

Table 1.1[1] Tissue-specific expression of glucose transporters

ISOFORM	TISSUE SPECIFIC EXPRESSION	PHYSIOLOGICAL ROLE
GLUT-1 (K_m=2mM)	Erythrocytes, placenta, brain, nervous tissue, muscle and adipose tissues	basal transporter in various tissues
GLUT-2 (K_m=11mM)	Liver, kidney, pancreatic cells, small intestine (basolateral membrane)	low affinity transporter expressed in tissues that handle large quantities of glucose (i.e. glucose efflux from the liver during gluconeogenesis)
GLUT-3 (K_m=1.4mM)	Brain parenchymal cells, nerve cells, muscle cells	scavenges glucose when plasma levels are low
GLUT-4 (K_m=5mM)	Skeletal muscle, heart, adipose tissue	glucose transporter regulated by insulin
GLUT-5 (K_m=N/A)	Small intestine (lumen), human skeletal muscle	fructose transporter
GLUT-6 (K_m=N/A)	N/A	N/A
GLUT-8	Human skeletal muscle, heart, small intestine, brain	unknown

[1] adapted from Gould and Holman, 1993 and modified.

[2] K_m defined as the glucose concentration at half maximal glucose uptake rate.

GLUT-4 is often referred to as the insulin-regulatable glucose transporter. Insulin has been shown to induce GLUT-4 translocation in adipocytes (Cushman and Wardzala, 1980; Suzuki and Kono, 1980), skeletal (Klip et al., 1987; Hirshman et al., 1990) and heart muscle (Egert et al., 1999). Somewhat after finding GLUT-4 translocated by insulin in resting muscle, it was found that GLUT-4 was also translocated in contracting skeletal muscle (Douen et al., 1990; Goodyear et al., 1991; Ploug et al., 1992). This occurred without activating levels of insulin present. More recently, it was established that there are distinct pools of intracellular GLUT-4. These separate GLUT-4 pools may be sensitive to the stimulus provided by either insulin or contraction (Lemieux et al., 2000). Thus, it appears that GLUT-4 is the important glucose transporter for providing glucose to both working and resting muscles.

Recently, GLUT-5 a fructose transporter (Burant et al., 1992), GLUT-6 (a pseudogene not expressing protein) and GLUT-8 (Doege et al., 2000) have also been identified. Although the proteins GLUT-1 to GLUT-8 are highly conserved in amino acid sequence (39-65% homology), each one displays a tissue-specific expression pattern and has distinct catalytic activities which allows these key glucose transporter proteins to contribute uniquely to whole body glucose homeostasis.

In some tissues, several GLUTs are co-expressed. Muscle expresses both GLUT-1 and GLUT-4. In skeletal muscle, the K_m of GLUT-4 has been estimated at ~ 5 mM and the K_m for GLUT-1 at ~1.4 mM. Based on these values, the optimal catalytic efficiency of GLUT-1 has been predicted to occur at a concentration of glucose lower than the normal blood glucose levels of approximately 4 - 6 mM. This would appear to make GLUT-1 more suited than GLUT-4 for glucose transport activity when blood glucose levels are very low. GLUT-1 levels in muscle are very low (Klip and Marette, 1992). GLUT-4 appears to operate more effectively at higher glucose levels, therefore an increase in the

membrane abundance of GLUT-4 transporters can rapidly provide muscles with glucose. The increase in cell surface GLUT-4 concentrations, after GLUT-4 translocation, lowers blood glucose rapidly in the post-prandial state. To date, it is generally believed that the confinement of GLUT-1 to surface membranes mediates basal glucose transport, and that the translocation of GLUT-4 is predominantly responsible for insulin-stimulated and contraction-stimulated glucose transport in skeletal muscle.

GLUT-4 translocation has been the dogmatic model for insulin-stimulated glucose transport regulation in skeletal muscle ever since antibodies raised against the glucose transporter proteins were used to verify that membrane availability of GLUT-1 and GLUT-4 fulfill the roles of basal glucose transport and insulin-regulatable glucose transport, respectively. However, glucose transporter catalytic activity might also be altered by insulin. James et al. showed that a 10-fold increase in plasma membrane associated GLUT-4 (James et al., 1988) was induced by insulin, but this did not correspond with the 15-30 fold increase in radio-labeled glucose transport (James et al., 1986; Toyoda et al., 1987). Others proposed that insulin augments the catalytic activity of individual transporters by increasing the affinity of glucose transporters for glucose (Jhun et al., 1992; Suzuki, 1988). Phosphorylation of plasma membrane GLUT-4 in adipocytes has been shown to decrease the transporter's intrinsic activity in this cell type. GLUT-4 activity is restored with dephosphorylation (Reusch et al., 1993). Thus, an "intrinsic activation" hypothesis has also emerged.

New techniques with antibodies raised against glucose transporters and photolabels that attach to glucose transporters have enabled a more detailed analysis of glucose transporter recycling events. Investigators have been able to elucidate the relative changes in GLUT-1 and GLUT-4 in the plasma membrane as well as being able to determinine the rates of appearance and loss of glucose transporters at the cell surface

(Clark et al., 1991; Satoh et al., 1993; Lund et al., 1993; Reynolds et al., 1997). Satoh used a non-permeable bismannose photolabel to mark and track GLUT-4 recycling events and concluded that insulin up-regulates protein trafficking events so that the rate of GLUT-4 exocytosis increases while the rate of GLUT-4 endocytosis remains unchanged (Satoh et al., 1993).

IV. INSULIN SIGNALLING ACTIVATES GLUCOSE TRANSPORT

Insulin and the Insulin Receptor

Insulin is a hormone secreted from β-cells of the pancreas when blood glucose levels increase. Insulin lowers the blood glucose concentration by stimulating glucose uptake. The receptor for insulin is a transmembrane protein tyrosine kinase that is upregulated by insulin binding. Two α-subunits are covalently linked with two β -subunits so that the protein spans the plasma membrane. Insulin binding to a region within the α-subunits activates tyrosine kinase activity on the β-subunits resulting in autophosphorylation of tyrosine residues within this intracellular region of the receptor (Lee and Pilch, 1994). Autophosphorylation and transphosphorylation provide an effective means of receptor activity amplification (Avruch et al., 1990).

Post-insulin receptor signalling

Although many studies have demonstrated that the increase in glucose transport in response to insulin corresponds to increases in insulin binding to its receptor, it has been suggested that full stimulation of glucose transport occurs with only 10-20% of the insulin receptors occupied by the hormone (Le Marchand-Brustel et al., 1978; Simpson and Cushman, 1986). Tyrosine kinase activity is essential for insulin-mediated signal transduction. However, only 14% of receptor tyrosine kinase activity is required for maximal glucose transport response in adipocytes (Klein et al., 1991). The "spare

receptors" likely provide the insulin stimulus for functions other than glucose transport activation, possibly related to insulin's mitogenic properties which promote protein synthesis for growth and development (Simpson and Cushman, 1986). It is likely that direction of the insulin signal for glucose transport activation occurs downstream of insulin receptor tyrosine kinase activity. Some principal substrates for the insulin receptor's tyrosine kinase activity have been identified.

Insulin receptor substrates

A family of insulin receptor substrate (IRS) proteins exists. The first known was denoted IRS-1(Sun et al., 1991); subsequently the discoveries of IRS-2, Grb2-associated binder-1(Gab-1), and IRS-3 soon followed (Sun et al., 1995; Holgado-Madruga et al., 1996; Lavan et al., 1997). IRS-1 (depicted in Fig. 1.1) has been shown to have a role in signalling to increase glucose transport (Kanai et al., 1993; Okada et al., 1994). Depression of phosphorylated IRS-1 levels in 3T3-L1 adipocytes correlates with a decrease in insulin-stimulated glucose transport (Rice and Garner, 1994) suggesting that the active protein involved in signalling glucose transport is the phosphorylated form. The IRS-1 contains a Tyrosine-X-X-Methionine consensus sequence (Sun et al., 1991). Phosphorylation on tyrosine (Y) residue within YXXM motifs allow IRS-1 to associate with *src*-homologous-(SH2) containing signalling proteins (Sun et al., 1991).

Phosphatidylinositol-3'-kinase

IRS-1 couples insulin-stimulated IR activity to the activation of phosphatidylinositol 3'-kinase (PI3-kinase). The 85 kDa PI3-kinase subunit (p85) contains the necessary SH-2 domain (Carvalho, 1996) to allow an IRS-1–PI3-kinase signalling complex to form. The p110 subunit of PI3-kinase has activatable catalytic activity (Okada et al., 1994). Wortmannin is a compound known to block the activity of

the p110 subunit. PI3-kinase inhibition by wortmannin has been frequently used to help decipher its metabolic effects. When used at low concentrations, wortmannin specifically and irreversibly blocks PI3-kinase activity (IC_{50} ~ 3nM)(Ui et al., 1995). Nanomolar concentrations of wortmannin have been shown to completely inhibit PI3-kinase and prevent the stimulation of glucose transport by insulin in rat adipocytes (Okada et al., 1994) and skeletal muscle (Le-Marchand-Brustel et al., 1995), suggesting that PI3-kinase may be important for insulin-induced glucose transport activation in adipose cells and muscle. Furthermore, nanomolar concentrations of wortmannin were reported to prevent insulin-stimulated increases in 3T3-L1 cell-surface glucose transporters without changing the total cellular quantity of this transporter protein (Clarke et al., 1994), further implicating PI3-kinase activity as a signal that activates glucose transport. Cell fractionation experiments in studies by Heller-Harrison et al. (1996) showed that levels of the PI3-kinase reaction products PI(3,4)P_2 and PI(3,4,5)P_3 were higher in membranes of GLUT-4-containing vesicles, which were isolated from cells that were first subjected to insulin. Collectively, these studies could indicate that insulin-activated PI3-kinase activity promotes GLUT-4 translocation and the phospholipid products of the PI3-kinase reaction might act as second messengers to induce this response.

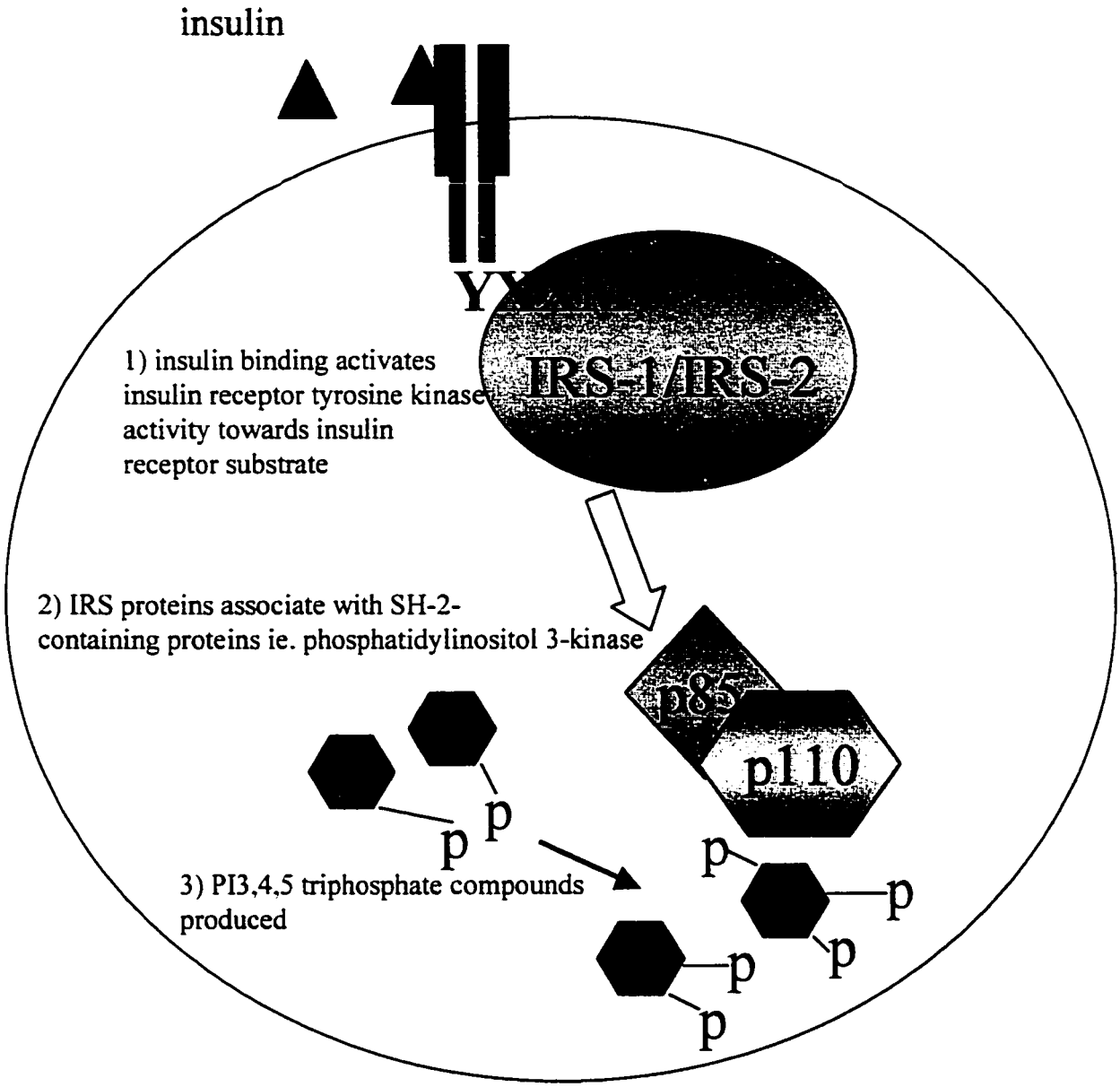


Figure 1.1. Insulin receptor activity is linked to the activation of PI3-kinase

Beyond PI3-kinase: a potential role for Akt

Isakoff et al. demonstrated that PI3-kinase alone is insufficient for glucose transport activation (Isakoff et al., 1995). Another molecule beyond PI3-kinase is likely involved. In 1991 the cellular homologue viral-AKT oncogene was cloned from a transforming retrovirus AKT8 in a mouse cell line (c-AKT) (Bellacosa et al., 1991). Coincidentally, two other groups cloned the same cDNA while searching for new genes of novel cell signalling kinases that might have been related to the superfamily of protein kinases A and C (RAC kinase) (Coffer and Woodgett, 1991; Jones et al., 1991). These observations led to the discovery of a common protein product. This protein is now usually referred to as Akt (or sometimes protein kinase B).

In mammalian tissues, three distinct Akt isoforms are encoded by 3 distinct genes (Altomare et al., 1995; Brodbeck et al., 1999; Nakatani et al., 1999). These genes are 85% homologous and are expressed in a variety of tissues (Kandel and Hay, 1999). All isoforms are activated by insulin (Walker et al., 1998). Akt-1 and Akt-2 may function more similarly than Akt-3. Both Akt-1 and Akt-2 are highly abundant and are found in greater quantity than Akt-3 in muscle and adipose tissues (Walker et al., 1998). Akt-3 is highly expressed in brain and testis (Brodbeck et al., 1999; Nakatani et al., 1999). Fittingly, Akt-1 and Akt-2 are activated to a similar extent in the insulin sensitive tissues (i.e. skeletal muscle and adipose), and more so than Akt-3 (Walker et al., 1998). Moreover, the structural organization of Akt -1 and -2 isoforms are dissimilar to the Akt-3 variant. Both Akt-1 and -2 isoforms have a pleckstrin homology (PH) domain, a catalytic fragment and a putative regulatory segment, whereas Akt-3 has all of these except a PH

domain (Kandel and Hay, 1999). Phosphatidylinositol dependent kinases (PDKs 1 and 2) help to activate Akt by phosphorylating specific regulatory serine and threonine sites (Stokoe et al., 1997). One notable difference between Akt-1 and Akt-2 is that the regulatory serine (SER^{473}) and threonine (THR^{308}) amino acid residues of Akt-1 are situated at different positions in the protein sequence of Akt-2 (SER^{474} and THR^{309}) (Kandel and Hay, 1999), suggesting that slight isoform-specific differences for controlling Akt activation by the phosphatidylinositol-dependent kinases PDK-1 and PDK-2 may exist.

Akt is downstream from PI3-kinase towards glucose transport activation

Since it was demonstrated that PI3-kinase alone is insufficient for glucose transport activation (Isakoff et al., 1995), efforts have intensified to determine whether Akt kinase lies downstream of PI3-kinase in the insulin signalling cascade (Fig. 1.2). When a dominant negative Akt protein is expressed in rat adipocytes, in place of the native protein, insulin-stimulated GLUT-4 translocation is reduced (Cong et al., 1997). Notably, when L6 muscle cells are treated with wortmannin at concentrations specific for PI3-kinase, this enzyme's activity is irreversibly inhibited (Ui et al., 1995) and insulin-stimulated Akt activity is prevented (Ueki et al., 1998). Concurrent over-expression of a constitutively active Akt protein in L6 muscle cells overrides the wortmannin-induced inhibition of PI3-kinase and restores insulin-stimulated glucose transport (Ueki et al., 1998), implying that Akt activation beyond PI3-kinase stimulates glucose transport. Since these studies, Akt has also been proposed to lie in an insulin signalling pathway that

activates glucose transport by increasing GLUT-4 translocation in skeletal muscle (Krook et al. 1997; Song et al. 1999; Wilkes and Bonen, 2000), as illustrated in Fig. 1.3.

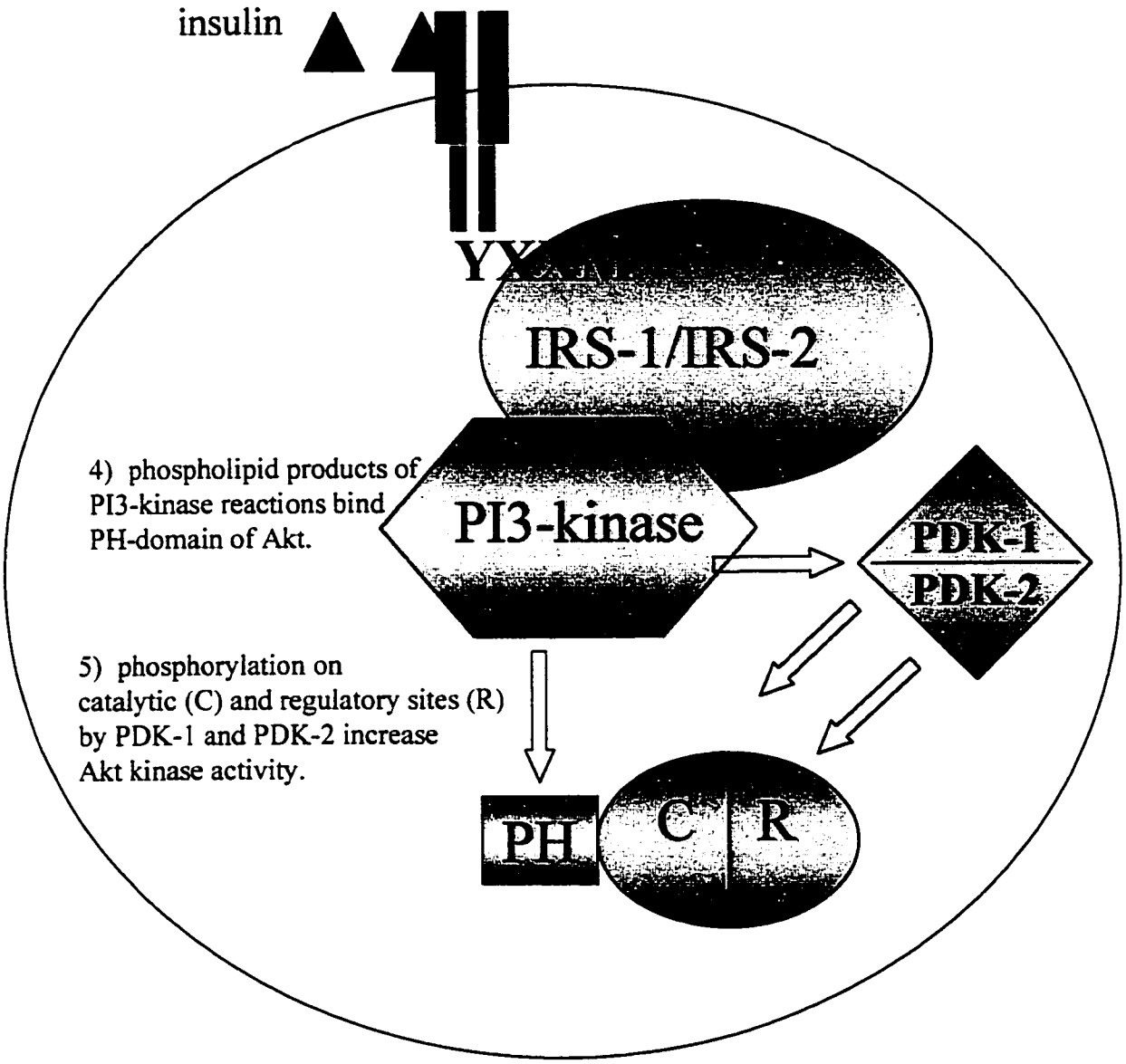


Figure 1.2. PI3-kinase-dependent signals activate Akt

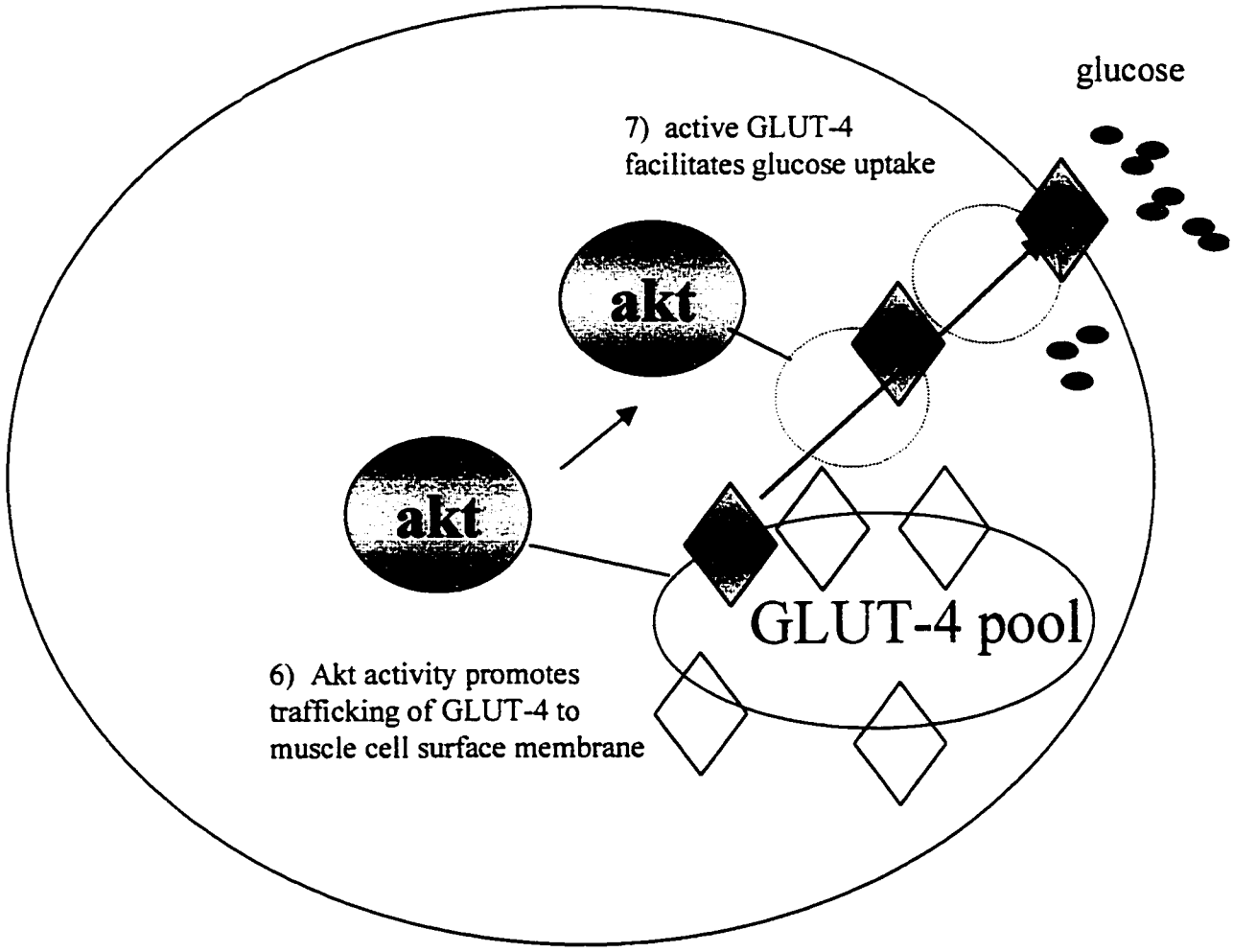


Figure 1.3. GLUT-4 translocation induced by Akt activity

V. LIFESTYLE-RELATED FACTORS INFLUENCING INSULIN SENSITIVITY AND TYPE II DIABETES

Insulin sensitivity may be diminished in a large number of individuals because of poor lifestyle-related choices. The high incidence of insulin resistance in the Western world can be partially attributed to the North American diet being high in fat (~35-40% total energy from fat) (Himsworth, 1935; Parker et al., 1993; Mayer et al., 1993) and to a habitually sedentary lifestyle (Heimrich et al., 1991; Manson et al., 1992). Some studies have suggested that the reduced sensitivity to insulin, or insulin resistance, that occurs when consuming a high fat diet may be compensated for with higher post-prandial and fasting blood insulin concentrations (Parker et al., 1993; Mayer et al., 1993). A hyperinsulinemic state in humans can be futile, as excessive insulin secretion may ultimately contribute to the worsening of insulin resistance. Individuals with chronic hyperinsulinemia accompanied by defective insulin secretion are thought to eventually develop full blown non-insulin-dependent (type 2) diabetes (Kahn, 1994).

Surprisingly, individuals with type 2 diabetes can have insulin resistant muscles with normal total GLUT-4 content (Pedersen et al., 1990; Handberg et al., 1990). This leaves open the possibility that a defect in insulin-stimulated glucose transport in human diabetic muscle may be due to impairment(s) in insulin signalling mechanism(s). Bjornholm (1997) reported that insulin-stimulated activation of PI3-kinase is decreased in skeletal muscle of humans with type 2 diabetes (Bjornholm et al., 1997). In contrast, the

improvement in insulin sensitivity that results from increased physical activity could also involve the alteration of insulin-mediated signalling. Acute bouts of exercise, which do not increase muscle GLUT-4 content (Hansen et al., 1998), can still enhance insulin-stimulated glucose transport (Hansen et al., 1998). Therefore, the enhanced insulin sensitivity found during the post-exercised period (Hansen et al., 1998; Richter, et al. 1989; Mikines et al., 1988) could be a result of acute bouts of exercise conveying short lived improvements to insulin signalling step(s).

High-fat-feeding causes insulin resistance in rats without GLUT-4 reduction

One model for diet-related, human diabetes is the high-fat-fed rat. Rats fed specific diets that are high in fat content (≥ 59% calories) display insulin resistant skeletal muscle. The advantage of an animal model is that insulin resistance can be induced in a predictable (Storlein et al., 1991) and reproducible (Wilkes et al., 1998) manner which is detectable after a reasonably short period of feeding (3-4 weeks), without serious reductions in total muscle GLUT-4 (Rosholt et al., 1994; Hansen et al., 1998; Han et al., 1997; Zierath et al., 1997).

GLUT-4 translocation has been shown to be decreased in muscle from rats fed a high-fat diet, when total GLUT-4 is unchanged (Zierath et al., 1997; Hansen et al., 1998) suggesting that insulin signalling in high-fat-fed rat muscle may be insufficient. Zierath (1997) reported that the activation of PI3-kinase via IRS-1 was decreased in muscle made insulin resistant by high-fat-feeding and suggested that these early defective signals contributed to the reduced insulin-stimulated GLUT-4 translocation and resulting impaired

glucose transport (Zierath et al., 1997). Therefore, insulin resistance induced by high-fat-feeding could be due to a reduction in GLUT-4 translocation resulting from an inadequate generation of insulin-activated signals. In this thesis, it was examined whether an insulin signalling defect includes the loss of Akt-derived signalling influences in muscle which has been made insulin resistant by high-fat-feeding. Whether Akt is central to the insulin resistance induced in high-fat-fed rats was explored as a possibility in the studies included in this thesis.

However, it is not completely certain whether reduced GLUT-4 translocation can be due to a disruption in the insulin signalling pathway. Hansen et al. (1998) were of the opinion that early insulin signals are intact in muscle made insulin resistant by high-fat-feeding. They found normal insulin-induced tyrosine phosphorylation of IRS-1 and suggested that the defective GLUT-4 translocation found after high-fat-feeding was due to an impediment in GLUT-4 trafficking rather than a diminishment in insulin signalling *per sé* (Hansen et al., 1998). Maybe defective glucose transport activaiton could be a result of defective GLUT-4 trafficking and the insulin signalling steps to initiate this process are still in place. This possibility was considered in this thesis. The alternative idea may be that the insulin signalling responses through Akt becomes more effective when insulin resistance is induced by high-fat-feeding. In studies by Elchebly et al. insulin action was improved prior to high-fat-feeding by first enhancing IR / IRS-1 tyrosine phosphorylation (Elchebly et al., 2000). They used a genetic 'knockout out' model of protein tyrosine phosphatase-1B (PTP-1B), an enzyme which downregulates IR activity by removing phosphates from insulin receptor sites (1146, 1150, 1151) deemed important for insulin

receptor tyrosine kinase activity. Negative signalling influences exerted by PTP-1B were eliminated in mice by breeding animals to be missing key portions of the PTP-1B gene. High-fat-fed, PTP-1B null mice had normal insulin concentrations, lower glucose concentrations and a greater sensitivity to insulin than PTP-1B wild type mice fed the same high-fat diet. Therefore, it appears from studies by Elchebly and coworkers that the insulin resistance caused by the consumption of a high-fat diet could be prevented if insulin signalling was first enhanced. Diets high in mixed oils are thought to be protective against glucose intolerance (Storlien et al., 1991). Therefore, changes in insulin signalling might possibly improve glucose uptake under select conditions of high-fat-feeding, when mixed oil-based diets which are more favorable for glucose tolerance, are provided. This was also considered as a possibility in this thesis. Therefore, studies were conducted to investigate whether Akt might be upregulated in an attempt to alleviate the reductions in insulin-stimulated glucose transport in muscle from high-fat-fed rats.

Muscle training increases GLUT-4 while muscle inactivity reduces GLUT-4

A. Training:

GLUT-4 protein content is increased in muscle with exercise training or in electrically-stimulated muscle that contract chronically for 7 days. This is likely due, in part, to intramuscular signals generated by adenylate cyclase and / or muscle metabolites; possibly linked to changes in cyclic adenosine 3',5'-monophosphate (cAMP) and high energy phosphates (Yaspelkis III et al., 1997; Yaspelkis III et al. 1998). GLUT-4 protein levels and insulin-stimulated glucose transport have been reported to increase rapidly (in 1

day) in epitrochlearis muscles of rats engaged in swim training for 6 hours (Ren et al., 1994), suggesting that a rapid increase in GLUT-4 induced by training could improve insulin sensitivity.

The extent to which insulin-stimulated glucose transport improves with exercise is closely correlated with increases in muscle GLUT-4 content (Brozinick Jr. et al., 1993; Etgen et al., 1993; Friedman et al., 1990). In theory, an expansion of the total GLUT-4 pool caused by training increases internal GLUT-4 availability for recruitment to cell surface membranes by the translocation mechanism (Brozinick Jr. et al., 1993; Etgen et al., 1993; Friedman et al., 1990; Reynolds et al., 1997). Reynolds has demonstrated repeatedly that training-induced increases in GLUT-4 can selectively improve insulin-stimulated GLUT-4 translocation, since hypoxia-induced GLUT-4 translocation was unaffected in their studies (Reynolds et al., 1997 and 1998). This suggestion was based on a 2 signalling pathway model which implies that GLUT-4 recruitment to surface membranes in skeletal muscle can be induced by an insulin–stimulated pathway and also by a separate hypoxia (contraction)-activated pathway which converge on separate insulin-sensitive and hypoxia (contraction)- sensitive GLUT-4 pools, respectively (Brozinick et al., 1992; Gao et al., 1994). However, an increase in the insulin-sensitive GLUT-4 content of muscle caused by habitual physical activity may, by itself, not fully improve the insulin-stimulated glucose transport response. Recent evidence has suggested that physical activity can also affect insulin signalling, since insulin-activated PI3-kinase was found to be upregulated in response to exercise (Zhou et al., 1997) and exercise training (Koval et al., 1999).

Full activation of upstream signals may not be necessary for maximal insulin-stimulated glucose transport (Kurowski et al., 1999; Heart et al., 2000). Therefore, the studies in this thesis examined Akt. It was hypothesized that Akt activity could also affect insulin-stimulated glucose transport rates in conjunction with the increased amount of total GLUT-4 produced in trained muscle.

B. Muscle inactivity:

Reduced muscle activity may be expected to impair insulin-stimulated glucose transport by lowering the total GLUT-4 content of muscle. Denervation has often been used as a model for muscle inactivity. A considerable number of studies have shown that when muscle activity was completely eliminated for 3 days, by severing the motor nerve to rat hindlimb muscles by sciatic nerve sectioning, insulin resistance was pronounced and GLUT-4 content was appreciably reduced (Handberg et al., 1996; Megeney et al., 1993; Megeney et al., 1994; Megeney et al., 1995). Large reductions ($\leq$ 80%) in total GLUT-4 were observed after 3 days of denervation (Megeney et al., 1994).

Reductions in total GLUT-4 content have not always occurred in muscles in which insulin sensitivity was decreased after a period of inactivity. Houmard (1993) reported that there was a decrement in insulin-stimulated glucose transport in muscle of normally active individuals after a 14 day period of inactivity, but these individuals showed no appreciable losses in total muscle GLUT-4 protein (Houmard et al., 1993). Moreover,

muscles denervated for 24 hours displayed an obvious resistance to insulin (Elmendorf et al., 1997; Henriksen, et al., 1991; Turinsky, 1997) when total GLUT-4 was unchanged (Coderre et al., 1992; Henriksen et al., 1991). These studies suggest that there may be unknown defect(s) that lower insulin-stimulated glucose transport in inactive muscles. Such putative defects occur in the absence of any reductions in muscle GLUT-4 quantity.

Insulin resistance in denervated muscles could be due, in part, to altered tyrosine phosphorylation-induced signalling capabilities. Henriksen (1991) found that denervated muscles were resistant to vanadate, a phosphotyrosine phosphatase inhibitor that can stimulate glucose transport by preventing dephosphorylation of tyrosine residues from insulin signalling proteins (i.e. IRS-1). Furthermore, Sowell (1991) previously reported that concentrations of select pools of diacylglycerol (DAG), byproducts of phospholipid metabolism with glucose transport modulating effects, were altered in denervated muscles (Sowell et al., 1991). However, these studies (Henriksen et al., 1991; Sowell et al., 1991) do not clarify whether direct insulin signalling mechanisms can be affected by muscle denervation. An effect of muscle denervation on PI3-kinase was observed by Elmendorf and coworkers. They found insulin-activated PI3-kinase to be diminished after 3 days of muscle denervation (Elmendorf et al., 1997). This is in contrast to the normal PI3-kinase activation observed in 24 h denervated muscle (Elmendorf et al., 1997; Chen et al., 1993). Since total GLUT-4 was substantially reduced by 3 days (Megeney et al., 1993), it was still uncertain from these studies whether an impairment in insulin signalling alone could have also lowered the amount of GLUT-4 translocated by insulin. Akt activation has been shown to be decreased when the insulin signals upstream of Akt are still intact (Kurowski

et al., 1999; Heart et al., 2000). Therefore, studies on denervated muscle in the present thesis used a short-term denervation model (24 hours), where neither total GLUT-4 nor PI3-kinase activation is altered, to explored the possibility that a diminshed insulin-activated Akt decreases insulin-stimulated glucose transport in denervated muscle. Short term (24 h) denervation-induced insulin resistance was hypothesized to be due to defective signalling at the level of Akt.

VI. HYPOTHESES

Based on the foregoing review the following hypotheses were examined.

<u>High-fat diet induced insulin reisstance</u>

a. Normal glucose tolerance observed after feeding a high mixed-oil diet may reflect reciprocal changes in either the presence or degree of insulin resistance in insulin sensitive tissues.

b. Insulin activates Akt more rigorously in high-fat-fed rat muscle in an attempt to upregulate glucose transport.

<u>Altered muscle activity models</u>

c. Reductions in muscle activity caused by denervation will induce insulin resistance by disrupting Akt.

d. Improvements to insulin responsiveness caused by chronic muscle activity are modulated by Akt-derived signalling influences.

Chapter 2:

Differential insulin resistance induced by a high unsaturated (mixed)-fat-based diet: decreased insulin-stimulated glucose transport in muscle but not adipose tissue

INTRODUCTION

In rodents and humans, dietary intake of high amounts of fat have been shown to have adverse effects on insulin sensitivity and to contribute to the development of glucose intolerance and overt diabetes (recently reviewed by Storlien et al.1996). Much of the work reported in the literature has used a model where the absolute amount of dietary fat is considered to be the central variable affecting insulin sensitivity. Fat intakes ranging from approximately 40% to 75% of total kcal, usually in the form of saturated fat, safflower oil or corn oil (both high in n-6 polyunsaturated fatty acids), have been reported to reduce whole body insulin-stimulated glucose uptake (Han et al., 1995; Khoursheed et al., 1995; Kraegen et al., 1991; Lui et al., 1996; Miller et al., 1985; Rocchini et al., 1997; Storlien et al., 1987 and 1993). This is accompanied by reductions in insulin stimulated glucose transport in individual skeletal muscles (Han et al., 1995; Kim-J.K. et al., 1996; Kraegen et al., 1991 and 1986; Lui et al., 1996; Miller et al., 1985; Storlien et al., 1991 and 1993) and adipose tissue (Kahn et al., 1994; Maegawa et al., 1986; Storlien et al., 1993), along with enhanced hepatic glucose production (Khoursheed et al., 1995; Kraegen et al., 1991; Storlien et al., 1987). Thus, high dietary fat intake will alter the regulation of glucose transport in insulin-sensitive tissues, leading to changes in whole body glucose metabolism.

In addition to the amount of dietary fat being an important determinant of insulin sensitivity, there is also evidence that variation in fatty acid composition may independently affect insulin action and alter insulin sensitivity. Storlien et al. (1987) demonstrated that when a portion of the fat from a high polyunsaturated diet (60% total

kcal as fat) was replaced with fish oil (6% kcal of total kcal) containing long chain n-3 fatty acids, glucose transport in skeletal muscle and hepatic glucose output were restored to levels observed in chow-fed rats. Saturated fat combined with these large amounts of linolenic acid (C18:3, n-3) similarly alleviated insulin resistance in muscle and in the whole body (Storlien et al., 1991). Monounsaturated fatty acids, when fed at high levels, may not lead to the reductions in insulin sensitivity observed with the high polyunsaturated fat diets (Garg et al., 1992; Parillo et al., 1996) and may improve indices of glucose tolerance in NIDDM patients (Low et al., 1996). Thus, different dietary fatty acids appear to have varying effects on insulin sensitive tissues, and this may affect the expression of glucose tolerance.

The purpose of this study was to examine direct measures of insulin resistance in several insulin-dependent tissues in high fat fed rats consuming diets of 60% kcal from fat but with different fatty acid combinations, and to relate these measures to whole body glucose tolerance. Initial studies in our laboratory had used a modified high fat version (60% kcal fat) of the American Institute of Nutrition 1993 (HF-MIXED) diet as a model of insulin resistance. It was predicted that 3 weeks of feeding this diet would produce a similar degree of insulin resistance and glucose intolerance as had been observed with a safflower oil-based, high fat diet (Storlien et al., 1991 and 1987), because the HF-MIXED diet contained a high degree of polyunsaturated and saturated fatty acids, and a relatively low amount of n-3 fatty acids, in addition to the large proportion of calories as fat. Quite surprisingly in fact, in preliminary studies there were no differences in glucose tolerance observed in rats fed the HF-MIXED diet vs. those fed a control, 60% carbohydrate diet

(Bell et al., 1996). This prompted the consideration of the possibility that this combination of fatty acids in the HF-MIXED diet may lead to tissue specific changes in glucose transport. It was hypothesized that normal glucose tolerance observed after feeding the HF-MIXED diet may reflect reciprocal changes in either the presence or degree of insulin resistance in insulin sensitive tissues.

METHODS

Animals and Diets:

Male Sprague Dawley rats weighing approximately 200g were individually housed with a 12:12 hour light:dark cycle and were randomly assigned to one of three dietary groups: high carbohydrate (LF, 20 kcal % protein, 10% fat and 70% carbohydrate), high fat-safflower oil (HF-SAFF, 20% kcal protein, 59% fat, 20% carbohydrate), or the high fat-mixed oil (HF-MIXED, 20% kcal protein, 59% fat diets, 20% carbohydrate). The source of fat in the LF and HF-SAFF diets was safflower oil, while the HF-MIXED diet contained a mixture of soybean oil (12% of kcal), hydrogenated coconut oil (24% of kcal), and high oleic sunflower oil (24% of kcal). Diets used in this study are outlined in Table 1.

Prior to the start of the experimental diets, food intake (g/day) on standard rat chow was determined for 3 days. Rats were fed their respective experimental diets at 98% of *ad libitum* chow energy intake daily; access to water was unlimited at all times. Food was generally consumed in its entirety by all animals each day. Rats were weighed a minimum of 3 times weekly to ensure that growth rates in all dietary groups were similar.

Table 2.1. Composition of diets used in this study.

Ingredient	High Carbohydrate (LF)	High Fat - safflower oil (HF-SAFF)	High Fat - mixed oils (HF-MIXED)
		g/kg diet	
Casein	188	254	200
Cornstarch	438	169	227
Sucrose/Maltose dextrin[1]	219	85	132
Wheat Bran/Cellulose[1]	38	51	50
Safflower Oil	41	339	--
Soybean Oil	--	--	70
High Oleic Sunflower Oil	--	--	135
Hydrogenated Coconut Oil	--	--	135
Gelatin	14	19	--
Salt Mix	50	67	35
Vitamin Mix	9.7	13	10
Vitamin E acetate (500 IU/g)	--	.31	--
DL-Methionine / L-cystine1	2.3	3	3
Choline Bitartrate	--	--	2.5
% kcal			
CHO	69	20	27
Fat	10	59	59
Protein	21	21	15
Caloric Value (kcal/g)	3.82	5.20	5.16

Following 3 weeks of feeding, rats underwent an intravenous glucose tolerance test (details below). Rats recovered from this procedure for 5 days before glucose transport was directly assessed in skeletal muscle and adipose tissue. All procedures used in this study were approved by the Animal Care Committee at the University of Waterloo, and followed the Guidelines of the Canadian Council on Animal Care.

Intravenous Glucose Tolerance Tests (IVGTT)

One subgroup of rats (n=10 rats/dietary group) underwent the IVGTT's in the anesthetized state, while a second subgroup of rats (n=5 rats/dietary group) were conscious during their IVGTT's. All rats were fasted overnight prior to their IVGTT. For the anesthetized group, rats were anaesthetized with a tail vein injection of sodium pentobarbital (65 mg/kg body weight, less 10%) and buprenorphine (Temsgesic, 0.003 mg/100 g body weight) was administered subcutaneously as an analgesic. Animals were kept on a heating blanket during both the surgery and experiment to prevent hypothermia during anesthesia (Lang et al., 1987). The jugular vein was fitted with a catheter and animals were allowed to rest for 30 minutes. Two basal blood samples were drawn at 5 minute intervals, and then a bolus of glucose (300 mg / kg, 10% solution) was injected into the catheter and immediately rinsed with heparinized saline (16 units/mL) to prevent clotting. Blood samples (approximately 0.2 mL) were collected into NaF / heparinized microcentrifuge tubes at 1, 2.5, 3.5, 5, 6.5, 8, 12 and 18 minutes post glucose dose and placed on ice. Samples were centrifuged (Eppendorf microcentrifuge, 12,000 rpm, 4 minutes), and plasma was separated from red cells. Plasma for glucose and insulin determinations was stored frozen at -20 °C until used. Glucose was determined by

glucose spectrophotometric method while insulin was determined by RIA using a rat-specific antibody. Following the IVGTT, the jugular catheters were tied off, rats were allowed to recover under a heating lamp and were returned to their cages later the same day. Rats ate normal quantities of food 24-48 hours after surgery and body weights were back to pre-operative levels by 2-3 days after surgery.

For the conscious group, rats were anesthetized under halothane, given buprenorphine, fitted with the jugular catheter, and then regained consciousness. Rats rested quietly in the experimental cage for 30 minutes before the basal blood samples were drawn. Glucose administration, timing of samples post-glucose bolus, sample separation and storage, and animal recovery was identical to the protocol outlined for the anesthetized rats.

Glucose tolerance (Kg) was determined as the slope of the log of blood glucose vs. time using the samples obtained from the 2.5 to 12 minute period after glucose injection. In both the conscious and anesthetized groups, Kg showed significant effects of diet treatment ($P<0.001$), but the effects of anesthesia were not statistically different ($P>0.05$), therefore values from the anesthetized and conscious groups were combined. The acute insulin response to glucose was calculated as the area under the insulin curve from 1 to 6.5 minutes after glucose injection and was corrected for the pre-stimulus baseline levels. Plasma from basal (fasting) samples were also used for determination of triglycerides and free fatty acids.

3-O-methyl glucose transport into perfused hindlimbs

Rats were anesthetized by i.p. injection of sodium pentobarbital (65 mg / kg body weight), and the epididymal fat pads were quickly removed for assessment of glucose transport into isolated adipocytes. Rats were surgically prepared for hindlimb perfusion as described by Ruderman et al. (1971) with modifications previously made in our laboratory (McDermott et al., 1989). Briefly, the gastrointestinal tract was removed and all major blood vessels within the abdominal cavity were tightly sutured. Heparin (500 mU/mL) was injected into the vena cava of each rat (0.5 mL) prior to cannulation to inhibit clotting. The descending aorta was cannulated (20 gauge angiocath) to deliver perfusate to the hindlimb and inferior vena cava was cannulated (14 gauge angiocath) to remove the effluent to complete a one pass perfusion system. A cell-free perfusate containing Krebs-Henseleit buffer with 4% bovine serum albumin (BSA) under constant gassing (95% O_2 / 5% CO_2) was connected to the indwelling cannulae via tygon tubing which was connected to a pump while the rat lay in a warm chamber (30°C). Over the first 20 minutes of the perfusion protocol, the flow rate was gradually increased to 20 mL/minute once flow back pressure stabilized between -80 to -100 mm Hg. This flow rate is sufficient to perfuse and oxygenate the entire hindlimb (unpublished observations). After 20 minutes, 30 mM 3-O-methylglucose (3-O-MG) containing 5μCi ^{3}H-3-O-MG and 2.5 μCi ^{14}C-L-glucose was added to the perfusate and the hindlimb was perfused for an additional 5 minutes. Addition of the ^{14}C-L-glucose the perfusate allowed us to account for the non-transported diffusion component of glucose transport into skeletal muscle. To assess insulin-stimulated glucose transport, insulin (160 μU / mL, porcine insulin, Iletin II regular) was added to the buffer at the start of the perfusion and was maintained at this concentration for the

duration of the experiment. This concentration of insulin has been previously shown by our laboratory and others to maximal glucose transport maximally in skeletal muscle of normal, chow fed rats using this perfusion system (Megeney et al., 1992). Muscles were categorized based on their oxidative potential, and results pooled accordingly (Megeney et al., 1993).

2-deoxyglucose transport into adipocytes

At the time of the hindlimb perfusion experiment, epididymal fat pads were rapidly excised, weighed, minced and adipocytes were isolated by collagenase digestion according to the method of Rodbell (1964), with modifications by Wilkes et al. (1996). Following collagenase digestion (which contained 100 nM N°-phenylisopropyl adenosine (PIA)), isolated adipocytes were washed 3 times with phosphate buffered saline (PBS) with 1 mM $MgCl_2$ and 0.68 mM $CaCl_2$, 1 mg/mL BSA, and 1mM pyruvic acid, pH 7.4. Adipocytes were counted in a hemocytometer and resuspended to 5 X 10^5 cells/mL. Adenosine deaminase was added to cell suspensions in all treatments to remove endogenous adenosine. Adipocytes were treated in round bottom test tubes with insulin (Iletin II regular) ranging from 0 (basal) – 40 μU / mL (final concentrations) for 30 minutes. in a shaking waterbath (37°C, 100 oscillations/minute). Nonspecific transport was measured in cells preincubated with phloretin (0.3 mM) after 25 minute of incubation and radioactivity from this condition was subtracted from all other treatments to obtain specific transport; non-specific transport was confirmed using cytocholasin B (personal observations). In some incubations 50 μM final concentration of the PI3-kinase specific

inhibitor LY294002 (LY) was added at the beginning of the preincubation and 16 μU / mL insulin was added after 27 minutes of incubation, to demonstrate inhibition of insulin-stimulated glucose transport.

Following the preincubation, 150 μL aliquots of adipocytes were layered over 100 μL of light mineral oil / silicon oil (57 : 43) in 400 μL polyethylene microcentrifuge tubes. Glucose (10 mM) containing 2-deoxy-1,2-^{3}H-glucose (2-DG, 50 μL, 0.5 μCi/tube) was added to the adipocytes and transport was allowed to occur for 3.5 minutes. Glucose transport was stopped by centrifugation for 6 sec at 14,000 rpm (Eppendorf Microcentrifuge). The top layer containing the adipocytes was cut off using a razor blade into polypropylene scintillation vials and radioactivity associated with adipocytes was quantified by liquid scintillation counting.

Determination of Fatty Acid Composition of Adipose Tissue

Fatty acid composition was determined in samples of epididymal adipose tissue from a subset of rats (n=5 rats /group) using the method described by Wainwright et al. (1992). Homogenized adipose tissue was extracted using chloroform : methanol (1:1, v:v), in the presence of 0.02% BHT (w/v). After separation, samples were dried under nitrogen, and the total lipids fractionated by thin-layer chromotography using silica gel plates and a chloroform / methanol / acetic acid/ water (50/30/4/2) solvent system. The fatty acids of the resulting phospholipid fractions were methylated with 14% boron trifluoride in methanol and analyzed by gas chromotography. Fatty acids were identified by comparison of their retention time with those of authentic standards.

Statistical Analysis

Body weight, food intake, fat pad weight, and glucose tolerance data were assessed for differences among dietary groups using a one-way ANOVA. Differences in glucose transport among different muscles and in adipocytes at the various concentrations of insulin were assessed using a repeated measures one-way ANOVA, with muscle type and insulin concentration as the repeated factor, respectively. Significant effects were further analyzed using Tukey's t-test; for all analyses, a P-value of <0.05 was accepted as statistically significant.

RESULTS

Body weights of rats fed the HF-MIXED, HF-SAFF, and LF diets are shown in Figure 2.1. Body weights did not differ among any of the dietary groups at any time during the feeding protocol. Rats in all dietary groups consumed similar amounts of food (kcal/day) throughout the study. Mean intake for the different dietary groups was LF: 91 ± 0.77 kcal / day, HF-SAFF: 91 ± 1.56 kcal / day, and HF-MIXED: 91 ± 1.04 kcal / day. Food intake did not differ significantly among dietary groups.

Final body weights, fat pad weights, fasting glucose, insulin, triglyceride and free fatty acid concentrations, characteristics of glucose tolerance and the acute insulin response to glucose are presented in Table 2.2. Final body weights measured on the day of the hindlimb perfusions did not differ significantly among the experimental treatment groups. Despite similar body weights and similar caloric intakes among dietary groups, fat

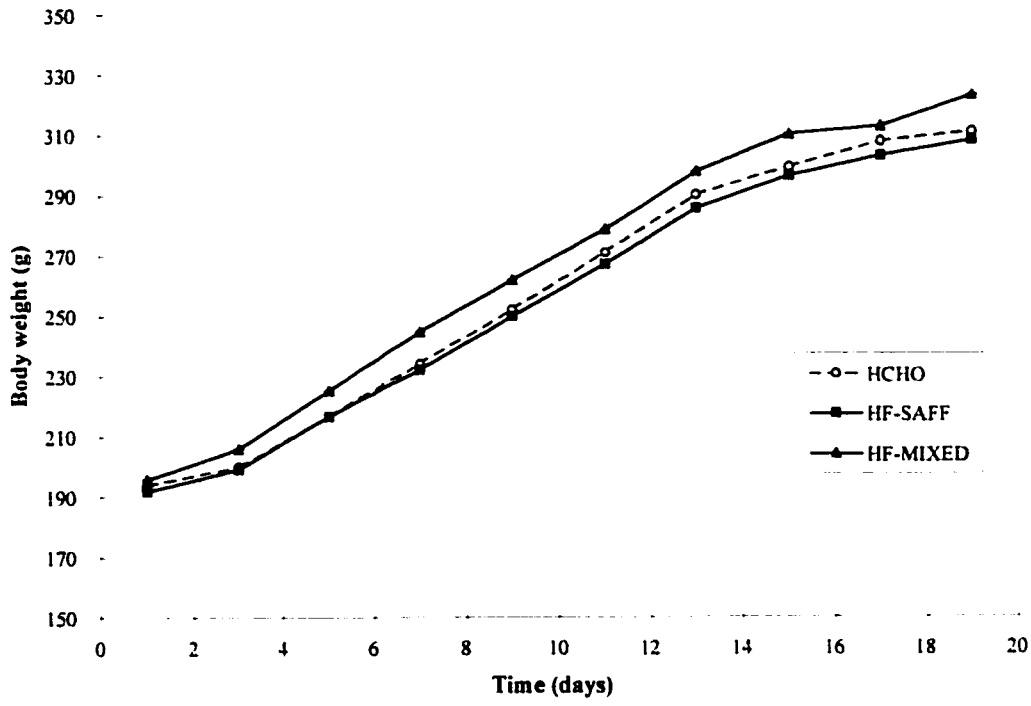


Figure 2.1. Mean body weights (± SEM) of groups of rats fed the LF, HF-SAFF or HF-MIXED diets for 3 - 4 weeks (n=5). Body weights did not differ significantly among dietary groups at any time during the study.

Table 2.2. Final body weights, fat pad weights, fasting plasma triglyceride, free fatty acid and glucose concentrations, characteristics of glucose tolerance (Kg), and the acute insulin response to glucose (AIRg) in rats following 3 - 4 weeks of experimental diets.

Measurement[1,2]	LF (n=5)	HF-SAFF (n=5)	HF-MIXED (n=5)
Final Body Weight (g)	309 ± 4	317 ± 5	321 ± 4
Fat Pad Weight (g)	3.49 ± 0.19[a]	3.55 ± 0.11[a]	4.65 ± .025[b]
Glucose (mM)[3]	7.1 ± 1.7	6.7 ± 0.2	6.5 ± 0.2
Insulin (ng/mL)	0.68 ± 0.08	0.54 ± 0.12	0.58 ± 0.08
Triglycerides (mg/dL)	17.1 ± 5.2	13.4 ± 3.9	16.9 ± 4.4
Free Fatty Acids(mmol/L)	0.85 ± 0.26	0.78 ± 0.22	0.74 ± 0.19
Kg (%/min)[4]	3.70 ± 0.4[a]	2.70 ± .04[b]	3.90 ± .02[a]
Acute Insulin Response to Glucose (AUC - basal)	11.5 ± 1.7	13.7 ± 2.6	17.8 ± 2.3

[1] All values are mean ± SEM
[2] Values within a row with different superscripts differ significantly. P<0.01
[3] significant main effect of anesthesia, P<0.001
[4] significant main effect of diet (P<0.02), but no significant effect of anesthesia and no significant interaction, P>0.05.
[5] AUC = area under curve

pads of rats in the HF-MIXED group weighed significantly more than fat pads from rats in the other 2 groups ($P<0.005$). Fasting plasma levels of glucose, insulin, triglycerides and free fatty acids did not differ significantly among experimental treatment groups.

Glucose tolerance (Kg), calculated as the slope of the log of blood glucose concentrations over time for 2.5 - 12 minutes after the bolus glucose injection, showed a significant main effect of diet such that Kg was significantly reduced in the HF-SAFF group relative to the LF-fed and HF-MIXED-fed groups. Glucose tolerance in rats fed the HF-MIXED diet did not differ from those in the LF group. Insulin secretion in response to i.v. glucose, measured as area under the curve minus basal, did not differ among rats from the 3 dietary groups.

The fatty acid composition of rat adipose tissue reflected the amount of those fatty acids found in the various diets (Table 2.3). In the LF and HF-SAFF dietary groups, fatty acid composition of adipose tissue was similar except for a significant absolute increase in the amount of C18:2,n-6 fatty acids in the HF-SAFF-fed group, reflective of the approximately 8-fold increase in n-6 polyunsaturated fatty acid found in the HF-SAFF diet. The absolute increase in C18:2,n-6 in adipose tissue from HF-SAFF rats resulted in a dramatic increase in the n-6:n-3 ratio observed in fat tissue from rats in the HF-SAFF group relative to the LF-fed rats. In adipose tissue from rats fed the HF-MIXED diet, fatty acids of the C10, C14 and C18:1, n-9 families were significantly elevated compared with adipose tissue from the other dietary groups, while C18:2, n-6 and the C20:, n-6 families were relatively reduced ($P<0.05$). Fatty acids in the n-3 family (C18:3,n-3 and

Table 2.3. Fatty acid composition[1] of adipose tissue from rats fed experimental diets for 3-4 weeks (n = 4-6).

Fatty Acid	LF	HF-SAFF	HF-MIXED
C10	$0.3 \pm 0.04^{2,a}$	0.3 ± 0.02^{a}	10.4 ± 0.7^{b}
C12	1.7 ± 0.10^{a}	2.0 ± 0.30^{a}	2.2 ± 0.17^{a}
C14	2.2 ± 0.05^{a}	1.7 ± 0.80^{a}	5.1 ± 0.5^{b}
C18:1n-9	26.5 ± 5.9^{a}	22.7 ± 5.0^{a}	40.4 ± 1.7^{b}
C18:2n-6	31.8 ± 4.0^{a}	51.0 ± 7.3^{b}	18.0 ± 1.8^{c}
C20:n-6	0.6 ± 17.0	0.7 ± 0.12	0.3 ± 0.06
C18:3n-3	0.8 ± 0.10^{a}	0.7 ± 0.14^{a}	1.8 ± 0.2^{b}
C22:6n-3	0.1 ± 0.01^{a}	0.11^{a}	0.2 ± 0.03^{b}
n-6:n-3[3]	36.8^{a}	72.8^{b}	9.1^{c}

[1] fatty acid composition of adipocytes from epididymal fat pad expressed as a relative percent

[2] values with differing superscripts are significantly different from each other, $P<0.05$

[3] n-6:n-3 ratio determined from the fatty acids reported here

C22:6,n-3) were approximately twice as abundant in adipocytes from HF-MIXED-fed rats compared with cells from rats in the HF-SAFF group despite a similar total quantity of energy from fat, and is therefore reflective of the quantity of n-3 fatty acids from the soybean oil and not from de novo synthesis (HF-MIXED significantly different from LF and HF-SAFF, $P<0.05$). This elevation in n-3 content in tissue from rats fed the HF-MIXED-based diet decreased the n-6:n-3 ratio relative to rats in the other 2 dietary groups.

Figure 2.2 shows that the capacity of skeletal muscle for glucose transport under both insulin stimulating and non-stimulating conditions (basal) was altered by feeding a high absolute quantity of energy as fat. Basal glucose transport was significantly increased in oxidative (soleus, red gastrocnemius, red tibialis anterior) and mixed fiber-type muscles (extensor digitorum longis and plantaris) from HF-MIXED and HF-SAFF groups relative to rats fed the LF diet ($P<0.03$). In glycolytic muscle (white tibialis anterior and white gastrocnemius), basal glucose transport was elevated in rats fed the HF-SAFF relative to rats in the LF diet group ($P<0.04$). While glucose transport in glycolytic muscles from the HF-MIXED-fed rats was intermediate between the groups fed the HF-SAFF and LF diets and did not differ significantly from either of the other 2 dietary groups.

Insulin stimulated glucose transport, which was quantified as the amount of glucose transport above basal in the presence of a maximally stimulating concentration of insulin, was reduced in both the HF-MIXED and HF-SAFF-fed groups relative to the LF group ($P<0.04$). Insulin-stimulated glucose transport was similar across muscle

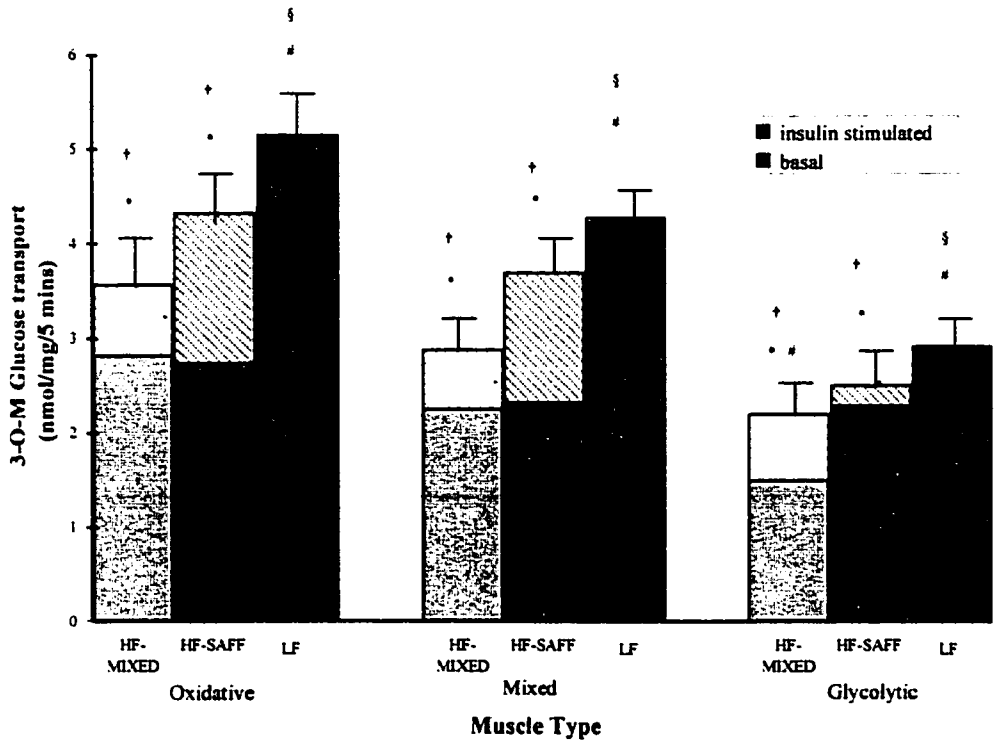


Figure 2.2. Mean 3-O-methyl-glucose transport (± SEM), measured in the absence of insulin (basal transport, filled bars) and in the presence of 160 μU / mL insulin (insulin-stimulated, hatched bars) into hindlimb muscles of rats fed experimental diets for approximately weeks. Muscles were grouped as follows: oxidative muscles: soleus, red gastrocnemius and red tibialis anterior; mixed muscles: extensor digitorum longus and plantaris; glycolytic muscles: white gastrocnemius and white tibialis anterior. Basal transport: bottom set of symbols. Bars within muscle types with different symbols differ significantly from each other, ($P<0.04$). Insulin-stimulated transport: top set of symbols. Bars within muscle types with different symbols differ significantly from each other, $P<0.02$. ^{14}C-L-glucose used to determine extracellular water space and passive diffusion: dpm / mg was not different between dietary groups ($P=0.6$). n=12 rats were fed LF, HF-MIXED, or HF-SAFF diets ; 6 rats were perfused with insulin and 6 rats without insulin (basal).

types of HF-MIXED-fed rats, yet within each muscle group it was reduced compared with HF-SAFF-fed rats. Total glucose transport (basal plus insulin-stimulated transport) was significantly reduced in oxidative, mixed fiber and glycolytic muscles of all rats fed the HF-MIXED-based diet relative to those fed the LF diet ($P<0.03$).

Insulin-stimulated glucose transport into adipocytes is shown in Figure 2.3. Glucose transport by isolated adipocytes did not differ among dietary groups following pre-treatments without insulin (o) and at lower concentrations of insulin (4 and 8 μU/mL). At higher concentrations of insulin, adipocytes from rats fed the HF-MIXED and LF diets had significantly higher rates of glucose transport compared to adipocytes isolated from rats fed the HF-SAFF diet ($P<0.001$). Insulin-stimulated glucose transport was completely blocked in the presence of LY294002, but the reduction was simliar across all dietary treatments.

DISCUSSION

Results from this study demonstrate that the total fat content of the diet was not the sole factor affecting glucose tolerance, and that the combination of dietary fatty acids plays a significant role in affecting this measure. Varying the fatty acid composition of a high fat diet from a single source of predominantly n-6 polyunsaturated fatty acids to include a large portion of oleic acid, short chain saturated fatty acids, and an improved n-6:n-3 ratio allowed normal glucose tolerance to be maintained in the presence of insulin resistance in skeletal muscle. This supports the hypothesis that the HF-MIXED diet has

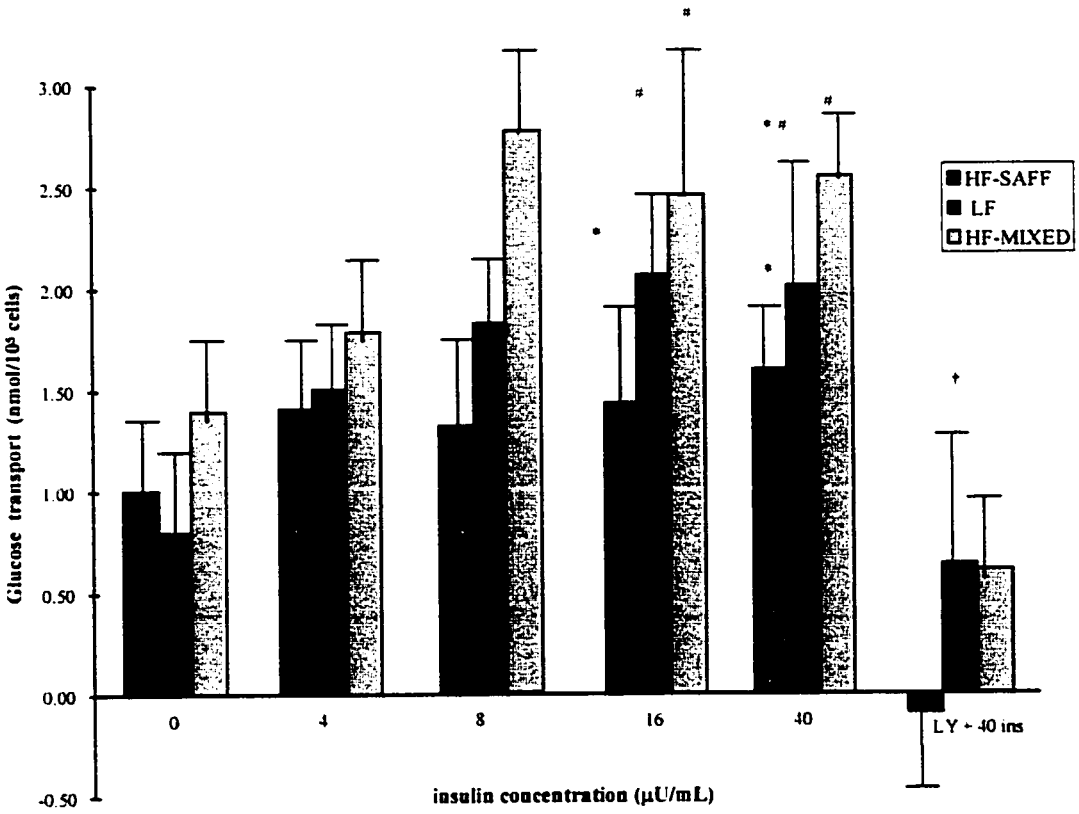


Figure 2.3. Mean 2-deoxyglucose transport (± SEM) into adipocytes following preincubation of cells with various concentrations of insulin. Adipocytes were isolated from epididymal fat pads of animals fed experimental diets for 3-4 weeks. In some incubations, LY294002 and 16 μU / mL insulin were added during the preincubation as described in the Methods section. Within each concentration of insulin tested, data points with different symbols are significantly different from each other, $P<0.05$. Ly + insulin resulted in a significant reduction in glucose transport relative to all other conditions tested (main effect of Ly + insulin, $P<0.05$), but did not differ by dietary group. n=3-5 rats per treatment in each group.

differential effects on insulin resistance in the insulin sensitive tissues skeletal muscle and adipose tissue. Varying the fatty acid composition in this way had a tissue-specific effect on insulin-stimulated glucose transport in muscle and fat cells, with insulin resistance being observed only in muscle but not in adipocytes. Moreover, basal glucose transport in skeletal muscle increased. This suggests that in certain tissues, insulin resistance may be accompanied by compensatory changes in an attempt to offset diminished glucose transport. Together these observations suggest that in the rat, high-fat-feeding induces changes in both skeletal muscle and adipose but that the variation in dietary fat composition contributes significantly to the overall ability of the animal to maintain glucose homeostasis.

A large proportion of a glucose load is disposed of in insulin sensitive tissues, with muscle accounting for more than 80% of the insulin-mediated glucose disposal in healthy chow-fed rodents (DeFronzo et al., 1981). It is not known whether the different dietary interventions may affect the relative contribution of muscle, fat and other insulin-sensitive tissues to insulin-mediated glucose disposal. Our data provide some insight into how diet may affect the partitioning of glucose transport and the physiological significance of these changes. In our study, glucose intolerance was observed only when insulin resistance in skeletal muscle and adipose were both present (following HF-SAFF feeding), but not when insulin resistance was observed in only skeletal muscle (i.e. following HF-MIXED feeding). Thus, glucose tolerance was preserved in rats with uncompromised glucose transport into adipocytes. The epididymal fat pad mass was increased in the HF-MIXED group relative to the other 2 diet groups, thus the larger fat mass in these animals may

account for an absolute increase in the amount of glucose disposed and may have helped to preserve glucose tolerance. Since adipocyte cell size and the absolute number of adipocytes per fat pad were not assessed in this study, it is not clear whether the increased fat pad mass reflects an increased cell number, cell size, or both. Results from this study suggest that glucose transport per adipocyte was increased in the cells from rats fed the HF-MIXED diet relative to those fed the HF-SAFF diet, although in the absence of cell volume measurements this cannot be confirmed in this study. Other investigators have reported that the rate of glucose transport into isolated adipocytes is similar regardless of whether results are expressed per cell or per unit cell volume (Fickova et al., 1998).

The recent suggestion that particular fatty acids may act as natural ligands for peroxisome proliferator activated receptor-γ (PPARγ) (Kliewer et al., 1997; Spiegelman, 1998), points to another possible mechanism through which different dietary fats may contribute to maintenance of normal glucose tolerance. PPARγ, when activated, induce adipogenesis and also improve insulin sensitivity (Spiegelman, 1998). It is unclear whether PPARγ activation is able to reverse insulin resistance induced by high-fat-feeding (Khoursheed et al., 1995; Stevenson et al., 1996) although newly formed, smaller adipocytes are more sensitive to the effects of insulin (Hallakou et al., 1997). Thus, it is conceivable that if PPARγ were differentially activated by the 2 high fat diets used in this study due to differing proportions of available fatty acids, then glucose tolerance may be preserved in the face of increased fat mass. This may be an area for future research.

Another alternative may be that insulin sensitivity must be reduced in all, or some critical number of, insulin-sensitive tissues before glucose tolerance is disturbed. Others have shown that with high-fat-feeding, hepatic insulin resistance develops more rapidly than insulin resistance in skeletal muscle (Kraegen et al., 1991), therefore we assume that this is also present in our study. Hepatic insulin sensitivity was not assessed in this study, and therefore the effects of the HF-MIXED diet on the liver remains unknown.

Feeding of either high fat diet (HF-MIXED or HF-SAFF) to rats resulted in a reduction in insulin-stimulated glucose transport in all muscle groups relative to LF-fed rats. This effect is well documented in animals consuming diets with a fat content over 50% of total calories (Han et al., 1995; Khoursheed et al., 1995; Kraegen et al., 1991; Lui et al., 1996; Miller et al., 1985; Rocchini et al., 1997; Storlien et al., 1987 and 1993) and may be related to the large amount of stored lipids which accumulates within the muscle cells (Pan and Storlien, 1993; Pan et al., 1997; Storlien et al., 1991). In fact, both the high polyunsaturated fat diet (HF-SAFF) and the monounsaturated / SCFA soybean oil-based diet (HF-MIXED) similarly reduced insulin sensitivity in skeletal muscle in these rats. Using a clamp technique, Storlien et al. (1991) observed reversal of insulin resistance in skeletal muscle after including 6% of kcal from long chain n-3 fatty acids in a high polyunsaturated fat diet or after including 6% of kcal from short-chain n-3 fatty acids along with a high saturated fat diet. The HF-MIXED-based diet used in this study contained approximately 10-times less (0.8% kcal) n-3 fatty acid (linolenic acid) than the high fish oil diet used by Storlien's group. Whether the lower level of n-3 fatty acid found in the HF-MIXED in combination with the high saturated short chain and

monounsaturated fatty acids diet was just not adequate enough to alleviate muscle insulin resistance is unknown.

It is also unknown whether changes in the muscle cell membrane fluidity and / or composition under these varied dietary conditions could have modulated insulin-stimulated glucose transport through changes in cell content of phospholipid signalling molecules. It is evident from studies by others that high-fat-feeding changes insulin signalling patterns. Feeding a high fat, beef tallow-base diet with ratios of saturated / monounsaturated fat similar to the HF-MIXED diet results in a reduction in the gene expression of the insulin receptor substrate-1 (IRS-1) and phosphatidylinositol (PI)-3 kinase in rat skeletal muscle compared to muscle from rats fed a high safflower oil diet (similar to the HF-SAFF diet) (Rocchini et al., 1997) contributing to glucose intolerance in these animals. Furthermore, both the expression of the insulin-regulatable glucose transporter, GLUT-4 (mRNA and protein) and insulin-stimulated PI3-kinase activity were dramatically reduced in skeletal muscle after feeding a high lard-based diet to mice (Kahn and Pedersen, 1993; Zierath et al., 1997). Whether the reduction in insulin responsiveness in muscle of high-fat-fed groups is due, in part, to changes in insulin signalling will be examined by future studies.

In skeletal muscle, when insulin was not present in the perfusate an increased glucose transport was observed in all the muscle groups (oxidative, mixed fiber, and glycolytic muscles) from HF-SAFF-fed rats, and in two muscle groups (oxidative and mixed muscle) from HF-MIXED-fed rats relative to LF-fed rats. In both high fat groups the elevation in basal glucose transport was most apparent in oxidative muscle. Thus, both

groups of high-fat-fed rats were able to achieve a higher glucose transport during the 5 minute perfusion period than would have been possible with a normal basal transport component coupled with the compromised insulin-stimulated transport. This could be an adaptive response to the development of insulin resistance in this tissue since oxidative muscles as a group have the highest insulin sensitivity in normal, healthy rats (Megeney et al., 1993). The upregulation of basal transport in oxidative muscle may result from altering the diet fatty acid composition since Storlien et al. saw elevated basal transport in oxidative muscle after supplementing a high saturated fat diet with n-3 fatty acids (Storlien et al., 1993). The weak positive relationship observed between non-insulin stimulated glucose transport and oxidative capacity in muscle from LF-fed rats is also present in muscle from rats fed the HF-MIXED diet, albeit the level of basal transport is higher in this latter group. In contrast, the HF-SAFF-fed rats showed a fairly constant increased basal level of transport, and this was irrespective of the fiber composition of these muscles. Subtle differences between the effects of the HF-SAFF and HF-MIXED diet may be due to the nature of the cellular mechanisms responsible for upregulating basal glucose transport. Kahn and Pedersen (1993) observed reduced muscle GLUT-1 mRNA following 7 weeks of feeding an 80% fat diet to rats while in rats fed a high safflower oil diet (60% kcal) for 4 weeks, Stevenson et al. (1996) reported no change in the amount of GLUT-1. In a different model of insulin resistance, the denervated rat, we have observed increases in basal glucose transport to be accompanied by increases in GLUT-1 expression (Handberg et al., 1996). The cellular mechanisms and / or transporter proteins involved in the upregulation of basal transport remains to be determined in this particular high-fat feeding model. Interestingly, as in the denervated rat model, the increase in basal

transport following high-fat-feeding does not fully restore the muscle's total capacity for glucose transport when insulin is available. This suggests that the basal compensatory mechanisms are only partially capable of offsetting the reduction glucose transport when insulin is present. This may help account for the normal fasting glucose concentrations in the same animals that expressed glucose intolerance following a glucose challenge.

In contrast to its effects in skeletal muscle, the HF-MIXED diet had no adverse effect on insulin-stimulated glucose transport into isolated adipocytes, while HF-SAFF feeding reduced insulin-stimulated glucose transport in isolated adipocytes relative to LF-fed rats. The absolute fold increase in glucose transport following insulin stimulation compared with basal levels of transport was approximately 2 fold, and most likely reflects the combination of the specific incubation conditions used in this study along with properties inherent to cells from animals of this particular size and age fed purified diets. Still, the relative impairment in glucose transport by adipocytes following HF-SAFF feeding but not HF-MIXED feeding suggests that in adipose tissue, unlike skeletal muscle, the total amount of dietary fat was not the important factor affecting glucose transport, but the fatty acid composition of the diet had an important impact on this process. The results here suggest that in the presence of large amounts of dietary fat, glucose transport can proceed normally in adipocytes if fatty acid profiles allow, and points to an interactive effect between fatty acid classes and amount of dietary fatty acid as determinants of glucose transport in fat cells.

Membrane fatty acid composition of adipocytes reflected dietary fatty acids intake after feeding of the experimental diets. Whether changes in fatty acid profiles in adipocytes from rats fed the HF-MIXED diet containing the mixture of saturated, n-6 and n-3 polyunsaturated, and monounsaturated fatty acids leads to changes in numbers of glucose transporters or restores some aspect of the insulin signal transduction - glucose transporter translocation pathway (Kahn et al., 1994) is not known. If the proportion of membrane n-3 fatty acids is important in determining glucose transport in adipocytes, then this study suggests that in adipocytes, unlike in muscle, only a small amount of dietary n-3 fatty acid is required to remedy the impaired glucose transport induced by a high fat diet. Understanding of these observations, as well as their application to humans will require more detailed understanding of the relationships among dietary availability of particular fatty acids, responsiveness of membrane composition to diet, and biochemical or molecular mechanisms controlling incorporation of specific fatty acids into membranes.

In summary, this study demonstrates that dietary fatty acid composition of a high fat diet is critical in determining the effects on tissue-specific insulin resistance and whole body glucose tolerance in rats. When the fatty acid composition of a high fat diet is changed from a single source of predominantly n-6 polyunsaturated fatty acids to include a large portion of oleic acid, short chain saturated fatty acids, and an increased n-3:n-6 ratio, normal glucose tolerance is maintained despite the presence of insulin resistance in skeletal muscle. It is conceivable then that overall glucose tolerance in rats may be sustained when one of the compartments mediating glucose homeostasis becomes less responsive to insulin. Varying the fatty acid composition to the HF-MIXED diet appears to have tissue-

specific effects on glucose transport such that although hormonally regulated glucose transport in skeletal muscle is adversely affected, basal glucose transport in skeletal muscle is increased and glucose transport into adipose tissue is apparently spared. These results underline the importance of examining dietary effects in multiple tissues and at both the cellular and whole body levels in order to gain insight into how diet affects the interaction among tissues to achieve glucose homeostasis. The extent to which our observations are specifically related to a small amount of n-3 fatty acids or other fatty acids such as monounsaturates or some combination remains to be determined, but may be important for understanding the development and reversal of insulin resistance, glucose intolerance and impairment of glucose homeostasis in animal models and in humans.

Chapter 3:

Reduced insulin-stimulated glucose transport in denervated muscle is associated with impaired Akt-1 activation

INTRODUCTION

Skeletal muscle is a major site for insulin-dependent disposal of circulating glucose. In this tissue, insulin signalling to stimulate glucose transport occurs through a network of protein-protein interactions (Holman and Kasuga, 1997; White, 1998) that promote GLUT-4 glucose transporter translocation from intracellular sites to T-tubules and plasma membranes (Dombrowski et al., 1996). IRS proteins (IRS-1 / IRS-2) couple insulin receptor (IR) activity to metabolic events controlled by phosphatidylinositol-3' kinase (PI3-kinase) (Backer et al., 1992 and 1993). PI3-kinase activation is a key signal that promotes GLUT-4 translocation (Cheatham et al., 1994; Quon et al., 1995; Tanti et al., 1996). However, insulin-stimulated PI3-kinase activation alone is insufficient to stimulate glucose transport (Isakoff et al., 1995) implying that additional signalling protein(s) beyond PI3-kinase are involved in stimulating glucose transport.

One target of PI3-kinase is a plecktrin homology domain containing serine / threonine kinase Akt (protein kinase B, or related to A and C (rac) protein kinase). Akt lies downstream of PI3-kinase in the insulin signalling pathway (Burgering and Coffer, 1995; James et al., 1996) and becomes active when phosphatidylinositol-dependent kinases PDK-1 and PDK-2 phosphorylate Akt on specific kinase activation sites (Cohen et al., 1997). Over-expression of constitutively active Akt protein increases GLUT-4 translocation independently from insulin stimulation in 3T3-L1 adipocytes (Kohn et al., 1995) and rat adipocytes (Cong et al., 1997). Insulin-induced Akt-1 activation is thought to promote an increase in surface glucose transporters in insulin responsive cells and tissues (Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997). In the skeletal muscle

from patients with NIDDM the Akt-1 kinase activity is reported to be reduced in concert with a reduction in insulin-stimulated glucose transport (Krook et al., 1998). Furthermore, in insulin resistant skeletal muscle of hyperglycemic Goto-Kakizaki rats, insulin-stimulated Akt-1 kinase activity is also reduced (Krook et al., 1997; Song et al., 1998). However, when the blood glucose concentrations in Goto-Kakizaki rats were normalized with phlorizin treatment, Akt-1 kinase activity was restored and insulin-stimulated glucose transport was improved (Krook et al., 1997; Song et al., 1998). Thus, Akt-1 may be a central component of insulin resistance that can develop in skeletal muscle.

Previous studies (Handberg et al., 1996; Megeney et al., 1993 and 1994; Coderre et al., 1992; Elmendorf et al., 1997; Henriksen et al., 1991; Turinsky and Damrau-Abney, 1987) have shown that when muscle activity is completely eliminated by severing the motor nerve (denervation), insulin-stimulated glucose transport is markedly reduced. The insulin resistance observed in denervated muscle develops rapidly, since insulin-stimulated glucose transport rates are decreased within a few hours after the muscles have been denervated (~6 hours) (Turinsky and Damrau-Abney, 1987). After 3 days of denervation the large reductions in the total GLUT-4 content (up to 80%) (Handberg et al., 1996; Megeney et al., 1993 and 1995) and reduced insulin-stimulated PI3-kinase activity (Elmendorf et al., 1997) can account for a considerable fraction of the reduced insulin-stimulated glucose transport. However, the reduction in insulin-stimulated glucose transport observed after 24 hours of denervation (Elmendorf et al., 1997; Henriksen et al., 1991) occurs before there is a decrease in the total amount of GLUT-4 protein (Block et

al., 1991; Coderre et al., 1992; Henriksen et al., 1991) or PI3-kinase activation (Chen et al., 1993, Elmendorf et al., 1997). To date, no mechanism has been identified to account for insulin resistance in 24 h denervated muscles.

Because Akt-1 has been implicated as a key insulin signalling protein for regulating GLUT-4 translocation, we examined whether the loss of insulin-stimulated glucose transport in short-term (24 h) denervated muscle, in which GLUT-4 content is not altered, is associated with 1) impaired insulin-stimulated Akt-1 activation and 2) reduced GLUT-4 translocation. It is proposed that reductions in muscle activity caused by denervation will induce insulin resistance by disrupting Akt.

METHODS

Animals:

Male Sprague Dawley rats weighing 200-230g were housed in a temperature controlled environment and were maintained on a 12 hour reverse light / dark cycle. The animals were provided with a rat chow diet *ad libitum* and tap water. All experimental procedures were approved by the Committee on Animal Care at the University of Waterloo.

Hindlimb muscle denervations:

The hindlimb muscles in one limb were denervated as we have previously described (Megeney et al., 1993). Briefly, rats were anesthetized under halothane gas after a subcutaneous injection of buprenorphine (0.03 mg/kg) analgesic. A small superficial incision was made on one leg, the sciatic nerve was located, and approximately

3 mm of the nerve was removed. A sham operation was performed on the contralateral leg. Incisions in both legs were closed with surgical clips and a topical disinfectant was applied to the skin. After rats had recovered (within one hour) food and water were provided. Experiments were performed 24 hours later on overnight fasted rats.

Animal preparation:

Rats were anesthetized with 65 mg/kg sodium pentobarbitol, placed on a heating pad at a low temperature setting, and hindlimb muscles were exposed by carefully cutting free the exterior skin. Soleus muscles were removed for determination of glucose transport and surface GLUT-4 measurements. Approximately 15 minutes later the descending aorta in rats was injected with 0.1ml saline (+0.1% BSA) plus insulin or the equivalent volume of saline (+ 0.1% BSA) alone, followed by a rapid dissection of tibialis anterior (TA) muscle at two specific time points (3 and 5 minutes) in different experiments. After both, the denervated and sham TA muscles were dissected from hindlimbs the red TA (RTA) portion was quickly separated from the white TA compartment and immersed in liquid N_2 and kept at -80°C for later use

INSULIN-STIMULATED GLUCOSE TRANSPORT

Glucose transport in incubated soleus strips:

Soleus muscles were cut into thin lengthwise strips suitable for *in vitro* incubations (Bonen et al., 1994). Strips were pre-incubated for 1 hour at 29 °C in Krebs-Henseleit buffer (KHB) with 32mM mannitol, 8mM D-glucose, and 0.1% bovine serum albumin (BSA). Porcine insulin was added to the incubation medium at a maximal stimulating concentration of 400μU/ml for 30 minutes. Prior to glucose transport measurements, D-glucose was removed by washing strips twice for 5 minutes each in a glucose free KHB

with 38 mM mannitol and 2 mM pyruvate. The uptake of 2-deoxyglucose was determined with 2mM pyruvate, (1.5μCi) 2-deoxy-D- [^{3}H]glucose (1 mM) and (0.1 μCi) ^{14}C-mannitiol (37mM) for 10 minutes. Strips were removed rapidly, rinsed in 0.9% ice-cold saline, cut free of tendons and snap frozen in liquid N_2. Muscles were stored at -80 °C until analyzed for ^{14}C and ^{3}H in digested muscle extract (Bonen et al., 1990 and 1992).

GLUT-4 AND AKT-1 DETERMINATION BY WESTERN BLOTTING

Total Akt-1 and total GLUT-4 protein:

For GLUT-4 determinations, soleus muscle was prepared as described elsewhere (Hansen et al., 1998; Johannsson et al., 1996). For Akt-1 determinations RTA muscles (50-80 mg) were homogenized in ice-cold homogenization buffer (50mM TRIS pH 7.5, 110 mM sodium tetra-pyrophosphate, 11mM EDTA, 110 mM sodium fluoride, 10 mM sodium orthovanadate, 1% triton-X-100, 200 mM PMSF, 10 mg/mL aprotinin, 1mg/mL leupeptin, and 1 mg/mL pepstatin A). Western blotting was used to determine GLUT-4 and Akt-1 content. Briefly, samples were separated by SDS-polyacrylamide electrophoresis on a 12% gel and transferred to an immobilon membrane by electromembrane transfer for 90 minutes. Membranes were blocked overnight in 5% non-fat-dry milk (NFDM) made in Tris-buffered saline (TBS, pH 7.6). Proteins were detected by incubating blocked membranes with an anti-GLUT-4 polyclonal immuno-A purified antibody (1:7000) or anti-rat Akt-1 antibody (1:1000) followed by horse radish peroxidase (HRP)-conjugated anti-rabbit IgG diluted (1:2000) in TBS, pH 7.6, for GLUT-4 detection, or HRP-labeled anti-sheep IgG diluted (1:2000) in 5% NFDM for Akt-1 detection. GLUT-4 and Akt-1 were visualized using an enhanced chemiluminescence

system according to the manufacturer's instructions. Western blots were quantified using a Macintosh LC with an Abaton scanner and appropriate software.

Surface detectable GLUT4:

The method for detection of surface GLUT-4 in skeletal muscle was adapted from a procedure used with rat adipocytes (Czech and Buxton, 1993; Wilkes et al., 1996). This method is based on the principle that exogenous trypsin cuts surface accessible GLUT-4 at a predicted trypsin cleavage site in the exofacial loop of the GLUT-4 transporter. Trypsin cleaved GLUT-4 appears on a blot at a lower molecular weight than native GLUT-4 protein when the fragmented GLUT-4 is detected with antibodies to the GLUT-4 carboxy-terminus (Czech and Buxton, 1993). GLUT-4 fragments migrate to 35 kDa instead of the 46 kDa position of non-trypsinized native GLUT-4. We have previously used this procedure to detect surface GLUT-4 in adipocytes (Wilkes et al., 1996).

To apply the method to skeletal muscle, soleus muscles were cut into strips and pre-incubated in vials with KHB for 45 min at 35°C. Insulin (400μU/ml) was added to half the vials for 10 minutes. All vials received 2mM KCN for another 20 minutes to stop surface membrane proteins recycling with internal protein pools. Strips were treated with 1 mg/ml trypsin and continued to incubate for 30 more minutes. Strips were washed (2 X 5 minutes) in a buffer free of exogenous trypsin with 1 mg/ml soybean trypsin inhibitor (SBTI) added, then quickly frozen in liquid N_2 and stored at -80 °C for later use. Strips were prepared with the standard homogenization protocol described above for GLUT-4 detection by Western blotting except the homogenization buffer also contained 1 μg/ml

SBTI. Blots were quantified using a scanner connected to a Macintosh computer with appropriate software.

Akt-1 serine^{473} phosphorylation with in vivo insulin treatment:

Saline and insulin injected rats were used to determine basal (non-insulin-stimulated) and activated Akt-1 [SER^{473}] phosphorylation levels respectively. Muscles were homogenized in ice-cold buffer 1 (150 mM NaCl, 50 mM TRIS pH 7.5, 30 mM sodium pyrophosphate, 10 mM sodium fluoride, 1mM dithiothreitol (DTT), 10%v/v glycerol, 1% triton-X-100, 1 mg/mL bacitracin, 200 mM PMSF, 10 mg/mL aprotinin, 1mg/mL leupeptin, 1 mg/mL pepstatin A, 1 μM microcystin). Homogenates were spun at 15,000 rpm (70Ti, Sorvall) for 1 h at 4°C. Samples were resolved on a 6% gel by SDS-PAGE and transferred to an immobilon membrane by electromembrane transfer for 90 minutes. Membranes were blocked in 5% non-fat dry milk (1 hour), probed with anti-phospho[SER^{473}]-Akt-1 antibody (1 hour) followed by HRP-labeled anti-sheep IgG secondary antibody (1 hour). Phospho[SER^{473}]Akt-1 was visualized and quantified as described above.

AKT-1 KINASE ACTIVITY

Measurements of Akt-1 kinase activity in vivo:

Akt-1 kinase activity was determined as described by Krook et al. (Krook et al., 1997). For these experiments, rats were injected with 0.1U or 1U insulin to generate physiological or supraphysiological plasma insulin concentrations, respectively. RTA muscles were dissected 5 minutes after injection, at a time when insulin injections are

known to maximally activate muscle Akt-1 (Walker et al., 1998). Therefore, the dissection was completed rapidly (within 1 minute) and muscles were frozen in liquid N_2 quickly. To determine plasma insulin levels from injected rats, 3 blood samples of ~0.1ml each were withdrawn. Muscles were homogenized as described above for Akt-1 [SER ^{473}] phosphorylation measurements. Anti-Akt-1 antibody was agitated with protein-G sepharose beads in Buffer 1 for 1 hour to form an anti-Akt-1-protein-G immune complex. Aliquots of muscle homogenate were rotated with protein-G sepharose beads (50µl/ml) and pulsed in a bench top Sorvall (12, 000g) to reduce non-specific binding. The activated Akt kinase was immunoprecipitated from pre-cleared sample (500µg) by rotation with the anti-Akt-protein-G immune complex overnight at 4°C. Akt-1 protein immunoprecipitates were collected by centrifugation, washed 4 X in buffer 2 (1 M NaCl, 25 mM HEPES pH7.6, 1 mM DTT, 0.1% BSA, 10% glycerol, 1% tritonX-100) to remove unbound protein and washed 2 times in kinase buffer 3 (50mM TRIS pH 7.5, 10mM $MgCl_2$, 1 mM DTT). The final buffer was carefully removed, beads completely dried and resuspended in 30µl kinase buffer 3 supplemented with 100µM cold ATP, 2µCi [γ-^{32} P]ATP, 17nM protein kinase A inhibitor, and 100µM Akt-specific crosstide peptide (GRPRTSSFAEG). After 30 minutes gentle shaking at 30°C reactions were terminated by placing tubes into ice. [^{32}P]-incorporation into crosstide peptide was determined by resolving 25 µl reaction mixture on a 40% urea-based acrylamide gel. Autoradiographs were generated by exposing the dry gel to scientific imaging film X-OMAT™ AR and the band corresponding to the peptide substrate was quantified by densitometry.

Protein Assay:

All protein concentrations were determined in triplicate by the bicinchoninic acid assay using BSA as a standard.

Plasma insulin:

Insulin levels were determined using an insulin radioimmunoassay kit by following the manufacturer's directions.

Statistical Analysis:

Data were analyzed using one-way and two-way analyses of variance as well as t-tests. as was appropriate for a given experiment. All data are reported as means ± SEM.

RESULTS

GLUCOSE TRANSPORT:

Glucose transport was determined in soleus muscles *in vitro.* In addition, incubated control and denervated soleus muscles were also used to compare total GLUT-4 and the surface GLUT-4 in basal and insulin-stimulated muscles.

Glucose transport in incubated soleus strips. In the absence of insulin (basal) 2-deoxyglucose transport was not different between 24 hour denervated and control soleus muscle ($P>0.05$) (Fig. 3.1). Insulin treatment increased soleus muscle glucose transport; 2.2-fold in control ($P<0.01$) but only 1.7-fold in denervated soleus muscles (28% reduction; $P<0.01$) (Fig.3.1). In a separate experiment, when insulin-stimulated glucose transport was determined by hindlimb perfusion the denervated RTA muscle showed a

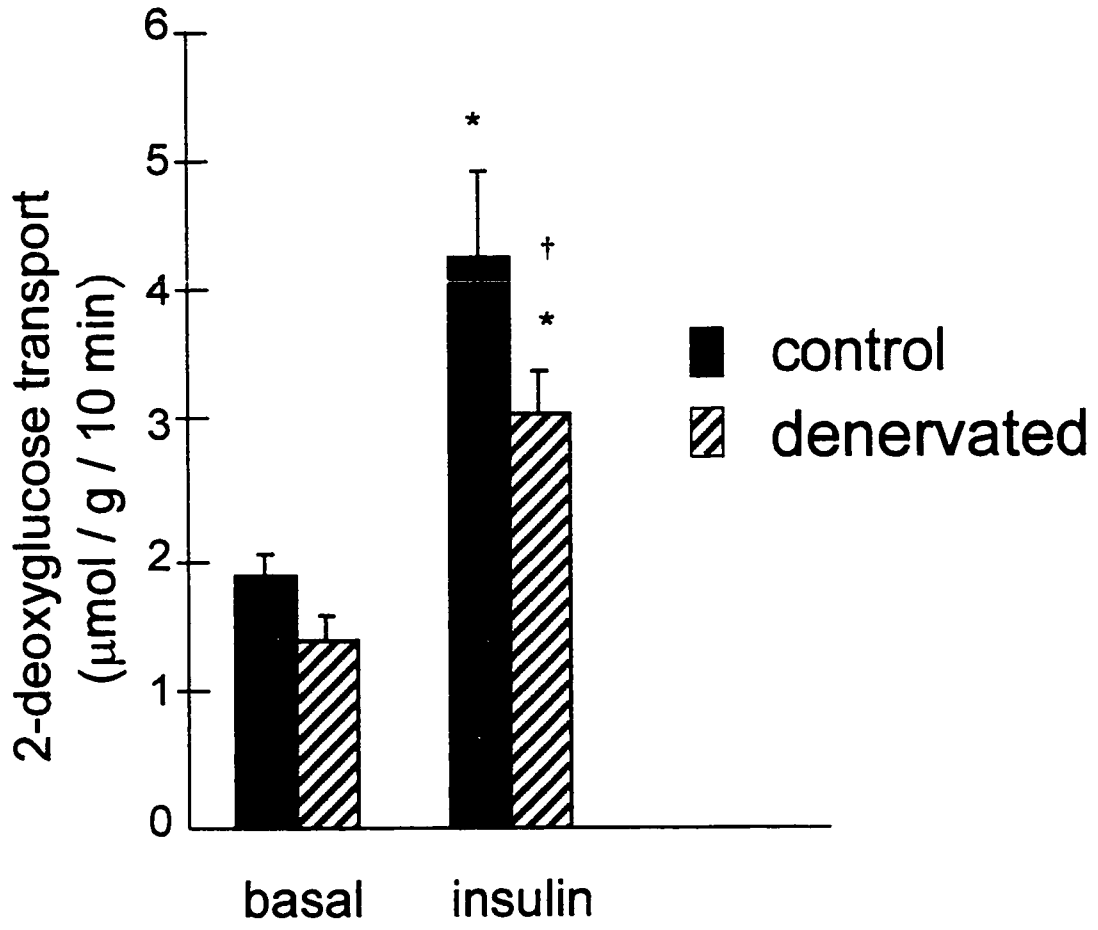


Figure 3.1. Insulin-stimulated glucose transport in vitro in soleus muscle from 24 hour denervated rats. Values are means ± SEM. n = 8-12 soleus strips for basal and insulin-treated muscle from denervated and control legs.

*Insulin-treated groups are significantly different than their respective basal $P<0.01$.

†, Denervated group (hatched bars) treated with insulin alone compared to same treatment in control group (solid bars) $P<0.05$.

similar reduction (-27%) in glucose transport (4.0 ±0.28 vs. 2.92 ±0.21μmol / g / 5 minutes, $P<0.05$).

INSULIN SIGNALLING THROUGH AKT-1:

Insulin was injected *in vivo* to activate muscle Akt-1. Basal Akt-1 kinase activity (Fig. 3.2) was not significantly different than non-specific activity, (i.e. non-specific = enzyme activity measured in protein-G beads which were not conjugated with the Akt-1 antibody (personal observations)). Basal Akt-1 kinase activity was set at 100% for both groups.

Insulin-stimulated Akt-1 kinase activity:

Plasma insulin concentrations were elevated to high physiological levels or to supraphysiological levels following insulin injections (Table 3.1). In control muscle, 0.1 U insulin injections increased Akt-1 kinase activity (Fig. 3.2) 2.3-fold ($P<0.05$) while 1U insulin injections increased Akt-1 kinase activity 3.4-fold ($P<0.01$). In denervated muscle, Akt-1 kinase activity was found to be 22% lower ($P<0.05$) and 26% lower ($P<0.01$) after 0.1 U insulin and 1 U insulin injections respectively.

Akt-1 [SER^{473}] phosphorylation and total immunoreactive Akt-1 protein:

Insulin- stimulated Akt-1 phosphorylation occurred in a time-dependent manner (Fig. 3.3). Phospho[SER^{473}] Akt-1 protein level was significantly higher in control muscle at 3 minutes ($P<0.05$) than denervated muscle. In addition, the total Akt-1 protein (Fig. 3.4) was decreased (-37%) in denervated muscle ($P<0.05$).

Table 3.1. Plasma insulin before and 7 minutes after exogenous insulin administration by intravascular injection (mean ± SEM)

	plasma insulin (μU/mL)	
Injection treatment	Pre	Post
Saline (n=3)	2.8±1.6	0.7±0.2
0.1 U insulin (n=6)	4.8±0.9	101±30.6[a]
1 U insulin (n=6)	4.1±1.1	5334±2081[a,b]

[a] $p<0.05$ pre vs. post
[b] $P<0.05$ 0.1 U post vs. 1 U post
sample size indicated (n)

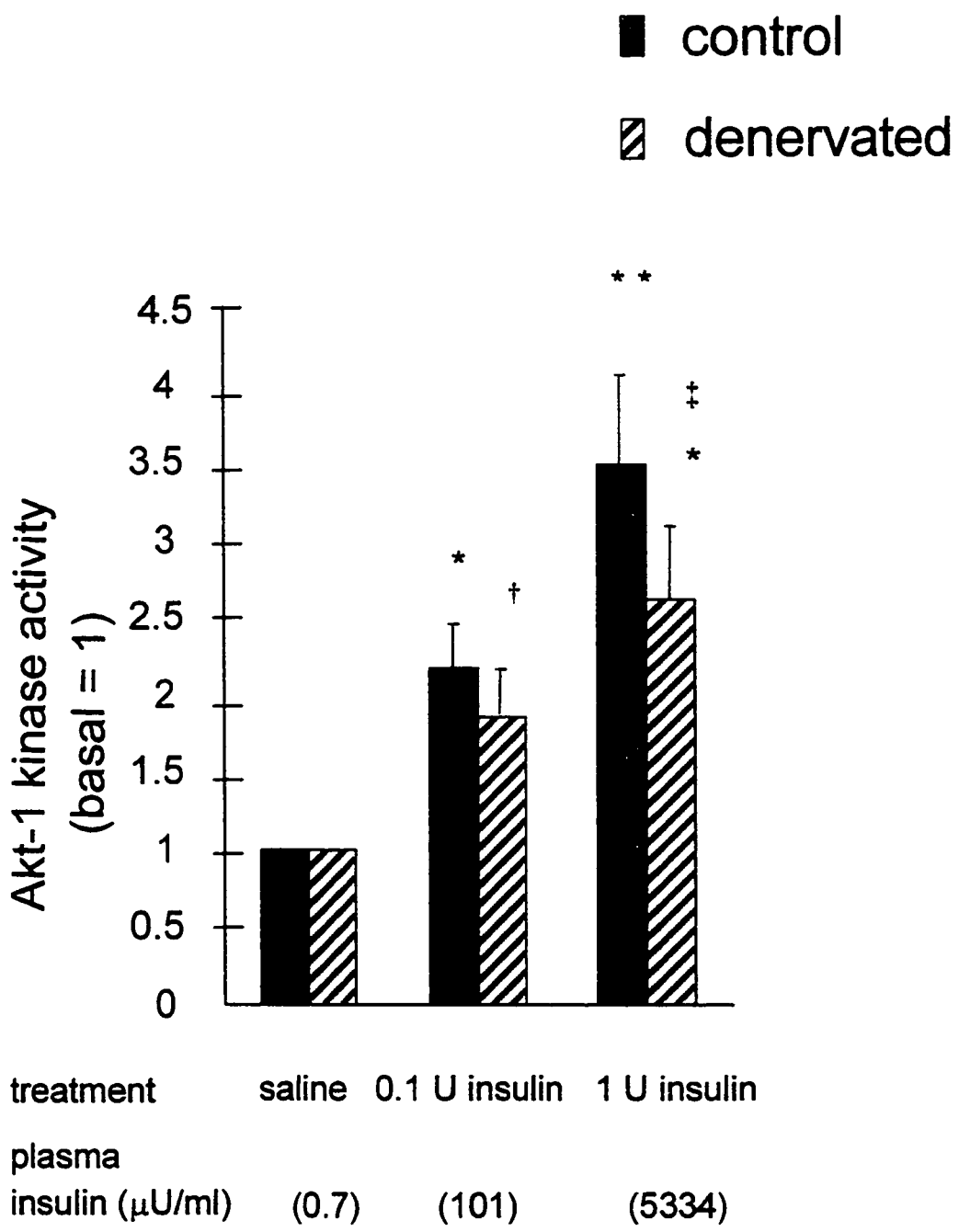


Figure 3.2. Insulin activation of Akt-1 kinase in vivo in RTA muscle from 24 hour denervated rats. Saline treatment is set = 1 for both groups (n = 3). Significant differences are denoted *P<0.05, **P<0.01 vs. saline. For insulin-treated animals, data from insulin treatments are paired (n=6). Means (±SEM) in control group are different from denervated group †P<0.05, ‡P<0.01.

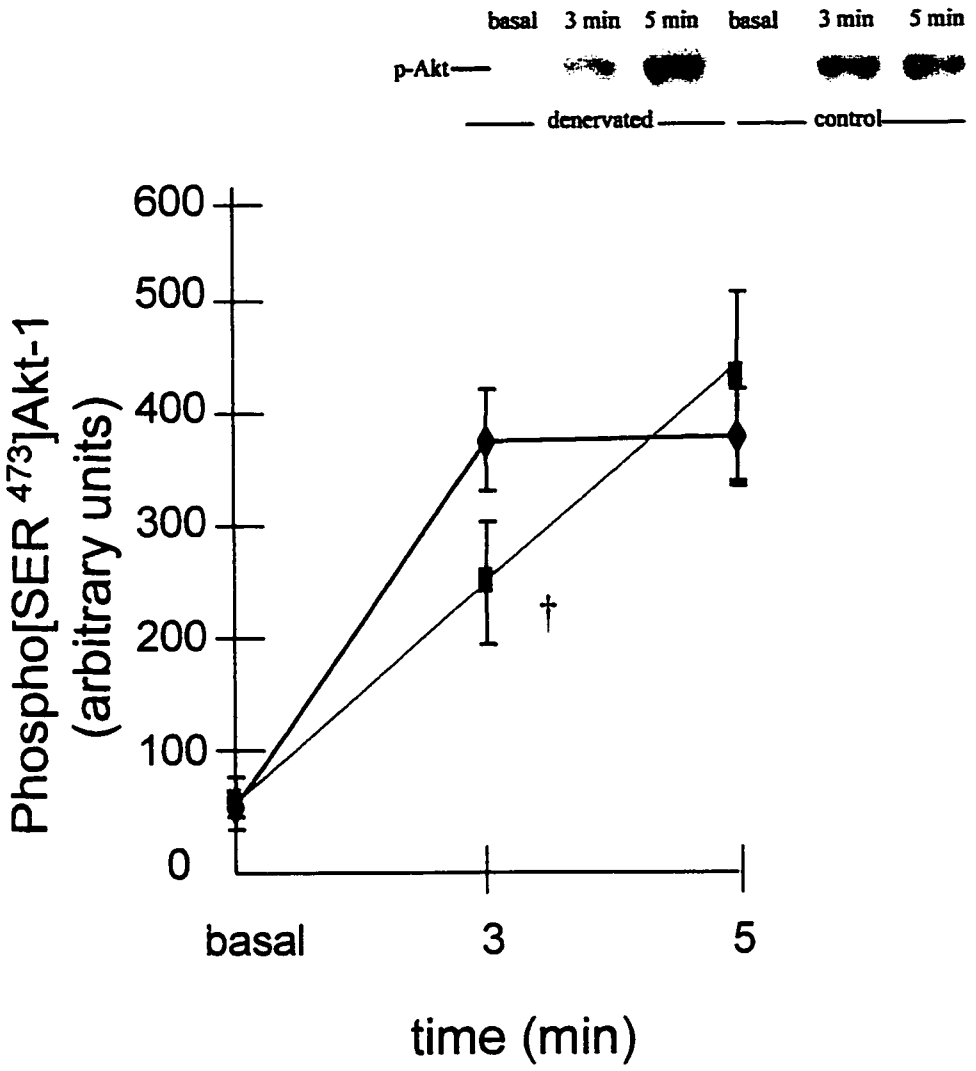


Figure 3.3. The effect of insulin injections on Akt-1 SER ^{473} phosphorylation in RTA muscle from 24 hour denervated rats.

Blot shows Akt-1 SER ^{473} bands from one representative experiment. The Akt-1 SER ^{473} band is not detectable in saline-injected animals. Basal arbitrary scanning units represent background. Values are represented as means ± SEM of arbitrary scanning units. n = 6 RTA muscles at 3 minutes, n = 3 RTA muscles at 5min.

Control (triangles) and denervated (squares) groups are significantly different at the time point denoted, †$P<0.05$.

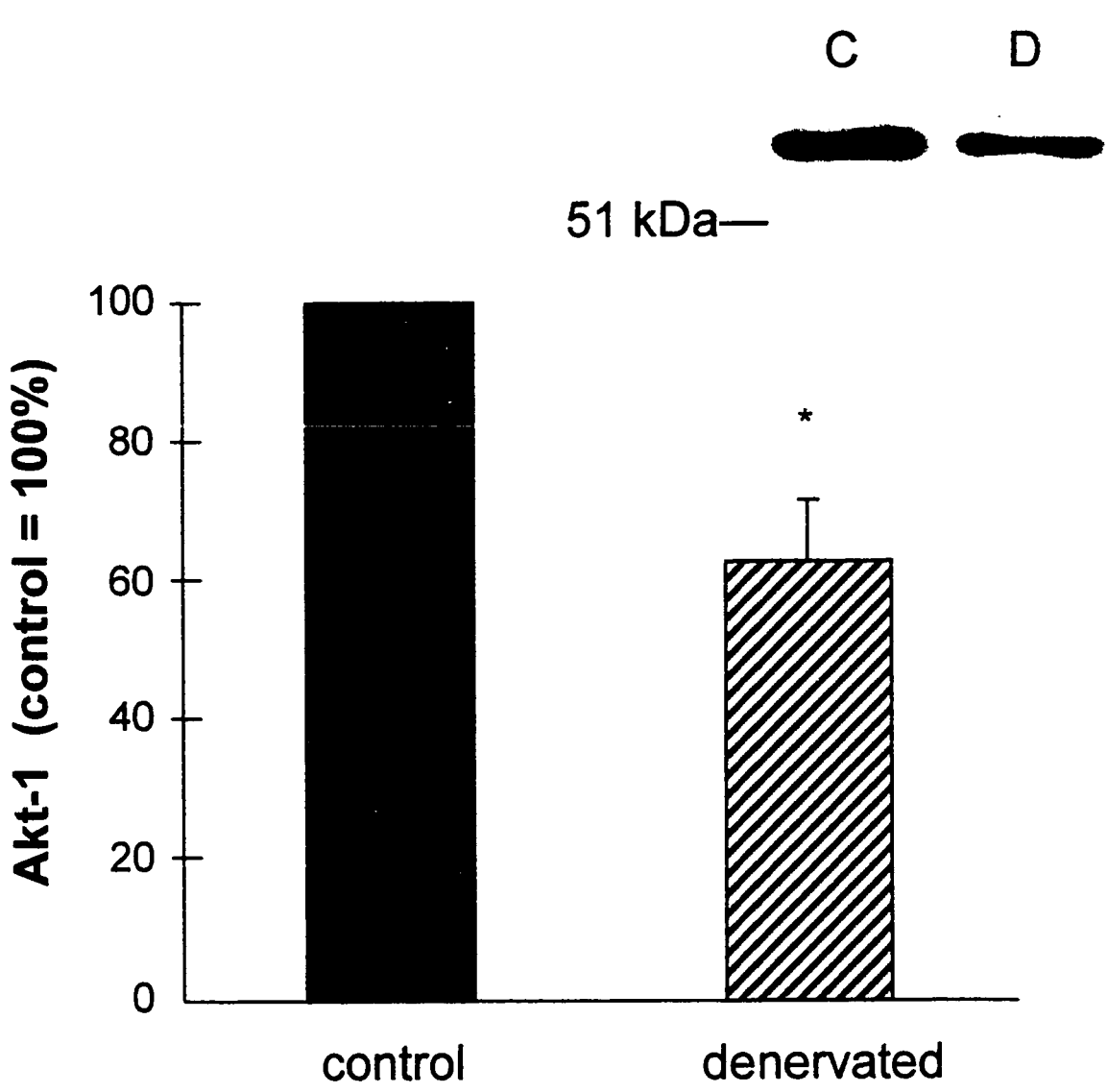


Figure 3.4. Total immunoreactive Akt-1 protein in RTA muscle from 24 hour denervated rats. (mean ± SEM) *P<0.05, control vs. denervated (n=6).

TOTAL GLUT-4 AND TRANSLOCATED SURFACE GLUT-4

Soleus GLUT-4 availability: surface detectable GLUT-4 vs. total GLUT-4.

The level of total GLUT-4 was comparable in denervated and control soleus muscle ($P>0.05$) (Fig. 3.5A). In a separate experiment total GLUT-4 in RTA muscle was also not different in denervated muscle compared to control muscle (personal observations). The increased surface GLUT-4 availability after insulin stimulation (translocated GLUT-4) was determined by a trypsin cleavage method (Fig. 3.5B). Insulin increased surface GLUT-4 1.8-fold ($P<0.05$) in control muscle where as no change in surface GLUT-4 was detected in denervated muscle (Fig. 3.5C).

DISCUSSION

This study has shown that insulin-stimulated glucose transport is decreased (Fig. 3.1) in short term (24 h) denervated muscle. This decrease is not due to a reduction in total GLUT-4, consistent with previous work (Block et al., 1991; Coderre et al., 1992). Instead, denervation-induced insulin resistance is attributed to an impairment in Akt-1 activation which is associated with a concomitant reduction in insulin-stimulated GLUT-4 translocation.

Insulin activates Akt-1 through earlier signals that first activate PI3-kinase (Burgering and Coffer, 1995). Increasing Akt-1 kinase activity *in vivo* is accomplished by the phosphatidylinositol-dependent kinases (PDK-1 and PDK-2) (Coffer et al., 1998). PDK-1 and PDK-2 are proposed to phosphorylate THR^{308} and SER^{473} respectively on

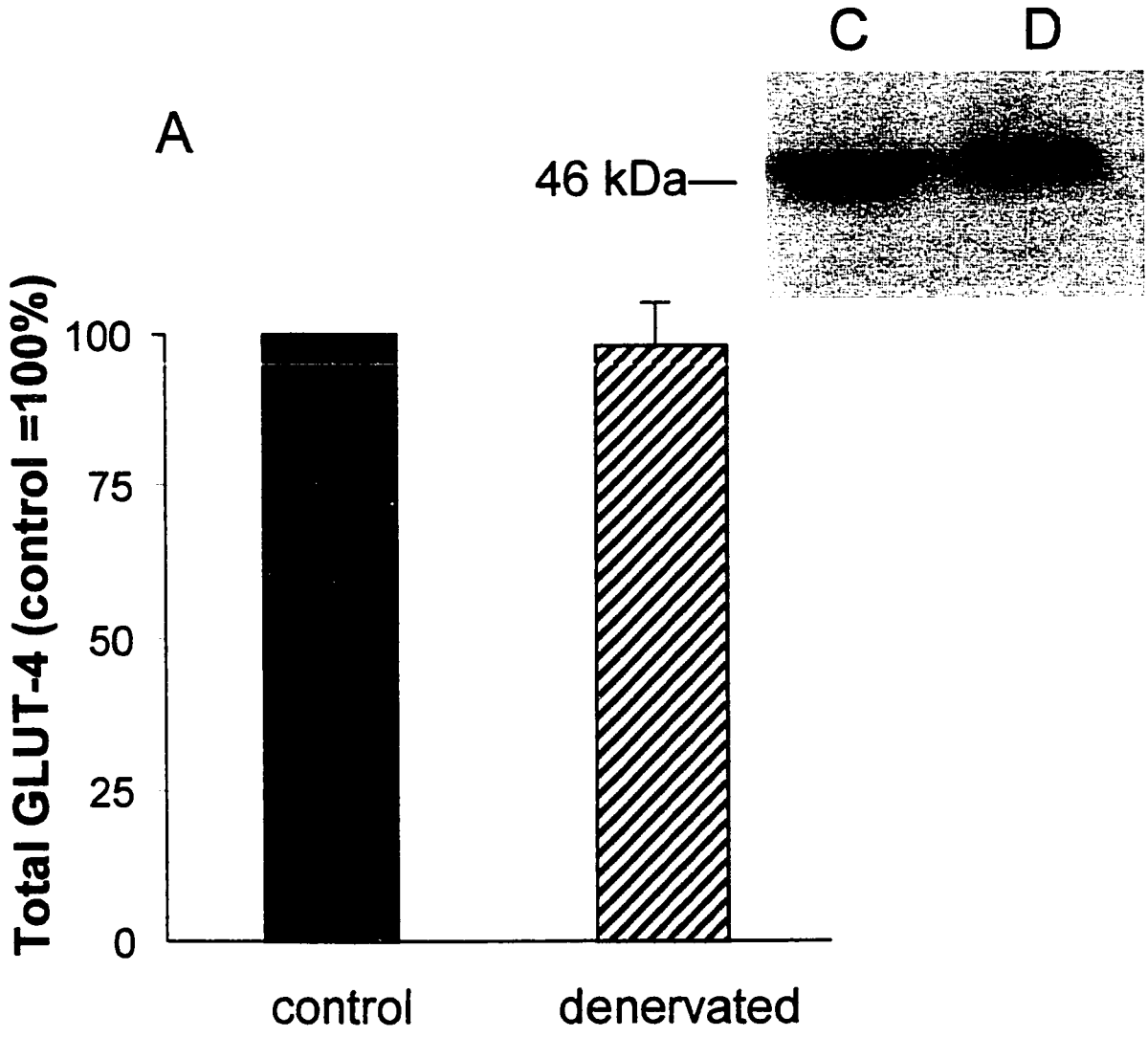
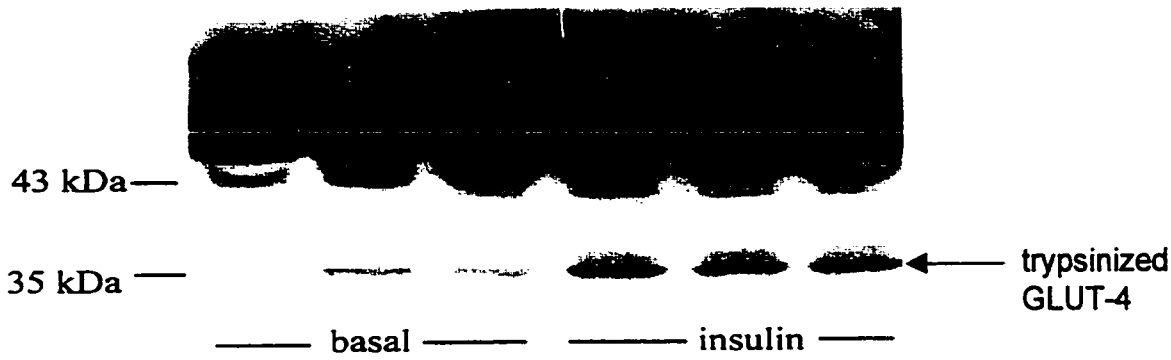


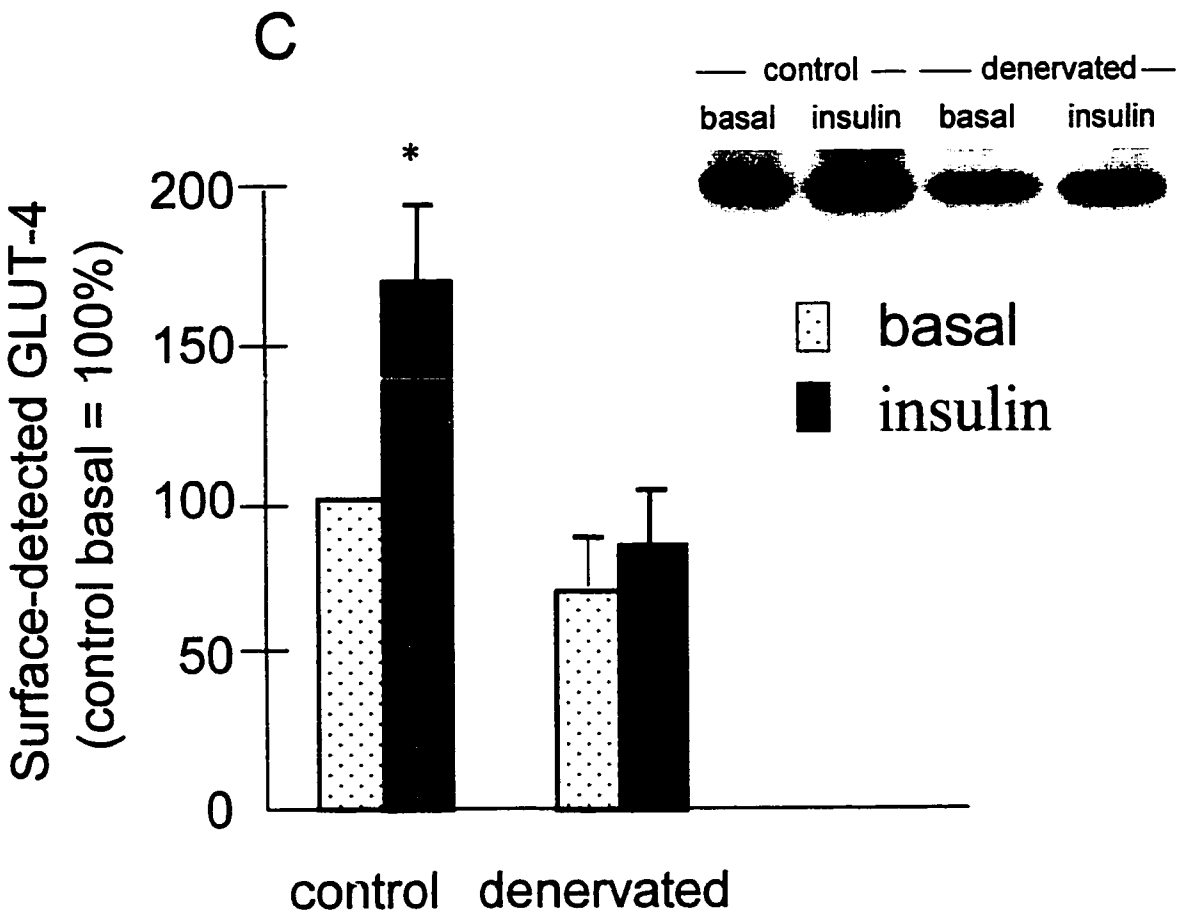
Figure 3.5. Trypsin-accessible surface GLUT-4 and total GLUT-4 in soleus muscle from 24 hour denervated rats.

A) Total GLUT-4 in control (C) and denervated (D) soleus muscles, n = 5 ($P>0.05$). Means ± SEM.

B



B) The trypsin cleavage method determines the effect of insulin on surface GLUT-4 availability (see methods). Anti-GLUT-4 immunoreactive bands at ~46kDa represent native GLUT-4 (overexposed bands) and at 35kDa represent surface (trypsin-accessible) GLUT-4 in muscles from control animals: (basal, lanes 1-3 and insulin, lanes 4-6). Trypsin-accessible GLUT-4 was significantly increased (+97%) by insulin ($P<0.05$).



C) Quantification of scanned trypsin-cleaved bands (means ± SEM). *P<0.05 basal vs. insulin, n = 8-10 soleus strips for basal and insulin-treated muscle from denervated and control legs.

Akt-1 (see review by Coffer et al., 1998). Intravenous insulin injections increase Akt-1 kinase activity in rat muscle until maximum activity is seen after 5 minutes (Walker et al., 1998). In our studies, maximal Akt-1 kinase activity was observed after 3 minutes in control muscle (personal observations).

Recently, Akt-1 activation has been implicated as a key step in insulin signalling in skeletal muscle (Krook et al., 1997 and 1998; Song et al., 1999). Two studies (Krook et al., 1997; Song et al., 1999) showed that the decrease in insulin-induced Akt-1 activity and glucose transport was due to a prevailing hyperglycemia because both Akt-1 activity and glucose transport were restored to control levels once blood glucose was normalized. Moreover, decreases in Akt activity and glucose transport activation, reportedly caused by hyperglycemia (Kurowski et al., 1999), may be due to changes in insulin signalling events downstream from PI3-kinase. Kurowski et al. (1999) showed that with hyperglycemia insulin-stimulated glucose transport is reduced in skeletal muscle (-30%). In these muscles, phosphotyrosine- immunoprecipitated PI3-kinase activity and both IRS-1 and -2 associated PI3-kinase activity were not altered while marked reductions in Akt activation (-60%) were observed. Therefore, insulin resistance in skeletal muscle, in some instances (i.e. during hyperglycemia), may be a consequence of reducing the signals from Akt.

Similar to hyperglycemia, the insulin resistance induced by denervation may involve a defect beyond PI3-kinase. In previous studies there appeared to be no defects in insulin signalling pathways (up to PI3 kinase) in short term (24 h) denervated muscles (Chen et al., 1993; Elmendorf et al., 1997; Wilkes and Bonen, 1998). Earlier data support

the idea that PI3-kinase activation occurs normally in muscles denervated for 24 hours, since we found that the amount of insulin-stimulated PI3-kinase regulatory subunit (p85) immunoprecipitated by anti-phosphotyrosine antibodies was not decreased by denervation (Wilkes and Bonen, 1998). However, data here show that muscle denervation decreased insulin-stimulated Akt-1kinase activity (Fig. 3.2). This decrease is attributable to an impaired Akt-1 activation. Denervation decreased the rate (-36%) of muscle Akt-1 phosphorylation (Fig. 3.3) and muscle Akt-1 protein (-37%) content (Fig. 3.4). Based on this work, it may be that an inadequate insulin signalling response through Akt-1 may contribute to the insulin resistance in 24-hour denervated muscles.

The lower insulin-stimulated Akt-1 kinase activity induced by denervation could have occurred if impairments to the insulin signalling mechanism, prior to Akt-1 in the insulin signalling pathway, contributed to impairments in Akt-1 activation in denervated muscle. But, since insulin receptors (IR) bind insulin with a normal affinity (Burant et al., 1984) and denervation does not appear to affect insulin-activated IR tyrosine kinase activity 24 hours after muscle denervation (Elmendorf et al., 1997), it is unlikely that the reduced Akt-1 activation in 24-hour denervated muscle was due to poor IR activation. Moreover, our data show that supraphysiological plasma insulin concentrations did not further increase Akt-1 kinase activity in the denervated muscle (Fig. 3.2). Rather, the reductions in Akt-1 kinase activity (~25 % lower) were similar at both high physiological and supraphysiological insulin concentrations. Thus, the lower insulin-stimulated Akt-1 activation response in the denervated muscle was more likely due to a signalling change at a post-IR level rather than from insufficient IR tyrosine kinase activation.

Another possibility is that denervations had uncoupled upstream signals from Akt-1 that would impair the ability of insulin to activate Akt-1 *in vivo*. Since insulin-stimulated PI3-kinase activity is not reported to be affected in muscle denervated for 24 hours (Chen et al., 1993; Elmendorf et al., 1997; Wilkes and Bonen, 1998), PI3-kinase-activated signals required to activate Akt-1 *in vivo* were likely sufficient in denervated muscles. Furthermore, PDK-2 was unlikely to be affected by denervation, since Akt-1 [SER^{473}] phosphorylation still occurred in denervated muscle, albeit at a slower rate (Fig. 3.3). It is more likely that reductions in insulin-stimulated Akt-1 kinase activity observed in 24-hour denervated muscle were a consequence of lower Akt-1 protein availability in these muscles (Fig. 3.4). Interestingly, Akt-1 SER^{473} phosphorylation in denervated muscle reached control levels by 5 minutes. Since phosphorylation on THR^{308} also contributes to Akt-1 activation (Alessi et al., 1996), and both the SER^{473} and THR^{308} sites require phosphorylating to obtain full Akt-1 kinase activation (Alessi et al., 1996), these data suggest that a diminished THR^{308} phosphorylation on Akt-1 may account for the reduced Akt-1 kinase activation in 24-hour denervated muscles.

Conceivably, an impairment in Akt-1 activation in denervated muscles may attenuate signals which normally induce GLUT-4 translocation. Notably, there was a discrepancy in the % reduction between insulin-induced Akt-1 activation (-25%) and GLUT-4 translocation. It is possible that additional insulin-activated mechanisms such as GLUT-4 trafficking or GLUT-4 membrane insertion steps were also affected by denervation.

The reduction in glucose transport in denervated muscle was less than would have been expected given that GLUT-4 at the surface was not increased by insulin. Undoubtedly, reduced GLUT-4 translocation contributed to insulin resistance in our studies. A number of studies have shown that glucose transport into the cell occurs in direct proportion to the surface GLUT-4 (Lund et al., 1993 and 1995). However, insulin may also stimulate glucose transport without necessarily increasing the number of surface GLUT-4 transporters (Sweeney et al., 1999). A variety of more recent studies have revitalized the idea that the intrinsic activity of surface GLUT-4 can be increased or decreased (Dauterive et al., 1996; Fisher and Frost, 1996; Han and Bonen, 1998; Hansen et al., 1996; Quon et al., 1995; Sweeney et al., 1999). Published work from our laborartory has shown that epinephrine can markedly lower glucose transport despite an insulin-induced increase in plasma membrane GLUT-4 (Han and Bonen, 1998).

The results obtained in the soleus and red TA muscles with different insulin administration procedures can be integrated. It has previously been shown that the soleus and plantaris muscles, with markedly different fiber types, respond similarly to denervation (Turinsky and Damrau-Abney, 1998). Two highly oxidative muscles such as the soleus and red TA would therefore be expected to respond similarly to denervation. Moreover, both muscles here were exposed to maximally stimulating concentrations of insulin, either *in vitro* (soleus) or *in vivo* (RTA). Nevertheless, it is recognized that the delivery of insulin differs *in vivo* and *in vitro*. Therefore, the extent of GLUT-4 translocation or

signalling per gram of muscle may be different between these two means of providing insulin.

In summary, these data demonstrate that there is partial reduction in insulin-stimulated glucose transport along with a similar partial reduction in insulin-stimulated Akt-1 activity in denervated muscle. These data indicate that short-term (24 hours) denervation decreased Akt-1 activation and impaired insulin-stimulated GLUT-4 appearance at the muscle cell surface.

Chapter 4:

Glut-1 levels and Akt activation are increased in rat muscle made insulin resistant by high-fat-feeding: compensatory changes in basal glucose transport

INTRODUCTION

In an earlier study (Chapter 2) it was found that rats became glucose intolerant after they consumed a diet high in safflower-oil (HF-SAFF) relative to two other groups of rats fed on either a high-mixed (safflower, sunflower, coconut oil)-based diet (HF-MIXED) or a low fat (LF) diet. It was also observed that there was a reciprocal effect on glucose transport regulation, since insulin-stimulated 3-O-methylglucose (3-OMG) transport was decreased and basal 3-OMG was found to be increased in all groups of skeletal muscle examined. Furthermore, high-fat diets had different tissue-specific effects on insulin-stimulated glucose transport. Adipose cells isolated from the epididymal fat of HF-MIXED-fed rats had retained normal sensitivity to insulin, while fat cells from HF-SAFF became insulin resistant (Wilkes et al., 1998). Based on these observations it was suggested that the normal whole body glucose tolerance in HF-MIXED-fed rats (Chapter 2) occurred as a consequence of multiple physiological changes, not only due to sustained insulin responsiveness of adipose cells, but also as a result of the increased rate of basal glucose transport in skeletal muscle. These early findings reported in Chapter 2 could indicate that high fat diets might have a differential effect on the glucose transporters in muscle which regulate basal and insulin-stimulated glucose transport in an attempt to compensate for muscle insulin resistance. This prompted the current studies which investigate the possibility that basal 2-deoxyglucose transport is up-regulated in response to high-fat diets that are known to decrease insulin-stimulated 3-OMG transport in skeletal muscle.

Glucose transport is regulated by a variety of GLUT transporter proteins (reviewed by Mueckler, 1994). In muscle GLUT-4 generally resides in an intracellular pool and translocates to the cell surface following insulin stimulation (Lemieux et al., 2000). GLUT-1 is believed to be confined to the plasma membrane where it facilitates basal glucose transport in muscle tissue (Marette et al., 1992). Much is known about the response of insulin-stimulated glucose transport and GLUT-4 under a variety of physiological conditions, while less is known about changes in basal glucose transport and GLUT-1. An increase in GLUT-1 protein expression has been shown to occur in muscle made insulin resistant by denervation for 3 days (Handberg, 1996; Megeney et al., 1994; 1995). Moreover, low glucose availability appears to be a positive regulator of GLUT-1 protein expression, since a reduction of glucose in media of cultured 3T3-L1 adipocytes has been shown to increase GLUT-1-mediated glucose transport (Tordjman, 1990). In early developing pigs, the provision of a low energy diet was found to increase GLUT-1 gene expression in skeletal muscle (Katsumata et al., 1999).

Akt has been suggested to promote increases in the quantity of cell surface glucose transporters by inducing the translocation of GLUT-4 (Kohn et al., 1995; Wang et al, 1999; Cong et al., 1997; Tanti et al., 1997) and by upregulating GLUT-1 biosynthesis (Harjduch, Barthel et al. 1999, Taha et al., 1999) (see Fig. 4.1). Therefore, Akt could have a central signalling role in mediating cell surface glucose transporter availability in different ways by promoting a) GLUT-4 translocation and b) GLUT-1 biosynthesis.

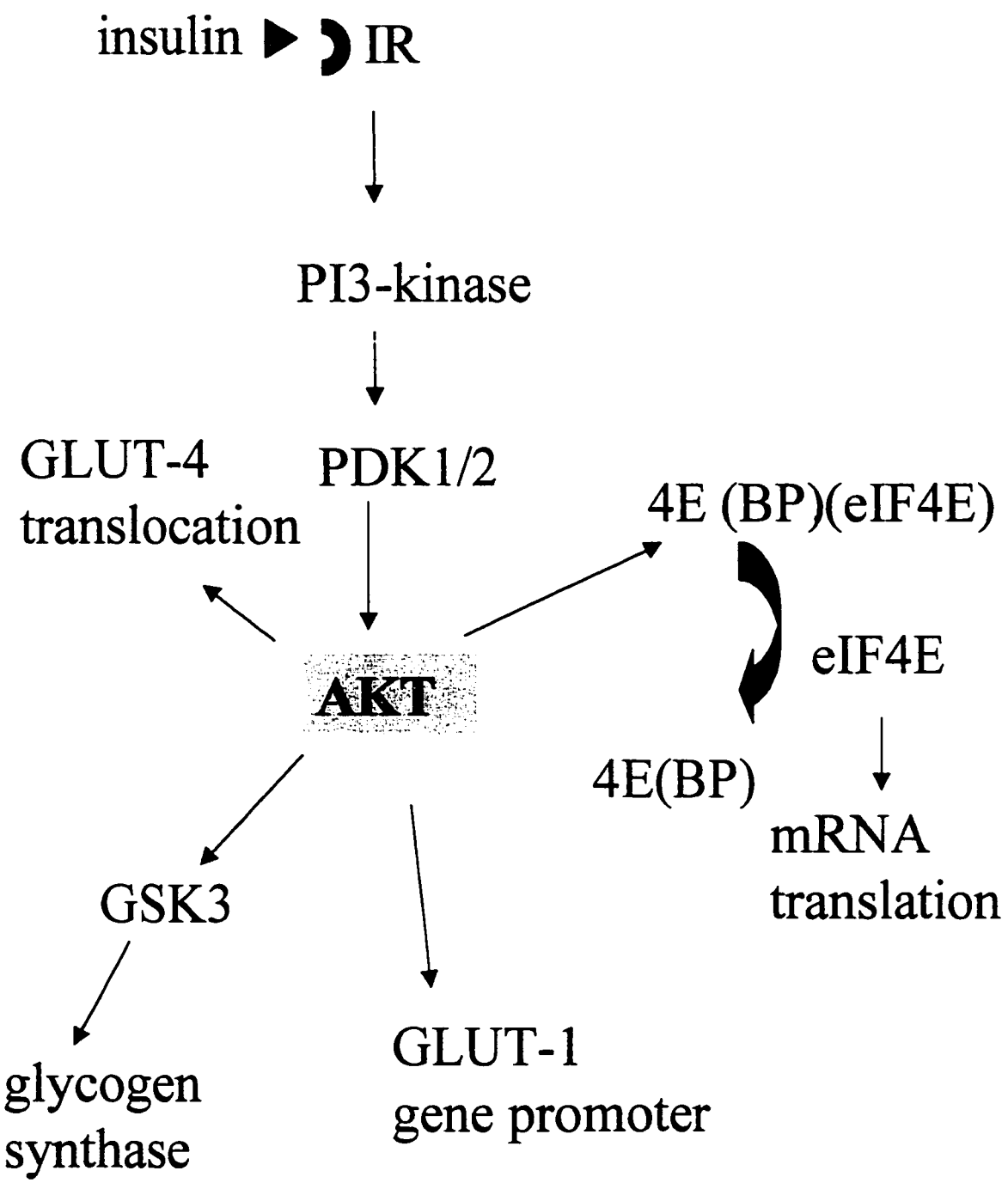


Figure 4.1. Akt is found within insulin signalling pathways which modulate glucose utilization.

Since basal glucose 3-OMG transport was previously found to be increased in the muscles of rats provided one of two high-unsaturated-fat diets (Chapter 2), the current studies re-examined whether 1) basal glucose transport (assessed with 2-DG) in muscle is increased independently of fat subtypes in high-unsaturated fat-fed rats. Furthermore, since the type of dietary fat (safflower or mixed oils) impacted differentially on whole body glucose tolerance in the previous study (Chapter 2), rats may be expected to upregulate basal glucose transport in muscle differently if provided with the HF-SAFF or the HF-MIXED diet. It is hypothesized that the increased basal glucose transport occurs in high-fat-fed rats, in part, by 2) an upregulation of GLUT-1 protein levels. Also, given that Akt may be involved in increasing surface GLUT-1 and GLUT-4 glucose transporter availability, it is also hypothesized that 3) signalling at the level of Akt will be increased in muscle from high-fat-fed rats to compensate for insulin resistance induced by high-fat-feeding. It is proposed that insulin activates Akt more rigorously in high-fat-fed rat muscle in an attempt to upregulate glucose transport.

METHODS

Animal use:

Male Sprague Dawley rats were randomly assigned to the high carbohydrate (LF, 20 kcal % protein, 10% fat and 70% carbohydrate), the high fat-safflower oil (HF-SAFF, 20% kcal protein, 59% fat, 20% carbohydrate), or the high fat-mixed oil (HF-MIXED, 20% kcal protein, 59% fat, 20% carbohydrate) diets for 3 weeks as outlined in Chapter 2. The body weight of high-fat fed and low fat control rats increased over the feeding period.

Final body weights of rats were similar at the end of the feeding period as it was previously shown (Wilkes et al., 1998).

2-deoxyglucose transport in incubated soleus strips:

Soleus muscles were cut into thin lengthwise strips suitable for *in vitro* incubations (Bonen et al., 1994). Strips were pre-incubated for 1 hour at 29 °C in Krebs-Henseleit buffer (KHB) with 32mM mannitol, 8mM D-glucose, and 0.1% bovine serum albumin (BSA). After 50 minutes one incubation vial received 50 μM LY294002 (LY) by adding 6μl of LY already dissolved in DMSO to the incubation medium. To keep the concentration of DMSO constant between all treatments 6 μl DMSO alone was added to the remaining vials. Porcine insulin was added to the incubation medium at a maximal stimulating concentration of 400μU/ml for 30 minutes. Prior to glucose transport measurements, D-glucose was removed by washing muscle strips twice for 5 minutes each in a glucose free KHB with 38 mM mannitol and 2 mM pyruvate. The uptake of 2-deoxyglucose was determined with 2mM pyruvate, (1.5μCi) 2-deoxy-D- [^{3}H]glucose (1 mM) and (0.1 μCi) ^{14}C-mannitiol (37mM) for 20 minutes. Strips were removed rapidly, rinsed in 0.9% ice-cold saline, cut free of tendons and snap frozen in liquid N_2. Muscles were stored at -80 °C until analyzed for ^{14}C and ^{3}H in digested muscle extract (Han and Bonen, 1998; Johannsson et al., 1996).

GLUT-1 AND GLUT-4 DETERMINATION BY WESTERN BLOTTING

Total GLUT-1 and total GLUT-4 protein:

Soleus muscle was prepared as described elsewhere (Han and Bonen, 1998; Johannsson et al., 1996). Briefly, samples were separated by SDS-polyacrylamide electrophoresis on a 12% gel and transferred to an immobilon membrane by electromembrane transfer for 90 minutes Membranes were blocked overnight in 5% non-fat-dry milk (NFDM) made in Tris-buffered saline (TBS, pH 7.6). Proteins were detected by incubating blocked membranes first with an anti-GLUT-1 diluted (1:5000) or anti-GLUT-4 diluted (1:7000) polyclonal immuno-A purified antibodies followed second by horse radish peroxidase (HRP)-conjugated anti-rabbit IgG diluted (1:2000) in TBS (pH 7.6) for GLUT-4 or in 5% NFDM for GLUT-1. GLUT-1 and GLUT-4 were visualized using an enhanced chemiluminescence system according to the manufacturer's instructions. Western blots were quantified using a Macintosh LC with an Abaton scanner and appropriate software.

AKT-1 KINASE ACTIVITY

Measurements of Akt-1 kinase activity in vivo:

Muscles were homogenized and maximal Akt-1 kinase activity was determined as previously described in Chapter 3. For these experiments, the rat's descending aorta was injected with 1U insulin and the red tibialis anterior (RTA) muscles were dissected 5 minutes after injection and quickly frozen in liquid N_2 (within 1 minute). Anti-Akt-1 antibody was agitated with protein-G sepharose beads in buffer 1 for 1 hour to form an anti-Akt-1-protein-G immune complex. Aliquots of muscle homogenate were rotated with protein-G sepharose beads (50µl/ml) and pulsed in a bench top Sorvall (12, 000g) to reduce non-specific binding. The activated Akt kinase was immunoprecipitated from pre-

cleared sample (500μg) by rotation with the anti-Akt-protein-G immune complex overnight at 4°C. Akt-1 protein immunoprecipitates were collected by centrifugation, washed 4 X in buffer 2 to remove unbound protein and washed 2 times in kinase buffer 3. The final wash was carefully removed, beads completely dried and resuspended in 30μl kinase buffer 3 supplemented with 100μM cold ATP, 2μCi [γ-^{32} P]ATP, 17nM protein kinase A inhibitor, and 100μM Akt-specific crosstide peptide (GRPRTSSFAEG). After 30 minutes gentle shaking at 30°C reactions were terminated by placing tubes into ice. [^{32}P]-incorporation into crosstide peptide was determined by resolving 25 μl reaction mixture on a 40% urea-based acrylamide gel. Autoradiographs were generated by exposing the dry gel to scientific imaging film X-OMAT™ AR and the band corresponding to the peptide substrate was quantified by densitometry.

Akt-2 band shift assay:

Akt activation was determined by Akt-2 band motility shift assay. In the absence of insulin, Western blots revealed 2 bands: an obvious lower (60 kDa) band and a more faint upper (>60 kDa) band. Phosphorylation causes Akt to 'shift' to the higher molecular weight (>60 kDa) band. The degree of Akt phosphorylation is highly correlated with Akt activity (Alessi et al., 1998). Akt activation was taken as a percent band shift by dividing densitometry values obtained through scanning the upper band for each by the scanner values of the lower band and multiplying by 100. When underphosphorylated bands were visually absent 100% activation was assumed. Blots were generated as already outlined for GLUT-4 with some modifications. Briefly, samples were resolved on a large (20 X 22 cm) 6% gel by SDS-PAGE for maximal separation and transferred to an immobilon

membrane by electromembrane transfer for 90 minutes. Membranes were blocked in 5% non-fat dry milk (1 hour), probed with anti-Akt-2 antibody (1 hour) followed by HRP-labeled anti-sheep IgG secondary antibody (1 hour). Akt-2 was visualized and quantified as described above for GLUT-4.

Protein Assay:

All protein concentrations were determined in triplicate by the bicinchoninic acid assay using BSA as a standard.

Statistical Analysis

Data were analyzed using one-way and two-way analyses of variance, as was appropriate for a given experiment. All data are reported as means ± SEM.

RESULTS

GLUCOSE TRANSPORT

Basal glucose transport:

Under basal (non-insulin-stimulated) conditions soleus muscle glucose transport in HF-MIXED-fed rats and in HF-SAFF-fed rats was significantly higher ($P<0.05$) than glucose transport in soleus muscle from LF-fed-rats (Table 4.1).

Insulin-stimulated glucose transport:

The amount of glucose transport stimulated by insulin (Δi-b) was determined by subtracting mean values for basal glucose transport from mean values for glucose

transport with insulin (Table 4.1). In muscle from low-fat-fed rats Δi-b was similar to Δi-b for HF-MIXED-fed. The Δi-b for HF-SAFF was significantly lower than Δi-b in the low fat and HF-SAFF groups ($P<0.05$) (Table 4.1).

LY294002 (LY)-inhibitable glucose transport:

Inclusion of 50 μM LY, a PI3-kinase inhibitor, prevented the insulin-induced increase in glucose transport in all three groups ($P<0.01$). This concentration of LY fully inhibits PI3-kinase in perfused skeletal muscle (Wojtaszewski, et al., 1999) as well as downstream Akt activation in isolated muscle strips (personal observation). LY-inhibited glucose transport was found to be significantly higher in muscle from HF-MIXED-fed rats than in muscles from LF and HF-SAFF. The LY-inhibitable glucose transport in muscle from HF-SAFF was significantly lower than basal glucose transport in this group ($P<0.05$) (Table 4.1).

GLUCOSE TRANSPORTERS

Total GLUT-1 was increased by ~80% in soleus muscle from both groups of high-fat-fed rats compared with those fed the LF diet ($P<0.05$; Fig. 4.2). Total GLUT-4 decreased marginally (-15%) in HF-MIXED ($P<0.05$), and total GLUT-4 was not revealed to be significantly different in soleus muscle of HF-SAFF (Fig. 4.3).

ACTIVATION OF AKT WITH INSULIN

Insulin was administered into arterial circulation of unconscious rats by intravascular injections and RTA muscles were dissected for Akt-1 kinase measurements

Table 4.1. 2-DG uptake (μmol/g/20 minutes) in isolated soleus muscle strips from rats fed LF, HF-MIXED or HF-SAFF diets. Incubations occurred in the absence of insulin (basal), with 400μU/ mL insulin, and 400 μU/mL insulin + 50 μM LY294002 (LY).

Group	Basal(b)	Insulin(i)	Insulin + LY	Δ i-b
LF (n=5-8)	1.5 ± 0.14 [a]	7.0 ± 0.46 [*a]	1.4 ± 0.16 [a]	5.5 ± 0.32 [a]
HF-MIXED (n=7-10)	3.8 ± 0.55 [b]	9.4 ± 0.84 [*b]	3.3 ± 0.68 [b]	5.6 ± 0.29 [a]
HF-SAFF (n=8)	4.0 ± 0.42 [b]	6.1 ± 0.70 [*a]	2.0 ± 0.33 [a ⊥]	2.1 ± 0.28 [b]

Values are MEANS ± SEM. Within a dietary group, *differs from basal and insulin + LY treatments, $P<0.01$ and ⊥differs from HF-SAFF basal, $P<0.05$. Within a treatment condition, values with different superscripted letters differ significantly from each other, $P<0.01$.

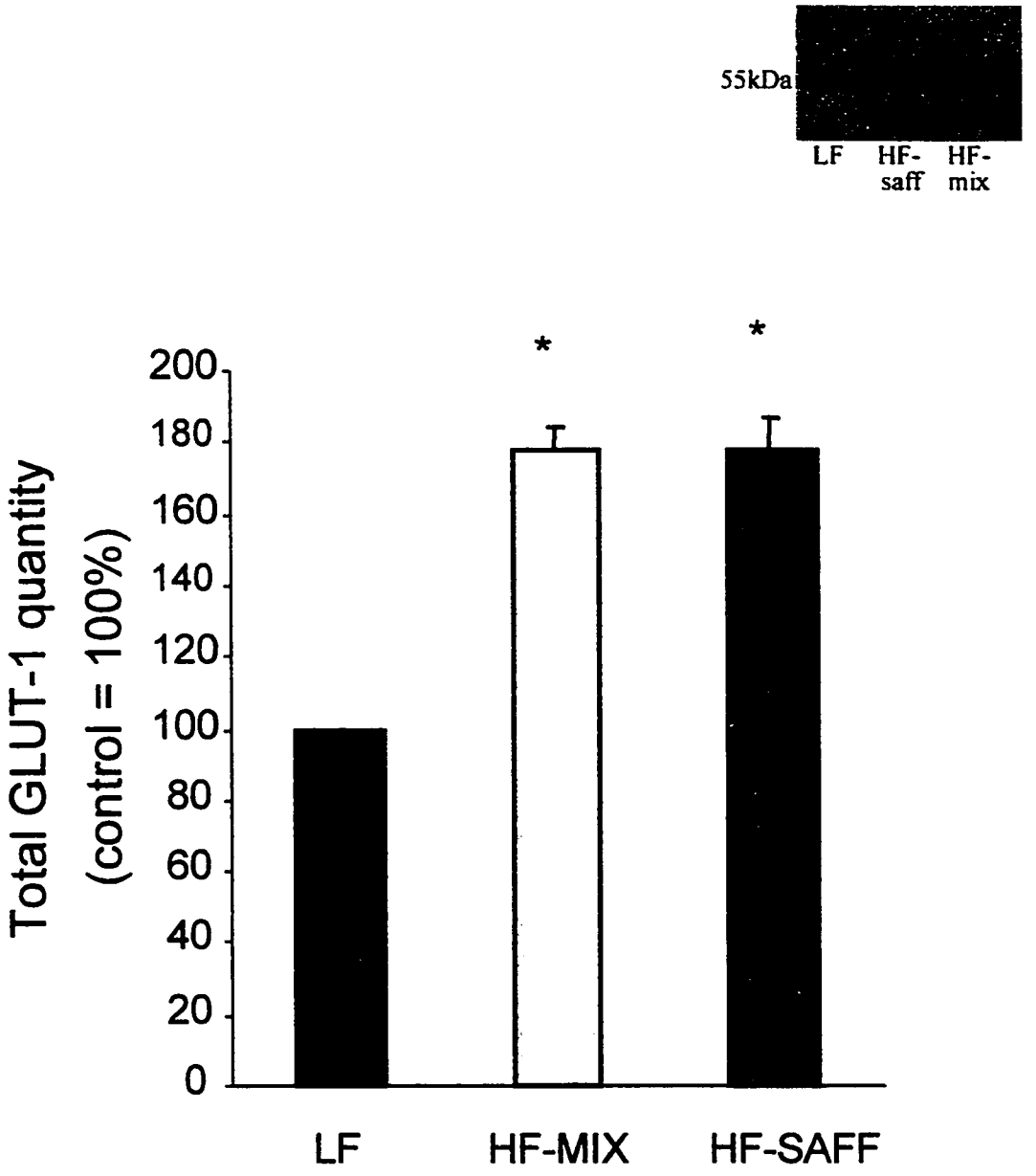


Figure 4.2. Total quantity of GLUT-1 protein in soleus muscle of LF, HF-MIXED, HF-SAFF (means ± SEM). n=6 rats per group. *P<0.05 vs. LF group.

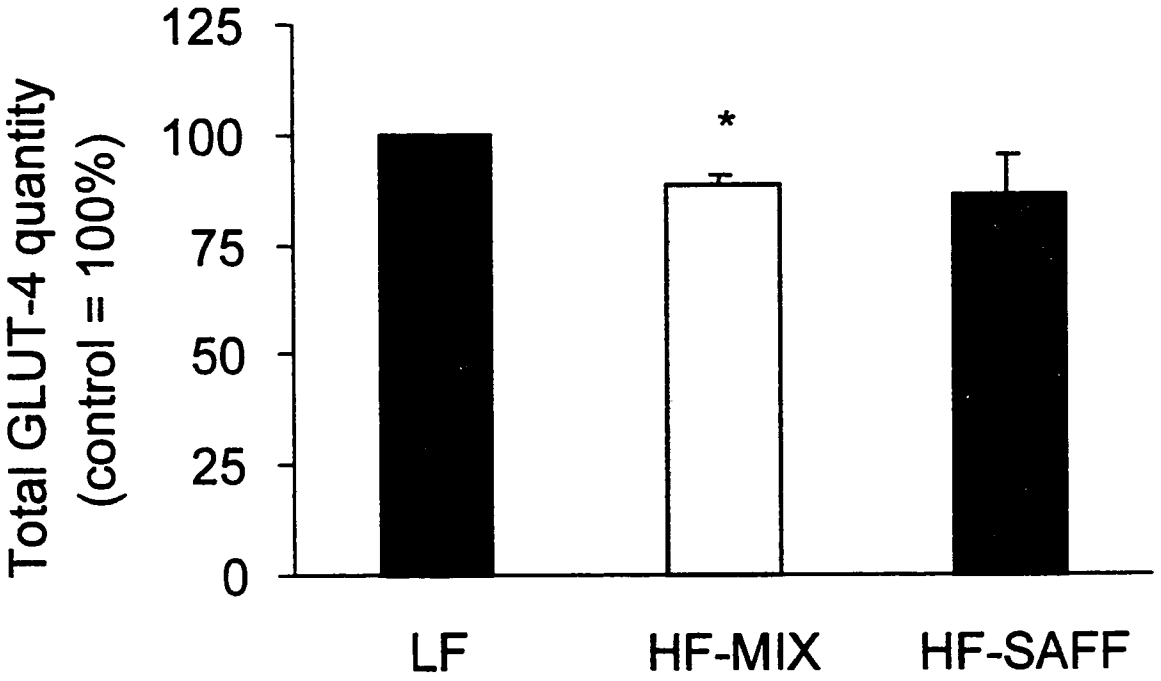


Figure 4.3. Total quantity of GLUT-4 protein in soleus muscle of LF, HF-MIX, and HF-SAFF (means ± SEM). n=6 rats per group. *P<0.05 vs. LF group.

as previously described in Chapter 3. Basal Akt-1 kinase activity was determined in muscles of rats injected with saline. Basal Akt activity did not differ significantly amongst the 3 dietary groups ($P>0.05$). Insulin injections increased Akt-1 kinase activity in all groups of rats ($P<0.01$). Insulin-activated Akt kinase was found to be significantly higher in muscle from HF-MIXED (+44%) and HF-SAFF (+61%) than LF controls ($P<0.05$, Fig.4.4). Total Akt-1 protein was not altered by high-fat-feeding (Fig. 4.5). Akt motility shift assay was used to determine Akt-2 activation in RTA muscles. The degree of Akt-2 activation by insulin was similar in all 3 dietary groups (Fig. 4.6, $P>0.05$).

DISCUSSION

In this study I confirmed that feeding rats high (59%kcal) unsaturated fat-based diets increases basal glucose transport. This replicates and extends the observations previously made in Chapter 2. In the present study basal 2-DG transport (+260%) and GLUT-1 protein (+80%) were both increased in HF-SAFF and HF-MIXED-fed rats. There was also a more vigorous insulin-induced Akt activation in oxidative muscle from high-fat-fed rats compared with low-fat controls. Together these results, along with the previous results in Chapter 2, may suggest that an increase in basal glucose transport could be an adaptive response to increase non-insulin mediated glucose transport under the conditions of insulin resistance caused by high-fat-feeding.

Recently, an issue surrounding the physiological importance of GLUT-1 was raised. Ebeling et al. (1998) proposed that GLUT-1 protein might be detrimental to

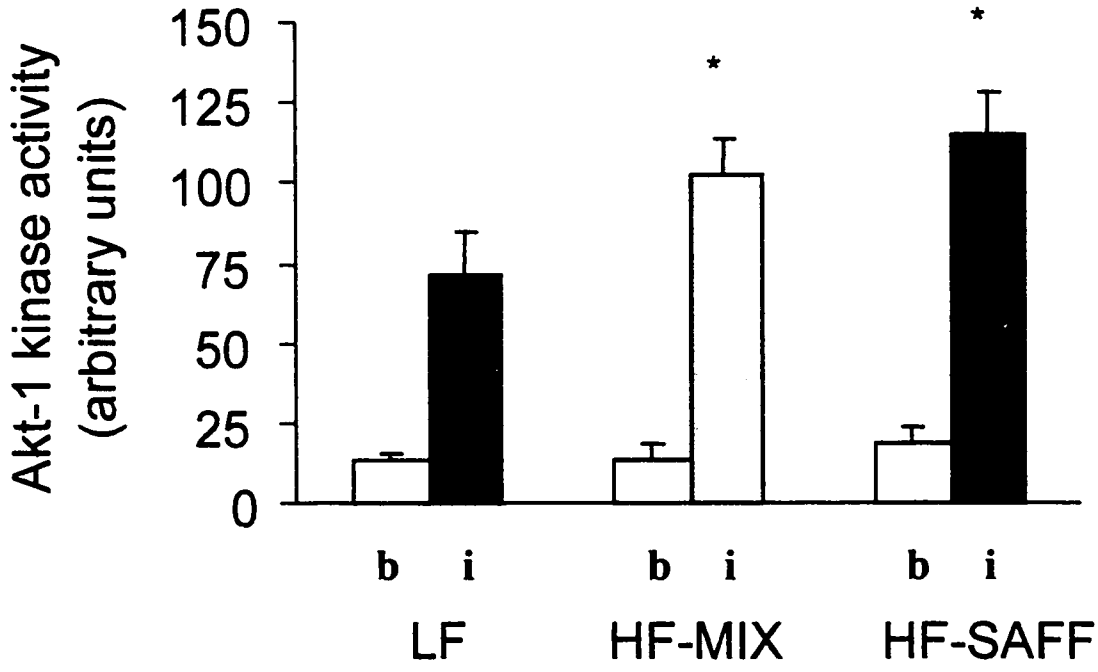


Figure 4.4. Akt-1 kinase activity in RTA muscle of LF, HF-MIXED, and HF-SAFF (means ± SEM). n=5-6 rats per group. Insulin (i) vs. basal (b) in all groups, $P<0.05$. Insulin treatments of HF-MIX and HF-SAFF groups significantly different than insulin treatment of LF group, *$P<0.05$.

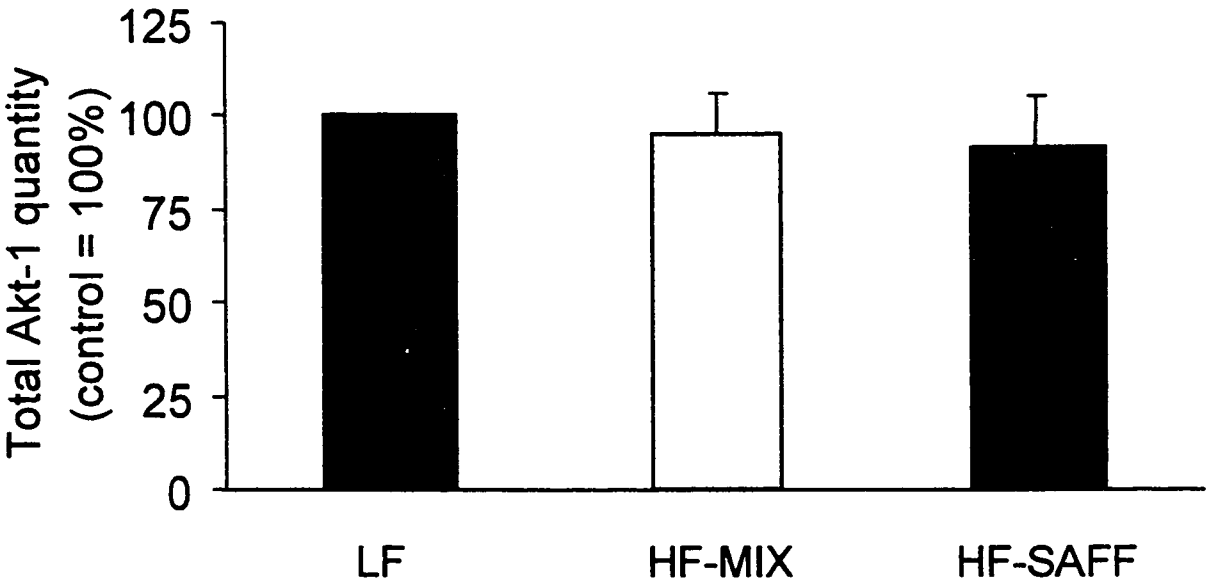
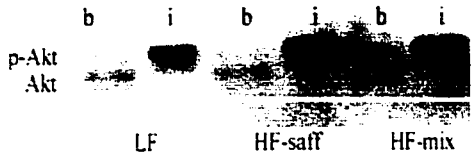


Figure 4.5. Total Akt-1 protein quantity in RTA muscle of LF, HF-MIXED, and HF-SAFF (means ± SEM). n= 4-5 rats per group (P>0.05).



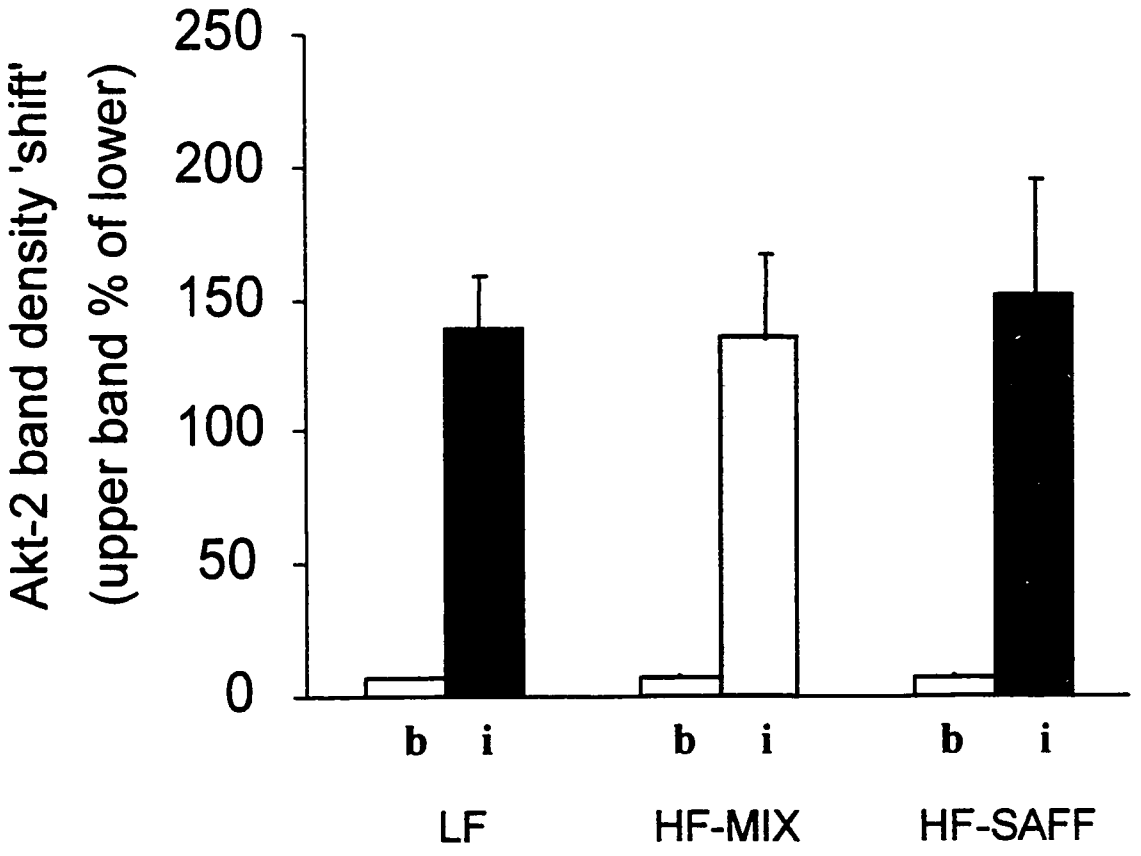


Figure 4.6. Akt-2 activation in RTA muscles of LF, HF-MIXED, and HF-SAFF (means ± SEM). n=5-6 rats per group. Insulin (i) vs. basal (b) in all groups, $P<0.05$. No differences amoung groups ($P>0.05$).

insulin sensitivity. In this paper the authors indicated that glucose transport via the GLUT-1 glucose transporter could actually promote a state of insulin resistance (Ebleling et al., 1998). Their opinion was based on observations that were provoked by hyperglycemia and GLUT-1 overexpression models of insulin resistance, in which excessive glucose influx into non-stimulated cells (i.e. cells not exposed to insulin or made to contract) is thought to be toxic to the cell. Hyperglycemia is thought to promote glucose toxicity in skeletal muscle by augmenting glucose flux through hexosamine synthetic pathways (Baron et al., 1995). As well, transgenic mice that overexpress GLUT-1 in skeletal muscle could easily increase intramuscular glucose concentrations to undesirable (glucose toxic) levels. Buse et al. showed metabolic activity of hexosamine producing pathways to be increased in muscle from transgenic mice overexpressing GLUT-1 (Buse et al., 1996). However, under more realistic physiological conditions GLUT-1 may compensate for insulin resistance when GLUT-4 activation is suboptimal. Earlier studies by Charron and Kahn showed that GLUT-1 expression can be increased in muscle by fasting and these higher glucose transporter levels can return to control levels upon refeeding suggesting that glucose transporter availability is highly adaptable (Charron and Kahn, 1990).

The etiology behind the insulin resistance following the consumption of diets high in unsaturated fat could differ from the insulin resistance caused by glucose toxicity. Hyperglycemia-induced insulin resistance is associated with impaired Akt activation (Kurowski et al., 1999; Krook et al., 1997; Heart et al., 2000). In studies by Krook, the

impaired insulin-induced Akt activity found in the muscle of hyperglycemic diabetic rats was corrected, and insulin-stimulated glucose transport was restored, when urinary glucose excretion was promoted with phlorizin to normalize blood glucose concentrations (Krook et al., 1997). Insulin-activated Akt has also been found to be impaired in skeletal muscle subjected to 4 h hyperglycemia *in vitro* (Kurowski, 1999) or in 3T3-L1 adipose cells exposed 6 h to high 50 mM glucosamine concentrations (Heart et al., 2000). These studies (Kurowski et al., 1999; Krook et al., 1997; Heart et al., 2000) could indicate that a reduction in insulin-activated Akt-derived signals might be central to insulin resistance caused by glucose toxicity. In contrast, our findings seem to suggest that the insulin resistance in muscle observed after high-fat-feeding may be dissimilar to that caused by excessive glucose availability, since Akt activation in muscle was not impaired, but rather, was found to be elevated after high-fat-feeding instead.

In high-fat-fed rats an increased Akt activation may be important for increasing GLUT-1 levels. Insulin-activated Akt seems to upregulate GLUT-1 biosynthesis at multiple levels; by increasing both GLUT-1 gene transcriptional and GLUT-1 mRNA translational events. Akt-1 has been shown to increase GLUT-1 gene transcription in cultured Hepa1c1c7 heptoma mouse cells (Barthel et al., 1999). Other studies in 3T3-L1 adipocytes have shown that GLUT-1 mRNA translation can be increased by activating a PI3-kinase / Akt / 4E-BP1 signalling pathway (Taha et al., 1999). Akt is thought to be important for phosphorylating 4E-BP1 (or PHAS-1 (phosphorylated heat- and acid-stable protein)) *in vivo* (Gingras et al., 1997). 4E-BP1 has been found to hyperphosphorylate and subsequently dissociate from the mRNA initiation factor eIF-4E in insulin-stimulated

skeletal muscle (Kimball et al., 1997). Therefore, GLUT-1 mRNA translation may also be upregulated by an Akt-dependent mechanism in skeletal muscle. Greater use of Akt-dependent signalling pathways to increase GLUT-1 might be responsible for the increased GLUT-1 levels observed in muscle of high-fat-fed rats in the present studies.

The increased basal glucose transport was far above the rise in GLUT-1 protein levels indicating glucose transport was increased by means other than elevating GLUT-1 protein expression. Upregulation of basal glucose transport independent of GLUT-1 availability appears to have occurred differentially in the 2 groups of high-fat-fed rats. PI3-kinase inhibition lowered muscle insulin-stimulated glucose transport of LF- and HF-MIXED-fed rats, yielding glucose transport rates in these two groups which were equivalent to their respective basal glucose transport levels. In contrast, the insulin-stimulated glucose transport blocked by PI3-kinase inhibition in HF-SAFF was found to be decreased to a level markedly below (-50%) the basal glucose transport level of HF-SAFF-fed rats. PI3-kinase inhibition is known to decrease GLUT-4 translocation (Quon et al., 1995). This could suggest that higher cell surface levels of GLUT-4 contributed to some part of the higher rates of basal glucose transport which were observed in HF-SAFF-fed rats. Reductions in total GLUT-4 were only slight (-15%) in high-fat-fed rats suggesting GLUT-4 translocation was likely not limited by total muscle GLUT-4 content. Total Akt-1 protein was not altered (Fig. 4.5) nor was Akt-2 activation (Fig. 4.6). This could indicate some signals before Akt-1 may have been upregulated to promote increases in cell surface GLUT-4 transporters in HF-SAFF-fed rats. PI3-kinase-dependent insulin

signals were suggested to be enhanced in livers of rats provided with a diet high in saturated fat (58% lard) for 2 weeks (Anai et al., 1999).

Besides increased GLUT-1, it is not entirely clear what else could have contributed to an upregulation of basal glucose transport in HF-MIXED-fed rats. However, some speculation can be made based on previous findings reported in Chapter 2. One noteworthy observation is that insulin-stimulated 3-OMG transport was previously found to be impaired in HF-MIXED-fed rats (Chapter 2), where as it was found here that insulin-stimulated 2-DG transport occurred normally in these rats (Table 4.1). This could suggest that post-transporter steps (i.e. hexokinase) involved in the phosphorylation of glucose allowed normal glucose transport to occur in HF-MIXED-fed rats. Whether upregulated hexokinase activity does indeed contributed to the increased basal glucose transport in HF-MIXED-fed rats remains to be determined.

In summary, insulin-activatable Akt-1 kinase activity was found to be increased in oxidative muscle of rats fed diets high in unsaturated fat. These observations are in sharp contrast to observations made in denervated muscle where a reduction in insulin-stimulated Akt activation was found to be associated with an impairment in insulin-stimulated glucose transport (Chapter 3). Nevertheless, the present data do indicate that there are adaptive changes in insulin signalling and GLUT-1 protein expression in high-fat-fed rats. Perhaps the augmented Akt activation observed in these studies represents an attempt by muscle cells to increased the surface glucose transporter availability by

promoting GLUT-1 biosynthesis and increasing surface GLUT-4 content when insulin-stimulated glucose transport is made defective by high-fat-feeding.

Chapter 5:

Red and white muscle differences in Akt activation are revealed in chronically-active tibialis anterior muscle

INTRODUCTION

Insulin stimulates glucose transport in tissues such as the heart, adipocytes and skeletal muscle. Insulin acts by first binding to the extracellular α-subunit of its receptor resulting in the autophosphorylation of tyrosine residues and the activation of a tyrosine kinase in the β-subunit of the insulin receptor (IR). The IR tyrosine kinase phosphorylate various insulin receptor substrate (IRS) proteins such as IRS-1 and -2 which couple IR activity to key glucose transport activating kinases. Phosphatidylinositiol 3'-kinase (PI3-kinase) is a key signalling protein which is necessary for insulin to promote GLUT-4 translocation. However, its activation alone in the insulin signalling cascade is not sufficient to stimulate GLUT-4 translocation (Isakoff et al., 1995).

Recently, the serine/threonine kinase Akt (protein kinase B, or related to A and C (rac) protein kinase), a downstream target of PI3-kinase, has been implicated with this signalling role. Over-expression of a constitutively active Akt kinase has been shown to increase GLUT-4 translocation independently of insulin stimulation. Akt-derived signals generated by insulin may be disturbed in some insulin resistant muscles; for example, when insulin resistance is induced by hyperglycemia or denervation. In rats with hyperglycemia, insulin-stimulated glucose transport was reduced and Akt activation was sub-optimal. Normalization of circulating blood glucose levels by phlorizin restored both insulin-stimulated glucose transport and insulin-stimulated Akt activation (Krook et al, 1997; Song et al., 1999). In denervated muscles, reduced Akt activation was associated with impaired GLUT-4 translocation (Wilkes and Bonen, 2000). Collectively, these studies (Krook et al., 1997; Song et al., 1999;Wilkes and Bonen, 2000) demonstrate that when

insulin signalling is disturbed at the level of Akt there is a concomitant change in the effectiveness of insulin to stimulate glucose transport.

Recent studies have examined whether insulin signalling in muscle is directly affected by exercise training. Insulin-stimulated PI3-kinase activation was shown to increase in trained quadricep muscles in humans (7 days @ 70% VO2max) (Koval et al., 1999) and Akt activation was shown to be enhanced in muscle of swim trained rats (6 h / day for 5 days) (Chibalin et al., 2000) suggesting that a heightened insulin signalling response occurs with prolonged muscle activity. However, determining whether these signalling changes alone impact on glucose transport has been problematical. One reason for this appears to be that there is a concomitant increase in muscle GLUT-4 expression that occurs with exercise training. Many studies suggest that glucose transport in muscle is directly proportional to the size of the total GLUT-4 pool. Thus, it might seem that the induction of similar levels of GLUT-4 in different muscles would result in similar rates of insulin-stimulated glucose transport. However, in previous work, it has been observed that in chronically active white tibialis anterior skeletal muscle total GLUT-4 content may be increased more than insulin-stimulated glucose transport (Johannsson et al., 1996). This may suggest that an increase in insulin-stimulated glucose transport in chronically active muscle is not completely dependent on greater amounts of GLUT-4. Based on previous evidence indicating that Akt impacts on insulin responsiveness in muscle, it may be that adequate Akt activation is also required in chronically active muscles. Therefore, in the present studies, we induced the same levels of total GLUT-4 in red and white muscles via chronic electrical stimulation. In these muscles we examined insulin-

stimulated glucose transport, Akt kinase activity and Akt activation. It is hypothesized that improvements to insulin responsiveness caused by chronic muscle activity are modulated by Akt-derived signalling influences.

METHODS

ANIMAL USE

Animal selection:

Male Sprague-Dawley rats weighing 250- 300g were used for these experiments. Rats were provided with rat chow (Purina) and water *ad libitum*. Rats were kept on a reverse 12 h light (6 pm to 6 am) and 12 h dark cycle (6 am to 6 pm). Ethical approval was obtained by the committee on animal care at the University of Waterloo.

Rat surgery and chronic electrical stimulation of tibialis anterior muscle:

Muscles were stimulated as previously described (Johannsson et al., 1996). Rats were anesthetized under halothane gas after a subcutaneous injection of buprenorphine (0.03/mg/kg) analgesic. Stainless steel electrodes were sutured to underlying muscles on either side of the peroneal nerve. These electrodes were passed subcutaneously from the thigh, exteriorized at the nape of the neck, and attached to a miniature electronic stimulator firmly secured to the rat's back. Rats were housed individually for at least 5 days to permit for adequate recovery. Afterwards, simulators were turned on to stimulate the peroneal nerve (10 Hz, 50 μs duration) which innervates the red and white tibialis anterior (TA) muscles. Every day TA muscles were electrically activated by turning stimulators on at 9 am for 5, 15, 60, 180, or 360 minutes / day. Once the electrical

stimulation protocols were finished (on day 7) stimulators were turned off. Experiments were performed the next day. Rats were fasted overnight by removing food from the cages at 6 pm. The first experimental phase involved stimulating muscles for 5-360 minutes / day to generate chronically active muscles for glucose transport determination by hindlimb perfusions. The total GLUT-4 content was also determined in these stimulated muscles (n=4-8 animals/group). This enabled us to ascertain insulin responsiveness and GLUT-4 levels in muscles that undergo varied amounts of stimulation time / day and to determine that the amount of stimulation time that induces equal amounts of GLUT-4 in RTA and WTA is 360 minutes / day. In the second phase of experiments rats (n=9) were electrically stimulated for 360 minutes /day. Rats in this group received 0.4 U insulin by *in vivo* injection to activate muscle Akt.

ASSESSMENT OF INSULIN RESPONSIVENESS IN RELATION TO GLUT-4 LEVELS

Glucose transport:

Basal and insulin-stimulated glucose transport was determined using a cell-free, perfused hindlimb preparation (Megeney et al., 1992; Wilkes et al., 1998). The animals were surgically prepared under anesthesia (65 mg pentobarbital sodium / 100 g body weight). The descending aorta was cannulated (20 gauge angiocath) to deliver perfusate to the hindlimb and the inferior vena cava was cannulated (14 gauge angiocath) to remove the effluent to complete a one pass perfusion system. A cell-free perfusate containing Krebs-Henseleit buffer with 4% bovine serum albumin (BSA) under constant gassing (95% O_2 / 5% CO_2) was connected to the indwelling cannulae via tygon tubing that was

connected to a pump while the rat lay in a warm chamber (30°C). Over the first 20 minutes of the perfusion protocol, the flow rate was gradually increased to 20 ml / minutes to stabilize the pressure between -80 to -100 mm Hg. This flow rate is sufficient to perfuse and oxygenate both the experimental and the contralateral hindlimb muscles simultaneously (unpublished observations). To assess insulin-stimulated glucose uptake, insulin 160 μU/ml was added to the buffer at the start of the perfusion and was maintained at this concentration for the duration of the experiment. Insulin (porcine insulin, Iletin II regular) has previously been infused at this concentration to maximally stimulate glucose transport in this perfusion system (Wilkes et al., 1998). After 20 minutes, 30 mM 3-O-methylglucose (3-O-MG) containing 5μCi ^{3}H-3-O-MG and 2.5 μCi ^{14}C-L-glucose was added to the perfusate and the hindlimb was perfused for an additional 5 minutes. Inclusion of the ^{14}C-L-glucose in the perfusate allowed us to account for the non-transported diffusion component of glucose transport into skeletal muscle. Upon completion, TA from both hindlimbs were quickly excised, RTA separated from WTA and both muscles washed in cold saline and frozen in liquid N_2.

GLUT-4:

GLUT-4 protein was determined in RTA and WTA muscle samples using Western blotting procedures as previously described (Megeney et al., 1993 and 1994). Briefly, samples were separated by SDS-polyacrylamide electrophoresis on a 12% gel miniapparatus and transferred to an immobilon membrane by electromembrane transfer for 90 minutes Membranes were blocked overnight in 5% non-fat-dry milk (NFDM) made in Tris-buffered saline (TBS, pH 7.6). GLUT-4 was detected by incubating blocked

membranes with an anti-GLUT-4 polyclonal immuno-A purified antibody (1:7000) followed by horse radish peroxidase (HRP)-conjugated anti-rabbit IgG diluted (1:2000) in TBS, pH 7.6. GLUT-4 was visualized using an enhanced chemiluminescence system according to the manufacturer's instructions. Western blots were quantified using a Macintosh LC with an Abaton scanner and appropriate software.

INSULIN SIGNALLING

Akt activation in vivo and muscle preparation:

For these experiments, rats were injected with saline (+0.1% BSA) or 0.4U insulin mixed in saline (0.1% BSA) to activate Akt as previously reported in Chapter 3. TA muscles were dissected 5 minutes after injection. Dissections were completed rapidly and muscles were frozen in liquid N_2 quickly. Muscles were homogenized in ice-cold buffer 1 (150 mM NaCl, 50 mM TRIS pH 7.5, 30 mM sodium pyrophosphate, 10 mM sodium fluoride, 1mM dithiothreitol (DTT), 10%v/v glycerol, 1% triton-X-100, 1 mg/mL bacitracin, 200 mM PMSF, 10 mg/mL aprotinin, 1mg/mL leupeptin, 1 mg/mL pepstatin A, 1 μM microcystin). Homogenates were spun at 15,000 rpm (70Ti, Sorvall) for 1 h at 4°C.

Measuring Akt-1 kinase activity:

Akt-1 kinase activity *in vivo* was determined as previously described in Chapter 3. Anti-Akt-1 antibody was agitated with protein-G sepharose beads in Buffer 1 for 1 hour to form an anti-Akt-α-protein-G immune complex. Aliquots of muscle homogenate were rotated with protein-G sepharose beads (50μl/ml) and pulsed in a bench top Sorvall (12, 000g) to reduce non-specific binding. The activated Akt kinase was immunoprecipitated

from pre-cleared sample (500μg) by rotation with the anti-Akt-protein-G immune complex overnight at 4°C. Akt-1 protein immunoprecipitates were collected by centrifugation, washed 4 X in buffer 2 (1 M NaCl, 25 mM HEPES pH7.6, 1 mM DTT, 0.1% BSA, 10% glycerol, 1% tritonX-100) to remove unbound protein and washed 2 times in kinase buffer 3 (50mM TRIS pH 7.5, 10mM $MgCl_2$, 1 mM DTT). The final buffer was carefully removed, beads completely dried and resuspended in 30μl kinase buffer 3 supplemented with 100μM cold ATP, 2μCi [γ-^{32}P]ATP, 17nM protein kinase A inhibitor, and 100μM Akt-specific crosstide peptide (GRPRTSSFAEG). After 30 minutes gentle shaking at 30°C reactions were terminated by placing tubes into ice. [^{32}P]-incorporation into crosstide peptide was determined by resolving 25 μl reaction mixture on a 40% urea-based acrylamide gel. Autoradiographs were generated by exposing the dry gel to scientific imaging film X-OMAT™ AR and the band corresponding to the peptide substrate was quantified by densitometry.

Akt-2 band shift assay:

Akt activation was determined by Akt-2 band motility shift assay. Western blots were generated as already outlined for GLUT-4 with some modifications. Briefly, samples were resolved on a large (20 X 22 cm) 6% gel by SDS-PAGE for maximal separation and transferred to an immobilon membrane by electromembrane transfer for 90 minutes. Membranes were blocked in 5% non-fat dry milk (1 hour), probed with anti-Akt-2 antibody (1 hour) followed by HRP-labeled anti-sheep IgG secondary antibody (1 hour). Akt-2 was visualized and quantified as described above for GLUT-4. In the absence of insulin an obvious lower (60 kDa) band and a more faint upper (>60 kDa) band were

present. Phosphorylation causes Akt to 'shift' to the higher molecular weight (>60 kDa) band. The degree of Akt phosphorylation is highly correlated with Akt activity (Alessi et al., 1998). Akt activation was taken as a percent band shift by dividing densitometry values obtained through scanning the upper band for each by the scanner values of the lower band and multiplying by 100.

Protein Assay:

All protein concentrations were determined in triplicate by the bicinchoninic acid assay using BSA as a standard.

Statistical Analysis:

Data were analyzed using repeated measures analyses of variance, and paired t-tests, as was appropriate for a given experiment.

RESULTS

Rats lost a small amount of body weight for 2 days after surgery. Afterwards rats steadily regained weight for 5 days ($P<0.05$). Rats underwent chronic electrical stimulation once weight loss was fully regained ($P<0.05$) so that full weight recovery was attained first before electrical stimulation began (Fig. 5.1).

GLUCOSE TRANSPORTERS

Total GLUT-4 determination at various stimulation times:

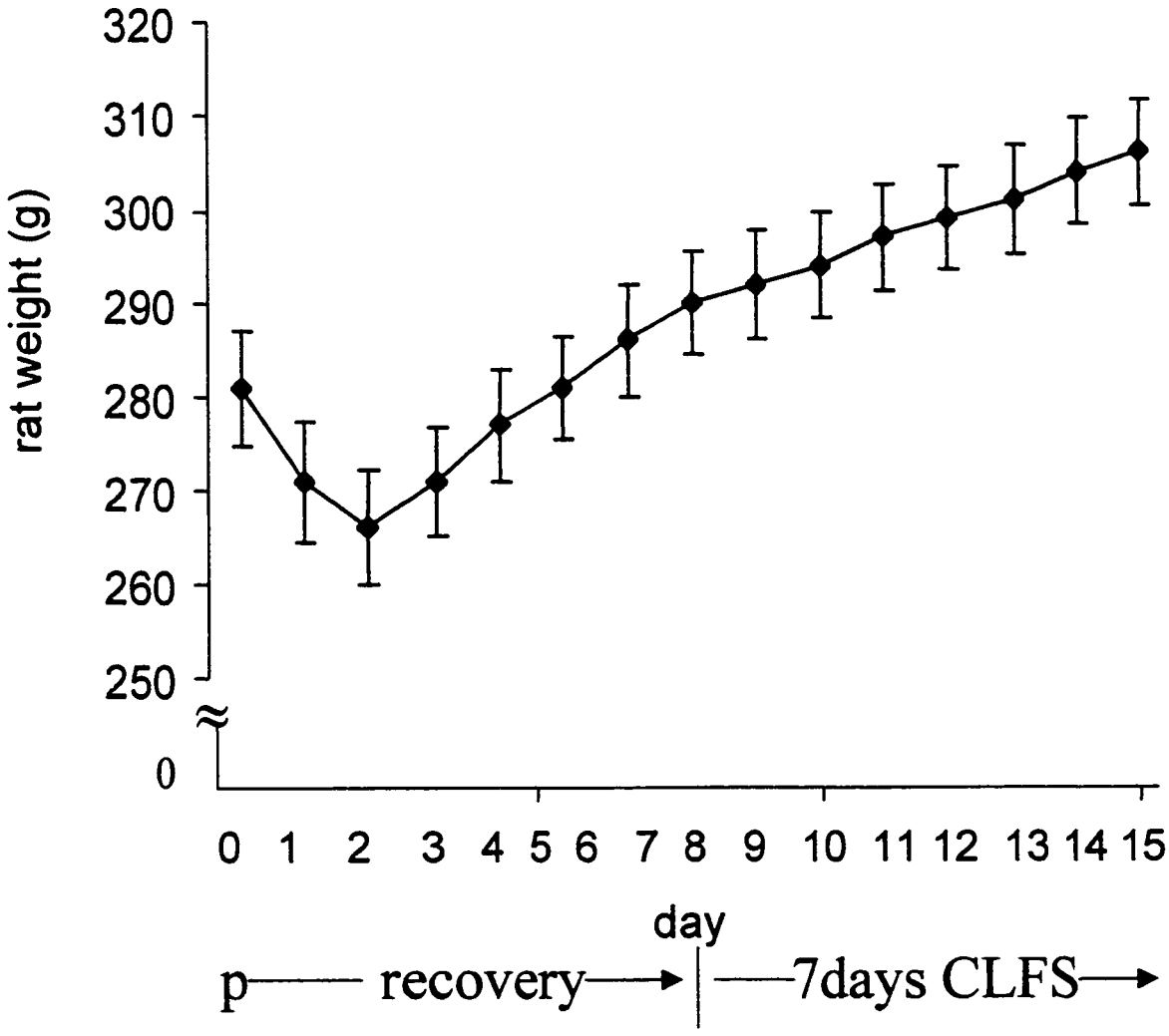


Figure 5.1. Weights of experimental rats: pre (p)-surgery, recovery (7 days) and during chronic low frequency stimulation (CLFS) (7 days). means ± SEM for n=4-6 rats.

In control muscles GLUT-4 was greater in RTA than in WTA ($P<0.05$; Table 5.1). Total GLUT-4 content was measured by Western blotting after all of the perfusions were complete. Time-dependent increases in stimulation increased GLUT-4 in both muscles ($P<0.05$) (Fig. 5.2). Short stimulation times of 5 and 15 minutes / day did not alter GLUT-4 content in RTA. Electrical stimulation for 15 minutes / day increased GLUT-4 content of WTA by 1.8-fold over GLUT-4 in control ($P<0.05$). Maximal increases in RTA GLUT-4 occurred with 60 minutes / day (1.6-fold over GLUT-4 in control RTA, $P<0.05$); GLUT-4 increased by 2.5-fold in WTA with 60 minutes stimulation / day ($P<0.05$). In RTA, total GLUT-4 content was not further altered with longer stimulation times of 180 or 360 minutes / day ($P>0.05$ vs. 60 minutes / day). Longer electrical stimulation of 180 minutes / day induced some further increase (+30%) in WTA GLUT-4 compared with GLUT-4 in WTA after 60 minutes stimulation / day ($P<0.05$). Stimulation of 360 minutes / day resulted in a similar GLUT-4 content of the RTA and WTA muscles ($P>0.05$, Fig.5.2).

GLUCOSE TRANSPORT

Control muscles:

Table 5.1 shows glucose transport in control muscles. Basal glucose transport was 1.6-fold greater in RTA than in WTA ($P<0.05$). Insulin increased 3-OMG transport in both RTA and WTA muscles ($P<0.05$). Insulin-stimulated 3-OMG transport was 2.1 times greater in RTA than in WTA ($P<0.05$).

Table 5.1. Basal and insulin-stimulated 3-OMG transport and total GLUT-4 in control red tibialis anterior (RTA) and control white tibialis anterior (WTA) muscles (mean ± SEM).

	3-0MG transport (μmol /g / 5 min)		GLUT-4 (arbitrary units)
Muscle	Basal	Insulin	GLUT-4
RTA	0.43 ± 0.05	5.91 ± 0.28†	100
WTA	0.27 ± 0.4 *	2.76 ± 0.24 *†	36 ± 4

* WTA vs. RTA, P<0.05
† insulin vs. basal, P<0.05
n = 16-26 muscles

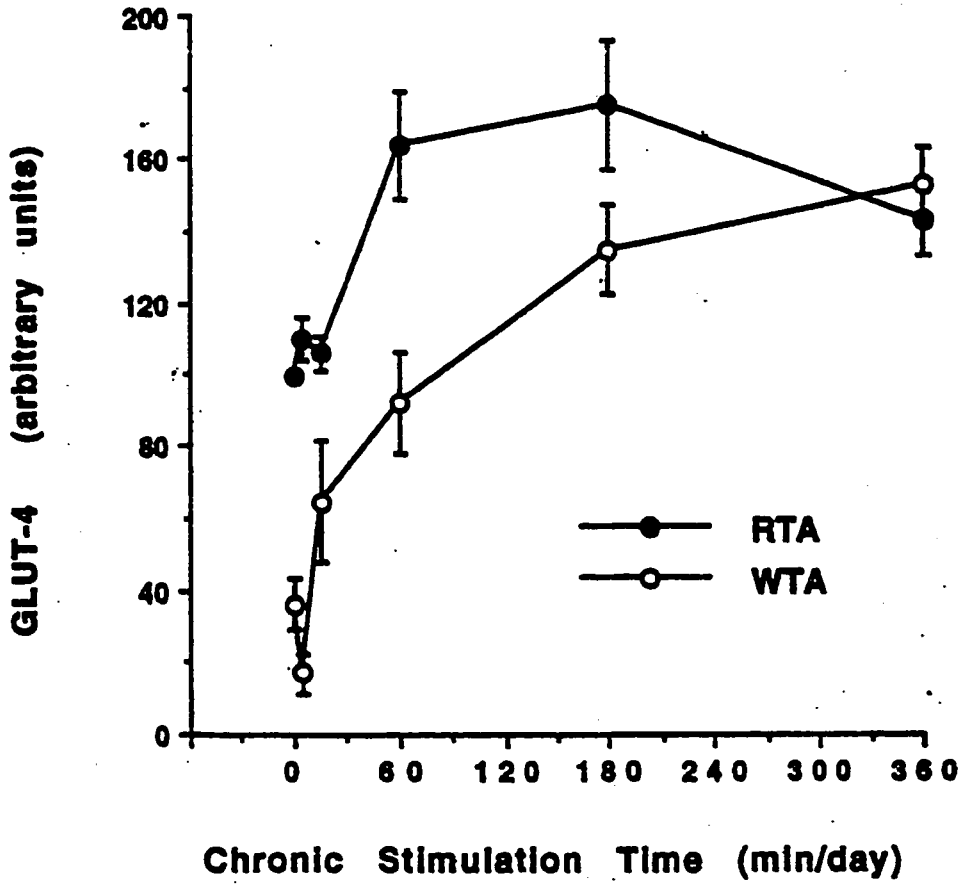


Figure 5.2. Chronic electrical low frequency stimulation time-dependent increase in GLUT-4 protein expression in red tibialis anterior (RTA) and white tibialis anterior (WTA) muscles (means ± SEM). n = 4-5 muscles for each stimulation time point.

Basal 3-OMG transport in chronically-active TA muscles:

Basal 3-OMG transport increased in both RTA (+30%) and WTA (+19%) muscles compared with their respective controls ($P<0.05$). After 360 minutes / day of electrical Fig. 2 (GLUT-4 time dpe increase) stimulation 3-OMG transport in the WTA remained 1.83-fold lower than basal 3-OMG transport in the RTA ($P<0.05$; Fig. 5.3A).

Insulin-stimulated 3-OMG transport in chronically-activate TA muscles:

Both RTA and WTA muscles showed increased insulin-stimulated 3-OMG transport with chronic electrical stimulation ($P<0.05$) (Fig. 5.3B). For both muscles maximal increases in insulin-stimulated 3-OMG transport were observed with 180 minutes stimulation / day ($P<0.05$) with no further increases when the stimulation time was extended to 360 minutes / day ($P>0.05$).

EFFECT OF 360 MINUTES STIMULATION / DAY ON GLUT-4, INSULIN-STIMULATED GLUCOSE TRANSPORT AND AKT

Total GLUT-4 content was observed to be markedly increased (+117%) in WTA and only moderately increased (+44%) in RTA ($P<0.05$; Fig. 5.4A). Electrical stimulation for 360 minutes / day increased insulin-stimulated glucose transport by 45% in RTA and 79% in WTA ($P<0.05$; Fig. 5.4B).

Akt-1 kinase activity after in vivo insulin administration.

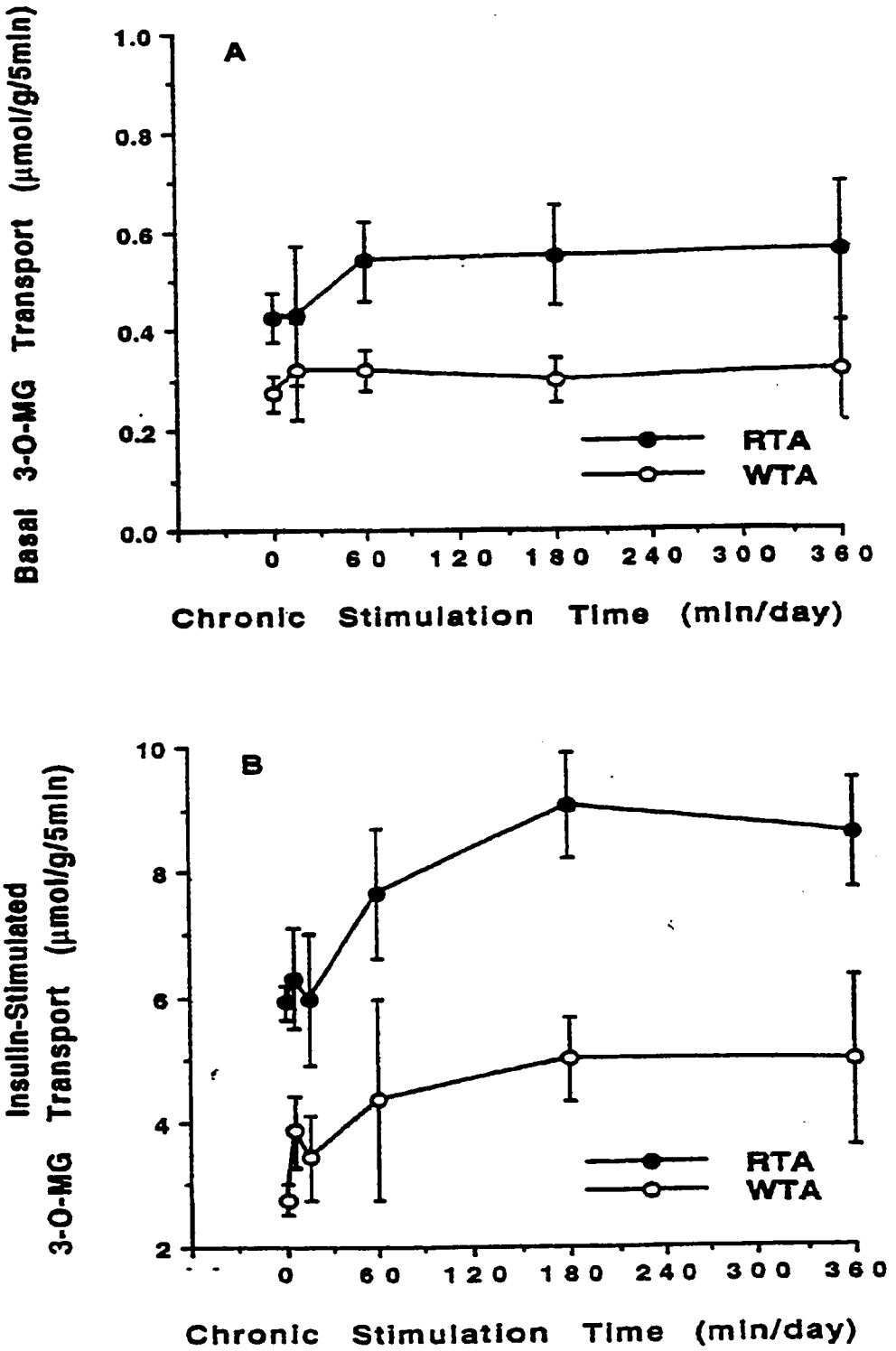


Figure 5.3. Basal (A) and insulin-stimulated (B) 3-O-methyl glucose transport (mean ± sem). RTA significantly different than WTA at all stimulation times tested, $P<0.05$. n = 5-6 muscles at each stimulation time point.

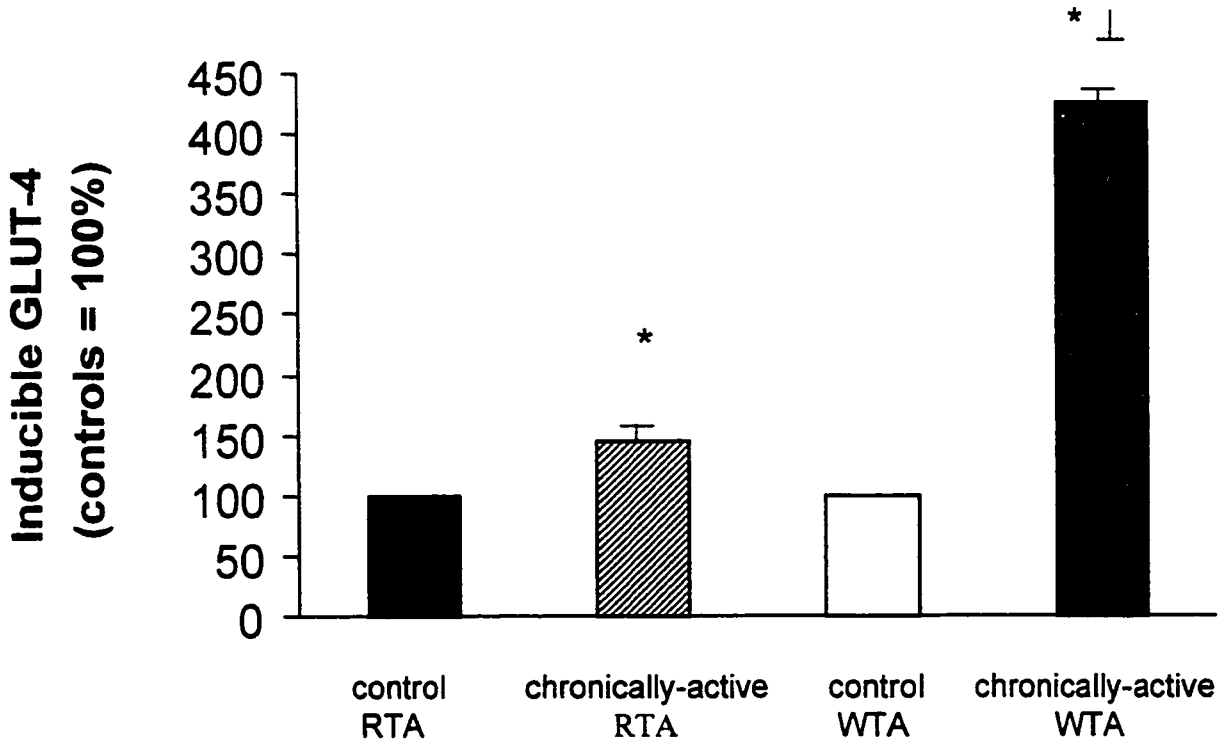


Figure 5.4A. GLUT-4 protein expression inducible by 360 minutes of CLFS / day for 7 days (means ± SEM). n = 5-6 muscles for each group.

* chronically-active muscle vs. respective control muscle, $P<0.05$.
⊥ chronically-active white vs. chronically-active red, $P<0.05$.

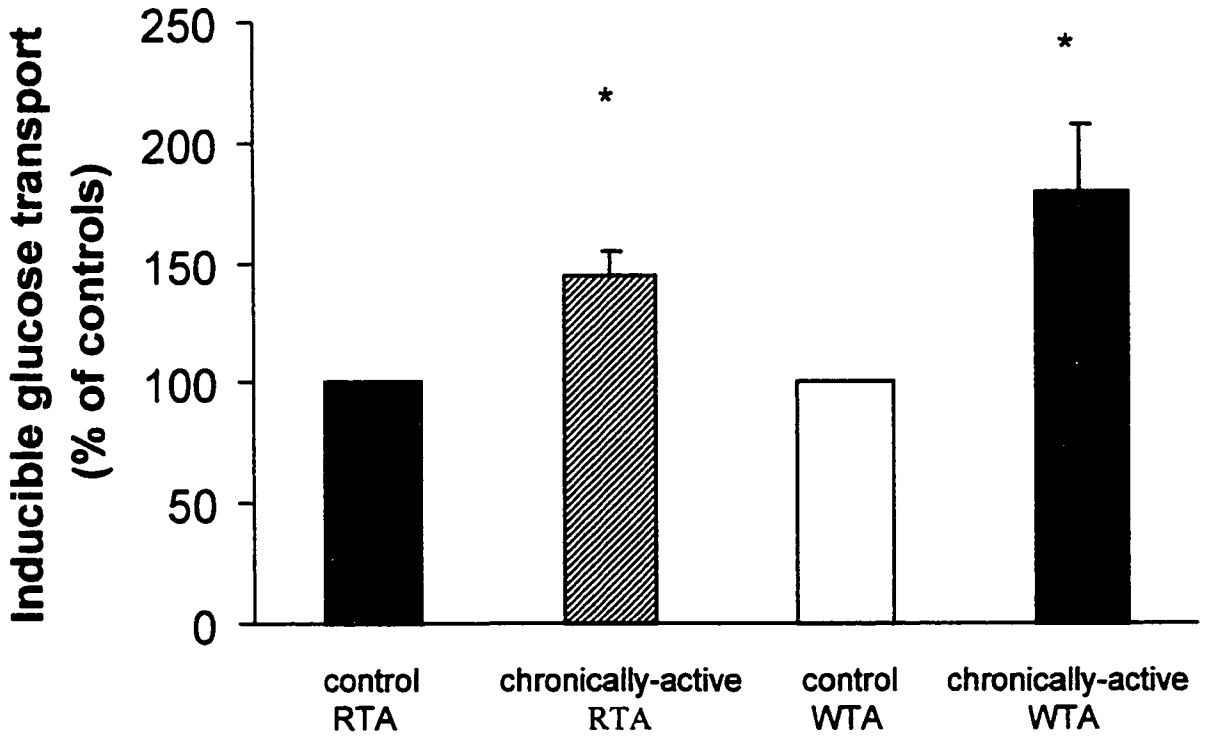


Figure 5.4B. Insulin-stimulated glucose transport inducible by 360 minutes of CLFS / day for 7 days (means ± SEM). n = 5-6 muscles for each group.

* chronically-active muscle vs. respective control muscle, $P<0.05$

Basal Akt-1 kinase activity was not easily detected in either red or white muscles since bands generated were too faint to scan. Insulin treatments increased Akt-1 kinase activity by 92% in chronically-active RTA muscle compared to Akt-1 kinase activity in control RTA muscles ($P<0.01$). Similar to the control RTA muscles, the Akt-1 kinase activity in control WTA muscles was found to be increased by insulin treatments ($P<0.05$). But in contrast to the chronically-active RTA muscles, Akt-1 kinase activity in chronically-active WTA muscles was found to be reduced (-36%) ($P<0.01$; Fig. 5.4C).

Akt-2 activation after in vivo insulin administration:

Band shift assays were performed to demonstrate insulin-induced activation of Akt (Fig. 5.5). Motility shift (activation) of Akt-2 in RTA and WTA was slight under basal conditions and not significantly different in the chronically-active muscles ($P>0.05$). Total Akt-2 protein levels were not altered by chronic electrical stimulation (Fig. 5.6). Insulin activated Akt-2 in all control muscles ($P<0.01$). In chronically active RTA, Akt-2 activation was 2.3-fold greater than in control RTA ($P<0.05$). In contrast, Akt-2 activation was found to be lower (-37%) in chronically active WTA than in control WTA ($P=0.059$).

DISCUSSION

The objective of this study was to compare two actions induced by insulin (Akt activation and glucose transport) in red and white muscles that are similar in GLUT-4 content. We found that after chronic electrical stimulation (360 minutes / day) for 7 days, red tibialis anterior muscle (RTA) and white tibialis anterior muscle (WTA) displayed 1)

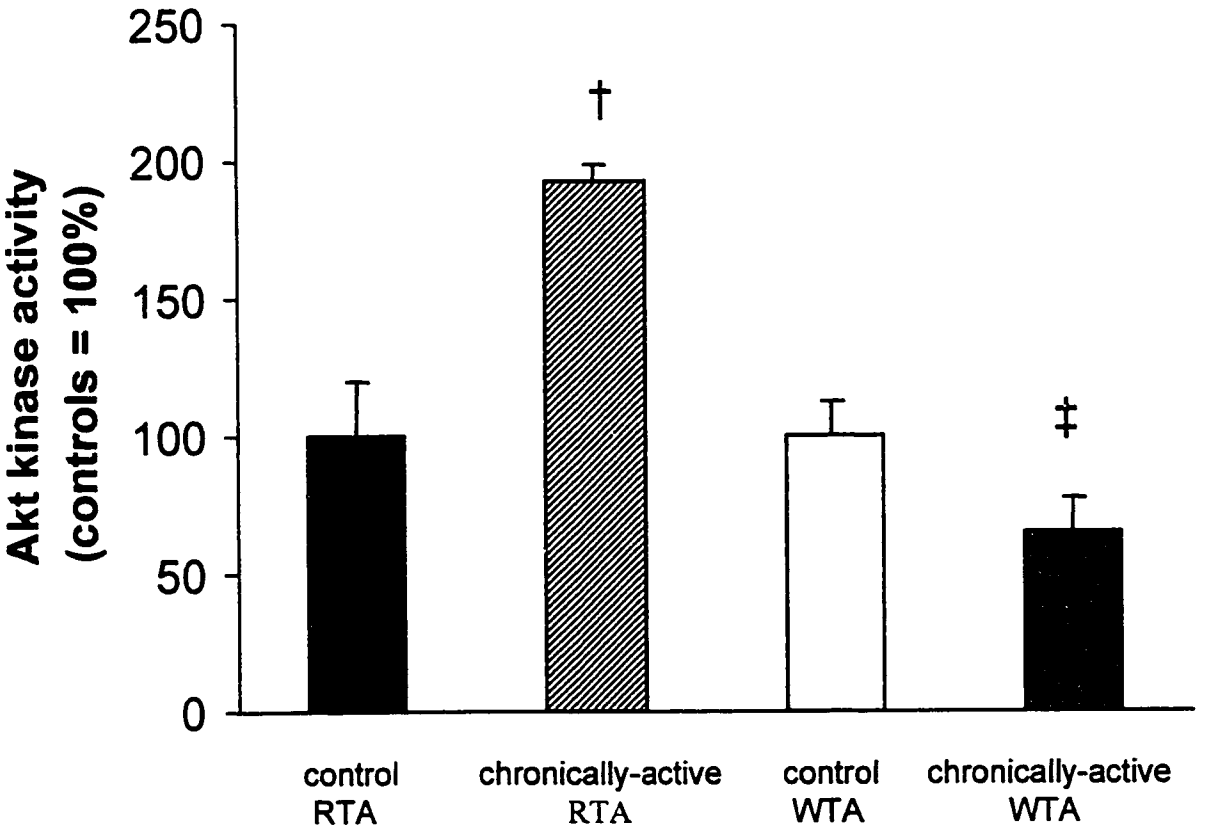


Figure 5.4C. Akt kinase activity in control muscles and muscles chronically-active for 360 minutes / day for 7 days (means ± SEM). n=5-6 muscles for each group.

† chronically-active red vs. control red, $P<0.05$

‡ chronically-active white vs. control white, $P<0.05$

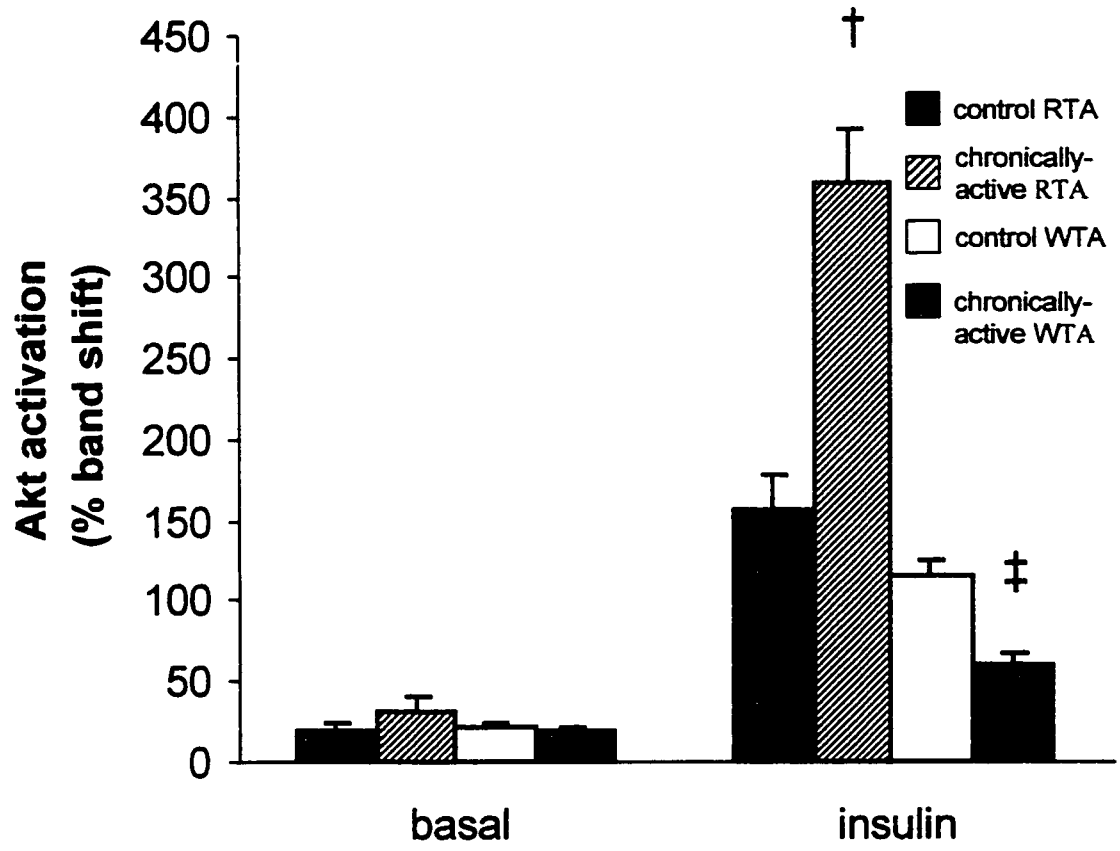


Fig. 5.5. Insulin-stimulated Akt activation in control muscles and muscles chronically-active for 360 minute / day for 7 days (means ± SEM). n = 5-6 muscles for each group.

Insulin vs. basal, $P<0.05$ for control red, chronically-active red, control white, and chronically-active white muscles.

† chronically-active red vs. control red, $P<0.05$

‡ chronically-active white vs. control white, $P<0.05$

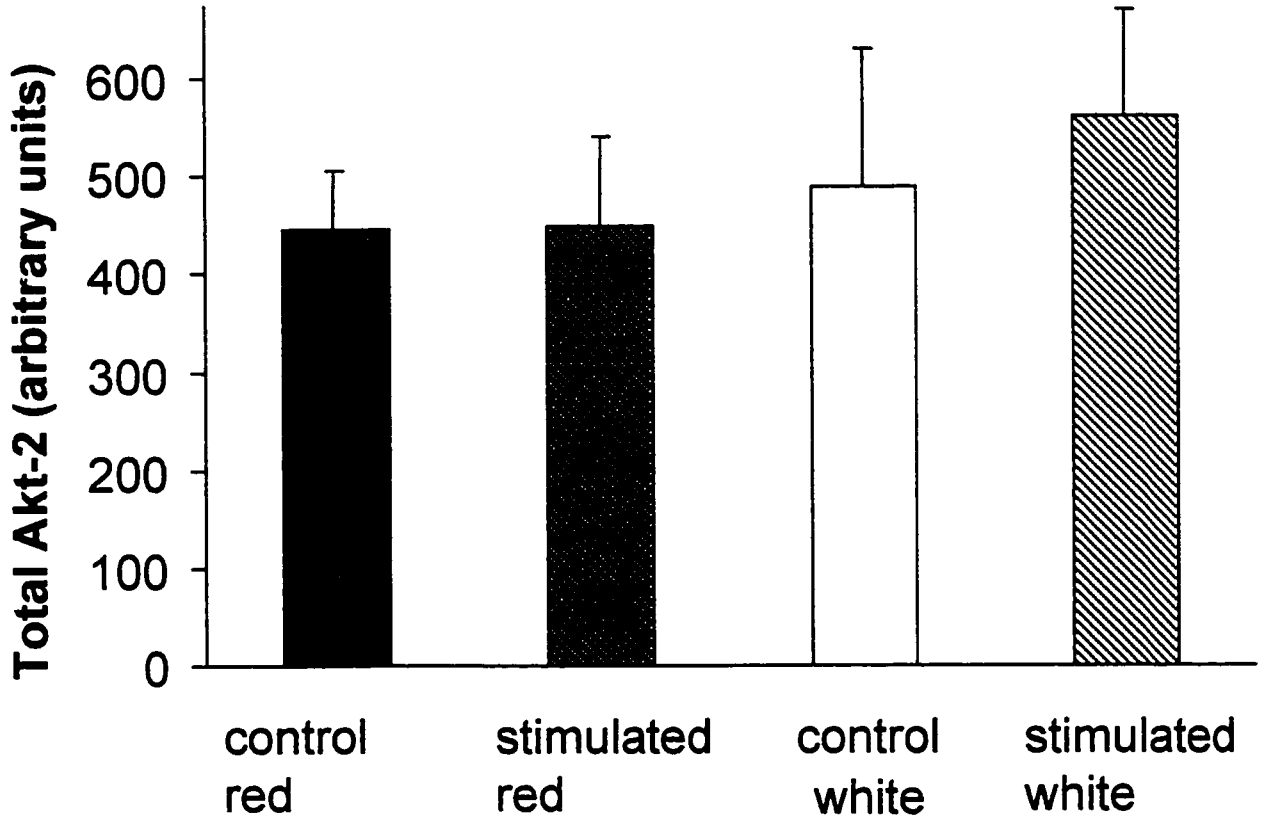


Figure 5.6. Total Akt-2 in control muscles and muscles chronically-active for 360 minutes / day for 7 days (means ± SEM). n = 5-6 muscles for each group.

similar levels of total GLUT-4 content but 2) different rates of insulin-stimulated glucose transport (RTA>WTA) and 3) a differential effect on Akt activation. Insulin-induced Akt activation was increased in chronically-active RTA while, unexpectedly, this was suppressed in chronically-active WTA.

Increasing GLUT-4 in red and white muscles can be achieved with relative ease by chronic electrical stimulation of rat hindlimb muscles (Johannsson et al., 1996). The advantage of this model is that all muscle fibers in a given muscle can be made to contract at the same intensity while the muscles of the contralateral limb serve as controls. This technique offers an effective means by which to vary GLUT-4 levels in skeletal muscle of living animals because the amount of expressed GLUT-4 protein is dependent on the duration of electrical stimulation (present study and Johannsson et al., 1996). The 7 day electrical stimulation protocol employed in our studies does not alter muscle fiber composition (McCullagh et al., 1997). Longer periods of stimulation (18-56 days) have been used to promote muscle fiber remodeling in TA muscles in rats (Termin et al, 1989; Bonen et al., 2000). Moreover, since WTA muscles are more responsive to chronic electrical stimulation than RTA muscles (Johannsson et al., 1996), it was possible to elevate total GLUT-4 to levels comparable with GLUT-4 in RTA (Fig. 5.2).

It has long been thought that muscle activity-induced increases in GLUT-4 protein content in muscle enhance insulin responsiveness. Muscle GLUT-4 content is highly correlated with glucose transport rates in insulin-stimulated muscles (Megeney et al., 1993). These studies infer that the translocation of more GLUT-4 in insulin-stimulated

muscles is a direct result of the increased intracellular GLUT-4 availability. A substantial induction of GLUT-4 occurred in WTA, while improvements in insulin-stimulated glucose transport resulting from prolonged muscle activity were not nearly as dramatic. These discrepancies between the inducible GLUT-4 observed and the degree to which insulin-stimulated glucose transport was found to be improved in WTA may suggest significantly more GLUT-4 is produced with electrical stimulation than is actually recruited (i.e. translocated) by insulin. The amount of GLUT-4 which is translocated by insulin stimulation is normally in good relation to the amount of glucose transport stimulated by insulin (Lund et al., 1993 and 1995). It may be that in muscle made chronically-active for 360 minutes / day for 7 days, the lower insulin-stimulated glucose transport rates observed in WTA were due to less efficient recruitment of GLUT-4, due in part, to inadequate generation of Akt-derived signals. In 24 h denervated muscles, GLUT-4 translocation is reduced or absent when insulin-induced Akt activation is impaired (Wilkes and Bonen, 2000).

Oxidative muscles express more insulin receptors (Bonen et al., 1981) and elicits higher IR tyrosine kinase activity (James et al., 1986) than receptors associated with glycolytic muscles. Recently, PI3-kinase, Akt and glucose transport activation were compared *in vitro* in soleus (~80% slow oxidative fibers / 20% fast oxidative glycolytic fibers) and EDL muscles (50% fast oxidative glycolytic / 50% fast glycolytic). Song et al. (1999) found that the time course for PI3-kinase and Akt activations by insulin were qualitatively similarly, but the maximal activity attained was greater in soleus muscle for both PI3-kinase and Akt. Moreover, Turinsky and Damrau-Abney (1999) showed Akt

activation was not increased in plantaris muscles to the level of activation observed in soleus muscles after 0.1 U insulin administration. These findings support the idea that insulin-signalling influences may contribute to fiber-type specific differences in glucose transport activation.

The present studies are unique, since the total amount of GLUT-4 present could not explain the discrepancy between maximal insulin-stimulated glucose transport in chronically-active RTA and chronically-active WTA muscles. Interestingly, glucose transport improved (+45%) in line with the amount GLUT-4 increase (+44%) in RTA, whereas the improvement in insulin-stimulated Akt activity was slightly greater (+92%) in this muscle group. This could suggests that both a change in Akt-mediated insulin signalling along with a concomitant increase in GLUT-4 content together contribute to improving insulin-stimulated glucose transport of chronically-active RTA muscle. In contrast, the results in the WTA could suggest that increasing total GLUT-4 is necessary to increase insulin-stimulated glucose transport, but this increase alone is not sufficient to fully activate glucose transport. It also appears necessary to have an adequate insulin signalling response at the level of Akt. Given a key role proposed for Akt in insulin-stimulated GLUT-4 translocation, lessening the activation of Akt in chronically-active WTA may limit the increase in glucose transport in this muscle. Compared to the chronically-active RTA, it appears that the differential effect of muscle activity on reducing insulin-stimulted Akt activation in chronically-active WTA is what might modulate the rise in insulin-stimulated glucose transport in WTA with similar GLUT-4 availability to RTA.

In summary, the findings reported here show that a) higher insulin-stimulated glucose transport rates in RTA muscles can be associated with greater insulin-activated Akt. But, b) in WTA muscles, with levels of GLUT-4 increased to levels comparable to GLUT-4 in RTA muscles, insulin-stimulated glucose transport remained lower than insulin-stimulated glucose transport in RTA muscles. In chronically-active WTA insulin-stimulated Akt activation was found to be depressed. Therefore, it is concluded that full activation of glucose transport by insulin in chronically-active muscles is not solely dependent on the availability of total GLUT-4 but also on the adequate activation of the key signalling protein Akt.

Chapter 6:

SUMMARY

It is becoming increasingly apparent that an intact insulin signalling system is vital for insulin to effectively stimulate glucose transport. Therefore, there is a great deal of interest on the impact of metabolic and physiological changes on those parts of the insulin signalling pathway that regulate glucose transport. This was previously not possible because, in the past, any insulin-activated signals with clearly defined roles were relatively unknown. For instance, in some earlier studies it was first presumed that cAMP acted as a second messenger for insulin. The belief that cAMP was directly responsible for promoting glucose transport implicated cAMP regulating enzymes (i.e. adenylate cyclase) as targets of insulin receptor activity. Later on, the observations that diacylglycerol (DAG) concentrations could be increased by insulin stimulation implicated phospholipase C as another potential signal that might also activate the glucose transport system. However, since it was found that insulin requires *src*-homologous containing proteins coupling IR activity by IRS proteins (see introduction), it has been possible to verify that the enzymes which increase cAMP and DAG levels are not the intermediate signals that link insulin receptor activity to the induction of GLUT-4 translocation.

Soon after the discovery of PI3-kinase, this insulin signalling intermediate was acknowledged to be a signalling protein linking IR activity to glucose transport activation. PI3-kinase converts IR tyrosine kinase activity to signals that increase GLUT-4 translocation in insulin sensitive tissues such as skeletal muscle. However, although PI3-kinase is required, the activation of PI3-kinase itself is insufficient to activate glucose transport on its own (Isakoff et al., 1995). Several research groups have since proposed

that molecules known to follow PI3-kinase in insulin signalling pathways might also be involved in stimulating glucose transport activation.

My attention has been focused on Akt. This signalling protein is likely to have a pivotal effect on cellular glucose utilization. Akt has been shown to promote glucose transport by increasing GLUT-4 translocation (Kohn et al., 1996; Hajduch et al., 1998) and GLUT-1 biosynthesis (Hajduch, 1998). As well, Akt is thought to lie within a signalling pathway important for increasing glycogen synthesis in skeletal muscle. This illustrates that Akt appears to have a central role in regulating muscle glucose utilization. Therefore, I examined Akt in muscles in which insulin responsiveness was altered by various physiological and metabolic perturbations.

In the first study on Akt (Chapter 3) it was found that a reduction in muscle activity caused by muscle denervation led rapidly to a loss of insulin-stimulated Akt-1 kinase activity. The reduced insulin-stimulated Akt-1 activation observed was found to be associated with a severe reduction in GLUT-4 translocation and, as well, a lowering of glucose transport. Prior work had shown that long term denervation (>24 h) decreased muscle GLUT-4 concentrations (Megeney et al., 1993). However, these studies with short term denervations (<24 h) demonstrate that before GLUT-4 is reduced, insulin signalling at the level of Akt could be disrupted. Thus, it appears that the denervation-induced impairment in Akt activation is a means by which an inactive muscle reduces insulin-mediated glucose utilization. Muscles with lower activity levels presumably require less glucose. Therefore, decrements in a more flexible insulin signalling system

rather than lost GLUT-4 from a rigid GLUT-4 pool could represent a means to rapidly diminish insulin-stimulated glucose transport, when the muscle's requirement for glucose is instantly lowered by denervation. Alternatively, there may be as yet unidentified neurotrophic substances that serve to maintain signalling proteins.

The second study on Akt (Chapter 4) reports on the effect of feeding a high-fat-diet on Akt. The high-fat-feeding protocol is outlined in Chapter 2. In HF-MIXED-fed rats, insulin-stimulated 3-O-methyl-glucose transport was observed to be decreased (Chapter 2). The impairment in insulin-stimulated 3-O-methylglucose glucose transport found (Chapter 2) seemed to be some what paradoxical with the normal glucose tolerance test results (Chapter 2) given that skeletal muscle is the most important tissue for insulin-mediated glucose disposal (DeFronzo et al., 1981). This was not the case for the HF-SAFF-fed rats. These rats showed a decrease in insulin-stimulated 3-O-methylglucose transport which was accompanied more characteristically by whole body glucose intolerance. In these earlier studies (Chapter 2) it was suggested that the additional adipose mass as well as the increased muscle basal glucose uptake may have accounted for normal disposal of glucose in HF-MIXED-fed rats.

In contrast to denervation-induced insulin resistance, the insulin resistance induced in muscle by high-fat-feeding was accompanied by a concomitant increase in muscle Akt activation (Chapter 4). These studies implicate Akt as a perturbed signal when insulin resistance is caused by the consumption of a high-fat diet. However, these studies do not implicate Akt as a defective signal that lowers insulin-stimulated glucose transport in

muscle of high-fat-fed rats. One interpretation is that the increased Akt-1 activation may have occurred to compensate for insulin resistance in the muscle of high-fat-fed rats, analogous to the hyperinsulinemia that occurs during the development of insulin resistance in humans. In both instances (increased Akt or increased hyperinsulinemia), these compensatory effects to insulin resistance would act to alleviate the reduction in insulin-stimulated glucose uptake in tissues important for blood glucose removal (ie. skeletal muscle) and enable animals to retain normal circulating blood glucose concentrations.

The finding that basal glucose transport increases in both groups of high-fat-fed rats prompted an inquiry as to whether there are additional changes in the glucose uptake system that occur in skeletal muscle with high-fat-feeding (Chapter 4). The increase in Akt-1 kinase activation and total GLUT-1 found with high-fat-feeding (Chapter 4) was suggested to have occurred in order to compensate for insulin resistance. Interestingly, in this set of studies it was found that the muscle of HF-MIXED-fed rats displayed normal insulin-stimulated 2-deoxyglucose transport, while muscle from the HF-SAFF-fed rats still displayed an overt resistance to insulin (Chapter 4). These findings could suggest that compensatory mechanisms in skeletal muscle, which attempt to offset insulin resistance caused by high-fat-feeding, were more effective in HF-MIXED-fed rats, and not completely effective in HF-SAFF-fed rats. Adaptive changes in muscles of HF-MIXED-fed rats were likely more effective since this dietary group consumed a diet with a very different fatty acid composition. Select biochemical features of the HF-MIXED diet may have allowed for more normal insulin actions of insulin-sensitive tissues to occur (see introduction in Chapter 2).

Studies on chronically-active RTA and WTA muscles demonstrate that Akt activation, as well as changes in GLUT-4 content, could explain, in part, discrepancies between insulin-stimulated glucose transport in chronically-active red and white muscle (Chapter 5). These studies showed insulin-stimulated glucose transport to be greater in chronically-active RTA than in chronically-active WTA when total GLUT-4 content and Akt activation were concomitantly increased in chronically-active RTA muscle. In contrast, the ability to induce Akt activation with insulin in the chronically-active WTA was suppressed. Yet, chronic electrical stimulation induced a very dramatic increase in GLUT-4 protein expression in WTA to the level equivalent with the total GLUT-4 level in chronically-active RTA. It is well known that normal red muscle is substantially more insulin responsive (demonstrated in Chapter 2) and contains impressively more GLUT-4 than normal white muscle (Megeney et al., 1993). The studies in Chapter 5 could indicate that fiber-type differences in signal transduction mechanisms in chronically-active muscle might also contribute to fiber-type differences in glucose transport regulation independent of muscle total GLUT-4 content.

Clearly there are differences in insulin-stimulated Akt activation in denervated muscle, chroncially-active muscle and muscle from high-fat-fed rats which should be noted. In previous studies with chronic electrical stimulation or denervation as models of altered muscle activity, changes in total GLUT-4 content have been used to explain effects on maximal insulin-stimulated glucose transport activity (Megeney, 1993; Johannsson, 1996). However, in my studies, denervation and chronic white TA muscle activation

produced muscles with total GLUT-4 content that did not easily predict the insulin-stimulated glucose transport potential. Therefore, it seems as though the decrement in Akt activation in denervated muscle and in chronically-active white muscle limited or at the very least, restricted insulin-stimulated glucose transport when there was more than an adequate total GLUT-4 content. It may be interesting to speculate that when GLUT-4 content of muscle is mismatched with insulin responsiveness, then signals from Akt are changed in order to fine tune the insulin-stimulatable glucose transport system mediated by the GLUT-4 transporter.

Unlike in denervated muscle, Akt in high-fat-fed rat muscle did not explain insulin resistance, since Akt-1 activation was increased and Akt-2 activation was normal. A possible explanation for this is that insulin-stimulated glucose transport is diminished in muscle of high-fat-fed rats due to an impairment in insulin signalling situated downstream from Akt in the insulin signalling pathway, or in the GLUT-4 trafficking / activation steps beyond Akt. As such, the insulin stimulation of Akt-1 could have increased in an attempt to correct a deficiency in post-Akt signals or compensate for faulty steps in GLUT-4 trafficking. Whether GLUT-4 translocation was increased by an enhancement of Akt-1 activation is unknown. Furthermore, an elevation of Akt-1 kinase activity may have had alterior or additional affects on glucose transport activation. Because Akt has also been implicated in GLUT-1 biosynthesis (Hajduch et al., 1998), the increased Akt-1 kinase activity observed could have been responsible for the higher GLUT-1 levels detected in muscle of high-fat-fed rats (Chapter 4). Although this is not definitive, the work in this

thesis begins to establish that there is possibility of a linkage between the synthesis of GLUT-1 and the activity of Akt-1 in muscle made insulin resistant by high-fat-feeding.

In conclusion, this thesis supports a role for Akt in insulin-stimulated glucose transport. However, the present studies in this thesis show that when muscle Akt is affected by select models of insulin resistance and muscle models of improved insulin action, the Akt responses are more complex than have previously been recognized. These studies indicate that skeletal muscle Akt makes key contributions to muscle glucose transport by exploiting glucose transporter availability when the insulin responsiveness of this tissue is altered by high-fat-feeding or muscle activity changes.

RECOMMENDATIONS

It may be of interest to feed rats high-fat diets for longer periods of time to allow animals to become obese. This would allow for determining whether insulin resistance deteriorates further and permit for addressing the question of whether Akt activation decreases in obese high-fat-fed rats?

Muscle denervation interupts both muscle activity and neurotrophic flow. It would be of considerable interest to determine whether nerve-derived factors maintain Akt activity in inactive muscles. This may be ascertained using tetrodotoxin treatment to block sodium channels while retaining axoplasmic flow.

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