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DIETARY FAT AND EXERCISE INFLUENCE REGULATION OF GLUCOSE METABOLISM IN SKELETAL MUSCLE

by

SHA LIU

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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TO MY WIFE AND MY SON FOR THEIR LOVE AND ENCOURAGEMENT

ABSTRACT

Skeletal muscle is the major site for glucose disposal. Insensitivity of skeletal muscle to insulin is a primary feature in insulin-resistance states. Previous studies have shown that a diet high in ω-3 fatty acid improves the action of insulin in high-fat diet induced insulin-resistance in skeletal muscle. The possible mechanism of this was investigated in the present study. It was also determined whether a high-fat diet would induce IGF-I resistance. Furthermore, possible interaction between dietary fat and exercise was explored.

A diet high in ω-3 fatty acids significantly increased the content of ω-3 fatty acid in muscle plasma membrane phospholipid, and insulin binding to intact sarcolemma vesicles was increased by ~14 fold ($p<0.0001$). Increased binding was due to increased receptor number at the low affinity, high capacity binding site. When the receptors were solubilized, the increased insulin binding due to the diet high in ω-3 fatty acid disappeared, indicating the importance of the membrane phospholipid environment to the improved receptor binding by dietary manipulation. Similarly, diet high in ratio of polyunsaturated/saturated fatty acid also increased insulin binding to sarcolemmal vesicles ($p<0.05$).

The possibility of a high-fat induced IGF-I resistance in epitrochlearis muscle was investigated. Rats were fed either a low-fat (5% w/w) or high-fat (20% w/w) diet. The high-fat diet reduced both responsiveness and sensitivity of IGF-I-stimulated glucose transport, to a similar degree in the reduction of insulin-stimulated glucose transport

($p<0.05$). IGF-I binding was also decreased by the high-fat diet, which might be responsible for reduced action of IGF-I ($p<0.05$).

The effects, and possible interactions, of the level of dietary fat and exercise on glucose transport in skeletal muscle were also studied. Exercise-stimulated glucose transport was maintained for rats with insulin resistance induced by high-fat feeding. However, exercise failed to increase insulin sensitivity for high-fat fed rats. There was a significant interaction between the level of dietary fat and exercise ($p<0.05$), with basal glucose transport significantly higher for the high-fat fed exercised rats than that of low-fat fed exercised rats ($p<0.05$).

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CHAPTER I: GENERAL INTRODUCTION

Skeletal muscle is the most abundant tissue in human beings. Numerous studies have shown that the physiological functions of this tissue are far beyond being the powerhouse for mechanical movements of the body. Skeletal muscle serves as a reservoir for amino acids, actively participates in the metabolism of amino acids, and is very important for the disposal of glucose. Skeletal muscle has a fundamentally important role in the maintenance of normal glucose homeostasis (Sinacore and Gulve, 1993). It was reported that ~75% of glucose removed from the blood goes into this tissue (DeFronzo, 1987).

Insulin-resistance is a metabolic state in which normal or higher concentrations of insulin produce a less than normal biological function (DeVries et al., 1989). Impaired glucose transport into skeletal muscle plays an important role in whole body insulin-resistance (DeFronzo, 1987). In this chapter, factors affecting glucose transport into skeletal muscle will be reviewed, with brief introductions to some of the key factors in glucose transport, such as the glucose transporter, insulin and IGF-I. Studies on the regulation of glucose transport in the other insulin-sensitive tissues, i.e., adipocytes and liver, are very useful for the understanding of regulation of glucose transport in skeletal muscle. Therefore, knowledge in regulation of glucose transport and metabolism in these tissues was included in this chapter.

INSULIN AND INSULIN RECEPTOR

INSULIN

Insulin is a polypeptide consisting of an A and B chain of 21 and 30 amino acids, respectively. The two chains of the dimer are linked through a pair of disulfide bonds; an intrachain disulfide bond connects the sixth and eleventh amino acids within the A chain. In skeletal muscles, insulin has multiple anabolic functions. It stimulates glucose transport and metabolism (Bak and Pedersen, 1991), increases amino acids uptake (Dardevet et al., 1994), and promotes protein synthesis and reduces protein degradation (Hasselgren and Fisher, 1992).

INSULIN RECEPTOR

The first step in the action of insulin is binding to its receptor, which is a high molecular weight membrane glycoprotein (Jacobs and Cuatrecasas, 1981). The insulin receptor consists of two different subunits, an α-subunit (135,000 dalton) and a β-subunit (90,000 dalton). Two of each of these subunits are linked by disulfide bonds, constituting a heterotetramer with an approximate total weight of 350,000 daltons (Jacobs and Cuatrecasas, 1981). The α-subunit is extracellular and contains the insulin binding site, and the region of amino acids 83-103 and the so-called "cysteine rich region" (amino acids 205-316) are important for binding (Haring, 1991). The β-subunit is a transmembrane protein; the intracellular portion contains tyrosine kinase activity which catalyses autophosphorylation after activation by insulin (Roth and Cassell, 1983). The receptor has two main functions: one is to recognize the insulin molecule by binding it

with a high affinity and specificity; the second is to transmit a transmembrane signal leading to the biochemical responses (Kahn, 1976).

INSULIN RECEPTOR TYROSINE KINASE

Formation of the receptor-ligand complex induces a conformational change in the extracellular domain, e.g., altered chemical accessibility and chromatographic properties. The structural alteration translates into activation of the intracellular tyrosine kinase.

The β-subunit of the receptor consists of 620 amino acids. It contains a 194 amino acid extracellular domain which is glycosylated, a 23 amino acid transmembrane part, a 403 amino acid cytoplasmic sequence that contains a well-preserved tyrosine kinase domain, and a C-terminal tail. The three tyrosines at 1158, 1162 and 1163 in the preserved tyrosine kinase region contain 50-60% of the phosphate after insulin stimulation, and are crucial for autoactivation (for review see Haring, 1991).

It was proposed that the unoccupied α-subunit functions as an inhibitor of the β-subunit (Shoelson et al., 1988). The insulin binding-induced conformational change of the α-subunit is transduced to the β-subunit and leads to relief from kinase inhibition.

FACTORS AFFECTING INSULIN ACTION

A variety of factors influence insulin-dependent metabolism, particularly glucose uptake, in insulin target tissues. These factors include diet, particularly the level and composition of dietary fat; disease status, such as obesity and diabetes; progress through the life cycle (aging) is a another factor; finally, exercise is a potent influence on insulin action in the whole body through its influence on skeletal muscle. This collection of influences on insulin-dependent glucose metabolism together shape the overall action of

insulin in any individual. Some of these factors increase glucose metabolism through increase of insulin-dependent responses (such as low-fat diet, diet high in polyunsaturated/saturated fatty acid ratio, exercise), while others are associated with insulin-resistance (such as high-fat diet, saturated fat diet, old age, obesity, insulin-dependent and non-insulin-dependent diabetes mellitus). The mechanisms for altered action of insulin by different factors involve changed insulin receptor binding or its receptor tyrosine kinase activity (e.g., dietary factors and obesity), or may or may not involve changes at the receptor level (exercise, diabetes). In light of the multiple levels of modification of the action of insulin on glucose transport by different factors, the factors affecting the action of insulin will be reviewed below, starting with a consideration of alteration at the receptor level, and also at the level of glucose transport (see REGULATION OF GLUCOSE TRANSPORT).

FACTORS AFFECTING INSULIN RECEPTOR FUNCTION

Dietary fat. The level and type of dietary fat affect insulin action in its target tissues. It is worthwhile to point out that the term low- or high-fat diet is a relative one, especially in animal experiments. In some studies, the low- and high-fat diets contained 5% and 20% (w/w) fat (or 11% and 40% of total energy), respectively (e.g., Field et al., 1988); while in other studies, the low- and high-fat diets contained 5% and 41% (w/w) fat (or 11% and 67% of total energy), respectively (e.g., Grundleger and Thenen, 1982). Since typical Western diets contain ~35-40% (% of total energy) fat, using this level of fat in the high-fat diet might be more appropriate. In terms of degree of unsaturation, some of the studies employed a polyunsaturated/saturated fatty acid ratio (P/S ratio) of

0.25 for low P/S diet and 1.0 for high P/S diet, which represent the P/S ratio in typical North American diet and the P/S ratio recommended for diabetic patients (Field et al., 1985; American Diabetes Association, 1987), respectively; on the other hand, unphysiologically high P/S (8.0) diet has been fed (Awad, 1981). It is preferable to avoid using unphysiological dietary fat in terms of either the level of fat or the composition.

Numerous studies have shown that diets high in fat decreases insulin binding to insulin sensitive tissues. In 1978, Olefsky and Saekow reported a decreased insulin binding to adipocytes from rats fed a diet with 67% calories (~40% w/w) as fat for 10 days, compared with insulin binding to adipocytes from control animals fed a diet with 22% calories as fat (Olefsky and Saekow, 1978). The decrease was observed at all insulin concentrations used (0.2-100 ng/ml), but at higher insulin concentrations the decrease was more marked. Feeding a similar high-fat diet also induced a 35% decline in insulin binding to rat soleus muscle, compared to carbohydrate fed rats (Grundleger and Thenen, 1982). The decreased insulin binding was due to decreased insulin receptor number, and no change in receptor affinity was observed. In addition to reduced insulin binding, Watarai et al. (1988) reported that insulin-stimulated phosphorylation of the β-subunit of the insulin receptor was decreased to almost 50% in liver of rats fed a high-fat diet, compared to that from low-fat fed rats. It was also reported that a diet high in sucrose decreased insulin binding to both skeletal muscle and adipocytes (Maegawa et al., 1986).

Insulin binding to its receptor is also influenced by dietary composition of fatty acids. It has been established in adipocytes that the type of fat consumed can alter membrane phospholipid composition (Field et al., 1988). Reconstitution of solubilized

erythrocyte insulin receptors into highly unsaturated phospholipid vesicles, compared to saturated vesicles, increased the number of available binding sites (Gould et al., 1982). Field et al. (1988) demonstrated that feeding rats diets different in polyunsaturated/saturated fatty acid ratio (P/S) resulted in differences in insulin binding. Rats were fed a diet containing 20% (w/w) fat for six weeks. Insulin binding to adipocytes was higher for animals fed on a high P/S diet (P/S=1), compared to those fed on a low P/S diet (P/S=0.25). Similar results were observed in a later study (Field, 1990). It was concluded that the amount of insulin bound to adipocytes increased as the content of polyunsaturated fatty acids in phosphotidylethanolamine increased; insulin binding decreased with increasing content of saturated and monounsaturated fatty acids in phosphotidylethanolamine (for review, see Clandinin et al., 1993). Improved insulin binding was reported to correlate with altered membrane fatty acid composition and modified membrane fluidity (Ginsberg et al., 1982; Field et al., 1990). However, the dietary modification on fatty acyl composition of skeletal muscle plasma membrane phospholipid has not been documented, and it is not known if modified fatty acyl composition in plasma membrane will result in a change in insulin receptor function.

Diabetes. It was reported that patients with non-insulin-dependent diabetes mellitus (NIDDM) had decreased maximum specific insulin binding and receptor concentration (Panze et al., 1992). Insulin receptor was also reported to be reduced in patients with insulin-dependent diabetes mellitus (IDDM, Bak et al., 1989). However, where diabetes was induced by injection of streptozotocin in animal models, insulin binding to adipocytes (Field et al., 1988) and liver (Blondel et al., 1990) was not altered, and insulin binding

to crude plasma membrane of skeletal muscle was increased (Nishimura et al., 1989). Decreases in insulin-stimulated tyrosine kinase activity and autophosphorylation were also reported (Bak et al., 1989; Blondel et al., 1990), suggesting the existence of post-receptor defect(s).

Obesity. Evidence suggest that both insulin binding and insulin receptor kinase activity are altered by obesity. Olefsky et al. (1976) reported that muscle membranes from old, obese rats bound less insulin, compared to membranes from younger, lean animals. In isolated soleus muscles, it was found that insulin binding to muscles from gold thioglucose-induced obese mice bound less insulin than muscles from lean controls (Le Marchand-Brustel et al., 1978). For obese human subjects, it was found that insulin binding to abdominal muscle was decreased due to reduced number of binding sites (Caro et al., 1987). In addition to insulin binding, the ability of insulin to stimulate the autophosphorylation of the β-subunit was not altered. However, the insulin receptor kinase activity was decreased in obese subjects, using Glut4:Tyr1 as exogenous phosphoacceptor (Caro et al., 1987).

Exercise. There are conflicting reports on whether acute exercise increases insulin binding to its receptors in skeletal muscles. In rats swum for 90 min, insulin binding to membranes from soleus and EDL was significantly increased (Webster et al., 1986). However, Bonen and Tan (1989) found that, after 2-3 hours of acute treadmill exercise, insulin binding to isolated soleus was not altered, and insulin binding to isolated EDL was lowered, compared to muscles from resting controls. In contrast, insulin-stimulated glucose uptake into the exercised muscles were found increased (Bonen and Tan, 1989).

Treadway et al. (1989) studied the effect of 45 min treadmill running on insulin receptor functions. It was found that none of the receptor functions, i.e., insulin receptor binding, receptor autophosphorylation and exogenous kinase activity, was affected by the acute exercise. It is apparent that exercise might increase the action of insulin at a post-receptor level.

INSULIN-LIKE GROWTH FACTOR I (IGF-I)

IGF-I

IGF-I is a polypeptide with 70 amino acids (human). The amino acid sequence of IGF-I reveals a high degree of similarity to insulin and therefore IGF-I exhibits some affinity for the insulin receptor (Hadly, 1984). The IGF-I precursor is similar to insulin in that they contain A and B domains which are homologous to the A and B chains of insulin. In contrast to the situation with insulin, the C domain of the IGFs is not removed during prohormone processing. Thus, the mature IGF-I is a single-chain polypeptide (LeRoith and Roberts, 1993). IGF-I has endocrine, paracrine and autocrine actions (Langford and Miell, 1993). In skeletal muscle, IGF-I increases glucose transport and utilization, stimulates amino acid uptake and protein synthesis (Dimitriadis et al., 1992; Dardevet et al., 1994), influences glutamine metabolism (Parry-Billings et al., 1993), and plays a role in muscle growth and repair (Allen and Rankin, 1990). In muscle cells, IGF-I plays an important role in muscle proliferation and differentiation (Johnson and Allen, 1990).

IGF-I RECEPTOR AND BINDING

IGF-I and insulin receptors are structurally very similar. Two α- and two β-subunits joined by disulfide bridges form the mature receptor which spans the plasma membrane. The α-subunits lie entirely extracellularly and are responsible for binding the ligand, while the β-subunits are transmembrane polypeptides which contain a tyrosine kinase domain in their intracellular portion. For review, see Rechler and Nissley (1985).

Because of the sequence homology and structure similarities between insulin and IGF-I, it is possible that insulin interacts with IGF-I receptor. Burguera et al. (1991) found that it took an insulin concentration as high as $1*10^{-6}$ M to displace 50% the binding of [^{125}I]IGF-I, compared with only $1*10^{-9}$ M for IGF-I to displace 50% IGF-I bound, in receptors from human liver, muscle and adipocytes.

In spite of the similarity between insulin and IGF-I, the factors affecting IGF-I binding might be different from those affecting insulin binding. Poggi et al. (1979) reported a unchanged IGF-I binding to soleus muscle of goldthioglucose-obese mice, compared with lean controls. This was in contrast with insulin binding which was significantly decreased in obese mice. For human obese non-insulin-dependent diabetes mellitus (NIDDM) patients, insulin binding to receptors prepared from skeletal muscle was decreased by ~30%; however, IGF-I binding was not changed (Livingston et al., 1988). Those studies indicate that insulin and IGF-I binding are affected differently. It is not clear if IGF-I receptor functions will be affected under other insulin resistant stages, such as a high-fat diet induced insulin resistance.

IGF-I BINDING PROTEINS (IGF-BPs)

It was proposed that the major form of plasma IGF-I was a 150-kDa complex made up of 3 subunits: IGF-I, a specific IGF-BP and an acid-labile subunit (Furlanetto, 1980). Smaller amounts of IGF circulated in a 40- to 50-kDa complex made up of IGF-I and IGF-BP, and only a very small proportion of the plasma IGF-I was free.

IGF-I bound to BPs has a vastly increased half-life in plasma (Kaufman et al., 1977). This is particularly true of the 150-kDa complex composed of IGF-I, IGF-BP and

a acid-labile component, which represents the majority of plasma IGF-I. Most estimates of the half life of the 150-kDa IGF-I complex are in terms of hours rather than the few minutes for free IGF peptides. One consequence of this long half-life is that the total circulating level of IGF-I in plasma is close to 1,000 ng/ml, which is 100- to 1000-fold higher than most peptide hormones (Hintz, 1990).

There is evidence that the IGF-BPs can have both inhibitory and stimulatory effects on the action of IGF-I. Bagley et al. (1989) have shown that inhibition of the action of IGF peptide analogues was directly related to their ability to bind to IGF-BPs. The more IGF peptide that is bound the less biological action is seen. This data is consistent with the hypothesis that only free IGF-I is biologically active. On the other hand, Blum et al. (1989) have shown a potentiation of IGF action in the presence of IGF-BPs. Further investigation is needed to determine the effect of IGF-BPs on the action of IGF-I.

GLUCOSE TRANSPORT

GLUCOSE TRANSPORT

Glucose is transported across cell membranes via facilitated diffusion, which is distinguished from active or co-transport of sugars across the cell membrane in two important ways: 1) although glucose transport via the passive mechanism is bidirectional, net transport always occurs in the direction of high to low glucose concentration; and 2) transport of glucose via the passive mechanism does not require obligatory coupling to the symport or cotransport of a cation (Carruthers, 1990).

One of the widely accepted models explaining how glucose transport occurs is Widdas's alternating-conformer model (Widdas, 1952). The glucose carrier was proposed to contain mutually exclusive saturable sugar influx and efflux sites. At any point of time, a carrier can be available for sugar influx or sugar efflux but not for both simultaneously. When sugar binds to one of these sites to form the carrier-sugar complex, a conformational change is promoted that results in sugar translocation, dissociation of sugar from the carrier at the opposite side of the membrane, and the exposure of a glucose transport site at the opposite side of the membrane. The carrier would them be available for transport in the opposite direction or to isomerize back to its original state, a sugar binding site exposed at the original side of the membrane.

GLUCOSE TRANSPORTERS

The isolation and characterization of cDNA clones encoding five different functional facilitative glucose carriers indicates that facilitative glucose transport is not

the property of a unique protein but rather is a feature of a family of structurally related proteins (Bell et al., 1990). Based on the order in which they were described, they have been designated as GLUT1, GLUT2, GLUT3, GLUT4 and GLUT5 (Bell et al., 1990). Another designation whereby each isoform is identified based on the tissue in which it is very abundant and that conveys a minimum of physiological information, i.e., erythrocyte, liver, brain, muscle/fat, and small intestine, respectively, has been proposed (Pilch, 1990). The sizes of the glucose transporters vary from 492 to 524 amino acids (Silverman, 1991).

The insulin-sensitive tissues, fat and muscle, express the GLUT1 and GLUT4 transporter isoformers, and GLUT4 is at least 10-fold more abundant in fat and skeletal muscle than is GLUT1 (James et al., 1989; Zorzano et al., 1989; Holman et al., 1990). The level of GLUT4 expressed in insulin-sensitive tissues of the rat (brown fat >heart >red muscle >white muscle >white fat) parallels the magnitude of the insulin-mediated increase in glucose transport observed in these tissues (James et al., 1989). This suggests that GLUT4 is the isoform primarily responsible for acutely insulin-stimulated glucose transport.

REGULATION OF GLUCOSE TRANSPORT

1) Insulin and IGF-I. Insulin rapidly increases glucose uptake and metabolism in fat and skeletal muscle (Charron and Kahn, 1990). This response, especially in skeletal muscle, is crucial to lowering blood glucose levels in the postprandial state. Glucose uptake is the rate-limiting step for its metabolism in skeletal muscle (Berger et al., 1975), and insulin exerts its stimulatory effect principally via increasing this initial step in

glucose uptake. Essentially all of the GLUT4 is present in intracellular compartments in the basal state, but a proportion of GLUT1 is found in the plasma membrane, where it likely contributes to basal transport (Holman et al., 1990). Insulin induces the redistribution of both GLUT1 and GLUT4 from their intracellular storage sites to the cell surface, resulting in augmented glucose transport (Zorzano et al., 1989; Holman et al., 1990). This effect occurs within minutes and is rapidly reversible upon insulin withdrawal. It appears that most of the insulin-stimulated increase in glucose transport can be attributed to this translocation phenomenon (Holman et al., 1990). Insulin may also increase intrinsic activity of the glucose transporter. Sternlicht et al. (1988) found that the Vmax for glucose transport was increased by 3.2-fold after maximum insulin stimulation, with no change in Km. The number of glucose transporters, as assessed by cytochalasin-B binding, increased by only 1.7-fold, with no change in Kd. This result led Sternlicht et al. (1988) to conclude that insulin increased glucose transport by both translocation and activation of glucose transporters. Similar results were reported by Klip et al. (1987) and Goodyear et al. (1991). In addition, chronic exposure to insulin increased GLUT1 mRNA and protein in 3T3L1 adipocytes (Tordjman et al., 1989), suggesting that insulin also stimulates glucose transport by modulating glucose transporter gene expression.

In addition to insulin, IGF-I is a potent peptide hormone stimulating glucose metabolism in skeletal muscles. Poggi et al. (1979) found that IGF-I stimulated glucose uptake, glycolysis, and glycogen synthesis in mouse soleus muscle. IGF-I was found to be 4-9% as potent as insulin and nearly as effective as insulin. The insulin-like effect on glucose transport was further demonstrated in isolated rat soleus muscle (Dimitriadis et

al., 1992), isolated rat epitrochlearis muscles (Dardevet et al., 1994) and in cultured muscle cells (Bilan et al., 1992). Since IGF-I has acute insulin-like metabolic effects, administration of IGF-I has shown to lower serum glucose level (Zenobi et al., 1992). Studies are in progress to determine the effect of a treatment with IGF-I in patients with insulin-dependent diabetes mellitus whose endogenous IGF-I production is reduced (Bondy et al., 1994). Insulin-like growth factor I treatment may reduce glucose and triglyceride levels in adults with non-insulin-dependent diabetes mellitus and in some patients with extreme insulin resistance. Thorough study of the actions of IGF-I under different metabolic and pathological states may contribute to the therapeutic application of IGF-I.

2) Exercise. a). Acute exercise. Numerous studies have shown that electrical stimulation of muscle or a single bout of exercise stimulates glucose uptake in skeletal muscles (for review see Ivy, 1987). Exercise-stimulated glucose transport consists of two phases (Garetto et al., 1984). The initial effect lasts for a few hours and does not require the presence of insulin (Young et al., 1987; Cartee et al., 1989). The duration of this phase seems to be correlated with glycogen resynthesis and can be prolonged by dietary manipulations, such as feed restriction or feeding animals lard, to delay glycogen resynthesis (Young et al., 1983; Garetto et al., 1984). This effect of acute exercise is additive to that of insulin (Nesher et al., 1985; Constable et al., 1988; Wallberg-Henriksson et al., 1988), indicating the mechanisms for stimulation of glucose uptake by insulin and the initial phase of exercise are different. The exact mechanism involved in this exercise-stimulated insulin-independent glucose transport is not completely

understood; however, it seems this increase in glucose uptake is regulated by local factors (Richter et al., 1989; Gulve et al., 1990; Hespel and Richter, 1990). Similar to insulin-stimulated glucose transport, translocation of glucose transporters to plasma membrane was suggested by an increase in cytochalasin-B binding in sarcolemma vesicles with acute exercise (Hirshman et al., 1988; Douen et al., 1989). Recent evidence suggests that calcium may be involved in activating the glucose transport system in muscle with acute exercise (Youn et al., 1991). Since the mechanisms for exercise- and insulin-stimulated glucose transport might be different, it is reasonable to postulate that exercise-stimulated glucose transport remains intact in insulin-resistant stages, e.g., high-fat induced insulin-resistance. However, this area has not been explored.

After the initial acute increase in glucose transport, there is a secondary phase in which the action of insulin, especially insulin sensitivity, is enhanced. This is shown by the observation that the effects of insulin and exercise were more than additive (Wallberg-Henriksson et al., 1988; Gulve et al., 1990). Annuzzi et al. (1991) observed the effect 24 h after exercise and showed that the effect was specific for muscles directly involved in exercise. Although insulin is thought to act via the insulin receptor tyrosine kinase, as discussed earlier, Treadway et al. (1989) found no increase in receptor kinase activity after acute exercise. Increased insulin binding by acute exercise was reported (Webster et al., 1986), but could not be confirmed (Bonen and Tan, 1989). It is still unknown how insulin sensitivity is increased by acute exercise.

Acute exercise also enhances IGF-I-stimulated glucose transport in skeletal muscle. Henriksen et al. (1992) demonstrated that, 3.5 hours after a single bout of swimming, both

submaximal (5 nM) and maximal (20 nM) IGF-I-stimulated glucose transport were increased ~one-fold in epitrochlearis muscles, compared with resting controls. In comparison, the same study found glucose uptake was enhanced only at submaximal (0.2 nM), but not maximal (13.3 nM) insulin concentration.

One report has shown that acute exercise increases susceptibility of muscle glucose transport to activation by various stimuli (Cartee and Holloszy, 1990). The stimulatory effects of H_2O_2, hypoxia and insulin-mimetic agent vanadate, on glucose transport in epitrochlearis muscle were enhanced in swimming-exercised rats. It is apparent that the stimulatory effect of exercise is not restricted to hormone-stimulated glucose transport.

b). Exercise training. Exercise training for several weeks increased maximal insulin- or insulin plus contraction-stimulated glucose transport by ~40% (Rodnick et al., 1992; Slentz et al., 1992). When different intensities of exercise training (with equal work) were employed, it was found that the increase in muscle glucose uptake was exercise intensity specific: muscles from high-intensity trained rats transported more glucose than that from low-intensity trained rats (Cortez et al., 1991). GLUT4 protein was increased by training to a similar extent as was glucose uptake (Rodnick et al., 1992; Slentz et al., 1992). These results indicate that training does result in adaptations in rats not seen with a single bout of exercise. Ploug et al. (1990) reported increased protein and mRNA for GLUT1, in addition for GLUT4. However, this could not be confirmed (Rodnick et al., 1992).

3) Diet. Numerous studies have demonstrated that diets high in fat cause impaired insulin-stimulated glucose transport. The pioneering work of Himsworth (1934)

demonstrated that high-fat low-carbohydrate diet reduced glucose tolerance. Olefsky and Saekow (1978) found that feeding animals a diet with 67% of energy from fat resulted in a decrease in insulin-stimulated glucose transport in adipocytes. Grundleger and Thenen (1982) fed lean Zucker rats diets with 67% of energy either as fat or carbohydrate for 10 days, and insulin-stimulated glucose transport in soleus muscle was decreased for rats fed the high-fat diet. Basal glucose transport was not changed by feeding the high-fat diet. After 10 to 12 weeks of feeding rats either a high-fat (40% energy as fat) or low-fat (9% as fat) diet, maximally insulin-stimulated glucose transport into sarcolemmal vesicles was significantly lower for rats fed the high-fat diet (Grimditch et al., 1987). These results clearly show that the level of dietary fat influences insulin-stimulated glucose transport in insulin sensitive tissues.

The mechanism underlying the effects of the high fat diet on glucose transport was investigated. In isolated rat adipocytes, it was found that the high-fat diet (67% energy as fat) induced decrease in insulin stimulated glucose transport is closely associated with decreased insulin binding (Olefsky and Saekow, 1978). In isolated rat soleus muscle, the high fat diet (67% energy as fat) decreased both insulin binding and glucose transport, indicating the decreased insulin binding might be responsible for decreased glucose transport (Grundleger and Thenen, 1982). One study reported that prolonged treatment (4 hours) of rat adipocytes with palmitate induced insulin-resistance (Hunnicutt et al., 1994), indicating circulating free fatty acids may play a role in development of insulin-resistance. Pedersen et al., (1991) reported that a diet high in fat (80% of energy) induced a 90% decrease in GLUT4 protein and a 62% decrease in GLUT1 protein in adipocytes, as well

as decrease in their mRNA levels. Insulin-stimulated glucose transport was significantly decreased. Interestingly, the basal glucose transport was elevated in face of the decreased GLUT1 protein. However, it is unknown if these changes were induced by high-fat diet per se, or due to humoral changes (e.g., changes in plasma insulin level and/or insulin resistance) associated with high-fat feeding.

In addition to the quantity of fat consumed, the quality of dietary fat (i.e., fatty acid composition) affects insulin-stimulated glucose transport. Field et al. (1990) reported that, at all insulin concentrations tested, adipocytes from animals fed the high P/S diet transported significantly more glucose than did those from animals fed the low P/S diet. Storlien et al. (1991) have shown that glucose metabolism in skeletal muscle was sensitive to dietary fatty acid composition. Glucose metabolism for muscles from rats fed a diet high in fat (59% of energy as fat, with a P/S ratio ~1.5) was significantly lower than glucose metabolism in muscles from rats fed a low-fat diet. When a portion of the fat in the high-fat diet was substituted with polyunsaturated ω-6 or ω-3 fatty acids (P/S ratio was raised from ~1.5 to ~11), glucose metabolism in muscles from rats fed the diet high in polyunsaturated fatty acids was similar to that from rats fed the low-fat diet. It also has been shown that, while the level of dietary fat remained constant (59% of energy) and the P/S ratio low (0.27), 6% ω-3 fatty acid (of total calories) will restore the decreased insulin-dependent glucose uptake induced by the high-fat diet (Storlien et al., 1991), suggesting the ω-3 polyunsaturated fatty acids might be more potent in preventing high-fat diet induced insulin-resistance than ω-6 polyunsaturated fatty acids. In isolated muscle preparations, Sohal et al. (1992) found that muscles from rats fed a high-fat diet rich in

long chain ω-3 fatty acids had more insulin-stimulated glucose transport than muscles from rats fed a high-fat diet without long chain ω-3 fatty acids. The improvement in insulin-stimulated glucose transport is likely due to the improved insulin binding by dietary polyunsaturated fatty acids. However it has not been demonstrated in skeletal muscle (see FACTORS AFFECTING INSULIN RECEPTOR FUNCTION above).

4) Diabetes. a). Insulin-dependent diabetes mellitus. There are many studies in which insulin deficiency was induced by streptozotocin in rats that show severe insulin resistance in insulin sensitive tissues. In adipocytes, insulin-stimulated glucose transport was lower for diabetic rats than that for normal controls (Field et al., 1990). In isolated sarcolemmal vesicles, both basal and insulin-stimulated glucose transport was reduced for the diabetic rats (Barnard et al., 1990). The decreased glucose uptake was due to fewer glucose transporters in the sarcolemmal vesicles. Daily insulin injections corrected the basal transport but did not completely normalize the insulin-stimulated glucose transport (Barnard et al., 1990). Another study also reported that skeletal muscle GLUT4 protein and mRNA levels were decreased for streptozotocin-induced diabetic rats (Camps et al., 1992).

b). Non-insulin-dependent diabetes mellitus (NIDDM). NIDDM is closely associated with decreased insulin-stimulated glucose uptake. Using isolated muscle strips, Dohm et al. (1988) found no difference in basal glucose transport between non-obese controls, obese individuals, and obese NIDDM patients. Insulin-stimulated glucose uptake in the controls by ~3 fold, compared with a small effect in the obese and no response in the NIDDM patients. For lean patients with NIDDM, Scheck et al. (1991) found glucose

transport in isolated sarcolemmal vesicles was comparable with healthy lean controls; however, serum insulin levels were three times as high as the controls, indicating the existence of insulin-resistance.

In contrast to insulin, the action of IGF-I is not impaired in diabetic states. Rossetti et al. (1991) reported an unaltered IGF-I-stimulated whole-body glucose utilization in partially (90%) pancreatectomized diabetic rats, compared to a 30% decrease for the action of insulin. In addition, the administration of IGF-I corrected the intracellular defects in glycogen synthesis and glycolysis. Jacob et al. (1991) also reported a normal IGF-I stimulated whole-body glucose uptake in face of a decreased insulin action in spontaneously diabetic BB/w rats. Further study has been recommended to evaluate the efficacy and safety of IGF-I treatment for diabetic patients (Bondy et al., 1994).

5) Other factors. a). Obesity. Insulin-stimulated glucose transport is decreased in obese subjects. For soleus muscles isolated from obese mice, maximally insulin-stimulated glucose uptake was markedly decreased, compared to glucose transport in muscles from lean controls (Le Marchand-Brustel et al., 1978). Using hindlimb perfusion, Sherman et al. (1988) found that glucose uptake was significantly depressed with or without presence of different concentrations of insulin in muscles from obese Zucker rat, compared to their lean littermates. Decreased insulin-stimulated glucose transport was also documented in muscles from obese human subjects (Dohm et al., 1988).

Similar to the action of insulin, IGF-I stimulated glucose transport was also decreased in the isolated soleus muscle of obese mice, compared with lean controls (Poggi et al., 1979); and IGF-I was completely ineffective in stimulating glucose transport in

muscles from obese human subjects (Dohm et al., 1990). There was a 50% decrease in IGF-I-stimulated glucose transport in obese Zucker rats, compared with lean controls (Jacob et al., 1992). These data indicate that IGF-I-stimulated glucose uptake is impaired in the obese state.

b). Aging. A decline in insulin-stimulated glucose transport, as well as amino acid uptake, was documented for epitrochlearis muscles from adult rats (6-8 months), compared with transport activities for muscles from young animals (1 month, Dardevet et al., 1994). Gulve et al. (1993) demonstrated ~60% decrease in both basal and insulin-stimulated glucose uptake in epitrochlearis muscles for 4 month old rats, compared with muscles from 1 month old rats. The decrease in glucose transport is at least partially due to decreased GLUT4 in the muscle (Gulve et al., 1993). For review of decreased hormone-stimulated glucose transport, see Nishimura et al. (1988). IGF-I-stimulated glucose transport was also lowered in epitrochlearis muscles from adult rats, which was accompanied by dramatic decrease in IGF-I receptor number (Dardevet et al., 1994).

In conclusion, glucose transport in insulin sensitive tissues, especially skeletal muscle, is affected by a variety of factors. Glucose transport, even for a healthy human being, is regulated by the sum of the effects of different factors, such as plasma level of insulin and IGF-I, their receptor function, physical activity, age and obesity. It is preferable, therefore, to determine the effects of different factors and the possible interactions among them, e.g., the effects of dietary fat and exercise, which has not been thoroughly studied.

HYPOTHESES

It was hypothesized that:

1. Dietary supplementation of long chain ω-3 fatty acids would enrich the content of ω-3 fatty acid in plasma membrane phospholipid in skeletal muscle;

2. An increase in the content of ω-3 fatty acid in plasma membrane phospholipid in skeletal muscle would be associated with an increase in insulin binding to plasma membrane vesicles;

3. High-fat feeding would be associated with a decrease in IGF-I-stimulated glucose transport, as well as a decrease in insulin-stimulated glucose transport, in skeletal muscle;

4. High-fat diet induced IGF-I-resistance would be associated with decreased IGF-I binding to skeletal muscle;

5. Glucose transport stimulated by acute exercise would remain intact in muscles resistant to insulin;

6. The effects of insulin, acute exercise and high-fat diet on glucose transport would interact with each other.

OBJECTIVES

The objectives of this thesis research were:

1. To isolate purified plasma membrane from rat skeletal muscle and to determine if fatty acyl composition of phospholipid is modified by diets with different fatty acid compositions (Chapter II).

2. To determine hormone binding characteristics for insulin and IGF-I, including Kd, Bmax, in muscle plasma membranes from rats fed diets with different fatty acid compositions (Chapter II).

3. To measure dose-response for IGF-I- and insulin-stimulated glucose uptake in rat skeletal muscle, and measure IGF-I- and insulin-stimulated glucose transport in muscles from rats fed a low- or a high-fat diets (Chapter III).

4. To determine IGF-I binding to skeletal muscles from rats fed a low- or a high-fat diet (Chapter III).

5. To measure glucose transport activity in muscles from rats fed a low- or a high-fat diet, with or without acute exercise prior to the study, and with or without the presence of insulin during incubation (Chapter IV).

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CHAPTER II: DIETARY ω-3 AND POLYUNSATURATED FATTY ACIDS MODIFY FATTY ACYL COMPOSITION AND INSULIN BINDING IN SKELETAL MUSCLE SARCOLEMMA[1]

INTRODUCTION

Insulin resistance is a metabolic state in which normal or high concentrations of insulin produce a less than normal biological response (DeVries et al., 1989). Defects in insulin binding, tyrosine kinase and post-kinase cascades have been postulated, however the mechanism is not completely understood (Haring, 1991). Insulin initiates its biological effects by binding to its receptors on target tissues (DeVries et al., 1989). There is evidence suggesting that impaired insulin binding is closely associated with insulin resistant states, such as those associated with feeding high fat diets (Olefsky and Saekow, 1978), obesity (Olefsky et al., 1976; Marchand-Brustel et al., 1978; Caro et al., 1987) and non-insulin-dependent diabetic mellitus (NIDDM) (Trischitta et al., 1989).

Insulin binding to its receptor can be altered by changing fatty acid composition in phospholipids in artificial membranes (Gould et al., 1982). In cultured (Ginsberg et al., 1981) and isolated cells (Field et al., 1988), membranes enriched in unsaturated fatty acids tend to bind more insulin than membranes enriched in saturated fatty acids. It has been postulated that alteration in membrane fluidity induced by enrichment of polyunsaturated fatty acids might be involved in the change observed in insulin binding (Ginsberg et al., 1981). Composition in cell membrane phospholipids can be altered by dietary fatty acids

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(Clandinin et al., 1991) by mechanisms that can involve altering activity of enzymes of fatty acid desaturation (Garg et al., 1988), membrane cholesterol content and the biosynthesis of membrane phosphatidylcholine de novo (Hargreaves and Clandinin, 1987). In rats and in humans, consumption of a high-P/S (polyunsaturated/saturated fatty acid ratio, P/S=1.0) diet increased the content of polyunsaturated fatty acids in adipocyte membrane phospholipids, compared with the low-P/S diet (P/S=0.25) (Field et al., 1985; Field et al., 1988). In agreement with in vitro studies (Ginsberg et al., 1981), the elevated content of polyunsaturated fatty acids in adipocyte membrane was associated with increased insulin binding (Field et al., 1988). By feeding a high-P/S diet, it was demonstrated in adipocytes that glucose transport was also augmented by the high-P/S diet, along with increased insulin binding (Field et al., 1990).

Skeletal muscle is an important site of insulin action. The insensitivity of this tissue to insulin plays an important role in the development of whole body insulin resistance (Klip and Paquet, 1990). Feeding rats a high-fat diet can lead to skeletal muscle insulin resistance, even when polyunsaturated ω-6 fatty acids are also fed (Storlien et al., 1987, 1991). When a portion of the ω-6 fatty acids was replaced by fish oil high in long chain ω-3 fatty acids (22:5ω-3 and 22:6ω-3) the muscle insulin resistance induced by the high-fat diet was prevented (Storlien et al., 1987, 1991). Recently, we reported that maximum insulin stimulated glucose uptake in isolated skeletal muscle was also higher in rats fed fish oil (Sohal et al., 1992). However, the mechanism for how ω-3 fatty acids prevent insulin resistance in skeletal muscle is unknown. Membrane phospholipid content of long-chain (22:5 and 22:6) ω-3 fatty acid in skeletal muscle is apparently closely

related with insulin-stimulated glucose metabolism (Storlien et al., 1991). Feeding rats a high-fat diet also seems to decrease the amount of insulin binding to skeletal muscle (Maegawa et al., 1986). Thus, we hypothesized that improved insulin binding to skeletal muscle of rats fed a high ω-3 fatty acid diet might be responsible for the prevention of insulin resistance. Furthermore, we hypothesized that the differences in insulin binding may be attributable to ω-3 fatty acids per se, or due to the high degree of fatty acid unsaturation produced in the plasma membrane fraction.

EXPERIMENTAL

MATERIALS

Benzamidine and iodoacetamide was purchased from Aldrich Chemical Company Inc. (Milwaukee, Wis 53233, USA). Porcine insulin, aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), bacitracin, wheat germ agglutinin (WGA), BSA and rabbit γ-globulin was purchased from Sigma Chemical Co. (St Louis, Mo 63178, USA). ^{125}I-insulin (porcine) was purchased from Du-Pont New England Nuclear (Boston, MA, USA).

ANIMAL CARE AND TREATMENT

Studies were carried out in accordance with the Canadian Council on Animal Care guidelines. Female Sprague-Dawley rats were obtained from a colony maintained at the University of Alberta and were fed semipurified diets starting at 30 to 35 days of age. Rats were housed in individual wire mesh cages in a room that was maintained at 23 °C on a 12 h light/dark cycle. Animals were given food and water ad libitum and were fed one of the experimental diets for 6 weeks.

Animals were fed semipurified nutritionally complete diets containing (% w/w): 27% high protein casein, 38% carbohydrates (cornstarch), 20% fat, 1% vitamin mixture (Association of Analytical Chemists), 5% mineral mixture (Bernhart-Tomerelli), 0.3% choline, 0.6% inositol, 0.3% methionine, and 8% non-nutritive cellulose (Field et al., 1988). In experiments where a different dietary level of ω-3 fatty acid was studied, 20 rats were randomly assigned to two treatment groups: low ω-3 and high ω-3 diets. Diets differed only in the composition of fat providing two different levels of long chain ω-3

fatty acids. The high ω-3 diet contained long chain ω-3 fatty acids (2.4% 20:5ω-3, 0.2% 22:5ω-3 and 0.7% 22:6ω-3) as well as α-linolenic acid (18:ω-3, 0.3%). The low ω-3 fatty acid diet contained only α-linolenic acid (C18:3ω-3, 0.8%) as the source of ω-3 fatty acid. Fatty acid composition of the diets is illustrated (Table II-1). In experiments where the dietary ratio of polyunsaturated to saturated fatty acids balance was studied, the experimental design was similar to that described above except that the diets used provided a P/S ratio of either 1.0 (high P/S) or 0.25 (low P/S) (Table II-1) as described previously (Field et al., 1988).

MEMBRANE PREPARATION

Rats were killed by carbon dioxide asphyxiation. Plasma membrane was prepared using differential and density gradient centrifugation according to Ohlendieck et al. (1991). After those steps, membrane was further purified by wheat germ agglutination. Membrane isolated using this procedure is enriched in the sarcolemmal protein dystrophin, but does not contain T tubule membrane or junctional sarcoplasmic reticulum as assessed by immunochemical methods (Ohlendieck et al., 1991). The agglutination step was incorporated into the binding protocol for membrane fractions used in insulin binding studies. Membrane protein content was determined after solubilization with 1 M-NaOH by the method of Bradford (1976) using BSA as standard protein (Sigma, fraction V). About 10 mg of membrane protein was recovered after the density gradient centrifugation step from 100 g mixed hindlimb muscle dissected from rats of each treatment group. The membrane was suspended in Krebs-Ringer HEPES buffer (118 mM-NaCl, 5 mM-KCl, 1.2

mM-$MgSO_4$ and 50 mM-HEPES, pH 7.6), quickly frozen in liquid nitrogen and stored at -50°C for further analysis.

INSULIN BINDING TO SKELETAL SARCOLEMMA

Insulin binding to sarcolemmal vesicles was measured as described by Grimditch et al. (1985) and Kergoat et al. (1988) with some modifications. Membrane protein (100 µg) was incubated in a microcentrifuge tube with 0.03 nM ^{125}I-insulin (about 1200 Bq/tube) in Krebs-Ringer HEPES buffer containing 1% (w/v) BSA and 1mg/mL bacitracin. The final volume was 200 µl. After 20 h incubation at 4°C the binding reaction was stopped by centrifugation after agglutination of the vesicles with wheat germ agglutinin (WGA) as follows: the reaction medium was mixed with 100 µl (1mg/ml) WGA; after 20 min incubation on ice, bound insulin was separated by centrifugation at 17,000 g for 5 min using a IEC Centra-M centrifuge at 4°C. The supernatant was discarded and the pellet was washed twice with chilled 0.25 M-sucrose. The tip of the tube containing the pellet was cut off and placed in a test tube for radioactive counting in a Packard Cobra Auto-Gamma counter. Counts were converted to dpm. Non-specific binding was defined as radioactivity bound to membrane in the presence of 10 µM unlabelled insulin and was typically ~ 30% of total binding. Specific binding was obtained by subtracting nonspecific binding from total binding.

INSULIN BINDING TO SOLUBILIZED RECEPTOR

Solubilized receptor was prepared according to Whitson and Kaplan (1985). The receptor from 0.1 mg membrane protein was incubated with 1200 Bq ^{125}I-insulin in the buffer as described in the assay of insulin binding to sarcolemma. After a 20 hour

incubation at 4°C, 100 µl of 0.3% (w/v) r-globulin and 300 µl of 25% (w/v) polyethylene glycol was added to the binding mixture. This mixture was placed on ice for 20 min and centrifuged at 17,000 g for 5 min using a IEC Centra-M centrifuge at 4°C. The final pellet was counted as described above.

MEMBRANE LIPID EXTRACTION, PHOSPHOLIPID SEPARATION AND FATTY ACID DETERMINATION

Lipid was extracted from plasma membrane obtained after WGA agglutination by a modified Folch procedure (Field et al., 1988). Individual phospholipids were separated in a one-dimensional solvent system using silica gel H plate (ANALTECH, Newark, Del, USA). Phospholipid fatty acid methyl esters were prepared using 14% (w/v) BF_3/methanol reagent (Morrison and Smith, 1964) and separated by automated gas-liquid chromatography (Vista 6010 GLC and Varian 604 chromatography data system, Varian Instruments, Georgetown, Ontario, Canada). Chromatography was performed using a fused silica BP20 capillary column (25mx0.25mm inner diameter; Varian Instruments, Georgetown, Ontario, Canada) under conditions described in detail earlier (Field et al., 1988).

DATA ANALYSIS

The data presented was obtained from four separate feeding studies and preparations of membrane. All analyses were done in duplicate. Rosenthal analysis was completed using the software LIGAND (Biosoft, 22 Hills Road, Cambridge, CB2 1JP, UK). The parameters obtained were statistically compared using Student's t-test.

RESULTS

METHODOLOGICAL CONSIDERATIONS

In preliminary experiments, membrane fractions obtained after density gradient centrifugation were used in insulin binding assays. In these studies, nonspecific binding varied from 45-50% of total insulin bound to membrane fractions precipitated by ultracentrifugation at ~140,000g for 5 min. We then used WGA to precipitate membrane and demonstrated that does not precipitate free insulin (data not shown). The non-specific binding was lowered by this procedure to ~30%. The absolute amount of specifically bound insulin was the same using the two precipitation methods, however both the absolute amount of total and non-specifically bound insulin was lower using the WGA method.

EFFECTS OF DIETS ON THE FATTY ACYL COMPOSITION OF SKELETAL MEMBRANE PHOSPHOLIPIDS

Fatty acyl composition of individual membrane phospholipids was obtained using WGA-purified membrane prepared from a pool of 10 rats per dietary treatment. Dietary content of long chain ω-3 fatty acids exhibited profound effects on the membrane phospholipid fatty acid composition (Table II-2).

Effects of high and low ω-3 diet

In phosphatidylcholine, total ω-3 fatty acid content was 6.5% for rats fed the high ω-3 fatty acid diet, compared with only 1.5% for rats fed low ω-3 fatty acid diet, a more than three fold increase (Table II-2). The content of 20:5ω-3, 22:5ω-3 and 22:6ω-3 were 0.9, 1.6 and 3.9% for animals fed the high ω-3 fatty acid diet vs 0.1, 0.4 and 0.9% for

animals fed the low ω-3 fatty acid diet, respectively. Feeding the high ω-3 diet decreased the 20:4ω-6 content from 13.2% observed for the low ω-3 group to 5.8% for the high ω-3 group.

In phosphatidylserine, the amount of total ω-3 fatty acid was higher in rats fed the high ω-3 diet (23.1%) than rats fed the low ω-3 diet (10.3%). This was mainly due to the increase in the contents of 22:5ω-3 and 22:6ω-3 fatty acids (5.0% and 17.3% for high ω-3 vs 1.9% and 8.1% for low ω-3 group, respectively). The content of 20:5ω-3 was similar in both groups (0.3% vs 0.4%). The amount of 20:4ω-6 was decreased from 9.3% to 3.8% by feeding the high ω-3 diet.

In phosphatidylinositol, the total amount of ω-3 fatty acid was higher in rats fed the high ω-3 diet than obtained for animals fed the low ω-3 diet (11.4% vs 4.2%). The content of all of the long chain ω-3 fatty acids was increased by feeding the diet high in ω-3 fatty acids (Table II-2). In contrast, the content of 20:4ω-6 was 50% higher in rats fed low ω-3 diet (10.2% vs 15.8%).

In phosphatidylethanolamine, the total ω-3 fatty acid content was increased 51% by feeding the diet high in ω-3 fatty acids (22.3% vs 14.8%, Table II-2). This represented the smallest percentage increase obtained among all phospholipid fractions. The individual long chain ω-3 fatty acids were slightly higher in rats fed the diet high in ω-3 fatty acids with the exception of 20:5ω-3, which was 10 fold higher in rats fed the high ω-3 diet. The content of 20:4ω-6 was lowered by feeding the high ω-3 diet (from 16.1% to 4.5%).

Effects of high and low P/S diet

In all phospholipid fractions the content of saturated and monounsaturated fatty acids was not altered by the diet fed (Table II-2). The P/S ratios of the membrane were similar in both dietary treatments, except for phosphatidylethanolamine where P/S ratio tended to be lowered for animals fed the high ω-3 diet.

Both the content of individual fatty acids and the P/S ratio in phospholipids were not altered by the diets with different P/S ratio (Table II-3).

EFFECT OF HIGH AND LOW ω-3 DIETS ON INSULIN BINDING TO SKELETAL MUSCLE SARCOLEMMA

Skeletal sarcolemma from animals fed the high ω-3 fatty acid diet bound more insulin than that from rats fed the low ω-3 fatty acid diet over a wide concentration range of insulin (0.1 to 100nM). This difference in binding was especially marked at high insulin concentrations (Fig II-1a). Rosenthal analysis revealed two insulin binding sites on the skeletal muscle sarcolemma. Binding parameters were derived from Rosenthal analysis, including Bmax and 1/Kd for high and low affinity binding sites (Table II-4a). There was no significant difference due to diet in either parameter at the high affinity binding sites, however the Bmax was 14-fold higher for the low affinity, high capacity binding site in membrane of rats fed the high ω-3 fatty acid diet ($p<0.0001$) (Table II-4a).

When insulin receptors were solubilized the receptors from animals fed a high ω-3 fatty acid diet tended to bind more insulin at high insulin concentrations than the receptors from animals fed a low ω-3 diet. From the Rosenthal analysis it appears that the binding curves for the two diets were not identical (Fig II-1b). The Bmax observed was not significantly different for membrane from animals fed each diet (Table II-4a).

EFFECT OF HIGH AND LOW P/S DIETS ON INSULIN BINDING TO SKELETAL MUSCLE SARCOLEMMA

Skeletal sarcolemma from animals fed a high P/S diet bound more insulin than observed for animals fed a low P/S diet, especially at high insulin concentrations (Fig II-2a). Rosenthal analysis demonstrated that the Bmax was 2.3-fold higher in the membrane from rats fed a high P/S diet compared to those fed a low P/S diet at the low affinity binding site (Table II-4a). The other binding parameters were not significantly different between animals fed the high vs the low diets.

After insulin receptors were solubilised, the receptors from animals of both high and low P/S diet treatments bound similar amounts of insulin at all insulin concentrations and exhibited similar binding parameters (Fig II-2b and Table II-4a).

EFFECT OF HIGH AND LOW ω-3 DIETS ON IGF-I BINDING TO SKELETAL MUSCLE SARCOLEMMA

Membrane from rats fed a diet high in ω-3 fatty acids bound more IGF-I than that from rats fed a diet low in ω-3 fatty acids (Fig II-3). Rosenthal analysis revealed that the Bmax of the high ω-3 group was more than two-fold higher than that of the low ω-3 group at the low affinity binding site ($p<0.05$, Fig II-3 and Table II-4b). The other binding parameters were not significantly different from each other between the two dietary groups.

DISCUSSION

EFFECT OF DIETARY FAT ON FATTY ACYL COMPOSITION IN SKELETAL MUSCLE MEMBRANE PHOSPHOLIPIDS

In skeletal muscle, it was shown that fish oil, high in long chain ω-3 fatty acids, changed the fatty acyl composition of muscle total phospholipids, especially the content of 22:5ω-3 and 22:6ω-3 (Storlien et al., 1991). However, this work was conducted on muscle phospholipids isolated from a Folch extract of whole tissue, and would therefore represent all muscle membranes rather than sarcolemma specifically. These authors did not further differentiate the effects of dietary fat on individual skeletal membrane phospholipid fractions. This separation is critical to interpretation, since it has been demonstrated that subcellular membrane types contain different content of phospholipid fractions which respond to dietary fat manipulation to varying degrees (Field et al., 1990).

The present study demonstrates that the fatty acyl composition of individual phospholipids of skeletal muscle plasma membrane is markedly sensitive to dietary long chain ω-3 fatty acid supply and that the content of specific fatty acids responds to dietary manipulation differently in individual phospholipids. When rats were fed diets containing about 4% long chain ω-3 diet, the ω-3 fatty acids as percentage of total fat acids were doubled in all four phospholipid classes, and the three individual long chain ω-3 fatty acids (20:5ω-3, 22:5ω-3 and 22:6ω-3) were increased by 2 to 10 fold. The increase of long chain ω-3 fatty acids in the membrane was greatest in phosphatidylcholine, phosphatidylserine and phosphatidylinositol, and relatively smaller in phosphatidylethanolamine. In terms of degree of unsaturation, each phospholipid class had

a characteristic P/S ratio, and this was not affected by ω-3 fatty acids in the diet, except for phosphatidylethanolamine, which tended to be lowered by the diet high in ω-3 fatty acids.

In addition to the increase in the content of long chain ω-3 fatty acids, there was a concomitant decrease in the amount of 20:4ω-6 in the rats fed a diet high in ω-3 fatty acids (Table II-2). Since the content of saturated fatty acids and total polyunsaturated fatty acids was not significantly affected, the major effect of feeding animals with fish oil was the selective replacement of membrane phospholipid 20:4ω-6, with long chain ω-3 fatty acids. In a study where the only source of dietary fatty acid was fish oil, the content of long chain ω-3 fatty acids in muscle phosphatidylethanolamine was increased to 23%, and 20:4ω-6 became undetectable (Jackson et al., 1988). These are in agreement with the finding that ω-3 fatty acids are preferably elongated and desaturated, compared with ω-6 fatty acids (Garg et al., 1989).

The P/S ratio of muscle membrane phospholipid was insensitive to the diet. The fatty acyl composition of skeletal muscle plasma membrane phospholipid was not different between animals fed the diets with different P/S ratios of 0.25 and 1.05. In purified adipocyte plasma membrane, the diet high in P/S ratio (2.0, compared to 0.2) significantly changed the membrane fatty acyl composition: increased the content of 20:4ω-6 and decreased amount of saturated fatty acids, hence increased the P/S ratio (Field et al., 1990). This difference could be due to the cell type or to the more polyunsaturated diet used in that study. However, in skeletal muscle, it was shown that the content of saturated fatty acids and 20:4ω-6 in total muscle phospholipids was not

sensitive to diets with different degrees of saturation, even with a P/S ratio of up to 9.2 (Storlien et al., 1991). It seems that changing the P/S ratio is more difficult than changing the ω-3/ω-6 ratio in skeletal muscle plasma membrane phospholipids. It might be possible that, for skeletal muscle, even the low P/S (0.25 used here) diet provided enough 18:2ω-6 to maintain an optimal level of polyunsaturated fatty acids (mainly 20:4ω-6), and that providing a diet with a higher P/S ratio will not increase the degree of unsaturation further.

MEMBRANE FATTY ACYL COMPOSITION AND INSULIN AND IGF-1 BINDING

Skeletal muscle is an important tissue for insulin action and becomes insulin resistant under different physiological or pathological conditions, such as obesity, high fat feeding and diabetes (Olefsky et al., 1976; Caro et al., 1987; Grimditch et al., 1987). It has been shown that dietary long chain ω-3 fatty acids may improve insulin action in this tissue (Storlien et al., 1987; Sohal et al., 1992). Storlien et al. (1991) have proposed that there is a correlation between muscle phospholipid 22:5ω-3 and 22:6ω-3 levels and tissue insulin-sensitivity. When the content of 22:5ω-3 and 22:6ω-3 was increased from 3.1% to about 10% in skeletal muscle total phospholipid, insulin-stimulated glucose metabolism was enhanced in parallel by more than three-fold (Storlien et al., 1991). How these fatty acids improve insulin action is not clear. The present study provides an explanation for the observation that dietary polyunsaturated fatty acids, particularly long chain ω-3 fatty acids, increase insulin-dependant glucose transport in skeletal muscle (Sohal et al. 1992; Storlien et al. 1991). Feeding rats diet either high in ω-3 fatty acid or of high P/S content improved insulin binding to skeletal sarcolemma (Fig 1 and 2). Insulin binding to these

membranes was higher at all insulin concentrations studied and Rosenthal analysis revealed that the receptor number at the low affinity binding site was significantly higher. Enhanced insulin binding to skeletal sarcolemma resulting from feeding diets high in ω-3 or ω-6 fatty acid is consistent with improvement of insulin binding to adipocytes induced by feeding a high P/S diet (Field et al., 1988; Field et al., 1990). Together these observations suggest that dietary polyunsaturated fatty acids may have a generalized effect on insulin action via modification of hormone binding to insulin-sensitive tissues. In view of the large effect of fish oil on muscle insulin binding, it is unfortunate that the effect of ω-3 fatty acids has not been studied on adipocyte insulin binding and insulin sensitivity. In agreement with the outcome of in vitro studies where membrane fatty acyl P/S composition has been manipulated (Ginsberg et al., 1981; Gould et al., 1982), the present study provides further evidence that dietary fatty acids within the range of composition consumed by humans has a profound effect on insulin action.

In terms of improving insulin binding to skeletal sarcolemma, long chain ω-3 fatty acids seem to be a more potent factor than ω-6 polyunsaturated fatty acids. Because the P/S ratio and the content of ω-3 fatty acids in the low ω-3 diet was similar to that in the low P/S diet, the low ω-3 diet and the low P/S diet were comparable (Table II-1). Feeding the high ω-3 diet increased insulin binding 14 fold over the low ω-3 diet, compared with an improvement of 2.7 fold in insulin binding resulting from the high P/S diet. This result is consistent with the report that ω-3 fatty acids are more effective in preventing insulin resistance induced by high fat diets than ω-6 polyunsaturated fatty acids (Storlien et al., 1991). The lack of the diet P/S ratio on membrane phospholipid composition is in

contrast with the observed changes in insulin binding. The relative amounts of different phospholipid may in this case have been a factor (Field and Clandinin, 1989), since improved insulin binding was lost after membrane solubilization.

The importance of the phospholipid environment to insulin binding is addressed further by the solubilized receptor binding experiment. After receptors were solubilized, the effect of dietary fatty acids on insulin binding disappeared. There was no difference in insulin binding to solubilized receptors from rats fed high versus low P/S diets and the difference between insulin binding to the solubilized receptors from rats fed high and low ω-3 diets was not significant. Thus it is clear that insulin binding to its receptor is significantly affected by the lipid environment around the receptor. In light of this conclusion, it should be pointed out that caution should be exercised in interpreting results of ligand binding using solubilized receptors from muscles in different physiological and pathological states where sarcolemmal fatty acid and /or phospholipid composition may be altered (e.g. Turinsky 1986).

IGF-I and insulin have similar anabolic functions in skeletal muscle, especially for glucose uptake and metabolism (Dimitriadis et al., 1992). The receptors for these two hormones share functional similarity and structural homology (Abbott et al., 1992). Our results indicated that IGF-I binding to skeletal sarcolemma was altered by changes in dietary fatty acid composition. To our knowledge this is the first demonstration that IGF-I receptor function is sensitive to dietary ω-3 fatty acids. The observation that binding activity of IGF-I, like that of insulin, is affected by dietary fatty acids implies that this might be a general phenomenon for other receptor binding activities. It remains to be

determined whether ω-3 fatty acids increase IGF-I-dependent glucose transport in skeletal muscle.

SEPARATING INSULIN BOUND TO SKELETAL MEMBRANE FROM FREE INSULIN USING WGA

Wheat germ agglutinin (WGA) is a lectin with an affinity for N-acetyl-β-D-glucosamine residues (Charuk et al., 1989). WGA is used in purification of skeletal and cardiac membranes to separate right-side out vesicles from the inside out vesicles and to reduce contamination by intracellular membranes (Charuk et al., 1989; Ohlendieck et al., 1991). The present study incorporated the membrane WGA purification step into insulin binding procedures to successfully separate bound insulin from free insulin. This procedure gave identical values for specific binding, but lowered the non-specific binding compared with identical assays using ultracentrifugation to separate bound and free insulin. This procedure could be a valuable addition to studies of insulin binding to intact membranes where the level of nonspecific binding usually reported to be high (Grimditch et al., 1985). Another advantage of this procedure is that WGA-agglutinated plasma membrane vesicles can be pelleted at the end of binding assays by relatively low speed centrifugation (~17,000 x g), whereas non - agglutinated vesicles require ultracentrifugation (~140,000 x g).

CONCLUSION

1. Feeding rats long chain ω-3 fatty acids significantly increased the content of 22:5ω-3 and 22:6ω-3 in skeletal muscle membrane phospholipids. The fatty acyl compositions of individual phospholipids were affected differently. Feeding rats with high and low P/S diets did not significantly affect membrane phospholipid fatty acyl composition.

2. Both dietary long chain ω-3 and polyunsaturated fatty acids can increase insulin binding to skeletal sarcolemma, due to increase in the Bmax of the low affinity, high capacity binding site. The effect of the long chain ω-3 fatty acids is more potent than that of ω-6 polyunsaturated fatty acids.

3. When receptors were solubilized from the membrane lipid the increase in insulin binding disappeared in both dietary groups, indicating that the altered membrane fatty acyl composition is responsible for the modified insulin binding by dietary polyunsaturated fatty acids..

4. Precipitating insulin bound to skeletal sarcolemma using WGA can reduce the non-specific binding.

TABLES AND FIGURES

Table II-1 Fatty acid composition of the diets.

Diets	High ω-3	Low ω-3	High P/S	Low P/S
Fatty acids				
C14:0	3.5	3.7	1.5	2.8
C16:0	24.7	27.0	15.3	22.5
C18:0	48.2	35.2	25.1	47.5
C18:1	3.3	9.3	9.3	4.4
C18:2ω-6	13.4	10.5	44.3	17.4
C18:3ω-3	0.3	0.8	1.4	1.2
C20:0	0.5	0.3	0.4	0.5
C20:5ω-3	2.4	ND	ND	ND
C22:5ω-3	0.2	ND	ND	ND
C22:6ω-3	0.7	ND	ND	ND
SAT	77.4	68.8	43.9	75.9
MONO	4.9	10.4	9.7	5.1
PUFA	17.6	11.9	46.3	19.4
ω-6	13.5	10.9	44.8	17.6
ω-3	4.2	1.0	1.5	1.4
P/S ratio	0.23	0.18	1.05	0.25

Values are % (w/w) for the fat in the diet. SAT: total saturated fatty acids; MONO: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; P/S ratio: the ratio of polyunsaturated over saturated fatty acids; ω-6: total ω-6 fatty acids; ω-3: total ω-3 fatty acids; ND: not detectable.

Table II-2 Effects of high and low ω-3 diets on the fatty acyl composition of skeletal muscle plasma membrane phospholipids.

	PC		PE	
	High ω-3	Low ω-3	High ω-3	Low ω-3
C16:0	36.7	33.9	7.5	4.5
C18:0	14.2	15.2	20.7	20.9
C18:1	8.7	7.1	5.8	5.0
C18:2ω-6	22.8	24.1	19.4	20.3
C20:4ω-6	5.8	13.2	4.5	16.1
C20:5ω-3	0.9	0.1	2.2	0.2
C22:5ω-3	1.6	0.4	2.6	2.1
C22:6ω-3	3.9	0.9	13.7	10.6
SAT	53.3	50.8	37.5	30.0
MONO	9.8	8.0	7.8	5.7
ω-6	30.7	39.7	32.4	49.5
ω-3	6.5	1.5	22.4	14.8
P/S	0.70	0.81	1.46	2.14

	PS		PI	
	High ω-3	Low ω-3	High ω-3	Low ω-3
C16:0	5.2	6.6	16.0	13.1
C18:0	47.2	46.1	32.9	33.4
C18:1	9.3	8.8	12.4	9.4
C18:2ω-6	4.3	4.3	8.7	7.7
C20:4ω-6	3.8	9.3	10.2	15.8
C20:5ω-3	0.3	0.4	2.9	1.5
C22:5ω-3	5.0	1.9	2.8	ND
C22:6ω-3	17.3	8.1	5.8	2.6
SAT	54.9	56.4	51.6	51.8
MONO	10.0	8.8	15.6	13.1
ω-6	11.9	24.6	21.4	31.0
ω-3	23.1	10.3	11.4	4.2
P/S	0.64	0.62	0.64	0.68

Values are % of total fatty acids in each membrane fraction. Fatty acids were determined using membranes pooled from 10 rats per dietary treatment. ND: not detectable; SAT: total saturated fatty acids; MONO: total monounsaturated fatty acids; ω-6: total ω-6 fatty acids; ω-3: total ω-3 fatty acids; P/S: polyunsaturated/saturated fatty acid ratio.

Table II-3 Effects of high and low P/S diets on the fatty acyl composition of skeletal muscle plasma membrane phospholipids.

	PC		PE	
	High P/S	Low P/S	High P/S	Low P/S
C16:0	35.9	37.7	7.2	8.6
C18:0	10.9	10.1	35.8	33.6
C18:1	5.0	5.7	4.2	4.4
C18:2ω-6	20.7	24.8	11.4	9.5
C18:3ω-3	0.7	0.2	0.3	ND
C20:4ω-6	20.0	17.9	23.8	23.4
C20:5ω-3	0.1	0.2	0.3	ND
C22:5ω-3	0.4	0.5	2.5	3.7
C22:6ω-3	0.8	1.1	8.3	11.2
SAT	47.3	48.5	43.2	43.2
MONO	5.5	6.1	5.5	5.4
ω-6	45.9	43.5	40.0	36.6
ω-3	1.4	1.9	11.4	14.8
P/S	1.00	0.94	1.19	1.19

	P		PI	
	High P/S	Low P/S	High P/S	Low P/S
C16:0	5.4	8.4	2.9	3.6
C18:0	53.3	51.2	51.0	52.1
C18:1	4.1	6.6	2.2	2.4
C18:2ω-6	5.3	4.7	3.4	2.4
C18:3ω-3	ND	ND	0.1	0.1
C20:4ω-6	9.2	10.2	35.2	33.7
C20:5ω-3	ND	0.8	0.1	0.1
C22:5ω-3	2.1	1.1	0.6	0.8
C22:6ω-3	11.0	8.7	0.7	0.8
SAT	59.8	60.3	54.4	56.2
MONO	5.2	7.5	3.0	3.1
ω-6	21.8	21.6	41.2	39.0
ω-3	13.1	10.6	1.4	1.8
P/S	0.58	0.53	0.78	0.73

Values are % of total fatty acids in each membrane fraction. Fatty acids were determined using membranes pooled from 10 rats per dietary treatment. SAT: total saturated fatty acids; MONO: total monounsaturated fatty acids; P/S: the ratio of

polyunsaturated over saturated fatty acids; ND: not detectable; ω-6: total ω-6 fatty acids; ω-3: total ω-3 fatty acids.

Table II-4 Insulin (A) and IGF-I (B) binding to membranes and solubilized receptors prepared from rats fed high and low ω-3 or P/S diets.

A. Insulin binding

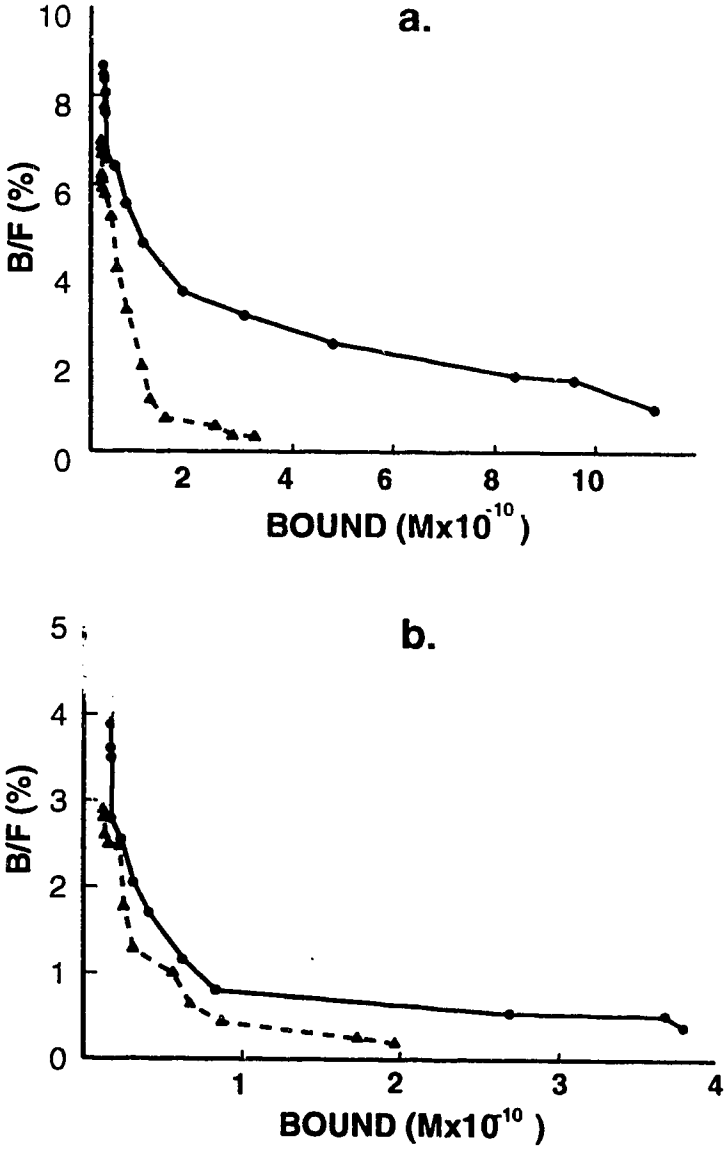
	High affinity site		Low affinity site	
	Bmax	1/Kd	Bmax	1/Kd
High ω-3 Membrane	$5.0\pm1.0\times10^{-11}$	$17\pm4.8\times10^{8}$	$75\pm8.5\times10^{-10}$	$6.6\pm1.0\times10^{6}$
Low ω-3 Membrane	$9.2\pm2.5\times10^{-11}$	$8.5\pm1.6\times10^{8}$	$5.2\pm2.3\times10^{-10***}$	$82\pm6.2\times10^{6}$
High ω-3 Receptor	$2.7\pm0.62\times10^{-11}$	$23\pm10\times10^{8}$	$8.6\pm2.5\times10^{-10}$	$79\pm36\times10^{6}$
Low ω-3 Receptor	$4.1\pm8.8\times10^{-11}$	$8.3\pm2.2\times10^{8}$	$3.1\pm0.97\times10^{-10}$	$100\pm620\times10^{6}$
High P/S Membrane	$2.4\pm0.57\times10^{-11}$	$25\pm12\times10^{8}$	$2.7\pm0.48\times10^{-10}$	$19\pm8\times10^{6}$
Low P/S Membrane	$3.0\pm0.43\times10^{-11}$	$13\pm29\times10^{8}$	$1.2\pm0.32\times10^{-10**}$	$9.9\pm100\times10^{6}$
High P/S Receptor	$5.4\pm0.77\times10^{-11}$	$6.9\pm1.2\times10^{8}$	$5.2\pm2.4\times10^{-10}$	$5.3\pm3.7\times10^{6}$
Low P/S Receptor	$4.6\pm0.4\times10^{-11}$	$7.7\pm8.1\times10^{8}$	$2.4\pm0.24\times10^{-10}$	$14\pm3.1\times10^{6}$

B. IGF-I binding to membrane

	High affinity site		Low affinity site	
	Bmax	1/Kd	Bmax	1/Kd
High ω-3	$1.6\pm.5\times10^{-11}$	$7.1\pm4.9\times10^{8}$	$5.0\pm0.5\times10^{-10}$	$9.9\pm1.0\times10^{6}$
Low ω-3	$1.2\pm.5\times10^{-11}$	$81\pm67\times10^{8}$	$2.1\pm0.3\times10^{-10*}$	$2.7\pm4.0\times10^{6}$

Values are binding parameters ± standard error of the mean. Kd: dissociation constant, expressed as M; Bmax: total receptor concentration, expressed as sites/mg protein; ***: $p<0.0001$, compared with the binding to membrane from rats fed high ω-3 diet; **: $p<0.05$, compared with membrane prepared from rats fed high P/S diet; *: $p<0.05$, compared with membrane from rats fed high ω-3 diet.

FIGURE II-1 Rosenthal plot of insulin binding to (a) membranes and (b) receptors prepared from rats fed high and low omega-3 diets.

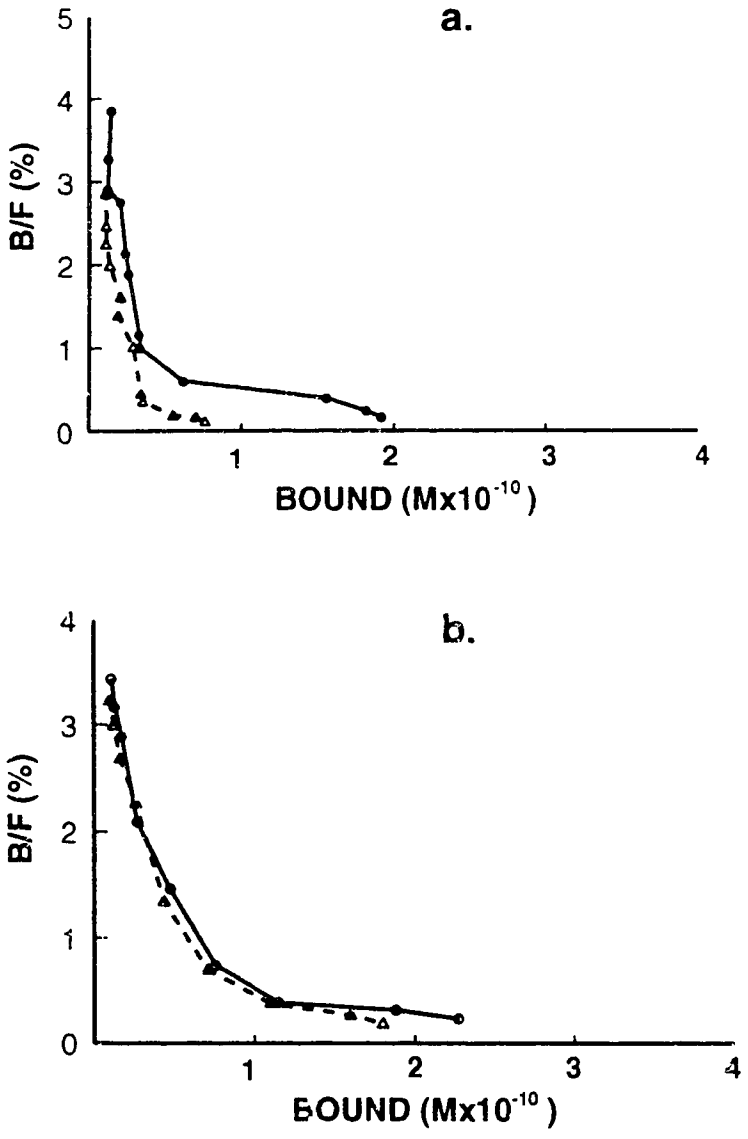


B/F: the amount of bound insulin over that of free insulin.

—●— : high omega-3 diet.

- - ▲ - - : low omega-3 diet.

FIGURE II-2 Rosenthal plot of insulin binding to (a) membranes and (b) receptors prepared from rats fed high and low P/S diets.

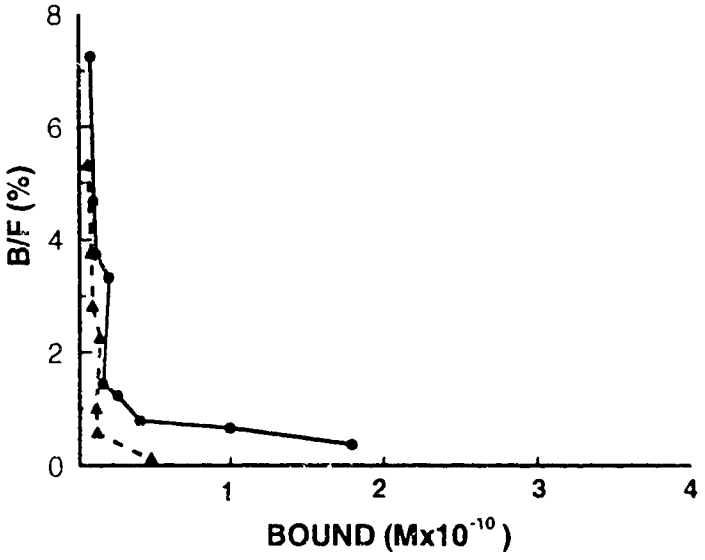


B/F: the amount of bound insulin over that of free insulin.

—o— : high P/S diet.

- - -△- - - : low P/S diet.

FIGURE II-3 Rosenthal plot of IGF-I binding to (a) membranes and (b) receptors prepared from rats fed high and low omega-3 diets.



B/F: the amount of bound IGF-I over that of free IGF-I.

—●— : high omega-3 diet.

- - ▲ - - : low omega-3 diet.

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CHAPTER III: PARALLEL IGF-I AND INSULIN RESISTANCE IN MUSCLES OF RATS FED A HIGH FAT DIET[1]

INTRODUCTION

Skeletal muscle is the most abundant insulin-sensitive tissue and plays an important role in insulin-dependent glucose transport. Under certain physiological and pathological conditions, muscle tissue becomes resistant to insulin, i.e., normal or high concentrations of insulin produce a less than normal biological response. These conditions include obesity, hypertension, diabetes, as well as consumption of high fat diet, such as a typical Western diet.

IGF-I shares structural and functional similarities with insulin. In skeletal muscle, IGF-I increases glucose transport and metabolism, stimulates amino acid uptake and promotes protein synthesis (Dimitriadis et al., 1992). Due to the hypoglycemic effect of IGF-I, it is of interest to investigate the effect of IGF-I on glucose transport in insulin-resistant conditions. Obese Zuker rats are resistant to insulin and IGF-I to the same degree in vivo (Jacob et al., 1992). Muscles from obese human subjects are also resistant to IGF-I (Dohm et al., 1990). IGF-I elicits rates of glucose utilization in insulin-resistant pancreatectomized diabetic rats similar to that of healthy controls (Rossetti et al., 1991). Dardevet et al. (1994) showed that insulin and IGF-I resistance were evident in muscle of old rats, but the degree of resistance varied depending upon the metabolic process concerned (i.e. glucose uptake, amino acid uptake and protein synthesis). Poggi et al.

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(1979) reported decreased IGF-I and insulin stimulated glucose transport in soleus muscle of obese mice, even though IGF-I binding was intact. These results indicate that insulin and IGF-I resistance may not always happen simultaneously; or even if they do, the mechanism might be different.

Feeding rats a high fat diet, especially with saturated fat, induces insulin resistance (Storlien et al., 1991). High fat feeding causes marked decrease in both insulin sensitivity and responsiveness in adipocytes and skeletal muscle (Olefsky et al., 1978; Grundleger and Thenen, 1982; Field et al., 1988). Interestingly, if the dietary fat was mainly polyunsaturated fatty acids in a high fat diet, particularly long chain ω-3 fatty acids, insulin action in muscles could be restored (Storlien et al., 1991). Our research suggest that this improvement might be due to increase in insulin binding to its receptor (Liu et al., 1994). Moreover, insulin-like growth factor I (IGF-I) binding to skeletal muscle was also augmented by feeding rats diets high in long chain ω-3 polyunsaturated fatty acids (Liu et al., 1994).

However, the action of IGF-I on glucose transport has not been studied when insulin-resistance is induced by feeding animal a high fat diet. In light of our recent study showing that IGF-I binding to skeletal muscle is sensitive to dietary fatty acids (Liu et al., 1994), we hypothesized that the action of IGF-I on glucose transport in skeletal muscle is reduced by feeding a diet with increased fat content. The present study was thus designed to investigate if: a high fat diet could induce IGF-I resistance in skeletal muscle; the IGF-I resistance was due to impairment to IGF-I sensitivity or responsiveness; IGF-I resistance is associated with decreased IGF-I binding to skeletal muscle.

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

Studies were carried out in accordance with the Canadian Council on Animal Care guidelines. Weanling female Sprague-Dawley rats were obtained from a colony maintained at the University of Alberta. Five rats per plastic cage were housed in a room that was maintained at 23°C on a 12 hour light/dark cycle. Animals were given food and water ad libitum. Prior to study, the rats were on their diets for six weeks. Six weeks of feeding of the diets with different fatty acid composition have shown to be effective in terms of inducing chemical changes in cell membrane phospholipid, as well as altered hormone actions (Field et al., 1988; Liu et al., 1994). After six weeks of feeding, rats were fasted overnight. Rats were humanely killed by CO_2 asphyxiation and epitrochlearis muscles were dissected for the study (detailed below).

Rats were fed either a high fat (20% w/w fat, 42% of total energy) or a low fat (5% w/w fat, 13% of total energy) semipurified nutritionally complete diet, formulated according to McCargar et al. (1989). The composition of the diets is indicated in Table III-1. The diets were formulated to provide non-fat essential nutrients at similar nutrients/energy density for the high versus low fat diets fed. In a previous study (McCargar et al., 1989), animals fed a broad range of dietary fat levels were studied (19-52% of total energy as fat), and there were no differences between treatments in total caloric intake, protein intake or body weight gain among the different dietary fat levels. All of the diet ingredients were from Teklad except corn starch (Best Foods Canada Inc.), beef tallow (Canada Packers Inc.), safflower oil (Tosca) and flax seed oil (Gold Top).

Since saturated fatty acids induce insulin resistance, a low dietary polyunsaturated to saturated fatty acid ratio (P/S ratio, 0.17) was selected for the study (Field et al., 1990). Both diets had the same fatty acid composition (Table III-2).

In the first experiment, the effect of diet fat level on IGF-I stimulated 3-*O*-methyl-glucose transport was examined at sub-maximal and maximal hormone concentrations, based on results obtained in a preliminary experiment (see results). Insulin stimulated glucose transport was also studied for comparison. In a second experiment, the effect of diet fat level on IGF-I binding to epitrochlearis muscles was studied. IGF-I binding was performed at the same hormone concentrations used in the 3-*O*-methyl-glucose transport studies.

MEASUREMENT OF 3-*O*-METHYL-GLUCOSE TRANSPORT IN MUSCLE

Glucose transport activity was measured in epitrochlearis muscle using the method described by Young et al. (1986) with some modifications. In a preliminary experiment, linearity of 3-*O*-methyl-glucose uptake was first confirmed on rats fed the low fat diet. 3-*O*-methyl-glucose uptake by epitrochlearis muscle was linear for up to 35 min under the experimental condition (see below). The standard error for 3-*O* methyl-glucose transport estimate was decreased when longer period of incubation (30 min) was adopted, compared with shorter incubation time (e.g., 10 min, data not shown). Dose-response curves for both IGF-I and insulin stimulated 3-*O*-methyl-glucose transport were determined and hormone concentrations stimulating maximal and half-maximal 3-*O*-methyl-glucose transport were used in subsequent glucose transport and IGF-I binding studies. The hormone concentrations to stimulate half-maximal glucose transport were

calculated by regression of the glucose transport data to the Michaelis-Menten equation. The maximal effective concentrations were determined first by visual inspection of the dose-response curve, and them confirmed by statistical comparison of the amount of 3-*O*-methyl-glucose transported obtained at the highest hormone concentrations using student *t*-test. Of the two epitrochlearis muscles from each rat in the main experiment, one muscle was incubated without hormone to determine the basal glucose transport, the other was incubated with hormone, and the hormone stimulated 3-*O*-methyl-glucose uptake was calculated as the difference (Henriksen et al., 1992). The percentage stimulation of glucose transport for different groups was then calculated.

Muscles were pre-incubated in 3 ml KRB buffer (119 mM NaCl, 25 mM $NaHCO_3$, 4.82 mM KCl, 1.0 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.24 NaH_2PO_4, 2.0 mM HEPES, pH 7.4) containing 0.1% BSA (Sigma, RIA grade), 8 mM glucose, 32 mM mannitol, with or without different concentrations of either IGF-I (Bachem) or insulin (Sigma) in a shaking water bath (29°C) for one hour. Incubation medium was continuously bubbled with 95%O_2/5%CO_2. Following pre-incubation, the muscles were transferred into 3 ml KRB of the same composition except without glucose and with 40 mM mannitol and incubated at 29°C for 10 min. The muscles were then transferred to 3 ml KRB containing either 8 mM 3-*O*-[^{3}H]methyl-glucose or [^{3}H]L-glucose (both from New England Nuclear, 300 μCi/mmol), 32 mM mannitol and IGF-I or insulin at the same concentrations as in the pre-incubation media. Before use, 3-*O*-[^{3}H]methyl-glucose and [^{3}H]L-glucose were purified to remove ^3H_2O by evaporation to dryness. After 30 min incubation at 29°C, the muscles were washed in 3 ml KRB at 0°C three times 10 min to

remove 3-*O*-[^{3}H]methyl-glucose or [^{3}H]L-glucose from the extracellular space (Poggi et al., 1979). The muscles were then solubilized in 1 ml Soluene-350 (Packard) and counted in 15 ml Hionic-Fluor (Packard) using a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer.

In preliminary experiments, it was found that using the 0°C washing protocol alone could not remove all of the radio-labelled L-glucose from the muscles. This unwashable radioactivity represents L-glucose which either non-specifically binds to the muscle surface or passively diffuses into muscle fibres, since L-glucose can not be transported by the glucose transporter. The chemical similarity between L- and D-glucose makes L-glucose a good candidate to estimate how much untransported D-glucose is trapped in the muscle tissue. To correct for non-specifically bound and passively diffused 3-*O*-[^{3}H]methyl-gluc[illegible] muscles (from both diet treatments) were incubated with [^{3}H]L-glucose [illegible]cose transport studies, and the amount of [^{3}H]L-glucose remaining [illegible] washing was subtracted from the amount of 3-*O*-[^{3}H]met[illegible]d no influence on [^{3}H]L-glucose uptake by incubated epitrochl[illegible]

MEASUREMENT OF IGF-I BINDING TO SKELETAL MUSCLE

Before each use, ^{125}I-IGF-I was purified as following: a resin column (Bio Rad Anion Exchange AG1x8, 50-100 mesh) was flushed with 1 ml 1% BSA in 0.05 M PO_4 buffer (pH 7.5) followed by 3 ml 0.05 M PO_4 (pH 7.5) buffer; these steps were then repeated once before loading the ^{125}I-IGF-I in KRB (pH7.4) onto the column. The column was then eluted with 2 ml 1% BSA in 0.05 M PO_4 (pH 7.5) and the effluent was collected for the study of IGF-I binding.

IGF-I binding to skeletal muscle was performed according to Poggi et al. (1979). Epitrochlearis muscles were dissected, pre-incubated in 3 ml KRB containing 2 mM pyruvate and 2% BSA at 20°C for one hour to wash out residual IGF-I. Muscles were then transferred to 2 ml medium of the same composition, with the presence of IGF-I at the same concentrations as in the 3-*O*-methyl-glucose transport experiment and ^{125}I-IGF-I (New England Nuclear, 300,000 dpm/tube). Muscles were incubated for four hours at 20°C. During the process of pre-incubation and incubation, the incubation medium was bubbled with 95%O_2/5%CO_2. At the end of the experiment, muscles were washed three times 10 minutes at 0°C as described for the 3-*O*-methyl-glucose transport study. Six muscles from each treatment were incubated with or without 25 μM IGF-I to determine non-specific binding. Specific IGF-I binding was then calculated by subtracting the non-specific binding from the total binding obtained from each muscle. The calculation was indicated below:

Specific IGF-I binding=Total IGF-I bound (fmol/mg tissue)-nonspecifically IGF-I bound (fmol/mg tissue)

The samples were counted in a Packard Cobra ›-Gamma counter.

OTHER MEASUREMENTS

Dietary lipids were extracted using a procedure described by Folch et al. (1957) with some modifications (Field et al., 1988). Fatty acid methyl esters were prepared using 14% (w/vol) BF_3/methanol reagent (Morrison et al., 1964) and separated by automated gas-liquid chromatography (Vista 6010 GLC and Varian 604 chromatography data system, Varian Instruments, Georgetown, Ontario, Canada). Chromatography was performed using a fused silica BP20 capillary column (25mx0.25mm inner diameter; Varian Instruments, Georgetown, Ontario, Canada) under conditions described in detail earlier (Field et al., 1988).

STATISTICAL ANALYSIS

Data are expressed as group means±standard error of the mean. All data were compared statistically using the Student *t*-test.

RESULTS

Under the experimental conditions described above, muscle 3-*O*-methyl-glucose uptake was linear for up to 35 min (Fig III-1). The hormone concentration at which maximal stimulation of 3-*O*-methyl-glucose transport was produced was determined as ≥25 nM for IGF-I and ≥20 nM for insulin (Fig III-2a and III-2b). The hormone concentrations at which half-maximal stimulation of 3-*O*-methyl-glucose transport was produced were determined as 2.32 nM for IGF-I and 0.79 nM for insulin (Fig III-2a and III-2b). Hormone concentrations of 2.5 and 25 nM for IGF-I and 0.8 and 20 nM for insulin were used in the subsequen studies.

There was no significant effect of diet fat level on basal 3-*O*-methyl-glucose uptake, with 631±54 pmol/mg/30 min (n=10) for animals fed the low fat diet and 602±52 pmol/mg/30 min (n=10) for animals fed the high fat diet.

Half-maximal effective IGF-I (2.5 nM) stimulated 3-*O*-methyl-glucose by 52±7.6% (n=10) in muscles from rats fed the low fat diet, compared with only 8.3±11.2% (n=10) in muscles from rats fed the high fat diet ($p<0.01$, Fig III-3). In comparison, in muscles from rats fed the low fat diet 3-*O*-methyl-glucose transport responded to half-maximal effective insulin (0.8 nM) by 60±10.4% (n=10), while muscles from rats fed the high fat diet exhibited a 3-*O*-methyl-glucose transport only 17±14.5% (n=10) higher than basal ($p<0.05$, Fig III-3).

Maximal effective IGF-I (25 nM) stimulated 3-*O*-methyl-glucose transport 137±12% (n=10) in muscles from rats fed the low fat diet, compared with 98±13% (n=10) for muscles from rats fed the high fat diet ($p<0.05$, Fig III-3). Insulin, at 20 nM,

produced a 146±11% (n=10) increase in 3-*O*-methyl-glucose transport for low fat diet fed rats, compared with 99±20% (n=10) for the high fat fed rats ($p<0.05$, Fig III-3).

To investigate if the lowered IGF-I stimulated 3-*O*-methyl-glucose transport for high fat fed rats was due to a shift to the right of the dose-response curve, muscles from high fat fed rats were incubated at either 25 or 50 nM IGF-I. Muscles incubated with these two concentrations of IGF-I showed virtually identical 3-*O*-methyl-glucose transport: 1535±95 pmol/mg/30 min (n=10) for 25 nM IGF-I and 1549±76 (n=10) for 50 nM IGF-I ($p>0.05$).

When muscles were incubated with 2.5 nM IGF-I, IGF-I binding to isolated epitrochlearis muscles was lowered by high-fat feeding, from 0.73±0.03 fmol per mg wet tissue (n=12) for the low fat fed group, to 0.61±0.03 (n=12) for the high fat fed group ($p<0.01$, Table III-3), which represents a ~16% decline. At 25 nM IGF-I, the binding was 5.25±0.30 for the low fat fed group and 4.38±0.44 for the high fat fed group. The decline in IGF-I binding with the presence of 25 nM IGF-I was also ~16%, but was not statistically significant (Table III-3). To investigate if IGF-I binding was the only factor involved in modified 3-*O*-methyl-glucose uptake induced by high fat diet feeding, we divided the value of stimulated 3-*O*-methyl-glucose transport per mg tissue (i.e., 3-*O*-methyl-glucose uptake with IGF-I stimulation minus that without IGF-I stimulation) obtained in the first experiment by the value of IGF-I binding per mg tissue (second experiment; Table III-3). In this way, it could be examined how much 3-*O*-methyl-glucose uptake was stimulated by each unit IGF-I bound. At an IGF-I concentration of 2.5 nM, each fmol of IGF-I bound stimulated 359 pmol 3-*O*-methyl-glucose uptake for muscles

from rats fed the low fat diet, compared with 242 pmol for muscles from rats fed the high fat diet. In the presence of 25 nM IGF-I, each fmol of IGF-I bound stimulated 134 pmol of 3-*O*-methyl-glucose transport for rats fed the low fat diet, compared with 95 pmol for rats fed the high fat diet (Table III-3).

DISCUSSION

The fat level in the high fat diet was selected to represent the fat content of typical Western diets (20% w/w). In addition to fat level, the fatty acid composition of the diet also affects tissue insulin sensitivity. A low P/S ratio diet was chosen because saturated fat has been shown to be more potent in inducing insulin resistance (Storlien et al., 1991; Field et al., 1988). The fatty acid composition and P/S ratio in each diet were kept constant since different fatty acids may have diverse effects on tissue hormone binding and sensitivity and/or responsiveness (Liu et al., 1994; Field et al., 1990). Considering that multiple experimental factors such as dietary fat level, carbohydrate content, level of saturation of dietary fat and muscle fibre type influence hormone stimulated glucose transport, we first constructed dose-response curves for both IGF-I- and insulin-stimulated glucose transport using rats fed the low fat diet. From these curves, the ED50 value (hormone concentration at which half-maximal stimulation of glucose transport is produced) was determined as 2.32 nM for IGF-I stimulated 3-*O*-methyl-glucose uptake, which was similar to results obtained by Dardevet et al. (1994), but lower than 10 to 14 nM reported for mouse soleus muscle (Poggi et al., 1979) or 5 nM for epitrochlearis of chow - fed rats (Henriksen et al., 1992). As reported (Poggi et al., 1979), the ED50 for IGF-I was greater than for insulin (i.e. IGF-I is less potent than insulin at submaximal concentrations). In comparison, IGF-I (25 nM) was as effective as insulin (20 nM) in maximally stimulating glucose transport in skeletal muscle.

This present study demonstrates, for the first time, that a high fat diet induces IGF-I resistance in rat skeletal muscle. Impaired 3-*O*-methyl-glucose transport was

observed at both submaximal and maximal effective IGF-I levels, indicating a down shift in the IGF-I stimulated glucose transport curve. As expected, feeding the high fat diet also caused reduced insulin-stimulated 3-*O*-methyl-glucose transport which was observed at both submaximal and maximal insulin concentrations. This is comparable with the report that feeding high fat diet decreases glucose transport at all insulin concentrations in rat adipocytes (Olefsky et al., 1978). Since the dose-response curve and the maximally effective IGF-I concentration (25 nM) were determined initially on rats fed the low fat diet, the decreased 3-*O*-methyl-glucose transport at 25 nM IGF-I could indicate either a down-shift of the dose-response curve or a shift to the right. To clarify this, muscle 3-*O*-methyl-glucose uptake was compared at 25 and 50 nM IGF-I in animals fed the high fat diet. Since 50 nM IGF-I did not elicit a greater degree of 3-*O*-methyl-glucose transport than did 25 nM IGF-I, it is evident that feeding animals a diet high in fat induces a decrease in the maximal response of IGF-I stimulated 3-*O*-methyl-glucose transport (i.e. a down-shift of the dose-response curve).

The phenomenon of insulin resistance has been studied for a long time, while little attention has been paid to IGF-I resistance. Due to the structural and functional similarities between insulin and IGF-I, it is suggested that IGF-I may be therapeutically useful in insulin resistance (Dohm et al., 1990; Zenobi et al., 1992). Indeed, IGF-I timulated *in vivo* glucose utilization was similar for both pancreatectomized insulin resistant rats and normal control rats, and IGF-I administration corrected the intracellular defects in muscle glycogen synthesis and whole-body glycolysis (Rossetti et al., 1991). In skeletal muscles from obese Type 2 diabetic subjects, IGF-I receptor binding and IGF-I

stimulated tyrosine kinase activity were not impaired, compared with a 30% decrease in insulin binding and 40% reduction in insulin-stimulated tyrosine kinase activity (Livingston et al., 1988). The metabolic responses to infused IGF-I were identical for both diabetic and non-diabetic BB/w rats (Jacob et al., 1991).

However, there is evidence indicating that IGF-I and insulin resistance may happen simultaneously. In soleus muscle of obese mice, both insulin- and IGF-I-stimulated 2-deoxyglucose uptake were markedly depressed compared to lean controls (Poggi et al., 1979). In obese Zucker rats, IGF-I-stimulated glucose uptake was 50% lower than that of lean controls, accompanied by a similar reduction for insulin-stimulated glucose transport (Jacob et al., 1992). A recent study demonstrated that insulin resistance in skeletal muscle developed during aging was associated with IGF-I resistance (Dardevet et al., 1994). Interestingly, while the degree of IGF-I and insulin resistance of glucose uptake were similar in magnitude in old rats, hormone resistance of amino acid uptake was very much greater for IGF-I than for insulin.

In the present study we have shown that insulin and IGF-I resistance occur in parallel and to a similar degree in skeletal muscle from rats fed a high fat diet. It is possible that when insulin resistance is associated with a lack of insulin (e.g., pancreatectomized animals and BB rats), IGF-I stimulated glucose transport is normal; but that when insulin resistance is mainly a result of a receptor or post-receptor defect, such as Type 2 diabetes, obesity, aging and high fat feeding, the action of IGF-I on glucose transport also becomes impaired.

In this study, IGF-I binding to rat skeletal muscle was decreased by ~17% for animals fed a diet high in fat. This observation indicates that impaired IGF-I binding might be responsible, at least partially, for high fat diet induced IGF-I resistance. It is of interest to express 3-*O*-methyl-glucose uptake relative to IGF-I binding in our study (Table III-3), although statistical comparison was not possible because 3-*O*-methyl-glucose transport and IGF-I binding studies were carried out on different muscles from different rats. At both IGF-I concentrations, the high fat diet seemed to lower the amount of hormone stimulated 3-*O*-methyl-glucose transport per unit IGF-I bound by ~30%. This points to the possibility that, in addition to impaired IGF-I binding, IGF-I resistance might be caused by reduced activity of its receptor tyrosine kinase or by post-receptor defect(s).

IGF-I, like insulin, initiates its biological functions by binding to its receptor and activating the tyrosine kinase in the cytoplasmic portion of the receptor (Yarden and Ullrich, 1988). The mechanism for IGF-I or insulin resistance may involve reduced receptor binding activity, decreased tyrosine activity and/or altered post-receptor events. However, IGF-I and insulin resistance may differ significantly in many aspects, such as the circumstances when resistance occurs, or the site of defect(s). The action of IGF-I remains normal in certain insulin resistance models (Rossetti et al., 1991; Livingston et al., 1988). When IGF-I resistance occurs along with insulin resistance, the site of defect(s) may not be the same. Soleus muscles from obese mice had normal IGF-I binding capacity, while IGF-I resistance was obvious (Poggi et al., 1979). This was in contrast to the observation that both the binding and action of insulin were impaired in obese mice (Le Marchand-Brustel et al., 1978). When muscles from obese human subjects with or

without NIDDM became completely resistant to IGF-I, IGF-I binding to skeletal muscle was not altered (Dohm et al., 1990). Considering that insulin resistance in obesity, high fat diet and NIDDM is associated with decreased hormone binding (Olefsky and Saekow, 1978; Le Marchand-Brustel et al., 1978; Nagegawa et al., 1986), the mechanisms for insulin and IGF-I resistance may be different, even though they may occur simultaneously.

The first study of the relationship between dietary fat and insulin action dated back to early this century (Himsworth, 1935). However, the mechanism(s) by which a high fat diet induces insulin and IGF-I resistance remains unclear. Insulin binding and insulin-stimulated glucose transport are influenced by membrane fatty acid composition, which in turn is modified by dietary fat (Field et al., 1988; Liu et al., 1994), so that an effect of membrane composition may be a contributing factor. Feeding animals a diet high in fat reduced GLUT4 mRNA level in rat soleus muscle (Kim et al., 1994), and if this change is associated with decreased levels of transporter protein, it could in part explain simultaneous insulin and IGF-I resistance. A recent study indicated that prolonged exposure (4 h) to the saturated fatty acid palmitate (16:0) could induce insulin resistance in adipocytes, mainly via a post-receptor mechanism (Hunnicutt et al., 1994), suggesting a direct effect of circulating fatty acid levels. Further studies are needed to investigate whether these mechanisms contribute to insulin and IGF-I resistance induced by a high fat diet.

TABLES AND FIGURES

Table III-1 Composition of the diets[1]

Ingredients	High Fat Diet	Low Fat Diet
(Data are expressed as g/kg diet)		
Casein	265	225
Starch	385	610
Fat	200	50
Vitamin Mix[2]	10	8.5
Mineral Mix[3]	50	42
Choline	3	2.5
Inositol	6	5
L-Methionine	3	2.5
Cellulose	78	54.5

[1] Diets were formulated according to McCargar et al. (1989).

[2] Vitamin mix A.O.A.C. (Association of Official Agricultural Chemists, Washington D.C., 1960), Teklad.

[3] Teklad, see Bernhart and Tomarelli, 1966.

Table III-2 Fatty acid composition of the diet

Fatty Acids	%
C14:0	2.29
C16:0	23.57
C18:0	55.26
C18:1(9 and 7)	3.60
C18:2(6)	10.75
C18:3(3)	2.83

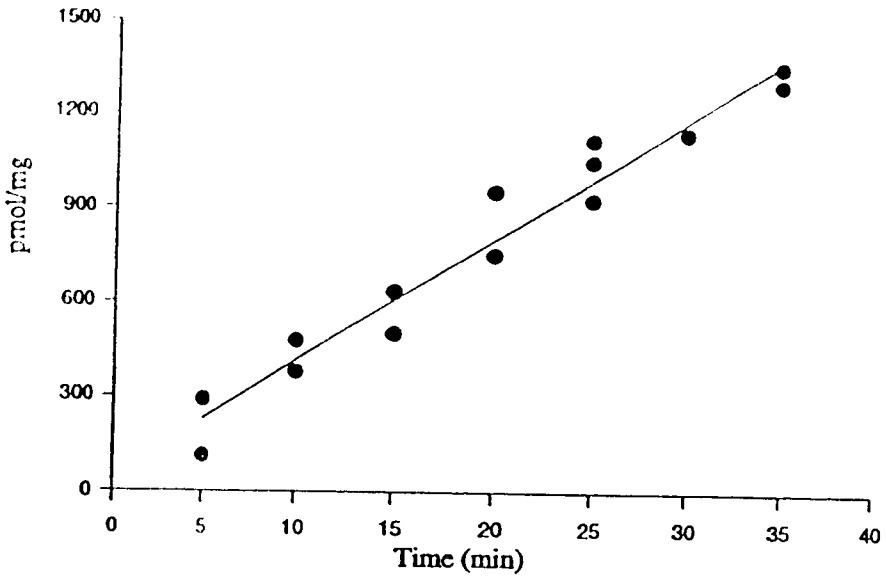
The dietary fat is composed of 7.7 g of flaxseed oil, 20 g of safflower oil, and 72.3 g beef tallow per 100 g of fat. Fatty acid composition was determined in duplicate by gas liquid chromatography.

Table III-3 Effect of high and low fat feeding on IGF-I binding to skeletal muscle

IGF-I (nM)	Dietary fat level	IGF-I binding (fmol/mg)	Stimulated glucose transport per unit IGF-I bound (pmol/fmol)**
2.5	low	0.73±0.03	359
2.5	high	0.61±0.03*	242
25	low	5.25±0.30	134
25	high	4.38±0.44	95

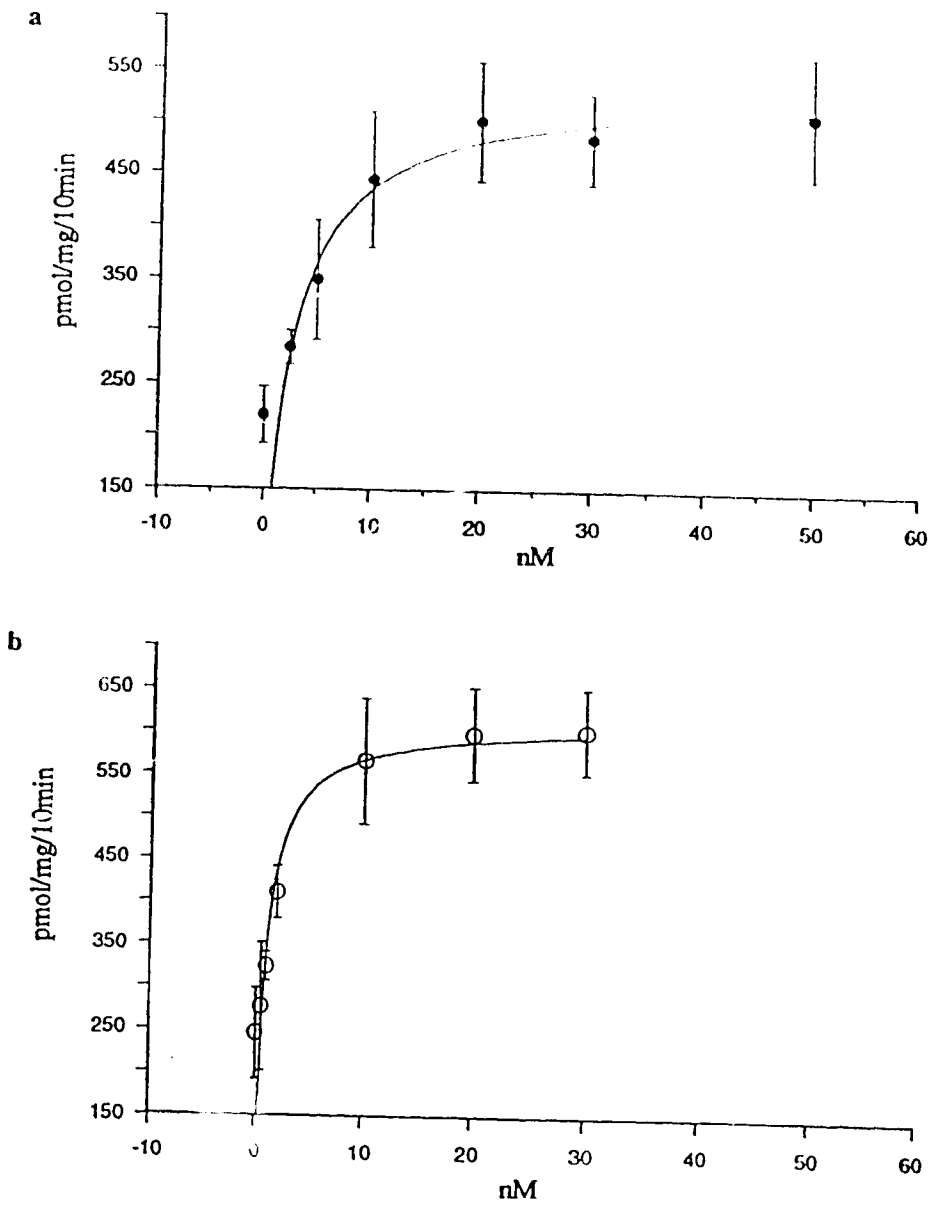
Epitrochlearis muscles were incubated in 2 ml incubation medium containing ^{125}I-IGF-I (300,000 dpm/tube), with or without 2.5 or 25 nM IGF-I for four hours at 20°C to measure IGF-I binding (see Experimental for detail). *: significantly lower compared with low fat group incubated at the same hormone concentration (n=12), $p<0.05$. We divided the value of stimulated 3-*O*-methyl-glucose transport per mg tissue (i.e. 3-*O*-methyl-glucose uptake with IGF-I stimulation minus that without IGF-I stimulation) obtained in the first experiment, by the value of IGF-I binding per mg tissue (second experiment; Table III-3). In this way we estimated 3-*O*-methyl-glucose uptake per unit IGF-I bound. **: statistical comparison was not possible because 3-*O*-methyl-glucose transport and IGF-I binding studies were carried out on muscles from different rats.

Fig III-1: Time course for 3-O-methyl-glucose uptake



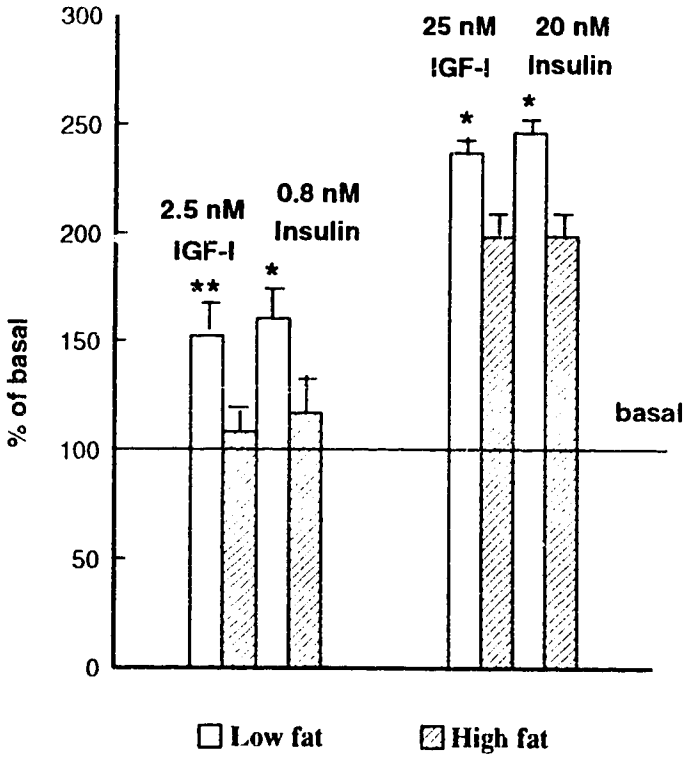
Epitrochlearis muscles from rats fed the low fat diet were incubated in 3 ml KRB, 8 mM 3-*O*-methyl-glucose, in the presence of a maximally effective insulin concentration (20 nM) in the incubation media. Each point represents one muscle.

Fig III-2: Dose-response curves for 3-O-methyl-glucose uptake



Epitrochlearis muscles from rats fed the low fat diet were incubated in 3 ml KRB, 8 mM 3-*O*-methyl-glucose, with the addition of various concentrations of either IGF-I (a) or insulin (b) for 10 min

FIGURE III-3 IGF-I and insulin stimulated 3-O-methyl -glucose uptake for rats fed the low- and the high-fat diet



3-O-methyl-glucose uptake was measured under the experimental conditions specified in detail in Experimental. Epitrochlearis muscles from rats fed either the low- or the high-fat diet were incubated with or without different concentrations of IGF-I and insulin for 30 min. Two epitrochlearis muscles were dissected from each rats: one was incubated without hormone to determine the basal glucose transport, and the other was incubated with hormone to determine hormonal effect. A percentage increase due to hormone stimulation was calculated from each pair. *: significantly higher corresponding high fat group, $p<0.05$; **: significantly higher than the corresponding high fat group, $p<0.01$.

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CHAPTER IV: DIETARY FAT MODIFIES EXERCISE-DEPENDENT GLUCOSE TRANSPORT IN SKELETAL MUSCLE

INTRODUCTION

Exercise is a potent factor promoting glucose uptake in skeletal muscle (Wallberg-Henriksson and Holloszy, 1985; Constable et al., 1988; King et al., 1989). Exercise apparently increases glucose uptake by two different mechanisms or phases (Wallberg-Henriksson et al., 1988). Immediately after exercise, insulin-independent glucose uptake may be increased by more than 4-fold (Young et al., 1987). The exercise-stimulated insulin independent glucose uptake and insulin-stimulated glucose uptake are additive, suggesting that exercise and insulin stimulate muscle glucose transport via different mechanisms (Nesher et al., 1985; Wallberg-Henriksson et al., 1988; Constable et al., 1988). A few hours after acute exercise when most of the insulin-independent increase in glucose transport has worn off, the sensitivity and responsiveness of glucose transport to insulin are increased (Wallberg-Henriksson et al., 1988; Gulve et al., 1990). Acute exercise shifted the insulin dose response curve up and to the right, so that insulin responsiveness (the maximal insulin - stimulated response) of muscle glucose transport increased by ~22% and insulin sensitivity (glucose uptake stimulated by half maximal concentrations of insulin) increased by about 50% (Wallberg-Henriksson et al., 1988).

Most studies of the interaction between hormones and exercise on muscle glucose transport have been focused on healthy subjects or experimental animals showing no abnormalities of carbohydrate metabolism (Constable et al., 1988; Wallberg-Henriksson

et al., 1988; Henriksen et al., 1992). Little is known regarding whether exercise-stimulated glucose transport, including both insulin-dependent and insulin-independent components, is modified in different physiological or pathological states, such as insulin-resistance (Wallberg-Henriksson, 1992). Insulin-resistance is a metabolic stage when normal or high concentrations of insulin produce a less than normal biological response. Insulin-resistant conditions include obesity, diabetes, as well as consumption of a diet high in fat, such as a typical Western diet. A high fat diet is a factor known to cause insulin resistance in insulin-sensitive tissues (Olefsky 1978; Storlien et al., 1991). In our recent studies (Liu et al., 1995), a high fat diet (20% w/w) caused a downward shift of the insulin dose response curve for muscle glucose uptake, compared with a low fat (5% w/w) diet. At submaximal insulin concentrations (0.8 nM), glucose transport was decreased by ~75%; at maximally-stimulating insulin concentration (20 nM), glucose transport was decreased by 25%. To our knowledge, there is no prior report regarding the effect of an insulin-resistant state induced by feeding a high-fat diet on acute exercise-stimulated glucose transport in skeletal muscle. Such information would be of interest in development of dietary and exercise interventions to modify the pathology of insulin-resistant states.

The present study was designed to investigate the impact of high fat feeding on acute exercise-stimulated glucose transport in rat epitrochlearis muscle, with or without the presence of different concentrations of insulin. The first part of the experiment was carried out to study if the exercise-stimulated insulin-independent glucose transport was impaired by the high fat feeding. The second part of the experiment was designed to

investigate if the exercise enhanced sensitivity and responsiveness to insulin were affected by the high fat diet.

METHODS AND MATERIAL

ANIMAL TREATMENT

Experiments were carried out in accordance with the Canadian Council on Animal Care guidelines. Weanling female Sprague-Dawley rats were obtained from a colony maintained at the University of Alberta. Five rats per plastic cage were housed in a room that was maintained at 23°C on a 12 hour light/dark cycle. Animals were given food and water for ad libitum consumption. Prior to study, the rats were on their diets for six weeks. Six weeks of feeding of the diets with different dietary fat have shown to be effective in terms of inducing chemical changes in cell membrane phospholipid, as well as altered hormone actions (Field et al., 1988; Liu et al., 1994). After six weeks of feeding, rats were fasted overnight. Rats were humanely killed by CO_2 asphyxiation and epitrochlearis muscles were dissected.

DIETS

Rats were fed either a high fat (20% w/w fat, 42% of total energy) or a low fat (5% w/w fat, 13% of total energy) semi-purified nutritionally complete diet, according to McCargar et al. (1989). The composition of the diets is indicated in Table IV-1 (Liu et al., 1995). The diets were formulated to provide non-fat essential nutrients at similar nutrients/calorie ratio for the high versus low fat diets fed. In a previous study (McCargar et al., 1989), animals fed a broad range of dietary fat levels were studied (19-52% of total calories as fat), and there were no differences between treatments in total caloric intake, protein intake or body weight gain among the different dietary fat levels. All of the diet ingredients were from Teklad except corn starch (Best Foods Canada Inc.), beef tallow

(Canada Packers Inc.), safflower oil (Tosca) and flax seed oil (Gold Top). Both diets had the same fatty acid composition (Table IV-2).

EXERCISE

Half of the rats from each diet treatment were exercised by swimming in a protocol similar to that described by Wallberg-Henriksson et al. (1988). In brief, barrels were filled with water to a depth of ~50 cm. Water temperature was maintained between 30-35°C. One rat swam in each barrel. During the first 25 min they swam without weights; a weight equivalent to ~1.5% body weight was then attached to the tail of the rat. Then they swam three times 25 min with 5 min rest period intervals. Some of the swimmers were killed right after exercise to study the effect of the high fat diet on the insulin-independent component of exercise-stimulated glucose uptake; other swimmers were returned to cages with water, but not feed, and killed 3.5 hours after exercise to study the insulin-dependent component of exercise-stimulated glucose transport.

3-O-METHYL-GLUCOSE TRANSPORT STUDY

3-*O*-methyl-glucose (3-MG) transport was studied using the method described by Young et al. (1986) with some modifications (Liu et al., 1995). Muscles were pre-incubated in 3 ml Krebs-Ringer buffer (KRB) (119 mM NaCl, 25 mM $NaHCO_3$, 4.82 mM KCl, 1.0 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.24 NaH_2PO_4, 2.0 mM HEPES, pH 7.4) containing 0.1% BSA (Sigma Chemical Co., RIA grade), 8 mM glucose, 32 mM mannitol, with or without insulin (Sigma) in a shaking water bath (29°C) for one hour (Liu et al., 1995). The incubation medium was continuously bubbled with 95%O_2/5%CO_2. Following pre-incubation, the muscles were transferred into 3 ml KRB of the same

composition but without glucose and with 40 mM mannitol and incubated at 29°C for 10 min. The muscles were then transferred to 3 ml KRB containing either 8 mM 3-*O*-[^{3}H]methyl-glucose or [^{3}H]L-glucose (both from New England Nuclear, final specific radio-activity ~50 μCi/mmol), 32 mM mannitol and insulin at the same concentrations as in the pre-incubation media and incubated for 10 min. [^{3}H]L-glucose was used here to correct for non-specifically bound and passively diffused 3-*O*-[^{3}H]methyl-glucose in the muscles which could not be washed away (Liu et al., 1995). Before use, 3-*O* [^{3}H]methyl-glucose and [^{3}H]L-glucose were purified to remove ^3H_2O by evaporation to dryness. Muscles were washed in 3 ml [illegible]B at 0°C three times 10 min to remove 3-*O*-[^{3}H]methyl-glucose or [^{3}H]L-glucose from the extracellular space. The muscles were then solubilized in 1 ml Soluene-350 (Packard) and counted in 15 ml Hionic-Fluor (Packard) using a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer.

It has been shown that the exercise-induced increase in insulin sensitivity is detectable several hours after acute exercise (Wallberg-Henriksson et al., 1988; Cartee and Holloszy, 1990). To study this component of glucose transport, exercised rats were killed 3.5 hours after swimming, and muscles were incubated with or without half-maximally effective (0.8 nM) insulin levels for insulin-independent and insulin-dependent glucose transport determinations. For the exercise-stimulated insulin-independent glucose transport, it has been shown that this component is at its maximum immediately after acute exercise (Wallberg-Henriksson et al., 1988). To study this component of glucose transport, the exercised rats were killed immediately after swimming. Half of the muscles dissected were incubated with maximally effective (20 nM) insulin concentration to 1) demonstrate

a decreased responsiveness to insulin for rats fed a high-fat diet; 2) detect possible interaction of dietary fat with maximally effective insulin concentration and acute exercise. The maximally (20 nM) and half-maximally (0.8 nM) effective insulin concentrations in stimulating muscle 3-*O*-methyl-glucose transport under our experimental conditions were determined in animals fed a semi-purified low-fat diet (5% w/w) in a previous study (Liu et al., 1995).

DATA AND STATISTICS

Analysis of variance (general linear model) for the original data, multiple comparison (pdiff) for insulin-stimulated 3-MG uptake (the difference for 3-MG uptake value with the presence of insulin minus that without insulin for muscles from the same rat), and student *t*-test for L-glucose uptake, were performed using SAS (SAS Institute Inc., 1982)). Data are expressed either as least-squares mean (lsmean) and standard error of the lsmean, or mean and standard error of the mean.

RESULTS

3-MG UPTAKE IMMEDIATELY AFTER EXERCISE

Muscle 3-MG transport immediately after acute exercise is shown in Table IV-3. Values for uptake in the presence and absence of added insulin are shown, in addition the insulin-stimulated uptake was calculated by subtracting uptake value determined in the absence of insulin from the uptake value determined in the presence of insulin for the two muscles from each animal. There was a significant effect on 3-MG uptake in skeletal muscle for both insulin and acute exercise ($p=0.0001$). Dietary fat level overall did not have any effect on 3-MG transport ($p=0.18$), however an interaction between insulin and dietary fat level was significant ($p=0.03$). This interaction can be interpreted to indicate that the level of dietary fat did not alter insulin-independent glucose transport. However, when incubated with 20 nM insulin, consuming high fat diet was associated with a significant decrease in insulin-stimulated 3-MG uptake. This is confirmed by calculating insulin-stimulated 3-MG uptake as the difference between the correspondent groups incubated with or without insulin: 20 nM insulin stimulated 3-MG uptake by 442 pmol/mg/10 min 3-MG for muscles from rats fed the low-fat diet, compared with 288 pmol/mg/10 min from rats fed the high-fat diet ($p=0.0023$). There was no interaction between the level of dietary fat and exercise. These data indicate that muscles of rats fed a high-fat diet have a considerable impairment of insulin-dependent glucose uptake, but no impairment of glucose uptake in response to acute exercise.

3-MG UPTAKE 3.5 HOURS AFTER EXERCISE

Muscle 3-MG uptake measured 3.5 hours after acute exercise are shown in Table IV-4. The effects of both 0.8 nM insulin and acute exercise on 3-MG transport were significant (p=0.0001 and 0.0006, respectively). The level of dietary fat overall did not have any effect on 3-MG uptake, however an interaction between insulin and the level of dietary fat was significant (p=0.009). This interaction was due to a lowered insulin-stimulated 3-MG uptake for muscles from rats fed the high-fat diet: insulin-stimulated 3-MG transport was decreased from 231 pmol/mg/10 min for low-fat sedentary rats to 83 pmol/mg/10 min for high-fat sedentary rats ($p<0.05$). Interestingly, the interaction between level of dietary fat and exercise was significant (p=0.035). It seems the interaction arose from the groups incubated without insulin. Analysis of variance was then performed for the groups incubated either with or without insulin, separately. The interaction was significant for the groups incubated without insulin (p=0.021), with 3-MG uptake more than 100% higher for the high-fat exercised rats compared with other groups. There was no interaction for the groups incubated with 0.8 nM insulin (p=0.437). Insulin-stimulated 3-MG tended to be higher for low-fat exercised rats than low-fat sedentary rats (365±54 vs 231±45 pmol/mg/10 min, respectively, p=0.065). These results indicate that insulin resistance in muscles of animals fed a high-fat diet is not modified by acute exercise. Muscles of exercised rats fed the high-fat diet had a high rate of glucose uptake overall at 3.5 hours after exercise, because of their ability to maintain elevated insulin-independent 3-MG uptake.

One explanation for an elevated rate of glucose uptake in the absence of insulin would be an increased passive diffusion or non-specific binding of 3-MG to muscles from

exercised rats fed the high-fat diet. In order to test this possibility, we exercised both high-fat fed and low-fat fed rats and incubated epitrochlearis muscles 3.5 hours after acute exercise with [^{3}H]L-glucose. Glucose transporters specifically transport D-glucose, L-glucose can not be transported by the glucose transporters. Therefore, any [^{3}H]L-glucose left in the muscles, after incubation and washing, would have either passively diffused into the muscles or non-specifically bound to the surface of the muscles. The incubation and washing protocols were identical to that for the 3-MG uptake study. There was no significant difference between the amount of L-glucose retained in the muscles from the two groups (214±14.8 pmol/mg/10 min for low-fat exercised rats and 246±27 for high-fat exercised rats, respectively), indicating the apparently enhanced basal 3-MG uptake was not due to an elevation in passive diffusion or non-specific binding of 3-MG to skeletal muscle for high-fat exercised rats.

DISCUSSION

Glucose uptake by insulin-sensitive tissues is influenced by a variety of factors, such as the level and composition of dietary fat, the level of physical activity, obesity, as well as specific disease states such as diabetes (Olefsky and Saekow, 1978; Field et al., 1990; Dohm et al., 1990; Rodnick et al., 1992). Some of these factors may cause a decrease in glucose transport, such as a diet high in saturated fat (Storlien et al., 1991; Liu et al., 1995), obesity (Dohm et al., 1990) and diabetes (Field et al., 1990); while others may improve glucose uptake, e.g. acute and chronic exercise (Goodyear et al., 1990; Rodnick et al., 1992a) and diets high in polyunsaturated fatty acids (Field et al., 1990; Storlien et al., 1991; Sohal et al., 1992). Most studies on glucose transport have concerned effects of single factors (Goodyear et al., 1990; Storlien et al., 1991), with few studies focused on interactions of different factors, such as dietary fat and diabetes (Field et al., 1990) and exercise and diabetes (Wallberg-Henriksson, 1992). Because the mechanisms of altered glucose uptake are not fully characterized, it is far from clear what the interaction will be if two or more of the factors are present simultaneously under controlled experimental conditions. It would therefore seem valuable to probe for potential interactions between dietary factors, physical activity, genetic factors and disease. This information might be very useful for the planning of specific interventions in insulin-resistant states. The present study was designed to investigate the interaction between the level of dietary fat and exercise, the two lifestyle factors which affect most people, on muscle glucose transport. The choice of the high-fat diet for this study is within the range

of fat levels consumed by Western people, and has been shown to induce insulin-resistance in skeletal muscles of laboratory rats (Liu et al., 1995).

A novel discovery of this study is that basal glucose transport remained higher for the high-fat exercised group than the low-fat exercised group 3.5 hours after a bout of exercise. It was reported that most of the insulin-independent component of acute exercise-stimulated glucose transport would have worn off 2-3 hours after exercise (Young et al., 1987; Cartee et al., 1989). In this study, the insulin-independent 3-MG transport completely returned to normal levels 3.5 hours after exercise for rats fed the low-fat diet. However, the 3-MG transport rate was ~120% higher for the high-fat fed exercised rats, compared with high-fat fed sedentary rats. We have shown that this augmented action in 3-MG transport is likely due to carrier-mediated uptake, not due to an elevation in passive diffusion or non-specific binding of 3-MG to muscles from high-fat fed exercised animals. This result was not expected, because exercise-stimulated glucose transport (insulin-independent component) was reported to be decreased for streptozocin-induced insulin-resistant diabetic rats (Wallberg-Henriksson, 1992). The mechanism for this elevated basal glucose transport for high-fat fed exercised animals is unknown. Since there is evidence indicating that exercise-stimulated glucose uptake is regulated by local glucose metabolism (Richter et al., 1989; Gulve et al., 1990; Hespel and Richter, 1990), it could be postulated that muscle glucose metabolism might be affected by high-fat feeding for the exercised rats; and the altered glucose metabolism, in turn, caused increased glucose transport. Further study is needed to investigate the exact mechanism for this phenomenon.

Acute exercise did not increase insulin sensitivity for high-fat fed exercised rats 3.5 hours after exercise, compared with high-fat fed sedentary rats. This might indicate that the high-fat diet induces insulin-resistance at the same site where acute exercise normally increases insulin sensitivity. The mechanism for increased insulin sensitivity by acute exercise is not clear, and there are conflicting reports regarding whether or not altered insulin binding is involved in the increased insulin sensitivity after a bout of exercise (Webster et al., 1986; Bonen and Tan, 1989; Treadway et al., 1989). Several authors reported that exercise-stimulated glucose transport is regulated by local factors, such as tissue glucose metabolism or glycogen repletion (Richter et al., 1989; Gulve et al., 1990; Hespel and Richter, 1990). Sites for the high-fat induced insulin-resistance have been proposed, including decreased insulin binding to its receptors (Olefsky and Saekow, 1978; Grundleger and Thenen, 1982) or lowered numbers of glucose transporters (Pedersen et al., 1991). It has been reported that exposure of isolated adipocytes to saturated fatty acids for 4 hours induces insulin-resistance (Hunnicutt et al., 1994), raising the possibility that a high-fat diet induces insulin-resistance via alteration of local substrate metabolism. It is possible that the both acute exercise and a high-fat diet induce changes in insulin-sensitivity via alteration in local metabolism, and the change induced by acute exercise can not correct or compensate for that induced by a high-fat diet. However, it should be pointed out that basal 3-MG transport was higher for the high-fat fed exercised rats, compared with high-fat fed sedentary rats. Also, 3-MG uptake in the presence of 0.8 nM insulin was similar for low-fat fed exercised rats vs high-fat fed exercised rats. There is a possibility that increased insulin-sensitivity by acute exercise

and altered basal glucose transport for the high-fat fed exercised rats share the same mechanism, hence the effects of the two are not additive.

Our results indicate that a diet high in fat causes a decrease in insulin-dependent glucose transport, however it did not affect insulin-independent glucose transport; indeed the exercise-stimulated insulin-independent glucose transport was markedly activated at 3.5 hours after exercise. It is known that there are two types of glucose transporter in skeletal muscle: GLUT1 is not sensitive to insulin and is responsible for basal glucose transport, and GLUT4 is sensitive to insulin and is thought to be responsible for insulin-dependent and exercise-dependent glucose transport (Barnard and Youngren, 1992). Therefore, the result from the present study implies that a high-fat diet impairs GLUT4 mediated-glucose transport, but not GLUT1 mediated-glucose transport. However, it is not clear whether the high-fat diet induced decrease in GLUT4 mediated-glucose transport involves a decline in the number of GLUT4, as indicated by one study (Kahn and Pedersen, 1993), or is caused solely by defect(s) in the insulin signal transduction cascade. The present study demonstrated that exercise-stimulated insulin-independent glucose transport was normal for high-fat fed rats. If a single pool of GLUT4 is responsible for both exercise- and insulin-stimulated glucose transport (Rodnick et al., 1992b), it is likely that the number of GLUT4 is not affected by high-fat feeding, and defect(s) in the insulin signal transduction cascade is responsible for impaired insulin action.

Insulin stimulated less 3-MG uptake for rats fed the high-fat diet, measured both immediately and 3.5 hours after acute exercise in the presence of either 0.8 nM or 20 nM insulin. Insulin concentrations of 0.8 nM and 20 nM represent half-effective and

maximally-effective doses in terms of stimulating muscle 3-MG uptake under our experimental conditions (Liu et al., 1995). This result is compatible with earlier reports for adipocytes and muscles of sedentary animals in which a high-fat diet induced a decrease in both sensitivity and responsiveness to insulin (Olefsky and Saekow, 1978; Liu et al., 1995). It has been shown that insulin-resistance induced by a high-fat diet could be corrected by changing dietary fatty acid composition while keeping the level of fat unchanged (Storlien et al., 1991). One of the aims of the present study was to investigate if acute exercise could normalize the high-fat diet induced insulin resistance. The lack of interaction between dietary fat level and exercise, for insulin-stimulated glucose transport, indicates that acute exercise does not correct the impaired insulin-stimulated glucose transport for rats fed the high fat diet. On the other hand, 3-MG uptake was higher for the exercised groups than the sedentary groups for rats fed the high-fat diet. This result is compatible with the suggestion that chronic exercise compensates, rather than corrects, for insulin resistance induced by a high-fat diet (Kraegen et al., 1989).

There was no interaction between exercise and dietary fat level in the statistical analysis for 3-MG uptake measured immediately after acute exercise, suggesting that exercise-stimulated glucose transport (insulin-independent component) was not impaired in muscles resistant to insulin. It has been postulated that exercise (insulin-independent component) and insulin stimulate glucose transport by different mechanisms because 1) the effects of maximal effective insulin and exercise on glucose transport are additive (Nesher et al., 1985; Constable et al., 1988; Wallberg-Henriksson et al., 1988); 2) exercise and insulin may recruit glucose transporters from different intracellular pools (Douen et

al., 1989). If the mechanism(s) for insulin and exercise stimulated glucose transport are indeed different, then it would be possible that muscle tissue may not be resistant to exercise when it is insulin-resistant. Further investigation is required to determine the effects of exercise and diet on glucose uptake and metabolism in different insulin-resistant status.

TABLES

Table IV-1 Composition of the diets[1]

Ingredients	High Fat Diet	Low Fat Diet
(Data are expressed as g/kg diet)		
Casein	265	225
Starch	385	610
Fat	200	50
Vitamin Mix[2]	10	8.5
Mineral Mix[3]	50	42
Choline	3	2.5
Inositol	6	5
L-Methionine	3	2.5
Cellulose	78	54.5

[1] Diets were formulated according to McCargar et al. (1989).

[2] Teklad Vitamin mix A.O.A.C. (1960).

[3] Teklad, Bernhart and Tomarelli (1966).

Table IV-2 Fatty acid composition of the diet

Fatty Acids	%
C14:0	2.29
C16:0	23.57
C18:0	55.26
C18:1(9 and 7)	3.60
C18:2(6)	10.75
C18:3(3)	2.83

The dietary fat is composed of 7.7 g of flaxseed oil, 20 g of safflower oil, and 72.3 g beef tallow per 100 g of fat. Fatty acid composition was determined in duplicate by gas liquid chromatography.

Table IV-3 Effect of dietary fat level and acute exercise on 3-MG transport: immediately after exercise.

Fat level	Low		High				Statistical					
Exercise	-	+	-	+	n	SE	Ins	Fat	Exe	Ins*Exe	Ins*Fat	Fat*Exe
(-)insulin	175	324	200	358	10	50	0.0001	0.182	0.0001	0.627	0.033	0.8385[A]
(+)insulin	588	796	482	652	10	50						
Insulin-stimulated[B]	413[a,b]	471[a]	283[b]	294[b]	10	47		0.002[C]	0.465[C]			

3-MG uptake (pmol/mg/10 min) in rat epitrochlearis muscles was measured with or without 20 nM insulin, as described in METHODS AND MATERIAL. Data are expressed as lsmeans. Ins: insulin; Fat: level of dietary fat; Exe: exercise; A: statistical values in this row were from analysis of variance for glucose transport with two levels of insulin, two levels of exercise and two levels of dietary fat (2*2*2); B: lsmeans for the difference between the two muscles from the same rat incubated with or without insulin (insulin-stimulated 3-MG uptake); C: analysis of variance for insulin-stimulated 3-MG uptake; n: number of muscles in each group; SE: standard error of lsmean; a, b: values without the same letter are significantly different from each other ($p<0.05$).

Table IV-4 Effect of dietary fat level and acute exercise on 3-MG transport: 3.5 hours after exercise

Fat level	Low		High		Statistical					
Exercise	-	+	-	+	Ins	Fat	Exe	Ins*Exe	Ins*Fat	Fat*Exe
(-)insulin	187±56 (10)	179±59 (9)	157±63 (8)	408±56 (10)	0.0001	0.761	0.0006	0.480	0.009	0.0353[A]: 0.021[B] 0.437[C]
(+)insulin	417±56 (10)	547±63 (8)	241±63 (8)	473±56 (10)						
Insulin-stimulated[D]	231±45[a] (10)	365±54[a] (7)	83±50[b] (8)	65±45[b] (10)		0.0001[E]	0.244[E]			

3-MG uptake (pmol/mg/10 min) in rat epitrochlearis muscles was measured with or without 0.8 nM insulin, as described in METHODS AND MATERIAL. Data are expressed as lsmeans±SE. Numbers in bracket indicate the number of muscles in each group. Ins: insulin; Fat: level of dietary fat; Exe: exercise; A: statistical values in this row were from analysis of variance for glucose transport with two levels of insulin, two levels of exercise and two levels of dietary fat (2*2*2); B: variance analysis for the groups incubated without insulin; C: variance analysis for the groups incubated with 0.8 nM insulin; D: lsmeans for the difference between the two muscles from the same rat incubated with or without insulin (insulin-stimulated 3-MG uptake); E: analysis of variance for insulin-stimulated 3-MG uptake; a, b: values without the same letter are significantly different from each other ($p<0.05$). P value for insulin-stimulated glucose transport between low-fat sedentary and low-fat exercised rats is 0.065.

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CHAPTER V: CONCLUSION

SUMMARY

The data from this thesis provides evidence that the composition of dietary fatty acid plays a significant role in determining insulin receptor binding activity in skeletal muscle plasma membrane (Chapter II). This is in agreement with the results for adipocytes (Field, 1988; Field, 1990). Putting this data together with the observation that dietary ω-3 fatty acid and a diet with a high P/S ratio increased insulin-stimulated glucose metabolism in skeletal muscle (Storlien et al., 1991; Sohal et al., 1992), one may postulate that increased insulin binding might be responsible for increased insulin action in skeletal muscle. In addition to insulin binding, IGF-I binding to skeletal muscle plasma membrane was also increased by polyunsaturated ω-3 fatty acids, indicating that the effect of dietary fat on hormone binding is not limited to insulin. When binding was performed on solubilized receptors, the effect of dietary fat on insulin binding disappeared. This observation suggests that dietary ω-3 fatty acids increase insulin receptor binding without increasing the amount of the receptor, and supports the theory that altered membrane fluidity induced by diet changes in the fatty acyl composition in phospholipids is responsible for improved hormone binding to its receptor (Ginsberg et al., 1982; Gould et al., 1982).

It has long been known that a high-fat diet induces a decrease in insulin-stimulated glucose transport (Olefsky and Saekow, 1978). In this thesis, I have shown that a high-fat diet also causes an impaired IGF-I-stimulated glucose transport in skeletal muscle (Chapter III). This result further confirmed the observation from Chapter II that the effect

of dietary fat on hormone action is not limited to insulin. The magnitude of the depression was similar for insulin- and IGF-I-stimulated glucose transport. Decreased IGF-I binding was demonstrated in isolated epitrochlearis muscles (Chapter II and Chapter III), which leads to the conclusion that IGF-I binding to its receptor in skeletal muscle is sensitive to both dietary fatty acid composition (Chapter II) and the level of dietary fat (Chapter III). The fact that IGF-I binding was decreased, by high fat feeding, to a less degree than IGF-I-stimulated glucose transport suggests the existence of a post-receptor defect(s). These results indicate that the mechanisms for a high-fat diet induced alteration in the actions of insulin and IGF-I are similar in skeletal muscle.

The effects and interaction of two lifestyle factors, i.e., acute exercise (swimming) and the level of dietary fat, on muscle glucose transport were also studied (Chapter IV). Exercise-stimulated insulin-independent glucose transport was normal for rats which were made resistant to insulin by high-fat feeding, when measured immediately after swimming. This observation supports the hypothesis that insulin and acute exercise stimulates muscle glucose uptake through different mechanisms. Three and a half hours after swimming, there was a significant interaction between the level of dietary fat and exercise: basal glucose transport was significantly higher for the high-fat fed exercised rats, when glucose transport had returned to resting level for low-fat fed exercised rats. The mechanism for this interesting phenomenon is not clear. Half-maximal effective (0.8 nM) insulin-stimulated glucose transport was similar for high-fat sedentary and high-fat exercised rats, indicating a lack of exercise-stimulated increase in insulin sensitivity.

DISCUSSION

There is a good understanding of the phenomenon and the mechanism for regulation of glucose metabolism by dietary fat in adipocytes (Field et al., 1988; Field et al., 1990). However, the mechanism for altered glucose uptake by dietary fat in muscle is not well studied, despite the fact that skeletal muscle plays an important role in glucose disposal. This thesis provides some insights in the mechanism of how dietary fat alters insulin action on muscle. Dietary lipid composition is clearly capable of modifying the fatty acid composition of skeletal muscle plasma membrane phospholipid. The data in this thesis show that the content of ω-3 fatty acid in plasma membrane phospholipid is particularly susceptible to influence by diet, at least compared with the P/S ratio of the same lipids. The modified insulin binding to its receptor is linked to changes in the phospholipid fatty acyl composition, and is unlikely due to an altered gene expression for insulin receptors (Chapter II). This result clearly demonstrates the importance of the phospholipid environment for the normal functions of insulin and IGF-I receptors which are embedded in the membrane lipids.

Altered membrane composition may have an influence on other proteins and functions of the sarcolemma. There are many integral membrane proteins of skeletal muscle. Muscle expresses receptors for a variety of hormones, including glucocorticoids, growth hormone, cathecholamines, androgens and estrogens. It remains to be determined whether membrane lipid composition has an impact on the function of these receptors or the post-receptor responses. Sarcolemma contains acetyl choline receptor concentrated in the neuro-muscular junction. A wave depolarization or action potential is a key

component of excitation contraction coupling; this function is dependant upon voltage-dependent ion channels found in the plasma membrane. Muscle is also characterized by extensive intracellular membrane systems, including the T-tubular system, triads, junctional and fenestrated sarcoplasmic reticulum, endoplasmic reticulum, Golgi, and nuclear membranes. Studies need to be extended to investigate the possible influence of modified membrane fatty acyl composition on the functions of these systems.

The actions of insulin and IGF-I in skeletal muscle changed in concert in response to dietary fat manipulation: in muscles of rats fed a high fat diet, a similar degree of resistance to these hormones in glucose uptake was observed. Apparently similar mechanisms are involved in this response since both insulin and IGF-I binding to the tissue were reduced. In other insulin-resistant states, IGF-I resistance may or may not be observed (Poggi et al., 1979; Dohm et al., 1990; Jacob et al., 1992). In some cases resistance is clearly limited to one factor (Rossetti et al., 1991); in other cases resistance may be observed to both factors but the mechanisms are apparently different (Poggi et al., 1979; Dohm et al., 1990). Results obtained on insulin and IGF-I resistance may offer insights into the common and differential mechanisms by which tissues respond to these factors. The results in this thesis suggest that the hormone-binding characteristics of insulin and IGF-I receptors are affected to a similar degree by the lipid environment of the receptor. A diet high in fat also appeared to reduce hormone binding for both factors, but it would be necessary to find out the mechanism for reduced hormone binding in both cases to know whether, for example, an element such as receptor gene expression was the common target of a high fat diet. The influence of dietary lipid level and composition on

the full cascade of postreceptor events leading to all of the metabolic pathways influencing insulin and IGF-I remains to be clarified. The ability of muscle to carry out hormone-stimulated glucose uptake and metabolism requires two main agonists, insulin and IGF-I, which act through their respective receptors and post-receptor cascades. These cascades may converge or have common elements, but since response of the tissue to these two factors may change in concert or differentially, there must be unique elements in their pathways of signal transduction. Factors which modify tissue responses to insulin and IGF-I, such as diet, disease and exercise, also act at a specific site (or sites) in these pathways of signal transduction. Based on present knowledge, it is not possible to predict to what extent the factors which increase hormone-dependent glucose uptake will be effective in the simultaneous presence of factors which depress this function. Empirical approaches to assess the nature of interactions, as used in this thesis, can be employed to explore the full range of potential interactions amongst dietary fat (level of fat and fatty acid composition), metabolic states (healthy, obese, diabetes), physical activity (sedentary, exercise) and agonist (insulin, IGF-I).

Skeletal muscle is the major site for glucose disposal (DeFronzo, 1987). Glucose transport in skeletal muscle is influenced by a variety of factors, such as the level and composition of dietary fat, physical activity, obesity and diabetes. Dietary fat and physical activity are the two lifestyle factors which affect most people. The interaction between these two factors, along with hormonal factors such as insulin and IGF-I, deserves special attention from researchers interested in glucose transport, insulin-resistance and diabetes mellitus.

Data from this thesis provides evidence that a high-fat diet causes an IGF-I resistance, as well as insulin-resistance. To further understand the similarities and differences between insulin and IGF-I stimulated glucose transport or insulin- and IGF-I-resistance, the ability of IGF-I to stimulate glucose transport remains to be determined in other insulin-resistant animal models, such as ?? and corpulant rats. In addition, a diet high/low in fat may modify the action of IGF-I in skeletal muscles under different physiological states. Furthermore, the effect of exercise on IGF-I-stimulated glucose transport in skeletal muscle is poorly understood (Henriksen et al., 1992), compared to the effect of exercise on insulin-stimulated glucose transport. By employing the treatments of diet (high/low fat level, high/low P/S ratios), exercise (acute and chronic) and IGF-I (different concentrations) on animals of different insulin-resistant models, there are many experiments one may plan to further explore the action of IGF-I under different physiological and pathological conditions.

In addition to decreased IGF-I binding, the high-fat diet induced IGF-I resistance apparently involves either defect(s) in the signal transduction cascade or decreased number of glucose transporter. To investigate the biochemical mechanism(s) in future studies, one may consider to determine the IGF-I receptor autophosphorylation and phosphorylation activities, measure the amount of both GLUT1 and GLUT4 proteins in skeletal muscle (one study has indicated that GLUT1 was also responsible for IGF-I-stimulated glucose transport in muscle, see Bilan et al., 1992).

One question remains to be answered in further study is what are the physiological importance and biochemical mechanism for the elevated insulin-independent glucose

transport in muscles from the exercised high-fat fed rats. It is postulated that the elevated glucose transport for these rats might be a compensation mechanism. The rate of glucose transport for the insulin-resistant muscles from rats fed the high-fat diet may not meet the increased muscle demand for glucose either during or after the acute exercise. The elevated glucose transport by the insulin-resistant exercised muscles enables them to transport more glucose, compared to the resting-control insulin-resistant muscles, to cope with the increased demand for glucose associated with exercise. The fact that this elevation in glucose transport was seen only 3.5 hours, not immediately, after acute exercise suggests that the elevated glucose transport might be related to glycogen repletion process. It is hypothesized that a lower glycogen level, at 3.5 hours after exercise, is responsible for the observed elevation in glucose transport. Therefore, measurement of muscle glycogen level should be included in future studies. Specifically, glycogen level in epitrochlearis muscles should be measured before exercise, immediately after exercise and 3.5 hours after exercise for both high- and low-fat fed rats.

The experimental results obtained here may have implications for healthy people, athletes and individual with diabetes. However, the result in this thesis revealed interaction between diet and exercise, and make it clear that caution is required in extrapolating results from animals studied under constant conditions of diet, health or activity. On the other hand, data from this thesis justify analogous experimentation on human subjects to determine whether the same effects of diet and interactions occur in man. Recent research in human subjects has shown that variations in insulin sensitivity are correlated with differences in fatty acyl composition of muscle phospholipid (Borkman

et al., 1993). Future studies in humans could focus on the interactions among dietary fat, exercise and insulin-resistant states, as well as the effect of IGF-I in different physiological states. Further studies of this kind in normal, obese or diabetic human subjects might show that physical activity, manipulation of dietary fat and IGF-I have great potential in the treatment of insulin-resistance.

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CHAPTER VI: APPENDIX

APPENDIX I: SARCOLEMMA PREPARATION

This procedure is a modification of Ohlendick et al. (1991, J. Cell Biol. 112: 135-148).

BUFFERS USED IN THE PREPARATION

BUFFER A

20 mM tetrasodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 76.8 nM aprotinin, 1.1 uM leupeptin, 0.7 uM pepstatin A, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM phenylmethylsulfonylfluoride (PMSF), pH 7.0

BUFFER B

0.303 M sucrose, 20 mM Tris-maleate, 0.6 M KCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0

SUCROSE BUFFER

0.878 M sucrose, 0.6 M KCl, 20 mM Tris-maleate, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0

DILUTION BUFFER

0.6 M KCl, 20 mM Tris-maleate, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0

STORAGE BUFFER

118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 50 mM HEPES, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.6

BUFFER C

50 mM sodium phosphate, 0.16 M NaCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.4

BUFFER D

0.303 M sucrose, 20 mM Tris-HCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.4

BUFFER E

0.2 M N-Acetyl-D-Glucosamine, 0.303 M sucrose, 20 mM Tris-HCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.4

CRUDE MEMBRANE PREPARATION

(The procedure described here is to be performed at 4°C)

1. Grind 150 g of muscle in a meat grinder.

2. Put the ground muscle in a Waring blender with 7 volumes (~one litre) of buffer A.

3. Homogenize the ground muscle for 30 seconds. Repeat this step 3 times.

4. Centrifuge the homogenate in JA-10 rotor at 14,000 g (9,000 rpm) for 15 min.

5. Filter the supernatant from step 4 through six layers of cheesecloth.

6. Homogenize the pellet from step 4 in buffer A (3 times volume of the original muscle sample, or 450 ml).

7. Centrifuge the homogenate from step 6 as described in step 4.

8. Filter the supernatant from step 7 through six layers of cheesecloth and combine it with the supernatant from step 5.

9. Centrifuge the combined supernatant with JA-14 rotor at 30,000 g (14,000 rpm) for 30 min.

10. Solid KCl is added to the supernatant from step 9 to a final concentration of 0.6M.

11. Centrifuge the KCl treated supernatant in a Beckman 60Ti rotor for 35 min at 142,000g (37,500 rpm).

12. Suspend the pellet in 200 ml buffer B and centrifuge it again as described in step 11.

13. The pellet is resuspended in 30 ml of buffer B and 5-ml aliquots are loaded on top of 35 ml sucrose buffer in ultraclear ultracentrifuge tubes (40 ml capacity). Centrifuge the tubes in an SW27 rotor at 112,000 g (25,000 rpm) for 17 h.

14. Collect the crude surface membrane fraction at the 0.303/0.878 M sucrose interface, dilute it with dilution buffer, and centrifuge it in a 60Ti rotor for 30 min at 142,000g.

15. The pellets are suspended in 10 ml of buffer C. After determining the protein concentration, dilute the sample to 1 mg protein/ ml.

PURIFICATION OF SKELETAL SARCOLEMMA BY WHEAT GERM AGGLUTINATION

(The procedure described here is to be performed at 4°C)

1. Gently mix the crude membrane with an equal volume of 1 mg/ml wheat germ lectin in buffer C.

2. Incubate the mixture for 10 min on ice.

3. Pellet the solution in a centrifuge (Eppendorf model 5414) for 90 s at 14,000 g.

4. Discard the supernatant and resuspend the pellet in buffer D and centrifuge it for 90 s at 14,000 g (Eppendorf model 5414).

5. Repeat step 4 for two more times.

6. The pellets are then resuspended and deagglutinated by incubating for 20 min in 18 mL of 0.2 M N-acetyl-D-glucosamine in buffer D. The deagglutinated suspension is centrifuged in the Eppendorf centrifuge for 90 second at 14,000 g and the supernatant pelleted at 150,000 g for 20 min (60Ti, 38,600 rpm), resuspended in buffer D and centrifuged for 20 min at 150,000 g as described above. The final pellets are resuspended in 4 mL of buffer C and stored frozen in liquid N.

APPENDIX II: FATTY ACID COMPOSITION ANALYSIS

LIPID PREPARATION

A) DIETARY LIPIDS

LIPID EXTRACTION

To 0.5 g of diet, 20 ml of $CHCl_3$:MeOH (2:1) and 4 ml of KCl (0.1 M) is added. After vortexing vigorously for 5 min, the sample is filtered through Whatman #1 filter paper and left in cold room in a separation funnel overnight to separate. The lower layer ($CHCl_3$) containing the lipids is collected. The $CHCl_3$ is evaporated under nitrogen.

SAPONIFICATION AND METHYLATION OF THE LIPIDS

Two ml of 0.5 N KOH in methanol is added into the sample and heated to 100°C for two hours. After the sample is cooled to room temperature, 2 ml hexane and 1.5 ml 14% BF3 in methanol is added and the sample is heated to 110°C for two hours. After cooling down to room temperature and addition of 1 ml of water, the sample is shaken vigorously, and let sit to separate for 10 min. The hexane (top layer) containing the lipids is transferred into a gas chromatography vial and dried under nitrogen. The sample is then ready for analysis by gas chromatography.

B) MEMBRANE LIPIDS

EXTRACTION

To 0.4 ml (1 mg membrane protein/ml) membrane, the following solvents are added in sequence, with vortexing for 30 seconds after each addition: 0.8 ml methanol, 2 ml $CHCl_3$:MeOH (1:1), 2.7 ml $CHCl_3$, 2.5 ml $CHCl_3$:MeOH (2:1), and 1.6 ml KCl (0.1 M).

To all solvents add 1 drop ethoxyquin. Leave the sample in cold room overnight to separate. The lower layer ($CHCl_3$) is removed and evaporated under N_2. Reconstitute with 100 µl $CHCl_3$, the sample is then ready for thin-layer-chromatography separation.

PHOSPHOLIPID SEPARATION

The solvent system:

$CHCl_3$	MeOH	2-Propanol	KCl (0.25%)	Triethylamine
60 ml	18 ml	50 ml	12 ml	36 ml

A Silica Gel H plate (ANAYECH) is placed in oven at 110°C for one hour to activate it. Divide the plate into six to eight lanes with a sharp needle. Place the sample and standards onto the plate, using glass capillary tube, at ~one inch from the bottom of the plate. Place the plate into the tank containing the solvent (~half an inch deep) until the solvent almost reaches the top of the plate (about 1.5 to 2 hours). Take the plate out and put in the 110°C oven for 2 min. Spray with 0.1% 8-anilind-1-naphthalene-sulfonic acid (ANSA) on the plate to visualize the phospholipid fractions under UV. Identify each fraction by comparing with standards, and scratch each fraction out and place them into culture tubes. Add a known amount (5 µg) of C17:0 as internal standard.

METHYLATION

Add 1.5 ml 14% BF_3 in methanol and 2 ml of hexane. Heat the tubes in sandbath for one and half hours at 110°C. After cooling down to room temperature, 1 ml iter was added to each tube, vortexed, and let sit in a cold room (4°C) overnight. Transfer the top layer (hexane, containing the lipids) to a microvial and dry it down under nitrogen. The sample is then ready for GC analysis.

APPENDIX III: GLUCOSE TRANSPORT MEASUREMENT

KREBS RINGER BICARBONATE MEDIUM (KRB)

KRB (in mM) of 119 NaCl, 4.82 KCl, 1.25 $MgSO_4$, 1.24 NaH_2PO_4; 25 $NaHCO_3$, 2.0 N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)/NaOH (pH 7.4). Note: thoroughly bubble medium with 95% O_2, 5% CO_2. Adjust pH to 7.4 before use.

Preincubation medium

KRB containing: -Bovine serum albumin (insulin - free) 0.1% (w/w)

-D-glucose 6 mM

-Mannitol 32 mM

-Insulin or IGF-I

Wash medium

KRB containing: -Bovine serum albumin (insulin - free) 0.1% (w/w)

-Mannitol 40 mM

-Insulin or IGF-I

Incubation medium

KRB containing: -Bovine serum albumin (insulin - free) 0.1% (w/w)

-3-O-methyl glucose 8 mM

-3-O-[3H]methyl glucose 50 uCi/mmol (70,000 -80,000 dpm/ 100 ul). Note: 3-O-[^{3}H]methyl glucose tends to be contaminated with

[^{3}H]H2O. Immediately prior to use the concentrated isotope - containing solution is evaporated to dryness under gentle heating to remove this contaminant. The 3-O-[^{3}H]methyl-glucose may then be suspended in the incubation medium.

-Mannitol 32 mM

-Insulin or IGF-I

Final wash medium

KRB.

GENERAL PROCEDURE

Glucose transport is carried out using a method described by Young et al. (1986) with some modifications. Epitrochlearis muscles are dissected intact and weighed. All incubations are carried out in a temperature - controlled shaking water bath and incubation media continuously bubbled with a mixture of 95% O_2, 5% CO_2. Immediately after dissection muscles are preincubated in individual flasks containing 3 ml of Preincubation medium for 30 minutes at 29 oC. Muscles are then transferred to fresh tubes containing Wash medium, and incubated for 10 minutes at 29 °C. Muscles are thereafter transferred to fresh tubes containing Incubation medium and incubated for 10 - 30 minutes at 29 °C. Glucose uptake measures are carried out in the linear portion of uptake; this must be determined in preliminary experimentation for specific study conditions. After incubation, muscles are washed 3 times for 10 minutes in ice cold KRB

(4 °C) in a shaking icewater bath, to wash out excess 3-O-methyl glucose from the extracellular space. Muscles are then blotted and transferred to glass scintillation vials, solubilized in 1 ml Soluene 350 (Packard) and counted in 15 ml Hionic Fluor (Packard) with a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer.

In preliminary experiments, it was found that using the 0°C washing protocol alone could not remove all of the radio-labelled L-glucose from the muscles. This unwashable radioactivity represents L-glucose which either non-specifically binds to the muscle surface or passively diffuses into muscle fibres, since L-glucose can not be transported by the glucose transporter. The chemical similarity between L- and D-glucose makes L-glucose a good candidate to estimate how much untransported D-glucose is trapped in the muscle tissue. To correct for non-specifically bound and passively diffused 3-*O*-[^{3}H]methyl-glucose, ten muscles (from both diet treatments) are incubated with [^{3}H]L-glucose on each day of glucose transport studies, and the amount of [^{3}H]L-glucose remaining in the muscles after washing is subtracted from the amount of 3-*O*-[^{3}H]methyl-glucose (e.g., an individual muscle has a 3-*O*-[^{3}H]methyl-glucose of 550 pmol/mg. The average [^{3}H]L-glucose uptake from the ten muscles on that day is 150 pmol/mg. Then the corrected 3-*O*-[^{3}H]methyl-glucose uptake for that muscle is 550-150=400 pmol/mg).

PREPARATION OF THE MEDIUM

1. KRB preparation (24 ml/sample).
2. Gas the KRB with 95% O_2 and 5% CO_2 for 30 min.
3. Adjust pH to 7.4.
4. Add BSA (0.1 %, w/v) to half of the KRB prepared (12ml/sample, buffer A).

5. Take 80% of buffer A into another flask, and add mannitol to it to 40 mM (0.73g/100ml, buffer B).

6. 40% of buffer B is ready as the washing buffer. This buffer can be directly aliquoted to the tubes for basal glucose transport measurement. For the addition of IGF-I or insulin, a small beaker or flask should be used. The buffer with lower hormone concentration should be prepared first..

7. Mix the rest of buffer B with certain amount of buffer A (4:1) to make the mannitol concentration to 32 mM (buffer C). Half of this is for pre-incubation, and another half for incubation.

8. Add D-glucose (MW 180.2) to the buffer C for pre-incubation to 8 mM (14.4 mg/10 ml). This medium is ready for pre-incubation after addition of IGF-I or insulin (see step 6).

9. Add 3-O-methyl-glucose to the buffer C for incubation to 8 mM (15.536 mg/10 ml, buffer D).

10. Dry down an appropriate amount of the original stock 3-*O*-[^{3}H]methyl-glucose (0.4 μCi/ml buffer D). This is done by let the microcentrifuge tube containing the sample to sit on a hot stirrer (at the lowest temperature set, with four layers of paper towel between the surface of the stirrer and the sample) in a fumehood inside the radiation room.

11. Add the 3-*O*-[^{3}H]methyl-glucose to the buffer D. Take 100 μl of this solution and count. The radioactivity should be around 70,000~80,000 dpm/100 μl. After addition of IGF-I or insulin, this buffer is ready for incubation.

12. Mark three sets of tubes (for pre-incubation, washing and incubation, respectively) and fill the tubes with corespondent medium.

MUSCLE INCUBATION

1. The timing of muscle incubation and transferring is pre-scheduled, with about 3 min and 40 second for each rats

2. Fill 3 petri-dishes with KRB (0.1% BSA) and gas the medium with 95% O_2 and 5% CO_2.

3. The temperature of the water bath is set at 29°C. Connect the pre-incubation tubes with the oxygen apparatus and gas them with 95% O_2 and 5% CO_2.

4. Dissect three rats and put the muscles in the three petridishes before starting to weigh them.

5. Start the timer after transfer of the first muscle into pre-incubation medium.

6. Personnel: the first person is responsible for dissection and later on transferring muscles from incubation tubes to the final washing tubes; the second person is responsible for weighing muscles and the transfers in the final washings; the third person is responsible for killing animals and transferring muscles from pre-incubation to washing tubes; the fourth person is responsible for transferring muscles from washing to incubation tubes.

7. Rinse the spatala every time before and after a transfer in a beaker with at least one litre distilled water, because usually different tubes have different hormone concentrations.

8. After the final washings, the muscles were transferred into glass scintillation vial, and 1 ml SoluEne was added to each vial. The vials were capped tightly. Shake the vials on a shaker for ~12 hours to dissolve.

9. Add 5 ml Hionic-Fluor and 10 regular scintillation cocktail to each vial. Leave the vials in the scintillation counter for at least one hour before starts counting.