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THE EFFECT OF EXERCISE TRAINING ON SKELETAL MUSCLE GLUCOSE TRANSPORTER ISOFORM GLUT4 CONCENTRATION IN THE OBESE ZUCKER RAT

by

Eric Anthony Banks, B.S.

THESIS

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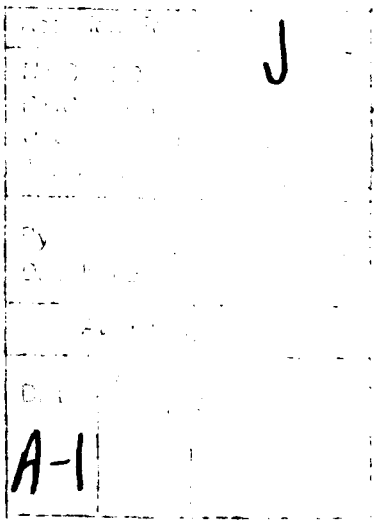
of the Requirements
for the Degree of
Master of Arts



THE UNIVERSITY OF TEXAS AT AUSTIN

May 1991

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I'd like to thank my fellow graduate students in the Exercise Metabolism laboratory for their help.

Eric A. Banks

ABSTRACT

THE EFFECT OF EXERCISE TRAINING ON SKELETAL MUSCLE GLUCOSE TRANSPORTER ISOFORM GLUT4 CONCENTRATION IN THE OBESE ZUCKER RAT

BY

ERIC ANTHONY BANKS, B.S.

SUPERVISING PROFESSOR: JOHN L. IVY

Exercise training has been demonstrated to improve skeletal muscle insulin resistance, however, the mechanisms for this improvement have not been fully chararcterized. The effects of high (HT) and low (LT) intensity exercise training were therefore examined on insulin-stimulated 3-O-methyl-D-glucose (3-OMG) transport and the concentration of glucose transporter isoform GLUT4 in the red (fast-twitch oxidative) and white (fast-twitch glycolytic) quadriceps of the obese Zucker rat. Sedentary obese (SED) and lean (LN) rats served as controls. 3-OMG transport was determined during hindlimb

perfusion in the presence of 8 mM 3-OMG, 2 mM mannitol, 0.3 mM pyruvate and 500 μU/ml insulin. GLUT4 concentration was determined by Western blot analysis. HT and LT rats displayed greater red quadriceps 3-OMG transport rates and GLUT4 concentrations than SED rats. Citrate synthase activity, which is an oxidative enzyme marker for skeletal muscle exercise training, highly correlated with GLUT4 concentration (r=0.73). No differences in red quadriceps 3-OMG transport or GLUT4 concentration were observed between HT and LT rats. There were also no differences in white quadriceps 3-OMG transport, GLUT4 concentration or citrate synthase activity among HT, LT and SED rats. The 3-OMG transport rate of LN rats was two-fold greater than SED rats despite similar GLUT4 concentration. These results suggest muscle insulin resistance in the obese Zucker rat is not due to a reduced GLUT4 concentration. However, the improved insulin resistance seen with exercise training appears to be due in part to an increased GLUT4 concentration which is only observed in muscle fibers recruited during training.

KEY WORDS: glucose, GLUT4, insulin, insulin resistance, diabetes, exercise training, muscle fiber-type

TABLE OF CONTENTS

TABLE LIST

FIGURE LIST

ARTICLE

INTRODUCTION

The genetically obese Zucker rat (*fa/fa*) is a well documented animal model for the study of skeletal muscle insulin resistance (Bray, 1977). The resistance can be attributed to reduced insulin sensitivity and responsiveness (Sherman et al., 1988). Insulin sensitivity is defined by the percent of maximal biological response elicited by a submaximal concentration of insulin. The higher the percent maximal response at a given submaximal concentration the greater the insulin sensitivity. Alternately, insulin responsiveness is defined by the biological response achieved with a maximally stimulating insulin concentration. The greater the biological response the better the responsiveness (Kahn, 1978).

Exercise training was shown to improve muscle insulin resistance in the obese Zucker rat. The improved resistance was due to increased muscle insulin responsiveness while muscle insulin sensitivity remained constant (Ivy et al., 1989). In skeletal muscle, the maximal insulin response is achieved with only 20 to 30% of the receptor sites occupied (LeMarchand-Brustel et al., 1978). However, decreases of only 25 to 35% in

insulin binding have been demonstrated for the muscle of the obese Zucker rat (Crettaz et al., 1980; Crettaz et al., 1983). Therefore, it is likely the increase in muscle insulin responsiveness following exercise training occurred at a post insulin receptor level and may have involved a transport process modification. It has also been demonstrated improvement in muscle insulin responsiveness is fiber-type and exercise intensity specific. During low intensity training only the fast-twitch red fibers showed improved insulin-stimulated glucose transport, whereas high intensity training was needed to show improvement in fast-twitch white fibers (Ivy et al., 1989; Cortez et al., in press). Furthermore, only muscles with increased citrate synthase activity demonstrated improved insulin-stimulated glucose transport. These observations suggest muscle recruitment is required for improvement in muscle insulin resistance.

Muscle glucose transport is controlled specifically by glucose transporter proteins on the plasma membrane. Activation of glucose transport by insulin is associated with an increase in plasma membrane transporters. This process is postulated to occur by a rapid reversible translocation of glucose transporters from low density microsomes to the plasma membrane. Five different

glucose transporters have been identified, with the two predominant muscle isoforms being GLUT1 and GLUT4. The GLUT1 isoform is found in small amounts on the plasma membrane and its concentration is not significantly increased by insulin exposure. However, the plasma membrane GLUT4 concentration is readily increased by insulin and was initially referred to as the insulin-regulatable glucose transporter (IRGT). The possibility of an insulin-regulatable transporter was first implied by the weak cross-reaction between antibodies specific for either the HepG2/rat brain transporter (GLUT1), or the rat adipocyte transporter (GLUT4) (Wang, 1987; Oka et al., 1988). This was confirmed through the use of a monoclonal antibody specific for the rat adipocyte transporter. This monoclonal immunolabels a 43K relative molecular mass species present only in tissues that exhibit insulin-regulatable glucose transport, including skeletal muscle (James et al., 1988).

Using a rat diaphragm incubation procedure, Wardzala & Jeanrenaud (1981) were the first to demonstrate insulin stimulates glucose transporter redistribution from an intracellular pool to the plasma membrane in muscle. This observation was later verified in hindlimb muscle from rats exposed to insulin injection in vivo and

following maximally stimulating insulin concentrations during hindlimb perfusion. In addition, insulin significantly stimulated the translocation of GLUT4 but not GLUT1 in skeletal muscle. Recently, Kern et al. (1990) reported the GLUT4 concentration in rat fast-twitch red muscle was five-fold greater than that of fast-twitch white muscle, and suggested the difference in transporter concentration was responsible for the difference in the insulin responsiveness of the two muscle fiber types. Similar observations were made by Henriksen et al. (1990) using a variety of rat hindlimb muscles.

Although it is known the obese Zucker rat has depressed insulin stimulated glucose transport, the relationship between glucose transporter concentration and muscle insulin resistance has not been clearly established. Friedman et al. (1990) reported the GLUT4 concentrations in the gastrocnemius muscle of obese and lean Zucker rats were the same, and low intensity exercise training increased the level of GLUT4 in both. It was suggested the improved muscle insulin resistance of the obese Zucker rat after exercise training was due to an increase in functional glucose transporters. However, the physiological significance of increased

transporter number was not examined. Nor was it determined if the increase in glucose transporter concentration was fiber type specific. The purposes of the present study were to determine if muscle insulin resistance in the obese Zucker rat is related to reduced GLUT4 levels, and if improved muscle insulin resistance following exercise training is associated with increased GLUT4 levels.

METHODS

Animal care and housing

Twenty-two female obese (fa/fa) and 6 lean Zucker rats (Animal Model CORE Facility, Obesity Research Center, Vassar College, Poughkeepsie, NY) 4 weeks of age were housed 2-3 per cage and fed normal rat chow (Ralston Purina, St. Louis, MO) and water ad libitum for 2 weeks. Animal room temperature was maintained at 21°C with an artificial 12 hour light/dark cycle. After this familiarization period, rats were individually housed, given powdered chow and familiarized with the treadmill. In week 4, obese rats were randomly assigned to 1 of 3 groups: 1)sedentary control (SED), 2) low intensity trained (LT) and 3) high intensity trained (HT). The lean littermate served as a second control.

Exercise training

Rats assigned to training groups were run on a rodent motor-driven treadmill, up an 8% grade, 5 days/week for 6 to 8 weeks. Treadmill speed and duration were rapidly

increased during the first few weeks to have both groups running, 40 min at 18 meters/min by the end of week 2. Beginning the 3rd week of training, the LT group continued to run at 18 meters/min with the duration gradually increased each week to 120 min/day. The HT group was interval trained, with the maximum intensity reaching 24 meters/min for 18 minutes per exercise bout (5) with a 3 minute rest between each bout. Treadmill speeds of 18 meters/min and 24 meters/min were selected because they represented a work intensity that required 65-70% and 80-85% maximum oxygen consumption (VO_2max) respectively. Whereas exercise at 70% VO_2max was shown to increase oxidative capacity only in red oxidative fibers, exercise at 85% VO_2max was shown to increase oxidative capacity in white glycolytic and red oxidative (Dudley et al., 1982) fibers. Both trained groups performed similar amounts of work each day during the run sessions.

Hindlimb perfusion

At the end of 6 weeks, following a 12 hour fast and 48-56 hours after the last training session, rats were anesthetized with an intraperitoneal injection of

pentobarbital sodium (6.5 mg/100 g body weight). The surgical technique and perfusion apparatus was similar to that used by Ruderman et al. (1971) as modified by Ivy et al. (1989). The surgical procedure limited perfusate flow to the right leg by cannulating the right iliac artery and inferior vena cava to the tip of the femoral artery and iliac vein, respectively. The perfusion medium consisted of Krebs-Henseleit buffer (KHB), pH 7.4 containing 4 grams dialyzed bovine serum albumin and blood bank time-expired human erythrocytes at a concentration of 12 grams hemoglobin/100 ml perfusate. The expired erythrocytes were rejuvenated according to procedures described by Valeri (1974).

Once cannulas were inserted, the hindlimb was washed out with 10 ml of KHB and the rat killed by an intracardiac pentobarbital sodium injection. Immediately after, the cannulas were placed in line with the perfusion system and the hindlimb allowed to stabilize during a 5 minute nonrecirculating washout with perfusate containing 8 mM glucose and 500 μU/ml insulin. The perfusate was gassed constantly with a mixture of 95% oxygen and 5% carbon dioxide. Perfusate flow rate was set at 5 ml/min during the stabilization period, and the subsequent perfusion during which muscle glucose

transport rates were determined. For muscle glucose transport determination, the perfusate contained 2 mM mannitol (60 μCi D-[1-^{14}C] mannitol/mmol), 8 mM 3-O-methyl-D-glucose (32 μCi [^{3}H]3-OMG/mmol) and 0.3 mM pyruvate. Transport was measured in the presence of 500 μU/ml insulin. The duration of perfusion was set at 8 minutes. Immediately after perfusion, the right leg quadriceps were excised, blotted with KHB dampened gauze, and frozen at -80^{o}C until analyzed for 3-OMG glucose transport, citrate synthase activity, and GLUT4 content.

Glucose transport measurement and tissue analysis

The quadriceps were thawed on ice and surgically separated into red and white portions. The portions were further divided into two 150 mg pieces for the three individual tissue analyses. For glucose transport activity one piece was homogenized in 1.0 ml of 10% trichloroacetic acid (TCA) at 4^{o}C and then centrifuged at 13,600 g for 15 min. Two 300 μl aliquots of supernatant were transferred to 10 ml of Scintiverse E for counting. An arterial perfusate aliquot was similarly added to 10% TCA, and 300 μl of the deproteinated acid extract transferred to a vial containing 10 ml Scintiverse, for

determination of specific activity. Samples were counted in a liquid scintillation spectrophotometer (Beckman model LS 350) preset for simultaneous counting of $^{3}H/^{14}C$. Sample quenching was determined by the quenching amount occurring in prepared standards of [^{3}H]3-OMG and D-[1-^{14}C] mannitol (concentrations based on expected sample counts). The intramuscular [^{3}H]3-OMG accumulation, which is indicative of glucose transport into muscle, was calculated by subtracting the concentration of [^{3}H]3-OMG in the extracellular space from the total muscle [^{3}H]3-OMG concentration. Extracellular space was quantified by measuring the [^{14}C] mannitol concentration in the homogenate (Narahara & Ozand, 1963).

Citrate synthase activity and GLUT4 quantitation were determined from the same muscle homogenates. The muscle portions (red and white) were weighed (approximately 150 mg for each) then homogenized on ice at high speed (VirTishear homogenizer) for three 15 second bursts in HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4, 1:20, w/v). For citrate synthase a 300 μl aliquot was further diluted 1:10 in 1 M Tris and 0.4% Triton X-100, pH 8.1, then centrifuged at 13,600 g for 5 minutes. Citrate synthase activity was determined spectrophotometrically by measuring the appearance of the

free sulfhydryl (SH) group from released coenzyme A (CoASH). Activity was expressed as μmol/g wet weight/min (Srere, 1969).

For GLUT4 quantitation muscle homogenates were solubilized 1:1 in 2x Laemmli buffer (125 mM Tris, 20% glycerol, 2% SDS, .008% bromophenol blue, pH 6.8) for 15 minutes (Laemmli, 1970). Proteins were measured according to the method of Bradford (1976). A 75 μg protein aliquot for each sample was then subjected to electrophoresis on a 12.5% polyacrylamide resolving gel. The apparent molecular weight of the GLUT4 was estimated from the mobility of molecular weight standards on the acrylamide gel. Immediately after electrophoresis the proteins were transferred (Western blot) from SDS gels to polyvinylidene difluoride sheets (PVDF) at 150 mA for 11 min using a semi-dry transfer unit (Bio-Rad). The semi-dry transfer buffers were modified from Kyhse-Anderson (1984). After transfer, PVDF sheets were incubated in 5% nonfat dry milk (in Tween Tris buffered saline, pH 7.5) for 1 hour at room temperature. They were then washed and incubated in GLUT-4 rabbit anti-rat antibody buffer (1:500) for 1 hour at room temperature. The GLUT-4 antibody (F349) was donated by Dr. Mike Mueckler, Washington University Medical School. After GLUT-4

antibody incubation, sheets were washed then incubated with ^{125}I labeled goat anti-rabbit antibody (each sheet exposed to 0.3 μCi/ml) for 1 hour (at 37^{o}C). Lastly, sheets were washed, air dried, and prepared for autoradiography. The autoradiographs were then used to identify the respective bands on the PVDF sheets. The bands were cut from the sheets and counted for radioactivity (Beckman Gamma 5500 Counter).

Statistical analysis

A one way analysis of variance (ANOVA) was performed on all variables (citrate synthase activity, glucose transport, GLUT4 content). Fisher's protected least significant difference was used to identify differences between means. Significance was set at $p < 0.05$ for all tests and values were expressed as means ± standard error.

RESULTS

Citrate synthase activity

There was no significant difference between the

citrate synthase activities in the red quadriceps of HT and LT obese rats (Fig 1). However, both HT and LT obese rats had significantly greater red quadriceps citrate synthase activities than SED obese and LN rats. Additionally, the SED obese rats had significantly greater red quadriceps citrate synthase activity than LN rats. Red quadriceps citrate synthase activities for HT and LT rats were almost twice those of LN rats. The SED obese rats had approximately 1.3 times greater red quadriceps citrate synthase activity than LN rats. There were no significant differences among groups (HT, LT, SED, LN) for white quadriceps citrate synthase activity (Fig 1). The red quadriceps/white quadriceps (RQ/WQ) ratio for citrate synthase activity was approximately 5.3 for HT and LT, 4.4 for SED, and 3.8 for LN rats (Fig 1).

Glucose transport

There were no significant differences in 3-O-methyl glucose (3-OMG) transport rates among HT and LT obese rats, and LN rats in the red quadriceps. However, transport rates in red quadriceps of HT and LT obese rats, and LN rats were significantly greater (approximately 1.7 times) than SED obese rats (Fig 2).

There was also a significant correlation between red quadriceps 3-OMG transport rate and red quadriceps GLUT4 content in the obese Zucker rats (r=0.63) (Fig 3). There were no significant differences among groups for white quadriceps 3-OMG transport rates (Fig 2).

GLUT4 Content

GLUT4 concentrations for red and white muscles were expressed as percentages of a rat heart standard. There was no significant difference in GLUT4 content in the red quadriceps between HT and LT rats. There was also no significant difference between SED and LN rats. However, HT and LT rats had significantly greater (approximately 1.4 times) red quadriceps GLUT4 content than SED and LN rats (Fig 4). GLUT4 content in the red quadriceps of obese rats significantly correlated with red quadriceps citrate synthase activity (r=0.73) (Fig 5). There were no significant differences in GLUT4 content among groups for the white quadriceps (Fig 3). The RQ/WQ ratio for GLUT4 was approximately 3.0 for HT, LT, and SED rats. For LN rats the ratio was approximately 2.2 (Fig 4).

DISCUSSION

High and low intensity exercise training increased citrate synthase activity in the red quadriceps (RQ) of the obese Zucker rat. Associated with this increase was an increase in RQ submaximal insulin-stimulated 3-OMG transport and GLUT4 concentration. However, there were no significant differences in the white quadriceps (WQ) citrate synthase activities among HT, LT, and SED rats. In addition, no increase in 3-OMG transport rate or GLUT4 level was observed for the white quadriceps after training.

Exercise training has been demonstrated to increase citrate synthase activity in the muscle fibers recruited for the particular exercise (Dudley et al., 1982; Torgan et al., 1989). Only the red quadriceps displayed this training adaptation. There were no significant differences in RQ citrate synthase activity between HT and LT rats. However, HT and LT rats had significantly greater RQ citrate synthase activity than SED and LN rats. There were also no significant differences in muscle protein content (milligrams of protein per gram of muscle) among HT, LT, SED and LN rats. This suggests the improvements seen with training are not due to increased

muscle mass. Since citrate synthase activity, 3-OMG transport and GLUT4 levels all increased with exercise training, it suggests recruitment is required for these changes to occur. Obligatory recruitment is supported by our finding that increased GLUT4 levels correlated well with increased citrate synthase activity.

Willems et al. (1991) reported submaximal insulin-stimulated glucose uptake was greater in the perfused hindlimb muscles of HT than LT rats. HT and LT rats had significantly greater glucose uptakes than SED rats as well. However, glucose transport was not examined. In agreement with our results, Cortez et al. (in press) found HT and LT rats had significantly greater red gastrocnemius 3-OMG transport than SED rats. In contrast, they found HT rats had greater white gastrocnemius 3-OMG transport than LT and SED rats, whereas we found no improvement in WQ 3-OMG transport in HT rats. The differences between our findings and Cortez et al. (in press) may be due to the difference in muscles investigated.

There were no significant differences in 3-OMG transport, GLUT4 level, or citrate synthase activity for the white quadriceps among HT, LT, SED, and LN rats. This suggests high intensity training was insufficient to

recruit the white quadriceps. Our results agree with the findings of Willems et al. (1991) who reported no difference in citrate synthase activity for the red and white quadriceps between HT and LT rats. Our results also partly agree with Cortez et al. (in press) who found no significant difference in citrate synthase activity for the red and white gastrocnemius between HT and LT rats. Both studies suggest the red quadriceps are maximally recruited with low intensity training. In contrast, Dudley et al. (1982) reported high intensity exercise training increased WQ citrate synthase activity. Again in contrast, Cortez et al. (in press) found HT and LT rats had significantly greater white gastrocnemius citrate synthase activity than SED and LN rats. These differences may be due to the species of rat used (Dudley et al., 1982) and the muscle examined (Cortez et al., in press).

We observed a significant correlation between 3-OMG transport rate and GLUT4 content. Although Friedman et al. (1990) measured muscle GLUT4 concentration in the obese Zucker rat, they did not relate this to insulin-stimulated glucose transport. Henriksen et al. (1990) examined the relationships among muscle fiber-type, GLUT4 content and 2-deoxyglucose transport rate stimulated

maximally with insulin and/or contraction in lean Wistar rats. They reported significant correlations between GLUT4 levels and 2-deoxyglucose transport stimulated either by contraction alone or insulin in combination with contraction. However, these correlations were made using mean values combined from different muscle groups (epitrochlearis, soleus, extensor digitorum longus, flexor digitorum brevis). Despite the differences in methods, our results agree with Henriksen et al. (1990) in that glucose transport highly correlates with muscle GLUT4 concentration.

Skeletal muscle insulin resistance in the obese rat is associated with a defect in the glucose transport process (Ivy et al., 1986; Sherman et al., 1988; Ivy et al., 1989; Willems, et al., 1991; Cortez et al., in press). In muscle homogenates, we found LN rats had the same GLUT4 levels as SED rats but LN rats had significantly greater 3-OMG transport. Likewise, Friedman et al. (1990) reported no significant difference in gastrocnemius GLUT4 levels between lean and obese Zucker rats. They also found exercise training (low intensity) for 18 and 30 weeks increased GLUT4 levels by 1.7 and 2.3 fold, respectively, above SED rats. These results suggest the exercise training induced improvement in

insulin-stimulated, 3-OMG transport in the obese Zucker rat is partly due to an increase in muscle GLUT4 concentration. However, the findings that muscle GLUT4 concentrations of LN and SED rats were the same indicates muscle insulin resistance in the obese Zucker rat is not due to a reduced GLUT4 concentration.

Horton et al. (1990) investigated the effects of basal and maximal insulin treatment to recruit transporters to the plasma membrane and/or increase transporter intrinsic activity in the obese Zucker rat. For lean rats, maximal insulin stimulation increased glucose transport V_{max}. This reflected an increase in active transporters and turnover number. For obese rats, maximal insulin treatment also increased V_{max}. However, it was proportional to increased turnover number with no significant change in transporter number. Based on our results and those of Horton et al. (1990), muscle insulin resistance in the obese Zucker rat is most likely due to a reduced GLUT4 translocation.

In summary, we have demonstrated high and low intensity exercise training increase GLUT4 levels and submaximal insulin stimulated glucose transport activity in skeletal muscle of the obese Zucker rat. These adaptations were also associated with an increase in

citrate synthase activity. The positive correlations between citrate synthase activity and these adaptations suggest they are stimulated by muscle fiber recruitment. The ratio of glucose transport rate to GLUT4 content was higher in lean than obese rats. Furthermore, there was no significant difference in muscle GLUT4 levels of sedentary lean and obese rats. This suggests insulin-stimulated GLUT4 translocation is defective in the obese Zucker rat. The improvement in glucose transport seen with exercise training is at least partly due to an increase in the active GLUT4 level.

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FIGURE LEGEND

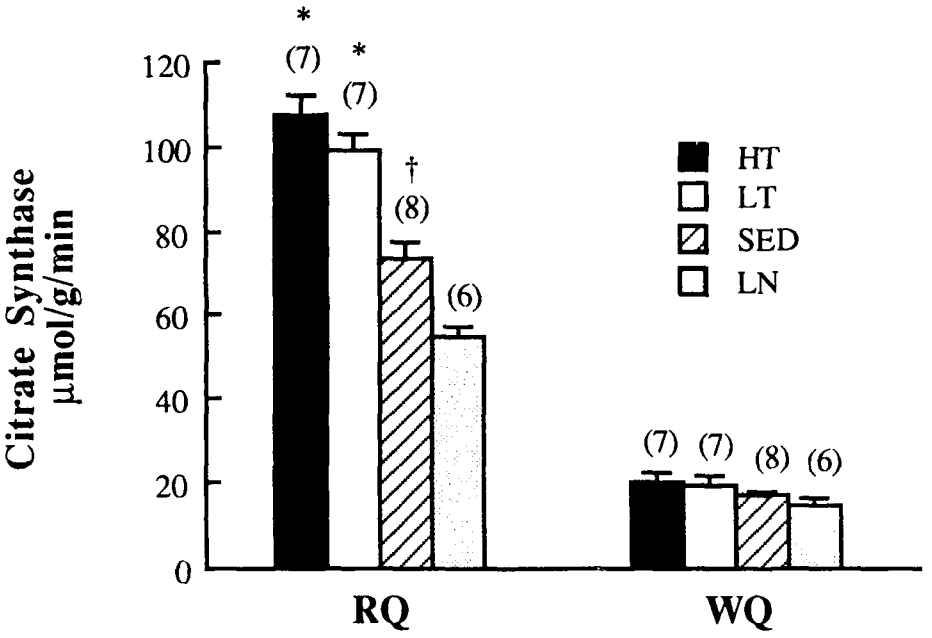
Figure 1. Citrate synthase activity in the red (RQ) and white (WQ) quadriceps for high intensity trained (HT), low intensity trained (LT), sedentary obese control (SED), and lean sedentary control (LN) rats. Bars represent means ± SE in μmol/g/min. Numbers in parentheses represent numbers of observations. * $p < 0.05$ from SED and LN; † $p < 0.05$ from LN.

Figure 2. 3-O-methylglucose transport in the red (RQ) and white (WQ) quadriceps for HT, LT, SED, and LN rats. Bars represent means ± SE in μmol/g/hr. Numbers in parentheses represent numbers of observations. * $p < 0.05$ from SED.

Figure 3. Correlation between RQ 3-O-methyl-D-glucose transport and RQ %GLUT4 in the obese Zucker rat.

Figure 4. RQ and WQ GLUT4 content expressed as a percentage of a rat heart standard for HT, LT, SED, and LN rats. Bars represent means ± SE in percents. Numbers in parentheses represent numbers of observations. * $p < 0.05$ from SED and LN.

Figure 5. Correlation between RQ citrate synthase activity and RQ %GLUT4 in the obese Zucker rat.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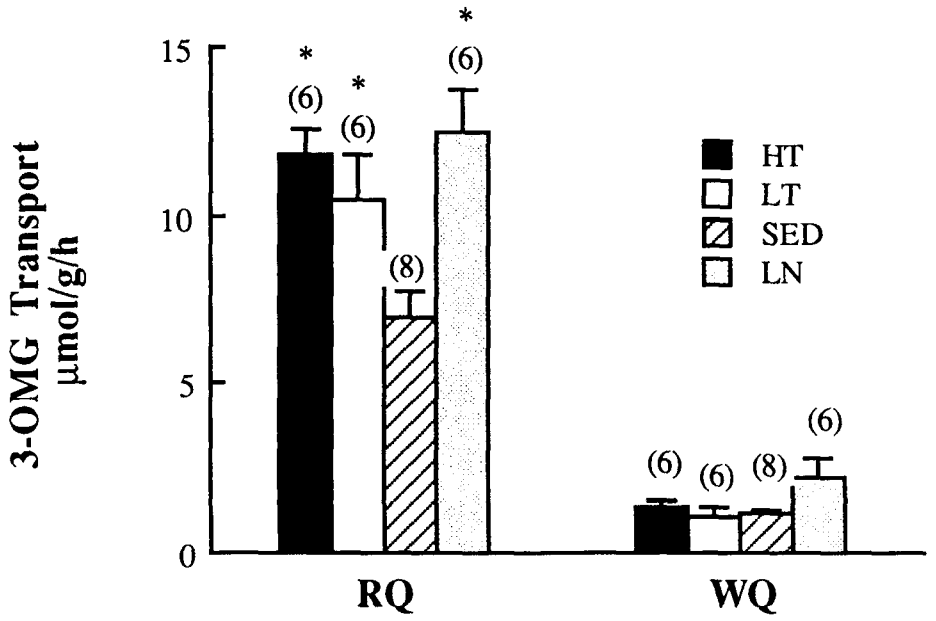
3-OMG Transport
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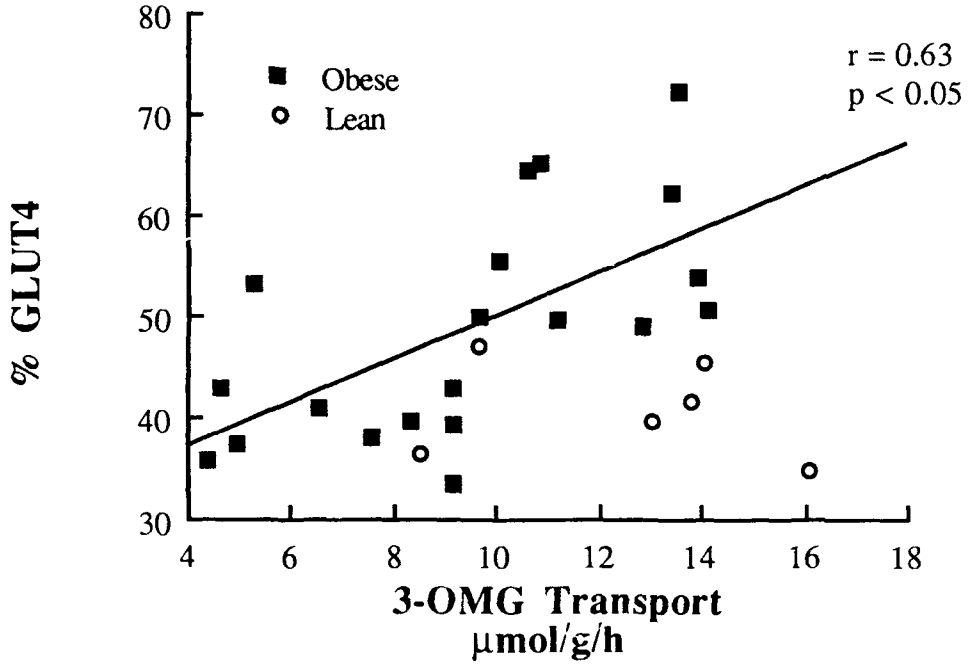
80
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% GLUT4
Obese
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r = 0.63
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3-OMG Transport
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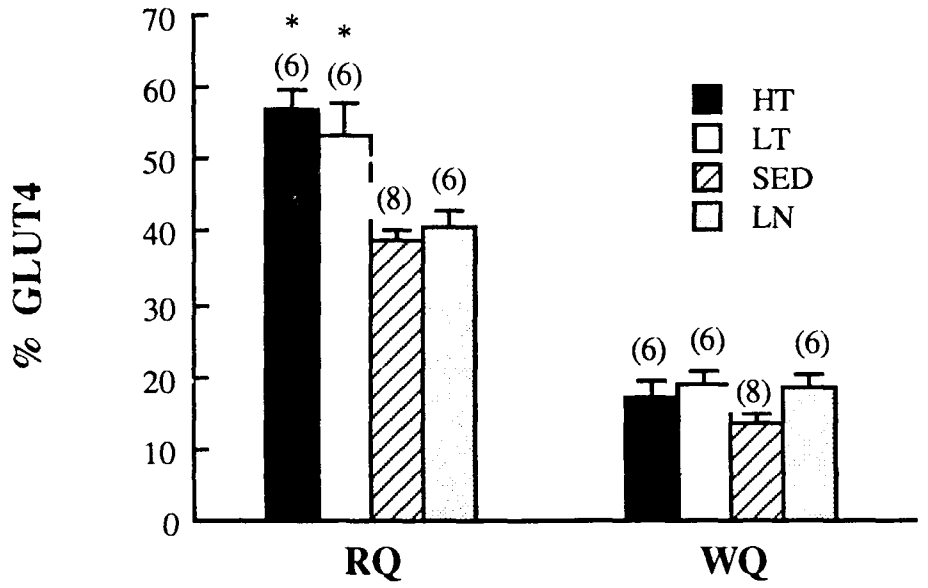
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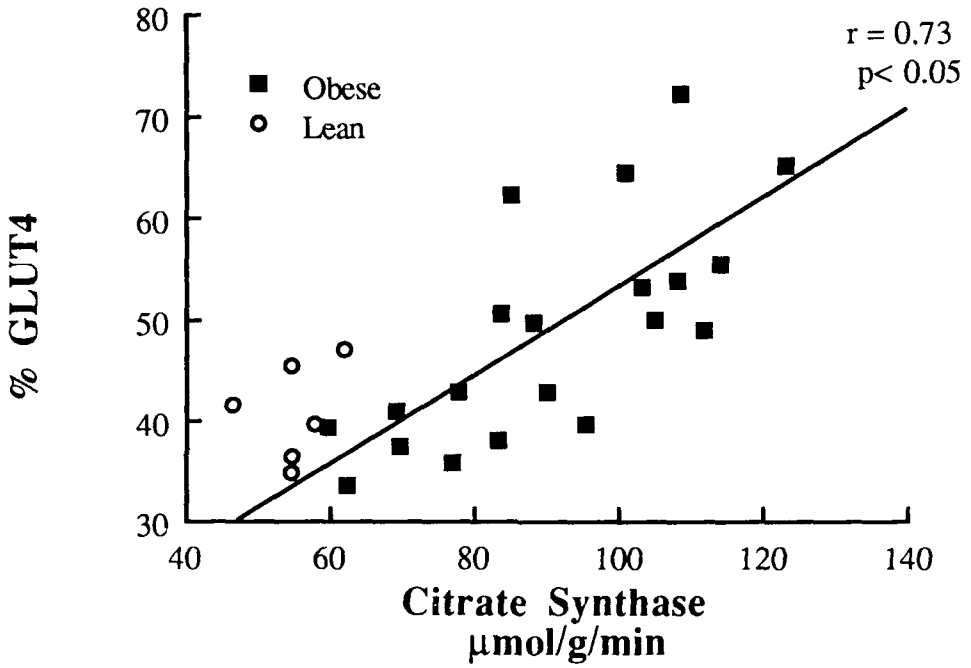
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% GLUT4
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r = 0.73
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Citrate Synthase
µmol/g/min

LITERATURE REVIEW

Introduction

The genetically obese Zucker rat (*fa/fa*) is a well documented animal model for skeletal muscle insulin resistance (Bray, 1977). The locus for this resistance has been associated with a glucose transport defect occurring in all of the basic skeletal muscle fiber types. The muscles of the obese Zucker rat were also demonstrated to be less insulin sensitive and less insulin responsive than its lean littermate. Exercise training has been demonstrated to improve insulin responsiveness and increase insulin regulatable glucose transporter (GLUT4) levels in the obese Zucker rat. The relative amount of GLUT4 protein has been associated with insulin responsiveness and its expression is believed to be defective in the obese Zucker rat.

This literature review will initially focus on splanchnic versus peripheral glucose disposal, and cellular glucose transport kinetics. It will then examine the synergistic interactions of insulin and exercise in muscle, and the relationship between exercise training and skeletal muscle insulin resistance in the obese Zucker rat. Next, it will address the identification, characterization, topology,

and function of the different glucose transporter isoforms. Lastly, the effects of insulin and exercise on transporter redistribution, GLUT4 isoform expression, and insulin responsiveness will be reviewed.

Splanchnic and peripheral glucose disposal

Skeletal muscle is quantitatively the most important tissue in glucose disposal. Several studies on various animal models first suggested the splanchnic region was the major glucose disposal area. Although some studies contradicted the splanchnic disposal theory, they all relied on indirect investigative techniques which made their findings inconclusive. Felig and Wahren (1975) gave the first direct evidence supporting splanchnic glucose disposal in man. They used the hepatic venous catheter technique to demonstrate the contribution of splanchnic tissues in clearing an oral glucose load. From their results, they concluded 60% of the oral glucose load was retained by the liver. Katz et al. (1983) also used hepatic, along with femoral, venous catheterization and leg blood flow measurements to follow oral glucose disposal in man. However, they demonstrated in man over 67% of an oral glucose load bypassed the

splanchnic region. Furthermore, Katz et al. concluded the peripheral tissues (primarily muscle) quantitatively played the dominant role in oral glucose disposal.

In a related study, Ferrannini et al. (1985) used hepatic vein catheterization and double isotope tracers to follow oral glucose disposal in man. Specifically, they investigated the main determinants of oral glucose tolerance: rate of oral glucose appearance, splanchnic and peripheral glucose uptake, and suppression of hepatic glucose production. Ferrannini et al. found after an oral glucose load in healthy subjects endogenous glucose production was suppressed about 50%, while splanchnic and peripheral glucose uptake were stimulated. The increase in splanchnic uptake was achieved mainly through augmented glucose availability rather than increased splanchnic glucose extraction. Peripheral (primarily muscle) glucose uptake accounted for the majority of glucose disposal. They also estimated that in the post-absorptive state, approximately 25% of glucose use took place in insulin-dependent peripheral tissues (75 to 85% occured in the brain and other obligatory tissues). Ferrannini et al. (1985) confirmed the findings of Katz et al. (1983) by illustrating the importance of skeletal muscle in oral glucose disposal in man. DeFronzo et al.

(1985) demonstrated the importance of skeletal muscle in infused glucose disposal as well. They used hepatic and femoral vein catheters to follow infused tritiated glucose disposal in non-insulin dependent (type II) diabetic patients. Their results verified peripheral tissue was the major site for infused glucose disposal.

Peripheral glucose uptake in the rat hindlimb is more responsive to insulin activation following exercise training (Berger et al., 1979; Mondon et al., 1980; Crettaz et al., 1983). Physical activity level is also associated with increased insulin-mediated peripheral glucose uptake in humans of different ages and fitness (Bjorntop et al., 1972; Le Blanc et al., 1979; Seals et al., 1984). In addition, an increase in physical activity was found to increase insulin-stimulated glucose clearance in humans with pathological conditions like obesity (Bjorntop et al., 1977) and diabetes (Wallberg-Henriksson et al., 1982; Trovati et al., 1984). DeFronzo et al. (1985) suggested skeletal muscle was the most important site in non-insulin dependent diabetes. Skeletal muscle is also the most logical site for the increased peripheral insulin action associated with physical training (Horton, 1986). In summary, muscle appears to be the major site for insulin-stimulated

glucose clearance.

Cellular glucose transport kinetics

Skeletal muscle glucose transport is a passive process of facilitative diffusion. Glucose is transported from the extracellular environment into the cell by plasma membrane localized carrier proteins. Evidence for these carrier proteins was provided by several experiments which revealed glucose transport exhibited saturation kinetics, stereospecificity, competitive inhibition and counter transport (Regan & Morgan, 1964; Morgan et al., 1964; Park et al., 1968; Morgan & Whitfield, 1973; Klip, 1982). There is a hyperbolic relationship between glucose transport rate and extracellular glucose concentration (Morgan et al., 1961). This means the transporting mechanism is saturable and has a finite number of glucose-binding sites. Narahara & Ozand (1963) approximated the Michaelis constant (K_m) for glucose transport from the outer to inner membrane surface to be 5-10 mM in skeletal muscle and several other tissues. Therefore, transport in these tissues under physiological conditions follows nearly first order kinetics.

Since transport in muscle is not at equilibrium, it is

consequently under regulatory control (Newsholme & Leech, 1983). Insulin and contraction are the major physiological regulators for muscle glucose transport. They both increase transport maximal velocity (V_{max}) without significantly changing the K_m (Holloszy & Narahara, 1965; Narahara & Ozand, 1963). Changes in the carrier proteins (glucose transporters) could account for the increased transport. However, kinetic studies cannot distinguish between increased transport due to increased carrier proteins and increased transport due to increased intrinsic activity of the carrier proteins. Sternlicht et al. (1988) determined the glucose transport kinetics for skeletal muscle sarcolemma and quantitated the number of cytochalasin B binding sites in the plasma membrane. Their results verified insulin increased glucose transport by changing transport V_{max} without altering the K_m. Sternlicht found the increased V_{max} resulted from a combination of increased total transporters and increased transporter turnover.

Synergistic interactions of insulin and exercise in muscle

Several studies have examined the synergistic effects of insulin and exercise on rat skeletal muscle glucose transport in vitro. In the studies described next, the non-metabolizable glucose analog, 3-O-methyl-D-glucose, was used to follow transport. An additive effect of exercise combined with a maximally stimulating concentration of insulin was observed in some of these investigations (Garetto et al., 1984; Wallberg-Henriksson et al., 1986; Zorzano et al., 1986). Glucose transport can also be increased by in vitro electrical stimulation of muscle to contract (Nesher et al., 1985; Wallberg-Henriksson et al., 1985). Constable et al. (1988) concluded the effect of exercise on muscle glucose transport was mediated specifically through a process initiated by contraction. They also proposed unphysiologically high concentrations of insulin induce cell membrane changes prohibiting the contraction/insulin additive effect.

It has been hypothesized contraction and insulin stimulate transport synergistically and through different mechanisms involving a common intermediate. Therefore,

to understand the mechanisms controlling muscle glucose transport, it is important to differentiate the effects of insulin from those of muscle contraction. Insulin sensitivity is defined by the percent of maximal biological response elicited by a submaximal concentration of insulin. The higher the percent maximal response at a given submaximal concentration the greater the insulin sensitivity. Alternately, insulin responsiveness is defined by the biological response achieved with a maximally stimulating insulin concentration. The greater the biological response the better the responsiveness (Kahn, 1978) (see Fig 1). In skeletal muscle, a maximal insulin response is achieved with only 20-30% of the insulin receptor sites occupied (Herberg et al., 1977; LeMarchand-Brustel et al., 1978). However, greater than 35 to 40% reductions in receptor populations have not been observed under physiological conditions. Insulin resistance can therefore be viewed in terms of decreased insulin sensitivity and/or responsiveness to distinguish pre- and post-insulin receptor defects.

Studies by Soman et al. (1979) and James et al. (1984) were the first to demonstrate exercise training in man and the rat improved insulin action in vivo.

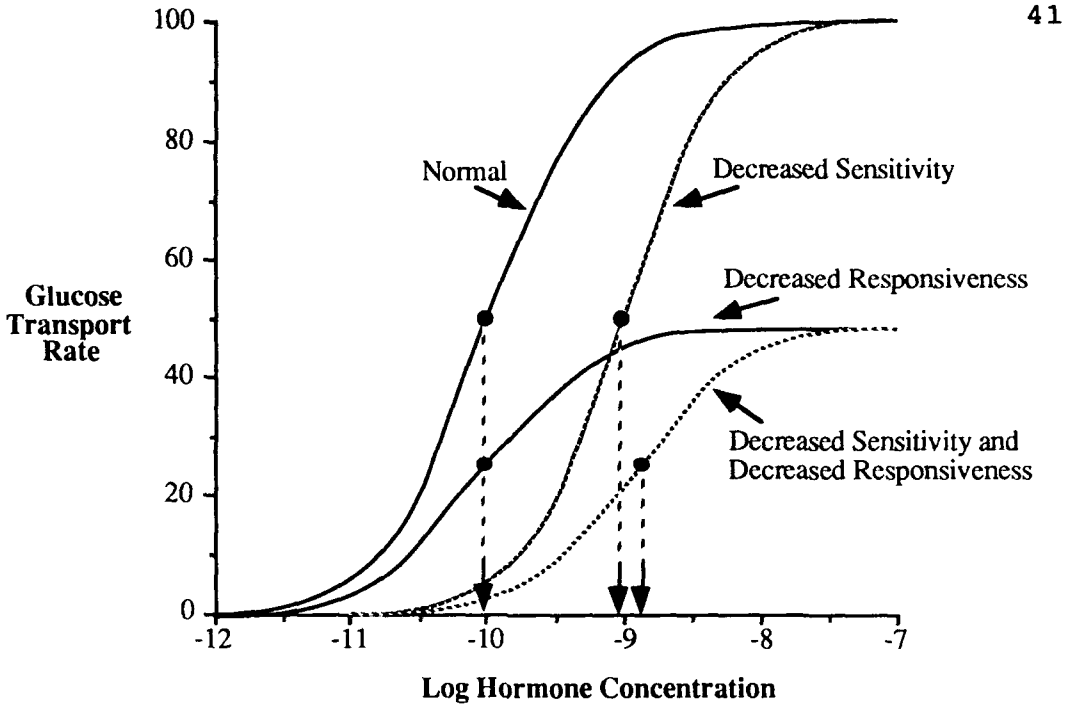

Fig. 1. Insulin sensitivity vs. insulin responsiveness. Broken arrows indicate insulin concentration that produces a half-maximal response. Modified from Kahn 1978.

In a follow up study, James et al. (1985) using the euglycemic clamp, investigated how exercise training affected in vivo insulin action in individual rat tissues. They observed the major effects of training involved improved insulin action of muscle. Since muscle was the main site for insulin stimulated glucose disposal, James et al. (1985) concluded skeletal muscle was also primarily responsible for the increased whole body insulin action observed in the rat following exercise training. The enhanced insulin action of the muscle resulted from increased insulin sensitivity and responsiveness, and was also fiber-type specific. However, James et al. (1985) did not consider possible systemic factors that might have altered muscle insulin action in vivo. Evidence of a possible systemic stimulus was the observation that enhanced insulin sensitivity occurred in both oxidative (soleus and red gastrocnemius) and glycolytic muscle (white gastrocnemius). In vitro studies have suggested the training intensity used by James et al. (1985) was insufficient to recruit the fast-twitch glycolytic fibers. Therefore, they should not have demonstrated an improved insulin response.

Skeletal muscle insulin resistance and exercise training in the obese Zucker rat

The obese Zucker rat is genetically predisposed to develop chronic insulin resistance, hyperglycemia, hyperinsulinemia and impaired glucose tolerance. Ivy et al. (1986) investigated the effects of exercise training plus a high carbohydrate diet on skeletal muscle insulin resistance in the obese Zucker rat. They concluded the two treatments acted in combination to decrease muscle insulin resistance. Ivy et al. (1986) further hypothesized the decreased resistance was related to an increase in the number of active glucose transporters. A study by Sherman et al. (1988) investigated whether muscle insulin resistance in the obese Zucker rat was caused by a defective glucose transport process and if the resistance was fiber-type specific. In the rat, skeletal muscle is classified into three basic fiber-types: slow-twitch red, which has intermediate oxidative and low glycolytic capacity; fast-twitch red, which has high oxidative and high glycolytic capacity; and fast-twitch white, which has low oxidative and high glycolytic capacity (Baldwin et al., 1973; Barnard et al., 1971). Using three different insulin concentrations

(physiological, submaximal, maximal) and in the absence of insulin, Sherman et al. (1988) observed glucose transport was significantly lower in all fiber-types of obese rats as compared to lean rats. They therefore deduced muscle insulin resistance in the obese Zucker rat resulted from a defective glucose transport process common to all fiber-types. Since exercise training was demonstrated to improve muscle insulin resistance, Ivy et al. (1989) examined the effects of training on muscle glucose transport in the obese Zucker rat. The rate of 3-O-methyl-D-glucose transport was examined using three different insulin concentrations (like Sherman et al., 1988) and under basal conditions (without insulin) in the three basic skeletal muscle fiber types. The transport rate in the red gastrocnemius (fast-twitch oxidative glycolytic) was significantly higher in the trained when compared to the sedentary obese rat, without insulin and with the different insulin concentrations. The plantaris (mixed fibers) of the trained obese rat also showed improvement in the absence of insulin as well as with physiological and maximal insulin concentrations. Training had no effect on the glucose transport rate of the soleus (slow-twitch oxidative) or white gastrocnemius (fast-twitch glycolytic). These results suggested

improved resistance coincided with improved glucose transport, improvement was fiber-type specific, and muscle recruitment was necessary to see these effects. Furthermore, the enhanced transport resulted from increased insulin responsiveness and not increased insulin sensitivity. Willems et al. (1990) took this hypothesis a step further by examining if the magnitude of the training response was exercise intensity specific. They compared glucose uptake between low and high intensity exercise trained obese Zucker rats, which performed an equal amount of work during training. It was observed, rats undergoing the high intensity protocol had greater improvements in their muscle insulin resistance than rats undergoing the low intensity protocol. The results suggested enhanced muscle insulin resistance in the Zucker was exercise intensity dependent.

In a similar study, the effects of high and low intensity exercise training on hindlimb glucose transport in the obese Zucker rat were examined (Cortez et al., in press). The rates of muscle glucose uptake were significantly higher in both groups of trained rats when compared to the rates of the sedentary controls. Additionally, glucose uptake rates of the high intensity

group were significantly greater than those of the low intensity group. The 3-O-methyl-D-glucose transport rates were greater in the fast-twitch red fibers of the high and low intensity groups when compared to those of sedentary control group. However, the high intensity training significantly enhanced transport in the fast-twitch white fibers, whereas low intensity training did not. The results suggested muscle insulin resistance improvement was fiber-type and exercise intensity dependent and muscle recruitment was necessary for this improvement. Since exercise training improves muscle insulin resistance and glucose transport it most likely affects the glucose transport regulatory proteins as well.

Identification of glucose transport proteins

The existence of an insulin-regulatable glucose transporter (IRGT) was demonstrated simultaneously by Cushman & Wardzala and Suzuki & Kono in 1980. Their studies on rat adipocytes showed insulin caused translocation of glucose transporters from an intracellular compartment to the plasma membrane. Wardzala & Jenrenaud (1981, 1983) later proved insulin

caused the same kind of transporter translocation in rat diaphragm muscle (see Fig 2). These studies used cytochalasin B binding to determine glucose transport in various cellular preparations. Cytochalasin B binding cannot distinguish between different glucose transport proteins but immunochemical techniques can. Evidence for a specific IRGT was suggested by the weak cross-reactivity between antibodies specific for the HepG2 or rat brain transporter and the rat adipocyte transporter (Wang, 1987; Oka et al., 1988).

Direct evidence was obtained when James et al. (1988) developed a monoclonal antibody specific for the rat adipocyte transporter. It immunolabeled a protein (relative molecular mass of 43K) identified only in tissues demonstrating insulin-dependent glucose transport. In a follow up study on rat adipocytes, the monoclonal was used to immunoprecipitate a 43K protein that had been photoaffinity labeled with cytochalasin B. The monoclonal was also used to immunolabel a plasma membrane protein whose concentration increased fivefold after cellular insulin treatment (James, Strube & Mueckler, 1989).

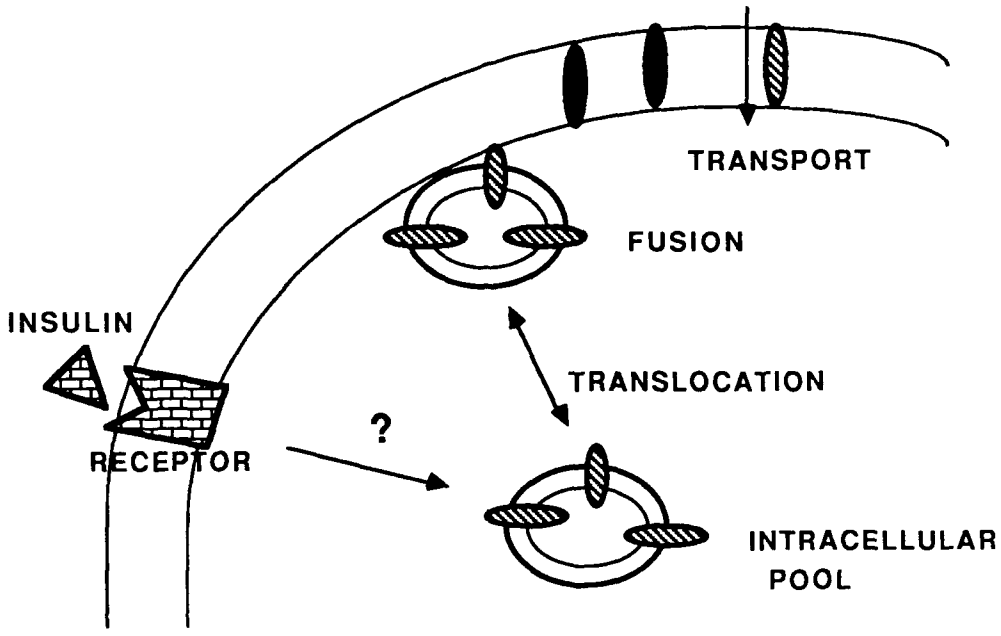

Fig. 2. Insulin induced glucose transporter translocation

Collectively, the immunochemical results suggested the HegG2 transporter most likely existed in low levels, if it was expressed at all, in insulin regulatable tissues.

Glucose transporter characterization

Glucose transport occurs by facilitated diffusion controlled by specific transport proteins in the plasma membrane. There are two types of glucose transporters, sodium dependent and sodium independent. Five different sodium independent, facilitative mammalian glucose transporter (GLUT) isoforms have been identified (see Table 1). GLUT1 was originally identified from human erythrocytes. It has also been identified in HepG2 (human hepatoma) cells and rat brain (Jung, 1975; Mueckler et al., 1985; Birnbaum et al., 1986). The GLUT2 isoform is expressed in the liver, β-cell, kidney and small intestine. The GLUT3 isoform is found in brain and along with GLUT1, may be responsible for basal (constitutive) glucose transport. Several investigators have confirmed the existence of GLUT4 (IRGT) expressed only in insulin sensitive tissues. Lastly, GLUT5 is the predominant isoform in the small intestine (Bell et al., 1990).

TABLE 1.

Glucose transporter isoforms and distribution

Designation	Number of amino acid residues	Distribution
SGLT1 (Na^+-glucose cotransporter)	664	Small intestine
GLUT1 (erythrocyte)	492	Placenta, brain, kidney, and colon
GLUT2 (liver)	524	Liver, β-cell, kidney, and small intestine
GLUT3 (brain)	496	Many tissues, including brain, placenta, and kidney
GLUT4 (muscle/fat)	509	Skeletal muscle, heart, and fat
GLUT5 (small intestine)	501	Small intestine (jejunum)

Overall, glucose transporters are widely dispersed and present on the surface of nearly all mammalian cells.

Since five different functional isoforms (and their cDNA clones) have been characterized, facilitative glucose transport is a property of a structurally related protein family. The isoforms range in size from 492 to 524 amino acid residues. In human isoforms, there is 39-65% identity and 50-76% similarity between amino acid sequences. There is also a significant degree of sequence conservation between predicted amino acid sequences of human GLUT1 and GLUT4 with corresponding rat isoforms. This suggests many regions of GLUT1 and GLUT4 are functionally important for either glucose transport or for regulation of transport activity. In contrast, only 81.5% identity exists between human and rat GLUT2 sequences, which is the limit of sequence divergence when comparing related human and rat proteins (Bell et al., 1990).

Transporter topology and function

Mueckler et al. (1985) proposed a model for the two-dimensional orientation of the GLUT1 isoform in plasma membrane. They based this model on hydropathic and

secondary structure predictions. Similar analyses of other isoforms suggest they may have like topological arrangement. The model predicts the protein spans or traverses the plasma membrane twelve times (designated segments M1 to M12). The amino and carboxy termini are on the cytoplasmic side and may vary in length and sequence, depending on the isoform. An exofacial loop of 33 (GLUT's 1,3,5), 37 (GLUT4), or 67 (GLUT2) amino acid residues connect segments M1 and M2. These connecting loops contain a putative site for asparagine-linked glycosylation in all isoforms. This site is glycosylated in GLUT1 and may be similarly modified in the other isoforms (Mueckler et al., 1986). Peptide specific antisera and proteolytic digestion have been used to study the orientation of GLUT1 in the plasma membrane. These studies have demonstrated the region spanning segments M6 and M7 (largely hydrophilic) and the carboxy-terminal are on the cytoplasmic side of the plasma membrane (Mueckler et al., 1985; Davies et al., 1987; Haspel et al., 1988). Lastly, research has indicated the binding site for cytochalasin B is near or in segment M9 (Cairns et al., 1987; Holman et al., 1987) (see Fig 3).

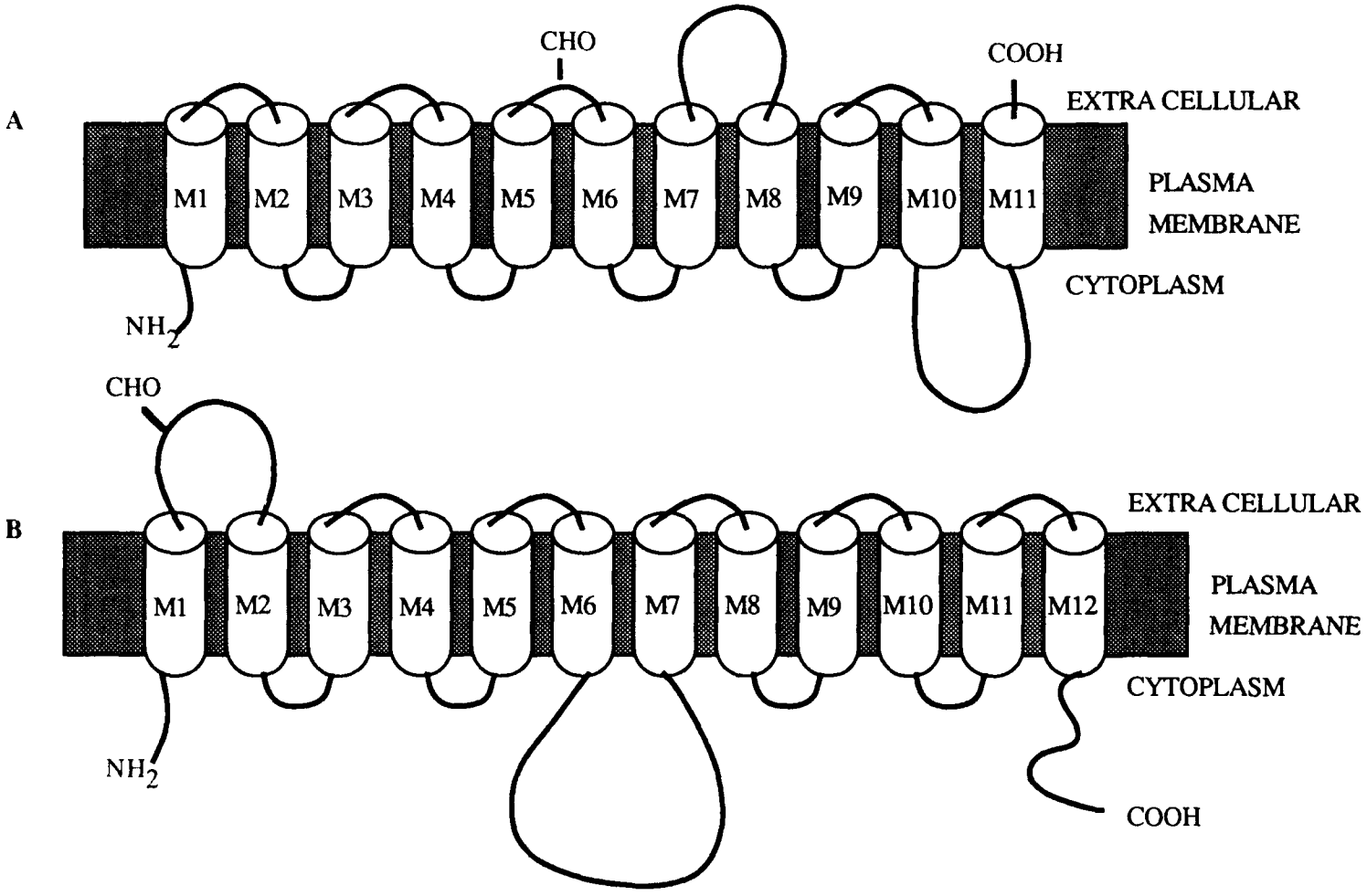

Fig. 3. Glucose Transporter Topology. Model for sodium-dependent glucose-cotransporter (A) and facilitative glucose-transporter (B). Modified from Bell et al. 1990.

Amino acid sequence comparison of the five human isoforms suggest the short loop sequences that connect the transmembrane regions are the most highly conserved regions of these proteins. The conserved regions most likely have a common function in each isoform. Several of the conserved amino acids probably form part of a channel or pore through which glucose moves. Although the residues involved have not been determined, the conserved polar amino acids in the putative transmembrane segments, specifically M7, are likely choices (Bell et al., 1990). Glucose movement could be mediated through the relatively hydrophobic protein core by interaction of the hydroxyl and amide side chains of the conserved asparagine, glutamine, serine and threonine residues. Less well conserved regions, like the amino and carboxy ends, and the intracellular M6 to M7 loop, may contribute to isoform-specific characteristics. These characteristics may include kinetic constants, hormone sensitivity, and intracellular handling (Kasanicki et al., 1990).

Effects of insulin on transporter redistribution

Several investigators have confirmed the hypothesis insulin induces a translocation of glucose transporters from an intracellular compartment to the plasma membrane in adipocytes. Klip et al. (1987) were the first to confirm the results of Wardzala & Jeanrenaud (1980) and demonstrate translocation in skeletal muscle in vitro. Klip et al. (1987) examined glucose transport and transporter translocation in rat hindlimb skeletal muscle exposed to insulin during hindlimb perfusion. Transporters were quantitated on isolated plasma and intracellular membrane fractions with cytochalasin B.

Kern et al. (1989) examined the relationship between insulin stimulated glucose transport, transporter number and transporter redistribution in rat skeletal muscle. At all insulin concentrations used, red muscle displayed a 2.5-fold higher 3-O-methyl-D-glucose transport rate than white muscle. Red muscle contained 1.9 times as many cytochalasin B binding sites in the plasma membrane as compared to white muscle. In the intracellular membrane, red contained 2.4 times as many cytochalasin B binding sites as compared to white muscle. The glucose transporter intracellular/extracellular membrane ratio

was 4.1 for red but only 2.5 for white muscle. Kern et al. (1989) noted the maximal glucose transport rate in red and white muscle correlated well with total glucose transporter number. Also, the greater insulin responsiveness correlated well with the greater proportion of glucose transporters in the intracellular membrane. Since glucose transport was less insulin responsive in white than red muscle fibers, these results suggested the number and membrane content of glucose transporters may play a role in insulin resistance (discussed later).

Effects of acute exercise on transporter redistribution

As discussed earlier, skeletal muscle responds in an exercise-dependent manner and one of these responses is a change in plasma membrane tranporter number. Few investigators have examined the effects of long term exercise training on transporter redistribution. Most studies have focused on the effects of acute (single or short bout) exercise. In acute exercise investigations, the data (glucose transport, transporter number etc..) is collected within 24 hours of an exercise bout. This is an essential distinction because acute effects are seen

up to 24 hours (but not beyond 48 hours) following exercise cessation. Since there are no published long term studies on transporter redistribution, the acute exercise effects are reviewed for comparison.

Hirshman et al. (1988) reported an acute exercise bout increased plasma membrane transporter number two-fold. However, this could not explain the four- to five-fold increase observed in glucose transport. Hirshman et al. (1988) did not examine where the transporters originated. They hypothesized exercise may have resulted in transporter redistribution as well as a conformational change that increased transporter intrinsic activity. Fushiki et al. (1989) examined the origin of acute exercise induced transporters in the plasma membrane. They followed glucose transporter redistribution in plasma and intracellular membrane fractions taken from the hindlimb muscles of untrained rats given one exercise bout. Like insulin, acute exercise appeared to increase transport V_{max} without significantly changing transport K_m. Fushiki et al. (1989) also found part, but not all, of the observed increase in glucose transport during contraction resulted from an increase in plasma membrane glucose transporters. This suggested glucose transporter intrinsic activity may also have been involved in the

increased glucose transport seen in exercising muscle.

The results of a study by Douen et al. (1990) showed exercise, insulin treatment, and exercise followed by insulin treatment, all increased the GLUT4 isoform level in the plasma membrane. The increase was also concomitant with a decrease in intracellular GLUT4. However, when compared to exercise alone, exercise followed by insulin did not decrease the GLUT4 isoform level in the intracellular membrane fraction. This suggested exercise possibly modulated insulin-induced transporter redistribution in skeletal muscle. Again when compared to exercise or insulin alone, enhanced glucose uptake due to exercise followed by insulin treatment, was not accompanied by an increase in plasma membrane transporters. These results suggested insulin increases transporter intrinsic activity which is magnified by acute exercise. It has also been postulated insulin and exercise recruit glucose transporters from different intracellular compartments. Douen et al. (1989) reported insulin and exercise increased skeletal muscle plasma membrane transporter number in the normal rat. However, only insulin decreased intracellular transporter number at the same time. The inability of exercise to access the insulin-sensitive intracellular

pool suggested there was a second exercise-sensitive pool. An alternate explanation was contractile activity somehow exposed and activated hidden transporters.

GLUT4 isoform expression and insulin responsiveness

More evidence supporting the existence of a separate exercise sensitive pool of transporters was provided in a subsequent study by Douen et al. (1990). Their results confirmed GLUT4 plasma membrane levels increased in response to acute exercise and insulin treatment in normal rat skeletal muscle. More importantly, their results indicated the exercise-recruited transporters were also GLUT4 but did not originate from the insulin-sensitive intracellular membrane fraction. In a previously discussed study, Kern et al. (1990) proposed insulin responsiveness in rat skeletal muscle was determined by GLUT4 protein level. This study suggested glucose transport in specific skeletal muscle fiber types is at least partially, if not wholly, dependent on the amount of GLUT4 present. They attributed the difference in glucose transport of red and white muscle to the five-fold difference in their GLUT4 concentrations. In addition, the steady-state red muscle GLUT4 mRNA level

was twice as high compared to white muscle. This suggested red muscle preferentially expressed more GLUT4 mRNA and therefore, more GLUT4 protein than white muscle.

Henriksen et al. (1990) postulated a relationship between GLUT4 level, fiber-type, and maximal glucose transport stimulated simultaneously by insulin and contraction. Their results showed GLUT4 level and glucose transport correlated closely in all muscle fiber-types when transport was maximally stimulated. This suggested GLUT4 protein level may be the main determinant for cellular glucose transport capacity. Based on this concept, Henriksen proposed the differences in maximal glucose transport between the different fiber-types was related to their respective GLUT4 concentrations. Rodnick et al. (1990) examined the effects of exercise training on GLUT4 levels in rats allowed to exercise (run) voluntarily. They measured GLUT4 levels in whole muscle homogenates but did not examine GLUT4 membrane redistribution. They postulated the increased GLUT4 level in the plantaris was directly related to a training adaptation that did not represent an increase in total muscle protein. No increase in total transporter number was observed in the soleus, when expressed per unit muscle protein. Rodnick suggested the increase in

insulin mediated glucose uptake seen with exercise training was related to the increase in GLUT4 level.

Exercise training has been observed to improve muscle insulin responsiveness in the insulin resistant obese Zucker rat. Friedman et al. (1990) investigated the effects of exercise training on GLUT4 levels in the obese Zucker rat as compared to its lean littermates. GLUT4 levels in the untrained lean and obese rats were approximately the same. The skeletal muscle insulin resistance of the obese Zucker rat had no apparent affect on GLUT4 protein expression. Using two training groups (18 and 30 weeks), they found GLUT4 protein levels increased by 1.7 and 2.3 fold respectively, when compared to sedentary obese rats. This suggested exercise training stimulated GLUT4 expression and GLUT4 may be responsible for increased insulin responsiveness observed with training.

Insulin resistance in rats made diabetic by streptozotocin treatment may also be related to GLUT4 protein levels. Strout et al. (1990) examined the effects of vanadate treatment on streptozotocin diabetic rats. Immunoblot analyses showed a 70% decrease in skeletal muscle GLUT4 expression in diabetic rats compared to healthy controls. Vanadate treatment

normalized glucose homeostasis and restored GLUT4 expression to 87% of controls. Additionally, a 55% decrease in the steady state level of GLUT4 mRNA was detected by Northern Blot analysis of total skeletal muscle RNA. Vanadate treatment increased GLUT4 mRNA levels 187% over normal. These results further demonstrated insulin responsiveness may be a function of GLUT4 expression.

SUMMARY

Skeletal muscle is the major site for glucose disposal and insulin stimulated glucose clearance. Skeletal muscle glucose uptake and transport are regulated physiologically by the hormone insulin and by muscle contractile activity. Transport is accomplished through facilitated diffusion mediated by five different tissue-specific transporter isoforms. Insulin and exercise increase the plasma membrane level of the GLUT4 (insulin-regulatable) isoform. Associated with this increase is an increase in glucose transport V_{max} without a significant change in the K_m. Specifically, insulin induces a redistribution or translocation of GLUT4 from an intracellular pool to the plasma membrane. However, exercise cannot access this insulin-sensitive pool which suggests an exercise recruitable compartment may exist.

The obese Zucker rat is a common animal model for examining insulin resistance. Exercise training has been effective in improving whole body insulin resistance in the obese Zucker rat. This improvement is believed to be a result of enhanced skeletal muscle insulin responsiveness. The exercise training effect has also been demonstrated to be muscle fiber-type and exercise

intensity specific. Accordingly, training has been shown to increase the level of GLUT4 protein in a fiber-type specific manner. Red fibers show a greater improvement than white fibers. However, most studies (Klip, Fushiki, Douen) have only investigated the effects of acute exercise in the non-insulin resistant rat. Recently, Friedman determined the effects of low intensity exercise training on GLUT4 levels in the insulin resistant muscle of the obese Zucker rat. However, no studies have investigated the effects of low and high intensity exercise training on GLUT4 levels in the various muscle fiber-types of the obese Zucker rat. Finally, the physiological significance of increased GLUT4 concentration following exercise training has not been directly assessed.

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DETAILED METHODS

Animal care and housing

Twenty-two female obese (*fa/fa*) and 6 lean Zucker rats (Animal Model CORE Facility, Obesity Research Center, Vassar College, Poughkeepsie, NY) 4 weeks of age were housed 2-3 per cage and fed normal rat chow (Ralston Purina, St. Louis, MO) and water ad libitum for 2 weeks. Animal room temperature was maintained at 21^{o}C with an artificial 12 hour light/dark cycle. After this familiarization period, rats were individually housed, given powdered chow and familiarized with the treadmill. In week 4, obese rats were randomly assigned to 1 of 3 groups: 1)sedentary control (SED), 2) low intensity trained (LT) and 3) high intensity trained (HT). The lean littermate served as a second control.

Exercise training

Rats assigned to training groups were run on a rodent motor-driven treadmill, up an 8% grade, 5 days/week for 6 to 8 weeks. Treadmill speed and duration were rapidly increased during the first few weeks to have both groups running, 40 min at 18 meters/min by the end of week 2. Beginning the 3rd week of training, the LT group

continued to run at 18 meters/min with the duration gradually increased each week to 120 min/day. The HT group was interval trained, with the maximum intensity reaching 24 meters/min for 18 minutes per exercise bout (5) with a 3 minute rest between each bout. Treadmill speeds of 18 meters/min and 24 meters/min were selected because they represented a work intensity that required 65-70% and 80-85% maximum oxygen consumption (VO_2max) respectively. Whereas exercise at 70% VO_2max was shown to increase oxidative capacity only in red oxidative fibers, exercise at 85% VO_2max was shown to increase oxidative capacity in white glycolytic and red oxidative (Dudley et al., 1982) fibers. Both trained groups performed similar amounts of work each day during the run sessions.

Hindlimb perfusion

At the end of 6 weeks, following a 12 hour fast and 48-56 hours after the last training session, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg/100 g body weight). The surgical technique and perfusion apparatus was similar to that used by Ruderman et al. (1971) as modified by Ivy et

al. (1989). The surgical procedure limited perfusate flow to the right leg by cannulating the right iliac artery and inferior vena cava to the tip of the femoral artery and iliac vein, respectively. The perfusion medium consisted of Krebs-Henseleit buffer (KHB), pH 7.4 containing 4 grams dialyzed bovine serum albumin and blood bank time-expired human erythrocytes at a concentration of 12 grams hemoglobin/100 ml perfusate. The expired erythrocytes were rejuvenated according to procedures described by Valeri (1974).

Once cannulas were inserted, the hindlimb was washed out with 10 ml of KHB and the rat killed by an intracardiac pentobarbital sodium injection. Immediately after, the cannulas were placed in line with the perfusion system and the hindlimb allowed to stabilize during a 5 minute nonrecirculating washout with perfusate containing 8 mM glucose and 500 μU/ml insulin. The perfusate was gassed constantly with a mixture of 95% oxygen and 5% carbon dioxide. Perfusate flow rate was set at 5 ml/min during the stabilization period, and the subsequent perfusion during which muscle glucose transport rates were determined. For muscle glucose transport determination, the perfusate contained 2 mM mannitol (60 μCi D-[1-^{14}C] mannitol/mmol), 8 mM

3-O-methyl-D-glucose (32 μCi [^{3}H]3-OMG/mmol) and 0.3 mM pyruvate. Transport was measured in the presence of 500 μU/ml insulin. The duration of perfusion was set at 8 minutes. Immediately after perfusion, the right leg quadriceps were excised, blotted with KHB dampened gauze, and frozen at -80^{o}C until analyzed for 3-OMG glucose transport, citrate synthase activity, and GLUT4 content.

Glucose transport measurement and tissue analysis

The quadriceps were thawed on ice and surgically separated into red and white portions. The portions were further divided into two 150 mg pieces for the three individual tissue analyses. For transport determination one piece was homogenized in 1.0 ml of 10% trichloroacetic acid (TCA) at 4^{o}C and then centrifuged at 13,600 g for 15 min. Two 300 μl aliquots of supernatant were transferred to 10 ml of Scintiverse E for counting. An arterial perfusate aliquot was similarly added to 10% TCA, and 300 μl of the deproteinated acid extract transferred to a vial containing 10 ml Scintiverse, for determination of specific activity. Samples were counted in a liquid scintillation spectrophotometer (Beckman model LS 350) preset for simultaneous counting of ^{3}H/^{14}C.

Sample quenching was determined by the quenching amount that occurred in prepared standards of [^{3}H]3-OMG and D-[1-^{14}C] mannitol (concentrations based on expected sample counts). The intramuscular [^{3}H]3-OMG accumulation, which is indicative of glucose transport into muscle, was calculated by subtracting the concentration of [^{3}H]3-OMG in the extracellular space from the total muscle [^{3}H]3-OMG concentration. Extracellular space was quantified by measuring the [^{14}C] mannitol concentration in the homogenate (Narahara & Ozand, 1963).

Citrate synthase activity

Citrate synthase is a tricarboxylic acid cycle enzyme that catalyzes the formation of citrate and coenzyme A (CoASH) from acetyl-CoA and oxaloacetate. Citrate synthase activity was determined spectrophotometrically by measuring the appearance of the free sulfhydryl (SH) group from released CoASH. The free SH group was detected by its reaction with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB). The reaction was followed at 412 nm where the mercaptide ion absorbs strongly. The starting materials do not absorb at this wavelength.

Oxaloacetate was used to initiate the citrate synthase reaction. The reaction was conducted at 37°C and followed for at least 3 minutes to ensure linearity (Srere, 1969).

Citrate synthase activity and GLUT4 quantitation were determined from the same muscle homogenates. The muscle portions (red and white) were weighed (approximately 150 mg for each) then homogenized on ice at high speed (VirTishear homogenizer) for three 15 second bursts in HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4, 1:20, w/v). For citrate synthase a 300 µl aliquot was further diluted 1:10 in 1 M Tris and 0.4% Triton X-100, pH 8.1, then centrifuged at 13,600 g for 5 minutes. The supernatant was analyzed with a spectrophotometer (Beckman DU 6) and activity was expressed as µmol/g wet weight/min.

GLUT4 quantitation

Muscle homogenates were solubilized 1:1 in 2x Laemmli buffer (125 mM Tris, 20% glycerol, 2% SDS, .008% bromophenol blue, pH 6.8) for 15 minutes (Laemmli, 1970). Protein was measured by the method of Bradford (1976). A 75 ug protein aliquot for each sample was then subjected

to electrophoresis on a 12.5% polyacrylamide resolving gel for 1 hour and 15 minutes at a current of 50 mA. Electrophoresis was accomplished with a Mini-Protean II dual slab cell (Bio-Rad, Richmond, CA). The apparent molecular weight of the GLUT4 was estimated from the mobility of molecular weight standards on the acrylamide gel. In addition, a rat heart homogenate was used as a standard to quantitate GLUT4 content. A 75 ug protein aliquot of heart homogenate was run separately for comparison on each gel. Each gel contained 1 lane for the heart, with 8 lanes for the muscle samples. Each sample was run in duplicate.

Immediately after electrophoresis, the proteins were transferred (Western blot) from the gel to polyvinylidene difluoride membranes (Bio-Rad PVDF protein sequencing membranes) at 150 mA for 11 minutes. The transfer was accomplished with a semi-dry electrophoretic transfer cell (Bio-Rad Trans-Blot SD). The semi-dry transfer buffers were modified from Kyhse-Anderson (1984). After transfer, membranes were soaked in deionized water for 10 minutes at room temperature, removed from the water and then stored at -20°C. For immunoblotting, the membranes were thawed at room temperature for 5 minutes. Once thawed, the membranes were gently agitated (blocked) in

5% nonfat dry milk (Carnation) in Tween-Tris buffered saline (TTBS) (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) at room temperature for 1 hour. All of the next steps were done with gentle agitation as well.

After blocking, the milk solution was decanted and the membranes were washed twice with TTBS for 10 minutes. The membranes were incubated in the primary antibody solution at room temperature for 1 hour. The primary antibody solution was prepared from rabbit antiserum F349 (donation from Dr. Mike Mueckler, Washington University) diluted 1:500 in 1% milk/TTBS. Next, the primary antibody was decanted and the membranes washed twice with TTBS for 5 minutes at room temperature. The membranes were then incubated in the secondary antibody for 1 hour at 37°C.

The secondary antibody, [^{125}I] goat anti-rabbit IgG (New England Nuclear), was aliquoted into small samples to avoid repeated freeze-thawing and stored at -70°C. The secondary antibody solution was prepared by diluting the samples in 1% milk/TTBS to a concentration of 0.3 μCi/ml. After incubation, the secondary antibody solution was decanted into a radioactive waste jug. The membranes were washed twice for 30 minutes at 37°C in sarcosyl buffer (50 mM Tris, 1 M NaCl, 5 mM EDTA, 0.4%

(w/v) N-laurylsarcosine, pH 7.5). The buffer was decanted and the membranes were washed twice in deionized water for 10 minutes at room temperature. After the final water wash, the membranes were air dried on bench paper and marked with fluorescent paint for identification following autoradiography.

For autoradiography, membranes were placed in an x-ray film cassette (Dupont Cronex Hi Plus intensifying screen) with film (Kodak XAR-5) for 36-40 hours at -70°C. The film was developed immediately after the cassette was removed from the freezer. The film was used to identify the bands of interest on the membranes (matched with the fluourescent paint markings). The bands were traced on the membranes according to the film bands, cut from the membranes and counted for radioactivity (Beckman Gamma 5500). For each size sample band a like background band was cut to correct for background cpm. GLUT4 contents for the muscle samples were expressed as percentages of the heart standard for each individual membrane.

Statistical analysis

A one way analysis of variance (ANOVA) was performed on all variables (glucose transport, citrate synthase

activity, GLUT4 content). Fisher's protected least significant difference was used to identify differences between means. Significance was set at $p < 0.05$ for all tests and values were expressed as means ± standard error.

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APPENDIX A

SEMI-DRY WESTERN TRANSFER BUFFERS

SEMI-DRY WESTERN TRANSFER BUFFERS

Anode 1: 300 mM Tris, pH 10.4
0.05% sodium dodecyl sulfate (SDS)
10% methanol
10 mM β-mercaptoethanol

Anode 2: 25 mM Tris, pH 10.4
0.05% sodium dodecyl sulfate (SDS)
10% methanol
10 mM β-mercaptoethanol

Cathode: 25 mM Tris, pH 9.4
40 mM α-amino hexanoic acid
0.05% sodium dodecyl sulfate (SDS)
10% methanol
10 mM β-mercaptoethanol

APPENDIX B

CITRATE SYNTHASE ASSAY

CITRATE SYNTHASE ASSAY

Prepare the following reagents:

1. 1 mM DTNB (free acid):

   4.0 mg DTNB in 10 ml 0.1 M Tris-HCl, pH 8.1 (protect from light)

2. Acetyl CoA:

   10 mg Acetyl CoA dissolved in 1 ml H_2O or 20 mg CoA + 1.8 ml H_2O + 0.2 ml $KHCO_2$ + 0.026 ml acetic anhydride (add in order)

3. Oxaloacetate 10 mM:

   13.2 mg oxaloacetate in 10 ml 0.1 M Tris-HCl

Muscle homogenization:

1. Homogenize 1:20 in HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4)
2. Dilute homogenate 1:10 in 0.1 M Tris-HCl + 0.4% Triton X-100 pH 8.1; let react 10 min
3. Centrifuge; use supernatant for citrate synthase activity determination

Procedure:

1. Set spectrophotometer at 412 nm
2. Warm cuvettes to 37°C
3. Add the following in order:

Tris-HCl buffer, pH 8.1	0.77 ml
Acetyl CoA	0.03 ml
DTNB	0.10 ml
	0.90 ml

4. Add 0.025 or 0.050 ml homogenate supernatant (amount of homogenate depends on activity level since measurements need to be made at peak activity where it is also linear).
5. Add 0.05 ml oxaloacetate to all cuvettes (except for blank) to start citrate synthase reaction (1 blank may be used for up to 20 min to measure possible deacylase activity).
6. Follow absorbance change for 3 to 5 min until maximal linear rate is reached.

Calculations:

If 0.025 ml is used, the factor is 1147 (40/1 x 10/1 x 1/13.6 x 0.975/0.025)

If 0.050 ml is used, the factor is 588.2 (40/1 x 10/1 x 1/13.6 x 1/0.05)

APPENDIX C

KREBS - HENSELEIT BUFFER

KREBS - HENSELEIT BUFFER

Prepare following stocks:

Stock I

Reagent	Conc(mM)	x10(M)	MW(g)	g/L
NaCl	116	1.160	58.44	67.79
KCl	4.6	0.046	74.56	3.429
KH_2PO_4	1.16	0.0116	136.09	1.578
$NaHCO_3$	25.3	0.253	84.01	21.25

Stock II

Reagent	Conc(mM)	x10(M)	MW(g)	g/L
$CaCl_2 \cdot 2H_2O$	2.5	0.025	147.02	3.675
$Mg_2SO_4 \cdot 7H_2O$	1.16	0.0116	246.48	2.859

To make 1 liter of buffer:

1. Mix 100 ml stock I with 790 ml deionized H_2O
2. Gas with 5% CO_2/95% O_2 for 30 min
3. Add 100 ml of stock II adjust pH to 7.4
4. Bring to volume with deionized H_2O and filter

APPENDIX D

ALBUMIN

ALBUMIN

Prepare the following:

1. 15 liters Krebs-Henseleit buffer (KHB).
2. Layer appropriate amount of albumin (approximately 10 grams per rat) over enough KHB to make a 20% albumin solution and stir until completely dissolved.
3. Cut 8 to 9 inches of Spectrophor 2 dialysis tubing and wet unclamped end with KHB. Pour in albumin solution, clamp and leak check.
4. Place albumin solution in remaining 15 liters of KHB and stir refrigerated for 48 hrs.
5. Filter albumin solution through 25 mm glass fiber filter; then through millipore (0.45 μ) filter with glass fiber filter.
6. Filter albumin solution through 45 mm, 0.45 μ millipore filter.
7. Dilute albumin solution to 12%.
8. Measure concentration with refractometer.

APPENDIX E

RED BLOOD CELL REJUVENATION

RED BLOOD CELL REJUVENATION

Use time expired red blood cells from Blood Bank (approximately 1 unit of red cells needed for each 300 ml of perfusion medium) and store in refrigerator.

Prepare stock 100 mM phosphate buffer, pH 7.2:

1. 1 liter 100 mM Na_2HPO_4, dibasic (14.2 g/L).
2. 1 liter 100 mM Na_2HPO_4, monobasic (12.0 g/L).
3. Adjust pH of 600 ml dibasic with monobasic until 7.2.

Prepare rejuvenation solution:

Solution	1000 ml	500 ml
phosphate buffer	500 ml	250 ml
100 mM glucose	18.0 g	9.0 g
50 mM Na pyruvate	5.5 g	2.75 g
50 mM inosine	13.4 g	6.7 g
5 mM adenine	0.68 g	0.34 g
0.9% NaCl	9.0 g	4.5 g

1. Bring to final volume with deionized H_2O.
2. Filter through 0.45 μ millipore filter.

Prepare buffered 0.9% NaCl:

1. Bring 400 ml phosphate buffer to 4000 ml with 0.9% NaCl.
2. Filter through 0.45 μ millipore filter.

Procedure:

1. Add 50 ml filtered rejuvenation solution to each unit red blood cells.
2. Add 100 ml filtered, buffered 0.9% saline to each unit red blood cells.
3. Mix and incubate 1 hr, 37°C (30 min each side).

Washing red blood cells:

1. Filter blood through silk screen mesh into 250 ml centrifuge bottles (Beckman).
2. Centrifuge at 800 x g (2000 rpm) for 15 min in large plastic bottles (260 ml).
3. Remove supernatant and fibrous grey layer from top of cells.
4. Resuspend in equal volume filtered, buffered 0.9% saline, mix gently repeat steps 2 through 4 twice.
5. Resuspend in KHB, add 0.25 ml of 2 M glucose to each bottle.

6. Store refrigerated overnight.
7. On morning of perfusion, make fresh KHB, spin stored blood at 2500 rpm for 15 min.
8. Aspirate and resuspend in KHB, twice.
9. Cells now have hemoglobin concentration of 28-30 g/100 ml.
10. Dilute to 12 g/ml with KHB.
11. For transport, check glucose level (glucose analyzer); if greater than 32 mg/100 ml wash again in KHB.

APPENDIX F

INDIVIDUAL RAT DATA

RAT	3-OMG TRANSPORT µmol/g/hr		% GLUT4		CITRATE SYNTHASE ACTIVITY µmol/g/min	
	RED	WHITE	RED	WHITE	RED	WHITE
52 HT	12.86	1.46	49.16	11.96	111.90	23.35
50 HT	9.64	1.27	50.04	11.87	105.00	27.31
67 HT	10.05	1.39	55.57	13.59	114.30	12.94
60 HT	13.43	2.17	62.15	20.93	84.85	16.47
68 HT	13.92	0.60	53.72	23.35	108.10	15.12
48 HT	10.90	1.30	65.18	17.01	123.20	28.82
64 HT			63.84	23.76	105.20	20.06
43 LT	14.11	0.81	50.60	14.01	83.36	15.48
2 LT	13.54	1.66	72.39	19.04	108.50	32.07
25 LT	8.30	0.20	39.77	18.39	95.24	16.32
13 LT	10.60	0.21	64.50	22.42	100.80	15.33
15 LT	5.28	1.91	53.21	22.14	103.10	17.14
40 LT	11.19	1.67	49.58	24.06	88.27	15.40
5 LT			42.79	12.94	114.80	21.52
56 SED	4.37	1.01	35.71	10.68	76.83	18.95
58 SED	4.63	0.85	42.92	15.72	89.88	17.03
54 SED	4.97	0.78	37.51	11.95	69.74	13.07
55 SED	9.14	0.97	33.59	11.93	62.37	13.70
65 SED	9.15	0.86	43.02	7.41	77.60	15.48
49 SED	6.55	1.57	41.04	21.58	69.05	19.62
6 SED	7.59	1.41	37.96	14.27	83.21	21.09
33 SED	9.18	1.73	39.32	13.28	59.41	14.45
27 LN	8.51	1.12	36.42	15.89	54.67	21.88
C LN	13.80	3.32	41.72	17.12	46.16	9.94
26 LN	14.05	1.48	45.40	18.34	54.35	14.45
30 LN	16.09	4.44	34.80	16.03	54.41	15.42
29 LN	13.02	2.14	39.54	19.43	57.74	13.42
28 LN	9.69	0.77	47.11	25.61	61 34	11.74