

# Glucose uptake and GLUT-4 protein distribution in skeletal muscle of the obese Zucker rat

J. T. BROZINICK, JR., G. J. ETGEN, JR., B. B. YASPELKIS III, AND J. L. IVY  
*Exercise Physiology and Metabolism Laboratory, Department of Kinesiology,  
The University of Texas at Austin, Austin, Texas 78712*

**Brozinick, J. T., Jr., G. J. Etgen, Jr., B. B. Yaspelkis III, and J. L. Ivy.** Glucose uptake and GLUT-4 protein distribution in skeletal muscle of the obese Zucker rat. *Am. J. Physiol.* 267 (Regulatory Integrative Comp. Physiol. 36): R236–R243, 1994.—The rates of muscle glucose uptake of lean and obese Zucker rats were assessed by hindlimb perfusion under basal conditions (no insulin), in the presence of a maximally stimulating concentration of insulin (10 mU/ml), and after muscle contraction elicited by electrical stimulation of the sciatic nerve. After perfusion, plasma and microsomal membranes were isolated from selected hindlimb muscles for determination of GLUT-4 protein distribution. Under basal conditions, rates of glucose uptake were similar for lean and obese rats despite plasma membranes from lean rats containing 82% more GLUT-4 protein than obese rats. Insulin stimulation resulted in significant increases in plasma membrane GLUT-4 protein concentration in lean but not obese rats. Glucose uptake of lean rats ( $35.3 \pm 4.7 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ) in the presence of insulin was approximately fourfold greater than that of obese rats ( $8.8 \pm 1.3 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ ), but this difference in glucose uptake could not be completely accounted for by the difference in plasma membrane GLUT-4 protein concentration. Stimulation by contraction resulted in significant increases in plasma membrane GLUT-4 protein concentration in both lean and obese rats and similar rates of glucose uptake. These results suggest that the muscle insulin resistance of the obese Zucker rat is due to 1) a reduced plasma membrane GLUT-4 protein concentration, which results in part from an impairment in the insulin-stimulated GLUT-4 protein translocation process, and 2) a defect in the insulin-stimulated activation of this protein. However, contraction-stimulated glucose uptake, GLUT-4 protein translocation, and activation are normal in the obese Zucker rat.

glucose transporter; 2-deoxyglucose; muscle contraction; insulin; plasma membranes

THE GENETICALLY OBESE Zucker rat is a well-documented model for muscle insulin resistance (9, 19, 20, 31, 35). The locus of this insulin resistance is thought to reside in the glucose transport process and occurs in all three muscle fiber types of the rat (9, 19, 31). It has recently been demonstrated that skeletal muscle expresses a glucose transport protein (GLUT-4), which is responsible for facilitated glucose uptake in response to both insulin and muscle contraction (10, 11, 18). Activation of glucose uptake is thought to occur in part through the rapid reversible translocation of GLUT-4 from an intracellular pool to the plasma membrane (10, 11, 15, 18).

Recent evidence from our laboratory (2, 7) and others (13) has shown that the difference in insulin-stimulated muscle glucose uptake between lean and obese Zucker rats cannot be attributed to a difference in total muscle GLUT-4 protein concentration. However, the total muscle GLUT-4 protein concentration may not be as

important as its distribution or possibly its intrinsic activity, i.e., transporter turnover number. In this regard, King et al. (21) recently compared the effects of an intraperitoneal injection of insulin on muscle GLUT-4 protein translocation and intrinsic activity in lean and obese Zucker rats. It was found that the GLUT-4 protein translocation process in the obese Zucker rat was defective, but that transporter intrinsic activity was normal and responded normally to insulin. With the use of this in vivo model, however, it is not possible to compare the direct effects of insulin on GLUT-4 protein translocation or transporter intrinsic activity between lean and obese Zucker rats because responses of counter-regulatory hormones to the insulin injection may differ between these phenotypes. Therefore, the first purpose of the present study was to directly assess the effects of insulin on GLUT-4 protein translocation in muscle of lean and obese Zucker rats and to determine if a defect in the translocation process could account for the muscle insulin resistance of the obese rat.

Muscle contraction, in the absence of insulin, has been shown to stimulate glucose uptake (7, 16, 27). Stimulation of glucose uptake by contraction occurs through a pathway distinct from the insulin pathway and provides a second method for stimulating glucose uptake into muscle (8, 27). Despite the well-documented skeletal muscle insulin resistance of the obese Zucker rat, recent evidence from our laboratory has shown that skeletal muscle glucose uptake in the obese Zucker rat is not resistant to contraction stimulation (7). However, it is unknown if contraction causes similar increases in plasma membrane GLUT-4 protein concentration in lean and obese rats. Therefore, the second purpose of the present study was to determine if contraction causes similar increases in GLUT-4 protein translocation in lean and obese rats and to compare the increases in plasma membrane GLUT-4 protein concentration and contraction-stimulated glucose uptake in these rats.

## METHODS

**Experimental animals.** Nineteen female obese Zucker rats (*fa/fa*) and seventeen lean littermates (*Fa/Fa*), 14 wk of age, were randomly assigned to a basal (no insulin), insulin, or muscle contraction group. All rats were housed three to a cage and provided laboratory Chow and water ad libitum. The temperature of the animal room was maintained at 21°C, and an artificial 12:12-h light-dark cycle was set with the lights on from 2000 to 0800 h.

**Surgical preparation and hindlimb perfusion.** After an overnight fast and between 1100 and 1600 h, rats were anesthetized with an intraperitoneal injection of 6.5 mg/100 g body wt pentobarbital sodium. The surgical procedure for hindlimb perfusion of the rats and the perfusion apparatus used were similar to those previously described by Ruderman

et al. (28). Additionally, both sciatic nerves of the contraction-stimulated rats were surgically exposed and attached to miniature electrodes. After completion of the surgical preparation, cannulas were inserted into the abdominal aorta and vena cava of the rats and their hindlimbs were washed out with 50 ml of Krebs-Henseleit buffer (pH 7.4). Immediately thereafter the cannulas were placed in line with the perfusion system and the hindlimbs were allowed to stabilize during a 15-min nonrecirculating washout period. The perfusion medium consisted of Krebs-Henseleit buffer (pH 7.4) containing 4% dialyzed bovine serum albumin (Cohn fraction V, US Biochemical), 30% time-expired washed human red blood cells, 0.3 mM pyruvate, and 0.5 mM glucose. Perfusate flow rate during the washout period was 15 ml/min. After the washout perfusate samples were taken from the venous line for determination of insulin, and the rats were killed with an intracardiac injection of pentobarbital sodium. Insulin analysis indicated that the washout procedure effectively lowered the insulin concentration of the hindlimbs below the level of detection ( $<2.5 \mu\text{U/ml}$ ).

For determination of basal- and insulin-stimulated glucose uptake, the perfusate was changed to one containing 28 mM glucose, 2-deoxy-D-[ $^3\text{H}$ ]glucose (2-DG;  $7.5 \mu\text{Ci/mmol}$  glucose), 2 mM mannitol ( $60 \mu\text{Ci D-[}^{14}\text{C]mannitol/mmol}$ ), and 0.3 mM pyruvate. The concentration of glucose used was selected because it was approximately fourfold higher than the average Michaelis constant for insulin-stimulated muscle glucose uptake (24), thereby ensuring that the maximum velocity of uptake was measured. When insulin-stimulated glucose uptake was determined, the perfusate also contained 10 mU/ml insulin. Perfusion times were 10 min for basal and 7 min for insulin- and contraction-stimulated rats, and the perfusate flow rate was maintained at 15 ml/min (7).

For contraction-stimulated glucose uptake, the skin was reflected from both hindlimbs of the rat during the initial 15-min perfusion period. A section of the calcaneus, with the Achilles tendon still attached, was then clipped from the foot. Next, the triceps surae of both hindlimbs were reflected away from the tibia and jewelers' chains clipped to both calcaneus-Achilles tendon interfaces. The chains were attached to an apparatus that allowed for adjustment of muscle tension, with one chain in line with an isometric force transducer (Harvard Instruments, Millis, MA), which was in turn connected to a chart recorder. Both hindlimbs of the stimulated rats were then immobilized in a specially designed acrylic cradle, and the muscles of the triceps surae group were adjusted to achieve maximum twitch tension.

After the 15-min washout period, muscle contraction was initiated by stimulating the sciatic nerves with 200-ms trains of 100 Hz, with each impulse in a train being 0.1 ms. The trains were delivered at a rate of 1/s at a supramaximum voltage (8–12 V) for  $2 \times 5$  min separated by a 1-min rest period. Immediately before the initiation of contraction the flow rate was increased to 25 ml/min to ensure proper oxygen and substrate delivery to the hindlimb muscles. Once the second stimulation period was completed, the perfusate was changed over to that used for the measurement of basal glucose uptake, and the flow rate was reduced to 15 ml/min. Time of perfusion was set for 7 min. All perfusions were performed at  $37^\circ\text{C}$ , and all perfusates were continuously gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ .

Immediately after each perfusion, the hindlimbs of the rats were washed out with 30 ml of Krebs-Henseleit buffer to remove intravascular erythrocytes. The plantaris (10% slow-twitch red, 65% fast-twitch red, 25% fast-twitch white; 32), and portions of red (fast-twitch red) and white (fast-twitch white) gastrocnemius were then removed from the left leg and

blotted on gauze dampened with Krebs-Henseleit buffer. The muscles were freeze-clamped in tongs cooled in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until analyzed for 2-DG uptake. These muscles were chosen because they best represent the hindlimb muscle fiber-type composition of the rat, which is mainly comprised of fast-twitch red and white fibers (1) and because the average glucose uptake of these muscles is representative of hindlimb glucose uptake (8, 30, unpublished results).

*Determination of average 2-DG uptake.* The plantaris and red and white portions of gastrocnemius from the left leg were weighed, homogenized in 1 ml of 10% trichloroacetic acid (TCA) at  $4^\circ\text{C}$ , and centrifuged in a microcentrifuge (Fisher Scientific, Houston, TX) for 10 min. Duplicate 0.3-ml samples of the supernatant were transferred to 20-ml scintillation vials containing 10 ml of Scintiverse E (Fisher Scientific, Pittsburgh, PA) and vortexed. For determination of perfusate specific activity, well-mixed samples of the arterial perfusate were obtained during perfusion. The samples were deproteinized in 10% TCA and treated the same as the muscle samples. The samples were counted for radioactivity in a LS-350 liquid scintillation spectrophotometer (Beckman Industries, Fullerton, CA). Efficiency and channel crossover were determined by counting  $^3\text{H}$  and  $^{14}\text{C}$  standards of known disintegrations per minute. The accumulation of intracellular 2-[ $^3\text{H}$ ]DG, which is indicative of muscle glucose uptake, was calculated by subtracting the concentration of 2-[ $^3\text{H}$ ]DG in the extracellular space from the total muscle 2-[ $^3\text{H}$ ]DG concentration. The extracellular 2-DG was quantified by measuring the concentration of [ $^{14}\text{C}$ ]mannitol in the muscle sample.

*Membrane preparation.* Plasma and intracellular membranes were prepared from perfused hindlimb muscle innervated by the sciatic nerve. Approximately 6 g of mixed rat skeletal muscle consisting of the biceps femoris, flexor halicis longus, semimembranosus, semitendinosus, and tibialis anterior from both legs, and the plantaris, and red and white gastrocnemius from the right leg were removed at the end of perfusion, cleaned of fat and connective tissue, and weighed (Table 1). This muscle was minced in a buffer consisting of 255 mM sucrose, 100 mM tris(hydroxymethyl)aminomethane (Tris), and 0.2 mM EDTA at pH 7.6. The minced muscle was next homogenized in a Brinkman P1-10 polytron at a setting of 4 and then homogenized again with a Potter-Elvehjem Teflon-glass tissue grinder and brought up to a volume of 25 ml with homogenization buffer. A 0.5-ml aliquot was removed for measurement of marker enzymes and protein, and the homogenate was then centrifuged at  $34,000 g$  for 20 min. The resulting pellet was used for purification of plasma membranes, whereas the supernatant was used for the purification of microsomal membranes according to Hirshman et al. (18).

Each plasma and microsomal membrane suspension was weighed to determine the exact volume. An aliquot of each suspension was removed for marker enzymes and protein determinations, and the remainder was used for Western blotting. All samples were stored at  $-80^\circ\text{C}$  until assayed.

*Protein and marker enzyme assays.* Homogenate and membrane protein contents were determined for each preparation (Table 1) by the Coomassie brilliant blue method (Bio-Rad protein assay, Richmond, CA) as described by Bradford (6), using crystalline bovine serum albumin as the standard.  $\text{K}^+$ -stimulated-*p*-nitrophenolphosphatase (KpNPPase) specific activity was measured as a marker for plasma membranes by the method of Bers et al. (5). To determine if there was sarcoplasmic reticulum contamination of the plasma membrane fraction, both homogenate and membrane fractions were assayed for  $\text{Ca}^{2+}$ -stimulated adenosinetriphosphatase (ATPase) activity by the method of Sciler and Fleischer (30).

Table 1. *Body and muscle weights used in membrane preparations and protein recovery*

	Lean			Obese		
	Basal (7)	Contraction (10)	Insulin (5)	Basal (7)	Contraction (8)	Insulin (5)
Body wt, g	207.5 ± 7.6*	208.0 ± 6.3*	185.5 ± 6.4*	372.2 ± 16.1	350.7 ± 25.7	393.8 ± 2.6
Muscle wt, g	6.94 ± 0.30	6.74 ± 0.30	5.90 ± 0.27	5.68 ± 0.27	6.09 ± 0.16	6.15 ± 0.27
Total protein						
Homogenate, mg	854.7 ± 43.0	824.7 ± 45.3	780.7 ± 28.7	740.2 ± 20.4	725.61 ± 28.1	822.5 ± 36.7
Plasma membrane, µg/mg CH protein	1.63 ± 0.18	1.61 ± 0.08	1.78 ± 0.05	2.03 ± 0.25	2.10 ± 0.19	2.37 ± 0.21
Microsomal membrane, µg/mg CH protein	0.83 ± 0.19	0.81 ± 0.13	1.71 ± 0.22*†	1.09 ± 0.07	0.81 ± 0.13	1.11 ± 0.06

Values are means ± SE; no. for each group in parentheses. CH, crude homogenate. \*Significantly different from obese ( $P < 0.05$ ). †Significantly different from lean basal and lean contraction.

**Western blotting.** Samples of the membrane suspensions (50 µl) were diluted 1:1 with Laemmli sample buffer (26). Plasma membrane (15 µg) or microsomal membrane sample protein (30 µg), along with molecular weight markers (Bio-Rad), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis run under reducing conditions using a 12% resolving gel. Samples from lean and obese rats were run on the same gels. All membrane samples were run on duplicate gels. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) sheets (Bio-Rad) by the method of Towbin et al. (33) utilizing the buffer system of Kyhse-Andersen (25) and a Bio-Rad SD semidry transfer unit. All subsequent incubations were carried out in Tween-Tris buffered saline (TTBS; 0.05% Tween-20) composed of 20 mM Tris and 500 mM NaCl (pH 7.5, at 25°C). After transfer, the PVDF sheets were blocked in TTBS and 5% nonfat dry milk at pH 7.5. Next, the sheets were washed in TTBS for 20 min and then incubated for 1 h with the polyclonal GLUT-4 antibody F349 (donated by Dr. Mike Mueckler, Washington University, St. Louis, MO) at a titer of 1:500 in TTBS, 1% milk, and 0.02% sodium azide. The PVDF was then washed in TTBS for 20 min followed by incubation with <sup>125</sup>I-goat-anti-rabbit immunoglobulin G (New England Nuclear, Boston, MA) at a concentration of 0.3 µCi/ml for 1 h. The sheets were then washed and air dried. Antibody binding was visualized by autoradiography that was performed at -70°C with Kodak X-omat AR film and Dupont Cronex Lightning Plus intensifying screens for 24 h. Labeled bands were traced, cut out, and counted in a Beckman model 5500 gamma counter. Each band was corrected for background radiation, and the counts were expressed as a percentage of a standard (100 µg of heart homogenate protein) run on each gel. Average counts/min above background for the heart standard when counting plasma and microsomal membrane GLUT-4 were 3,159 ± 104 and 3,575 ± 256, respectively. Background was < 10% of the total counts.

**Statistical analysis.** The data were analyzed by multivariate analysis of variance using the MANOVA procedure (SPSS/PC + 4.0) with a priori comparisons using the appropriate contrasts to test the effects of phenotype and treatment, and interactions between these main effects. A level of  $P < 0.05$  was considered to be statistically significant. All values were expressed as means ± SE.

## RESULTS

Body weight and the weight of the muscle used in the plasma and microsomal membrane preparations are presented in Table 1. Lean rats were significantly lighter than obese rats, but there was no difference in muscle mass recovered or total protein recovered between phenotypes. Plasma and microsomal membrane protein concentrations were similar between lean and obese

rats, with the exception that the microsomal protein concentration of the insulin-stimulated lean rats was significantly elevated above that of basal and contraction-stimulated lean rats.

Average basal, insulin-, and contraction-stimulated glucose uptakes are shown in Fig. 1. Under basal conditions and after muscle contraction, glucose uptake was not significantly different between lean and obese rats. In contrast, insulin-stimulated glucose uptake was fourfold higher in lean than in obese rats.

Specific activities, recoveries, and degrees of purities of the plasma membrane marker enzyme KpNPPase are given in Table 2. KpNPPase activity was enriched ~40-fold in the plasma membrane fraction and ~10-fold in the microsomal membranes, compared with the crude homogenate. Degree of enrichment did not differ between lean and obese rats. Recovery of KpNPPase was ~7% in the plasma membranes and ~1% in the microsomal membranes. There were no differences in percent recovery between lean and obese rats, and muscle contraction and insulin had no effect on percent recovery in lean or obese rats. KpNPPase specific activity in the plasma membrane was significantly lower in the obese rats. The reason for this is unknown, but recent evidence has suggested that streptozotocin diabetic rats, which also exhibit skeletal muscle insulin resistance,

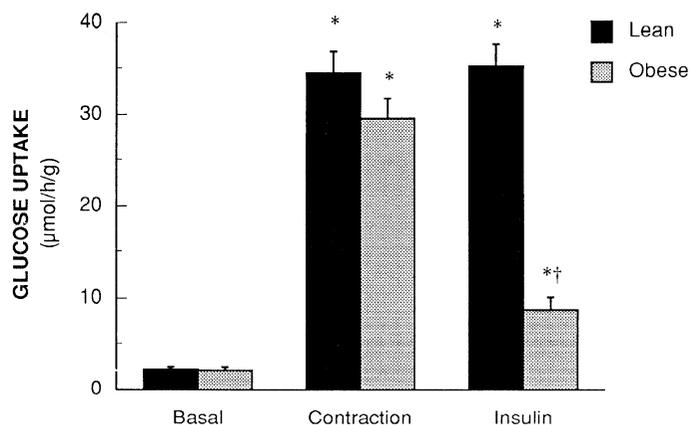


Fig. 1. Hindlimb glucose uptake, determined from glucose uptakes of plantaris and red and white portions of gastrocnemius, for lean and obese Zucker rats under basal conditions, insulin stimulation, and after muscle contraction. Bars represent means ± SE. No. of observations are same as indicated in Table 2. \*Significantly different from basal. †Significantly different from lean ( $P < 0.05$ ).

Table 2. *KpNPPase activities, percent recoveries, and degrees of enrichment of membrane fractions from basal, contracted, and insulin-stimulated muscle from lean and obese rats*

	Lean			Obese		
	Basal (7)	Contraction (10)	Insulin (5)	Basal (7)	Contraction (8)	Insulin (5)
Homogenate sp act, nmol·mg <sup>-1</sup> ·min <sup>-1</sup>	4.04 ± 0.16	3.66 ± 0.15	4.38 ± 0.42	3.76 ± 0.17	3.53 ± 0.21	3.98 ± 0.28
Plasma membrane						
Sp act, nmol·min <sup>-1</sup> ·mg <sup>-1</sup>	157.8 ± 12.4*	166.6 ± 9.0*	165.4 ± 20.6*	112.3 ± 10.6	138.10 ± 11.6	116.6 ± 12.3
Recovery, %	6.3 ± 0.56	7.15 ± 0.38	6.66 ± 1.02	6.11 ± 0.48	7.57 ± 0.60	6.96 ± 0.99
Enrichment, no. of times	40.7 ± 4.9	45.9 ± 2.6	37.8 ± 6.16	30.7 ± 3.7	39.8 ± 3.5	30.2 ± 4.2
Microsomal membrane						
Sp act, nmol·min <sup>-1</sup> ·mg <sup>-1</sup>	45.1 ± 4.3	36.0 ± 5.3	43.3 ± 3.1	36.1 ± 4.2	44.0 ± 3.9	34.7 ± 4.7
Recovery, %	0.88 ± 0.12	0.72 ± 0.09	1.76 ± 0.39	1.05 ± 0.13	1.21 ± 0.13	0.97 ± 0.13
Enrichment, no. of times	11.7 ± 1.4	9.2 ± 1.3	10.0 ± 1.2	9.9 ± 1.2	12.6 ± 1.3	8.8 ± 1.3

Values are means ± SE; no. for each group in parentheses. KpNPPase, K<sup>+</sup>-stimulated *p*-nitrophenolphosphatase. \*Significantly different from obese (*P* < 0.05).

have a decreased skeletal muscle Na<sup>+</sup>-K<sup>+</sup>-ATPase activity (22).

Specific activities, degrees of enrichment, and percent recoveries of the sarcoplasmic reticulum marker enzyme Ca<sup>2+</sup>-ATPase are given in Table 3. These activities demonstrate that there was virtually no contamination of the plasma membrane preparation with sarcoplasmic reticulum, as the degrees of enrichment of Ca<sup>2+</sup>-ATPase were < 1 for both lean and obese rats. Although the average Ca<sup>2+</sup>-ATPase activity of the obese rats was higher than that of the lean rats, the difference was not statistically significant (*P* = 0.07). The reason for this marginal difference in Ca<sup>2+</sup>-ATPase activity between the lean and obese rats resulted from several of the membrane preparations from the obese rats having a high Ca<sup>2+</sup>-ATPase activity relative to that of membrane preparations from the lean rats. In the majority of samples from both lean and obese rats, Ca<sup>2+</sup>-ATPase activity was undetectable. In addition, excluding the samples with the high Ca<sup>2+</sup>-ATPase activities from the statistical analysis had no effect on the plasma membrane GLUT-4 protein results. Ca<sup>2+</sup>-ATPase activity was not detectable in microsomal membranes.

Figures 2 and 3 are representative autoradiograms showing <sup>125</sup>I-labeled antibody binding to GLUT-4 protein bands from plasma membranes and low-density microsomes. Under basal conditions, plasma membranes from the lean rats had an 82% greater GLUT-4 protein concentration than plasma membranes from obese rats (Figs. 2 and 4). Insulin stimulation signifi-

cantly increased plasma membrane GLUT-4 protein concentration above that of basal in lean but not obese rats. Muscle contraction significantly increased plasma membrane GLUT-4 protein concentration in both lean and obese rats. However, because of their initial lower basal membrane concentration, the plasma membrane GLUT-4 protein concentration of the obese rats remained significantly lower than that of the lean rats.

Under basal conditions there were no significant differences in microsomal GLUT-4 protein concentration between lean (Figs. 3A and 5) and obese rats (Figs. 3B and 5). Insulin stimulation caused a significant decline in microsomal GLUT-4 protein concentration in lean rats (27%) but resulted in only a slight nonsignificant decline in microsomal GLUT-4 protein concentration in obese rats (11%). Muscle contraction did not cause a significant change in microsomal GLUT-4 protein content in either lean or obese rats.

The ratio of hindlimb glucose uptake to plasma membrane GLUT-4 protein was determined as an estimate of GLUT-4 protein intrinsic activity. Because this is an indirect measure of intrinsic activity, it will be referred to as functional activity (see Table 4 for details). There was no significant difference in basal plasma membrane GLUT-4 protein activity between the lean and obese rats. Contraction stimulation resulted in significant increases in plasma membrane GLUT-4 protein activity that were similar in both lean and obese rats. In contrast, insulin stimulation only increased the plasma membrane GLUT-4 protein activity of the lean rats.

Table 3. *Ca<sup>2+</sup>-stimulated ATPase specific activities, percent recoveries, and degrees of enrichment of membrane fractions from basal, contracted, and insulin-stimulated muscle from lean and obese rats*

	Lean			Obese		
	Basal (7)	Contraction (10)	Insulin (5)	Basal (7)	Contraction (8)	Insulin (5)
Homogenate sp act, nmol·mg <sup>-1</sup> ·min <sup>-1</sup>	126.7 ± 15.6	153.3 ± 13.6	139.6 ± 24.8	113.1 ± 11.7	123.2 ± 13.7	111.9 ± 10.6
Plasma membrane						
Sp act, nmol·min <sup>-1</sup> ·mg <sup>-1</sup>	0.91 ± 0.91	13.84 ± 13.84	10.52 ± 10.52	56.7 ± 47.9	42.2 ± 22.6	74.6 ± 56.7
Recovery, %	0.001 ± 0.001	0.01 ± 0.01	0.02 ± 0.02	0.17 ± 0.15	0.31 ± 0.18	0.17 ± 0.11
Enrichment, no. of times	0.01 ± 0.01	0.08 ± 0.08	0.12 ± 0.12	0.59 ± 0.50	0.10 ± 0.06	0.66 ± 0.45
Microsomal membrane sp act, nmol·min <sup>-1</sup> ·mg <sup>-1</sup>	ND	ND	ND	ND	ND	ND

Values are means ± SE; no. for each group in parentheses. ND, not detectable.

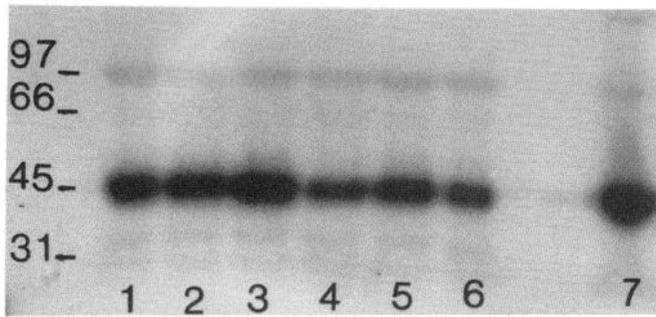


Fig. 2. Autoradiogram showing  $^{125}\text{I}$ -labeled GLUT-4 protein bands in skeletal muscle plasma membrane from lean (lanes 1-3) and obese (lanes 4-6) Zucker rats. Plasma membranes are from basal (lanes 1 and 4), contraction-stimulated (lanes 2 and 5), and insulin-stimulated (lanes 3 and 6) muscle. Lane 7 shows GLUT-4 protein from rat heart standard (100  $\mu\text{g}$  homogenate protein). All lanes were equally loaded with 15  $\mu\text{g}$ /sample plasma membrane protein. Locations of 97-, 66-, 45-, and 31-kDa markers are indicated in left margins.

This resulted in a significant difference in plasma membrane GLUT-4 protein activity between obese and lean rats perfused with insulin.

#### DISCUSSION

The skeletal muscle of the obese Zucker rat is highly insulin resistant, which is due in part to a decrement in both insulin sensitivity and responsiveness (19, 31, 35). The insulin resistance is common to all three fiber types of the rat and is associated with a defect in the glucose transport process (9, 19, 31). In the present study average insulin-stimulated glucose uptake was approximately fourfold higher in the lean rats than in the obese rats. This confirms earlier results from our laboratory

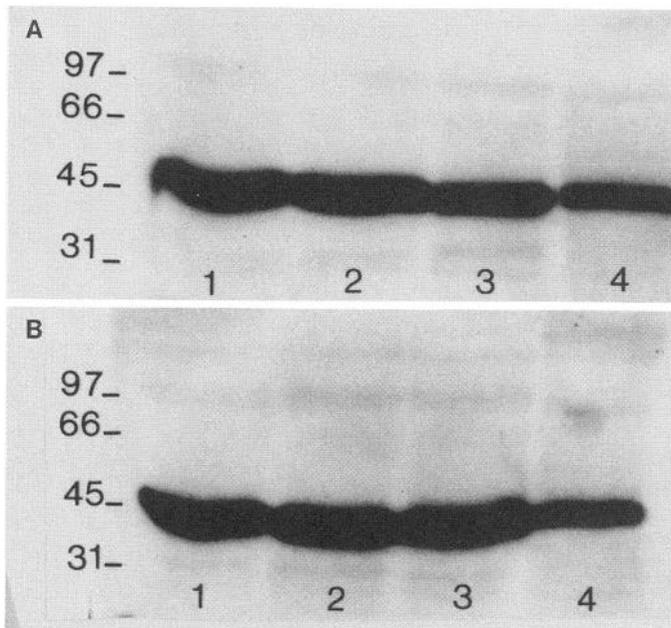


Fig. 3. Autoradiograms showing  $^{125}\text{I}$ -labeled GLUT-4 protein bands in low-density microsomes prepared from skeletal muscle from lean (A) and obese (B) Zucker rats. Microsomal membranes are from basal (lane 1), contraction-stimulated (lane 2), and insulin-stimulated (lane 3) muscle. Lane 4 shows GLUT-4 protein from rat heart standard (100  $\mu\text{g}$  homogenate protein). All lanes were equally loaded with 30  $\mu\text{g}$ /sample microsomal membrane protein. Locations of 97-, 66-, 45-, and 31-kDa markers are indicated in left margins.

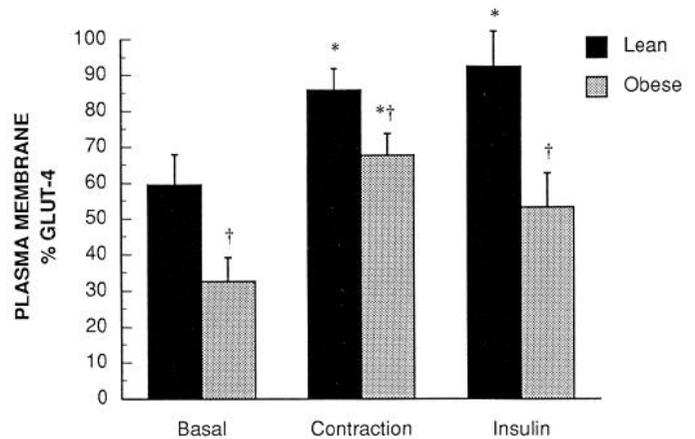


Fig. 4. Plasma membrane GLUT-4 protein content of lean and obese Zucker rats expressed as percentage of heart standard. Bars represent means  $\pm$  SE. No. of observations are same as indicated in Table 2. \*Significantly different from basal.  $\dagger$ Significantly different from lean ( $P < 0.05$ ).

that the maximal capacity of insulin-stimulated glucose uptake is impaired in the obese Zucker rat (7).

Activation of glucose uptake by insulin involves the rapid reversible translocation of specific glucose transporter proteins to the plasma membrane from an intracellular pool (10, 11, 16, 18, 23). Six different isoforms of this protein have been identified, but in skeletal muscle the two predominate isoforms are termed GLUT-1 and GLUT-4 (4, 11). The GLUT-1 isoform is found in small amounts in the plasma membrane, and its concentration is not significantly increased by insulin (10, 11, 15). Insulin, however, increases the plasma membrane GLUT-4 protein concentration, and this suggests that it is primarily the GLUT-4 isoform that is involved in the regulation of insulin-stimulated glucose uptake (10, 11, 18).

Results from Henriksen et al. (17) indicated that the maximal insulin- or contraction-stimulated skeletal muscle glucose uptake is correlated with the total muscle GLUT-4 protein concentration in normal rats. Subsequent studies from our laboratory have shown

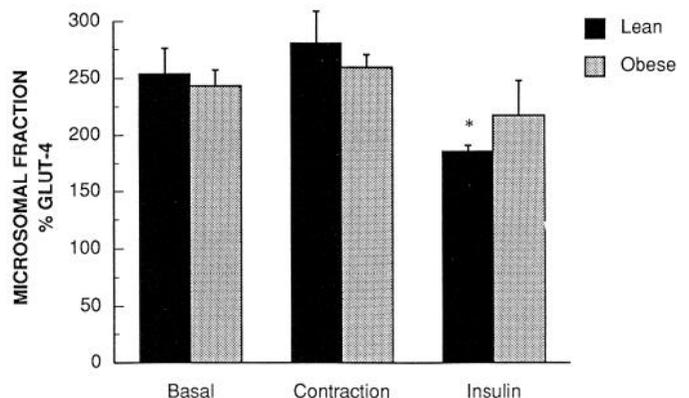


Fig. 5. Microsomal membrane GLUT-4 protein concentration of lean and obese Zucker rats expressed as percentage of rat heart standard. Values are expressed relative to 15  $\mu\text{g}$  microsomal protein so that comparisons can be made to plasma membrane GLUT-4 protein concentrations. Bars represent means  $\pm$  SE. No. observations are same as indicated in Table 3. \*Significantly different from basal ( $P < 0.05$ ).

Table 4. *Estimated functional activity of plasma membrane GLUT-4 protein from lean and obese rats*

	Basal	Contraction Stimulated	Insulin Stimulated
Lean	3.42 ± 0.82	30.70 ± 1.85†	25.56 ± 2.35†
Obese	3.93 ± 0.70	30.63 ± 4.66†	8.42 ± 1.12†*

Values are means ± SE in units of nmol glucose uptake · %GLUT-4<sup>-1</sup> · h<sup>-1</sup>. Functional activity was calculated by dividing hindlimb glucose uptake (μmol · g wet wt<sup>-1</sup> · h<sup>-1</sup>) by %GLUT-4 protein/gram muscle. %GLUT-4 protein/gram muscle was determined by multiplying %GLUT-4 protein per milligram plasma membrane protein by milligram plasma membrane protein per gram muscle. Results were same as presented when %plasma membrane recovery and purity of membrane preparation (fold enrichment) were taken into consideration. \*Significantly different from corresponding lean value. †Significantly different from corresponding basal value ( $P < 0.05$ ).

this relationship also exists in the insulin-resistant obese Zucker rat (2, 7). However, for a given muscle GLUT-4 protein concentration, the obese rat would be predicted to have an insulin-stimulated muscle glucose uptake one-fourth that of its lean littermate. These results indicate that although total GLUT-4 protein concentration is important in the control of insulin-stimulated glucose uptake, other factors such as a decrement in GLUT-4 protein translocation or intrinsic activity (transporter turnover number) are more important in the etiology of the insulin resistance in the obese rat.

To better evaluate the etiology of muscle insulin resistance in the obese Zucker rat, we measured GLUT-4 protein in membrane fractions isolated from lean and obese Zucker rats. Western blotting of basal plasma and microsomal membranes from lean and obese rats showed that the basal plasma membranes of lean rats had 82% greater GLUT-4 protein concentration than those of obese rats, whereas basal microsomal GLUT-4 protein levels were not different between lean and obese rats. Although this differs from previous studies in which it was reported that skeletal muscle of lean and obese rats have similar total GLUT-4 levels, it is not totally inconsistent with previous results from our laboratory. We have consistently found a small but significantly lower GLUT-4 protein concentration in fast-twitch glycolytic fibers (type IIB) and slightly lower GLUT-4 protein concentrations in slow (type I)- and fast-twitch oxidative fibers (type IIA) of obese rats compared with lean rats (2, 7). Therefore, the difference in plasma membrane GLUT-4 protein between lean and obese rats may represent a magnification of the difference in total GLUT-4 protein concentration of the three basic skeletal muscle fiber types when expressed per milligram membrane protein rather than per milligram of homogenate protein.

The possibility also exists that the difference in basal plasma membrane GLUT-4 protein concentration between lean and obese rats resulted from differential responses of the membranes to the isolation procedure. It should be noted that the degree of enrichment of the plasma membranes of the lean rats was ~25% greater than that of obese rats. Therefore, a more pure membrane preparation might account for the higher plasma membrane GLUT-4 protein determination in the lean

rats. We have several reasons, however, why we do not believe the differences in plasma membrane GLUT-4 protein between the lean and obese rats was due to a difference in membrane purity. First, the difference in basal plasma membrane GLUT-4 protein concentration was much greater than that which could be accounted for by a difference in membrane purity. Second, when individual preparations from lean and obese rats with the same degree of enrichment were compared, the lean rats always had the higher GLUT-4 protein concentration. Third, King et al. (21) have previously reported a lower, albeit not statistically different, plasma membrane GLUT-4 protein concentration in obese compared with lean Zucker rats.

The finding that the lean and obese rats had similar rates of basal glucose uptake, yet a large disparity in basal plasma membrane GLUT-4 protein concentration, may indicate that basal glucose uptake is under the control of the GLUT-1 isoform of the glucose transporter and that plasma membrane GLUT-4 protein in the absence of insulin or muscle contraction is relatively inactive. This is supported by the recent finding that no difference exists in GLUT-1 protein levels between lean and obese Zucker rats (21). Furthermore, the hypothesis that GLUT-4 protein is relatively inactive in the basal state agrees with recent results in adipocytes showing that GLUT-4 transporters that are not transporting glucose may be in close association with the plasma membrane (29, 34). The finding that isoproterenol can inhibit insulin-stimulated glucose transport in adipocytes without altering glucose transporter distribution also supports this hypothesis (34).

Insulin stimulation produced a significant increase in plasma membrane GLUT-4 protein concentration in lean but not in obese rats. The actual difference in magnitude of GLUT-4 protein translocation between lean and obese rats was ~40%. However, because of the initial differences in basal plasma membrane GLUT-4 protein concentrations, the lean rats had plasma membrane GLUT-4 protein levels that were 74% greater than obese rats after insulin stimulation. Western blot analysis of basal microsomal membranes from lean and obese rats showed no differences in GLUT-4 protein concentration. Insulin stimulation resulted in a significant 27% decline in GLUT-4 protein concentration in lean microsomal membranes; however, microsomal membranes from obese insulin-stimulated rats only showed a nonsignificant 11% decline in GLUT-4 protein concentration. Although the GLUT-4 microsomal responses are compatible with the GLUT-4 plasma membrane responses, the difference in microsomal GLUT-4 protein response between lean and obese rats should be viewed with caution due to differences in protein recovery in the microsomal fractions. These results, however, suggest that the insulin resistance of the obese rat is due in part to a deficient amount of GLUT-4 protein associated with the plasma membrane after insulin stimulation. This appears to be the consequence of an abnormally low basal plasma membrane GLUT-4 protein concentration as well as a defect in insulin-stimulated translocation of GLUT-4 protein from its intracellular storage site to the plasma membrane. The nature of the defect in transloca-

tion is unknown but could involve a defect in the transduction of the hormone signal from the insulin receptor (3), the transporter transit system, or vesicle fusion process.

Examination of the functional activity of GLUT-4 protein in the plasma membrane as assessed by the ratio of glucose uptake to plasma membrane GLUT-4 protein content (Table 4) indicated that in addition to a defect in GLUT-4 protein translocation, there was also a defect in the ability of insulin to activate or increase the functional activity of the GLUT-4 protein at the plasma membrane of obese rats. These results differ from those of King et al. (21), who measured plasma membrane glucose transporter turnover number as a marker of transporter intrinsic activity in isolated plasma membrane vesicles of lean and obese Zucker rats. In agreement with this study, these authors found that the obese rat had an impairment in insulin-stimulated glucose transporter translocation; however, in contrast with our results, the ability of insulin to increase glucose transporter activity was the same in their lean and obese rats. The differences in the results of the present study and those of King et al. (21) are unclear but could be related to the fact that the glucose uptake measurements in the study by King et al. (21) were made in isolated membrane vesicles *in vitro*, whereas in the present study glucose uptake was measured *in situ* during hindlimb perfusion. This would suggest that factors that modulate the functional activity of the GLUT-4 protein may be removed during the membrane isolation procedure. Nevertheless, the results of the present study, in agreement with the results of King et al. (21), provide good evidence that insulin-stimulated GLUT-4 protein translocation is defective in the muscle of the obese rat and that glucose uptake is a multistep process, involving both GLUT-4 translocation and activation.

Like insulin, muscle contraction is believed to stimulate skeletal muscle glucose uptake by causing the translocation of GLUT-4 protein to the plasma membrane, although muscle contraction is thought to stimulate translocation through a mechanism separate from that of insulin (10, 11, 15, 16, 18, 27). In agreement with recent results from our laboratory (7), the present results indicate that contraction-stimulated glucose uptake was not different between the lean and obese rats. These results provide strong evidence that contractile activity stimulates skeletal muscle glucose uptake through a pathway that is distinct from that of insulin. Furthermore, contractile activity caused a similar translocation of GLUT-4 protein to the plasma membrane in both lean and obese rats. However, due to a reduced basal plasma membrane GLUT-4 protein concentration, the plasma membrane GLUT-4 protein concentration of contraction-stimulated obese rats was 27% lower than lean rats. This difference, however, was marginal, and the ratio of glucose uptake to plasma membrane GLUT-4 protein concentration was not different between the lean and obese rats (Table 4). These results suggest that contraction-stimulated GLUT-4 protein translocation and activation are normal in the skeletal muscle of the obese Zucker rat.

Although muscle contraction increased GLUT-4 protein translocation to the plasma membrane, the exact location of the intracellular pool of contraction-sensitive GLUT-4 transporters is still in question (11, 15, 16). In the present study, contraction stimulation did not result in a significant decline in microsomal GLUT-4 protein concentration in lean or obese rats. This is in agreement with the results of Douen et al. (10, 11), who demonstrated that exercise did not result in a decline in microsomal GLUT-4 protein concentration in skeletal muscle of normal rats. Furthermore, recent results from our laboratory have shown that muscle contraction via neural stimulation did not result in a decline in microsomal GLUT-4 protein concentration in normal rats (12). These results provide evidence for the existence of two intracellular pools of GLUT-4 protein in mammalian skeletal muscle, one sensitive to mobilization by muscle contraction and one sensitive to mobilization by insulin.

In contrast with these findings, Fushiki et al. (14), using the membrane preparation of Klip et al. (23), and Goodyear et al. (15), using the same membrane isolation technique as was used in this study, found that an acute exercise bout resulted in a decrease in intracellular GLUT-4 protein concentration, concurrent with an increase in plasma membrane GLUT-4 protein. A possible explanation for the difference in findings could be the different procedures used for the activation of muscle contraction. Fushiki et al. (14) and Goodyear et al. (15) used acute treadmill exercise instead of electrical stimulation to induce muscle contraction. Furthermore, the rats in the study by Goodyear et al. (15) were examined in the postprandial state when plasma insulin levels would have been elevated, whereas, in the present study, muscle contraction was induced during hindlimb perfusion in the absence of insulin. Acute exercise has been shown to increase insulin sensitivity, and the effects of insulin and muscle contraction have been shown to have an additive effect on skeletal muscle glucose uptake (8, 27). Therefore, it is possible that during the studies of Fushiki et al. (14) and Goodyear et al. (15) the presence of insulin during exercise could have had an effect on intracellular GLUT-4 protein translocation.

Other possible reasons for the differences in results could be due to differences in membrane preparations and the length of time the muscle was exposed to stimulation. This last possibility could account for the different findings of Douen et al. (10, 11) and Fushiki et al. (14), who exercised their rats in the fasted state and used similar membrane isolation procedures. However, Douen et al. (10, 11) exercised their rats for 45 min, whereas the rats of Fushiki et al. (14) were exercised for 2 h.

In summary, it was found that unlike insulin-stimulated glucose uptake, contraction-stimulated glucose uptake was not impaired in the muscle of the obese Zucker rat. The muscle insulin resistance in the obese rat was associated with a reduced plasma membrane GLUT-4 protein concentration and the inability of insulin to activate or increase the functional activity of the GLUT-4 transporter. Muscle contraction increased

GLUT-4 protein translocation and functional activity to a similar extent in lean and obese rats. Finally, insulin, but not muscle contraction, reduced the intracellular GLUT-4 protein concentration, suggesting that there exists insulin-sensitive and contraction-sensitive pools of GLUT-4 protein in mammalian skeletal muscle.

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Present address of J. T. Brozinick, Jr.: National Inst. of Health, Bldg. 10, Rm. 5N102, Bethesda, MD 20892.

Address for reprint requests: J. L. Ivy, Dept of Kinesiology, Bellmont Hall 222, Univ. of Texas, Austin, TX 78712.

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