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DIVERGENCE BETWEEN GLUT4 mRNA AND PROTEIN ABUNDANCE IN SKELETAL MUSCLE OF INSULIN RESISTANT RATS

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Summary: Hyperglycemia and skeletal muscle insulin resistance coexist in uncontrolled type 2 diabetes mellitus. Similar defects in insulin action were observed in glucose-infused, normal rats, a model of glucose toxicity. In these rats insulin-stimulated glucose uptake by skeletal muscle was decreased due to a post-receptor defect. We investigated whether the impaired glucose uptake resulted from a decrease in the abundance of the predominant muscle glucose transporter (GLUT4) mRNA and/or protein. GLUT4 protein abundance in the hyperglycemic rats was not different from the control group despite a 50% decrease in muscle glucose uptake. GLUT4 mRNA abundance was 2.5-fold greater in the hyperglycemic rats as compared to the control animals. We conclude that the coexistence of hyperglycemia and hyperinsulinemia results in (1) a defect in GLUT4 compartmentalization and/or functional activity and (2) a divergence between GLUT 4 mRNA levels and translation.

The coexistence of hyperglycemia and skeletal muscle insulin resistance is well-documented in patients with uncontrolled non-insulin-dependent diabetes mellitus (NIDDM) (1,2). This relationship has generated the concept of glucose toxicity whereby a high plasma glucose concentration impairs insulin action in peripheral tissues (3,4). We have documented that glucose infusion in normal, intact rats reproduces many of the defects in insulin action observed in humans with uncontrolled NIDDM (5). In these rats, insulin-stimulated muscle glucose uptake is reduced by 50% at both physiologic and pharmacologic insulin levels.

Muscle glucose uptake occurs via facilitative diffusion mediated by a specific transport protein. The glucose transport protein exists as a family of protein isoforms with differential tissue distribution (6,7). Two of these isoforms, GLUT1 and GLUT4, exist in skeletal muscle. GLUT1 is a ubiquitous isoform occurring in low abundance in skeletal muscle. Recent evidence suggests that it may be localized to the perineural sheath rather than within the muscle fiber, *per se* (8). GLUT4 is the predominant muscle isoform and both its functional activity and expression are regulated by insulin (9-12). Reduced abundance of GLUT4 protein in muscle has been reported in one study of obese patients with or without NIDDM (13) but was not observed in another study on patients with obesity or NIDDM (14). Antithetical data has also been reported in studies on animal models of impaired insulin action. Muscle GLUT4 protein abundance does not change in

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spontaneously obese (db/db) insulin resistant mice (15). GLUT4 mRNA and protein levels decrease in muscle of rats treated with streptozotocin (16).

We investigated whether the reduced insulin-stimulated glucose uptake that we observe in muscle of hyperglycemic-hyperinsulinemic rats resulted from a decrease in the abundance of GLUT4 mRNA and/or protein. The GLUT4 mRNA was increased 2.5-fold with no change in the GLUT4 protein abundance in muscle of the insulin resistant rats as compared to control animals.

Materials and Methods

Materials. Male Sprague-Dawlely rats were purchased from Harlan, Madison, WI. Rnase A, RNase T1, and mineral oil were purchased from Sigma Chemical Company, St. Louis, MO. Ribonucleotides and One-Phor-All Plus buffer were purchased from Pharmacia, Sweden. Molecular biology grade formamide and Whatman glass fiber filters (GF/C) were purchased from Fisher Scientific, Pittsburgh, PA. Herring sperm DNA, RNasin, SP6 and T7 RNA polymerases, and pGEM3Z were purchased from Promega, Madison, WI. The plasmid containing GLUT1 cDNA, pSGT, was purchased from the American Type Culture Collection, Rockville, MD. The plasmid containing a GLUT4 insert was kindly provided by Graeme I. Bell, Howard Hughes Medical Institute, University of Chicago, Chicago, IL. NucTrap push columns were purchased from Stratagene, LaJolla , CA. [^{32}P]CTP was purchased from Dupont-NEN, Boston, MA. Antisera to GLUT1 and GLUT4 were purchased from East Acres Biologicals, Southbridge, MA. [^{125}I]Protein A and nitrocellulose membrane (Hybond C) were purchased from Amersham, Arlington Heights, IL. The bicinchoninic acid protein assay reagents were purchased from Pierce Chemicals, Rockford, Il.

Animal and Tissue Preparation. Age-matched male Sprague-Dawley rats (200-240 g) were individually housed in metabolic cages under a 12:12 h light-dark cycle and were given food and water ad libitum. Indwelling jugular catheters were implanted. Following 2-3 d for recovery, the rats were continuously administered 50% glucose (60-66 mg/kg/min) or saline via the indwelling jugular catheter for 72 h (17,18). The rats were then anesthetized with pentobarbital sodium (50 mg/kg via the venous catheter). Venous blood was sampled from the vena cava and the vena cava was excised. Hindlimb muscle was removed. Muscle used to prepare total RNA was quickly trimmed of visible fat and connective tissue, frozen in liquid nitrogen, and stored at -80 C until processed. Muscle used to prepare the total membrane fraction was placed on ice until processing.

RNA Extraction. The frozen muscle was powdered using a stainless steel mortar and pestle chilled in liquid nitrogen. Total RNA was extracted from the frozen muscle powder (19) using a single-step method (20). All solutions used in the extraction of total RNA were made with 0.01% diethylpyro-carbonate-treated water. An ethanol precipitate containing total RNA was collected by centrifugation, mixed with 4 M lithium chloride and re-centrifuged to remove glycogen which interfered with subsequent extraction steps (21). The final total RNA extract was dried and then dissolved in diethylpyrocarbonate-treated water. RNA concentration was determined by measuring the absorbance of the solution at 260 nm. The RNA solutions were stored at -80 C until analyzed.

Glucose Transporter mRNA Analysis. GLUT1 and GLUT4 mRNA abundance in total RNA from muscle was measured using quantitative solution hybridization/RNase protection assays (22). GLUT1 or GLUT4 synthetic mRNA (0-8.3 fg/μl) or samples of total muscle RNA (0-660 ng/μl) were hybridized with [^{32}P]-cRNA probes for GLUT1 or GLUT4 (100,000 cpm/tube) in a solution containing 40 mM Tris (pH 7.5), 4 mM EDTA, 40% formamide, and 0.6 M NaCl in a total volume of 30 μl. Following hybridization (16 h, 68 C), the hybridization mixture was treated with RNase A (20 μg) and RNase T1 (1 μg) for 1 h at 37 C. The protected RNA (double-stranded cRNA-mRNA) was precipitated with 6 M trichloroacetic acid using herring sperm DNA as a carrier and the precipitate was collected on glass fiber filters. The filters were washed with 3% trichloroacetic acid containing 1% sodium pyrophosphate, rinsed with 95% ethanol, and dried. Radioactivity on the filters was determined using liquid scintillation spectroscopy. GLUT1 or GLUT4 mRNA abundance (pg/μg total RNA) in the muscle sample was calculated from a standard curve constructed using GLUT1 or GLUT4 synthetic mRNA standards hybridized and processed using the same conditions as the unknown samples.

Membrane Isolation. A total muscle membrane fraction was prepared by mincing muscle with scissors in a 100mM Tris (pH 7.6) solution containing 255 mM sucrose, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1000 U/ml aprotinin followed by homogenization using a Polytron (setting 3, two 30 s bursts). The homogenate was centrifuged at 8000 g (10 min; 4 C). The resulting supernatant was centrifuged at 200,000 g (90 min;4 C). The membrane pellet was dissolved in homogenization buffer containing 1% triton. Protein concentration was determined using the bicinchoninic acid assay (23).

Glucose Transporter Protein Analysis. Membrane proteins were separated on an 11% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (24). A rat heart total membrane standard was run on each gel and used to correct for variations in the western blots. The proteins were then electrophoretically transferred to a nitrocellulose sheet. After blocking non-specific sites on the sheet (2 h at 22 C in blocking buffer containing Tris-buffered saline (pH 7.4), 5% non-fat dry milk, 0.2% triton, and 0.02% sodium azide), the sheet was incubated with antisera against GLUT4 (1:250) or GLUT1 (1:2500) for 16 h at 4 C. The blot was washed in Tris-buffered saline containing 0.2% Triton, incubated with [^{125}I]-protein A (40,000 cpm/ml blocking buffer) for 2 h at 22 C, rewashed as above, and air dried. An autoradiogram of each blot was used to localize labeled bands. These were cut from the blot and counted in gamma counter. The counts per band were normalized for total membrane protein loaded per gel lane.

Statistics. All results are presented as means ± SE. Statistical comparisons between means were made using Student's *t* test for unpaired data (25). Differences between means were judged to be significant at $P<0.05$.

Results

Plasma glucose and insulin concentrations were similar in the two groups of rats prior to the saline or glucose infusions (Table 1). After 72 h, the plasma glucose and insulin concentrations were significantly elevated in the glucose-infused rats as compared to the saline-infused animals. The body weight of the two groups was not significantly different at the beginning of the infusions.

GLUT4 mRNA abundance was significantly elevated in the glucose-infused animals (Fig. 1). GLUT 1 mRNA abundance was unchanged in the two groups; averaging 1.09±0.14 and 1.02±0.07 pg/μg total RNA (mean±SE, n=16) in the saline- and glucose-infused groups,

TABLE 1. Physiological values from saline control and glucose-infused rats

Parameter	0 h	72 h
Body weight, g		
Saline control	263±5	258±6
Glucose infused	280±10	267±9
Plasma glucose, mM		
Saline control	9.7±0.3	8.7±0.3
Glucose infused	9.4±0.4	14.9±0.8*†
Plasma Insulin, pM		
Saline control	271±47	247±47
Glucose infused	258±40	1633±190*†

Values are means±SE of 9 animals/group. *$P<0.001$ as compared to saline control, same time. †$P<0.001$ as compared to glucose infused, 0 h.

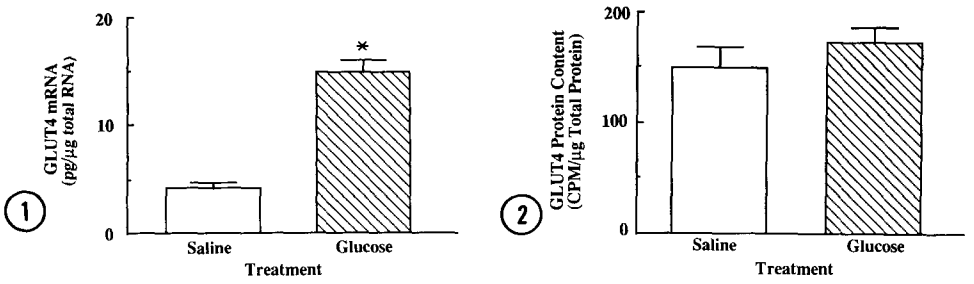

FIG. 1. GLUT4 mRNA abundance was increased by 72 h glucose infusion. After 72 h of glucose (60-66 mg/kg/min; hatched bar) or saline (open bar) infusion, total RNA was extracted from hindlimb skeletal muscle. The GLUT4 abundance (pg/μg total RNA) was quantitated using solution hybridization. Values are means ± SD (n = 8 rats per group). *P<0.001 as compared to the saline infused group.

FIG. 2. GLUT4 protein abundance was unchanged following 72 h glucose infusion. After 72 h of glucose (60-66 mg/kg/min; hatched bar) or saline (open bar) infusion, a total membrane fraction was prepared from hindlimb muscle. Relative GLUT4 protein abundance (CPM/ total protein) was determined using a western blotting protocol and detection of GLUT4 using a specific anti-serum. Values are means ± SE (n = 8 rats per group).

respectively. Total RNA extraction was not significantly different in the two groups (303±33 versus 243±33 μg/g tissue powder in the saline- and glucose-infused rats, respectively; n=16).

The abundance of GLUT4 protein was not significantly different in the two groups (Fig. 2). GLUT1 protein abundance was similar in the glucose-and saline-infused groups. Total membrane protein averaged 3.0±0.4 and 3.0±0.2 mg/g wet weight (mean±SE; n=16) in the saline- and glucose-infused rats, respectively.

Discussion

Combined hyperglycemia and hyperinsulinemia impairs insulin-stimulated glucose uptake in muscle by inducing a functional defect in insulin action. This conclusion is base on our observation that there was no difference in the abundance of GLUT4 protein in muscle from saline- and glucose-infused rats. Because the abundance of the protein did not change, the impaired insulin-stimulated glucose uptake could result from a defect in insulin-mediated translocation of GLUT4 from an intracellular membrane pool to the plasma membrane or in insulin-mediated activation of GLUT4. Alternatively, hyperglycemia plus hyperinsulinemia might interfere with signal transduction at the insulin receptor. Further studies are required in order to identify the cellular mechanism(s) involved.

Our results contrast with another animal model of insulin resistance, the hyperinsulinemic rat. In these animals, four days of insulin infusion results in decreased insulin-stimulated glucose utilization by muscle and in decreased GLUT4 protein abundance (26). The plasma insulin concentration in the hyperinsulinemic rats is in the range of the 72h plasma insulin level in the glucose-infused rats (Table 1), averaging 1380±90 pM and 1633±190 pM, respectively. The plasma glucose concentration in the hyperinsulinemic rats averages 6.3±0.3 mM as compared to 14.9±0.8 mM in the 72h glucose-infused rats. Cumulative glucose uptake into muscle would be expected to be much greater in the hyperglycemic-hyperinsulinemic rats than in the hyperinsulinemic rats. This difference in net glucose uptake could explain the maintenance of

GLUT4 protein abundance. The increased intracellular concentration of glucose or of a glucose metabolite might prevent the decrease in GLUT4 initiated by hyperinsulinemia alone.

Hyperglycemia in the presence of hyperinsulinemia causes a divergence between steady-state GLUT4 mRNA levels and translation. The increase in GLUT4 mRNA could result from increased transcription or from increased stability of GLUT4 mRNA along with a partial block in GLUT4 mRNA translation. Because we quantitated total GLUT4 mRNA, increased transcription combined with a partial block in transport of the mRNA from the nucleus into the cytoplasm could also explain the increased GLUT4 mRNA abundance with no change in GLUT4 protein abundance that we observed. Alternatively, GLUT4 protein degradation might be increased along with increased transcription and translation. This would result in an increase in GLUT4 mRNA with no net change in GLUT4 protein abundance.

In the insulin resistant, hyperinsulinemic rat, GLUT4 mRNA abundance decreases in parallel with the decline in insulin-stimulated glucose utilization and GLUT4 protein abundance (26). The decrease in GLUT4 mRNA abundance may be a direct effect of the increased plasma insulin concentration. For example, refeeding of fasted rats returns plasma insulin concentration to control levels (27) and the muscle GLUT4 mRNA abundance decreases from the increased level observed in fasted rats (28). GLUT4 mRNA abundance is also reduced in L_6 cells incubated in the presence of 100 nM insulin for 24 h (29). The increase in GLUT4 mRNA abundance observed in the present study may result from an increase in the intracellular concentration of glucose or a glucose metabolite opposing the decrease in GLUT4 mRNA abundance in response to hyperinsulinemia alone. Glucose or a glucose metabolite could directly or indirectly increase transcription or stabilize GLUT4 mRNA and alter GLUT4 mRNA translation leading to increased GLUT4 mRNA with no change in GLUT4 protein.

The present study supports the concept that insulin resistance can result from a functional defect in the glucose transport system and may not require a decrease in the abundance of GLUT4 protein or mRNA. Whether the defect occurs in insulin signal transduction, in insulin-mediated GLUT4 translocation to the plasma membrane, or in activation of GLUT4 by insulin remains to be identified. This study coupled with results from studies on fasted,refed rats (28), on streptozotocin treated rats (16), and on hyperinsulinemic rats (26) suggests that insulin resistance probably occurs through multiple mechanisms involving transcriptional, translational, or post-translational defects in insulin regulation of GLUT4. The results reported here also suggest that glucose and insulin may have differential effects on the expression and functional activity of GLUT4 in vivo.

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