Insulin-mediated translocation of GLUT-4-containing vesicles is preserved in denervated muscles

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Zhou, Min, Gino Vallega, Konstantin V. Kandror, and Paul F. Pilch. Insulin-mediated translocation of GLUT-4-containing vesicles is preserved in denervated muscles. Am J Physiol Endocrinol Metab 278: E1019–E1026, 2000.—Skeletal muscle denervation decreases insulin-sensitive glucose uptake into this tissue as a result of marked GLUT-4 protein downregulation (~20% of controls). The process of insulin-stimulated glucose transport in muscle requires the movement or translocation of intracellular GLUT-4-rich vesicles to the cell surface, and it is accompanied by the translocation of several additional vesicular cargo proteins. Thus examining GLUT-4 translocation in muscles from denervated animals allows us to determine whether the loss of a major cargo protein, GLUT-4, affects the insulin-dependent behavior of the remaining cargo proteins. We find no difference, control vs. denervated, in the insulin-dependent translocation of the insulin-responsive aminopeptidase (IRAP) and the receptors for transferrin and insulin-like growth factor II/mannose 6-phosphate, proteins that completely (IRAP) or partially co-localize with GLUT-4. We conclude that 1) denervation of skeletal muscle does not block the specific branch of insulin signaling pathway that connects receptor proximal events to intracellular GLUT-4-vesicles, and 2) normal levels of GLUT-4 protein are not necessary for the structural organization and insulin-sensitive translocation of its cognate intracellular compartment. Muscle denervation also causes a twofold increase in GLUT-1. In normal muscle, all GLUT-4 is present at the cell surface, but in denervated muscle a significant fraction (~25.1 ± 6.1%) of this transporter is found in intracellular vesicles that have the same sedimentation coefficient as GLUT-4-containing vesicles but can be separated from the latter by immunoadsorption. These GLUT-1-containing vesicles respond to insulin and translocate to the cell surface. Thus the formation of insulin-sensitive GLUT-1-containing vesicles in denervated muscle may be a compensatory mechanism for the decreased level of GLUT-4.

denervation; rats; skeletal muscle

SKELETAL MUSCLE IS THE MAJOR SITE FOR INSULIN-MEDIATED GLUCOSE DISPOSAL IN HUMANS (7) AND RODENTS (25). The failure of normal insulin levels to stimulate muscle glucose uptake, i.e., insulin resistance, is a major contributor to or cause of type II diabetes mellitus (16, 41). The glucose transporter isoform GLUT-4 is the prime mediator of postprandial muscle glucose uptake (4, 6, 36). GLUT-4 resides in intracellular membrane vesicles in the basal state, and these are translocated to the sarcolemma and T-tubules in response to insulin, thus accounting for the increased clearance of glucose into this tissue (8, 24, 35, 37, 47). In addition to GLUT-4, there is a small amount of GLUT-1 expressed in skeletal muscle, most or all of which is permanently present in the sarcolemma (and/or T-tubules) where it mediates basal glucose transport (12, 47). The molecular basis for insulin-stimulated vesicle movement remains incompletely understood (6) as does the cause for most cases of insulin resistance (16, 41). However, rat hindlimb denervation leads to a type of insulin resistance as a result of marked GLUT-4 downregulation, despite a slight increase in the levels of muscle GLUT-1 (1, 5, 13, 14). Therefore, this model of insulin resistance can be useful in understanding insulin-regulated glucose transport under conditions of altered transporter expression.

In addition to GLUT-4, a number of cargo proteins have been identified as being partially co-localized with GLUT-4 in intracellular vesicles from rat adipocytes, and they translocate to the cell surface in response to insulin. Among these are the insulin-responsive aminopeptidase (IRAP) (20, 23) and sortilin (28, 34), which were identified by microsequencing isolated vesicle proteins after their immunoadsorption with anti-GLUT-4 antibody. The insulin-like growth factor II/mannose 6-phosphate (IGF-II/Man-6-P) and transferrin receptors were identified immunologically (21, 22), as were several other proteins involved in membrane trafficking and fusion, such as secretory component-associated membrane proteins (SCAMPs) (27, 44) and vesicle-associated membrane proteins (VAMPs) (3). We have confirmed that all of the above-named proteins are co-localized with GLUT-4 in vesicles from skeletal muscle (18, 48) and that the sedimentation behavior of vesicles from fat and muscle are identical (18), suggesting that there are no major differences in the composition or trafficking behavior of GLUT-4 in these two tissues.

As noted above, in hindlimb skeletal muscle from the rat, GLUT-4 mRNA and protein expression decrease dramatically after 3 days of denervation. However, there are no notable changes in the levels of other component proteins of GLUT-4-containing vesicles such as IRAP, the transferrin receptor, the IGF-II/Man-6-P receptor, SCAMPs, and VAMP 2 (48). These proteins are still co-localized in the same membrane compart-
ments as in normal extensor digitorum longus (EDL) muscle as determined by immunoadsorption. Thus the denervated muscle represents a type of tissue-specific knockout (or marked reduction) of GLUT-4 and allows us to determine whether this causes an alteration in the behavior of the GLUT-4-deficient vesicles. Here, we show that there is no detectable change in the insulin response of the remaining proteins. Moreover, GLUT-1 shows intracellular sequestration and a modest amount of insulin-responsive translocation to fractions corresponding to the cell surface in apparent compensation for the reduced GLUT-4 levels.

**EXPERIMENTAL PROCEDURES**

**Animals.** Male Sprague-Dawley rats (150–175 g) were purchased from Taconic Breeding Laboratory (Germantown, NY). The animals were fasted overnight and then injected with insulin (1.5 units/animal) or with the buffer alone via the portal vein 12 min before being killed, as previously described (5). All rats were anesthetized with pentobarbital sodium (60 mg/kg body wt) by intraperitoneal injection. The EDL muscle was denervated by sciatic nerve section 3 days before muscle isolation and fractionation. For the sham operation, the sciatic nerve was visualized but not touched.

**Antibodies.** We used monoclonal anti-GLUT-4 antibody 1F8 (17), monoclonal anti-SCAMPs antibody (44), monoclonal anti-IRAP antibody (Zymed Laboratory), monoclonal antidiarylpropyridine-receptor antibody (a kind gift of Dr. Kevin Campbell, University of Iowa), polyclonal anti-GLUT-1 antibody (a kind gift of Dr. M. Czech, University of Massachusetts Medical School), polyclonal anti-GLUT-1 antibody (a kind gift of Dr. R. Jahn, Yale University School of Medicine), O-dithylylaminoethyll (DEAE)-cellulose purified anti-IGF-II/Mankan-6-P receptor polyclonal antibody (a kind gift of Dr. M. Czech, University of Massachusetts Medical School), monoclonal anti-GLUT-1 antibody (a kind gift of Dr. C. Carter-Su, University of Michigan), and monoclonal anti-β1 integrin antibodies (a kind gift of Dr. Carles Enrich, Universitat de Barcelona). Polyclonal anti-IRAP antibody was generated by QCB (Hopkinton, MA) in rabbits against University de Barcelona). Polyclonal anti-IRAP antibody was generated by QCB (Hopkinton, MA) in rabbits against

**Subcellular fractionation of skeletal muscle.** Rat EDL muscle (5 muscles/experimental condition) was fractionated as we have described (48). Briefly, after cell homogenization and removal of the nonhomogenized tissue by low-speed centrifugation, we carry out a spin at 9,000

**Gel electrophoresis and immunoblotting.** Protein samples were electrophoresed according to Laemmli (26) and transferred to an Immun-Blot polyvinylidene fluoride membrane (Bio-Rad). After transfer, membranes were blocked with 10% nonfat dry milk in PBS for 1 h at room temperature and incubated with specific antibodies. Secondary antibodies (Sigma) were conjugated to horseradish peroxidase. Blots were developed by means of an enhanced chemiluminescence detection system (Du Pont NEN), and films were scanned with a computing densitometer (Molecular Dynamics) for quantitative analysis.

**RESULTS**

GLUT-4-vesicles translocate to the cell surface in response to insulin in control and denervated EDL muscle as detected by subcellular fractionation. Three days after denervation of the sciatic nerve, rats were injected with insulin or buffer via the portal vein. Control and denervated EDL muscles from the same rat were removed 12 min after injection and fractionated, as described in **EXPERIMENTAL PROCEDURES**. As we have previously demonstrated, the intracellular pools of recycling proteins are present in the gradient fractions, whereas pellets P1 and P2 contain the cell surface domains (plasma membrane and T-tubules).
where GLUT-4 and other proteins are translocated on insulin stimulation. As shown in Fig. 1, GLUT-4 protein expression in denervated EDL muscle is decreased in intracellular membrane vesicles (gradient fractions) and in all surface membrane (P1 and P2) compared with controls, which is consistent with the published data from our own and other laboratories cited in the introductory section. The sedimentation coefficient of GLUT-4-containing vesicles is not changed after denervation. Interestingly, the effect of denervation on movement of GLUT-4 to the P2 fraction appears greater than that for the P1 fraction for unknown reasons.

Insulin administration causes a profound decrease in GLUT-4 content in the fractions of the gradient and a corresponding enrichment in P1 and P2 in both control and denervated EDL muscle (Fig. 1). A similar effect of insulin can also be seen in the case of other major component proteins of GLUT-4-vesicles, IRAP, the transferrin receptor, and the IGF-II/Man-6-P, receptor (Fig. 2). As we have previously shown, almost all IRAP and a considerable amount of the two receptors co-localize with GLUT-4 in normal muscle, and they move to the surface membranes in response to insulin (48). Thus the data of Fig. 2 suggest that, in denervated EDL muscle, insulin can induce translocation of the GLUT-4-containing vesicular compartment to the cell surface despite the reduced amount of GLUT-4.

GLUT-4-vesicles translocate to the cell surface in response to insulin in control and denervated EDL muscle as detected by cell surface biotinylation. Cell impermeable biotinylation reagents, such as sulfo-NHS-biotin and cleavable sulfo-NHS-SS-biotin, have been used for the analysis of protein traffic in insulin-sensitive tissues in our and other laboratories (19, 39). These reagents react with lysine residues and/or amino termini in the extracellular domains of cell surface proteins, and they do not react with intracellular proteins unless the latter move to the cell surface. Thus increased biotinylation of an intracellular membrane protein, stimulated by insulin, for example, strongly indicates its translocation to the site accessible for biotinylation, i.e., the cell surface. Thus control and denervated EDL muscles were incubated with sulfo-NHS-SS-biotin in the absence and in the presence of insulin and were then processed for electrophoresis, as described in EXPERIMENTAL PROCEDURES. As shown in Fig. 3A, insulin treatment of EDL muscle increases the specific biotinylation of the transferrin receptor by 48 ± 11% in control EDL and 51 ± 8.3% in denervated EDL. Insulin also stimulated biotinylation of IRAP by 26 ± 4.2% in control EDL and 33 ± 7.1% in denervated EDL. These data confirm the results of Fig. 2 and document that insulin's effect on translocation of IRAP and the transferrin receptor is not changed in denervated EDL compared with normal muscle. We could not use this approach in the case of GLUT-4 and the IGF-II/Man-6-P receptor, because GLUT-4 is biotinylated to a very low degree, most probably because there is only one lysine in its extracellular portion that is minimally accessible for reaction, whereas antigenicity of the IGF-II/Man-6-P receptor was destroyed by 2-mercaptoethanol.

Formation of intracellular GLUT-1-containing vesicles in denervated EDL muscle. As noted in the introductory section, it has previously been shown that the expression of GLUT-1, unlike that of GLUT-4, is increased in denervated muscles. However, the subcellular distribution of the de novo synthesized GLUT-1 protein has not been addressed in these previous studies. Figure 4A demonstrates that, in control EDL muscle, GLUT-1 is present almost exclusively at the cell surface. In denervated EDL, the amount of GLUT-1 is greatly increased, and a significant fraction of these transporters (25.1 ± 6.1%) is subsequently found in the part of the gradient corresponding to intracellular vesicles. These data suggest that GLUT-1 may be targeted to a specific intracellular vesicular compartment in denervated muscles that does not exist in normal muscle. As is the case for
fat cells (18), GLUT-1-containing vesicles have a sedimentation coefficient indistinguishable from GLUT-4-containing vesicles (Fig. 4B). However, when we performed immunoabsorption with anti-GLUT-4 monoclonal antibody 1F8, we found that GLUT-1 is largely excluded from GLUT-4-containing vesicles. As shown in Fig. 5, under conditions when 88 ± 4.1% of GLUT-4 is immunoabsorbed, no GLUT-1 is found in the eluate from immunobeads.
We thus conclude that in denervated EDL muscle, GLUT-1 is not targeted to GLUT-4-containing vesicles, but rather to a different vesicular population.

Insulin causes translocation of GLUT-1-containing vesicles in denervated EDL muscle. We then asked whether or not these GLUT-1-containing vesicles respond to insulin in denervated EDL. Using the same centrifugation protocol for monitoring the translocation of GLUT-4-containing vesicles, we examined the translocation of GLUT-1-containing vesicles in the denervated EDL muscle. The results are presented in Fig. 6, where insulin stimulation is shown to cause a decrease of intracellular GLUT-1-containing vesicles and an increased level of GLUT-1 protein in the fractions corresponding to the cell surface. It is clear that these GLUT-1-containing vesicles respond to the signals generated by insulin. However, the extent of translocation of these vesicles is much less than that of GLUT-4-containing vesicles, and the nature and composition of the GLUT-1-containing vesicles remain to be determined.

**DISCUSSION**

We have previously demonstrated that denervation of hindlimb muscle causes a considerable downregulation of GLUT-4 protein, whereas the levels of expression of other component proteins of GLUT-4 vesicles remain the same (48). As is noted in the introductory section, many investigators have shown that GLUT-4 expression is downregulated by denervation and that insulin-sensitive glucose uptake is markedly attenuated (1, 5, 13, 14). The question remained, however, as to whether the lack of an insulin response in denervated muscle was due mainly to GLUT-4 downregulation (32) or whether there were signaling (see below) and/or vesicular trafficking defects as well. Thus we show that, in denervated EDL muscle, GLUT-4 vesicles maintain insulin responsiveness and are translocated to the cell surface after insulin administration. As previously suggested (13), the marked decrease in the GLUT-4 protein in denervated muscle may be partially compensated by increased expression of GLUT-1, which is expressed both at the cell surface and in intracellular vesicles that have a sedimentation coefficient indistinguishable from GLUT-4-containing vesicles but can be separated from the latter by immunoadsorption.

Thus we believe that the normal level of GLUT-4 is not necessary for insulin-sensitive translocation of its cognate intracellular compartment in skeletal muscle. This conclusion is consistent with our previous studies that show that, during adipocyte differentiation, the formation of insulin-responsive vesicles precedes expression of GLUT-4 (10). These data also suggest that the

![Fig. 3. IRAP and transferrin receptors translocate to cell surface in response to insulin in control and denervated EDL muscle, as revealed by in vitro cell surface biotinylation. Isolated EDL muscles from control and denervated animals were incubated with sulfo-NHS-SS-biotin in absence and in presence of insulin and were processed as in EXPERIMENTAL PROCEDURES. An aliquot of Triton X-100 supernatant from microcentrifuge spin (1 mg) was incubated with ImmunoPure Immobilized Streptavidin overnight at 4°C with rotating. Biotinylated proteins adsorbed by Streptavidin beads were eluted from them with Laemmli sample buffer containing 2-mercaptoethanol (2, 29). Eluates were analyzed by Western blot with antibodies to IRAP and transferrin receptor. A: Western blot analysis; B: quantitation of results, expressed as relative percentage with data from muscle without insulin treatment having assigned value of 100. Results are means ± SE of 3 separate experiments. *P < 0.05, significant difference from basal values according to Student's t-test.

![Fig. 4. Expression of GLUT-1 is upregulated in intracellular vesicles and at cell surface in denervated EDL muscle. Muscle membranes from control and denervated EDL from same rat were prepared and analyzed for GLUT-1 by Western blotting (see EXPERIMENTAL PROCEDURES). A: representative result of 3 independent experiments; B: quantitation of results with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. Squares, GLUT-4; circles, GLUT-1; filled, control EDL; open, denervated muscle. GLUT-4 distribution in gradients from control EDL is shown as reference. Results are expressed as relative percentage with data from control muscle having assigned value of 100. Results are means ± SE of 3 separate experiments. *P < 0.05, significant difference from basal values according to Student's t-test.

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The presence of GLUT-4 in vesicles may not be absolutely necessary for the formation and functioning of this compartment. Additional evidence for this conclusion comes from studies using transgenic animals, where GLUT-4-vesicles from fat were shown to accommodate much more GLUT-4 protein than in normal tissue without any major changes in their composition and insulin responsiveness (45). Thus the specific content of GLUT-4 protein in vesicles may vary to a considerable degree in both directions without major losses in physiological functions of this compartment.

Our results also indicate that the insulin signaling pathway that leads to translocation of GLUT-4 vesicles is not markedly affected by denervation, and the loss of GLUT-4 is, therefore, likely to be the major reason for reduced insulin-stimulated glucose uptake under this physiological condition. It has been shown, however, that insulin-dependent regulation of glycogen synthesis and amino acid transport is significantly impaired early after denervation (42, 43, 46). Those studies showed that the effect of denervation on these processes in skeletal muscle is dependent on muscle fiber type and time after denervation. Thus the reduced insulin-mediated glucose uptake due to the loss of GLUT-4 expression may contribute to the decreased insulin-stimulated glycogen synthesis seen in denervated muscle, but there are likely to be other defects in insulin action that are caused by denervation. Presently, it is not clear where insulin-dependent signal transduction might diverge to glycogen synthesis on the one hand and GLUT-4 translocation on the other. It is clear, however, that GLUT-4 translocation in skeletal muscle can be regulated by exercise independently of the insulin signaling pathway (11).

Basal glucose uptake in denervated muscles is increased, which is consistent with our data showing upregulation of GLUT-1 at the cell surface (Fig. 4).
significant fraction of this transporter (25.1 ± 6.1%) is, however, found in intracellular vesicles. In rat adipose tissues, unlike skeletal muscle, about half of GLUT-1 resides at the plasma membrane in the basal state with the rest of GLUT-1 being sequestered inside the cells where it can translocate to the cell surface in response to insulin (15, 49). The component proteins of these vesicles besides GLUT-1 are unknown; it is also unknown whether the GLUT-1-containing vesicles in denervated skeletal muscle are the same as those in adipocytes. However, because the GLUT-1-containing vesicles in denervated EDL are insulin responsive, they may be useful for studying signal transduction to a vesicle population in the absence of a large background of GLUT-4-containing vesicles.

As is noted several places elsewhere in this paper, GLUT-4 and GLUT-1 protein and mRNA levels are differentially affected by denervation. The transcriptional mechanisms that underlie this phenomenon remain unknown but may involve myocyte-specific enhancer factors in the case of GLUT-4 (30). During muscle development in vivo (40), as well as a function of the differentiation state of cultured muscle cell lines (33, 38), the expression of GLUT-1 decreases, and GLUT-4 concomitantly increases. In denervated muscle, the regulation of these two transporters is reversed; moreover, denervation is associated with the upregulation of several myogenic transcription factors as well as the α3-subunit of acetylcholine receptor, and these same proteins are downregulated during postnatal development (9). Thus denervation may induce partial dedifferentiation of muscle due to the absence of selective gene regulation by nerve stimuli and may represent an early event in 3T3-L1 adipocyte differentiation. Mol Biol Cell 10: 1581–1594, 1999.


REFERENCES


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