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Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level

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Glucose transport in skeletal muscle is mediated by two distinct transporter isoforms, designated muscle/adipose glucose transporter (Glut4) and erythrocyte/HepG2/brain glucose transporter (Glut1), which differ in both abundance and membrane distribution. The present study was designed to investigate whether differences in insulin responsiveness of red and white muscle might be due to differential expression of the glucose transporter isoforms. Glucose transport, as well as Glut1 and Glut4 protein and mRNA levels, were determined in red and white portions of the quadriceps and gastrocnemius muscles of male Sprague–Dawley rats (body wt. approx. 250 g). Maximal glucose transport (in response to 100 nM-insulin) in the perfused hindlimb was 3.6 times greater in red than in white muscle. Red muscle contained approx. 5 times more total Glut4 protein and 2 times more Glut4 mRNA than white muscle, but there were no differences in the Glut1 protein or mRNA levels between the fibre types. Our data indicate that differences in responsiveness of glucose transport in specific skeletal muscle fibre types may be dependent upon the amount of Glut4 protein. Because this protein plays such an integral part in glucose transport in skeletal muscle, any impairment in its expression may play a role in insulin resistance.

INTRODUCTION

Skeletal muscle comprises a large percentage of the body mass and is the primary tissue responsible for the disposal of an oral glucose load (Defronzo *et al.*, 1985). Skeletal muscle is composed of a mixture of different fibre types (Nemeth & Pette, 1981) which are generally classified by their contractile and metabolic characteristics, as well as by colour. Red muscle fibres can be either slow- or fast-contracting and have a high oxidative capacity, whereas white fibres are fast-contracting and glycolytic. Insulin sensitivity and the maximal rate of glucose transport are also different between red and white muscle fibres (James *et al.*, 1985; Richter *et al.*, 1988), with white muscle fibres being less sensitive and less responsive to insulin than red muscle fibres. Differences in glucose transport between skeletal muscle fibre types have been proposed to be an important factor responsible for various insulin-resistant states (Krotkiewski & Björntrop, 1986; Lillioja *et al.*, 1987). Elucidation of the mechanisms responsible for the differences in glucose transport between different skeletal muscle fibre types may help us to understand the mechanisms responsible for insulin-resistant states.

It is now recognized that glucose transport in skeletal muscle is mediated by two distinct isoforms, designated muscle/adipose glucose transporter (Glut4) and erythrocyte/HepG2/brain glucose transporter (Glut1). We have previously reported that glucose transport in human muscle strips incubated *in vitro* is impaired in obese individuals compared with lean controls, and this most probably plays an important role in the whole-body insulin resistance seen with obesity (Dohm *et al.*, 1988). Likewise, muscle from streptozotocin-diabetic rats has a reduced rate of glucose transport (Wallberg-Henriksson, 1987) and contains fewer glucose transporters than that from control animals (Ramlal *et al.*, 1989). Ramlal *et al.* (1989) concluded that this decrease in the number of glucose transporters is at least partially responsible for the decrease in glucose metabolism seen in the diabetic condition.

Because decreased insulin responsiveness of glucose transport observed in streptozotocin-induced diabetes is caused by decreased levels of the glucose transporter protein, we hypothesized that the mechanism responsible for the differences in red and white muscle glucose transport may also be due to different levels of the glucose transporter protein. We measured glucose transport and the levels of glucose transporter protein and mRNA in red and white skeletal muscle from rats.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats weighing approx. 250 g were used in all three phases of this experiment. Animals were housed individually, maintained on a 12 h light/dark cycle (light from 03:00 to 15:00 h) and provided with food and water *ad libitum*. Rats used for hindquarter perfusion were anaesthetized with Innovar-Vet (0.04 mg/100 g) and prepared for perfusion. Animals used for quantification of glucose transporter protein and mRNA isolation were killed after an overnight fast. Gastrocnemius and quadriceps muscles were quickly removed and separated by visual inspection into red and white portions, and were either quick-frozen in clamps cooled in liquid N_2 for glucose transporter protein determination or immediately homogenized for mRNA analysis.

3-*O*-Methylglucose transport into perfused hindquarter

The hemicorpus was prepared for perfusion using methods previously described (Dohm *et al.*, 1980). The perfusion medium (4% BSA, 1.0 mM-pyuvate, 30% washed bovine red blood cells and Krebs–Henseleit buffer) was gassed with O_2/CO_2 (19:1), and contained the appropriate insulin concentration. The flow rate was maintained at 18 ml/min. After discarding the first 50 ml of perfusate flowing through the animal, the remaining 100 ml volume was recirculated. At the end of a 30 min pre-perfusion period, 20 mM-sorbitol and 5.0 mM-3-*O*-methylglucose, containing 0.1 μCi of [U-^{14}C]sorbitol/ml and 2.0 μCi of 3-*O*-

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[^{3}H]methylglucose/ml (final concentrations) were added to the media. When no insulin was present in the perfusion medium, a 30 min perfusion was necessary to accumulate enough radioactivity to accurately measure transport rates. Perfusions were continued for only 10 min after the end of pre-perfusion when insulin was present. The red and white portions of the gastrocnemius and quadriceps muscles were quickly removed, frozen in liquid N_2, homogenized in 10% trichloroacetic acid, centrifuged and the supernatant counted for radioactivity. Accumulation of 3-*O*-[^{3}H]methylglucose was corrected for the [^{14}C]sorbitol space.

Quantification of glucose transporter protein

Approx. 100 mg of red or white quadriceps femorus muscle was homogenized (1:6, w/v) using a Polytron homogenizer at a setting of 7 for 15 s. Homogenization buffer contained 25 mM-Hepes, 4 mM-EDTA, 25 mM-benzamidine and 1 μM of each of leupeptin, pepstatin and aprotinin. Triton X-100 was added to a final concentration of 1%, then samples were incubated for 1.5 h at 4 °C and centrifuged in a Beckman 42.2 Ti rotor at 150000 *g* for 35 min. Supernatants were removed and protein content was determined (Pierce BCA). Laemmli sample buffer (50 μl) containing 5% dithiothreitol was mixed with 150 μl of supernatant protein and brought to a 100 μl volume with buffer containing 25 mM-Tris, 0.19 M-glycine and 1% SDS. SDS/PAGE (8% gels) was used to separate proteins (Laemmli, 1970), which were then transferred to Immobilon membranes by electrotransfer. The membrane was incubated in the primary antibody (2 μg of either ECU1 or ECU4/ml) followed by ^{125}I-labelled goat anti-(rabbit IgG). Autoradiography was carried out for 48 h and the resulting autoradiograph was analysed by densitometry. The polyclonal antibody ECU1 was raised in rabbits against the *C*-terminal peptide (prepared by Dr. Dave Klapper, UNC, Chapel Hill, CA, U.S.A.) of Glut1 by the method of Haspel *et al.* (1988). ECU4 was raised against the *C*-terminal peptide of Glut4 (James *et al.*, 1989) by a similar procedure. The antibodies were purified from rabbit serum by passage over a Protein A–Sepharose column and used directly. Verification of their specificity was obtained by comparison of these autoradiographs with those generated using the 1F8 (generously supplied by Dr. Paul Pilch, Boston University, Boston, MA, U.S.A.) and anti-(erythrocyte glucose transporter) antibodies (generously supplied by Dr. Christine Carter-Su, University of Michigan, Ann Arbor, MI, U.S.A.).

Isolation of RNA

RNA was isolated by the method of Chirgwin *et al.* (1979). Briefly, red or white muscle samples were removed from the animal, weighed and homogenized in tissue guanidinium isothiocyanate. This crude preparation was spun, and the supernatant was removed, heated and CsCl added. This solution was then layered over 9 ml of a 5.7 M-CsCl solution and spun overnight. The supernatant was removed, the pellet was resuspended and RNA was extracted. After quantification, RNA species were separated on an agarose/formaldehyde gel and blotted on to Hybond N filters (Amersham). Northern analysis was performed using the cDNA inserts for B-actin (Cleveland *et al.*, 1980) as a control, and Glut1 (HepG2/erythrocyte) and Glut4 (heart/skeletal muscle/adipocyte) glucose transporters (Kaestner *et al.*, 1989).

RESULTS

Glucose transport

Transport rates for red portions of the gastrocnemius and quadriceps muscles were found to be very similar, as were those of white portions of gastrocnemius and quadriceps (Fig. 1), and therefore are grouped as red and white for the remainder of this discussion. Under basal conditions (zero insulin), glucose transport rates in red and white muscles were not different (Fig. 1). When insulin was added to the perfusion medium, transport rates of the two types of muscle diverged. At a physiological insulin concentration (1 nM), red muscle transported twice as much glucose as white muscle ($P < 0.01$). This increased to a 3.6-fold difference at 100 nM-insulin ($P < 0.001$). The degree of insulin stimulation of glucose transport was also different between fibre types. White muscle demonstrated a 6-fold increase from basal transport to that at maximal insulin concentration, whereas the red muscle was able to increase glucose transport by 17-fold.

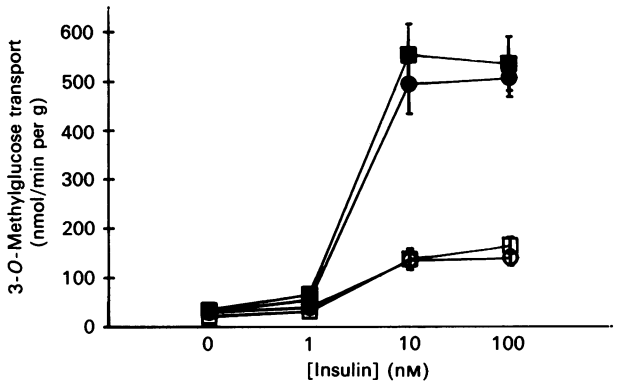

Fig. 1. Glucose transport in red and white skeletal muscle

Dose–response curves for red and white gastrocnemius (●, ○) and vastus (■, □) muscles ($n = 10$ at each insulin concentration) are shown: ●, ■, red muscle; ○, □, white muscle. Significant differences between the fibre types were achieved at all insulin concentrations ($P \leqslant 0.05$).

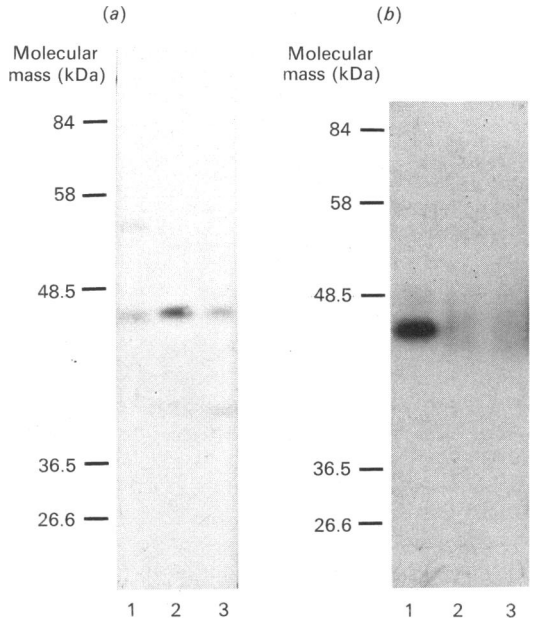

Fig. 2. Glut4 and Glut1 protein levels in red and white skeletal muscle

Western analysis of the Glut4 protein content using the polyclonal antibody ECU4 (*a*) and of the Glut1 protein content using the polyclonal antibody ECU1 (*b*) (50 μg of protein/lane) was carried out as described in the Materials and methods section. Lane 1, brain; lane 2, red skeletal muscle; lane 3, white skeletal muscle. Exposure time for autoradiography was 48 h in both cases. These results are representative of data from six separate rats in which the results were identical.

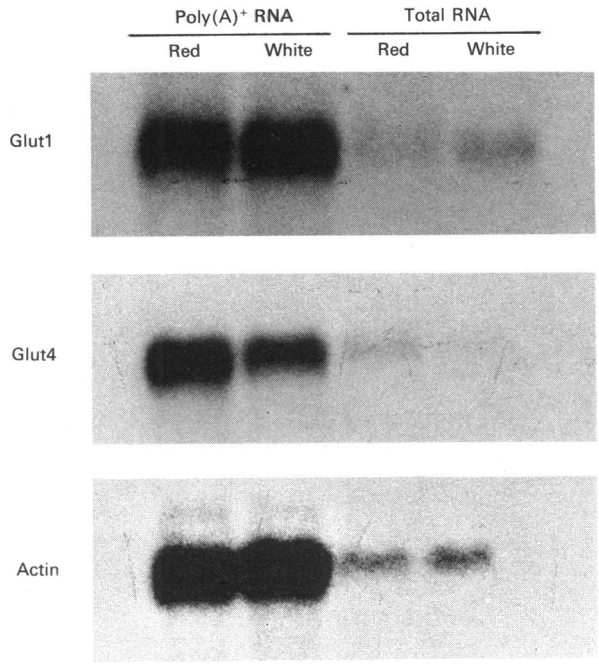

Fig. 3. Glut1 and Glut4 mRNA content in red and white skeletal muscle

Analysis of total RNA (20 μg/lane) and poly(A)$^+$ RNA (7.5 μg/lane) was carried out as described in the Materials and methods section. The total amount of RNA isolated (μg/g of tissue) was identical for both red and white muscle. Hybridizations were carried out overnight in prehybridization solution containing 0.02% each of BSA, polyvinylpyrrolidone and Ficoll, and with the Glut1, Glut4 or B-actin cDNA probe (1×10^7 c.p.m./ml). Hybridization and washing were carried out under high-stringency conditions. The blot was sequentially probed with the three cDNAs. Exposure time for autoradiography to obtain the Glut1 panel was 18 h, whereas only a 1 h exposure was required to obtain the Glut4 panel. The data are from a representative experiment of four with different preparations of RNA. In all cases, the results were identical.

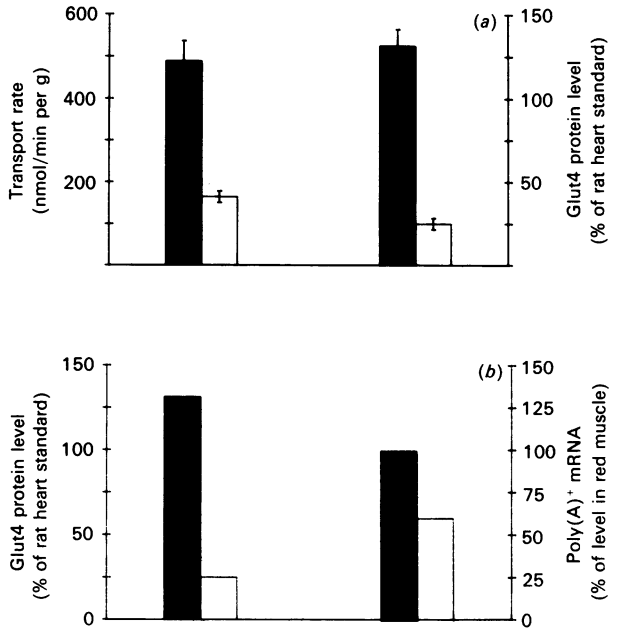

Fig. 4. Comparisons between maximal transport rate, Glut4 protein level and Glut4 mRNA in red (■) and white (□) muscle

(*a*) Similarities between maximal transport rate (100 nM-insulin), as measured in the perfused hindquarter ($n = 10$ per group; left-hand bars), and Glut4 protein level ($n = 6$ per group; right-hand bars), as determined by Western analysis ($P \leqslant 0.001$ for the difference between red and white muscle for both parameters). (*b*) Glut4 protein level (left-hand bars) is compared with Glut4 mRNA levels (right-hand bars), as determined by Northern analysis.

This difference was also evident at physiological insulin concentrations, white muscle showing a 1.4-fold increase in transport and red muscle demonstrating a 2.1-fold increase.

Glut4 and Glut1 transporter proteins in red and white muscle

Fig. 2 shows the autoradiographs from representative Western blots using both antibodies. Densitometric scanning revealed 5.2 times more Glut4 protein in red than in white muscle. In contrast, no difference in Glut1 protein was seen between the fibre types (Fig. 2*b*). As reported previously by James *et al.* (1989), brain contained an abundance of Glut1 transporter and very little Glut4 transporter (Figs. 2*a* and 2*b*).

Glut4 and Glut1 mRNA levels in red and white muscle

A Northern blot representative of four separate trials is shown in Fig. 3. Total RNA and poly(A)$^+$ RNA gave similar results. Scanning densitometry of the autoradiographs found total Glut1 mRNA levels to be slightly higher in white than in red muscle (22%), but poly(A)$^+$ was present in equal amounts in both muscles, as was the control mRNA, actin (Fig. 3). By contrast, levels of Glut4 poly(A)$^+$ mRNA and total RNA were 2-fold greater in red compared with white (Figs. 3 and 4). Attention should be drawn to the different exposure times needed to visualize these different messages (see the Fig. legends).

DISCUSSION

The findings of the present study suggest that glucose transport in specific skeletal muscle fibre types is at least partially, if not entirely, dependent upon the total amount of Glut4 glucose transporter protein, since glucose transport and Glut4 were both greater in red than in white muscle. Data from the Western analysis agree with previously reported cytochalasin B binding data (Kern *et al.*, 1989), in that more total transporters were found in red than white muscle. More specifically, this difference is due to the Glut4 transporter isoform (5 times more). Moreover, the steady-state red muscle Glut4 mRNA level is 2 times higher than that in white muscle, suggesting that red skeletal muscle preferentially expresses more Glut4 mRNA as well as protein (Fig. 4). Because this protein plays such an integral part in glucose transport in skeletal muscle, it suggests that the greater expression of Glut4 in red skeletal muscle may explain the cellular mechanism for the observed difference in insulin responsiveness in red and white muscle, and this same mechanism may be responsible for various insulin-resistant states.

These results beg the question of which factors regulate the expression of the Glut4 transporter in the two fibre types. Their innervation is very different (Edington & Edgerton, 1976), as is their oxidative capacity (Romanul, 1965; Nemeth & Pette, 1981) and capillarization (Lillioja *et al.*, 1987). After chronic nerve stimulation or endurance training, white fibres take on characteristics more like those of red muscle. Perhaps the neural input during endurance activity (already present in the red muscle) serves to regulate expression of a number of genes (Williams *et al.*, 1987), which could include the Glut4 gene.

The relationship between capillary density, fibre types and glucose transport could possibly be explained by the recent results of Vilaro' *et al.* (1989). Using immunofluorescence, they found the Glut4 transporter to be largely present in the endothelial cells of non-fenestrated capillaries, and conspicuously absent within the skeletal muscle itself. Red muscle is more

vascularized than white muscle and thus this finding would be consistent with the observations reported here. However, preliminary experiments in our laboratory using immunocytochemistry (Dudek *et al.*, 1990) have been unable to confirm the findings of Vilaro' *et al.* (1989), and by contrast found Glut4 to be present exclusively within the muscle cell.

An alternative way to look at this situation is to think of white muscle as having a depressed rate of Glut4 synthesis, resulting in an 'insulin-resistant' state. A recent article by Mueckler (1990) proposes a scenario in which a reduction in skeletal muscle glucose transporters could result in insulin resistance and non-insulin-dependent diabetes. He suggests that factors regulating glucose transporters in skeletal muscle could explain insulin resistance. As mentioned earlier, streptozotocin-diabetic rats demonstrate a decrease in the total number of glucose transporters, and this has been suggested to be responsible for the diminished glucose uptake seen in diabetic muscle (Ramlal *et al.*, 1989). These results are very similar to the results shown here between red and white muscle.

In summary, the differences between red and white muscle described in this paper indicate that the level of the Glut4 transporter protein appears to be a major determinant in the muscles' ability to respond to insulin. Discovery of mechanisms by which skeletal muscle regulates this key protein may provide important clues and information regarding the mechanism(s) of insulin resistance.

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REFERENCES

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Biochemistry **18**, 5294–5299

Cleveland, D. W., Lopata, M. A., MacDonals, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) Cell **20**, 95–105

Defronzo, R. A., Gunnarsson, R., Bjorkman, O., Olsson, M. & Wahren, J. (1985) J. Clin. Invest. **76**, 149–155

Dohm, G. L., Kasperek, G. J., Tapscott, E. B. & Beecher, G. R. (1980) Biochem. J. **188**, 255–262

Dohm, G. L., Tapscott, E. B., Pories, W. J., Dabbs, D. J., Flickinger, E. G., Meelheim, D., Fushiki, T., Atkinson, S. M., Elton, C. W. & Caro, J. F. (1988) J. Clin. Invest. **82**, 486–494

Dudek, R. W., Friedman, J. E., Whitehead, D., Leggett-Frazier, N. & Dohm, G. L. (1990) Diabetes **39** (suppl. 1), 82A

Edington, D. W. & Edgerton, V. R. (1976) The Biology of Physical Activity, pp. 51–72, Houghton Mifflin Co., Boston

Haspel, H. C., Rosenfeld, M. G. & Rosen, O. M. (1988) J. Biol. Chem. **263**, 398–403

James, D. E., Jenkins, A. B. & Kraegen, E. W. (1985) Am. J. Physiol. **248**, E567–E574

James, D. E., Strube, M. & Mueckler, M. (1989) Nature (London) **338**, 83–87

Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. H. & Lane, M. D. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3150–3154

Kahn, B. B., Charron, M. J., Lodish, H. F., Cushman, S. W. & Flier, J. S. (1989) J. Clin. Invest. **84**, 404–411

Kern, M., Tapscott, E. B. & Wells, J. A. (1989) Diabetes **38**, 65A

Krotkiewski, M. & Björntrop, P. (1986) Int. J. Obesity **10**, 331–341

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lillioja, S., Young, A. A., Culter, C. L., Ivy, J. L., Abbott, W. G. H., Zawadzki, J. K., Yki-Järvinen, H., Christin, L., Secomb, T. W. & Bogardus, C. (1987) J. Clin. Invest. **80**, 415–424

Mueckler, M. (1990) Diabetes **39**, 6–11

Nemeth, P. & Pette, D. (1981) J. Physiol. (London) **320**, 73–80

Ramlal, T., Rastogi, S., Vranic, M. & Klip, A. (1989) Endocrinology (Baltimore) **125**, 890–897

Richter, E. A., Hansen, B. F. & Hansen, S. A. (1988) Biochem. J. **252**, 733–737

Romanul, F. C. A. (1965) Arch. Neurol. **12**, 497–509

Vilaro', S., Palacin, M., Pilch, P. F., Testar, X. & Zorzano, A. (1989) Nature (London) **343**, 798–800

Wallberg-Henriksson, H. (1987) Acta Physiol. Scand. **131** (suppl.), 564

Williams, R. S., Garcia-Moll, M., Mellor, J., Salmons, S. & Harlan, W. (1987) J. Biol. Chem. **262**, 2764–2767

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