

## Diverse Effects of Glut 4 Ablation on Glucose Uptake and Glycogen Synthesis in Red and White Skeletal Muscle

Antine E. Stenbit, Rémy Burcelin, Ellen B. Katz, Tsu-Shuen Tsao, Nadine Gautier,\* Maureen J. Charron, and Yannick Le Marchand-Brustel\*

Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461-1602; and \*INSERM U 145, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 02, France

### Abstract

The ability of muscles from Glut 4-null mice to take up and metabolize glucose has been studied in the isolated white EDL and red soleus muscles. In EDL muscles from male or female Glut 4-null mice, basal deoxyglucose uptake was lower than in control muscles and was not stimulated by insulin. In parallel, glycogen synthesis and content were decreased. Soleus muscles from male Glut 4-null mice took up twice more deoxyglucose in the absence of insulin than control muscles, but did not respond to insulin. In females, soleus deoxyglucose uptake measured in the absence of hormone was similar in Glut 4-null mice and in control mice. This uptake was stimulated twofold in Glut 4-null mice and threefold in control mice. Basal glycogen synthesis was increased by 4- and 2.2-fold in male and female null mice, respectively, compared to controls, and insulin had no or small (20% stimulation over basal) effect. These results indicate that while EDL muscles behaved as expected, soleus muscles were able to take up a large amount of glucose in the absence (males) or the presence of insulin (females). Whether this is due to a change in Glut 1 intrinsic activity or targeting and/or to the appearance of another glucose transporter remains to be determined. (*J. Clin. Invest.* 1996; 98:629–634.) Key words: Glut 4-null mice • insulin effect • isolated muscles • deoxyglucose • glucose transporters

### Introduction

Glut 4 is of particular importance in glucose homeostasis because it catalyzes the rate-limiting step for glucose uptake and metabolism in skeletal muscle, the main site of glucose disposal, and in fat tissues (1–4). We have recently produced mice (Glut 4-null) in which the murine Glut 4 gene has been disrupted (5). The resulting mice display various abnormalities. They are growth retarded, exhibit decreased longevity, possess

severely reduced adipose tissue deposits and cardiac abnormalities (5). However, unexpectedly, glucose homeostasis was only slightly perturbed in the Glut 4-null mice. Indeed, female mice were normoglycemic and male mice were only moderately hyperglycemic. Both animals exhibited hyperinsulinemia in the fed state, as a sign of insulin resistance. These *in vivo* results suggested that the Glut 4-null mice could compensate for the lack of insulin sensitive glucose transporters by a mechanism which did not involve the overexpression of another known Glut isoform in skeletal muscle.

In the present study, we looked for the ability of the muscles isolated from Glut 4-null mice to take up glucose. Since glucose metabolism is very dependent on the muscle type (6), two leg muscles have been used which contain substantially different proportions of fiber types in rodents: the red soleus muscle, which is continuously active and contains more than 84% slow oxidative fibers (slow twitch), and the extensor digitorum longus (EDL)<sup>1</sup> muscle, which consists primarily of pale fast-twitch glycolytic oxidative fibers (7, 8). Further, muscles were isolated from both male and female animals since male mice seemed more susceptible to develop glycemic abnormalities than female animals. We found that the muscles' ability to take up glucose and to synthesize glycogen varied both depending on sex and on muscle fiber type, but that red muscles were able to take up a significant amount of glucose.

### Methods

**Materials.** Bovine serum albumin (fraction V) was from Interger (Purchase, NY). [ $\gamma$ -<sup>32</sup>P]ATP and [<sup>3</sup>H]deoxyglucose were purchased from ICN Biomedicals (Irvine, CA) or Amersham Corp. (Arlington Heights, IL). All other chemicals and biochemicals were from Sigma Chem. Co. (St. Louis, MO) or Merck Sharpe & Dohme (Rahway, NJ). Antiphosphotyrosine antibodies were obtained from Upstate Biomedicals, Inc. (Lake Placid, NY). Insulin (Umulin) was obtained from Eli Lilly, Inc. (Indianapolis, IN).

**Animals.** Mice were obtained and genotyped as previously described in (5). Control mice (CD1 or [+/-]) and mice homozygous for the Glut 4 mutation (-/-) were used at 10–12 wk of age. They were maintained at a constant temperature (22°C), on a 12 h light cycle until the time of experiment and were fed *ad libitum*.

**Isolation and incubation of muscles.** After cervical dislocation of the mice, the two muscles (soleus and extensor digitorum longus, EDL) were rapidly isolated (7) and tied to stainless steel clips by the tendons. All incubations were carried out at 37°C under an atmosphere of 95% O<sub>2</sub>:5% CO<sub>2</sub> in 1 ml of Krebs-Ringer bicarbonate

Address correspondence to Y. Le Marchand-Brustel, INSERM U 145, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 02, France. Phone: (33) 93 37 77 99; FAX: (33) 93 81 54 32; E-mail: lemarcha@unice.fr

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1. Abbreviations used in this paper: EDL, extensor digitorum longus; IRS 1, insulin receptor substrate 1.

buffer (pH 7.3) supplemented with 1% bovine serum albumin (fraction V, pH 7; Intergen) and 2 mM sodium pyruvate.

**Determination of glucose transport and glycogen synthesis.** Glucose transport was measured as follows: after a preincubation period of 15 min, muscles were incubated for 60 min without or with 10 nM insulin. They were then incubated for 10 min in the same medium supplemented with 2- $^3\text{H}$ deoxyglucose (0.1 mM, 0.5  $\mu\text{Ci/ml}$ ). Afterwards, muscles were washed for 30 min in ice-cold saline buffer and dissolved in 1 N NaOH before scintillation counting for  $^3\text{H}$ -labeled radioactivity was performed. Sample aliquots were used for protein determination. For the determination of glycogen synthesis, muscles were incubated for 60 min without or with 10 nM insulin in the same medium without pyruvate but with 3- $^3\text{H}$ glucose (5 mM, 1  $\mu\text{Ci/ml}$ ). Upon completion, muscles were dissolved in 1 N NaOH, aliquots of the alkaline solution were spotted onto Whatmann papers (2  $\text{cm}^2$ ). Papers were dropped into ice-cold 60% ethanol, and washed extensively (three washes of 20 min each) in 60% ethanol before counting. An aliquot of the solution was used for the determination of unlabeled glycogen content. Glycogen was precipitated with 66% ethanol using  $\text{Na}_2\text{SO}_4$  as a carrier. Glycogen was digested with amyloglucosidase, and the released glucose was measured by enzymatic determination as previously described (9).

**Determination of receptor autophosphorylation and receptor tyrosine kinase activity.** Receptor tyrosine kinase activity to polyglutamate/tyrosine was measured after immobilization on microtiter wells of an antibody to the insulin receptor (10). After incubation for 40 min without or with 100 nM insulin, muscles were homogenized by sonication in 0.5 ml of solubilization buffer (50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM sodium vanadate, 30 mM PNPP, 1 mM PMSF, 10  $\mu\text{g/ml}$  aprotinin, 50  $\mu\text{M}$  leupeptin, 1% Triton X-100, pH 7.4), and solubilization was allowed for 30 min at 4°C. The samples were centrifuged for 15 min at 10,000 g and the supernatant used in the microwell assay system (10, 11). Briefly, microtiter wells were coated with a non-species-specific insulin receptor antibody (AB-3; Oncogene Science, Mineola, NY) and homogenates (40  $\mu\text{l/well}$ ) added for 16 h at 4°C. Wells were washed and tyrosine kinase activity of the bound receptors was measured using polyglutamate/tyrosine as a substrate (11).

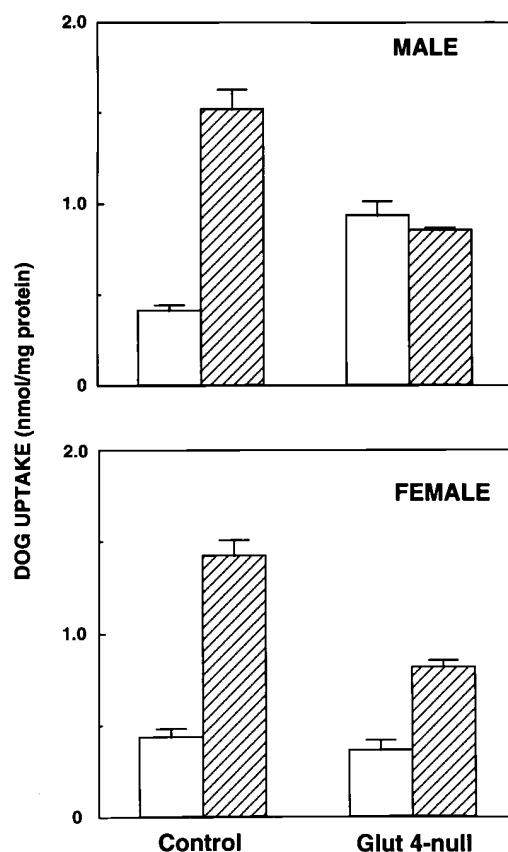
For the determination of receptor autophosphorylation, another aliquot of muscle homogenate was treated with Laemmli buffer and proteins (50  $\mu\text{g}$ ) were separated by SDS-PAGE (12). After transfer to nitrocellulose, phosphotyrosine containing proteins were revealed by antibodies to phosphotyrosine, and  $^{125}\text{I}$ -protein A as previously described (13).

**Immunoblotting of glucose transporter isoforms.** Muscles were homogenized by sonication as described above, and treated with Laemmli buffer. Muscle proteins (30 or 15  $\mu\text{g}$  for Glut 1 and Glut 4, respectively) were separated by SDS-PAGE and transferred to nitrocellulose. Uniformity of loading was assessed by Ponceau S staining of the membranes and transfer efficiency was determined by Coomassie blue staining of the gels. The membranes were blocked with TBS (10 mM Tris buffer, pH 7.4, 150 mM NaCl), 0.1% Tween 20, 3% bovine serum albumin, and 2% nonfat dry milk for 30 min at 37°C. The membranes were then incubated with the polyclonal antibody MC2A to Glut 4 (14) at a dilution of 1:1,000 or with the polyclonal antibody to Glut 1 (East Acres, South Bridge, MA) at 1:500 dilution overnight at 4°C. Membranes were washed at room temperature three times with TBS, 0.1% Tween 20 for Glut 4 or as follows for Glut 1: twice with 10 mM Tris, pH 7.5, 150 mM NaCl, once in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, once in 20 mM Tris, pH 7.5, 300 mM NaCl, again in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, and twice in 10 mM Tris, pH 7.5, 150 mM NaCl. Membranes were blocked again in the above blocking solution, and the antibody binding was revealed with the Enhanced Chemiluminescence Reagent (Amersham Corp.) with HRP-coupled goat anti-rabbit IgG (1:2,500), (Southern Biotechnology, Birmingham, AL). The relative amounts of Glut 1 and Glut 4 were quantitated using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA).

**Statistics.** All results were expressed per milligram of muscle protein (determined by BCA assay; Pierce, Rockford, IL). Determinations were performed with the number of muscles indicated in the figure legends and are presented as means  $\pm$  SEM. Comparisons were performed using the Student's *t* test for unpaired data when muscles from various groups of mice were used (control mice vs Glut 4-null mice). Student's *t* test for paired data was used when two muscles were isolated from the same mouse, one muscle incubated without insulin and the controlateral muscle with insulin.

## Results

**Glucose uptake in muscles isolated from Glut 4-null mice.** Two soleus and two EDL muscles were isolated from each mouse and were incubated in the absence (basal condition) or in the presence of insulin used at a concentration (10 nM) which is maximally effective in normal muscles (15, 16). Results were expressed per milligram of muscle protein; however, the results would be similar whether they were expressed per mg of muscle or per total muscle. Indeed, there was no change in the macroscopic appearance of the muscles, and, as shown in Table I, there was no difference in the protein content of the soleus muscles between normal and Glut 4-null mice while the



**Figure 1.** Glucose uptake in isolated soleus muscles of control and Glut 4-null mice. Soleus muscles were isolated from male and female control and Glut 4-null mice and incubated for 60 min in Krebs Ringer bicarbonate buffer, 1% BSA, 2 mM pyruvate, without (empty bars) or with 10 nM insulin (hatched bars).  $^3\text{H}$ 2-Deoxyglucose (0.1 mM, 0.5  $\mu\text{Ci/ml}$ ) was then added for 10 min to measure glucose uptake as described in methods. Values are means  $\pm$  SEM of six to eight muscles from six to eight different animals.

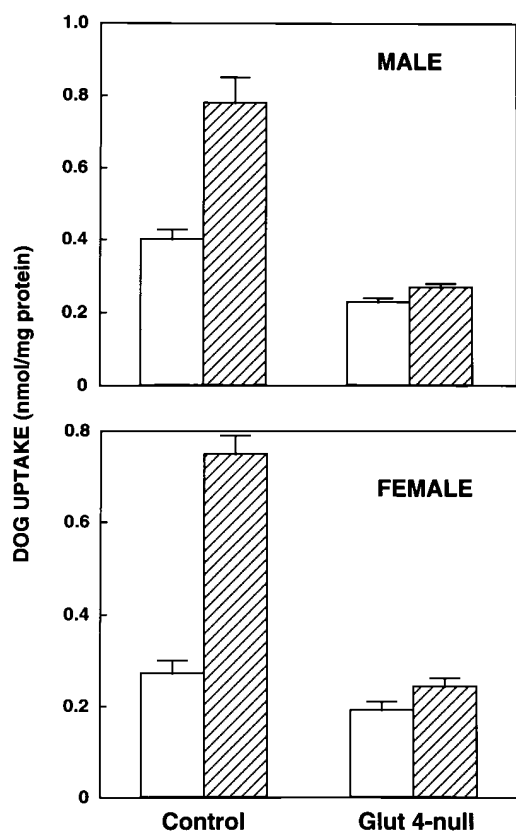


Figure 2. Glucose uptake in isolated EDL muscles of control and Glut 4-null mice. EDL muscles were isolated from male and female control and Glut4-null mice and incubated as described in Fig. 1. Values are means  $\pm$  SEM of six to eight muscles from six to eight different animals.

EDL protein content was 10% less in the female null-mice compared to control mice. Deoxyglucose uptake was measured as an index of glucose uptake and phosphorylation and the results obtained in soleus and EDL muscles are shown in Figs. 1 and 2, respectively. Soleus muscles from male Glut 4-null mice took up 2.3 more glucose than control mice in the absence of insulin. They did not respond to insulin while control mice displayed a 3.7-fold increase when stimulated with insulin. In contrast, soleus muscles from female Glut 4-null mice did not show any significant alteration in basal glucose uptake and this uptake was increased 2.25-fold, when stimulated with insulin. The results obtained in the white EDL muscles were different. Basal glucose uptake was decreased by 50% in male and 30% in female null mice. Further, EDL muscles were totally insulin resistant for glucose uptake, while a two to three-fold stimulation by insulin was observed in control mice. Similar results were obtained when the insulin concentration was increased to 100 nM (data not shown).

**Glycogen synthesis in muscles from Glut 4-null mice.** The ability of muscles to convert glucose into glycogen was assessed. In these experiments, a physiological glucose concentration (5 mM) was used, while uptake was previously measured at 0.1 mM 2-deoxyglucose. The results shown in Table II paralleled those observed for glucose uptake. In the absence of insulin, soleus muscles from Glut 4-null mice accumulated more glycogen than muscles from control animals (4-fold and

Table I. Characteristics of the Muscles

	Males		Females	
	Control	Glut 4-Null	Control	Glut 4-Null
Muscle protein (mg)				
Soleus	0.81 $\pm$ 0.02	0.82 $\pm$ 0.03	0.72 $\pm$ 0.02	0.68 $\pm$ 0.02
EDL	1.06 $\pm$ 0.04	0.96 $\pm$ 0.05	0.89 $\pm$ 0.01	0.83 $\pm$ 0.02*
Muscle glycogen ( $\mu$ g/mg protein)				
Soleus	28.6 $\pm$ 8.2	20.8 $\pm$ 2.2	15.1 $\pm$ 3.4	26.1 $\pm$ 2.0*
EDL	24.5 $\pm$ 4.5	10.7 $\pm$ 0.7*	22.6 $\pm$ 3.1	14.9 $\pm$ 2.1*

Soleus or EDL muscles were isolated from control and Glut4-null mice. Protein and glycogen contents were measured at the end of the incubation as described in the methodology. Values are means  $\pm$  SEM of 8–10 muscles (protein content) or 4–5 muscles (glycogen content). \*Difference between control and Glut 4-null mice significant with  $P < 0.05$ .

2.2-fold in male and female mice, respectively), while EDL muscles synthesized very small amounts of glycogen. Further, none of the muscles responded to insulin, except soleus from female null mice which showed a 20% increase over basal ( $P < 0.05$ , using Student's *t* test for paired data). In accordance with these results, glycogen content was lower in EDL muscles of Glut 4-null mice than in controls (Table I).

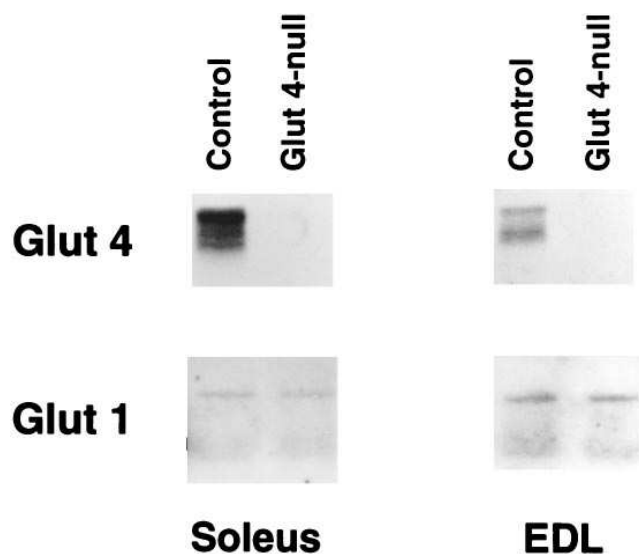
**Glucose transporter immunoblotting.** Our previous studies did not reveal any significant increase in Glut 1 isoform in muscles of Glut 4-null mice (5). However, since blotting experiments were performed on total muscle homogenate, we verified whether the results were similar in red and white muscles. As shown in Fig. 3 and as expected, Glut 4 was absent from all muscle membranes of Glut 4-null mice. Further, there was no significant increase in Glut 1 isoform in any of the muscles studied.

**Insulin receptor autophosphorylation and tyrosine kinase activity.** Since muscles from male animals never responded to

Table II. Glycogen Synthesis in Muscles from Control and Glut 4-Null Mice

	Control		Glut 4-null	
	Basal	Insulin	Basal	Insulin
Soleus muscles				
Male	14.7 $\pm$ 2.4	111.1 $\pm$ 20.2	64.1 $\pm$ 11.6*	65.4 $\pm$ 4.9*
Female	10.6 $\pm$ 1.6	93.2 $\pm$ 7.0	26.6 $\pm$ 6.8*	31.9 $\pm$ 8.1*
EDL muscles				
Male	13.2 $\pm$ 4.0	66.2 $\pm$ 8.1	5.5 $\pm$ 1.6	5.1 $\pm$ 1.0*
Female	9.2 $\pm$ 1.3	36.6 $\pm$ 1.9	6.7 $\pm$ 1.9	9.2 $\pm$ 1.9*

Soleus or EDL muscles were isolated from male and female control and Glut 4-null mice. They were incubated for 60 min in Krebs Ringer bicarbonate buffer, 1% BSA, without (basal) or with 10 nM insulin and [ $^3$ H]-Glucose (5 mM, 1  $\mu$ Ci/ml). At the end of the incubation, muscles were dissolved and glycogen was precipitated and counted as described in the methodology. Values are expressed as nmol glucose incorporated into glycogen per mg muscle protein and per hour and are presented as means  $\pm$  SEM of five (female) and four (male) muscles. \*Difference significant with at least  $P < 0.05$  versus control. †Insulin effect versus basal significant with  $P < 0.05$  using Student's *t* test for paired data.

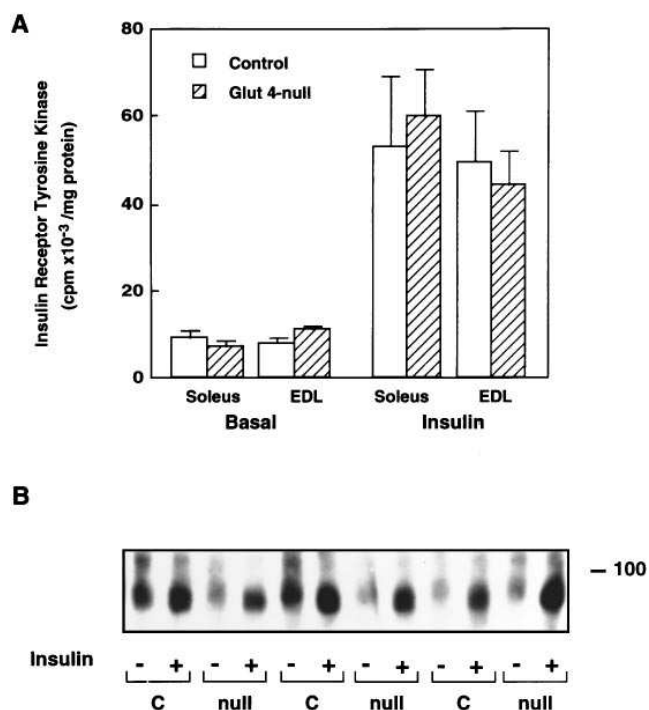


**Figure 3.** Immunoblotting of glucose transporter isoforms in muscles of Glut 4-null mice. Sonicated homogenates of soleus and EDL muscles were prepared. Proteins (15 or 30  $\mu$ g, for Glut 4 and Glut 1, respectively) were separated by SDS-PAGE and immunoblot analysis of Glut 4 (top) and Glut 1 (bottom) was performed according to the methodology. The immunoblots are representative of five male normal or Glut 4-null mice for each muscle type. No Glut 4 was detected in either the soleus or the EDL of the Glut 4-null mice. No difference was detected in either the soleus or the EDL expression of Glut 1 when the quantification was performed as described in methods. Arbitrary densitometric units: EDL,  $1.95 \pm 0.15$  and  $1.93 \pm 0.11$ ; soleus,  $2.25 \pm 0.31$  and  $2.21 \pm 0.45$  (mean  $\pm$  SEM of 4 Glut 4-null and normal muscles, respectively).

the addition of insulin, we looked for the ability of insulin to stimulate its receptor autophosphorylation and its tyrosine kinase activity. Muscles were incubated without or with insulin before homogenization and solubilization in the presence of phosphatase and protease inhibitors. One aliquot sample was immunoadsorbed with antibodies to the insulin receptor and used to measure kinase activity towards polyglutamate/tyrosine (Fig. 4 A), while another sample was analyzed by SDS-PAGE, and proteins immunoblotted with antibodies to phosphotyrosine (Fig. 4 B). As evident in Fig. 4 A, there was no difference in basal or in insulin-stimulated tyrosine kinase activity towards the synthetic substrate. Further the autophosphorylation of the insulin receptor  $\beta$  subunit was similar in control and Glut 4-null mice (Fig. 4 B).

## Discussion

We have studied the ability of muscles lacking Glut 4 to take up deoxyglucose and to metabolize glucose into glycogen in the absence or presence of insulin. In respect to glucose transport in vitro, the behavior of red and white muscles completely differed in Glut 4-null mice. As expected, the in vitro glucose transport and glycogen synthesis were decreased in both the male and female EDL muscles under basal and insulin-stimulated conditions. Two distinct observations have been made in the soleus muscle of the Glut 4-null mice, possibly involving two different mechanisms. First, the basal glucose uptake in the soleus of the male Glut 4-null mice was twice the uptake



**Figure 4.** Insulin receptor tyrosine kinase activity and autophosphorylation in muscles from control and Glut 4-null mice. Soleus or EDL muscles were isolated from male control and Glut 4-null mice and were incubated for 40 min without (Basal) or with 100 nM insulin. Muscles were homogenized in stopping buffer. (Top) Insulin receptor were immunoadsorbed on microtiter well coated with an antibody to the insulin receptor. Kinase activity was then measured on polyglutamate/tyrosine as described in the methodology. Results are expressed in cpm  $^{32}$ P incorporated into the substrate per milligram of muscle homogenate protein, and are presented as means  $\pm$  SEM of three muscles from three mice. (Bottom) Proteins (50  $\mu$ g) were separated by SDS-PAGE, transferred, and blotted with antibodies to phosphotyrosine as described in methodology. The autoradiogram obtained with three EDL muscles from control and Glut 4-null mice is shown. Quantification of the spots corresponding to the  $\beta$  subunit: Control,  $1,062 \pm 198$  and  $1,470 \pm 207$  cpm; Glut 4-null,  $676 \pm 60$  and  $1512 \pm 351$  in basal and insulin-stimulated conditions, respectively, means  $\pm$  SEM of three values.

observed in control mice. This unexpected observation was confirmed when glycogen synthesis was measured. Thus, glucose was able to enter the soleus muscles, not only at the low (0.1 mM) glucose concentration used for glucose uptake measurement but also at the higher (5 mM) glucose concentration used for glycogen synthesis determination. Secondly the basal glucose uptake in the soleus muscle of Glut 4-null female mice was normal and these muscles responded to the action of insulin. Although blunted compared to normal, the insulin stimulated glucose uptake in the female Glut 4-null soleus muscles was twofold higher than basal. Basal glycogen synthesis measured in female soleus was higher than in control, but could still respond slightly to insulin addition.

Since the cloning of Glut 4, no direct proof of the functional role of the transporter was obtained, until recently when mice overexpressing Glut 4 have been obtained. Indeed, overexpression of Glut 4 throughout the body or specifically in skeletal muscle led to a slight increase in basal glucose uptake

but mostly to an elevated response to insulin (17–20). The results of the present study in Glut 4-null mice indicate that Glut 4 is necessary in the EDL for an insulin-stimulated glucose uptake. By contrast, it does not appear to be the case in the soleus muscle. It remains unclear why glucose uptake was normal (female mice) or increased (male mice) in soleus muscles of Glut 4-null mice. One can propose at least two different explanations. The first possibility is that Glut 1 may be playing an important role in the absence of insulin. The Glut 1 transporter is relatively low in abundance in normal muscle since photolabeling of the glucose transporters in muscle suggests that in the basal state, the amount of Glut 1 in the plasma membrane is about half of the amount of Glut 4 (21). It was generally hypothesized that Glut 1 would be responsible for the basal uptake of glucose (4, 22). This was confirmed by the fact that muscles from mice overexpressing Glut 1 showed an increased basal glucose uptake in muscle, but no response to insulin (23). This is compatible with the predominant location of Glut 1 in the plasma membrane even in the absence of insulin which has been described in adipose and muscle cells (21, 24, 25). Although Glut 1 expression is not increased in muscles of Glut 4-null animals, Glut 1 could be more “efficient” in Glut 4-null mice. While in most situations an increase in glucose transport is related to a larger number of transporters at the plasma membrane level, several lines of evidence support the concept that glucose transporter activity can be modulated once transporters are inserted in the plasma membrane. This is for example the case in adipocytes from fasted-refed rats or from insulinopenic animals treated with insulin (25, 26), in cells treated with protein synthesis inhibitors (27, 28), or in adipocytes treated with phorbol esters (29). It is conceivable that a change in Glut 1 intrinsic activity could occur in soleus muscles from Glut 4-null mice as a consequence of the continuous work imposed on this postural muscle.

The second possibility for the increased glucose transport in soleus muscles of Glut 4-null male mice and for the insulin sensitive uptake in female soleus muscles is that an unknown transporter is specifically expressed in their red muscles. It could be observed that the knock-out of IRS 1 was compensated by the alternative expression of IRS 2 (30, 31). Such a compensatory phenomenon could appear in red muscles from Glut 4-null mice. This transporter would be able to be translocated in response to insulin in female mice. This is not the case in male mice, perhaps because all the transporters would be already present at the plasma membrane in the absence of insulin. Indeed, the value of glucose uptake in male soleus muscle in the absence of insulin was similar to the value observed in female soleus in the presence of insulin. A similar lack of insulin response in muscles from male Glut 4-null mice was observed at 10 and 100 nM insulin, indicating that the lack of insulin response was not due to a decreased sensitivity of the muscles to insulin. This suggests that whatever the nature of the transporter which is acting in these muscles, it does not translocate in response to insulin in male animals, although insulin receptor autophosphorylation and its tyrosine kinase activity were normally stimulated by the hormone.

Most muscles are not composed of one fiber type but are mixed. It is thus possible that in the intact mice, red fibers, which are able to take up more glucose than the white fibers, play a major role in the maintenance of a normal blood glucose. The lack of insulin stimulated *in vitro* glucose uptake in white muscle can partially explain the whole body intolerance

seen in the Glut 4-null mice (5). Further, the animals display high circulating insulin levels. It is possible that, even under normoglycemic conditions, insulin reduces hepatic glucose production more in the Glut 4-null mice than in the control animals. The higher levels of liver Glut 2 may be responsible for increased hepatic glucose uptake in Glut 4-null mice suggesting that the liver is probably playing an important role in the maintenance of a normal glycemia in those mice. Examination of glucose utilization during clamp studies, although technically difficult to perform in conscious mice, should help in understanding the precise role of the liver and of muscles in glucose homeostasis in Glut 4-null mice.

In summary, the consequence of the absence of Glut 4 in muscle glucose uptake differs between male and female and also with the type of muscle. Glut 4 is necessary for insulin-stimulated glucose uptake in the EDL muscle, and the lack of Glut 4 is not compensated by any mechanism. In the male soleus muscles, the basal glucose uptake is twice that of controls. The reason for this elevation is not known, however, it could be due to an increase in Glut 1 “efficiency” or an increase in Glut 1 at the plasma membrane or to an unknown glucose transporter. Whatever the transport system involved in this elevation, it does not respond to insulin addition. In the soleus from female Glut 4-null mice, glucose uptake which is normal in basal condition can be stimulated by insulin. Whether this is due to an unknown glucose transport system remains to be determined.

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