Expression of the glucose transporter GLUT4 in the muscular dystrophic *mdx* mouse

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Glucose transporter protein levels have been investigated in *mdx* and control (C57BL/10) mice. Crude membrane fractions (microsomes plus plasma membranes) were prepared from skeletal muscle, heart, diaphragm and brain of 5–6-week-old and 6–7-month-old control and *mdx* mice. Using Western blot analysis with C-terminal-specific anti-peptide antibodies, we investigated the glucose transporters GLUT4 in the different muscle tissues and GLUT1 in brain. In skeletal tissue from the hindlegs, GLUT4 was increased by ~55% in *mdx* mice compared with control mice at both ages studied. In the diaphragm, the amount of GLUT4 protein was unchanged in young *mdx* mice, and was decreased by $37.4 \pm 4.7\%$ in older mice compared with age-matched control mice. No difference was observed between *mdx* and control mice in the amounts of GLUT4 and GLUT1 in heart and brain preparations respectively. To determine whether the change in GLUT4 protein observed in the diaphragm and

skeletal muscle of *mdx* mice was regulated through changes at the level of glucose transporter mRNA, Northern blot analyses were performed. In skeletal muscle, GLUT4 mRNA level per tissue was not different between the two groups of mice at both ages studied. In contrast, the decrease in the amount of GLUT4 protein observed in the diaphragm of 6–7-month-old *mdx* mice was accompanied by a decrease in the GLUT4 mRNA level. In conclusion, the levels of GLUT4 protein were modified in muscle tissues from *mdx* compared with control mice, and these modifications were different depending on the muscle involved and the age of the mice. An increase in the amount of GLUT4 protein in the skeletal muscle of *mdx* mice was not due to changes at the mRNA level. The diaphragms of 6–7-month-old *mdx* mice exhibited decreases in GLUT4 protein and mRNA levels that were not detected in young animals (5–6 weeks old).

INTRODUCTION

The *mdx* mouse contains an X-chromosome-linked recessive myopathic mutation [1] and is widely used as a model for human Duchenne muscular dystrophy (DMD). The *mdx* mouse dystrophin gene presents a nonsense point mutation which causes premature termination of the polypeptide chain [2]. Dystrophin, a 400 kDa protein with amino acid sequence identity to the spectrin family of membrane cytoskeletal proteins [3,4], is found in the sarcolemmal membrane of normal skeletal muscle [5]. It is absent from the skeletal muscle of both DMD humans and *mdx* mice [6–9]. Although muscular dystrophies are characterized by muscle degeneration, a major difference between the *mdx* mouse and the DMD human syndromes is the successful muscle fibre regeneration that occurs in the *mdx* mice, which restores muscle histology and function to approximate normality [10–12]. This explains the lack of muscle weakness and the normal life-span of *mdx* mice. Further, while there is an almost total replacement of some muscles by fat and fibrous tissue in human DMD, the *mdx* mouse does not exhibit such fibrosis. Nevertheless, the evolution of the *mdx*-associated disease is not identical in all muscles. Necrosis and regeneration are present, although variable, in most muscles throughout the life-span of the animals [10]. By contrast, the *mdx* mouse diaphragm exhibits a pattern of progressive degeneration, fibrosis and severe functional deficit comparable with that of DMD limb muscle [13].

Previous studies have shown that glycogen accumulation is increased in *mdx* mouse muscle [14,15], suggesting an abnormality of carbohydrate metabolism. Glucose is a major energy source for skeletal muscle. Its passage across the plasma membrane is

mediated by a family of five glucose transporter isoforms that have a tissue-specific expression [16]. Two transporters are found in skeletal muscle: (a) the major transporter, GLUT4, which occurs exclusively in muscle and adipose tissue, and which is thought to be responsible for insulin-stimulated glucose transport [17–21], and (b) the ubiquitous GLUT1 glucose transporter, which is the major brain transporter and is also present in very small amounts in skeletal muscle [22,23]. The aim of the present study was to investigate GLUT4 expression at both the protein and the mRNA levels in the *mdx* mouse. Since the myopathic syndrome differs in the various muscle types, we studied the heart, the diaphragm and the hindleg muscular mass. Further, given the abrupt onset of muscle fibre degeneration/regeneration around 6 weeks of age, young (5–6-week-old) and old (6–7-month-old) mice were investigated in order to search for a correlation between the state of degeneration/regeneration and the expression of GLUT4.

MATERIALS AND METHODS

Animals

Breeding pairs of *mdx* and control C57BL/10 mice were generously provided to us by J. Koenig (Laboratoire de Neurobiologie Cellulaire, Bordeaux II, France). All breeding was by brother–sister mating. Homozygous females (*mdx/mdx*) and hemizygous males (*mdx/-*) were used interchangeably when they were 5–6 weeks old or 6–7 months old. Mice were maintained at 23 °C on a 12 h-light/12 h-dark cycle and were fed *ad libitum* with laboratory chow (M 25 biscuits; Extralabo, Provins, France). They were anaesthetized with pentobarbitone sodium

Abbreviations used: DMD, Duchenne muscular dystrophy; SSC, 0.15 M NaCl/0.015 M sodium citrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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(50 mg/kg, intraperitoneal), blood samples were drawn from the inferior vena cava, and animals were killed by cervical dislocation. For membrane and RNA preparations, tissues (skeletal muscle, heart, diaphragm and brain) were rapidly dissected out, immediately frozen in liquid nitrogen and stored at -80°C .

Blood determinations

Following centrifugation of blood, plasma was used for glucose and insulin determinations as previously described [24]. Creatine kinase activity (which was used to confirm the phenotype of the *mdx* mice) was measured using the Sigma diagnostics kit (Sigma Chemical Co., St. Louis, MO, U.S.A.; colorimetric procedure no. 520).

Membrane preparation and glucose transporter immunoblotting

Total muscle (microsomal plus plasma) membranes were prepared as described [25,26]. Briefly, skeletal muscle mass from the hindleg, diaphragm and heart was removed, weighed and homogenized in NaHCO_3 buffer (20 mM, pH 7.0) containing 250 mM sucrose, 100 mM phenylmethanesulphonyl fluoride and 5 mM NaN_3 , in a Polytron apparatus. Homogenates were centrifuged at 1000 *g* for 10 min at 4°C . The supernatant was saved, and the pellet was re-homogenized and submitted to a second centrifugation at 1000 *g*. The first and second supernatants were pooled and centrifuged at 9000 *g* for 10 min, and the membranes were obtained by centrifugation of the 9000 *g* supernatant at 170000 *g* for 70 min. Brains were homogenized in 20 mM Hepes, pH 7.4, 250 mM sucrose and 1 mM EDTA. Membranes were obtained as described for the muscles, with the exception that the 9000 *g* centrifugation step was omitted. The protein content of the preparations was determined by the Bio-Rad assay with BSA as standard. Equal amounts of protein were subjected to SDS/PAGE. After transfer to nitrocellulose (Hybond C extra; Amersham), the blots were saturated in PBS, pH 7.4, containing 5% dried skimmed milk for 2 h at room temperature. The blots were then incubated overnight at 4°C with affinity-purified antibodies against the C-terminal sequence of the GLUT4 glucose transporter [26]. After three washings (10 min each) in PBS containing 1% Triton X-100, the sheets were incubated for 2 h at room temperature with ^{125}I -Protein A (4×10^5 c.p.m./ml). Blots were washed as above and the protein-antigen complexes were revealed by autoradiography at -80°C . Following autoradiography, the pieces of nitrocellulose corresponding to the glucose transporter were excised and their radioactivity content was determined in a γ -radiation counter.

RNA preparation and Northern blots

Total tissue RNAs were extracted using a guanidine thiocyanate method [27]. RNA concentration and purity were assessed by measuring the absorbance at 260 and 280 nm. For Northern blot analysis, 20 μg of total RNA was size-fractionated by electrophoresis through a 1% agarose/0.66 M formaldehyde gel. After electrophoresis, RNA abundance and integrity were confirmed by ethidium bromide staining of the 28 S and 18 S ribosomal bands by u.v. shadowing. The RNA was transferred to Amersham C extra membranes, which were baked for 2 h at 80°C . Blots were prehybridized for 2 h at 42°C in a solution containing 42% deionized formamide, $8 \times$ Denhardt's solution, 40 mM Tris/HCl, pH 7.5, 350 mM NaCl, 0.08% sodium pyrophosphate,

0.8% SDS, 7.5% dextran sulphate and 0.1 mg/ml denatured salmon sperm DNA [28]. The labelled denatured cDNA probe was added for 16 h at 42°C . Following hybridization, the blots were washed twice for 30 min in $2 \times \text{SSC}/0.1\%$ SDS at 42°C and twice for 30 min in $0.1 \times \text{SSC}/0.1\%$ SDS at 55°C . For the 18 S oligonucleotide probe, blots were hybridized for 16 h at 42°C in $0.1 \times$ Denhardt's solution, $5 \times \text{SSC}$, 0.05 M sodium phosphate, pH 6.5, 0.1% SDS and 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA in the presence of 10^4 c.p.m. of labelled probe. Then blots were washed twice for 10 min in $2 \times \text{SSC}$ at room temperature. The blots were exposed to Amersham MP films for autoradiography and the signals were quantified by scanning densitometry (GS-300 scanning densitometer; Hoefer, San Francisco, CA, U.S.A.).

cDNA and oligonucleotide probes

The rat GLUT4 cDNA probe was obtained by isolating a 2 kb insert from a Bluescript plasmid kindly provided to us by Dr. D. E. James (University School of Medicine, St. Louis, MO, U.S.A.). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 1.3 kb cDNA obtained from Dr. C. Dani (Centre de Biochimie, Nice, France). The probes were labelled using the random priming procedure with [α - ^{32}P]dCTP to a specific radioactivity of $(1-5) \times 10^8$ c.p.m./ μg [29]. The oligonucleotide probe, provided by Dr. J. Girard (CEREMOD, Meudon, France), was 24-mer long and the sequence is complementary to the 18 S RNA. The probe was labelled at the 5' end with T4 kinase and ^{32}P to a specific radioactivity of 2.5×10^8 c.p.m./ μg .

Statistical analysis

Individual membrane and RNA preparations were obtained from four to eight animals. All preparations, Western blots and Northern blots were performed in parallel for *mdx* and C57BL/10 control mice. Blots were repeated at least twice for each preparation. All results are presented as means $\pm$ S.E.M., the number of values being indicated in the legends to Tables. Statistical significance was assessed by Student's *t* test for unpaired comparisons.

RESULTS

Characteristics of control and *mdx* mice

The *mdx* and control mice were studied at two different stages of development, i.e. at 5-6 weeks and at 6-7 months of age. The mean body weight of 6-7-month-old *mdx* mice was significantly increased compared with controls in both female and male groups (Table 1). This difference was significant in the female group only in the 5-6-week-old mice. Glycaemia and insulinaemia were not markedly different between control and *mdx* mice. As expected, creatine kinase activity was elevated in *mdx* mice.

Glucose transporter expression in different tissues from control and *mdx* mice

To study the expression of glucose transporters in the myopathic mice, crude membranes were prepared from skeletal muscle, diaphragm, heart and brain of 5-6-week- and 6-7-month-old mice. After SDS/PAGE of proteins and transfer to nitrocellulose, sheets were immunoblotted using specific antibodies against GLUT4 for muscle tissues and GLUT1 for brain preparations. To allow comparison between control and *mdx* mice, identical amounts of proteins were analysed. Figure 1 shows the immuno-

Table 1 Characteristics of control and *mdx* mice

Control (C57B1/10), *mdx/mdx* female and *mdx*— male mice were anaesthetized, and blood was sampled for determination of glucose, insulin and creatine kinase, as described in the Materials and methods section. Values are means $\pm$ S.E.M. of six to 16 mice in each group. Statistically different results between *mdx* and sex-corresponding control values are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

	Control		<i>mdx</i>	
	Female	Male	Female	Male
(a) 5–6-week-old mice				
Body weight (g)	17.2 $\pm$ 0.23	20.7 $\pm$ 0.24	18.8 $\pm$ 9.5***	21.4 $\pm$ 0.5
Glycaemia (mM)	12.3 $\pm$ 1.1	13.9 $\pm$ 0.9	12.5 $\pm$ 0.6	14 $\pm$ 0.7
Insulinaemia (ng/mg)	0.97 $\pm$ 0.08	0.76 $\pm$ 0.09	1.3 $\pm$ 0.09**	1.3 $\pm$ 0.07***
Creatine kinase (i.u./l)	283 $\pm$ 69	173 $\pm$ 21	3519 $\pm$ 728***	3856 $\pm$ 431***
(b) 6–7-month-old mice				
Body weight (g)	25.8 $\pm$ 0.47	31.3 $\pm$ 0.38	29 $\pm$ 0.7***	36.7 $\pm$ 0.29***
Glycaemia (mM)	8.6 $\pm$ 0.7	9.9 $\pm$ 1.6	6.6 $\pm$ 0.4*	8.5 $\pm$ 0.5
Insulinaemia (ng/ml)	0.51 $\pm$ 0.02	0.71 $\pm$ 0.15	0.61 $\pm$ 0.07	1.21 $\pm$ 0.19*
Creatine kinase (i.u./l)	33.5 $\pm$ 3.5	54.6 $\pm$ 5.9	8120 $\pm$ 766***	9157 $\pm$ 792***

Figure 1 Immunodetection of glucose transporters in hindleg skeletal muscle, heart and brain from 6–7-month-old control and *mdx* mice

Crude membranes were prepared as described in the Materials and methods section. Identical amounts of membranes (60 μ g) were subjected to SDS/PAGE. Proteins were transferred to nitrocellulose and immunoblotted with an anti-GLUT4 antibody (for muscle tissues) or with an anti-GLUT1 antibody (for brain). Autoradiograms obtained from three different preparations from three control and three *mdx* mice are presented.

detection of GLUT4 in skeletal and heart muscles, and of GLUT1 in brain membrane preparations obtained from 6–7-month-old control and *mdx* mice. The glucose transporters GLUT4 and GLUT1 appeared as 45–50 kDa proteins which were occasionally resolved as a doublet. The relative mobilities of the GLUT4 and GLUT1 proteins were similar in preparations from control and *mdx* mice. By contrast, the amount of GLUT4 protein was increased in membranes from the hindleg skeletal muscle from *mdx* compared with control mice, while the amounts of GLUT4 in heart and GLUT1 in brain were unchanged. As shown in Table 2, where the quantification of autoradiograms obtained with four to eight different membrane preparations is

Table 2 Quantification of GLUT4 protein in different muscles from control and *mdx* mice

Crude membrane preparations were prepared from hindleg skeletal muscle, diaphragm and heart of young (5–6 weeks) or old (6–7 months) control and *mdx* mice. Identical amounts of proteins were subjected to SDS/PAGE. Proteins were transferred to nitrocellulose and immunoblotted with an antibody to GLUT4 as described in the Materials and methods section and in the legends to Figures 1 and 2. Quantification of GLUT4 protein was performed by excising the nitrocellulose pieces corresponding to the transporter and counting for radioactivity, or by scanning densitometry. Values are expressed as percentages of the values obtained in control mice and are presented as the means $\pm$ S.E.M. of 4–8 different membrane preparations obtained from different mice. Statistically different results from control values are indicated by * $P < 0.01$, ** $P < 0.005$.

Muscle type	Young mice		Old mice	
	Control	<i>mdx</i>	Control	<i>mdx</i>
Hindleg	100 $\pm$ 8.5	163.2 $\pm$ 16.7**	100 $\pm$ 9.4	154.8 $\pm$ 12.3**
Diaphragm	100 $\pm$ 7.0	96.6 $\pm$ 11.6	100 $\pm$ 6.5	62.6 $\pm$ 7.8*
Heart	100 $\pm$ 5.3	106 $\pm$ 10.9	100 $\pm$ 15.0	115 $\pm$ 17

presented, the level of GLUT4 protein in hindleg skeletal muscle was increased by 55–60% in *mdx* mice, irrespective of age. The results were different in diaphragm preparations (Figure 2 and Table 2). Indeed, the amount of GLUT4 was not modified in the diaphragms of 5–6-week old *mdx* mice, while it was decreased by 40% in diaphragms from 6–7-month-old *mdx* mice compared with age-matched controls.

GLUT4 mRNA levels in muscles from control and *mdx* mice

We next determined whether the modifications in the amount of GLUT4 protein observed in hindleg skeletal and diaphragm muscles from *mdx* mice were regulated through changes at the level of GLUT4 mRNA. Total RNAs were extracted from skeletal muscle and diaphragm of control (C57BL/10) or *mdx* mice and analysed by Northern blotting. Blots were hybridized successively with the GLUT4 cDNA probe and with an oligo-probe specific for 18 S RNA, to take into account differences in the amounts of RNA loaded on to the gels. Probing Northern blots with the GLUT4 cDNA yielded a single 2.7 kb transcript in both tissues and in both groups of mice (Figures 3 and 4). In

Figure 2 GLUT4 immunodetection in diaphragms of control and *mdx* mice

Crude membranes were prepared from young (5–6 weeks old) and old (6–7 months old) control and *mdx* mice. Identical amounts of membranes (60 μ g) were subjected to SDS/PAGE and proteins were then transferred to nitrocellulose and immunoblotted with an anti-GLUT4 antibody as described in the Materials and methods section. Three different preparations from three mice in each group are presented.

Figure 3 GLUT4 mRNA levels in hindleg skeletal muscle from 5–6-week-old control and *mdx* mice

Hindleg skeletal muscle was used for total RNA extraction, and Northern blot analysis was performed as described in the Materials and methods section. Identical amounts of total RNA (20 μ g) from four control and three *mdx* mouse preparations were loaded. Hybridization was performed successively with cDNA probes for GLUT4 and GAPDH, and with an oligoprobe for 18 S RNA. Autoradiograms were exposed for 48 h for GLUT4 and 8 h for 18 S RNA and GAPDH at -80°C .

hindleg skeletal muscle (Figure 3), the amount of GLUT4 transcript/ μ g of RNA was lower in *mdx* than in control mice. Quantification of the autoradiograms by scanning (Table 3) showed a $\sim 30\%$ decrease in *mdx* mice when GLUT4 mRNA was expressed per μ g of RNA. However, since the total RNA content increased by $\sim 40\%$ in the skeletal muscle tissue of *mdx* mice (Table 3), the total GLUT4 mRNA content was similar in the hindleg muscles of *mdx* and control mice. When the same RNA preparations were hybridized with a probe for GAPDH, an enzyme involved in glycolysis, the pattern of its mRNA expression was parallel to that of GLUT4 mRNA. The expression of GLUT4 mRNA was measured in the diaphragm muscles from 6–7-month-old control and *mdx* mice at a time when the GLUT4 protein was markedly decreased. The Northern blot presented in Figure 4 clearly shows that GLUT4 mRNA was diminished in diaphragms of *mdx* mice compared with control mice when the same amount of total RNA was loaded. As total RNA content

Table 3 Quantification of GLUT4 mRNA levels in skeletal muscle from control and *mdx* mice

Total RNAs were extracted from 5–6-week-old control and *mdx* mice. Total RNA contents were determined by measuring the absorbance at 260 nm. RNAs were analysed by Northern blotting, and hybridized with a GLUT4 cDNA probe and an 18 S oligoprobe as described in the Materials and methods section and in the legend to Figure 3. Signals were quantified by scanning densitometry of autoradiograms. Results are means $\pm$ S.E.M. of five control and four *mdx* RNA preparations used in two different Northern blot hybridization experiments.

	Control	<i>mdx</i>
Total RNA content (μ g/g of tissue)	910 $\pm$ 40	1350 $\pm$ 150
GLUT4 mRNA (arbitrary units/ μ g of RNA)	1354 $\pm$ 74	947 $\pm$ 145

Figure 4 GLUT4 mRNA levels in diaphragm from 6–7-month-old control and *mdx* mice

RNA extraction and Northern blot analyses were performed as described in the Materials and methods section. Total RNA (20 μ g) from four RNA preparations obtained from four mice in each group was loaded. Hybridization was performed as described in Figure 3 with a cDNA probe for GLUT4 and with an oligoprobe for 18 S RNA. Autoradiograms were exposed for 8 h at -80°C .

was similar in the diaphragms of *mdx* and control mice, the amount of GLUT4 mRNA in diaphragms of *mdx* mice was decreased when results were expressed per μ g of total RNA or per total tissue.

DISCUSSION

We have studied GLUT1 and GLUT4 glucose transporter expression in different muscles and in the brains of control and *mdx* mice. In accord with previous reports, the *mdx* mice did not show striking alterations in development [1,10], with their mean body weight even being increased compared with control mice at 6–7 months of age [30]. High activities of the muscular enzyme creatine kinase were present in the serum of *mdx* compared with control mice, as previously described [1,31–33]. This measurement was used in our study to confirm the phenotype of each mouse.

Glucose transporter expression was altered in some muscles from *mdx* compared with C57B1/10 control mice, but no modifications were observed in the brain. These changes were clearly dependent on the muscle studied. Indeed, the amount of GLUT4 was unchanged in the heart, which is moderately or not at all involved in the mouse syndrome [10,34]. By contrast, GLUT4 expression was increased in the hindleg skeletal muscle in both young and old mice, but was decreased in the diaphragms of old mice. These results indicate that GLUT4 expression is

clearly not related to dystrophin deficiency, which occurs in all muscles, including the heart [7–9,35]. It seems to be more closely related to the ability of muscle to regenerate. The necrosis and regeneration of skeletal muscle in *mdx* mice has been well documented [10–12], with acute necrosis and regeneration being most marked in hindleg muscles at 1–2 months [10]. This is accompanied by increases in protein synthesis and degradation rates in hindlimb skeletal muscles [36]. The increase in the amount of GLUT4 protein observed in skeletal muscle is thus associated with a marked capacity for muscular regeneration. The results obtained with diaphragms are consistent with the relationship between the regeneration capacity of the muscles and GLUT4 expression. Indeed, in diaphragms of young *mdx* mice, foci of myofibre degeneration, necrosis, mineralization and regeneration are present, but are not extensive [13], and the amount of GLUT4 was normal. During the development of the syndrome, in contrast to *mdx* limb muscles, the diaphragm undergoes progressive degeneration and fibrosis [13]. This was associated with a decrease in GLUT4 content in the diaphragms of 6–7-month-old *mdx* mice compared with their age-matched controls.

Northern blot analyses were performed to investigate whether the changes in GLUT4 levels were regulated via changes at the mRNA level. In the diaphragm, the decrease in GLUT4 protein content observed in 6–7-month-old *mdx* mice compared with control mice was paralleled by a decrease in the GLUT4 mRNA level. By contrast, the increase in the amount of GLUT4 protein found in *mdx* skeletal muscle was not accompanied by a similar change in GLUT4 mRNA, suggesting post-transcriptional regulation with an increase in synthesis rate and/or a higher stability of the protein.

The changes in the total number of GLUT4 transporters in hindleg skeletal muscle do not appear to modify glucose uptake in these muscles. Indeed, glucose uptake was unchanged in soleus muscles of *mdx* mice compared with control mice ([14]; C. Olichon-Berthe, unpublished work) and, furthermore, *mdx* mice did not present any gross alteration of glycaemia or insulinaemia. It should be noted that in muscles under basal conditions GLUT4 molecules are mostly intracellular, and they are incorporated into the plasma membrane only when muscles are stimulated by insulin or exercise [37,38]. Since the membrane preparations analysed in the present study represent both intracellular membranes and plasma membranes, the GLUT4 concentration could be unchanged at the plasma membrane level despite an increase in the total pool of GLUT4 in *mdx* mice. The difficulty in obtaining pure plasma membranes and intracellular membranes precludes the possibility of testing this hypothesis. It is also conceivable that the intrinsic activity of GLUT4 is decreased in *mdx* skeletal muscle.

In conclusion, glucose transporter expression was altered in *mdx* mice compared with control mice, and alterations could be related to the ability of the tissue to regenerate. Indeed, the amount of GLUT4 was increased in skeletal muscle in which regeneration is marked. By contrast, GLUT4 expression was decreased in the diaphragms of 6–7-month-old *mdx* mice, and seems to parallel the progressive degeneration occurring in this tissue.

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