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ORIGINAL ARTICLE

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GLUT-4 expression is not consistently higher in type-1 than in type-2 fibres of rat and human vastus lateralis muscles; an immunohistochemical study

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Abstract In whole muscle homogenates, the glucose transporter-4 (GLUT-4) content is reported to be higher in muscles consisting predominantly of oxidative (type-1) muscle fibres than in muscles consisting predominantly of glycolytic (type-2) fibres. From these findings, it has been deduced that in rat muscle, oxidative fibres have an intrinsically higher level of GLUT-4 protein than glycolytic fibres. No data is available concerning human muscle. Moreover, the fibre-type-specific expression of GLUT-4 has not yet been examined directly. In this study, the relative abundance of GLUT-4 protein expression in individual fibres of different types within a muscle was compared directly in immunohistochemical assays. The human vastus lateralis muscle and a selection of rat muscles were studied using a novel GLUT-4 antiserum. It is concluded that the pattern of fibre-type-specific GLUT-4 expression differs between human and rats and varies between the different muscles studied, indicating that non-fibre-type-specific factor(s) affect expression of GLUT-4. The observation that within a muscle a fibre-type-specific expression of GLUT-4 was observed indicates that fibre-type-specific factors contribute to GLUT-4 expression as well. Thus, it can be postulated that both fibre-type-dependent and fibre-type-independent factors affect GLUT-4 expression.

Keywords GLUT-4 · Fibre type · Immunofluorescence microscopy · Skeletal muscle activity

Introduction

Skeletal muscle is responsible for the major part of insulin-stimulated plasma glucose disposal [3]. The insulin and contraction regulatable glucose transporter, GLUT-4, is expressed abundantly in skeletal muscle of humans and rodents. Its expression in skeletal muscle correlates positively with insulin-stimulated glucose disposal in man [13]. In rat, GLUT-4 protein content is higher in muscles comprising predominantly oxidative fibres ("red muscle"), than in muscles consisting predominantly of glycolytic fibres ("white muscle") [6, 9, 11, 16, 17]. Accordingly, insulin-stimulated glucose uptake is highest in red muscle of the rat in vitro [6, 9, 11, 16, 17]. In humans, insulin-stimulated, whole-body glucose uptake correlates positively with the percentage of oxidative fibres in the vastus lateralis muscle and inversely with the percentage of glycolytic fibres [15]. This has been taken to suggest that in humans, as in rats, muscle GLUT-4 content is higher in oxidative than in glycolytic muscle fibres [10]. However, in a more recent study, no correlation between oxidative fibre content and GLUT-4 protein expression was found in vastus lateralis muscle of athletes or controls [7].

Individual rat muscles often exhibit a preponderance of either glycolytic or oxidative fibres, justifying the subdivision into red and white muscles [1]. In contrast, fibre type composition is much less variable between human skeletal muscles [20]. Thus, extrapolation from the relationship between muscle fibre type and GLUT-4 protein expression in whole-muscle homogenates from rats to humans is a rather indirect and possibly invalid approach. A more direct assessment of this relation is therefore essential. In the present study, this was achieved by concurrently determining muscle fibre type and the abundance of GLUT-4 protein expression by immunofluorescence staining in serial muscle sections. We investigated the hypothesis that in human skeletal muscle, the fibre type/GLUT-4 protein expression relationship is similar to that reported in whole-muscle homogenates from rats, namely a higher expression of GLUT-4 in oxidative than in glycolytic fibres. Rat muscles were investigated using to the same procedures to confirm the observations of earlier studies on whole-muscle homogenates [6, 9, 11, 17].

Immunostaining was performed using a previously unpublished, custom-raised, affinity-purified polyclonal

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rabbit antibody (GLUT4-BW). The procedures followed for raising and characterising this antibody will therefore be discussed here as well.

Materials and methods

Subjects and biopsy procedure

Ten sedentary male human volunteers, aged 45–60 years, were recruited by an advertisement in a local newspaper. All gave their written, informed consent before entering the study, which was approved by the medical ethics committee of Maastricht University. All subjects had refrained from any vigorous exercise for at least 48 h, before, in the morning and following an overnight fast, biopsies were taken using a Bergström-needle, modified according to [5], from the vastus lateralis muscle. Skin and muscle fascia were anaesthetised using xylocaine (without adrenaline) and an incision of approximately 0.7–1.0 cm was made through skin and fascia. All biopsies were taken about 20 cm proximally from the patella. Further procedures of biopsy sample treatment will be described subsequently.

Animals and muscle sampling

Male Wistar rats (n=4) and male Lewis rats (n=3), aged 12 weeks, were used in this experiment. They were housed individually, with a 12-h dark/light cycle and provided with food and water ad libitum up to 2 h before muscle dissection. Rats were anaesthetised by injection of pentobarbitone sodium (Narcovet, 1.0 ml/kg body weight, i.p.), and soleus, extensor digitorum longus (EDL), vastus lateralis, tibialis anterior and medial and lateral gastrocnemius muscles were dissected carefully. All procedures were approved by the Institutional Animal Care and Use Committee of Maastricht University, and complied with the principles of laboratory animal care.

Muscle tissue processing

After biopsy (human) or dissection (rat), muscle tissue was frozen immediately in isopentane cooled to its melting point with liquid nitrogen. Orientation of the tissue was such that fibres were cut perpendicular to the muscle fibres. These samples were stored in an aluminium cryovial at –70 °C. Abundance of GLUT-4 expression (immunofluorescence assay) and muscle fibre typing {myosin adenosine 5′-triphosphatase (ATPase) staining [2]} were carried out on serial, transverse, 4-µm-thick cryosections.

Production and characterisation of the antibody used

The antibody (GLUT4-BW) used in the present study was raised in a New Zealand white rabbit by injecting a commercially synthesised (Biogenesis, New Fields, UK) peptide, comprising the final 12 amino acids of the C-terminus of the human GLUT-4 protein and diluted in Freund's adjuvant, into the popliteal lymph node. Prior to injection of the antigen, blood was sampled to obtain pre-immune serum. The first antigen injection was boosted after 6 weeks by a second injection. Then, 2 weeks after the booster, the animal was sacrificed by exsanguination. The serum was affinity purified and stored in small aliquots at –70 °C.

To characterise the antibody, immunoblotting and immunofluorescence assays were performed on both human and rat samples. Muscle biopsies (~100 mg) were homogenised in ice-cold buffer: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 10 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, pH 7.4 (HES buffer), containing 1 mM phenylmethylsulphonylfluoride (Merck, Darmstadt, Germany) and 400 U/ml Trasylol (Bayer, Leverkusen, Germany) and centrifuged 15 min at 3,000 g. The supernatant was ultracentrifuged at 150,000 g for 90 min at 4 °C. The membrane fraction (pellet) was suspended in 50 µl phosphate-buffered saline (PBS) and stored at –70 °C until further processing. Polyacrylamide sodium dodecylsulphate (SDS)-gel electrophoresis was performed essentially according to Laemmli [14]. In short, 25 µl membrane fraction was boiled for 4 min in an equal volume of SDS sample buffer containing 2.3% SDS (BDH, Poole, UK) and 5% β-mercaptoethanol (Bio-Rad, Hercules, Calif., USA) and subsequently centrifuged for 5 min. Polyacrylamide slab gels containing 0.1% SDS were loaded with the samples on a Mini Protean-3 Electrophoresis Cell (Bio-Rad). After electrophoretic separation, the proteins were immunoblotted using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The separated polypeptides were transferred to a nitrocellulose membrane (Bio-Rad) by blotting for 60 min at 100 V in cold (4 °C) buffer containing 25 mM tris(hydroxymethyl)aminomethane (TRIS, Merck), 192 mM glycine (Merck) and 20% methanol (Merck) [22]. For detection of GLUT-4 we used the polyclonal rabbit antiserum GLUT4-BW. Nitrocellulose sheets were pre-incubated for 60 min in blocking buffer containing 5% non-fat dry milk (Bio-Rad) and 0.05% Tween20 (Sigma, St. Louis, Mo., USA) in PBS. Incubation with anti GLUT-4, diluted 1:1,000 in blocking buffer, was carried out overnight at room temperature while gently shaking. After three washing steps with 0.05% Tween20 in PBS, blots were incubated for 60 min at room temperature with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin (Ig) (SWARPO, Dako, Glostrup, Denmark), diluted 1:10,000 in blocking buffer. Blots were subsequently washed for 90 min in 0.05% Tween20/PBS and for 10 min in PBS. The chemiluminescence assay was performed using a Super Signal West Dura Extended kit (Pierce, Rockford, Il., USA). Cross-reaction of the GLUT4-BW antibody with other proteins was checked for by examining the entire 94- to 5-kDa range for additional bands. The molecular weight of the band visualised by chemiluminescence was estimated by comparison with reference values.

Specificity of the GLUT-4 antibody in immunostaining was checked for by pre-incubation of the antibody with the peptide. The pre-immune serum was tested also. Cross-reactivity of the secondary antibody was tested by omission of the primary antibody. For sake of comparison with other findings [18] we included longitudinal sections as well (Fig. 1B). In addition, the same incubation procedure was followed after replacing the primary GLUT4-BW antibody by three distinct, commercially available GLUT-4 antibodies. These antibodies were a rabbit-anti-insulin-regulatable-glucose-transporter (manufacturer's code: RaIRGT, East Acres Biologicals, Southbridge, Mass., USA) in a 1:25 dilution; a polyclonal antibody in a 1:50 dilution raised in a rabbit against a 12-amino-acid peptide at the C-terminus of GLUT-4, with inclusion of a cysteine at the N-terminus to accommodate protein conjugation (code: 4670-1704, Biogenesis, Poole, UK) and a polyclonal antibody in a 1:25 dilution, raised in a goat against an epitope within a C-terminal cytoplasmic domain of human GLUT-4 with unknown amino acid sequence [code: Glut4 (C-20) sc-1608, Santa Cruz Biotechnology, Santa Cruz, Calif., USA].

Muscle sectioning and staining

Frozen muscle tissue was cryosectioned at –20 °C (CM3050, Leica, Nussloch, Germany). After trimming the block face, transverse sections of 4 µm were cut serially and every ninth and tenth section thaw-mounted sequentially on one of two uncoated glass slides. Sections were air-dried before storing (at –70 °C) or staining. Labelling of the sections on the one slide started with 5 min fixation in methanol (–20 °C) followed by 1′ acetone fixation and air-drying. After each step, sections were washed 3 times for 5 min in PBS. Sections were incubated overnight at 4 °C with GLUT4-BW [1:10 in 1% bovine serum albumin (BSA)/PBS]. Sections were incubated subsequently for 30 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit Ig (GARFITC, SBA, Birmingham, Ala., USA) or with a FITC-conjugated donkey-anti-goat (DAGFITC, SBA) antibody. There-

after sections were mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 0.5 µg/ml 4′,6′-diamino-2-phenylindole (DAPI, Merck) for deoxyribonucleic acid (DNA) staining.

The second slide with the corresponding serial section was subjected to muscle fibre typing using the ATPase staining method [2]. Sections were immersed in acetate buffer at either pH 4.3, pH 4.45, or pH 4.6 at room temperature for 45 s, washed in three rinses of TRIS buffer at pH 7.8 and three rinses of distilled water. Sections were then incubated for 25 min in fresh ATP-glycine buffer (pH 9.4), quenched 3 times in calcium chloride (1%), washed in three rinses of distilled water, incubated 3 min in cobalt chloride 1%, washed in three rinses of distilled water, incubated 1 min in ammonium sulphate and washed in three rinses of distilled water. Nuclei were stained for 1 min with haematoxylin solution according to Mayer (Fluka, Buchs, Switzerland). After rinsing the slides for 20 min in tap water, slides were dehydrated in an ethanol series of 50–100% and 2 times xylol 100% and embedded in Entellan (Merck).

All sections were examined and photographed using a Leica DM fluorescence microscope, coupled to a CCD camera and image processing system (MetaSystem, Heidelberg, Germany). Exposure time of the fluorescent sections was maintained constant. Sections stained for ATPase for fibre typing were photographed using identical magnification. On all sections at least four corresponding fields, comprising at least 250 different fibres, were matched offline by visual inspection. Subsequently these fields were examined by comparing the relative abundance of GLUT-4 protein expression within the distinguishable fibre types (types 1, 2A and 2B).

Results

In both human and rat vastus lateralis muscles, GLUT-4 protein was detected with Western blotting using the novel polyclonal rabbit GLUT4-BW antibody at a molecular mass of some 43 kDa (Fig. 1A). This affinity-purified GLUT-4 antiserum is directed against the final 12 amino acids at the C-terminus of the human GLUT-4. The GLUT4-BW antiserum was characterised and tested for specificity by immunoblotting and immunofluorescence assays on different muscles of different species. As indicated in Fig. 1A, cross-reaction of the primary antibody with proteins other than GLUT-4 is negligible in the 94- to 95-kDa range in both human (lanes 1 and 2) and rat (lanes 3 and 4) muscle samples.

Pre-incubation with the peptide against which the antibody was raised yielded no detectable labelling, nor did the samples incubated with pre-immune serum instead of the GLUT4-BW antibody (results not shown). In addition, omission of the GLUT4-BW antibody from the immunofluorescence labelling procedure completely abolished the fluorescent signal (results not shown). A representative longitudinal section (obtained from human muscle) is shown in Fig. 1B, revealing a pattern of strings of GLUT-4 aggregates parallel to the fibre axis as has been observed before in a preparation of single whole fibres from the rat soleus muscle [18].

In our hands, the Santa Cruz Glut4 (C-20) sc-1608 antibody yielded no detectable immunofluorescence labelling of human or rat muscle sections. The other two commercially available antibodies yielded results similar to our custom-raised GLUT4-BW antibody i.e. GLUT-4 protein expression is more abundant in type-2 fibres

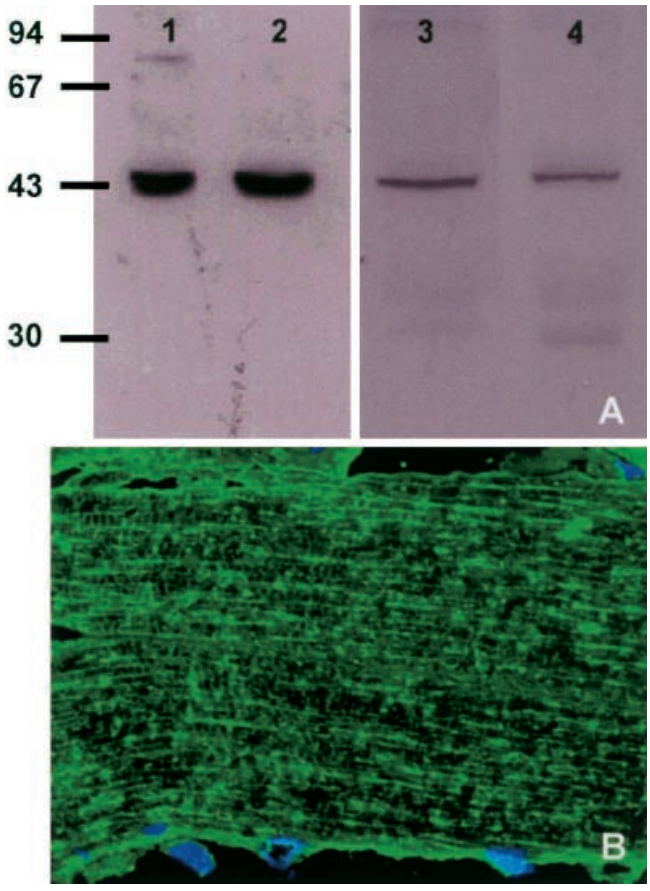

Fig. 1 **A** Western blots using the polyclonal rabbit glucose transporter-4 (GLUT4)-BW antiserum. The *numbers* on the *left* indicate the migration of the marker proteins (kDa) in the 12% polyacrylamide sodium dodecylsulphate (SDS) gel. The GLUT4-BW antibody recognises GLUT-4 at 43 kDa in membrane fractions from two randomly chosen human vastus lateralis samples (lanes *1* and *2*) as well as in membrane fractions from rat extensor digitorum longus (EDL) (lane *3*) or vastus lateralis (lane *4*) muscles. **B** Immunofluorescence staining of GLUT-4 in a longitudinal section of human vastus lateralis muscle, detected with the polyclonal antibody GLUT4-BW. GLUT-4 protein staining appears as the *dotted lines* aligned with the fibre axis. Muscle cell nuclei appear blue due to deoxyribonucleic acid staining with 4′,6′-diamino-2-phenylindole

(compare Fig. 2A with B). This finding was consistent in all sections examined and independent of the primary antibody used [compare Fig. 2C and D (East Acres antibody RaIRGT) and 2E and F (Biogenesis antibody 4670-1704) with 2A and B (GLUT4-BW antibody)].

Abundance of GLUT4 expression in muscle sections

The results reported here represent consistent observations after examining three duplicate (serially cut) sections per subject with a minimum of four microscopic fields (comprising ~250 muscle fibres). Immunofluorescence studies of transverse sections of human vastus lateralis muscle revealed a distinct “chessboard” pattern of

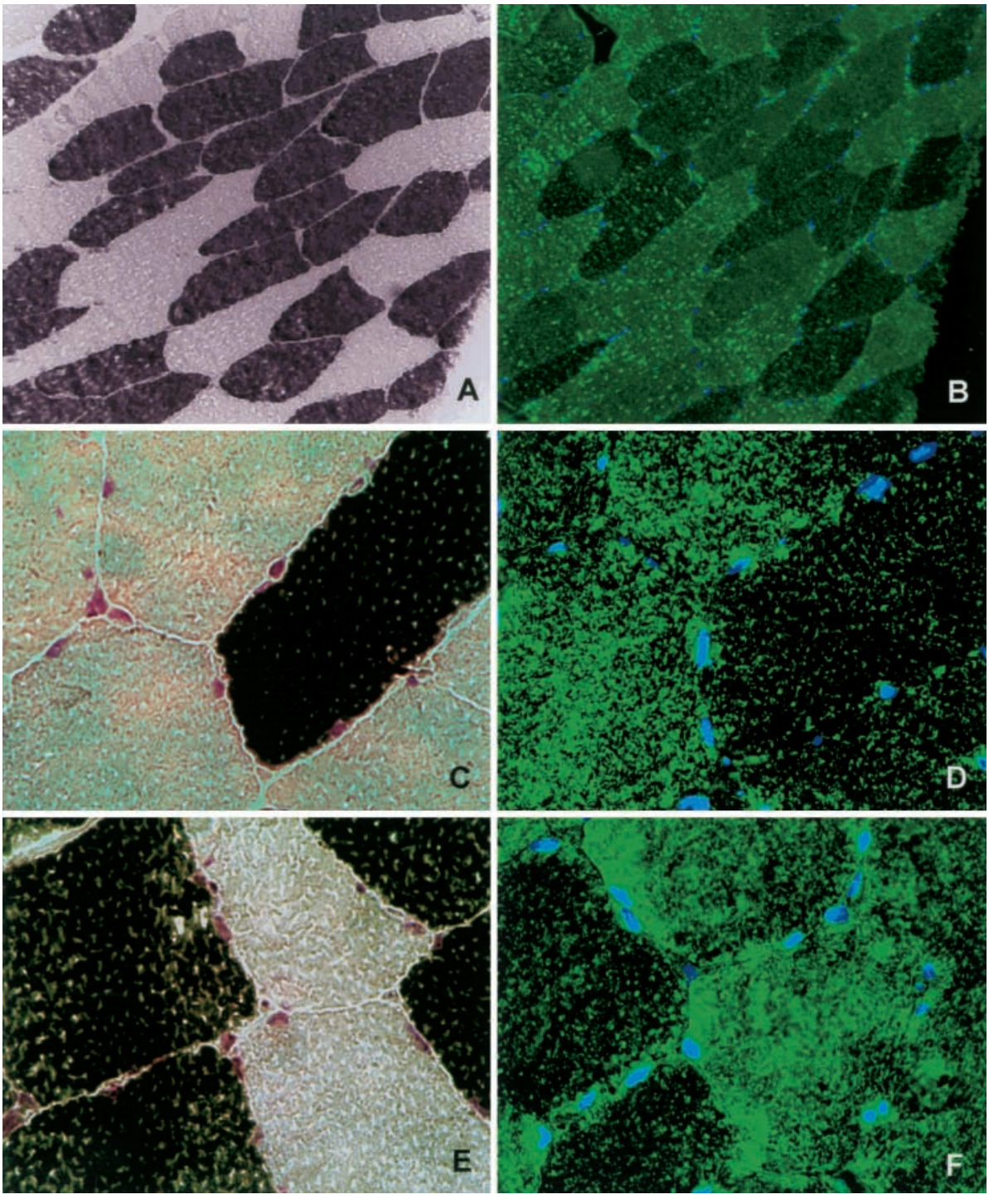

Fig. 2 **A**, **C**, **E** Myosin adenosine 5′-triphosphatase (ATPase) staining of human vastus lateralis muscle. Pre-incubation was at pH 4.3, therefore type-1 fibres appear dark, type-2 fibres appear light. **B** Immunofluorescence staining of GLUT-4 in a transverse serial section of human vastus lateralis muscle, using the GLUT4-BW antibody. **D**, **F** Immunofluorescence using the commercially available GLUT4 antibodies RaIRGT from East Acres or 4670-1704 from Biogenesis, respectively. Photomicrographs shown here comprise a representative selection of all sections examined and were obtained after randomly imaging the ATPase stained sections with off-line matching with corresponding field in fluorescence

fibres stained either strongly or weakly for GLUT-4 (Fig. 2B). ATPase staining of corresponding slides (Fig. 2A) showed that slow-twitch (type-1) fibres had a low expression of GLUT-4 compared with glycolytic fibres (types 2A, 2B). No consistent distinction could be made between types-2A and -2B fibres with respect to GLUT-4 protein labelling.

In both the medial (Fig. 3B) and lateral (data not shown) gastrocnemius muscle of the rat, GLUT-4 expression relative to fibre type was opposite to that

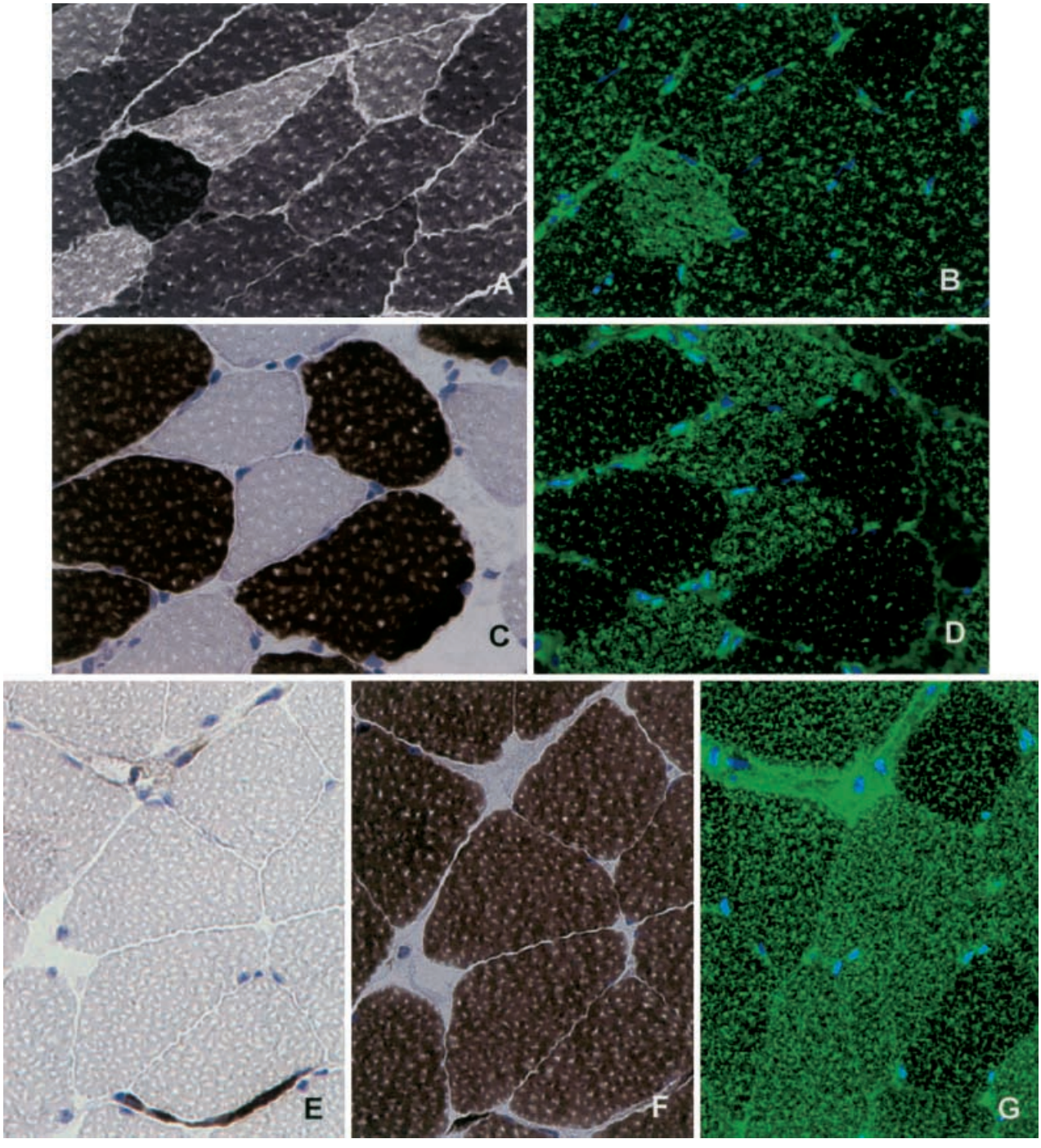

Fig. 3 **A** Myosin ATPase staining of a section of rat medial gastrocnemius muscle. Pre-incubation was at pH 4.45, therefore type-1 fibres appear dark, type-2A fibres appear light and type-2B fibres appear intermediately stained. **B** Immunofluorescence staining of GLUT-4 in the corresponding section of rat medial gastrocnemius muscle. **C** Myosin ATPase staining of a transverse section of rat soleus muscle. Pre-incubation was at pH 4.45, therefore type-1 fibres appear dark, type-2A fibres appear light. No type 2B fibres are visible. **D** Immunofluorescence staining of GLUT-4 in the corresponding section of rat soleus muscle. **E**, **F** Myosin ATPase staining of serial transverse sections of rat vastus lateralis muscle. **E** Pre-incubation at pH 4.3, after which both types-2A and -2B fibres appear light. **F** Pre-incubation at pH 4.6, after which type-2A fibres appear light, type-2B fibres dark. Thus, all fibres in this section are type 2B. **G** Immunofluorescence staining of GLUT-4 in a serial section of rat vastus lateralis muscle. Photomicrographs shown here comprise a representative selection of all sections examined and were obtained after randomly imaging the ATPase stained sections with off-line matching with corresponding field in fluorescence

observed in human vastus lateralis muscle; that is, GLUT-4 fluorescence labelling was highest in type-1 fibres (Fig. 3B). In contrast, in all other rat muscles studied: soleus (Fig. 3C and D), EDL (data not shown), tibialis anterior (data not shown) and vastus lateralis muscle (Fig. 3E–G), GLUT-4 expression was highest in type-2 fibres and lowest in type-1 fibres. In none of the muscles could a clear difference be observed in GLUT-4 expression between types-2A and -2B fibres. However, in rat vastus lateralis muscle, small differences in GLUT-4 labelling were observed between individual type-2 fibres, irrespective of their subclass (Fig. 3E–G).

Discussion

The GLUT-4 content in different muscle fibre types has almost exclusively been deduced from measurement of GLUT-4 protein content in muscle homogenates of different muscles of the rat [6, 8, 9, 11, 17]. These studies have shown conclusively that red muscles, such as soleus, have a higher GLUT-4 protein expression than white muscle, such as EDL. Accordingly, in vitro insulin-stimulated glucose uptake is highest in red muscle groups [6, 8, 9]. Although this approach is most likely valid in rodents that possess a homogenous intramuscular fibre type distribution, extrapolation of these findings to humans is hampered by the more heterogeneous intramuscular fibre type distribution. A direct assessment of the relationship between muscle fibre type and GLUT-4 expression is therefore preferable.

Here we studied the relationship between GLUT-4 expression and muscle fibre typology directly by (immuno)histochemical staining of serial sections, rather than by studying GLUT-4 expression in whole muscle homogenates of muscles characterised as “red” or “white”, based on which GLUT-4 expression is believed to be highest in type-1 fibres.

At present, we are aware of only three studies showing GLUT-4 immunofluorescence in rat [16, 18] and human [23] skeletal muscle. In the one study [18] GLUT-4 localisation is elegantly shown in longitudinal muscle sections as well. The subcellular localisation of GLUT-4 in longitudinal and cross sections as detected by the GLUT4-BW antiserum is perfectly in line with observations by others [16, 18, 23]. The GLUT4-BW antiserum detected a 43-kDa band in gels after Western blotting without cross-reaction with proteins of distinct molecular mass, this is in accordance with previous investigations [9, 10, 11]. Since the GLUT4-BW antibody, presented here for the first time, successfully passed all specificity checks performed, together with the observation that similar results were obtained using commercially available GLUT-4 antibodies, we conclude that the GLUT4-BW antibody specifically recognises GLUT-4 in skeletal muscle sections of different species without cross-reacting with other proteins.

Contrary to our hypothesis, we found that GLUT-4 protein was more abundantly expressed in glycolytic than in oxidative fibres of human vastus lateralis muscle. This relationship is thought to be the opposite in rodents. However, species-specific differences have been suggested previously in this regard: in both bovine and goat muscle, GLUT-4 expression decreases with increasing oxidative capacity in different muscle groups [10]. Therefore, we also examined rat skeletal muscle using to the same procedures and found that the relation between GLUT-4 expression and fibre type is dependent on the muscle group studied. In medial gastrocnemius muscle, which expresses mostly type-2 fibres, GLUT-4 expression is highest in type-1 fibres, while in the other muscles studied here, this relationship was the opposite.

As a part of an elaborate study of the expression and localisation of glucose transporters in red and white muscle of the rat by Marette et al. [16], immunofluorescence labelling has revealed that in the medial gastrocnemius, type-1 fibres contain more GLUT-4 protein than type-2 fibres. This finding is in agreement with our data in rat gastrocnemius, but we have shown here for the first time that in a selection of other rat muscles, as well as in human vastus lateralis muscle, type-2 fibres show the highest expression of GLUT-4 protein. This may seem incompatible with the findings of the previously cited studies on whole-muscle homogenates that report a lower expression of GLUT-4 in rat medial gastrocnemius than in e.g. rat soleus muscle [6, 9, 11, 16, 17]. However, the more abundant expression of GLUT-4 in type-1 fibres within the medial gastrocnemius compared with the type-2 fibres is most likely not reflected in Western blots of whole-muscle homogenates due to the low number (less than 4%) of type-1 fibres in the medial gastrocnemius of the rat [1, 21]. Therefore, we feel that studies of GLUT-4 expression employing whole-muscle homogenates yield results different to those reported here largely due to differences in the experimental procedure (i.e. studying GLUT-4 content indirectly in so-called oxidative muscles vs. studying GLUT-4 content directly in muscle fibres with known fibre typing).

It is our belief that differences in GLUT-4 content between different muscles depend on factors other than fibre type composition. Observations from previous studies illustrate this. Hardin et al. have shown that, although marathon runners had a higher muscle GLUT-4 content than sedentary controls, insulin sensitivity and muscle GLUT-4 expression are not dependent upon muscle fibre type composition in either group [7]. Etgen et al. measured GLUT-4 protein expression in the epitrochlearis, containing ~85% type-2 fibres, and soleus, containing ~85% type-1 fibres, muscles in the obese Zucker rat before and after training [4]. GLUT-4 protein content in the epitrochlearis muscle after endurance training approaches the pre-training GLUT-4 levels in soleus muscle. This shows that although the epitrochlearis predominantly expresses type-2 fibres, its GLUT-4 content can increase to a level found in muscle consisting almost exclusively of type-1 fibres within 2 weeks of exercise training. Muscle fibre type composition is determined largely genetically [12]. However, GLUT-4 protein ex-

pression can be up-regulated within hours after acute exercise [19], a time span too short to affect fibre type composition. This suggests at least a partial independence of GLUT-4 expression and muscle fibre composition. Chronic stimulation (10 days) of rat white muscle with a stimulation pattern resembling nerve activity of a nerve innervating red muscle increases GLUT-4 expression, suggesting that the activity pattern of the motor nerve is decisive for GLUT-4 expression [4].

From these observations, a strict coupling between GLUT-4 content and muscle fibre type composition seems unlikely. It is therefore not surprising that the relative abundance of GLUT-4 in different fibre types varies among muscles, or indeed per muscle portion studied. Those muscle fibres with the highest glycolytic demand within a muscle will probably have the highest expression of GLUT-4. The glycolytic demand of a given muscle fibre will depend on the recruitment pattern of its motor units and its capacity to utilise substrates through pathways other than glycolysis. For some (or probably most) muscles, the type-2 fibres will have the highest glycolytic capacity, and thus GLUT-4 protein content. However, a muscle that is activated primarily as a fast-twitch glycolytic muscle will derive its energy largely from high-energy phosphate stores such as phosphocreatine. If a few type-1 fibres are expressed within such a muscle (as seen in the medial or white portion of the gastrocnemius; Fig. 3A and B), they will probably have to adapt to the muscle's function by up-regulating their glycolytic capacity. Thus, GLUT-4 protein content within such a muscle will be highest in type-1 fibres. Although this mechanism is a putative one, it could explain the findings of our (and indeed other) studies. It may prove interesting to direct future studies toward the falsification of this theory.

In summary, GLUT-4 content within the same fibre type differs between distinct muscles: in rat gastrocnemius muscle GLUT-4 expression was highest in type-1 fibres while in several other rat muscles, as well as human vastus lateralis, GLUT-4 expression was highest in the type-2 fibres. Based on the immunohistochemical studies described in this paper we conclude that the pattern of fibre type specific GLUT-4 expression may differ between rat and human muscles, that it varies between distinct fibre types within a muscle and that it varies between muscles within the same species. Fibre-type-specific expression of GLUT-4 within a muscle indicates that fibre-type-dependent factors affect the expression of GLUT-4 while differences in the fibre-type-specific GLUT-4 expression between muscles point towards fibre-type-independent factors affecting GLUT-4 expression. We hypothesise that, in addition to fibre-type specific-factors, other factors such as recruitment pattern and glycolytic demand, determine GLUT-4 expression in muscle.

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