# Glucose transporters and *in vivo* glucose uptake in skeletal and cardiac muscle: fasting, insulin stimulation and immunoisolation studies of GLUT1 and GLUT4

Edward W. KRAEGEN,*§ Judith A. SOWDEN,* Michael B. HALSTEAD,* Peter W. CLARK,* Kenneth J. RODNICK,† Donald J. CHISHOLM* and David E. JAMES‡

*Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, NSW 2010, Australia,
and †Department of Medicine and ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO, 63110, U.S.A.

Our aim was to study glucose transporters GLUT1 and GLUT4 in relation to *in vivo* glucose uptake in rat cardiac and skeletal muscle. The levels of both transporters were of a similar order of magnitude in whole muscle tissue (GLUT1/GLUT4 ratio varied from 0.1 to 0.6), suggesting that both may have an important physiological role in regulating muscle glucose metabolism. GLUT4 correlated very strongly ($r^2 = 0.97$) with maximal insulin-stimulated glucose uptake ($Rg'_{max.}$, estimated using the glucose clamp plus 2-deoxy[$^3$H]glucose bolus technique) in six skeletal muscles and heart. A distinct difference in regulation of the two transporters was evident in heart: in 5 h-fasted rats, basal glucose uptake and GLUT1 levels in heart were very high and both were reduced, by 90 and 60% respectively, by 48 h fasting. However, in heart (and in red skeletal muscle), neither GLUT4 levels nor $Rg'_{max.}$ were reduced by 48 h fasting. GLUT1 was shown to be specifically expressed in cardiac myocytes, because intracellular vesicles enriched in GLUT4 contained significant levels of GLUT1. In conclusion, the high association of muscle GLUT4 content with insulin responsiveness in different muscles, and the preservation of both with fasting, supports a predominant role of GLUT4 in insulin-mediated glucose uptake. GLUT1 may play an important role in mediating cardiac muscle glucose uptake in the basal metabolic state. Marked changes in GLUT1 expression with alterations in the metabolic state, such as prolonged fasting, may play an important role in cardiac glucose metabolism.

## INTRODUCTION

It has been established that there are at least two glucose transport proteins in muscle, namely GLUT1 and GLUT4 (see [1]), and, on the basis of intracellular targeting of the transporters analogous to that found in many other tissues, it has been suggested that these may be differentially involved in basal and insulin-stimulated muscle glucose transport respectively [2]. This has not been clearly resolved, however, and in particular it is not clear what is the functional role, if any, of GLUT1 in muscle glucose transport; this uncertainty has been exacerbated by the absence of definite evidence for the presence of GLUT1 in the actual muscle myocyte [3]. Also, while GLUT4 is clearly important for insulin-stimulated muscle glucose transport [4], it is possible that, eventually, other transporters will be found which are also involved. We have examined roles of GLUT1 and GLUT4 by relating transporter content to *in vivo* glucose fluxes in different muscle types of the rat, and have also performed immunoisolation studies in an attempt to provide definite evidence for the presence of GLUT1 in the muscle myocyte. The physiological states chosen for study were maximal insulin stimulation and prolonged fasting. Under maximal insulin stimulation it is likely that most muscle GLUT4 is located on the cell surface and can facilitate glucose transport [5]. Our rationale is that the heterogeneity in insulin-stimulated glucose uptake among different skeletal-muscle types [6] and between cardiac and skeletal muscle [7] can be used with transporter measurement to infer functional involvement of the glucose-transport proteins. This has been used in a limited way in comparisons between red and white hindlimb muscle of the rat [4,8,9]. However, a conclusion that GLUT4 is the sole transporter contributing to heterogeneity in muscle insulin responsiveness would be considerably strengthened by the demonstration of a high correlation between maximal capacity for insulin-mediated glucose uptake and muscle GLUT4 content over a wide range of muscle types. Prolonged fasting was chosen as a suitable physiological perturbation to assess differential involvement of GLUT1 and GLUT4. There are major changes in basal muscle glucose uptake with prolonged fasting. This is most apparent in metabolically active muscles; for example, after 48 h fasting, heart glucose uptake decreases to less than 10% of its level in the postabsorptive animal [10,11]. Over this period there is, if anything, only a small fall in GLUT4 content in heart [12], but the change in GLUT1 is not known. Regarding GLUT4 and fasting, it is notable that there is a transport/transporter discrepancy [3] in findings inferring muscle insulin resistance [13] and a lack of reduction in skeletal-muscle GLUT4 with fasting [3]. Interpretation of the influence of prolonged fasting on insulin-stimulated muscle glucose uptake is also complicated by the inference from isolated soleus [14] or perfused-rat-hindlimb data [15] that insulin sensitivity is not decreased and may in fact increase with prolonged fasting.

In view of other recent studies examining the cell type containing GLUT1 in muscle [3,16], we felt that a case for a functional role of GLUT1 in muscle glucose transport would be enhanced by evidence for the presence of GLUT1 in the actual muscle cell (myocyte) *in vivo*. Evidence for this was obtained in the myocardium using an immunoisolation procedure, making

Abbreviations used: $^3$H-2DG, 2-deoxy[2,6-$^3$H]glucose; EDL, extensor digitorum longus; *Rg'*, glucose metabolic index; NEFA, non-esterified fatty acids; BCA, bicinchoninic acid; ANOVA, analysis of variance; *GIR*, glucose infusion rate.

* To whom correspondence should be addressed.

use of a prior finding of exclusive localization of GLUT4 to the myocyte [17]. We suggest, on the basis of the physiological and biochemical data presented here, that GLUT1 may play a functional role in cardiac muscle glucose transport.

## EXPERIMENTAL

### Animals

Adult male Wistar rats (300–380 g) with free access to water and, when not fasting, to rat chow (65% energy intake as carbohydrate) were used in these studies. Rats were housed in a temperature-controlled room (22 ± 1 °C) with a 12 h/12 h light/dark cycle (lights on at 06.00 h). Rats for metabolic studies were housed in individual cages after cannula implantation.

### Euglycaemic clamp studies in 5 h- and 48 h-fasted rats

Rats for euglycaemic hyperinsulinaemic clamp studies were fitted with cannulae introduced into the carotid artery and jugular vein as previously described [18]. Studies were conducted 3–5 days after catheter implantation, on animals in the unrestrained sedentary conscious state following food withdrawal either 5 or 48 h previously. Food withdrawal for the 5 h group took place at the start of the light cycle (06.00 h) and thus was over a period when, with unrestricted feeding, there would have been very little food intake [19]. Euglycaemic hyperinsulinaemic clamps were performed on the 5 h- or 48 h-fasted groups as described previously [18]. Briefly, a continuous infusion of porcine insulin (Actrapid; Novo Industrie, Copenhagen, Denmark) was given for 2 h at a dose of 4.1 or 33 munits/min per kg to achieve plasma insulin concentrations in the upper physiological or maximal insulin ranges respectively [18]. Potassium (0.6–1.0 mmol/h per kg) was also infused during the high insulin-infusion rate to prevent insulin-induced hypokalaemia. The glucose infusion rate required to maintain euglycaemia during the second hour of the clamp ($GIR_{60-120}$) was taken as the steady-state net whole-body glucose-disposal rate. Blood samples (0.4 ml) were obtained for insulin determination in all clamp studies at 0, 60 and 120 min. Cells were resuspended and returned following the 0 and 60 min samples.

Insulin action and glucose metabolism within individual tissues *in vivo* were studied as described previously [18]. The non-metabolizable glucose analogue 2-deoxy[2,6-$^3$H]glucose (50 $\mu$Ci) ($^3$H-2DG) was administered as an intravenous bolus at 75 min after the commencement of the clamp or basal study. Blood sampling was as described previously [18]. At completion of the clamp, rats were anaesthetized (pentobarbitone; 60 mg/kg, intravenously) and heart and brain (cortex) samples and the following hindquarter muscles were rapidly removed and frozen: soleus (containing mainly slow-twitch oxidative fibres), superficial or white parts of the gastrocnemius and quadriceps (containing mainly fast-twitch glycolytic fibres), deep red part of the gastrocnemius and quadriceps (containing mainly fast-twitch oxidative-glycolytic fibres) and plantaris and extensor digitorum longus (EDL) (containing a mixture of fast-twitch red and white fibres, but predominantly fast-twitch glycolytic) [6]. An estimate of tissue glucose uptake (the glucose metabolic index, *Rg'*) was calculated from tissue accumulation of phosphorylated $^3$H-2DG [18].

### Basal glucose-uptake studies

Simplified studies, using a single jugular catheter, were also performed in 5 h- and 48 h-fasted rats by administering $^3$H-2DG as described above, but without insulin or glucose infusion, to examine the effects of fasting on basal *Rg'*. Tissues were taken for glucose-transporter measurement as described below.

### Analytical methods: *in vivo* metabolic studies

Blood and plasma glucose concentrations were determined with a glucose analyser (model 23AM; Yellow Springs Instruments). Plasma samples for determination of insulin and non-esterified fatty acids (NEFA) were kept at −20 °C until assayed. Insulin was assayed by a double-antibody radioimmunoassay. Enzymic colorimetric kits were used to determine plasma concentrations of NEFA (NEFAC code no: 279-75409; Wako Chemical Co., Osaka, Japan).

Plasma samples for determination of tracer concentration were deproteinized immediately in 2.8% $ZnSO_4$ and saturated $Ba(OH)_2$. An aliquot of supernatant was evaporated to remove $^3H_2O$ and redissolved in an aqueous scintillant (Picofluor 30; Packard Instruments, Rockville, MD, U.S.A.). Radioactivity counting was performed in a liquid-scintillation spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.).

### Total protein extraction and GLUT1 and GLUT4 protein analysis

This method was described previously [20]. In brief, muscles were removed, after brief anaesthesia, from adult male rats (300–400 g) that had been fasted for 5, 24 or 48 h. The tissues were rapidly frozen and stored at −90 °C until further treated by homogenization for 15 s in 30 vol. (v/w) of ice-cold buffer containing 20 mM Hepes, 1 mM EDTA and 250 mM sucrose. Protein concentrations were determined using the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, U.S.A.) according to manufacturer's instructions.

Muscle homogenates (25 $\mu$g of protein) were solubilized in Laemmli buffer containing 2% SDS and were electrophoresed using a 10% polyacrylamide resolving gel. Prestained molecular-mass markers were run in adjacent lanes. Proteins were transferred from gels to nitrocellulose using a Mini Trans apparatus (Bio-Rad) at 300 mA for 1 h.

Nitrocellulose sheets were incubated in PBS, pH 7.4 with 5% non-fat dry milk (Carnation) and 0.02% $NaN_3$ at 4 °C overnight. Nitrocellulose filters were immunoblotted with anti-GLUT4 or anti-GLUT1 antisera. These antisera were raised against synthetic peptides encompassing the C-terminus [4]. The sheets were then washed in PBS with 1% Triton X-100 for 30 min at 22 °C and incubated with $^{125}$I-labelled Protein A (0.25 $\mu$Ci/ml) in PBS with 1% powdered milk for 60 min at 37 °C. The sheets were washed again and air-dried. Autoradiography of the sheets was performed at −70 °C with Kodak X-Omat AR film using du Pont intensifying screens for 48 h. The relative levels of protein were determined by densitometry.

### Total RNA extraction and Glucose-Transporter-mRNA estimation

Procedures were performed as previously described [21]. In brief, RNA was extracted from approx. 200 mg of tissue using the method of Chomczynski [22]. Samples (15 $\mu$g) of total RNA were loaded on to 1%-agarose gels and electrophoresed under reducing conditions. Polaroid negatives were taken for densitometric evaluation of loading variation. The RNA was transferred on to Nitrocellulose filters under the recommended conditions. Filters were probed with a nick-translated 2 kb *Eco*R1 fragment of the HepG2 GT cDNA (GLUT1) as previously described [21]. The filters were washed and autoradiographed at −70 °C. The filters were stripped by consecutive washings in boiling 0.1%

SDS, and probed with the nick-translated GLUT4 probe (IRGTSK-2) at high stringency [21]. After washing, the filters were autoradiographed at −70 °C. The levels of mRNA were determined by densitometric analysis of the autoradiographs and corrected for minor loading variation.

### Immunoisolation of GLUT4 vesicles

After an overnight fast, rats were anaesthetized with sodium pentobarbital (50 mg/kg body wt., intraperitoneal injection). The skin was removed, and the gastrocnemius muscle was rapidly frozen *in situ* using an aluminium clamp cooled to the temperature of liquid $N_2$. The heart was excised, thoroughly rinsed in PBS, and frozen using the precooled aluminium clamp. Frozen muscles were weighed and pulverized at the temperature of liquid $N_2$. The muscle powder was homogenized in ice-cold buffer (5 ml/g of muscle) containing 20 mM Hepes, 1 mM EDTA, and 250 mM sucrose (HES), pH 7.4, by using a two-step process. The tissue was initially homogenized using a Tekmar Tissuemizer (2 × 10 s) at 75% of maximum output and then subjected to ten passes of a tight-fitting Potter–Elvehjem homogenizer at 800 rev./min. The homogenate was centrifuged for 15 min at 3000 *g* in a Sorvall SS-34 rotor at 4 °C. The resultant pellet from this centrifugation was resuspended in 10 vol. of HES buffer, rehomogenized using the Potter–Elvehjem homogenizer and centrifuged at 3000 *g* for 15 min as described above. Two additional extractions of the 3000 *g* pellet were performed, and all the supernatants were combined and centrifuged at 184000 *g* in a Beckman 50.2 Ti rotor for 90 min. The resulting high-speed pellet was resuspended in HES buffer to a final concentration of 5–8 mg/ml and kept at 4 °C. This membrane fraction was used as the starting material for subsequent immunoisolation of intracellular GLUT4 vesicles. Protein concentrations were determined using the BCA reagent according to manufacturer's instructions.

Magnetic beads (4.5 μm diameter) with conjugated sheep anti-mouse IgG bound to the surface were used for immunoadsorption (Dynal, Great Neck, NY, U.S.A.). In each of the steps described below, beads were separated from the supernatant using a magnetic particle concentrator. Beads were blocked with BSA (10 mg/ml in PBS) for 30 min at 22 °C and washed three times for 20 min, each at 4 °C., with PBS, pH 7.4. Ascites fluid (1F8) was added to the beads (1 ml/4 × $10^6$ beads) and incubated for 14 h ta 4 °C in PBS containing BSA (1 mg/ml), 1 mM EDTA and 0.02% $NaN_3$ using slow end-over-end rotation (25 rev./min). Beads were washed three times for 20 min each at 4 °C in PBS. Control beads were treated identically, except that 1F8 was not added. Beads were then incubated with the 184000 *g* muscle pellet suspension for 4 h at 4 °C in PBS/BSA (1 mg/ml)/EDTA (1 mM), pH 7.4. This incubation was performed in a volume of 1 ml containing membranes (100 μg/ml) and 2 × $10^7$ beads. The beads were washed twice for 20 min at 4 °C with 0.5 ml of PBS. All supernatants were saved and pelleted at 200000 *g* for 30 min in a Beckman T1-100 rotor. Both magnetic beads and pelleted membranes were resuspended in PBS and prepared for SDS/PAGE. Immunoblotting was performed with antisera specific for GLUT1, GLUT4 and $Na^+/K^+$-ATPase (obtained from B. Mercer, Washington University, and raised against an N-terminal synthetic peptide derived from the $a_1$ subunit of the rat $Na^+/K^+$-ATPase).

Additional data could come from the reciprocal experiment of co-immunoisolation of GLUT4 using the GLUT1 antibody. However, we have been unable to perform this; there appears to be a technical problem in immunoprecipitating GLUT1-containing vesicles, since we were unable to immunoprecipitate significant levels of GLUT1 using the procedure. This may relate to the antigen density in the vesicles being a critical determinant of immunoprecipitation efficiency [23,24].

### Statistical analysis

All experimental data are expressed as means ± S.E.M. Statistical analyses were performed using unpaired two-tailed Student's *t* tests or a one-way analysis of variance (ANOVA) as appropriate. Probability values $P < 0.05$ were considered significant.

## RESULTS

### Muscle glucose uptake and transporter content in briefly fasted rats

In the present studies we have estimated either glucose uptake or GLUT1/GLUT4 content in whole muscle under different experimental conditions. Like all previous studies of muscle glucose metabolism, we do not distinguish between different cell types present in the whole muscle. Thus changes that are described and correlations made refer to measurements on this basis. To estimate whole-muscle glucose uptake we have employed the *in vivo* deoxyglucose technique [18]; while not a direct measure of glucose transport, it probably closely reflects this process, because considerable evidence suggests that this is the rate-limiting step [25]. To measure the relative levels of GLUT1 and GLUT4 in muscle we have employed a quantitative immunoblotting technique. By using saturating concentrations of primary antibody the amount of labelled secondary antibody bound is proportional to the total amount of antigen present. This technique has been extensively validated [26]. Over the muscles sampled (Table 1) the GLUT1/GLUT4 ratio was found to vary from 0.1 to 0.6. This compares well with other methods of quantification [27].

Table 1 shows GLUT1 and GLUT4 in the muscles sampled, with basal and maximally insulin-stimulated *in vivo* glucose uptakes obtained in similar muscles (glucose uptake estimated as glucose metabolic index, *Rg'*, based on the deoxyglucose–euglycaemic-clamp technique performed in the awake state [18]). There was considerable variation in GLUT4 levels, and in insulin-stimulated *Rg'*, among individual muscles, with a high degree of correlation between these parameters ($r^2 = 0.97$, $P < 0.0001$). Heart muscle had the highest insulin-stimulated *Rg'*

**Table 1 Relative muscle content of GLUT1 and GLUT4 compared with *in vivo* glucose uptake (glucose metabolic index *Rg'*) at basal insulin levels and under maximal insulin stimulation (euglycaemic clamp during 33 munits/min of insulin infusion) per kg in 5 h-fasted rats**

Abbreviations used: Red, red quadriceps or gastrocnemius; White, white quadriceps or gastrocnemius. Quantification of absorbances (*A*) was performed over four experiments. Glucose-uptake data are means for five to seven rats. Results are means ± S.E.M.

| | Glucose metabolic index (*Rg'*) (μmol/min per 100 g) | | Glucose-transporter content (*A*) | |
|---|---|---|---|---|
| Muscle | Basal | Maximal insulin stimulation | GLUT1 | GLUT4 |
| Heart | 37.0 ± 2.9 | 52.1 ± 4.0 | 20.6 ± 1.4 | 76.4 ± 4.6 |
| Diaphragm | 5.3 ± 0.6 | 41.6 ± 4.9 | 9.3 ± 0.9 | 58.8 ± 6.6 |
| Soleus | 5.4 ± 0.8 | 32.7 ± 3.1 | 7.1 ± 0.8 | 53.2 ± 3.6 |
| Red | 2.2 ± 0.2 | 29.0 ± 3.1 | 9.8 ± 1.5 | 46.4 ± 1.9 |
| Plantaris | 1.8 ± 0.2 | 22.8 ± 4.6 | 9.8 ± 1.2 | 29.2 ± 4.1 |
| EDL | 3.4 ± 0.6 | 20.5 ± 4.9 | 11.2 ± 1.0 | 28.0 ± 3.9 |
| White | 1.3 ± 0.1 | 10.5 ± 1.6 | 11.2 ± 1.3 | 19.8 ± 3.6 |

**Table 2 Effect of fasting on metabolic parameters and tissue RNA and protein yields**

Results are means ± S.E.M. ($n$ = 4–6).

| Group | Fasting period... 5 h | 48 h |
|---|---|---|
| Final body weight (g) | 347 ± 6 | 309 + 6* |
| Plasma glucose (mM) | 6.9 ± 0.2 | 5.5 ± 0.2* |
| Plasma insulin (munits/l) | 17 ± 2 | 2 ± 1* |
| Plasma NEFA (mmol/l) | 0.65 ± 0.08 | 0.78 ± 0.06 |
| RNA yield ($\mu$g/mg wet wt. of tissue) | | |
| Heart | 0.68 ± 0.01 | 0.58 ± 0.08 |
| Diaphragm | 0.45 ± 0.07 | 0.62 ± 0.11 |
| Red quadriceps | 0.78 ± 0.05 | 0.75 ± 0.04 |
| White quadriceps | 0.44 ± 0.02 | 0.45 ± 0.02 |
| Brain | 0.76 ± 0.07 | 0.84 ± 0.09 |
| Protein yield ($\mu$g/mg wet wt. of tissue) | | |
| Heart | 171 ± 15 | 204 ± 7 |
| Soleus | 182 ± 8 | 177 ± 10 |
| Red quadriceps | 217 ± 4 | 212 ± 6 |
| White quadriceps | 195 ± 5 | 225 ± 14 |

* $P < 0.01$ compared with 5 h-fasted group

**Table 3 Effect of 48 h-fasting on GLUT1 and GLUT4 protein and basal glucose uptake ($Rg'$)**

All parameters are expressed as a percentage of measurements in 5 h-fasted rats. Results are means ± S.E.M. ($n$ = 4–7); *$P < 0.05$; **$P < 0.01$ (versus 5 h-fasted values). Abbreviation: ND, not determined.

| Muscle | Fasting period (h) | Basal glucose uptake ($Rg'$) | Protein level (%) GLUT1 | GLUT4 |
|---|---|---|---|---|
| Heart | 24 | ND | 64 ± 9* | 100 + 9 |
| | 48 | 3 ± 0.3** | 41 ± 7* | 87 ± 7 |
| Soleus | 24 | ND | 75 ± 6* | 107 ± 9 |
| | 48 | 51 ± 18 | 56 + 9* | 98 ± 3 |

and GLUT4 content, followed by diaphragm and the various rat hindlimb muscles of decreasing order of oxidative capacity. In contrast with the pattern for GLUT4, there was little variation in GLUT1 among skeletal muscles, and the major feature of the relationship between GLUT1 and basal $Rg'$ was the high levels of both GLUT1 and basal $Rg'$ in the heart compared with other muscles. It should be noted that transporter content is expressed per unit of tissue protein, whereas $Rg'$ is expressed per unit of tissue wet weight. However, protein yields did not differ appreciably among different muscles (see, e.g., Table 2).

### Influence of fasting on basal glucose uptake and transporter mRNA and muscle content

Prolonged fasting was used to provide different conditions to assess further the relationship in selected individual muscles between glucose transporters and basal and insulin-stimulated glucose fluxes. The effect of 48 h compared with 5 h fasting on body weight, plasma glucose, serum insulin and serum NEFA are shown in Table 2. There was a significant reduction in body weight, plasma glucose and plasma insulin, while NEFA rose, but not significantly. Consistent with previous data on the effects of fasting on 300–400 g adult rats [28], there was no significant alteration in the total muscle or brain RNA or protein yield in 5 h- versus 48 h-fasted animals (Table 2). Subsequent transporter mRNA data are expressed per unit of total RNA, but response patterns were similar when expressing data per mg of tissue (results not shown). As a control to the muscle data (see below), brain GLUT1 mRNA was also determined, but this was unaffected by fasting (48 h fasting 100% of 5 h-fasted).

Prolonged (48 h) fasting induced a 97% decline in heart basal $Rg'$ ($P < 0.002$), a 70% decline in diaphragm basal $Rg'$ ($P < 0.01$), a 50% decline in soleus basal $Rg'$ ($P < 0.2$) and a smaller mean change in red and white quadriceps. Changes observed in heart and soleus are compared in Table 3 with changes in transporter content; altered basal $Rg'$ paralleled the change in GLUT1, but not GLUT4 protein levels, following fasting (Table 3; see also the representative radiographs in Figure 1 for heart). GLUT1 protein levels were significantly reduced by 59 and 44% respectively in heart and soleus from the 48 h- as against the 5 h-fasted animal. Smaller but significant reductions in GLUT1 were found in heart and soleus muscle after 24 h fasting. We did not detect any significant change in GLUT4 protein in heart or soleus muscle following either a 24 h or 48 h fast (Figure 1; Table 3).

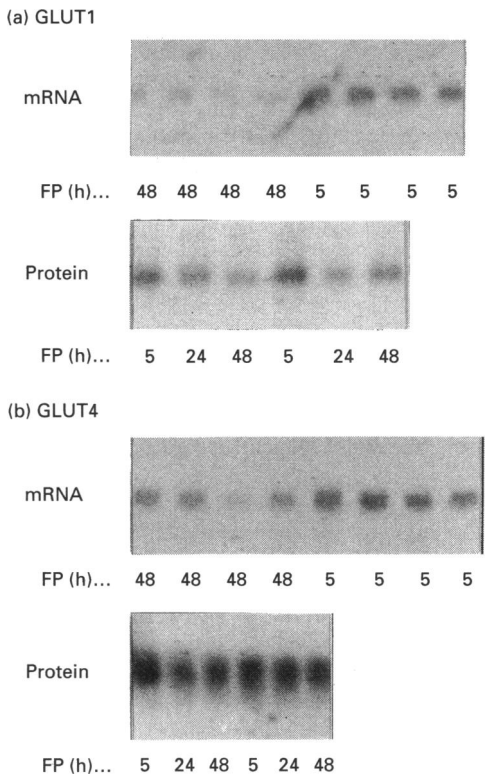


**Figure 1 Representative radiographs of Northern blots of GLUT1 (a) and GLUT4 (b) mRNA levels and Western blots of GLUT1 and GLUT4 protein in heart muscle after 5, 24 or 48 h periods of fasting (FP)**

Consistent with the GLUT1 protein data after 48 h fasting there was a decline of 86% in GLUT1 mRNA levels in heart with 48 h fasting, with no change in brain, taken as a control tissue (Table 4). The levels of GLUT1 mRNA were much lower in skeletal muscle than heart, and so we were unable to obtain quantifiable GLUT1 mRNA data in skeletal muscle. There was

**Table 4 Effect of 48 h fasting on GLUT1 and GLUT4 mRNA, expressed as a percentage of measurements in 4 h-fasted rats**

Results are means ± S.E.M. ($n$ = 4–7); *$P$ < 0.05 (versus 5 h-fasted values).

| mRNA (muscle) | Content (%) |
|---|---|
| GLUT1 | |
| Heart | 14 ± 2* |
| Brain | 99 ± 2 |
| GLUT4 | |
| Heart | 44 ± 5* |
| Diaphragm | 69 ± 19 |
| Red quadriceps | 97 ± 21 |
| White quadriceps | 131 ± 19 |

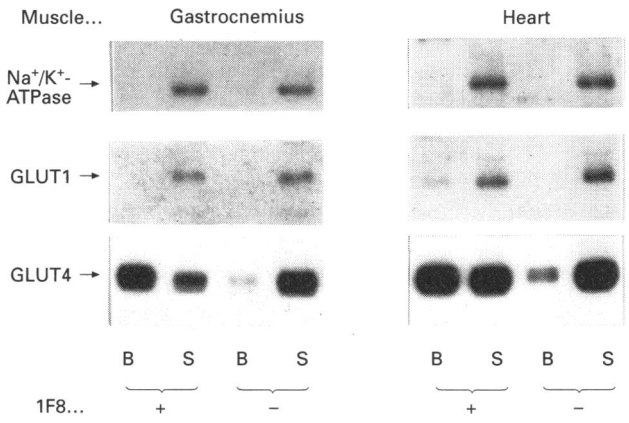


**Figure 2 Immunoisolation of intracellular vesicles containing GLUT4 from non-stimulated skeletal muscle (gastrocnemius) and heart**

Heart and skeletal-muscle homogenates were centrifuged at 3000 ***g*** for 15 min. The supernatant was centrifuged at 184000 ***g*** for 90 min. The resulting pellet was resuspended in HES buffer and incubated with 1F8-coupled or control magnetic beads in PBS/0.1% BSA for 4 h at 4 °C. Washed beads (B) and the non-adsorbed membranes (S) were subjected to SDS/PAGE and immunoblotted with antibodies specific for the $Na^+/K^+$-ATPase, GLUT1 and GLUT4.

**Table 5 Effect of degree of fasting (5 or 48 h) on *in vivo* basal and insulin-stimulated glucose uptake (glucose metabolic index, *Rg′*) during the euglycaemic clamp**

Results are means ± S.E.M. ($n$ = 5–7); *$P$ < 0.05; **$P$ < 0.01 (compared with 5 h-fasted values).

| | *Rg′* (μmol/min per 100 g) | | | |
|---|---|---|---|---|
| Muscle fasting time | Basal | Insulin infusion... | 4.1 munits/min per kg | 33 munits/min per kg |
| Heart | | | | |
| 5 h | 54.6 ± 7.8 | | 85.6 ± 7.3 | 52.1 ± 4.0 |
| 48 h | 2.8 ± 0.6** | | 55.3 ± 8* | 66.7 ± 5.3 |
| Diaphragm | | | | |
| 5 h | 10.4 ± 1.7 | | 26.1 ± 1.2 | 44 ± 4.9 |
| 48 h | 2.6 ± 0.4** | | 22.6 ± 1.5 | 37.6 ± 3.9 |
| Soleus | | | | |
| 5 h | 12.5 ± 2.8 | | 24.9 ± 3.7 | 32.7 ± 3.1 |
| 48 h | 9.2 ± 2.3 | | 26 ± 3.3 | 34.4 ± 1.9 |
| Red gastrocnemius | | | | |
| 5 h | 3.0 ± 0.5 | | 12.1 ± 2.1 | 29.0 ± 3.1 |
| 48 h | 2.5 ± 0.5 | | 14.2 ± 2.3 | 23.9 ± 2.4 |
| White gastrocnemius | | | | |
| 5 h | 1.3 ± 0.2 | | 2.1 ± 0.2 | 9.1 ± 1.0 |
| 48 h | 1.0 ± 0.1 | | 3.4 ± 0.5* | 7.8 ± 0.9 |

a significant 56% fall in GLUT4 mRNA in heart with fasting that did not correlate with GLUT4 protein. A similar discrepancy between GLUT4 protein and mRNA in fasted-rat skeletal muscle has been noted [29] and may be due to greater stability of the GLUT4 protein under these conditions. However, supporting the GLUT4 protein data in soleus muscle, GLUT4 mRNA levels were not significantly altered with fasting in a variety of skeletal muscles, including red and white quadriceps (Table 4).

### Effect of fasting on *in vivo* insulin-mediated glucose uptake in skeletal muscle and heart

As we were unaware of *in vivo* studies examining the effects of prolonged fasting on insulin-mediated glucose uptake in individual muscles of the rat, we report a further series of basal and euglycaemic hyperinsulinaemic clamp studies (2 h duration) to investigate this. Three different insulin levels were selected to represent the basal level, a midrange level (around the $ED_{50}$ for red skeletal muscle [6]), and an insulin infusion adequate to produce maximal insulin stimulation of muscle glucose uptake. Basal and insulin-stimulated *Rg′* are shown in Table 5 for five muscles in the 5 h- and 48 h-fasted groups. Basal glucose uptake was reduced by 48 h fasting in heart and diaphragm and to a lesser extent in other muscles. {The fasting-induced decline in soleus was less than in the earlier study (Table 3); we commonly observe some variability in this postural muscle in conscious rats, which is probably related to degree of activity during the clamp; nevertheless, other work [11] clearly supports a decline in basal soleus *Rg′* with fasting.} In contrast with basal *Rg′* there was no evidence for impairment of insulin-stimulated *Rg′* in any of the tissues examined. It is possible that insulin-sensitivity in white gastrocnemius may have marginally increased with fasting, but further data points would be necessary to confirm this. The dose–response curve in heart is more complicated in the 5 h fasted rat, with a previously reported [30] anomalous reduction in *Rg′* at very high insulin levels. The reasons for this are not clear. Nevertheless, it is apparent that the heart retains insulin-responsiveness with prolonged fasting and that *Rg′* at a supra-physiological insulin level is not diminished in 48 h- compared with 5 h-fasted animals. Whole-body insulin action is consistent with the individual muscle dose–response data in Table 5; the glucose infusion rates (*GIR*) required to maintain equivalent levels of glycaemia (mean plasma glucose during clamp, 6.6 ± 0.2 mM, both groups) were not significantly different (e.g. *GIR* at midrange insulin level in 5 h group, 15.3 ± 1.4 mg/min per kg; 48 h group, 14.4 ± 1.0 mg/min per kg).

### Presence of GLUT1 in the muscle cell (myocyte)

GLUT4 is exclusively localized to myocytes in heart and skeletal muscle [5,31], whereas GLUT1 is present in other cells associated with muscle tissue [3,16]. To examine whether GLUT1 is also in the myocyte, we immunoisolated vesicles containing GLUT4 from muscle homogenates using the monoclonal antibody 1F8 [4]. A substantial proportion (60–80%) of the total GLUT4 in

the heart and skeletal-muscle membrane fractions was immunoadsorbed using 1F8. Control beads contained less than 5% of total GLUT4. Protein determinations on the immunoadsorbed fractions indicated that the GLUT4 vesicles were enriched 15–20-fold compared with the starting material using this procedure. The absence of the $Na^+/K^+$-ATPase, a cell-surface marker, from the immunoadsorbed fraction from heart and skeletal muscle indicated that these vesicles were of intracellular origin. Approx. 20% of the GLUT1 present in the original heart membrane fraction co-precipitated with GLUT4 (Figure 2). The remaining GLUT1 was recovered in the non-adsorbed fraction. By contrast, there was no detectable GLUT1 in the GLUT4 vesicles isolated from skeletal muscle. Because GLUT4 is exclusively localized to myocytes in heart [5,17,31], these data clearly indicate that GLUT1 is also expressed in cardiac myocytes.

## DISCUSSION

Tissues that exhibit insulin-sensitive glucose transport (fat, skeletal and cardiac muscle) contain two different glucose transporters (GLUT1 and GLUT4). Findings presented here further strengthen the argument [4] for the importance of GLUT4 in the regulation of insulin-stimulated glucose transport in muscle. We additionally sought evidence consistent with GLUT1 having some functional role in muscle glucose transport. Our evidence does not resolve this issue in skeletal muscle, but provides evidence of a role for GLUT1 in cardiac muscle, namely: (*a*) significant levels of GLUT1, though less than GLUT4, in heart; (*b*) definite evidence that GLUT1 is expressed in cardiac myocytes (Figure 2); (*c*) much higher basal glucose uptake and GLUT1 levels in heart than in any other sampled muscle, but GLUT4 levels and maximal *in vivo* glucose uptake in proportion to that found in diaphragm or red oxidative hindlimb muscle (Table 1); (*d*) significant suppression of both basal glucose uptake and GLUT1 levels in heart with 48 h verus 5 h fasting, but no change in GLUT4 level or glucose uptake under maximal insulin stimulation (the latter consistent with skeletal muscle); (*e*) support from published data in rat muscle [2,32] and many other cells (e.g. [26]) on the subcellular localization of each transporter, supporting differential roles of GLUT1 and GLUT4.

On the basis of relative levels we consider it possible that both GLUT1 and GLUT4 play an important role in regulating muscle glucose metabolism. Concerning the presence of both transporters in the actual myocyte, immunocytochemical localization of GLUT4 in muscle has recently indicated myocyte-specific GLUT4 labelling with a predominance of intracellular GLUT4 in both non-stimulated heart and skeletal muscle [5,17]. Co-localization studies now show intracellular GLUT4 vesicles from heart contain approx. 20% of total GLUT1 in the starting membrane fraction. This is important, as it clearly shows GLUT1 in cardiac myocytes. Finding of GLUT1 in other cells in muscle sections [3] does not preclude a proportion of GLUT1 residing in myocytes, particularly in cardiac muscle, which has a higher GLUT1 content than skeletal muscle (Table 1).

It is significant that the GLUT4 vesicles from basal cardiac muscle contained 60% of total GLUT4, but only 20% of total GLUT1 present in the starting material. Further work is necessary to determine the cellular location of the additional GLUT1. However, it seems reasonable that, as has been recently reported in skeletal muscle [32] and many other cells (see [26]), all or part of the additional GLUT1 is present at the cell surface in non-stimulated cardiac myocytes to facilitate the basal glucose needs of the cell. Absence of GLUT4 from the cardiac myocyte cell surface under basal conditions [17] suggests that basal glucose utilization is mediated via an alternative transporter (e.g. GLUT1). However, our studies also indicate a small intracellular GLUT1 fraction in non-stimulated cardiac myocytes. Either insulin or exercise trigger translocation of muscle GLUT4 from intracellular vesicles to the cell surface [17]. Because GLUT1 and GLUT4 co-localize in cardiac muscle, it is likely that various stimuli trigger the movement of both transporters to the cell surface. This is not inconsistent with the current view that most of the GLUT1 resides at the cell surface in the basal state.

We performed a series of experiments to examine the relationship between glucose transporter levels and basal/insulin-stimulated glucose utilization in cardiac and skeletal muscles. In 5 h fasted rats there was a high correlation ($r^2 = 0.97$) between muscle GLUT4 levels and maximal insulin-mediated glucose uptake ($Rg'_{max.}$): the strong correlation supports the dominant role of GLUT4 in muscle-cell glucose transport, and, as $Rg'$ estimates rate of glucose flux to the phosphorylation step, supports glucose transport as the rate-limiting step in glucose uptake [25]. Conversely GLUT1 levels were virtually constant across skeletal muscles, but were greater in heart, correlating with differences in basal glucose uptake. Basal $Rg'$ is 10–20-fold higher in heart than in skeletal muscle and GLUT1 levels in heart are 2-fold higher than in all skeletal muscles examined; in contrast, GLUT4 levels were similar among heart and tonically active muscles such as diaphragm. It therefore seems possible that the higher cardiac GLUT1 content contributes to differences in glucose uptake between heart and skeletal muscle and that GLUT1 may play a significant role in myocardial glucose uptake.

The fasting responses in cardiac muscle importantly showed a clear association of GLUT1 levels and basal glucose uptake. Fasting for 48 h markedly reduced basal $Rg'$ and GLUT1 levels in heart, whereas maximal insulin responses and GLUT4 levels were unchanged compared with 5 h-fasted animals. Fasting for 24 h also reduced heart GLUT1, but not GLUT4 levels. The finding of little or no change in heart GLUT4 level with fasting agrees with another recent report where GLUT1 was not measured [12]. On the basis of our findings and knowledge of the intracellular targeting of GLUT1 [2], it seems likely that GLUT1 influences basal glucose transport in the heart. However, additional factors may be important; for example, fasting reduced cardiac GLUT1 expression by 60%, but $Rg'$ by over 90% (Table 3). It will be important to determine whether intracellular sequestration of GLUT1 is an additional contributor to the fall in cardiac muscle basal glucose uptake. We do not exclude a role for GLUT4 in the basal state; in particular, tonic activity of 'working' muscles may trigger movement of GLUT4 to the membrane, as with exercise-stimulated translocation of GLUT4 [2].

Response to prolonged fasting was chosen as a manipulation to vary basal and insulin-stimulated responses in individual muscles. However, because the literature did not allow prediction of fasting effects on *in vivo* insulin-stimulated responses, further physiological studies were performed. When equivalent conditions of glycaemia and hyperinsulaemia are produced (euglycaemic clamp), there was no reduction in whole-body or muscle insulin-sensitivity. This accords with previous *in vitro* data [14,15]. Given that glycogen synthesis contributes substantially to glucose uptake at insulin levels at 100–150 munits/l [33], the findings also agree with a report of no impairment in insulin-mediated glycogen synthesis in quadriceps muscle of fasted rats [34]. Methodological differences may explain why Penicaud et al. [13] found whole-body insulin resistance with fasting; they used a longer fast and assessed insulin action under anaesthesia at different glycaemic levels in fed and fasted animals. Thus, whereas it has been considered that maintenance of muscle GLUT4 mRNA and

protein levels was at variance with *in vivo* physiological data [35], we show a good concordance between muscle GLUT4 and physiological responses.

Previous studies have shown slightly increased skeletal-muscle GLUT4 levels with a prolonged fast [29,35]. Severity of nutritional deprivation may significantly contribute to this difference with our findings. In these other studies smaller animals (150 g) were fasted for longer periods; in our studies total muscle RNA levels were unaltered by fasting, as previously reported with 48 h fasting of 300–400 g adult rats [28]. However, in the other studies [29,35] total RNA levels fell, consistent with reduced muscle protein synthesis [28].

The possibility of an important role of GLUT1 in skeletal muscle deserves comment. We did not detect any GLUT1 in intracellular GLUT4 vesicles in skeletal muscle, and so we have no proof of the presence of GLUT1 in the skeletal-muscle myocyte. However, this might reflect sensitivity of the technique in view of the low levels of GLUT1 in skeletal versus cardiac muscle. Further studies are needed to resolve this issue, but the substantial reduction in GLUT1 in soleus muscle, though from a lower baseline, suggests changes could be functionally significant. Regardless of the situation in skeletal muscle, our data strongly suggest a role for GLUT1 in regulating glucose metabolism in cardiac muscle, and marked changes in GLUT1 expression may be important in the adjustment of cardiac glucose metabolism in metabolic states such as fasting.

This research was supported by grants from the National Health and Medical Research Council of Australia (to E.W.K. and D.J.C.), the National Institutes of Health (DK-42503 to D.E.J.) and the Washington University Diabetes Research and Training Center (to D.E.J.). D.E.J. is a recipient of a Career Development Award from the Juvenile Diabetes Foundation.

## REFERENCES

1 Mueckler, M. (1990) Diabetes **39**, 6–11
2 Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., Holloszy, J. O. and Klip, A. (1990) J. Biol. Chem. **265**, 13427–13430
3 Kahn, B. B., Rossetti, L., Lodish, H. F. and Charron, M. J. (1991) J. Clin. Invest. **87**, 2197–2206
4 James, D. E., Strube, M. and Mueckler, M. (1989) Nature (London) **338**, 83–87
5 Rodnick, K. J., Slot, J. W., Studelska, D. E., Hanpeter, D. E., Robinson, L. J., Geuze, H. J. and James, D. E. (1992) J. Biol. Chem. **267**, 6278–6285
6 James, D. E., Jenkins, A. B. and Kraegen, E. W. (1985) Am. J. Physiol. **248**, E567–E574
7 James, D. E., Burleigh, K. M., Chisholm, D. J. and Kraegen, E. W. (1985) J. Mol. Cell. Cardiol. **17**, 981–985
8 Richardson, J. M., Balon, T. W., Treadway, J. L. and Pessin, J. E. (1991) J. Biol. Chem. **266**, 12690–12694
9 Kern, M., Wells, J., Stephens, J., Elton, C., Friedman, J., Tapscott, E., Pekala, P. and Dohm, G. (1990) Biochem. J. **270**, 397–400
10 Issad, T., Penicaud, L., Ferre, P., Kande, J., Baudon, M. and Girard, J. (1987) Biochem. J. **246**, 241–244
11 Holness, M. J. and Sugden, M. C. (1990) Biochem. J. **270**, 245–249
12 Camps, M., Castello, A., Munoz, P., Monfar, M., Testar, X., Palacin, M. and Zorzano, A. (1992) Biochem. J. **282**, 765–772
13 Penicaud, L., Kande, J., Le Magnen, J. and Girard, J. (1985) Am. J. Physiol. **249**, E514–E518
14 LeMarchand-Brustel, Y. and Freychet, P. (1979) J. Clin. Invest. **64**, 1505–1515
15 Brady, L. J., Goodman, M. N., Kalish, F. N. and Ruderman, N. B. (1981) Am. J. Physiol. **240**, E184–E190
16 Handberg, A., Kayser, L., Hyer, P. and Vinten, J. (1992) Am. J. Physiol. **262**, E721–E727
17 Slot, J. W., Geuze, H. J., Gigengack, S., James, D. E. and Lienhard, G. E. (1991) Proc. Natl. Acad. Sci. **88**, 7815–7819
18 Kraegen, E. W., James, D. E., Jenkins, A. B. and Chisholm, D. J. (1985) Am. J. Physiol. **248**, E353–E362
19 Holness, M. J., Howard, R. M. and Sugden, M. C. (1992) Biochem. J. **286**, 395–398
20 Rodnick, K. J., Holloszy, J. O., Mondon, C. E. and James, D. E. (1990) Diabetes **39**, 1425–1429
21 Wake, S. A., Sowden, J. A., Storlien, L. H., James, D. E., Clarke, P. W., Shine, J., Chisholm, D. J. and Kraegen, E. W. (1991) Diabetes **40**, 275–279
22 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
23 Gruenberg, J. and Howell, K. E. (1985) Eur. J. Cell Biol. **38**, 312–321
24 Robinson, L. J. and James, D. E. (1992) Am. J. Physiol. **263**, E383–E393
25 Ziel, F. H., Venkatesan, N. and Davidson, M. B. (1988) Diabetes **37**, 885–890
26 Piper, R. C., Hess, L. J. and James, D. E. (1991) Am. J. Physiol. **260**, C570–C580
27 Calderhead, D. M., Kitagawa, K., Lienhard, G. E. and Gould, G. W. (1990) Biochem. J. **269**, 597–601
28 Goodman, M. N. and Ruderman, N. B. (1980) Am. J. Physiol. **239**, E269–E276
29 Bourey, R. E., Koranyi, L., James, D. E., Mueckler, M. and Permutt, M. A. (1990) J. Clin. Invest. **86**, 542–547
30 James, D. E., Kraegen, E. W. and Chisholm, D. J. (1985) J. Clin. Invest. **76**, 657–666
31 Slot, J. W., Moxley, R., Geuze, H. J. and James, D. E. (1990) Nature (London) **346**, 369–371
32 Marette, A., Richardson, J. M., Ramial, T., Balon, T. W., Vranic, M., Pessin, J. E. and Klip, A. (1992) Am. J. Physiol. **263**, C443–C452
33 Kraegen, E. W., Storlien, L. H., Jenkins, A. B. and James, D. E. (1989) Am. J. Physiol. **256**, E242
34 Kruszynska, Y. and McCormack, J. (1989) Biochem. J. **258**, 699–707
35 Charron, M. J. and Kahn, B. B. (1990) J. Biol. Chem. **265**, 7994–8000

Received 29 December 1992/11 May 1993; accepted 26 May 1993