Failure of insulin-regulated recruitment of the glucose transporter GLUT4 in cardiac muscle of obese Zucker rats is associated with alterations of small-molecular-mass GTP-binding proteins

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Cardiac ventricular tissue of lean and genetically obese (*fa/fa*) Zucker rats was used to study the expression, subcellular distribution and insulin-induced recruitment of the glucose transporter GLUT4 and to elucidate possible molecular alterations of the translocation process. Hearts were removed from basal and insulin-treated (20 min) lean and obese Zucker rats, and processed for subcellular fractionation and Western blotting of proteins. In obese rats, the total GLUT4 content in a crude membrane fraction was reduced to 75±8% ($P = 0.019$) of lean controls. In contrast, GLUT4 abundance in plasma membranes was not significantly different between lean and obese rats with a concomitant decrease (47±3%) in the microsomal fraction of obese animals. In plasma membranes of lean animals insulin was found to increase the GLUT4 abundance to 294±43% of control with a significantly ($P = 0.009$) reduced effect in the obese group (139±10% of control). In these animals insulin failed to recruit GLUT4 from the microsomal fraction, whereas the hormone induced a significant decrease (41±4%) of microsomal GLUT4 in lean controls. In GLUT4-enriched membrane vesicles, obtained from cardiac microsomes of lean rats, a 24 kDa GTP-binding protein could be detected, whereas no significant labelling of this species was observed in GLUT4 vesicles prepared from obese animals. In addition to the translocation of GLUT4, insulin was found to promote the movement of the small GTP-binding protein rab4A from the cytosol (decrease to 61±13% of control) to the plasma membrane (increase to 177±19% of control) in lean rats with no effect of the hormone on rab4A redistribution in the obese group. In conclusion, cardiac glucose uptake of insulin-resistant obese Zucker rats is subject to multiple cellular abnormalities involving a reduced expression, altered redistribution and defective recruitment of GLUT4. We show here an association of the latter defect with alterations at the level of small GTP-binding proteins possibly leading to an impaired trafficking of GLUT4 in the insulin-resistant state.

INTRODUCTION

The genetically obese Zucker rat (*fa/fa*) represents a well-characterized model of insulin resistance associated with extreme obesity, hyperinsulinaemia, and impaired glucose tolerance (for review, see [1]). One of the most prominent features of this insulin resistance consists of a pronounced reduction of insulin-stimulated glucose uptake into skeletal [2,3] and cardiac [4,5] muscle. Multiple cellular defects may contribute to this abnormality including altered insulin transmembrane signalling pathways [6], or defects of the glucose uptake process itself. The major part of insulin action on glucose uptake is mediated by the transporter isoform GLUT4, which in the basal state is sequestered in intracellular tubulo-vesicular structures found in the trans-Golgi region, and is shifted to the plasma membrane in response to insulin [7,8]. It is well accepted that the effectiveness of insulin to stimulate muscle glucose transport correlates positively with the muscle GLUT4 content [9,10]. Furthermore, insulin resistance due to insulin deficiency [11,12] or denervation [13] results in a reduced expression of muscle GLUT4 protein. A 40% reduction of GLUT4 in soleus and gastrocnemius muscle of obese Zucker rats was recently reported by Handberg et al. [14]. However, most studies on Zucker rat skeletal muscle and other hyperinsulinaemic animal models of insulin resistance have observed an unaltered level of GLUT4 expression [15–18], suggesting that impaired functional activity and/or transporter translocation may be underlying the insulin resistance of obesity.

In a recent study on obese Zucker rat skeletal muscle, King et al. [16] reported the complete failure of insulin to promote GLUT4 translocation to the plasma membrane despite an unaltered expression and activation of the glucose transporter. These observations support the notion that the defect of insulin action could be located at the level of cellular factors mediating the process of GLUT4 recruitment. Precise knowledge of the GLUT4 translocation machinery is presently lacking. However, several proteins which co-localize with GLUT4 in its subcellular compartment have been identified, and these proteins are thought to play a key role in GLUT4 trafficking [19–21]. Using cardiac tissue, we recently reported the presence of an insulin-sensitive 24 kDa G-protein in GLUT4-enriched membrane vesicles and the insulin-induced translocation of rab4A to the plasma membrane [22], giving rise to the hypothesis that these G-proteins could be involved in the subcellular trafficking of GLUT4, at least in the heart. Very recently, Ricort et al. [23] reported a parallel defect in GLUT4 and rab4 movement in insulin-resistant 3T3-L1 adipocytes, suggesting the putative involvement of small G-proteins in the impaired GLUT4 recruitment in insulin-resistant states.

The present investigation was initiated in order to elucidate cellular and molecular alterations which may contribute to the insulin resistance of cardiac glucose uptake in genetically obese Zucker rats. We have approached this problem by: (i) measuring the protein expression and subcellular distribution of the glucose transporter GLUT4, and (ii) monitoring the insulin-regulated

Abbreviations used: CF, crude fraction; PM, plasma membrane fraction; MM, microsomal membrane fraction; DTT, dithiothreitol.

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recruitment of this transporter isoform under *in vivo* conditions. Furthermore, we have addressed the question of a possible association between the insulin-resistant state and abnormalities of small G-proteins previously recognized to represent putative components of the GLUT4 translocation machinery [22–24]. The data show a depletion of the intracellular cardiac GLUT4 pool to about one-half, resulting from a reduced expression and redistribution of the transporter in obese rats. Cardiac insulin resistance can be explained by the failure of GLUT4 recruitment from the remaining transporter pool. It is suggested that alterations of GLUT4-trafficking components may be involved in the pathogenesis of this defect.

MATERIALS AND METHODS

Chemicals

[α-^{32}P]GTP (3000 Ci/mmol) was purchased from New England Nuclear (Germany). ^{125}I-labelled Protein A (30 mCi/mg) was from Amersham (Germany). Reagents for SDS/PAGE were supplied by Pharmacia and Sigma (Germany). Insulin (Actrapid HM, 100 units/ml) was supplied by Novo (Germany). Immunobead goat anti-(rabbit immunoglobulin) was purchased from Bio-Rad (Germany). The polyclonal GLUT4 antiserum was a product of Calbiochem (Germany). All other chemicals were of the highest grade commercially available. Rabbit antiserum against recombinant human rab4A was generated by immunization with baculovirus-expressed purified protein (M. McCaffrey and B. Goud, unpublished work).

Membrane preparation and characterization

Genetically obese (*fa/fa*) male Zucker rats (12–16-weeks-old, 480–520 g) and their age-matched lean (FA/?) controls (280–320 g) were kindly provided by Professor L. Herberg, Düsseldorf, Germany. Blood samples were collected for determinations of glucose and insulin as outlined previously [5]. Plasma and microsomal membranes from cardiac ventricles were prepared as recently described by us [25]. Briefly, ventricular tissue was removed and homogenized in a buffer containing 10 mM Tris/HCl, 0.1 mM PMSF and 2.6 mM dithiothreitol (DTT) by application of an Ultraturrax for 60 s. Homogenization was continued by 10 strokes in a glass–Teflon homogenizer followed by 3 x 3 strokes in a tight-fitting Potter–Elvehjem homogenizer. After centrifugation at 3000 *g* the supernatant was centrifuged at 200000 *g* for 90 min to pellet the crude membrane fraction (CF) and to obtain the cytosol (supernatant). Further purification was achieved by applying this fraction to a discontinuous gradient consisting of 0.57, 0.72, 1.07 and 1.43 M sucrose buffer and centrifugation at 40000 *g* for 16 h. Membranes were harvested from each sucrose layer and stored at −70 °C. Protein was determined using a modification of the Bio-Rad protein assay with BSA as a standard. Ouabain-sensitive Na^+/K^+-ATPase and Ca^{2+}/K^+-ATPase were used as marker enzymes for sarcolemma and microsomal membranes, respectively, and determined as described [25]. Membranes recovered from the 0.72 M sucrose layer were enriched 5–7-fold in the activity of the Na^+/K^+-ATPase and considered as a plasma membrane fraction (PM), whereas membranes obtained in the 1.07 M sucrose layer showed a 0.5-fold activity of this marker enzyme, when compared with the homogenate. Furthermore, Ca^{2+}/K^+-ATPase increased 4-fold in this fraction. The 1.07 M membrane fraction was hence termed microsomal membranes fraction (MM). For the insulin-stimulated studies, rats received a tail vein injection of regular insulin (4 units/100 g) and hearts were removed 20 min later.

Immunoblotting

Protein samples were separated by SDS/PAGE using 10% horizontal gels and transferred to nitrocellulose filters. Filters were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween and 10% milk powder. Thereafter, filters were incubated for 16 h at 4 °C with a 1:500 dilution of the GLUT4 antiserum or the rab4 antiserum. After extensive washing with PBS containing 0.05% Tween, filters were incubated for 2 h with ^{125}I-Protein A (0.3 μCi/ml). Filters were again extensively washed, air-dried and exposed to Hyperfilm-MP films using intensifying screens. Autoradiograms were analysed using laser scanning densitometry. In addition, blots were visualized on a FUJIX BAS 1000 bio-imaging analyser (Fuji, Japan). Quantification was performed on a SPARCstation (Sun Microsystems, U.S.A.) using image analysis software. Significance of reported differences was evaluated using the null hypothesis and *t*-statistics for unpaired data.

Immunoadsorption of GLUT4-containing vesicles

GLUT4-enriched membrane vesicles were prepared essentially as previously described [22]. Briefly, microsomal membranes were incubated for 14 h with immunobeads at 4 °C in PBS, pH 7.4, containing PMSF (0.1 mM), DTT (2.6 mM), EDTA (1 mM) and BSA (0.4%). After centrifugation, GLUT4 antiserum was added to the supernatant and the membranes were sonicated for 15 s. Control membranes were treated identically except that the antiserum was not added. After a 2 h incubation at 4 °C the membranes were pelleted, resuspended and incubated for an additional 2 h with immunobeads. After centrifugation, the beads were washed three times with PBS, and the vesicle proteins were eluted with Laemmli sample buffer [26].

Labelling of small GTP-binding proteins

Protein samples were subjected to SDS/PAGE (12% horizontal gels), transferred to nitrocellulose filters, and GTP-binding was determined as previously described [25]. Blots were washed for 10 min in buffer containing 50 mM Tris/HCl, pH 7.5, 0.3% Tween 20 and 2 μM $MgCl_2$. Incubation was then continued for 30 min at room temperature in the same buffer in the presence of [α-^{32}P]GTP (1 μCi/ml), 1 nM GTP and 10 μM ATP. After several washing steps in the same buffer without [α-^{32}P]GTP, blots were air-dried and visualized on a Fujix BAS 1000 bio-imaging analyser and quantified as outlined above.

RESULTS

Expression and subcellular redistribution of GLUT4

Obese Zucker rats used in the present investigation exhibited the well-known pattern of normoglycaemia and hyperinsulinaemia, as shown in Table 1. In lean animals, administration of insulin (4 units/100 g body weight) was associated with a 56% decrease of plasma glucose within 20 min. This response was much less pronounced in obese rats, reflecting the insulin resistance of these animals (Table 1). Using our previously published procedure [22], a plasma membrane preparation of comparable purity was obtained from cardiac tissue of both lean and obese rats (Table 1).

GLUT4 protein expression was determined by immunodetection of GLUT4 in a crude membrane fraction obtained

Table 1 Characteristics of animals and plasma membrane purification

Plasma glucose and insulin were determined as outlined in the Materials and methods section. Insulin (4 units/100 g body weight) was administered by tail vein injection and glucose was determined 20 min later. PM enrichment was quantified by monitoring the ouabain-sensitive Na^+/K^+-ATPase in the homogenate and the PM sample, as detailed in the Materials and methods section. Values are the mean ± S.E.M. for the number of experiments in parentheses.

	Lean rats	Obese rats
Weight (g)	315 ± 14 (9)	478 ± 16 (18)
Plasma glucose before insulin (mg/dl)	206 ± 13 (8)	211 ± 21 (8)
Plasma glucose after insulin (mg/dl)	91 ± 5 (8)	164 ± 8 (8)
Plasma insulin (ng/ml)	3.8 ± 0.3 (6)	39.5 ± 11.8 (5)
PM enrichment (relative to homogenate)	6.8 ± 1.3 (4)	6.2 ± 0.4 (4)

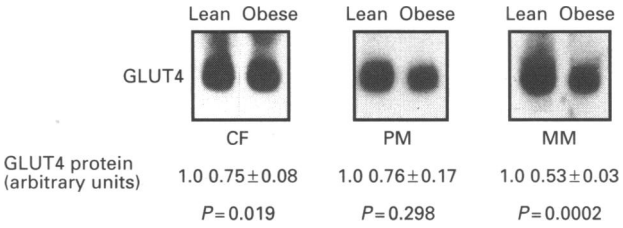

Figure 1 Protein expression and subcellular distribution of GLUT4 in cardiac muscle of lean and obese Zucker rats

Portions (15 μg) of crude membranes (CF), plasma membranes (PM) and microsomal membranes (MM) were subjected to SDS/PAGE and immunoblotted with antiserum against GLUT4, as detailed in the Materials and methods section. The nitrocellulose sheets were then incubated with ^{125}I-Protein A and submitted to autoradiography. Quantification of autoradiograms was performed with a bio-imaging analyser. All results are expressed relative to the amount of GLUT4 protein present in lean controls. Data are mean values ± S.E.M. of nine (CF) or four (PM, MM) separate experiments.

from hearts of lean and obese Zucker rats (Figure 1). The data show that the total amount of this transporter isoform was slightly (25%), but significantly, reduced in the obese group. GLUT4 abundance in the PM was subject to a higher variation and did not reflect the decrease of GLUT4 expression (Figure 1), suggesting the possibility of a redistribution of GLUT4 from the intracellular storage site to the plasma membrane. This was confirmed by quantification of GLUT4 in the MM fraction of obese rats, showing a 47% reduction of GLUT4 content when compared with lean controls (Figure 1). Thus, reduced protein expression of GLUT4 and an additional redistribution of the transporter may equally contribute to a significant depletion of the intracellular GLUT4 pool in cardiac muscle of obese Zucker rats.

Effect of insulin on GLUT4 recruitment

The data reported so far raised the question of whether the diminished GLUT4 pool is still accessible to insulin-induced recruitment. This, indeed, has been observed in skeletal muscle of insulin-deficient rats [27]. Therefore, lean and obese Zucker rats were subjected to a 20 min insulin treatment, the heart was removed, and after subcellular fractionation the GLUT4 abundance was determined in the PM and MM samples. Representative Western blots are shown in Figure 2. As can be seen from the data, in lean animals insulin was found to induce a

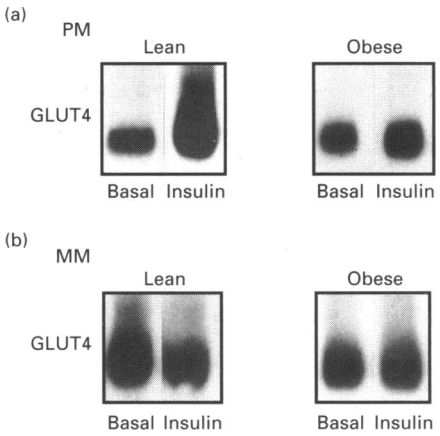

Figure 2 Insulin-induced translocation of GLUT4 in lean and obese Zucker rats

Plasma membranes (**a**) and microsomal membranes (**b**) were prepared from cardiac ventricle of basal and insulin-treated lean and obese Zucker rats. Membranes (20–30 μg) were analysed by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with an antiserum against GLUT4. After incubation with ^{125}I-Protein A, the filters were subjected to autoradiography. Representative blots are shown.

Table 2 Quantification of insulin-induced redistribution of GLUT4 in lean and obese Zucker rats

GLUT4 was immunodetected in plasma membranes and microsomal membranes obtained from basal and insulin-treated Zucker rats as outlined in Figure 2. The resultant autoradiographs were scanned by laser densitometry or quantified by image analysis software, as described in the Materials and methods section. The effect of insulin is expressed by the relative increase or decrease of abundance of GLUT4 signal intensity (insulin/basal). Data presented are the mean ± S.E.M. with the number of experiments in parentheses.

	Relative abundance of GLUT4 (insulin/basal)	
	Lean	Obese
Plasma membranes	2.92 ± 0.42 (7) $P = 0.002$	1.39 ± 0.10 (6) $P = 0.0064$
Microsomal membranes	0.59 ± 0.04 (3) $P = 0.005$	0.83 ± 0.10 (3) $P = 0.24$

pronounced translocation of GLUT4 to the plasma membrane with a concomitant decrease in the microsomal fraction. This response was only marginally detectable in the obese group. Quantification of Western blots revealed a nearly 3-fold increase in the GLUT4 content in the PM of lean control rats in response to insulin, whereas in obese rats GLUT4 increased by only 39 ± 10% (Table 2). In parallel, insulin-mediated recruitment of GLUT4 from the MM of lean rats was found to be 41 ± 4% with an insignificant effect (17 ± 10%) in the obese group (Table 2). These data show that cardiac insulin resistance of obesity involves a reduced GLUT4 pool, which, in addition, appears to be largely unable to respond to insulin-regulated recruitment.

Co-localization of small G-proteins with GLUT4 and redistribution of rab4A

The failure of insulin-induced GLUT4 recruitment in obese rats may result from impaired insulin signalling, alterations of the translocation machinery or both. We have recently described a

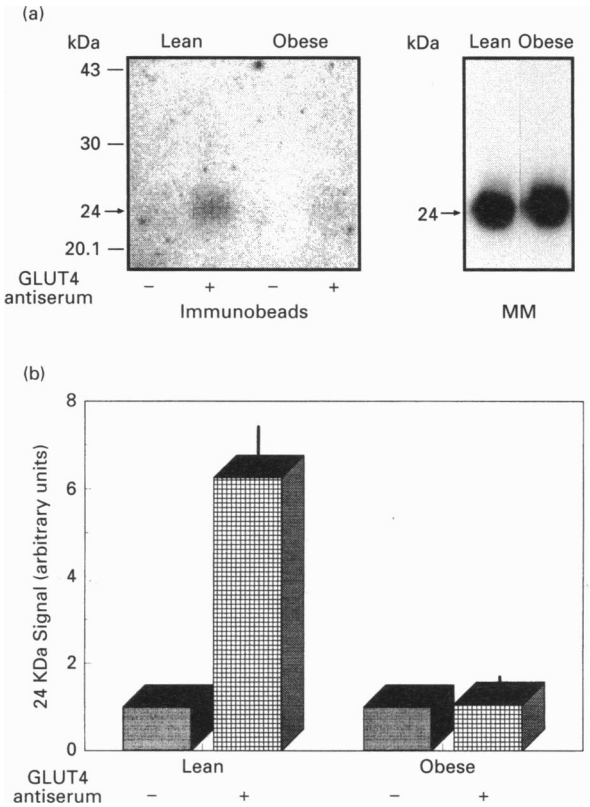

Figure 3 Labelling of small GTP-binding proteins in GLUT4 vesicles or microsomal membranes of lean and obese Zucker rats

(**a**) Microsomal membranes (0.5–1 μg/μl) were pre-adsorbed to immunobeads followed by incubation in the absence or presence of GLUT4 antiserum. Immunoprecipitation was then performed by addition of immunobeads as described in the Materials and methods section. Aliquots of the eluted vesicle proteins or microsomal membranes were then analysed by SDS/PAGE and subjected to ligand blotting with [α-^{32}P]GTP as outlined in the Materials and methods section. Bands were visualized on a bio-imaging analyser (left) or by autoradiography (right). (**b**) GLUT4-containing vesicles were subjected to ligand blotting as outlined above. The 24 kDa signals were then quantified with a bio-imaging analyser. The intensity of the bands is given in relative units with the signal of control vesicles set as 1.0. The data are mean values ± S.E.M. of four separate GLUT4 vesicle preparations from both lean and obese animals.

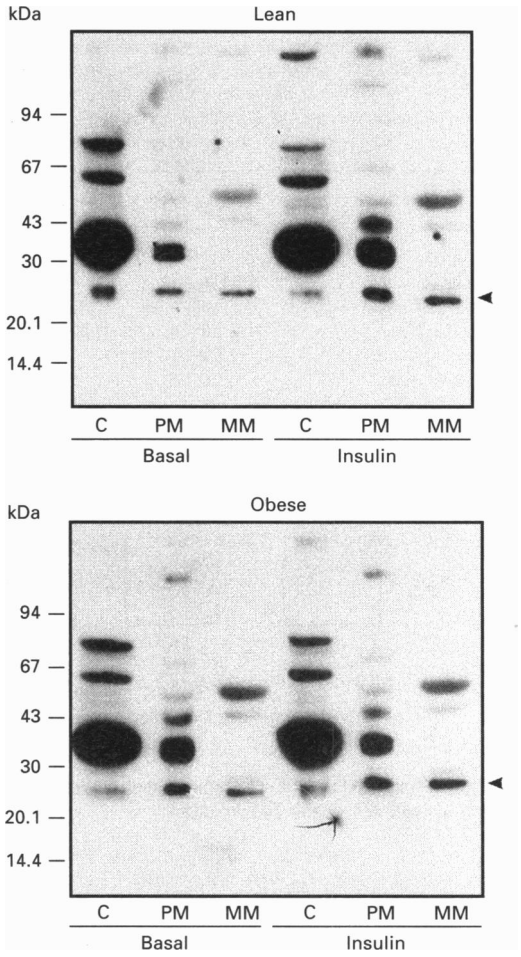

Figure 4 Insulin-induced translocation of rab4A in lean and obese Zucker rats

Subcellular fractions were prepared from cardiac tissue of basal and insulin-treated lean and obese Zucker rats. 30 μg of cytosol (C), plasma membranes (PM) and microsomal membranes (MM) were analysed by SDS/PAGE (8–18% gradient gel), transferred to nitrocellulose, and immunoblotted with an antiserum against rab4A. After incubation with ^{125}I-protein A, the nitrocellulose sheets were subjected to autoradiography. Representative autoradiograms out of six (lean) or four (obese) separate experiments are presented. Position of rab4A is marked by an arrow.

24 kDa insulin-sensitive small G-protein, which co-localizes with cardiac GLUT4 [22] and may serve to dock the insulin signal to GLUT4 vesicles and to the exocytotic movement of the transporter. GLUT4 vesicles obtained from lean and obese rats were now subjected to ligand blotting with [α-^{32}P]GTP in order to evaluate a possible alteration of this protein. As illustrated in Figure 3(a), the 24 kDa G-protein which was clearly visible in controls, was undetectable in obese rats, despite an unaltered amount of small G-proteins in the MM sample of these animals (Figure 3a). Quantification of 24 kDa signals was performed by bio-image analysis and compared with control vesicles prepared in the absence of GLUT4 antiserum (Figure 3b). The data clearly show that no significant labelling of a 24 kDa species took place in GLUT4 vesicles of obese rats. It should be noted that these data were not corrected for the size of the intracellular GLUT4 pool (see Figure 1), since the amount of GLUT4 vesicle protein is not precisely known for the two groups. However, taking into account a signal to background ratio of 6:1 for the 24 kDa protein in lean controls (Figure 3b), the 40–50% reduction of the GLUT4 pool cannot explain the complete failure of labelling the 24 kDa species in the obese group. The specificity of this defect is demonstrated by an unaltered amount of small G-proteins in the microsomal fraction of lean and obese rats (Figure 3a).

We recently reported that the translocation of cardiac GLUT4 after *in vivo* administration of insulin is paralleled by an extensive shift of the small G-protein rab4A from the cytosol to the plasma membrane, suggesting that rab4A could be involved in GLUT4 exocytosis [22]. We now tested the effect of insulin on the redistribution of rab4A in cardiac muscle of lean and obese Zucker rats. Western blot analysis of various subcellular fractions using rab4A antiserum is presented in Figure 4. As shown in our previous report [22], this antiserum is highly reactive against recombinant 24 kDa rab4A and detects a protein band of identical size in cardiac microsomal membranes. Several non-specific protein bands of higher molecular mass became visible also (Figure 4). However, no consistent changes of these bands in response to insulin could be detected. Further, valid analysis

Table 3 Quantification of insulin-induced rab4A redistribution in lean and obese Zucker rats

Rab4A was immunodetected in cytosol and plasma membranes of basal and insulin-stimulated Zucker rats, as outlined in Figure 4. Quantification was performed with a bio-imaging analyser and the effect of insulin is expressed by the relative decrease or increase of abundance of rab4A signal intensity (insulin/basal). Data presented are the mean ± S.E.M. with the number of experiments in parentheses.

	Relative abundance of rab4A (insulin/basal)	
	Lean	Obese
Cytosol	0.61 ± 0.13 (6) $P = 0.01$	0.83 ± 0.20 (4) $P = 0.47$
Plasma membranes	1.77 ± 0.19 (6) $P = 0.029$	1.24 ± 0.15 (4) $P = 0.22$

of rab4A could be performed as seen by identical electrophoretic mobility and clear separation of the 24 kDa signal under all conditions (Figure 4). In lean controls the 24 kDa rab4A (see arrow) was most abundant in the cytosol with insulin producing a translocation of rab4A from the cytosol to the plasma membranes. In obese rats, rab4A exhibited a different subcellular distribution, being most abundant in the PM sample (Figure 4). Under these conditions insulin was unable to shift rab4A to the plasma membrane (Figure 4). The quantification of six (lean) and four (obese) independent experiments is summarized in Table 3. Insulin was found to induce a 40% reduction of rab4A in the cytosol of lean rats with a concomitant 77% increase in the PM sample. No significant effect of insulin on the redistribution of rab4A in obese rats could be detected (Table 3).

DISCUSSION

The molecular mechanisms leading to the profound insulin resistance of glucose uptake in cardiac and skeletal muscle of various obese animal models have remained incompletely understood. Alterations of insulin receptor signalling at the level of kinase and post-kinase signal transduction at least partly underly this process [6,28,29]. The present investigation has evaluated the contribution of the glucose transporter GLUT4 to cardiac insulin resistance of genetically obese Zucker rats by (i) measuring the protein expression and subcellular distribution of GLUT4, (ii) quantification of GLUT4 translocation after *in vivo* insulin treatment, and (iii) analysis of cellular elements previously recognized to represent putative components of the GLUT4 translocation machinery [22–24].

The present study shows a slight but significant reduction of total GLUT4 protein content in the heart of obese Zucker rats. This finding is in principal agreement with the data of Handberg et al. [14], who reported a 40% decrease of GLUT4 protein expression in crude membrane fractions of both soleus and red gastrocnemius muscles using Western blotting and immunofluorescence microscopy. Other investigators were unable to detect a reduction of GLUT4 protein expression in skeletal muscle of Zucker rats [15–17]. This may be related to a different level of glycaemia, insulinaemia, etc., as shown by reduced GLUT4 expression in obese hypoinsulinaemic diabetic Zucker rats [30].

Most studies on GLUT4 protein expression in muscles of obese rodents have determined the total abundance of GLUT4 [14–18,30] but have not considered a possible redistribution of GLUT4. Using our established protocol for subcellular fractionation of cardiac muscle [22,25] we have obtained plasma and microsomal membranes from lean and obese rats of very similar purity (see Table 1), making it possible to perform a valid comparison of GLUT4 distribution between these animal groups. Our data show a reduction of the intracellular GLUT4 pool to about one-half in obese rats. This clearly exceeds the reduced protein expression of the transporter and strongly suggests a redistribution of GLUT4, most probably to the plasma membrane. Interestingly, in skeletal muscle of hyperglycaemic lean type-2 diabetic patients a redistribution of GLUT4 from the plasma membrane has been detected in the basal state [31]. It may be speculated that this redistribution of the transporter results from metabolic abnormalities (hyperglycaemia, hyperinsulinaemia) of the different insulin-resistant states. In obese rats, at least in the heart, both a reduced expression of GLUT4 and a redistribution of this transporter concomitantly contribute to a marked depletion of the intracellular pool. In light of the well known correlation between glucose transport stimulation and GLUT4 concentration in muscle tissue [9,10], the reduction of the intracellular cardiac GLUT4 pool may be considered as one major factor leading to the resistance of insulin-stimulated glucose uptake in this tissue [4,5].

A second key finding of the present study relates to the observation of a complete failure of insulin-mediated GLUT4 recruitment from the cardiac GLUT4 pool in obese Zucker rats. Taking into account that GLUT4 translocation is of major importance for insulin stimulation of glucose transport in cardiac myocytes [7,32], defective GLUT4 recruitment may be considered as the primary cause of cardiac insulin resistance in obesity. Our data are in excellent agreement with the findings of King et al. [16], who reported the complete failure of GLUT4 translocation in skeletal muscle of obese Zucker rats. On the other hand, using the same tissue Galante et al. [33] reported a normal GLUT4 translocation in this animal model, requiring further clarification. It may be argued that the translocatable part of the GLUT4 pool has already been redistributed to the plasma membrane in the basal state in the obese group, and that the inability of the remaining transporters to be recruited from the pool reflects a different species of GLUT4 which is not accessible to translocation. This, however, seems most unlikely, since (a) a 50% reduction of the GLUT4 pool has been observed in skeletal muscle of insulin-deficient rats, and (b) a complete normal recruitment of these transporters could be detected in response to insulin [27]. Thus, the inability of GLUT4 to be recruited from its intracellular storage site must be related to an altered insulin signalling and/or a defect in the translocation machinery.

Very recent investigations on the characterization of GLUT4 trafficking in target cells have led to the concept that the majority of GLUT4 is localized in specific membrane vesicles which cycle to and from the cell surface in an insulin-dependent manner [8,34]. GLUT4-containing vesicles are therefore thought to represent a crucial part of the translocation machinery and the identification of proteins co-localizing with GLUT4 has provided initial insights into the putative mechanisms of GLUT4 trafficking [19–22,35]. This laboratory has recently reported the co-localization of cardiac GLUT4 with an insulin-sensitive 24 kDa G-protein, which is different from rab1A, rab2, rab4A, rab6 [22] and rab3D (I. Uphues and J. Eckel, unpublished work). In light of the well known function of small GTP-binding proteins for the control of cellular traffic [36], the 24 kDa protein could be involved in the exocytosis of GLUT4 in agreement with the original hypothesis by Baldini et al. [37] of a G-protein-mediated recruitment of GLUT4. Our present investigation shows that this G-protein appears to be affected by the insulin-resistant state, most probably leading to the inability to detect

this species in GLUT4 vesicles obtained from obese rats. The molecular nature of this defect remains presently unclear and may involve a reduced expression of the 24 kDa G-protein, a reduced affinity towards GTP or an altered membrane attachment. The latter requires a defined post-translational processing, as documented for rab [38] and ras proteins [39]. Nevertheless, our data clearly indicate that a component of GLUT4 vesicles, which is of potential importance for GLUT4 exocytosis, is defective in insulin-resistant cardiac muscle. Attempts to purify and clone this protein are presently under way.

In addition to the still unidentified 24 kDa G-protein, rab4A has been implicated in GLUT4 exocytosis in cardiac muscle [22] and adipose tissue [24,40]. Evidence supporting this view has been obtained from a simultaneous movement of both GLUT4 and rab4A under identical experimental conditions [22,24]. We show here that in obese Zucker rats rab4A is already redistributed to the plasma membrane in the basal state and that exogenous insulin fails to promote the translocation of rab4A from the cytosol. Taking into account a redistribution of GLUT4 in obese animals (see above), it may be concluded that rab4A and GLUT4 exhibit a very similar behaviour both in the normal and the insulin-resistant state, supporting the view that these two proteins are functionally related [22,24,40]. Our findings principally agree with the data of Ricort et al. [23], who reported a parallel defect in GLUT4 and rab4 movement in insulin-resistant 3T3-L1 adipocytes, although in these cells rab4A is shifted from microsomal membranes to the cytosol [23]. It may be speculated that the hyperinsulinaemia of the obese Zucker rat is responsible for the redistribution of rab4A; alternatively, phosphorylation/dephosphorylation reactions [41] or modifications of post-translational processing of rab4 [38] could also explain the altered subcellular distribution of this G-protein. Defective insulin signalling [6] and/or the reduced amount or structural alterations of rab4A available in cardiac cytosol of obese animals may underly the lack of insulin-mediated rab4A-translocation. This important issue needs further investigation. Taking into account the involvement of rab4A in endosome recycling processes [42], possibly including GLUT4 [22], we suggest that an abnormality of rab4A could represent a molecular location of cardiac insulin resistance.

In summary, we provide evidence that the insulin resistance of cardiac glucose uptake is based on at least two major defects at the level of GLUT4 involving a reduced intracellular pool of this transporter isoform and the failure of insulin to recruit GLUT4 from this reduced pool. The latter defect is associated with alterations of at least two small GTP-binding proteins, which are potential components of the translocation machinery including the exocytosis and possibly the recycling of GLUT4. Additional studies are now required to evaluate if molecular abnormalities of these small G-proteins represent an early event in the pathogenesis of insulin resistance.

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REFERENCES

1 Bray, G. A. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. **36**, 148–154
2 Kemmer, F. W., Berger, M., Herberg, L., Gries, F. A., Wirdeier, A. and Becker, K. (1979) Biochem. J. **178**, 733–741
3 Sherman, W. M., Katz, A. L., Cutler, C. L., Withers, R. T. and Ivy, J. I. (1988) Am. J. Physiol. **255**, E374–E382
4 Zaninetti, D., Crettaz, M. and Jeanrenaud, B. (1983) Diabetologia **25**, 525–529
5 Eckel, J., Wirdeier, A., Herberg, L. and Reinauer, H. (1985) Endocrinology **16**, 1529–1534
6 Folli, F., Saad, M. J. A., Backer, J. M. and Kahn, C. R. (1993) J. Clin. Invest. **92**, 1787–1794
7 Slot, J. W., Geuze, H. J., Gigengack, S., James, D. E. and Lienhard, G. E. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7815–7819
8 Satoh, S., Nishimura, H., Clark, A. E., et al. (1993) J. Biol. Chem. **268**, 17820–17829
9 Kern, M., Wells, J. A., Stephens, J. M., et al. (1990) Biochem. J. **270**, 397–400
10 Henriksen, E. J., Bourney, R. E., Rodnick, K. J., Koranyi, L., Permutt, M. A. and Holloszy, J. O. (1990) Am. J. Physiol. **259**, E593–E598
11 Kahn, B. B., Rossetti, L., Lodish, H. F. and Charron, M. J. (1991) J. Clin. Invest. **87**, 2197–2206
12 Strout, H. V., Vicario, P. P., Biswas, C., Saperstein, R., Brady, E. J., Pilch, P. F. and Burger, J. (1990) Endocrinology **126**, 2728–2732
13 Henriksen, E. J., Rodnik, K. J., Mondon, C. E., James, D. E. and Holloszy, J. O. (1991) J. Appl. Physiol. **70**, 2322–2327
14 Handberg, A., Kayser, L., Hoyer, P. E., Voldstedlund, M., Hansen, H. P. and Vinten, J. (1993) Diabetologia **36**, 481–486
15 Friedman, J. E., Sherman, W. M., Reed, M. J., Elton, C. W. and Dohm, G. L. (1990) FEBS Lett. **268**, 13–16
16 King, P. A., Horton, E. D., Hirshman, M. F. and Horton, E. S. (1992) J. Clin. Invest. **90**, 1568–1575
17 Banks, E. A., Brozinick, J. T., Jr., Yaspelkis, B. B., III, Kang, H. Y. and Ivy, J. L. (1992) Am. J. Physiol. **263**, E1010–E1015
18 Koranyi, L., James, D., Mueckler, M. and Permutt, M. A. (1990) J. Clin. Invest. **85**, 962–967
19 Cain, C. C., Trimble, W. S. and Lienhard, G. E. (1992) J. Biol. Chem. **267**, 11681–11684
20 Thoidis, G., Kotliar, N. and Pilch, P. F. (1993) J. Biol. Chem. **268**, 11691–11696
21 Kandror, K. and Pilch, P. F. (1994) J. Biol. Chem. **269**, 138–142
22 Uphues, I., Kolter, T., Goud, B. and Eckel, J. (1994) Biochem. J. **301**, 177–182
23 Ricort, J.-M., Tanti, J.-F., Cormont, M., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994) FEBS Lett. **347**, 42–44
24 Cormont, M., Tanti, J.-F., Zahraoui, A., Van Obberghen, E., Tavitian, A. and Le Marchand-Brustel, Y. (1993) J. Biol. Chem. **268**, 19491–19497
25 Russ, M., Wichelhaus, A., Uphues, I., Kolter, T. and Eckel, J. (1994) Eur. J. Biochem. **219**, 325–330
26 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
27 Klip, A., Ramlal, T., Bilan, P. J., Cartee, G. D., Gulve, E. A. and Holloszy, J. O. (1990) Biochem. Biophys. Res. Commun. **172**, 728–736
28 Slieker, L. J., Roberts, E. F., Shaw, W. W. and Johnson, W. T. (1990) Diabetes **39**, 619–625
29 Le Marchand-Brustel, Y., Grémeaux, T., Ballotti, R. and Van Obberghen, E. (1985) Nature (London) **315**, 676–679
30 Friedman, J. E., De Vente, J. E., Peterson, R. G. and Dohm, G. L. (1991) Am. J. Physiol. **261**, E782–E788
31 Vogt, B., Mühlbacher, C., Carrascosa, J., Obermaier-Kusser, B., Seffer, E., Mushack, J., Pongratz, D. and Häring, H. U. (1992) Diabetologia **35**, 456–463
32 Kolter, T., Uphues, I., Wichelhaus, A., Reinauer, H. and Eckel, J. (1992) Biochem. Biophys. Res. Commun. **189**, 1207–1214
33 Galante, P., Maerker, E., Scholz, R., Rett, K., Herberg, L., Mosthaf, L. and Häring, H. U. (1994) Diabetologia **37**, 3–9
34 Czech, M. P. and Buxton, J. M. (1993) J. Biol. Chem. **268**, 9187–9190
35 Kandror, K. V. and Pilch, P. F. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8017–8021
36 Goud, B. and McCaffrey, M. (1991) Curr. Opin. Cell Biol. **3**, 626–633
37 Baldini, G., Hohmann, R., Charron, M. J. and Lodish, H. F. (1991) J. Biol. Chem. **266**, 4037–4040
38 Li, G. and Stahl, P. D. (1993) Arch. Biochem. Biophys. **304**, 471–478
39 Hancock, J. F., Paterson, H. and Marshall, C. J. (1990) Cell **63**, 133–139
40 Cormont, M., Tanti, J.-F., Zahraoui, A., Van Obberghen, E. and Le Marchand-Brustel, Y. (1994) Eur. J. Biochem. **219**, 1081–1085
41 Van der Sluijs, P., Hull, M., Huber, L. A., Male, P., Goud, B. and Mellman, I. (1992) EMBO J. **11**, 4379–4389
42 Van der Sluijs, P., Hull, M., Zahraoui, A., Tavitian, A., Goud, B. and Mellman, I. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6313–6317

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