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Insulin-responsive glucose transporters are concentrated in a cell surface-derived membrane fraction of 3T3-L1 adipocytes

Klaus Lange and Ursula Brandt

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, FRG

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The recently proposed mechanistic concept of a receptor-regulated entrance compartment for hexose transport formed by microvilli on 3T3-L1 adipocytes predicted a preferential localization of glucose transporters in these structures. The cytochalasin B-binding technique was used to determine in basal and insulin-stimulated cells the distribution of glucose transporters between plasma membranes, low density microsomes (LDM) and two cell surface-derived membrane fractions prepared by a hydrodynamic shearing technique. The shearing procedure applied prior to homogenization yielded a low density surface-derived vesicle (LDSV) fraction which contained nearly 60% of the cellular glucose transporters and the total insulin-sensitive transporter pool. The rest of the glucose transporter population was localized within the plasma membrane (5%) and the LDM fraction (37%). Pretreatment of the cells with insulin (20 mU/ml for 10 min) reduced the transporter content of the LDSV fraction by 40% and increased that of the plasma membrane fraction 4-fold. The transporter containing LDSV fraction was clearly differentiated from the LDM fraction by its low specific galactosyltransferase activity and its insulin-sensitivity. Scanning electron microscopy revealed that the LDSV fraction contained a rather uniform population of spherical vesicles of 100–200 nm in diameter.

Glucose transporter; Adipocyte(3T3-L1); Insulin action; Microvilli

1. INTRODUCTION

Recent studies on 3T3-L1 adipocytes yielded evidence that hexose transport occurred through a small surface-located entrance compartment separated from the cytoplasm by a diffusion barrier [1,2]. This entrance compartment was proposed to be localized within the microvilli which were shown to grow out from the apical surface of 3T3-L1 preadipocytes after induction of the adipose conversion by addition of dexamethasone and isobutylmethylxanthine [2]. Insulin action as well as the 2-DG-induced endogenous transport-regulating mechanism caused a conspicuous transformation of microvilli into large saccular surface protrusions, thereby modulating the effectiveness of the diffusion barrier between the entrance compartment and the cytoplasm. Such changes of the shape of microvilli were accompanied by a significant increase of the cellular 3-*O*-methylglucose (3-OMG) distribution space [2]. The involvement of microvilli was also shown for the glucose-induced inhibition of 3-OMG uptake in 3T3-L1 adipocytes (hexose curb) [2]. As a consequence arising from the concept of a (receptor-)regulated entrance compartment of hexose transport, a considerable portion of the cellular transporter pool should be localized in the microvillar membranes. In order to test this proposition, we examined the transporter distribution between two surface-derived membrane fractions obtained by a hydrodynamic shearing technique, the plasma membrane (PM) and the low density microsomal (LDM) fraction of basal and insulin-stimulated cells.

Correspondence address: K. Lange, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, FRG

2. MATERIALS AND METHODS

2.1. *Cell culture*

3T3-L1 cells were cultured and differentiated as described earlier [2]. Differentiation was tested by determination of the insulin-induced stimulation of 3-OMG uptake [2]. A 5-fold uptake stimulation was observed for well-differentiated cells. Prior to the experiments, cell cultures were maintained in serum-containing Dulbecco's modified Eagle's medium (DMEM) with 5 mM glucose for 24 h and for the last 2 h in serum-free DMEM with 5 mM glucose. Monolayer cultures were treated with 100 nM insulin for 10 min in the presence of 0.02% (w/v) bovine serum albumin which was also added to the control cultures.

2.2. *Preparation of subcellular membrane fractions*

PM and LDM fractions were prepared essentially according to [4,5]. 3T3-L1 adipocytes cultured in plastic flasks (150 cm^2) were washed once with 1 mM Hepes buffer, pH 7.4, containing 1 mM EDTA and 0.255 M saccharose (buffer A) and gently detached. Cells collected from 10 flasks were suspended in 20 ml of buffer A, homogenized (25 strokes) with a 30 ml glass/teflon homogenizer (about 90 μm clearance). Centrifugation at 16000 × g for 20 min yielded a pellet which was washed once with buffer A and again sedimented at 16000 × g for 20 min; the supernatants were combined and centrifuged at 180000 × g for 80 min. The resulting pellet represents the LDM fraction. The pellet of the 16000 × g centrifugation steps was finely suspended in 15 ml of buffer A and given on the top of 15 ml of 10 mM Hepes buffer, pH 7.4, containing 1 mM ED-

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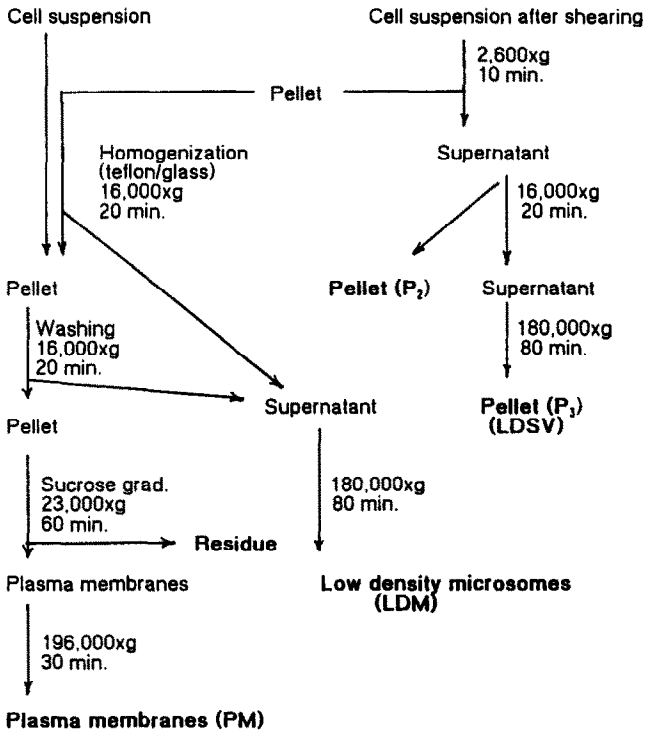

Fig.1. Preparation of subcellular membrane fractions from 3T3-L1 adipocytes.

TA and 1.12 M saccharose. After centrifugation at 23000 × *g* for 60 min the PM fraction was collected from the interface of the 2-step saccharose gradient, diluted with buffer A and again sedimented at 196000 × *g* for 30 min (fig.1). The pellets of the saccharose gradient centrifugation (residue, fig.1) did not contain significant amounts of D-glucose-inhibitable cytochalasin B-binding sites.

2.3. *Preparation of surface-derived membrane fractions*

The shearing technique described by Carothers Carraway et al. [3] was adapted to the 3T3-L1 adipocyte system as follows. A cell suspension was prepared as described above. Prior to homogenization, the cell surface projections were removed from the 3T3-L1 cells by gently pressing the suspended cells with a 20 ml syringe through hypodermic needles of the following size: no.1 (0.9 × 40 mm, 0.6 mm i.d.) and then no.2 (0.8 × 38 mm, 0.5 mm i.d.). Each step was repeated once. The resulting cell suspension was centrifuged at 2600 × *g* for 10 min. Determination of the viability by Trypan blue exclusion showed that about 80–90% of the cells remained intact. In order to prepare the plasma membrane and the LDM fraction from the cells after shearing, the pellet was resuspended in 20 ml of buffer A and further treated as described above for the cell suspension. The supernatant was centrifuged at 16000 × *g* for 20 min yielding the pellet P_2 and the supernatant. The latter was centrifuged at 180000 × *g* for 80 min to obtain the pellet P_3, called LDSV (low density surface-derived vesicle) fraction (fig.1).

2.4. *Cytochalasin B binding assay*

According to Cushman and Wardzala [6], membrane suspensions (100 μg protein) were incubated for 20 min at 4°C with cytochalasin E (2.2 μM) in the presence of 500 mM of D- or L-glucose, and then after addition of tritium-labeled cytochalasin B (29–300 nM) for a further 30 min at 4°C. The membranes were sedimented at 178000 × *g* for 10 min in a Beckman-Airfuge.

2.5. *Determination of galactosyltransferase (GalTase) activity*

GalTase activity was determined according to [7] by measuring incorporated activity from [^{14}C]UDP-galactose into ovalbumin.

2.6. *Preparation of samples for scanning electron microscopy*

A suspension of P_3 vesicles in 0.15 M phosphate buffer (PB) was given on a laminin-coated (2 μg/cm^2) plastic coverslip and allowed to settle for 1 h. The coverslip was then briefly washed with PB containing 0.1% bovine serum albumin, fixed with 5% (v/v) glutaraldehyde in PB for 10 min and washed again with PB. The specimen was then dehydrated in 30%, 50%, 75%, 95% and 100% (v/v) ethanol/water for 5 min each. After exchange of ethanol with hexamethyldisilazane according to [8], the samples were air-dried and coated with gold in a commercial sputter-coater and examined in a Cambridge scanning electron microscope.

3. RESULTS

The cytochalasin B-binding technique was applied for the quantification of glucose transporters. Since both transporter isoforms occurring in adipocytes bind cytochalasin B with indistinguishable affinity [9–11], this method is believed to be a most reliable technique for determination of hexose carriers.

As shown in table 1, the LDSV fraction prepared from unstimulated 3T3-L1 adipocytes by a hydrodynamic shearing technique and two subsequent centrifugation steps contained the highest specific transporter concentration of all fractions prepared and more than 60% of the total number of transporters found in these cells. As judged by the Trypan blue exclusion test, 80–90% of the cells remained intact after the shearing procedure (data not shown). The glucose transporter-containing subcellular membrane fractions containing PMs and LDMs, were prepared from cells with and without the shearing pretreatment using the method of McKeel [5], modified by Gibbs et al. [4]. An outline of the whole preparation protocol is shown in fig.1. The LDM fraction prepared without the shearing step prior to homogenization contained more than 90% of the whole cellular transporter pool (table 1). In accordance with the findings of Gibbs et al. [4], insulin treatment transferred a large part of these LDM transporters into the plasma membrane fraction, thereby increasing its transporter content almost 4-fold. When unstimulated cells were submitted to the

Table 1

Subcellular distribution of D-glucose-inhibitable cytochalasin B binding activity (pmol/fraction) in 3T3-L1 adipocytes according to the fractionation protocol in fig.1

	D-Glucose-inhibitable cytochalasin B binding (pmol/fraction) ± SD				
	PM	LDM	P_2	P_3 (LDSV)	Σ
Control	18.2 ± 5.8	318 ± 4			336
Insulin	69.8 ± 2.4	214 ± 21			284
Shearing	13.0 ± 2.1	99 ± 17	n.d.[a]	158 ± 1	270
Insulin + shearing	55.3 ± 2.4	102 ± 5	12.7 ± 5.2	98 ± 5	268

[a] Not determinable

Σ = sum of the activities of all fractions determined. All determinations were carried out as triplicates

Table 2

Subcellular distribution of specific D-glucose-inhibitable cytochalasin B binding (pmol/mg membrane protein) and specific galactosyltransferase activity (dpm/μg membrane protein) in 3T3-L1 adipocytes

	GalTase activity (dpm/μg protein) ± SD				Specific CB-binding (pmol/mg protein) ± SD			
	PM	LDM	P_2	P_3 (LDSV)	PM	LDM	P_2	P_3 (LDSV)
Control	694 ± 4	368 ± 1	–	–	4.9 ± 1.6	17.5 ± 0.2		
Insulin	1000 ± 6	361 ± 3	–	–	20.4 ± 0.7	13.5 ± 1.4		
Shearing	1004 ± 8	643 ± 9	184 ± 5	161 ± 4	5.6 ± 0.9	12.4 ± 2.2	n.d.[a]	27.2 ± 0.2
Insulin + shearing	986 ± 20	736 ± 17	224 ± 7	184 ± 5	18.2 ± 2.3	14.2 ± 1.6	1.2 ± 0.6	18.9 ± 1.0

[a] Not determinable

All determinations were carried out as triplicates

shearing procedure prior to homogenization, the cell surface-derived LDSV fraction P_3 contained nearly 60% of the cellular transporters, and the transporter content of the LDM fraction decreased to 30%. Stimulation of the adipocytes with insulin reduced the number of transporters in the LDSV fraction by 50% and increased the transporter content of the plasma membrane fraction 4-fold. This increase in transporter content of the plasma membrane is in good agreement with the insulin-induced 4–5-fold stimulation of 3-*O*-methylglucose uptake usually observed in 3T3-L1 adipocytes (data not shown). The maximum transporter concentration (based on mg protein) was also found in the LDSV fraction prepared from basal cells (table 2). After insulin treatment (20 mU/ml for 10 min), the transporter concentration in this fraction decreased by 30% and that of the plasma membrane fraction increased more than 3-fold. These experiments were repeated several times yielding always the same results with respect to the above main statements.

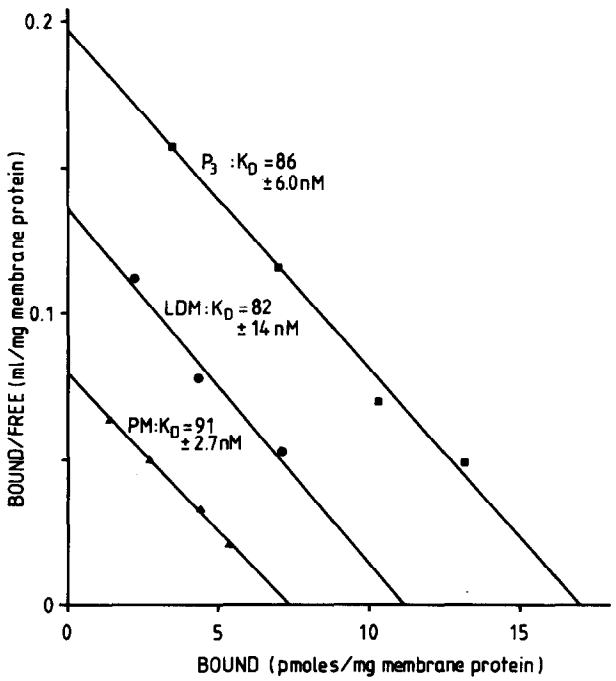

Fig.2. Scatchard analysis of D-glucose-inhibitable equilibrium binding of cytochalasin B to subcellular fractions of 3T3-L1 adipocytes at 4°C. The plasma membrane (PM), the LDM, and the surface-derived fractions P_2 and P_3 were prepared as described from untreated cells (see section 2). Plots of D-glucose-inhibitable binding were constructed by subtracting the binding curve obtained in the presence of 500 mM D-glucose along the radial axes of constant cytochalasin B concentration from that obtained in the presence of 500 mM L-glucose (K_d = dissociation constant in nM). Each point represents the mean of 3 determinations.

Nearly identical K_d values of 80–90 nM were found for the D-glucose-inhibitable cytochalasin B binding sites in LDSV, LDM, and plasma membrane fractions of unstimulated cells (fig.2). These values are in accordance with those of Greco-Perotto et al. [12] and Joost et al. [13] for rat adipocytes (90–100 nM).

Determination of the GalTase activity revealed that the LDS fraction contained the lowest specific activity of this enzyme of all fractions (table 2).

4. DISCUSSION

Since the publications of Cushman and Wardzala [6] as well as Suzuki and Kono [15], insulin is assumed to increase the number of glucose transporters in the plasma membrane through a rapid and reversible translocation from a large intracellular pool associated with the LDM fraction. However, the experimental procedure used for preparation of plasma membrane and microsomal fractions involves a homogenizing step which may severely alter the original structure of the plasma membrane. In particular, one cannot exclude that the shearing forces exerted on the cell surface during homogenization may cause vesiculation of surface protrusions such as microvilli and lamellipodia thus producing a special population of vesicles which subsequently may appear in the microsomal fraction. The presented results demonstrate that shearing forces which were much smaller than those exerted by use of a teflon/glass homogenizer (about 90 μm clearance compared to 600 μm i.d. of the used hypodermic needle) are able to pull off microvilli and lamellipodia

from the cell surface and to generate a distinct population of vesicles which may subsequently be found within the microsomal fraction, although their original location was on the cell surface.

Several lines of evidence suggest that the isolated insulin-responsive transporters of the LDSV fraction did not originate from intracellular sources. First, the insulin-responsive transporter pool was not partially but completely removed from the LDM fraction and appeared in the LDSV fraction after the shearing pretreatment. Second, the very low specific GalTase activity of the LDSV fraction (fig.2) indicates that the insulin-sensitive portion of the glucose transporters in this fraction did not arise from plasma membrane or microsomal contaminants. Third, as shown in fig.3, the LDSV fraction consisted of a population of vesicles of a rather unique size with an average diameter of 100–200 nm. This vesicle size is in good agreement with the dimensions of the spherical cell surface protrusions observed on 3T3-L1 cells in the early phase of adipose conversion and with that of the tips of the microvilli evolving from these protrusions during cultivation in glucose-containing medium [2]. In contrast, the inside-out vesicles, recently isolated by Biber and Lienhard [14] by an immunoadsorption technique, are much smaller having a diameter of 50 nm. Since the antibody used for vesicle isolation was raised against the cytoplasmic COOH-terminal peptide of the erythrocyte/HepG2 transporter, it is safe to assume that these vesicles are orientated inside-out and consequently of intracellular origin. The LDSVs described here should have the opposite orientation. They could not have been isolated by the immunoadsorption technique. The small LDM vesicles most probably represent an intracellular, condensed precursor form of the LDSVs. The low GalTase activity in both types of vesicles is in accordance with this notion [13].

The presented data yield strong evidence for the existence of a special cell surface-located, insulin-sensitive transporter pool which is large enough to account for the rapid initial 4–5-fold stimulation of 3-OMG transport occurring with a half-time of about 2 min after insulin addition. Moreover, these findings confirm the previously supposed mechanism of a receptor-regulated entrance compartment of hexose transport and offer an explanation for the discrepancies between the degree of insulin-induced stimulation of hexose uptake and the small amount of transporter protein translocated to the cell surface of 3T3-L1 adipocytes [4,16–19] or rat adipocytes [13,20]. This mechanistic

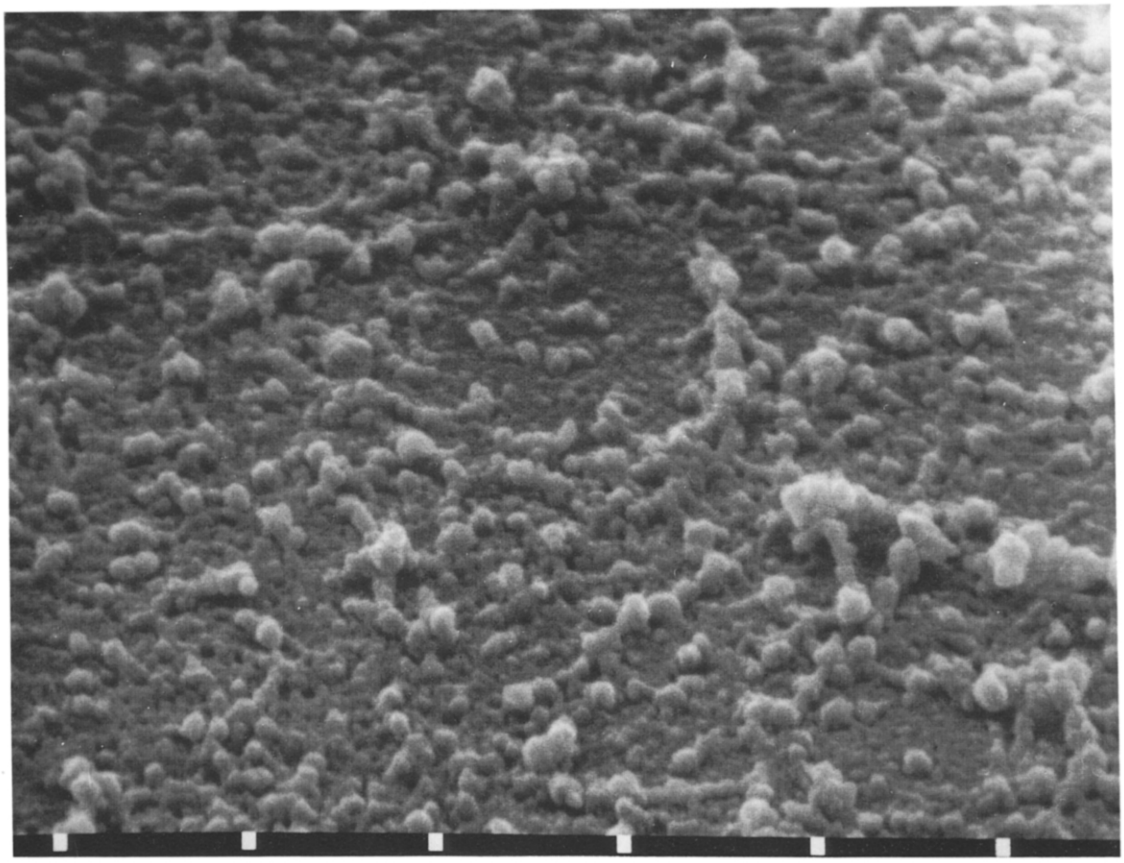

Fig.3. Electron scanning micrograph of the LDSVs isolated in the fraction P_3. LDSVs sedimented on a laminin-coated plastic coverslide (see section 2). Numerous vesicles between 100 and 200 nm in diameter are tightly bound to the finely structured laminin coat. Distance between 2 ticks at the bottom of the picture = 1 μm.

concept was also supported by the recent immunoelectron microscopic demonstration that glucose transporters of rat pancreatic β cells are restricted to certain domains of the plasma membrane especially to microvilli facing adjacent endocrine cells [21].

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