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Biochemical characterization and subcellular distribution of the glucose transporter from rat brain microvessels

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This study describes the biochemical characterization and subcellular distribution of glucose transporters from isolated rat brain cortical microvessels. The D-glucose inhibitable [^{3}H]cytochalasin B binding assay was used to quantitate glucose transporter binding sites in plasma membranes, high-density microsomes and low-density microsomes prepared from basal and insulin-stimulated cells. Incubation with insulin for 30 min increased the number of glucose transporters in the high-density microsomes by around 33% but had no effect on the number of glucose transporters in the plasma membrane or low-density microsomes. Prolonged incubation with insulin (2 h), however, resulted in a small but significant redistribution of glucose transporters to the low-density microsomes. Preincubation of cells with cycloheximide blocked this insulin-induced increase in glucose transporter number, suggesting that this effect of insulin was due to the synthesis of new glucose transport proteins. Specific labeling of glucose transporters was achieved by photoincorporation of [^{3}H]cytochalasin B. Labeled membranes from all fractions contained a single D-glucose inhibitable peak, migrating with a molecular size of 55 kDa on SDS-polyacrylamide gel electrophoresis. Isoelectric focusing of the 55 kDa protein revealed one major peak of D-glucose inhibitable radioactivity focusing at pH 6.0 in all fractions.

Introduction

The prime requirement of brain and nervous tissue for high levels of glucose both as a metabolic fuel and as a source of growth makes it especially important to understand the molecular events that govern glucose homeostasis in these tissues. Moreover, the degenerative changes that occur in the microvasculature in response to diabetes could have significant consequences for glucose handling by the brain.

The first step in the intracellular metabolism of glucose is its entry into the cell [1]. In most tissues this is accomplished by a carrier mediated process [1], which in muscle and fat is under stringent regulation by insulin [2,3]. Recent studies have indicated that insulin stimulates glucose transport in rat adipocytes [2,3], diaphragm [4] and cardiac muscle [5] by the redistribution of glucose carriers from an intracellular site to the plasma membrane. It is well established that glucose uptake by brain and nerve cells is mediated by the transfer of hexoses across the endothelial cells that comprise the blood brain barrier [6–9]. Whether or not this process is under acute regulation by insulin is open to question and conflicting reports appear in the literature [10–17]. In this report we have undertaken to define the subcellular distribution and

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molecular properties of glucose transporters from brain microvessels and to examine how these are modified by insulin.

Materials and Methods

Materials

Male Sprague-Dawley rats (150–175 g) were obtained from Charles River Laboratories. Animals were fed laboratory chow ad libitum. [^{3}H]Cytochalasin B (spec. act. 10–15 Ci/mmol) and [^{14}C]sucrose (spec. act. 673 mCi/mmol) were from New England Nuclear. Carrier ampholytes were from Pharmacia, all other reagents for isoelectric focusing and electrophoresis were from Bio-Rad. Tris and all other reagent-grade chemicals were from Sigma.

Methods

Isolation of rat brain microvessels. Rat brain cortex microvessels were isolated by a modification of the methods of Goldstein et al. [18] and Frank and Pardridge [19]. Sixty to seventy male Sprague-Dawley rats were guillotined. The brains were immediately removed and placed into ice-cold Krebs Ringer Hepes 1% bicarbonate buffer (KRBH) (pH 7.4) containing 1% bovine serum albumin. Pieces of cortical tissue (5 g) were removed from the brain, placed in a hand held homogenizer and gently homogenized in 50 ml of KRBH buffer in a Dounce homogenizer (clearance 0.25 mm) using 12 up and down strokes. This procedure disrupted the majority of nonvascular tissue. The homogenate was centrifuged at $1000 \times g_{max}$ for 10 min and the pellet resuspended in KRBH buffer containing 25% bovine serum albumin. After centrifugation at $1000 \times g_{max}$ for a further 10 min the myelin, which floats to the top, was removed. The pellet was resuspended in KRBH buffer containing 1% bovine serum albumin and filtered through a 202 μm nylon mesh to separate microvessels from nonvascular material and cell debris. The filtrate was passed through a 1.2×1.5 cm column of 0.25 mm diameter glass beads. Nuclei were not retained by the beads and could be removed by washing with buffer. The microvessels which adhered to the glass beads were removed by gentle agitation in buffer. After the beads settled, the microvessels were collected by centrifugation of the decanted supernatant ($500 \times g_{max}$ for 10 min). All steps were carried out at 4°C. Each preparation of brain microvessels were examined using phase contrast-microscopy which indicated that the isolated microvessels were virtually free of contamination by glia or neurons. Alkaline phosphatase, a specific marker enzyme of brain microvessels [18], was determined by the method of Garen and Levinthal [20]. Typical preparations showed a 25-fold enrichment of alkaline phosphatase in brain microvessels compared to brain cortex homogenate. Freshly isolated microvessels resuspended into KRBH buffer containing 3% bovine serum albumin (60 ml) were incubated with or without insulin (2 nM) at 37°C for 30 min or 2 h. In some experiments microvessels were preincubated with cycloheximide, 10 μg/ml, for 1 h prior to the addition of insulin.

Subcellular fractionation of microvessels. After incubation with insulin, microvessels were centrifuged at $500 \times g_{max}$ for 5 min. The resulting pellet was resuspended in ice-cold 20 mM Tris, 1 mM EDTA, 250 mM sucrose (pH 7.4) buffer (TES) containing 2.5 μg/ml aprotinin, leupeptin and pepstatin. The microvessels were homogenized by sonication (two times 2 min) on ice with a Branson cell disruptor model 200, set at 40 units. The resulting homogenate was centrifuged at $17\,000 \times g_{max}$ for 30 min. The supernatant was saved for the preparation of microsomal membrane fractions. The pellet, which contained plasma membranes and mitochondria, was resuspended into TES buffer, layered on a 41% sucrose cushion and centrifuged at $101\,000 \times g_{max}$ for 1 h. Plasma membranes, which band at the 41% sucrose interface, were collected by aspiration and diluted with TES buffer. The purified plasma membranes were pelleted by centrifugation at $48\,000 \times g_{max}$ for 45 min. The resulting pellet was washed in TES buffer and collected by centrifugation at $17\,000 \times g_{max}$ for 30 min. The plasma membranes were resuspended into TES and stored at −80°C. The initial supernatant was centrifuged at $48\,000 \times g_{max}$ for 30 min yielding a pellet of high-density microsomes. The supernatant was then centrifuged at $430\,000 \times g_{max}$ for 90 min yielding a pellet of low-density microsomes. All pellets were resuspended in TES buffer and stored at −80°C.

Determination of glucose transporter number. The

number of D-glucose inhibitable cytochalasin B binding sites in the subcellular fractions was determined as previously described [26].

2-Deoxyglucose uptake assay. Microvessels were incubated with 2-deoxy-D-[1,2(n)-^{3}H]glucose, spec. act. 30 Ci/mmol, at a concentration of 0.1 mM in Krebs Ringer Hepes buffer (pH 7.5) containing 1% bovine serum albumin. Cells were incubated at 37 °C for 3 min and the reaction was rapidly terminated by spinning cells in a microcentrifuge (1 min $10000 \times g$) and aspirating off the supernatant. The microvessels were then removed and the radioactivity determined. The portion of cellular uptake due to diffusion and trapping of label in the extracellular water space was measured with L-Glucose[1-^{14}C]. All transport data have been corrected for this.

Photochemical crosslinking of membranes and SDS-PAGE. Membrane fractions prepared as described above were resuspended in 50 mM phosphate buffer (pH 7.4) to a final concentration of 1–5 mg of membrane protein per ml. After incubation for 10 min at 4 °C [^{3}H]cytochalasin B (10 μM) the membranes were photochemically crosslinked as previously described [27]. Experiments involving D- or L-glucose were carried out as above except that the sugar (500 mM) was incubated with the membranes for 30 min prior to the addition of cytochalasin B.

Membranes covalently crosslinked to [^{3}H]cytochalasin B were solubilized by treatment with Laemmli's sample buffer [28] containing 8 M urea for 10 min at room temperature. The solubilized membranes were then centrifuged ($160000 \times g_{max}$ for 15 min for low-density microsomes, $48000 \times g_{max}$ for 15 min for high-density microsomes and $20000 \times g_{max}$ for 15 min for plasma membranes) and the supernatant was analyzed by SDS-PAGE on 5 mm (internal diameter) cylindrical gels or on 1.5 mm slab gels. Resolving gels of 9 or 10% acrylamide were used. Electrophoresis was carried out at 3 mA per gel for the cylindrical gels and at 30 mA per gel for the slab gels. After electrophoresis, the cylindrical gels were serially sliced with a Hoefer vibrating gel slicer. The slices were incubated in 20 ml scintillation vials, two or three slices per vial, with 1.5 ml NCS (Amersham) at 60 °C. The solubilized gel slices were cooled, 15 ml of scintillation fluid (3a20 Research products International) and 50 μl of acetic acid were added, and the vials were counted in a scintillation counter. The radioactive profile obtained was used as a guide to locate the position of the glucose transporter in duplicate gels. Similar procedures were carried out in slab gels. Prestained molecular weight marker proteins (Bethesda Research Laboratories) were used to estimate the molecular weight of the radiolabeled membrane proteins,

Proteins corresponding to a molecular mass of 50–65 kDa were excised from the slab gels and electrophoretically extracted as described by Hunkapiller et al. [29].

Isoelectric focusing. The extracted and concentrated SDS-solubilized glucose transporter was subjected to isoelectric focusing on cylindrical polyacrylamide gels. Gel composition and isoelectric focusing conditions were as previously described [30]. Duplicate gels were run; one was processed for scintillation counting as above, slices from the remaining gel were extracted for 1 h in 1 ml of water (four slices per tube) and the pH was determined [31].

Results

Subcellular membrane fractions from the microvessels were prepared as described under Materials and Methods. The purity of the isolated subcellular membrane fractions was determined by measuring the distribution of specific marker enzyme in each fraction, and Table I shows the specific activities of various marker enzymes characteristic of different subcellular organelles. The plasma membrane fraction was highly purified by the procedure described. Adenylate cyclase was used as the marker enzyme of plasma membranes and its specific activity was increased 75-fold over the original homogenate Table I. Rotenone insensitive cytochrome *c* reductase, a marker enzyme characteristic of the endoplasmic reticulum, was most enriched in the high-density microsomes. UDPgalactose : *N*-acetylglucosamine galactosyltransferase, a marker enzyme characteristic of membranes of the Golgi apparatus, was most enriched in the low-density microsomes. The almost 5-fold enrichment of this enzyme in low-density microsomes over homogenate is comparable to the fold enrichment observed in the same fraction

TABLE I

DISTRIBUTION OF MARKER ENZYME ACTIVITIES FROM SUBCELLULAR FRACTIONS OF RAT BRAIN MICROVESSELS

Values presented are the averages of two separate experiments. PM, plasma membranes; HDM, high-density microsomes; LDM, low-density microsomes.

Fraction	NaF-stimulated adenylate cyclase (pmol/min per mg)		NADH cytochrome *c* reductase (μmol/h per mg)		Galactosyl transferase (nmol/2 h per mg), basal
	basal	insulin	basal	insulin	
Homogenate	2.9	3.1	0.2	0.18	3.0
PM	225	221	0.4	0.6	6.9
HDM	100	98	4.2	4.5	11.8
LDM	18	23	1.2	1.0	14.0

prepared from rat adipocytes [32]. Insulin did not affect the the activity of cytochrome *c* reductase or adenylate cyclase in any of the membrane fractions (Table I).

The number of glucose transporters in each of the membrane fractions derived from basal and from insulin-stimulated cells was assessed by the D-glucose-inhibitable cytochalasin B binding assay. Scatchard analysis of the data from three separate binding experiments is shown in Fig. 1. All three membrane fractions from brain microvessels contain glucose transporter binding sites. Unlike the situation in adipose cells [2] and muscle [4], insulin (30 min, 37°C) has no affect on this distribution or on the apparent affinity of the glucose transporters (Fig. 1). However, insulin does increase the number of transporters in the high-density microsomal fraction by 60% ($P < 0.01$). This increase in glucose transporters in the high-density microsomes does not appear to be accom-

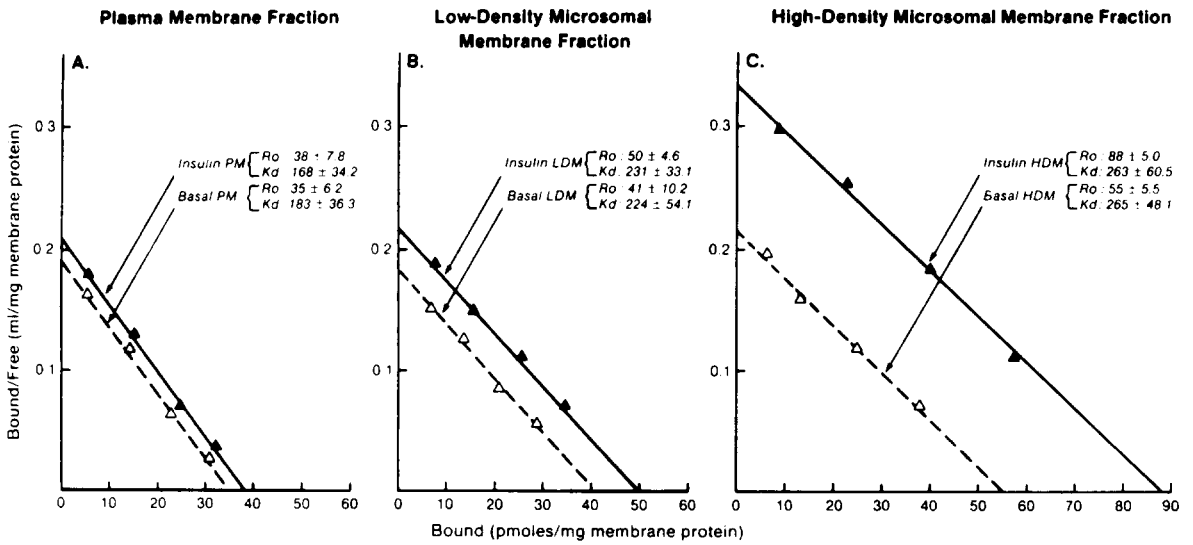

Fig. 1. Scatchard analysis of D-glucose inhibitable [^{3}H]cytochalasin B binding to plasma membranes (A); low-density microsomes (B); and high-density microsomes (C); from rat brain microvessels. Cells were incubated with (▲) or without (△) insulin (7 nM) for 30 min at 37°C before subfractionation. Derived Scatchard plots were determined by subtracting the binding at each of four cytochalasin B concentrations measured in the presence of 500 mM D-glucose from that measured in the absence of D-glucose. Results shown are the mean of triplicate determinations from three separate experiments. (K_d, dissociation constant in nM; R_0, D-glucose-inhibitable binding sites in pmol/mg membrane protein).

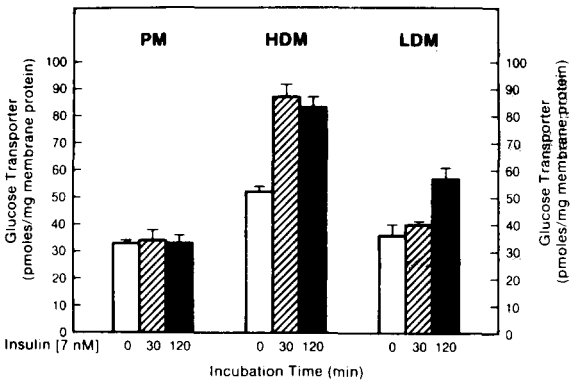

Fig. 2. The effect of prolonged insulin incubation on the distribution of glucose transporters in brain microvessel plasma membranes (PM), high-density microsomes (HDM) and low-density microsomes (LDM). (1) Open boxes represent incubation of microvessels for 120 min without any additions (basal) prior to subcellular fractionation; (2) hatched boxes represent 90 min incubation as in (1), followed by 30 min with insulin; (3) solid boxes represent microvessels incubated for 120 min with insulin. The vertical bar represents mean ± S.E., data from three separate experiments.

panied by changes in numbers of glucose transporter in either plasma membrane or low-density microsomes. Interestingly, ore prolonged exposure of the microvessels to insulin (2 h) caused a small but significant ($P < 0.01$) increase in glucose transporters in the low-density microsomes (Fig. 2). Plasma membranes were unaffected by this longer incubation with hormone. In confirmation of earlier studies insulin did not stimulate glucose transport in the isolated microvessels as assessed by 2-deoxyglucose uptake, basal glucose transport 1.35 pmol of 2-deoxyglucose taken up per second compared to 1.19 pmol of 2-deoxyglucose taken up per second for insulin-treated cells, respectively.

The increase in glucose transporter numbers in brain microvessel high-density microsomes induced by insulin is of interest, particularly since it does not appear to result from a redistribution of glucose transporters by the hormone from other membrane fractions as is the case in adipose cells and in muscle [2–4]. For this reason we sought to characterize the molecular properties of the glucose transporter in order to determine whether we could detect biochemical changes induced by insulin. Analysis of the [^{3}H]cytochalasin B labeled membrane fractions of brain microvessels by SDS-PAGE is shown in Fig. 3. The membrane proteins were treated as described under the figure legend. SDS-PAGE analysis of the labeled membrane fractions revealed a single D-glucose inhibitable (40–65%) peak, migrating with a molecular weight of 55 kDa. Only the high-density micro-

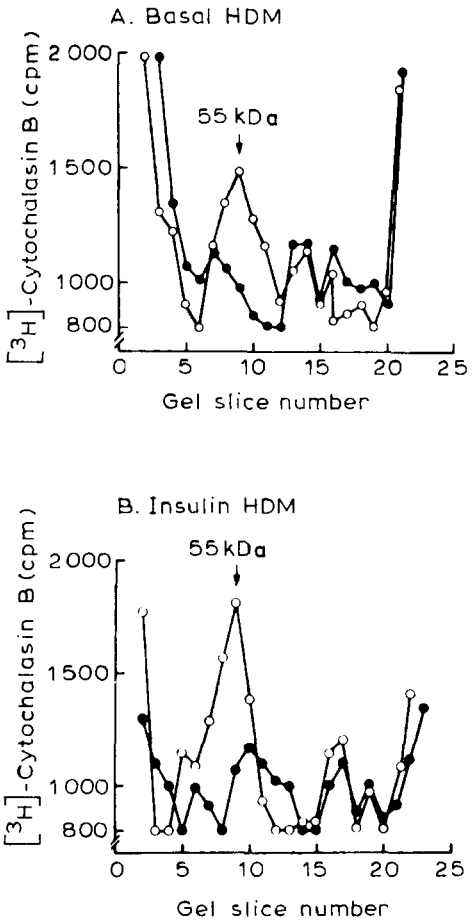

Fig. 3. Electrophoretic profile of [^{3}H]cytochalasin B-labeled high-density microsomes (HDM) from rat brain microvessels incubated in the presence of either D- (●) or L-glucose (○) with or without insulin. Membranes were prepared as described under Materials and Methods, and preincubated with 500 mM D-glucose or L-glucose prior to the addition of [^{3}H]cytochalasin B for 10 min at 4°C. Membranes were irradiated with ultraviolet light at 254 nm for 5 min. Membrane protein (200 μg) was applied to 9% gels and analysed by SDS-PAGE. Prestained marker proteins were used as standards, arrow indicates the molecular weight of the glucose transporter (55 kDa). Experiments were carried out three times and the data shown represent a typical experiment.

somes are shown here for clarity; labeling of the other fractions was identical. Insulin had no effect on the SDS-PAGE profile other than to increase the amount of the 55 kDa D-glucose inhibitable protein in the high-density microsomal membrane fraction. Previous studies by Dick et al. [17] revealed a similar molecular weight for the brain microvessel glucose transporter.

Additional assessment of the molecular properties of the glucose transporter from brain microvessel subcellular membrane fractions was carried out by subjecting the SDS-PAGE purified 55 kDa membrane protein to isoelectric focusing. In rat adipocyte membranes analyzed in this manner we previously observed a heterogeneity of glucose transporters in the intracellular pool while the plasma membrane glucose transporters were uniform in charge properties [33]. In contrast, in all three subcellular membrane fractions from brain microvessels, (only the high-density microsomal fraction profile is shown) only one major D-glucose inhibitable peak, focusing at pH 6.0, was observed (Fig. 4) in the presence or absence of insulin. Thus, based on the criteria of molecular size and charge properties we could detect no discernable biochemical difference between the glucose transporters from the three brain microvessel membrane fractions, and no effect of insulin on these properties.

Based on these findings we sought to determine whether the increase in glucose transporters induced by insulin could be due to an increase in de novo protein synthesis. To address this question we incubated brain microvessels under the following conditions: (1) Basal; cells were incubated for 90 min at 37°C with no additions, (2) Insulin; cells were incubated for 60 min without insulin and then for a further 30 min with 2 nM insulin, (3) Cycloheximide; cells were incubated in the presence of 10 μg/ml cycloheximide for 60 min

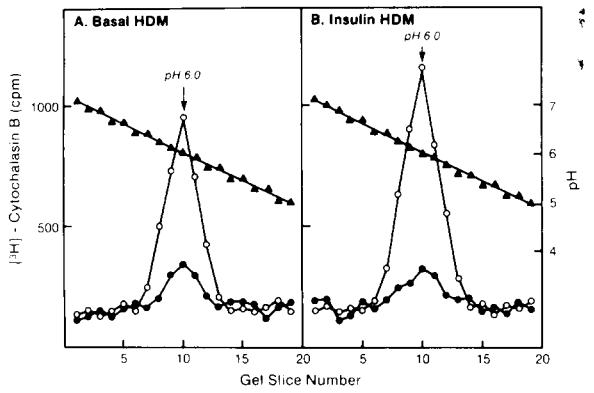

Fig. 4. Isoelectric focusing of the SDS-PAGE purified, [^{3}H]cytochalasin B-labeled high-density microsomal glucose transporters from basal (Panel A) and insulin (Panel B) brain microvessels. The radiolabeled membrane proteins migrating at 45–65 kDa were excised from an SDS-polyacrylamide gel and after elution were subjected to isoelectric focusing as described under Materials and Methods. Membranes were preincubated with 500 mM D-glucose (●) or L-glucose (○). Arrows indicate the isoelectric points of the focused protein peaks; (▲) pH. Identical profiles were obtained for the plasma membrane and low-density microsome membrane fractions with or without insulin (data not shown).

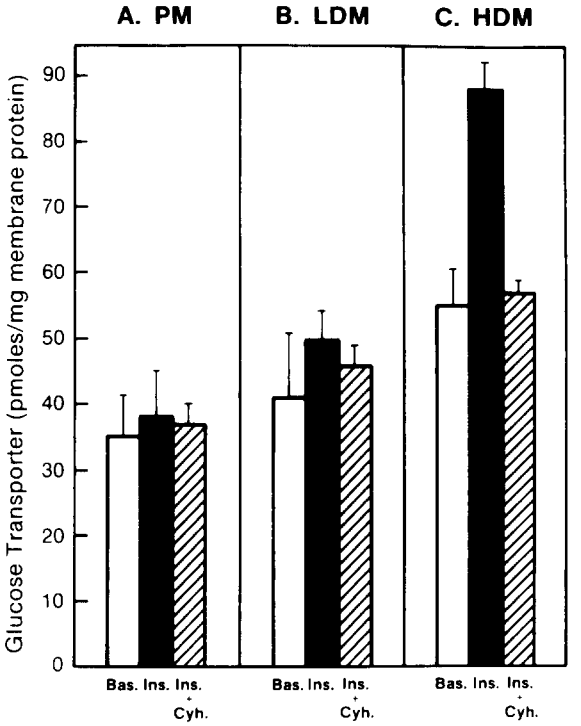

Fig. 5. The effect of cycloheximide on the content of specific D-glucose inhibitable [^{3}H]cytochalasin B binding sites in subcellular fractions from rat brain microvessels. Panel A, plasma membranes (PM); Panel B, low-density microsomes (LDM); and Panel C, high-density microsomes (HDM). Open boxes represent membranes prepared from cells incubated with no additions. Solid boxes represent membranes prepared from cells incubated with insulin. Hatched boxes represent membranes prepared from cells incubated with cycloheximide prior to incubation with insulin. The vertical bar represents mean ± S.E., data from three separate experiments.

followed by a further 30 min with 2 nM insulin. Subcellular membrane fractions were then prepared and the number of glucose transporters in each of the fractions was determined using the D-glucose inhibitable cytochalasin B binding assay. The results of these experiments are shown in Fig. 5. Here we can see that cycloheximide alone has no effect on glucose transporter number in either plasma membranes or in low-density microsomes. In contrast, however, cycloheximide totally inhibited the insulin-induced increase in cytochalasin B binding sites in the high-density microsomes.

Discussion

The movement of glucose across brain cell membranes is dependent on its rapid transfer across the blood brain barrier [7]. Since brain and nervous tissue depend almost exclusively on glucose metabolism for cell growth and energy the endothelial glucose transport system, which supplies this need, thus assumes a role of major importance. Although insulin stimulates glucose transport in target tissues such as fat and muscle [2–4], and insulin receptors have been demonstrated in brain cells [34], the role of the hormone in regulating glucose homeostasis in the brain has so far been ill defined. Numerous reports have sought to link insulin to the possible regulation of glucose flux in brain and nervous tissue [10–16]. Of particular relevance to the current studies, Pillion et al. [16] recently reported that insulin stimulated glucose metabolism in isolated brain microvessels. Similarly, Daniel et al. [35] measuring radioactive glucose uptake in living animals concluded that insulin significantly increased the net overall gain of glucose by the brain. Important as these, and several other studies are, it has so far not been possible to dissociate the effects of insulin on glucose transport from its overall effects on glucose metabolism.

To address these issues, and to characterize the blood brain barrier glucose transport protein, we developed a subcellular fractionation scheme to isolate specific membrane fractions from rat brain microvessels in high purity (Table I). Using this protocol we have determined the distribution of glucose carriers in these subcellular fractions using the D-glucose-inhibitable cytochalasin B binding assay. The results show that although the distribution of glucose transporters is largely independent of any effect of insulin, the hormone does stimulate a significant increase in the numbers of carriers in the high-density microsomal membrane fraction (Fig. 1). The fact that insulin does not acutely alter the distribution or the affinity of the glucose transporter, as measured by the cytochalasin B binding assay, argues against any stimulatory effect of the hormone on glucose transport in brain microvessels. Further support for this notion comes from the finding that isolated brain microvessels incubated with or without insulin exhibited identical rates of 2-deoxyglucose uptake. Thus brain microvessels appear to be functionally different, in this respect at least, to cultured glial cells in which insulin has been reported to stimulate 2-deoxyglucose uptake [15]. Prolonged incubation of microvessels with insulin did, however, result in a small but significant increase in glucose carriers in low-density microsomes.

The fact that insulin stimulates an increase in glucose transporters in the high-density microsomal fraction, independent of its failure to stimulate glucose transport, was an unexpected finding. One possible explanation for this was that insulin treatment of microvessels altered the biochemical structure of the high-density microsomal transporter in a way that allowed it to bind more cytochalasin B, giving the appearance of an increase in glucose transporter number. Changes in the glycosylation and/or phosphorylation state of the glucose transporter [33] for instance, could alter its molecular properties. We thus chose to determine whether the glucose carriers from the subcellular fractions were biochemically identical by examining their molecular mass and charge properties using a modified two-dimensional electrophoresis procedure. Membrane proteins from basal and insulin treated microvessels were preincubated with D- or L-glucose, photolabeled with [^{3}H]cytochalasin B and subjected to SDS-PAGE. The protein profiles were remarkably similar; a major band of D-glucose sensitive [^{3}H]cytochalasin B binding protein migrated with a molecular mass of 55 kDa in each case (Fig. 3). Furthermore, when the 55 kDa band was eluted from the SDS

gel and subjected to isoelectric focusing it gave rise, in each instance, to a D-glucose sensitive peak having an isoelectric point of pH 6.0 (Fig. 4). Thus, on the basis of size and charge properties glucose transporters from basal and insulin-treated microvessels were indistinguishable. The insulin-induced increase in high-density microsome glucose transporter numbers was also observed by antibody detection using antiserum raised against the human erythrocyte glucose transporter (data not shown). This would appear to rule out insulin-induced biochemical changes of the glucose carrier as an explanation for the increase in high-density microsomal glucose transporter numbers.

It has recently been shown that insulin stimulates DNA synthesis in cerebral microvessels [36] and the rate of postnatal brain protein synthesis [37]. Since high-density microsomes consist mainly of endoplasmic reticulum, which are intimately involved in protein synthesis, we reasoned that the insulin-induced increase of glucose transporter numbers in this membrane fraction could be explained by effects on de novo protein synthesis. To test this idea, we prepared subcellular membrane fractions from brain microvessels incubated in the presence and absence of insulin and cycloheximide. Cycloheximide had no effect on glucose transporter numbers in either plasma membranes or in low-density microsomes, but it totally inhibited the insulin-induced increase in glucose transporter number observed in high-density microsomes (Fig. 5).

At the doses used here, cycloheximide mainly inhibits protein synthesis, however, the drug may also affect other cellular processes such as protein turnover rates. While we cannot rule out such an effect here, we think it extremely unlikely since neither the plasma membrane nor low-density microsome glucose transporters were affected by cycloheximide treatment of the microvessels. Furthermore, the effect of insulin on protein synthesis was not general since it did not alter the marker enzyme levels in the subcellular fractions (Table I); only glucose transporter number was increased.

It is intriguing to speculate both on the possible fate of the increased numbers of glucose carriers induced by insulin in the high-density microsomes and also their possible function. It is possible that prolonged exposure to insulin will induce translocation of glucose carriers from high-density microsomes to other cellular sites, since we observed a small increase in carrier sites in the low-density microsomes after a 2 h incubation with insulin. The fact that we saw no decrease in the high-density microsomes at this longer time of incubation could suggest that newly synthesized transporters are moving from the high-density microsomes to the low-density microsomes such that the movement exactly balances the synthesis of new transporters.

Perhaps the glucose carriers induced by insulin in high-density microsomes are in some way sequestered from the mainstream of glucose transporter recycling, intracellular processing, and trafficking, and represent a nascent pool of carriers awaiting modification in some way that will enable them to participate fully as active carrier proteins.

In summary, these studies have yielded new information on the rat brain microvessel glucose transporter: (1) glucose uptake in these cells is not stimulated by insulin, (2) consistent with this, insulin does not acutely alter the distribution of hexose carriers in these cells though it does increase the number of carriers in the high-density microsomes, (3) based on molecular size and charge properties the hexose carriers appear to be identical and insulin does not modify their properties, and (4) the insulin-induced increase in hexose carriers in high-density microsomes most likely represents an increase in de novo protein synthesis, since it can be blocked by cycloheximide.

Acknowledgments

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