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Biochimica et Biophysica Acta 1285 (1996) 255–266

BBA

Modulation of cultured brain, adrenal, and aortic endothelial cell glucose transport

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Received 15 July 1996; accepted 6 August 1996

Abstract

Studies of glucose transporter activity and anti-glucose transporter (GLUT1) immunoblots were performed on different endothelial cell primary cultures (brain capillary, adrenal capillary and aortic) to determine their response to glucose deprivation. Cell cultures were exposed to glucose deprivation (0.5 mM) for 48 h periods and refed (11.0 mM) for 36 additional hours. Control cultures were kept in 11.0 mM glucose for the duration of these studies. Measurements of 2-[^{3}H]deoxy-D-glucose uptake and membrane fraction purification were performed every 12 h during these timecourses. Baseline cytochalasin-B sensitive uptake of 2-deoxy-D-glucose was near three times larger in brain capillary endothelial cells than in adrenal or aortic endothelial cultures. In all three endothelial cell cultures, 2-deoxy-D-glucose uptake increased during glucose deprivation, and returned to control values upon refeeding. Aortic and adrenal cortical endothelia expressed the starvation induced increases 12 h sooner than brain capillary endothelia. Return to control values was also 12 h faster in these cultured endothelia. Immunoblot studies showed that in all three endothelial cell cultures the increases in transporter activity during glucose starvation correlate with increased membrane expression of GLUT1. Quantitative analysis of the anti-GLUT1 immunoblots indicated that induction of GLUT1 following glucose starvation was slower in brain capillary endothelia than in aortic or adrenal endothelia. The slower response by brain capillary endothelial cells may be related to the higher transport rate of glucose in these cells.

Keywords: Glucose transport; Endothelium; Blood-brain barrier; GLUT1

1. Introduction

The blood-brain barrier (BBB) is the anatomic and physiologic complex of structures and functions which controls flux of substances into the central nervous system (CNS) interstitium. This barrier function is thought to arise from special properties of the brain capillaries themselves, which express interendothelial tight junctions, have few fenestrations, and demonstrate a low rate of fluid-phase pinocytosis when compared with extracerebral capillaries [1,2]. The BBB effectively prevents small hydrophilic molecules from gaining access to the CNS. Those which do cross from the plasma space into the CNS interstitium do so because of specific transport proteins in

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PII S0005-2736(96)00172-1

both lumenal and ablumenal membranes of the capillary endothelial cells. Transport proteins for hexoses, amino acids, purines, and monocarboxylic acids have all been identified in the endothelia of the mammalian BBB [3].

Because glucose is the primary metabolic fuel of the CNS, BBB glucose transport protein function and regulation may play a crucial role in the maintenance of brain function. The primary glucose transporter found in mammalian BBB endothelia, GLUT1 [4–6], is a member of a family of facilitative hexose transporter proteins [7–11], and is non-insulin dependent. Experimental evidence suggests that BBB GLUT1 is regulated by metabolic factors in vivo. Hyperglycemia, resulting from experimental diabetes mellitus in rats, is associated with a significant decrease in brain glucose uptake [12]. Later studies suggest that this diminished brain glucose uptake is associated with decreased BBB glucose transporter density [13]. Diminishing available glucose appears to produce the converse situation: an increase in brain glucose uptake is associated with hypoglycemia in hyperinsulinemic rats [14]. Cultured cell studies have provided additional insight into the molecular basis for these changes in BBB function: glucose deprivation has been shown to increase glucose transport and GLUT1 expression in cultured brain capillary endothelium [15], in agreement with the in vivo studies. Serum glucose concentration may thus play a role in the modulation of BBB GLUT1 function.

The endothelium of brain capillaries possesses a physiologically unique role when compared with endothelium from other organs, in that the non-barrier forming endothelial cells of these latter organs do not appear to control glucose flux or utilization as tightly. Transporter proteins in these extracerebral capillary beds may function simply to subserve the metabolic needs of the endothelial cells themselves, and not to have the additional role of allowing for trans-endothelial cell solute flux. It is thus of interest to evaluate if the regulation of GLUT1 differs in endothelium from blood-brain barrier when compared with non-barrier forming endothelium. A cultured cell model of the blood-brain barrier and two examples of non-barrier forming endothelial cells from adrenal cortex and aorta were chosen to compare the influence of glucose deprivation on glucose transporter activity and expression.

2. Materials and methods

2.1. Materials

General laboratory chemicals, protease inhibitors, and colorimetric alkaline phosphatase and gamma-glutamyl Transpeptidase test kits were purchased from Sigma, St. Louis, MO. Hanks' Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), antibiotics, trypsin, and L-glutamine were purchased from GIBCO., Grand Island, NY. Defined equine serum and bovine calf serum were obtained from Hyclone, Logan, UT. Type II collagenase was obtained from Worthington, Frechold, NJ. Nylon meshes were obtained from Tetko, Lancaster, NY. Di-acetylated-LDL was obtained from Biomedical Technologies, Stoughton, MA. Cell culture filtration supplies were purchased from Nalgene, Rochester, NY. Cell culture plasticware were obtained from Costar, Cambridge, MA. 2-[^{3}H]Deoxy-D-glucose and ^{125}I-labeled goat anti rabbit Fc polyclonal antibodies were purchased from New England Nuclear, Boston, MA. U-[^{14}C]Sucrose was obtained from Amersham, Arlington Heights, IL. Anti-GLUT1 antibodies were purchased from East Acres Biologicals, Southbridge, MA. Ultima Gold scintillation fluid and vials were purchased from Packard, Meriden, CT. Nitrocellulose membranes (Nitrobind, 0.45 micron) were purchased from Micron Separations, Westborough, MA, and Whatman #2 qualitative filter papers were obtained from Whatman Paper, Maidstone, England. The Biometra fast-blot semi-dry electroblotter was purchased from Immunetix, Cambridge, MA. X-OMAT-AR scientific imaging film and Kodak X-omatic cassettes were purchased from Eastman-Kodak, Rochester, NY. Sarcoma-180 cells were a gift of Dr. C. Haudenschild, Department of Pathology, Boston University School of Medicine.

2.2. Brain capillary endothelial cell cultures

Brain capillary endothelial cells were cultured using a modification of the method of Carson and Haudenschild [16]. Tissues were obtained from the local slaughterhouse immediately following death. Brains were aseptically removed from bisected calvaria and put into sterile HBSS supplemented with 11.0 mM D-glucose, 100 mU/ml penicillin, 100

μg/ml streptomycin, and 250 ng/ml amphotericin B, at pH 7.4 and 22°C. The cortical grey matter was aspirated away using a Pasteur pipette, homogenized and passed through serial meshes (149, 78, and 37 micron nylon mesh). Only material from the 78 and 37 micron mesh was utilized. Capillaries were gently removed from the meshes with a stream of HBSS, containing 1% bovine serum albumin, and treated with collagenase (0.1% in DMEM, penicillin, streptomycin, and amphotericin B, 37°C, for 2.5 h) followed by trypsin (1% in HBSS, 37°C, 20 min). The resulting cell suspension was washed twice and plated onto gelatin coated cell culture dishes at a density of $3–5 \cdot 10^4$ cells or capillary fragments/cm^2. Gelatin coating of the tissue culture plastic involved overlaying the plastic with a 5 mm layer of gelatin solution (1% in HBSS and antibiotics) and overnight incubation. Prior to use, the coated tissue culture plastic was washed three times with DMEM, overlayed with culture medium (see below) and allowed to equilibrate for 1 h at 37°C. Culture medium for all cells in these studies (unless otherwise noted) consisted of 20% confluent Sarcoma-180 conditioned medium containing 10% bovine calf serum (dialyzed 3×12 h against glucose-free DMEM, 1/20, at 4°C prior to use), 2% extract from bovine retina, 500 μg/ml heparin, 100 mU/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and 15% defined equine serum in glucose free DMEM, pH 7.4, to which 11 mM D-glucose was added. All cultures were maintained in 5% CO_2 at 37°C. After 2–3 days the adherent cells were treated with pancreatin (0.25% in HBSS and antibiotics, 37°C, 15 min) which selectively released the endothelial cell colonies. These released endothelial cell colonies were exposed to trypsin (1% in HBSS, 37°C, 10 min) and plated onto gelatin coated 24 well culture plates (for 2-DG uptake studies) or onto gelatin coated 100 mm culture dishes (for membrane fraction purification). This plating is referred to throughout this study as a primary culture. Cultures were evaluated for endothelial cell purity as discussed below.

2.3. Adrenal cortical capillary endothelial cell cultures

Cultures of calf adrenal cortical capillary endothelium were prepared by a modification of the method of Folkman et al [17]. Unless otherwise noted, all isolation steps were carried out aseptically in the same solution used for brain capillary isolations. The adrenal capsules were bisected, which was followed by gentle removal and mincing of cortical contents with a #10 scalpel and forceps. This minced tissue was then treated with collagenase (0.1% in DMEM, 37°C, 1 h) and the resulting suspension poured through a 112 micron nylon mesh. Capillary fragments, red blood cells, and adrenal parenchymal cells passed through the mesh and were plated out under conditions identical to those used for brain capillary cultures.

2.4. Aortic endothelial cell cultures

Cultures of calf aortic endothelium were prepared by the method of Haudenschild [18]. These were isolated by gently scraping the lumenal surface of calf aortas, after which treatment with collagenase (0.1% in DMEM, 37°C, 30 min) released single cell suspensions which were plated onto gelatin coated culture dishes. The cells were plated at the same density and grown in identical medium as brain capillary endothelial cells.

2.5. Endothelial cell marker studies

The measurement of endothelial cell surface enzymatic markers was used to verify cell identity and de-differentiation in brain capillary cultures with increasing culture passage. Alkaline phosphatase and gamma-glutamyl transpeptidase are two well characterized brain capillary enzymes that were measured for this purpose. These enzymes have been localized to the brain endothelial cells themselves [19,20]. Quantitative measurement of these enzymes was performed using a colorimetric, kinetic method available commercially. Brain capillary endothelial cells were homogenized in HBSS at 4°C and samples taken for the measurement of enzyme activity and protein content. The quantitative measure of total sample enzyme activity was then normalized per milligram protein and compared with similar studies of brain capillaries themselves.

2.6. Di-acetylated-LDL uptake studies

Brain capillary, adrenal cortical and aortic endothelia were routinely screened for di-acetylated-low

density lipoprotein uptake (DiAc-LDL), an endothelial cell marker [21]. Endothelial cells plated onto gelatin coated glass coverslips were incubated with acetylated-low density lipoprotein (10 μg/ml in standard culture medium) for 4 h at 37°C. At the end of this period the cells were washed three times with probe-free DMEM to remove all excess, non-cell bound DiAc-LDL. The uptake of DiAc-LDL by cell cultures was visualized and photographed using a fluorescence microscope, with 514 nm and 550 nm wavelength filters for excitation and emission respectively. Photographs were taken using automatic exposure control on 1600 ASA speed film.

2.7. Glucose starvation of cultured cells

Glucose deprivation of endothelial cell cultures was conducted by changing the standard culture medium (11 mM D-glucose) to one containing only 0.5 mM D-glucose. Cultures were washed three times for 1 min each with glucose-free saline to remove residual cell culture glucose. With the exception of the glucose, the glucose deprivation culture medium was identical to the standard culture medium. Despite the lack of glucose in the medium, the cell cultures were provided with ample substrate with which to continue oxidative and anaerobic metabolism, including sodium pyruvate, and both essential and non-essential amino acids. Within a given experiment, glucose deprived and control endothelial cell cultures were always from the same primary culture, and were timed and refed simultaneously. All cultures were refed daily with fresh culture medium. The rate of 2-DG uptake and membrane fraction purification were performed every 12 h during these experiments.

2.8. Measurement of 2-deoxy-glucose uptake

Uptake of 2-DG was done using modifications of the methods of Vinters et al. [22] and Walker et al. [23]. Cells were plated into gelatin coated 24-well tissue culture dishes 5–7 days prior to uptake studies, and were always allowed to reach confluence. All uptake studies were conducted in glucose-free HBSS. Prior to uptake studies, culture medium was removed by aspiration and cell monolayers were washed twice in 1.5 ml of HBSS for 5 min at room temperature. The preincubation buffer was then aspirated and 0.25 ml of 100 nM 2-[^{3}H]deoxy-glucose (2-[^{3}H]DG) in HBSS was added to each well. The rate of 2-deoxy-glucose uptake was determined by incubating the separate groups of wells with 2-[^{3}H]DG for varying times. Tritiated 2-DG was used in these studies because it is transported into the cytosol and phosphorylated by intracellular hexokinase, but is not further metabolized [24]. This property, in combination with the use of 100 nM 2-DG (a concentration far below the estimated Km for either the glucose transporter or hexokinase [25]) allows linear, non-saturated cell uptake kinetics to be observed. Estimation of non-transporter mediated 2-DG uptake and cell surface binding was performed by conducting parallel studies using cell monolayers which were pre-incubated with phloretin (1.0 mM), or cytochalasin B (100 μM), which are agents known to block the glucose transporter [26,27]. Uptake in all studies was terminated by rapidly washing the cell monolayers with three volumes of ice-cold HBSS containing 100 μM phloretin. This step removes all non-transported 2-DG from the cell monolayer in addition to effectively 'trapping' any already transported but not phosphorylated 2-DG within the cultured cell cytoplasm. Cell monolayers were solubilized in 0.5 M NaOH for 12 h at 22°C, and aliquots were then taken for measuring protein and isotope content. Protein was measured by the method of Bradford [28] using a Perkin-Elmer Lambda-2 UV/VIS spectrophotometer. 2-[^{3}H]DG was measured in Ultima-Gold scintillation medium on a Packard 1900CA Tri-Carb liquid scintillation analyzer with automatic quench control. All uptake studies were normalized per μg protein. Specific carrier-mediated uptake was calculated by subtracting the 2-DG uptake rate remaining after phloretin or cytochalasin-B preincubation from the total 2-DG uptake rate. This estimation of specific carrier-mediated 2-DG uptake was carried out on control and glucose deprived endothelial cell primary cultures from brain capillary, adrenal cortical capillary, and aorta.

2.9. Western blot analysis

Membrane fractions were purified from endothelial cell cultures by washing 3 times with HBSS at 4°C, followed by homogenization in 1.0 mM Tris/0.5 mM PMSF at pH 7.4 on a polytron (3 × 15 s). The homogenate was then centrifuged at 1500 × g for 5

min at 4°C, and the pellets discarded. The supernatants were then centrifuged at 35 000 × g at 4°C in a Beckmann Ultracentrifuge using a Ti-70 rotor. These supernatants were discarded, and the pellets solubilized in 0.25 M Sucrose containing Antipain (2 μg/ml), Pepstatin A (0.7 μg/ml), PMSF (0.5 mg/ml), Benzamidine (2 mM), and Soybean Trypsin Inhibitor (0.1 mg/ml). Samples were stored at −80°C until further use. Membrane fractions samples were size-fractionated using SDS-PAGE analysis. Gels in these studies were of the stacking type, with initial 3.9% acrylamide (125 mM Tris, pH 6.8, 3.9% acrylamide, 0.1% SDS, 0.5% ammonium persulfate, 0.001% TEMED) followed by 12.5% acrylamide (377 mM Tris, pH 8.8, 12.5% acrylamide, 0.1% SDS, 0.05% ammonium persulfate, 0.001% TEMED). Electrode buffer formulation was: 50 mM Tris, 384 mM glycine, 0.1% SDS, and 0.002% EDTA. Electrophoresis was carried out at 100 V for 4–6 h at room temperature. Proteins were transferred onto nitrocellulose membranes with a semi-dry electroblotting system. This system was set up as follows: anode, filter paper 1, filter paper 2, filter paper 3, nitrocellulose membrane, acrylamide gel, filter paper 4, and cathode. The filter papers were saturated with transfer buffer as follows: filter papers 1 and 2: 0.3 M Tris-HCl, 20% methanol, pH 10.4; filter paper 3: 0.025 M Tris-HCl, 20% methanol, pH 10.4, and filter paper 4: 0.025 M Tris-HCl, 20% methanol, 40 mM aminocaproate, pH 9.4. Transfer was accomplished by running 100 milliamperes through this apparatus for 3 h. Nitrocellulose membranes were incubated in blocking solution for 12 h at 4°C. Blocking solution consisted of 5% Carnation non-fat dry powdered milk, 1.5% BSA, 0.01% Na-Azide, in TNT (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). Primary antibody (rabbit anti-GLUT1, 1:2000) was then added to this solution and incubated for 12 h at 4°C, followed by four 15 min washes; 1st wash: 0.1% BSA in TNT, 2nd wash: 0.1% BSA, 0.1% Triton X-100, in TNT, and the 3rd and 4th washes: 0.1% BSA in TNT. All washes were performed at 4°C. Nitrocellulose membranes were then incubated in ^{125}I-conjugated goat anti-rabbit Fc 2 antibody (1:1500, in blocking solution), for 2 h at 4°C, followed by five 30 min washes, as follows, 1st wash: 0.1% BSA, 0.1% Triton X-100, in TNT, second wash: 0.1% BSA in TNT, third wash: 0.1% BSA, 0.1% Triton X-100, in TNT, and fourth and fifth washes: 0.1% BSA in TNT. Nitrocellulose membranes were then autoradiographed with Kodak X-100 X-ray film, in Kodak X-ray casettes. Exposure was conducted at −80°C, and lasted from 3–48 h, depending on signal strength. Following exposure and development of films, the nitrocellulose blots were quantitated by cutting out the regions of the nitrocellulose which contain the antigen-antibody complexes, as determined by overlay of autoradiographs and nitrocellulose membranes on a light box. These nitrocellulose fragments were quantitated on a Beckmann G200 gamma counter. All nitrocellulose pieces cut from a given gel were the same size. Similar size, non-band containing pieces of nitrocellulose were also cut and counted, and used to correct for background.

Nonspecific binding was shown to be minimal by using a different primary antibody: rabbit anti-GLUT4 (insulin-regulated glucose transporter), and then incubating the preparation with the usual secondary antibody (goat anti-rabbit Fc antibody). The binding here was negligible. Similarly, Western blots incubated with goat anti-rabbit Fc antibody alone showed minimal binding.

Antibody and sample stardardization curves were run using cultured brain endothelial cell membrane samples and the primary rabbit anti-GLUT1 antisera to insure that the experimental samples were not in the plateau phase for the assay, but in the relatively linear portion of the curve.

2.10. Statistics

Statistical analysis of the 2-deoxy-glucose uptake studies and quantitated western blot studies was performed using Statworks (version 2.0, Cricket Software, Philadelphia, PA) running on a Macintosh IIsi computer. One-way ANOVA's were performed on the data to determine the degree of statistical significance of differences between control and experimental groups.

3. Results

3.1. Endothelial cell cultures

All three primary cultures exhibited typical endothelial cell morphology when viewed under phase

contrast microscopy. Fluorescence microscopy of these cultures preincubated with DiAc-LDL showed them to be generally free of contaminating, non-LDL binding cells (Fig. 1). Uptake of DiAc-LDL decreased after the third or fourth passage in both brain capillary and aortic endothelial cell cultures (data not shown). The brain capillary endothelial cultures exhibited alkaline phosphatase and gamma-glutamyl transpeptidase activity, which are brain endothelial cell markers [19,20]. Compared with freshly isolated brain capillaries, confluent cultures of brain capillary endothelial cells progressively lost enzyme activity after the third or fourth passage (data not shown). Glucose transporter activity also diminished with increasing passage, although not to the same extent as the enzymes. Based on these data, only primary cultures of brain capillary, adrenal capillary and aortic endothelium were used for comparative studies of glucose transport protein activity and expression.

3.2. Glucose transport activity induction during glucose deprivation

Parallel 48 h glucose deprivation studies were conducted in cultures of brain capillary endothelium, adrenal capillary endothelium, and endothelium from descending aorta. Fig. 2 shows that during glucose deprivation, all three primary cell cultures demonstrate an increase in 2-DG uptake rate. However, this increase was observed to be slower in the brain

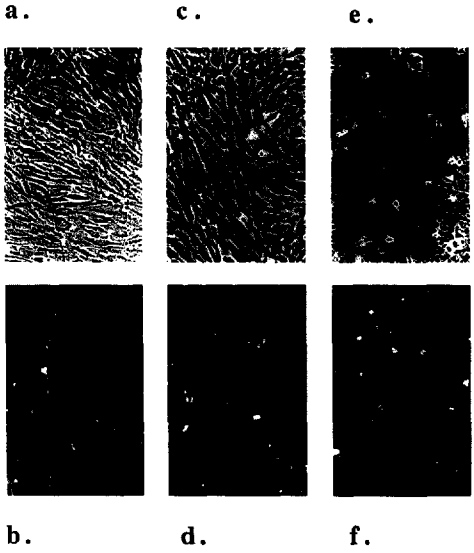

Fig. 1. Phase contrast (top) and fluorescence microscopy (bottom) of primary endothelial cell cultures, grown under identical conditions. a,b: brain capillary; c,d: adrenal capillary; and e,f: aortic endothelium. Bottom photographs were obtained after treatment with diacetylated-low density lipoprotein to assess endothelial cell purity.

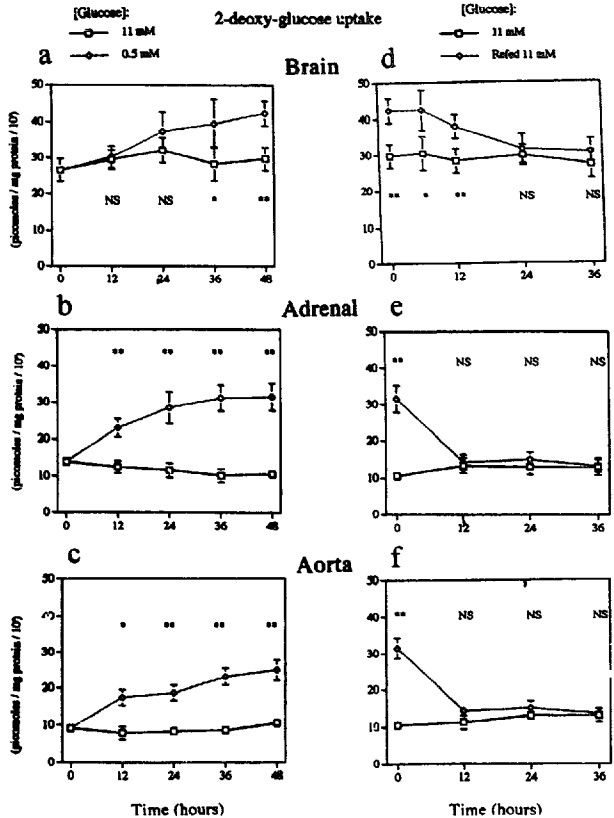

Fig. 2. Changes in glucose transporter activity with glucose deprivation and refeeding in primary endothelial cultures. a and d: brain capillary endothelial cells; b and e: adrenal cortex capillary endothelium; c and f: aortic endothelium. Data shown are mean ± S.E. from three separate primary cultures of each type, with each average coming from 6 individual data points per study. Time zero in the right-hand graphs represents the time at which the 48 h glucose deprived cultures were refed with 11 mM glucose. *: $P < 0.05$, **: $P < 0.01$, NS: not significant.

capillary cultures. The adrenal and aortic endothelium (Fig. 2b,c) both demonstrated a significant increase in glucose transport activity after 12 h of glucose deprivation, whereas brain capillary endothelia (Fig. 2a) required up to 24 h longer to exhibit increased transport activity. Baseline 2-[^{3}H]DG uptake was about three times larger in brain capillaries (29 ± 1 pmol/mg per 10 min) than in the adrenal capillaries (12 ± 1 pmol/mg per 10 min) or aortic endothelia (9 ± 1 pmol/mg per 10 min). These differences were unlikely to be due to differences in total protein synthesis by the cell cultures, as the cultures generally produced similar amounts of total protein. The relative increase in 2-DG uptake was also different in the cultured brain endothelial cells. The brain capillary endothelia showed a 55% increase in 2-DG uptake (to 42 ± 3 pmol/mg per 10 min) whereas the adrenal capillary and aortic endothelia demonstrated a 167% increase (to 32 ± 4 and 25 ± 3 pmol/mg per 10 min, respectively). The increase in transporter activity following 48 h of glucose deprivation was reversible following glucose refeeding (Fig. 2d,e,f). As with the induction of transporter activity, the adrenal and aortic endothelia (Fig. 2e,f) both returned to control levels of activity within 12 h of refeeding (differences between experimental and control groups were no longer significant at 12 h), whereas the brain endothelial cultures (Fig. 2d) required 12 h longer.

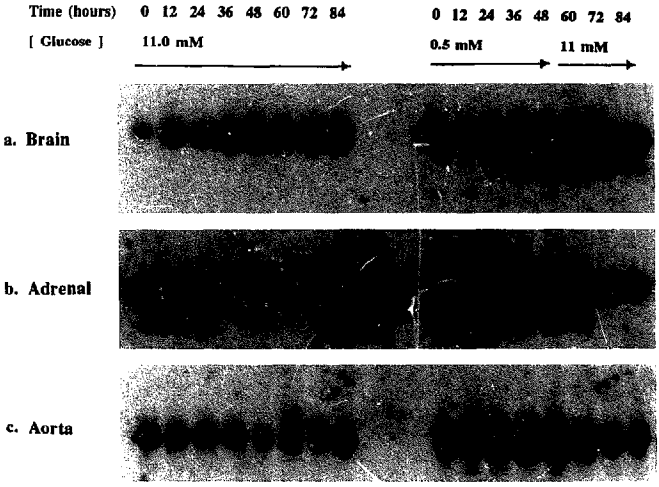

Fig. 3. Autoradiograms of Western blot studies of GLUT1 expression by glucose deprived and control primary cultures of brain, adrenal, and aortic endothelial cells. Cell membrane fractions were purified from experimental and control groups at the times specified in the figure. 20 μg of membrane fraction (determined spectrophotometrically) was size fractionated with SDS-PAGE technique, using stacking gel (3.8%/12.5% acrylamide). Proteins were then transferred unto nitrocellulose membrane, blocked for non-specific antibody interactions, and incubated with rabbit anti-GLUT1 polyclonal antibody (1:2000) at 4°C for 24 h. Nitrocellulose membranes were then washed 4 ×, and incubated with ^{125}I-conjugated, goat anti-rabbit IgG-Fc fragments (1:1500), at 4°C for 2 h. Following 5 washes, nitrocellulose blots were exposed to Kodak X-100 film at −80°C and developed. See Section 2 for discussion of membrane fraction purification, SDS-PAGE gel protocol, and buffer formulation.

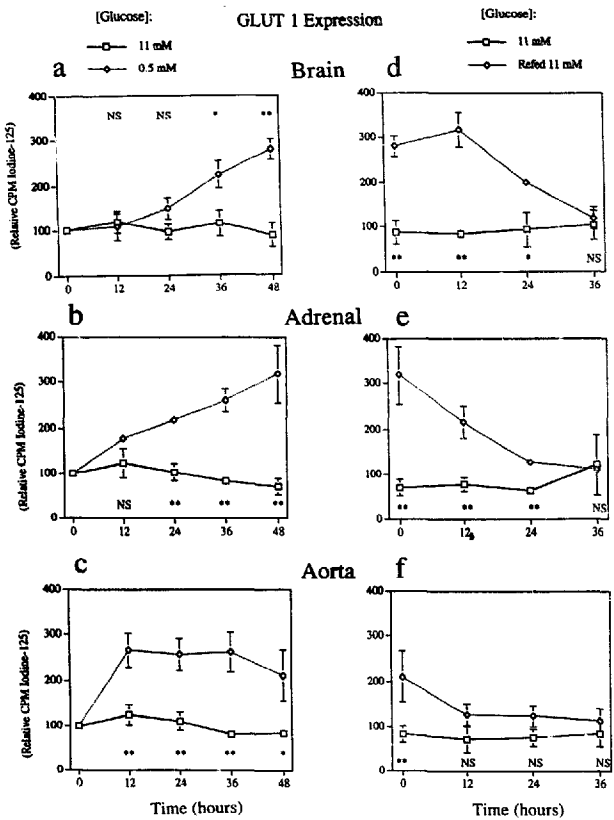

Fig. 4. Quantitation of Western blot studies of GLUT1 expression by glucose deprived and refed primary cultures of different endothelial cells (3 blots/endothelial cell type). All values are normalized with respect to GLUT1 expression at $t = 0$, which is assigned a value of 100. a and d: brain capillary endothelium; b and e: adrenal capillary endothelium; c and f: aortic endothelium. Time zero in the right-hand graphs represents the time at which the 48 h glucose deprived cultures were refed with 11 mM glucose. *: $P < 0.05$, **: $P < 0.01$, NS: not significant.

3.3 Glucose transporter protein induction by glucose deprivation

Studies of GLUT1 expression by these different cell cultures were conducted to correlate measurements of glucose transporter activity with transporter density. Fig. 3 demonstrates three representative Western blot studies of GLUT1 expression by primary cultures of brain capillary, adrenal capillary, and aortic endothelium during glucose deprivation and refeeding, in addition to their non-stressed controls. These autoradiographs demonstrate that all three cultures increase their GLUT1 expression during glucose deprivation, and return to control values following refeeding. Baseline GLUT1 expression by all three endothelial cell cultures generally varied somewhat from the level at 0 h (Fig. 3), hence data from multiple studies were combined to quantitate statistical significance.

All Western blots of glucose-deprived endothelial cells demonstrated an additional, lower molecular weight band reactive with anti-GLUT1 antisera (Fig. 3). This finding was consistent in all of these studies, although the degree to which the second band appeared varied somewhat from study to study. This finding has been reported in other studies of this type [29], and has been postulated to represent accumulation of a poorly glycosylated pool of GLUT1 protein, possibly due to the lack of substrate present under glucose-deprived experimental conditions. This lower molecular weight band generally disappears following glucose refeeding (Fig. 3), further supporting this hypothesis.

Autoradiographs from three separate primary culture studies (for each endothelial cell type) were combined per figure, as shown in Fig. 4. The induction of GLUT1 begins within 12 h in the cultures of adrenal and aortic endothelia, but the brain endothelial cells require an additional 12 h before this change is evidenced (Fig. 4a,b,c). Differences between control and experimental groups of brain endothelial cells (Fig. 4a) were not statistically significant at 12 nor 24 h. In adrenal capillary endothelial cells the differences were statistically significant at 24, 36, and 48 h (Fig. 4b). Differences in aortic endothelial cultures (Fig. 4c) were statistically significant at 12, 24, 36, and 48 h. These comparative behaviors correlate with similar differences in the kinetics of induction of glucose transporter activity in these cultures. However, the quantitative Western blot data for refeeding of 48 h glucose deprived cultures differed somewhat from the 2-deoxy-glucose uptake observations of these cells. The brain capillary and adrenal capillary endothelial cultures (Fig. 4d,e) retained their elevated GLUT1 expression for up to 24 h following refeeding, whereas the aortic cultures GLUT1 expression returned to control values within 12 h (Fig. 4f). Differences between experimental and control values in brain endothelial cell cultures (Fig. 4d) were significant at 12 and 24 h, but no longer at 36 h following refeeding. Adrenal endothelial cells showed a similar lag in return to control values after refeeding, unlike the relatively rapid suppression of glucose transporter activity (see Fig. 2e). The differences between control and experimental groups of adrenal endothelium (Fig. 4e) were statistically significant at 12 and 24 h, but no longer at 36 h. The results of GLUT1 suppression during refeeding correlated well with the studies of transporter activity in aortic endothelial cells. Differences between control and experimental groups in aortic endothelial studies (Fig. 4c) were not statistically significant at 12 h, indicating a rapid return to control levels of GLUT1 expression.

In contrast to the studies of 2-DG transport induction, the brain endothelial cell cultures demonstrated near 200% increase in GLUT1 expression during glucose deprivation. A possible explanation for the discrepancy between 2-DG transport and GLUT1 expression increases is discussed later. By comparison, the adrenal and aortic endothelial cell cultures exhibited increases in GLUT1 expression during glucose deprivation of 220% and 120%, respectively.

4. Discussion

The reversible induction of glucose transport activity and GLUT1 expression during glucose deprivation demonstrated in these studies is not unique to cultured brain capillary endothelial cells. Indeed, all endothelial cell cultures in this study demonstrated this behavior, as have numerous other cell culture systems [23,29–31]. Takakura et al. [15] have shown a similar induction of glucose transport and GLUT1 by glucose deprivation in cultured brain capillary

endothelium. Using 3-*O*-[^{3}H]methyl-D-glucose (3MG) these authors showed that at least 12 h were required to observe a significant increase in 3MG uptake after glucose starvation and that the response was dependent upon protein synthesis. After 48 h the increase in 3MG uptake was 81%, somewhat higher than the value of 55% found for 2-DG in the present study. Refeeding returned 3MG uptake to control levels within 48 h, as in the present study. Takakura et al. [15] also analyzed the response to glucose starvation of GLUT1 expression. 24 h were required to observe a significant increase in GLUT1 expression, which reached a maximum increase of 57% after 36 h. This relative increase in GLUT1 expression contrasts with the values found in the present study of 130% at 36 h and 175% at 48 h (see Fig. 4a). The differences could be due to the different techniques employed to measure GLUT1 expression levels. Not all the GLUT1 measured by the Western blot may necessarily be active, or on the membrane at all. Our samples were whole cell preparations and thus, if there is any GLUT1 confined to intracellular compartments, it will be raising the Western blot estimates of transporter density. In fact, it has been shown that brain endothelial cells in situ have 40% of their total cell glucose transporter in cytoplasmic compartments [32]. This may also explain why the changes in GLUT1 expression levels found in this study are greater than those seen in 2-DG transport after glucose deprivation.

Our main interest was to compare the regulation of glucose transport in brain capillary endothelium with that of extracerebral capillaries, in response to changes in extracellular glucose concentration. The results show that the induction of 2-DG uptake and GLUT1 expression in adrenal and aortic cultures were consistently more rapid than in brain endothelial cell cultures. During glucose deprivation, adrenal and aortic endothelial cells exhibit measurable increases in 2-DG and GLUT1 expression within 12 h, whereas brain endothelial cells require 12 h longer (Figs. 2–4). The return of 2-DG uptake to control values during refeeding was similarly slower in brain endothelial when compared with the extracerebral endothelial cell cultures (Fig. 2). The return of cultured brain endothelial GLUT1 expression to control levels also differed from the trends observed in the extracerebral endothelial cells, requiring 12–24 additional hours (Fig. 4). Thus, it appears that cultured brain endothelial cells differ from the other cultured cell systems in demonstrating relatively slow response of measurable GLUT1 induction and supression. There are several possible explanations for these differences. The rapidity with which adrenal and aortic endothelia induce glucose transport and GLUT1 compared with brain endothelia may reflect different levels of glycolysis by the cell cultures. Evidence exists that glucose-6 phosphate (G6P), a direct product of the glycolytic pathway, plays a central role in the signalling of cytosolic glucose levels within cells [29]. Differing glycolytic activities may thus be expressed as the time lag observed for cultured brain endothelial cells to begin the synthesis of additional glucose transport protein. If brain endothelial cultures metabolize existing G6P stores at a slower rate than the adrenal or aortic cultures, G6P depletion may occur more slowly, thus accounting for the observed time delay in these studies. Another possible explanation involves the higher level of baseline glucose transport activity in the cultured brain endothelial cells when compared with the adrenal and aortic cultures. The detection and synthesis of new glucose transport protein may well occur at similar rates in all three cultured endothelial cells; however, the greater baseline level of glucose transport activity in the brain cultures may make it such that detecting a measurable increase requires more time, because the proportional increase in these cells is smaller. The different kinetics of glucose transport and GLUT1 induction in these cell cultures may also reflect distinct rates of GLUT1 synthesis and degradation. Further studies are required to determine if a different half life for synthesis and/or degradation of the brain endothelial GLUT1 protein accounts for the observed differences, or if differences in glycolytic activity or baseline GLUT1 activity are responsible.

The larger question remains how this study might relate to these processes in vivo. The degree of glucose transporter activity increase in the brain capillary endothelial cultures is equivalent to the brain glucose uptake observed in vivo under hypoglycemia [14]. Furthermore, this increase required 2–3 days to come about. In comparison, studies of glucose deprivation in cultured neuronal cells have shown a rapid increase in transporter activity and expression [23,33], which behave more like the adrenal and aortic en-

dothelial cell cultures in this study. Thus it appears that in vivo and in vitro, brain capillary endothelial cells exhibit a relatively slow induction of glucose transport during glucose deprivation. It is possible that given the high capacity of the BBB system and the high affinity for glucose of the neuronal transporters, that it is not necessary to upregulate the former by a large amount or rapidly during glucose starvation. Thus, the moderate upregulation seen in the cultured endothelium may well represent the fact that these cells are maximally or near-maximally upregulated in vivo.

Acknowledgements

We wish to thank Drs. Javier Navarro, Christian C. Haudenschild, Kathleen Thomas, and Benjamin Kaminer for their support and insightful discussions regarding this research. This work was supported by an American Heart Association Medical Student Research Fellowship, a Grunebaum Cancer Research Fellowship, and by the Department of Physiology at Boston University School of Medicine.

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