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Hexose uptake in primary cultures of bovine brain microvessel endothelial cells. II. Effects of conditioned media from astroglial and glioma cells

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Regulation of glucose uptake by an astroglial cell secreted factor(s) was studied in primary cultures of brain microvessel endothelial cells (BMECs). Uptake of a non-metabolizable glucose analog, 3-*O*-[^{3}H]methyl-D-glucose ([^{3}H]3MG), was measured after the BMECs were treated with media conditioned by primary cultures of rat astrocytes (Astrocyte Conditioned Media: ACM) or rat C6 glioma cells (Glioma Cell Conditioned Media: GCM). Uptake of [^{3}H]3MG was significantly increased by ACM (30–50%) and GCM (60–200%) treatments, whereas conditioned medium from 3T3 fibroblasts (3T3) caused no significant effect. The elevation in [^{3}H]3MG uptake increased with increasing time of exposure of BMECs to these conditioned media (CM), and the effect was shown to be reversible. Glucose depletion of CM was shown not to be a factor. The presence of cycloheximide, a protein synthesis inhibitor, during treatment of the BMECs with ACM and GCM blocked the increase in [^{3}H]3MG uptake by the cells. These results suggested that ACM or GCM treatment elevated de novo synthesis of brain-type glucose transporter (GLUT1). Indeed, enhanced GLUT1 expression by these treatments in BMECs was demonstrated directly by enzyme-linked immunosorbent assay (ELISA) using antibodies against human GLUT1. After trypsinization of ACM and GCM, both conditioned media still induced significant stimulation of [^{3}H]3MG uptake by BMECs. A significant increase in [^{3}H]3MG uptake was also observed when ACM or GCM was exposed to BMECs through a dialysis membrane with a molecular weight cutoff of 1000. To examine whether the effects were specific to brain endothelial cells, [^{3}H]3MG uptake experiments were performed employing aortic endothelial cells (AECs), pulmonary microvessel endothelial cells (PMECs), and 3T3 cells. ACM treatment did not alter 3MG uptake by these cells, suggesting that the ACM effect was specific to BMECs. On the other hand, [^{3}H]3MG uptake by AECs and PMECs treated with GCM was significantly enhanced. The present study demonstrated that some factor(s) of relatively small molecular weight, which was released from astrocytes or glioma cells, stimulated glucose uptake by enhancing GLUT1 synthesis in BMECs.

Introduction

Unlike peripheral endothelium, brain microvessel endothelium, an integral part of the blood-brain barrier (BBB), is characterized by the presence of highly resistant, intercellular tight junctions, minimal pinocytotic activity, and the virtual absence of fenestrations [1]. These characteristics endow the endothelium with the ability to restrict the passage of most water-soluble molecules from the cerebrovascular circulation to the brain.

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Abbreviations: ACM, astrocyte conditioned medium; BBB, blood-brain barrier; BMECs, brain microvessel endothelial cells; BSA, bovine serum albumin; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; GCM, glioma cell conditioned medium; GLUT, glucose transporter; HRP, horseradish peroxidase; 3MG, 3-*O*-methyl-D-glucose.

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It is widely accepted that brain microvessel endothelial cells (BMECs) form the structural and functional basis of the blood-brain barrier (BBB) [2]. However, some BBB functions are thought to be regulated by a kind of glial cells, called astrocytes, which surround BMECs with their foot processes [3].

Cell culture of BMECs offers a good opportunity to examine how astrocytes regulate the functions of BMECs in vivo. For example, induction of specific marker enzymes including γ-glutamyl transpeptidase (γ-GTP) [4–7] and Na^+,K^+-ATPase [7,8], modulation of tight junctions [9,10], changes in permeability [11–13], and regulation of nutrient transport [14–16] have all been demonstrated in passaged BMECs when they are co-cultured with glial cells or treated with astrocyte-conditioned medium.

Our laboratory has developed an in vitro BBB model system consisting of primary cultures of bovine BMECs [17,18]. This in vitro model system has been extensively characterized as a useful tool for the study of uptake, transendothelial transport, and metabolism [17–22]. Recently, our laboratory demonstrated that glucose is taken up by cultured bovine BMECs by a carrier-mediated, facilitated diffusion mechanism similar to that found in the BBB in vivo (Takakura et al., unpublished data). Furthermore, the level of glucose but not the level of insulin were shown to affect glucose uptake by this in vitro BBB model system (Takakura et al., unpublished data). The present paper describes the effects of astroglial cells on glucose uptake in the same in vitro BBB model.

Materials and Methods

Chemicals

3-*O*-[^{3}H]Methyl-D-glucose ([^{3}H]3MG; 60 Ci/mmol) and L-[1-^{14}C]glucose (47 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, CA) and from NEN Chemicals (Boston, MA), respectively. All other reagents were of the highest grade commercially available.

Isolation and culture of BMECs

Bovine brain microvessel endothelial cells (BMECs) were isolated and grown in primary culture as described by Audus and Borchardt [17,18]. Briefly, approximately $3 \cdot 10^6$ cells were grown to confluence in 6-well culture plates which had been pretreated with rat tail collagen and fibronectin in culture medium comprised of a 1:1 mixture of minimum essential medium and Eagle's modified F-12 nutrient mix (MEM/F-12 mix), 10 mM Hepes, 13 mM sodium bicarbonate (pH 7.4), 10% plasma-derived equine serum (Hyclone, Logan, UT), 100 μg/ml heparin, 50 μg/ml gentamicin, and 2.5 μg/ml amphotericin B. Cells were refed every 2 days after seeding and monolayers were formed by 9–11 days after plating.

Astrocyte culture

Astrocytes were isolated from one-day old rat pups and cultured according to the method of Dr. Lieberburg, Athena Neurosciences, Inc, San Carlos, CA (personal communication) as modified from the literature [23]. Cerebral cortex was dissected away from the brain of pups under sterile conditions and the cortex material was minced with a scalpel, mixed with DNAse (40 μg/ml in serum-free Dulbecco's modified Eagle medium (DMEM), 2 ml/brain) for 2–4 min and the pieces were allowed to settle over 2–3 min. The supernatant was removed and the remaining pieces were mixed with trypsin (0.24 mg/ml, 2 ml/brain) and shaken for 30 min at 37°C. Afterwards, the pieces were again allowed to settle and the supernatant was removed. The pieces were triturated with a second DNAse solution (same concentration as before but with serum-containing DMEM). The supernatant was collected after pieces settled (2–3 min) and spun down in a tabletop centrifuge for 10 min. The resulting pellet was dispersed and divided into the same number of T-25 flasks as there were rat brains originally used. The cells were allowed to grow for one week with two or three media changes (10% calf serum (Hyclone, Logan, UT) in DMEM). The flasks were then shaken for 8–10 h at 100 rpm in a 37°C incubator. Cells left attached were rinsed with serum-free culture medium, trypsinized loose (0.1% trypsin, 0.025% EDTA in phosphate-buffered saline (PBS) (pH 7.4), and plated at approximately 30000 cells/cm^2. In order to confirm that the isolated cells were astrocytes, glial fibrillary acidic protein (GFAP), a marker specific for astrocytes [24], was immunolocalized using immunoperoxidase staining (Histogen Immunohistology Kit, Biogenex Laboratories, San Ramon, CA). The cells were fixed for 10 min at −20°C with 95% ethanol and 5% acetic acid and then stained according to the kit instructions. The culture was shown to be > 98% pure for astrocytes.

C6 glioma cell culture

C6 glioma cells were obtained from the American Type Culture Collection (Rockville, MD). This established cell line was originally derived from a rat with a chemically induced glial tumor. The cells were passaged weekly at a ratio of 1:20 with the same trypsin/EDTA solution described above and cultured in DMEM with 10% calf serum. When used to condition medium, the cells were plated out at approximately 30000/cm^2.

Aorta endothelial cell (AEC) culture

AECs were isolated from bovine aorta as described elsewhere [25]. The lumenal surface of the aorta was scraped with a scalpel and the scrapings were treated with a 0.1% collagenase solution for 30 min at 37°C.

After centrifugation, the pellet was distributed into culture plates coated with collagen and cultured with the MEM/F-12 mix plus 10% horse serum. After approximately 4 h, the plates were gently washed once with medium to remove contaminating cells that had not attached and refed with fresh medium. The cultures were fed every 2–3 days.

Pulmonary microvessel endothelial cell (PMEC) culture

MECs were a kind gift from Dr. P. Del Vecchio, Albany Medical College, Albany, NY. This is a cell line of characterized endothelial cells from bovine pulmonary microvessels obtained at the 18th passage and designated as B_3B_5. The cells were passaged weekly with DMEM with 10% calf serum. The PMECs were plated at approximately 20000/cm^2 and reached confluency in approximately 6–8 days.

3T3 fibroblast culture

3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and passaged once per week at a split ratio of 1:20. The 3T3 fibroblasts were fed with MEM/F-12 mix plus 10% fetal bovine serum (Hyclone, Logan, UT).

Uptake study of glucose analogues by BMECs

Uptake studies were carried out using confluent cell monolayers in 6-well plates as described previously (Takakura et al., unpublished data). Briefly, cells were washed with glucose-free buffer (0.1% bovine serum albumin (BSA), 1 mM pyruvate, 4 mM KCl, 4.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 150 mM NaCl, 15 mM Hepes (pH 7.4) and incubated with the buffer for 20 min at 37°C. After the preincubation, the buffer was removed and buffer containing [^{3}H]3MG (1 μCi/ml) and L-[^{14}C]glucose (0.25 μCi/ml) was added. Uptake was stopped with the addition of ice-cold PBS containing 0.02 mM cytochalasin B. The cell monolayers were then washed with PBS and solubilized with 0.1 M NaOH overnight at room temperature. Aliquots were taken for determination of radioactivity and protein content. Specific uptake of [^{3}H]3MG was calculated after subtraction of non-specific uptake or uptake of L-[^{14}C]glucose from the total uptake of [^{3}H]3MG. Protein was measured with a Bio-Rad protein assay kit [26] using BSA as a standard.

Conditioned medium (CM) experiments

Astrocytes, glioma cells, or 3T3 fibroblasts were cultured for 1–2 days in the medium appropriate for that cell type and then fed with fresh MEM/F-12 medium with 10% horse serum, except the 3T3 cells which were fed with MEM/F-12 medium with a 1:1 mixture of calf serum and fetal bovine serum (10% final total volume). After 9 days, the CM was removed and stored at −20°C for up to one month. BMECs, PMECs, AECs, and 3T3 cells were fed with a 1:1 mixture of CM and culture medium after the third or fourth day in culture. This was repeated every 2–3 days until the cells reached confluency. After 10–11 days for BMECs or 6–8 days for AECs, PMECs, or 3T3, the 3MG uptake experiments were performed.

Reversibility of ACM and GCM effects

To study reversibility of the effect of ACM or GCM treatment, refeeding experiments were done. BMECs were treated with CM for a specified period of time as described above and were then refed with culture medium. Control BMECs were incubated with culture medium only. [^{3}H]3MG uptake was measured at 0, 24, and 48 h after refeeding.

Glucose supplement experiments

To show that glucose depletion from the medium during conditioning was not a factor, glucose concentrations were adjusted to the control level (7.3 mM). Glucose concentrations in ACM and GCM were measured by a diagnostic kit using hexokinase (Sigma, St. Louis, MO).

Effect of protein synthesis inhibition

Cycloheximide, a protein synthesis inhibitor, was used to determine if protein synthesis was involved in the effect of CM. Prior to uptake experiments, confluent BMECs were cultured with or without CM in the presence or absence of cycloheximide (0.05 μg/ml) for 48 h.

Determination of GLUT1 expression in BMECs

Total cellular expression of GLUT1 was measured by ELISA using anti-human GLUT1 antibodies and detergent-permeabilized BMECs as described previously (Takakura et al., unpublished data).

Polyclonal antibodies were raised against a synthetic peptide corresponding to residues 477–492 from the C-terminus of the human erythrocyte GLUT1 as previously described [27]. The specificity of the anti-GLUT1 antibodies for the bovine GLUT1 was demonstrated by ELISA using BMECs and the anti-GLUT1 antibodies and by Western blot analysis [28].

For ELISA, bovine BMECs were plated at 100000 cells/cm^2 in collagen- and fibronectin-coated 96-well plates. At near confluence on day 6, ACM or GCM treatment was begun as described above. All subsequent steps were done at room temperature. The cells were fixed for 15 min in 4% (w/v) paraformaldehyde in Ca,Mg-free PBS, rinsed and incubated for 30 min in PBS containing 1% (w/v) BSA and 0.05% (v/v) Nonidet P-40(NP-40). Following a 1 h incubation with the appropriate dilution of rabbit anti-GLUT1 in PBS/BSA/NP-40, the cells were rinsed three times and incubated for 1 h with 50 μl of a 1:4000 dilution

of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; American Qualex, La Mirada, CA). After three washes, 100 μg of the peroxidase substrate, *o*-phenylenediamine dihydrochloride, in 100 μl of 0.02 M citric acid-0.07 M sodium phosphate dibasic buffer at pH 6.3 that contained 0.02% (v/v) H_2O_2 was added per well. Color development was stopped by the addition of 50 μl of 1 M HCl and the optical density was read at 490 nm by using an EIA Autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

Trypsinization procedure

The factor(s) secreted by the astrocytes or glioma cells was tested for sensitivity to trypsin. Astrocytes or C6 glioma cells were cultured as usual with DMEM with 10% calf serum. After 1–2 days the cells were refed with MEM/F12 mix with 10% calf serum until day 5 of culture. Then, the cells were washed two times with serum-free medium and refed with serum-free medium for an additional 4 days. The CM was collected and 2000 U/ml of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TCPK)-treated trypsin was added and mixed for 1 h at room temperature. An equivalent amount of immobilized trypsin inhibitor was then added and mixed with the treated CM for 30 min at room temperature. Control serum-free medium was also treated with trypsin and trypsin inhibitor. The glass particles with the immobilized trypsin inhibitor were allowed to settle, the supernatant was removed, 10% calf serum was added, and the media were stored at −20°C.

Molecular weight cutoff experiment

To determine the approximate molecular weight of the putative factor(s) in CM, BMECs were exposed to CM through dialysis membranes with molecular weight cutoffs of 1000 and 10000. The dialysis membrane was placed over the opening of a Transwell ™ (Costar, Cambridge, MA) insert with the polycarbonate membrane removed and tied in place with surgeon's silk. The modified insert was soaked in 50% ethanol for 24 h and washed with sterile distilled water over 4 days and then soaked in culture medium prior to use. BMECs were grown in 6-well culture plates as described above. At day 6 in culture, the insert was placed in the well and culture medium (2.5 ml) was added to the bottom chamber and CM (2.5 ml) was added to the upper chamber of the insert. After 2 days the media were replenished and [^{3}H]3MG uptake was measured at day 10.

Statistical analysis

All data were analyzed by Student's *t*-test.

Results

Effect of CM on [^{3}H]3MG uptake by BMECs

Treatment of BMECs with CM resulted in a significant increase in [^{3}H]3MG uptake by the cells at 5 mM compared with control cells (Fig. 1). The effect of GCM was more dramatic than that of ACM. An approximate 30–50% increase was obtained with ACM while GCM stimulated [^{3}H]3MG uptake by 50–200%.

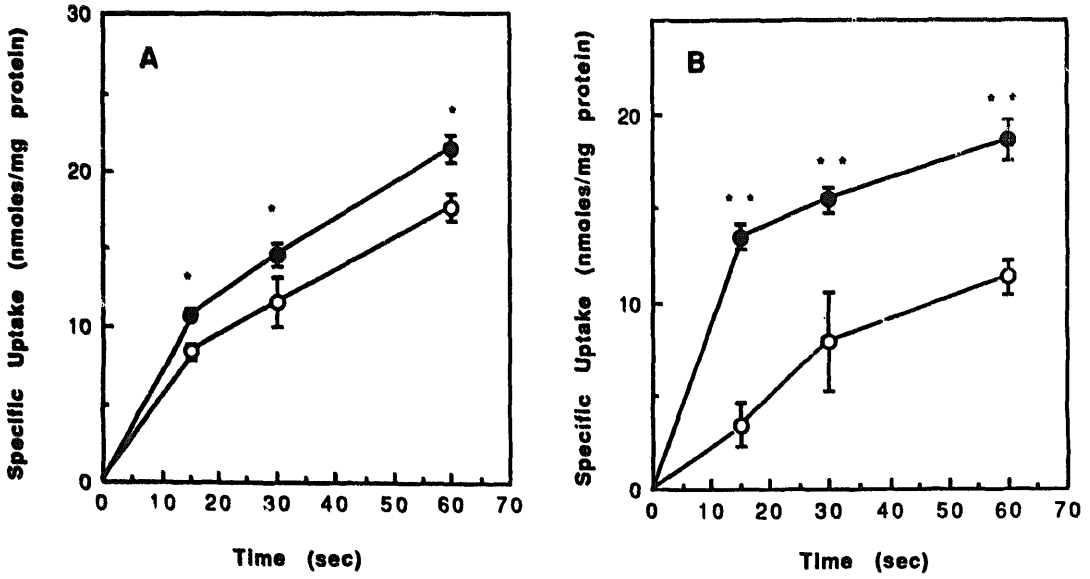

Fig. 1. Effect of ACM (A) and GCM (B) on [^{3}H]3MG uptake by BMECs. BMECs were incubated with CM for 6 and 4 days, respectively, before measuring uptake. Uptake of [^{3}H]3MG was measured at 37°C and a concentration of 5 mM in control (○) and ACM- and GCM-treated (●) BMECs. Each value was expressed as specific uptake corrected by non-specific uptake using L-[^{14}C]glucose. Data are mean ± S.D. (bars) values ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

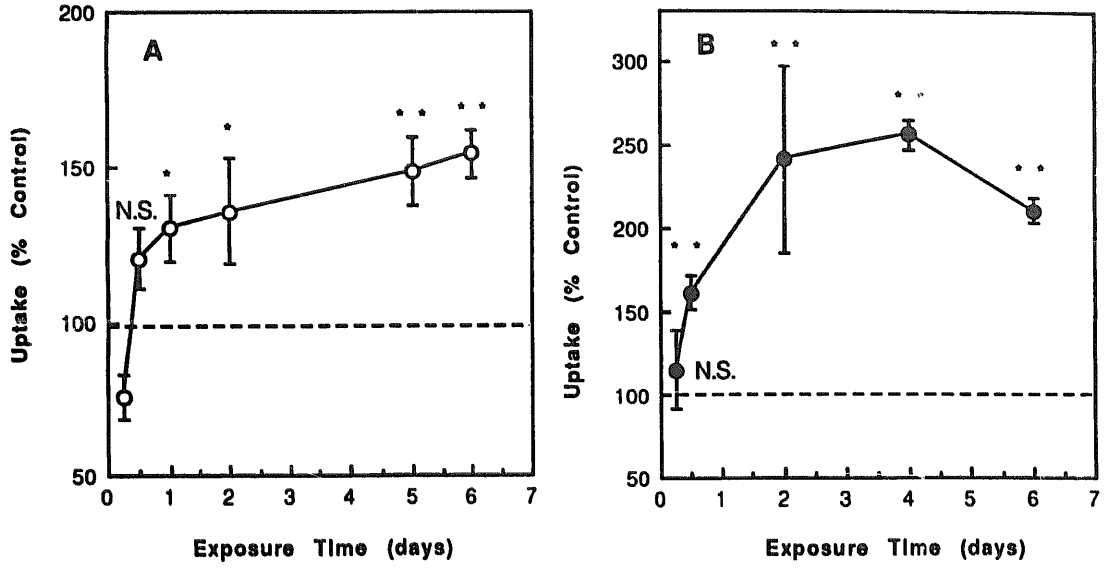

Fig. 2. Effect of time of exposure to ACM (A) and GCM (B) on [^{3}H]3MG uptake by BMECs. BMECs were treated with ACM and GCM for various time periods from 6 h to 6 days. Uptake of [^{3}H]3MG (5 mM, 15 s) was measured in BMECs and data were expressed as mean ± S.D. (bars) values (n = 3) as a percentage of the control. * $P < 0.05$, ** $P < 0.01$. n.s., not significant.

The equilibrium water space for [^{3}H]3MG was determined from the uptake at 10 min, and no significant differences were observed between the values for control BMECs and for CM-treated BMECs. Thus, the increase in [^{3}H]3MG uptake was due to an enhanced initial rate of uptake of the hexose and not to an increase in the water space.

To see whether exposure time had an effect, BMECs were treated with CM for different lengths of time. Both effects were dependent upon exposure time as shown in Fig. 2. At least 24 and 12 h treatments were required for a significant effect of ACM and GCM, respectively. This increase in glucose uptake in response to CM returned to control levels within 1–2 days after feeding with culture medium (data not shown). These results showed that the effects induced by CM were reversible and required the continuous presence of the factor(s) for maintenance.

The glucose concentration of ACM was determined to be approximately 20% of the control whereas no glucose was detected in GCM. We found previously that low glucose concentrations significantly increased [^{3}H]3MG uptake by BMECs (Takakura et al., unpublished data). Therefore, glucose supplementation was

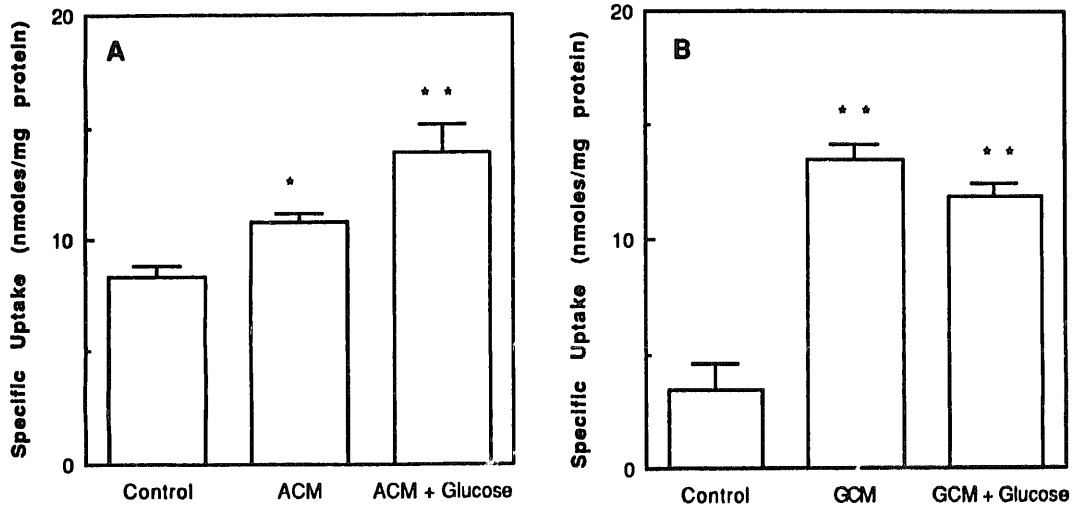

Fig. 3. Effect of glucose supplementation on enhanced [^{3}H]3MG uptake induced by ACM (A) and GCM (B) in BMECs. BMECs were incubated with ACM and GCM for 6 and 4 days respectively, and with or without glucose supplementation. Uptake of [^{3}H]3MG (5 mM, 15 s) was measured and data were expressed as mean ± S.D. (bars) values (n = 4). * $P < 0.05$, ** $P < 0.01$.

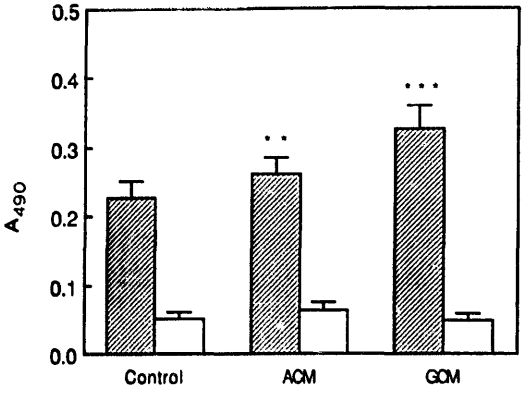

Fig. 4. Effect of ACM and GCM on GLUT1 expression in BMECs. The amount of total cellular GLUT1 was determined by ELISA using anti-human GLUT1 antibodies and detergent-permeabilized BMECs. After exposure to ACM and GCM for 2 days, BMECs were fixed and permeabilized with nonionic detergent and rabbit anti-GLUT1 antibodies (shaded bar) or normal rabbit serum (open bar) were added at a dilution of 1:1000. Goat anti-rabbit IgG conjugated to HRP was added and colorimetric analysis was done using a substrate of HRP. Data are expressed as mean ± S.D. (bars) values ($n = 12$) of optical density at 490 nm. * $P < 0.01$, *** $P < 0.001$.

carried out to confirm if glucose depletion from the medium during conditioning was responsible for induction of glucose uptake. The stimulation of [^{3}H]3MG uptake induced by CM was not changed by the addition of glucose to control concentrations (Fig. 3). Thus, this experiment showed that glucose starvation was not the signal that caused the increase in glucose uptake.

To determine whether protein synthesis was required for stimulation of glucose uptake by CM, BMECs were incubated with 0.05 μg/ml cycloheximide for 48 h during CM treatment. Elevation of [^{3}H]3MG uptake was not observed under these conditions (data not shown), suggesting that protein synthesis, most likely GLUT1, was required for stimulation of glucose uptake.

Effect of CM on GLUT1 expression

The results from [^{3}H]3MG uptake studies following treatments with ACM and GCM suggested that de novo synthesis of GLUT1 might be enhanced. To examine this possibility, GLUT1 expression by control and CM-treated BMECs was measured directly by ELISA using antibodies against the brain-type GLUT1. Total cellular GLUT1 expression was enhanced by 16 and 45% over control cells following 48 h treatments with ACM and GCM, respectively (Fig. 4).

Characterization of the factor(s) in ACM and GCM

To see if the astrocyte cell factor responsible for inducing glucose transport was a trypsin-sensitive protein, CM were treated with 2000 U/ml of trypsin for 1 h at room temperature. Both ACM and GCM exhibited a significant enhancement of glucose uptake after trypsinization (Fig. 5). These results showed that the factor(s) was insensitive to trypsin and suggested that it might not be proteinaceous.

For further characterization of the factor(s), molecular weight cutoff experiments were done. Significant increases in [^{3}H]3MG uptake were elicited when either ACM or GCM were used with BMECs separated by dialysis membranes with molecular weight cutoffs of 1000 and 10000 (Fig. 6). The results implied that the factor(s) is a substance with a molecular weight of less than 1000.

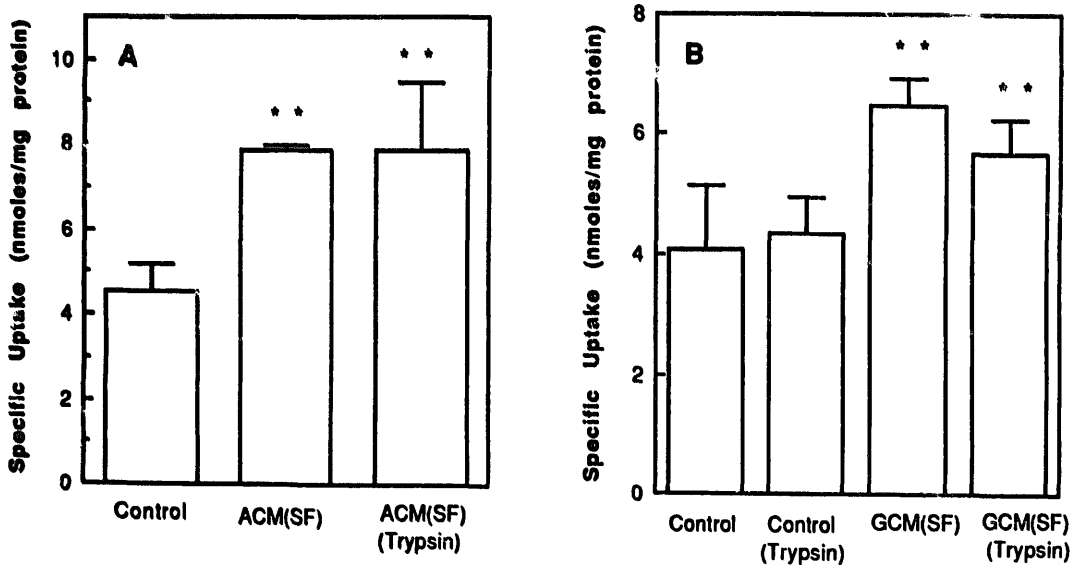

Fig. 5. Effect of trypsinization on the ability of ACM (A) and GCM (B) to stimulate [^{3}H]3MG uptake by BMECs. Serum-free ACM and GCM and culture medium were treated with trypsin (2000 U/ml) for 1 h at room temperature. After neutralization with immobilized trypsin inhibitor, calf serum was added to the media and these were incubated with BMECs for 4 days. Uptake of [^{3}H]3MG (5 mM, 15 s) was measured in BMECs and data were expressed as mean ± S.D. (bars) values ($n = 4$). ** $P < 0.01$.

TABLE I

Effect of ACM, GCM, and 3T3CM on 3MG uptake (5 mM, 15 s) by BMECs, aortic endothelial cells (AECs), pulmonary microvessel endothelial cells (PMECs) and 3T3 fibroblasts (3T3)

All data are expressed as the mean ± S.D. of three wells. Data for each individual treatment were analyzed by Student's *t*-test against the control. n.s., not significant.

Cell	Type of CM	Days of exposure	Uptake (% control)	*P*
BMECs	ACM	2	135.9 ± 17.4	< 0.05
		6	154.3 ± 7.7	< 0.01
	GCM	2	241.7 ± 55.9	< 0.01
		6	211.6 ± 7.8	< 0.01
	3T3CM	2	104.4 ± 18.2	n.s.
		6	78.2 ± 4.3	< 0.05
AECs	ACM	3	104.8 ± 8.6	n.s.
	GCM	3	185.5 ± 14.5	< 0.01
PMECs	ACM	2	95.1 ± 13.3	n.s.
	GCM	2	132.5 ± 9.4	< 0.05
3T3	ACM	2	89.7 ± 21.4	n.s.
	GCM	2	89.2 ± 9.9	n.s.

Specificity of CM

To examine whether the effect on glucose transport by CM was specific to brain endothelium, several experiments were performed using other types of cultured cells, that is, AECs, PMECs, and 3T3. The results are summarized in Table I.

In contrast to ACM and GCM, conditioned medium prepared from 3T3 cells (3T3CM) failed to increase [^{3}H]3MG uptake by BMECs, suggesting that the non-glial cell did not secrete the factor(s).

[^{3}H]3MG uptake by AECs, PMECs, and 3T3 fibroblasts was measured using the same technique as for BMECs to see if ACM and GCM had the same effect on these cultured cells. ACM showed no significant effect on [^{3}H]3MG uptake by AECs, PMECs, or 3T3, suggesting that the effect of ACM was specific to BMECs. On the other hand, GCM significantly increased [^{3}H]3MG uptake by all of the vascular cells; GCM stimulated [^{3}H]3MG uptake not only by BMECs but also by AECs and PMECs. A significant effect of GCM was not observed in [^{3}H]3MG uptake by 3T3 fibroblasts, a non-endothelial cell.

Discussion

Because of the unique characteristics of brain microvessel endothelium, the movement of most water-soluble molecules across the BBB is highly restricted. On the other hand, several specific transport systems across the BBB have been identified for water-soluble nutrients, including hexoses, monocarboxylic acids, amino acids, amines, purines, and nucleotides [29].

Among these, glucose is an important source of energy for the brain, and its transport across the BBB has been well studied in vivo [30–35] and in vitro [36–40]. These studies showed that glucose transport across the BBB was performed by a carrier-mediated, facilitated diffusion mechanism. Our recent studies have demonstrated that glucose uptake by primary cultures of bovine BMECs also involves the same mechanism (Takakura et al., unpublished data). In this study, the effects of astroglia cells on the uptake of glucose and the expression of the GLUT1 were investigated in an attempt to clarify the mechanism by which glucose transport across the BBB is regulated.

Although astrocytes encircle the BMECs in vivo, both cells are separated physically by basement mem-

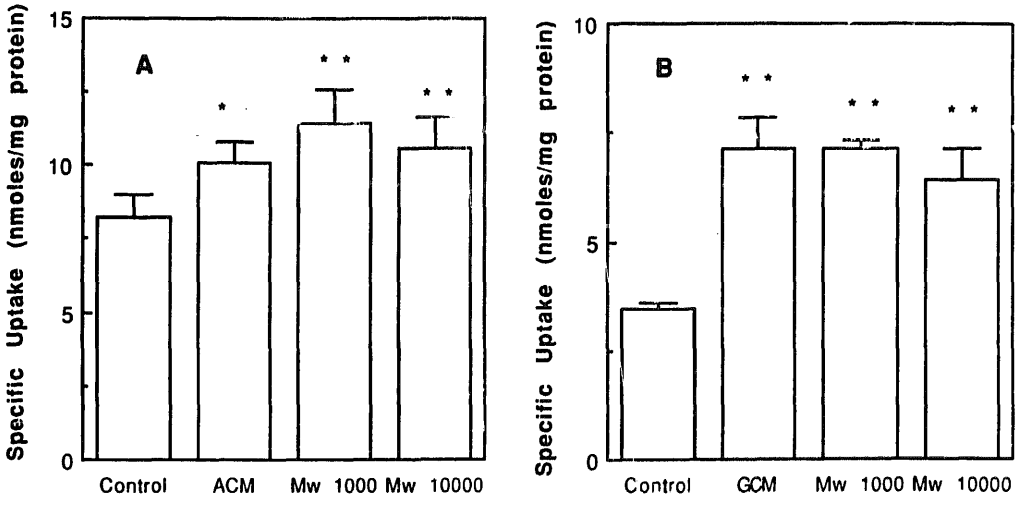

Fig. 6. Molecular weight cutoff experiments for ACM (A) and GCM (B). BMECs were exposed to ACM or GCM for 4 days through dialysis membranes with molecular weight cutoffs of 1000 and 10000. Uptake of [^{3}H]3MG (5 mM, 15 s) was measured in BMECs and data were expressed as mean ± S.D. (bars) values (n = 6 for ACM; n = 4 for GCM).

brane. Therefore, some soluble factors secreted from astrocytes might be expected to have an influence on the BBB functions. Based on these considerations, conditioned medium from astroglial cells has been used to study the regulation of some properties of BMECs in vitro [6,10,14,16]. We used two cell types of astroglial cell origin, a rat glial tumor cell line and C6 glioma cells, and normal mouse astrocytes for the preparation of CM.

The results obtained in this study have shown that one or more factor(s) released from astroglial cells, both astrocytes and glioma cells, stimulated glucose uptake by primary cultures of BMECs. Furthermore, it was directly demonstrated by ELISA using anti-GLUT1 antibodies that enhanced expression of GLUT1 was involved.

Preliminary characterization showed that the factor(s) in ACM and GCM was insensitive to trypsin and had a molecular weight of less than 1000. These results suggested that glucose uptake stimulation might be mediated by a non-proteinacious molecule(s) with small molecular weight. However, we can not rule out the possibility that this factor is a trypsin-resistant peptide. Regardless, this finding is inconsistent with the results of Maxwell et al. [16], who also demonstrated that ACM and GCM stimulated glucose uptake by passaged mouse BMECs and speculated that this involved increased GLUT synthesis. However, treatment of ACM with trypsin destroyed its effectiveness, leading the authors to conclude that the enhancement of glucose uptake by the cells was induced by a protein released by the astrocytes. Although the reason for the difference between these results is unclear, the state of the BMECs used and the method of preparation of CM might be involved.

At present, it is not clear if the factor(s) from astrocytes and from glioma cells are identical, although a similarity in characteristics of ACM and GCM was shown. GCM was more effective than ACM in that the magnitude of stimulation was greater and the onset of induction was earlier. Such results are consistent with the possibility that astrocytes and glioma cells secrete the same factor(s) but glioma cells, being transformed cells, secrete a larger amount of it. Alternatively, it may be that the cells secrete different factors, since a different spectrum of specificity was observed (see Table I). However, the difference in specificity could also be explained by factor concentration.

Our laboratory has reported that permeability of primary cultures of bovine BMECs to radiolabeled inulin was decreased by 50% when the BMECs were treated with ACM and GCM [11]. In this study, the factor(s) which caused the decreased permeability was shown to be trypsin-insensitive and smaller than 1000. These results are similar to those in the present study and suggest that the factor(s) may be the same. However, more detailed studies are required for the identification of the factor(s).

In most cases, the regulation of BBB functions by astroglial cells in vitro has been studied using passaged BMECs, which are known to lose certain BBB characteristics. For example, Arthur et al. [10] reported that passaged rat BMECs, which no longer possess tight junctions, formed numerous tight junctions which were identical to those displayed in vivo when these cells were exposed to rat brain astrocyte-conditioned medium. In addition, γ-GTP activity is lost in early passage of mouse BMECs, but this enzyme is induced in BMECs when co-cultured with glioma cells [4]. Moreover, polarity of Na^+-dependent neutral amino acid transport, which was not observed in passaged BMECs, was induced upon co-culture with glioma cells [15]. These findings are consistent with the role of astroglial cells in the regulation of BBB functions which had been lost during passage in culture.

Our in vitro BBB model system consisting of primary cultures of BMECs retains some in vivo BBB characteristics. γ-GTP was demonstrated in BMECs histochemically and biochemically [17,21]. Furthermore, BMECs were shown to be polarized with regard to ricin recycling [41], transferrin transport [42], and angiotensin II responsiveness [43]. Therefore, it would be of interest to study the effect of astroglial cells on the BMEC characteristics in vitro using the primary cultures which already possess in vivo BBB characteristics. Other aspects, including transport of peptides and polarity of responsiveness by BMECs, are currently being investigated in our laboratory using the same in vitro system in an attempt to better understand astroglial cell regulation of BBB functions.

In summary, we conclude that glucose uptake by primary cultures of bovine BMECs was regulated by some factor(s) secreted from astroglial cells. Preliminary data indicate that this factor(s) is insensitive to trypsin and appears to be of low molecular weight. Quantification of GLUT1 by ELISA strongly suggested that stimulation of glucose uptake by the factor(s) was due, at least in part, to increased de novo synthesis of GLUT1. Thus, the usefulness of this in vitro model system for studies of the regulatory mechanisms of BBB functions at the cellular level was demonstrated.

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