Biotin Uptake and Transport Across Bovine Brain Microvessel Endothelial Cell Monolayers

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Primary cultures of bovine brain microvessel endothelial cells (BMECs) were used to characterize blood–brain barrier (BBB) uptake and transport of biotin. Both the uptake and the transcellular transport of either radiolabeled or fluorescein-conjugated biotin by confluent monolayers of BMECs were measured. Biotin uptake (K_m = 123 μM) and bidirectional transport across BMEC monolayers was a saturable process and could be competed for by unlabeled biotin, biocytin, and biotinmethyl ester. Pantothenic and nonanoic acid were found not to be effective competitors for either biotin uptake or transport. The metabolic inhibitor, 2-deoxyglucose, had only small effects on the saturable apical-to-basolateral transport and apical uptake of biotin by BMECs. In contrast, basolateral-to-apical transport of biotin was substantially attenuated by 2-deoxyglucose pretreatment. Results supported the existence of specific and saturable uptake and efflux carrier systems for biotin in BMEC monolayers. The function of these systems was dependent to some degree on the metabolic status of the BMECs. Our findings confirm the existence of a biotin uptake system at the BBB *in vivo* and provide the first indication of an efflux system for biotin in BMECs.

KEY WORDS: biotin; blood–brain barrier; brain microvessel endothelium; transport.

INTRODUCTION

Biotin (vitamin H) plays a central role in fatty acid synthesis and the metabolism of carbohydrate lipids, proteins, and other compounds. Generally, biotin is covalently incorporated into various enzymes and is involved in carboxylation reactions in the brain and other tissues (1,2).

The availability of biotin to the central nervous system has been shown to be controlled and facilitated by a specific, saturable blood–brain barrier (BBB) carrier (3). On the basis that the BBB regulates delivery of biotin in some form to the brain, biotin-conjugated peptides and antisense oligonucleotides have been considered in drug delivery strategies (4). In addressing cellular level mechanisms of biotin-conjugate transport, however, studies to date have been conducted with isolated brain capillary suspensions. While suspensions of isolated brain capillaries allow characterization of the uptake of substances, these systems do not permit the study of the transcellular transport events and may have other limitations with respect to metabolic activity (5).

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In this study, we have used an *in vitro* model to characterize the role of the endothelial component of the BBB in regulating biotin delivery to the brain. Both the uptake and the transcellular transport of either radiolabeled or fluorescein-conjugated biotin were measured. Findings from this study will contribute to a better understanding of vitamin and nutrient transfer across the BBB and in the potential development of nutrient conjugates in drug delivery strategies.

MATERIALS AND METHODS

Materials

Biotin [(+)-[8,9-^{3}H(*N*)]vitamin H; 1.0 mCi/mL)] was purchased from Dupont Co. (Boston, MA). (+)-Biotin, biocytin, biotinmethyl ester, pantothenic acid, and nonanoic acid (pelargonic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). 5-[(*N*-(5-*N*-(6-(Biotinoyl)aminohexanoyl)amino)pentyl)thioureidyl]fluorescein (fluorescein-biotin) was purchased from Molecular Probes, Inc. (Eugene, OR). Horse serum was purchased from HyClone Laboratories, Inc. (Logan, UT). Eagle's minimum essential medium (MEM) and Ham's nutrient mixture F-12 were purchased from Hazelton Biologics, Inc. (Lenexa, KS). ScintiVerse E scintillation cocktail was purchased from Fisher Scientific (St. Louis, MO).

Uptake of Biotin by Bovine Brain Microvessel Endothelial Cells

Bovine brain microvessel endothelial cells (BMECs) were isolated from the gray matter of the cerebral cortices and grown to confluent monolayers in primary culture as previously detailed (6–8). These cells have been characterized as an appropriate *in vitro* model of the endothelial component of the BBB (6–8).

For biotin uptake studies, BMECs were seeded into rat-tail collagen-coated and fibronectin-treated 24-well plastic plates. Monolayers were confluent in approximately 10 days. The BMEC monolayers were then washed three times with Hank's balanced salt solution (HBSS) containing 10 m*M* HEPES buffer, pH 7.4 (9). The monolayers were incubated for 1 min at 37°C in 1 mL of the desired concentrations of unlabeled biotin (1–2000 μM) and 0.5 μCi/well [^{3}H]biotin in HBSS. After the 1-min incubation, the monolayers were washed three times with 1 mL of ice-cold HBSS. The monolayers received 0.5 mL/well of a 0.25% aqueous trypsin–EDTA mixture and solubilized at 37°C overnight on a Mini-Orbital shaker table (Bellco Technology, Vineland, NJ) set at low speed. Cell-associated radioactivity was assayed by liquid scintillation spectrometry following addition of the cell material to 9 mL of cocktail.

To investigate the uptake of [^{3}H]biotin by BMECs in the presence of potential competitors, 250 μM biotin, biocytin, biotinmethyl ester, pantothenic acid, or nonanoic acid was added to the incubation mixture described above for 1–30 min at 37°C. The cell-associated [^{3}H]biotin was assayed as described above.

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Biotin Transport Across Bovine Brain Microvessel Endothelial Cell Monolayers

BMECs were grown onto rat tail collagen and fibronectin-coated polycarbonate membranes, 3-μm pore, placed in 100-mm culture dishes (5,7,10). The BMEC monolayer-covered membranes were placed between side by side diffusion cells (Crown Glass Co., Somerset, NJ) to facilitate the study of the bidirectional transport of biotin. Each chamber of the diffusion cell apparatus contained 3.0 mL of a phosphate-buffered saline (PBSA) buffer (129 m*M* NaCl, 2.5 m*M* KCl, 7.4 m*M* Na_2HPO_4, 1.3 m*M* KH_2PO_4, 0.63 m*M* $CaCl_2$, 0.74 m*M* $MgSO_4$, 5.3 m*M* glucose), pH 7.4. The BMEC monolayers were oriented in the diffusion cell apparatus such that the cells faced the donor chamber (apical side) and the polycarbonate membrane (basolateral side) faced the receptor chamber. The diameter of the diffusion area was 9 mm. The water jacket surrounding the donor and receptor chambers was thermostated at 37°C and the contents of each chamber continuously stirred at 600 rpm with magnetic stir bars. The donor chamber received either 25 μ*M* fluorescein-biotin alone or together with concentrations of 0–1000 μ*M* unlabeled biotin. Other competitors used in the studies were 250 μ*M* biocytin, biotinmethyl ester, nonanoic acid, or pantothenic acid. In some experiments, the fluorescein-biotin and competitors were added to the receptor chamber and the contents of the donor chamber assayed (as the receiver) with time. A 0.2-mL aliquot from the receptor chamber was taken at times between 0 and 70 min. A 0.2-mL aliquot of PBSA buffer was added back into the receptor chamber to maintain constant volumes across the monolayers. In other experiments, the metabolic poison, 2-deoxyglucose, was added to the buffer as a replacement for glucose. Assessment of biotin passage across the BMEC monolayers was then repeated as described above. An SLM AMINCO Subnanosecond Lifetime Fluorometer (Urbana, IL) was used to assay sample fluorescence in microcuvettes following dilution with 1.8 ml of PBSA. The wavelength of excitation was 491 nm and emission was monitored at 520 nm.

Apparent permeability coefficients were calculated using the formula $P = X/(A \times t \times C_d)$, where P is the apparent permeability coefficient (cm/sec), X is the amount of substance (mol) in the receptor chamber at time t (sec), A is the diffusion area (0.636 cm^2), and C_d is the concentration of the substance in the donor chamber (mol/cm^3). The flux ($mol/cm^2/sec$) of a substance across the monolayers was calculated at the linearly regressed slope through linear data (e.g., sampling times, 1–70 min). (The concentration of substance in the donor chamber remained >90% of the initial value over the time course of the experiments.)

Statistics

All experiments were carried out in replicates of three to five different monolayers and data are expressed in figures and tables as the mean ± standard deviation. Data were tested for difference from the controls at the 0.05 level of significance by an analysis of various (ANOVA) and Sheffe's post hoc test (Abstat Software, Ver. 6.0, Anderson-Bell Corp., Parker, CO).

RESULTS

Biotin uptake by BMEC monolayers was concentration dependent and saturable over a range of 0–1000 μ*M* as shown in Fig. 1. Concentrations higher than 1000 μ*M* resulted in a reduced uptake of biotin (not shown). Uptake studies were conducted at 4°C to block cellular internaliza-

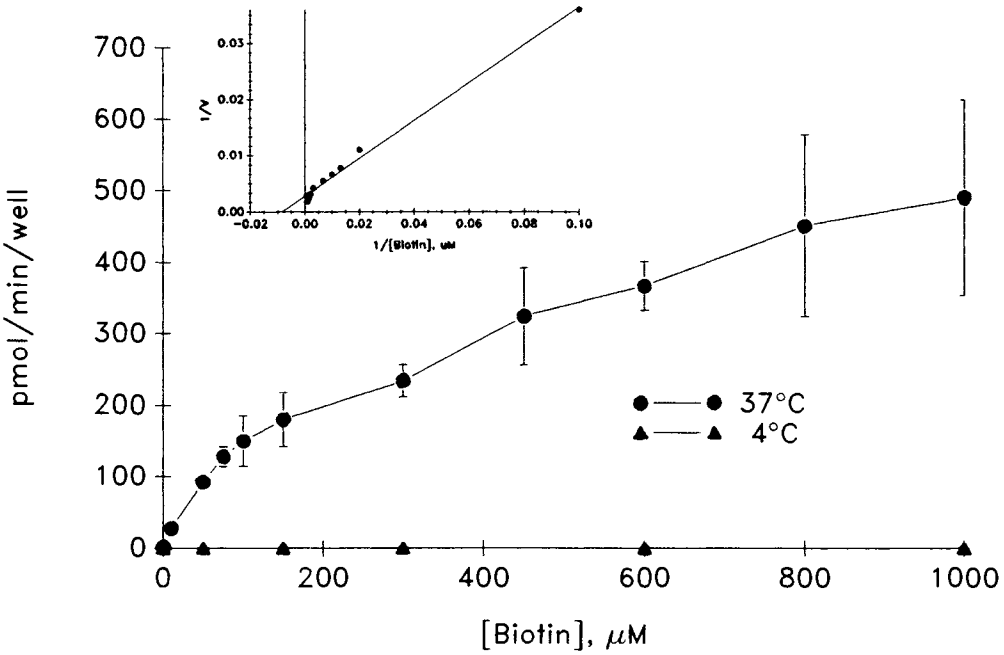

Fig. 1. Concentration-dependent uptake of [^{3}H]biotin by primary cultures of bovine brain microvessel endothelial cell (BMEC) monolayers. Confluent BMEC monolayers were incubated with indicated concentrations of biotin for 1 min at 37°C. Data points represent the means ± SD (n = 4). Inset: Lineweaver–Burke plot of data.

tion of biotin. Results shown in Fig. 1 indicated that biotin uptake at a low temperature was insignificant and suggested a negligible contribution of surface bound biotin to the total cell-associated material. Therefore, biotin associated with the cells at 37°C likely represented internalized biotin and not surface-bound material. On analysis by a Lineweaver–Burke plot (Fig. 1, inset), the apparent K_m was estimated to be 123 μM and the V_{max} 375 pmol/min/well or 3.0 nmol/min/mg cell protein.

The time-dependent uptake of biotin in the presence of potential uptake competitors, biotin and biotinmethyl ester, is shown in Fig. 2. The uptake of [^{3}H]biotin was significantly inhibited when present in combination with unlabeled biotin ($P < 0.01$; not shown), biocytin ($P < 0.01$), or biotinmethyl ester ($P < 0.01$), at 250 μM (Fig. 2). In contrast, pantothenic acid did not significantly inhibit ($P > 0.05$) the uptake of [^{3}H]biotin by BMEC monolayers except at the 30-min incubation time ($P < 0.01$). Nonanoic acid effects on BMEC uptake of biotin were significant at 1 min ($P = 0.04$) and 30 min ($P < 0.01$).

Due to the potential costs and amounts of radiolabeled biotin required for studies in a side-by-side diffusion apparatus, we opted to use fluorescein-biotin to characterize the transmonolayer processing of biotin. In transmonolayer transport studies, the time-dependent passage of 25 μM fluorescein-biotin across the monolayers was linear over a time range of 1 to 70 min and was inhibited by increasing concentrations of unlabeled biotin (not shown). Figure 3 shows a summary of the concentration-dependent inhibition of the flux of 25 μM fluorescein-biotin across the BMEC monolayers. The transport of biotin across the monolayers was bidirectional, with the apical-to-basolateral direction greatest. The concentration of unlabeled biotin required to inhibit one-half maximal passage (IC_{50}) of fluorescein-biotin across the monolayers was estimated by nonlinear regression curve-fitting of data in Fig. 3 as 270 μM for the apical-to-basolateral direction and 215 μM for the basolateral-to-apical direction.

Permeability coefficients for the passage of fluorescein-biotin, both apical-to-basolateral and basolateral-to-apical directions, across the BMEC monolayers were calculated in the presence or absence of other competitors and are shown in Fig. 4. As observed in the uptake studies, the bidirectional passage of 25 μM fluorescein-biotin was significantly inhibited ($P < 0.01$) by structurally related molecules, unlabeled biotin, biocytin, and biotinmethyl ester. Generally, the basolateral-to-apical passage of biotin across the monolayers was lower and less sensitive to the competitors. However, neither pantothenic acid ($P > 0.05$) nor nonanoic acid ($P > 0.05$) significantly altered the passage of fluorescein-biotin in either direction across the monolayers.

Following a 5-min preexposure to 50 mM 2-deoxyglucose, the bidirectional passage of fluorescein-biotin was significantly reduced overall ($P < 0.01$) as also shown in Fig. 4. In the apical-to-basolateral studies, competition with related molecules was still observed. In the basolateral-to-apical studies, however, the metabolic poison almost completely blocked biotin passage across the monolayers and competition between labeled biotin and related molecules was not significant ($P > 0.05$) To examine further the apparent asymmetry of biotin passage across the BMEC monolayers suggested by data in Figs. 3 and 4, the apparent permeability coefficients for the bidirectional passage of fluorescein-biotin across the BMEC monolayers was determined when 250 μM unlabeled biotin was placed in the opposite or receptor chamber. Table I summarizes results of the fluorescein-biotin transport against a high concentration of biotin and indicated that, in the apical-to-basolateral direction, the biotin passage was inhibited significantly ($P < 0.01$) by ≈59%. The basolateral-to-apical passage of biotin was significantly inhibited also ($P < 0.01$) but by a lesser degree, ≈32%.

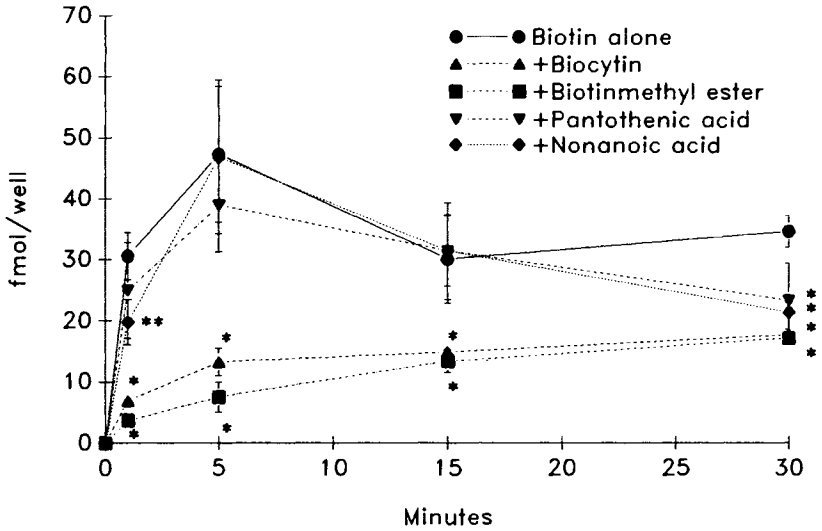

Fig. 2. Effect of potential competitors on the time-dependent uptake of [^{3}H]biotin by primary cultures of bovine brain microvessel endothelial cell (BMEC) monolayers. Confluent BMEC monolayers were incubated with 25 μM [^{3}H]biotin (0.5 μCi/well) and 250 μM indicated competitor at 37°C for various times. Data points represent the means ± SD ($n = 4$). (*) Significantly different ($P < 0.01$) from biotin alone; (**) significantly different ($P < 0.04$) from biotin alone.

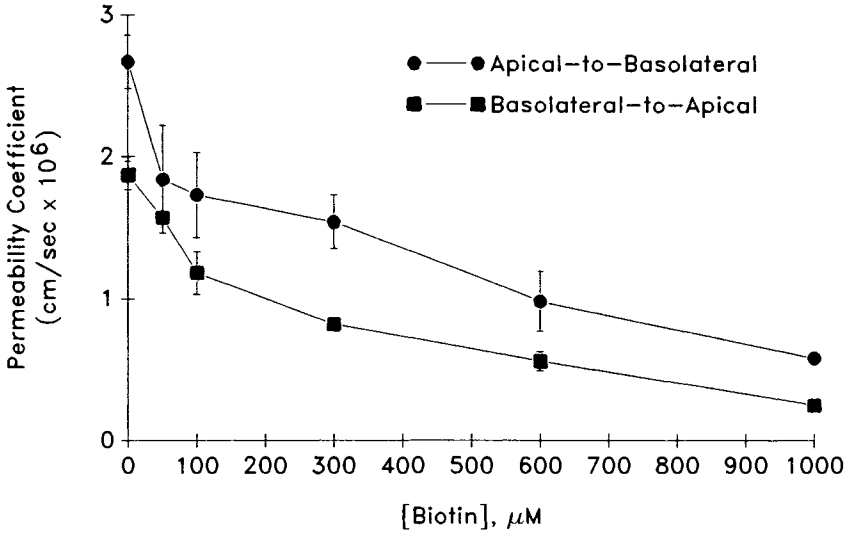

Fig. 3. Effect of increasing biotin concentration on the bidirectional passage of 25 μ*M* fluorescein-biotin across primary cultured bovine brain microvessel endothelial cell monolayers at 37°C. Data points represent the means ± SD (n = 3–4).

DISCUSSION

We have used an *in vitro* model to characterize biotin uptake and transport characteristics at the endothelial component of the BBB. This study supported and confirmed saturable biotin uptake and transport across the BBB. In general, the half-saturation concentration for biotin in microorganisms is <1 μ*M* (11) and 20 μ*M* or higher in mammalian tissues (2,12,13). The K_m of 123 μ*M* for [^{3}H]biotin uptake and the approximate IC_{50} of 270 and 215 μ*M* for fluorescein-biotin passage apical-to-basolateral and basolateral-to-apical, respectively, across the monolayers were in good agreement with the *in vivo* K_m of 100 μ*M* for BBB uptake of biotin reported by Spector and Mock (3).

Biotin-like molecules containing the ring structure, biocytin and biotinmethyl ester, competed for both [^{3}H]biotin uptake and the bidirectional passage of fluorescein-biotin across BMEC monolayers. Previous work by Spencer and Brody (14) similarly demonstrated the ability of biocytin and biotinmethyl ester to compete for biotin transport in the small intestine of hamster. The presence of one intact ring, either the thiophane or the ureido ring, along with the valeric chain has appeared to be necessary for transport by cell biotin carriers (12). Piffeteau and Gaudry (11) also reported that an intact biotin ring structure was necessary for transport in microorganisms. One other study has further indicated that a free carboxyl group was necessary for biotin carrier recognition (13). Conversely, Spector and Mock (3) had reported that pantothenic acid and nonanoic acid were the more potent inhibitors of biotin uptake at the BBB. Here we observed pantothenic acid and nonanoic acid as significant inhibitors of biotin uptake, but only consistently at longer incubation times. As noted, unlabeled biotin, biocytin, and biotinmethyl ester inhibition of biotin uptake by the BMEC monolayers was immediate and substantial on short-term incubation. The transport studies further confirmed sensitivity of biotin passage across the monolayers to structurally related molecules. Presumably, the initial transport of nutrients into the cell occurs rapidly (i.e., on a time scale of a minute or less). The subsequent accumulation in cells of nutrient on longer incubation may be the result of a summation of uptake, intracellular compartmentalization, and back flux (15). This might suggest that pantothenic acid and nonanoic acid, which required longer incubation times to inhibit biotin uptake by BMEC monolayers, could indeed be inhibitors by blocking the cell uptake of biotin through an alternative mechanism. Spector *et al.* (16) have also described a pantothenic acid uptake carrier at the BBB which was inhibited by both biotin and nonanoic acid. The existence of this latter carrier may have some role in this system but has not been investigated in the *in vitro* model. The availability of other biotin analogues or biotin conjugates should assist in further characterization of a distinct BBB biotin carrier.

Pardridge (4) recently addressed the potential application of biotin conjugates for delivering therapeutic entities to the brain. Uptake studies were conducted with isolated brain capillary suspensions but failed to demonstrate large amounts of uptake when a single trace concentration of biotin was added to the suspensions in the absence of an exogenous protein, avidin (4). Avidin was not included in experiments shown here and the amount of biotin taken up by BMEC monolayers relative to the concentration of biotin added was very low (0.1–0.6%), consistent with the studies in brain capillaries. In contrast, however, we observed a decreased transcellular passage of 25 μ*M* fluorescein-biotin in a concentration-dependent manner by the presence of avidin (33–333 μg/mL) in our studies (data not shown). A significant difference in this study was the use of fluorescein-biotin rather than [^{3}H]biotin in the transmonolayer transport study. Fluorescein-biotin may not interact with avidin in a manner similar to [^{3}H]biotin, thus affecting the uptake process.

Most nutrient carrier systems at the BBB are facilitated and not energy dependent (17). In this study, we used a metabolic poison to try and classify biotin transport as either

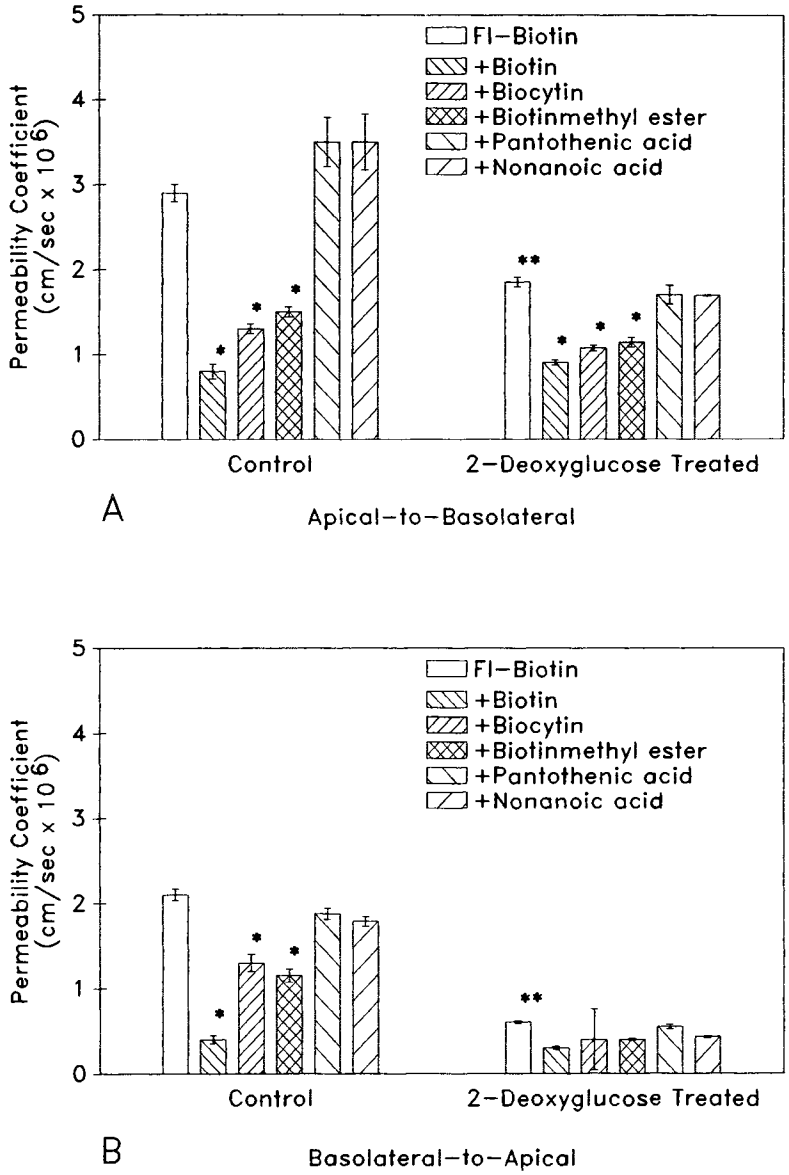

Fig. 4. Effect of 250 μ*M* selected competitors on the apparent permeability coefficient for the passage of 25 μ*M* fluorescein (Fl)-biotin across primary cultures of bovine brain microvessel endothelial cell monolayers at 37°C. (A) Apical-to-basolateral passage of fluorescein-biotin; (B) basolateral-to-apical passage of fluorescein-biotin. Data points represent the means ± SD (n = 3–4). (*) Significantly different ($P < 0.01$) from Fl-biotin with and without 2-deoxyglucose treatment; (**) significantly different ($P < 0.01$) from Fl-biotin without 2-deoxyglucose treatment.

facilitated or active. The 2-deoxyglucose treatment reduced transfer of fluorescein-biotin across the BMEC monolayers, however, the inhibition of transport by unlabeled biotin, biocytin, and biotinmethyl ester remained unaffected. The 2-deoxyglucose concentration used was sufficient to retard BMEC energy-dependent systems substantially as shown in our earlier work (18,19). In that earlier work, the transmonolayer passage of passive permeability marker was also attenuated by the poison, perhaps attributable to cell swelling and a reduction in paracellular diffusion (19). Therefore, the partially reduced apical-to-basolateral transport of biotin in the presence of 2-deoxyglucose may actually represent the biotin transported by cell carriers, less the biotin passing through paracellular pathways. On the other hand, the basolateral-to-apical transport of biotin appeared quite sensitive to the poison. A bidirectional system for biotin is consistent with many of the other nutrient carrier systems. However, unlike most nutrient carrier systems (17), the sensitivity to a metabolic poison suggested that the bidirectional passage of biotin across BMECs was dependent to some degree on the metabolic status of the cells. Worth noting is that microorganisms have biotin countertransport carriers with an active component in one direction and a passive nature in the opposite direction across the membrane (11).

Table I. The Effect of Biotin on Fluorescein-Biotin (Fl-Biotin) Passage Across Bovine Brain Microvessel Endothelial Cell Monolayers at 37°C

[Biotin], apical compartment (μM)	Permeability coefficient* (cm/sec × 10^6)[a]	[Biotin], basolateral compartment (μM)
Apical-to-basolateral passage of Fl-biotin		
25	2.7 ± 0.1	0
25	1.1 ± 0.3*	250
Basolateral-to-apical passage of Fl-biotin		
0	1.9 ± 0.1	25
250	1.3 ± 0.2*	25

[a] Apparent permeability coefficients expressed as the means ± SD of five different monolayers.

* Significantly different ($P < 0.01$) from conditions where biotin was absent.

The 2-deoxyglucose studies strongly suggested the existence of asymmetry in the transport of biotin. In isolated cell suspensions, varying intra- and extracellular substrate concentrations under nonequilibrium conditions has been successfully used to delineate the asymmetry of transporters in membranes (20,21). Using this approach, we found that apical-to-basolateral fluorescein-biotin passage across the monolayers was about twofold more sensitive to the presence of biotin in the opposite chamber than was basolateral-to-apical fluorescein-biotin transport under similar conditions. The biochemical mechanism providing for such asymmetry of BMEC biotin transport is not known but, based on our studies, seems to be due to one or more factors. For instance, the differing energy requirements of individual carriers was one factor. The other factor seems to be related to smaller, significant differences in the bidirectional biotin transport, where an apparent existence of unequal numbers of carriers on apical and basolateral surfaces of the BMECs could have resulted in different maximum transport capacities of the apical-to-basolateral and the basolateral-to-apical systems. These characteristics have been observed with other BMEC carriers with functional polarity (22,23).

The nature and role of the asymmetry of BMEC biotin transport have implications for both drug delivery schemes and under conditions of inborn errors of biotin-dependent enzymes (24). In animal models, depleting the central nervous system of biotin is possible (25–27) but difficult relative to organs such as the liver (28). Explanations include the fact that biotin is covalently associated with enzymes, thus, upon entering the central nervous system, may be trapped. The relatively energy-independent and higher apical-to-basolateral transport capacity for biotin in BMECs would seem to assist, in part, in preventing depletion of biotin on the central nervous system side of the BBB. Perhaps the efflux system might function to remove slowly but continually and, as a consequence, regulate free biotin concentrations in the central nervous system under normal conditions. A better understanding of the mechanisms directing the distribution of biotin across the BBB will benefit from a broader systematic study of sidedness with a selection of competitors for biotin transport.

In summary, we have demonstrated the existence of a saturable, relatively low-affinity, and bidirectional transport system for biotin in BMEC monolayers. This system will provide the basis for ongoing investigations of the potential utility of biotin and nutrient carrier systems to facilitate nutrient and drug delivery to the central nervous system.

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