Blood-Brain Barrier Transport of Reduced Folic Acid

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Purpose. The brain is relatively resistant to folic acid deficiency, indicating specialized transport systems may exist for this vitamin localized within the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) in vivo. The present studies quantify the BBB transport of [^{3}H]-methyltetrahydrofolic acid (MTFA) in vivo and in isolated human brain capillaries in vitro.

Methods. BBB transport of [^{3}H]-MTFA was compared to that of [^{14}C]-sucrose, a plasma volume marker, following either intravenous injection or intracarotid perfusion in anesthetized rats. Competition by 10 μM MTFA or 10 μM folic acid was examined to determine whether folic acid is also transported by the MTFA uptake system.

Results. The BBB permeability-surface area (PS) product of [^{3}H]-MTFA, 1.1 ± 0.3 μL/min/g, was 6-fold greater than that of [^{14}C]-sucrose following intravenous injection. The BBB PS product determined by intracarotid arterial perfusion was not significantly different from the BBB PS product calculated following intravenous injection. A time- and temperature- dependent uptake of [^{3}H]-MTFA in human brain capillaries was observed. The uptake of [^{3}H]-MTFA by either rat brain in vivo or by human brain capillaries in vitro was equally inhibited by 10 μM concentrations of either unlabeled MTFA or unlabeled folic acid.

Conclusions. (1) A saturable transport system exists at the BBB for folic acid derivatives and since this transport is equally inhibited by either folic acid or MTFA, it is inferred that this transport system is the folic acid receptor, and not the reduced folic acid carrier. (2) The presence of a folate transport system at the BBB may offer an endogenous transport system for brain drug delivery of conjugates of folates and drugs that do not normally cross the BBB in vivo.

KEY WORDS: folate receptor; folic acid; blood-brain barrier; drug delivery.

INTRODUCTION

Folic acid, and the biologically active metabolite, 5'methyltetrahydrofolic acid (MTFA) are transported across cell membranes by one of at least 3 different classes of transport systems: (a) reduced folate carrier (RFC), which has a preferential affinity for MTFA rather than folic acid; (b) the folate receptor, which has an equal affinity for either MTFA or folic acid; and (c) folic acid export pumps (1). The folate receptor is up-regulated in cells exposed to reduced folate availability (1).

The brain is relatively resistant to folic acid deficiency, suggesting the presence within the central nervous system (CNS) of transport systems for folic acid analogues (2). Spector and colleagues (2,3) demonstrated that folic acid and MTFA were both rapidly taken up by choroid plexus either in vivo or in vitro, but that only MTFA was selectively transported across the choroid plexus to distribute into cerebral spinal fluid (CSF). The uptake of [^{3}H]-folic acid by choroid plexus in vitro was inhibited by low concentrations of either unlabeled folic acid or unlabeled MTFA (3). This comparable sensitivity of the folate transport system to either folic acid or MTFA suggests that the folate receptor, rather than the reduced folic acid carrier, was responsible for choroid plexus transport of the folates. Recent studies have provided biochemical evidence for immunoreactive folate receptor in the choroid plexus in human brain as well as certain brain tumors (4). The folate receptor in brain was only localized at the choroid plexus, and not in brain parenchyma, as judged by Western blotting (4). However, if folate receptor in brain was localized only at the capillary endothelium, this would not be detected in Western blotting of whole brain homogenates, because the capillary endothelial volume (1 μL/g) constitutes only 0.1% of the brain. The presence of a folate transport system at the choroid plexus allows for rapid distribution of circulating folates into CSF (2); however, the distribution of blood folates into brain parenchyma would require a transport system at the BBB. Therefore, the present studies examined the transport of labeled MTFA at the BBB in vivo using both intravenous injection/pharmacokinetic and internal carotid artery perfusion methods. Parallel studies are also performed with isolated human brain capillaries, used as an in vitro model system of BBB transport.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats weighing 260 to 330 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Human brain capillaries isolated from postmortem autopsy human brain were prepared and stored in liquid nitrogen as previously reported (5). (6S)-5-Methyltetrahydrofolic acid (MTFA) [3',5',7,9-^{3}H] was purchased from Moravek Biochemicals, Inc (Brea, CA). The specific activity was 30 Ci/mmol, and the radiochemical purity was 97.1%. [^{14}C]-sucrose (0.4 Ci/mmol) was from DuPont NEN Products, Inc. (Boston, MA). 5-methyltetrahydrofolic acid, folic acid, and all other chemicals were from Sigma Chemical Corp. (St. Louis, MO).

Pharmacokinetics

Rats were anesthetized with 100 mg/kg ketamine and 2 mg/kg xylazine intraperitoneally. The left femoral vein was cannulated with a PE50 cannula and injected with 0.2 mL Ringer-Hepes solution (pH = 7.4) containing 5 μCi of [^{3}H]-MTFA and 2 μCi of [^{14}C]-sucrose. Blood samples (0.25 mL) were collected via heparinized PE50 cannula implanted in the left femoral artery at 0.25, 1, 2, 5, 15, and 30 minutes after intravenous injection of the isotope. The blood volume was replaced with an equal volume of saline. After 30 minutes, the animals were decapitated for removal of the brain and other

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ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; CSF, cerebrospinal fluid; %ID/g, percentage of injected dose delivered per gram of tissue weight; PS, permeability-surface area; AUC, area under the plasma concentration curve; CL, plasma clearance; V_{ss}, systemic volume of distribution; MRT, mean residence time; V_d, organ volume of distribution; IV, intravenous; ICAP, internal carotid artery perfusion; MTFA, 5-methyltetrahydrofolic acid; FA, folic acid.

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organs. The plasma and organ samples were solubilized with Soluene-350 (Packard Instrument Company, Downer's Grove, IL) and neutralized with glacial acetic acid prior to liquid scintillation counting.

Pharmacokinetic parameters were calculated by fitting the plasma radioactivity data to a biexponential equation:

$$A(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

where A(t) = % injected dose (ID)/mL plasma. The biexponential equation was fit to plasma data using a derivative-free nonlinear regression analysis (PARBMDP, Biomedical Computer P-Series, developed at UCLA Health Sciences Computing Facilities). The data were weighted using weight = 1/(concentration)^2, where concentration = %ID/mL plasma. The organ volume of distribution (V_d) of the [^{3}H]-MTFA and [^{14}C]-sucrose at 30 minutes after i.v. injection was determined from the ratio of disintegrations/minutes (dpm)/g tissue divided by the dpm/μL of terminal plasma. The pharmacokinetic parameters such as plasma clearance (CL), the initial plasma volume (V_C), steady state volume of distribution (V_{ss}), steady state area under the plasma concentration curve ($AUC|_0^\infty$), and mean residence time (MRT) were determined from the A_1, A_2, k_1, k_2, as described previously (6). The organ clearance or permeability-surface area (PS) product was determined as follows:

$$PS = \frac{[V_d - V_o]A(T)}{AUC|_0^\infty}$$

$$AUC|_0^t = \frac{A_1(1 - e^{-k_1 t})}{k_1} + \frac{A_2(1 - e^{-k_2 t})}{k_2}$$

where A(T) = the terminal plasma concentration, and V_O = the plasma volume for the respective organs reported previously (7): V_O = 225, 150, 131, 119, and 10 μL/g for lung, heart, kidney, liver, and brain, respectively. The organ uptake, expressed as % injected dose (ID) per g organ, was calculated from:

$$\%ID/g|_0^t = PS(AUC)|_0^t$$

Internal Carotid Artery Perfusion Study

Rats were anesthetized with ketamine/xylazine and the right internal carotid artery was cannulated with stretched PE50 tubing; the ipsilateral pterygopalatine, superior thyroidal, and occipital arteries were closed by electrocoagulation as described previously (8). At the initiation of the perfusion, the ipsilateral common carotid artery was ligated and the internal carotid artery was perfused with Krebs-Henseleit buffer containing 0.1% bovine serum albumin, 10 mM sodium L-ascorbate (to maintain reduction of MTFA), 0.125 μCi/mL [^{14}C]-sucrose (0.275 nM), and 0.25 μCi/mL [^{3}H]-MTFA (8.3 nM) at a perfusion rate of 1.25 mL/min. In some experiments, unlabeled MTFA or FA was added to a final concentration of 10 μM. The perfusate was filtered through a 0.45 μm Millex-HV filter (Millipore, Bedford, MA) and the pH was adjusted to pH = 7.2 with HCl after gassing with 95% O_2:5% CO_2. The blood volume was maintained relatively constant by simultaneously withdrawing blood at a rate of 1.0 mL/min via a cannula implanted in the left femoral artery. After a 5 or 10 minute perfusion, the animals were decapitated and the brain was removed and solubilized in Soluene-350 for liquid scintillation counting. The BBB PS product for the [^{3}H]-MTFA was calculated from:

$$PS = \frac{V_d - V_O}{t}$$

where V_d and V_O are the brain volumes of distribution of [^{3}H]-MTFA and [^{14}C]-sucrose, respectively, after 5 or 10 minutes (t) of internal carotid artery perfusion. The volume of distribution (V_d) of the [^{3}H]-MTFA or the [^{14}C]-sucrose in the brain was calculated from the ratio of dpm/g brain divided by the dpm/μL of the perfusate.

Binding of [^{3}H]-MTFA to Human Brain Capillaries

The incubation buffer was Ringer/N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (Hepes) solution containing 0.1% bovine serum albumin and 10 mM sodium L-ascorbate; 50 L of [^{3}H]-methyltetrahydrofolic acid (1 μCi/mL) was pipetted into plastic 12 × 75 mm tubes, and was preheated either at 37°C or 4°C for 10 min. Some of the tubes that were preheated at 37°C were designed for inhibition studies; 5-methytetrahydrofolic acid or folic acid was added to achieve a final concentration of 10 μM. The incubation was initiated by addition of 50 μL (about 125 μg_p) of human brain capillary preparation. The incubation was allowed to proceed for 2 min, 5 min, 15 min or 30 min. At the end of the incubation, the solution was transferred into 0.5 mL microcentrifuge plastic tubes, and centrifuged at 8,000 rpm at room temperature for 45 sec. The tube pellet was cut off and solubilized in 0.5 mL of 1N NaOH at 60°C for 30 min; 0.1 mL of the sample was used for protein assay (Pierce Chemical Co., Rockford, IL), while 0.3 mL of the sample was added to 10 mL of Ultima-Gold cocktail (Parkard Instrument Company, Downer's Grove, IL) for radioactivity liquid scintillation counting. The results were presented as % bound per mg protein of brain capillaries, as repeated previously (5).

RESULTS

The plasma profile of [^{3}H]-MTFA (Fig. 1) was biexponential following a bolus i.v. injection to the rat for the observation

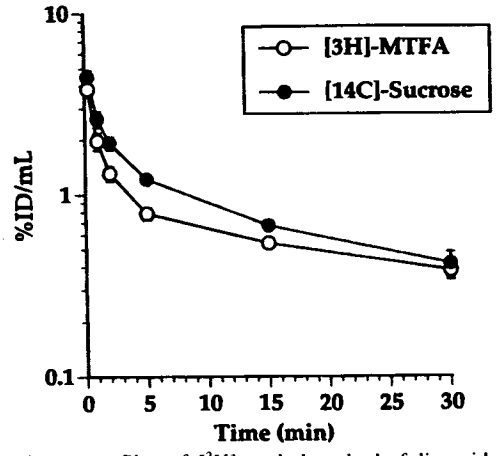

Fig. 1. Plasma profiles of [^{3}H]-methyltetrahydrofolic acid (MTFA) and [^{14}C]-sucrose in the rat following an i.v. dose. Each point on the graph represents the mean ± SEM of three rats.

Table 1. Pharmacokinetic Parameters for [^{3}H]-MTFA and [^{14}C]-sucrose in Rats at 30 min Following Intravenous Injection

Parameter	[^{3}H]-MTFA	[^{14}C]-sucrose
A_1 (%ID/ml)	3.76 ± 0.21	3.72 ± 0.35
A_2 (%ID/ml)	0.865 ± 0.034	1.34 ± 0.07
k_1 (min^{-1})	1.11 ± 0.08	0.951 ± 0.156
k_2 (min^{-1})	0.028 ± 0.002	0.041 ± 0.007
$t_{1/2}$ 1 (min)	0.632 ± 0.052	0.772 ± 0.132
$t_{1/2}$ 2 (min)	25 ± 2	18 ± 4
$AUC_{0-30'}$ (%ID min/ml)	21 ± 2	27 ± 2
$AUCD_{0-\infty}$(%ID min/ml)	35 ± 4	39 ± 6
V_c (ml/kg)	67 ± 3	62 ± 3
V_{ss} (ml/kg)	292 ± 12	186 ± 7
CL (ml/min/kg)	32 ± 3	23 ± 4

Note: Mean ± S.E. (N = 3 rats).

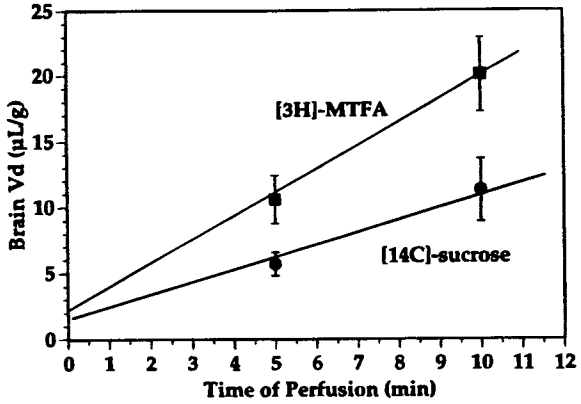

Fig. 2. Brain volume of distribution (Vd) of [^{3}H]-methyltetrahydrofolic acid (MTFA) and [^{14}C]-sucrose in the rat following internal carotid artery perfusion for 5 and 10 min. Each point on the graph represents the mean ± SEM of three animals.

period of 30 min, and was very similar to that of [^{14}C]-sucrose, a plasma marker. The similarity between [^{3}H]-MTFA and [^{14}C]-sucrose was confirmed by comparison of their pharmacokinetic parameters which are listed in Table 1. However, as shown in Table 2, brain uptake of [^{3}H]-MTFA was 4.2 times that of [^{14}C]-sucrose (0.0228 ± 0.0050 vs. 0.0054 ± 0.0015% ID/g). Although renal uptake of the two compounds was similar, the hepatic uptake of [^{3}H]-MTFA was 33 times higher than that of [^{14}C]-sucrose (Table 2).

The brain uptake of [^{3}H]-MTFA was linearly increased over the time of the intracarotid arterial perfusion in rats, and was twice as high as that of [^{14}C]-sucrose (Fig. 2). The BBB PS products calculated from the 5 and 10 min perfusion data are 0.98 ± 0.21 and 0.88 ± 0.17 μL/min/g, respectively. The presence of either 10 μM unlabeled MTFA or folic acid in the perfusate reduced the brain uptake of the [^{3}H]-MTFA to a level that was not significantly different from [^{14}C]-sucrose (Fig. 3). The brain volume of distribution of [^{14}C]-sucrose, a plasma marker, was unchanged in the presence of either MTFA or FA (Fig. 3).

Table 2. Organ Clearance of [^{3}H]-MTFA and [^{14}C]-sucrose in Rats at 30 min Following Intravenous Injection

Organ	Parameter	[^{3}H]-MTFA	[^{14}C]-sucrose
Brain	Vd(μL/g)	73 ± 19	23 ± 4
	PS(μL/min/g)	1.12 ± 0.30	0.199 ± 0.052
	%ID/g	0.0228 ± 0.0050	0.0054 ± 0.0015
Lung	Vd(μL/g)	883 ± 62	644 ± 81
	PS(μL/min/g)	11.8 ± 0.772	6.51 ± 1.89
	%ID/g	0.246 ± 0.003	0.185 ± 0.066
Heart	Vd(μL/g)	832 ± 50	230 ± 11
	PS(μL/min/g)	12.3 ± 0.5	1.18 ± 0.12
	%ID/g	0.256 ± 0.009	0.0323 ± 0.0039
Liver	Vd(μL/g)	2694 ± 351	193 ± 15
	PS(μL/min/g)	46.2 ± 4.5	1.08 ± 0.15
	%ID/g	0.957 ± 0.060	0.0291 ± 0.0033
Kidney	Vd(μL/g)	12751 ± 1881	8262 ± 2950
	PS(μL/min/g)	226 ± 26	130 ± 58
	%ID/g	4.66 ± 0.23	3.75 ± 1.91

Note: Mean S.E. (N = 3 rats).

The binding of [^{3}H]-MTFA to human brain capillaries was time and temperature dependent, and was saturable, as the presence of either 10 μM unlabeled MTFA or FA inhibited the specific binding completely. Another study using graded doses of MTFA or FA revealed that both MTFA and FA were equally effective in the inhibition of binding of MTFA to human brain capillaries at nanomolar concentrations (data not shown). In parallel studies with isolated bovine brain capillaries, no specific uptake of [^{3}H]-MTFA was observed.

DISCUSSION

The results of the present studies are consistent with the following conclusions. First, MTFA is rapidly cleared from blood at a rate about 50% faster than sucrose (Fig. 1), and this is due to rapid glomelular filtration by the kidney (Table 2). Second, the BBB PS product for MTFA is 6-fold greater than that of sucrose when measured after intravenous injection (Table 2), and this value is not significantly different from the BBB PS product determined following intracarotid artery perfusion (Results). Third, BBB transport of MTFA is equally inhibited

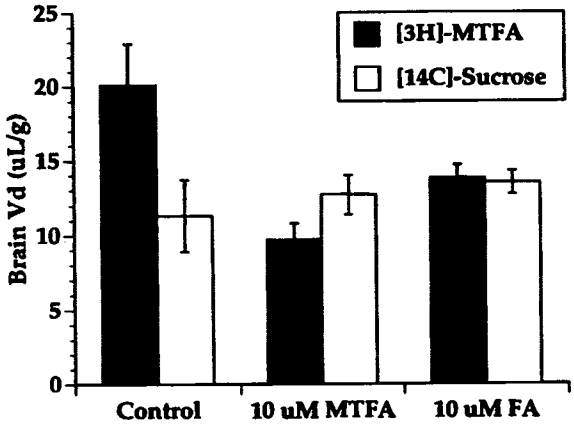

Fig. 3. Brain V_d of [^{3}H]-MTFA and [^{14}C]-sucrose in the rat following 10 min of internal carotid artery perfusion in the presence or absence of 10 μM MTFA or 10 μM folic acid (FA). Each point on the graph represents the mean ± SEM of three animals.

by either 10 μM MTFA or 10 μM folic acid in the rat in vivo (Fig. 3) or in human brain capillaries in vitro (Fig. 4). The equivalent inhibition by either MTFA or folic acid suggests that BBB transport of MTFA is mediated by a BBB folate receptor. Since isolated brain capillaries are patent (10), it is assumed the uptake studies with isolated brain capillaries reflect transport across both luminal and abluminal endothelial membranes.

The systemic clearance of MTFA is comparable to that of biotin reported previously (9). However, the brain uptake of [^{3}H]-biotin is more than 10-fold greater than that of MTFA (0.28 ± 0.03% ID/g vs. 0.023 ± 0.005% ID/g) owing to a 10-fold greater BBB PS product for biotin, 10.8 ± 1.0 μL/min/g vs. the BBB PS product of MTFA, 1.12 ± 0.30 μL/min/g (Table 2). Therefore, the BBB biotin carrier is 10-fold more active than the BBB transport of MTFA. Nevertheless, the BBB transport of MTFA has a PS product 6-fold greater than that of sucrose (Table 2). This selective transport of MTFA, relative to sucrose, is also seen in myocardium and liver (Table 2) but much less so in lung or kidney (Table 2). The comparable estimates for BBB PS products for MTFA with either the intravenous injection/pharmacokinetic method or the internal carotid artery perfusion method suggests there is no significant plasma protein binding of MTFA, which would adversely affect the estimation of the PS product following intravenous injection, but not affect the estimate following internal carotid artery perfusion. This correlation between the two methods is consistent with low serum plasma protein binding of MTFA previously reported (11). The virtually identical estimates of the BBB PS product for MTFA also suggests there is minimal release to plasma of [^{3}H]-metabolites of MTFA, since this would also affect the estimation of the PS product with the intravenous injection method, but not the arterial perfusion technique. Previous studies indicate <10% of the injected MTFA is metabolized at 30 minutes after intravenous injection (2). The magnitude of the PS product of MTFA is only 1% of the rate of cerebral blood flow in the ketamine-anesthetized rat (12), which means anesthesia should not affect the PS measurement.

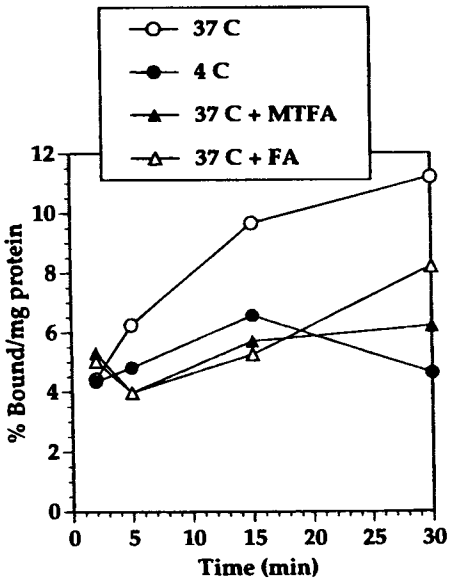

Fig. 4. The binding of [^{3}H]-MTFA to human brain capillaries incubated at either 4°C or 37°C, and in the presence or absence of 10 μM MTFA or folic acid (FA). Each point on the graph represents the mean value of duplicate incubations.

The present studies do not provide biochemical evidence that proves the BBB transport of MTFA is mediated by the folic acid receptor as opposed to the RFC. However, the RFC selectively transports reduced folates and has a very weak affinity for folic acid (1). The present studies show that BBB transport either in vivo or in isolated brain capillaries in vitro is inhibited to a comparable degree by either MTFA or folic acid. These studies suggest that the folate receptor is expressed at the BBB and mediates the BBB transport of circulating MTFA or folic acid. Since folate reductase is not present in brain, the brain cannot utilize folic acid, and the principal folate utilized by brain metabolism is the reduced form of the vitamin (2).

The expression of the folate receptor at the BBB could provide an endogenous transport system for drug delivery to the brain wherein either folic acid or MTFA is conjugated to a drug that does not normally cross the BBB. If MTFA transport across the BBB is in fact mediated by the BBB folate receptor, then this process is one of receptor-mediated transcytosis similar to the receptor-mediated endocytosis of folic acid and folic acid drug conjugates observed in cells in tissue culture (13). The BBB PS product of MTFA is about 50% less than the BBB PS product of an anti-transferrin receptor (TfR) monoclonal antibody (MAb) that undergoes receptor-mediated transcytosis through the BBB in vivo via the endogenous BBB transferrin receptor (14). However, owing to the rapid renal clearance of a small molecule such as MTFA, the plasma AUC of the TfRMAb is much higher than the plasma AUC of MTFA. Consequently, the brain uptake of the TfRMAb, 0.26% ID/g (14), is 11-fold greater than the brain uptake of MTFA (Table 2). Nevertheless, it is possible that certain folate/drug conjugates could be delivered to brain in pharmacologically significant amounts via the endogenous BBB folate transport system.

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