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BLOOD-BRAIN BARRIER TRANSPORT OF CAFFEINE: DOSE-RELATED RESTRICTION OF ADENINE TRANSPORT

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Summary

We studied the transport of ^{14}C-caffeine across the blood-brain barrier (BBB) by measuring brain ^{14}C:^{3}H ratios five seconds after rats received the caffeine, with $^{3}H_2O$, by intracarotid injection. Caffeine was found to enter the brain by both simple diffusion and saturable, carrier-mediated transport. This latter observation suggested to us that caffeine's transport might involve macromolecules that are structurally similar to caffeine. Hence, we examined caffeine's ability to inhibit the BBB transports of ^{14}C-adenosine and ^{14}C-adenine. Caffeine caused a dose-dependent inhibition of ^{14}C-adenine transport but no clear change in that of ^{14}C-adenosine. At very high blood levels ($K_i = 9.8$ mM), caffeine may restrict the availability of circulating purines to the brain. This effect may be important neonatally, when carrier-mediated adenine transport apparently is maximal.

With the exception of ethanol, caffeine (1,3,7-trimethylxanthine) is probably the most widely-consumed agent affecting the central nervous system. Caffeine is thought to enter the brain readily because of its high degree of lipid solubility. Oldendorf (1) found that caffeine has an olive-oil:water partition coefficient of approximately 1:10, and attributed its nearly complete clearance (>80%) in a single circulatory passage through the brain vasculature to this high degree of lipid solubility. Rapoport has also shown a high degree of correlation between lipid solubility, as predicted by oil:water or octanol: water coefficients, and drug penetration into the brain (2).

Drugs acting on the brain must pass through the cerebral capillary endothelial cells, which make up the blood-brain barrier. Like metabolic substrates, drugs may enter and leave the brain by simple diffusion, facilitated diffusion, or active transport. A high degree of lipid solubility predicts ready passage into the brain by simple diffusion. Important substrates for

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brain metabolism which are not very lipid soluble may nonetheless enter the brain by facilitated diffusion if transport carriers for them, presumably located within cerebral endothelial cells, happen to exist. Such transport carriers have been described for glucose (3), amino acids (4), amines (5,6) (e.g., choline), monocarboxylic acids (7)(e.g., lactate), thyroid hormones (8), purines (9), and purine nucleosides (9). Some substances (e.g., glucose) enter the brain by both simple diffusion and carrier-mediated facilitated diffusion.

We present evidence that caffeine enters the brain both by a carrier-mediated (saturable) transport mechanism, probably catalyzing facilitated diffusion, and by simple diffusion, probably based on its high lipid solubility. Moreover, the macromolecule that mediates its transport may be the same as the adenine carrier of Cornford and Oldendorf (9), inasmuch as caffeine competes with adenine for brain uptake.

Methods

Animals: All animals used in these studies were male Sprague-Dawley albino rats weighing 150-350 g and obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts). The animals were stored in a temperature-controlled animal facility and exposed to light between 7:00 h and 19:00 h. All experiments were performed in the late afternoon. Animals were housed in our animal facility for at least a three-day period prior to use in an experiment. They had access to standard rat chow (Big Red Lab Chow, 23% protein) and water *ad libitum*.

Brain Uptake Index (BUI) Method: Animals were anesthetized with 40 mg/kg sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, Illinois) intraperitoneally and supplemental small doses of diethyl ether as needed during the neck dissection. A median ventral neck incision was made with a pair of scissors, and the mandibular salivary glands, pretracheal fascia, sternohyoid, and other strap muscles were separated by blunt dissection. The neurovascular bundle on the left side was isolated, and the common carotid artery gently separated from the vagus and surrounding fascia with a tweezers. A ligature was then placed beneath the carotid artery, and the animal was positioned on a guillotine. The artery was pierced with a sharp 27-gauge 5-cm needle (VitaNeedle Corporation, Needham, Massachusetts) without interrupting blood flow, and the 200-µl test solution was injected rapidly, causing momentary blanching of the tissues supplied by the external carotid artery. Five seconds after injection of the bolus, the animal was decapitated and its ipsilateral forebrain rostral to the pons quickly removed and prepared for scintillation counting. This preparation involved expressing roughly equal amounts of each brain sample through a 20-gauge needle into two scintillation vials containing 1 ml of NCS tissue solubilizer (Amersham Corporation, Arlington Heights, Illinois). The vials were then placed in a Dubnoff metabolic shaker containing water warmed to 50°C and shaken for 2-3 hours until the tissue was completely dissolved. Then, 15 ml of Betafluor (National Diagnostics, Somerville, New Jersey) were added and the samples counted in a Packard Tricarb (Model 3300) liquid scintillation spectrophotometer.

The BUI measures the unidirectional flux of a ^{14}C-labelled test compound (e.g., caffeine or adenine) into the brain in comparison with that of a freely diffusable reference standard; i.e., $^{3}H_2O$ (10). The dpm of ^{14}C and ^{3}H in each sample and in 4-ml aliquots of the standard injection solution are derived from raw cpm using previously-prepared quench curves generated with Beckman sealed quench standards (Beckman Instruments, Inc., Palo Alto, California). The carotid injection solution containing the radionuclides was Ringer's solution buffered with 10 mM HEPES (N-hydroxy-N'-ethylpiperazine-ethanesulfonic acid, Sigma Chemical Company, St. Louis, Missouri). For experiments in which caffeine was

the labelled test compound, the BUI was calculated as follows:

$$\text{BUI (\%)} = \frac{{}^{14}\text{C dpm}/{}^{3}\text{H dpm brain tissue}}{{}^{14}\text{C dpm}/{}^{3}\text{H dpm injectate}} \times 100$$

The BUI is converted to the percent extraction (E) in a single circulatory passage by multiplying it by the actual percent extraction of $^{3}H_2O$ (86% in five seconds)(6). Thus,

$$E_{test} = BUI_{test} \times E_{H_2O}$$

For compounds like caffeine with a high degree of extraction across the blood-brain barrier, it is necessary to correct for the resulting diminution in their concentrations in order to estimate their mean concentrations in brain capillaries and to estimate their kinetic parameters of transport. Assuming the fall in caffeine's concentration would be logarithmic (11), we used the method for estimation suggested by Pardridge (12), i.e.,

$$\overline{C} = \frac{\ln(1-E)}{E} \times C_a$$

where $\overline{C}$ is the average capillary concentration and C_a is the average arterial or bolus concentration. For compounds with relatively low degrees of extraction (e.g., adenine and adenosine), it is necessary to correct for the residual radioactivity present within blood plasma or cells in order to obtain accurate assessments of transport kinetics (6) and brain uptakes. This can be accomplished by using a short-lived gamma emitter, like ^{113m}Indium ($t\frac{1}{2}$ = 100 minutes) which is chelated to ethylene-diaminetetraacetic acid (EDTA) to confine it to the intravascular space. The apparent brain extraction of ^{113m}In (or Indium BUI) is equal to:

$$\text{Indium BUI} = \frac{{}^{113m}\text{In dpm}/{}^{3}\text{H dpm brain tissue}}{{}^{113m}\text{In dpm}/{}^{3}\text{H dpm injectate}} \times 100$$

The indium BUI is subtracted from the BUI for the test compound; the difference is then multiplied by E_{H_2O} in order to obtain the test compound's percent extraction (E_{test}). Thus, for low uptake compounds like adenine and adenosine, the percent extraction is:

$$E_{adenine} = (BUI_{adenine} - BUI_{Indium}) \times E_{H_2O}$$

Radionuclides were obtained from New England Nuclear Corporation (Boston, Massachusetts) and included 8-^{14}C-adenine (NEC-055H, 45 mCi/mmol in 0.1 N HCl, Lot #1059-064), 1-methyl-^{14}C-caffeine (NEC-412, 48.5 mCi/mmol in ethanol, Lot #796-133), $^{3}H_2O$ (NET-001B), 1 mCi/g. Other radionuclides used were obtained from Amersham (Springfield, Illinois) and included U-^{14}C-adenine in aqueous solution containing 2% ethanol (286 mCi/mmol, CFA 435, batch 15) and U-^{14}C-adenosine in aqueous solution with 2% ethanol (559 mCi/mmol, CFB 54, batch 32). Indium was obtained by eluting a ^{113m}Sn generator (CEA/Sorin, Gif-Sur-Yvette, France) column with 0.1 N HCl.

Statistical analysis was performed using one-way analysis of variance and unpaired t-tests. Multiple comparisons were done using the Newmann-Keuls test.

Results

Circulating radiolabelled caffeine had a high degree of extraction across cerebral capillaries (Table I). Adding increasing concentrations (10-100 mM) of unlabelled caffeine to the injection solution caused a dose-related decrease in brain extraction of the ^{14}C-caffeine (Table I). Experiments were

performed with the addition of ^{113m}In chelated to EDTA as well to subtract for vascular radioactivity. These data are not shown, as they yielded identical results to those shown in Table I.

TABLE I

Blood-Brain Barrier Extraction of Radiolabelled Caffeine

Inhibitor	N	Bolus Concentration	% Extraction (± SD)
None	4	--	82.0 ± 4.8
Caffeine	5	10	74.9 ± 6.8
Caffeine	4	50	69.7 ± 4.8*
Caffeine	11	100	69.4 ± 12.6*

*$p < .05$ by one-way ANOVA differs from control value.

The data relating the caffeine concentration in the bolus injection to the uptake of the ^{14}C-labelled caffeine were plotted as for an enzymatic reaction with transport velocity as ordinate and substrate concentration as absicca. From the resulting inverse rectangular hyperbolic plot, we derived kinetic parameter estimates for transport of caffeine. The K_m for the saturable component of caffeine transport was 5.4 mM. Assuming normal cerebral blood flow for a barbiturate anesthetized rat of 0.56 ml/g/min (24), the maximal saturable transport velocity of caffeine would be 2.9 μM/g/min.

It seemed possible to us that this saturable component of caffeine transport was related to caffeine's structural similarity to purines or purine nucleosides, compounds which have been described as having independent transport carriers at the blood-brain barrier. We therefore examined the extent to which unlabelled caffeine could inhibit the saturable transports of ^{14}C-adenosine or ^{14}C-adenine into the rat's brain. Addition of caffeine (25 or 50 mM) to intracarotid injection solutions containing $^{3}H_2O$, ^{113m}In chelated to EDTA, and ^{14}C-adenosine failed to inhibit adenosine transport (Table II). Data for

TABLE II

Lack of Effect of Caffeine on Brain Adenosine Transport

Inhibitor	N	Bolus Concentration (mM)	% Extraction (± SD)
None	5	--	5.3 ± 2.2
Caffeine	5	24	5.0 ± 1.3

25 mM caffeine failing to inhibit ^{14}C-adenosine transport are shown in the table. Similar lack of effect on brain adenosine transport was also found for higher caffeine concentrations.

On the other hand, caffeine did cause a dose-dependent inhibition of brain ^{14}C-adenine transport (Table III). A plot of ^{14}C-adenine's transport velocity against caffeine concentration again yielded an inverse rectangular hyperbola. The data were fitted to the equation:

$$y = P_1 + P_2 e^{-P_3 t}$$

by regression analysis with a least squares method using the non-linear BMDP 3R program on an IBM 370 computer. In this equation, "y" represents the extraction of radiolabelled adenine and "t" is the rate of decline of the

saturable radiolabelled transport component with increasing unlabelled caffeine concentration. The best fit of the equation was $P_1 = 0$, $P_2 = 4.5 \pm 0.32$, $P_3 = 0.07 \pm 0.019$ (constant ± asymptomatic standard deviation). The kinetic constant (K_i) represents the concentration of caffeine needed to produce half-maximal inhibition of brain adenine transport derived from this equation and was 9.8 ± 1.5 mM.

TABLE III

Caffeine Inhibition of Brain Adenine Transport

Inhibitor	N	Bolus Concentration (mM)	% Extraction (± SD)
None	6	---	5.3 ± 1.7
Caffeine	5	0.1	4.3 ± 0.7
Caffeine	4	0.5	4.5 ± 2.0
Caffeine	5	1.0	4.1 ± 2.0
Caffeine	5	5.0	3.8 ± 1.6
Caffeine	6	10.0	2.6 ± 1.2*
Caffeine	6	25.0	0.9 ± 0.5*
Caffeine	6	50.0	1.1 ± 0.5*

*$p < .05$ by one-way ANOVA differs from control.

Discussion

These data show that caffeine enters the brain by a combination of transport mechanisms -- simple diffusion and carrier-mediated transport. A possible explanation for the saturable (carrier-mediated) transport of the drug caffeine could be its structural similarity to the nucleic acid precursors -- purines and purine nucleosides. To test this hypothesis, caffeine's ability to restrict the entry of adenosine and adenine into the brain was examined. While caffeine produced no diminution of adenosine transport, it did inhibit the blood-brain barrier transport of ^{14}C-adenine in a dose-dependent fashion. Thus, caffeine can restrict the entry of adenine (and perhaps other purines) into the brain. Very high blood caffeine levels would be required to interfere with the supply of purines to the brain from the blood, as indicated by an inhibition constant of 9.8 mM.

Burg (13) reported that peak caffeine blood levels after a 25 mg/kg dose of caffeine in four nonhuman animal species were 8-22 μg/ml (approximately 0.1 mM). Robertson et al (14) studied the effects of a 250 mg dose of caffeine (roughly 3 mg/kg) in humans and found blood levels peaked within one hour. Peak values ranged from 4.2 - 26 μg/ml. Thus, humans seem to have a lower clearance of caffeine and achieve greater blood levels for a given dose than do several animal species. Clearly, large doses of caffeine must be ingested, even in humans, to produce blood levels near the K_i for brain adenine transport estimated from these studies.

To evaluate caffeine's effects on brain adenine transport, the kinetic characteristics of the purine transport system and plasma levels of adenine must be taken into account. Cornford and Oldendorf (9) found the K_m for brain adenine transport to be 27 μM. Thus, caffeine has an affinity for the purine transport system 300 times less than adenine. Rustrum (15) recently reported an HPLC method for separation and measurement of purine and pyrimidine nucleosides and bases in rat plasma. Blood adenine levels were found to be about 8 μM. This value is somewhat higher than others in our laboratory have determined using a different HPLC system (personal communication, R. von Borstel). Nonetheless, these estimates indicate the purine carrier is not saturated with

adenine. Therefore, changing blood adenine levels or competition by other compounds for the same transport carrier would be expected to influence transport of adenine into the brain and possibly transport of other purines. This interpretation fits with studies showing adenine loading produced increases in ATP pools from which c'AMP is generated within brain parenchyma (16,17). Increasing or decreasing adenine transport into the brain could then alter the formation of c'AMP, an important neuromodulatory compound for which adenine may act as a precursor.

Our studies presented here used ^{113m}In chelated to EDTA as a vascular space marker to calculate brain extraction of ^{14}C-adenine and ^{14}C-adenosine. We confirmed the utility of this radionuclide as an important control in the study of compounds with a low rate of brain uptake. Studies on compounds like ^{14}C-caffeine with a high rate of brain extraction seemed not to show significant differences in extraction or kinetic analysis whether or not Indium was used. Since caffeine may alter vascular tone, one might ask whether changes in cerebral blood flow could explain some of our findings. The use of very short experimental times (five seconds after injection animals were sacrificed) should obviate the potential problem of altered cerebral blood flow. More importantly, compounds like caffeine and tritiated water have a similar degree of brain extraction and, thus, are unlikely to be differentially affected using the BUI technique (personal communication, W.H. Oldendorf).

The transport of adenine into the brain is known to be modified by development. Recent work by Braun et al (18) has shown that the immature blood-brain barrier extracts adenine from the blood at a rate four times that of adult animals. The higher capacity for purine transport into the neonatal brain complements the higher rate of brain nucleic acid synthesis in the fetus and newborn (19). The higher transport capacity in neonates might also produce an enhanced susceptibility to caffeine inhibition of brain purine transport, a hypothesis which can be tested.

It is not known if caffeine levels ever reach high enough concentrations to interfere with the brain's supply of adenine from the blood. It is not even certain if there is an ongoing need by the brain for an exogenous supply of purine bases (20,21). Lesch-Nyhan syndrome, a rare heritable disorder of purine metabolism, produces an interference with purine recycling with secondary effects on purine biosynthesis (22). This disorder produces neurological manifestations including a characteristic and bizzare self-mutilation. Interestingly, one manifestation of extreme chronic caffeine intoxication in rodents is self-mutilation (23). One might speculate that interference with brain purine supply or metabolism might also be common to these two situations. On the basis of the findings in this paper, further investigation into the effects of caffeine on brain purine metabolism and transport seems warranted, perhaps especially so in the neonate.

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