Comparison of blood-brain barrier permeability in mice and rats using in situ brain perfusion technique

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MATERIALS AND METHODS

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THE BLOOD-BRAIN BARRIERS (BBB) and blood-cerebrospinal fluid barriers restrict the transport of drugs from blood to brain interstitial fluid (5). The BBB consists of a continuous layer of endothelial cells joined by tight junctions. Drug distribution to the brain requires penetration through endothelial cells, and this occurs more easily for lipophilic drugs than for hydrophilic drugs (22). For drugs that are transported by passive diffusion, there is a good correlation between lipophilicity and permeation rate across the BBB (14). However, some drugs show exceptional behavior (3, 14), and this has been ascribed to the existence of specific uptake transporters, e.g., those contributing to the exceptionally high permeation of glucose and amino acids into the brain (9, 19), and specific efflux transporters, e.g., P-glycoprotein, which accounts for the low apparent permeation of vincristine and cyclosporin A into the brain (23, 25). Therefore, drug distribution to the brain cannot be predicted solely on the basis of lipophilicity. A further complication is the existence of species differences in active transport mechanisms.

To determine permeation rates across the BBB, an intraventricular injection technique (14), an in situ brain perfusion technique (28), a multiple indicator diffusion technique (24), a brain uptake index technique (18), and a brain efflux index technique (12) have been developed. Such mice should be useful for studies on drug transport and drug interactions at the BBB, and some results using the intraventricular injection technique have been reported (15, 16). However, there is still insufficient comparative information to establish the usefulness of the mouse as an experimental animal for drug distribution studies. Therefore, we decided to compare the distributions of various drugs to the brain in mice and rats in detail. For this purpose, we chose to use the in situ brain perfusion technique, because it is not necessary to consider the effects of plasma protein binding or the metabolism.

In this study, we applied the in situ brain perfusion technique to determine drug permeation across the BBB under normal physiological conditions. In addition, to examine the species differences in drug transport, we compared the permeability coefficient-surface area product (PS) values in mice and rats of drugs that are transported by passive diffusion and drugs that are transported by specific transport mechanisms at the BBB.

Reagents and animals. Iodo-[14C]antipyrine (67.5 mCi/mm), [3H]methotrexate (15.0 Ci/mmol), [3H]quinine (15.0 Ci/mmol), and [3H]quinine (14.5 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). [14C]tobutamide (63.0 mCi/mmol), [14C]mannitol (19.7 mCi/mmol), [14C]phenylalanine (44.8.0 mCi/mmol), [14C]diazepam...
Brain uptake of a drug during the perfusion is given by Eq. 4 (back diffusion was ignored) or Eq. 5 (back diffusion was considered), where \( C_{br\,art} \) is the brain arterial concentration (pmol/ml), \( K_1 \) is influx clearance (ml \cdot g^{-1} \cdot s^{-1}), and \( k_2 \) is efflux rate constant (s^{-1}). During perfusion, \( C_{br\,art} \) equals \( C_{pf} \) and is fixed, and therefore we may divide Eq. 5 by \( C_{pf} \) to obtain Eq. 6, where \( K_0 \) (ml/g) is the ratio of \( C_{brain} \) to \( C_{pf} \).

\[
dC_{brain}/dt = K_1 \times C_{br\,art} - k_2 \times C_{brain} \tag{4}
\]

\[
dC_{brain}/dt = K_1 \times C_{br\,art} - k_2 \times C_{brain} \tag{5}
\]

\[
dK_p/dt = K_1 - k_2 \times C_p \tag{6}
\]

To determine \( K_1 \) and \( k_2 \), the \( K_0 \) of the tracer was fitted to Eqs. 4 and 6 by means of a nonlinear least-squares procedure (MULTI) (30), and we judged the suitability of the equation by whichever obtained a smaller Aikaike information crite-
rion (AIC) (30). The PS values were calculated from Eq. 7 (28)

$$PS = -F_d \times \ln (1 - K_d/F_w)$$ (7)

Measurement of the octanol-water partition coefficient. The octanol-water partition coefficients were measured by the method described below. n-Octanol and 0.1 mM phosphate buffer (pH 7.4) were used as the organic and the aqueous phases, respectively. The initial concentrations of ^3H^-labeled and ^14C^-labeled compounds in the aqueous phases were 333 and 16.7 nCi/ml, respectively. An aliquot (3 ml) of drug-containing aqueous phase was mixed with an equivalent volume of octanol and shaken vigorously for 1 h at 37°C. The organic and the aqueous phases were then separated by centrifugation at 738 g for 5 min. The organic and the aqueous phases (1 ml each) were placed in a scintillation vial and prepared for scintillation counting by addition of 15 ml of scintillation cocktail. The radioactivity was measured with a liquid scintillation counter.

Data analysis. The PS values were plotted against the values of the octanol-water partition coefficients divided by the square root of the molecular weight. In this plot, the data for eight compounds reported by Takasato et al. (28), i.e., sucrose, mannitol, urea, glycerol, ethylene glycol, trimethyl-ene glycol, thiourea, and antipyrine, and the regression line for these compounds were additionally superimposed. Moreover, the PS values were compared between mice and rats. A slope, an intercept, a coefficient of correlation ($r^2$), and a $P$ value were determined by multiple regression analysis.

RESULTS

Perfusion pressure and rate. In anesthetized mice after ligation of the pterygopalatine artery, the systolic pressure was 126 ± 8 (means ± SE) mmHg, and the diastolic pressure was 113 ± 7 (means ± SE) mmHg at the carotid artery. As shown in Fig. 1, the carotid pressure increased in proportion to the perfusion rate from 0.6 to 1.2 ml/min. On the basis this relationship, a perfusion rate of 1.0 ml/min was selected for the in situ brain perfusion in this study. At this rate, the time required to replace the blood in cortical arteries with perfusate after starting the pump was 2 s ($n = 5$). Therefore, 2 s was subtracted from the total perfusion time, and the corrected perfusion times are used for calculation.

Intravascular volume and cerebral perfusion fluid flow. A shown in Fig. 2, the distribution volume of ^3H^-inulin was almost constant (11.4 ± 0.4 µl/g; means ± SE, $n = 9$) after perfusion for 15–60 s. This distribution volume of ^3H^-inulin was used to correct the intravascular volume. Cerebral perfusion fluid flow calculated from brain uptake data of ^14C^-diazepam for 5 s was 71.3 ± 6.7 µl·g$^{-1}$·s$^{-1}$ (means ± SE, $n = 3$).

Contribution of systemic blood to total flow. To determine the contribution of the systemic blood flow to the right cerebral hemisphere flow during perfusion, perfusate without the radiotracer was passed and iodo[^14C]-antipyrine was intravenously injected. The perfused mouse was killed 30 s later. The values of $A$, $B$, $D$, and $E$ were obtained by fitting the femoral arterial concentration of iodo[^14C]-antipyrine-time profile to Eq. 3 ($A = 5.38 \pm 2.58$ pmol/ml, $B = 3.74 \pm 1.15 \times 10^{-1}$ s$^{-1}$, $D = 3.75 \pm 1.72$ pmol/ml, and $E = 3.74 \pm 1.88 \times 10^{-2}$ s$^{-1}$; means ± SD, $n = 3$). By using these parameters and the parenchymal brain concentration of iodo[^14C]-antipyrine, we obtained a value of 3.2 ± 1.1% (means ± SE, $n = 3$) as the contribution of systemic blood to total flow, according to Eq. 2.

PS values of mice and rats. Figure 3 shows the brain uptake (apparent distribution volume)-time profiles of 21 drugs in mice and rats. For several drugs (e.g., phenylalanine, quinine, and thiourea), the distribution volumes increased linearly with time up to 60 s. For others (e.g., iodoantipyrine), the uptake rate decreased with time. Table 1 summarizes the values of molecular weight, octanol-water partition coefficient (pH 7.4), $K_1$, $k_2$, and PS values (Eqs. 4–7) in mice and rats. In addition, as shown in Fig. 4, we plotted the PS values in mice and rats against the values of the octanol-water partition coefficients divided by the square root of the molecular weight. The line shows the relationship for eight drugs that were transported only by passive diffusion across the BBB in rats ($\log(PS) = -1.17 + 0.848 \times \log(\text{partition coefficient/square root (molecular weight)})$; $r^2 = 0.882, P < 0.001$) in a previous study (28). In this study, drugs that were transported only by passive diffusion (e.g., sucrose, mannitol, thiourea, and iodoantipyrine) lie on the line, whereas drugs that were transported into the brain by facilitated uptake...
systems (e.g., alanine, phenylalanine, and glucose) lie above the line, and drugs that were subject to active efflux (e.g., vincristine and quinidine) lie below the line.

As shown in Fig. 5, a good correlation (1:1) was obtained between the PS values in mice and in rats \[ \log(PS_{\text{mouse}}) = (-0.499 \pm 0.0215) + (0.884 \pm 0.068) \times \log(PS_{\text{rat}}); r^2 = 0.898, P < 0.0001, \text{means} \pm \text{SE} \]. There were no significant differences in the PS values between mice and rats.

**DISCUSSION**

In this study, we used the in situ brain perfusion technique to compare the PS values of various drugs at the BBB between mice and rats. Several techniques have been established to measure PS values at the BBB quantitatively (1, 18, 28). To examine transport saturation, competition, and inhibition by altering the concentration of solute or inhibitor in the perfusate and to investigate the effects of pH, osmolality, and ionic content (21), the in situ brain perfusion technique is particularly suitable, because the PS value at the BBB can be determined accurately over a $10^4$-fold range from a minimum of $10^{-5}$ ml $\cdot$ g$^{-1}$ $\cdot$ s$^{-1}$, and the drug concentration in the fluid of brain arteries is equal to that in the perfusate. This technique has several other advantages, as described below. Because the contribution of systemic blood flow to the right
cerebral hemisphere flow was only 3.2%, serum protein binding can be neglected. The effects of metabolism in other tissues, such as the liver, can be ignored because the perfusate is introduced directly into the cerebral hemisphere. For instance, the \( PS \) values of trimethylene glycol and glycerol at the \( \text{BBB} \) measured by using the intravenous injection technique are larger than those using the in situ brain perfusion technique, because these compounds are immediately converted in the liver to glucose and \( \text{CO}_2 \), which readily permeates into the brain (4, 8, 10). Therefore, we considered that the in situ brain perfusion technique was the most suitable technique to determine the \( PS \) values at the \( \text{BBB} \).

Fig. 4. Comparison of the experimental permeability coefficient-surface area (\( PS \)) values at the \( \text{BBB} \) with the predicted values based on the octanol-water partition coefficient divided by the square root of the molecular weight. ● Data obtained from the previous report (28). The experimental values in rats (○) and mice (△) were determined by using the in situ brain perfusion technique. The correlation line for the data from Ref. 28 was also indicated (straight line). Numbers correspond to the drug numbers in Table 1 and Fig. 3. Specific transport systems were known (to be implicated) on \( \text{BBB} \) for the compounds 3, 4, or 5 (influx) and 11, 12, 13, 20, or 21 (efflux).

Fig. 5. Comparison of the \( PS \) values at the \( \text{BBB} \) of various drugs between rats and mice. Numbers correspond to the drug numbers in Table 1 and Fig. 3. A least-squares fit of the equation \( \log(PS_{\text{BBB}}) = A + B \log(PS_{\text{influx}}) \) to the data is shown (straight line). Best-fit values are \( A = -0.499 \pm 0.0215 \) and \( B = 0.884 \pm 0.068; \) correlation coefficient \( r^2 = 0.898, P < 0.0001 \). Values are means ± SE. The dashed line shows 1:1 line.
It is important to select an appropriate perfusion rate for mice. If the perfusion rate is too slow, the contribution of systemic blood flow from vertebral arteries to total flow in the right hemisphere increases, despite ligation of the right common carotid artery. In this case, the advantages of this technique, i.e., no protein binding and the fluid in brain arteries being identical with the perfusate, are attenuated. However, if the perfusion rate is too fast, the perfusion pressure in microvessels is too high and integrity of the BBB is compromised. We considered 1.0 ml/min to be an appropriate perfusion rate, because the carotid perfusion pressure generated by this rate is higher than the systolic pressure and lower than the pressure required to destroy the BBB in rats (160–190 mmHg) (11). This rate in mice (assuming that the weight of the right cerebral hemisphere is 0.13 g) is comparable with the value predicted (1.1 ml/min) from the relationship between brain weight (0.6 g) and perfusion rate (4.98 ml/min) in rats. The perfusion pressure caused by this perfusion rate was considered not to injure the BBB in mice, because the distribution volume of $[^{3}H]$luminol showed no significant change during perfusion for 15–60 s (9–13 μl/g) and was close to the brain capillary volume (8 μl/g from Ref. 20 and 6–9 μl/g from Ref. 28) previously reported. The cerebral perfusion fluid flow calculated from the brain uptake of $[^{14}C]$diazepam in 5 s was $7.13 \times 10^{-2}$ ml·g$^{-1}$·s$^{-1}$ (the cerebral perfusion fluid flow measured in rats is $6–13 \times 10^{-2}$ ml·g$^{-1}$·s$^{-1}$, see Ref. 28) and is sufficiently higher than cerebral blood flow (17). Furthermore, the contribution of systemic blood flow to total flow was only 3% in mice during the perfusion into the external carotid artery, similar to the values (3–5%) reported in rats (28). We conclude that the right cerebral hemisphere of mice was adequately perfused at this rate.

As shown in Fig. 3, the apparent distribution volume-time profiles of various drugs, obtained by in situ brain perfusion studies in mice and rats, were classified into two groups. In one group, the rate of increase of the apparent brain distribution volume did not alter with time (linear type), and in the other group, it decreased with time (saturation type), indicating that the efflux rate of the drug from the brain to the blood is rapid in the latter group. The $PS$ values at the BBB obtained in mice were plotted against the values of the octanol-water partition coefficients divided by the square root of the molecular weight (Fig. 4). As for drugs that do not have a specific transport system, it was reported that the permeability coefficients at the BBB and the octanol-water partition coefficients have a good correlation (6, 14, 27), and a correlation line was derived from Takasato’s report (Fig. 4, solid line) (28). Data for drugs that are thought to be transported only by passive diffusion, such as sucrose, mannitol, thiourea, and iodoantipyrene (28), lie on the line. On the other hand, values for drugs that are transported into the brain by facilitated diffusion, such as alanine (2), phenylalanine, and glucose (9, 19), lie above the line, whereas values of drugs that are actively transported out of the brain by P-glycoprotein, such as vincristine (25), vinblastine (7), digoxin (26), and quinidine (13), lie below the line. Similar results were obtained in rats, in accordance with previous reports (3, 18). Other drugs giving values below the line (e.g., tolbutamide and glibenclamide) might also be actively transported out of the brain. We have previously reported that tolbutamide was transported by a carrier-mediated system that differs from P-glycoprotein (28). Moreover, our preliminary studies indicate that glibenclamide may be a substrate of P-glycoprotein (unpublished data).

As shown in Fig. 5, a good correlation between the $PS$ values of drugs in mice and rats was obtained, indicating that there is no significant species difference in the permeation of drugs across the BBB between mice and rats. This observation is consistent with a prior comparison of rats and neonatal rabbits (6). However, species differences in the $PS$ values of some drugs (e.g., vincristine and warfarin) were noted in the present study. One possibility is that species differences in the transport systems of rats and mice may differ. A second possibility is that for drugs with small $K_{p}$ values, individual differences of vascular volume may influence the estimated $K_{p}$ measurement.

Recently, P-glycoprotein gene knockout mice were developed and used to investigate the transport mechanisms of drugs or toxic compounds (15, 16). In the future, knockout mice lacking other transporter genes may be available to investigate the transport mechanisms of endogenous compounds, drugs, and toxic compounds. However, the in situ brain perfusion technique in rats remains useful because of the amount of reported data available for comparison. Our findings in mice should further extend this utility.

In conclusion, a good relationship was obtained between the perfusion rate and the carotid perfusion pressure in the in situ mouse brain. In our studies, we find that a perfusion rate of the 1.0 ml/min is optimal, providing a pressure higher than the systolic blood pressure without impairing the integrity of the BBB. At this rate, the intravascular volumes and cerebral perfusion fluid flows in mice were similar to those in rats. In addition, the contribution of systemic blood to total flow in the hemisphere was very small. The $PS$ values at the BBB determined by using the in situ brain perfusion technique in mice and rats were compared with the predicted values based on the relationship between lipophilicity and molecular weight. The $PS$ values for drugs transported by passive diffusion were almost equal to the predicted values, whereas the $PS$ values of drugs transported into the brain by facilitated diffusion were greater than the predicted values, and the $PS$ values of drugs actively transported out of the brain were smaller than the predicted values. Finally, there was a very good correlation (1:1) in the $PS$ values between mice and rats, indicating that the $PS$ values of various drugs are very similar in these two species despite the likely involvement of different transport mechanisms.
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