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DIALYZABLE SERUM COFACTOR(S) REQUIRED FOR THE PROTEIN-MEDIATED TRANSPORT OF DL-PROPRANOLOL INTO RAT BRAIN*

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Abstract—To elucidate the characteristics of promotion factor(s) in rat serum required for the protein-mediated transport of drugs into the brain, we examined the brain uptake of DL-propranolol as a model drug using the *in vivo* brain uptake index (*BUI*) method in rats. The protein-mediated transport was not observed in rats injected with the buffer solution containing either various concentrations of purified rat α_1-acid glycoprotein (α_1-AGP) or rat albumin. When the filtrate from rat serum was used as an injection vehicle to which a physiological concentration of purified rat serum protein(s) was added, the protein-mediated transport of DL-propranolol was observed in the rat brain. Moreover, the ability of protein-mediated transport of DL-propranolol was reduced in rats injected with the dialyzed serum compared with the undialyzed serum. These results suggest that the dialyzable promotion factor in serum is required for the protein-mediated transport of DL-propranolol into the brain.

Although it has generally been assumed that only the drug unbound to serum protein is available for entry into the tissue [1, 2], recent reports have indicated that serum protein-bound ligands are also available for uptake into tissues such as liver [3–7] and brain [8–11]. This phenomenon is called protein-mediated transport. In our previous studies with an *in vivo* tissue-sampling single-carotid injection technique, it was also found that albumin and α_1-acid glycoprotein could help the transport of basic drugs such as imipramine and desipramine into the rat brain [12]. Besides, in our most recent studies [13] with the same method, the protein-mediated transport of DL-propranolol was also observed in the rat brain in the presence of rat serum in the injectate.

Therefore, the present studies were designed to compare the effects of crude serum proteins and purified proteins on the protein-mediated brain uptake of DL-propranolol.

MATERIALS AND METHODS

Chemical. DL-[4-^{3}H]Propranolol hydrochloride (20 Ci/mmol) was purchased from Amersham International plc; *N*-[1-^{14}C]butanol (1.0 mCi/mmol), Protosol (tissue solubilizer) and Bioflour (scintillation fluid) were purchased from New England Nuclear (Boston, MA). The human α_1-acid glycoprotein (α_1-AGP, fraction VI) and rat albumin (fraction V) were purchased from the Sigma Chemical Co., St. Louis, MO. Rat serum α_1-AGP purified from rat serum in our laboratory was used (see later). All other reagents were commercially available and of analytical grade.

Purification of rat α_1-AGP. Rat serum α_1-AGP was purified basically according to the method of Hao and Wickerhauser [14]. In brief, blood samples collected from groups of fourteen and eighteen healthy male rats were allowed to stand for 1 hr and centrifuged at 3000 rev/min for 15 min. The pooled serum was dialyzed against 0.025 M acetate buffer (pH 4.1) overnight and then was applied to a column of DEAE-cellulose which was equilibrated with the same buffer, using a pump at a rate of 55 ml/hr. The column was then washed with the same buffer and subsequently was eluted with 0.1 M NaCl/buffer (0.025 M acetate). The eluate adjusted to pH 4.0 was then applied to a column of CM-cellulose. The column was eluted by 0.125 M acetate buffer, and the eluted fractions were collected. The protein solution was concentrated by ultrafiltration through a Diaflo PM 10 membrane (Amicon Co., Lexington, MA). The purity was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Preparation of injection solution. The *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Ringer's solution (5 mM HEPES), equilibrated with 95% O_2–5% CO_2 gas (pH 7.4) and containing various amounts of human α_1-AGP, rat α_1-AGP, rat albumin, rat serum and dialyzed rat serum, was used as the injection solution. The dialyzed rat serum was obtained by dialyzing the pooled serum against the HEPES-buffered Ringer's solution (pH 7.4) at 4° overnight. The injection solution contained 5–10 μCi/ml DL[^{3}H]propranolol and 1–2 μCi/ml [^{14}C]butanol (the reference compound) in the transport studies of DL[^{3}H]propranolol. The pH values of all the injection solutions were adjusted to pH 7.4 right before the experiments.

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Brain transport studies. The brain transport of DL[^{3}H]propranolol was measured by the tissue-sampling single-carotid injection technique developed by Oldendorf [15]. Male Wistar albino rats (200–300 g) were used. The rats had free access to tap water and standard laboratory chow except before experiments when they were fasted for 24 hr. Each animal was anesthetized with intraperitoneal pentobarbital (62 mg/kg) 15 min prior to the carotid injection. This dose produced unresponsiveness to painful stimulation with preservation of spontaneous respiration (*ca.* 55–60 breaths/min). The animal was placed in a supine position, and the left common carotid artery was isolated. The test compound (DL[^{3}H]propranolol) and the internal reference compound ([^{14}C]butanol), a freely diffusible substance, were injected rapidly (~0.5 sec) into the common carotid artery as an approximately 200 μl bolus of the injection solution via a sharp 27-gauge needle. Because the rate of injection (~0.5 sec) exceeds the rate of carotid blood flow, the injection solution traverses the brain microcirculation as a bolus without significant mixing with the circulating blood [11, 16, 17]. The animal was decapitated 15 sec after injection. This period is sufficient for a single pass of the bolus through the brain, but short enough to minimize the efflux of the labeled compound from the brain or the recirculation of the labeled compound [6].

Sample analysis. The cerebral hemisphere ipsilateral to the injection was removed from the cranium, was solubilized in triplicate in 1.5 ml Protosol (New England Nuclear, Boston, MA) at 50° for overnight in an incubator, and was decolorized with 33% H_2O_2; 10 ml of Bioflour (New England Nuclear) was added before double-isotope liquid scintillation counting. An aliquot of the injection solution spiked to the control brain tissue was treated similarly. The serum concentration of α_1-AGP was determined by radial immunodiffusion [18] using rabbit antirat α_1-AGP antiserum.

Equilibrium dialysis method. The unbound fraction (*fu*) of DL[^{3}H]propranolol in the various injection solutions was measured by equilibrium dialysis at 37° using HEPES-buffered Ringer's solution (pH 7.4) in semimicrocells (Kokugo-Gomu Co., Tokyo) with semipermeable membrane (Spectrum Medical Industries Inc., Los Angeles, CA). After equilibration was attained at 6 hr, the drug concentrations in the protein side and the buffer side were measured in a liquid-scintillation spectrometer. The correction of the drug binding for the volume shift was carried out according to the method reported by Lima *et al.* [19].

Data analysis. The data analysis was carried out with the same equations, derived in other previous reports [20–22], and in our previous studies [12]. Briefly, the brain uptake index (*BUI*) was calculated as follows:

$$BUI = \frac{^3H/^{14}C \text{ dpm (in brain)}}{^3H/^{14}C \text{ dpm (in injection solution)}} = \frac{E_T}{E_R} \quad (1)$$

where E_T and E_R are the extraction ratio of the test compound and the reference compound, respectively, 15 sec after injection. The E_T or E_R represents the maximal extraction of the unidirectional influx into the brain minus the efflux of the test or reference compound during the period between the bolus flow through the brain (2–5 sec after injection) and the decapitation (15 sec after injection). With regard to the reference compound, [^{14}C]butanol, the maximal extraction ($E_{R,\max}$) was reported to be 100% [20, 21]. The relationship between $E_{R,\max}$ and the extraction at 15 sec $E_{R(15s)}$ is defined at [21]

$$E_{R(15s)} = E_{R,\max} \cdot e^{-k_{efflux} \cdot t'} \quad (2)$$

where k_{efflux} is the efflux rate constant for [^{14}C]butanol (0.665 min^{-1}) [13]. Substitution of the values for $E_{R,\max}$ and k_{efflux} and using $t' = 10$ sec (the time between bolus entry into the brain and decapitation) in Eq. 2 indicated $E_{R(15s)} = 90\%$ for [^{14}C]butanol. The test drug propranolol is retained by the brain and returns to the blood very slowly ($t_{1/2} = 5$ min) [13]. Therefore, the drug extraction ratio measured in the present studies represents the maximum extraction of the unidirectional influx into the brain.

The apparent blood–brain barrier (BBB) permeability-surface area product (PS_{app}) was calculated as follows: If we assume that only the unbound drug could be transported across the BBB, the Kety–Renkin–Crone equation [22] could be described by [12]

$$PS_{app} = PSu_{app} \cdot fu = -Q \cdot \ln(1 - E_T) \quad (3)$$

where *fu* is the unbound fraction determined *in vitro* by the equilibrium dialysis method, Q is the brain blood flow (the value measured by the efflux rate constant of [^{14}C]butanol was 0.585 ml/min/g brain) [13], and PSu_{app} is the BBB permeability-surface area product for the unbound drug in the brain capillary, which is obtained by dividing the PS_{app} value by *fu*. The simulation for E_T of DL-propranolol with the varied protein concentration was performed according to Eq. 4, which is obtained by rearranging Eq. 3.

$$E_T = 1 - \exp\left(-\frac{fu \cdot PSu}{Q}\right) \quad (4)$$

In these simulations, the *PSu* values obtained by the brain uptake studies in the absence of injectate protein, and the average *fu* values determined *in vitro* by the equilibrium dialysis method, were used (see Table 1).

Statistical analysis. All means are presented with their standard errors (mean ± SE). Student's *t*-test was utilized to estimate the significant difference with $P = 0.05$ as the minimal level of significance.

RESULTS

The effects of various concentrations of proteins in the buffer injection solution on the extraction ratio of DL-propranolol in rat brains were determined (Table 1, Fig. 1). The addition of proteins to the buffer injection solution led to a decrease in the brain extraction ratio of DL-propranolol. There were no marked disparities between the observed values and those predicted, assuming only the unbound drug was transported. In other words, as shown in

Table 1. Comparison of the transport parameters of DL-propranolol by the brain capillary of rats for the serum filtrate containing physiological concentrations of the purified serum proteins used as the carotid injection solution*

Expt. No.	Injectate (protein concn)	*BUI*† (%)	E_T	*fu*‡	PS_{app}§ (ml/min/g)	PSu_{app}‖ (ml/min/g)
(1) Buffer + human α_1-AGP	(0%)	91.92 ± 4.23	0.827 ± 0.041	1	1.027 ± 0.121	1.027 ± 0.121
	0.1%	40.30 ± 1.16	0.363 ± 0.011	0.244	0.263 ± 0.009	1.082 ± 0.039
	0.3%	23.01 ± 2.38	0.207 ± 0.021	0.127	0.144 ± 0.007	1.161 ± 0.060
(2) Buffer + rat α_1-AGP	(0%)	90.32 ± 2.85	0.812 ± 0.021	1	0.981 ± 0.123	0.981 ± 0.123
	0.01%	61.49 ± 5.84	0.553 ± 0.053	0.491	0.483 ± 0.068	0.984 ± 0.138
	0.03%	42.30 ± 3.07	0.298 ± 0.006	0.297	0.282 ± 0.027	0.949 ± 0.092
	0.1%	26.00 ± 1.57	0.234 ± 0.014	0.132	0.149 ± 0.014	1.140 ± 0.105
(3) Buffer + rat albumin	(0%)	83.06 ± 2.84	0.748 ± 0.026	1	0.813 ± 0.059	0.813 ± 0.059
	3%	64.40 ± 2.10	0.579 ± 0.019	0.544	0.509 ± 0.027	0.935 ± 0.050
	5%	63.84 ± 5.21	0.575 ± 0.047	0.507	0.511 ± 0.067	1.009 ± 0.133
	10%	58.32 ± 2.64	0.525 ± 0.477	0.441	0.444 ± 0.056	1.005 ± 0.126

* Data are expressed as mean ± SE of four rats.

† Brain uptake index (*BUI*) was calculated by

$$BUI = \frac{^3H/^{14}C \text{ dpm (in brain)}}{^3H/^{14}C \text{ dpm (in injection solution)}} = \frac{E_T}{E_R}$$

where E_T and E_R are the extraction ratio of the test compound and the reference compound respectively (see Materials and Methods).

‡ Unbound fraction in the injectate.

§ The apparent BBB permeability-surface area product (see Materials and Methods).

‖ Calculated by PS_{app}/fu.

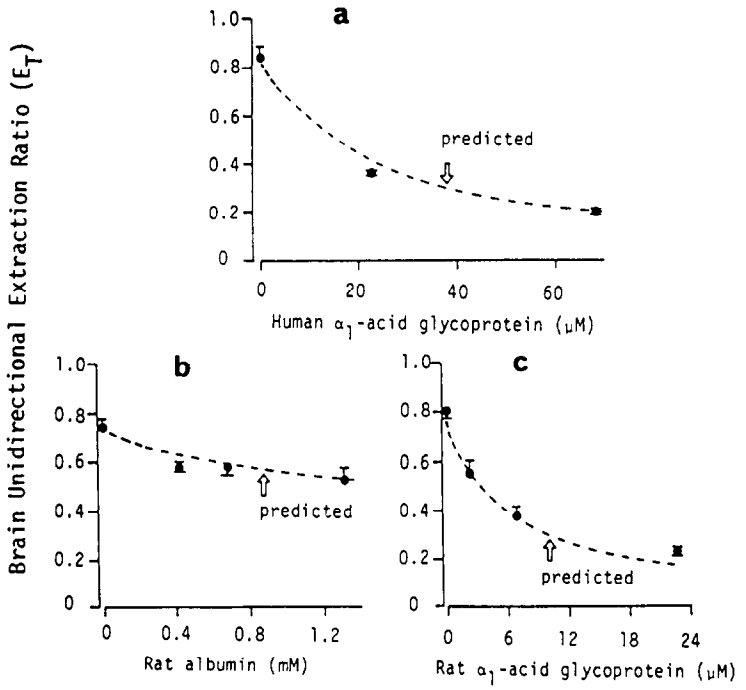

Fig. 1. Predicted and observed brain unidirectional extraction ratio (E_T) of DL-propranolol for various concentrations of proteins in carotid injection solution. Panel a: human α_1-acid glycoprotein; panel b: rat albumin; and panel c: rat α_1-acid glycoprotein. The closed circle represents the observed value (mean ± SE, N = four rats per point), and the dotted line was calculated by Eq. 4. The value of *fu* was determined *in vitro* by an equilibrium dialysis method, and the value of *PSu* was determined by the brain transport study in the absence of injectate proteins.

Table 2. Parameters for the brain uptake of DL-propranolol for the serum filtrate containing physiological concentrations of the purified serum proteins as the injectate*

Injectate	*BUI* (%)	E_T	*fu*	PS_{app} (ml/min/g)	PSu_{app} (ml/min/g)
Filtrate (F)	70.47 ± 2.20	0.634 ± 0.020	0.960	0.605 ± 0.030	0.653 ± 0.032
F + α_1-AGP	76.07 ± 5.27	0.685 ± 0.048	0.737	0.746 ± 0.073	1.013 ± 0.098†
F + albumin	65.97 ± 2.17	0.594 ± 0.020	0.595	0.552 ± 0.026	0.928 ± 0.043‡
F + (α_1-AGP + albumin)	55.14 ± 2.63	0.496 ± 0.024	0.348	0.425 ± 0.025	1.221 ± 0.071‡

* Data are expressed as mean ± SE for four rats. The concentrations of α_1-AGP and albumin were 15 mg/dl and 3.3 g/dl respectively.
† $P < 0.05$, compared to filtrate (F).
‡ $P < 0.01$, compared to filtrate (F).

Table 1, the addition of these purified serum proteins to the buffer injection solution did not change the PSu_{app} value of propranolol. This is contrary to our previous findings [13] that the PSu_{app} value for propranolol was increased by the coinjection of rat serum. These results suggested that the protein-mediated brain uptake of propranolol can be seen only for serum but not for purified proteins. Therefore, the effects of various proteins added to the serum filtrate for the injection solution on the brain transport of DL-propranolol were examined, and the results are summarized in Table 2. The transport parameters (BUI, E_T and PS_{app}) were reduced owing to the decrease in the unbound fraction of drug caused by addition of the serum protein, except for the case of the injection solution containing only α_1-AGP. However, the PSu_{app} value corrected for the binding increased significantly when the protein(s) was added to the serum filtrate for the injection solution. The effect of the dialyzed serum on the brain transport of DL-propranolol is shown in Table 3. Compared to the case of the undialyzed serum, the brain transport parameters, particularly the PSu_{app} value, for DL-propranolol were reduced significantly by the injection of the dialyzed serum.

DISCUSSION

Previous studies with the *in vivo* tissue-sampling single-carotid injection method have shown that the transport of DL-propranolol into rat brain can be mediated by rat serum [13]. As shown in Fig. 1, the protein-mediated transport, however, could not be observed in rats receiving the buffer injection solution containing purified proteins (human α_1-AGP, rat albumin or rat α_1-AGP). On the other hand, using the filtrate from rat serum as an injection vehicle to which a physiological concentration of purified rat serum protein(s) was added, the PSu_{app} value of DL-propranolol in rat brain was increased significantly, suggesting the protein-mediated transport of DL-propranolol (Table 2). Furthermore, the extent of protein-mediated transport of DL-propranolol was reduced, when the dialyzed serum was used instead of the normal rat serum as the injection solution (Table 3). These results may suggest that the unknown dialyzable substance(s) in the rat serum would be required for the protein-mediated transport of DL-propranolol in rat brain. There have been some reports suggesting the protein-mediated transport of metabolic substances [3, 5, 10], hormone [8], and drugs [4, 7, 9], into the liver and the brain. Some investigators [5, 23] suggested that the protein-mediated transport phenomenon may be attributed to the reduction of the binding affinity of ligand to serum proteins via the interactions of the complex of ligand–serum protein with the cell surface. If this speculation is correct, these interactions between the DL-propranolol–serum protein(s) complex with the surface of capillary endothelial cells may demand a certain substance(s) in rat serum as a cofactor.

Our previous studies [12] and other studies [11] showed that the phenomenon of the protein-mediated brain uptake could be observed for some ligands such as imipramine, steroid hormones, when

Table 3. Effect of dialyzed serum on the transport parameter of DL-propranolol by the brain capillary of rats*

Injectate	*BUI* (%)	E_T	*fu*	PS_{app} (ml/min/g)	PSu_{app} (ml/min/g)
Buffer	73.23 ± 2.11	0.659 ± 0.019	1	0.640 ± 0.030	0.640 ± 0.030
Serum	51.99 ± 3.75†	0.468 ± 0.034†	0.140	0.389 ± 0.035‡	2.801 ± 0.239‡
Dialyzed serum	37.92 ± 4.02‡§	0.341 ± 0.036‡§	0.186	0.265 ± 0.029‡§	1.423 ± 0.159‡‖

* Data are expressed as mean ± SE of four rats.
† $P < 0.05$, compared to buffer.
‡ $P < 0.01$, compared to buffer.
§ $P < 0.01$, compared to serum.
‖ $P < 0.05$, compared to serum.

serum protein(s) was used as the injectate. The studies by Pardridge *et al.* [9] showed this phenomenon for the brain uptake of *l*-propranolol in the buffer injectate containing human α_1-AGP. In the present studies, however, we could not find the protein-mediated transport for DL-propranolol in the buffer injectate containing purified serum protein(s) (Fig. 1). The reason for this discrepancy is not known at present. It may be related to the differences in either rat strains or the ligands used. That is, Pardridge *et al.* [9] used l-propranolol and Sprague–Dawley rats whereas we used DL-propranolol and Wistar rats.

The PSu_{app} value of DL-propranolol in rats injected with serum filtrate containing a physiological concentration of purified rat albumin and α_1-AGP was lower than that in rats injected with rat serum (Tables 2 and 3). Such a lower PSu_{app} value may be the result of a lack of the other components like β-lipoprotein which are one of the binders in serum for DL-propranolol [24]. It is also possible that the protein configuration may be altered during the purification procedure, so that these purified proteins could not exert the same effect as did the crude proteins in serum.

As shown in Table 3, the PSu_{app} value of DL-propranolol was reduced by the dialyzed serum. This result does not seem to be due to the increase in the unbound fraction caused by the volume change accompanied by dialysis, since the PSu_{app} value still yields a lower calculated value (1.89 ml/min/g brain), even if the *fu* value is assumed unchanged.

In summary, when the normal rat serum, dialyzed rat serum or rat serum filtrate containing purified serum proteins was used as the injection vehicle, the protein-mediated transport of DL-propranolol was observed in rat brain, but the degree of protein-mediated transport was much greater for normal rat serum than for dialyzed serum and serum filtrate. In contrast, when the buffer solution containing purified serum proteins was used as the injection vehicle, no protein-mediated transport of DL-propranolol was found. These findings suggest that the dialyzable promotion cofactor(s) in serum is required for the protein-mediated transport of DL-propranolol.

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