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The effect of polysorbate 80 on brain uptake and analgesic effect of D-kyotorphin

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Summary

The effect of the non-ionic surfactant, polysorbate 80, on the blood-brain barrier was investigated by using an in situ brain perfusion technique. It was confirmed that the brain perfusion technique allowed quantitative measurement of the cerebrovascular transport of drugs with varying degrees of lipophilicity and D-glucose. A linear relation was obtained between cerebrovascular PA products of drugs and their octanol/water partition coefficients. Transport of D-glucose, which is known to be a carrier-mediated process, was observed to be concentration-dependent and the K_m, V_{max} and K_d values could be obtained by non-linear least-squares regression. It was shown in perfusion experiments that the intravascular volume calculated from [^{14}C]inulin was increased significantly by intravenous administration of polysorbate 80. The content of D-kyotorphin also showed a tendency to increase. These effects of polysorbate 80 were also confirmed by the analgesic effect of D-kyotorphin in mice in vivo. D-Kyotorphin at 300 mg/kg showed no analgesic activity whereas it did display a significant level following administration with polysorbate 80. These results suggested that polysorbate 80 affected the blood-brain barrier and enhanced the brain uptake and analgesic activity of D-kyotorphin.

Introduction

The transport of material between blood and tissue within the central nervous system is different from that within most other organs. In contrast to systemic capillaries, brain microvessel endothelial cells are joined together by intercellular tight junctions (zonulae occludens), contain few endocytic vesicles, and are devoid of fenestrae (Rapoport, 1976; Reese and Carnovsky, 1967). Therefore, these endothelial cells restrict the transport of water-soluble solutes into brain and form the transport barrier between blood and brain. This barrier system is generally called the blood-brain barrier. Although the uptake of water-soluble substances is restricted, there are specific carrier systems for nutrients necessary for the brain, such as amino acids, hexoses, amines, purines, nucleosides and monocarboxylic acids (Pardridge and Oldendorf, 1977). Additionally, some hydro-

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philic drugs such as α-methyldopa, L-DOPA, melphalan and baclofen are transported by the neutral amino acid transport system (Pardridge, 1985; Van Bree et al., 1988; Greig et al., 1987). However, most of the water-soluble drugs do not penetrate sufficiently deeply into the brain to exert their pharmacological actions.

It has been reported that non-ionic surfactants may increase biomembrane permeability to several drugs: Florence et al. reported that the non-ionic surfactant, polysorbate 80, increased the brain level of methotrexate (Azmin et al., 1985b). The brain uptake of methotrexate was also enhanced by entrapment in non-ionic surfactant vesicles (Azmin et al., 1985a). Some other substances have been demonstrated to enhance the brain uptake of drugs (Nishihata et al., 1985; Banks and Kastin, 1983; Eibl, 1984). In order to develop effective and safe enhancers for drug delivery to the brain, the quantitative measurement of their effect and the characterization of the mechanism of action are necessary. In particular, not only pharmacokinetical but also pharmacodynamical evaluation is thought to be important.

Kyotorphin is an analgesic dipeptide (L-Tyr-L-Arg) isolated originally from bovine brain and its analgesic activity is based on the release of endogenous Met-enkephalin (Takagi et al., 1979). Kyotorphin is hydrolyzed rapidly by enzymes in serum (Matsubayashi et al., 1984) after systemic administration and is considered not to cross the blood-brain barrier because of its hydrophilicity. Therefore, kyotorphin shows no analgesic activity after systemic administration, although it does exhibit potent activity following intracisternal injection. The analogue, D-kyotorphin (L-Tyr-D-Arg), is also a Met-enkephalin releaser (Takagi et al., 1982). It has more potent analgesic activity, since it is stable in serum and brain homogenate due to its resistance to degradation by enzymes (Matsubayashi et al., 1984).

In the present study, we have carried out a detailed investigation of the effect of polysorbate 80 on the blood-brain barrier through the use of an in situ brain perfusion technique. In addition, we have also examined the analgesic activity of D-kyotorphin for the purpose of its delivery to the brain.

Materials and Methods

Permeability of drugs across the blood-brain barrier

Drug permeability through the blood-brain barrier was evaluated by the in situ brain perfusion technique (Takasato et al., 1984). Briefly, male Wistar rats weighing 210–260 g were anesthetized with intraperitoneal sodium pentobarbital. The right occipital and superior thyroid arteries were ligated and cut. After ligation of the pterygopalatine artery, the right external carotid artery was catheterized for retrograde infusion. Just before starting perfusion, the right common carotid artery was ligated and the perfusion fluid (142 mM NaCl, 28.0 mM $NaHCO_3$, 4.2 mM KH_2PO_4, 1.7 mM $CaSO_4$, 1.0 mM $MgSO_4$, 6.0 mM glucose) containing 0.5–1.0 μCi/ml ^{3}H-labeled drug and 0.3 μCi/ml [^{14}C] inulin was infused retrogradely into the right external carotid artery by an infusion pump (JP-V-W, Furue Science, Tokyo). [^{14}C]Inulin is a marker of intravascular space. The infusion rate was 5.0 ml/min. At this flow rate, no damage was caused to the blood-brain barrier and mixing of the perfusion fluid with the systemic blood flow was minimized. Regional perfusion fluid flow rate was measured using [^{3}H]diazepam prior to perfusion experiments. After about 50 s perfusion, the rat was decapitated and the brain was removed from the skull. The right cerebral hemisphere was dissected, weighed and digested at room temperature in 0.5 ml of Soluene 350 (Packard, Downer Glove, IL). Liquid scintillation cocktail (Univer Gel II, Nakarai Chemical, Kyoto) was added and the radioactivity was determined on a liquid scintillation counter (LSC900, Aloka, Tokyo). The content of each tracer was calculated with the appropriate correction being made for counting efficiency and quenching. Cerebrovascular PA products were calculated from the brain tracer concentration corrected for intravascular tracer with [^{14}C]inulin.

Effect of polysorbate 80 on the blood-brain barrier

Rats were prepared for perfusion experiments as described above. 9% (w/v) polysorbate 80 solution (5 ml/kg) was administered intravenously via the right femoral vein. 10 min after administration, the perfusion experiment was initiated and

the intravascular volume and PA products were obtained as described above.

Partition coefficient of D-kyotorphin

Water-saturated octanol (3 ml) was added to perfusion fluid (3 ml) containing D-[^{3}H]kyotorphin (L-[^{3}H]Tyr-D-Arg, 1 μCi/ml) and D-kyotorphin (1 mg/ml) and tubes were incubated at 37 °C. Tubes were then subjected to 6 periods (each of 30 s duration) of vigorous shaking at 5-min intervals. After the final period of shaking, tubes were incubated at 37 °C for 1 h. During this incubation time, D-kyotorphin remains stable (Matsubayashi et al., 1984). Samples of the aqueous (0.1 ml) and octanol (1 ml) phases were taken and prepared for scintillation counting. The partition coefficient was calculated using the following equation:

$$\text{Partition coefficient} = {}^3\text{H dpm}_{\text{octanol}} / \left(10 \times {}^3\text{H dpm}_{\text{water}}\right)$$

Analgesic effect of D-kyotorphin

The analgesic effect of D-kyotorphin was evaluated via the writhing test. Male mice (ddY strain, 20–22 g) were administered intravenously via the tail vein with D-kyotorphin in 6% polysorbate 80 (0.15 ml) and then 3% acetic acid (0.4 ml) intraperitoneally. Writhing syndromes were counted for 10 min beginning at 5 min after injection of 3% acetic acid. For control groups, physiological saline, D-kyotorphin or 6% polysorbate 80 were administered intravenously.

Reagents

[^{14}C]Inulin and [^{3}H]tyrosine were purchased from New England Nuclear (Boston, MA). [^{3}H]Diazepam was obtained from Amersham-Searle (Arlington Heights, IL). D-[^{3}H]Kyotorphin (spec. act. 11.3 Ci/mmol) was synthesized from [^{3}H]tyrosine using immobilized enzyme (Kitabatake et al., 1987). All other reagents used were of analytical grade commercially available.

Results

In situ brain perfusion technique

Mean values for regional cerebral perfusion fluid flow calculated from the brain uptake of [^{3}H]diazepam are listed in Table 1. Perfusion fluid flows in the left hemisphere were significantly smaller, suggesting that the right hemisphere was mainly perfused. In order to assess the validity of the technique, cerebrovascular PA products of some drugs of varying degrees of lipophilicity (6-mercaptopurine, 5-fluorouracil, acyclovir,

TABLE 1

Cerebral perfusion fluid flows in right and left hemisphere

Each value represents the mean ± S.E. of at least 4 animals. Perfusion fluid flow was calculated from perfusion experiment with [^{3}H]diazepam as (dpm/g brain)/(dpm/ml perfusate)/(perfusion time 15 s)

Brain region	Perfusion fluid flow (ml/s per g tissue)
Cerebral cortex	0.1121 ± 0.0051
Hippocampus	0.1080 ± 0.0070
Amygdala	0.1038 ± 0.0065
Striatum	0.0703 ± 0.0102
Thalamus	0.1016 ± 0.0093
Midbrain	0.0708 ± 0.0080
Hypothalamus	0.0948 ± 0.0122
Right hemisphere	0.1025 ± 0.0037
Left hippocampus	0.0051 ± 0.0024
Left hypothalamus	0.0110 ± 0.0018

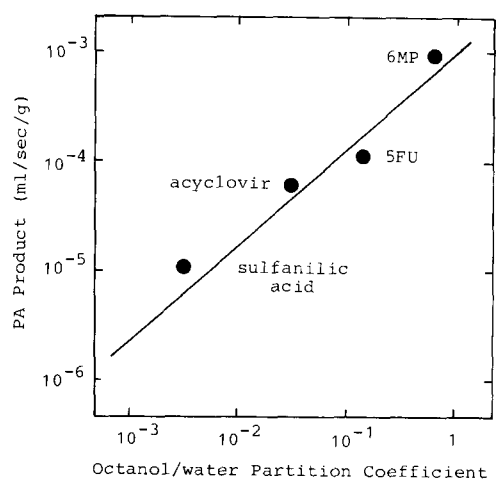

Fig. 1. Relation of cerebrovascular PA products to octanol/water partition coefficients. Each point represents the mean of 3 animals. 6MP, 6-mercaptopurine; 5FU, 5-fluorouracil. The line is the least-squares fit and is given as log PA = −2.96 + 0.917 × log(partition coefficient).

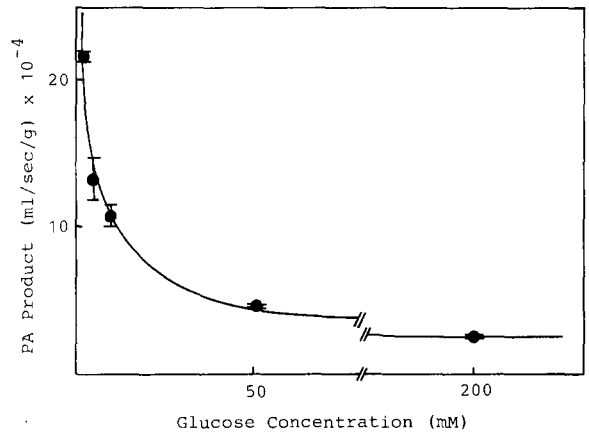

Fig. 2. Saturable glucose transport across the blood-brain barrier in cerebral cortex. Each point represents the mean ± S.E. of 3 animals. The curve was predicted from best-fit parameters (K_m = 6.02 mM, V_{max} = 13.29 nmol/s per g, $K_d = 1.98 \times 10^{-4}$ ml/s per g).

TABLE 2

Kinetic constants for blood-brain barrier transport of [^{3}H]glucose

K_m, V_{max} and K_d represent half-saturation constant, maximum transport rate and passive diffusion constant, respectively. There are no statistically significant differences among brain regions.

Brain region	K_m (mM)	V_{max} (nmol/s per g)	K_d (10^{-4} ml/s per g)
Cerebral cortex	6.02 ± 1.92	13.29 ± 2.64	1.98 ± 0.34
Hippocampus	6.02 ± 0.51	14.85 ± 0.81	2.00 ± 0.09
Amygdala	4.62 ± 1.08	11.19 ± 1.66	1.95 ± 0.22
Striatum	8.54 ± 2.39	20.06 ± 3.89	1.78 ± 0.37
Thalamus	6.50 ± 0.92	16.89 ± 1.60	2.18 ± 0.20
Midbrain	8.02 ± 1.27	16.83 ± 1.86	1.56 ± 0.19
Hypothalamus	6.16 ± 1.08	11.04 ± 1.38	1.60 ± 0.16
Right hemisphere	6.70 ± 1.54	14.50 ± 2.11	1.91 ± 0.24

sulfanilic acid) were determined and the relation between the partition coefficient and PA value is shown in Fig. 1. It is known that the brain uptake of drugs of low molecular weight depends directly on the lipophilicity and a linear relationship between the cerebrovascular permeabilities and octanol/water partition coefficients is obtained (Rapoport et al., 1979; Levin, 1980; Takasato et al., 1984). The line in Fig. 1 was obtained from a least-squares fit as log PA = $-2.96 + 0.917 \times \log P_c$. Additionally, cerebrovascular transport of D-glucose, which is transported by facilitated diffusion, was also determined. Fig. 2 illustrates the relation between PA and D-glucose concentration. The PA value decreases with increasing glucose concentration. The kinetic parameters were derived by fitting these data to the following equation using the non-linear least-squares regression program 'MULTI' (Yamaoka et al., 1981).

$$PA \times C_{pf} = \frac{V_{max} \times C_{pf}}{K_m + C_{pf}} + K_d \times C_{pf}$$

where V_{max}, C_{pf}, K_m and K_d represent the maximum transport rate, D-glucose concentration in the perfusion fluid, half-saturation constant and the constant for passive diffusion, respectively. The results are set out in Table 2. K_m is a little lower than the published value obtained by the brain uptake index method (Pardridge, 1983). It has been reported that the K_m for neutral amino acids was overestimated by the brain uptake index method due to mixing of the injectate with circulating blood (Smith et al., 1985) and efflux of amino acids from the brain. The K_m value obtained may be reasonably satisfactory.

Permeability of D-kyotorphin through the blood-brain barrier

The cerebrovascular PA products of D-kyotorphin derived from perfusion experiments

TABLE 3

Regional cerebral PA values for D-[^{3}H]kyotorphin

Each value represents the mean ± S.E. of 3 animals.

Brain region	PA (10^{-4} ml/s per g)
Cerebral cortex	5.34 ± 0.08
Hippocampus	6.15 ± 0.43
Amygdala	6.26 ± 0.27
Striatum	6.40 ± 0.35
Thalamus	6.38 ± 0.41
Midbrain	4.12 ± 0.85
Hypothalamus	4.93 ± 0.92
Right hemisphere	5.37 ± 0.22

are listed in Table 3. The PA values are very low (5×10^{-4} ml/s per g tissue) and there are no differences between brain regions. The octanol/perfusion fluid partition coefficient of D-kyotorphin was determined to be 0.0215 ± 0.00094. The PA value expected from this coefficient based on the linear relation (Takasato et al., 1984) amounts to 3×10^{-4} (ml/s per g tissue), in agreement with the obtained one.

Effect of polysorbate 80 on the blood-brain barrier

Apparent intravascular volumes (% v/w) calculated from [^{14}C]inulin are shown in Fig. 3. On administration of polysorbate 80, the apparent intravascular volume showed a significant increase of 25%. These results showed that polysorbate 80 increased the brain uptake of inulin which was used as intravascular marker with an M_r value of 5000. The brain contents of D-kyotorphin (% v/w) are also shown in Fig. 3. The brain content also had the tendency to increase.

Effect of polysorbate 80 on the analgesic effect of D-kyotorphin

D-Kyotorphin exhibited no significant analgesic activity following administration at 100 mg/kg despite being administered with polysorbate 80. However, after administration at 300 mg/kg, the writhing number fell drastically, decreasing by 44% (Fig. 4). Additionally, this decrease disappeared following s.c. administration of 50 μg/kg naloxone (data not shown). D-Kyotorphin (300 mg/kg) alone exhibited no analgesic activity and polysorbate 80 exerted no influence on the evaluation of the analgesic effect. These results suggested that D-kyotorphin showed the analgesic effect by enhancement of the brain uptake by polysorbate 80.

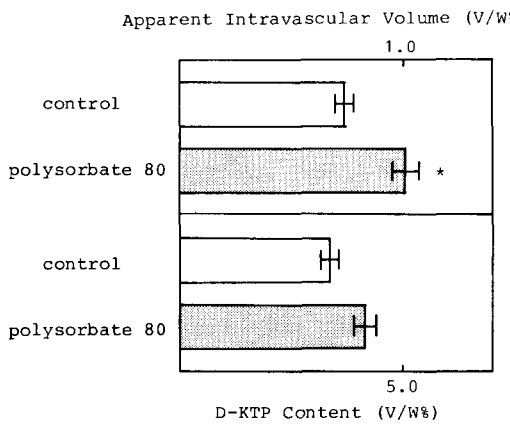

Fig. 3. Effect of polysorbate 80 on apparent intravascular volume (upper) and D-kyotorphin (D-KTP) content (lower) after brain perfusion experiment. Each column represents the mean ± S.E. of 3 animals. * $p < 0.05$ vs control.

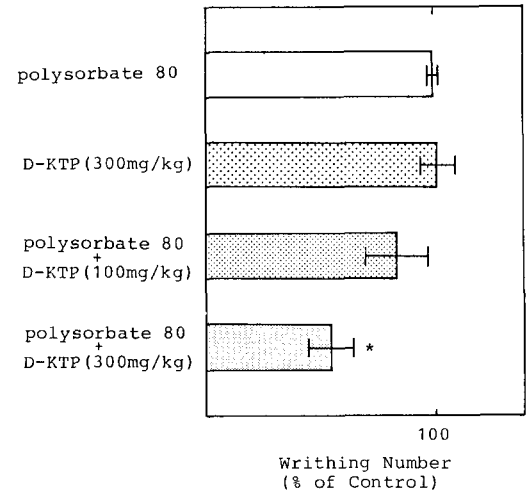

Fig. 4. Effect of polysorbate 80 on analgesic effect of D-kyotorphin. Data are expressed as % of control (saline i.v.). Each column represents the mean ± S.E. of at least 3 animals. * $p < 0.05$ vs control.

Discussion

In this report, we have examined the effect of polysorbate 80 on the blood-brain barrier by the in situ brain perfusion technique and the analgesic effect of D-kyotorphin in vivo. In situ brain perfusion is a newly developed method possessing several advantages. This technique allows the quantitative measurement of the cerebrovascular permeability of solutes spanning a wide range of lipophilicity. Since HCO_3-buffered physiological saline is perfused, absolute control of the solute concentration in brain capillary is possible. Changes of cerebral blood flow directly affect drug disposition in the brain but cerebral perfusion fluid flow is believed to remain constant when using an infusion pump. Therefore, this

technique is most suitable for evaluation of carrier-mediated transport. The K_m value for neutral amino acid transport obtained by the brain uptake index method was higher than that yielded by in situ brain perfusion, due to mixing of injectate with blood and efflux of amino acids from the brain during transit through the tissue. A limitation of this method is, perhaps, the perfusion time. The perfusion time is limited to 1 min during perfusion with HCO_3-saline and 5 min during that with whole or artificial blood, since long perfusion may cause hypoxia (Takasato et al., 1984). Therefore, it appears that a slight change in blood-brain barrier permeability in a pathological condition or under the influence of other drugs cannot be detected easily.

The results demonstrated that the uptake of [^{14}C]inulin was increased significantly and that of D-kyotorphin showed a tendency to increase. Since surfactants are one of the most important groups of adjuvants in pharmaceutical preparations, the effect of polysorbate 80 on intestinal absorption was studied extensively. The intestinal absorption of lipophilic drugs was increased when the polysorbate 80 concentration was below the critical micellar concentration (CMC) but decreased above the CMC owing to the formation of an unabsorbable micelle-drug complex. The increased degree of absorption at concentrations below the CMC was due to an increase in permeability of the biological membrane (Gibaldi and Feldman, 1970). Intestinal absorption of salicylic acid was also enhanced by pretreatment with polysorbate 80, suggesting that polysorbate 80 exerts a direct effect on biological membranes (Kaneda et al., 1974). The effect of polysorbate 80 on the blood-brain barrier is perhaps based on the direct action of polysorbate 80 on the plasma membrane of endothelial cells. Oleic acid, the metabolite of polysorbate 80, has been shown to alter membrane permeability (Azmin et al., 1985a). In our experiments, the participation of oleic acid in this process cannot be ignored, however the size of its contribution will be small, since all experiments were performed 10 min (a comparatively short time) after administration of polysorbate 80.

The results also showed that polysorbate 80 enhanced the analgesic effect of D-kyotorphin. In this case, we must consider two factors; the penetrability of D-kyotorphin into brain parenchyma and its stability in plasma and brain interstitial fluid. Since D-kyotorphin is stable in plasma and brain homogenate, as mentioned above, the latter factor does not require to be taken into account. Hence, the permeability of D-kyotorphin was affected by polysorbate 80.

The ED_{50} value of intracisternal D-kyotorphin was determined as 22.8 ng/mouse by the writhing test (Shiomi et al., 1981). If polysorbate 80 affects the blood-brain barrier in the same way, 100 mg/kg of D-kyotorphin should exhibit an analgesic effect by polysorbate 80. We considered the possibility that D-kyotorphin might bind to some proteins in plasma and that binding saturation might occur after a dose of 300 mg/kg has been administered. We determined the plasma protein binding of D-kyotorphin by ultrafiltration. In the plasma of mice administered with 300 mg/kg D-kyotorphin, the fraction of free D-kyotorphin was about 100% and no different from that in 100 mg/kg. It is the concentration in the receptor site that determines the pharmacological action of drugs. 100 mg/kg D-kyotorphin may be insufficient even when polysorbate 80 is administered.

It has been reported that other substances such as salicylate (Nishihata et al., 1985), aluminium (Banks and Kastin, 1983; Kim et al., 1986) and alkyl glycerides (Eibl, 1984) enhance brain uptake of drugs. Salicylate increases the brain concentration of cefmetazol, the increase being dependent on the brain non-protein sulfhydryls (Nishihata et al, 1985). Salicylate is also known to increase intestinal absorption of a number of drugs, the underlying mechanism being believed to involve a decrease in non-protein sulfhydryls in the enterocyte. Aluminium also enhances the uptake of proteins and sucrose (Banks and Kastin, 1983; Kim et al., 1986). It is suspected to be one of the causes of senile dementia of the Alzheimer type, however the mechanism is poorly understood. Alkyl glycerides (Eibl, 1984) are newly synthesized for the enhancement of biomembrane permeability. The effect on the blood-brain barrier is short-lived (1 min). It is not reasonable to use these enhancers from the standpoint of the physiological significance of the blood-brain barrier, however short-

acting ones such as alkyl glycerides may be applicable if used appropriately.

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