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Blood-brain-barrier Transport of Lipid Microspheres Containing Clinprost, a Prostaglandin I_2 Analogue

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Abstract

Because the permeability of the blood-brain barrier to lipid microspheres (LMs) has not hitherto been demonstrated, blood-brain-barrier permeability to LM containing the prostaglandin I_2 analogue clinprost has been evaluated for an in-vitro system of primary cultured monolayers of bovine brain capillary endothelial cells (BCECs), by a capillary depletion study in rats and by an in-situ brain perfusion study in normal and 4-vessel-occluded fore brain ischaemic rats.

Although energy-dependency was not observed in [^{3}H]clinprost uptake by BCECs, in accordance with results for simple diffusional transport, uptake of [^{3}H]clinprost contained in lipid microspheres (denoted [^{3}H]clinprost(LM)) was significantly inhibited by the endocytosis inhibitor, dansylcadaverine. The transport of LM into BCECs by endocytosis was also confirmed by fluorescence microscopy and flow-cytometric analysis using LM labelled with a fluorescent probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). The absolute uptake of DiI(LM) by BCECs, measured by HPLC, was, however, almost 1/10 that of [^{3}H]clinprost(LM), results which suggest the superiority of simple diffusion of clinprost over endocytosis of its LM form in the uptake of clinprost(LM) by BCECs. In the capillary-depletion study with rat-brain-perfused [^{3}H]clinprost(LM) from the internal carotid artery, the parenchyma apparent distribution volume was about 45 times larger than that of the capillary, showing that [^{3}H]clinprost(LM) was transported through the blood-brain barrier into the brain. The permeability coefficients of [^{3}H]clinprost and [^{3}H]clinprost(LM) determined by in-situ brain perfusion in normal rats were considerably higher than those of the active metabolite [^{3}H]isocarbacyclin and its LM form. In addition, the Blood-brain-barrier permeabilities to [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms in ischaemic rats were almost identical to those in normal rats.

It was concluded that clinprost(LM) was transported through the blood-brain barrier by endocytosis of LM, simple diffusion of clinprost released from LM, and transport of isocarbacyclin generated by hydrolysis of clinprost. The blood-brain-barrier permeability of clinprost(LM) is not reduced in ischaemic conditions, because the simple diffusion of clinprost released from LM contributed mainly to clinprost(LM) transport.

Lipid microspheres (LMs), oil droplets consisting of soybean oil and lecithin, are used as carriers for lipophilic drugs; successful pre-clinical (Yamaguchi et al 1984; Sim et al 1986) and clinical (Mizushima & Hoshi 1993) use of this dosage form have recently been achieved. Mizushima et al (1987) demonstrated that administration of the stable prostaglandin I_2 analogue, clinprost (Shibasaki et al 1983) in lipid microspheres dramatically increased its in-vivo antithrombotic activity compared with that of the corresponding aqueous solution. It has been reported that after intravenous injection in animals LM are accumulated in the inflammatory area and injured vascular lesions (Mizushima & Hoshi 1993). LM uptake by cultured human umbilical vein endothelial cells has also been demonstrated using LM containing a fluorescent probe (Suzuki et al 1992). These pharmaceutical characteristics of LM would explain the enhanced biological activity of clinprost.

It was recently shown that clinprost(LM) has therapeutic efficacy in cerebral infarction (Hoshi & Mizushima 1990) and in peripheral vascular disorders. The permeability of the blood-brain barrier to LM has not, however, been clarified. In this study we have examined the blood-brain-barrier permeability of clinprost(LM) and its transport mechanisms in the in-vitro system of primary cultured bovine brain capillary endothelial cells (BCECs) and in in-vivo studies using normal and ischaemic rats.

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Materials and Methods

Chemicals

[11β^3H]Clinprost (sp. act. 0·68 TBq mmol^{-1}) was donated by Teijin (Tokyo, Japan) and [11β^3H]isocarbacyclin was obtained by hydrolysis of [^{3}H]clinprost in alkaline solution as described previously (Minagawa et al 1994a). The chemical structures and labelling positions of [^{3}H]clinprost and [^{3}H]isocarbacyclin are shown in Fig. 1. Soybean oil (Ajinomoto, Tokyo, Japan) and egg yolk lecithin (Asahi Chemical Industry, Tokyo, Japan) were intravenous grade. [^{14}C(U)]Sucrose (sp. act. 0·1 GBq mmol^{-1}) was obtained from New England Nuclear (Boston, MA, USA). Diisopropylfluorophosphate (DFP) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Wako Pure Chemicals (Osaka, Japan); dansylcadaverine was obtained from Fluka Chemie (Buchs, Switzerland). All other chemicals were commercial products of reagent grade.

Preparation of LM formulation

Emulsions of 10% (w/v) soybean oil-in-water with 1·2% (w/v) lecithin with mean diameters of approximately 200 nm

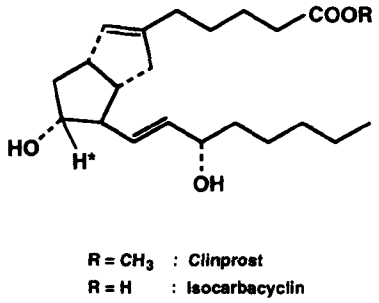

FIG. 1. Chemical structures of [^{3}H]clinprost and [^{3}H]isocarbacyclin (*^{3}H labelling position).

containing [^{3}H]clinprost (2 μg mL^{-1}), isocarbacyclin (equivalent to 2 μg mL^{-1} [^{3}H]clinprost) or DiI (100 μg mL^{-1}) were prepared with a French press (Aminco Instrument, MD, USA) by the method described previously (Minagawa et al 1994b). The particle-size distribution was determined by photon correlation spectroscopy (Nicomp Model 370, Nicomp Instruments Division Pacific Scientific, CA, USA).

Uptake experiments using primary cultured BCECs

The isolation and culture of BCECs and the uptake studies were performed using methods described by Terasaki et al (1991a). BCECs isolated from the cerebral grey matter of bovine brains were cultured on 4-well multi-dishes (2 cm^2; Nunc, Denmark) at 37°C in 95% air–5% CO_2 until the cells reached confluence (10–12 days). This was followed by the uptake studies. The primary cultured BCECs were washed with incubation solution (3 × 1·0 mL) of composition (mM): NaCl 141, KCl 4·0, $CaCl_2$ 2·0, H_2SO_4 1·0, HEPES 10, and D-glucose 10, pH 7·4, at 37°C or 4°C. The cultured cells were preincubated at 37°C or 4°C for 10 min in the incubation solution (250 μL). Immediately after preincubation the solution was removed by suction, and incubation solution (250 μL) containing [^{3}H]clinprost (35 nM), [^{3}H]isocarbacyclin (35 nM), the LM forms diluted 150 times (35 nM [^{3}H]clinprost or [^{3}H]isocarbacyclin) or [^{14}C]sucrose (0·1 mM) was added to each well. To study the effects of the inhibitors DFP (1·0 mM), NaN_3 (10 mM), dansylcadaverine (500 μM) or unlabelled clinprost (35 μM), preincubation and incubation were performed in the presence of each inhibitor or unlabelled drug. At the designated time cells were washed with ice-cold incubation solution (3 × 1·0 mL) to terminate the uptake reaction. Cells in each well were solubilized in NaOH (1 N; 300 μL) at room temperature for 60 min. Samples (100 μL) were withdrawn for determination of the protein content by the method of Lowry et al (1951) using bovine serum albumin (BSA; Wako) as standard. The remaining solution in each well was neutralized with HCl (5 N; 60 μL) and transferred to a counting vial containing Clear-Sol I scintillation fluid (4·0 mL; Nacalai Tesque, Kyoto, Japan). Its radioactivity was measured by liquid scintillation counting (LSC-1000; Aloka, Tokyo, Japan).

Uptake was expressed as the cell-to-medium concentration ratio (R):

$$R = A_{app}/C - V \quad (1)$$

where A_{app} is the apparent uptake of ^{3}H-labelled substrate (mg protein)$^{-1}$ in BCECs, C is the substrate concentration in the medium, and V is the volume of medium absorbed by BCECs (mg protein)$^{-1}$ measured using [^{14}C]sucrose.

Analysis of DiI-LM uptake by primary cultured BCECs

DiI-LM uptake into the cultured BCECs was determined using the method of Suzuki et al (1992), with minor modifications. Briefly, BCECs cultured on dishes (3·5 cm diameter, 8 cm^2; Costar, Cambridge, MA, USA) were incubated in DiI-LM diluted 20-fold (5 μm DiI mL^{-1}; 1·0 mL). The preincubation and incubation conditions in the presence or absence of inhibitors were as described above. BCECs were washed with ice-cold incubation solution (3 × 2·0 mL) to terminate the uptake and were incubated in phosphate-buffered saline (PBS; 0·5 mL) of composition (mM): NaCl 136·9, KCl 2·68, Na_2PO_4 8·1 and KH_2PO_4 1·47, pH 7·4) containing trypsin (0·05% w/v) and EDTA (0·02% w/v). The cells were suspended in PBS (2·0 mL) containing BSA (1·0% w/v) and centrifuged at 2000 rev min^{-1} for 5 min at 4°C. After washing three times the cells were fixed with 2·0% (w/v) formaldehyde in PBS for 30 min at 4°C for flow-cytometric analysis. DiI-LM-labelled cells were analysed on an EPICS-XL flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an argon laser, at excitation and emission wavelengths of 488 and 570 nm, respectively. Mean fluorescence intensity was obtained from 5000 BCECs in each sample. Appropriate cell fractions for analysis of BCECs were selected by a gating method with two-dimensional display of the forward scatter and side scatter of analysed cells (Saltzman et al 1975). The cut-off fluorescence intensity was set so that 99% of the control cell autofluorescence was negative. DiI-LM uptake by BCECs was analysed by sigma minus plot.

The absolute uptake of DiI-LM by BCECs was measured by high-performance liquid chromatography (HPLC). DiI was extracted from BCECs with isopropanol by the method of Stephan & Yurachek (1993). The DiI extracts were evaporated to dryness, the residues were dissolved in mobile phase (200 μL) and 100-μL volumes were assayed with a Jasco (Tokyo, Japan) HPLC system comprising an AS-350 auto-injector, a Tri Rotor-V pump and an 821-FP fluorescent detector (excitation 525 nm, emission 565 nm). The reversed-phase isocratic assay was performed on a 4·6 × 150 mm, 5 μm Wakopak column (Wako) with 0·1% trifluoroacetic acid in acetonitrile as mobile phase; the flow rate was 1·0 mL min^{-1} at room temperature.

Fluorescence microscopy

The uptake of DiI-LM by the primary cultured BCECs was visualized by fluorescence microscopy as reported previously (Suzuki et al 1992). BCECs cultured on glass cover slips were incubated with DiI-LM (1 μg mL^{-1}) at 37°C by the method described above. The cells were washed with ice-cold PBS (3 × 0·5 mL) and fixed with 2·0% formaldehyde for 30 min at 4°C. The cover slips were inverted over the slide glasses and covered with glycerol gelatine. The cells were examined by microscopy (IMT-2; Olympus, Tokyo, Japan) in phase-contrast and fluorescence modes. DiI was visualized with the standard rhodamine excitation and emission filters. Photographs were taken using an Olympus OM-4Ti camera with Fuji (Tokyo, Japan) Super G Ace 400 film.

Capillary depletion study

A capillary depletion study was performed using the method described by Shimura et al (1992). [^{3}H]Clinprost(LM) diluted 15 times or [^{14}C]sucrose (2·7 mM) were infused at 50 μL min^{-1} for 10 min from the right external carotid artery into the common carotid artery of male Fisher rats (F344; Japan Charles River Co., Shiga, Japan), 150–190 g, whose pterygopalatine, occipital and superior thyroid arteries were cauterized. After the termination of the infusion, blood (1·0 mL) was collected from the abdominal aorta to determine the plasma level of radioactivity, and the right hemisphere of the brain without the charotid plexus was isolated for the capillary depletion technique (Triguero et al 1990; Terasaki et al 1991b). The apparent distribution volume (μL g^{-1}) for [^{3}H]clinprost(LM) or [^{14}C]sucrose in the capillary and parenchyma fractions were calculated by dividing the amount of [^{3}H]clinprost(LM) or [^{14}C]sucrose (g cerebral hemisphere)$^{-1}$ by their concentration in the plasma.

In-situ brain perfusion studies in normal and ischaemic rats

The blood-brain-barrier permeability of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms was measured by the internal carotid artery perfusion method (Takasato et al 1984) in normal Fisher rats and in 4-vessel occluded forebrain ischaemic rats (Pulsinelli & Brierley 1979) as described previously (Sakata et al 1994). Both ^{3}H-labelled compound (42 nM) and [^{14}C]sucrose (27 μM) were perfused at 4·98 mL min^{-1} for 30 s from the right external carotid artery. For ischaemic rats brain perfusion was initiated 20 min after forebrain ischaemia. At the end of perfusion the rats were decapitated and the hemisphere ipsilateral to the perfused side was solubilized in 1·5 mL of Solvable (New England Nuclear) at 60°C for 3 h. The samples were decoloured with 30% H_2O_2, neutralized as described above and the radioactivity was measured by means of the LSC-1000. The blood-brain-barrier permeability, expressed as the cerebrovascular permeability surface area product (PS), was determined by use of the equation (Takasato et al 1984):

$$PS = -f_{pf} \cdot \ln[1 - q_b(c_{pf} \cdot t \cdot f_{pf})] \quad (1)$$

where f_{pf} is the perfusion rate, q_b is the amount of ^{3}H-labelled compound or [^{14}C]sucrose (g cerebral hemisphere)$^{-1}$, C_{pf} is the concentration of ^{3}H-labelled compound or [^{14}C]sucrose in the perfusate, and t is the net perfusion time (30 s). PS values of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms were corrected for the vascular space by use of the PS of [^{14}C]sucrose.

Results

Uptake of clinprost, isocarbacyclin and their LM forms by BCECs

Fig. 2 shows time-courses for the uptake of [^{3}H]clinprost, [^{3}H]clinprost(LM), [^{3}H]isocarbacyclin and [^{3}H]isocarbacyclin(LM) by the cultured monolayers of BCECs in the absence or presence of DFP, which inhibits hydrolysis of clinprost by esterase in BCECs. Although the accumulation of [^{3}H]clinprost and [^{3}H]clinprost(LM) in the absence of DFP was 16 times higher than that of [^{3}H]isocarbacyclin at 5 min, and decreased gradually up to 60 min, their uptake in the presence of DFP was time-dependent, and steady-state levels were established by 60 min. No influence of DFP was observed on the uptake of [^{3}H]isocarbacyclin. The uptake of [^{3}H]clinprost(LM) in the presence of DFP at 60 min was 1·5, 7·5 and 30 times higher than that of [^{3}H]clinprost, [^{3}H]isocarbacyclin(LM) and [^{3}H]isocarbacyclin, respectively.

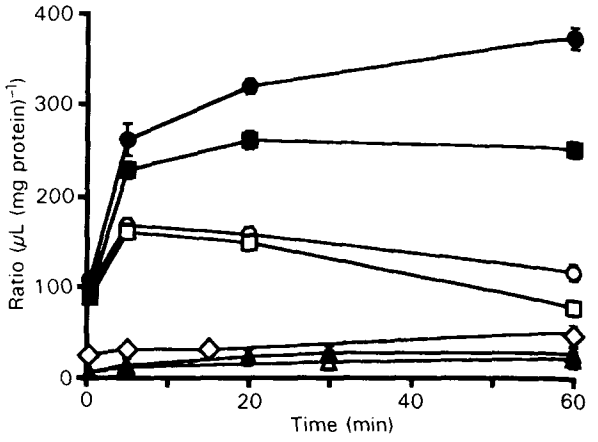

FIG. 2. Effects of esterase inhibitor DFP on uptake of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms by cultured monolayers of BCECs. Cells were preincubated at 37°C for 30 min in the absence or presence of DFP (1 mM). The uptake of [^{3}H]clinprost(LM) diluted 150 times (○, ●), [^{3}H]clinprost (□, ■, 35 nM), [^{3}H]isocarbacyclin(LM) diluted 150 times (◇) and [^{3}H]isocarbacyclin (△, ▲, 35 nM) was measured in the absence (open symbols) or presence (closed symbols) of the inhibitor. Each point represents the mean ± s.e. of three or four experiments.

The effects of temperature and inhibitors on the uptake of [^{3}H]clinprost and [^{3}H]clinprost(LM) by BCECs was determined. As shown in Table 1, the initial uptake of [^{3}H]clinprost (10 s) at 4°C was significantly lower than the control value. The activation energy, calculated from the [^{3}H]clinprost uptake at both 4°C and 37°C, was 3·8 kcal mol^{-1}, which accorded with that for simple diffusive transport (< 4 kcal · mol^{-1}). The metabolic inhibitor NaN_3, which depletes cellular ATP, and unlabelled clinprost (35 mM) did not affect the uptake of [^{3}H]clinprost. The uptake of [^{3}H]clinprost(LM) by BCECs, determined up to 20 min, decreased significantly at 4°C, and was inhibited by an endocytosis inhibitor, dansylcadaverine (500 μM; Table 2).

The uptake of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms by BCECs in human plasma was examined under conditions where [^{3}H]clinprost entrapment of LM was reduced

Table 1. Effects of temperature, metabolic inhibitor and unlabelled clinprost on the uptake of [^{3}H]clinprost by cultured monolayers of BCECs.

	Cell-to-medium ratio (μL (mg protein)$^{-1}$)
Control (37°C)	49·2 ± 2·4
4°C	25·0 ± 1·7*
NaN_3 (10 mM)	53·8 ± 0·70
Clinprost (35 μM)	51·7 ± 1·6

Cells were preincubated at 37°C or 4°C for 30 min in the absence or presence of NaN_3 or unlabelled clinprost. The uptake of [^{3}H]clinprost (35 nM) was measured for up to 10 s. Each value represents the mean ± s.e. of three or four experiments. *$P < 0·05$ compared with the control.

Table 2. Effects of dansylcadaverine and temperature on the uptake of [^{3}H]clinprost(LM) by cultured monolayers of BCECs.

	Cell-to-medium ratio (% of control)
Control (37°C)	100·0 ± 5·0
4°C	76·1 ± 4·7*
Dansylcadaverine (500 μM)	81·7 ± 2·8*

Cells were preincubated at 37°C or 4°C for 30 min in the absence or presence of dansylcadaverine. The uptake of [^{3}H]clinprost(LM) (diluted 150 times) was measured for up to 20 min. Each value represents the mean ± s.e. of three or four experiments. *$P < 0.05$ compared with the control.

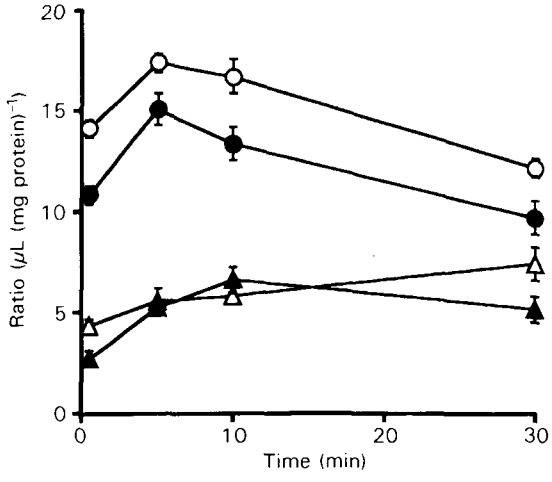

FIG. 3. Time-courses for uptake of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms by cultured monolayers of BCECs in human plasma. Cells were preincubated in the plasma for 30 min at 37°C. The uptake of [^{3}H]clinprost(LM) diluted 150 times (○), [^{3}H]clinprost (●, 35 nM), [^{3}H]isocarbacyclin(LM) diluted 150 times (△) and [^{3}H]isocarbacyclin (▲, 35 nM) was measured at 37°C. Each point represents the mean ± s.e. of three or four experiments.

by dilution of LM, protein binding and hydrolysis of [^{3}H]clinprost (Minagawa et al 1994b; Fig. 3). The time-courses for the uptake of [^{3}H]clinprost and [^{3}H]clinprost(LM) showed maximum values at 5 min and decreased time-dependently. The uptake of [^{3}H]clinprost(LM) by BCECs in human plasma at 5 min was approximately one tenth that in the culture medium (Fig. 2). The uptake of [^{3}H]clinprost(LM) at each point during 30 min was, however, from 1·2 to 1·5 times higher than that of [^{3}H]clinprost, and was from 2·5 to 5 times higher than those of [^{3}H]isocarbacyclin and [^{3}H]isocarbacyclin(LM).

Uptake mechanisms of LM by BCECs

The uptake of DiI-LM by the cultured monolayers of BCECs was evaluated by photomicroscopy, flow cytometry and HPLC. As shown in Fig. 4, phase-contrast and fluorescence photomicrographs of BCECs incubated with DiI-LM revealed a distinctive perinuclear distribution of fluorescent granules. Representative histograms of fluorescence for numbers of BCECs after incubation with DiI-LM measured by flow cytometry are shown in Fig. 5A. The proportion of cells labelled with DiI (% of positive) increased in the course of incubation up to 4 h. Fig. 5B illustrates the time-course of DiI-

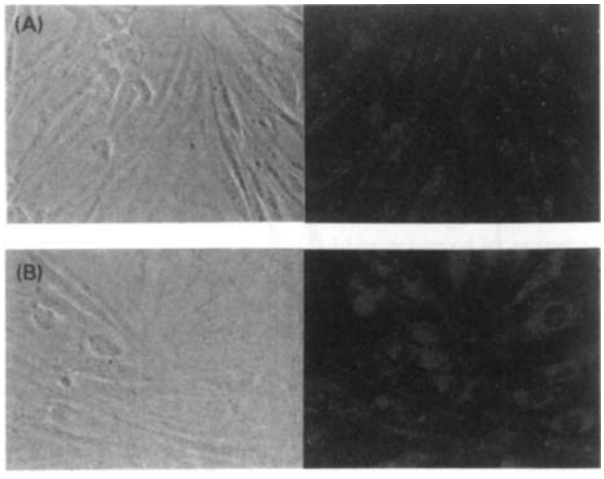

FIG. 4. Phase-contrast (left) and fluorescence (right) photomicrographs of cultured monolayers of BCECs incubated with DiI-LM (1 μg DiI mL^{-1}) at 37°C for 4 h (A) and 12 h (B).

Table 3. Effects of temperature, dansylcadaverine and unlabelled LM on the uptake of DiI-LM by cultured monolayers of BCECs.

	Percentage of cells positive (% of control)
Control (37°C)	100·0 ± 1·67
4°C	24·7 ± 0·98*
Dansylcadaverine (500 μM)	33·8 ± 5·18*
Unlabelled LM	6·93 ± 0·43*

Cells were preincubated at 37°C or 4°C for 30 min in the absence or presence of dansylcadaverine or unlabelled LM. The uptake of DiI-LM (5 μg mL^{-1}) was measured for 1 h. Each value represents the mean ± s.e. of three or four experiments. *$P < 0.05$ compared with the control.

LM accumulation by BCECs at 37°C and 4°C. The flow-cytometric time-profiles were analysed, as shown in Fig. 5C, by sigma minus plot, where the uptake after 4 h was taken as X_∞. The uptake rate constant at 4°C was almost half that determined at 37°C. Table 3 shows the effects of dansylcadaverine, unlabelled LM and temperature on the uptake of DiI-LM by BCECs. The incubation of DiI-LM with BCECs at 4°C resulted in a significant decrease in uptake to 24·7% of the control value. Dansylcadaverine and unlabelled LM also significantly reduced the uptake of DiI-LM. These results indicate that DiI-LM is transported in BCECs by endocytosis.

The absolute uptake of DiI-LM by BCECs at 37°C for up to 1 h, measured by HPLC, was 12·5 ± 0·72 μL (mg protein)$^{-1}$ (mean ± s.e., n = 3) which was almost one tenth that of [^{3}H]clinprost(LM) (data not shown).

Blood-brain-barrier permeability of clinprost(LM) in normal and ischaemic rats

The transport of [^{3}H]clinprost(LM) through the blood-brain barrier was evaluated using the capillary depletion method and the internal carotid artery perfusion method in normal and ischaemic rats. Table 4 shows the apparent distribution volume, of [^{3}H]clinprost(LM) and [^{14}C]sucrose in the rat brain parenchyma and capillary obtained in the capillary depletion

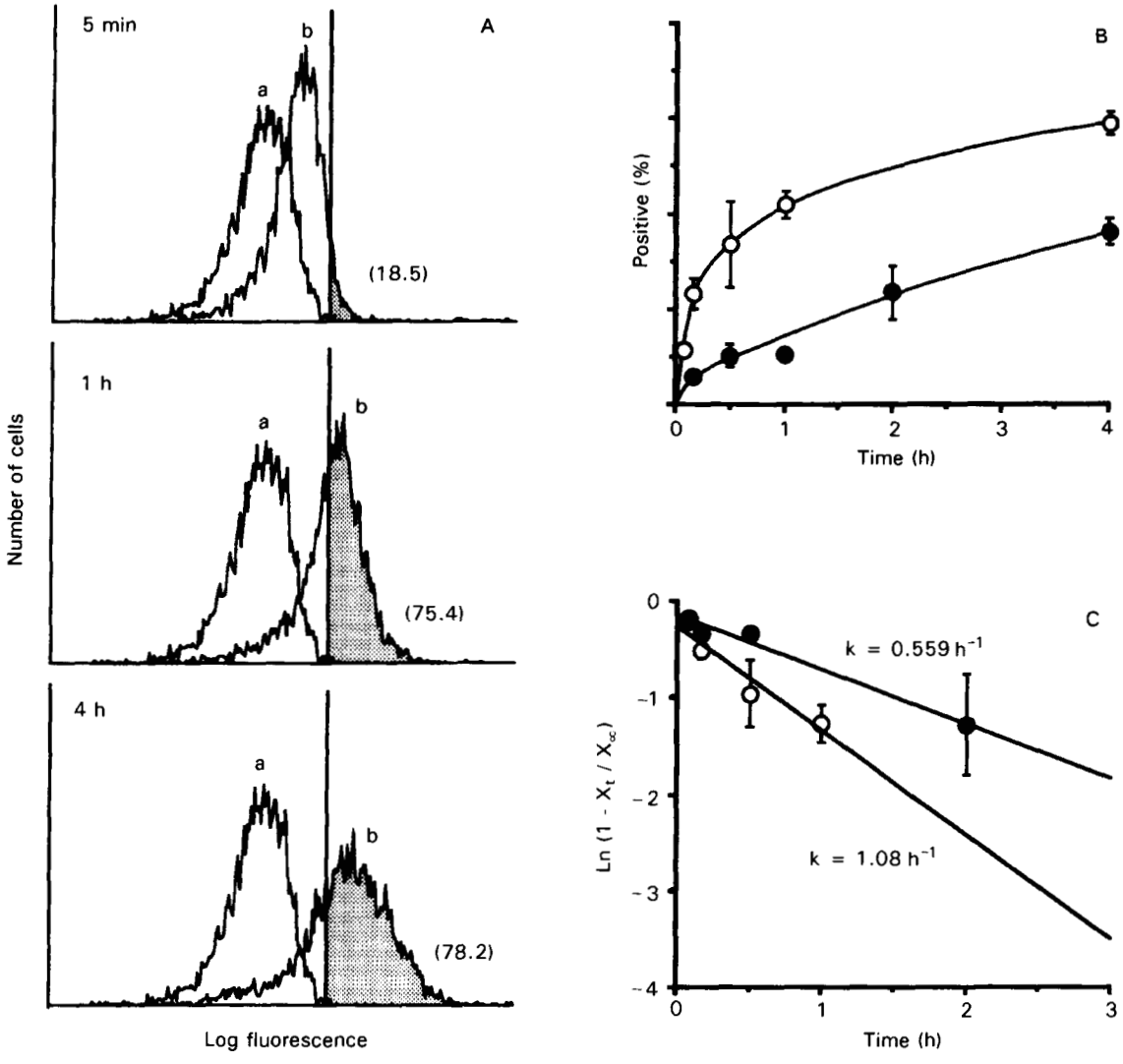

FIG. 5. Flow-cytometric analysis of DiI-LM uptake by cultured monolayers of BCECs. A. The distribution of fluorescence for cell populations incubated in the absence (a) or presence (b) of DiI-LM (5 μg DiI mL^{-1}). Each histogram represents analysis of 5000 cells from a representative experiment. The vertical lines in the figures were set to calculate percentage of positive cells as shaded area (figures in parentheses). B. Time-courses of DiI-LM uptake measured at 37°C (○) or 4°C (●). C. X_∞ for sigma minus plot was from the uptake after 4 h. Each point in B and C represents the mean ± s.e. of three or four experiments.

study. The parenchyma apparent distribution volume for [^{3}H]clinprost(LM) was about 45 times larger than that of the capillary, and was about 180 times larger than the parenchyma apparent distribution volume for [^{14}C]sucrose. These results revealed that [^{3}H]clinprost(LM) is transported through the blood-brain barrier into the brain.

Table 4. Apparent distribution volume of [^{3}H]clinprost(LM) and [^{14}C]sucrose in the rat brain hemisphere measured by the capillary depletion method.

	Apparent distribution volume (μL (g brain)$^{-1}$)	
	Parenchyma	Capillary
[^{3}H]Clinprost(LM)	4352 ± 343	95·6 ± 17
[^{14}C]Sucrose	23·7 ± 2·8	0·89 ± 0·09

[^{3}H]Clinprost(LM) diluted 15 times and [^{14}C]sucrose (2·7 nM) were infused at 50 μL min^{-1} into the internal carotid artery. Homogenate of brain hemisphere was separated into parenchyma and capillary. Each value represents the mean ± s.e. of four experiments.

As shown in Table 5, the permeability coefficients of [^{3}H]clinprost and [^{3}H]clinprost(LM) in normal rats were considerably higher than those of [^{3}H]isocarbacyclin and [^{3}H]isocarbacyclin(LM), which values were slightly higher than that of [^{14}C]sucrose. The permeability coefficients of [^{3}H]clinprost, [^{3}H]isocarbacyclin and their LM forms for ischaemic rats were closely similar to those for normal rats.

Discussion

The mechanisms of uptake of lipid microspheres in the reticuloendothelial system (Yanagikawa 1982), inflammatory lesions (Mizushima & Hoshi 1993) and human umbilical vein endothelial cells (Suzuki et al 1992) have been demonstrated to be either phagocytosis or endocytosis. Neither the blood-brain-

Table 5. Cerebrovascular permeability coefficients of [^{3}H]clinprost(LM), [^{3}H]clinprost, [^{3}H]isocarbacyclin(LM), [^{3}H]isocarbacyclin and [^{14}C]sucrose by the in-situ brain perfusion method in normal and ischaemic rats.

	Permeability coefficient (mL min^{-1} (g brain)$^{-1}$)	
	Control	Ischaemia
[^{3}H]Clinprost(LM)	5·29 ± 0·22	4·89 ± 0·48
[^{3}H]Clinprost	3·11 ± 0·37	4·54 ± 0·42
[^{3}H]Isocarbacyclin(LM)	0·156 ± 0·007	nd
[^{3}H]Isocarbacyclin	0·058 ± 0·009	0·058 ± 0·005
[^{14}C]Sucrose	0·032 ± 0·003	0·038 ± 0·001

Cerebrovascular permeability of [^{3}H]clinprost(LM) diluted 150 times, [^{3}H]clinprost (42 nM) and [^{3}H]isocarbacyclin(LM) diluted 150 times, [^{3}H]isocarbacyclin (42 nM) and [^{14}C]sucrose (27 nM) were measured at 37°C for 30 s at a perfusion rate of 4·98 mL min^{-1}. Each value represents the mean ± s.e. of 3–6 experiments. nd = not determined.

barrier permeability of LM nor its transport mechanisms have, however, been reported. This study was performed to clarify systematically the transport of LM through the blood-brain barrier, using the pharmaceutical formulation clinprost(LM), which has therapeutic efficacy against cerebral infarction (Hoshi & Mizushima 1990).

In the presence of DFP the accumulation of [^{3}H]clinprost(LM) by the cultured monolayers of BCECs at 60 min was 1·5 times higher than that of [^{3}H]clinprost. Because neither the inhibitory effect of NaN_3 on the initial uptake of [^{3}H]clinprost nor its concentration- and temperature-dependencies were observed [^{3}H]clinprost uptake was thought to occur by simple diffusion. For [^{3}H]clinprost(LM), although uptake by BCECs decreased significantly at 4°C and was inhibited by the endocytosis inhibitor, dansylcadaverine, their inhibitory effects were not remarkably high. We have reported previously that [^{3}H]clinprost was released from LM by dilution with aqueous solution (Minagawa et al 1994b). It was, accordingly, suggested that the uptake of [^{3}H]clinprost(LM) by BCECs occurred by simple diffusion of [^{3}H]clinprost released from LM, as well as endocytosis of its LM form.

It has been demonstrated that there is little liberation of DiI from labelled LM to the culture medium during uptake experiments with cultured monolayers of human umbilical vein endothelial cells (Suzuki et al 1992). The uptake of DiI-LM by BCECs decreased at 4°C and was remarkably inhibited by dansylcadaverine in the flow-cytometric studies. Fluorescence photomicrographs showed perinuclear distribution of DiI-LM, indicating strongly that DiI-LM was transported in BCECs by endocytosis. The absolute accumulation of DiI-LM in BCECs determined by HPLC was, however, almost one tenth that of [^{3}H]clinprost(LM). In addition, a microautoradiograph of BCECs incubated with [^{3}H]clinprost(LM) showed almost uniform distribution of radioactivity in cells with partial perinuclear accumulation (data not shown). These results suggest the greater likelihood of simple diffusion of [^{3}H]clinprost over endocytosis of its LM form in the uptake of [^{3}H]clinprost(LM) by BCECs. We have previously reported that [^{3}H]clinprost entrapment by LM was reduced by protein binding and hydrolysis of the drug, and by dilution of LM in serum (Minagawa et al 1994c). Uptake studies with BCECs in human plasma were, therefore, performed to elucidate phenomena occurring in the body after injection of clinprost(LM). Whereas the uptake of [^{3}H]clinprost(LM) by BCECs in human plasma decreased to about one tenth that in the culture medium, it was from 1·2 to 1·5 times that of [^{3}H]clinprost and from 2·5 to 5 times higher than that of [^{3}H]isocarbacyclin. No elevation of [^{3}H]isocarbacyclin uptake was observed in the experiment with its LM form. These findings indicate that desirable delivery to the brain could be achieved by preparing the LM form of clinprost, but not that of isocarbacyclin.

In-vivo brain-uptake studies were performed to evaluate the permeability of clinprost(LM) in normal and ischaemic rats. Perfusion of [^{3}H]clinprost(LM) from the internal carotid artery was employed in the studies, because [^{3}H]clinprost hydrolysis activity in rat blood is considerably higher than that in man, as reported previously (Minagawa et al 1995). The parenchyma apparent distribution volume for [^{3}H]clinprost(LM) obtained by the capillary depletion method was about 45 times larger than that of capillary, suggesting that [^{3}H]clinprost(LM) is transported through the blood-brain barrier into the brain. The permeability coefficients of [^{3}H]clinprost and [^{3}H]clinprost(LM), observed in brain perfusion studies in ischaemic and normal rats, were remarkably higher than those of [^{3}H]isocarbacyclin and its LM form. These observations are consistent with those of in-vitro studies using BCECs. The ATP content of the brains was depleted to 3% of the control level by 20 min ischaemia in 4-vessel occluded forebrain ischaemic rats (Ohnishi et al 1995). [^{3}H]clinprost(LM) permeability through the blood-brain barrier in ischaemic rats was, however, closely similar to that in normal rats, because the simple diffusion of [^{3}H]clinprost released from LM contributed mainly to [^{3}H]clinprost(LM) transport. It is conceivable that clinprost(LM) might have therapeutic promise for the treatment of cerebrovascular diseases in which ATP for active transport is diminished in the brain.

Attempts have recently been made to avoid the trapping of LM by the reticuloendothelial system by using surface-modified LM (Kimura et al 1986), small LM (Takino et al 1993) and negatively charged LM (Takino et al 1994). By various pharmaceutical modifications, LM will be advanced as a drug carrier for a variety of lipophilic drugs. Finally, our discovery that LM is taken up by endocytosis by BCECs will be helpful to the development of new LM formulations for drug delivery to the brain.

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