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In-vitro Characterization of Blood-brain Barrier Permeability to Delta Sleep-inducing Peptide

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Abstract—The diffusion of delta sleep-inducing peptide (DSIP) across the blood-brain barrier (BBB) has been investigated with an in-vitro model comprised of primary cultures of brain microvessel endothelial cell (BMEC) monolayers. The BMEC monolayers were mounted in a side-by-side diffusion apparatus and the transendothelial flux of DSIP analysed by HPLC with UV detection at 280 nm. The transendothelial flux of the peptide was linear with time and increasing concentrations of DSIP (non-saturable), but was not altered by reduced temperature. The apparent permeability coefficient for DSIP penetration of BMEC monolayers was in a range similar to water-soluble substances (e.g. fluorescein, fluorescein isothiocyanate dextrans) that penetrate the blood-brain barrier to a limited degree based on molecular weight. DSIP flux across the BMEC monolayers was also found to be bidirectional, insensitive to metabolic inhibitors, and not altered by high concentrations of tryptophan. Little degradation (apparent $t_{\frac{1}{2}}$ about 10 h) of DSIP to major metabolites, tryptophan (trp) and des-trp DSIP, occurred over the time of the diffusion experiments. The results of these studies support and confirm observations in-vivo indicating that intact DSIP crosses the BBB by simple transmembrane diffusion.

Until recently, significant penetration of the blood-brain barrier (BBB) by peptides and proteins has been considered unlikely (Banks & Kastin 1987). However, there now appears evidence that leu-enkephalin (Zlokovic et al 1987), insulin (Pardridge et al 1985a; Duffy & Pardridge 1987), vasopressin (Banks et al 1987a), small tyrosinated peptides with molecular weights less than 1000 (Banks et al 1987b), D-[Ala^1]-Peptide T-amide (Barrera et al 1987), and transferrin (Fishman et al 1987), have specific carrier mechanisms mediating passage of these molecules across the BBB. In addition, several neuropeptides may cross the BBB to a limited degree by non-saturable transmembrane diffusion (Banks & Kastin 1987).

The therapeutic application of peptides and proteins in the future will depend, in part, on development of a more precise understanding of the mechanisms by which these molecules might cross biological barriers (e.g. nasal and gastrointestinal mucosa, and the BBB) to effect drug delivery (Lee 1986; Audus & Borchardt 1987a). The complexity of the whole animal as an experimental model limits conclusions about cellular level transport and metabolic processes at the BBB. Thus, development of in-vitro models of epithelial and endothelial barriers to drug delivery presents an opportunity to characterize at the cellular level, mechanisms of peptide and protein transport (Audus & Borchardt 1987a; Audus et al 1987). The purpose of this study was to demonstrate the use of an in-vitro system in the characterization of the passage of a model peptide, delta sleep-inducing peptide (DSIP; Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu, mol. wt $\approx$850), across the BBB. In-vivo studies by other investigators suggest that DSIP crosses the BBB by non-saturable transmembrane diffusion (Banks & Kastin 1987).

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Materials and Methods

Bovine brain microvessel endothelial cells (BMECs) were isolated from grey matter of the cerebral cortex as previously detailed and characterized by Audus & Borchardt (1986a, 1987b).

Isolated BMECs suspended in culture medium were seeded into either 100 mm culture dishes, alone or containing translucent, 13 mm diameter, polycarbonate (PC) membrane supports with a 3·0 μm pore (Nucleopore; Pleasanton, CA). The culture dishes with and without PC membrane supports were pretreated with rat-tail collagen and fibronectin (Audus & Borchardt 1986b, 1987b). After allowing the BMECs to form confluent monolayers in culture (8–12 days after seeding), dishes containing BMEC monolayers alone were used for degradation studies and dishes containing BMEC monolayers grown on PC membrane supports were used for transendothelial transport studies described below.

Culture dishes containing BMEC monolayers were washed with PBSA three times at 37°C. Ten mL of a 110 μM concentration of DSIP in PBSA was added to washed culture dishes with BMECs (experimental) or without BMECs (control). At various times over a 5 h incubation at 37°C, samples were removed from experimental and control dishes and analysed by HPLC with UV detection as described below.

From culture dishes containing confluent BMEC monolayers grown on PC membrane supports, the PC membrane supports were removed and placed in a Side-Bi-Side (Crown Glass, Inc.; Somerville, NJ) diffusion apparatus thermostated at either 37° or 4°C. Both sample chambers of the diffusion apparatus contained 3·0 mL phosphate buffered saline (129 mM NaCl, 2·5 mM KCl, 7·4 mM Na_2PO_4, 1·3 mM KH_2PO_4, 0·63 mM $CaCl_2$, 0·74 mM $MgSO_4$, 5·3 mM glucose, 0·1 mM ascorbic acid), pH 7·4 (PBSA), constantly stirred with magnetic stirring bars at 600 rev min^{-1} driven with the diffusion apparatus consoles (Crown Glass, Inc.; Somerville, NJ). At various times after pulsing the donor chamber (i.e.

sample chamber on the side of the membrane support containing the BMEC monolayers) with an aliquot of either DSIP (Peninsula Laboratories, Inc.; Belmont, CA) or tryptophan, or both, 100 μL aliquots were removed from the receptor chamber and analysed by HPLC as described below. Equivalent amounts of fresh PBSA were added to the sample chamber to maintain a constant volume across the monolayer. In other experiments, collagen-coated PC membrane supports without BMEC monolayers were used in diffusion studies exactly as described above.

Samples were mixed with equivalent amounts of mobile phase (11% aqueous acetonitrile adjusted to pH 3·0 with H_3PO_4) and injected immediately into an HPLC system consisting of a Waters 6000A pump with a flow rate of 1·2 mL min^{-1}; Waters 440 UV-detector set at 280 nm interfaced with a Hewlett Packard 3390A integrator; and an Alltech 150 × 4·6 mm, C18, 5 μm column.

The permeability of the BMEC monolayers to a membrane impermeant molecule was assessed by pulsing the donor chamber with fluorescein or a fluorescein isothiocyanate conjugated dextran (Sigma Chemical Co.; St. Louis, MO) to obtain a 10 to 100 μM final concentration and the receptor chamber sampled at various times as described above. The 100 μL samples were diluted with 1·4 mL of PBSA and the fluorescence observed with an SLM-Aminco 4800 spectrofluorometer at 520 nm with an excitation of 490 nm. The fluorescence response of fluorescein was observed to be linear over a concentration range of 0·1 to 5 nM.

Apparent permeability coefficients were calculated by the following equation:

$$P\ (cm/min) = X/(A \times t \times C_D)$$

where P is the apparent permeability coefficient, X is the amount of substance in moles in the receptor chamber at time, t in min, A is the diffusion area (i.e., 0·636 cm^2), and C_D the concentration of substance in the donor chamber in mol cm^{-3} (C_D remains > 90% of the initial value over the time of the experiments). Data points collected at time points of 5 to 60 min (linear portion of time-dependence plots) were used to compute apparent permeability coefficients.

Results

Fig. 1 shows a sample chromatogram of standard solutions of DSIP and the major metabolite, tryptophan. The UV-detection of DSIP was linear over a working range of 0·5 to 10 μg mL^{-1}. The stability of DSIP (110 μM) when incubated in the presence and absence of BMEC monolayers in PBSA was followed for 5 h at 37°C. Little degradation of DSIP in the presence of BMECs was observed (apparent $t_{\frac{1}{2}}$ of approximately 10 h) during the 1 h of the diffusion experiments (data not shown). On incubation in PBSA without BMECs at 37°C, DSIP degradation was not observed over the same time (data not shown). Tryptophan appearance in incubation medium, when DSIP was incubated with BMEC monolayers, was observed with the amount appearing less than would be expected from the corresponding disappearance of DSIP (data not shown).

DSIP passage across BMEC monolayers grown on PC membranes was linear with time and the micromolar concentration range examined (Fig. 2). By comparison, the passage

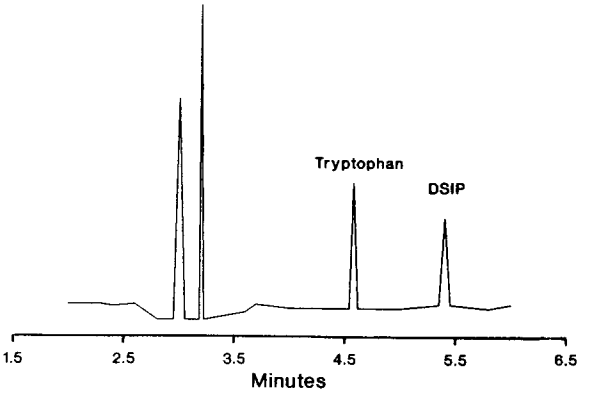

FIG. 1. HPLC chromatogram for standard solutions of delta sleep-inducing peptide (0·01 mM) and major metabolite, tryptophan (0·1 mM).

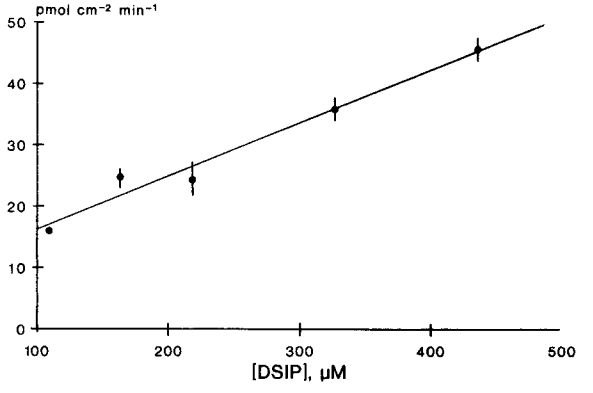

FIG. 2. Concentration-dependent flux of delta sleep-inducing peptide across brain microvessel endothelial cell monolayers grown on collagen-coated polycarbonate membranes. Data points represent the means of at least triplicate determinations ± standard error.

of a molecule known to cross the BBB by a saturable carrier-mediated mechanism, tryptophan, crosses the BMEC monolayers at a much higher rate than DSIP (Table 1). Passage of the DSIP across the monolayers was also insensitive to temperature, while tryptophan passage across the monolayers was attenuated by reduced temperature (Table 1).

The rate of DSIP passage across the monolayers was similar in both directions (i.e., forward and reverse) and was

Table 1. Influence of temperature on the apparent permeability coefficients for delta sleep-inducing peptide (DSIP) and tryptophan passage across collagen-coated polycarbonate membranes in the presence (P) and absence ($P_{W/0\ cells}$) of brain microvessel endothelial cell monolayers.

	P	$P_{W/0}$ cells	*P_{cells}
DSIP (cm $min^{-1} \times 10^4$)**			
4°C	1·2 ± 0·1	8·6 ± 0·3	1·4
25°C	1·1 ± 0·2	8·8 ± 0·7	1·3
37°C	1·1 ± 0·2	12·4 ± 0·5	1·2
Tryptophan (cm $min^{-1} \times 10^4$)**			
4°C	3·4 ± 0·2	15·9 ± 0·5	4·3
37°C	19·4 ± 1·1	30·5 ± 1·7	56·4

* $1/P_{cells} = 1/P - 1/P_{W/0\ cells}$.
** All data presented are the means of at least triplicate determinations ± standard error.

Table 2. Influence of selected treatments on the apparent permeability coefficient for delta sleep-inducing peptide (DSIP) and tryptophan passage across brain microvessel endothelial cells grown on polycarbonate membranes.

	*DSIP cm $min^{-1} \times 10^4$	*Tryptophan cm $min^{-1} \times 10^4$
Control (Forward)	1·1 ± 0·3	19·8 ± 1·1
Reverse	1·0 ± 0·1	16·1 ± 0·3
2-Deoxyglucose 50 mM	1·7 ± 0·2	19·0 ± 0·7
Tryptophan 500 μM	1·4 ± 0·1	—

* Data presented are the means of at least triplicate determinations ± standard error.

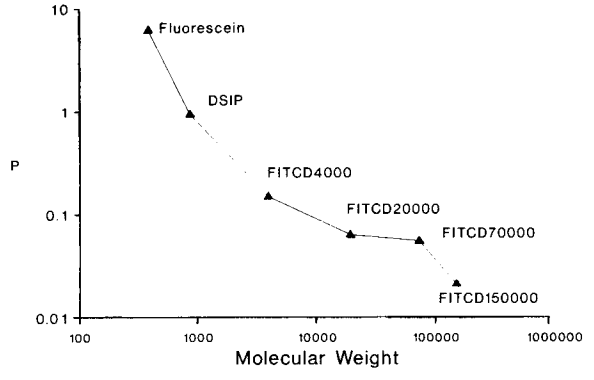

FIG. 3. Relationship between apparent permeability coefficient (P; cm $min^{-1} \times 10^4$) and molecular weight for permeation of selected substances across brain microvessel endothelial cell monolayers. Fluorescein-isothiocyanate conjugated dextrans (FITCD) with average molecular weights of 4000 to 150000 are presented as FITCD4000, FITCD20000, etc.

not substantially altered by the metabolic inhibitor, 2-deoxyglucose (Table 2). Although unlikely, apparent DSIP penetration of the BMEC monolayers by the tryptophan carrier was further ruled out by demonstration of the inability of tryptophan to alter DSIP diffusion across the monolayers (Table 2). Finally, when compared to other molecules crossing BMEC monolayers to a limited extent by passive mechanisms (e.g., fluorescein, and fluorescein-isothiocyanate conjugated dextrans), the apparent permeability coefficient for DSIP appears to be predictable based on molecular size (Fig. 3).

Discussion

The extent of DSIP degradation as a result of interactions with intact BMEC monolayers was determined to be insignificant (apparent $t_{\frac{1}{2}}$ about 10 h) within the time of the transendothelial transport studies. In contrast, rapid degradation of DSIP (i.e., the release of the N-terminal tryptophan) has been observed following incubation of the peptide with either whole rat or mouse brain homogenates (Marks et al 1977; Huang & Lajtha 1978). In a previous study, we have shown that aminopeptidase activity is expressed in primary cultures of BMEC monolayers (Baranczyk-Kuzma & Audus 1987). Therefore, the slow release of N-terminal tryptophan from DSIP may be attributable to known BMEC aminopeptidase activity. Since we used intact BMEC monolayers, however, limited penetration of DSIP into intracellular compartments expressing aminopeptidase activity may explain the differences we observe here compared to brain homogenates. Pardridge et al (1985b) have provided evidence to suggest that binding and subsequent uptake of neuropeptides at the BBB may precede effective degradation. Noteworthy also, is the fact that the stability of DSIP observed here, is consistent with the stability of DSIP found in characterization of binding sites for the peptide on intact brainstem neuron cultures (Hosli et al 1983). An alternative explanation for differences in the stability of DSIP is that the whole brain homogenates contain many different cell types which may or may not express greater degrees of aminopeptidase activity than the BBB alone. Certainly, our previous work suggests that aminopeptidase activity is not predominantly located at the BBB in homogenates of cerebral cortical gray matter (Baranczyk-Kuzma & Audus 1987).

The hydrophobic components of the secondary conformation of peptides are considered to be primary considerations in the interactions of peptides with biomembranes (Kaiser & Kezdy 1987). From NMR studies in an aqueous environment at 20°C, DSIP has been reported to exist as a folded structure with the ionogenic groups extended and the beta-COOH centrally positioned (Mikhaleva et al 1982). In part, the secondary conformation of DSIP in which the charged groups are shielded from interactions with biomembranes could be implicated to explain the ability of DSIP to undergo slow transmembrane diffusion. However, at higher temperatures other conformations of the peptide are likely to exist (Mikhaleva et al 1982) which may or may not allow favourable interactions of the DSIP with biomembranes permitting substantial transmembrane diffusion. Thus, the observed temperature-independence for DSIP permeation across the BMEC monolayers probably reflects the results of both conformational changes in the peptide as well as changes in the cell membranes.

Based upon results from in-vivo studies, demonstrating the lack of specificity and saturability of brain uptake, Banks & Kastin (1987) have suggested that intact DSIP crosses the BBB by simple transmembrane diffusion. The major metabolite of DSIP degradation (Marks et al 1977; Huang & Lajtha 1978; Hosli et al 1983), tryptophan, is normally transported across the BBB by the large neutral amino acid (LNAA) transport system (Oldendorf 1971). In this study, we confirmed that the rate of tryptophan transfer across the monolayers was much more rapid than DSIP and consistent with the kinetics of the neutral amino acid carrier system (Audus & Borchardt 1986b; van Bree et al 1988) versus simple transmembrane diffusion. In addition, the rapid rate and saturability of the passage across the BBB typical for leu-enkephalin (Zlokovic et al 1987) and vasopressin (Banks et al 1987a), and neutral amino acids (Audus & Borchardt 1986b; van Bree et al 1988), were not evident for DSIP over the concentration range examined here. The micromolar concentration range tested in this study was considered appropriate based on the kinetic studies of Pardridge et al (1985b), where the K_m for somatostatin uptake by bovine brain microvessels was approximately 76 μM. Tryptophan in concentrations five-fold greater than DSIP also failed to inhibit passage of the peptide across the BMEC monolayers indicating that the intact peptide was not recognized as a

substrate for the LNAA carrier system. Previous studies have demonstrated that amino acids compete for transport across the BMEC LNAA carrier (Audus & Borchardt 1986a).

As demonstrated in a previous study, the contribution of fluid-phase (i.e., non-specific and non-saturable) endocytosis and subsequent transcytosis to overall BMEC monolayer permeability is small and substantially decreased in the presence of either low temperature or metabolic inhibitors (Guillot et al 1987). In that study, neither metabolic inhibitors nor low temperature attenuated DSIP passage across the monolayers to the degree that would be observed if the mechanism involved fluid-phase transcytosis. Thus, potential contributions of fluid-phase transcytotic mechanisms to transport of DSIP across the BMEC were also ruled out. In addition, Gonzalez-Mariscal et al (1984) have demonstrated reversible, temperature-induced decreases (i.e. lowering the temperature from 37°C to 3°C) as great as 300%, in the permeability of epithelial cells that result from the sealing of tight intercellular junctions. The lack of temperature sensitivity observed for the permeation of DSIP through BMECs suggests that diffusion of the peptide through intercellular junctions may also not be significant.

Carrier systems exist at the BBB which facilitate transport of peptides from brain-to-blood. For example, brain-to-blood carriers at the BBB include those for small tyrosinated peptides (Banks et al 1987b) and vasopressin (Banks et al 1987a). The probable explanation that only small amounts of systemically administered vasopressin are transported from blood-to-brain across the BBB, is apparently the more rapid brain-to-blood reverse transport (Banks et al 1987a). The rate of passage of DSIP across the BMEC monolayers in both directions was observed to be similar, ruling out the possibility of different mechanisms contributing to the distribution of the peptide across the monolayers.

BMEC monolayers exhibit tight intercellular junctions in primary culture (Audus & Borchardt 1986a, 1987b). The restricted permeability of BBB endothelium to membrane impermeant molecules greater in size than La^{3+} (mol. wt 139; Stokes Radius 10 Å) is attributable to the presence of tight intercellular junctions (Bouldin & Krigman, 1975; Cserr & Bundgaard 1984). The apparent permeability coefficient for DSIP diffusion across the BMEC monolayers was determined to be lower than that of fluorescein (mol. wt 376; Stokes Radius 5·5 Å), a molecule that crosses the BBB to a limited extent both in-vivo (Gulati et al 1985) and in-vitro (Audus et al 1988). When DSIP penetration of the BMEC monolayers was compared with other relatively membrane impermeant molecules of varying molecular weights (i.e., fluorescein-isothiocyanate dextrans), in this study, permeation across the BMECs appeared to be predictable based on the molecular weight of the peptide.

Evidence here rules out carrier systems, significant intercellular diffusion, endocytosis, and bidirectional carrier mechanisms to account for the distribution of DSIP across BMEC monolayers as an in-vitro BBB model. We conclude that the results suggest slow, simple transendothelial diffusion as a mechanism by which DSIP crosses the BBB. Our results support and confirm the findings of other investigators with in-vivo models.

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