Leucine Enkephalin Effects on Brain Microvessel Endothelial Cell Monolayer Permeability

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INTRODUCTION

The transport of leucine enkephalin (YGGFL) in the basolateral-to-apical direction across the blood-brain barrier (BBB) via the small, N-tyrosinated peptide transport system has been well-characterized by Banks and Kastin (1,2) and Banks et al. (3,4). Less well-defined, perhaps, is the possibility of an apical-to-basolateral transport system (5). Opioid interactions with the BBB *in vivo* also include the observed property of YGGFL analogs to increase barrier permeability to low molecular weight, membrane impermeant molecules (6), a category which includes the peptide itself.

The mechanisms mediating apparent YGGFL-induced BBB permeability increases noted above are not precisely known. Baba et al. (6) reported that it was μ-opioids that preferentially alter BBB permeability through the μ-type receptor. Opioid agonists which bind to μ-receptors (i.e., morphine, YAGFL, and [D-Ala^2, Me-Phe^4, Gly(ol)^5] enkephalin) have been reported to stimulate neuronal histamine release (7), and recent studies have suggested neuronal histamine may be the mediator of opioid-enhanced BBB permeability (8). Therefore, opioids could mediate BBB permeability changes either through direct stimulation of a receptor or more indirectly through biochemical mediators released following receptor stimulation.

This study was prompted by the observed enhancement of the trans-brain microvessel endothelial cell (BMEC) monolayer flux of impermeant markers when attempting to elucidate the characteristics of enkephalin carriers in BMEC monolayers in our laboratory. The purpose of this note is to report observed BMEC permeability changes to the inert, membrane impermeant molecule ^{14}C-sucrose following exposure to YGGFL. A well-characterized *in vitro* model of the endothelial component of the BBB composed exclusively of primary cultures of bovine BMEC monolayers (9-13) was used to examine concentration, temperature, energy, metabolite, and opiate antagonist effects on the YGGFL-induced increases in permeability.

MATERIALS AND METHODS

Materials

^{14}C-Sucrose (350 mCi/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Leucine enkephalin and all other peptides and fragments, naloxone, and 2-deoxyglucose were purchased from Sigma Chemical Co. (St. Louis, MO). Tyr-MIF-1 was a gift from Dr. William Banks. All other reagents were of the highest grade commercially available.

Cell Isolation and Culture

Bovine brain microvessel endothelial cells (BMECs) were isolated from gray matter of the cerebral cortex as previously detailed (10-13). The purity of these BMEC cultures has been shown to be approximately 99.5% endothelial in nature (14) and the monolayers are functionally polarized in primary culture (15,16). The BMECs were stored in 10% DMSO in culture medium at -70°C until use. The BMECs were thawed, rinsed, suspended in culture medium [45% (v/v) minimum essential medium, 45% (v/v) F-12 nutrient mixture (Ham), 10% (v/v) equine serum, 100 μg/ml heparin, 50 μg/ml gentamycin, and 2.5 μg/ml amphotericin B], and seeded at 50,000 cells/cm^2 on to collagen- and fibronectin-coated polycarbonate membranes (3 μm pores) in 100 mm tissue culture dishes. Cells were then incubated at 37°C with 5% (v/v) CO_2 and 95% humidity, as previously detailed (10-13). Confluent monolayers formed within 10 to 14 days.

Transendothelial Transport Studies

Confluent BMEC monolayers on polycarbonate membranes were lifted out of the tissue culture dishes and placed into Side-Bi-Side diffusion Cells (Crown Glass Co., Somerville, N.J.). The system was thermostated at 37°C or 5°C by means of a thermal jacket and a circulating water bath. The receiver chamber was filled with phosphate buffered saline, pH 7.4, supplemented with 0.74 mM $MgSO_4$, 0.63 mM $CaCl_2$, and 5.3 mM Glucose (PBSA). The donor chamber was filled with various effector compounds dissolved in PBSA. The chambers were stirred continuously by teflon stir bars at approximately 600 rpm (11,13). At the beginning of each experiment, ^{14}C-sucrose, was added to the donor chamber and allowed to mix for approximately 2 minutes. Sample aliquots of 200 μl were withdrawn to be assayed for initial concentration values. At various times between 0 and 60 minutes, 200 μl aliquots were withdrawn from the receiver chamber to determine the flux of the marker molecule. After each sample was taken, the sample volume was replaced with fresh PBSA. Approximately 10 ml of Scintiverse E Scintillation Cocktail (Fischer Scientific, St. Louis, MO) was added to each of the radiolabelled samples. These samples were then mixed thoroughly, allowed to settle, and assayed by scintillation spectrometry (Beckman Model 6800, Fullerton, CA). Flux was determined from the slope of the linear regression line of the amount of marker transported into the receiver chamber versus time, dM/dt. Permeability coefficients for the permeation of solutes across the BMEC monolayers and polycarbonate membranes, were calculated using the equation $P = Flux / C_D / SA$, where P = apparent permeability coefficient in cm/min, Flux = dM/dt in mol/min, C_D = initial donor concentration in M, and SA = surface area available for permeation, constant at 0.636 cm^2.

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Statistics

Results were presented as means ± SEM. Statistical analysis was performed with either one-way or two-way analysis of variance (ANOVA) using Minitab 8.2 software (Minitab Statistical Software Release I, Macintosh version; Minitab Inc.; State College, Pennsylvania, PA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

A non-metabolizable, membrane-impermeant marker, ^{14}C-sucrose, was used to quantitate the transmonolayer permeability of BMEC monolayers. The probe crossed collagen-coated polycarbonate membranes, both with and without BMEC monolayers, in a linear fashion throughout the 60 minute experimental time period (data not shown). The transfer rate for ^{14}C-sucrose across collagen-coated polycarbonate membranes was significantly decreased in the presence of BMEC monolayers. The apparent permeability coefficient for ^{14}C-sucrose in this system was $3.6 \pm 0.4 \times 10^{-3}$ cm/min, in both the apical-to-basolateral and basolateral-to-apical directions. These values served as our baseline control values for BMEC monolayers at 37°C and were repeated and reproduced for each subsequent treatment below.

Leucine enkephalin significantly enhanced the transfer of ^{14}C-sucrose across BMEC monolayers relative to untreated controls, as demonstrated in Figure 1. The permeability increases were bi-directional and concentration-dependent. A maximal increase was observed at 50 μM YGGFL in both directions, corresponding to approximately 225% of the control. At concentrations greater than 50 μM, YGGFL failed to exert a significant effect on ^{14}C-sucrose permeability.

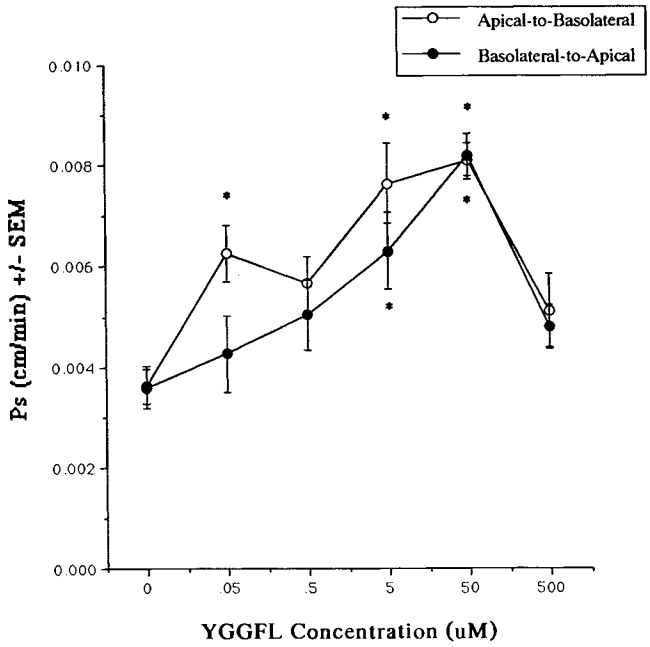

FIGURE 1. Concentration-Dependent Effects of Leucine Enkephalin (YGGFL) on the Apparent Permeability Coefficient (Ps) for the ^{14}C-Sucrose Passage Across Bovine Brain Microvessel Endothelial Cell Monolayers at 37°C. Data represent the means ± SEM for n ≥ 4 experiments. * Indicates statistically significant difference from untreated control, $p < 0.05$.

As listed in Table 1, results demonstrated the bi-directional, temperature- and energy-dependence of YGGFL's effects. While 5 μM YGGFL effectively increased BMEC permeability to ^{14}C-sucrose in both directions at 37°C, the peptide had no significant effect at either 5°C or following exposure to 50 mM 2-deoxyglucose, a metabolic poison. Results in Table 1 also indicate that only the intact YGGFL molecule effectively enhanced ^{14}C-sucrose permeability. When BMEC monolayers were exposed to 5 μM concentrations of the YGGFL metabolites tyrosine (Y), tyr-gly-gly (YGG), and gly-gly-phe-leu (GGFL), no significant effects on ^{14}C-sucrose transport in either direction were observed. Similarly, higher concentrations of these agents did not produce significant changes in sucrose transfer across the BMEC monolayers (not shown). Interestingly, the data in Table 1 demonstrated that the metabolically-stable YGGFL analog, YAGFL, stimulated ^{14}C-sucrose transfer across BMEC monolayers in both directions, to similar levels as YGGFL. Conversely, the anti-opiate peptide, Tyr-MIF-1 (YPLG-NH_2) and an analog (YAGF-NH_2), both of which share a basolateral-to-apical transport system with YGGFL, had no apparent effect on ^{14}C-sucrose transport. The δ- and μ-opioid receptor antagonist, naloxone alone, at 50 μM concentrations, had no significant effect on ^{14}C-sucrose transport across BMEC monolayers (data not shown). However, as shown in Table 1, naloxone did inhibit YGGFL's ability to enhance BMEC monolayer permeability to ^{14}C-sucrose in both directions and suggested a putative role for opiate receptors.

DISCUSSION

The application of BMEC monolayers to examine permeability properties of the endothelial component of the

Table 1. Effect of Leucine Enkephalin and Leucine Enkephalin Metabolites and Analogs on the Apparent Permeability Coefficient (P) for ^{14}C-Sucrose Passage Across Bovine Brain Microvessel Endothelial Cell Monolayers. Data represent the means ± SEM for n ≥ 4.

	P ± SE (cm/min × 10^3)	
Treatment	Apical-to-Basolateral	Basolateral-to-Apical
Studies at 5°C		
Control	2.0 ± 0.1*	1.9 ± 0.1*
+ 5 μM YGGFL	2.8 ± 0.4	2.8 ± 0.3
Studies at 37°C		
Control	3.6 ± 0.4	3.6 ± 0.5
+ 50 μM 2-deoxyglucose (2DG)	2.9 ± 0.7	3.5 ± 1.4
+ 50 μM 2DG + 5 μM YGGFL	4.1 ± 0.4	2.3 ± 0.2
+ 5 μM Y	4.3 ± 0.6	4.7 ± 0.5
+ 5 μM YGG	3.3 ± 0.8	2.3 ± 0.3
+ 5 μM GGFL	3.3 ± 0.6	4.8 ± 0.7
+ 5 μM YGGFL	5.9 ± 0.8*	6.3 ± 0.7*
+ 5 μM YAGFL	6.2 ± 0.2*	5.6 ± 0.4*
+ 5 μM YPLG-NH_2	4.1 ± 0.2	2.9 ± 0.4
+ 5 μM YAGF-NH_2	3.4 ± 0.4	2.7 ± 0.2
+ 50 μM Naloxone + 5 μM YGGFL	2.9 ± 0.3	1.7 ± 0.9

* Data statistically different from Control (37°C), $p < 0.05$.

BBB *in vitro* has been characterized and used extensively (12,13,16,17). Findings in this short study, indicated that the transfer of ^{14}C-sucrose across BMEC monolayers was significantly increased upon exposure to YGGFL. In *in vivo* studies, Baba et al. (6) demonstrated that morphine enhanced the passage of sodium fluorescein across the BBB in mice. The maximum stimulation of sodium fluorescein transfer across the BBB occurred in response to a subcutaneous, 50 mg/kg dose of morphine HCl, and resulted in a 3.6-fold increase in permeability. Other μ-opiate stimulators, including YGGFL analogs, were less effective. Considering YGGFL's preference for the δ-opiate receptor over the μ-opiate receptor, it would be expected to exhibit a less potent enhancer effect on BBB permeability and is then, consistent with our *in vitro* results. Therefore, qualitatively, the magnitude of our *in vitro* findings parallel *in vivo* reports of opioid stimulated permeability enhancement at the BBB.

Leucine enkephalin-induced changes in BMEC monolayer permeability to sucrose were both temperature- and energy-dependent. While cellular changes at 5°C resulted in significantly decreased control ^{14}C-sucrose permeability coefficients, the lower temperature also rendered YGGFL's permeability-modulating ability ineffective. Other experiments performed by incorporating 2-deoxyglucose in the donor chamber similarly demonstrated that altering cellular energy-producing processes (16) prevented YGGFL-stimulated ^{14}C-sucrose transfer. Together, these observations suggested the peptide-induced changes in BMEC monolayer permeability reflected alterations in an energy-dependent cellular mechanism.

The BBB has a substantial aminopeptidase activity associated with both the endothelial cell membrane and cytosol (18). The primary metabolites of YGGFL at the BBB *in vivo* and *in vitro* are Y and GGFL (19). Neither these YGGFL metabolites, nor YGG, had any effect on ^{14}C-sucrose transfer across BMEC monolayers, which suggested that the intact peptide was required for action. Moreover, the metabolically stable YGGFL analog, YAGFL, increased ^{14}C-sucrose transfer across BMEC monolayers in both directions to levels very similar to YGGFL. This too suggested that the intact molecule was necessary for peptide-induced changes in BMEC monolayer permeability to sucrose. We found 5 μM YAGFL caused approximately a 1.7-fold increase in ^{14}C-sucrose transport *in vitro*. This increase was comparable to the *in vivo* report by Baba et al. (6) that administration of 0.1 mg i.c.v. of YAGFL to mice resulted in a 1.6-fold increase in BBB permeability to sodium fluorescein. In the presence of naloxone, an opioid antagonist with affinity for both the μ- and δ-opiate receptors, YGGFL effects on BMEC permeability were completely inhibited. These results strengthen the argument that YGGFL modulated BMEC monolayer permeability through an opioid receptor although the type or class remains unknown. Again, this was consistent with the *in vivo* observations (6).

Tyr-MIF-1 (YPLG-NH_2), the anti-opiate peptide, and an analog that shares a basolateral-to-apical transport system with YGGFL (1-4), had no effect on the BMEC transport of ^{14}C-sucrose. Based on these findings, we would suggest that transport of YGGFL and permeability modulating effects were separate interactions of opioids with BMECs. The difference may be in the concentration ranges at which these interactions occur, with perhaps the higher YGGFL concentrations mediating the permeability effects on the BMEC monolayers.

The effects of YGGFL on BBB permeability could involve biochemical mediators released on receptor stimulation. Oishi et al. (8), for instance, demonstrated that morphine induced enhancement in the permeability of the BBB in mice was inhibited by the histamine H_2-antagonists cimetidine and ranitidine, but not by histamine H_1-antagonists. They suggested that the activation of H_2-receptors by neuronally released histamine was involved in the increased permeability observed upon administration of some μ-opiate agonists. The *in vitro* system discussed here is not an innervated system. However, histamine stores and synthetic enzymes do exist within brain endothelia (20). Furthermore, other investigators have shown that histamine-induced effects on brain capillaries are mediated by H_2 receptors (21,22). The possibility remains for opioid-induced release of histamine which might have been responsible in part for the observed increase in BMEC monolayer permeability to sucrose. In preliminary experiments, we were unable to demonstrate that cimetidine could block YGGFL's effects on sucrose transfer across the BMEC monolayers. While this observation suggests the absence of a role for histamine *in vitro,* more extensive concentration-dependent studies are required to rule out the possible role of BMEC-derived histamine as a mediator for opioid effects on BBB permeability.

In conclusion, YGGFL-stimulated increases in BMEC permeability were characterized. The permeability modulating properties of YGGFL were found to be similar to literature reports of μ-opioid agonists actions on BBB permeability in *in vivo* models. Because YGGFL can be considered a small molecule, and because permeability enhancement depends on YGGFL concentration, it is likely that YGGFL may facilitate it's own passage across the BBB at certain concentrations. Our *in vitro* studies also suggested that YGGFL alters BBB permeability through an opioid receptor mediated mechanism as observed *in vivo*. This study provides a basis for further investigation of the type of mediating opioid receptor, the role of histamine, and the transcellular permeability pathways altered by YGGFL in BMEC monolayers.

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