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PRELIMINARY REPORT

Transfer of Opiorphin Through a Blood-Brain Barrier Culture Model

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Opioid peptides are potent analgesics with therapeutic potential in the treatment of acute and chronic pain. Their efficacy is limited by peptidases (enkephalinases). Opiorphin pentapeptide (QRFSR) is the first characterized human endogenous inhibitor of enkephalinases. The peptide is able to increase the binding and affinity of endogenous opiates to mu opioid receptors; thus, the mechanism of opiorphin may provide a new therapeutic approach in pain management. The analgesic effect of opiorphin was proven in several earlier published *in vitro* and *in vivo* studies. Our aim was to test the transfer of opiorphin through a blood-brain barrier model for the first time. The flux of opiorphin was tested on a blood-brain barrier culture model consisting of rat brain endothelial, glial and pericyte cells. Brain endothelial cells in this triple co-culture model form tight monolayers characterized by transendothelial electrical resistance measurement. Relative quantity of the peptide was estimated by mass spectrometry. The transfer of opiorphin through the blood-brain barrier model was estimated to be ~3%, whereas the permeability coefficient was $0.53 \pm 1.36 \times 10^{-6}$ cm/s ($n = 4$). We also observed rapid conversion of N-terminal glutamine into pyroglutamic acid during the transfer experiments. Our results indicate that opiorphin crosses cultured brain endothelial cells in the absence of serum factors in a significant amount. This is in agreement with previous *in vivo* data showing potentiation of enkephalin-mediated antinociception. We suggest that opiorphin may have a potential as a centrally acting novel drug to treat pain. © 2015 IMSS. Published by Elsevier Inc.

Key Words: Opiorphin, Peptidase inhibitor, Blood-brain barrier, Brain endothelial cell, Permeability, LC-MS.

Introduction

Opioid analgesics are still one of the most effective drugs against pain; however, their clinical usefulness is limited by several side effects including physical dependence, respiratory depression, gastrointestinal effects and tolerance (1). New opioid peptides could have therapeutic potential for central nervous system (CNS) diseases, but they have a short half-life and low metabolic stability (2,3). Opiorphin (QRFSR) is an endogenous peptide that inhibits Zn-dependent metallo-ecto-peptidases, neutral endopeptidase (NEP EC3.4.21.11) and aminopeptidase (AP-N EC3.4.11.2) (4). These enzymes metabolize opioid peptides such as enkephalins and their derivatives *in vivo* and *in vitro* (5). Opiorphin as an enkephalinase inhibitor exerts analgesic and antidepressive effects by the protection of endogenous enkephalins released after pain stimuli (6,7). Opiorphin is the only natural enkephalinase-inhibitor characterized in humans and has similar pain-suppressive potency to morphine but without adverse effects (4,6). The efficacy of opiorphin has been verified by *in vitro* methods and its analgesic activity was also shown in different *in vivo* pain studies (4,6,8). According to our previous *in vitro* maximal binding and affinity measurements, opiorphin is able to increase the binding and affinity of endogenous opiates to opioid receptors (9).

In this study we were interested in the opiorphin transfer across the BBB. Opioid peptides have restricted penetration to the CNS across the BBB (10,11). The transfer of opioid peptides through the BBB was studied previously with isotopically labeled peptides showing a penetration index $<0.01\%$ (12–14). Specific features of the BBB, mainly interendothelial tight junctions and efflux transporters (15), as

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well as peptidase activity in blood, brain microvessels and brain tissue may be responsible for limiting the transfer of these potential biotherapeutics from the blood to brain.

The aim of the study was to test the transfer of unlabeled opiorphin across a well-characterized culture model of the BBB. Mass spectrometry was used to detect the passage of the peptide across the BBB *in vitro*.

Materials and Methods

Materials

All reagents used in the study were purchased from Sigma-Hungary Ltd. (Budapest, Hungary) unless otherwise indicated. Wistar rats were obtained from the animal facility of the BRC. All animals were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publications No. 80−23) and as approved by the local authority, Csongrád County Animal Health and Food Control Station (Permit number: XVI./834/2012).

Peptide Synthesis

Opiorphin was synthesized manually using Fmoc (fluorenylmethyloxycarbonyl) solid phase synthesis on N^{α}-Fmoc-Arg(Pmc)-Wang resin. N^{α}-Fmoc-protected amino acids were used. The side chain protecting groups used to build the peptide sequence were the following: trityl (Trt) for Gln, tert-butyl (t-Bu) for Ser and 2,2,5,7,8-pentamethylchroman (Pmc) for Arg. The couplings were performed by 1-hydroxybenzotriazole (HOBt) and N,N′-diisopropylcarbodiimide (DIC). The coupling efficiencies were monitored by the Kaiser test. The Fmoc groups were removed by a solution of 20% piperidine in dimethylformamide. After assembly of the peptide sequence, a cocktail of TFA/TIS/H_2O (trifluoroacetic acid/triisopropylsilane/water, 95: 2.5: 2.5) was used to remove the side chain protecting groups and to cleave the peptide from the resin. The resin was filtered and the filtrate was cooled at −20°C. After precipitating with diethyl ether, the peptide was redissolved in water and lyophilized. The crude peptide was purified using a semipreparative RP-HPLC column (Altima HP C_{18}, 1 cm × 25 cm, 5 μm particle size). The homogeneity of the final peptide was determined by analytical RP-HPLC (Altima HP C_{18} 0.46 cm × 25 cm, 5 μm particle size) column, retention time: 6.5 min. ESI-MS analysis confirmed the molecular mass of the peptide ($[M+H^+]_{found}$: 693.5; ($[M+H^+]_{theoretical}$: 693.4 Da).

Blood-Brain Barrier Model

Primary brain endothelial cells, astrocytes and pericytes were isolated from 1-month-old Wistar rats. Cell isolation and the preparation of the co-culture BBB model was performed as previously described (16). Brain endothelial cells and pericytes were seeded on the opposite surfaces of collagen IV and fibronectin coated Costar Transwell polycarbonate inserts (12 mm diameter, 0.4 μm pore size; Corning, Corning, NY) and kept in co-culture with glial cells to reach good barrier properties for the permeability measurements (Figure 1A). The tightness of the model was checked by transendothelial electrical resistance (TEER) measurement using an EVOM resistance meter and STX-2 electrodes (World Precision Instruments, USA). TEER of coated, cell-free filters was subtracted from measured TEER values of the BBB model. TEER of rat brain endothelial cell layers was 593 ± 47 Ω cm^2 (mean ± SD; n = 12) in agreement with our previous data (17).

Bidirectional Permeability Assay

To measure the transfer of opiorphin across the BBB model, cell culture inserts were transferred to 12-well plates containing 1.5 mL Ringer-Hepes solution (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, 25 mM glucose and 10 mM Hepes, pH 7.4) in the lower or acceptor compartments. The peptide was dissolved in distilled water to yield a 10-mM solution, which was further diluted in Ringer-Hepes buffer. In the upper or donor chambers, culture medium was replaced by 0.5 mL Ringer Hepes containing opiorphin at 10 μM concentration or permeability markers fluorescein (10 μg/mL; molecular weight: 376 Da) or

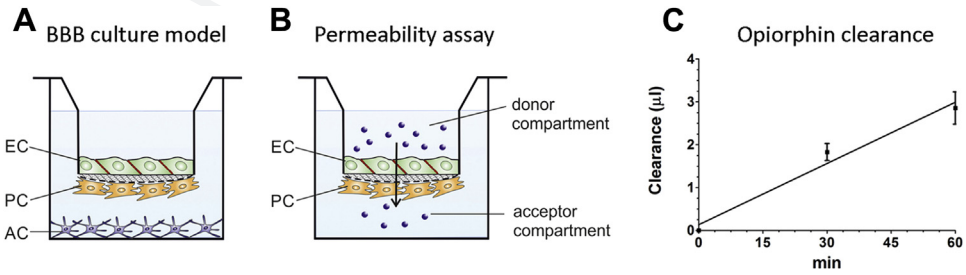

Figure 1. Culture model of the blood-brain barrier (A). The model is a co-culture of three cells types, primary rat brain endothelial cells (EC), rat pericytes (PC) and rat astrocytes (AC). In the permeability assay (B) culture inserts with EC and PC cells are used. Blue circles represent opiorphin, our test molecule. The direction of the transfer from the donor to the acceptor compartment is indicated by an arrow. Clearance of opiorphin across the culture model (C) (mean ± SD, n = 4).

bovine serum albumin (1%, molecular weight: 65 kDa) bound to Evans blue (165 µg/mL) (Figure 1B). To measure peptide flux from the upper to lower compartment (blood to brain direction) the inserts were transferred at 30 and 60 min to new wells containing Ringer-Hepes solution. Opiorphin in samples from the donor and acceptor compartments ($n = 4$) were detected by mass spectrometry. Evans blue-albumin content of samples was measured at 584 nm excitation and 680 nm emission wavelengths (Fluostar Optima, BMG Labtechnologies, Germany). Fluorescein concentrations were determined by the same instrument using 485 nm excitation and 520 nm emission wavelengths. Clearance and the apparent permeability coefficient (P_{app}) were calculated as described earlier (16) by the following equations:

$$\text{Cleared volume } (\mu l) = \frac{C_A \times V_A}{C_D}$$

where C is concentration of the peptide in the acceptor or donor compartments and V is the volume of the acceptor compartment (1.5 ml).

$$P_{app}(\text{cm/s}) = \frac{\Delta[C]_A \times V_A}{A \times [C]_D \times \Delta t}$$

where $\Delta[C]_A$ is the concentration difference of the peptide in the acceptor compartments after 1 h and C_D is the concentration in the donor compartments at 0 h, and V_A is the volume of the acceptor compartment (1.5 mL), and A is the surface area available for permeability (1.1 cm^2). The quantity of opiorphin transfer was calculated from areas under curve from the chromatograms representing the intact opiorphin peptide.

Mass Spectrometry

LC-MS experiments were performed on a nanoAcquity UPLC (Waters) on-line coupled to an Orbitrap-Elite (Thermo Scientific) hybrid tandem mass spectrometer operated in the positive ion mode. Five µl of the samples containing the peptide was injected onto a trapping column (Waters Symmetry C18; 180 µm × 20 mm, 5 µm particle size) and after washing with 1% solvent B for 5 min was transferred onto the separating column (Dionex Acclaim PepMap; 75 µm × 25 cm, 2 µm particle size, 100 Å pore size) developing a linear gradient of 1−35% solvent B in 10 min using a flow rate of 200 nl/min (solvent A: 0.1% formic acid/water; solvent B: 0.1% formic acid/acetonitrile). For MS experiments, mass range of m/z: 200−600 was monitored at resolution of 60000 using internal calibration to the background polysiloxane ion (m/z: 445.120024). Relative quantity of opiorphin was estimated using the extracted ion chromatogram (XIC) of m/z: 347.1932 (±5 ppm) peak intensity corresponding to the doubly charged opiorphin peptide cation.

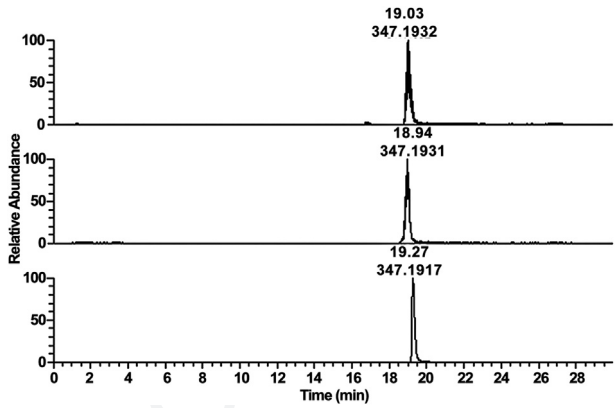

Figure 2. Extracted ion chromatograms of m/z: 347.1932 ± 5 ppm corresponding to the doubly charged peptide ion of opiorphin. Upper panel: sample from the receiver compartment ("brain side") after 30 min incubation, middle panel: sample from the receiver compartment after 60 min incubation, bottom panel: sample from the donor compartment ("blood side") after 60 min incubation. Peak labels denote retention time and m/z value.

Results

MS/MS characterization of the synthetic opiorphin peptide was performed using both collision-induced dissociation and higher energy collisional activation. The observed m/z value of the protonated peptide ions and fragmentation pattern were in good agreement with those expected for the QRFSR sequence. Signal intensities in the receiver compartment samples were $1-2 \times 10^6$ as opposed to 1.5×10^8 in the donor compartment samples (Figure 2).

The relative quantity of opiorphin that penetrated through the BBB model was calculated to be 2.91 ± 1.13% based on the area under curve of the doubly charged opiorphin peptide ion m/z 347.1932. As a comparison, 0.41 ± 0.03% of fluorescein and 0.03 ± 0.01% of albumin crossed the brain endothelial cell layers in parallel inserts from the same cell isolation under identical assay conditions.

The clearance of opiorphin is shown in Figure 1C. P_{app} of opiorphin was $0.53 \pm 1.36 \times 10^{-6}$ cm/s ($n = 4$). In parallel inserts from the same cell isolation under identical assay conditions the P_{app} of fluorescein was $1.03 \pm 0.09 \times 10^{-6}$ cm/s ($n = 4$) and that of albumin $0.07 \pm 0.04 \times 10^{-6}$ cm/s ($n = 4$).

We also observed rapid conversion of N-terminal glutamine into pyroglutamic acid both in 0.1% formic acid and in Ringer-Hepes buffer used for BBB penetration experiments. We monitored the pyroglutamic acid-containing peptide using its calculated molecular mass [monoisotopic MW: 675.3453 yielding a doubly charged protonated peptide ion m/z:338.6799 (z = 2)].

Discussion

We measured the transfer of opiorphin across a BBB culture model for the first time. The resistance data and P_{app}

values for passive hydrophilic permeability markers fluorescein and albumin were in accordance with our previous data and indicated a tight barrier (16,17). Instead of radiolabeling we used the native form of the peptide and a sensitive method, LC-MS, to detect opiorphin. A mass spectrometry method was already applied for the quantification of opiorphin in human saliva (18). Our data indicate that opiorphin crosses the BBB *in vitro* in the absence of plasma factors at a slow rate, which is lower than that of the paracellular marker fluorescein but higher than that of the passive transcellular marker albumin. At the same time the extent of penetration was significant, ~3% in contrast to the low amount of the paracellular markers. As a comparison, numerous endogenous peptides or regulatory proteins have $<0.1\%$/g uptake in brain and are still effective in the CNS after peripheral administration (12−14,19). Among the opiates, the centrally active morphine has an uptake of only $<0.02\%$/g brain (20).

The amount of opiorphin transferred across the BBB culture model indicates that a specific transport mechanism, a peptide transport system or receptor-mediated transcytosis, may be involved in its transfer. Six transport systems (PTS1-6) have been identified for peptides at the BBB, which transport enkephalins, arginine vasopressin, or pituitary adenylate cyclase-activating polypeptides, among others (11). The peptide/histidine transporter 2 (PHT2, SLC15a3) was described at the BBB carrying di- and tripeptides, whereas larger peptides or proteins like insulin or transferrin cross the BBB by receptor-mediated transcytosis (15). To reveal if carriers or transporters participate in opiorphin transfer, further experiments are needed.

We observed the conversion of N-terminal glutamine into pyroglutamic acid in the peptide samples. Peptide N-terminal glutamines are prone to cyclization yielding pyroglutamic acid. This reaction can be catalyzed enzymatically by cyclization of L-glutamine and L-glutaminyl peptides (21), but the reaction also happens spontaneously, especially at acidic pH values. The presence of glutaminyl cyclase is described in brain but not known at the level of brain capillaries and there are no data on how pyroglutamate formation modifies the transport of peptides across the BBB. The tripetide thyrotropin-releasing hormone contains a pyroglutamate and has a slow, but *in vivo* measurable penetration across the BBB (22).

The relative quantity of opiorphin penetration through the BBB culture model is obviously more elevated than values obtained with iv-administered labeled opioid peptides (14). A possible explanation for this difference is that in our culture system no serum factors were present during the experiments, which is a limitation of the model. Peptidases are active in blood and rapidly cleave the native opiorphin peptide, which has a metabolic half-life of 6 min in human plasma; therefore, enzyme activity limits the transfer of opiorphin to the CNS *in vivo* (4,23,24). *In vivo* data suggest that even in the presence of serum peptidases opiorphin can cross the BBB in sufficient amounts to raise the concentration of endogenous opioid ligands by inhibiting enkephalinases; thus, it can be appropriate for producing central effects.

In conclusion, our results indicate that opiorphin crosses cultured brain endothelial cells. These data are in agreement with observations that opiorphin potentiates enkephalin-mediated antinociception and exerts antidepressant-like effects. We suggest that opiorphin may have a potential for further development as a centrally acting novel drug for the treatment of pain or depression.

Acknowledgments

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