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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.

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

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<0.01% (12–14). Specific features of the BBB, mainly interendothelial tight junctions and efflux transporters (15), as 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).

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

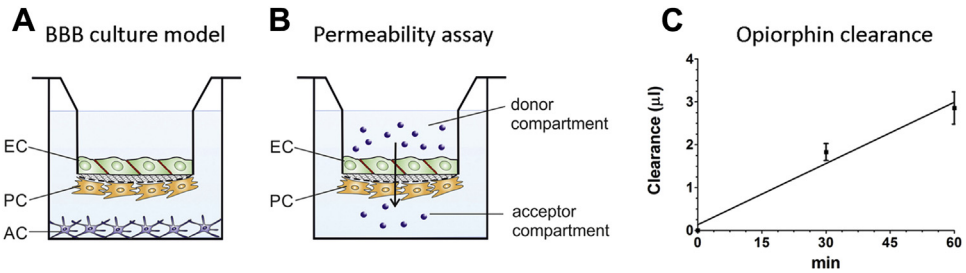

Figure 1. Culture model of the blood-brain barrier (A). The model is a co-culture of three cells types, primary rat brain endothelial cells (EC), rat pericytes (PC) and rat astrocytes (AC). In the permeability assay (B) culture inserts with EC and PC cells are used. Blue circles represent opiorphin, 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, $n = 4$). (A color figure can be found in the online version of this article.)