Naltrexone Transport by a Proton-Coupled Organic Cation Antiporter in hCMEC/D3 Cells, an in Vitro Human Blood–Brain Barrier Model

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Naltrexone is a mu-opioid receptor antagonist used in the treatment of opioid and alcohol dependence. The blood–brain barrier (BBB) transport characteristics of naltrexone was investigated by means of hCMEC/D3 cells, a human immortalized brain capillary endothelial cell line. In hCMEC/D3 cells, naltrexone is taken up in a concentration-dependent manner. Furthermore, naltrexone uptake significantly decreased in the presence of H+/organic cation (OC) antiporter substrates, during the little alteration exhibited by substrates of well-identified OC transporters classified into SLC22A family. Although naltrexone uptake by hCMEC/D3 cells was partially affected by changes of ionic conditions, it was markedly decreased in the presence of the metabolic inhibitor sodium azide. Furthermore, when treated by ammonium chloride, naltrexone uptake by hCMEC/D3 cells was altered by intracellular acidification and alkalization, suggesting the involvement of oppositely directed proton gradient in naltrexone transport across the BBB. The results obtained in the present in vitro study suggest the active transport of naltrexone from blood to the brain across the BBB by the H+/OC antiporter.

Key words  blood–brain barrier; membrane transport; organic cation; naltrexone

INTRODUCTION

The distribution of compounds from the circulating blood to the brain is strictly limited to maintain the brain homeostasis. The regulatory interface of the brain is known as the blood–brain barrier (BBB). The BBB is a complex cellular system composed of capillary endothelial cells connected by tight junctions, with the polarized expression of various transport systems including P-glycoprotein (P-gp/MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2). Due to this barrier, many pharmacologically active compounds cannot reach the brain in sufficient concentrations at the acceptable dose, lowering the success rate of developing central nervous system (CNS) drugs.

Parallel artificial membrane permeability assay (PAMPA)-BBB, P-gp and BCRP-expressing transport assay, and in silico analysis have been developed to improve CNS drug development. Such assays were assumed to be the best way to screen candidates at the discovery stage of drug development. However, it is also known that these assay systems screen out the commercially available antipsychotic drugs risperidone and paliperidone, because they are substrates of P-gp.

Additionally, it is known that several cationic drugs, such as the first-generation histamine H₂ blockers and CNS-active opioid agonists, could achieve drug approval. Diphenhydramine and oxycodone show notably higher brain unbound concentration than that of plasma. Cumulative study on drug transport revealed the involvement of H⁺/organic cation (OC) antiporter in their distribution into the brain.

However, the molecules that influence H⁺/OC antiporter remain unknown. The transport characteristics of H⁺/OC antiporter have been reported to be pH-dependent, sodium, membrane potential-independent, and energy-dependent. The functional expression of H⁺/OC antiporter was clearly suggested in the rat and human BBB. The uptake clearances of diphenhydramine and memantine in human in vivo studies were also estimated with in vitro human BBB model cells (hCMEC/D3 cells). The evidence indicated that the H⁺/OC antiporter has an important role on the active transport of cationic drugs into the brain in vivo. Therefore, it is important to explore new substrates of H⁺/OC antiporter to improve the development of CNS drugs.

Naltrexone is a mu-opioid receptor antagonist indicated for opioid and alcohol dependence. Moreover, it is used with oxycodone to reduce the risk of substance abuse. The purpose of this study was to investigate the transport mechanism of naltrexone at the human BBB.

MATERIALS AND METHODS

Substrates, Media and Assay Kits Naltrexone hydrochloride, EBM-2 medium and Micro BCA protein assay kit were purchased from Merck Millipore (Burlington, MA, U.S.A.), Lonza (Basel, Switzerland), and Thermo Fisher Scientific (Waltham, MA, U.S.A.), respectively. All other chemicals and reagents were commercially available products of reagent grade. Buffer and cell culture medium contents used in the present study are summarized in the Supplementary Materials.

Cell Culture and Cellular Uptake Assay hCMEC/D3 cells are human brain capillary endothelial cell line, immortalized by lentiviral transduction of the catalytic subunit of human telomerase and SV40-T antigen. EBM-2 medium and rat collagen type I-coated dishes were used for culturing hCMEC/D3 cells as described elsewhere (Supplementary Materials). In our cultivation, 10mM N-(2-hydroxyethyl)-
piperazine-\(N\)-2-ethanesulfonic acid (HEPES), 1% penicillin-streptomycin, 5\(\mu\)g/mL basic fibroblast growth factor (bFGF), 0.01% hydrocortisone, 0.025% hEGF, 0.025% R_{1-IFG}, 0.025% vascular endothelial growth factor (VEGF), and 2.5% fetal bovine serum were added into EBM-2 medium.

The uptake study was carried out according to methods described elsewhere.\(^{10,16}\) Briefly, at 3–4 d after seeding on collagen-coated plates (BIOCOAT, BD Biosciences, Franklin Lakes, NJ, U.S.A.), hCMEC/D3 cells were pre-treated with transport buffer for 20 min at 37°C. The uptake assay was initiated by adding a transport buffer with 1\(\mu\)M naltrexone. The assessment of the concentration dependent uptake assays were conducted at 37°C using buffers containing 5, 30, 100, 300, 1000, and 5000\(\mu\)M naltrexone instead of the buffer with 1\(\mu\)M naltrexone. The assays were then terminated by multiple washing using 1 mL ice-cold transport buffer.

In the present uptake assay, sodium azide (Na\(_3\)N\(_3\)) or ice-cold buffer was used to assess the energy-dependent transport. Replacement buffers were prepared to assess Na\(^+-\) and membrane potential-sensitive transport as described in our previous report (Supplementary Materials).\(^{10,16}\) To study altered intracellular pH, incubation and pretreatment with 30mM NH\(_4\)Cl was conducted to cause intracellular alkalization and acidosis, respectively.\(^{17,18}\) Additionally, several OCs, including nutrients and drugs, were tested at the concentration of 1 mM to assess the substrate recognition and specificity of naltrexone transport.

**Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)** The quantification of naltrexone with LC-MS/MS was carried out according to our previous reports, using naloxone as an internal standard. In the present study, Accela (Thermo Fisher Scientific) was connected with TSQ Quantum ultra (Thermo Fisher Scientific), and naltrexone and naloxone were detected by multiple reaction monitoring and the m/z transition was 342.2 to 324.2 and 328.1 to 310.2, respectively. The drugs were separated at the flow rate of 0.4 mL/min, using Synergi Hydro-RP (2.5 \(\mu\)m, 50 \(\times\) 3 mm, Phenomenex, Torrance, CA, U.S.A.) analytical column, heated to 40°C. The mobile phase consisted of 10mM ammonium acetate (pH 4.0) (A) and methanol (B). The organic modifier content B was kept at 5% for 1 min, increased linearly to 95% for 1–3 min, and kept at 95% for 3–5 min.

**Cell-to-Medium Ratio** The protein assay kit was used to determine the cellular protein amount after uptake assays. The data obtained was applied to calculate cell-to-medium ratio (\(\mu\)L/mg protein) to quantitatively evaluate naltrexone uptake, as described elsewhere.\(^{10,16}\) The kinetic parameters were estimated using GraphPad Prism (Graphpad Software, San Diego, CA, U.S.A.) software for nonlinear least-squares regression analysis. The data obtained in the initial uptake assay, including the initial uptake velocity (pmol/mg protein/30 s, \(V\)), and drug concentration (\(\mu\)M, \(S\)) were fitted to Eq. 1:

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V = \frac{V_{\text{max}} \times S}{K_m + S} + K_c \times S
\]  

(1)

The maximum uptake velocity (pmol/mg protein/30 s), Michaelis–Menten constant (\(\mu\)M), and non-saturable uptake clearance (pmol/mg protein/30 s) are represented by \(V_{\text{max}}\), \(K_m\), and \(K_c\), respectively.

In the present study, mean ± standard error of the mean (S.E.M.) was used to express the data. One-way ANOVA with Dunnett’s post-hoc test was used to evaluate the significant differences. A \(p\)-value <0.05 was considered to be statistically significant.

**RESULTS**

**Uptake of Naltrexone by hCMEC/D3 Cells** During the in vitro assay, the uptake of naltrexone by hCMEC/D3 cells linearly increased up to 1 min (Fig. 1a). The initial uptake rate was assessed at 30 s in subsequent studies. The initial uptake of naltrexone was found to be concentration-dependent (Fig. 1b). The kinetic parameter calculation showed that \(K_m\) value of naltrexone was 323 ± 84\(\mu\)M, and \(V_{\text{max}}\) was 10.7 ± 2.3 mmol/mg protein/30 s. Further calculation demonstrate a non-saturable uptake clearance \((P_{\text{diff}})\) of 1.32 ± 0.61\(\mu\)L/mg protein/30 s.

**Characteristics of Naltrexone Uptake** The uptake assay in hCMEC/D3 was carried out to examine the effect of several conditions on naltrexone uptake. Naltrexone uptake was significantly reduced to 45.8, 10.6, and 11.5% in a membrane...
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potential reduced condition (NaCl in transport buffer was replaced with KCl; K⁺-replacement), an energy depleted condition by NaN₃ (NaN₃), and at low temperature condition (4 °C), respectively (Fig. 2a). However, the uptake was not affected in a Na⁺-free condition. Moreover, the pretreatment of NH₄Cl (NH₄Cl-Pre), which causes an intracellular acidification, significantly increased the uptake of naltrexone to 227.5%. On the other hand, incubation with NH₄Cl (acute treatment; NH₄Cl-Acute), which causes an intracellular alkalization, significantly decreased the uptake to 6.6% (Fig. 2a).

**Naltrexone Uptake Inhibitory Effect by hCMEC/D3 Cells**

The effect of several compounds on naltrexone uptake was examined in hCMEC/D3 cells. The uptake was significantly inhibited by pyrilamine, oxycodone, memantine, tramadol, and clonidine, whereas no significant effect was observed in the presence of L-carnitine, tetraethylammonium (TEA) or 1-methyl-4-phenylpyridinium (MPP⁺) (Fig. 2b).

**DISCUSSION**

Naltrexone is known to block mu-opioid receptor in the brain. Therefore, it is often used to manage alcohol or opioid dependence. Considering the physicochemical property of naltrexone, it has a cLogP value of 0.36 and pKₐ value of 8.13, indicating a 5.37-time greater amount of charged form than uncharged form at physiological conditions. In addition, the unbound brain to plasma concentration ratio (K_p,un,brain) of naltrexone was previously reported to be more than unity in rats. These pieces of evidence indicate the possible involvement of some active transport system at the BBB in the distribution of naltrexone to the brain.

In the *in vitro* uptake assay, naltrexone was taken up by hCMEC/D3 cells in a concentration-dependent manner (Fig. 1b). Furthermore, the uptake was significantly reduced at metabolic inhibitor or low-temperature conditions (Fig. 2a),

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**Fig. 2. Characteristics of Naltrexone Uptake**

Uptake of naltrexone (1 µM) was measured for 30s. Each column represents the mean ± S.E. (n = 3–10). *p < 0.05, significantly different from control. (a) The transport was measured with the following conditions: cells were pre-incubated with NH₄Cl containing buffer (NH₄Cl-Pre); cells were incubated with buffer containing NH₄Cl (intracellular alkalization), N-methylglucamine⁺ (Absence of sodium ion gradient), potassium (K⁺-replacement), sodium azide (NaN₃); and cells were incubated with ice-cold buffer (4 °C). The raw value of cell-to-medium ratio in the control was 11.0 µL/mg protein. (b) The cells were incubated with buffer containing inhibitors. The concentration of inhibitor was 1 mM. TEA, tetraethylammonium; MPP⁺, 1-methyl-4-phenylpyridinium. The raw value of cell-to-medium ratio in the control was 31.7 µL/mg protein.
suggesting the involvement of carrier-mediated transport process in naltrexone transport at the BBB.

No significant differences were observed at sodium-free condition, and the significant but slight change was observed at membrane potential reduced conditions in naltrexone uptake (Fig. 2a). The oppositely directed proton gradient was suggested to drive the transport of naltrexone at the BBB, since naltrexone uptake was significantly increased by intracellular acidification and decreased by alkalization (Fig. 2a). These results suggested the possible involvement of H⁺/OC antiporter in naltrexone transport at the BBB, which is strongly supported by the results obtained in the study of inhibitory effects. The mRNAs of hOC1T1 (SLC22A1), hOCT3 (SLC22A3), hOCTN1 (SLC22A4), hOCTN2 (SLC22A5), hMATE1 (SLC24A1), hMATE2 (SLC24A2) and hPMAT (SLC29A4) were detected in hCMEC/D3 cells by quantitative RT-PCR analysis. The uptake was significantly reduced by typical substrates of H⁺/OC antiporter including pyrilamine, oxycodone, memantine, tramadol, and clonidine, in spite of no significant effect shown by the substrates of well-identified OC transporters, L-carnitine (a substrate and inhibitor of OCTN2), TEA (a typical substrate and/or inhibitor of OCTs), and MPP⁺ (a typical substrate and/or inhibitor of plasma membrane monoamine transporter as well as OCTs) (Fig. 2b).

The kinetic parameters analysis of naltrexone transport revealed a \( K_m \) value of 323 µM and \( V_{\text{max}} \) of 10.7 nmol/mg protein/30s (Fig. 1b). Furthermore, the clearances for saturable component \( (V_{\text{max}}/K_m) \) and non-saturable component \( (V_{\text{app}}) \) were calculated to be 33.1 and 1.32 µL/mg protein/30s, respectively. These values support the major contribution of H⁺/OC antiporter to naltrexone transport at the BBB, since 96.2% of total naltrexone uptake is accounted for by the saturable component.

Clinically, oxycodone, a mu-opioid receptor agonist, is used for the relief of moderate-to-severe pain. Naltrexone is adjunctively used to reduce the risk of oxycodone abuse. In this case, the unbound plasma concentrations of naltrexone and oxycodone are approximately 0.1 µM or less. Moreover, it is indicated that the distribution of these two ethical drugs to the brain would not interact competitively with each other, since their \( K_m \) values are much higher than 0.1 µM (Fig. 1b). Regarding their pharmacological effects, the \( K_m \) value of naltrexone for mu-opioid receptor is 0.265 nM. The clinical significance of the distribution of oxycodone and naltrexone is assumed to sufficiently inhibit mu-opioid receptors expressed in the brain, supporting the pharmacokinetic validity of the combined administration of oxycodone and naltrexone.

In the present study, the \textit{in vitro} uptake study with hCMEC/D3 cells identified naltrexone as a substrate of H⁺/OC antiporter at the BBB. This could contribute to clarifying the substrate recognition of H⁺/OC antiporter. The distribution of CNS drugs to the brain across the BBB is essential for them to exert their pharmacological effects. Drug discovery focusing on H⁺/OC antiporter is expected to contribute to the development of CNS drugs with more permeability and less interaction at the BBB. Therefore, clarifying the relationship between substrate structure and transport activity of H⁺/OC antiporter will aid in the development of new CNS drugs. The study of structure requirements using diphenhydramine analogs and the permeability improvement by chemical conjugation have been challenged, supporting the importance of H⁺/OC antiporter in improving the success rate of CNS drug candidates.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

REFERENCES


