Transport Characteristics of Tramadol in the Blood–Brain Barrier

ATSUSHI KITAMURA, KEI HIGUCHI, TAKASHI OKURA, YOSHIHARU DEGUCHI

Laboratory of Drug Disposition & Pharmacokinetics, Faculty of Pharma-Sciences, Teikyo University, Itabashi, Tokyo 173-8605, Japan

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ABSTRACT: Tramadol is a centrally acting analgesic whose action is mediated by both agonistic activity at opioid receptors and inhibitory activity on neuronal reuptake of monoamines. The purpose of this study was to characterize the blood–brain barrier (BBB) transport of tramadol by means of microdialysis studies in rat brain and *in vitro* studies with human immortalized brain capillary endothelial cells (hCMEC/D3). The $K_{p,uu,brain}$ value of tramadol determined by rat brain microdialysis was greater than unity, indicating that tramadol is actively taken up into the brain across the BBB. Tramadol was transported into hCMEC/D3 cells in a concentration-dependent manner. The uptake was inhibited by type II cations (pyrilamine, verapamil, etc.), but not by substrates of organic cation transporter OCTs or OCTN2. It was also inhibited by a metabolic inhibitor but was independent of extracellular sodium or membrane potential. The uptake was altered by changes of extracellular pH, and by ammonium chloride-induced intracellular acidification, suggesting that transport of tramadol is driven by an oppositely directed proton gradient. Thus, our *in vitro* and *in vivo* results suggest that tramadol is actively transported, at least in part, from blood to the brain across the BBB by proton-coupled organic cation antiporter. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: active transport; blood–brain barrier; drug transport; *in vitro* models; membrane transporter; microdialysis; organic cation transporter; tramadol

INTRODUCTION

Tramadol hydrochloride, (1RS,2RS)-2-[(dimethylamino)ethyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, is a widely used centrally acting analgesic. The analgesic effect following parenteral administration of tramadol is due to synergistic interaction between its agonist activity toward opioid receptors and its antinociceptive effect mediated by inhibition of neuronal reuptake of monoamines in the brain.[1] Thus, the analgesic activity should be dependent on the concentration of unbound tramadol in the vicinity of both μ-opioid receptors and monoamine transporters, such as serotonin transporter (SERT) and norepinephrine transporter (NET), in the brain. Since the concentration of unbound tramadol in the brain would be at least partly determined by the blood–brain barrier (BBB) transport characteristics of tramadol, an understanding of the transport characteristics is important for predicting the onset and duration of analgesic activity of tramadol.

The BBB dynamically regulates the transfer of endogenous nutrients, waste products, and drugs between blood and brain interstitial fluid (ISF),[2] depending upon the functions of various transporters and receptors localized on the brain capillary endothelial cell membrane.[2] Opioids, such as morphine and oxycodone,[3] and opioid-like analgesic peptides, such as H-Tyr-D-Arg-Phe-beta-Ala-OH (TAPA)[4] and ebiratide,[5] are transported through the BBB via both identified and unidentified transporters and receptors. Recently, we have shown that oxycodone is actively taken up into rodent brain capillary endothelial cells by proton-coupled organic cation (H^+/OC) antiporter.[3] Other organic cationic drugs, such as diphenhydramine,[6,7] pyrilamine,[3,7] nicotine,[8,9] and clonidine,[10] are also transported by the H^+/OC antiporter. Tramadol (p*K*a 9.41),[11] which contains a tertiary amine moiety, is present in cationic form at physiological pH. The tramadol concentration in brain is approximately five times higher than that in plasma[12] and the unbound brain to plasma ($K_{p,uu,brain}$) concentration ratio has been indirectly estimated to be greater than unity using the rat brain slice method.[12] Thus, tramadol may be actively transported into the brain by the H^+/OC antiporter across the BBB.

Functional transporters at the human BBB are important not only for pharmacotherapy to treat cerebral diseases, but also for development of new central nervous system (CNS)-acting drugs. Human immortalized brain capillary endothelial cells (hCMEC/D3)[13] retain many of the morphological and functional characteristics of the human BBB in terms of expression of tight-junction proteins, as well as various ABC and several SLC transporters.[2,14–16] Our laboratory has shown recently that H^+/OC antiporter is functionally expressed in hCMEC/D3,[6] and therefore *in vitro* uptake studies using hCMEC/D3 cells may be useful to predict tramadol concentration in the human brain, and hence its analgesic effect. However, it is first necessary to establish the transport mechanism of tramadol.

The aim of this study, therefore, was to examine the transport mechanism of tramadol *in vivo* and in hCMEC/D3 cells. First, we measured the $K_{p,uu,brain}$ value of tramadol using the rat brain microdialysis technique in order to obtain evidence for active uptake of tramadol through the BBB *in vivo*. We then investigated the mechanism of tramadol transport using hCMEC/D3 cells *in vitro*.

MATERIALS AND METHODS

Reagents

Tramadol hydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

Correspondence to: Yoshiharu Deguchi (Telephone: +81-3-3964-8246; Fax: +81-3-3964-8252; E-mail: deguchi@pharm.teikyo-u.ac.jp)

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Cell Culture

hCMEC/D3 cells had been immortalized by lentiviral transduction of the catalytic subunit of human telomerase and SV40-T antigen.[13] The cells were cultivated at 37°C in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 2.5% fetal bovine serum, 0.025% VEGF, 0.025% R3-IGF, 0.025% hEGF, 0.01% hydrocortisone, 5 μg/mL bFGF, 1% penicillin–streptomycin, and 10 mM HEPES on rat collagen type I-coated dishes in an atmosphere of 95% air and 5% CO_2.

Animals

Adult male Wistar rats purchased from Japan SLC (Shizuoka, Japan) were housed, two or three per cage, with free access to food and water. The room was maintained on a 12-h dark/12-h light cycle with controlled temperature (24 ± 2°C) and humidity (55 ± 5%). This study was conducted according to guidelines approved by the Experimental Animal Ethical Committee of Teikyo University.

Transport Studies

hCMEC/D3 cells used for the experiments were between passages 25 and 35. The cells were seeded on rat collagen I-coated 24-well plates (Becton Dickinson, Franklin Lakes, New Jersey) at a density of 0.2×10^5 cells/cm^2. At 3 or 4 days after seeding, the cells reached confluence. For uptake experiments, they were washed twice with 1 mL of transport buffer (122 mM NaCl, 3 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4) and preincubated with 0.25 mL of transport buffer for 20 min at 37°C. After preincubation, 0.25 mL of the transport buffer containing 5 μM tramadol was added to initiate uptake. The cells were incubated at 37°C for a designated time, and then washed three times with 1 mL of ice-cold incubation buffer to terminate the uptake. The cells were collected with a scraper in 200 μL of H_2O containing 100 nM propranolol as an internal standard and stored in a freezer set at −30°C until analysis.

Uptake was expressed as the cell-to-medium ratio (μL/mg protein or μL/(mg protein·min), obtained by dividing the uptake amount by the concentration of substrate in the transport medium. In order to estimate the kinetic parameters, tramadol uptake data (10, 25, 50, 75, 100, 200, 500, and 1000 μM, for 30 s) were analyzed using Michaelis–Menten plots based on the following equation:

$$V = \frac{V_{max} \times S}{K_m + S} + P_{diff} \times S \quad (1)$$

where V is the initial uptake rate of substrate (nmol/(mg protein·min)), V_{max} is the maximum uptake rate (nmol/(mg protein·min)), S is the concentration of tramadol in the medium (μM), K_m is the Michaelis–Menten constant (μM), and P_{diff} is non-saturable uptake clearance (μL/(mg protein·min)), respectively. The uptake data were fitted to the above equation by nonlinear least-squares regression analysis with Prism software (Graphpad, San Diego, California).

To delineate the energy requirements of the transport system, uptake of tramadol was carried out in the presence of a metabolic energy inhibitor, sodium azide (NaN_3). The uptake was measured after pretreatment with 0.1% NaN_3 for 20 min. In this experiment, 10 mM D-glucose in the transport medium was replaced with 10 mM 3-*O*-methylglucose to reduce metabolic energy. To examine the sodium ion dependency and the effect of reducing the membrane potential, NaCl was replaced with *N*-methylglucamine$^+$ and KCl, respectively. Uptake was also measured at medium pH values of 6.4, 7.4, and 8.4. The influence of intracellular pH (pH_i) was examined by pretreatment or incubation with 30 mM NH_4Cl to produce intracellular acidosis or alkalization, respectively.[17,18] To measure tramadol uptake at acidic pH_i, extracellular NH_4Cl was removed after the preincubation with 30 mM NH_4Cl because intracellular NH_3 rapidly diffuses out of the cells, resulting in accumulation of protons released from NH_4^+ during NH_3 generation in the cells. In the inhibition study, uptake was measured after incubation with tramadol in the presence of test compounds (tramadol, 1-methyl-4-phenylpyridinium (MPP^+), tetraethylammonium (TEA), carnitine, morphine, codeine, oxycodone, apomorphine, clonidine, pyrilamine, diphenhydramine, amantadine, memantine, verapamil, and quinidine) at the concentration of 1 mM. Tramadol uptake (10–1000 μM) was measured in the absence and presence of oxycodone (500 μM).

The cells were solubilized with an equal volume of 1 M NaOH at 37°C for 60 min and three volumes of H_2O were added. The cellular protein content was determined with a BCA protein assay kit (Pierce Chemical Company, Rockford, Illinois).

Plasma Protein Binding

Tramadol was added to 1 mL of blank rat plasma to give a concentration of 3 μM. Aliquots of spiked plasma were equilibrated for 20 min at 37°C, then ultrafiltered (MPS-1; Millipore Corporation, Billerica, Massachusetts) and centrifuged at 35°C for 5 min (1000*g*). The concentrations remaining in spiked plasma ($C_{p,tot}$) and ultrafiltrate ($C_{p,u}$) samples were measured by LC–MS/MS. The unbound fraction in plasma (f_u) was determined by dividing $C_{p,u}$ into $C_{p,tot}$.

In Vitro Microdialysis

In vitro brain microdialysis studies were carried out according to our previous report.[19] A CMA12 microdialysis probe (3 mm; CMA, Stockholm, Sweden) was inserted into a tube containing 1 μg/mL tramadol and antipyrine (reference compound) in Krebs-Ringer phosphate (KRP) buffer (120 mM NaCl, 2.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 1.4 mM Na_2HPO_4, pH 7.4) solution. KRP buffer was perfused for 240 min through the probe at a constant flow rate of 5 μL/min by means of a syringe infusion pump (Model 22; Harvard Apparatus, South Natick, Massachusetts). The dialysate was collected every 30 min and the concentrations of tramadol and antipyrine in the dialysate and the buffer were measured by LC–MS/MS.

Brain Microdialysis

In vivo brain microdialysis studies were carried out according to our previous report.[19] The rats were anesthetized with pentobarbital and a hole was drilled 2.7 mm lateral and 0.8 mm anterior to the bregma, and 3.8 mm ventral to the surface of the brain. A CMA12 guide cannula (CMA) was implanted into the striatum and fixed to the skull by a screw and dental cement (GC Fuji I, Tokyo, Japan). A CMA12 probe was inserted through the guide cannula 24 h after the surgery.

Forty-eight hours after surgery, brain microdialysis was performed. The rats were anesthetized with pentobarbital and SP31 polyethylene tubes (inner diameter; 0.5 mm, outer diameter; 0.8 mm; Natsume Seisakusho Company, Ltd., Tokyo,