Carrier-Mediated Transport of Nicotine Across the Inner Blood–Retinal Barrier: Involvement of a Novel Organic Cation Transporter Driven by an Outward H^+ Gradient

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ABSTRACT: The present study was carried out to investigate the blood-to-retina transport of nicotine across the inner blood–retinal barrier (BRB). Using the *in vivo* vascular injection method, the blood-to-retina influx clearance of nicotine across the BRB was determined as 131 μL/(min?g retina), which is much higher than that of a nonpermeable paracellular marker, and blood-to-retina transport of nicotine was inhibited by organic cations such as pyrilamine and verapamil. The nicotine uptake by a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells), an *in vitro* model of the inner BRB, exhibited time, temperature, and concentration dependence with a K_m of 492 μM. These results suggest the involvement of a carrier-mediated transport process in nicotine transport in the inner BRB. The nicotine uptake by TR-iBRB2 cells was stimulated by an outwardly directed H^+ gradient, and the uptake was significantly inhibited by bulky and hydrophobic cationic drugs, whereas inhibitors of organic cation transporters did not show inhibitory effect. These results suggest that the novel organic cation transport system driven by an outwardly directed H^+ gradient is involved in the blood-to-retina transport of nicotine across the inner BRB. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3069–3075, 2015

Keywords: blood–retinal barrier; nicotine; organic cation transporter; membrane transport; transporters; membrane transporter; drug transport; *in vitro* models; TR-iBRB2 cells

INTRODUCTION

Nicotine is a typical nicotinic acetylcholine receptor (nAChR) agonist which affects a variety of behaviors including nociception, cognition, and memory.[1] Besides the pharmacological evidence about the effects of nicotine, there is increasing evidence for carrier-mediated nicotine transport. The previous studies have shown the carrier-mediated transport of nicotine in mammalian tissues and cell lines, such as rat kidney, rabbit choroid plexus, LLC-PK1 cells, JAR cells, and Caco-2 cells,[2–6] and nicotine is reported as a substrate of multidrug and toxin extrusion protein 1 (MATE1/SLC47A1) and plasma membrane monoamine transporter (PMAT/SLC29A4), and an inhibitor of organic cation transporter family (OCT1-3/SLC22A1-3) and organic cation/carnitine transporter family (OCTN1-2/SLC22A4-5).[7–12] Recently, our studies have demonstrated that the nicotine influx transport in the rat blood–brain barrier (BBB) and the liver was carried out by the carrier-mediated process involving a novel organic cation transporter.[13,14] Although the physiological role is unknown, the nicotine transport system is assumed to play an important role in modulating the concentration of nicotine in various tissues including the central nervous system (CNS).

Abbreviations used: BRB, blood–retinal barrier; MPP^+, 1-methyl-4-phenylpyridinium; nAChR, nicotinic acetylcholine receptor; PAH, *p*-aminohippurate; RUI, retinal uptake index; TEA, tetraethylammonium; TR-iBRB2 cells, a conditionally immortalized rat retinal capillary endothelial cell line.

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The optic nerve system is thought to be a part of the CNS, and the retina is the specific tissue where light is directly focused on the cells. For a healthy vision, it is essential to maintain retinal homeostasis, and the paracellular transport of compounds between the circulating blood and retina is regulated by the inner and outer blood–retinal barriers (BRB), that are formed by tight junctions of the retinal capillary endothelial cells and retinal pigment epithelial cells, respectively. In addition, it has been found that various transporter molecules are expressed at the BRB, and carry out endobiotic and xenobiotic transport between the retina and the blood.[15] To achieve efficient and safe drug treatment of retinal diseases, it is important to increase our understanding of the blood-to-retina transport systems via the BRB as topical drug administration is known to be inefficient.[16]

Interestingly, previous studies have provided the pharmacological evidence that nicotine binds to retinal nAChRs to alter responses in the retina as the expression of nAChRs has been reported in retinal cells, such as amacrine and ganglion cells, of various species including rat, mouse, chick, rabbit, and pig.[17–21] In humans, it has been suggested that the nAChRs are predominantly expressed in the inner plexiform layer of the retina,[22] and the electroretinogram was affected by administration of nicotine gum.[23] In addition, previous *in vitro* studies have demonstrated that nicotine exerts a protective effect against glutamate-induced excitotoxicity via the nAChR in retinal ganglion cells,[24,25] and a recent study using rat glaucoma model revealed that an α7 nAChR agonist has a neuroprotective effect against loss of retinal ganglion cells,[26] showing that the nAChR agonist is a strong candidate for the drug therapy of glaucoma. These lines of evidence indicate the usefulness of nicotine or nAChR agonists for retinal diseases accompanied by neurodegeneration, and an improvement of our understanding

of the nicotine transport mechanism at the BRB is important for achieving efficient drug delivery from the blood to the retina.

In the present study, the properties of blood-to-retina transport of nicotine across the inner BRB were investigated by an *in vivo* vascular injection technique and a cellular uptake study using a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells), an *in vitro* model of the inner BRB, to clarify the nicotine transport system at the inner BRB.

MATERIALS AND METHODS

Animals

Wistar rats (6-week-old, male, 150–200 g) were purchased from Japan SLC (Hamamatsu, Japan) and kept in a controlled environment. All experiments were approved by the Animal Care Committee, University of Toyama, and conformed to the provisions of the Association for Research in Vision and Ophthalmology Statement.

Reagents

L-(-)-[N-methyl-^{3}H]Nicotine ([^{3}H]nicotine, 83.5 Ci/mmol) was purchased from PerkinElmer (Boston, Massachusetts). *n*-[1-^{14}C]Butanol, ([^{14}C]*n*-butanol, 2 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, Missouri). All other chemicals were commercially available and analytical grade.

Integration Plot Analysis

Integration plot analysis was performed according to previous reports in order to determine the apparent blood-to-retina influx clearance ($K_{\text{in, retina}}$) of [^{3}H]nicotine.[27,28] Ringer-HEPES solution (141 mM NaCl, 4.0 mM KCl, 2.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4) (400 μL) containing [^{3}H]nicotine (3 μCi/rat) was injected into the femoral vein of rats anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). After collection of blood samples, the rats were decapitated, and their retinas were removed. All samples were dissolved in 2 N NaOH, and the radioactivity was measured in a liquid scintillation spectrophotometer (LSC-7400; Hitachi Aloka Medical, Tokyo, Japan). The uptake rate of [^{3}H]nicotine was determined by using Eq. (1); the details are described in Supporting Information.

$$V_{\text{d}}(t) = K_{\text{in, retina}} \times \text{AUC}(t)/\text{C}_{\text{p}}(t) + \text{V}_{\text{i}} \quad (1)$$

Retinal Uptake Index Method

Nicotine uptake in the rat retina was determined by injecting [^{3}H]nicotine into the common carotid artery, as described previously.[29,30] Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and 200 μL Ringer-HEPES buffer containing both [^{3}H]nicotine (3.4 μCi/200 μL, 208 nM) and [^{14}C]*n*-butanol (0.4 μCi/200 μL, 1 μM) as a highly diffusible internal reference in the absence or presence of inhibitors was injected into the common carotid artery. Rats were decapitated 15 s after injection, and their retinas were collected. Each retina was solubilized in 2N NaOH and subsequently neutralized with 2N HCl. The ^{3}H and ^{14}C radioactivity in the retina and the injectate was measured using a liquid scintillation spectrophotometer (LSC-7400; Hitachi Aloka Medical). [^{3}H]Nicotine uptake by the retina was expressed as the retinal uptake index (RUI) value (Eq. (2)).

$$\text{RUI}(\%) = \left(\left[^3\text{H}\right]/\left[^{14}\text{C}\right](\text{dpm in the retina})\right) / \left(\left[^3\text{H}\right]/\left[^{14}\text{C}\right](\text{dpm in the injectate})\right) \times 100 \quad (2)$$

Uptake Study in TR-iBRB2 Cells

As described previously,[31] TR-iBRB2 cells, a conditionally immortalized rat retinal capillary endothelial cell line,[32] was cultured at 33°C to allow temperature-sensitive large T-antigen expression. Cells were washed three times with extracellular fluid (ECF) buffer [122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 0.4 mM K_2HPO_4, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM D-glucose, and 10 mM HEPES (pH 7.4)], and the uptake was initiated by applying [^{3}H]nicotine (0.5 μCi/mL, 6 nM) dissolved in ECF buffer at 37°C in the absence or presence of inhibitors. When examining the influence of extracellular pH on nicotine uptake, media with different pH values (pH 6.4, 7.4, and 8.4) were used. NH_4Cl with a concentration of 30 mM was used to increase the intracellular pH of TR-iBRB2 cells.[33,34] To examine the influence of intracellular acidification, extracellular NH_4Cl was removed after preincubation with 30 mM NH_4Cl for 20 min, because intracellular NH_3 rapidly diffuses out of cells, resulting in the accumulation of H^+ released from NH_4^+ in a process to produce NH_3.[35] Cells were rinsed three times with ice-cold buffer to terminate the uptake, and were solubilized with 1N NaOH. Subsequently, the solubilized solution was neutralized with 1N HCl. The radioactivity associated with cells was measured by liquid scintillation counting (LSC-7400; Hitachi Aloka Medical), and the cellular protein content was measured by a detergent-compatible protein assay (a DC protein assay kit; Bio-Rad, Hercules, California) with bovine serum albumin as a standard.

Data were analyzed as described previously.[13,34] The cellular uptake was expressed as shown in Eq. (3).

$$\text{Cell/medium ratio } (\mu\text{L/mg protein}) = \left(\left[^3\text{H}\right]\text{dpm in the cell per mg protein}\right) / \left(\left[^3\text{H}\right]\text{dpm in the medium per } \mu\text{L}\right) \quad (3)$$

The kinetic parameters for nicotine uptake by TR-iBRB2 cells were obtained from Eq. (4) as the Akaike's information criterion suggested that the equation was most suitable for data fitting in the present study,[36,37] and the kinetic for the inhibition of nicotine uptake by pyrilamine (50 μM) was analyzed according to Eq. (5).

$$V = (V_{\max} \times C)/(K_{\text{m}} + C) \quad (4)$$

$$V = (V_{\max} \times C)/(K_{\text{m}} \times (1 + I/K_{\text{i}}) + C) \quad (5)$$

where V, C, $V_{\max}$, K_{m}, I, and K_{i} are the uptake rate, the medium concentration, the maximum uptake rate, the Michaelis constant, the inhibitor concentration, and the inhibitory constant, respectively. To obtain kinetic parameters, the equation was fitted using the iterative nonlinear least-squares regression analysis program, MULTI.[37]

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