Journal of Pharmaceutical Sciences 105 (2016) 982–988

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Transport Mechanism of Nicotine in Primary Cultured Alveolar Epithelial Cells

Mikihisa Takano*, Machi Nagahiro, Ryoko Yumoto

Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical & Health Sciences, Hiroshima University, Minami-ku, Hiroshima 734-8553, Japan

ARTICLE INFO

Article history:
Received 17 June 2015
Revised 4 August 2015
Accepted 11 August 2015
Available online 7 September 2015

Keywords:
absorption
active transport
cell culture
membrane transport/transporters
pulmonary delivery/absorption

ABSTRACT

Nicotine is absorbed from the lungs into the systemic circulation during cigarette smoking. However, there is little information concerning the transport mechanism of nicotine in alveolar epithelial cells. In this study, we characterized the uptake of nicotine in rat primary cultured type II (TII) and trans-differentiated type I-like (TIL) epithelial cells. In both TIL and TII cells, [^{3}H]nicotine uptake was time and temperature-dependent, and showed saturation kinetics. [^{3}H]Nicotine uptake in these cells was not affected by Na^+, but was sensitive to extracellular and intracellular pH, suggesting the involvement of a nicotine/proton antiport system. The uptake of [^{3}H]nicotine in these cells was potently inhibited by organic cations such as clonidine, diphenhydramine, and pyrilamine, but was not affected by substrates and/or inhibitors of known organic cation transporters such as carnitine, 1-methyl-4-phenylpyridinium, and tetraethylammonium. In addition, the uptake of [^{3}H]nicotine in TIL cells was stimulated by pre-loading the cells with unlabeled nicotine, pyrilamine, and diphenhydramine, but not with tetraethylammonium. These results suggest that a novel proton-coupled antiporter is involved in the uptake of nicotine in alveolar epithelial cells and its absorption from the lungs into the systemic circulation.

Introduction

Nicotine is the main tobacco alkaloid that leads to smoking addiction. During cigarette smoking, nicotine is absorbed from the lungs into the systemic circulation, and then distributed to the brain and other organs, where nicotine induces multiple pharmacological and toxicological effects. Benowitz et al.[1] reported that the blood concentrations of nicotine rose quickly during smoking and peaked at the completion of smoking, presumably because of the large surface area of the small airways and alveoli of the lungs.

Nicotine is a weak base with a p*K*a value of 8.0. Though the precise pH in the alveolar lining fluid is difficult to measure, it has been suggested that alveolar lining fluid is slightly acidic than plasma.[2,3] Within such a pH range, nicotine is predominantly present as a positively charged molecule. Considering that the cell membrane lipid bilayer is generally impermeable to charged molecules, a specific transporter would be needed for the transport of nicotine across the cell membrane.

Thus far, there have been some reports studying the transport mechanisms ofnicotine in various types ofcells and tissues such as the human intestinal epithelial cell line Caco-2,[4] the kidney epithelial cell line LLC-PK_1,[5] blood-brain barrier and brain capillary endothelial cell line TR-BBB13,[6,7] and the human choriocarcinoma cell line JAR.[8] All of these reports suggest the involvement of a specific transport system for nicotine in these cells, although the transporter itself has not been identified yet. Surprisingly, however, little information is available concerning the transport mechanism of nicotine in the alveolar epithelial cells ultimately resulting in the pulmonary absorption of nicotine during cigarette smoking.

The alveolar region of the lungs comprises type I (TI) and type II (TII) epithelial cells. The squamous and extremely thin TI cells cover 90%–95% of the alveolar surface area, and are essential for physiological gas exchange. Generally, TI cells are assumed to play a dominant role in the pulmonary absorption process, because of their morphological characteristics described above. On the contrary, the cuboidal TII cells occupy only 5%–10% of the surface area, although TII cells have multiple physiological functions, including surfactant secretion into the alveolar lining fluid. In addition, TII cells serve as progenitors of TI cells, and transdifferentiate into TI cells to repair the alveolar epithelium during injury.[9,10] TII cells also undergo transdifferentiation into type I-like (TIL) cells during primary culture of the cells under *in vitro* conditions.[11] Accompanying

* *Correspondence to:* Mikihisa Takano (Telephone: +81-82-257-5315; Fax: +8182-257-5319).
E-mail address: takanom@hiroshima-u.ac.jp (M. Takano).

http://dx.doi.org/10.1002/jps.24627
0022-3549/

the transdifferentiation of TII cells into TIL cells is not only a change in cellular morphology, but also drastic changes in mRNA and protein expression profiles.[11,12] It has been reported that the expression and function of several membrane transporters for small molecules is different between TII and TIL cells.[13] For example, a proton-coupled peptide transporter, PEPT2, is exclusively expressed in TII cells but not in TIL cells, whereas P-glycoprotein is functional in TIL cells but not in TII cells.[12,14,15] Therefore, in order to understand the mechanism underlying nicotine absorption from the alveolar space, the characterization of nicotine transport in both TI and TII cells is prerequisite.

In vitro culture cells are indispensable tools to study the molecular mechanisms underlying the transport of nutrients and xenobiotics into and across the alveolar epithelial cells. Two types of *in vitro* model systems are widely used: primary cultured cells and established cell lines. Primary cultured cells would have morphological and functional characteristics close to those of alveolar epithelial cells in the intact alveoli, when compared with cell lines. Therefore, the observations obtained with this experimental model may be reliably translated into *in vivo* conditions. In this context, primary cultured cells may be the best *in vitro* model to study distal lung functions at present, although the ethical issues for using animal or human lungs need to be considered.[16-18] We have been studying the transport mechanisms of large and small molecules in the alveolar epithelial cells using rat primary cultured alveolar TII cells and transdifferentiated TIL cells.[11,12,19] In the present study, we investigated the mechanism of nicotine uptake using these primary cultured alveolar TIL and TII cells.

Materials and Methods

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and Ham's F-12 nutrition mixture (F-12) were purchased from MP Biomedicals (Solon, OH). Penicillin- streptomycin, fungizone, and trypsin (1:250, powder) were purchased from Life Technologies (Carlsbad, CA). Percoll was purchased from GE Healthcare Bio-Science (Piscataway, NJ). [^{3}H]Nicotine was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). *p*-Aminohippurate (PAH), L-carnitine, choline chloride, diphenhydramine, and verapamil were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Nicotine, propranolol, pyrilamine maleate, pyrimethamine, quinidine, and quinine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Procainamide and 1-methyl-4-phenylpyridinium (MPP^+) were purchased from Sigma-Aldrich (St. Louis, MO). Tetraethylammonium chloride (TEA) and clonidine hydrochloride were purchased from Tokyo Kasei Kogyo Company (Tokyo, Japan). Cotinine was purchased from TOYOBO Company, Ltd. (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Isolation, Purification, and Culture of Rat Alveolar TII Epithelial Cells

Experiments with animals were performed in accordance with the Guide for Animal Experimentation from Hiroshima University and the guidelines of the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University. Rat alveolar TII epithelial cells were isolated from specific pathogen-free Sprague-Dawley male rats and purified and cultured as described previously.[11] Briefly, the lungs were dispersed enzymatically with 0.25% trypsin and 250 μg/mL deoxyribonuclease I. The cell suspension obtained was purified by discontinuous Percoll density gradient centrifugation. The yield of purified alveolar TII cells per rat was approximately 30×10^6 cells, and the cell viability estimated by the trypan blue exclusion test was more than 90%. The isolated alveolar TII cells were grown at 37°C in culture medium under 5% CO_2, 95% air, with the culture medium being replaced every 2 days. The culture medium consisted of DMEM/F-12 (1:1) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. When the cells were used as primary cultured TII cells, isolated TII cells were seeded in a 24-well plate at a density of 54×10^4 cells/cm^2, and were cultured for 2 days.[11] When the cells were used as primary cultured TIL cells, isolated TII cells were seeded in a 24-well plate at a density of 22×10^4 cells/cm^2, and were cultured for 6 days. During this culture period, TII cells undergo transdifferentiation into TIL cells as described above.[11]

Uptake of [^{3}H]Nicotine in Alveolar Epithelial Cells

To examine the time dependence of [^{3}H]nicotine uptake, TIL and TII cells were incubated with [^{3}H]nicotine (50) μM; adjusted by adding unlabeled nicotine to the tracer level of tritiumlabeled nicotine) in HEPES buffer (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) for the given times at 37°C after preincubation for 10 min.

To examine the concentration dependence of nicotine uptake, TIL and TII cells were incubated with HEPES buffer (pH 7.4) containing [^{3}H]nicotine (50 μM) and various concentrations of unlabeled nicotine for 15 s at 37°C. The concentration dependence of nicotine uptake in TIL cells were also examined at extracellular pH 7.0 and 8.0. Curve fitting was performed by nonlinear regression analysis, using a Michaelis-Menten type equation, to determine the kinetic parameters of nicotine uptake.

To examine the Na^+ dependence of nicotine uptake, TIL and TII cells were incubated with HEPES buffer (pH 7.4) or Na^+- free HEPES buffer (145 mM choline chloride, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, 5 mM HEPES, pH 7.4) containing [^{3}H] nicotine (50 μM) for 15 s at 37°C.

To examine the effect of extracellular pH on [^{3}H]nicotine uptake, TIL and TII cells were incubated with [^{3}H] nicotine (50 μM) in MES buffer [145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM MES (pH 5.5, 6.0, or 6.5)] or HEPES buffer (pH 7.0, 7.4, or 8.0) for 15 s at 37°C. To examine the effect of intracellular pH on [^{3} H] nicotine uptake in TIL cells, the cells were treated with NH_4Cl as reported previously.[20] Briefly, TIL cells were preincubated with HEPES buffer in the absence (Control, Acute) or presence (Pre) of 30 mM NH_4Cl for 20 min. Then, the preincubation medium was aspirated, and the cells were incubated with [^{3}H]nicotine (50 μM) in HEPES buffer in the absence (Control, Pre) or presence (Acute) of 30 mM NH_4Cl for 15 s.

For inhibition studies, TIL and TII cells were incubated with [^{3}H] nicotine (50 μM) in HEPES buffer at 37°C in the absence or presence of various organic compounds; 1 mM verapamil, 1 mM quinidine, 1 mM propranolol, 3–1000 μM diphenhydramine, 1 mM procainamide, 1 mM quinine, 1 mM TEA, 10–1000 μM pyrilamine, 1 mM clonidine, 1 mM choline, 1 mM MPP^+, 1 mM cotinine, 1 mM carnitine, 1 mM PAH, and 100 nM pyrimethamine. IC50 values of diphenhydramine and pyrilamine on [^{3}H]nicotine uptake were estimated through curve fitting to the Hill equation, as described previously.[21]

For countertransport studies, TIL cells were preincubated for 30 min with or without 1 mM unlabeled nicotine, pyrilamine, diphenhydramine, or TEA, and then the uptake of [^{3}H]nicotine was determined for 15 s.

After incubation with [^{3}H]nicotine, the uptake was stopped by aspirating the uptake medium and washing the cells three times with ice-cold HEPES buffer. The cells were solubilized with 300 μL of 0.1 M NaOH by pipetting and collected in a microcentrifuge tube. The wells were rinsed with 100 μL of 0.1 M NaOH to improve the