In Vivo Evidence of Organic Cation Transporter-Mediated Tracheal Accumulation of the Anticholinergic Agent Ipratropium in Mice

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Received 25 March 2013; revised 12 April 2013; accepted 16 April 2013

Published online 19 May 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23603

ABSTRACT: Ipratropium bromide (IPR) is an anticholinergic used to treat chronic obstructive pulmonary disease (COPD), and is a substrate of organic cation transporters. The present study aimed to assess the contribution of organic cation transporters to tracheobronchial absorption of IPR *in vivo* by directly injecting [^{3}H]IPR into the tracheal lumen of mice and measuring its accumulation in tracheal tissue. RT-PCR and immunohistochemical analysis showed that Octn1, Octn2, and Oct2 were localized at epithelial cells in the respiratory tract. Electron-microscopic immunohistochemistry indicated that Octn1 and Octn2 were localized at the apical portions of ciliated epithelial cells of trachea. *In vitro* uptake studies in HEK293 cells expressing these transporters demonstrated that IPR is a preferred substrate of Octn2. Inhibition of mouse tracheal accumulation of [^{3}H]IPR by carnitine was concentration-dependent, reaching a maximum of 42% at 1 mM, whereas inhibition by 0.1 mM MPP^+ amounted to 62%. Tracheal accumulation of [^{3}H]IPR was unchanged when mice were simultaneously injected with Octn1 substrate ergothioneine and organic anion transporter substrate estrone sulfate. These results suggest that Octn2 is involved in membrane permeation of IPR in the respiratory tract *in vivo*. Targeting organic cation transporters may be an effective strategy for delivery of cationic anti-COPD drugs to patients. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3373–3381, 2013

Keywords: membrane transport/transporters; pulmonary delivery/absorption; OCT; OCTN; permeability; COPD; epithelial

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death in the world, and further increases in its prevalence and mortality are predicted over the next few decades. Medication is useful to prevent and control symptoms, to reduce the frequency of exacerbation, and to improve excise tolerance. Anticholinergics are among the most commonly prescribed bronchodilators for the treatment of COPD, and they are required to traverse bronchial epithelial cells to gain access to their pharmacological targets in bronchial smooth muscle after administration by inhalation. Therefore, their physicochemical properties, such as hydrophobicity and ionization state, are expected to be important determinants of their efficacy and bioavailability, if these compounds diffuse passively across the bronchial mucosa.

However, major anticholinergics, ipratropium bromide (IPR) and tiotropium bromide, which are chemically related to atropine,[1] are highly hydrophilic and unlikely to cross the plasma membrane by

Abbreviations used: CAR, carnitine; COPD, chronic obstructive pulmonary disease; ERG, ergothioneine; MET, metformin; MPP^+, 1-methyl-4-phenylpyridinium; OCTs/Octs, organic cation transporters; OCTN/Octn, organic cation/carnitine transporter; PBS, phosphate-buffered saline; QUI, quinidine; RT-PCR, reverse transcription-polymerase chain reaction.

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Journal of Pharmaceutical Sciences, Vol. 102, 3373–3381 (2013)

diffusion. Early pharmacokinetic studies indicated that their renal clearances are several fold greater than the glomerular filtration rate,[2,3] and we subsequently demonstrated that these anticholinergics are recognized and transported by human organic cation/carnitine transporters (OCTN1/*SLC22A4* and OCTN2/*SLC22A5*),[4] as well as human and rat organic cation transporters (OCT1/*SLC22A1* and OCT2/*SLC22A2*), indicating that vectorial transport mediated by these organic cation transporters plays a key role in efficient renal secretion of these compounds.[5]

Several classes of ion-coupled transporters for amino acids (e.g., $ATB^{0,+}$/*SLC6A14*) and oligopeptides (e.g., PEPT1/SLC15A1 and PEPT2/SLC15A2) are involved in the absorption of protein degradation products at the apical membranes of airway epithelial cells.[6,7] OCTNs have been detected in human bronchial cell lines and bronchial epithelial cells.[4,8] Furthermore, in rodents, Oct1, Oct2, and Oct3 (encoded by *Slc22a3*) are expressed in airway epithelial cells of trachea and bronchi, whereas Oct2 was mainly detected in the luminal membranes of ciliated cells in humans.[9–11] We previously showed that OCTN2 is involved in pulmonary absorption of IPR because silencing OCTN2 caused a significant reduction in IPR uptake by BEAS-2B cells[4]; therefore, in the present study we aimed to evaluate the contributions of OCTNs and OCTs to pulmonary absorption of IPR *in vivo*, by directly measuring tracheal tissue accumulation of IPR in mice after intratracheal injection of radiolabeled IPR. We also examined the expression of organic cation transporter genes at the mRNA and protein levels in mice. Here, we present the first evidence that organic cation transporters, Octn2 and Octs, contribute *in vivo* to tracheobronchial absorption of the anticholinergic bronchodilator IPR. This result indicates that these transporters are likely to be important determents of the pharmacological efficacy of cationic anticholinergic drug such as IPR.

EXPERIMENTAL

Chemicals

[^{3}H]Labeled and unlabeled IPR bromide (72 Ci/mmol) was provided by GlaxoSmithKline (Ware, Heartordshire, UK). [^{3}H]Carnitine (CAR) (0.010 Ci/mmol) and [^{14}C]MET (MET) (0.0259 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, Missouri) and Moravek Biochemicals (Brea California), respectively. MPP^+, ergothioneine (ERG), and quinidine (QUI) sulfate were obtained from Sigma–Aldrich (St. Louis, Missouri) and L-CAR was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

Table 1. Sequences of Oligonucleotide Primers Specific to Major Mouse Organic Transporter Genes

Transporter			
Protein	*Gene*	Sense (5′ to 3′)	Antisense (5′ to 3′)
Oct1	*Slc22a1*	ctctggctacaggagaacgac	gctccattatccttaccgcttg
Oct2	*Slc22a2*	tggcatcgtcacacctttcc	agctggacacatcagtgcaa
Oct3	*Slc22a3*	tctgccacactgatgcaact	tcagagttgtacccaacgacatt
Octn1	*Slc22a4*	ttcccacatctgaaccctct	gatccaagtggtacctgaaga
Octn2	*Slc22a5*	gtgatgaccctgatattccgt	tttcgtgggtgtgctgat

Cell Culture

Human Embryonic Kidney 293 (HEK293) cells stably transfected with cDNA of mouse Octn1 (HEK/mOctn1) or Octn2 (HEK/mOctn2), or plasmid vector alone (Mock cells), were previously established in our laboratory.[12] All cell lines were grown in Dulbecco's modified Eagle's medium (Wako Pure Chemicals) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2.

Evaluation of mRNA Expression by Reverse Transcription-Polymerase Chain Reaction

mRNA expression of murine Octn1, Octn2, Oct1, Oct2, and Oct3 in the trachea and bronchi was studied by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was prepared using Isogen reagent (Nippon Gene, Tokyo, Japan) from freshly isolated mouse tissues, and RT-PCR was performed using the gene-specific primers listed in Table 1. PCR products were electrophoresed and then visualized with ethidium bromide.

Immunohistochemistry

For light-microscopic immunohistochemistry, frozen sections were treated with 0.3% Tween 20 in phosphate-buffered saline (PBS) for 1 h for cell permeabilization, followed by 0.3% H_2O_2 in methanol for 10 min to inhibit intrinsic peroxidase activity. The sections were incubated with PBS containing 3% bovine serum albumin for 1 h for blocking, then incubated overnight at 4°C with antimouse Octn1, antimouse Octn2, or antirat Oct2 rabbit antibodies.[12,13] For the negative control, normal rabbit IgG was used. For Oct2, the Oct2 antibody was preabsorbed with blocking peptide for 1 h at room temperature. The sections were washed in PBS, incubated successively with horseradish peroxidase-conjugated antirabbit IgG antibody for 1 h (Dako, Glostrup, Denmark) and ImmPACT DAB Substrate (Vector Laboratories, Burlingame, California) for a few minutes, and then observed under an Olympus BX50 microscope (Olympus, Tokyo, Japan). For electron-microscopic immunocytochemistry, preembedding immune electron