Transport Mechanisms of Nicotine across the Human Intestinal Epithelial Cell Line Caco-2

ATSUKO FUKADA, HIDEYUKI SAITO, and KEN-ICHI INUI
Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan
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ABSTRACT
Ulcerative colitis is a disease more commonly seen in nonsmokers. Because nicotine was postulated to be a beneficial component of tobacco smoke for ulcerative colitis, various formulations of nicotine have been developed to improve the local bioavailability within the gastrointestinal tissue. In the present study, to characterize the disposition of nicotine in the intestines, we investigated intestinal nicotine transport using Caco-2 cells. Nicotine was predominantly transported across Caco-2 cell monolayers in a unidirectional mode, corresponding to intestinal secretion, by pH-dependent specific transport systems. The specific uptake systems appear to be distinct from organic cation transporters and the transport system for tertiary amines, in terms of their substrate specificity and the pattern of the interaction. These transport systems could play a role in the intestinal accumulation of nicotine from plasma and could also be responsible for the topical delivery of nicotine for ulcerative colitis therapy. These findings could provide useful information for the design of effective nicotine delivery.

Ulcerative colitis has been well known to be inversely related to tobacco smoke. Thus, ulcerative colitis usually occurs in nonsmokers, and especially among former smokers with ulcerative colitis, the disease typically begins after smoking cessation (Motley et al., 1987; Odes et al., 2001). Although the precise mechanisms of this protective role of tobacco smoke are not clear, nicotine, known to be a major psychoactive component of tobacco smoke, is suggested to play a major role in it. Recently, transdermal nicotine patches (Guslandi and Tittobello, 1994; Pullan et al., 1994; Bonapace and Mays, 1997) andnicotine gum (Lashner et al., 1990) have been documented to be effective for ulcerative colitis. However, the dose of nicotine to obtain adequate effectiveness for ulcerative colitis is complicated, because of the large first pass effect in the liver and the severe gastric and systemic adverse effects. In this aspect, some other formulas of nicotine such as sublingual nicotine tablets (Molander and Lunell, 2001), oral formulations of nicotine carbomers (Green et al., 1999), liquid enema (Zins et al., 1997), andrectal suppository formulations (Green et al., 1997;Dash et al., 1999) have been investigated. Considering the local delivery of nicotine such as enema to improve local bioavailability, it becomes more important to characterize the nicotine transport system across the epithelial cells in the large intestine and rectum itself.

The intestinal absorption and secretion mechanisms of lipophilic organic cations have been explained by the contribution of passive diffusion of nonionized compounds and also by specific carrier-mediated transport systems (Inui et al., 1992; Zhang et al., 1998). Multiple mechanisms appear to be involved in organic cation transport in both intestinal brush-border membrane vesicles and Caco-2 cells. The involvement of P-glycoprotein (Hsing et al., 1992; Hunter et al., 1993a,b) and some members of the amphiphilic solute facilitator (ASF) family such as OCT1, OCT2, or extraneuronal monoamine transporter (EMT) (Bleasby et al., 2000; Martel et al., 2001) have been reported. We previously demonstrated that diphenhydramine, an antihistamine, was transported across Caco-2 cell monolayers by H + -coupled specific transport systems that exist in both the apical and basolateral membranes (Mizuuchi et al., 1999, 2000a,b; Katsura et al., 2000). The direction of the transepithelial transport for tertiary amines such as diphenhydramine corresponded to intestinal secretion, indicating the existence of a secretory pathway for tertiary amines. In contrast, there has been little information about the intestinal transport of nicotine, which consists of a pyridine and an N-methyl pyrrolidine ring, thereby being a cyclic tertiary amine. After smoking, nicotine distributes throughout various tissues, including the brain, liver, skeletal muscles, kidney, and intestine, where the nicotine concentration rises to several times higher than that in plasma (Tsujimoto et al., 1975). Several studies also demonstrated

ABBREVIATIONS: ASF, amphiphilic solute facilitator; OCT, organic cation transporter; NMN, N1-methyl nicotinamide.
that an i.v. administration of nicotine resulted in a few percent of the dose recovering in the first 24-h sample of the feces (Fishman, 1963; Turner, 1969). However, the explicit mechanisms for absorption and secretion of nicotine in the intestine were still poorly understood.

In the present study, to characterize the disposition of nicotine in the intestines, we investigated the intestinal nicotine transport system in Caco-2 cells. To our knowledge, this is the first report demonstrating that the pH-dependent transcellular transport of nicotine is mediated by transport systems both in the apical and basolateral membranes, which are distinct from the transport systems for organic cations and tertiary amines.

**Experimental Procedures**

**Materials.** [3H]Nicotine (2571.5 GBq/mmol) and [3H]digoxin (703 MBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). D-[(14)C]Mannitol (1961 MBq/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). Nicotine tartrate dihydrate, tetraethylammonium bromide, cimetidine, chlorpheniramine maleate, and unlabeled mannitol were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Diphenhydramine hydrochloride was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Cotinine was obtained from Daiichi Pure Chemicals Co. (Ibaraki, Japan). Levofloxacin was kindly supplied by Daiichi Pure Chemicals Co. (Ibaraki, Japan). N1-Methylnicotinamide iodide and quinidine were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of the highest purity available.

**Cell Culture.** Caco-2 cells at passage 18, obtained from the American Type Culture Collection (Manassas, VA; ATCC HTB37), were maintained by serial passage in plastic culture dishes (Falcon; BD Biosciences, Franklin Lakes, NJ) as described previously (Inui et al., 1992). For the transport studies, Caco-2 cells were seeded on polycarbonate membrane filters (3-µm pores, 4.71- or 1.00-cm² growth area) inside Transwell cell culture chambers (Costar, Cambridge, MA) at a density of 6.3 × 10⁴ cells/cm². Transwell chambers were placed in six-well tissue culture plates with 2.6 ml of medium on the basolateral side and 1.5 ml of medium on the apical side. For the chambers placed in 12-well culture plates (1.00-cm² growth area), the volume of 1.0 ml outside and 0.33 ml inside medium were applied. The medium consisted of Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (BioReliance, Rockville, MD) and 1% nonessential amino acids (Invitrogen) without antibiotics. The cells were grown in an atmosphere of 5% CO₂, 95% air at 37°C, given fresh medium every 2 or 3 days, and used at 15 days of culture. In this study, cells between the 37th and 50th passage were used.

LLC-GAS-COL150 cells, stably transfected with human multidrug-resistant protein-1 cDNA (Taniigawara et al., 1992; Ueda et al., 1992), and LLC-PK1 cells (American Type Culture Collection, CRL 1392) as host cells were maintained by serial passage in plastic culture dishes as previously described (Ito et al., 1997). For the transport studies, LLC-GAS-COL150, and LLC- PK1 cells were seeded on polycarbonate membrane filters (3-µm pores, 4.71-cm² growth area) inside Transwell cell culture chambers at a density of 5.0 × 10⁵ cells/cm². The medium consisted of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum without antibiotics. The cells were grown in an atmosphere of 5% CO₂, 95% air at 37°C, given fresh medium every 2 days, and used at 6 days of culture.

**Measurements of Transcellular Transport and Cellular Accumulation.** Transcellular transport and accumulation of [3H]nicotine and [3H]digoxin were measured using monolayer cultures grown in Transwell chambers. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgSO₄, 5 mM N-glucose, and 5 mM HEPES (pH 7.4) or 5 mM MES (pH 6.0). The pH of the medium was adjusted with a solution of HCl or NaOH. After removal of the culture medium from both sides of the monolayers, the cell monolayers seeded on 4.71-cm² growth area were preincubated with 2 ml of incubated medium at 37°C for 10 min. Then, 2 ml of incubation medium containing the radioactive substrate was added to either the basolateral or the apical side, with 2 ml of nonradioactive incubation medium to the opposite side, and the monolayers were incubated for specified periods at 37°C. For the cell monolayers seeded on 1.00 cm² growth area, the volumes of 1 ml for the basolateral and 0.5 ml for the apical side were used.

D-[(14)C]Mannitol (0.2 µCi/ml) was used to calculate the paracellular flux and the extracellular trapping of [3H]nicotine (12.5 nM, 0.87 µCi/ml). [3H]Inulin (0.2 µCi/ml) was used to calculate the paracellular flux and the extracellular trapping of [3H]digoxin (100 nM, 1 µCi/ml). For the transport measurements, aliquots of the incubation medium on the other side were taken at specified times and the radioactivity was counted.

For the accumulation studies, the medium was removed by aspiration at the end of the incubation period, and the monolayers were rapidly washed twice with 2 ml (1.0 ml for a 1.0-cm² growth area) of ice-cold incubation medium on both sides. The filters with monolayers were detached from chambers, the cells on the filters were solubilized with 0.5 ml of 0.5 N NaOH, and the radioactivity in aliquots was counted. The radioactivity of the collected medium and the solubilized cell monolayers was determined in 5 ml of ACSII (Amer sham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) by liquid scintillation counting.

**Estimation of Kinetic Parameters.** The kinetics parameters for nicotine uptake in Caco-2 cells were estimated using the following equation: 

\[ V_0 = V_{\text{max}} S/K_m + S \]

where \( V_0 \) is the uptake rate of the nicotine (nmol/mg protein/15 s), \( S \) is the nicotine concentration (µmol/mg protein/15 s), and \( K_m \) is the coefficient of simple diffusion (µmol/mg protein/15 s). The uptake measurements were fitted to the above equation by nonlinear least-squares regression analysis.

**Statistical Analysis.** Statistical significance of differences between mean values was calculated using nonpaired t test or one-way analysis of variance followed by Scheffe’s test when multiple comparisons were needed. \( P < 0.05 \) was considered significant.

**Results**

**Transcellular Transport and Cellular Accumulation of Nicotine in Caco-2 Cells.** We first evaluated the transcellular transport and cellular accumulation of [3H]nicotine in Caco-2 cells. As shown in Fig. 1A, at neutral pH the transport from the basolateral to apical side was more than twice compared with the one from the apical to basolateral side. In the presence of an inward H⁺ gradient (apical side, pH 6.0; basolateral side, pH 7.4), the basolateral-to-apical transport of [3H]nicotine was significantly increased, much greater than the apical-to-basolateral transport, whereas the cellular accumulation of [3H]nicotine from both sides for 60 min was significantly decreased. Therefore, the pH-dependent [3H]nicotine transport corresponding to the intestinal secretion was observed in Caco-2 cell monolayers.

**Effect of The Apical pH on Nicotine Uptake.** Next, to determine the pH dependent manner of [3H]nicotine uptake in Caco-2 cells, we examined the effects of apical pH on the transcellular transport and cellular accumulations. As illustrated in Fig. 2, the basolateral-to-apical transport of [3H]nicotine was markedly increased by lowering the pH of the apical side (pH of the basolateral side was fixed to 7.4), accompanied by a decrease in the accumulation of monolay-
ers. These results suggested that the specific transport system accompanied with the pH of the apical side is involved in the efflux and the accumulation of nicotine in Caco-2 cells.

Effects of Various Organic Cations on Nicotine Uptake. The substrate specificities of the transport system(s) across the apical and basolateral membranes in Caco-2 cells were compared. The unlabeled nicotine showed potent inhibitory effects on [3H]nicotine uptake by Caco-2 cells from both sides, as did its major metabolite, cotinine (Fig. 3), whereas tetraethylammonium, cimetidine, and NMN, which appeared to be substrates of renal organic cation transporters (Urakami et al., 1998; Urakami et al., 2001), had no effects on the uptake. On the other hand, the accumulation of [3H]nicotine from both sides was markedly inhibited in the presence of quinidine and levofloxacin, a fluoroquinolone antibacterial drug. Overall, the similar substrate specificity of the transport systems was observed on the apical side and basolateral side of Caco-2 cells.

Kinetic Analysis of Nicotine Uptake. The accumulation of [3H]nicotine from the apical side and the basolateral side in Caco-2 cells was measured as a function of the substrate concentration. Since the specific accumulations from the apical and basolateral sides were linear for 30 s (data not shown), the accumulations for 15 s were taken in this experiment. As shown in Fig. 4, the uptake of [3H]nicotine from both the apical and basolateral sides showed saturability at high substrate concentrations. The Eadie-Hofstee plots after the correction of diffusion component showed a single-saturable process for nicotine accumulation in Caco-2 cells. The apparent V_max and K_m values for the apical nicotine uptake were 2.72 ± 0.35 nmol/mg protein/15 s and 0.91 ± 0.14 mM, respectively, whereas those for the basolateral nicotine up-

Fig. 1. Transcellular transport (A) and cellular accumulation (B) of [3H]nicotine by Caco-2 cell monolayers. Caco-2 cell monolayers were incubated at 37°C with 12.5 nM [3H]nicotine added to the apical side (triangles) or the basolateral side (circles) of the monolayers. The transcellular transport and cellular accumulation when the apical pH was adjusted to 6.0 are represented by closed symbols or closed columns. The appearance of radioactivity of the opposite side was periodically measured. After a 60-min incubation, the radioactivity of the solubilized cells was counted. Each symbol or column represents the mean ± S.E. of nine monolayers from three separate experiments.

Fig. 2. Effect of apical pH on the transcellular transport (A) and accumulation (B) of [3H]nicotine by Caco-2 cell monolayers. The cell monolayers were incubated for 15 min at 37°C with 12.5 nM [3H]nicotine added to the basolateral side and with the various levels of pH on the apical side. The appearance of the radioactivity of the apical side and accumulations were determined. Each point represents the mean ± S.E. of six monolayers from two separate experiments.

Fig. 3. Effect of various organic cations on [3H]nicotine accumulation from the apical side (A) and from the basolateral side (B) of Caco-2 cell monolayers. The cell monolayers were incubated for 15 min at 37°C with [3H]nicotine (12.5 nM) added to the apical side (A) or the basolateral side (B), in the absence (control) and presence of the other cationic drugs (5 mM) on the same side. Accumulation was measured at pH 7.4 at both the apical and the basolateral sides. Each column represents the mean ± S.E. of six monolayers from two separate experiments. P < 0.05, significantly different from control.

Fig. 4. Concentration dependence of [3H]nicotine uptake by Caco-2 cell monolayers. The cell monolayers were incubated for 15 s at 37°C with various concentrations of [3H]nicotine added to the apical side (A) or the basolateral side (B). The solid and broken lines represent the estimated total and nonsaturable transport, respectively. Inset shows Eadie-Hofstee plots of nicotine uptake after correction for the nonsaturable component. Each point represents the mean ± S.E. of 11 monolayers from four separate experiments. When error bars are not shown, they are smaller than the symbol. V, uptake rate (nmol/mg protein/15 s); S, nicotine concentration (mM).
Effects of Tertiary Amines on Nicotine Uptake. Since we previously demonstrated that there would be an H+-coupled tertiary amine transport system in Caco-2 cells, the effects of tertiary amines (1 mM) on the transepithelial transport and the accumulation of \[^{3}H\]nicotine from both sides of the Caco-2 cells were investigated. The tertiary amine compound, diphenhydramine, used as an antihistamine drug, showed a potent inhibitory effect on \[^{3}H\]nicotine uptake by Caco-2 cells from both sides (Fig. 5). Chlorpheniramine, an antihistamine drug, being the substrate of the tertiary amine transport system in Caco-2 cells, decreased the accumulation of \[^{3}H\]nicotine to the same extent.

Effect of Diphenhydramine on Nicotine Uptake. The effects of diphenhydramine on the accumulation of \[^{3}H\]nicotine from both sides of Caco-2 cells were compared with those with nicotine. As the concentration of diphenhydramine increased, the accumulation of \[^{3}H\]nicotine was depleted (Fig. 6). The IC\(_{50}\) on the apical membrane was 0.40 ± 0.10 mM, which was slightly smaller than that on the basolateral membrane, 0.52 ± 0.09 mM, whereas those for nicotine were 1.4 and 1.2 mM, respectively.

Inhibition Pattern of Diphenhydramine on Nicotine Uptake. To further investigate the effect of diphenhydramine on the accumulation of \[^{3}H\]nicotine from both sides of the Caco-2 cells, the inhibition patterns of diphenhydramine were analyzed. The Eadie-Hofstee plots showed a noncompetitive pattern for the inhibition of diphenhydramine (Fig. 7). The kinetic parameters from these plots are summarized in Table 1. The values of \(K_m\) from both the apical and basolateral sides of Caco-2 cells were not changed when 1 mM diphenhydramine was added to the same side of \[^{3}H\]nicotine.
and tetraethylammonium inhibited nicotine accumulation in specificities showed quite a difference; cotinine, cimetidine, value found in Caco-2 cells (0.84 mM), whereas the substrate (Takami et al., 1998) was slightly smaller than the transport systems in LLC-PK1 cells have been reported, in distinct characteristics of nicotine and tetraethylammonium were already well characterized both in the intestine (Zhang et al., 1998) and kidney (Inui et al., 2000). In the kidney, the involvements of ASF transporters such as OCT1 or OCT2 were reported to mediate the uptake of organic cations by Caco-2 cells (Bleasby et al., 2000; Martel et al., 2001). However, previous findings also suggested that the transport system for nicotine is different between Caco-2 and LLC-PK1 cells. We also confirmed that nicotine could not be the substrate for P-glycoprotein, an active efflux pump expressed in Caco-2 cells as well as in the intestine. Although the transport of digoxin in LLC-GA5-COL150 cells was higher than that in the host cell and was significantly inhibited by cyclosporin A, there was no difference between these two cell lines in the nicotine transport (Table 2). Therefore, it is more likely that the nicotine transport was mediated via the transport system, which would be distinct from those for organic cations and P-glycoprotein.

Previously, we reported that transport of tertiary amines was mediated by the pH-dependent specific transport system in Caco-2 cells (Mizuuchi et al., 1999, 2000a,b) and also in the brush-border membrane vesicles (Katsura et al., 2000). Our previous findings also suggested that the transport system for tertiary amines would specifically recognize the $N,N$-dimethyl or $N,N$-diethyl moieties in tertiary amine compounds (Mizuuchi et al., 2000a). In contrast, nicotine consists of an $N$-methyl pyrrolidine ring and, therefore, a cyclic tertiary amine. We found that diphenhydramine and chlorpheniramine, which were reported to be the typical substrates of the transport system for tertiary amines, strongly inhibited the nicotine accumulation from both sides of Caco-2 cells (Figs. 5 and 6). The uptake of these tertiary amines was reported to show profiles similar to those for nicotine in Caco-2 cells, in terms of the pH-dependent fashion and affinities ($K_m = 0.9$ mM for diphenhydramine) (Mizuuchi et al., 2000a). However, considering the inhibition pattern of diphenhydramine on the nicotine uptake in both the apical and basolateral sides of Caco-2 cells, the substrate recognition of the nicotine transport system appears to be different from that of tertiary amines (Fig. 7). When 1 mM diphenhydramine was added, only the $V_{max}$ values were significantly decreased, whereas $K_m$ values were not altered, suggesting noncompetitive inhibition. Therefore, the nicotine transport...
system is suggested to be independent of the tertiary amine transport system.

There has been overwhelming epidemiological evidence that smoking protects against ulcerative colitis (Motley et al., 1987; Odes et al., 2001). The mechanisms through which nicotine may affect the course of colitis may be relevant to the pathogenesis of this disease. It is also unknown how the nicotine in the plasma of smokers is delivered to the affected bowel to protect against the inflammation. Some possible mechanisms have been proposed, such as the stimulation of colonic mucus synthesis (Zijlstra et al., 1994; Thomas et al., 1997) and nicotinic oxide release (Green et al., 2000), the reduction in the intestinal blood flow (Srivastava et al., 1990), the influences to the immune system by endogenous steroid release (Kershbaum et al., 1968), and suppression of T helper-2 cell function as measured by the inhibition of inflammatory cytokines (Motley et al., 1990; Madretsa et al., 1996; Van Dijk et al., 1998). Nevertheless, the exact mechanisms responsible for the therapeutic benefit exerted by nicotine in ulcerative colitis are still to be determined. Furthermore, the primary step of the action for the beneficial effects on ulcerative colitis in smokers could be the delivery of nicotine to the affected bowels from plasma, but this has not yet been demonstrated.

The delivery system of nicotine in the affected bowels would be essential to clarify the beneficial nicotine effects on ulcerative colitis in smokers. The fact that nicotine was predominantly transported in a unidirectional mode from apical to basolateral in Caco-2 cells in a pH-dependent and concentration-dependent fashion (Fig. 1) suggests that the specific transport systems could mediate nicotine delivery from the plasma to the affected bowels in smokers, thereby affecting the therapeutic effects on ulcerative colitis. This secretory system of nicotine also corresponds to the findings that the intestinal secretion was observed in an in vivo study (Fishman, 1963; Turner, 1969). Therefore, the existence of a nicotine uptake system from the basolateral side of Caco-2 cells could be one of the considerable factors for the efficacy of smoking in ulcerative colitis. Furthermore, the study on the transport system of nicotine in the intestines might possibly provide information for the pathogenesis of ulcerative colitis, including the prevention of inflammation by nicotine at the cellular level.

Alternatively, to consider the safer nicotine preparations for more extensive clinical use for ulcerative colitis, the administration of nicotine at high doses topically into the colon might achieve therapeutic efficacy with only a modest rise in serum nicotine, avoiding the systemic side effects. For this purpose, various formulae as enemas have been developed. In the present study, the concentric accumulation from the apical side of Caco-2 cells was observed. Considering topical nicotine administration in the intestines, it is possible that the effective dose of nicotine could be delivered and accumulated from the lumen into the affected epithelial cells. However, at the large dose of nicotine administration in the lumen or at the alteration of pH in the lumen, the sharp rise of nicotine concentration in plasma might be observed, which could result in the adverse effects. Therefore, the present findings could be useful for future studies to develop safer nicotine preparations for effective ulcerative colitis therapy.

In conclusion, we revealed that specific transport systems were involved in the secretory transport of nicotine, which appeared to be distinct from the transport systems for organic cation and tertiary amines in Caco-2 cells, and also to be independent of the nicotine transport system in LLC-PK1 cells. The transport systems could play a role in the accumulation of nicotine from plasma in smokers to protect against inflammation in the bowels, and also in the nicotine delivery in the topical administration of nicotine for ulcerative colitis therapy. These results could be useful to assess the pathogenetic analysis of ulcerative colitis and the effective drug delivery of nicotine.

References


Address correspondence to: Professor Ken-ichi Inui, Ph.D., Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: inui@kuhp.kyoto-u.ac.jp