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

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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 its 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 compound 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) and nicotine 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), and rectal 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 amphibiphilic solute facilitator (ASF) family such as OCT1, OCT2, or extraneuronal monoamine transporters (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...
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-[14C]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 Kosy Co. (Tokyo, Japan). Cotinine 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-cm² growth area) inside Transwell cell culture chambers (Costar, Cambridge, MA) at a density of 6.3 x 10⁴ cells/cm². Transwell chambers were placed in six-well tissue culture plates with 2.6 ml of outside (basolateral) side and 1.5 ml of inside (apical) side medium. 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.

LCC-GAS-COL150 cells, stably transfected with human multidrug-resistant protein-1 cDNA (Tanigawara et al., 1992; Ueda et al., 1992), and LCC-PK 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, LCC-GAS-COL150, and LCC-PK 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 x 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.

**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 monolayer-
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 \( V_{\text{max}} \) and \( K_{\text{m}} \) values for the apical nicotine uptake were \( 2.72 \pm 0.35 \, \text{nmol/mg protein/15 s} \) and \( 0.91 \pm 0.14 \, \text{mM} \), respectively, whereas those for the basolateral nicotine up-
take were 1.53 ± 0.42 nmol/mg protein/15 s and 0.84 ± 0.20 mM, respectively.

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 transcellular transport and the accumulation of [3H]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 [3H]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 [3H]nicotine to the same extent.

Effect of Diphenhydramine on Nicotine Uptake. The effects of diphenhydramine on the accumulation of [3H]nicotine from both sides of Caco-2 cells were compared with those with nicotine. As the concentration of diphenhydramine increased, the accumulation of [3H]nicotine was depleted (Fig. 6). The IC50 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 [3H]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 Km 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 [3H]nicotine.

In contrast, a significant decrease in the Vmax values for the uptake from both sides was observed.

P-Glycoprotein on Nicotine Uptake in LLC-GA5-COL150 Cells. An active efflux pump, P-glycoprotein, was expressed in Caco-2 cells, as well as in the intestine (Hsing et al., 1992; Hunter et al., 1993ab), which was reported to strongly secrete some lipophilic organic cations. Therefore, we examined the transport of nicotine in the P-glycoprotein stably expressed cell line, LLC-GA5-COL150 cells, comparing it with the host, LLC-PK1 cells. The transport characteristics of nicotine were not changed between these two cell lines, and cyclosporin A, a typical inhibitor of P-glycoprotein, showed no effect on the nicotine transport (Table 2). In contrast, the transcellular transport of digoxin was significantly higher in LLC-GA5-COL150 cells, which was strongly inhibited by cyclosporin A (Table 2). These findings clearly suggested that nicotine could not be the substrate for P-glycoprotein.
and tetraethylammonium inhibited nicotine accumulation in Caco-2 cells (0.84 mM), whereas the substrate (1 mM) (Takami et al., 1998) was slightly smaller than the value found in Caco-2 cells (0.84 mM) (Takami et al., 1998) and kidney (Inui et al., 2000). In the kidney, the transport in Caco-2 cells could be mediated by the system family and this nicotine transporter suggested that nicotine transport in Caco-2 cells was not inhibited by either tertiary amines would specifically recognize the pH-partition theory. However, considering the pK_a values (6.2 and 10.9), and the unidirectional and saturable uptake of nicotine, a significant contribution of the specific transport system should also take part in nicotine secretion in addition to the passive diffusion.

We also investigated the substrate specificity of nicotine accumulation in Caco-2 cells. Previously, the putative involvements of AS1 transporters such as OCT1 or OCT2 were reported to mediate the uptake of organic cations by Caco-2 cells (Blesby et al., 2000; Martel et al., 2001). However, nicotine uptake by Caco-2 cells was not inhibited by either tetraethylammonium, cimetidine, or NMN, all of which were known to be typical substrates for organic cation transport systems, OCT1 and OCT2 (Urakami et al., 1998, 2001). This apparent difference in substrate specificity between the OCT family and this nicotine transporter suggested that nicotine transport in Caco-2 cells could be mediated by the system that is distinct from organic cation transport systems that were already well characterized both in the intestine (Zhang et al., 1998) and kidney (Inui et al., 2000). In the kidney, the distinct characteristics of nicotine and tetraethylammonium transport systems in LLC-PK_1 cells have been reported, in which pH-dependent transport of nicotine was unidirectional from the basolateral to apical side, corresponding to renal tubular secretion. The apparent K_m value of nicotine accumulation from the basolateral side of LLC-PK_1 cells (0.36 mM) (Takami et al., 1998) was slightly smaller than the value found in Caco-2 cells (0.84 mM), whereas the substrate specificities showed quite a difference; cotinine, cimetidine, and tetraethylammonium inhibited nicotine accumulation in LLC-PK_1 cells, although these had no influence in Caco-2 cells. Therefore, it is also suggested that the substrate specificity of the transport system for nicotine is different between Caco-2 and LLC-PK_1 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 on 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

### Table 1

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<tr>
<th></th>
<th>Apical Side</th>
<th>Basolateral Side</th>
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<tr>
<td></td>
<td>(K_m)</td>
<td>(V_{max})</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>nmol/mg/15 s</td>
</tr>
<tr>
<td>Control</td>
<td>0.91 ± 0.14</td>
<td>2.72 ± 0.35</td>
</tr>
<tr>
<td>+ Diphenhydramine</td>
<td>0.98 ± 0.25</td>
<td>0.69 ± 0.29*</td>
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* \(P < 0.05\), significantly different from controls.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>LLC-PK_1</th>
<th>LLC-GA5-COL150</th>
<th>LLC-PK_1</th>
<th>LLC-GA5-COL150</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/cm^2/h</td>
<td>pmol/cm^2/h</td>
<td>pmol/cm^2/h</td>
<td>pmol/cm^2/h</td>
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<tr>
<td>Control</td>
<td>1.37 ± 0.15</td>
<td>2.97 ± 0.03*</td>
<td>1.46 ± 0.05</td>
<td>1.48 ± 0.13</td>
</tr>
<tr>
<td>+ Cyclosporin A</td>
<td>1.29 ± 0.09</td>
<td>1.16 ± 0.03**</td>
<td>1.38 ± 0.05</td>
<td>1.45 ± 0.03</td>
</tr>
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* \(P < 0.05\), significantly different from the host. ** \(P < 0.01\), significantly different from its controls.

### Discussion

In the present study, we demonstrated that nicotine was predominantly transported in a unidirectional mode from basolateral to apical, corresponding to the intestinal secretion (Fig. 1). The nicotine transport was dependent on the pH at the apical side (Fig. 2), implying that the intestinal secretion of nicotine was increased at lower pH in the lumen. This pH dependence of nicotine transport might be partly explained by passive diffusion of the nonionized form according to the pH-partition theory. However, considering the pK_a values (6.2 and 10.9), and the unidirectional and saturable uptake of nicotine, a significant contribution of the specific transport system should also take part in nicotine secretion in addition to the passive diffusion.
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 nitric oxide release (Green et al., 2000), the reduction in the intestinal blood flow (Srivastava et al., 1990), the influence of 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; Madreetsma 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 system(s) 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 typically 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**


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