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Cultured Rabbit Corneal Epithelium Elicits Levofloxacin Absorption and Secretion

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Abstract

Evidence for carrier-mediated transport of levofloxacin in the isolated rabbit cornea has been found. However, it is not known whether this mechanism is located in the epithelium or the endothelium. To resolve this question, we have measured the kinetics of levofloxacin uptake in primary cultures of rabbit corneal epithelial cells.

The results indicate that levofloxacin accumulation was time dependent and a steady state was reached after 30 min. Maximal uptake occurred from a solution whose pH was 6·5. The uptake process was stereoselective and concentration dependent. In addition to the uptake, secretion of levofloxacin also occurred.

These results indicate that the corneal epithelium is the site of levofloxacin transport mechanisms, mediating both absorption and secretion.

The corneal epithelium of the eye acts as a barrier to the penetration of noxious agents but also limits drug penetration. These barrier properties are a consequence of the permeability of epithelial cellular and paracellular routes for drug permeation (Burstein & Anderson 1985; Järvinen et al 1995). Each route is different, hydrophilic drugs traverse more readily along the paracellular route whereas hydrophobic drugs show greater penetration through the cellular route (Schoenwald & Huang 1983; Wang et al 1991). It is becoming evident that in addition to the lipophilicity of drugs there is another determination for transcorneal drug penetration. This is suggested by the fact that drug transport occurs by both diffusion and a carrier-mediated process. We recently showed for the first time that there is a carrier system for the fluoroquinolone levofloxacin in the isolated rabbit cornea (Kawazu et al 1999). However, we were unable to determine whether it occurred in the corneal epithelium or the endothelium.

In this study, we report that a primary culture of rabbit corneal epithelial cells mediated levofloxacin uptake. The identification of this system in the epithelium should aid in the design of drugs for delivery into the eye.

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Materials and Methods

Materials

Levofloxacin and DR-3354, an optical isomer of levofloxacin, were kindly supplied by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Lomefloxacin hydrochloride was extracted from Lomebact tablets (Shionogi Pharmaceutical Co. Ltd, Japan). A primary culture of rabbit corneal epithelial cells was obtained from Kurabo Industries Ltd (Osaka, Japan) (Torishima et al 1996). Dulbecco's modified Eagle medium/Nutrient mixture F-12 (DMEM/F-12), foetal bovine serum and other tissue culture reagents were obtained from Gibco (Grand Island, NY). Epidermal growth factor, cholera toxin, hydrocortisone and insulin-transferrin sodium selenite media supplement were from Sigma Chemicals (St Louis, MO). Penicillin G and streptomycin were from Wako Pure Industries Ltd (Osaka, Japan). All other chemicals were commercial products of reagent grade.

Cell culture

Rabbit corneal epithelial cells were grown using DMEM/F-12 at pH 7·4 as described by Kawazu et al (1998). The culture medium was supplemented with 5% foetal bovine serum, 10 ng mL^{-1} epidermal growth factor, 0·1 μg mL^{-1} cholera toxin, 5 μg mL^{-1} insulin, 0·5 μg mL^{-1} hydrocortisone,

penicillin G 100 int. units mL^{-1} and streptomycin 100 μg mL^{-1}. For uptake studies, rabbit corneal epithelial cells were inoculated on culture dishes (16 mm diam.) at 8×10^3 cells cm^{-2}. The cells were cultured at 37°C under 95% air and 5% CO_2. The culture medium was replaced every day.

Uptake studies

Uptake experiments were performed after the rabbit corneal epithelial cells reached confluence after 8–9 days. Uptake of levofloxacin was measured by the method of Tsuji et al (1992, 1993). Briefly, the cultured cell layer was washed three times with 1 mL incubation buffer, Hank's balanced salt solution (HBSS), at 37°C. HBSS consisted of (mM): 1·3 $CaCl_2$, 5·0 KCl, 0·3 KH_2PO_4, 0·8 $MgCl_2$, 138 NaCl, 0·3 Na_2HPO_4, 5·6 D-glucose, and 10 MES for pH 5·5 and 6·5 or 10 HEPES for pH 7·4; osmolarity, 315 mOsm kg^{-1}. Cultured corneal epithelial cells were preincubated at 37°C for 30 min in the incubation solution. Immediately after the preincubation, the solution was removed by suction, and the incubation medium (250 μL) containing levofloxacin (in the standard experiment; 0·05 mM, pH 6·5) was added to each incubation well. To terminate the uptake reaction, corneal epithelial cells were washed with 1 mL ice-cold incubation medium at the designated time. Then, the mobile phase (described below) containing 1% Triton X-100 was added to each incubation well. The cells were disrupted by sonic oscillation (Ultrasonic Processor USP-600A: Shimadzu, Japan). The cellular debris was removed by centrifugation, and the supernatant sample was then analysed. Protein content in cultured cells was determined by the method of Lowry et al (1951). Levofloxacin concentrations in the medium and the corneal epithelial cells were obtained from the peak area of levofloxacin against that of internal standard, lomefloxacin hydrochloride. The samples were assayed by a high-performance liquid chromatography (HPLC) system (Gulliver: Jasco Corporation, Japan) on a reversed-phase TSKgel ODS-80T_S column (4·6 × 250 mm, particle size 5 μm, Tosoh, Japan). The mobile phase was a mixture of 0·01 M KH_2PO_4 (pH 3·0, adjusted by phosphoric acid) and acetonitrile (80 : 20, v/v). The flow rate was 1·0 mL min^{-1}. Retention times of the drugs were monitored with a spectrofluorometer (820-FP, Jasco Corporation; excitation wavelength 294 nm and emission wavelength 510 nm).

Analytical method

Uptake, expressed as the cell-to-medium concentration (C/M) ratio (μL (mg protein)$^{-1}$), was obtained by dividing the apparent uptake amount (mg protein)$^{-1}$ by the levofloxacin or DR-3354 concentration in the incubation medium. The energetic requirement of levofloxacin uptake by rabbit corneal epithelial cells was determined from the Arrhenius equation:

$$\log P_{app} = -[E_a/2{\cdot}303R][1/T] + A \qquad (1)$$

where E_a is the activation energy, R is the gas constant, T is the absolute temperature, and A is the integration constant. A plot of log (C/M ratio) against 1/T yields a straight line from whose slope (−E/2·303R) the activation energy can be determined (Grass & Robinson 1988). To estimate the kinetic parameters of concentration dependency for levofloxacin uptake, the uptake rate (V) was fitted to equation 2, consisting of both saturable, relative absorption (influx) and secretion (efflux), and nonsaturable linear terms, using the non-linear least-squares regression analysis program, MULTI (Yamaoka et al 1981):

$$V = [V_{max,influx}S/(K_{m,influx} + S)]_{influx} - [V_{max,efflux}S/(K_{m,efflux} + S)]_{efflux} + k_dS \qquad (2)$$

where V_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, K_m is the half-saturation concentration (Michaelis constant), and k_d is the first-order rate constant. All data are expressed as the mean ± s.d. Statistical analysis was performed using Student's two-tailed *t*-test. A difference between means was considered significant if $P < 0{\cdot}05$.

Results

Time course of levofloxacin uptake by rabbit corneal epithelial cells

To ascertain whether levofloxacin uptake by corneal epithelial cells has properties similar to those described for the in-vivo and isolated rabbit cornea, the effect of pH on levofloxacin uptake was determined. We showed previously that levofloxacin permeation in-vivo (Kawazu et al 1996) and in-vitro (Kawazu et al 1999) reached a maximum when the donor side pH was 6·5. Accordingly, we measured levofloxacin uptake over the same range studied by us in-vitro. As shown in Figure 1, accumulation of levofloxacin by corneal epithelial cells increased for 15 min before reaching saturation. The linear phase of uptake was more rapid in cells exposed to a solution of pH 6·5.

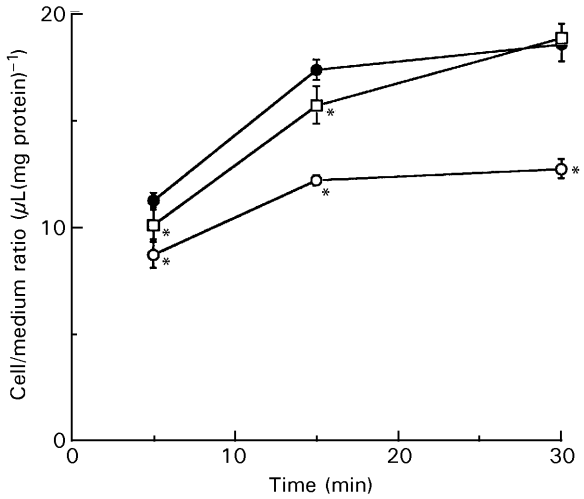

Figure 1. Time course of levofloxacin uptake by rabbit corneal epithelial cells. pH dependency of levofloxacin uptake. Uptake of levofloxacin (0·05 mM) was measured at 37°C. ○ pH 5·5, ● pH 6·5, □ pH 7·4. Each point represents the mean ± s.d. of four determinations. *$P < 0.05$ compared with the cell/medium ratio at pH 6·5 by Student's *t*-test.

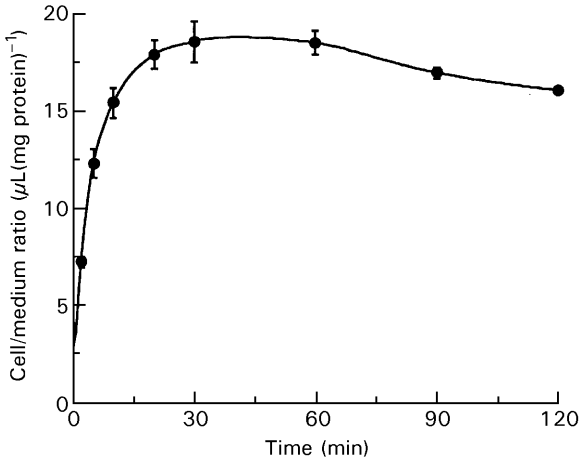

Figure 2. Time course of levofloxacin uptake by rabbit corneal epithelial cells. Uptake of levofloxacin (0·05 mM) was measured at 37°C, pH 6·5. Each point represents the mean ± s.d. of four determinations.

Figure 2 shows the time course of levofloxacin uptake by corneal epithelial cells at pH 6·5. Uptake of levofloxacin was time dependent and a steady state was reached within 30 min. Following attainment of the steady state, levofloxacin uptake gradually decreased. The kinetic parameters for uptake were evaluated at 5 min since uptake was linear during this period.

Effect of temperature on levofloxacin uptake

To evaluate the energy of activation, E_a, the effect of a stepwise reduction in temperature (37°C to 4°C) on uptake by corneal epithelial cells was determined. The effects are represented in an Arrhenius plot shown in Figure 3. The E_a for the levofloxacin uptake was 6·7 kcal mol^{-1}.

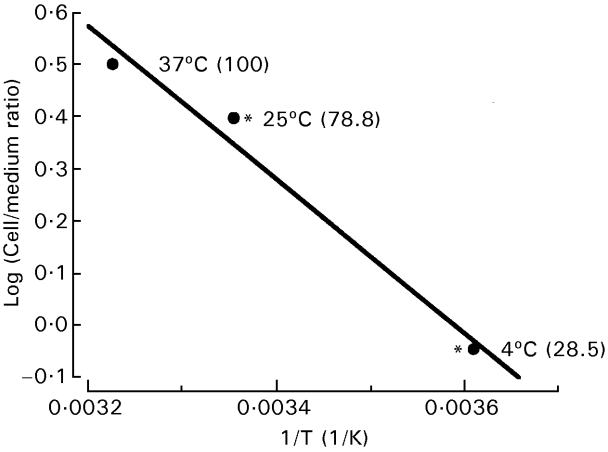

Figure 3. Arrhenius plot of the temperature dependence of levofloxacin uptake by rabbit corneal epithelial cells. Uptake of levofloxacin (0·05 mM) was measured at 4, 25, and 37°C, pH 6·5, for 5 min. Each point represents the mean of eight determinations. * $P < 0.05$ compared with the cell/medium ratio at 37°C by Student's *t*-test.

Stereoselectivity

Stereoselectivity of uptake by rabbit corneal epithelial cells was evaluated since such a finding is suggestive of carrier-mediated transport. The time course of levofloxacin was compared with that of its optical isomer, DR-3354 (Figure 4). The uptake of levofloxacin after 2·5 and 5·0 min was significantly greater than that for DR-3354. This result is consistent with a carrier-mediated process for levofloxacin uptake.

Concentration dependence

To evaluate the kinetics of levofloxacin uptake by rabbit corneal epithelial cells, the uptake was measured as a function of levofloxacin concentrations between 0·05 and 50 mM at pH 6·5. The results were analysed with an Eadie-Hofstee plot (Figure 5). Levofloxacin uptake was biphasic and concentration dependent. The uptake rate increased steeply up to 1 mM and then declined up to 5 mM before levelling off. The uptake after 5 min was significantly higher at 1 mM than at 0·05 or 5·0 mM. This result suggests that two different nonlinear events account for levofloxacin uptake. These events could reflect levofloxacin components of influx and efflux. The results of the evaluation of the kinetic parameters were: $V_{max,influx} = 42{\cdot}4$ nmol/5 min (mg protein)$^{-1}$; $K_{m,influx} = 3{\cdot}9$ mM; $V_{max,efflux} = 2{\cdot}65$ nmol/5 min (mg protein)$^{-1}$; $K_{m,efflux} = 0{\cdot}18$ mM; and $k_d = 7{\cdot}06$ μL/5 min (mg

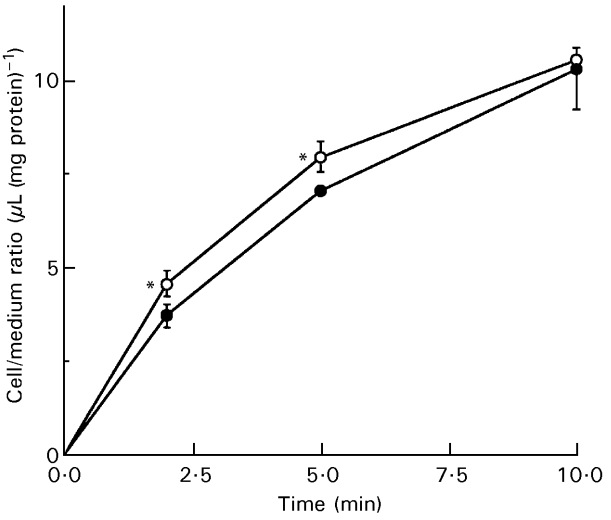

Figure 4. Time course of levofloxacin and DR-3354 uptake by rabbit corneal epithelial cells. Uptake of levofloxacin (○) and DR-3354 (0·05 mM; ●; (+)-isomer) was measured at 37°C, pH 6·5. Each point represents the mean ± s.d. of four to eight determinations. $^*P < 0.05$ compared with the cell/medium ratio of DR-3354 by Student's *t*-test.

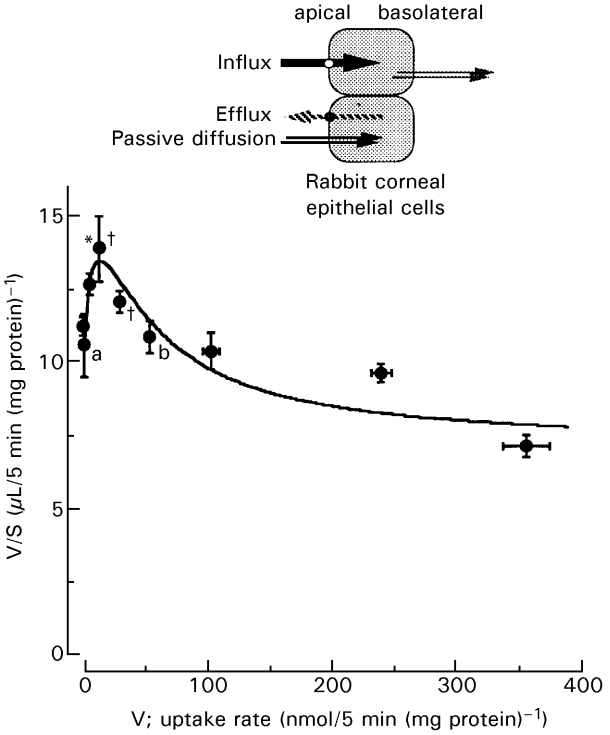

Figure 5. Eadie-Hofstee plots of levofloxacin uptake by rabbit corneal epithelial cells. Uptake of levofloxacin (0·05–50 mM) was measured at 37°C, pH 6·5. Each point represents the mean ± s.d. of four determinations. The solid line for V was calculated by fitting the data to the Michaelis–Menten equation. Extracted values for the kinetic parameters are: $V_{max,influx}$ of 42·4 nmol/5 min (mg protein)$^{-1}$, a $K_{m,influx}$ of 3·9 mM, a $V_{max,efflux}$ of 2·65 nmol/5 min (mg protein)$^{-1}$, a $K_{m,efflux}$ of 0·18 mM, and a k_d of 7·06 μL/5 min (mg protein)$^{-1}$. Schematic illustration is of the putative model for levofloxacin uptake by rabbit corneal epithelial cells based on our findings. * $P < 0.05$ compared with 0·05 mM, † $P < 0.05$ compared with 5 mM, by Student's *t*-test.

protein)$^{-1}$. Figure 5 also schematically illustrates the putative model for levofloxacin uptake by rabbit corneal epithelial cells based on our findings. The influx and efflux systems for levofloxacin should be located on the apical side of the corneal epithelium.

Discussion

Measurement of uptake rather than transepithelial permeation into cultured rabbit corneal epithelial cells was necessary because the culture system has higher paracellular permeability (Kawazu et al 1998). Levofloxacin is relatively hydrophilic and a high proportion of its flux would be by the paracellular route. Hence, it would be difficult to resolve any difference in unidirectional fluxes since the cellular component of the total flux would be exceedingly small. Therefore, it would not be possible to evaluate the existence of a carrier system based on the measurement of transepithelial fluxes. However, measurement of uptake by this system can clearly distinguish whether a carrier-mediated process is involved in levofloxacin permeation. This study was undertaken to determine whether the carrier-mediated levofloxacin transport described in the isolated rabbit cornea originates in the epithelium.

The results of our characterization of levofloxacin uptake showed that levofloxacin carrier-mediated transport occurred in the epithelium. Our results from this study indicate that levofloxacin uptake (tear-side to epithelium) was carrier mediated. The uptake was pH dependent and maximal at 6·5. We have shown (Kawazu et al 1999) that at this pH the transcorneal levofloxacin permeation in the isolated cornea from the stromal to the tear-side solution was maximal and significantly larger than levofloxacin permeation in the opposite direction. This study suggests that the carrier-mediated levofloxacin transporter resides in the epithelium. This is because uptake was significantly lower with DR-3354, the stereoisomer of levofloxacin, than with levofloxacin, indicating carrier-mediated uptake. The energetic requirement is consistent with carrier-mediated uptake. The value for E_a was 6·7 kcal mol^{-1}, which is larger than values of E_a associated with diffusion. For diffusion, the value for the E_a seldom exceeds 4 kcal mol^{-1} (Höfer 1981). The apparent $K_{m,influx}$ for uptake in cultured rabbit corneal epithelial cells was 3·9 mM, which corresponds very closely to the value of the K_m for transcorneal levofloxacin transport (i.e. 3·83 mM) (Kawazu et al 1999). Furthermore, our results agree

with those for intestinal absorption of ofloxacin (i.e. 3·80 mM) (Prieto et al 1988).

We suspect that in addition to an uptake mechanism there exists, in the epithelium, an efflux component (epithelium to tear-side) for levofloxacin permeation. This is indicated by the uptake of levofloxacin decreasing after reaching a maximum at 0·05 mM (Figure 2). Such a pattern was described in transport and uptake studies of azasetron, a 5-hydroxytryptamine-receptor antagonist, in Caco-2 cells (Tamai et al 1997). In that study, the decline was explained in terms of specialized transporters in both the absorptive and secretory direction. Similarly in rabbit corneal epithelial cells there may be levofloxacin transporters in both the absorptive (tear to stroma) and secretory (stromal to tear side) direction. Other evidence for a secretory mechanism is the kinetic analysis (c.f. Figure 5) based on measurement of the concentration dependence of levofloxacin uptake, best described by including an efflux component.

Regarding the identity of the carrier system, there are several possibilities. It is possible that the carrier-mediated influx pathway is a monocarboxylic acid transporter (Simanjuntak et al 1991; Takanaga et al 1994), whereas the efflux mechanism could be a P-glycoprotein (Griffiths et al 1993, 1994) or an organic cationic transporter (Okano et al 1990; Hirano et al 1995a, b; Ohtomo et al 1996; Rabbaa et al 1996). There is evidence that the rabbit corneal epithelium contains a monocarboxylic acid transporter based on the identification of lactate-cotransport in this layer (Bonanno 1990). At this time we cannot make a definitive assignment regarding which one of these alternatives describes the levofloxacin carrier system in the rabbit corneal epithelium. We conclude that in addition to passive diffusion, the corneal permeation of fluoroquinolones may be accounted for by a specific carrier-mediated transporter in the epithelium.

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