Transport of Acebutolol through Rabbit Corneal Epithelium

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The purpose of this study is to characterize transport of acebutolol through the corneal epithelium. Cultured normal rabbit corneal epithelial cells (RCEC) were used to investigate the drug transport. Primary RCEC were seeded on a filter membrane of Transwell-COL® insert coated with fibronectin and were grown in Dulbecco's modified Eagle's medium/nutrient mixture F-12 with various supplements. Measurements of acebutolol permeability through RCEC layer were carried out to assess transcellular permeability coefficient ($P_{transcell}$) in the absence or presence of inhibitors. Paracellular permeability coefficient ($P_{paracell}$) was calculated by permeability coefficient of hydrophilic drugs (P_{cell}). The transcellular permeability of acebutolol from apical side to basal side (A-to-B) showed concentration-dependency. The acebutolol flux in the A-to-B direction was smaller than that of opposite direction. Sodium azide, verapamil, and cyclosporin A enhanced the transcellular permeability of acebutolol in the A-to-B direction. Acebutolol permeability through an excised rabbit cornea was also increased by verapamil. Thus, it was suggested that acebutolol was actively secreted *via* P-glycoprotein in a corneal epithelium.

Key words cultured rabbit cornea; acebutolol; transporter; P-glycoprotein; corneal epithelium

In ophthalmic pharmacotherapy, the cornea is considered to be a major pathway for ocular permeation of topically applied drugs.[1,2] The cornea, however, is impermeable to hydrophilic compounds because of a poor paracellular route due to the epithelial tight junctions.[3,4] Passive diffusion across the lipid cell membrane primarily contributes to the penetration of most ophthalmic drugs through the intact cornea. Many drugs had an almost parabolic relationship between the corneal permeability and the drug lipophilicity.[5,6]

On the other hand, several transporters are expressed in ocular tissue. Jain-Vakkalagadda *et al*. identified an active transport system for L-arginine on rabbit corneal epithelium and human cornea and showed that amino acid ester prodrugs of acyclovir appeared to be substrates for this transporter.[7] Sodium dependent nucleoside transporter was functionally expressed on the rabbit cornea although acyclovir and idoxuridine were not substrates for this transporter.[8] We previously demonstrated an expression of P-glycoprotein (P-gp) on cultured normal rabbit corneal epithelial cells (RCEC).[9] Dey *et al*. reported that P-gp restricts topical erythromycin absorption across the cornea, which can be inhibited by known P-gp inhibitors.[10]

There was a relationship between the permeability of beta-blockers, which are often used in ophthalmic therapy, through an excised rabbit cornea and their lipophilicities.[6, 11] Among them, however, permeability of acebutolol was much lower than that expected by its lipophilicity. This results indicated the possibility that acebutolol was secreted by specific transporter in corneal epithelium.

Therefore, in the present study, the permeation mechanism of acebutolol in the corneal epithelium is investigated by RCEC system that we previously establised.[12]

MATERIALS AND METHODS

Materials and Animals Acebutolol, FITC-dextran (FD-4, molecular weight 4400), 6-carboxyfluorescein (6-CF) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Cyclosporin A (CyA) was kindly supplied from Sandoz Pharmaceuticals (Basel, Switzerland). Sodium azide (NaN_3) and verapamil hydrochloride (VER) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were commercial products of reagent grade.

Primary cultured cells were obtained from Kurabo Industries Ltd. (Osaka, Japan). Transwell-COL® cell culture chambers (pore size 0.4 μm, diameter 12 mm, surface area 1 cm^2) were purchased from Costar (Bedford, MA, U.S.A.). Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12), fetal bovine serum (FBS) and another culture reagents were purchased from GIBCO (Grand Island, NY, U.S.A.). Epidermal growth factor (EGF), choleratoxin (CTX), hydrocortisone (HCS), and insulin-transferrin sodium selenite media supplement (ISL) were purchased from Sigma Chemicals. Penicillin G and streptomycin were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Human fibronectin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). ^{14}C-Mannitol (specific activity, 2.11 GBq/mmol) was purchased from Amersham Life Science (Buckinghamshire, U.K.).

Male Nippon albino rabbits (Kitayama Labes, Ltd. Japan: 1.5—2.5 kg) were used in the permeability experiment through an excised rabbit cornea. They were housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co. Ltd., Tokyo, Japan). The rabbits were fasted for 24 h before use but had free access to water. All experiments in the present study conformed to Guideline for Animal Experimentation in Nagasaki University.

Cell Culture RCEC were cultured according to an ordinary method reported previously.[12] RCEC were grown using DMEM/F-12 at pH 7.4. The culture medium was supplemented with 5% FBS, 10 ng/ml EGF, 100 ng/ml CTX, 5 μg/

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ml ISL, 500 ng/ml HCS, and the antibiotics (penicillin G 100 IU/ml+streptomycin 100 μg/ml). Transwell-COL® insert was pre-coated with 4.0 μg human fibronectin as the attachment factor at room temperature for 30 min. RCEC were seeded at a density of 4×10^4 cells/cm^2 on filter membrane of Transwell-COL® insert and cultured at 37 °C under 95% air and 5% CO_2. The barrier of RCEC layer grown on the filter membrane was assessed by measuring the transepithelial electrical resistance (TEER) with a Millicell ERS electrical resistance meter (Millipore, Bedford, MA, U.S.A.).

Permeability Study Using RCEC In permeability study, RCEC grown on a filter membrane were washed three times with Hank's balanced salt solution (HBSS) (1.3 mM $CaCl_2$, 5.0 mM KCl, 0.3 mM KH_2PO_4, 0.8 mM $MgCl_2$, 138 mM NaCl, 0.3 mM Na_2HPO_4, 5.6 mM D-glucose, and 10 mM HEPES for pH 7.4) and preincubated for 30 min at 37 °C in a 5% CO_2 atmosphere before permeability experiments.

Drug permeability from apical side to basal side (A-to-B) was initiated by removing all of HBSS in apical side (0.5 ml) and being replaced with HBSS containing various concentrations of drugs at 37 °C. At 30, 60, 90, and 120 min, sample (0.9 ml) was collected from the basal side (1.5 ml) and was replaced with equal volume of HBSS. Drug permeability from basal side to apical side (B-to-A) was also initiated by being replaced with drug solution in basal side and sample (0.3 ml) was collected from the apical side at 37 °C. The integrity of the cell layer was checked at the beginning and at the end of the permeability experiments by determination of the TEER. The collected samples were supplied for drug determination with a high performance liquid chromatography (HPLC). Hydrophilic compounds such as ^{14}C-mannitol (1.85 KBq), 6-CF (50 μM), and FD-4 (50 μM) were also used as penetrants. Drug permeability through filter membrane without cell layer was examined in the same manner.

In permeability study with inhibitors, the cell layer was preincubated with inhibitors in both apical and basal sides for 30 min. The inhibitor was also present in both sides during the experiment. Sodium azide (NaN_3, 10 μM), verapamil hydrochloride (VER, 200 μM), and cyclosporin A (CyA, 30 μM) were used as inhibitors.

Calculation of Transcellular Permeability Coefficient The apparent permeability coefficient through overall membrane (P_{app}, cm/s) was calculated from a slope (flux, nmol/h) of drug amount profile in receiver side (P_{app}=slope/3600/surface area of the layer/initial concentration in the donor side).

The permeability coefficient of drugs through filter membrane (P_{filt}) was obtained from the drug flux through the fibronectin-coated filter membrane. The permeability coefficient of drugs through the RCEC layer (P_{cell}) was calculated by Eq. 1.

$$P_{cell}=P_{app}\times P_{filt}/(P_{filt}-P_{app}) \quad (1)$$

Further, the P_{cell} includes the permeability coefficients *via* transcellular route ($P_{transcell}$) and paracellular route ($P_{paracell}$). The $P_{transcell}$ was calculated by subtracting $P_{paracell}$ from P_{cell}. The $P_{paracell}$ of acebutolol was calculated from an inverse relationship between the P_{cell} and square root of the molecular weight in hydrophilic compounds.

Permeability Study Using an Excised Rabbit Cornea Rabbits were sacrificed with an injection of sodium pentobarbital into a marginal ear vein. The cornea was dissected and mounted in the Ussing chamber. HBSS solutions (4 ml) were added to the epithelial side and the endothelial side, respectively. The bathing solutions were bubbled with 5% CO_2 in air to maintain the pH at 7.4 and to provide adequate agitation of the solution. The Ussing chamber assembly was maintained at 37 °C by a water jacket, using a circulating water bath. After 30 min incubation, permeability was initiated by removing 0.4 ml of HBSS in epithelial side and being replaced with HBSS containing 1 mM acebutolol with or without 500 μM VER. At 15, 30, 45, 60, 90, and 120 min, aliquots (0.2 ml) were sampled from the endothelial side and replaced with the equal volume of HBSS. Acebutolol in the samples was assayed with an HPLC system. The apparent permeability coefficient through excised cornea (P_{app}, cm/s) was calculated from a slope (flux, nmol/h) of drug amount profile in receiver side (P_{app}=slope/3600/surface area of the layer/initial concentration in the donor side).

Drug Determination The samples of ^{14}C-mannitol were measured by using a liquid scintillation counter (Tri-Carb® Models 2100TR, Packard Co., Meriden, CT, U.S.A.). The samples of 6-CF and FD-4 were assayed by a spectrofluorophotometer (FP-770, JASCO Corporation, Tokyo, Japan; excitation and emission wavelengths; 492 and 524 nm for 6-CF; 495 and 514 nm for FD-4).

At *in vitro* permeability study, samples of acebutolol (0.3 ml) were mixed with methanol (0.6 ml) including internal standard (0.05 mM phenacetin). The mixture was centrifuged at 12000 ***g*** for 10 min and 50 μl of supernatant was injected into an HPLC system (LC-10AD, Shimadzu Co., Ltd., Kyoto, Japan) used in a reverse-phase mode. A stationary phase used was Cosmosil 5C_{18}-MS packed column (150 mm length×4.6 mm i.d., Nacalai Tesque Inc., Kyoto, Japan). Mixtures of acetonitrile and 10 mM KH_2PO_4 (85 : 15 v/v) were used as a mobile phase with a flow rate of 1.0 ml/min. Acebutolol was monitored with a UV spectrophotometric detector (240 nm).

Samples of acebutolol in permeability experiment through an excised rabbit cornea were assayed with another HPLC system (GULLIVER®: JASCO Corporation, Tokyo, Japan) used in a reverse-phase mode. A stationary phase used was Inertsil ODS-3V column (250 mm length×4.6 mm i.d., GL Sciences, Tokyo, Japan). Mixtures of methanol and 50 mM NaH_2PO_4 (43 : 57 v/v) were used as a mobile phase with a flow rate of 1.0 ml/min. Retention of acebutolol was monitored with a UV spectrophotometric detector (254 nm).

RESULTS

Using DMEM/F-12 containing 5% FBS, supplemented with EGF, CTX, ISL, and HCS, RCEC were readily attached to the matrix and began to spread. The density of seeding was 4×10^4 cells/cm^2 and led to rapid confluence. When allowed to become highly postconfluent, the cells became very tightly packed, and stratify on the surface of the membrane. The cells grew multilayer and had high transparency. The TEER increased to a maximum (143.8 ± 23.3 ohm×cm^2) around day 8 after inoculation. Cultured cells appeared to be more closely approximating *in vivo* corneal tissue in morphology.

There was an inverse linear relationship between square root values of molecular weight and P_{cell} values in hy-

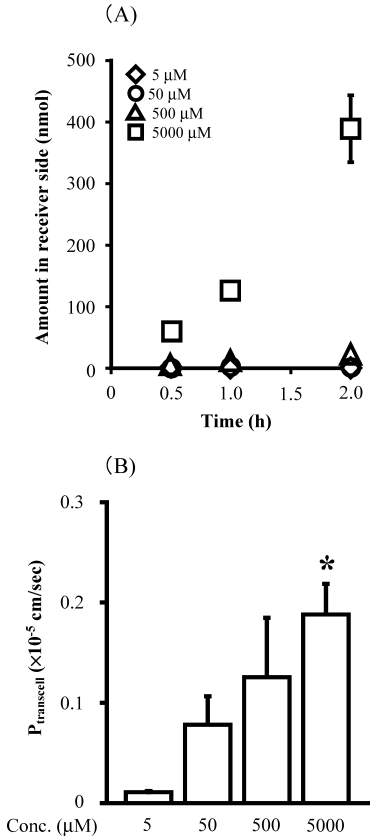

Fig. 1. Effect of Concentration on Acebutolol Permeation in the Apical Side to Basal Side (A-to-B) Direction

(A) Time course of acebutolol permeation. (B) Comparison of permeability coefficient through transcellular route ($P_{transcell}$). Each data represents the average of at least three experiments±S.E. (*p<0.05: significantly different from $P_{transcell}$ of 5 μM and 50 μM by Student's t-test).

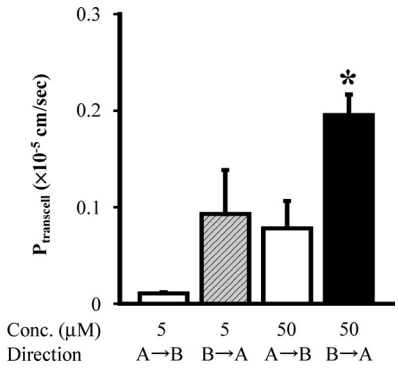

Fig. 2. Effect of Directions on Transcellular Permeability Coefficients ($P_{transcell}$) of Acebutolol

Each data represents the average of at least three experiments±S.E. (*p<0.05: significantly different from $P_{transcell}$ of 50 μM in the apical side to basal side (A-to-B) direction by Student's t-test).

drophilic drugs. The $P_{paracell}$ of acebutolol was calculated from the inverse relationship and the $P_{transcell}$ was determined by subtracting $P_{paracell}$ from P_{cell}. The permeation profiles of acebutolol in the A-to-B direction at various concentrations (5—5000 μM) are shown in Fig. 1A. The $P_{transcell}$ values of acebutolol were calculated from these profiles and are presented in Fig. 1B. The $P_{transcell}$ values of acebutolol significantly increased with an increase of drug concentrations. Figure 2 shows the $P_{transcell}$ values of acebutolol (5, 50 μM) in

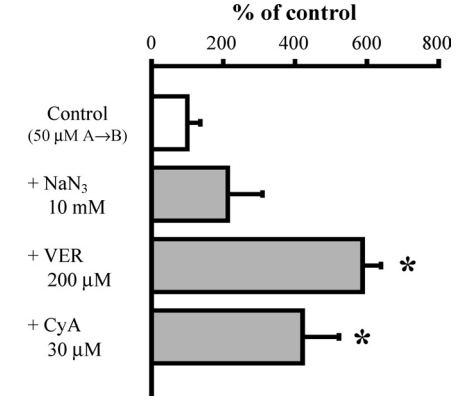

Fig. 3. Effect of Inhibitors on Transcellular Permeability Coefficients ($P_{transcell}$) of Acebutolol

Control is the $P_{transcell}$ at 50 μM in the apical side to basal side (A-to-B) direction. Each data represents the average of at least three experiments±S.E. (*p<0.05: significantly different from control by Student's t-test).

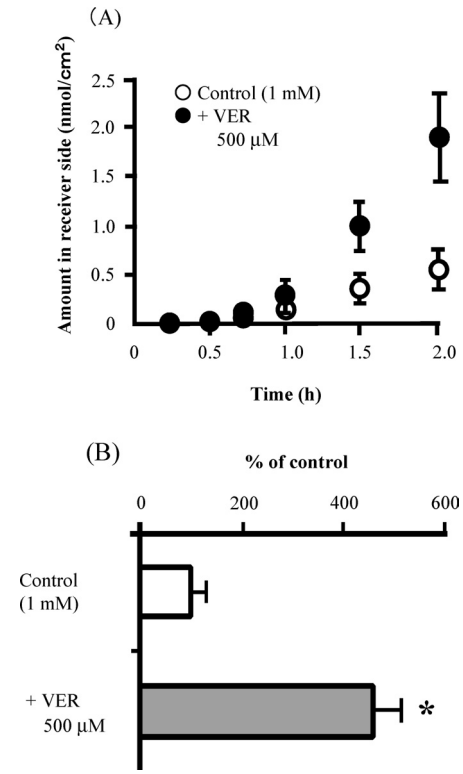

Fig. 4. Effect of VER on Acebutolol Permeation through Excised Rabbit Cornea

(A) Time course of acebutolol permeation. (B) Comparison of corneal permeability coefficient (P_{app}). Each data represents the average of at least three experiments±S.E. (*p< 0.05: significantly different from control, 100%, by Student's t-test).

different directions. The $P_{transcell}$ values of acebutolol in the A-to-B direction was smaller than that of opposite direction (B-to-A) at 5 and 50 μM. Figure 3 shows the effects of inhibitors on the transcellular permeability of acebutolol (50 μM) in the A-to-B direction. NaN_3, VER, and CyA enhanced the $P_{transcell}$ values of acebutolol.

Figure 4 shows the effect of VER on permeability of acebutolol using an excised rabbit cornea. Measurable lag time in permeation profile of acebutolol was observed (Fig. 4A). VER significantly enhanced the corneal permeability coefficient, P_{app}, of acebutolol through an excised cornea as shown

in Fig. 4B.

DISCUSSION

The corneal epithelium consists of 5—6 cell layers of non-keratinized squamous cells. The epithelium consists of a basal layer of columnar cells, 2—3 layer of wing cells, and 1 or 2 outermost layers of squamous, polygonal-shaped superficial cells.[13] The outer layer of the surface cells possess microvilli on their anterior surface that presumably help to anchor the precorneal tear film. The stratified corneal epithelium acts both as a protective barrier to prevent invasion of foreign substances. RCEC were well-developed microplicae and were multilayered.[12] Their cytoplasmic morphology and desmosomes were analogous to the *in vivo* situation.

Transcellular drug permeation was calculated by subtracting the paracellular permeation. Despite the leakiness of our model system, it is still appropriate system for evaluating transcellular drug permeability. In fact, the $P_{transcell}$ value of acebutolol (1.88×10^{-6} cm/s) was almost agreed with its permeability coefficients (1.10×10^{-6} cm/s) through an excised cornea reported previously.[6] This agreement indicates that acebutolol predominantly permeate *via* transcellular route across excised tight cornea.

The epithelial permeability of acebutolol was direction- and concentration-dependent, indicating the special secretion from basolateral side to apical side (Figs. 1, 2). The acebutolol permeability in the A-to-B direction was increased by metabolic inhibitor, NaN_3 (Fig. 3). However, the timolol and tilisolol permeability in the A-to-B direction was not increased by NaN_3 in the preliminary experiment. These results showed that active transporter mediated permeability of acebutolol in the B-to-A direction. This results is agreement with the previous report that acebutolol's permeability coefficient through a rabbit excised cornea was much lower than expected by its lipophilicity.[10] P-gp, one of ATP binding cassette (ABC) transporter, is well-known drug efflux pump and is expressed in various tissues.[14,15]

We previously demonstrated that P-gp appears to be located on cultured rabbit corneal epithelial cells using CyA as P-gp substrate.[7] Dey *et al*.[16] reported that cornea and corneal epithelial cells expresses the functionally active P-gp efflux pump using CyA and Rhodamin-123 as P-gp substrates. In Fig. 3, CyA and VER, which were transported by P-gp, significantly increased the permeability of acebutolol in the A-to-B direction, suggesting the contribution of P-gp to active secretion of acebutolol in corneal epithelium. VER also enhanced the permeability of acebutolol from epithelial side to endothelial side through an excised cornea as shown in Fig. 4. There was a relationship between the permeability of beta-blockers through an excised rabbit cornea and their lipophilicities.[6,11] Among them, however, permeability of acebutolol was much lower than that expected by its lipophilicity. This results indicated the possibility that acebutolol was secreted by specific transporter in corneal epithelium.

Thus, acebutolol was suggested to be actively secreted *via* P-gp in a corneal epithelium. The lower permeability of acebutolol through cornea and conjunctiva than that expected by its lipophilicity was considered to be caused by this secretion transporter. P-gp may play a role of protective barrier on foreign substances. RCEC system is also useful to characterize the drug permeation mechanism through corneal epithelium.

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