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THE INTERACTION OF CEPHALOSPORIN ANTIBIOTICS WITH RENAL CORTEX OF RATS: ACCUMULATION TO CORTICAL SLICES AND BINDING TO PURIFIED PLASMA MEMBRANES

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Abstract—The interaction of cephalosporin antibiotics with renal cortex of rats has been examined *in vitro* by means of cortical slice uptake and binding assay to plasma membranes and other subcellular fractions, including the comparative study with *p*-aminohippurate. The uptakes of cephalexin and cephaloridine by cortical slices were concentrative, and were strongly inhibited in the presence of 2,4-dinitrophenol, ouabain, nitrogen gas and probenecid. In the case of cefazolin, the degree of concentrative uptake and influence of inhibitors were low. Cephalexin, cephaloridine and *p*-aminohippurate specifically bound to the basolateral membranes, compared to brush border membranes and other subcellular fractions. Cefazolin binding to basolateral membranes was relatively small. These results suggest that the specificity of cephalexin and cephaloridine bindings to the basolateral membranes could be related to the interaction with the organic acid transport system at the antiluminal side. Thus, an examination of cephalosporin interaction with plasma membranes from renal cortex could offer an appropriate *in vitro* model system to study the renal transport of these antibiotics.

The cephalosporin antibiotics, the most widely used antimicrobial agents, are excreted principally by the kidney. In this [1] and other laboratories [2, 3], renal handlings of cephalosporins have been investigated by the clearance technique *in vivo*, indicating that the mechanisms responsible for the elimination of cephalosporins are glomerular filtration and tubular secretion. Certain of the cephalosporin antibiotics cause acute necrosis of the proximal renal tubule in a variety of mammalian species. The mechanism of this cytotoxic effect has been examined with cephaloridine, the most toxic cephalosporin so far studied [4, 5]. Since both transport and toxicity are prevented in the presence of competitive inhibitors for organic acid transport, e.g. probenecid, *p*-aminohippurate and benzylpenicillin, it has been concluded that the nephrotoxicity of cephaloridine is related to the high intracellular concentrations that result from its active transport [5–9]. However, it has been difficult to characterize the specific membrane events underlying the transport of the antibiotics in the intact tubule, because of the complexities involved in studying tubular transport processes that are bidirectional. The renal proximal tubule cell, which transports solutes bidirectionally across the tubular epithelium, is characterized by a plasma membrane that is differentiated into two distinct segments, the luminal brush border and the contraluminal basolateral membranes. The asymmetry is demonstrated by the findings that the two membranes differ morphologically, functionally and biochemically [10, 11].

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

Recently we established a simple method for the separation of basolateral membranes from brush border membranes in rat renal cortex [12]. In order to obtain more precise information about the mechanisms of renal cephalosporin excretion, the present studies were designed for the comparison of *in vitro* renal cortical uptake and of the interaction with subcellular fractions isolated from renal cortex, using the representative cephalosporins such as cephalexin, cephaloridine and cefazolin. Some comparative studies with *p*-aminohippurate are included in this report. The present data indicate that cephalexin and cephaloridine are actively accumulated by renal cortical slices, and specifically interact with basolateral membranes compared to brush border membranes and other subcellular fractions, although these characteristics for cefazolin are to a little extent. Corresponding studies of cephalosporin interaction with renal plasma membranes are currently absent from the literature.

MATERIALS AND METHODS

Preparation of cortical slices and subcellular fractionation. The excised kidneys from male Wistar albino rats, weighing 190–230 g, were placed as rapidly as possible into ice-cold saline. All subsequent steps were carried out on ice. The kidneys were decapsulated, and a thin slice of the renal cortex (approximately 0.3–0.4 mm thick) was prepared with a Stadie–Riggs microtome. In the case of subcellular fractionation of cortical tissues, the general procedures were the same as the isolation method of basolateral plasma membranes from renal cortex

recently described by us [12]. The minced cortical tissues were homogenized in 0.25 M sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.5, using a loosely fitting Dounce homogenizer, and the subcellular fractions were obtained under the following centrifugal conditions: pellet 1, 2400 *g* briefly; pellet 2, 2400 *g* for 15 min; pellet 3 (dark pellet) and crude plasma membranes (fluffy layer), 20,500 *g* for 20 min. After the Percoll density gradient centrifugation of crude plasma membranes, the top 8 ml of the gradient was collected as fraction I, then 5 ml as fraction II (basolateral membranes), 5 ml as fraction III, 4 ml as fraction IV (brush border membranes) and 8 ml as fraction V were collected. Finally an aliquot of fraction in each step of subcellular fractionation was suspended in 20 mM Tris–HEPES, pH 7.5 and centrifuged at 45,000 *g* for 30 min. The final pellet was resuspended in 20 mM Tris–HEPES, pH 7.5, by sucking the suspension 5 times through a fine needle with a plastic syringe. The preparation of purified brush border membranes was performed as previously described [12], according to calcium precipitation method of Evers *et al.* [13]. As confirmative data, $(Na^+ + K^+)$-ATPase, the marker enzyme for basolateral membranes, was enriched 22-fold in the basolateral membrane preparation (fraction II) compared with that found in the homogenate. Alkaline phosphatase and aminopeptidase, the marker enzymes for brush border membranes, were enriched 10-fold in purified brush border membrane preparation. In both preparations, the contamination by mutual membranes, mitochondria, lysosomes, endoplasmic reticulum and cytosol was small.

Uptake by cortical slices. Renal cortical slices were stored briefly in ice-cold oxygenated incubation buffer composed of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.5. Two slices, each weighing 30–40 mg, were randomly selected and placed for incubation in the flask containing 5 ml of the incubation buffer with various drugs. Duplicate flasks were prepared for each experimental point. The uptake of cephalosporin was measured under the following conditions: shaker speed 100 c/min, gas phase 100% oxygen, temperature 25°. After the incubation during 10–90 min, the slices were removed, blotted on filter paper, weighed and homogenized with 1 ml of 0.03 M phosphate buffer. After deproteinization with two volumes of methanol, cephalosporin concentrations of renal tissues and media were estimated by the method of high pressure liquid chromatography. The extracellular space of renal slices was determined by the addition of inulin to the medium in a separate series of experiments and was found to be 0.22 ± 0.02 (S.E.M.) ml/g wet wt. The dry weight of the slices was also determined in control experiments after drying the renal tissue in an oven at 100° overnight. The amount of cephalosporin in the intracellular part of slices was calculated as the difference between the cephalosporin content of renal tissue and that of the inulin space. This figure was then divided by the water content (wet weight of tissue minus inulin space minus dry weight) in order to obtain the tissue concentration of cephalosporin.

Binding studies. As the estimate of specific binding, the correction for nonspecific binding of cephalosporin was made by subtraction of the amount of cephalosporin binding in the presence of probenecid. In a routine assay, 200 μl of subcellular suspensions (Percoll density gradient fractions, 200–800 μg protein; other fractions, 1 mg protein) were preincubated for 5 min with or without probenecid (100 μl, 20 mM) in 20 mM Tris–HEPES, pH 7.5, at 4°. The binding reaction was then initiated at 4° by the addition of cephalosporins (100 μl, 2 mM) in 20 mM Tris–HEPES, pH 7.5. The final concentration for cephalosporins and probenecid were 0.5 and 5 mM, respectively. After the incubation during 10–60 min, the ligand bound to the membrane fractions were separated from the free ligand by centrifugation at 45,000 *g* for 30 min at 4°. Then, cephalosporin in the pellet was extracted with 200 μl of 0.03 M phosphate buffer, and deproteinized with two volumes of methanol. Since the time course studies had shown that binding was in equilibrium after 10 min incubation, a 10 min incubation period was used through the experiments. The binding assay was found to be linear with protein concentration in the range studied.

Analytical methods. Cephalosporins were analysed by a high pressure liquid chromatograph LC-3A (Shimadzu Co., Kyoto, Japan) equipped with a variable wavelength ultraviolet detector SPD-2A (Shimadzu Co.). The conditions used for high pressure liquid chromatography were as follows: column, Zorbax ODS 25 cm × 4.6 mm (DuPont Instruments, Delaware, U.S.A.); mobile phase, 0.03 M KH_2PO_4, pH 4.7/methanol = 80/20 for cortical slice uptake studies and 0.03 M phosphate buffer, pH 7.0/methanol = 85/15 for binding studies; flow rate, 1 ml/min; wave length, 262, 240 and 272 nm for cephalexin, cephaloridine and cefazolin, respectively; injection volume, 20–30 μl; temperature, 40°. *p*-Aminohippurate was diazotized coupled with 2-diethylaminoethyl-1-naphthylamine oxalate and the coloured material was determined spectrophotometrically at 550 nm. Protein was determined, after precipitation with ice-cold 10% (w/v) trichloroacetic acid, by the method of Lowry *et al.* [14] with bovine serum albumin as standard. Inulin was determined by the method of Dische and Borenfreund [15]. The marker enzymes were assayed as previously described [12].

Materials. Cephalexin, cephaloridine (Shionogi & Co., Osaka, Japan) and cefazolin (Fujisawa Pharmaceutical Co., Osaka, Japan) were kindly supplied. Tris and HEPES were obtained from Nakarai Chemicals (Kyoto, Japan). Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were of analytical grade and were obtained commercially.

RESULTS

Cephalexin, cephaloridine and cefazolin uptakes by renal cortical slices

The time course of cephalexin, cephaloridine and cefazolin uptake by renal cortical slices was measured in order to determine the general transport characteristics, such as uptake rates and tissue accumulation levels. The uptake of these drugs was concentrative,

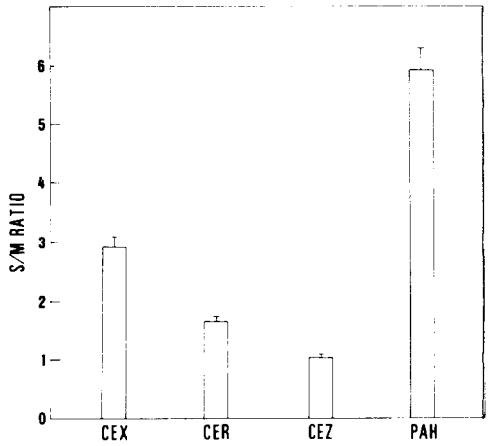

Fig. 1. Slice to medium concentration ratio (S/M) of cephalexin (CEX), cephaloridine (CER), cefazolin (CEZ) and *p*-aminohippurate (PAH). Cortical slices were incubated for 60 min in the incubation buffer containing the drugs at 0.1 mM. Each column represents mean ± S.E. of 3–7 experiments performed in four determinations.

and was reached in equilibrium after 60 min (data not shown). In Fig. 1, the distribution ratios (cortical tissue/incubation medium) at 60 min for cephalexin, cephaloridine, cefazolin and *p*-aminohippurate were summarized as 2.9, 1.7, 1.1 and 5.9, respectively.

In order to characterize the active transport of cephalexin, cephaloridine and cefazolin in the renal cortex, the concentration dependency of cephalosporin uptake was studied. As shown in Fig. 2, the saturation phenomenon in cephalexin and cephaloridine uptakes was more explicit than that in cefazolin.

Figure 3 shows the effect of various inhibitors on cephalosporin uptake. Cephalexin and cephaloridine uptakes were strongly inhibited in the presence of 2,4-dinitrophenol, ouabain and nitrogen, inhibitors of energy-coupled system, although the inhibitory effect on cefazolin uptake was relatively small. Probenecid, a competitive inhibitor for organic acid transport system in the renal tubules, also demonstrated similar inhibitory tendency to the cortical uptake of the cephalosporins.

Binding of cephalexin, cephaloridine and cefazolin to plasma membranes and subcellular fractions from renal cortex

In order to characterize the mechanism of concentrative cephalosporin accumulation by renal cortex, cephalosporin bindings to plasma membranes and other subcellular fractions were studied by the centrifugation method. Cephalosporin bindings to plasma membranes were specifically inhibited by probenecid, a potent inhibitor for organic acid transport. Therefore, in order to estimate the specific binding of cephalosporin, the correction for nonspecific binding and inulin space was made by subtraction of the amount of cephalosporin binding in the presence of 5 mM probenecid. As is evident from Fig. 4, cephalexin specifically interacted with fraction II (basolateral membranes), and considerably with fraction IV (brush border membranes) and crude plasma membranes. In contrast, there was not observed the significant interaction of cephalexin with other subcellular fractions such as pellet 1 (nuclei and debris), pellet 2 and 3 (mitochondrial fraction). Similar results were also obtained in cephaloridine binding assay (data not shown).

Furthermore, the specific bindings of cephalexin, cephaloridine and cefazolin to basolateral membranes and brush border membranes were compared in Fig. 5, including the comparative study with *p*-aminohippurate. As fraction IV had small contamination with basolateral membranes, more purified brush border membranes, obtained by calcium precipitation method, were used in this experiment. The bindings of cephalexin, cephaloridine and *p*-aminohippurate to basolateral membranes were significantly greater than those to brush border membranes. However, the binding of cefazolin was relatively small in both membranes.

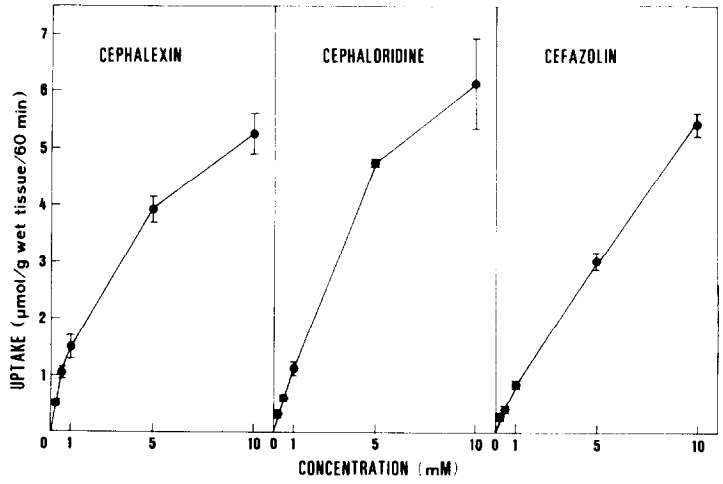

Fig. 2. Concentration dependency of cephalexin, cephaloridine and cefazolin uptakes by renal cortical slices. Slices were incubated for 60 min in the incubation buffer containing the drugs at the concentration of 0.2, 0.5, 1, 5 and 10 mM. Each point represents mean ± S.E. of four determinations from a typical experiment.

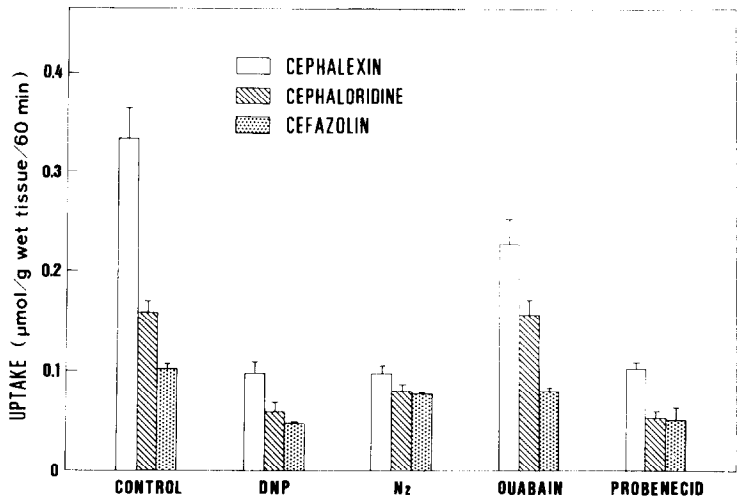

Fig. 3. Effect of various inhibitors on cephalexin, cephaloridine and cefazolin uptakes by renal cortical slices. Slices were incubated for 60 min in the incubation buffer containing the drugs at 0.1 mM in the presence of various inhibitors. The concentration of 2,4-dinitrophenol (DNP), ouabain and probenecid was 1 mM. Each column represents mean ± S.E. of at least 4 determinations from 1–3 experiments.

DISCUSSION

The present results demonstrate that cephalexin and cephaloridine were concentratively accumulated by renal cortical slices, and specifically interacted with basolateral membranes. The interaction of cephalosporins with plasma membranes from renal cortex offered an appropriate *in vitro* model system to study the renal transport of the antibiotics.

Renal tubular mechanisms for excretion of cephalosporins and their nephrotoxicity have been extensively investigated by Tune *et al.* [5–9]. Since both transport and toxicity of cephaloridine are prevented by inhibitors of organic acid transport, it has been concluded that the nephrotoxicity of cephaloridine is related to the high intracellular concentrations that result from its active transport. However, there is little information to characterize the specific membrane events underlying cephalosporin transport in the renal tubule. The accumulation studies for *p*-aminohippurate by renal cortical slices are widely used as a measure of tubular transport of *p*-amino-

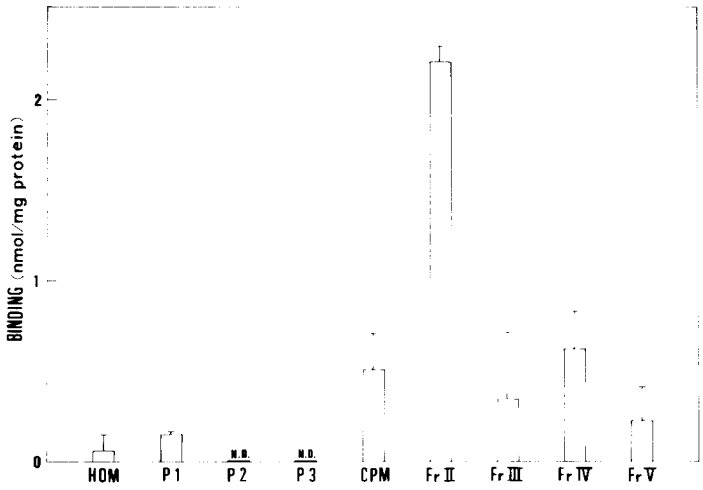

Fig. 4. Specific bindings of cephalexin to subcellular fractions from renal cortex. Cephalexin was added into the subcellular suspension after the preincubation for 5 min with or without probenecid in 20 mM Tris–HEPES, pH 7.5, at 4°. Then, 10 min later, the reaction mixture was centrifuged at 45,000 *g* for 30 min at 4° to separate the free ligand. The final concentration for cephalexin and probenecid were 0.5 and 5 mM, respectively. As the estimation of specific binding, the correction for nonspecific binding of cephalexin was made by subtraction of the amount of cephalexin binding in the presence of probenecid. HOM, homogenate; P1, pellet 1; P2, pellet 2; P3, pellet 3; CMP, crude plasma membranes; Fr II, fraction II (basolateral membranes); Fr III, fraction III; Fr IV, fraction IV (brush border membranes); Fr V, fraction V. Each column represents mean ± S.E. of 3 experiments performed in duplicate determinations.

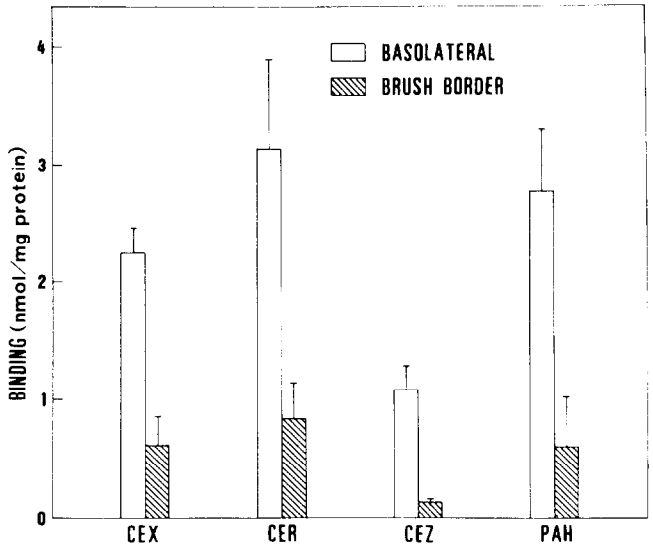

Fig. 5. Specific bindings of cephalexin (CEX), cephaloridine (CER), cefazolin (CEZ) and *p*-aminohippurate (PAH) to brush border and basolateral membranes from renal cortex. Specific bindings were determined as described for Fig. 4. Each column represents mean ± S.E. of 2–5 experiments performed in duplicate determinations.

hippurate *in vitro*. In the first series of experiments, therefore, we examined the uptake of cephalexin, cephaloridine and cefazolin by renal cortical slices. The cortical slice-to-medium concentration ratios of cephalexin, cephaloridine and cefazolin at the concentration of 0.1 mM were found to be 2.9, 1.7 and 1.1, respectively. The cortical uptakes of cephalexin and cephaloridine were significantly reduced by probenecid, 2,4-dinitrophenol, ouabain and anoxia. The results are strong evidence for the active transport of cephalexin and cephaloridine in the proximal tubule by an organic acid transport system, in agreement with the degree of *in vivo* renal uptake and renal clearance of cephalosporins [1–3].

In order to obtain more direct information about the accumulation mechanisms of cephalosporins in the renal cortex, we measured the interaction of the antibiotics with subcellular fractions of renal cortex. Cephalexin and cephaloridine specifically bound to the basolateral membranes, compared to brush border membranes and other subcellular fractions. Cefazolin binding to basolateral membranes was relatively small. Thus, the results of slice uptake experiments were more correlated with the binding with basolateral membranes than with brush border membranes. By use of the isolated brush border and basolateral membrane vesicles, Berner and Kinne [16] and Kinsella *et al.* [17], including our preliminary study [18], demonstrated that the translocation of *p*-aminohippurate across the basolateral membranes was via a carrier-mediated transport system. The specificity of cephalexin and cephaloridine binding to the basolateral membranes could be related to the interaction with the organic acid transport system at the antiluminal side. Furthermore, it should be emphasized that cephaloridine binding to basolateral membranes was to a similar degree or greater than that of cephalexin, although cortical uptake of cephaloridine was significantly less than that of cephalexin. Therefore, the high intracellular concentration of cephaloridine in the kidney *in vivo* may be explained in relation to the interaction with the basolateral membranes.

Pharmokinetic studies have demonstrated higher and more prolonged blood levels with cefazolin than with other cephalosporins [2, 3]. This could be explained largely by the greater binding of cefazolin to serum proteins and its lower rate of renal clearance. Furthermore, the mechanisms involved in the elimination of cefazolin were elucidated by the concurrent administration of probenecid, and as a result of the competitive inhibition of the tubular secretion, a prolonged half-life and elevated serum levels of cefazolin were observed [19]. However, based on the present data of binding assay which revealed a small binding of cefazolin to basolateral membranes, renal excretion of cefazolin could be largely attributed to glomerular filtration rather than tubular secretion.

In conclusion, cephalexin and cephaloridine were concentratively accumulated by renal cortical slices, and specifically interacted with basolateral membranes compared to brush border membranes and other subcellular fractions, although these characteristics were not shown to the same extent for cefazolin. An examination of cephalosporin interaction with plasma membranes from renal cortex could offer an appropriate *in vitro* model system to study the renal transport of the antibiotics.

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