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Report

Analysis of Hepatic Transport of Cefpiramide in Rats with Obstructive Jaundice by Using Isolated Hepatocytes

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The mechanism of the diminished biliary clearance of cefpiramide (CPM) in rats with obstructive jaundice (OJ) was investigated by using isolated hepatocytes. The kinetics of CPM uptake by hepatocytes isolated from normal rats and rats with OJ could be explained by the combination of saturable carrier-mediated and nonsaturable first-order rate processes. The maximum uptake rate (V_{max}) of the carrier-mediated process was significantly decreased in OJ, compared with normal hepatocytes, while the Michaelis constant (K_m) and the first-order rate constant (k_d) were not significantly different. This result indicated that the number of CPM transport carriers was decreased in OJ hepatocytes. Further, no CPM uptake occurred from the serum of OJ rats into normal hepatocytes. Partial recovery of CPM uptake after treatment of OJ serum with activated charcoal suggested the accumulation of inhibitors of CPM uptake in OJ serum.

KEY WORDS: obstructive jaundice; cefpiramide; hepatic uptake; isolated hepatocyte; carrier-mediated transport.

INTRODUCTION

Obstructive jaundice (OJ) is induced by the obstruction of common bile duct with gallstones or with malignant tumors. A patient in OJ state often suffers from bacillary bile duct inflammation, and thus, chemotherapy is performed with antibiotics that can be actively secreted into bile. However, our recent studies in rats with OJ (1) have revealed a large reduction of the biliary clearance of cefpiramide (CPM), a nonmetabolized antibiotic with normally high biliary clearance.

Transport processes across both sinusoidal and bile canalicular membranes in the liver are important in the biliary secretion process (2). Although the architecture and the polarity of the whole liver are lost when isolated hepatocytes are used, this simple system enables us to study plasma membrane transport processes without complicating factors such as hepatic blood and bile flows (3–5).

In this study, we have characterized the poor biliary clearance of CPM in the OJ state using isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

CPM was supplied by Yamanouchi Pharmaceutical Co., Ltd., Tokyo. [*Carboxy*-^{14}C]Inulin (3 μCi/mg) and a scintillation cocktail, ACS-II, were purchased from ICN Radiochemicals, Irvine, CA, and Amersham Japan, Tokyo, respectively. Collagenase (from clostridium histolytum, Type IV) and bovine serum albumin (BSA; Fraction V) were purchased from Sigma Chemicals Co., St. Louis, MO. All other reagents were commercial products of reagent grade.

Preparation of Rats with OJ

Male Wistar rats weighing about 300 g were used. Rats with OJ were prepared for experiments as reported previously (1). Briefly, under ethyl ether anesthesia, the abdominal midline was opened, the common bile duct was ligated, and the abdominal incision was closed. Three days after the operation, the rat was used as a model animal with OJ. Only the rat which exhibited a value of plasma direct bilirubin (D-Bil) level greater than 4 mg/dl was used as the one with OJ.

Preparation of Isolated Hepatocytes

Isolated hepatocytes were prepared according to the procedure of Moldeus *et al.* (6). Under ethyl ether anesthesia, heparin was injected from inferior vena cava. Then, medium A, containing 137 m*M* NaCl, 5.4 m*M* KCl, 0.8 m*M* $MgSO_4$, 0.3 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4, 26 m*M* $NaHCO_3$, 0.6 m*M* EGTA,[3] 13 m*M* HEPES,[3] and 2% BSA

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[3] EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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(pH 7.4), was perfused for 4 min from the portal vein (washing step), followed by the perfusion for 6 min of medium B, containing 137 m*M* NaCl, 5.4 m*M* KCl, 0.8 m*M* $MgSO_4$, 0.3 m*M* Na_2HPO_4, 0.4 m*M* KH_2PO_4, 26 m*M* $NaHCO_3$, 13 m*M* HEPES, 4.12 m*M* $CaCl_2$, and 0.08% collagenase (pH 7.4) (dispersion step). Thereafter, the medium B on the liver surface was completely washed out by medium C, containing 118 m*M* NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 13 m*M* HEPES, 2.6 m*M* $CaCl_2$, and 2% BSA (pH 7.4), and hepatocytes were suspended in the medium C (washing step). The fresh hepatocytes were completely resuspended in the incubation medium (modified Krebs–Henseleit buffer solution containing 118 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, 13 m*M* HEPES, and 10 m*M* glucose (pH 7.4)). The viability of the isolated hepatocytes was routinely checked by the lactate dehydrogenase latency test and the hepatocytes which exhibited a value greater than 92% were used. All uptake studies were performed within 5 hr after the cell isolation.

Activated-Charcoal Treatment of OJ Serum

The treatment of OJ serum with activated charcoal was carried out according to the method of Chen (7). Briefly, the activated charcoal was added to the serum of rats with OJ (50 mg/ml), the pH was lowered to 3.0 with 1 *N* HCl and the solution was mechanically stirred for 1 hr in an ice box. The charcoal was removed by centrifugation and the pH of the serum was adjusted back to pH 7.4 with 1 *N* NaOH.

Uptake Experiments

After incubating the hepatocytes in the incubation medium for 5 min at 37°C, uptake experiments were started by the addition of CPM dissolved in the incubation medium. In the case of uptake experiments from serum, CPM was dissolved in the serum. The transport reaction was stopped by placing the sample (0.2 ml) into a 0.4-ml plastic microcentrifuge tube containing 0.1 ml of silicone oil (d = 1.05: Aldrich Co., Milwaukee, WI) and 0.05 ml of 3 *M* KCl, followed by the centrifugation in the microcentrifuge. Then the samples were frozen immediately in a dry ice/ethanol bath. After separation of hepatocytes from the medium, the CPM concentration in cells was measured by HPLC.

The amount of CPM in the extracellular fluid adhering to the cell was corrected by performing separate experiments in which cells were incubated with [^{14}C]inulin.

Analytical Method

Cellular Protein. Cellular protein was measured by the method of Lowry *et al.* (8) with BSA as the standard.

CPM. For the determination of CPM in the frozen tubes, cells precipitated in 3 *M* KCl were cut off and were transferred into a 1.5-ml microcentrifuge tube containing 0.7 ml of 3% trichloroacetic acid and 0.1 ml of the incubation medium. Then the mixture was sonicated to release the intracellular CPM. After the sonicated sample was mixed well and centrifuged in the microcentrifuge, the supernatant fluid was filtered through a 0.45-μm-pore size membrane filter (Nihon Millipore Kogyo Co., Ltd., Yonezawa). An appropriate volume of the filtrate was injected into the liquid chromatograph.

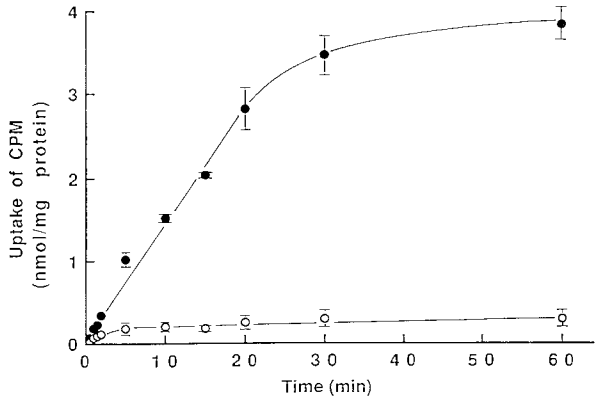

Fig. 1. Time course of cefpiramide uptake by hepatocytes isolated from normal rats (●) and rats with obstructive jaundice (○). Results are expressed as the mean ± SE at least three independent experiments. Initial concentration of cefpiramide was 0.5 m*M*.

The HPLC system consisted of a pump (LC-5A; Shimadzu, Kyoto), a column (4.6-mm I.D. × 150 mm) packed with Inertsil ODS (5 μm; Gasukuro Kogyo, Tokyo), and a spectrophotometric detector (SPD-2A; Shimadzu). The wavelength of the detector was set at 280 nm. The degassed mobile phase consisted of phosphate buffer (M/15; pH 7.0)–acetonitrile (82:18, by volume). The column temperature was kept at 40°C. The drug concentration was calculated from the calibration line constructed on the basis of peak-area measurements.

D-Bil. Plasma D-Bil was determined with commercial test kits (Sankyo Junyaku Co., Ltd., Tokyo).

[^{14}C]Inulin. The radioactivity was determined by liquid scintillation counting following the addition of a scintillation medium, ACS-II.

Data Analysis

Data are represented as the mean with the standard error (mean ± SE). Statistical analysis was carried out by Student's t test. The kinetic parameters of the uptake of CPM by hepatocytes were estimated by a nonlinear least-squares regression analysis program (MULTI) (9).

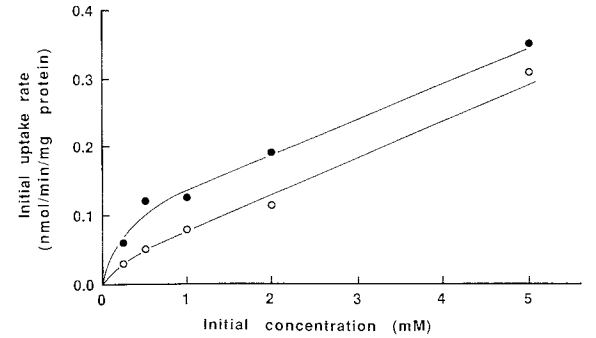

Fig. 2. Concentration dependency of initial uptake rate of cefpiramide in hepatocytes isolated from normal rats (●) and rats with obstructive jaundice (○). Results are expressed as the mean of three independent experiments. The solid lines were drawn by nonlinear least-squares regression analysis.

Table I. Kinetic Parameters of Cefpiramide Uptake by Hepatocytes Isolated from Normal Rats and Rats with Obstructive Jaundice (OJ)[a]

Hepatocytes	V_{max} (nmol/min/mg protein)	K_m (mM)	k_d (ml/min/mg protein)
Normal	0.119 ± 0.071	0.311 ± 0.341	0.0462 ± 0.0206
OJ	0.029 ± 0.020*	0.128 ± 0.256	0.0509 ± 0.0089

[a] Data are expressed as the mean ± SD of three independent experiments.
* Significantly different from normal (analysis of variance).

RESULTS

Time Course of CPM Uptake by Isolated Hepatocytes

Figure 1 illustrates the time courses of CPM uptake by hepatocytes isolated from normal rats and rats with OJ. In both hepatocytes, the uptake of CPM (initial concentration, 0.5 mM) was linear in the first 2-min incubation period and reached equilibrium after 30 and 5 min in normal and in OJ hepatocytes, respectively. The amount of CPM taken up in 60 min was 3.84 and 0.302 nmol/mg protein in hepatocytes from normal rats and rats with OJ, respectively.

Concentration Dependency of CPM Transport into Isolated Hepatocytes

Since linear initial uptakes of CPM were observed at all concentrations examined, the efflux of CPM from hepatocytes was considered to be negligible and the initial uptake rate of CPM was estimated from the slope of the linear regression line at the points at 30, 60, 90, and 120 sec. Figure 2 illustrates the concentration dependency of the initial uptake rate of CPM by hepatocytes from normal rats and rats with OJ. In both hepatocytes, the kinetics of CPM uptake consisted of two components, a saturable carrier-mediated process and an apparently nonsaturable first-order process. Thus, the uptake rate (v) can be expressed by the following equation with respect to the substrate concentration (s):

$$v = V_{max} \cdot s/(K_m + s) + k_d \cdot s$$

where V_{max} and K_m are the maximum uptake rate and the Michaelis constant for the saturable process, respectively, and k_d is the apparent first-order rate constant for the nonsaturable process. The kinetic parameters estimated by nonlinear least-squares regression analysis are listed in Table I. The V_{max} value in hepatocytes of rats with OJ was lowered significantly, while the K_m and the k_d values in hepatocytes of the diseased rats were not significantly different from those in normal rats.

Effect of Serum Components of Rats with OJ on CPM Uptake by Hepatocytes of Normal Rats

Table II shows the initial uptake rate of CPM by hepatocytes of normal rats from serum of normal rats, from serum of rats with OJ, and from activated-charcoal treated serum of rats with OJ at the initial concentrations of 0.25 and 1 mM. The uptake rates from serum of normal rats were 35.1 pmol/min/mg protein at 0.25 mM and 94.3 pmol/min/mg protein at 1 mM. On the other hand, no CPM uptake by hepatocytes of normal rats from serum of rats with OJ was observed. However, uptake recovered after the treatment of serum of the diseased rats with activated charcoal; however, the uptake rates after the treatment remained below those from normal serum at both 0.25 and 1 mM CPM.

DISCUSSION

We have recently demonstrated a large reduction of biliary CPM clearance in the OJ state (1). In the present study, we have further characterized the poor biliary clearance of CPM in OJ state using isolated hepatocytes. CPM uptake by hepatocytes isolated from rats with OJ in 1 hr was one-thirteenth that by hepatocytes from normal rats (Fig. 1). The V_{max} in hepatocytes of rats with OJ was reduced to 25% of control, while the K_m and the k_d were not significantly different in hepatocytes from diseased and normal rats. Tsuji *et al.* reported that in hepatocytes of normal rats, the V_{max}, K_m, and k_d values for the CPM uptake are 0.70 nmol/min/mg protein, 0.847 mM, and 0.047 nmol/min/mg protein/mM, respectively (10). The V_{max} and K_m values for the carrier-mediated transport process in the present study were somewhat lower than those reported by Tsuji *et al.*, possibly a result of different experimental conditions. Since both hepatocyte preparations exhibited values greater than 92% in the lactate dehydrogenase latency test, the estimated parameters reflect only viable hepatocytes. The results in Table I suggest that the number of carriers for CPM transport on the plasma membrane of hepatocytes of rats with OJ was reduced. This reduction of CPM carriers in hepatocytes could account in part for the poor biliary clearance of CPM in the OJ state. Reichen *et al.* reported that the V_{max} values for

Table II. Initial Uptake Rates of Cefpiramide in Hepatocytes of Normal Rats from Serum of Normal Rats, Serum of Rats with Obstructive Jaundice (OJ), and OJ Serum Treated with Activated Charcoal at the Initial Concentrations of 0.25 and 1 mM[a]

Serum	Initial uptake rate (pmol/min/mg protein)	
	0.25 mM	1 mM
Normal serum	35.1 ± 5.1	94.3 ± 9.1
OJ serum	0	0
OJ serum treated with activated charcoal	19.8 ± 2.9*	55.6 ± 7.7*

[a] Data are expressed as the mean ± SD of three independent experiments.
* Significantly different from normal serum (analysis of variance).

both taurocholate and ouabain were significantly reduced in hepatocytes from cirrhotic rats, which were induced by chronic exposure to phenobarbital and carbon tetrachloride (11). Using these kinetic parameters, we calculated two components, carrier-mediated and passive diffusion processes of each curve of CPM initial uptake rate (Fig. 3). Most CPM uptake by hepatocytes of rats with OJ occurs by a passive diffusion rather than carrier-mediated processes because of the reduction of CPM carriers in OJ hepatocytes.

Further, we investigated the effect of serum components of rats with OJ on CPM uptake by hepatocytes of normal rats. The CPM uptake was completely inhibited by serum of rats in the diseased state, which was recovered after the treatment of the serum with activated charcoal (Table II), suggesting the existence of inhibitors of CPM uptake in serum of rats with OJ. The inhibitors in the serum were not completely removed by the charcoal treatment, because the initial uptake rate did not fully recover. The inhibitors interfered not only with the carrier-mediated process but also with passive diffusion of the CPM uptake. It is possible that another specific transport system is involved in the CPM uptake process. Anyway, the mode of the inhibition remains unclarified. We have shown the presence of inhibitors of CPM binding to plasma protein in the plasma of rats with OJ (1). These findings suggest the presence of inhibitors not only for CPM binding to plasma protein but also for CPM transport to the liver in the blood of rats with OJ. However, it remains to be shown whether the inhibitors of the protein binding are identical to the inhibitors of the hepatic uptake or not.

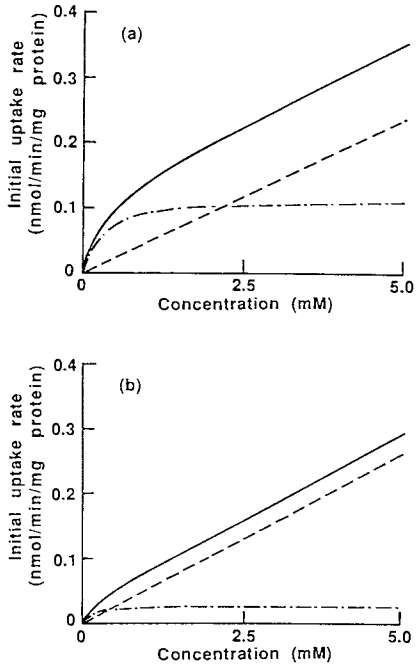

Fig. 3. Calculated curves for the initial uptake rate (——) which consists of a carrier-mediated process (– · –) and a passive diffusion process (– – –) in hepatocytes isolated from normal rats (a) and rats with obstructive jaundice (b).

The large reduction of biliary clearance of CPM in the OJ state is attributed mainly both to a defect of the transport carriers on the plasma membrane of the liver and to inhibitors of CPM uptake in the serum. Analysis of the contribution of dead liver cell mass to the decreased transport function is difficult, since we were unable to perform the uptake experiments in whole liver. The number of viable hepatocytes in OJ state may be much less than in the normal state. Also, the separate effects of the OJ state on CPM transport across the sinusoidal plasma membrane, intracellular transport, and translocation across the bile canalicular membrane into bile remain to be examined.

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