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Transport of Temocaprilat into Rat Hepatocytes: Role of Organic Anion Transporting Polypeptide[1,2]

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ABSTRACT

The mechanism for hepatic uptake of temocaprilat, an angiotensin-converting enzyme inhibitor that is predominantly excreted into bile was studied using isolated rat hepatocytes and COS-7 cells expressing the organic anion transporting polypeptide (oatp1). The uptake of temocaprilat into isolated rat hepatocytes exhibited saturation with a K_m of 20.9 μM and a V_{max} of 0.21 nmol/min/mg protein. This uptake was temperature sensitive and was significantly reduced by metabolic inhibitors, a sulfhydryl-modifying reagent and an anion-exchange inhibitor, although the replacement of Na^+ with Li^+ in the medium did not affect the uptake. [^{3}H]Temocaprilat uptake was inhibited by estradiol-17β-D-glucuronide and dibromosulphophthalein, typical substrates for the Na^+-independent organic anion transporter, in a concentration-dependent manner, whereas excess estradiol-17β-D-glucuronide did not completely inhibit the uptake. Temocaprilat uptake into COS-7 cells transfected with oatp1 cDNA revealed a concentration-dependency with a K_m of 46.7 μM, a value comparable with that obtained in isolated hepatocytes. The contribution of oatp1 to carrier-mediated hepatic uptake of temocaprilat was less than 50% by correcting the uptake clearance with that of estradiol-17β-D-glucuronide. A good linear correlation was observed for the inhibitory effect of angiotensin-converting enzyme inhibitors (benazeprilat, cilazaprilat, delaprilat and enalaprilat) between isolated hepatocytes and oatp1-expressing cells. These data suggest that oatp1, along with another transporter(s), mediates the uptake of angiotensin-converting enzyme inhibitors into rat hepatocytes.

Therapy with ACE inhibitors has become increasingly accepted over the past decade as a valuable option in the treatment of hypertension and congestive heart failure (Todd and Fitton, 1991). In general, ACE inhibitors are administered to patients as the prodrug (ethyl-ester). Recently, it was reported that treatment with an ACE inhibitor, temocapril · HCl (α-{(2S,6R)-6-[(1S)-1-ethoxy-carbonyl-3-phenyl-propyl]amino-5-oxo-2-(2-thienyl)perhydro-1,4-thiazepin-4-yl}acetic acid hydrochloride), improved forearm vasodilatory response to reactive hyperemia, suggesting a beneficial effect on endothelial function (Iwatsubo *et al.*, 1997). To achieve optimal pharmaco-therapeutic efficacy, the pharmacokinetic behavior of ACE inhibitors has been studied extensively and it has been demonstrated that, as far as their excretion is concerned, many active forms of ACE inhibitors such as captopril (Brogden *et al.*, 1988), enalaprilat (Todd and Goa, 1992), cilazaprilat (Deget and Brogden, 1991), ramiprilat (Frampton and Peters, 1995) and spiraprilat (Noble and Sorkin, 1995) are predominantly excreted into urine, whereas temocaprilat is excreted into bile after the oral administration of the respective prodrugs to humans; indeed, 36 to 44 and 17 to 24% of temocaprilat is excreted into feces and urine, respectively, 48 hr after oral administration of temocapril · HCl to humans (Suzuki *et al.*, 1993). In rats, more than 80% of the dose is excreted into bile after i.v. administration (Ishizuka *et al.*, 1997). The presence of an excretion route other than the urinary excretion provides a pharmacokinetic and pharmacodynamic advantage on temocapril over other ACE inhibitors, particularly in the treatment of patients with renal failure; in patients with severe renal insufficiency, the C_{max} and AUC of enalaprilat increased 6 and 13 times, respectively, compared with normal volunteers, whereas the change in C_{max} for temocaprilat was minimal

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ABBREVIATIONS: ACE, angiotensin-converting enzyme; oatp1, organic anion transporting polypeptide; Ntcp, Na^+/taurocholate co-transporting polypeptide; cMOAT, canalicular multispecific organic anion transporter; $E_2$17βG, estradiol-17β-D-glucuronide; BSP, bromosulfophthalein; DBSP, dibromosulfophthalein; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; FCCP, carbonyl cyanide *p*-(trifluoro-methoxy)phenylhydrazone; PCMBS, *p*-choloromercuriphenylsulfonic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; SD rats, Sprague-Dawley rats; EHBR, Eisai hyperbilirubinemic rats; DMEM, Dulbecco's modified Eagle's medium.

and the AUC only doubled in the same patient populations (Oguchi *et al.*, 1993).

One of the reasons for this efficient biliary excretion of temocapril is related to the transport properties of the cMOAT whose cDNA cloning and functional analysis have been performed by this and other laboratories (Paulusma *et al.*, 1996; Büchler *et al.*, 1996; Ito *et al.*, 1997; Madon *et al.*, 1997; Ito *et al.*, 1998). Previously, we examined the hepatobiliary excretion of temocaprilat in SD rats and EHBR whose cMOAT is hereditarily defective (Ishizuka *et al.*, 1997). We found that the clearance for the biliary excretion of temocaprilat after i.v. administration is lower in EHBR and that temocaprilat is taken up in an ATP-dependent manner by isolated bile canalicular membrane vesicles from SD rats but not from EHBR. Based on these findings, it was concluded that temocaprilat is a substrate of cMOAT. Kinetic analysis indicated that the K_m of temocaprilat for cMOAT is 92.5 μM, which was in marked contrast to the low affinity of other ACE inhibitors (Ishizuka *et al.*, 1997).

To compare the hepatobiliary excretion of temocaprilat with other ACE inhibitors, however, it is necessary to examine the uptake into hepatocytes from plasma across the basolateral membrane. It has been reported that many organic anions are transported into hepatocytes by active transport systems via Na^+-dependent and -independent mechanisms (Meier, 1995; Müller and Jansen, 1997). Recently, two kinds of organic anion transporters have been cloned (Hagenbuch *et al.*, 1991; Jacquemin *et al.*, 1994). One of these is the Na^+/taurocholate transporting polypeptide Ntcp by which several bile acids are transported (Hagenbuch *et al.*, 1991; Stieger *et al.*, 1994); another is the organic anion transporting polypeptide oatp1 that mediates the Na^+-independent transport of many amphipathic substrates (Jacquemin *et al.*, 1994; Bossuyt *et al.*, 1996).

In our study, the hepatic transport system(s) responsible for the uptake of temocaprilat was characterized in relation to that of other ACE inhibitors. Because we found that temocaprilat is taken up by isolated hepatocytes in an Na^+-independent manner, the role of oatp1 in the uptake of temocaprilat was investigated in transfected COS-7 cells.

Methods

Materials. [^{3}H]Temocaprilat (7.7 Ci/mmol) was synthesized by Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). $E_2 17\beta G$ (47.3 Ci/mmol) was purchased from Du Pont New England Nuclear Corp. (Boston, MA). The radiochemical purity of the [^{3}H]temocaprilat and [^{3}H]$E_2 17\beta G$ determined by HPLC with radiodetector on a Zorbax ODS column was more than 97% for both compounds using the following mobile phases; 30 (acetonitrile): 70 (2% acetic acid, pH 3.0) and 55 (1% triethylammonium acetate, pH 4.0): 45 (methanol), respectively. Unlabeled temocaprilat was synthesized in our laboratories. Benazeprilat, cilazaprilat, delaprilat and enalaprilat were synthesized by the Institute of Science and Technology Inc. (Tokyo, Japan). COS-7 (ATCC 1651, African green monkey kidney cells) were purchased from the American Type Culture Collection (Rockville, MD). Full-length cDNA for oatp1 cloned in the plasmid pSPORT1 (Jacquemin *et al.*, 1994) was excised with *Mlu*I to subclone it into the *Xho*I site in the pCAGGS vector (Niwa *et al.*, 1991) after converting to blunt ends. Rotenone, FCCP, PCMBS, DIDS and $E_2 17\beta G$ were purchased from Sigma Chemical Co. (St. Louis, MO) and DBSP was from the Societé d'Etudes et de Recherches Biologiques (Paris, France). Male SD rats (8 wk old) were purchased from SLC Co., Ltd. (Shizuoka, Japan). All other chemicals used were commercially available and of reagent grade. Animal experiments were carried out according to the guidelines provided by the Institutional Animal Care Committee of Sankyo Co., Ltd. (Tokyo, Japan).

Uptake into isolated rat hepatocytes. Hepatocytes were isolated from SD rats by the procedure described Baur *et al.* (1975), and were suspended in Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.4). Cell viability (>90%) was routinely checked by the trypan blue (0.4% w/v) exclusion test. The uptake study was performed as described in the previous report (Yamazaki *et al.*, 1993). Briefly, the study was initiated by addition of ligand to the preincubated (37°C for 3 min) cell suspension (2×10^6 cells/ml). At designated times, the uptake was terminated by separating the cells from the medium using a centrifugal filtration technique (Schwenk, 1980), and the radioactivity in the cell and medium was determined in a liquid scintillation spectrophotometer (LSC-3500, Aloka Co., Tokyo, Japan). To minimize the contribution of surface binding, initial uptake velocity was calculated by linear regression of the uptake at 30, 60 and 90 sec, each time point of which was determined by the duplicate or triplicate experiments. For the *cis*-inhibition experiment, unlabeled ligands ($E_2 17\beta G$ or DBSP) were added to [^{3}H]temocaprilat solution. Metabolic inhibitors, sulfhydryl-modifying reagent and anion exchange inhibitor, were added to the cell suspension 5 min before the addition of [^{3}H]temocaprilat to examine their effect. To estimate the Na^+-dependence of the uptake of temocaprilat, the experiments were performed in Krebs-Henseleit buffer with Na^+ being replaced by Li^+.

The effect of active metabolites of ACE inhibitors (benazeprilat, cilazaprilat, delaprilat and enalaprilat), dissolved in dimethylsulfoxide, on the uptake of [^{3}H]temocaprilat was also studied. The final concentration of dimethylsulfoxide was less than 4%. A control experiment was also performed in the presence of the maximum concentration (4%) of organic solvent. Analyses of variance followed by Dunnett's test was used to determine the significance of differences.

Uptake into COS-7 cells expressing oatp1. COS-7 cells were cultured on 35-mm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. At 80% confluence, cells were washed twice with DMEM without serum and then exposed to the solution containing plasmid ($pcXN_2$ with or without oatp1, 10 μg/ml) and LipofectAMINE (10 μl/ml, Gibco BRL, Gaithersburg, MD). Eight hours after infection, plasmid-LipofectAMINE solution was removed and replaced by DMEM containing 10% bovine serum. The transfected cells were cultured overnight on a 12-well plate. Cells were washed with Krebs-Henseleit buffer to initiate the uptake experiments after preincubation (37°C) for 5 min. At designated times, uptake was terminated by removing the medium, and cells were washed with ice-cold PBS. Cells were then dissolved in 1N NaOH, and the radioactivity was determined in a liquid scintillation spectrophotometer (LSC-3500, Aloka Co., Tokyo, Japan). Initial uptake velocity was calculated by linear regression of the uptake at 30 and 90 sec, each time point of which was determined by the duplicate or triplicate experiments. The effect of unlabeled compounds on the uptake of radiolabeled substrates into the COS-7 cells was examined using the method described for the isolated hepatocytes.

Determination of kinetic parameters. Uptake data were fitted to the following equation using the nonlinear least squares program, WinNonlin ver. 1.1 (Statistical Consultants Inc., Lexington, KY), to calculate the kinetic parameters:

$$V = \frac{V\max \cdot C}{K_m + C} + P_{dif} \cdot C \qquad (1)$$

where V is the initial uptake rate, V_{max} is the maximum uptake rate, K_m is the Michaelis constant, C is the ligand concentration in the medium and P_{dif} is the non-specific uptake rate. Uptake clearance (CL_{uptake}) was determined by the sum of V_{max}/K_m ratio and P_{dif}. For COS-7 cells, one nonlinear component model was used to fit the data subtracted nonspecific uptake portion by vector-transfected cells and the uptake clearance (CL_{uptake}) was determined by the V_{max}/K_m ratio.

Contribution of oatp1 to carrier-mediated hepatic uptake of temocaprilat. The contribution of oatp1 (R_{oatp1}) to carrier-mediated uptake of temocaprilat into hepatocytes was estimated from equation 2:

$$R_{oatp1} = \left(\frac{CL_{cos(temocaprilat)}}{CL_{cos(E_217\beta G)}}\right) / \left(\frac{CL_{hep(temocaprilat)}}{CL_{hep(E_217\beta G)}}\right) \quad (2)$$

where CL_{cos} is the clearance for the uptake into oatp1-expressing COS-7 cells determined by the V_{max}/K_m ratio and CL_{hep} is the uptake clearance into isolated hepatocytes determined by the V_{max}/K_m ratio.

Results

Uptake of temocaprilat into isolated hepatocytes. Uptake of temocaprilat was linear over at least 2 min, and was significantly reduced at low temperature (fig. 1). Temocaprilat uptake into hepatocytes revealed concentration dependency (fig. 2) with a K_m of 20.9 ± 8.0 μM, a V_{max} of 0.21 ± 0.03 nmol/min/mg protein and a P_{dif} of 1.9 ± 0.3 μl/min/mg protein (mean ± S.E., $N = 3$) (table 1). The uptake of $E_217\beta G$ also showed saturation (fig. 2) with a K_m of 6.5 ± 1.6 μM and a V_{max} of 0.47 ± 0.12 nmol/min/mg protein (mean ± S.E., $N = 3$) (table 1). Temocaprilat uptake was inhibited by pretreatment with metabolic inhibitors such as rotenone (30 μM) or FCCP (2 μM), sulfhydryl-modifying reagent (PCMBS, 100 μM) and anion exchange inhibitor (DIDS, 100 μM), although the replacement of Na^+ by Li^+ in the medium had no effect on temocaprilat uptake (table 2).

$E_217\beta G$ or DBSP, typical substrates for the Na^+-independent organic anion transporter, inhibited temocaprilat uptake in a concentration-dependent manner (fig. 3). Although a high concentration of DBSP completely inhibited the uptake of temocaprilat, its uptake was only partially inhibited by the addition of $E_217\beta G$.

Uptake of temocaprilat into COS-7 cells expressing oatp1. Uptake of temocaprilat into COS-7 cells was significantly increased by transfecting oatp1 cDNA (fig. 4). The concentration-dependent uptake of temocaprilat by oatp1-expressing COS-7 cells (fig. 5) was described with a K_m of 46.7 ± 15.9 μM and a V_{max} of 0.092 ± 0.022 nmol/min/mg protein (mean ± S.E., $N = 3$) (table 1). The uptake of $E_217\beta G$ also showed saturation (fig. 5) with a K_m of 11.0 ± 3.9 μM and a V_{max} of 0.32 ± 0.16 nmol/min/mg protein (mean ± S.E., $N = 3$) (table 1). Uptake parameters for temocaprilat and $E_217\beta G$ obtained from both isolated hepatocytes (fig. 2) and oatp1-transfected COS-7 (fig. 5) experiments are summarized in table 1.

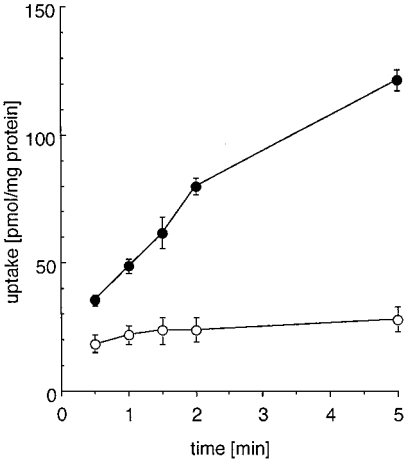

Fig. 1. Time-profiles for the uptake of temocaprilat by isolated rat hepatocytes. The uptake of [^{3}H]temocaprilat was measured by incubating isolated rat hepatocytes in Krebs-Henseleit buffer (pH 7.4) containing [^{3}H]temocaprilat (0.1 μM) at 37°C (●) or 4°C (○) after preincubation for 3 min. Each point represents the mean ± S.E. of three different preparations.

The contribution of oatp1 (R_{oatp1}) to carrier-mediated uptake of temocaprilat, calculated from equation 2, was 0.51, suggesting a significant contribution of oatp1 to the uptake of temocaprilat by rat hepatocytes.

Effect of other ACE inhibitors on the uptake of temocaprilat. We examined the effect of benazeprilat, cilazaprilat, delaprilat and enalaprilat on the uptake of temocaprilat into liver. Uptake of [^{3}H]temocaprilat was inhibited by these drugs (100 μM) in both isolated hepatocytes and oatp1-transfected COS-7 experiments (fig. 6). The degree of inhibition among ACE inhibitors exhibited a good linear correlation ($r^2 = 0.804$) between both experiments.

Discussion

The presence of the biliary excretion pathway confers a pharmacokinetic advantage on temocaprilat, particularly in the treatment of patients with renal failure. Although we found that temocaprilat is excreted into bile via cMOAT (Ishizuka *et al.*, 1997), it is necessary to study the uptake of temocaprilat into hepatocytes across the basolateral membrane to account fully for the efficient biliary excretion of temocaprilat. Although temocaprilat is administered as the prodrug (temocapril · HCl), investigation of the hepatic uptake mechanism of temocaprilat is essential because it has been revealed that almost all the drug in portal blood at 3 or 5 min after intraduodenal administration of temocapril · HCl to rats is converted to temocaprilat (unpublished observation) and no other metabolites are found in plasma or bile (Ishizuka *et al.*, 1997).

In our study, it was found that the uptake of temocaprilat by isolated rat hepatocytes is mediated by a Na^+-independent mechanism (table 2). The Na^+-independent transport system(s) on the sinusoidal membrane accounts for the hepatic uptake of many organic anions (Müller and Jansen, 1997); due to the broad substrate specificity, the putative transporter responsible for this uptake has been referred to as "multispecific organic anion transporter" (Meier, 1988). By examining the uptake into isolated hepatocytes, we and others have demonstrated that the substrates for this transporter include clinically important drugs such as DBSP (Blom *et al.*, 1981), pravastatin (Yamazaki *et al.*, 1993), benzylpenicillin (Tsuji *et al.*, 1986), grepafloxacin (Sasabe *et al.*, 1997) and conjugates of E3040 (Takenaka *et al.*, 1997). Even a small peptide like BQ-123 is also partially transported into liver by this transport system (Nakamura *et al.*, 1996).

Based on the expression cloning in *Xenopus laevis* oocytes, oatp1 has been cloned from rat liver as a transport carrier responsible for the Na^+-independent uptake of organic anions (Jacquemin *et al.*, 1994). This cloned oatp1 can, in fact, mediate the transport of a wide range of substrates as summarized by Meier *et al.* (1997). Because it was revealed that temocaprilat is taken up by isolated hepatocytes in a Na^+-independent manner, we investigated if this transport is mediated by oatp1 by examining the uptake into COS-7 cells transiently expressing this cloned transporter. As the uptake of temocaprilat was significantly increased by transfecting

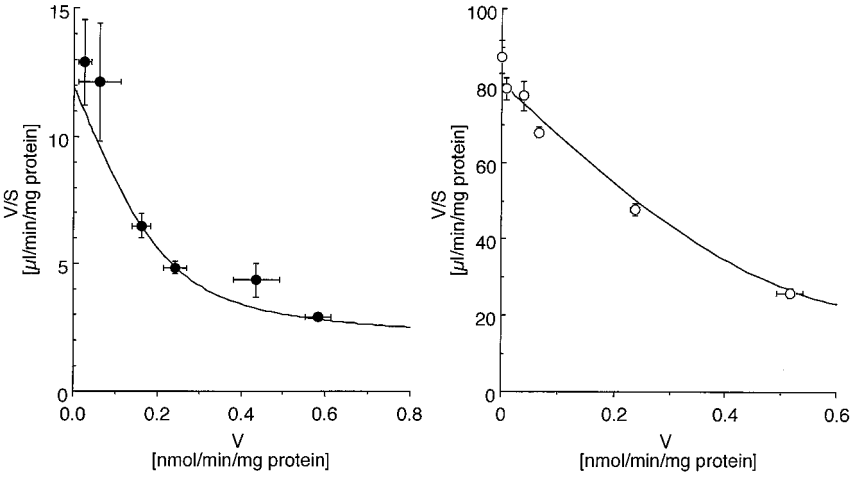

Fig. 2. Eadie-Hofstee plot for the uptake of temocaprilat (●) and $E_2$17βG (○) into isolated rat hepatocytes. Uptake of [^{3}H]temocaprilat (0.1 μM) and [^{3}H]$E_2$17βG (0.01 μM) into isolated hepatocytes was measured in the presence and absence of unlabeled temocaprilat and $E_2$17βG, respectively, in Krebs-Henseleit buffer (pH 7.4) at 37°C. Solid line represents the fitted line obtained from the kinetic parameters listed in table 1. Each point represents the mean ± S.E. of three to four different preparations.

TABLE 1
Kinetic parameters for the transport of temocaprilat and $E_2$17βG (mean ± S.E., N = 3)

	Hepatocytes				oatp1 COS-7		
	Vmax (nmol/min/mg)	Km (μM)	Pdif (μl/min/mg)	CLuptake (μl/min/mg)	Vmax (nmol/min/mg)	Km (μM)	CLuptake (μl/min/mg)
Temocaprilat	0.21 ± 0.03	20.9 ± 8.0	1.9 ± 0.3	14.0 ± 2.4	0.092 ± 0.022	46.7 ± 15.9	2.3 ± 0.4
$E_2$17βG	0.47 ± 0.12	6.5 ± 1.6	8.5 ± 4.5	81.3 ± 4.4	0.32 ± 0.16	11.0 ± 3.9	26.2 ± 5.6

The experimental data shown in figures 2 and 5 were analyzed to determine the kinetic parameters.

TABLE 2
Characteristics of temocaprilat uptake into isolated hepatocytes (mean ± S.E., N = 3)

Treatment	Uptake (% of Control)
Rotenone (30 μM)	22.9 ± 2.6
FCCP (2 μM)	28.7 ± 4.7
PCMBS (100 μM)	15.7 ± 1.7
DIDS (100 μM)	14.7 ± 3.2
4°C	4.3 ± 0.0
$Na^+ \rightarrow Li^+$	108.2 ± 1.5

The uptake of [^{3}H]temocaprilat (0.1 μM) by the isolated hepatocytes was studied in the presence of metabolic inhibitor, sulfhydryl-modifying reagent and anion exchange inhibitor. These drugs were added to the cell suspension 5 min before the addition of [^{3}H]temocaprilat. Effect of incubation temperature and Na^+ was also examined.

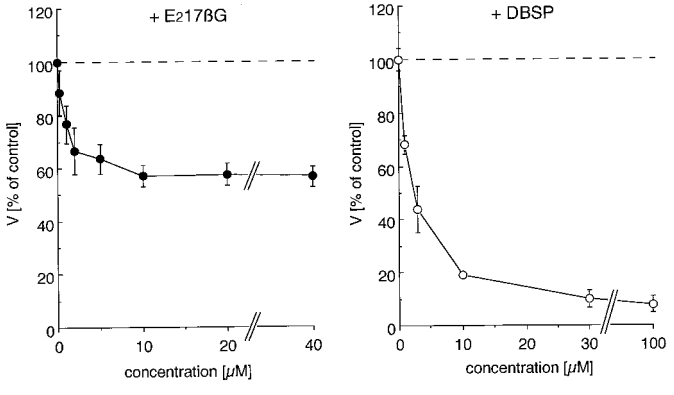

Fig. 3. Effect of $E_2$17βG and DBSP on the uptake of temocaprilat into isolated rat hepatocytes. Uptake of [^{3}H]Temocaprilat (0.1 μM) was measured in the presence of $E_2$17βG (left) and DBSP (right). Each point represents the percentage of the control value (mean ± S.E. of three different preparations).

oatp1 cDNA (fig. 4), we compared the uptake of temocaprilat with that of $E_2$17βG, a typical substrate for oatp1; based on studies using oatp1-injected oocytes, it has been shown that the Na^+-independent uptake of $E_2$17βG into rat basolateral membrane vesicles is mediated by oatp1 (Bossuyt *et al.*, 1996; Meier *et al.*, 1997). Kanai *et al.* (1996) determined the K_m value for $E_2$17βG in oatp1-expressing HeLa cells as 3 μM. We also found a concentration-dependent uptake of $E_2$17βG in oatp1-expressing COS-7 cells with a K_m of 11.0 μM (table 1), comparable with that reported by Kanai *et al.* (1996). In addition, the K_m for the uptake of $E_2$17βG by hepatocytes (6.5 μM, table 1) was comparable with these K_m values. These data indicate that COS-7 cells expressing oatp1 are suitable for estimating oatp1-mediated transport. At the present time, we do not have any good explanation to account for the low IC_{50} value of $E_2$17βG (0.8 μM) for the sensitive portion of temocaprilat uptake (fig. 3).

The contribution of oatp1 to carrier-mediated uptake of temocaprilat by isolated rat hepatocytes needs to be determined, however, because the uptake of temocaprilat was not completely inhibited by the addition of an excess of $E_2$17βG (fig. 3). We calculated this contribution as being approximately 50% by correcting the uptake clearance with that of $E_2$17βG (equation 2), suggesting the presence of another Na^+-independent organic anion transport system(s) to account for the uptake of temocaprilat.

To estimate the contribution of oatp1 to the uptake of temocaprilat by isolated hepatocytes, we used $E_2$17βG as a reference compound because of its high affinity for oatp1 among the reported substrates (Meier *et al.*, 1997). An underlying assumption with equation 2 is that $E_2$17βG is taken up into the hepatocytes predominantly by oatp1. This method has a limitation, however, because a recently cloned oatp1 homologue (oatp2) can also transport $E_2$17βG (Noé *et al.*, 1997). If we consider that oatp2 is also responsible for the

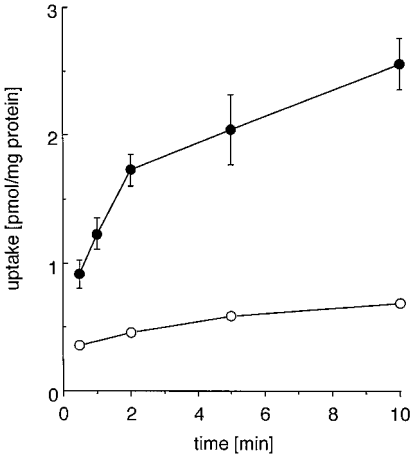

Fig. 4. Time-profiles for the uptake of temocaprilat by oatp1 (●) and vector-transfected (○) COS-7 cells. Uptake of [^{3}H]temocaprilat was measured by incubating COS-7 cells in Krebs-Henseleit buffer (pH 7.4) containing [^{3}H]temocaprilat (0.1 μM) after preincubation for 5 min. Each point represents the mean ± S.E. of three different preparations.

hepatic uptake of $E_2$17βG, the contribution of oatp1 calculated from equation 2 should overestimate the actual contribution, *i.e.*, the contribution of oatp1 to the uptake of $E_2$17βG should be less than 50%. These results are also consistent with the hypothesis that another transporter(s) also mediates the uptake of temocaprilat. This hypothesis is further supported by the results of hepatocellular uptake study of temocaprilat. A high concentration of DBSP completely inhibited the uptake of temocaprilat, however, its uptake was only partially inhibited by the addition of $E_2$17βG (fig. 3). This inhibitory effect by DBSP on the uptake of temocaprilat may not result from the toxicity to cells; we previously examined the effect of DBSP on the uptake of grepafloxacin which was taken up by isolated rat hepatocytes via an active transport system distinct from organic anion transporter (Sasabe *et al.*, 1997). The results showed that the transport of grepafloxacin was not affected by the addition of DBSP from 5 to 100 μM in the medium, although other drugs (such as quinidine and verapamil) inhibited the uptake of grepafloxacin. Therefore, the effect of DBSP on the uptake of temocaprilat shown in fig. 3 may not result from the toxicity to cells, but predominantly from its inhibitory effect on the transporter(s). Moreover, the effect of metabolic inhibitors on the uptake of temocaprilat was observed by the isolated hepatocytes (table 2). We found that the transport of temocaprilat was at least in part mediated by oatp1, which can act as a bicarbonate exchanger (Satlin *et al.*, 1997). The addition of metabolic inhibitor reduced the driving force for the uptake, resulting in a reduction of temocaprilat uptake.

To determined the kinetic parameters for the uptake of temocaprilat, we fitted the data obtained from isolated hepatocytes to equation 1. In our preliminary results, the uptake of temocaprilat into isolated hepatocytes at 4°C increased linearly against the medium concentration and the data (P_{dif}) obtained was comparable with the fitted values calculated from equation 1 (1.6 *vs.* 1.9, data not shown). We tried to calculate the uptake parameters using a model consisting of two transport components, in addition to the nonspecific component. However, meaningless values were obtained for some parameters because of the deviation in the data and because the number of parameters (five parameters containing K_{m1}, K_{m2}, V_{max1}, V_{max2} and P_{dif}) were excessive compared with the number of data points (six parameters).

The presence of multiple transport systems for the uptake of organic anions by the hepatocytes has been suggested previously and an oatp2 has been isolated recently (Noé *et al.*, 1997). To evaluate the extent to which oatp1 accounts for the hepatic uptake of BSP, Hagenbuch *et al.* (1996) used an antisense oligonucleotide. Oatp1-specific antisense oligonucleotides were coinjected with total rat liver mRNA into *Xenopus laevis* oocytes to measure the uptake of BSP. The results indicated that oatp1 accounts for only half of total BSP transport, also suggesting the presence of additional organic anion uptake systems in rat liver (Hagenbuch *et al.*, 1996). In addition, Horz *et al.* (1996) examined the uptake of bumetanide into oocytes injected with cRNA for ntcp or oatp1 and suggested the presence of an organic anion transport system that is different from these transporters. Thus, temocaprilat may be additionally transported by other organic anion transporter(s) including oatp2 (Meier *et al.*, 1997).

Other ACE inhibitors have also some affinity for oatp1, since they inhibited the uptake of temocaprilat into oatp1-expressing COS-7 cells (fig. 6). In addition, these ACE inhibitors may also have some affinity for the unidentified transporter(s), because the inhibitory effect of ACE inhibitors on the uptake of temocaprilat correlated well between isolated hepatocytes and oatp1-transfected cells (fig. 6). Recently, us-

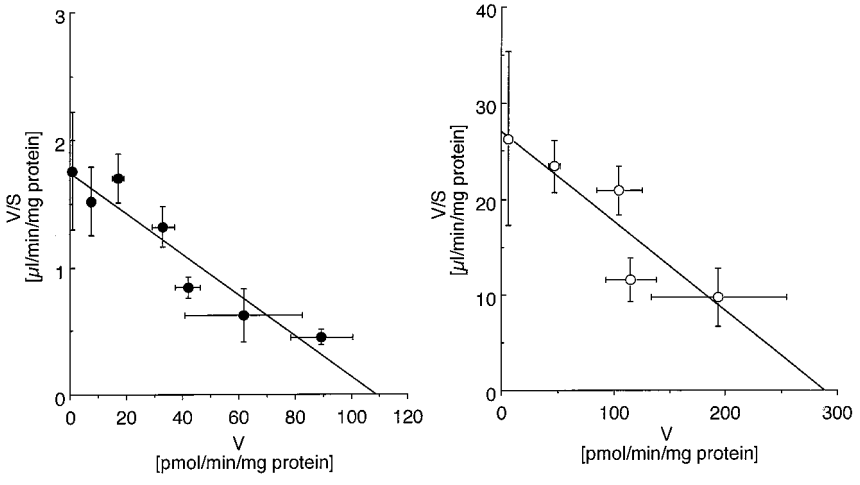

Fig. 5. Eadie-Hofstee plot for the uptake of temocaprilat (●) and $E_2$17βG (○) into oatp1-transfected COS-7 cells. Uptake of [^{3}H]temocaprilat (0.1 μM) and [^{3}H]$E_2$17βG (0.01 μM) into oatp1-transfected cells was measured in the presence and absence of unlabeled temocaprilat and $E_2$17βG, respectively. The initial uptake velocity for oatp1-mediated temocaprilat uptake was calculated by substracting the uptake for vector-transfected cells. Each point represents the mean ± S.E. of three to four different preparations.

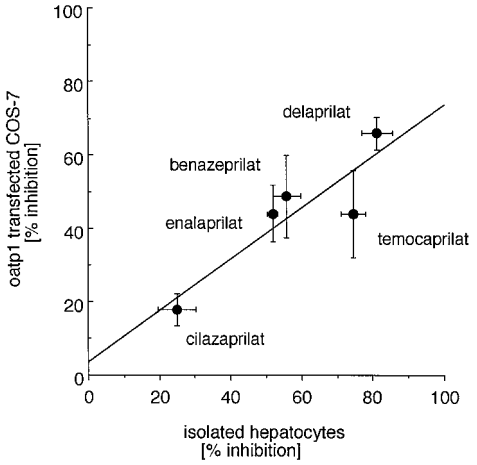

Fig. 6. Inhibitory effect of ACE inhibitors on the uptake of [^{3}H]temocaprilat (0.1 μM) into isolated hepatocytes and oatp1-transfected COS-7 cells. Uptake of [^{3}H]temocaprilat was measured in the presence of ACE inhibitors (100 μM) in both experiments. A good correlation ($R^2 = 0.804$) was observed between both experiments. Each point represents the percentage of the control value (mean ± S.E. of three different preparations).

ing a Hela cell line stably expressing oatp1, it was also reported that oatp1 mediates the uptake of enalapril although the uptake of enalaprilat was not studied (Pang *et al.*, 1997). Collectively, it is possible that oatp1 contributes to the sinusoidal uptake of these ACE inhibitors, although their predominant excretion pathway, except in the case of temocaprilat, is via urine. In our previous report, we demonstrated that temocaprilat is efficiently excreted into bile via cMOAT for which other ACE inhibitors, such as benazeprilat, cilazaprilat, delaprilat, enalaprilat and imidaprilat, have a low affinity (Ishizuka *et al.*, 1997). Taking these data into account, it is suggested that, although ACE inhibitors other than temocaprilat may be transported efficiently into hepatocytes, most of the drug may be released into the systemic circulation and, finally, excreted via urine. The affinity for cMOAT is the predominant reason accounting for the difference in the excretion pathway between temocaprilat and other ACE inhibitors.

In conclusion, our results indicate that temocaprilat is taken up by rat isolated hepatocytes via an Na^+-independent mechanism, approximately half of which is mediated by oatp1. Although other ACE inhibitors may be taken up by hepatocytes, the fact that they are almost exclusively excreted via urine may be accounted for by their low affinity for cMOAT.

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