UPTAKE OF ENALAPRIL AND EXPRESSION OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1 IN ZONAL, ISOLATED RAT HEPATOCYTES

TAWFIC NESSIM ABU-ZAHRA, ALLAN W. WOLKOFF, RICHARD B. KIM, AND K. SANDY PANG

Departments of Pharmaceutical Sciences (K.S.P.) and Pharmacology (T.N.A., K.S.P.), University of Toronto, Toronto, Ontario, Canada; the Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York (A.W.W.); and Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University, School of Medicine, Nashville, Tennessee (R.B.K.)

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ABSTRACT:

Sinusoidal entry is the first obligatory process preceding intracellular drug removal in liver. Transport of the angiotensin converting enzyme inhibitor enalapril (1–750 μM with [3H]enalapril), a substrate of Oatp1, the sodium-independent organic anion transporting polypeptide 1 cloned from rat liver, was studied in rat hepatocytes isolated from all zones of the liver (homogeneous) and from enriched perportal (PP) and perivenous (PV) hepatocytes prepared by collagenase perfusion and zone-selective destruction with digitonin, respectively. Uptake was linear over 1 min and was concentration-dependent. Transport by the homogeneous hepatocytes (in the presence and absence of Na+) and PP and PV cells was described by single saturatable components of similar kinetic constants (Km values of 344–461 μM and Vmax values of 9.5–11.6 nmol/min/106 cells; P > .05, ANOVA). The Km value for enalapril uptake in hepatocytes was of the same order of magnitude compared with that for Oatp1 expressed in HeLa cells transfected with cDNA-Oatp1 and Western blot analysis revealed similar levels of immunoreactive Oatp1 expression in PP and PV hepatocytes. However, enalapril was not taken up by Oatp2 nor by the human OATP expressed in recombinant vaccinia systems.

Hepatic transport is of paramount importance in the overall fate of drugs in the liver. In addition to the heterogeneity in drug-metabolizing activities, acinar transport is known to affect the ultimate removal of substrates (Gumucio, 1983; Meijer and Grothuis, 1991; Kwon and Morris, 1997). Recently, the angiotensin converting enzyme inhibitor (ACEi)1 enalapril [(S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-l-alanyl]-l-proline], was shown to be taken up by the organic anion transporting polypeptide 1 (Oatp1) cloned from rat liver in expression systems with a Michaelis-Menten constant (Km) of 214 μM (Pang et al., 1998). Oatp1 transport is a Na+-independent process (Jacquemin et al., 1994) involving the probable countertransport of bicarbonate (Satlin et al., 1997) and/or glutathione (Li et al., 1998). Oatp1, in particular, is capable of transporting endogenous organic anions such as the bile acid taurocholate (Jacquemin et al., 1994; Shi et al., 1995; Satlin et al., 1997) and the steroid hormone estrone-3-sulfate (Bosuuy et al., 1996). The cholephilic dye bromosulfophthalein (BSP; Jacquemin et al., 1994; Satlin et al., 1997) modified peptides exemplified by the thrombin inhibitor CRC-220 (Eckhardt et al., 1996), and more recently, the ACEis, enalapril (Pang et al., 1998) and temocaprilat (Ishizuka et al., 1998), have all been identified as exogenous substrates of Oatp1.

The identification of Oatp1-mediated uptake of the prototypic non-sulphydryl ACEi enalapril and not of the active diacid metabolite, enalaprilat, has provided an explanation of the high clearance of enalapril in the single pass perfused liver preparation (Pang et al., 1984). Studies by Pang et al. (1991) showed that the overall hydrolysis of enalapril to enalaprilat occurs predominantly in the perivenous (PV) region of the intact rat liver. It remains to be determined whether or not transport heterogeneity exists for enalapril because the overall removal within zonal regions may be due either to variation in intracellular enzyme activities and/or transport capacities among zonal hepatocytes.

It has been reported that transport proteins are heterogeneously distributed at the basolateral membrane of the liver. The human multidrug resistance associated protein 3 (MRP3) is more abundant in the perportal (PP) region (Kool et al., 1999), whereas Oatp2, another transporter of the Oatp family in rat liver (Kakyo et al., 1999; Reichel et al., 1999), the organic cation transporter, rOCT (Meyer-Wentrup et al., 1998), the sodium-dependent dicarboxylate/sulfate transporter, sDCT2 (Chen et al., 1999), and the cysteine (Saiki et al., 1992) and glutamate transporters in rat liver (Stoll et al., 1991) are more predominant in the PV region.

The studies presented herein were therefore conducted to test the hypotheses that uptake of enalapril into isolated rat hepatocytes is mediated by Oatp1 and is homogeneously distributed in rat liver. For hypothesis testing, enalapril uptake studies were conducted with homogeneous and zonal rat hepatocytes, and expression of Oatp1 among

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1 Abbreviations used are: ACEi, angiotensin converting enzyme inhibitor; BSP, bromosulfophthalein; Oatp1, organic anion transporting polypeptide 1 cloned from rat liver; OATP, organic anion transporting polypeptide cloned from human liver; PP, perportal; PV, perivenous; DIDS, 4,4′-diisothiocyanostilbene-2, 2′-disulfonic acid.

Send reprint requests to: Dr. K. S. Pang, Faculty of Pharmacy, University of Toronto, 19 Russell Street, Toronto, Ontario, Canada MSS 2S2. E-mail: ks.pang@utoronto.ca
zonal cells was further characterized by Western blot analysis. The role of other Oatps for enalapril transport was investigated in recombinant vaccinia expression systems for Oatp1, Oatp2, and the organic anion transporting polypeptide cloned from human liver (OATP).

**Experimental Procedures**

**Materials.** [1H]Enalapril was synthesized as described previously (Pang et al., 1998). Enalaprilat and unlabeled enalapril were obtained from Merck-Frosst (Montreal, PQ). [1C]Sucrose (specific activity, 0.05 mCi/ml) was purchased from DuPont (Boston, MA). Estrone-3-sulfate, harmol sulfate, DIDS (4,4′-disothiocyanostilbene-2, 2′-disulfonic acid), ouabain, rotenone, BSA, and alanine-proline were purchased from Sigma Chemical Co. (St. Louis, MO). KCN was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Collagenase A (clostridium histolyticum) was purchased from Boehringer Mannheim (Darmstadt, Germany). All other reagents and solvents were of HPLC grade.

**Expression Systems for Oatp1, Oatp2, and OATP.** The transporter cDNA constructs and transfection of the cDNAs have been described in detail in a previous report (Cvetkovic et al., 1999). Transport studies in HeLa cells transfected with 1 μg of plasmid DNA or the parental plasmid lacking any transporter sequence for the control were initiated by the addition of 0.4 ml Well of [1H]enalapril (5 μM). Uptake was allowed to occur at 37°C, then transport activity was stopped at 10 min as previously described (Cvetkovic et al., 1999). After lysing of the cells, the radioactivity associated with the cell lysate was counted by liquid scintillation spectrometry (Rackbeta model 1219; LKB Instruments, Gaithersburg, MD).

**Isolation of Rat Hepatocytes.** Isolated rat hepatocytes were prepared by the method of Tan et al. (1999) from male Sprague-Dawley rats (275–375 g; Charles River, St. Constant, PQ). Rats were anesthetized by i.p. injection of pentobarbital (65 mg/kg) in accordance with protocols approved by the Animal Care Committee of the Department of Comparative Medicine at the University of Toronto. All buffers were prepared with carbogen (95% O2/5% CO2; Matheson, Mississauga, ON). The portal vein was perfused with a Ca2+ free buffer [preperfusion buffer, consisting of Hanks’ buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH2PO4, 2H2O, 0.42 mM Na3HPO4, 12 H2O), plus 10 mM HEPES (pH 7.2), 0.5 mM EDTA, 4.2 mM NaHCO3, 5 mM glucose, and 0.65% BSA] in a single pass fashion at 30 ml/min for 10 min. Hepatocytes from all zones of the liver were prepared by perfusion with collagenase (Hanks’ buffer with 4 mM CaCl2 and 0.05% collagenase), which recirculated through the liver for 7 to 9 min at the flow rate of 25 ml/min. Zonally isolated hepatocytes were harvested following the procedure of Lindros and Penttila (1985) by zone-selective destruction with digitonin. The enrichment of hepatocyte populations was verified by marker enzymes (Tan et al., 1999): the activity of the PP marker enzyme alanine aminotransferase was found to be 2.13 ± 1.0 times higher in PP hepatocytes (433 ± 165 nmol/min/mg protein) than in PV hepatocytes (203 ± 80 nmol/min/mg protein). Conversely, glutamine synthetase, an enzyme expressed exclusively in the distal PV hepatocytes, exhibited significantly lower activity in PP hepatocytes (0.15 ± 0.06 nmol/min/mg protein) than in PV preparations (0.5 ± 0.10 nmol/min/mg protein). The viability of the resulting hepatocyte suspension was >90%, as assessed by trypan blue exclusion.

**Kinetic Uptake Studies.** Hepatocyte suspensions (1 ml of 2 × 106 cells/ml) were preincubated in a rotating water bath for 10 min at 37°C. Aliquots (500 μl) of the suspension were added to 100 μl of various solutions containing enalapril, tracer [1H]enalapril, and [1C]sucrose (an extracellular marker) in 1.15% KCl to result in enalapril concentrations of 1 to 750 μM in 1.67 × 106 cells/ml. After admixture, samples (100 μl) were retrieved over 1 min and centrifuged immediately (9650g) for rapid filtration through a layer of silicon oil, separating the hepatocytes from the extracellular medium. Samples were taken from both the supernatant and cell pellet for liquid scintillation counting (Model LS 6800; Beckman Canada, Mississauga, ON).

**Inhibitor Uptake Studies.** The ability of benzoic acid (100 μM), harmol sulfate (200 μM), estrone-3-sulfate (100 μM), or the dipeptides, alanine-proline (400 μM) and enalaprilat (400 μM), to cīs-inhibit enalapril uptake was examined by individually adding the compounds to the enalapril solution before the addition to hepatocytes. The metabolic inhibitor KCN (2 mM), the anion transport inhibitor, DIDS (2 mM), and the Na+/K+ ATPase inhibitor, ouabain (1 mM), were added to the cell suspension during the last 2 min of the 10-min hepatocyte preincubation interval, whereas the metabolic inhibitor, rotenone (30 μM), was added to the hepatocyte suspensions for preincubation for 30 min before the addition of enalapril. An equivalent volume of buffer was added for the control samples. All control samples were preincubated for 10 min except for the controls for rotenone, which were preincubated for 30 min. In all cases, the final concentration in the incubation mixture was 1 μM enalapril and 1.67 × 106 cells/ml. Data for the effects of treatment were expressed as percentages of the uptake rates of the controls.

**Western Blot.** Cells were pelleted from zonal hepatocyte suspensions and frozen at −70°C until use. Cells were then extracted with Na2CO3 to remove the loosely associated membrane proteins according to a previously described method (Bergwerk et al., 1996), with modifications. Briefly, cell pellets were suspended in 28 ml of 0.1 mM Na2CO3 containing the following mixture of protease inhibitors: 20 μg/ml BAME (Na-p-benzyl-l-arginine-methyl ester); 20 μg/ml soybean trypsin inhibitor; 20 μg/ml TAME (Na-p-tosyl-l-arginine-methyl ester); 1 μg/ml leupeptin; 1 mM phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; 1 mM EDTA; and 5 mM EGTA. The suspension was rotated at 4°C for 15 min and then centrifuged at 4°C for 1 h at 100,000g. The resulting pellet was resuspended in 0.4 ml of PBS by mild sonication. Protein concentration was determined by the BCA protein assay (Fierce, Rockford, IL) using BSA as standard. Immunoblot was performed using an antibody prepared to a synthetic peptide corresponding to a region of Oatp1 near the carboxy terminus as previously described (Bergwerk et al., 1996).

**Kinetic Analysis.** The [1C]sucrose counts associated with the pellet accounted for the volume of extracellular medium entrapped by hepatocytes. The uptake of [1H]enalapril was thus corrected for the extracellularly entrapped, and the specific activity of the radiolabeled enalapril was used to calculate the rate of enalapril uptake. The cumulative amounts taken up into cells (nmol/106 cells), after correction for the entrapped extracellular components, were plotted against the incubation time. Linear regression of the data yielded the slope that represents the initial uptake rate (v). When v was plotted against the corresponding concentration [S], the kinetic constants were obtained by regression of v versus [S] according to the following equations:

- For a system consisting of a single Michaelis-Menten component,

  \[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]  

  (1)

  for a system with saturable and nonsaturable components,

  \[ v = \frac{V_{\text{max}} [S]}{K_m + [S] + P_{\text{diff}} [S]} \]  

  (2)

  and for a system consisting of two saturable components,

  \[ v = \frac{V_{\text{max},1} [S]}{K_{m,1} + [S]} + \frac{V_{\text{max},2} [S]}{K_{m,2} + [S]} \]  

  (3)

  where \( K_m \) represents the Michaelis-Menten parameter, \( V_{\text{max}} \) denotes the maximal velocity, and \( P_{\text{diff}} \) is the unidirectional transmembrane clearance at the sinusoidal membrane.

**Fitting and Data Handling.** Data on the slopes (v) from each experiment were fitted individually to eq. 1, 2, or 3 by an iterative nonlinear least square procedure (Scientist v.2; Micromath Scientific Software, Salt Lake City, UT). Kinetic uptake data for each experiment were fit to all three equations. The Model Selection Criteria was used to determine the appropriateness of the equations: the greater the Model Selection Criteria value for a model, the better that model describes the data. A weighting scheme of 1/observation2 was used because this provided the best fit. The goodness of fit was judged by the S.D. of the parameter estimate or the r.v. (S.D.parameter estimate), residual plots, and the residual sum of squares.

**Statistics.** The data were presented as mean ± S.D. The means were
compared by use of ANOVA or the paired t statistic accordingly, with \( P < .05 \) denoting the level of significance.

**Results**

**Enalapril Uptake by Oatp1, Oatp2, and OATP.** The uptake of \([^{3}H]\)enalapril (5 \( \mu \)M) by Oatp2 and OATP was not significantly different from that of the control (Fig. 1). By contrast, uptake was significantly higher for Oatp1 in comparison to the control, as found previously (Pang et al., 1998).

**Enalapril Uptake into Hepatocytes.** Transport of enalapril among all hepatocyte preparations was linear over 1 min for all concentrations (see Fig. 2 for representative study). Similar saturation curves for enalapril uptake were evident with rat hepatocytes isolated from whole liver, in the presence and absence of sodium ion (Fig. 3), and with PP and PV hepatocytes (Fig. 4). The best fit in all cases was eq. 1, which described a single saturable component. The fitted \( V_{\text{max}} \) value in the presence of sodium for hepatocytes from whole liver was 11 \( \pm \) 1.5 nmol/min/10⁶ cells, and the \( K_{\text{m}} \) value was 344 \( \pm \) 52 \( \mu \)M. These values did not differ from those obtained for the uptake of enalapril in absence of sodium ion (\( V_{\text{max}} \) value of 11.6 \( \pm \) 4.2 nmol/min/10⁶ cells and \( K_{\text{m}} \) value of 446 \( \pm \) 179 \( \mu \)M), and were similar to those for PP and PV cells (\( V_{\text{max}} \) value of 9.5 \( \pm \) 2.8 and 10.4 \( \pm \) 2.0 nmol/min/10⁶ cells, and \( K_{\text{m}} \) values of 461 \( \pm \) 177 and 411 \( \pm \) 50 \( \mu \)M; \( P > .05 \), ANOVA, Table 1).

**Inhibitor Uptake Studies.** The uptake of 1 \( \mu \)M enalapril was unaffected by the metabolic inhibitors KCN (2 mM) and rotenone (30 \( \mu \)M) (\( P > .05 \), paired t test, Fig. 5). In addition, benzoic acid (100 \( \mu \)M), enalaprilat (400 \( \mu \)M), and alanine-proline (400 \( \mu \)M) failed to affect the uptake of enalapril (\( P > .05 \), paired t test, Fig. 5). However, the anion transport inhibitor DIDS (2 mM), temperature reduction, the Oatp1 substrate estrone-3-sulfate (100 \( \mu \)M) (Bossuyt et al., 1996), and surprisingly, harmol sulfate (200 \( \mu \)M), all significantly inhibited enalapril uptake (\( P < .05 \), paired t test, Fig. 5). Unexpectedly, ouabain (1 mM), another Oatp1 substrate (Noé et al., 1997), failed to exert a significant effect on the uptake of enalapril. This may be due to the high \( K_{\text{m}} \) value of ouabain (1700–3000 \( \mu \)M) (Noé et al., 1997; Reichel et al., 1999) and to the variability in the data (Fig. 5).

**Western Blotting.** The expression of Oatp1 in PP and PV hepatocytes, quantified by Western blot analysis, was similar (Fig. 6); integration of the optical density of the immunoreactive proteins revealed similar intensities of 0.79 \( \pm \) 0.13 and 0.88 \( \pm \) 0.33 for the PP and PV regions, respectively (\( P > .05 \), ANOVA).

**Discussion**

Oatp1 and Oatp2 (Jacquemin et al., 1994; Noé et al., 1997), the sodium/taurocholate cotransporting polypeptide (Hagenbuch et al., 1991), and the organic anion transporter 2 (Oat2) (Sekine et al., 1998) are cloned proteins expressed on the rat hepatocyte basolateral membrane that are capable of transporting organic anions. Presently, we
confirmed that sodium ion is not involved in the transport of enalapril in isolated rat hepatocytes (Table 1), excluding the role of sodium/taurocholate cotransporting polypeptide, as shown earlier (Pang et al., 1998). Moreover, uptake is not mediated by Oatp2, which is known to transport other anions such as the estrogen conjugates, estradiol 17β-glucuronide and estrone 3-sulfate (Noé et al., 1997), pravastatin glucuronide and estrone 3-sulfate (Noé et al., 1997), and for its glutathione conjugate (BSPGSH) (Schwarz et al., 1980), another Oatp1 substrate (Pang et al., 1998). Absence of inhibition may be due to the variability in the data and the relatively low affinity of ouabain for Oatp1 (Km value of 1700–3000 µM) (Noé et al., 1997; Reichel et al., 1999) compared with that for enalapril (Km value of 350–440 µM). Oatp2 will likely play a more important role than Oatp1 in the hepatic uptake of ouabain (Noé et al., 1997).

Although Oatp1 transport appears to be independent of ATP hydrolysis (Li et al., 1998), uptake may be affected when intracellular ATP is depleted. Uptake was further characterized with respect to temperature. An energy dependence was inferred because uptake was reduced drastically when the temperature was lowered (Fig. 5). However, the metabolic inhibitors rotenone (30 µM, preincubated for 30 min) and KCN (2 mM) were unable to reduce enalapril uptake in these studies. The lack of effect of rotenone or potassium cyanide on enalapril uptake is unexpected, because rotenone (30 µM) was known to reduce ATP levels by 90% when preincubated with hepatocytes over 30 min (Yamazaki et al., 1993). Indeed, conflicting results concerning the effects of cellular ATP depletion on Na+/K+-ATPase at similar concentrations (Sillau et al., 1998) were observed. The lack of effect of metabolic inhibitors antimycin A, rotenone, and carboxyl cyanide m-chlorophenylhydrazone (Schwenk et al., 1976), although uptake was reduced when 2-deoxyglucose and sodium azide were used for ATP depletion via glycolysis and mitochondrial respiration in hepatocytes (Yamazaki et al., 1993) and Oatp1-expressing HeLa cells (Shi et al., 1995). Uptake of temocaprilat, measured over 90 s, was inhibited by the electron transport inhibitor, rotenone (Ishizuka et al., 1998). The discrepancies are possibly explained by the fact that uptake of both temocaprilat and BSP are only partly mediated by Oatp1 in hepatocytes (Hagenbuch et al., 1996; Ishizuka et al., 1998).

There exist other sodium-independent systems on the basolateral membrane of hepatocytes that could potentially transport enalapril. Benzoic acid, a putative substrate of the monocarboxylate transporter MCT1 (Garca et al., 1995) and not of Oatp1 (Pang et al., 1998), failed to inhibit enalapril uptake (Fig. 5). The drug sulfate conjugate, harmol sulfate, which is transported into hepatocytes via an unknown mechanism, was inhibitory although harmol sulfate was not transported by Oatp1-expressing HeLa cells (Pang et al., 1998) and failed to inhibit
the uptake of estrone sulfate (Tan et al., 1999). The recently cloned novel liver-specific transport protein OAT2 (Sekine et al., 1998) that affects the uptake of salicylate, PGE2, dicarboxylates, and p-aminohippurate, is unlikely involved because there is a lack of inhibition of Oat2 transport by enalapril, and there is no known overlapping substrate specificity between Oat2 and Oatp1 (Kullak-Ublick et al., 1994; Sekine et al., 1998).

Inasmuch as Oatp1 is involved in the uptake of various pharmacologically important peptides and steroids, it becomes important to characterize its zonal expression and acinar function. No difference was observed for uptake between hepatocytes prepared from the PP and PV regions and whole rat liver (Fig. 4, Table 1). Additionally, Western blot analysis revealed a lack of quantifiable difference between Oatp1 protein expression in PP and PV hepatocytes (Fig. 6). This observation provides quantitative evidence for homogeneity of protein expression and is consistent with prior observations of the lack of noticeable unevenness in protein expression, qualitatively observed under confocal microscopy (Bergwerk et al., 1996), and findings of a lack of lobular heterogeneity for Oatp1 mRNA as measured by in situ hybridization (Dubuisson et al., 1996; Angeletti et al., 1998). By contrast, an enriched midzonal-PV distribution of Oatp2 was suggested recently (Kakyo et al., 1999; Reichel et al., 1999), and Oatp2 is not involved in enalapril uptake (Fig. 1). Because activities in zonal hepatocytes and Oatp1 are both homogeneously expressed across the acinus for the uptake of enalapril, and because similar \( K_m \) values are obtained for both systems, it appears that Oatp1 is likely the only transporter involved in the uptake of enalapril. Inasmuch as the transport of enalapril into rat hepatocytes is an early and mandatory process in the overall hydrolysis and excretion of both enalapril and enalaprilat, which subsequently return to the venous circulation, the PV preponderance in enalapril hydrolysis observed in the intact liver (Pang et al., 1991) may now be attributed to an enrichment in intracellular metabolic activity and not transport in the PV region. The PV preponderance of hydrolysis of enalapril by the 9000 \( \times g \) supernatant fraction of hepatocyte homogenates has recently been verified in incubation experiments (Abu-Zahra and Pang, 2000).

In conclusion, we showed that enalapril uptake by isolated rat hepatocytes is sodium-independent and is best described by a single saturable component model with a \( V_{\text{max}} \) value of 9.5 to 11.6 nmol/min/10^6 cells and a \( K_m \) value of 344 to 461 \( \mu \)M. The uptake of

The ability for the Oatp1 substrates [estrone-3-sulfate (100 \( \mu \)M) and ouabain (1 \( \mu \)M)], harmol sulfate (200 \( \mu \)M), the dipeptides [alanine-proline (400 \( \mu \)M) and enalaprilat (400 \( \mu \)M)], the monocarboxylic acid benzoic acid (100 \( \mu \)M), the nonspecific anion transport inhibitor DIDS (2 \( \mu \)M), temperature reduction, and the metabolic inhibitors (KCN (2 mM) and rotenone (30 \( \mu \)M)) to inhibit enalapril uptake was examined over 1 min. Values were expressed as a percentage of control ± S.D. (\( n = 3–6 \)). *, Denotes treatment values that are statistically different from controls, (\( P < .05 \), paired \( t \) test). Ala-Pro, alanine-proline dipeptide.
enalapril and the expression of Oatp1 were both found to be homogeneous across the liver acinus. Uptake of enalapril by isolated rat hepatocytes is consistent with Oatp1-mediated transport.

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References


