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Contribution of organic anion transporting polypeptide OATP-C to hepatic elimination of the opioid pentapeptide analogue [D-Ala^2, D-Leu^5]-enkephalin

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Abstract

The objective of this study was to examine the transport activity of the human organic anion transporter OATP-C (SLC21A6) for oligopeptides that are eliminated rapidly from the systemic circulation. We focused on an opioid peptide analogue, [D-Ala^2, D-Leu^5]-enkephalin (DADLE), a linear pentapeptide modified to be stable. [^{3}H]DADLE was taken up by rat isolated hepatocytes in a saturable manner and highly accumulated in the liver after intravenous administration to rats. The uptake of [^{3}H]DADLE by the isolated hepatocytes was inhibited by several organic anions and pentapeptides, but not by tetra- or tripeptides. When OATP-C was expressed in *Xenopus laevis* oocytes, a significant increase in uptake of [^{3}H]DADLE was observed. Moreover, the inhibitory effects of various compounds, including some peptides, on [^{3}H]estrone-3-sulfate uptake by OATP-C were similar to those observed in [^{3}H]DADLE uptake by rat isolated hepatocytes. In conclusion, it was demonstrated that OATP-C contributes to the rapid hepatic excretion of peptides and peptide-mimetic drugs.

Introduction

Many peptides and peptide-mimetic compounds are expected to be pharmacologically effective because they exhibit selective and potent biological activity towards target enzymes, receptors, or both. However, after systemic administration, many of these compounds are rapidly removed from the systemic circulation by enzymic degradation or hepatic and renal elimination, often with a half-life of less than half an hour. To overcome the susceptibility of biologically active peptides to hydrolyzing enzymes in circulating blood, derivatives can be obtained by chemical modification. Although such derivatives are stable to enzymic degradation, they are rapidly eliminated preferentially via the liver into bile, as observed in the cases of renin inhibiting peptides U-71038 (Greenfield et al 1989) and CGP 38 560 (Cumin et al 1990), opioid peptide [D-pen^2, D-pen^5]-enkephalin (DPDPE) (Chen & Pollack 1997) and endothelin antagonist BQ-123 (Nakamura et al 1996) in rats or marmosets. The extent of biliary excretion for U-71038, DPDPE and BQ-123 was 80–90% of the dose in rats, and most of the peptides were eliminated in intact form, but not as metabolites. Based on these pharmacokinetic features of peptides and peptide-mimetic compounds, several studies have demonstrated the presence of hepatic basolateral transporters responsible for the efficient uptake of those compounds by liver. Other peptides and peptide-mimetic compounds, including BQ-123 (Nakamura et al 1996), the somatostatin analogue octreotide (Terasaki et al 1995) and rennin-inhibiting peptides EMD51921 (Bertrams & Ziegler 1991) and ditekiren (Kim et al 1997), were also taken up by isolated rat hepatocytes via a carrier-mediated transport mechanism. The mechanisms involved were either sodium dependent or independent, depending on the test compounds, but were common in showing significant inhibition by organic anions such as sulfobromophthalein (BSP) and bile acids with apparent K_m values in the range 0.2–91 μM. These observations suggest an involvement of organic anion transporters in the hepatic uptake of peptides and peptide mimetics, though molecular identification of the responsible transporters remains incomplete.

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Previous studies on the hepatic transport of peptides as described above were conducted mainly in rats, and it is important to know which transporters are expressed in humans. Recently, various organic anion transporters expressed in human liver have been identified, including multidrug-resistance-associated proteins MRP (König et al 1999), organic anion transporting polypeptides OATP (Kullak-Ublick et al 1995), sodium-dependent bile acid transporter NTCP (Hagenbuch & Meier 1994), ATP-dependent bile acid transporter BSEP (Strautnieks et al 1998) and type-I inorganic phosphate transporter NPT1 (Uchino et al 2000). MRP2 and BSEP function in an ATP-dependent manner for efflux of anionic compounds, including bile acids, conjugated metabolites and anionic drugs, into bile, and therefore they are unlikely to be hepatic uptake transporters for peptides. NTCP might be involved in the transport of some peptides that show sodium dependence, as observed with octreotide and BQ-123 (Terasaki et al 1995; Nakamura et al 1996; Yamada et al 1997). Most peptides and peptide mimetics are likely to be taken up by the sodium-independent transporter that is responsible for bile-acid transport. Currently, OATP transporters are characterized as sodium-independent and multispecific organic anion transporters, and in particular, OATP-B (SLC21A9), OATP-C (SLC21A6) and OATP8 (SLC21A8) are expressed in human liver (Abe et al 1999; König et al 2000a; Tamai et al 2000), being localized at the basolateral membrane (Kullak-Ublick et al 2001; König et al 2000b).

Recently, human OATP-A (SLC21A3) was demonstrated to transport DPDPE and deltorphin II as substrates (Gao et al 2000), and both OATP-C and OATP8 were reported to transport DPDPE and BQ-123 (Kullak-Ublick et al 2001). They are different from native peptides, having a highly constrained cyclic structure. To understand the rapid clearance mechanism of peptides generally, it is important to know whether more native-type linear peptides are also taken up into the liver by OATP transporters. Recently, it was reported that cholecystokinin octapeptide (CCK-8), a linear peptide, could be transporterd by OATP8, whereas the peptide was not transported by OATP-C (Ismair et al 2001). Accordingly, in this study, we focused on [D-Ala^2, D-Leu^5]-enkephalin (DADLE), a biologically stable analogue of leucine enkephalin (Schulteis et al 1989). Among human OATP transporters, OATP-C is involved in hepatic clearance by accepting various compounds as substrate, and it remains unclear whether OATP-C can transport linear peptides. We conducted an in-vitro study with cells expressing human OATP-C and an in-vivo study in rats to examine whether DADLE is preferentially eliminated into bile. The peptide structure–transport activity relationship of OATP-C is discussed.

Methods

Animals

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University and were approved by the Committee of the Ethics of Animal Experimentation of Kanazawa University, Takara-machi Campus. Sprague-Dawley rats were purchased from SLC Co. (Shizuoka, Japan).

Materials

[^{3}H]Estrone-3-sulfate, ammonium salt (1961 GBq mmol^{-1}), [^{3}H][D-Ala^2, D-Leu^5]-enkephalin (DADLE, 1850 GBq mmol^{-1}), [^{3}H][D-penicillamine^2, D-penicillamine^5]-enkephalin (DPDPE, 1665 GBq mmol^{-1}) and [^{14}C]inulin (0.21 GBq g^{-1}) were purchased from NEN Life Science Products, Inc. (Boston, MA). RNA transcription kit was obtained from Stratagene (La Jolla, CA) and collagenase A was purchased from Roche Diagnostics Co. (Mannheim, Germany). DADLE and leucine-enkephalin were obtained from Peptide Institute, Inc. (Osaka, Japan). All other reagents for functional studies were purchased from Sigma Chemicals & Co. (St Louis, MO) and Wako Pure Chemical Industries (Osaka, Japan).

Pharmacokinetic analysis of [^{3}H]DADLE in rats

Sprague-Dawley rats, 7–8 weeks of age, weighing about 300 g, were used. [^{3}H]DADLE was injected intravenously at a dose of 66 pmol kg^{-1}. Serial blood samples of 300 μL were collected from the jugular vein of the unanaesthetized rats using heparinized capillary tubes at designated time intervals. The plasma was separated by centrifugation and the radioactivity was counted in a liquid scintillation counter (LSC-5100; Aloka, Japan). For determination of apparent tissue-to-plasma concentration ratio (Kp), the rats were sacrificed by decapitation at 5 min following a single intravenous injection of [^{3}H]DADLE. The tissues were isolated, rinsed well with ice-cold saline and weighed, and the associated radioactivity was measured by means of a liquid scintillation counter after solubilization of the tissues in Soluene-350 (Packard, USA).

Expression of OATP-C in *Xenopus laevis* oocytes

For transport experiments, Xenopus laevis oocytes were injected with in-vitro synthesized complementary RNA (cRNA) of OATP-C using T7 RNA polymerase as described previously (Tamai et al 2000). Briefly, defoliculated oocytes were injected with 50 nL of water containing 25 ng of cRNA, cultured for 3 days in modified Barth's solution (mM: 96 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$ and 10 HEPES, adjusted to pH 7.4 with NaOH) and used for uptake experiments. Uptake experiments were started by incubating the oocytes at 25 °C in medium 1 (mM: 100 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, adjusted to pH 7.4 with NaOH) containing radio-labelled test compound. At appropriate times, the oocytes were washed with ice-cold buffer, solubilized in 5% sodium dodecyl sulfate (SDS) solution and then the associated radioactivity was measured. As a reference, the same volume of water was injected into oocytes and the uptake was measured in the same manner.

Isolation and uptake experiments in rat hepatocytes

Rat hepatocytes were isolated according to the collagenase perfusing procedure as described previously (Moldeus et al 1978). After pre-incubation of isolated hepatocytes for 5 min at 37 °C, medium 2 (Krebs-Henseleit buffer containing (mM) 118 NaCl, 25 $NaHCO_3$, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 13 HEPES, 10 glucose and 2% of BSA) containing [^{3}H]DADLE with or without inhibitor was added. The initial uptake rate was determined from the slope of the linear regression line from the uptake points at 15, 30, 60 and 120 s after addition of the substrate. The transport reaction was stopped by centrifugal filtration of the mixture through a layer of a mixture of silicone oil (d = 1.07; Toray Dow Corning Co., Tokyo) and liquid paraffin (d = 0.86; Wako Pure Chemical Industries) with a density of 1.03. The amount of [^{3}H]DADLE in the extracellular fluid of cells was corrected on the basis of separate experiments to measure the apparent uptake of [^{14}C]inulin. All uptake studies were performed within 2 h of cell isolation to avoid a reduction of viability of the cells. Cellular protein was measured according to the method of Bradford by using a Bio Rad protein assay kit (Hercules, CA) with bovine serum albumin as the standard (Bradford 1976).

Statistical analysis

To estimate kinetic parameters for saturable transport, the uptake rate (v) was fitted to equation 1 by means of nonlinear least-squares regression analysis using the MULTI program (Yamaoka et al 1981).

$$V = V_{max} * s/(K_m + s) + k_d * s \quad (1)$$

where v and s are the uptake rate and concentration of substrate, respectively, K_m is the half-saturation concentration (Michaelis constant), V_{max} is the maximum transport rate for saturable process, and k_d is the apparently nonsaturable first-order rate constant. All data were expressed as means ± s.e.m with 3 or 4 independent experiments using isolated hepatocytes and rats, and with 5–10 independent experiments using oocytes. Statistical analysis was performed by using Kruskal–Wallis test, Mann–Whitney test or Student's t-test according to the experiments. The criterion of significance was taken to be $P < 0.05$. Cell-to-medium ratio was obtained by dividing the uptake amount by the concentration of test compound in the uptake medium.

Results

Transport activity of DPDPE and DADLE by OATP-C

To determine the transport activity of OATP-C for peptides, the uptake of [^{3}H]DPDPE via OATP-C and the inhibitory effects of several anionic compounds were examined using Xenopus laevis oocytes (Figure 1). Oocytes injected with cRNA of OATP-C showed increased uptake activity for [^{3}H]DPDPE compared with that by oocytes injected with water alone. Furthermore, the uptake was significantly inhibited by BSP, taurocholate and estrone-3-sulfate, typical substrates of OATP, at the concentration of 10 μM.

To clarify whether OATP-C might participate in the uptake of DADLE into human liver, an uptake study was performed using oocytes expressing OATP-C. The time course of [^{3}H]DADLE uptake by OATP-C is shown in Figure 2A. Significantly increased uptake was observed in OATP-C-expressing oocytes, and the uptake rates evaluated from the slopes of the uptake over 60 min by OATP-C-expressing oocytes and water-injected oocytes were 2.6 ± 1.7 and 0.98 ± 0.88 (mean ± s.e.m.) nL/oocyte/min, respectively, showing significant increase in the rate by OATP-C-expressing oocytes. Moreover, the uptake by OATP-C at 60 min was also significantly inhibited by 10 μM of estrone-3-sulfate to 32.3 ± 10.5% of the control (Figure 2B).

Inhibitory effects of various compounds on estrone-3-sulfate uptake by OATP-C

To more fully characterize the uptake of peptides and peptide mimetics via OATP-C, the inhibitory effects of various compounds on the uptake of [^{3}H]estrone-3-sulfate were examined (Table 1). Pentapeptides, DADLE and leu-enkephalin (YGGFL) significantly reduced the uptake of [^{3}H]estrone-3-sulfate at 0.3 mM, whereas tetrapeptide des-tyrosine DADLE (dAGFdL) exhibited weaker inhibitory potency, showing significant reduction at 1 mM, but not at

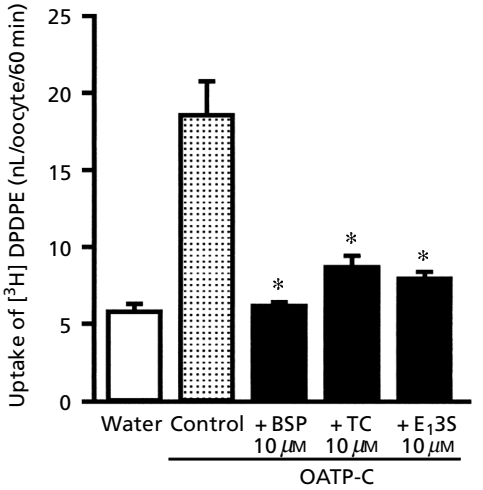

Figure 1 Xenopus laevis oocytes injected with OATP-C cRNA or water (open column) were incubated at 25 °C for 60 min in medium 1 (pH 7.4) containing [^{3}H]DPDPE (20 nM) with (closed column) or without (dotted column) 10 μM BSP, 10 μM taurocholate or 10 μM estrone-3-sulfate ($E_1$3S). Uptake was expressed as cell-to-medium ratio and each column represents the mean ± s.e.m. (n = 5–10). *P < 0.05, compared with control (Student's t-test).

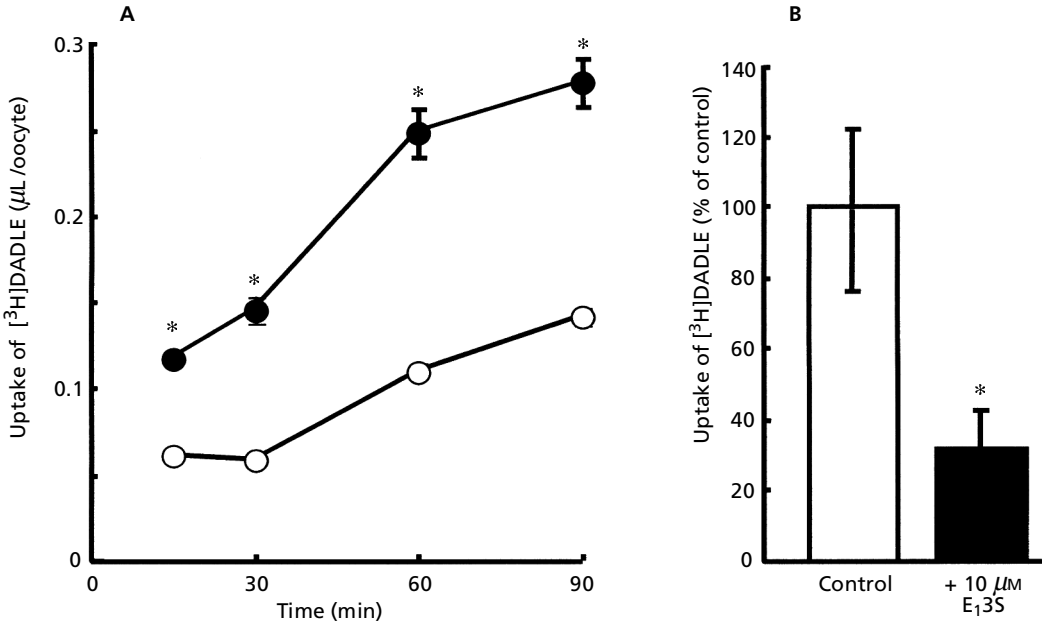

Figure 2 Time course of the uptake of [^{3}H]DADLE by Xenopus laevis oocytes expressing OATP-C (A) and inhibitory effect of estrone-3-sulfate (B). A. Xenopus laevis oocytes injected with OATP-C cRNA (closed circle) or water (open circle) were incubated at 25 °C in medium 1 (pH 7.4) containing [^{3}H]DADLE (0.4 nM). Uptake of [^{3}H]DADLE (20 nM) was measured at 25 °C for 90 min. Uptake was expressed as cell-to-medium ratio. B. Xenopus laevis oocytes were incubated for 60 min in medium 1 containing [^{3}H]DADLE (0.4 nM) with (closed column) or without (open column) 10 μM estrone-3-sulfate. OATP-C-specific uptake was calculated by subtracting uptake by water-injected oocytes from total uptake. Each point represents the mean ± s.e.m. (n = 5–10). When error bars are not shown, they are smaller than symbols. *P < 0.05 compared with uptake by water-injected oocytes (A) or the control (B) (Mann–Whitney test).

0.3 mM. Tripeptides YGG and YdAG, which are metabolites of enkephalin and DADLE, respectively, did not inhibit the uptake of [^{3}H]estrone-3-sulfate at up to 1 mM inhibitor concentration. These results suggest that the inhibitory effect of peptides increases with increase of peptide length. However, tetra- and penta-alanine and tetra- and penta-glycine showed negligible inhibitory effects at 5 mM. On the other hand, CCK-8 and ciclosporin were strongly inhibitory at low concentrations (10–30 μM). To confirm the affinity of OATP-C, various ionic compounds were examined as inhibitors of the uptake of [^{3}H]estrone-3-sulfate by OATP-C. Organic cations, quinidine and tetraethylammonium (TEA), and zwitterionic carnitine at 50 μM or 500 μM had no effect on [^{3}H]estrone-3-sulfate uptake. Among anionic compounds, p-aminohippuric acid (PAH), which is eliminated preferentially via kidney, showed a negligible effect at 500 μM, while BSP, which is eliminated via liver, significantly reduced the uptake of [^{3}H]estrone-3-sulfate to 8% of the control at 50 μM.

Hepatic disposition of DADLE in rats

To examine the hepatic disposition of DADLE, the time course of plasma concentration and the tissue-to-plasma concentration ratio (Kp) were measured after intravenous administration of [^{3}H]DADLE at a dose of 66 pmol kg^{-1} in rats (Figure 3, Table 2). Total radioactivity of [^{3}H]DADLE was rapidly eliminated from systemic circulation with an apparent half-life of 7.2 ± 1.4 min and total body clearance was calculated as 45.3 ± 1.3 mL min^{-1} kg^{-1}. Since the renal and hepatic plasma flow rate in rats is approximately 20 and 30 mL min^{-1} kg^{-1}, respectively, DADLE was eliminated rapidly, depending on the plasma flow rate, via kidney and liver. To evaluate the tissue distribution of DADLE, the apparent tissue-to-plasma concentration ratio (Kp) at 5 min after administration was measured in liver, heart, muscle and kidney (Table 2). The Kp value in liver (6.80) was the highest, followed by kidney (4.38), heart (1.92) and muscle (1.14).

Uptake of DADLE by rat isolated hepatocytes

Since a high accumulation of [^{3}H]DADLE was observed in the liver, transport experiments using isolated hepatocytes from rats were conducted to identify the mechanism of hepatic uptake of [^{3}H]DADLE. To determine the initial linear phase of [^{3}H]DADLE uptake, the time course of uptake of [^{3}H]DADLE was measured (Figure 4A). The uptake in rat isolated hepatocytes increased linearly for over 2 min. We therefore evaluated the transport characteristics in terms of the initial uptake of [^{3}H]DADLE by isolated hepatocytes in 2 min. Figure 4B shows the concentration dependence of the initial uptake of DADLE from 7.2 nM to 1 mM. The apparent uptake was saturable and the kinetic parameters, K_m, V_{max}, and k_d were determined to be 111 ± 53.3 (mean ± s.e.) μM, 0.60 ± 0.28 pmol

Table 1 Inhibitory effects of various compounds on [^{3}H]estrone-3-sulfate uptake by Xenopus laevis oocytes expressing OATP-C.

Inhibitor	Concn (μM)	Relative uptake (% of control)
Control	—	100 ± 13.4
Leu-enkephalin	100	84.9 ± 4.9
(YGGFL)	300	57.2 ± 4.2*
	1000	26.8 ± 2.4*
DADLE	100	78.3 ± 16.1
(YdAGFdL)	300	51.1 ± 11.0*
	1000	35.4 ± 6.8*
des-tyrosine-DADLE	100	100.2 ± 8.0
	300	94.8 ± 8.8
	1000	62.5 ± 4.4*
YGG	100	92.5 ± 12.0
	300	93.0 ± 8.0
	1000	77.2 ± 13.5
YdAG	100	96.8 ± 17.9
	300	100.5 ± 13.3
	1000	90.3 ± 13.3
(Alanine)4	5000	118.7 ± 8.9
(Alanine)5	5000	86.3 ± 7.4
(Glycine)4	5000	127.4 ± 12.9
(Glycine)5	5000	107.6 ± 6.1
CCK-8	10	40.5 ± 3.3*
	30	28.6 ± 3.9*
	100	9.1 ± 1.8*
Ciclosporin	10	6.1 ± 0.6*
Quinidine	500	81.0 ± 16.4
Carnitine	50	100.0 ± 22.1
PAH	500	106.7 ± 5.7
BSP	50	8.0 ± 0.7*

Xenopus laevis oocytes injected with cRNA of OATP-C were cultivated for 3 days and incubated at 25 °C for 30 min in medium 1 (pH 7.4) containing [^{3}H]estrone-3-sulfate (4 nM) with or without (control) inhibitors. OATP-C-specific uptake was calculated by subtracting uptake by water-injected oocytes from total uptake. Uptake was expressed as percent of the control uptake. Each value represents the mean ± s.e.m. (n = 5–10). *$P < 0.05$, compared with control (Student's t-test).

(mg protein)$^{-1}$ min^{-1} and 1.23 ± 0.38 μL (mg protein)$^{-1}$ min^{-1}, respectively. These results indicated the existence of a carrier-mediated transport mechanism for the uptake of DADLE by rat hepatocytes.

Inhibitory effects on DADLE uptake by rat isolated hepatocytes

To identify the transport system involved in hepatic uptake of DADLE in rats, the inhibitory effects of various compounds on [^{3}H]DADLE uptake by rat isolated hepatocytes were examined. Table 3 summarizes the results. A renally eliminated organic anion, PAH, zwitterionic carnitine or cationic TEA at 500 μM or 50 μM did not inhibit the [^{3}H]DADLE uptake. On the other hand, quinidine at 500 μM strongly reduced the uptake of [^{3}H]DADLE to 19% of the control. Estrone-3-sulfate, BSP, taurocholate, cholate and digoxin, typical substrates of organic anion transporting polypeptides (oatp), significantly decreased the uptake of [^{3}H]DADLE. The tetrapeptide des-tyrosine-DADLE (dAGFdL) and tripeptide YdAG, which are metabolites of DADLE, had no effect on [^{3}H]DADLE uptake at 500 μM.

Discussion

OATP-C is an organic anion transporter expressed predominantly in human liver and accepts various compounds as substrates, including metabolites of endogenous compounds and xenobiotics (Hsiang et al 1999; Tamai et al 2000; Cui et al 2001; Kullak-Ublick et al 2001). Previous studies demonstrated that some OATP family members transport peptides and peptide mimetics (Gao et al 2000, Cattori et al 2001), and the peptides are eliminated into bile via a specific transporter in rats (Terasaki et al 1995; Nakamura et al 1996; Chen & Pollack 1997). In this study, we examined whether rapid hepatic elimination of peptides, especially linear peptides, in man was due to, at least in part, OATP-C. Pentapeptide DPDPE was eliminated mainly via the liver, and the involvement of the hepatocyte basolateral uptake transporter has been suggested in rats (Gores et al 1986; Chen & Pollack 1997). In the present transport study, the increased uptake of [^{3}H]DPDPE by OATP-C-expressing oocytes compared with water-injected oocytes was observed (Figure 1), indicating that OATP-C is involved in the hepatic clearance of these peptides in man.

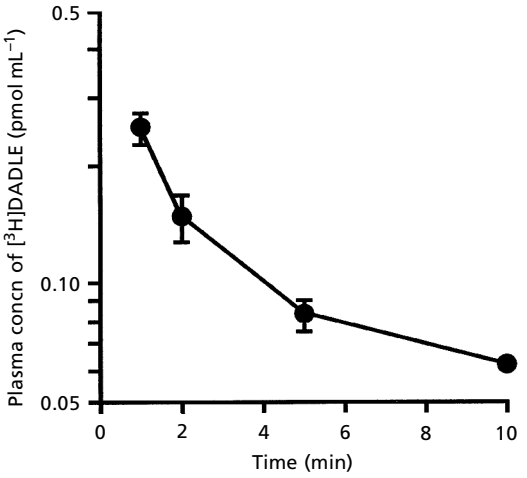

Figure 3 Plasma concentration–time curve of total radioactivity after a single intravenous administration of [^{3}H]DADLE to rats. [^{3}H]DADLE was administered at a dose of 66 pmol kg^{-1} via the femoral vein. Serial blood samples were collected from the jugular vein using a heparinized catheter. Each point represents the mean ± s.e.m. (n = 3). When error bars are not shown, they are smaller than symbols.

Table 2 Tissue-to-plasma concentration ratio (Kp) after intravenous administration of [^{3}H]DADLE to rats.

	Tissue-to-plasma concn ratio
Heart	1.92 ± 0.50
Muscle	1.14 ± 0.08
Kidney	4.38 ± 0.27
Liver	6.80 ± 0.99

The rats were sacrificed by decapitation at 5 min following a single intravenous injection of [^{3}H]DADLE (66 pmol kg^{-1}). The tissues were quickly isolated, rinsed well with ice-cold saline and weighed, and the associated radioactivity was measured. Each value represents the mean ± s.e.m. (n = 3).

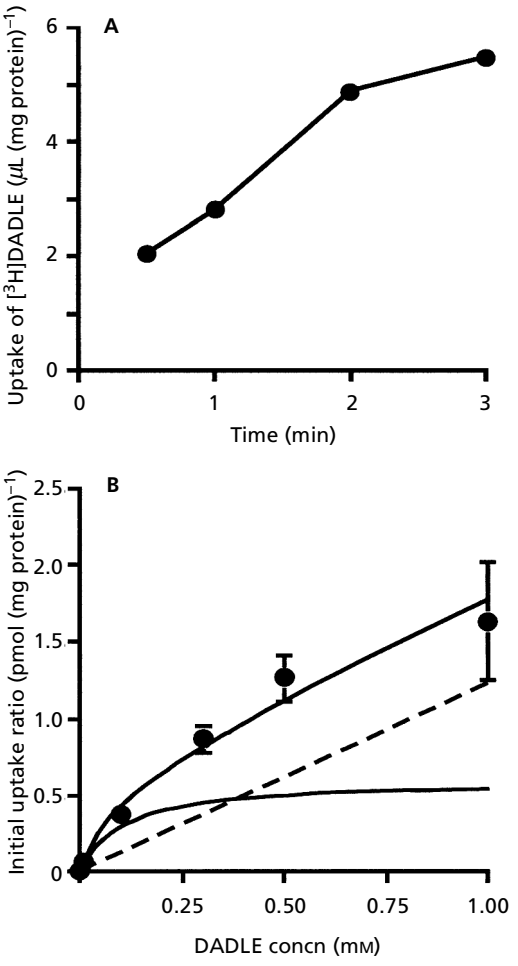

Figure 4 Time course of uptake of [^{3}H]DADLE (A) and concentration dependence of [^{3}H]DADLE uptake (B) by rat isolated hepatocytes. Isolated hepatocytes were preincubated at 37 °C for 10 min. The uptake of [^{3}H]DADLE (7.2 nM) was measured at 37 °C in medium 2 (pH 7.4) for 3 min (A). The results are net values after subtraction of extracellular space determined from the apparent uptake of [^{3}H]inulin at each time. Initial uptake of [^{3}H]DADLE was calculated from the slope of the uptake in 2 min at various concentrations of DADLE from 7.2 nM to 1 mM (B). The upper solid line represents the overall uptake rate, and the lower solid and dashed lines are the saturable and nonsaturable components of the uptake process, calculated from the kinetic parameters described in the text. Each point represents the mean ± s.e.m. (n = 3 or 4). When error bars are not shown, they are smaller than symbols.

Although it is clear that OATP/oatp can transport certain peptides, the mechanism and characteristics of hepatic extraction of linear peptides are not fully understood. Therefore, we focused on a linear pentapeptide, DADLE, to clarify whether OATP-C participates in the hepatic uptake of peptides more generally. DADLE is modified to be metabolically stable (Schulteis et al 1989), but remains similar to the native peptide in structure, in contrast to DPDPE, which is a cyclic molecule. Interestingly, significantly increased uptake of [^{3}H]DADLE in OATP-C-expressing oocytes was observed, which is the first demonstration that OATP-C can transport a linear pentapeptide (Figure 2). To further characterize the uptake process of peptides via OATP-C, we next examined the inhibitory effects of various compounds on the uptake of [^{3}H]estrone-3-sulfate. It is interesting that pentapeptides, leu-enkephalin and DADLE, significantly inhibited the uptake of [^{3}H]estrone-3-sulfate in a dose-dependent manner, whereas tetra- or tri-peptides were less effective inhibitors (Table 1). However, OATP-C was unable to recognize penta- or tetra-peptides consisting of relatively small amino acids, such as alanine or glycine. Moreover, OATP-C-mediated uptake was inhibited by large peptides, CCK-8 and ciclosporin, at low concentration. Since, OATP-C generally transports anionic compounds (in particular, comparatively bulky ones), the inhibitory effects of CCK-8 might be due to it being anionic or bulky as well as being a peptide. In contrast, penta- or tetra-peptides consisting of alanine or glycine

Table 3 Inhibitory effects of various compounds on [^{3}H]DADLE uptake by rat isolated hepatocytes.

Inhibitor	Concn (μM)	Relative uptake (% of control)
Control	—	100 ± 7.80
Estrone-3-sulfate	50	70.1 ± 5.87*
BSP	50	65.8 ± 6.81*
Taurocholate	50	31.3 ± 3.97*
Cholate	50	47.0 ± 14.1*
Digoxin	50	56.6 ± 6.50*
DADLE	500	28.0 ± 2.17*
des-tyrosine-DADLE	500	98.3 ± 4.85
YdAG	500	91.8 ± 4.53
PAH	500	106 ± 14.8
TEA	500	93.4 ± 9.12
Quinidine	500	19.3 ± 2.35*
Carnitine	50	97.6 ± 10.4

Isolated hepatocytes from rats were pre-incubated at 37 °C for 10 min. Uptake of [^{3}H]DADLE (7.2 nM) was measured at 37 °C in medium 2 (pH 7.4). The uptake was calculated from the slope of the uptake for 2 min with or without (control) inhibitors. Uptake was expressed as percent of the control uptake. Each value represents the mean ± s.e.m. (n = 3 or 4). *$P < 0.05$ compared with control (Kruskal–Wallis test).

might be too small to be recognized by OATP-C. Thus, the affinity for OATP-C might be restricted by many factors, such as molecular size, anionic property and peptide structure of substrates, and further study will be required to identify the substrate selectivity of OATP-C. Cholecystokinin is also known to be rapidly extracted by liver in the forms of octapeptide CCK-8 or tetrapeptide CCK-4 (Gores et al 1986), and Ismair et al (2001) showed that rat oatp4 and human OATP8, not OATP-C, can significantly transport CCK-8. This study showed the possibility of interaction of OATP-C with CCK-8 or CCK-4 even if they were not good substrates of OATP-C. The OATP/oatp family transports comparatively bulky compounds such as taurocholate, steroid-hormone conjugates, bilirubin and so on (Cattori et al 2001; Kullak-Ublick et al 2001). The result that OATP-C-mediated uptake was inhibited by comparatively bulky peptides is inconsistent with previous findings (Abe et al 1999; Kullak-Ublick et al 2001; Tamai et al 2001).

To evaluate the significance of OATP-C-mediated uptake of peptides, we examined the disposition of DADLE in rats to examine whether hepatic uptake of DADLE could be mediated by a transporter(s). The half-life of total radioactivity in plasma after intravenous administration of [^{3}H]DADLE was very short at approximately 7 min, and total body clearance was close to the plasma flow rate (Figure 3). Furthermore, the apparent Kp value determined for liver indicated that administered [^{3}H]DADLE was sufficiently extracted by the liver (Table 2). The result was similar to those for DPDPE and other peptides (Gores et al 1986; Terasaki et al 1995; Chen & Pollack 1997), and indicated that the hepatic uptake mechanism of DADLE does involve some transporters. The initial uptake rate of [^{3}H]DADLE into isolated rat hepatocytes exhibited saturation with a K_m value of 111 ± 53.3 μM (Figure 4). The K_m values of DPDPE for rat oatp1 and oatp2 were 48 μM and 19 μM, respectively, and that of deltorphin II for oatp1 was 137 μM (Gao et al 2000). Because the K_m of DADLE is in the range of these K_m values, some of oatp might contribute to the hepatic uptake of DADLE in rats.

Several peptides, octreotide and BQ123 are taken up by rat hepatocytes via a carrier-mediated system that is inhibited by bile acids and organic anions (Terasaki et al 1995; Nakamura et al 1996). To determine the characteristics of DADLE uptake by isolated rat hepatocytes, inhibition experiments were performed. As shown in Table 3, organic anions, estrone-3-sulfate, BSP, taurocholate, cholate and digoxin, which are substrates of the oatp family (Cattori et al 2001), inhibited [^{3}H]DADLE uptake significantly at 50 μM. As regards peptides, the metabolites of DADLE, the tetrapeptide des-tyrosine-DADLE (dAGFdL) and the tripeptide YdAG, did not inhibit the uptake of [^{3}H]DADLE at 500 μM, whereas DADLE itself significantly inhibited the uptake (Table 3). These characteristics are similar to those of the transport of peptides by OATP-C. Therefore, a common transport system for peptides seems to exist in rats and man. However, quinidine strongly inhibited the DADLE uptake by rat hepatocytes at 500 μM, whereas the inhibitory effect of quinidine on the uptake of [^{3}H]estrone-3-sulfate by OATP-C-expressing oocytes was weak and statistically insignificant at the same concentration (Tables 1 and 3). This observation may indicate that relative contribution of OATP-C in hepatic elimination is not significant. However, it is also true that there is species difference in OATP/oatp transporters between man and rats. Accordingly, at present it is not easy to quantitatively evaluate the role of OATP-C in the hepatic elimination of DADLE.

In conclusion, we have demonstrated that OATP-C expressed at the basolateral membrane of human liver transports biologically active linear peptides, DADLE. The current and recent observations by Kullak-Ublick et al (2001), that deltorphin II, DPDPE and BQ123 were transported by OATP-C and OATP8, suggest that rapid hepatic clearance of peptides could be ascribed to the hepatic organic anion

transporter OATP-C, as well as OATP8 in man. These lines of studies will be useful for the development of peptide-like drugs that are effective for clinical use.

References

Abe, T., Kakyo, M., Tokui, T., Nakagomi, R., Nishio, T., Nakai, D., Nomura, H., Unno, M., Suzuki, M., Naitoh, T., Matsuno, S., Yawo, H. (1999) Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. J. Biol. Chem. 274: 17159–17163

Bertrams, A., Ziegler, K. (1991) Hepatocellular uptake of peptides by bile acid transporters: relationship of carrier-mediated transport of linear peptides with rennin-inhibiting activity to multispecific bile acid carriers. Biochim. Biophys. Acta 1091: 337–348

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Cattori, V., van Montfoort, J. E., Stieger, B., Landmann, L., Meijer, D. K., Winterhalter, K. H., Meier, P. J., Hagenbuch, B. (2001) Localization of organic anion transporting polypeptide 4 (Oatp4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2 and Oatp3. Pflugers. Arch. 443: 188–195

Chen, C., Pollack, G. M. (1997) Extensive biliary excretion of the model opioid peptide [D-PEN^{2,5}]enkephalin in rats. Pharm. Res. 14: 345–350

Cui, Y., König, J., Leier, I., Buchholz, U., Keppler, D. (2001) Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. J. Biol. Chem. 276: 9626–9630

Cumin, F., Schnell, C., Richert, P., Raschdorf, F., Probst, A., Schmid, K., Wood, J. M., de Gasparo, M. (1990) Pharmacokinetics and tissue distribution of the renin inhibitor N-(2-(R)-benzyl-3-tert-butyl-sulfonyl -propionyl)-His-ChacVal-n-butylamine in marmosets. Drug Metab. Dispos. 18: 831–835

Gao, B., Hagenbuch, B., Kullak-Ublick, G. A., Benke, D., Aguzzi, A., Meier, P. J. (2000) Organic anion transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. J. Pharmacol. Exp. Ther. 294: 73–79

Gores, G. J., LaRusso, N. F., Miller, L. J. (1986) Hepatic processing of cholecystokinin peptides. I. Structural specificity and mechanism of hepatic extraction. Am. J. Physiol. 250: G344–G349

Greenfield, J. C., Cook, K. J., O'Leary, I. A. (1989) Disposition, metabolism, and excretion of U-71038, a novel rennin inhibitor peptide, in the rat. Drug. Metab. Dispos. 17: 518–525

Hagenbuch, B., Meier, P. J. (1994) Molecular cloning, chromosomal localization, and functional characterization of a human liver Na^+/bile acid cotransporter. J. Clin. Invest. 93: 1326–1331

Hsiang, B., Zhu, Y., Wang, Z., Wu, Y., Sasseville, V., Yang, W. P., Kirchgessner, T. G. (1999) A novel human hepatic organic anion transporting polypeptide (OATP-2). J. Biol. Chem. 274: 37161–37168

Ismair, M. G., Stieger, B., Cattori, V., Hagenbuch, B., Fried, M., Meier, P. J., Kullak-Ublick, G. A. (2001) Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides oatp4 and oatp8 of rat and human liver. Gastroenterology 121: 1185–1190

Kim, R. B., Perry, P. R., Wilkinson, G. R. (1997) High affinity uptake by isolated rat hepatocytes of a linear pseudo-hexapeptide, ditekiren. Biochim. Biophys. Acta 1328: 41–47

König, J., Nies, A.T., Cui, Y., Leier, I., Keppler, D. (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim. Biophys. Acta 1461: 377–394

König, J., Cui, Y., Nies, A. T., Keppler, D. (2000a) A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. Am. J. Physiol. 278: G156–G164

König, J., Cui, Y., Nies, A. T., Keppler, D. (2000b) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. J. Biol. Chem. 275: 23161–23168

Kullak-Ublick, G. A., Hagenbuch, B., Stieger, B., Schteingart, C. D., Hofmann, A. F., Wolkoff, A. W., Meier, P. J. (1995) Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. Gastroenterology 109: 1274–1282

Kullak-Ublick, G. A., Ismair, M. G., Stieger, B., Landmann, L., Huber, R., Pizzagalli, F., Fattinger, K., Meier, P. J., Hagenbuch, B. (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology 120: 525–533

Moldeus, P., Hogberg, J., Orrenius, S. (1978) Isolation and use of liver cells. Methods Enzymol. 52: 60–71

Nakamura, T., Hisaka, A., Sawasaki, Y., Suzuki, Y., Fukami, T., Ishikawa, K., Yano, M. Sugiyama, Y. (1996) Carrier-mediated active transport of BQ-123, a peptidic endothelin antagonist, into rat hepatocytes. J. Pharmacol. Exp. Ther. 278: 564–572

Schulteis, G., Weinberger, S. B., Martinez Jr., J. L. (1989) Plasma uptake and in vivo metabolism of [Leu]enkephalin following its intraperitoneal administration to rats. Peptides 10: 913–919

Strautnieks, S., Bull, L., Knisely, A., Kocoshis, S., Dahl, N., Arnell, H., Sokal, E., Dahan, K., Childs, S., Ling, V., Tanner, M., Kagalwalla, A., Nemeth, A., Pawlowska, J., Baker, A., Mieli-Vergani, G., Freimer, N., Gardiner, R., Thompson, R. (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. Nat. Genet. 20: 233–238

Tamai, I., Nezu, J.-I., Uchino, H., Sai, Y., Oku, A., Shimane, M., Tsuji, A. (2000) Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochem. Biophys. Res. Commun. 273: 251–260

Tamai, I., Nozawa, T., Koshida, M., Nezu, J., Sai, Y., Tsuji, A. (2001) Functional characterization of human organic anion transporting polypeptide OATP-B in comparison with liver-specific OATP-C. Pharm. Res. 18: 1262–1269

Terasaki, T., Mizuguchi, H., Itoho, C., Tamai, I., Lemaire, M., Tsuji, A. (1995) Hepatic uptake of octreotide, a long-acting somatostatin analogue, via a bile acid transport system. Pharm. Res. 12: 12–17

Uchino, H., Tamai, I., Yabuuchi, H., China, K., Miyamoto, K., Takeda, E., Tsuji, A. (2000) Faropenem transport across the renal epithelial luminal membrane via inorganic phosphate transporter Npt1. Antimicrob. Agents Chemother. 44: 574–577

Yamada, T., Ninuma, K., Lemaire, M., Terasaki, T., Sugiyama, Y. (1997) Carrier-mediated hepatic uptake of the cationic cyclopeptide, octreotide, in rats. Drug Metab. Dispos. 25: 536–543

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T. (1981) A pharmacokinetic analysis program (multi) for microcomputer. J. Pharmacobiodyn. 4: 879–885