Evaluation of the Uptake of Pravastatin by Perfused Rat Liver and Primary Cultured Rat Hepatocytes

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Purpose. We have already demonstrated that the HMG-CoA reductase inhibitor, pravastatin is actively taken up by isolated rat hepatocytes via a multispecific anion transporter (Yamazaki et al., Am. J. Physiol. 264, G36-44, (1993)). We further attempted the quantitative evaluation of this uptake in different experimental systems.

Methods. We have quantified the initial uptake of pravastatin by both primary cultured hepatocytes and by isolated perfused rat liver using the multiple indicator dilution (MID) method.

Results. The permeability surface area product for the influx (PSinf) of pravastatin evaluated in MID study was comparable with those reported previously in isolated rat hepatocytes and *in vivo*. Furthermore, the highly concentrative uptake (influx clearance >>efflux clearance) of pravastatin was confirmed by kinetic analysis of the dilution curves obtained in the MID study. On the other hand, the uptake by primary cultured cells was significantly lower than that by isolated cells, and the ability of hepatocytes to take up pravastatin showed a decrease with time in culture (0-96 hr). The Vmax for uptake diminished with increasing time in culture, while no significant change was observed in both Km and nonspecific diffusion clearance.

Conclusions. The MID method in isolated perfused liver which maintains the spatial and anatomical architecture can be used to quantitatively evaluate the initial uptake of pravastatin. Furthermore, the ability of hepatocytes to take up pravastatin is diminished in culture with time and this is caused by a decrease in Vmax.

KEY WORDS: multiple indicator dilution method; primary cultured hepatocytes; carrier-mediated uptake; active transport; HMG-CoA reductase inhibitor.

INTRODUCTION

We have demonstrated using freshly isolated rat hepatocytes that pravastatin is actively taken up by the liver via a Na^+-independent multispecific anion transporter (1). Furthermore, the hepatic permeability surface area product for hepatic uptake (influx) *in vivo* (PSinf, in vivo) obtained with intact rats by integration plot analysis was comparable with that *in vitro* (PSinf, cell), indicating that the carrier-mediated active transport system which we demonstrated *in vitro* is responsible for the hepatic uptake *in vivo* (1). In the present study, we compared quantitatively the hepatic uptake clearance of pravastatin estimated in multiple indicator dilution (MID) study (PSinf, MID) with PSinf, cell. If these two values are close, the initial uptake ability for pravastatin will exhibit good agreement among 3 different kinds of experimental systems (*in vivo,* perfused liver, *in vitro* freshly isolated cells). No study has been reported in which the systematic and quantitative comparison of the initial uptake ability for one ligand has been made among different kinds of experimental systems.

We have also previously examined pravastatin uptake by rat primary cultured (24h) cells (2). This also showed temperature-dependent, concentrative and active transport. However, comparison of the kinetic parameters obtained with both freshly isolated and primary cultured rat hepatocytes revealed that Km values were comparable, while the Vmax value for primary cultured hepatocytes was one-order of magnitude smaller than that for freshly isolated cells (1, 2). The possible reason for this apparent discrepancy may be the difference in the methods used for evaluating the initial uptake velocity, and/or in the intrinsic ability for the initial uptake of pravastatin between the two *in vitro* experimental systems. In the present study, the cellular uptake of pravastatin was compared between freshly isolated and primary cultured hepatocytes over the wide range of incubation times, evaluating the initial uptake velocity in its strict sense.

MATERIALS AND METHODS

Chemicals

[^{14}C] Pravastatin (10 mCi/mmol, purity 93.2 % checked by TLC analysis (see below)) and unlabeled pravastatin were obtained from Sankyo, Co., Ltd. [^{125}I]Bovine serum albumin (BSA, 3.5 μCi/μg) were purchased from New England Nuclear (Boston, MA). Collagenase, streptomycin and penicillin were purchased from Boehringer-Mannheim GmbH (Germany). Calf serum, fetal bovine serum and other culture media were from Gibco (New York, U.S.A.). All other chemicals were of reagent grade.

Liver Perfusion Study (Multiple Indicator Dilution Study)

Male Sprague-Dawley rats (300 g BW) were lightly anesthetized with ether. Perfusions were performed according to the method established in our laboratory (3-6). A single-pass liver perfusion was performed at 37°C, with a flow rate of 100 ml/min/kg BW. The perfusate consisted of BSA (3 % w/v) in Krebs-Ringer-bicarbonate buffer (pH 7.4) (erythrocyte-free). After a stabilization period of 20 min, 200 μl of fluid was injected into the portal vein as a bolus. This contained [^{125}I] BSA (0.1 μCi), an extracellular marker, and [^{14}C] pravastatin (2 μCi) and the hepatic venous outflow was collected at 1 sec intervals for 20 sec, then at 10 sec intervals for 60 sec. To compare [^{125}I] BSA and [^{14}C] pravastatin, the outflow radioactivity of each sample was normalized by dividing it by the injected radioactivity. Concentrations in the effluent were thus expressed as outflow fractions of the dose per ml.

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Analysis of the MID Curves

The data were analyzed in both a model-dependent (7) and -independent fashion (8). The relationship between the outflow fractions of [^{14}C] pravastatin and [^{125}I] BSA (Cs(t') and Cref(t'), respectively) was expressed as the natural logarithm of the ratio (Cref(t')/Cs(t')) vs. corrected time, where t' is the elapsed time, t minus the large vessel transit time, t_0, and an estimate of the t_0 value was obtained as reported previously (3, 4). The initial slope of this plot (designated as "ratio plot") calculated by linear regression analysis reflects the influx rate constant (K1) (9). Using this value as the initial estimate, we tried to calculate three kinetic parameters (K1, K2 (efflux rate constant) and K3 (sequential rate constant)) by fitting the Cref(t') and Cs(t') to the distributed model (7) using the iterative nonlinear least squares method. The details of the fitting have been reported previously (4). However, the reliable parameter we could obtain by fitting was only K1. We did not show the calculated values of K2 and K3, since they had large coefficients of variation, which can be easily predicted from the straight "ratio plot". Next, we calculated the influx clearance (PSinf), using the following equation:

$$PSinf = K1 \cdot Vext \quad (1)$$

where Vext is the volume accessible to the extracellular reference during its passage through the liver, which can be estimated by multiplying the flow rate by the mean transit time of [^{125}I] BSA (4). We also determined the hepatic availability (F) by a model-independent method as previously reported (4).

Cell Isolation and Culture

Hepatocytes were isolated from male Wistar rats (250 g BW) by the procedure of Moldeus et al (10). Cell viability was routinely checked (>90 %) by the trypan blue exclusion test. After preparation, freshly isolated cells were suspended in Williams' medium E (WE medium). Cell culture was performed as described previously (2). Transport experiments were done in cultured cells after an interval of 6, 24, 48, and 96 hr.

Cellular Uptake Experiments

Freshly Isolated Cells. Uptake of ligands was initiated by adding the ligand to the preincubated (37°C for 5 min) cell suspension (3×10^6 cells/ml). At designated times, the reaction was terminated by separating the cells from the medium using a centrifugal filtration technique (1). The details of the uptake experiments have been described previously (1).

Primary Cultured Cells. Uptake was initiated by adding the ligand to the medium after the culture dishes had been washed 3 times and pre-incubated with WE medium for 5 min at 37°C. The details of the uptake experiments have been described previously (2).

Determination of Protein Concentration and Radioactivity

Protein concentration was determined by the method of Lowry et al. (11) with bovine serum albumin as a standard. [^{14}C] Radioactivities were measured by liquid scintillation counting (model 2250A, Packard Instruments Corp., Downers Grove, IL, U.S.A.) and [^{125}I] radioactivities determined by gamma counting (model ARC-300, Aloka, Tokyo, Japan).

Determination of Kinetic Parameters

The initial uptake velocity (v_0) of pravastatin was calculated by carrying out a linear regression on the data obtained at 20, 40 and 60 sec. The kinetic parameters for pravastatin were estimated according to the following equation:

$$v_0 = Vmax \cdot S/(Km + S) + Pdif \cdot S \quad (2)$$

where Vmax is the maximum uptake velocity (pmol/min/mg), Km is the Michaelis constant (μM), Pdif is the nonspecific uptake clearance (μl/min/mg) and S is the pravastatin concentration in medium (μM). The above equation was fitted to the uptake data by an iterative nonlinear least-squares method using the MULTI program (12) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values, and the Damping Gauss Newton algorithm was used for the fitting (12).

Estimation of PSu,inf in ml/min/g Liver from PSu,inf in μl/min/mg Protein

Based on the kinetic parameters (Km, Vmax and Pdif) obtained by the described fitting procedure, the PSu,inf (ml/min/g liver) was calculated according to Ref. (13).

RESULTS

MID Study

Figure 1 (a) shows the outflow dilution curve for pravastatin in the MID study. According to the model-independent analysis (8), the hepatic extraction ratio was calculated to be 0.55, indicating that approx. half of the molecules were removed in a single-pass through the liver. The influx rate constant (K1), represented by the initial slope of the ratio plot (Fig. 1 (b)) was 0.072 sec^{-1}. With the value of Vext (0.29 ml/g liver) estimated from the moment analysis of the dilution curve of [^{125}I] BSA, PSinf,MID (for total ligand) was calculated to be 1.2 ml/min/g liver. Taking the unbound fraction of pravastatin (0.58) in the perfusate (3 % BSA) (1) into consideration, the value for PSu,inf,MID obtained was 2.1 ml/min/g liver.

As described in the "Materials and Methods", reliable K2 and K3 values could not be obtained. The difficulty in estimating K2 and K3 values can also be seen in the straight ratio plot in Fig. 1 (b).

Uptake of Pravastatin by Primary Cultured (24 h) Cells

The time course of pravastatin uptake by primary cultured (24 h) cells is shown in Fig. 2. The uptake increased in a linear fashion for 2 min, thus the initial uptake velocity was calculated from the data taken at 20, 40 and 60 sec. As a result, the inital uptake velocity from the data in Fig. 2 was calculated to be 75.8 ± 2.38 pmol/min/mg.

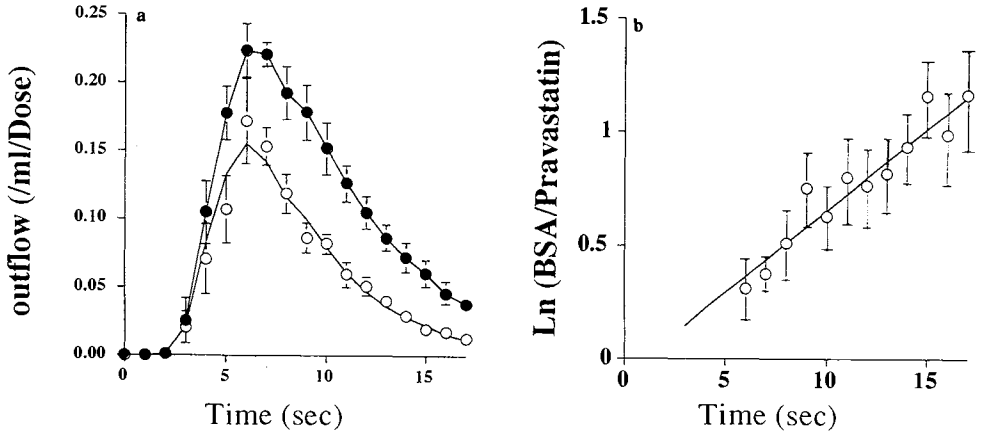

Fig. 1. Multiple indicator dilution analysis of Pravastatin. Each point and vertical bar represents the mean ± S.E. of 3 different experiments. The solid lines were obtained by fitting the data to the equation based on the distributed model (7). (a) Dose normalized venous outflow dilution curves. Ordinate represents the outflow fraction per milliliter. ○:^{14}C-Pravastatin, ●:^{125}I-BSA. (b) Plot of the natural logarithm of the ratio (^{125}I-BSA outflow fraction per milliliter/^{14}C-Pravastatin outflow fraction per milliliter) *vs* time (Ratio plot).

Effect of the Time in Culture on the Uptake of Pravastatin

The initial uptake velocity of pravastatin decreased with increasing time in culture (Fig. 3). In fact, the initial uptake velocity had already declined after only 6 h in culture. The saturability in terms of pravastatin concentration also changed with culturing time (Fig. 3). Determination of the apparent kinetic parameters of uptake showed that Vmax decreased with the time in culture, while Km (approx. 30 μM) was relatively stable (Table I). Table I represents also the effect of time in the culture on the carrier-mediated (Vmax/Km) and total (PSuinf = Vmax/Km + Pdif) uptake, respectively. Vmax/Km decreased significantly with time, while Pdif was maintained relatively stable during 96 h culture period.

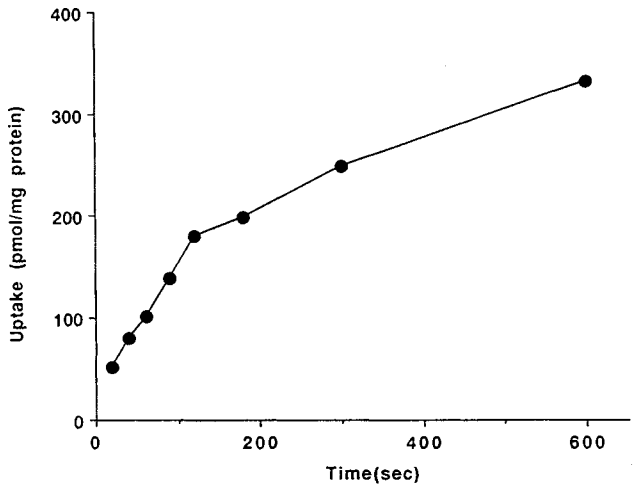

Fig. 2. Time course of [^{14}C] pravastatin uptake by primary cultured (24 hr) rat hepatocytes. Each point represents the mean value of 3 different cell preparations. The S.E. value of each point was less than 3 % of the mean value.

DISCUSSION

The MID method has the advantage of estimating the membrane permeability of a ligand in the liver, since it allows the determination of the influx, efflux, and sequestration processes of a ligand separately, under almost *in vivo* conditions (3-7). In addition, we have previously reported that the uptake clearance (PSinf,cell) obtained with isolated hepatocytes is similar to that (PSinf,MID) determined by the MID method for ligands with relatively low uptake clearance (6). Furthermore, for ligands with larger uptake clearances, the PSinf, MID values are lower than the PSinf,cell values and appear to reach an upper limit (approx. 15-20 ml/min/g liver) (6). We consider that one of the possible mechanisms for this phenomenon is that an unstirred water layer, which may exist in Disse's space in isolated perfused livers, limits the hepatic uptake velocity of ligands with extremely high membrane permeabilities (6). Based on the previous study using isolated hepatocytes (1), the PSu,inf,cell for unbound pravastatin was 2.6 ml/min/g liver. This value is much lower than the above upper limit (15-20 ml/min/g liver), and indicates *a priori* a good agreement between the isolated cell and MID systems (6). In fact, the PSu,inf,MID (2.1 ml/min/g liver) estimated in the present study is similar to PSu, inf,cells in both the previous (2.6 ml/min/g liver, (1)) and present studies (2.9 ml/min/g liver) (Table II). In addition, PSu,inf,cell is comparable with that *in vivo* (Table II) (1), revealing that the hepatic uptake abilities for pravastatin were comparable among 3 different experimental systems (*in vivo*, perfused liver, *in vitro* freshly isolated cells). Such a quantitative and systematic study to compare the initial uptake ability for one ligand among different experimental systems has never been reported before. The present results suggest that an uptake experiment with freshly isolated cells is useful for estimating the uptake clearance even for the ligands such as pravastatin, where the uptake is carrier-mediated and the uptake clearance value is relatively high (comparable with the hepatic blood flow).

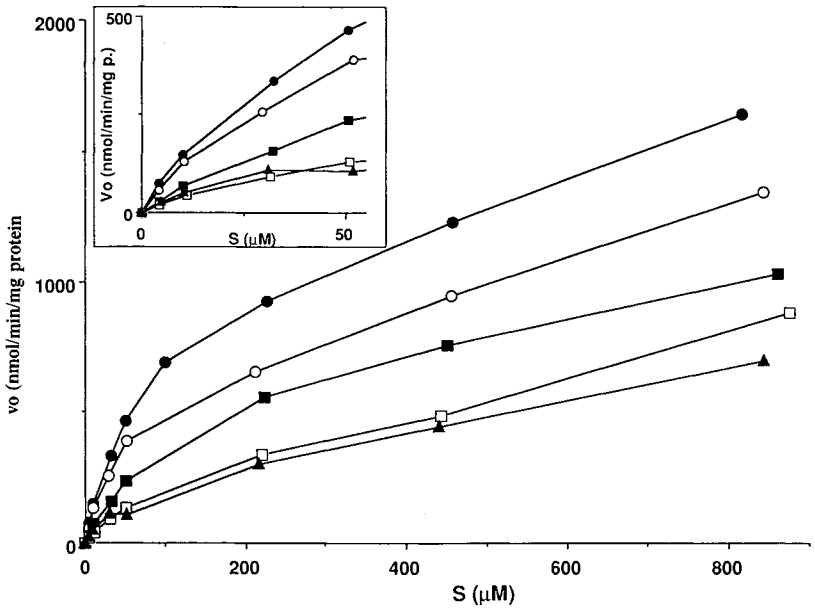

Fig. 3. Uptake of [^{14}C] pravastatin by rat hepatocytes in freshly isolated or primary culture for 6 - 96 h. Initial uptake velocity (v_o) plotted against pravastatin concentration (S). Each point represents the mean value of 2-3 different cell preparations. ●:0 h (freshly isolated hepatocytes), ○: 6 h culture, ■: 24 h culture, □:48 h culture, ▲: 96 h culture.

The value for the C/M ratio (16.3) at steady-state in the uptake study using isolated cells (1) indicates PSu,inf>>ft • PSu,eff, where ft is the unbound fraction in hepatocytes. Since the tissue binding of pravastatin is not very high (ft > 0.5, Yamazaki et al., submitted to Biopharmaceutics & Drug Disposition, the concentrative uptake (PSu,inf>>PSu,eff) should occur in isolated cells. Such vector transport of pravastatin is maintained by active transport (1). Unfortunately, we could not obtain a reliable value for PSeff in the MID study (See details in "Results"), but the concentrative vector uptake is clearly indicated by the ratio plot shown in Fig. 1 (b), which reveals a steady increase (no evidence of decline) with time, showing that virtually vector uptake (PSu,inf>>PSu,eff) occurs also in the liver-perfusion system.

The time course of uptake by primary cultured cells (24 h) reveals that the cellular accumulation increased linearly with time up to 2 min (Fig. 2). The initial uptake velocity estimated from the 20, 40, 60 sec data in the present study was 1.7 times the previously reported value (2). In our previous report, we evaluated the initial uptake velocity from the data at 10 min, and this is the most plausible explanation for the apparent discrepancy between the present and previous studies with regard to the uptake velocity of the primary cultured cells at the same culturing time (24 h). However, the initial uptake velocity in primary cultured cells determined in this strict sense was still 36 % of freshly isolated cells (Table I). To examine the reason for this difference, we evaluated the initial uptakes at different times in culture (6-96 h). As a result, the time-dependent decrease in the initial uptake velocity was clearly visible (Fig. 3). Even after 6 h of culture, at which time the cultured cells showed the greatest uptake ability among the 4 different times in culture, the absolute value was 70 % of freshly isolated cells, suggesting that during the culture, hepatocytes lose their ability to take up pravastatin. Furthermore, the carrier-mediated uptake clearance, reflected by Vmax/Km, showed a significant decrease with time in culture, while the non-specific diffusion clearance (Pdif) was relatively stable for 96 h (Table I). This suggests that the decrease in pravastatin uptake with time in

Table I. Effect of Time in Culture on the Uptake Clearance for Pravastatin by Rat Hepatocytes

Time in culture (h)	Km[a] (μM)	Vmax[a] (pmol/min/mg)	Vmax/Km (μl/min/mg protein)	Pdif[a] (μl/min/mg protein)	PSu,inf (Vmax/Km + Pdif) (μl/min/mg protein)
0	36.5 ± 12.6	816.2 ± 237.9	21.4	1.6 ± 1.4	23.0
6	39.7 ± 9.1	589.1 ± 92.2	14.9	1.3 ± 0.4	16.2
24	49.9 ± 15.7	354.1 ± 98.1	7.1	1.2 ± 0.3	8.3
48	39.2 ± 13.4	157.6 ± 43.7	4.0	1.0 ± 0.2	5.0
96	27.6 ± 13.6	140.1 ± 53.6	5.0	0.8 ± 0.3	5.8

[a] Each parameter was obtained by fitting the data shown in Fig. 3 to Eq. 2 as described in "Materials and Methods" (mean ± calculated S.D.).

Table II. Comparison of the Permeability-Surface Area Product (PSu,inf) for the Uptake of Unbound Pravastatin Determined in Four Experimental Systems (*in Vivo*, Perfused Liver, Freshly Isolated Cells and Primary Cultured Cells)

				Primary cultured cells	
	in Vivo	Perfused liver	Isolated cells	6 h	24 h
ml/min/g liver	1.1[a] – 1.9[b]	2.1[d]	2.6[c], 2.9[d]	2.0[d]	1.0[d]
μl/min/mg protein	—	—	20.4[c], 23.0[d]	16.2[d]	8.3[d]

PSu,inf, in ml/min/g liver for isolated cells and primary culture were calculated from those values in μl/min/mg protein according to Ref. 13.
[a] Ref. (1). Calculated using the sinusoidal perfusion model (16).
[b] Ref. (1). Calculated using the venous equilibrium model (17).
[c] Ref. (1).
[d] Present study.

culture is caused mainly by the reduction in active transport ability. Comparison of the change with time between Km and Vmax demonstrated a significant decrease in Vmax, with no corresponding change in Km (Table I). This strongly suggests that the decrease with time in culture may be due to the change in density, rather than the affinity, of the carrier-protein. To examine the possibility that the decrease in pravastatin uptake was due to the reduction in cell viability during cell culture, we also evaluated the uptake of other ligands at the same time in culture. The ligands we used were the analog of glucose, 3-O-methylglucose, which is taken up by facilitated diffusion (14), and the amino acid analog, alpha-aminoisobutyric acid, which is taken up by several carrier-mediated systems (A, ASC, and L (15)). There were no clear time-dependent changes in both uptakes during 96 h of cell culture. Based on this result, the decrease in pravastatin uptake with time in culture may not be due to a reduction in cell viability. Although the exact reason for the decrease in Vmax for pravastatin with culturing time is unknown, our results clearly indicate that careful interpretation of uptake data obtained with primary cultured cells should be required.

In conclusion, comparable values for pravastatin hepatic uptake clearance were obtained *in vivo*, perfused liver and *in vitro* freshly isolated cells. However, the initial uptake was less in primary cultured cells compared with freshly isolated cells, and the value significantly decreased during culture, possibly due to a reduction in the number of transporters. Freshly isolated hepatocytes rather than primary cultured hepatocytes are more suitable for evaluating the uptake of ligands by multispecific anion transport systems.

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