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CARRIER-MEDIATED UPTAKE OF PRAVASTATIN BY RAT HEPATOCYTES IN PRIMARY CULTURE

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Abstract—The transport mechanism of pravastatin, a new cholesterol-lowering drug, was compared *in vitro* with rat hepatocyte primary culture and mouse skin fibroblasts (L-cells). The uptake of ^{14}C-labeled pravastatin by cultured hepatocytes was temperature- and dose-dependent. The temperature-dependent uptake as a function of [^{14}C]pravastatin concentration showed saturation kinetics with $K_m = 32.2\ \mu M$ and a maximal uptake rate of 68 pmol/mg protein/min. The uptake of pravastatin was inhibited significantly by metabolic inhibitors such as rotenone, oligomycin A, antimycin A, 2,4-dinitrophenol and KCN. Unlabeled pravastatin as well as R-416 and R-195, structural analogues of pravastatin, effectively competed for the hepatic uptake of [^{14}C]pravastatin at 37°. These results indicate that pravastatin is taken up by the liver by an active transport. In contrast, the transport of pravastatin by L-cells was temperature-independent and non-saturable, suggesting that the uptake of pravastatin by L-cells is mediated by passive diffusion. The marked difference in the uptake mechanism of pravastatin between hepatocytes and L-cells may account for a unique feature of this drug in that the uptake and inhibition of cholesterol biosynthesis occur selectively in the liver.

Pravastatin is a new hydrophilic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA¶) reductase inhibitor obtained from a metabolite of compactin (ML-236B), a prototype of HMG-CoA inhibitors discovered in our laboratory [1]. This inhibitor is now clinically used as an effective plasma cholesterol-lowering agent like other analogues such as lovastatin and simvastatin. Since the primary objective of these HMG-CoA inhibitors is to suppress *de novo* cholesterol synthesis in the liver, leading to enhancement in low density lipoprotein catabolism, inhibitors lacking the property to suppress cholesterol synthesis in extrahepatic organs would be much safer for long clinical use. In this context, a previous study showed that *in vivo* effects of pravastatin in extrahepatic tissues were much weaker than those of lovastatin and simvastatin [2]. This might be ascribed to a difference in a physico-chemical property among these inhibitors; both lovastatin and simvastatin are highly lipophilic, thus being permeable across plasma membranes, whereas the hydrophilic nature of pravastatin makes it less permeable across plasma membranes [3].

Despite its hydrophilic property, however, pravastatin was effectively incorporated into the liver *in vivo*** as well as into hepatocytes *in vitro* [4], playing as effective an inhibitor for hepatic HMG-CoA reductase as the lipophilic inhibitors, lovastatin and simvastatin. These observations prompted us to examine the hepatic transport mechanism of pravastatin.

In the present paper, we studied the *in vitro* uptake of pravastatin with rat primary cultured hepatocytes and compared it with that of mouse fibroblasts (L-cells) as a non-hepatic cell source. The results indicate that the hepatic uptake of this drug is carrier-mediated, whereas the uptake by L-cells is due to simple diffusion, providing *in vitro* evidence for the *in vivo* occurrence of the drug action. Although similar results were also obtained by Mahoney *et al.* [5], our study was carried out independently and preliminarily presented [6] at the same time as the presentation of Mahoney *et al.* [5].

MATERIALS AND METHODS

Chemicals. ^{14}C-Labeled pravastatin was synthesized by Daiichi Pure Chemicals (Ibaragi, Japan) by the fermentation method**. The specific activity was 16 μCi/mg and the radiochemical purity was more than 98%. Unlabeled pravastatin and its analogs were synthesized in this laboratory. Collagenase, streptomycin, penicillin were purchased from Boehringer-Mannheim GmbH (Germany). Calf serum, fetal bovine serum and other culture media were obtained from Gibco (New York, U.S.A.). Dexamethasone was from the Wako Pure Chemical Co. (Osaka, Japan). Insulin, glucagon, trypsin inhibitor, rotenone, oligomycin A and antimycin A were purchased from the Sigma

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¶ Abbreviations: DNP, 2,4-dinitrophenol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, *N*-ethylmaleimide.

** Komai T, Kawai K, Tokui Y, Kuroiwa C, Shigehara E and Tanaka M, Disposition and metabolism of pravastatin sodium in experimental animals. *Eur J Drug Metab Pharmacokin*, submitted.

Chemical Co. (St Louis, MO, U.S.A.). Other chemicals were of the best grade available from commercial sources. These chemicals were used as saline solution except for dexamethasone, which was used as ethanol solution.

Experiments with hepatocyte primary culture. Male Wistar rats (200–220 g) were used as a donor of hepatocytes. Liver perfusion was performed as described by Moldeus *et al.* [7]. Briefly, the rat liver was perfused at 37° for 4 min with Ca^{2+}-free Hanks' solution (pH 7.4) containing 0.5 mM EGTA and for 6 min with Hanks' solution containing 0.1% collagenase, 0.005% trypsin inhibitor and 4 mM $CaCl_2$. Hepatocytes were liberated from the perfused liver by blunt dissection and dispersed by gentle shaking in Ca^{2+}-free Hanks' solution. After filtration through the gauge, the hepatocytes separated from non-parenchymal cells by centrifugation at 50 *g* for 1 min, a process that was repeated three times, each time after washing and resuspending the cells in Ca^{2+}-free Hanks' solution, and finally resuspended in culture medium. Cell suspensions with a viability greater than 90% assessed by trypan blue extrusion were cultured in Williams' medium E supplemented with 5% calf serum, 2 mM L-glutamate, 0.1 μM insulin, 0.01 μM glucagon, 20 μM dexamethazone, 100 U/mL of penicillin and 100 μg/mL of streptomycin. The cells were cultured in 35-mm × 6-well collagen-coated culture dishes (Corning). Hepatocytes (1×10^6 cells) in 2 mL of culture medium were dispersed on each well and incubated at 37° in a humidified atmosphere of air (95%) and CO_2 (5%) for 24 hr before use.

Immediately before uptake experiments, the incubation medium was replaced by Williams' medium E prewarmed at 37°. To each well (hepatocyte monolayer) was added ^{14}C-labeled pravastatin at the concentration specified and incubated at 37°. When effects of metabolic inhibitors or structural analogues were tested, these reagents were added to the medium 5 min prior to the addition of the radiolabeled pravastatin. After incubation with [^{14}C]pravastatin for a period specified, the medium was discarded and cells were washed three times with 1 mL of ice-cold Hanks' solution. The cells in each well were then dissolved in 1.0 mL of 1 N NaOH by a rubber policeman. Aliquots were used to determine the cellular radioactivity by a liquid scintillation counter (Packard 2250A) and cellular proteins by the method of Lowry *et al.* [8].

Experiments with L-cells. Mouse L-cells were seeded at about 2×10^5 cells per well (35 × 15 mm) and cultured for 3 days in Dulbecco's modified Eagle medium containing 5% fetal bovine serum. The cells were maintained at 37° in a humidified atmosphere of air (95%) and CO_2 (5%). Before the experiment, culture medium was replaced by 2 mL of serum-free Eagle medium containing various concentrations of [^{14}C]pravastatin. Cells were incubated for 10 min at 37° or 4°, washed three times with 1.0 mL of ice-cold Hanks' solution, and harvested with a rubber policeman. Then the cellular radioactivity as well as the protein concentration was determined as described above.

RESULTS

Uptake of pravastatin by rat cultured hepatocytes

When cultured hepatocytes were incubated at 37° with 22.4 μM of [^{14}C]pravastatin, the amounts of uptake determined as the cell-associated radioactivity were increased linearly with time up to 10 min and gradually reached a plateau within 20 min (Fig. 1). The uptake rate within 10 min was 0.045 nmol pravastatin/mg cell protein/min. Incubation time was fixed for 10 min for following studies.

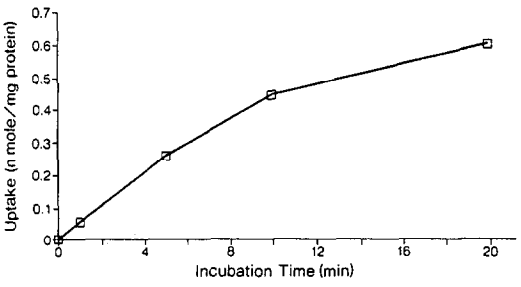

Fig. 1. Time-course of [^{14}C]pravastatin uptake by rat cultured hepatocytes. Hepatocytes were incubated with 22.4 μM [^{14}C]pravastatin at 37°.

Figure 2A shows the dose-dependent uptake of [^{14}C]pravastatin by hepatocytes. The hepatic uptake rate of the drug at 37° was increased as a function of the drug concentration in the medium, exhibiting a curvilinear pattern. The uptake rate at 4° under parallel conditions was less than 10% of that at 37° at 22.4 μM of [^{14}C]pravastatin, and the uptake rate at 4° was increased in proportion to the drug concentration in the medium. Unlike the hepatic uptake at 37°, a saturation pattern was not observed with the uptake at 4°. The subtraction of the uptake rate at 4° from that at 37° gave typical saturation kinetics (Fig. 2A, solid line). Double reciprocal plots of this curve gave a straight line (Fig. 2B), from which K_m = 32.2 μM and maximal uptake = 68 pmol/mg protein/min were calculated.

Effects of metabolic inhibitors and structural analogues on the hepatic uptake

The effect of various metabolic inhibitors on the hepatic uptake of [^{14}C]pravastatin (22.4 μM) was determined with 10 min incubation. The hepatic uptake of [^{14}C]pravastatin was suppressed by inhibitors for oxidative phosphorylation as shown in Fig. 3: 30 μM of rotenone (53%), 18 μM of antimycin A (76%) and 13 μM of oligomycin A (64%). Effects of metabolic poisons such as DNP and KCN were relatively weak (28% inhibition at 1 mM), whereas NEM exhibited a strong inhibition for the uptake: 55% at 0.1 mM (not shown in Fig. 3) and 75% at 1 mM, respectively. For the experiment with metabolic inhibitors, no significant decrease of cell viability was observed, when checked by lactate dehydrogenase leakage to the incubation medium by a method described by Moldeus *et al.* [7]. These findings indicate that the hepatic uptake of pravastatin is an energy-requiring process.

We then examined effects of structural analogues of pravastatin on the hepatic uptake of [^{14}C]-pravastatin at 37°. The uptake of [^{14}C]pravastatin (22.4 μM) for 10 min was inhibited by excess amounts

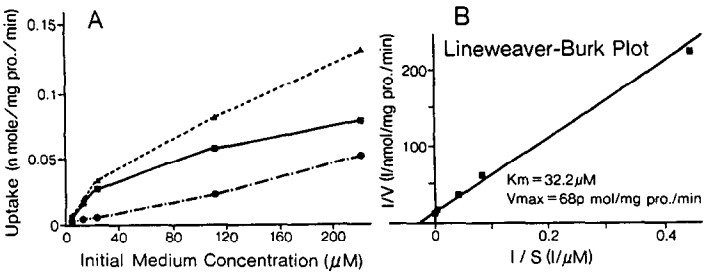

Fig. 2. Effect of initial medium concentration and incubation temperature on [^{14}C]pravastatin uptake by rat cultured hepatocytes (A) Uptake at 37° (▲), at 4° (●) and temperature-dependent uptake (■). (B) Lineweaver–Burk plot of temperature-dependent uptake.

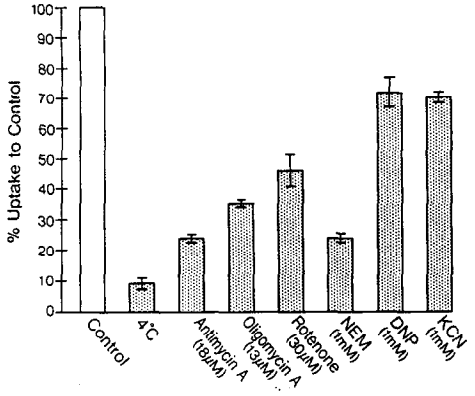

Fig. 3. Effect of metabolic inhibitors on [^{14}C]pravastatin uptake by rat cultured hepatocytes. Uptake rate of [^{14}C]-pravastatin at control was 0.051 ± 0.013 nmol/mg protein/min.

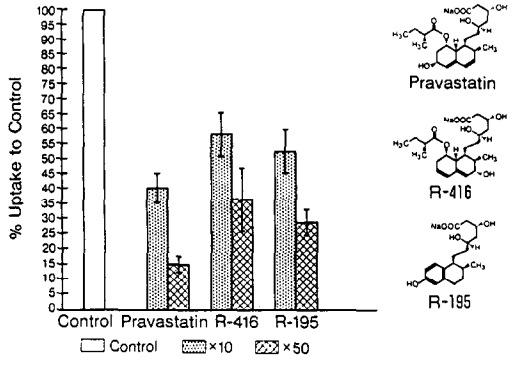

Fig. 4. Effect of structural analogues on [^{14}C]pravastatin uptake by rat cultured hepatocytes. Uptake rate of [^{14}C]-pravastatin at control was 0.051 ± 0.013 nmol/mg protein/min.

of unlabeled pravastatin: 60% by 10-fold excess and 85% by 50-fold excess (Fig. 4). Two other structural analogues of pravastatin, R-416 and R-195, were similarly effective in competing with [^{14}C]pravastatin

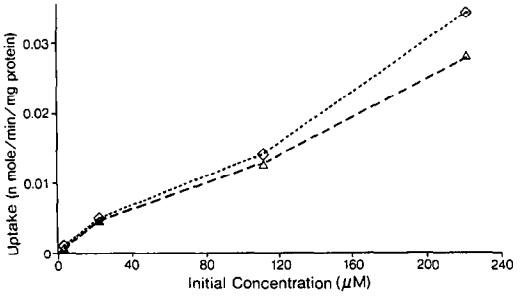

Fig. 5. Uptake of [^{14}C]pravastatin by mouse L-cells at 37° (◇) and at 4° (△).

for its hepatic uptake (Fig. 4). These data suggest that pravastatin is taken up by the hepatocytes by a structure-dependent mechanism.

Uptake of pravastatin by L-cells

The pravastatin uptake by hepatocytes was compared with that by L-cells as non-hepatic cells. The pravastatin uptake by L-cells as a function of the drug concentration was determined both at 37° and 4° (Fig. 5). The amount of uptake determined as the cell-associated radioactivity of [^{14}C]pravastatin was linearly increased with time up to 20 min and the uptake rate at 37° was 0.0049 nmol/mg protein/min with 22.4 μM of [^{14}C]pravastatin in the medium. This value is approximately 10%, when compared with that by cultured hepatocytes (Fig. 1). As shown in Fig. 5, the uptake rate of [^{14}C]pravastatin by L-cells at 37° increased linearly with pravastatin concentration and was indistinguishable from that at 4°.

DISCUSSION

Pravastatin, lovastatin and simvastatin are HMG-CoA reductase inhibitors which are now used clinically as plasma cholesterol-lowering agents. Pravastatin is the most hydrophilic and thus hardly penetrable across plasma membranes. In contrast, both lovastatin and simvastatin are lipophilic and their high membrane permeability endows them with

much easier access to HMG-CoA reductase. The difference between these inhibitors may well explain the observation that *in vivo* effects of pravastatin in extrahepatic tissues such as brain and muscle were much weaker than those of lovastatin and simvastatin [2]. However, this notion does not apply to the liver. Pravastatin is as effective as lovastatin and simvastatin in suppressing hepatic cholesterol synthesis [2]. Thus, it is likely that the effect of pravastatin on the liver may be more selective than the other lipophilic inhibitors. To understand the liver-selective action of pravastatin, its hepatic transport was investigated *in vitro* with rat primary cultured hepatocytes.

The uptake of pravastatin by hepatocytes was a temperature-dependent and saturable process (Figs 1 and 2). Furthermore, the uptake was inhibited by metabolic inhibitors for oxidative phosphorylation and respiratory coupling (Fig. 3). In addition, the hepatic uptake of [^{14}C]pravastatin was competed for not only by unlabeled pravastatin, but also by its structural analogues such as R-416 and R-195 (Fig. 5). These data taken together indicate that pravastatin uptake by rat hepatocytes is a carrier-mediated and energy-requiring process.

During the course of our study, Mahoney *et al.* [5] have provided evidence which supports our notion. In addition, they described lovastatin is also taken up via carrier-mediated transport by hepatic cells. However, in their experiments, lovastatin acid was used instead of lovastatin (lovasatatin is a lactone). Pravastatin is an acid form, whereas lovastatin and simvastatin are lactone forms. Thus, when lovastatin and simvastatin are studied, it will be important to tell whether they are in lactone or acid form. In our preliminary study, lovastatin and simvastatin were shown to be taken up by passive diffusion by hepatocytes, although both lovastatin acid and simvastatin acid were shown to be taken up via carrier-mediated transport by hepatocytes (data not shown here).

In sharp contrast to hepatocytes, pravastatin is taken up by L-cells by passive diffusion and its rate of uptake is lower by an order of magnitude compared with hepatocytes (Fig. 2A). This observation also supports our notion that the selective nature of pravastatin might be attributable to the difference in the transport mechanism between hepatic cells (carrier-mediated) and extrahepatic cells (passive diffusion).

Although the involvement of a carrier or an active mechanism is suggested by the present study, little is known about the ligand specificity of its hepatic carrier. It is well known that hepatocytes possess carrier proteins for various organic acids and bile acids [9, 10]. Examination of the hepatic carrier for these organic acids and for pravastatin, as well as the identification of endogenous ligands for the pravastatin carrier are important for understanding further the liver-selective action of pravastatin. These studies are under way in our laboratory.

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REFERENCES

1. Endo A, Kuroda M and Tsujita Y, ML-236A, ML-236B and ML-236C, new inhibitors of cholesterogenesis produced by penicillium citrinum. *J Antibiot* **29**: 1346–1348, 1976.
2. Koga T, Shimada Y, Kuroda M, Tsujita Y, Hasegawa K and Yamazaki M, Tissue-selective inhibition of cholesterol synthesis *in vivo* by pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochim Biophys Acta* **1045**: 115–120, 1990.
3. Watanabe H, Physicochemical properties of pravastatin, lovastatin, mevastatin and simvastatin. *Annu Rep Sankyo Res Lab* **42**: 117–120, 1990.
4. Tsujita Y, Kuroda M, Shimada Y, Tanzawa K, Arai M, Kaneko I, Tanaka M, Masuda H, Tarumi C, Watanabe Y and Fujii S, CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. *Biochim Biophys Acta* **877**: 50–60, 1986.
5. Mahoney EM, Child MJ and Smith-Monroy CA, Differential transport of pravastatin and lovastatin by hepatocytes and fibroblasts. *Circulation* **82**: Abstract No. 0023, 1990.
6. Komai T, Shigehara E, Tokui T, Kuroiwa C and Ishigami M, Transport mechanism of pravastatin by hepatocytes and its liver selective distribution. *J Jpn Atheroscler Soc* **18**: 1007, 1990.
7. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52**: 60–71, 1978.
8. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
9. Yamazaki M, Suzuki H, Sugiyama Y, Iga T and Hanano M, Uptake of organic anions by isolated rat hepatocytes: a classification in terms of ATP-dependency. *J Hepatol*, in press.
10. Yamazaki M, Sugiyama Y, Suzuki H, Iga Y and Hanano M, Utilization of ATP-dependent rat hepatocytes. *J Hepatol*, in press.