Cellular uptake of fluvastatin, an inhibitor of HMG-CoA reductase, by rat cultured hepatocytes and human aortic endothelial cells

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Aims To clarify the mechanism for cellular uptake of fluvastatin (FV) into rat primary cultured hepatocytes and human aortic endothelial cells (HAEC).

Methods Rat primary cultured hepatocytes and Endocell-AO as normal human aortic endothelial cells were used. Effects of incubation time, concentration- and temperature-dependency on cellular FV uptake were investigated after incubation with [¹⁴C]-FV and its enantiomers, (+)-FV and (–)-FV. Rat primary cultured hepatocytes were washed with either Na⁺-containing buffer or Na⁺-free buffer and incubated with metabolic inhibitors or bile acids. Intracellular radioactivity was measured by liquid scintillation counting. The determination of intracellular unchanged FV and its enantiomers was carried out by stereospecific h.p.l.c.

Results In rat cultured hepatocytes, concentration- and temperature-dependent saturable uptake of [¹⁴C]-FV was observed ($K_m = 37.6 \mu\text{M}$, $V_{\max} = 869 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$), suggesting a specific uptake mechanism. The uptake of each enantiomer also showed a specific uptake mechanism as observed for the racemate with no difference between enantiomers; (+)-FV, $K_m = 38.5 \mu\text{M}$, $V_{\max} = 611 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$, (–)-FV, $K_m = 41.5 \mu\text{M}$, $V_{\max} = 646 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$. In the presence of cholate and taurocholate, the uptake of FV was inhibited by 39–46%. Pravastatin inhibited FV uptake by 29%. In the absence of Na⁺, the uptake of FV was markedly inhibited 91–96% by bile acid. The uptake of FV into HAEC at 37° C and 4° C increased with the concentration of FV, but no saturable uptake was observed.

Conclusions FV transport system may be, at least in part, Na⁺- and ATP-dependent, and may have some features in common with the bile acid transport system and the organic anion transport system. Since saturable uptake was not observed in HAEC, FV appears to be taken up into these cells mainly via nonspecific simple diffusion.

Keywords: cellular uptake, fluvastatin, HMG-CoA reductase inhibitor, multispecific anion transporter, rat primary cultured hepatocytes, human aortic endothelial cells

Introduction

Fluvastatin ([$(\pm)$ -(3*RS*, 5*SR*, 6*E*)-sodium-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3, 5-dihydroxy-6-heptenoate; FV), is a hypolipidaemic agent with an indole moiety which inhibits specifically HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [1, 2]. Previous studies have shown that the highest level of radioactivity was found in the liver 2 h after oral administration of [¹⁴C]-FV in rats and was 51

times higher than that in whole blood. Unchanged drug in the liver accounted for 67–76% of the total radioactivity and was 40–75 times higher than the plasma levels [3].

In general, a compound is passively transported into various cells by simple diffusion. However, it has been known that some chemicals are taken up by hepatocytes through various specific active transport systems, including ATP- or Na⁺-dependent systems and an organic anion transporter-mediated system [4].

HMG-CoA reductase inhibitors such as pravastatin and simvastatin have been widely used clinically. In animals, both drugs are taken up by hepatocytes in high concentrations. However, the mechanisms responsible for the hepatocellular uptake of the two drugs are different.

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Simvastatin is taken up by hepatocytes because of its high lipophilicity, while pravastatin, a water-soluble organic anion, is transported efficiently by the organic anion transporter-mediated active transport system [5].

Tissue distribution of FV after administration in rats revealed that its peripheral distribution is similar to that of simvastatin while the hepatic distribution is similar to pravastatin [3]. However, little information is available on the mechanisms for the cellular uptake of FV.

In the present study, the mechanism for the hepatocellular uptake of FV was examined using rat primary cultured hepatocytes in order to define the reasons for the distinct tissue distribution of FV. FV has been shown to have an antiatherosclerotic effect without a significant change in serum lipid [6]. Therefore, the uptake of FV into human aortic endothelial cells (HAEC) was also examined and compared with the hepatocellular uptake.

Methods

Test compounds

[14 C]-FV and its [14 C]-enantiomers [(+)-FV; 3R,5S-isomer (–)-FV; 3S,5R-isomer] were synthesized by Daiichi Pure Chemicals, Co., Ltd (Ibaraki, Japan). The specific activity was 4.59 MBq for [14 C]-FV, and 4.71 MBq for [14 C]-(+)-FV and [14 C]-(–)-FV. The labelled compound was diluted accordingly with an unlabelled compound (supplied by Novartis Research Institute, USA; Lot no. 28). The radiochemical purity was shown to be >99% by high performance liquid chromatography (h.p.l.c., ODS column).

Preparation of Na⁺-containing buffer and Na⁺-free buffer

Krebs-Henseleit buffer was used as a Na⁺-containing buffer. The composition of Na⁺-free buffer was identical to the Na⁺-containing buffer except that NaCl and NaHCO₃ were replaced isotonicity with choline chloride (Sigma Chemicals, Co.) and choline bicarbonate (Sigma Chemicals, Co.). pH values of the Na⁺-containing buffer and Na⁺-free buffer were 7.77 and 7.71, and osmotic pressures were 286 and 283 mOsm kg^{–1}, respectively.

Cell preparation

Rat primary cultured hepatocytes were prepared by the method of Horiuchi [7] with minor modifications. Briefly, male Sprague-Dawley rats (5–7 weeks; Nippon Charles River, Ltd) were anaesthetized with pentobarbitone sodium (i.p.), and the portal vein was cannulated. After collagenase perfusion, the liver was resected and the parenchymal cells were isolated; viability was $\pm 90\%$. The cells were dispersed in WME (Gibco BRL) supplemented with 10% foetal bovine serum (FBS;

Whittaker Bioproducts, Ltd, Lot no. 4 M0055), 10^{-6} M insulin (Novo Nordisks Ltd, Manufacturing No. A211335) and 10^{-7} M dexamethasone (Wako Pure Chemical Industries, Ltd), seeded on collagen-coated dishes and incubated for 3–5 h. After incubation, the medium was changed. The cells were further cultured overnight, and used for uptake experiments.

Endocell-AO (Kurashiki Boseki Co., Ltd) were used as normal human aortic endothelial cells (HAEC). Quaternary cells were cultured in the growth medium supplied by the manufacture. The medium was changed every few days, and uptake experiments were performed when the cells reached confluence. These cultured cells have been shown to retain the morphological properties typical of aortic endothelial cells *in vivo*, such as tight junctions. Furthermore, the cells were confirmed to be aortic endothelial cells by the immunostaining method using factor VIII related antigen, acetylated LDL and smooth muscle α -actin (data not shown).

Both the rat primary cultured hepatocytes and HAEC were used for experiments immediately after separation from the incubation medium and washing three times with WME for rat primary cultured hepatocytes or DMEM for HAEC to remove FBS.

Effect of incubation time on hepatocellular FV uptake

Uptake studies of [14 C]-FV and its [14 C]-enantiomers into rat cultured hepatocytes were examined by the method reported previously [8, 9] with minor modifications. Briefly, the cultured hepatocytes were preincubated with WME (0.9 ml dish^{-1}) in a CO₂ incubator at 37° C for 10 min. 0.1 ml of [14 C]-FV ($5 \mu\text{g ml}^{-1}$ in WME) was added to each dish (FV = $1.15 \mu\text{M}$). After incubation at 37° C for 0, 5, 10, 20, 30 or 60 min, the cells were separated from the incubation medium and washed three times with cooled PBS (phosphate-buffered saline). After removing moisture, 0.1 N NaOH (0.7 ml dish^{-1}) was added to solubilize the cells. A scintillator (4 ml) was added to 0.5 ml of the solution, and radioactivity was determined (Tri-Carb 2500TR, Packard). Protein content was measured using the remainder of the solution [10].

Temperature-dependency of intracellular FV uptake

The cultured hepatocytes and HAEC were preincubated in a CO₂ incubator at 37° C or a refrigerator at 4° C for 10 min. 0.5 ml of [14 C]-FV or its [14 C]-enantiomers, which had been adjusted to 37° C or 4° C, was added to each dish (FV = $2.3\text{--}115 \mu\text{M}$). After incubation for 30 min at 37° C or 4° C, the intracellular uptake of [14 C]-FV was determined as described above.

Na^+ -dependency of intracellular FV uptake

The hepatocytes and HAEC were washed with either Na^+ -containing buffer or Na^+ -free buffer, and preincubated with the same buffer (0.5 ml dish^{-1}) for about 5 min in 5% CO_2 at 37°C . 0.5 ml of [^{14}C]-FV prepared with the same buffer was added to each dish ($\text{FV} = 2.3\text{--}115 \mu\text{M}$). After incubation for 30 min, the cells were washed with the buffer.

Effect of inhibitors and bile acids on intracellular FV uptake

After preincubation of the hepatocytes for 10 min at 37°C in the presence of metabolic inhibitors or bile acid ($10\text{--}1000 \mu\text{M}$, 0.9 ml dish^{-1}), 0.1 ml of [^{14}C]-FV was added to each dish ($\text{FV} = 1.15 \mu\text{M}$). The intracellular uptake of [^{14}C]-FV was determined after incubation at 37°C for 5 min.

Competition of [^{14}C]-FV uptake in the presence of unlabelled FV

To define the percentage of total uptake mediated by transport systems, the competition studies in the cultured hepatocytes were carried out with a fixed concentration of [^{14}C]-FV ($1.15 \mu\text{M}$) in the presence of unlabelled FV ($3.5\text{--}350 \mu\text{M}$) by the method of Tsuji *et al.* [11] with minor modifications. After preincubation with [^{14}C]-FV for 10 min at 37°C , various concentrations of unlabelled FV were added, and cells were incubated for 30 min at 37°C .

Effect of Na^+ on inhibition of intracellular FV uptake by inhibitors and bile acid

The cultured hepatocytes were preincubated with either Na^+ -containing buffer or Na^+ -free buffer for about 15 min at 37°C in the presence of an inhibitor or bile acid (1 mM). 0.1 ml of [^{14}C]-FV ($1.15 \mu\text{M}$) prepared with the buffer was added to each dish, and the cells were incubated for 5 min at 37°C . Subsequent treatment was as described above.

Determination of unchanged FV

In order to examine whether the [^{14}C]-FV taken up by the rat hepatocytes was metabolized or not, the unchanged FV was measured by a stereospecific h.p.l.c. as reported previously [12]. Briefly, after incubation of (+)-FV or (–)-FV with hepatocytes for 120 min at 37°C , unchanged FV in the cells and medium was extracted three times with *t*-butylmethyl ether. The organic phase was evaporated under a stream of N_2 and the residue was reconstituted with h.p.l.c. mobile phase

(acetonitrile/20 mM phosphate buffer = 15:100 v/v, pH 3.25). Aliquots were injected onto a chiral column (CHIRALCEL OJ, $5 \mu\text{m}$, 4.6 mm i.d. 250 mm, Daicel Chemical Industries Ltd) with a fluorescent detector (821-FP, JASCO).

Statistical evaluation of data

Data have been summarized as mean, $\pm$ s.d. as appropriate. Statistical significance was tested using a one-way analysis of variance, and the significance level (*P*) was set at 5% or less by a two-sided test.

Results

Intracellular uptake of [^{14}C]-FV increased with increasing incubation time (Figure 1). More than 30% of the FV added was taken up into the cells after 5 min of incubation, while the amount of FV in the cells reached a plateau after 30 min of incubation, with about 70% of the labelled drug in the cells.

As shown in Figure 2, the uptake of FV increased in a concentration-dependent manner, showing saturable uptake kinetics according to the Michaelis-Menten equation. The uptake of FV at 4°C was about one tenth of that at 37°C , but showed a linear increase with FV concentration (Figure 2a). The specific temperature-dependent uptake of FV was determined from the difference in the uptake at 4°C and 37°C . The Lineweaver-Burk plot formed a straight line, with K_m of $37.6 \mu\text{M}$ and $V_{\max}$ of $869 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ (Figure 2b). Each enantiomer also showed a specific concentration- and temperature-dependent saturable uptake as seen for the racemate, with K_m of ($38.5 \mu\text{M}$ for (+)-FV, $41.5 \mu\text{M}$ for (–)-FV) and $V_{\max}$ of ($611 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ for (+)-FV, $646 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ for (–)-FV) (data not shown). No

Figure 1 Time course of [^{14}C]-FV uptake by rat cultured hepatocytes. Each point is the mean of two determinations.

Figure 2 Effect of initial medium concentration and incubation temperature on [¹⁴C]-FV uptake by rat cultured hepatocytes. a) Uptake at 37°C (●) and 4°C (○) and temperature-dependent uptake (△). b) Lineweaver-Burk plot of temperature-dependent uptake. Each point is the mean of two determinations.

difference in the kinetics between enantiomers was observed. The uptake of FV into HAEC at 37°C and 4°C increased with the concentration of FV added, but no saturable uptake was observed (Figure 3).

The Scatchard plot of values obtained by subtracting the uptake at 4°C from that at 37°C, as described by Schwenk *et al.* [13], suggested the presence of two binding sites for FV with dissociation constants of 13.7 μM (K_{d1}) and 165 μM (K_{d2}) (data not shown).

It was considered that one quarter to one third of the total uptake was Na⁺-dependent in cultured hepatocytes (Figure 4a). In HAEC, Na⁺-dependent uptake accounted for 7–20% of the total uptake (Figure 4b).

Figure 3 Effect of initial medium concentration and incubation temperature on [¹⁴C]-FV uptake by human aortic endothelial cells (HAEC). Uptake at 37°C (●) and 4°C (○) and temperature-dependent uptake (△). Each point is the mean of two determinations.

As shown in Figure 5, in the presence of 100 μM 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) and 100 μM sulfobromophthalein (BSP), the uptake of FV was inhibited by 18% and 65% respectively. Inhibition was 40%, 4.3% and 20% in the presence of 100 μM 8-anilino-1-naphthalene sulfonic acid (ANS), 100 μM 2,4-dinitrophenol (DNP) and 1000 μM DNP, respectively. In the presence of 100 μM cholate and 100 μM taurocholate, the uptake of FV was inhibited by 39–46%. Pravastatin (100 μM) inhibited the FV uptake by 29%. Inhibition increased with the increase in the concentration of inhibitors.

Further, the uptake of [¹⁴C]-FV into rat cultured hepatocytes was significantly inhibited by the increase in the concentration of unlabelled FV. The 50% inhibition (IC_{50}) for FV uptake was estimated to be 23 μM, consistent with the K_m value (data not shown).

The uptake of [¹⁴C]-FV into primary cultured hepatocytes was examined in the Na⁺-containing and Na⁺-free buffers in the presence or absence of 1 mM metabolic inhibitors and bile acids. The results are shown in Figure 6. The total FV uptake was 335 pmol (mg protein)⁻¹, while the uptake in the absence of Na⁺ was 282 pmol (mg protein)⁻¹. The difference of 53 pmol (mg protein)⁻¹ was considered to represent Na⁺-dependent uptake, which accounted for 16% of the total uptake. In the presence of cholate or taurocholate, the total FV uptake was 130 and 141 pmol (mg protein)⁻¹, respectively. Cholate and taurocholate inhibited FV uptake by about 60%. The corresponding uptake in the absence of Na⁺ was 125 and 139 pmol (mg protein)⁻¹, respectively. Na⁺-dependent uptake of FV was 5 and 2 pmol (mg protein)⁻¹ in the presence of cholate and

Figure 4 Na⁺-dependency of uptake of [¹⁴C]-FV into rat cultured hepatocytes (a) and human aortic endothelial cells (b). Na⁺-dependent uptake (△) was estimated from differences between uptake in the presence (total uptake: ●) and absence (○) of Na⁺ in the medium. Each point is the mean of two determinations.

Figure 5 Effects of inhibitors on initial uptake of [¹⁴C]-FV into cultured hepatocytes. CA: sodium cholate, TCA: sodium taurocholate, PV: pravastatin-Inhibitor concentration: □ 10 μM ▨ 100 μM ■ 1000 μM. Each value represents mean ± s.d. of three experiments. Significantly different from the uptake in the absence of inhibitors. (*: $P < 0.05$, **: $P < 0.01$).

Figure 6 Effects of inhibitors on [¹⁴C]-FV uptake into rat cultured hepatocytes in the presence and the absence of Na⁺ ion. CA: sodium cholate, TCA: sodium taurocholate, PV: pravastatin. ▨ Na⁺-independent uptake ▨ Na⁺-dependent uptake. Each value represents the mean of two determinations.

taurocholate, and accounted for only 3.8 and 1.4% of the total uptake, respectively. The total FV uptake in the presence of pravastatin was 173 pmol (mg protein)⁻¹, about 50% inhibition. In the absence of Na⁺, the corresponding uptake was 123 pmol (mg protein)⁻¹. Thus, the Na⁺-dependent uptake of FV in the presence of pravastatin was 50 pmol (mg protein)⁻¹, 29% of the total uptake. Comparison of Na⁺-dependent uptake in the presence and absence of metabolic inhibitors revealed that the FV uptake was markedly inhibited 91–96% by cholate and taurocholate while the inhibition by pravastatin was negligible, 6%.

Discussion

The uptake of FV into rat primary cultured hepatocytes increased with increases in concentration of FV and temperature. The uptake showed saturable kinetics according to the Michaelis-Menten equation, suggesting that active transport is involved in the uptake of FV. A Lineweaver-Burk plot of the reciprocals of temperature-dependent specific uptake compared with those of FV concentrations yielded a K_m value of 37.6 μM, which was similar to that of pravastatin (32.3 μM) in rat primary cultured hepatocytes [8] and that (29 μM) in rat isolated

hepatocytes [9]. $V_{\max}$ of FV was calculated to be 869 pmol (mg protein) $^{-1}$ min $^{-1}$, 10 times higher than that of pravastatin (68 pmol (mg protein) $^{-1}$ min $^{-1}$) [8]. Scatchard plot of the values obtained by subtracting the uptake at 4°C from that at 37°C suggested the presence of at least two binding sites for FV in the cultured hepatocytes. In normal human aortic endothelial cells (HAEC), saturable uptake was not observed. These findings suggest that active transport is involved in the uptake of FV by hepatocytes while FV may be taken up by HAEC via nonspecific simple diffusion.

The percentage of unchanged drug was 85–90% within hepatocytes and 79–81% in the incubation medium, which indicates that the drug taken up into the cells exists predominantly as unchanged drug (data not shown).

There are several mechanisms responsible for the transport of compounds into hepatocytes, including bile acid transport system and organic anion transport system. The bile acid transport system can be further classified into at least two types, Na $^{+}$ -dependent and Na $^{+}$ -independent types. The organic anion transport system has been reported to be Na $^{+}$ -independent [13–17]. The results of experiments in the presence or absence of Na $^{+}$ revealed that the Na $^{+}$ -dependent transport process may be partly involved in the specific uptake of FV into cultured hepatocytes. In contrast, the contribution of the Na $^{+}$ -dependent process was negligible in HAEC, supporting the view that the uptake of FV by HAEC occurs mainly via nonspecific simple diffusion.

DIDS is an inhibitor of anion transporter [18]. BSP, an organic anion dye, is taken up into cells via an organic anion transporter. The uptake of BSP is independent of ATP or Na $^{+}$ [19]. In the presence of DIDS or BSP, the uptake of FV tended to be inhibited, suggesting that the anion transporter may be partly involved in the specific hepatocellular uptake of FV. ANS, which is taken up by hepatocytes in an ATP-dependent manner [19, 20], inhibited FV uptake. DNP, a metabolic inhibitor which depletes ATP [21], inhibited FV uptake. These findings suggested that hepatocellular uptake of FV is partly ATP-dependent.

Unconjugated bile acids including cholate are actively transported into hepatocytes and the contribution of Na $^{+}$ -dependent system is negligible [14–16]. On the other hand, hepatocellular uptake of conjugated bile acids such as taurocholate occurs via secondary active transport directed by the physiological Na $^{+}$ gradient (from blood to cell). In addition, Na $^{+}$ -independent uptake of taurocholate has been reported [17]. Since both cholate and taurocholate inhibited hepatocellular uptake of FV, it is possible that FV may be taken up into the cells via both mechanisms.

Pravastatin, another HMG-CoA reductase inhibitor, has been reported to be actively taken up by hepatocytes

in a Na $^{+}$ -independent manner, via a multispecific organic anion transporter [8]. Hepatocellular uptake of FV was inhibited pravastatin, suggesting that FV shares a partly common hepatocellular transport system to pravastatin.

Simvastatin, with a octanol/phosphate buffer (pH 7) partition coefficient (log P) of about 4.7, is nonspecifically taken up into cells via a simple diffusion mechanism. Pravastatin (log P; –0.23) is transported selectively into hepatocytes via a multispecific organic anion transporter, and the contribution of simple diffusion is small. In contrast, FV (log P; 1.5) is intermediate in cellular uptake between the two compounds [22, 23]. The results of this study suggested that FV is taken up by hepatocytes via both the nonspecific simple diffusion mechanism as seen for simvastatin and the specific uptake mechanism as seen for pravastatin. In addition, it is postulated that the specific hepatocellular transport system is partly Na $^{+}$ - and ATP-dependent and may have some properties in common with the bile acid transport system and the organic anion transport system.

It is concluded that the mechanism of cellular uptake of FV has some common features with that of simvastatin and pravastatin, and that FV is taken up by hepatocytes via the specific uptake mechanism.

Since the maximum plasma concentration ($C_{\max}$) of FV is about 250 ng ml $^{-1}$ (0.6 μ M) in human after oral administration of a clinical dose (30 mg), which is lower than K_m value (37.6 μ M), FV appears to have little effect on hepatic uptake of endogenous compounds (e.g. bilirubin, bile acid).

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