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Transport mechanism for lovastatin acid in bovine kidney NBL-1 cells: kinetic evidences imply involvement of monocarboxylate transporter 4

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Abstract

We previously indicated that lovastatin acid, a 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was transported by a monocarboxylate transporter (MCT) in cultured rat mesangial cells. In this study, to identify the MCT isoform(s) responsible for the lovastatin acid uptake, the transport mechanism was investigated using bovine kidney NBL-1 cells, which have been reported to express only MCT4 at the protein level. On RT-PCR analysis, the message of mRNAs for MCT1 and MCT4 was detected in the NBL-1 cells used in this study, which was confirmed by kinetic analysis of [^{14}C]L-lactic acid uptake, consisting of high- and low-affinity components corresponding to MCT1 and MCT4, respectively. The lovastatin acid uptake depended on an inwardly directed H^+-gradient, and was inhibited by representative monocarboxylates, but not by inhibitors/substrates for organic anion transporting polypeptides and organic anion transporters. In addition, L-lactic acid competitively inhibited the uptake of lovastatin acid and lovastatin acid inhibited the low affinity component of [^{14}C]L-lactic acid uptake dose dependently. The inhibition constant of L-lactic acid for lovastatin acid uptake was almost the same as the Michaelis constant for [^{14}C]L-lactic acid uptake by the low-affinity component. These kinetic evidences imply that lovastatin acid was taken up into NBL-1 cells via MCT4.

Keywords: Lovastatin acid; Transport; Monocarboxylate transporter 4; NBL-1 cell

1. Introduction

3-Hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, are safe and effective drugs for managing hypercholesterolemia (Mosley et al., 1989). The HMG-CoA reductase in the mevalonate pathway is the target molecule for statins, and furthermore, it exhibits another important role in the proliferation of various cells (Xu et al., 1996; Raiteri et al., 1997; Ishikawa et al., 1995; Ghosh et al., 1997; Yoshimura et al., 1998). Statins have been reported to inhibit their proliferation via inhibition of isoprenoids, the intermediate metabolites of the mevalonate pathway, by which small GTPase Rho and Ras are inactivated (Pahan et al., 1997; Endres et al., 1998; Danesh et al., 2002). Previously, we reported that simvastatin acid, lovastatin acid and pravastatin have an inhibitory effect on abnormal proliferation of cultured rat mesangial cells stimulated by fetal bovine serum (FBS) via inhibition of the mevalonate pathway,

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and that their inhibitory action depended on the uptake efficacy of the statins (Nagasawa et al., 2000). These findings suggested that statins may be potential drugs for mesangial proliferative glomerulonephritis, e.g. diabetic nephropathy.

Recently, we demonstrated, on the basis of the results of kinetic and mRNA expression analyses, that the uptake of lovastatin acid by rat mesangial cells is handled by H^+-coupled monocarboxylate transporter (MCT), and the involvement of MCT4, among the MCT isoforms, in the uptake was suggested (Nagasawa et al., 2002). However, because the expression of other MCT isoforms, that is, MCT1 and MCT2, in the mesangial cells, has been confirmed, the contribution of these isoforms to the uptake of lovastatin acid could not be ruled out (Nagasawa et al., 2002).

Wilson et al. (1998) reported that only MCT4 protein could be detected in bovine kidney NBL-1 cells, when they screened the expression of MCT isoforms, MCT1, MCT2, MCT3, MCT4, MCT5, MCT6, MCT7 and MCT8 by Western blot analysis using their specific antibodies. Thus, we thought the NBL-1 cells were suitable for demonstrating that MCT4 handles the uptake of lovastatin acid. However, in their report (Wilson et al., 1998), the expression of MCT isoforms was not examined at mRNA level by RT-PCR or Northern blot analysis, of which the sensitivity is higher than that by Western blot analysis, and the kinetic constants for L-lactic acid uptake were not calculated using an Eadie-Hofstee analysis, by which number of responsible components for total uptake is precisely estimated, and in fact, the affinity of L-lactic acid for MCT4 was apparently high comparing to that generally accepted.

Therefore, in this study, we firstly checked the expression of MCT isoforms in NBL-1 cells by RT-PCR analysis and by kinetic analysis using L-lactic acid as a representative substrate, and then, examined the involvement of MCT4 in the uptake of lovastatin acid.

2. Materials and methods

2.1. Chemicals

[^{14}C(U)]L-Lactic acid sodium salt (150 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc., St. Louis, MO, USA. Lovastatin was generously provided by Merck Sharp & Dohme Research Laboratories, Rahway, NJ, USA. Lovastatin acid was prepared from its lactone form by hydrolysis in a 0.05N NaOH solution, with stirring at 20 °C for 30 min. The hydrolyzed solution was adjusted to pH 7.4 with 0.2N HCl and then stored at 4 °C until use. The purity of lovastatin acid was >95%, as judged on HPLC. F-12 Nutrient Mixture (Ham) and FBS were purchased from GIBCO Laboratories, Grand Island, NY, USA and Funakoshi, Tokyo, respectively. All other reagents were of commercial or analytical grade requiring no further purification.

2.2. Cells

NBL-1 cells were obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation, Osaka, Japan, and cultured in Ham medium containing 10% FBS to about subconfluency (>80%) on a 12-well plate.

2.3. RT-PCR

Total RNA was extracted from NBL-1 cells with Sepazol RNA I reagent. One microgram of total RNA was reverse transcribed into cDNA using random priming and Moloney murine leukemia virus reverse transcriptase, as per the manufacturer's instructions, in a 20 μl reaction volume. Seven microliters of the cDNA was then PCR-amplified for 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 60 s. The reaction volume was 32 μl, comprising: 0.15 mM each of dATP, dCTP, dGTP and dTTP, 10× buffer, 0.15 μM primer and 15.6 U/ml Taq DNA polymerase. A PCR product was subjected to electrophoresis on an 1% agarose gel. Because the sequences of bovine MCT isoforms have not been reported, the 5′- and 3′-primers for MCT1, MCT2 and MCT4 were designed on the basis of the sequence preserved in the human, rat and mouse MCT isoforms. MCT1 (accession number of rat X86126) was analyzed with the 5′ primer 5′-CACCCCCCAGATGGAGGC-3′ and the 3′ primer 5′-AGTCTCCATACATGTCATTGAG-3′ to produce a 1202 bp product (bases 244–1445), MCT2 (accession number of rat X97445) with the 5′ primer 5′-TACTTCTATAGGAGGCGACCC-3′ and the 3′ primer 5′-AAGAACTGGGCAACACTCCAC-3′ to

produce a 744 bp product (bases 623–1366), and MCT4 (accession number of rat U87627) with the 5′ primer 5′-CCCTCTGGCCATGGGAG-3′ and the 3′ primer 5′-ACCTCCCCGTTTTTCTCAGG-3′ to produce a 1399 bp product (bases 79 to 1477).

2.4. Uptake experiments

Experiments were performed following the method of Nagasawa et al. (2000). In the uptake experiments on [^{14}C]L-lactic acid and lovastatin acid, cells were washed for three times with 1 ml of phosphate-buffered saline (PBS) warmed at 37 °C. Twenty-five mM 4-morpholinoethane-sulfonic acid (MES)—(pH 5.5–6.5) or 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES)—(pH 7.0–7.4) Hanks' balanced salt solution (HBSS) was used as the transport buffer. To deplete the cellular ATP, cells were preincubated in an appropriate transport buffer with 10 mM sodium azide (AZ) plus 10 mM 2-deoxy-D-glucose (DOG), and then used for uptake experiments. The uptake reaction was initiated by the addition of the indicated concentrations of lovastatin acid or [^{14}C]L-lactic acid plus non-labeled lactic acid, with or without inhibitors, to the preincubated cells. After appropriate time intervals, the reaction was terminated by the addition of ice-cold PBS, and the cells suspended in an appropriate ice-cold medium were stored at −80 °C until assaying.

2.5. Determination of concentrations of lovastatin acid and [^{14}C]L-lactic acid

The concentration of lovastatin acid in NBL-1 cells was measured by HPLC following the method described previously (Nagasawa et al., 2000). To quantify the radioactivity of [^{14}C]L-lactic acid taken up by the cells, the cells were solubilized with 1 M NaOH, neutralized with 5 M HCl, and then transferred to vials containing a liquid scintillation cocktail. The cellular protein content was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

2.6. Statistical analysis

The data are expressed as means ± S.E. Comparisons between two or more groups were performed by means of Student's unpaired *t*-test or analysis of variance (ANOVA, followed by Fischer's PLSD or Bonferroni/Dunn (Control) test), respectively, differences with a *P*-value of 0.05 or less being considered statistically significant.

3. Results

3.1. Expression of mRNA for MCT1, MCT2 and MCT4

To confirm the expression of mRNAs for MCT isoforms in NBL-1 cells, we performed RT-PCR analysis using the total RNA isolated from NBL-1 cells and the primer set specific to each MCT isoform. Because our previous study using rat mesangial cells suggested the possibility that lovastatin acid was transported by MCT4, but we could not deny the involvement of MCT1 and MCT2 (Nagasawa et al., 2002), and Halestrap and Price (1999) reported that only the MCT4 protein is expressed in NBL-1 cells, we checked the expression of mRNA for MCT1, MCT2 and MCT4. As shown in Fig. 1, the PCR products corresponding to MCT1 and MCT4, but not MCT2, were amplified under these conditions. Using these primer sets, we could amplify the MCT1, MCT2 and MCT4 from total RNA of rat cultured mesangial cell in which they are confirmed to be expressed in our previous study (data not shown). The size of each product was identical to that designed, and the sequence of PCR products for MCT1 and MCT4 was checked and was almost identical to the rat, mouse and human MCT1 and MCT4 (>90%), respectively (data not shown). Therefore, the PCR products observed here are the messages for MCT1 and MCT4.

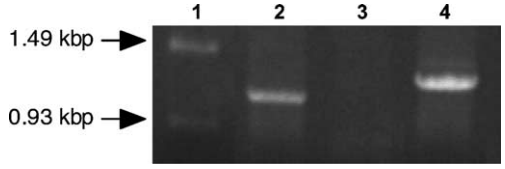

Fig. 1. Detection of mRNAs of MCT1, MCT2 and MCT4 in NBL-1 cells with the RT-PCR method. Lane 1, size marker; lanes 2–4, the RT-PCR product from NBL-1 cells using primers specific to MCT1, MCT2 and MCT4, respectively.

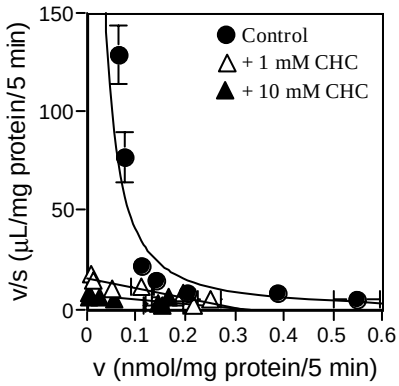

Fig. 2. Eadie-Hofstee plots of the concentration dependence of [^{14}C]L-lactic acid uptake by NBL-1 cells in the presence or absence of CHC. After cells had been preincubated in MES-HBSS (pH 6.0), they were incubated with the indicated concentrations of [^{14}C]L-lactic acid in the presence and absence of 1 or 10 mM CHC in MES-HBSS (pH 6.0) for 5 min at 37 °C. Dimethyl sulfoxide was used as a vehicle at the final concentration of 0.5%. Each point represents the uptake rate via the saturable component, and the mean ± S.E. for three experiments.

3.2. Concentration dependence of [^{14}C]L-lactic acid uptake

To clarify the characteristics of [^{14}C]L-lactic acid uptake by NBL-1 cells, the concentration dependence of the uptake was examined (Fig. 2). The initial rate of uptake of [^{14}C]L-lactic acid by NBL-1 cells showed clear saturation kinetics and negligible contribution of nonspecific diffusion, and Eadie-Hofstee analysis demonstrated that the uptake consisted of, at least, two components, the Michaelis constants (K_ms) for the low- and high-affinity components being calculated to be 37.3 ± 3.54 and 0.589 ± 0.144 mM, respectively, the maximum velocity (V_{max}s) being 747 ± 70.9 and 129 ± 4.36 nmol/mg protein per 5 min, respectively, V_{max}/K_ms being 20.1 ± 0.001 and 251 ± 68.2 μl/mg protein per 5 min, respectively, and the nonspecific diffusion constant (k_d) being 0.00456 ± 0.0005 μl/mg protein per 5 min.

3.3. Concentration dependence of lovastatin acid uptake

The kinetics of lovastatin acid uptake by NBL-1 cells was characterized by analysis of the concentration dependency of the uptake (Fig. 3). The uptake rate of lovastatin acid at 37 °C increased with increasing substrate concentration and tended to be saturated at high concentration, but at 0 °C, it linearly increased (Fig. 3A). A single component was involved in the uptake of lovastatin acid, which was estimated by subtracting the uptake at 0 °C from that at 37 °C (Fig. 3B). The apparent kinetic constants for lovastatin acid uptake was calculated to be as follows: K_m, V_{max}, V_{max}/K_m, and k_d were 0.207 ± 0.0142 mM, 11.5 ± 0.672 nmol/mg protein per 3 min, 55.7 ± 1.97 μl/mg protein per 3 min, and 12.5 ± 2.45 μl/mg protein per 3 min, respectively.

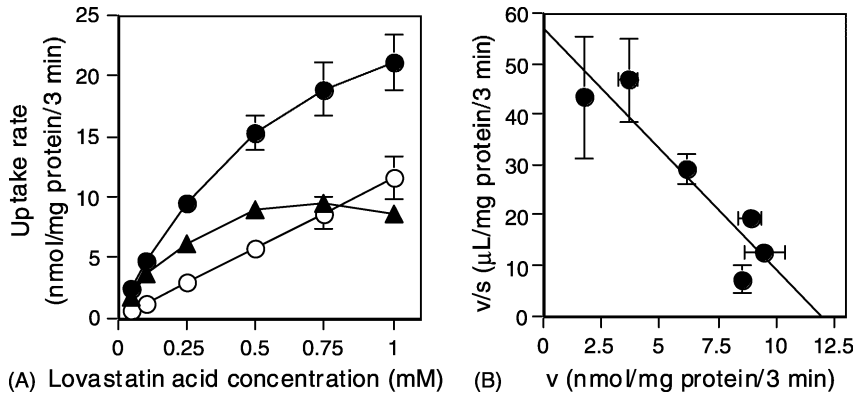

Fig. 3. Concentration dependence of lovastatin acid uptake by NBL-1 cells. After cells had been preincubated in HEPES-HBSS (pH 7.4), they were incubated with the indicated concentrations of lovastatin acid in HEPES-HBSS (pH 7.4) for 3 min at 37 °C (●) and 0 °C (○). Each point represents the mean ± S.E. for three experiments. The saturable component (▲) was calculated by subtracting the uptake rate at 0 °C from that at 37 °C. Panels A and B present the uptake rate vs. concentration profile, and Eadie-Hofstee plot for the saturable component, respectively.

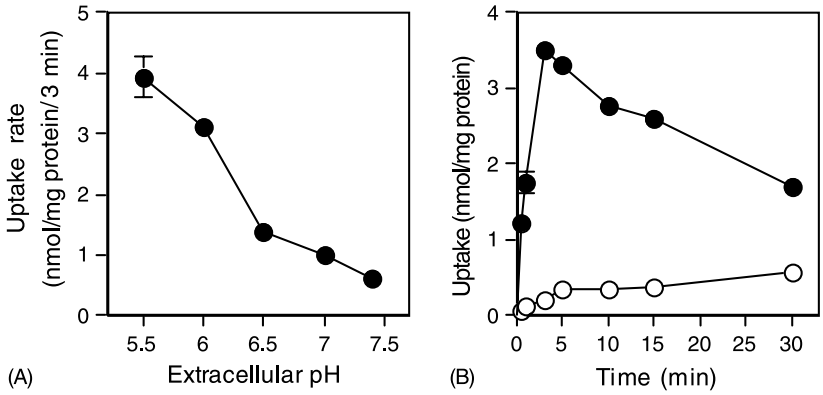

Fig. 4. Effect of the extracellular pH (A) and an inwardly directed H^+-gradient (B) on lovastatin acid uptake by NBL-1 cells. (A) Cells were preincubated in MES-HBSS (pH 5.5–6.5) or HEPES-HBSS (pH 7.0–7.4) at the designated pH for 10 min, and then incubated with 10 μM lovastatin acid for 3 min at 37 °C. (B) After cells had been pretreated with 10 mM AZ plus 10 mM DOG in glucose-free HEPES-HBSS (pH 7.4) for 20 min, they were incubated with 10 μM lovastatin acid in MES-HBSS (pH 5.5, ●) or HEPES-HBSS (pH 7.4, ○) containing 10 mM AZ plus 10 mM DOG for the indicated times at 37 °C. Each point represents the mean ± S.E. for three experiments.

3.4. Effects of the extracellular pH and a H^+-gradient on lovastatin acid uptake

Fig. 4A shows the extracellular pH dependence of lovastatin acid uptake by NBL-1 cells. In the pH range examined, the lovastain acid uptake linearly increased with decreasing extracellular pH, the uptake at pH 5.5 being about six-fold greater than that at pH 7.4.

We examined whether or not an inwardly directed H^+-gradient was the driving force for the uptake of lovastatin acid by NBL-1 cells. As depicted in Fig. 4B, the uptake of lovastatin acid by ATP-depleted NBL-1 cells with a H^+-gradient was apparently greater than that without the gradient, and showed an apparent overshoot phenomenon. This finding indicates that the lovastatin acid uptake by NBL-1 cells depends largely on an inwardly directed H^+-gradient as the driving force.

3.5. Effects of various compounds on lovastatin acid uptake

The effects of various monocarboxylates, substrates of organic anion transporting polypeptide (oatp) 1–3, and of organic anion transporter (oat) 1 and 3 on lovastatin acid uptake by NBL-1 cells were evaluated (Table 1). At pH 7.4, the uptake was inhibited by L-lactic acid, nicotinic acid, salicylic acid, and valproic acid, while taurocholate, *p*-aminohippurate (PAH), and benzylpenicillin had no effect. At pH 5.5, on the other hand, the lovastatin acid uptake by the cells was significantly inhibited by all the monocarboxylates. α-Cyano-4-hydroxycinnamic acid (CHC) showed slight but significant inhibition of lovastatin acid uptake by NBL-1 cells at 10 mM.

3.6. Effect of CHC on [^{14}C]L-lactic acid uptake

To evaluate the inhibitory effect of CHC on bovine MCT1 and MCT4, the effects of 1 and 10 mM CHC on [^{14}C]L-lactic acid uptake were investigated. As shown in Fig. 2, at both concentrations, CHC inhibited the uptake of [^{14}C]L-lactic acid by the cells, and the Eadie-Hofstee plots in the presence of 1 and 10 mM CHC were approximated closely with a single straight line. In this case, the K_m, V_{max}, and V_{max}/K_m values for the [^{14}C]L-lactic acid uptake were calculated to be 34.1 ± 18.7 mM, 424 ± 109 nmol/mg protein per 3 min, and 16.7 ± 3.93 μl/mg protein per 3 min for the 1 mM CHC group, and 65.8 ± 11.1 mM, 413 ± 24.6 nmol/mg protein per 3 min, and 6.67 ± 1.20 μl/mg protein per 3 min for the 10 mM CHC group, respectively.

3.7. Effects of salicylic acid on uptake of [^{14}C]L-lactic acid and lovastatin acid

To determine whether or not salicylic acid was transported by the same transporter as for L-lactic

Table 1
Effects of various compounds on lovastatin acid uptake by NBL-1 cells

Compound	Concentration (mM)	Uptake rate (% of control)	
		pH 7.4	pH 5.5
Control	–	100 ± 2.79	100 ± 2.50
Acetic acid	20	100 ± 3.86	75.6 ± 4.09**
Benzoic acid	20	95.0 ± 3.32	72.2 ± 14.4**
L-Lactic acid	20	64.9 ± 7.46***	57.5 ± 13.3***
(+)-Mandelic acid	20	129 ± 10.1	48.8 ± 5.14***
(−)-Mandelic acid	20	101 ± 3.08	45.7 ± 4.54***
Nicotinic acid	20	67.0 ± 5.80***	50.2 ± 5.50***
Propionic acid	20	81.6 ± 6.28	46.9 ± 6.02***
Salicylic acid	20	46.8 ± 4.18***	45.4 ± 1.68***
Valproic acid	20	65.0 ± 3.55***	25.6 ± 1.82***
Taurocholate	0.1	78.8 ± 9.46	–
PAH	0.3	124 ± 15.2	–
Benzylpenicillin	10	104 ± 7.18	–
Control (pH 6.0)	–	100 ± 5.81	–
CHC (pH 6.0)	10	73.2 ± 5.07*	–

Cells were preincubated in MES-HBSS (pH 5.5) or HEPES-HBSS (pH 7.4) for 10 min, and then incubated with 10 μM lovastatin acid in the presence or absence of the indicated concentration of an inhibitor in the respective medium for 3 min at 37 °C. In the experiments with CHC, dimethyl sulfoxide was used as the vehicle at the final concentration of 0.5%. Each value represents the mean ± S.E. for three to six experiments. The uptake rates in the control groups at pH 5.5, 6.0 and 7.4 were 4.47 ± 0.11, 3.35 ± 0.20 and 0.349 ± 0.010 nmol/mg protein per 3 min, respectively.

* $P < 0.05$ significantly different from each control value.

** $P < 0.01$ significantly different from each control value.

*** $P < 0.001$ significantly different from each control value.

acid and/or lovastatin acid, we examined the effects of preloading of cells with various concentrations of salicylic acid on the uptake of [^{14}C]L-lactic acid and lovastatin acid (Fig. 5). On preloading salicylic acid, the uptake of [^{14}C]L-lactic acid and lovastatin acid was significantly inhibited concentration dependently, indicating salicylic acid had a *trans*-inhibitory effect on their uptake.

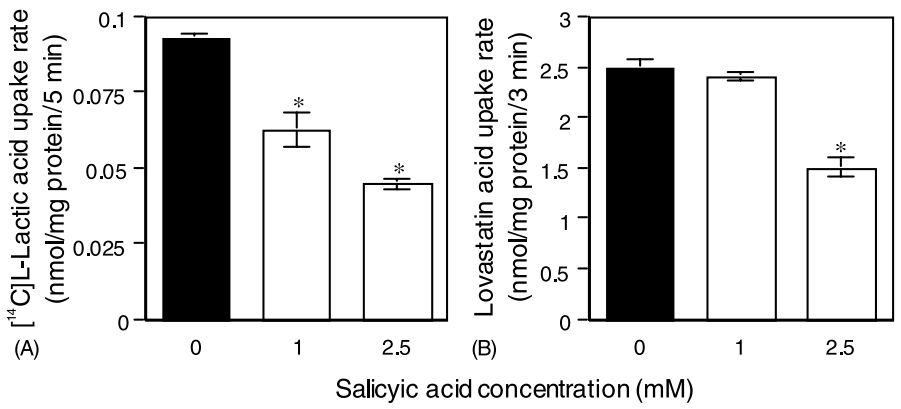

Fig. 5. Effect of preloaded salicylic acid on uptake of [^{14}C]L-lactic acid (A) and lovastatin acid (B) by NBL-1 cells. After cells had been loaded with the indicated concentrations of salicylic acid in MES-HBSS (pH 5.5) for 20 min, they were incubated with 20 mM [^{14}C]L-lactic acid (A) for 5 min or 10 μM lovastatin acid for 3 min in MES-HBSS (pH 5.5) at 37 °C. Each column represents the mean ± S.E. for three experiments. * $P < 0.01$, significantly different from each control (0 mM).

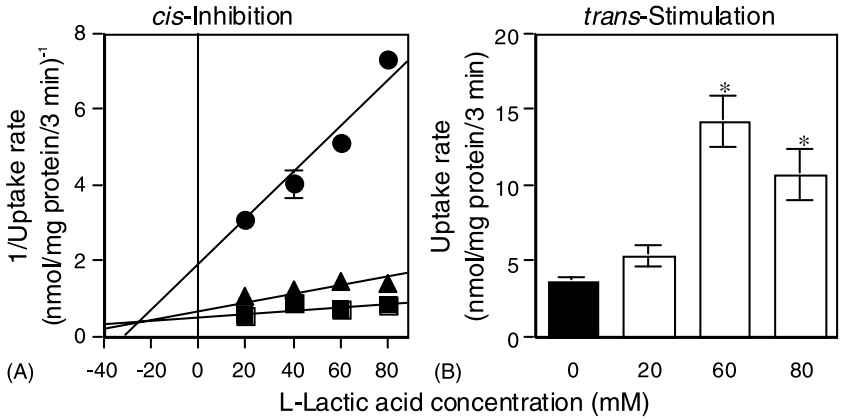

Fig. 6. *cis*-Inhibitory (A) and *trans*-stimulatory (B) effects of L-lactic acid on lovastatin acid uptake by NBL-1 cells. (A) After cells had been preincubated in MES-HBSS (pH 5.5) for 10 min, they were incubated with 10 (●), 25 (▲), or 50 (■) μM lovastatin acid and the indicated concentrations of L-lactic acid for 3 min at 37 °C. (B) After cells had been loaded with the indicated concentrations of L-lactic acid in MES-HBSS (pH 5.5) for 20 min at 37 °C, they were washed two times with PBS warmed to 37 °C, and then incubated with 50 μM lovastatin acid in MES-HBSS (pH 5.5) for 3 min at 37 °C. Each column represents the mean ± S.E. for three experiments. $^{*}P < 0.01$, significantly different from the control (0 mM).

3.8. Mutual effect of L-lactic acid and lovastatatin acid on their uptake

Fig. 6 shows the *cis*-inhibitory and *trans*-stimulatory effects of L-lactic acid on lovastatin acid uptake by NBL-1 cells. The uptake rate of lovastatin acid decreased with the simultaneous addition of L-lactic acid dose dependently, and Dixon plots revealed this is competitive inhibition, and the inhibition constant (K_i) of L-lactic acid for lovastatin acid uptake was estimated to be 24 mM (Fig. 6A).

The lovastatin acid uptake was significantly stimulated by preloading of the cells with L-lactic acid, demonstrating that a common transport system is involved in the uptake of the two compounds (Fig. 6B).

As depicted in Fig. 7, lovastatin acid had a dose-dependent inhibitory effect on [^{14}C]L-lactic acid uptake by NBL-1 cells.

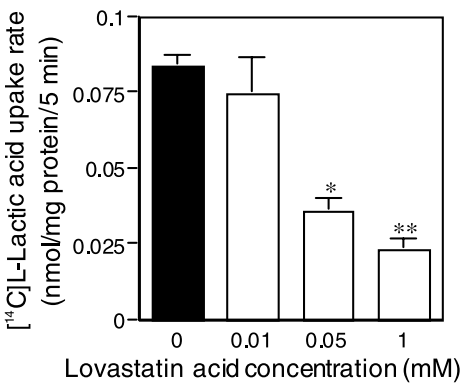

Fig. 7. Concentration-dependent effect of lovastatin acid on [^{14}C]L-lactic acid uptake by NBL-1 cells. After cells had been preincubated in MES-HBSS (pH 5.5) for 10 min, they were incubated with 20 mM [^{14}C]L-lactic acid and the indicated concentrations of lovastatin acid for 5 min at 37 °C. Each column represents the mean ± S.E. for three experiments. $^{*}P < 0.01$ and $^{**}P < 0.001$, significantly different from the control (0 mM).

4. Discussion

The large amount of evidence has demonstrated that the affinity of L-lactic acid for MCT isoforms, especially MCT1 and MCT4, is almost the same in various species (Halestrap and Price, 1999; Tamai et al., 1999; Juel and Halestrap, 1999; Fox et al., 2000). Therefore, we think that the findings obtained in this study exhibit universality beyond species.

In the NBL-1 cells, the message of mRNAs for MCT1 and MCT4 was observed (Fig. 1), and there was discrepancy between our findings and those of Wilson et al. (Wilson et al., 1998). This was thought to be due to the differences in culture conditions, evaluation

methods, that is, mRNA and protein levels, etc. As already mentioned, Wilson et al. reported the K_m value of L-lactic acid for bovine MCT4 with their NBL-1 cells was 10.1 mM (Wilson et al., 1998), and this value was apparently smaller than the generally accepted one (>20 mM) (Juel and Halestrap, 1999; Fox et al., 2000). We considered one of the reasons for this difference might be due to that they could not detect MCT1 actually functioned as a membrane protein by Western blot and this resulted in misjudgment of the K_m value of L-lactic acid by NBL-1 cells.

So, we examined the characteristics of L-lactic acid uptake by our NBL-1 cells. The uptake of [^{14}C]L-lactic acid was mediated by at least two saturable components, the K_m values for the high- and low-affinity components being calculated to be 0.589 and 37.3 mM, respectively, which were almost comparable to its K_m values for MCT1 (<5 mM) and MCT4 (>20 mM), respectively (Halestrap and Price, 1999; Tamai et al., 1999; Juel and Halestrap, 1999). Also, almost the same K_m values were obtained in the experiment at pH 5.5 (data not shown). On the basis of these kinetic evidences, it was suggested that in the NBL-1 cells used in this study, MCT1 and MCT4 function as membrane transporter proteins, and there was no or little expression of the other L-lactic acid-transportable protein.

Next, we characterized the uptake of lovastatin acid by NBL-1 cells. The uptake of lovastatin acid by the cells was mediated by a single system, and exhibited clear pH-dependence. Moreover, there was apparent overshooting of the lovastatin acid uptake in the presence of an inwardly directed H^+-gradient (Figs. 3 and 4). These results indicated that the uptake of lovastatin acid by NBL-1 cells is largely handled by a H^+-coupled transport system.

For evaluation of the contribution of MCT1 and MCT4 to lovastatin acid uptake by NBL-1 cells, an inhibition experiment was conducted using various monocarboxylates. All of the monocarboxylates apparently inhibited the uptake of lovastatin acid by the cells, and so the possibility of involvement of a MCT isoform in the lovastatin acid uptake was suggested. Although the inhibition at pH 7.4 was less than that at pH 5.5, this would be explained by the finding that the affinity of ligands for MCT isoforms and their transport activity are greater under acidic conditions than under neutral ones (Halestrap and Price, 1999).

It has been reported that pravastatin, an analog of lovastatin acid, is a substrate for rat oatp1 and human OATP2, and lovastatin acid might be a substrate for them (Hsiang et al., 1999), and the former is also one for rat oatp2 (Tokui et al., 1999) and 3 (Hasegawa et al., 2002). To confirm the contribution of the oatp and oat families to the lovastatin acid uptake by NBL-1 cells, we examined the effects of relatively specific substrates/inhibitors of them on the uptake. It has been reported that the K_m (or K_i) values of taurocholate for rat oatp1, 2 and 3, and human OATP2 are 60 (Hsiang et al., 1999), 35 and 18 (Abe et al., 1998), and 34 (Hsiang et al., 1999) μM, respectively, those of PAH for rat oat1 and oat3 47–60 and 398 μM (Hasegawa et al., 2002; Nagata et al., 2002), and those of benzylpenicillin for rat oat1 and oat3 418–800 and 53–83 μM, respectively (Hasegawa et al, 2002; Nagata et al., 2002), while the K_i value of benzylpenicillin for rOat1-mediated PAH transport is 1.7 mM (Jariyawat et al., 1999). These rat oat and oatp families are expressed in the rat kidney, although the expression levels differ with the transporter isoform (Sekine et al., 1997; Sweet et al., 1997; Simonson et al., 1994; Sekine et al., 1998; Kusuhara et al., 1999; Jacquemin et al., 1994; Abe et al., 1998). On the basis of these findings, we used taurocholate, PAH and benzylpenicillin at the concentration of 0.1, 0.3 and 10 mM, respectively, but they did not exhibit any inhibitory effects on the lovastatin acid uptake by NBL-1 cells. Thus, we think that there is no or little possibility of contribution of the oatp and oat families to the lovastatin acid uptake, and thus lovastatin acid uptake by NBL-1 cells is thought to be almost completely explained the uptake via MCT. We consider that this is supported by the finding that the system for lovastatin acid uptake consisted of a single component (Fig. 3), and was greatly dependent on an inwardly directed H^+-gradient (Fig. 4B).

CHC is a specific and competitive inhibitor for MCT1 and MCT4, the K_i values being reported to be 0.166 and 0.991 mM, respectively (Fox et al., 2000). As shown in Table 1, lovastatin acid uptake was significantly inhibited by 10 mM CHC, although the degree of the inhibition was low. This was thought to be due to the low affinity of CHC to MCT4. In fact, when its inhibitory effect on [^{14}C]L-lactic acid uptake was evaluated, only the uptake via a high affinity component, MCT1, was clearly inhibited by 1 and 10 mM CHC, while that via a low affinity one,

MCT4, was not affected by 1 mM CHC and was competitively, but only slightly, inhibited by 10 mM CHC (Fig. 2). This finding was considered to support the results shown in Table 1. We could not clearly explain why 10 mM CHC did not inhibit the uptake of [^{14}C]L-lactic acid and lovastatin acid completely. This might be due to difference in the sensitivity of MCT4 to the CHC inhibition between the cell species, but it is unclear at the present time, and so further detail investigations are needed to clarify this.

Recently, Emoto et al. (2002) proposed that salicylic acid, an NSAID, is a potent inhibitor of a H^+-coupled CHC-insensitive L-lactic acid transporter(s), and is transported mainly by a transport system distinct from that for L-lactic acid. Since lovastatin acid uptake was inhibited by salicylic acid and was slightly inhibited by CHC (Table 1) as the same as the result of Emoto et al. (2002), there was a possibility that lovastatin acid might be transported by the salicylic acid-transportable system. However, preloading of the cells with salicylic acid showed a *trans*-inhibitory effect on their uptake (Fig. 5). Thus, we think that the contribution of the salicylic acid-transportable system to the uptake of L-lactic acid and lovastatin acid by NBL-1 cells is strongly ruled out.

To confirm the same transport system contributes to the uptake of L-lactic acid and lovastatin acid, we performed *cis*-inhibitory and *trans*-stimulatory experiments. The uptake of lovastatin acid by NBL-1 cells was competitively inhibited by L-lactic acid and stimulated by the preloading of the cells with L-lactic acid (Fig. 6), and the K_i value of L-lactic acid for the lovastatin acid uptake was estimated to be 24 mM, which was almost identical to its own K_m value for MCT4 (37.3 mM). Furthermore, the low affinity component of [^{14}C]L-lactic acid uptake was inhibited by lovastatin acid dose dependently (Fig. 7). These results revealed that L-lactic acid and lovastatin acid are taken up by NBL-1 cells via the same transporter, MCT4. The K_ms for lovastatin acid and [^{14}C]L-lactic acid uptake, and the K_i of L-lactic acid for lovastatin acid uptake obtained here were comparable with those previously reported for rat mesangial cells (Nagasawa et al., 2002). Thus, it is considered that the MCT4 expressed in cow and rat has the same substrate specificity and affinity as to substrates, and transports lovastatin acid.

The cholesterol-independent "pleiotropic" effects of statins, most likely mediated through inhibition of Rho geranylgeranylation. Moreover, geranylgeranylation, and not farnesylation, has been reported to be primarily responsible for the release of the G_1/S phase block (Lefer et al., 2001). To express their beneficial "pleiotropic" effect, statins have to be incorporated into mesangial cells. Judging from our previous (Nagasawa et al., 2000, 2002) and present studies, lovastatin acid, simvastatin acid and pravastatin are transported into rat mesangial cells via MCT, probably MCT4, but the uptake efficacy of pravastatin is very low compared with the case of the others, and they inhibit the abnormal proliferation of rat mesangial cells stimulated by FBS. Taken together, lovastatin acid and simvastatin acid, but not pravastatin, are recommended for use for the treatment of mesangial proliferative glomerulonephritis, e.g. diabetic nephropathy. In fact, this idea was supported by initial clinical studies in which the effect of statins on the progression of diabetic nephropathy was encouraging (Grandaliano et al., 1993; Lam et al., 1995; Torolo et al., 1997).

In summary, the kinetic evidences obtained here imply that lovastatin acid was taken up by NBL-1 cells via MCT4, in which L-lactic acid uptake is mediated by MCT1 and MCT4.

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