J. Biochem. **132**, 395–400 (2002)

Effects of Pravastatin Sodium on Mevalonate Metabolism in Common Marmosets

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Received March 29, 2002; accepted June 26, 2002

In experimental animals and humans, the concentration of serum mevalonate (MVA), a direct product of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is considered to reflect the activity of whole-body sterol synthesis. The relationship between the concentration of serum MVA and the activity of sterol synthesis in tissues, however, has not been fully clarified. In the present study, we examined MVA metabolism by using pravastatin, a liver-selective inhibitor of HMG-CoA reductase, and common marmosets, a good model animal for studying lipid metabolism. In the time course study, the maximal reduction in the concentration of serum MVA was observed 2 h after a single oral administration of 30 mg/kg pravastatin to common marmosets. We, therefore, examined the relationship between the concentrations of serum and hepatic MVA, and sterol synthesis in some tissues at this time point. Sterol synthesis was determined *ex vivo* in tissue slices by measuring the incorporation of [^{14}C]acetate into digitonin-precipitable [^{14}C]sterols. Pravastatin at 0.03–30 mg/kg reduced dose-dependently the activity of hepatic sterol synthesis, whereas no significant reduction of sterol synthesis was observed in other tissues such as intestine, kidney, testis and spleen, even with the highest dose (30 mg/kg). The liver-specific inhibition of sterol synthesis caused parallel reductions in the concentrations of both serum and liver MVA. In addition, there were good correlations between the concentration of either serum or hepatic MVA and the activity of hepatic sterol synthesis. These data indicate that the major origin of serum MVA is the liver, and that the concentration of serum MVA reflects the concentration of hepatic MVA and the activity of hepatic sterol synthesis 2 h after a single oral administration of pravastatin in common marmosets.

Key words: common marmoset, HMG-CoA reductase inhibitor, mevalonate, pravastatin, sterol synthesis.

Hypercholesterolemia is a disease caused by corruption in cholesterol homeostasis in the whole body, which is maintained by absorption of cholesterol from the gut (*1–3*), hepatic cholesterol synthesis (*4–6*), and the uptake of cholesterol from the bloodstream *via* the low density lipoprotein (LDL) receptor pathway (*7–10*). Endogenous cholesterol is synthesized through the mevalonate (MVA) pathway (*11*), where cholesterol synthesis is regulated by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme for cholesterol synthesis (*12*).

It is important to determine the activity of whole-body sterol synthesis to diagnose hypercholesterolemia, and to evaluate the efficacy of lipid-lowering and anti-atherogenic drugs. Whole-body sterol synthesis has been measured by means of cholesterol intake/balance methods (*13, 14*) and kinetic approaches using labeled cholesterol (*15, 16*). These methods, however, are complicated and need prolonged measurement intervals. Recently, the blood concentrations of intermediate molecules of the MVA pathway such as MVA (*17–19*), squalene (*20*), and lathosterol (*21*) have been adopted as good indicators for measuring whole-body sterol synthesis. Jones *et al.* (*22*), in particular, have shown that there is a good correlation between the concentration of plasma MVA and the rate of incorporation of deuterium into plasma free cholesterol in humans. In addition, Parker *et al.* (*18*) and Naoumova *et al.* (*23*) have reported that HMG-CoA reductase inhibitors decrease plasma MVA in humans. These studies, however, did not demonstrate the relationship between the concentration of plasma MVA and the activity of sterol synthesis in tissues.

In the present study, we examine the MVA metabolism by using pravastatin, a liver-selective inhibitor of HMG-CoA reductase (*24–27*), and common marmosets, a good model animal for studying lipid metabolism (*28–31*). The data obtained show that the liver-selective inhibition of sterol synthesis by pravastatin causes parallel reductions in

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonate.

serum and hepatic MVA in these animals. The present study indicates that the origin of serum MVA is the liver, and that the concentration of serum MVA reflects the concentration of hepatic MVA and the activity of sterol synthesis in the liver 2 h after a single oral administration of pravastatin in common marmosets.

MATERIALS AND METHODS

Materials—Pravastatin was prepared as described previously (*32, 33*). ^{125}I-labeled protein A was purchased from Amersham International (Amersham, UK). [1-^{14}C]Acetate was obtained from New England Nuclear (Boston, MA, USA). All other chemicals were from Sigma Chemical (St. Louis, MO, USA) or Wako Pure Chemical Industries (Osaka).

Animals—Male common marmosets (250–400 g) were purchased from Charles River Japan (Kanagawa). The animals were housed individually in stainless steel hanging cages in a temperature- and light-controlled room (lights on from 7:00 a.m. to 7:00 p.m.), and had free access to water and a normal commercial chow diet (MD-7; Funabashi Farm, Chiba) (*28*). Animal experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo (Tokyo).

Administration of Pravastatin—The present study consisted of two different experiments. In the first experiment, we examined changes in the concentrations of serum MVA and total cholesterol after a single oral administration of 30 mg/kg pravastatin to common marmosets. In this experiment, the common marmosets were divided into 2 groups (n = 6) with comparable concentrations of mean serum MVA levels determined 7 days before the initiation of the drug administration. Blood was withdrawn to determine the initial values and then pravastatin was administered to the common marmosets at 9:00 a.m. Blood was withdrawn from a femoral vein at the indicated time-points after the drug administration. Over the experimental period, the common marmosets were fasted.

In the second experiment, the common marmosets were divided into 5 groups (n = 5) based on the serum MVA concentrations determined 7 days before initiation of the drug administration as described above. After blood sampling from a femoral vein to determine the initial values, pravastatin was orally administered once to these animals at the dose of 0.03, 0.3, 3, or 30 mg/kg. Two hours after the pravastatin administration, blood was withdrawn again, and then the marmosets were sacrificed under anesthesia with 40 mg/kg pentobarbital sodium (i.p.), and the liver, intestine, spleen, kidneys, and testes were excised. Changes in the concentrations of serum and hepatic MVA, and sterol synthesis in different tissues were determined as described below.

Pravastatin was dissolved in 0.5% carboxymethyl cellulose (CMC). Common marmosets in the control group received the vehicle (0.5% CMC) only.

Determination of Serum Total Cholesterol—Blood samples were kept at room temperature for 40 min, and serum was separated by centrifugation at 2,150 ×g for 10 min at 4°C. The concentration of serum total cholesterol was determined enzymatically with a HITACHI type 736 automatic analyzer (*25*).

Determination of Serum and Liver MVA—The concentrations of serum and liver MVA were determined using a gas chromatographic–mass spectrometer with minor modification (*34, 35*). In brief, 300 mg of liver was homogenized in 0.9 ml saline for 1 min with a Polytron homogenizer at the maximal speed and the homogenates were used for measurement of the liver MVA concentrations. After the addition of the internal standard, mevaonolactone-D4, to each sample, MVA was converted into the lactone form with hydrochloric acid, and the converted lactone was extracted using ethyl acetate. The extracts were evaporated to dryness and then dissolved in diethyl ether. Lipids in the solutions were absorbed on a silica centrifuge column, and then eluted with acetone. The dried extracts were trimethylsilylated with trimethyl chlorosilane and hexamethyl disilazone. Derivatized samples were analyzed with a mass spectrometer (Hewlett Packard model-5988). This involved electron capture and selected ion mode monitoring of ions at m/z ratios of 220 and 224 to detect the derivatised MVA and mevaonolactone-D4, respectively.

Determination of Sterol Synthesis in Tissue Slices—Sterol synthesis was determined *ex vivo* in tissue slices by measuring the incorporation of [^{14}C]acetate into digitonin-precipitable [^{14}C]sterols as described previously (*24–26*).

Statistical Analysis—Values are expressed as means ± SE. Statistical analysis was performed with Williams' test or the paired t-test. Comparison among the independent groups was performed with Student's t-test. The correlation analysis involved Pearson's correlation coefficient.

RESULTS

Changes in the Level of MVA after a Single Oral Administration of Pravastatin—Changes in the levels of serum

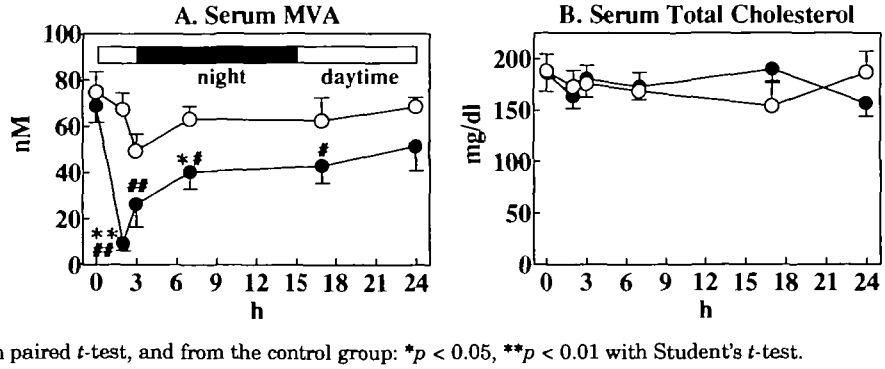

Fig. 1. **Effects of a single oral administration of pravastatin on serum MVA (A) and total cholesterol (B) in common marmosets.** Blood was withdrawn to determine the initial values and pravastatin (30 mg/kg) was administered to these animals (9:00 a.m.). Blood was withdrawn again at the indicated time points. The concentrations of serum MVA and total cholesterol were determined as described in the text. The symbols indicate: pravastatin 30 mg/kg (closed circles), and control (open circles). Each value represents the mean ± SE for 6 common marmosets. Significantly different from the initial values: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ with paired t-test, and from the control group: $^{*}p < 0.05$, $^{**}p < 0.01$ with Student's t-test.

MVA and total cholesterol after a single oral administration of pravastatin at 30 mg/kg are shown in Fig. 1. The pre-treatment values of serum MVA in the control and pravastatin 30 mg/kg groups were 74.8 ± 8.7 and 68.0 ± 7.0 nM, respectively. Over the 24 h monitoring period, there were no diurnal changes of serum MVA or total cholesterol in the control group. Pravastatin had decreased the concentration of serum MVA by more than 80% by 2 h after the administration (Fig. 1A). The reduced level of serum MVA returned almost to the pre-treatment level within 24 h after this dosage. On the other hand, a single oral administration of pravastatin at 30 mg/kg did not significantly change the level of serum total cholesterol (Fig. 1B).

The Relationship between the Serum and Hepatic MVA Levels, and the Activity of Sterol Synthesis in Tissues—Since pravastatin at 30 mg/kg maximally lowered the serum MVA concentration at 2 h after a single oral administration, we examined the relationship between the concentrations of serum and hepatic MVA, and the activity of sterol synthesis in some tissues at this time point. A single oral administration of pravastatin at a dose of 0.03–30 mg/kg reduced the activity of hepatic sterol synthesis in a dose-dependent manner (Fig. 2A). Pravastatin even at the highest dose (30 mg/kg), however, did not inhibit the activity of sterol synthesis in the intestine, spleen, kidneys and testes (Fig. 3). Pravastatin also decreased dose-dependently the concentrations of both serum (Fig. 2B) and liver (Fig. 2C) MVA, and these reductions occurred in a similar fashion.

Effects of Pravastatin on Total Cholesterol, LDL-Cholesterol, and LDL Receptor Protein—There were no changes in the level of total cholesterol after the oral administration of pravastatin at 0.03 and 30 mg/kg (Fig. 2D). There were no changes in the levels of LDL-cholesterol and LDL receptor protein with 3 and 30 mg/kg either (Table I).

The Correlation between Serum and Hepatic MVA, and the Activity of Sterol Synthesis in the Liver—Since a single oral administration of pravastatin reduced the concentrations of serum and hepatic MVA, and the activity of sterol synthesis in the liver, we examined the correlation among these three parameters (Fig. 4). There were good correlations between the activity of sterol synthesis in the liver and hepatic MVA ($r^2 = 0.724$; $p < 0.001$) (Fig. 4A), the activity of sterol synthesis in the liver and serum MVA ($r^2 = 0.797$; $p < 0.001$) (Fig. 4B), and the levels of hepatic MVA and serum MVA ($r^2 = 0.823$; $p < 0.001$) (Fig. 4C).

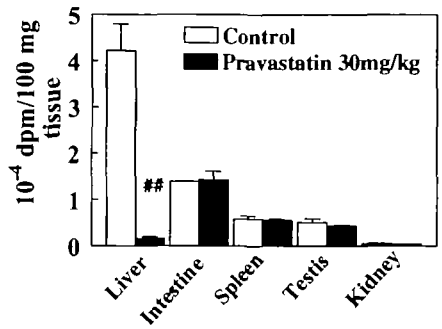

Fig. 3. **Effects of pravastatin on sterol synthesis in different tissues in common marmosets.** Blood samples were collected to determine the initial values of serum MVA and TC, and then pravastatin was orally administered to common marmosets at a dose of 30 mg/kg. Blood samples were collected again 2 h after the administration of pravastatin for determination of serum MVA and TC, the common marmosets were sacrificed under anesthesia, and the liver, intestine, spleen, kidneys, and testes were excised. Sterol synthesis in tissue slices was determined as described in the text. Each value represents the mean ± SE ($n = 5$). Each value in parentheses expresses the mean of the percent changes from the control group. Significantly different from the control group: $^{\#\#}p < 0.01$ with paired t-test.

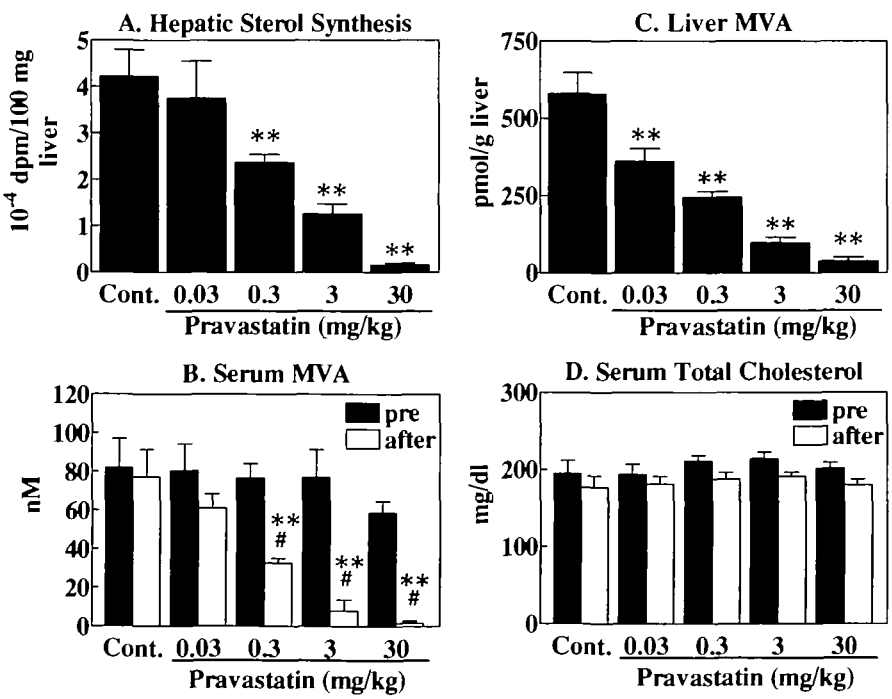

Fig. 2. **Effects of a single oral administration of pravastatin on hepatic sterol synthesis (A), serum MVA (B), liver MVA (C), and serum total cholesterol (D) in common marmosets.** Blood was withdrawn to determine the initial values of serum MVA and total cholesterol, and pravastatin at 0.03–30 mg/kg was orally administered to these animals. Two hours after the drug administration, blood was withdrawn again, the common marmosets were sacrificed, and tissues, such as the liver, intestine, spleen, kidneys and testes, were excised. The concentrations of serum MVA, hepatic MVA and serum total cholesterol were determined as described in the text. The activity of sterol synthesis in tissues was determined as described in the text. Each value represents the mean ± SE for 5 common marmosets. Significantly different from the initial values: $^{\#}p < 0.01$ with paired t-test, and from the control group: $^{**}p < 0.01$ with non-parametric Williams' test.

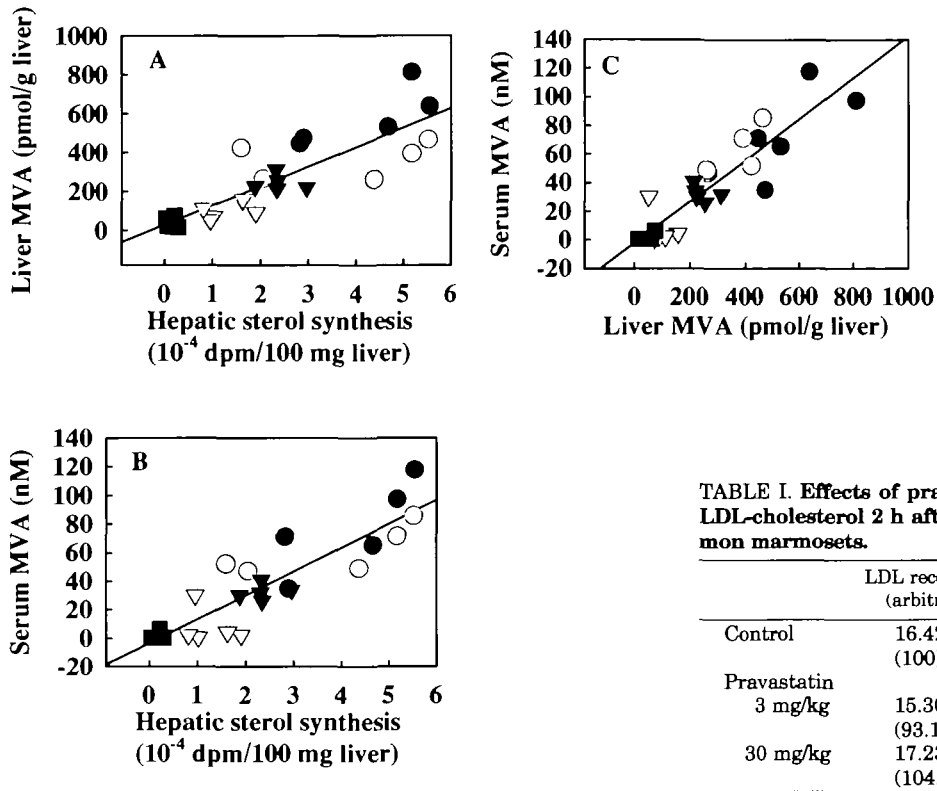

Fig. 4. **Correlations among the activity of hepatic sterol synthesis, the liver MVA level and the serum MVA level in common marmosets.** The symbols indicate: pravastatin 0.03 mg/kg (open circles), 0.3 mg/kg (closed inverted-triangles), 3 mg/kg (open inverted triangles), and 30 mg/kg (closed squares), and control (closed circles). (A) Relationship between hepatic sterol synthesis and liver MVA concentration ($r^2 = 0.724, p < 0.001$). (B) Relationship between hepatic sterol synthesis and serum MVA concentration ($r^2 = 0.797, p < 0.001$). (C) Relationship between liver MVA concentration and serum MVA concentration ($r^2 = 0.823$, $p < 0.001$).

TABLE I. **Effects of pravastatin on LDL receptor protein and LDL-cholesterol 2 h after a single oral administration to common marmosets.**

	LDL receptor protein (arbitrary units)	LDL-cholesterol (mg/dl)	
		Initial	2 h
Control	16.42 ± 2.64 (100)	102.8 ± 5.1 (100)	96.9 ± 13.2 (94.2 ± 3.4)
Pravastatin			
3 mg/kg	15.30 ± 5.07 (93.1)	115.3 ± 6.2 (100)	99.3 ± 4.8 (89.1 ± 7.1)
30 mg/kg	17.23 ± 2.40 (104.9)	116.7 ± 8.5 (100)	103.8 ± 7.7 (88.9 ± 1.3)

Blood samples were collected to determine the initial values of serum MVA and TC, and then pravastatin was orally administered to common marmosets at doses of 3 and 30 mg/kg. Blood samples were collected again 2 h after the administration of pravastatin for determination of serum MVA and TC, the common marmosets were sacrificed under anesthesia, and the livers were excised. LDL-receptor protein in the liver was determined by immunoblotting. Each value represents the mean ± SEM ($n = 5$). Each value in parentheses expresses the mean of the percent changes from the control group (LDL receptor protein), and from initial values (LDL-cholesterol).

DISCUSSION

There have been some papers describing methods for determining whole-body sterol synthesis, such as cholesterol intake/balance methods (*13, 14*), kinetic approaches (*15, 16*), and determination of the intermediate molecules of cholesterol synthesis (*17–21*). These methods, however, are complicated and need prolonged measurement intervals. Among these methods, monitoring of the serum MVA concentration (*17–19*) seems to be a good indicator for whole-body sterol synthesis (*18, 22, 23*). These studies, however, did not de-monstrate the major origin of serum MVA, or the relationship between the serum MVA concentration and the activity of sterol synthesis in tissues. In the present study, we addressed these issues using pravastatin, a liver-selective inhibitor of HMG-CoA reductase (*24–27*), and common marmosets, a good model animal for examining lipid metabolism (*28–31*).

In the present study, a single oral administration of pravastatin to common marmosets caused liver-selective inhibition of sterol synthesis: pravastatin inhibited dose-dependently the sterol synthesis in the liver (Fig. 2A), but not in other tissues, such as the intestine, another major tissue of sterol biosynthesis, kidneys, spleen and testes (Fig. 3). The liver-selective inhibitory action of pravastatin was consistent with our previous findings with rodent models (*24–27*). Under these conditions, pravastatin caused parallel and dose-dependent reductions in the concentrations of serum (Fig. 2B) and liver (Fig. 2C) MVA. In addition, there were good correlations among three parameters, *i.e.* the hepatic MVA level, serum MVA level and the activity of hepatic sterol synthesis (Fig. 4). We, therefore, conclude that the origin of serum MVA is the liver, and that the concentration of serum MVA reflects the concentration of hepatic MVA and the activity of hepatic sterol synthesis at 2 h after a single oral administration of pravastatin in common marmosets.

We should consider the possibility that pravastatin may enhance the decay of serum MVA, leading to a reduction in serum MVA: the increase in the utilization of MVA by the liver and nonhepatic tissues, and the increase in excretion of MVA from the body. Using radiolabeled MVA, McNamara *et al.* (*36*) found that approximately 40% of the metabolism of circulating MVA occurs in the kidneys, the remaining 60% of serum MVA being taken up by the liver and nonhepatic tissues for the synthesis of sterols (50%) and nonsterol products (10%), respectively. Since pravastatin cannot permeate the cell membrane in nonhepatic tissues (*26*), this agent did not significantly inhibit sterol synthesis in the nonhepatic tissues. The results suggested that pravastatin is less able to increase the uptake-protein of MVA in nonhepatic tissues. In addition, a single oral administration of pravastatin did not even change the levels of LDL receptor proteins in the liver, by which this agent is taken up. We, therefore, think that the reduction of serum MVA after a single oral administration of pravastatin is primarily due to the inhibition of hepatic sterol syn-

thesis.

The range of concentrations of serum MVA in common marmosets was 30–110 nM, which is similar to that in humans (*18*). No diurnal changes of serum MVA were seen in the control common marmosets fasted overnight (Fig. 1A). Kopito *et al.* (*37*) have also reported that in fasted humans, there was no diurnal change of serum MVA, although more than a five times diurnal change of serum MVA in fed humans was observed. In addition, Parker *et al.* (*18*) have shown that lovastatin, another HMG-CoA reductase inhibitor, maximally decreased plasma MVA in humans at 4–6 h after its administration: the decreased level of serum MVA recovered and returned to the initial level within 24 h. These data obtained in a human study are similar to those obtained in the present study (Fig. 1A), indicating that common marmosets are similar to humans regarding MVA metabolism as well as lipid metabolism (*28–31*).

We have reported that the 28-day administration of pravastatin to common marmosets at doses of 1–30 mg/kg dose-dependently reduced the level of LDL-cholesterol (*28*): Pravastatin significantly reduced LDL-cholesterol at doses of more than 3 mg/kg. In the present study, a single oral administration of pravastatin to common marmosets at a dose of more than 3 mg/kg reduced the level of serum MVA by more than 80% as compared to the initial level when monitored 2 h after the oral administration of this agent. These data might indicate that the reduction of the serum MVA level at 2 h after a single oral administration of pravastatin is a good indicator of the LDL-cholesterol lowering effect of this agent. It is of interest of how pravastatin affects the serum MVA level in comparison with the LDL-cholesterol lowering effect after repeated oral administration in these animal models and men. Further studies are necessary to clarify the clinical usefulness of serum MVA monitoring for examining the action of the statins in patients who exhibit variety in the efficacy as to lowering of serum cholesterol.

In summary, by using pravastatin, a liver-selective inhibitor of sterol synthesis (*24–27*), and common marmosets, a good animal model for studying lipid metabolism (*28–31*), we demonstrated that serum MVA mainly originated from the liver, and that changes in the level of serum MVA reflected the activity of hepatic sterol synthesis and the content of MVA 2 h after a single oral administration of pravastatin in common marmosets.

We are grateful to Drs. Yoshio Tsujita and Tomoyuki Fujioka for critical reading of the manuscript.

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