Regular Article

The HMG-CoA Reductase Inhibitor Pravastatin Stimulates Insulin Secretion through Organic Anion Transporter Polypeptides

Michiaki Abe1,a, Takafumi Toyohara1,b, Akiko Ishii2,c, Takehiro Suzuki1,d, Naoya Noguchi3, Yasutoshi Akiyama1, Hiromi O. Shiwaku1, Rie Nakagomi-Hagihara4, Guodong Zheng1, Eisuke Shibata1, Tomokazu Souma1, Tomohiko Shindo1, Hirohito Shima1, Yoichi Takeuchi1, Eikan Mishima1, Masayuki Tanemoto1, Tetsuya Terasaki5, Tohru Onogawa6, Michiaki Unno6, Sadayoshi Ito1, Shin Takasawa7 and Takaaki Abe1,8,9,*

1Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
2Diabetes Center, Tokyo Women’s Medical University School of Medicine, Tokyo, Japan
3Department of Advanced Biological Sciences for Regeneration, Tohoku University Graduate School of Medicine, Sendai, Japan
4Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi-Sankyo Co., Ltd., Tokyo, Japan
5Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan
6Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan
7Department of Biochemistry, Nara Medical University, Nara, Japan
8Division of Medical Science, Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan
9Department of Clinical Biology and Hormonal Regulation, Tohoku University Graduate School of Medicine, Sendai, Japan

a-dThese authors contributed equally to this study

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor pravastatin has been reported to have a beneficial effect on reducing the new onset of diabetes as well as lowering plasma lipids. Because pravastatin is a water-soluble organic anion, it cannot easily penetrate the lipid bilayer of the cell membrane. As the precise mechanisms of the effect of pravastatin on glucose metabolism and diabetes have not been clarified, we examined the roles of the organic anion transporter family on pravastatin-treated islet and adipocyte functions. Rat oatp1/slc01a1, oatp2/slc01a4 and oatp3/slc01a5 were expressed in the pancreas, and rat oatp3/slc01a5 was also detected in rat insulinoma cell line INS-1e. Pravastatin was transported not only by oatp1/slc01a1 and oatp2/slc01a4, but also by rat oatp3/slc01a5. Pravastatin uptake into INS-1e cells was detected and this transport was inhibited by sulfobromophthalein and rifampicin, both of which are known to inhibit oatp family-mediated uptake. In addition, pravastatin enhanced the glucose-stimulated insulin secretion from INS-1e cells. When fat-loaded db/db mice were treated with pravastatin, glucose intolerance and insulin resistance were prevented. In addition, insulin secretion from isolated islets was enhanced by pravastatin. These data suggest that pravastatin has pleiotropic effects on islets through membrane transport under high fat/glucose conditions.

Keywords: SLCO (SLC21); SLC transporters; HMG-CoA reductase inhibitors; xenobiotics; diabetes; disease; insulin; endobiotics; adipose; organ; islet; OATP; SLC transporters

Introduction

Pravastatin is a water-soluble 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor that has significant effects lowering total and LDL cholesterol and triglyceride levels. In addition to these lipid-lowering effects, the West of Scotland Coronary Prevention Study (WOSCOPS) demonstrated the effectiveness of
pravastatin therapy in inhibiting the new onset of diabetes. Because of its hydrophilic nature, pravastatin cannot easily penetrate the cell membrane. To exert its effects, pravastatin has to be transported into cells via some kind of membrane transporter. Recently, we isolated rat organic anion transporting polypeptide 2 (oatp2/slc1a4) as a transporter responsible for pravastatin uptake. In addition, LST-1/OATP-C/SLOC1B1 was also isolated and was identified as the molecule responsible for transporting pravastatin in human liver. However, no attempt was made to examine the transport of pravastatin into the islets or the effect of pravastatin on insulin secretion.

Here, we report the distribution of the oatp family in the pancreas; also the effects of pravastatin on insulin secretion are examined in vitro and in vivo.

Materials and Methods

Ethics statement/animals and experimental design: All research involving animals was conducted according to the national guidelines (Regulations and Guidelines on Scientific Care and Use of Laboratory Animals in Japan) and was also approved by the Tohoku University Animal Care Committee. Six-week-old, male db/db mice (+ Lepr+/−) were purchased from CLEA (Tokyo, Japan). Mice were fed a high-fat diet (15.3% fat, 424.5 cal/100 g; Quick fat, CLEA, Tokyo, Japan) and had free access to tap water. At 7 weeks of age, mice that showed diabetic symptoms were randomly assigned to 2 groups: the control group and the pravastatin group (n = 5–12 per group). In the pravastatin group, 200 mg/kg body weight/day pravastatin was administered by gavage.

Materials: Pravastatin was provided by Daiichi-Sankyo (Tokyo, Japan). Rat insulinoma cell line INS-1e was kindly provided by Dr. Hisamitsu Ishihara, Nihon University.

Immunohistochemistry: Antibodies against rat oatp1/slc1a1, oatp2/slc1a4 and oatp3/slc1a5 and mouse oatp3/slc1a5 were raised using peptides. Synthetic peptides used for immunization were as follows; ENETGGATTTGATTACACGC-3′, reverse 5′-ATGAGAC-A GTGGGCTTTTGGGAGA-3′), mouse oatp1/slc1a1 (forward 5′-TTAGCTGGCATCTCAGCCTG-3′, reverse 5′-TTTTAAATGTTCTGTCCGCTCA-3′), mouse oatp2/slc1a4 (forward 5′-AACTAGGAAGACCATTGCGCCTTGG-3′, reverse 5′-ATCCAGGCAATTGAGGTAACAG-3′), mouse oatp3/slc1a5 (forward 5′-CAGTTTTTACTCAAATGATTTTGGAA GTAGC-3′), HMG-CoA reductase (forward 5′-GCCGCATTTGCAAGAAGATCC-3′, reverse 5′-CTTATGCTGGAAGGATG-3′) and GAPDH (forward 5′-TGAAGGTCGGAGTCAACCGATTTG-3′, reverse 5′-CATGTGGGCCCATGAGTCACC-3′).

RT-PCR amplification was performed according to the following schedule: 94°C for 3 min, 94°C for 45 s, 57°C for 1 min, and 72°C for 2 min for 35 cycles, followed by further incubation for 10 min at 72°C.

Effects of drugs on glucose-stimulated insulin secretion (GSIS): INS-1e cells were grown in RPMI1640 and GSIS was measured as reported. In brief, cells ~80% confluence were incubated in the presence or absence of pravastatin for 48 h. Cells were then maintained for 1 h, washed twice and preincubated at 37°C for 1 h in a buffer containing (in mM): 135 NaCl, 3.6 KCl, 5 NaHCO3, 0.5 NaH2PO4, 0.5 MgCl2, 1.5 CaCl2, 10 HEPES, and 0.1% BSA, pH 7.4. Cells were then incubated with the buffer containing glucose for 60 min. The insulin concentration was measured using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden).

Functional characterization of rat oatp3/ slco1a5: Pravastatin uptake by oatp3/slc1a5 was determined as described. Briefly, cDNA for rat oatp3/slc1a5 was synthesized and injected into defolliculated Xenopus laevis oocytes. Injected oocytes were cultured for 2–3 days and the uptake (60 min) of [14C]pravastatin (30 μM) was measured (n = 4–6). Water-injected oocytes were used as a control (n = 4–6).

Cellular uptake of pravastatin: Pravastatin uptake in INS-1e cells was measured as reported. Briefly, cells were washed twice with Krebs-Henseleit (KH) buffer containing (in mM): 142 NaCl, 23.8 NaHCO3, 4.83 KCl, 0.96 KH2PO4, 1.2 MgSO4, 12.5 HEPES, 5 glucose and 1.53 CaCl2, pH 7.3). Then, the cells were incubated with [14C]pravastatin (30 μM) with or without sulfochromophthalein (BSP) or rifampicin for 5 min under linear conditions.

Cholesterol measurement: INS-1e cells (106 cells/well) or mouse islets (~60 islets/tube) were homogenized with 200 ml of chloroform with 1% Triton X-100 and the extract was centrifuged for 10 min at 15,000 rpm. The cholesterol content of the organic phase was measured using a Cholesterol/Cholesteryl Ester Quatitntion Kit (Biovision Labs, Mountain View, CA).
Glucose tolerance test and insulin tolerance test: Oral glucose tolerance testing (OGTT) was performed after overnight fasting. Two grams of glucose per kg body weight was administered orally. For insulin tolerance testing (ITT), 8-h fasted mice were injected with human regular insulin (2 U/kg body weight) intraperitoneally.

Measurement of insulin secretion from isolated islets: Islets were isolated as previously reported.\textsuperscript{13) }Ten islets were preincubated in RPMI1640 medium containing 10% FCS under 5% CO\textsubscript{2} for 2 h at 37°C. The insulin secretion was stimulated by incubating islets in a medium containing 20 mM glucose for 30 min.\textsuperscript{14) }The insulin concentration was determined using a mouse insulin kit (Morinaga, Yokohama, Japan).

Statistical analysis: Data are presented as means ± SEM. Differences between groups were calculated using the unpaired Student’s \( t \)-test. We considered \( P < 0.05 \) as statistically significant.

Results

Localization of rat oatps in the pancreas: Rat oatp1/slco1a1 was detected in the \( \beta \)-cells by immunohistochemical analysis (Fig. 1A). Rat oatp2/slco1a4 immunoreactivity was detected in \( \alpha \)-cells (Fig. 1B). In contrast, rat oatp3/slco1a5 immunoreactivity was detected widely in the islets as well as acinar cells (Fig. 1C). These signals were abolished using antibodies preabsorbed with antigen peptides (Supplemental Fig. 1). Insulin (Fig. 1D) and glucagon (Fig. 1E) immunoreactivities were also used to confirm the cellular identification. To further confirm the expression at the mRNA level, RT-PCR was performed. Individual specific bands were detected in the pancreas for rat oatp1/slco1a1 (Fig. 1F), oatp2/slco1a4 (Fig. 1G) and for oatp3/slco1a5 (Fig. 1H).

Fig. 1. Immunohistochemistry of rat pancreas
Sections were incubated with antibody against rat oatp1/slco1a1 (A), oatp2/slco1a4 (B) and oatp3/slco1a5 (C). Insulin (D) and glucagon-immunostaining (E) are also shown. Scale bar, 200 μm. RT-PCR of the pancreas and INS-1e cells: rat oatp1/slco1a1 (F), oatp2/slco1a4 (G), oatp3/slco1a5 (H) and GAPDH (I).
Fig. 2. Effects of pravastatin on INS-1e cells
(A) Radiolabeled pravastatin uptake by INS-1e cells with or without pravastatin (Prava) (1000 μM), rifampicin (300 μM) or BSP (300 μM).31) (B) GSIS in INS-1e cells. Insulin was measured at 3.3 mM glucose (3.3 G) and 11 mM (11 G). In addition, the effects of pravastatin (30 μM) or pravastatin (30 μM) and BSP (30 μM) (n = 3) on GSIS in INS-1e cells were also measured. (C) Effect of BSP on pravastatin-induced insulin secretion at 3.3 mM (3.3 G) glucose or 20 mM (20 G). Increased GSIS in INS-1e cells seen with pravastatin was abolished by BSP.*P < 0.05, pravastatin vs. control. (D) Total cholesterol (T-chol) contents in INS-1e cells treated or not treated with pravastatin.

In contrast, only the band for oatp3/slco1a5 was detected in INS-1e cells (Fig. 1H). The mRNA quality was confirmed by using GAPDH as a control. These data suggest that there is molecular divergence between tissue and the cell line.

Pharmacological characterization: So far, there are no reports on oatp3/slco1a5-mediated pravastatin transport, although pravastatin is a preferred substrate for the oatp family,5,7) so we examined the pravastatin uptake by oatp3/slco1a5. Using the oocyte expression system, radiolabeled pravastatin uptake was found to be four-fold that of water-injected control oocytes: 0.10 ± 0.007 vs. 0.47 ± 0.001 pmol/oocytes/min at 30 μM pravastatin (P < 0.05).

In vitro effect of pravastatin on INS-1e cells: Based on the expression of rat oatp3/slco1a5 in INS-1e, we next examined the pravastatin uptake by rat insulino-
Fig. 3. Effects of pravastatin on db/db mice
(A) Body weight changes. Open circles, water (n = 6); closed circles, pravastatin-treated mice (n = 6). (B) Plasma levels of total cholesterol (T-cho) and triglycerides (TG) after 1 week and 3 weeks of treatment. Open bars, water (n = 6); closed bars, pravastatin-treated db/db mice (n = 6). (C) Serum adiponectin levels after 4 weeks of treatment (n = 6). (D) Urinary protein after 5 weeks of treatment.*P < 0.05, pravastatin vs. water.

tent of INS-1e cells and found that the cholesterol level is not different between control and pravastatin-treated cells (Fig. 2D). This inhibitory effect of pravastatin transport by BSP was not caused by changes in cell viability because pravastatin and BSP had no effect on cell viability under these conditions (Fig. S2A), although rifampicin had a small toxic effect. Thus, the primary inhibitory effect of pravastatin by BSP may be achieved by uptake inhibition of oatp transporter.

Effect of pravastatin on high-fat loaded db/db mice: To confirm the beneficial effect of pravastatin on the metabolic state, we next examined the effects of pravastatin in diabetic db/db mice. The body weight was similar between pravastatin-treated and control groups (Fig. 3A). It has been reported that pravastatin decreased triglyceride levels without changing plasma cholesterol levels. Clinically, pravastatin administration also increased adiponectin levels and reduced urinary protein excretion. To confirm these effect of pravastatin in a diabetic model, we measured the relevant parameters.

Pravastatin administration in db/db mice significantly decreased triglyceride levels after 3 weeks without changing the plasma cholesterol level (Fig. 3B). The serum adiponectin level in the pravastatin-treated group was also significantly increased (Fig. 3C). In addition, the urinary protein level was markedly reduced in the pravastatin-treated group (Fig. 3D) without evident histological differences (data not shown), confirming the effects of pravastatin in diabetic db/db mice.

Effect of pravastatin on glucose metabolism: We next examined the effect of pravastatin on glucose metabolism in vivo. Pravastatin significantly suppressed the fasting plasma glucose level of high-fat-loaded db/db mice 5 weeks after administration (Fig. 4A). In addition, after 3 weeks of treatment, the serum glucose level dur-
Fig. 4. Effect of pravastatin on the glucose metabolism
(A) Fasting plasma glucose of control (n = 6) and pravastatin-treated db/db mice (n = 6). Open bars, control; closed bars, pravastatin-treated mice. (B) OGTT of db/db mice. (C) ITT of db/db mice. In parts B, C: open circles, water; closed circles, pravastatin-treated mice. (D) The serum level of insulin 0 and 60 min after glucose stimulation in water- or pravastatin-treated db/db mice. In part D: open and meshed bars, water-treated mice (n = 6); closed and hatched bars, pravastatin-treated mice (n = 6). *P < 0.05, pravastatin vs. water; #P < 0.05, 0 min vs. 60 min after glucose stimulation.

Islet Statin Transporters

Insulin secretion from the islets of db/db mice decreased during fat loading (Fig. 4D). Because diabetic mice suffer from pancreatic β-cell dysfunction, a decrease in GSIS in isolated islets from db/db mice is conceivable. However, 4 weeks after administration of pravastatin, the insulin levels compared with the controls increased both at 0 min and 60 min after glucose stimulation. Furthermore, the insulin level of the pravastatin-treated group 4 weeks after administration of pravas was decreased compared to the level at 2 weeks after administration. These data suggest that the improvement of OGTT results by pravastatin is attributed to the protective effect on insulin secretion of high-fat/glucose-loaded β-cells, in addition to improving insulin tolerance.

We next demonstrated the potentiating effect on insulin secretion in vivo by pravastatin in the isolated islets from high-fat/glucose-fed db/db mice. When islets were stimulated by 20 mM glucose, insulin secretion in the pravastatin-treated db/db group was significantly increased (Fig. 5A). Because it has been reported that the cholesterol level in the islet influences the β-cell function, we measured the cholesterol level in the isolated islets. The cholesterol level of pravastatin-treated mice islets was reduced (Fig. 5B). These data further suggest that lipid accumulation hampers insulin secretion under high glucose conditions. We also examined the existence of the oatp family in mouse pancreas. As shown in Figure 5C, mouse oatp2/slco1a4 (left panel) and oatp3/slco1a5 (right panel) mRNA were detected in the pancreas. Immunohistochemical analysis also detected mouse oatp3/slco1a5 immunoreactivity in the islets (Fig. 5D). Because HMG-CoA reductase was detected in the
islets and INS-1e cells (Fig. 5E), these data suggest that pravastatin transported into the islets decreases the cholesterol content following the improvement of insulin secretion. We measured the concentration of cellular cholesterol in INS-1e cells under normal cell culture conditions (Fig. 2D). On the other hand, in Figure 5B, we show results using islets collected from diabetic high-fat-fed db/db mice. The difference in the fat conditions between these two experiments could be responsible for the differences seen in the cellular cholesterol level, suggesting the possibility that statins could be very effective under high-cholesterol condition.

**Discussion**

Statins have a wide variety of pleiotropic effects on endothelial function, smooth muscle proliferation, and vascular inflammation. Pravastatin has a water-soluble nature and therefore it cannot easily penetrate the lipid bilayer without the aid of membrane transport system(s). Recent studies have elucidated the contribution of the oatp family to the membrane transport of pravastatin. Rat oatp1/slc1a1 accepts a broad range of amphipathic compounds, including bile acids, steroids, eicosanoids and peptidomimetics and is localized in the liver and kidney. Rat oatp2/slc1a4 is abundantly expressed in the liver and central nervous system. Rat oatp3/slc1a5 is highly expressed in the kidney, brain and retina and mediates transport of bile acids and thyroid hormones. Concerning pravastatin, recent studies elucidated that both oatp1/slc1a1 and oatp2/slc1a4 contribute to its membrane transport, and here, we report the evidence for oatp3/slc1a5-mediated pravastatin transport.
In histochemical analysis, immunoabsorption with antibodies with antigen peptides reinforced the specificity of the immunoreactivity and the clear expression of the oatp family in the islets (Supplementary Fig. 1). Thus, the existence of the oatp family and its uptake properties suggests that the membrane transport of pravastatin occurs in pancreas and islets (Figs. 1, 5).

In Figure 2A, 300 μM BSP slightly inhibited carrier-mediated pravastatin uptake into INS-1e cells. On the other hand, 30 μM of BSP completely abolished the increase in insulin secretion by pravastatin (Fig. 2B). In the uptake study, INS-1e cells were incubated with BSP with radiolabeled-pravastatin for only 5 min. On the other hand, in GSIS, INS-1e cells were treated with pravastatin for 48 h. The differences in experimental procedures or long-term exposure to inhibitor(s) may possibly yield further metabolic cellular inhibition even at low concentrations. A similar phenomenon in terms of the concentration difference between statin uptake inhibition (~μM order) and the inhibition of cholesterol biosynthesis cascade (~nM order) has been reported.

Further experiments are needed to clarify these differences in different assay systems.

Pravastatin reduced glucose levels during OGTT in db/db mice fed with a high-fat diet (Fig. 4B). Type 2 diabetes is marked by a relative inability of the pancreas to secrete sufficient insulin to meet metabolic demands. Although the pathogenesis of β-cell dysfunction remains obscure, one proposed mechanism is lipotoxicity, in which toxic lipid accumulation leads to β-cell apoptosis and the loss of insulin secretion.

Recently, Brunham et al. reported that lipid accumulation as a result of β-cell-specific disruption of cholesterol transporter ABCA1 reduced insulin secretion and influenced glucose homeostasis and also that lowering the blood glucose level increases insulin secretion. Because the accumulation of cholesterol in β-cells inhibits insulin secretion in the pathway before membrane depolarization, high KCl levels stimulate calcium-dependent increases in both insulin secretion and phospholipase C activation. In our study, high KCl levels also increase insulin secretion (data not shown). The expression of mouse oatp2/slc01a4 and oatp3/slc01a5 in the islets and the reduction of cholesterol content in isolated islets from high-fat-loaded db/db mice treated with pravastatin suggested that the transport of pravastatin and modulation of the cholesterol level in the islets may be responsible for the augmented insulin secretion and reduction of serum glucose levels. In our experiment, the stimulatory effect of pravastatin on insulin secretion occurred when the glucose concentration was high (Fig. 5A). Differences in environmental glucose conditions might have caused differences in the cholesterol level between INS-1e cells and isolated islets. In addition, there was no significant difference in the size of islet area between pravastatin-treated and non-treated mice (Supplementary Fig. 2B). These data further suggest the pleiotropic effect of pravastatin.

In conclusion, these findings suggest that the uptake of pravastatin into β-cells via oatp transporter(s) contributes to insulin secretion. Further studies on human transporter would be helpful to understand these effects in patients suffering from diabetes and obesity.

Acknowledgments: We thank Ms. Izumi Nakamura for secretarial assistance. This study was funded in part by research grants from the Ministry of Education, Science and Culture of Japan.

References


5) Tokui, T., Nakai, D., Nakagomi, R., Yawo, H., Abe, T. and


