Pitavastatin, a Potent Hydroxymethylglutaryl Coenzyme A Reductase Inhibitor, Increases Cholesterol 7-α-Hydroxylase Gene Expression in HepG2 Cells

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Background  The effect of pitavastatin on the mRNA levels of apolipoprotein (apo) A-I, peroxisome proliferator-activated receptor (PPAR), cholesterol 7α-hydroxylase (CYP7A1), and farnesoid X receptor (FXR) in HepG2 cells was examined to establish whether pitavastatin affects bile acid synthesis and if so, to determine a possible molecular mechanism.

Methods and Results  HepG2 cells were cultured in serum-free Dulbecco’s modified Eagle medium for 18 h before drug treatment. Total RNA was extracted at set times and mRNA levels were quantified by reverse transcription-real time polymerase chain reaction. Pitavastatin at 0.1, 1, 5, and 10 μmol/L increased the mRNA levels of apo A-I, PPAR, CYP7A1, and FXR in a dose-dependent manner. The mRNA levels of apo A-I, PPAR, CYP7A1, and FXR similarly increased with increasing doses of pitavastatin. Coincubation of mevalonate (4 mmol/L) with pitavastatin (5 μmol/L) reversed the inductive effects of pitavastatin on the mRNA levels of these genes, indicating that the inductive effects of pitavastatin were related to its inhibition of HMG-CoA reductase.

Conclusions  Pitavastatin increased the mRNA levels of CYP7A1 in HepG2 cells, suggesting that increased conversion of cholesterol to bile acids may be the mechanism for its potent low-density lipoprotein cholesterol-lowering effects. (Circ J 2004; 68: 1061 – 1066)

Key Words: CYP7A1; FXR; HepG2 cells; Low-density lipoprotein cholesterol; Pitavastatin; PPAR

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lowering cholesterol by statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is a potent approach to preventing coronary heart disease (CHD).1–4 Although their ability to reduce the morbidity and mortality associated with CHD has been well documented in several randomized clinical trials5,6 and large cohort studies7,8 monotherapy with first- and second-generation statins (ie, lovastatin, pravastatin, simvastatin, and fluvastatin) does not always achieve the therapeutic target for low-density lipoprotein cholesterol (LDL-C)7–9 in patients with familial hypercholesterolemia (FH).9,10 Among the third-generation of statins, the newly developed so-called super-statins, rosuvastatin and pitavastatin (ie, NK-104, itavastatin, nisvastatin), have been shown to produce even greater reductions in LDL-C and pitavastatin (ie, NK-104, itavastatin, nisvastatin), have been shown to produce even greater reductions in LDL-C and LDL-C.20 The reduction in the concentration of LDL-C in the absence of the LDL-R gene markedly decreases plasma concentrations of VLDL-C and LDL-C. In mice lacking both LDL-R and apolipoprotein (apo) E,22 and (3) overexpression of hepatic CYP7A1 in mice lacking the LDL-R gene markedly decreases plasma concentrations of VLDL cholesterol (VLDL-C) and LDL-C.23 The reduction in the concentration of LDL in the absence of the LDL-R gene or function by increasing CYP7A1 was caused, in large part, by intrahepatic diversion of cholesterol destined for circulating (VLDL and LDL) lipoproteins in plasma to bile acid synthesis and, therefore, to a decrease in the rate of LDL-C transfer into the plasma space.23 Pitavastatin has been shown to decrease apo B-100 secretion from HepG2 cells through increased intracellular degradation of newly synthesized protein24 and to strongly reduce VLDL secretion in WHHL rabbits25 and guinea pigs.26 Because of the striking parallel between the effects

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of pitavastatin and bile acid depletion on cholesterol metabolism in the absence of LDL-R, we hypothesized the existence of a common mechanism involving CYP7A1.

Because of the importance of CYP7A1 in cholesterol homeostasis, the effects of the inhibition of HMG-CoA reductase by old-generation statins on the regulation of bile acid synthesis in humans have been investigated in previous studies, but the results are controversial.27–32 Lovastatin had an inhibitory effect on CYP7A1 activity in rat liver;31,33 pravastatin had no significant effect on CYP7A1 activity in human subjects;34 simvastatin decreased CYP7A1 mRNA levels in guinea pigs35 but did not affect 7α-hydroxylation rates in human subjects;36 atorvastatin decreased CYP7A1 mRNA levels in guinea pigs35 showed no significant effects on plasma 7α-hydroxy-4-cholesten-3-one, a validated marker of bile acid synthesis, in FH heterozygotes39 and enhanced the effects of IBAT inhibition by PR835 on CYP7A1 mRNA and LDL-C levels in mice lacking both LDL-R and apo E.22 However, the superstatin, pitavastatin, has been shown to slightly increase the level of CYP7A1 mRNA in guinea pigs35 but there is still no information available regarding its effects on CYP7A1 in humans.

The enzymatic conversion of cholesterol into bile acids is regulated through feedback suppression by bile acids18,37 and feedforward induction by cholesterol18,38,39 mediated by the orphan nuclear receptors, farnesoid X receptor (FXR) and liver X receptor (LXR), respectively.18,40 The overexpression of peroxisome proliferator-activated receptor (PPAR), a nuclear receptor that regulates fatty acid metabolism, inhibits the transcription of the CYP7A1 gene in HepG2 cells44,45 and the activation of PPARα by fibrates or the PPARα agonist Wy14643 also decreases CYP7A1 activity or mRNA levels in humans and animals.46–48 Pitavastatin has been shown to increase apo A-I mRNA levels by increasing PPARα activity in HepG2 cells.49

Therefore, we examined the effects of pitavastatin on the mRNA levels of apo A-I, PPARα, CYP7A1, and FXR in HepG2 cells, an established model for studies of the regulation of the human CYP7A1 gene at the molecular level.42,50

### Methods

#### Materials

Pitavastatin (NK-104) was kindly provided by Kowa Inc, Fukuoka, Japan; DL-mevalonic acid lactone was purchased from Sigma (St Louis, MO, USA); human hepatoma HepG2 cells were obtained from RIKEN Cell Bank (RCB 0459, Japan); bovine serum albumin (BSA: fatty acid-free grade) was purchased from TRACE Biosciences NZ Ltd (New Zealand); and Dulbecco’s Modified Eagle Medium (DMEM) and other culture supplements were purchased from Gibco BRL (Invitrogen, Carlsbad, CA, USA).

#### Cell Culture and RNA Preparation

Human hepatoma HepG2 cells were maintained in DMEM containing 100units/ml penicillin and 100μg/ml streptomycin, and supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2. At approximately 60–70% confluence, the medium was replaced by a serum-free medium containing 0.5% BSA (fatty acid-free) for 18 h before treatment with NK-104 to avoid interference by serum components. Pitavastatin was dissolved in water. A stock solution of mevalonate salt (0.1 mol/L) was made by incubating DL-mevalonic acid lactone with 0.1N NaOH at 50°C for 2h51 and stored at −30°C until use. Cells were subsequently incubated with the indicated compounds for the indicated period of time. At the end of the treatment period, total RNA was extracted using RNAzol B Reagents (TEL-TEST, Inc, Friendswood, USA) according to the manufacturer’s instructions.

#### Quantification of mRNA Levels by Reverse Transcription (RT) and Real-Time Polymerase Chain Reaction (PCR)

cDNA was reverse-transcribed from the total RNA using a TaqMan Reverse Transcription Reagent Kit (PE Applied Biosystems, Roche Molecular Systems, Inc, NJ, USA). Oligo d(T) 16 was used for the primers. The RT reaction was performed in a reaction volume of 30μl at 25°C for 10min, 48°C for 30min, and 95°C for 5min (GeneAmp PCR System 9600, Perkin Elmer, USA), as described previously.52 Oligonucleotide primers for PCR amplification were designed from the GenBank cDNA sequences of the human apo A-1 (accession no. XM006435), PPARα (accession no. NM005036), CYP7A1 (accession no. NM000780), and FXR (accession no. NM005123) genes using Primer Express (Version 1.0, PE Applied Biosystems). Primers for a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from PE Biosystems (Warrington, UK). Real-time PCR of apo A-I, PPARα, CYP7A1, FXR, and GAPDH was performed using SYBR Green PCR Core Reagents (PE Biosystems) in the Gene

| Table 1 Sequences of the Primers Used for the RT-PCR Assays |
|-----------------|------------------|------------------|
| mRNA species    | Sense primers    | Antisense primers | Size of PCR product (bp) |
| Apo A-I         | 5'-CGTGATGTCCTGCAAGAGCAG-3' | 5'-GAGCCCTCAACTGGGACAC-3' | 56 |
| PPARα           | 5'-CTTCTGTCCTCGTGGGACTCA-3' | 5'-CCTCCAGAAGCTTGGTAGAC-3' | 79 |
| CYP7A1          | 5'-CAACCTATGAGACCTCCTCCAGTC-3' | 5'-CAGTCCTAAACATCAGCCTGGTAG-3' | 102 |
| FXR             | 5'-GAGCCCTTCCCTTTCGTTGCTACA-3' | 5'-TCTCCATGACATCAGCTAGCAGTCAG-3' | 143 |
| GAPDH           | 5'-GAAGGTGAGGTTCGGAGTGC-3' | 5'-GAAGATGTTGAGGTTGATTCT-3' | 228 |

Apo, apolipoprotein; PPARα, peroxisome proliferator-activated receptor; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Circulation Journal Vol.68, November 2004

Pitavastatin Increases CYP7A1 in HepG2 Cells

Amp 5700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with the primers described in Table 1. The amplification reactions were performed in a final volume of 50 l, with thermal cycling conditions of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, 15 s at 58°C, and 1 min at 72°C, as described previously.52 Samples were assayed in triplicate. The relative standard curve method was used to quantitate apo A-I, PPARα, CYP7A1, FXR, and GAPDH mRNA levels.53

Statistical Analysis
All of the statistical analyses were performed using the SAS (Statistical Analysis System) Software Package (Version 8.2, SAS Institute Inc, Cary, NC, USA) in Fukuoka University. Data are presented as the mean ± standard deviation. Significant effects of pitavastatin on gene expression were examined by an analysis of variance (ANOVA). The significance level was considered to be 5% unless indicated otherwise.

Results

Dose-Dependent Effects of Pitavastatin on mRNA Levels of Apo A-I, PPARα, CYP7A1, and FXR in HepG2 Cells

Human HepG2 cells were treated with different concentrations of pitavastatin (0.1, 1, 5, 10 l/mol/L) for 24 h, and the mRNA levels of apo A-I, PPARα, CYP7A1, and FXR were quantified. Treatment with pitavastatin increased the mRNA levels of apo A-I, PPARα, CYP7A1, and FXR in a dose-dependent manner, as assessed by an ANOVA (Fig 1). As shown in Fig 1, pitavastatin at 0.1 l/mol/L was already effective at increasing the apo A-I, PPARα, and CYP7A1 mRNA levels. Pitavastatin at 10 l/mol/L had the greatest effect on apo A-I, PPARα, CYP7A1, and FXR mRNA levels. The mRNA levels of apo A-I, PPARα, CYP7A1, and FXR similarly increased with increasing doses of pitavastatin, which suggests that pitavastatin not only increases reverse cholesterol transport by increasing apo A-I, but also may increase the conversion of cholesterol to bile acid by increasing CYP7A1.

Effects of Mevalonate on the Induction of Apo A-I, PPARα, CYP7A1, and FXR mRNA Levels by Pitavastatin in HepG2 Cells

To demonstrate that the increase in the apo A-I, PPARα, CYP7A1, and FXR mRNA levels with pitavastatin treatment is caused by the inhibition of HMG-CoA reductase by pitavastatin, we examined the effects of mevalonate (4 mmol/L), the product of HMG-CoA reductase, on the induction of apo A-I, PPARα, CYP7A1, and FXR mRNA levels by pitavastatin (5 l/mol/L). As shown in Fig 2, the apo A-I, PPARα, and CYP7A1 mRNA levels in cells treated with pitavastatin at 5 l/mol/L were higher than those in untreated cells. Simultaneous incubation of pitavastatin with mevalonate completely reversed the effects of pitavastatin on the apo A-I, PPARα, CYP7A1, and FXR mRNA levels (Fig 2). This result indicates that the inductive effects of pitavastatin on apo A-I, PPARα, CYP7A1, and FXR mRNA were caused by its inhibition of HMG-CoA reductase.

Discussion

The present study examined the effects of pitavastatin on mRNA levels of CYP7A1 in HepG2 cells and a surprising result was that pitavastatin dose-dependently increased CYP7A1 mRNA levels and mevalonate reversed the inductive effects of pitavastatin on CYP7A1 mRNA. This suggests that pitavastatin increases the conversion of cellular cholesterol to bile acids, which may contribute to its potent...
cholesterol-lowering effects. Our finding supports that of Aoki et al who reported that pitavastatin slightly increases CYP7A1 mRNA levels in guinea pigs\textsuperscript{35} and is in agreement with that of Galman et al\textsuperscript{22} who reported that atorvastatin enhances the effect of IBAT inhibition on CYP7A1 mRNA levels in mice lacking both LDL-R and apo E. However, our finding does not agree with those of other authors who have reported no significant effects of lovastatin\textsuperscript{54} pravastatin\textsuperscript{34} simvastatin\textsuperscript{35,36} or atorvastatin\textsuperscript{30,35} on CYP7A1.

The factors that contribute to the differences in the effects of the different statins on CYP7A1 are not fully understood. First, pitavastatin differs from simvastatin\textsuperscript{55} and pravastatin\textsuperscript{56} in both its potency in inhibiting sterol synthesis and the duration of this effect\textsuperscript{55} Suzuki et al showed in the guinea pig that pitavastatin at 1 mg/kg decreased hepatic sterol synthesis at 3 h to a similar extent as simvastatin at 15 mg/kg and that the effect of pitavastatin lasted up to 9 h whereas that of simvastatin was attenuated after 6 h and disappeared after 9 h\textsuperscript{53} Indeed, Pandak et al showed that CYP7A1 activity in rat was maximally decreased by lovastatin between 3 and 6 h and recovered after 27 h, and that the reduction of bile acid synthesis was closely correlated with the changes in the activity of CYP7A1\textsuperscript{33} However, there is no firm evidence that the half-lives of these drugs are related to their effects on CYP7A1. In fact, Aoki et al reported that both the long-lasting statins atorvastatin and simvastatin decreased CYP7A1 mRNA levels in guinea pigs\textsuperscript{35} Second, pitavastatin is more efficient at increasing the LDL-R in HepG2 cells than other statins, including pravastatin and atorvastatin\textsuperscript{17} Mazzella et al, who showed that simvastatin had no significant effect on bile acid synthesis in patients with nonfamilial hypercholesterolemia, suggested that the inhibition of cholesterol synthesis is not sufficient to reduce bile acid synthesis that may largely depend on the available preformed intrahepatic cholesterol\textsuperscript{57} In addition, the inhibitory effects of lovastatin on CYP7A1 activity in rats were abolished by cholesterol in the diet\textsuperscript{11} However, it is not likely that the effects of statins on CYP7A1 depend on their effects on the LDL-R, considering that atorvastatin enhanced the effects of IBAT inhibition on CYP7A1 mRNA levels in mice lacking both LDL-R and apo E\textsuperscript{52} and decreased the rate of bile acid synthesis when that bile acid was upregulated by either partial ileal bypass or bile acid sequestrants in patients with heterogeneous FH\textsuperscript{30} Third, pitavastatin differs from simvastatin in its effects on VLDL secretion. Pitavastatin at 3 mg/kg decreased VLDL-triglyceride and VLDL-apo B secretion in the guinea pig, whereas simvastatin at 30 mg/kg had no significant effect\textsuperscript{55} Although our finding supports the hypothesis that the suppressive effect of pitavastatin on VLDL secretion may be attributable to an increased conversion of cholesterol to bile acids, it is not clear whether or not the results obtained in vitro may be applied to the pathophysiology in human subjects, in whom bile acids are continuously reabsorbed in the intestine.

Feedback regulation of CYP7A1 by bile acids is known to be mediated by FXR and activation of FXR suppresses the CYP7A1 gene\textsuperscript{18} However, we observed a simultaneous increase in mRNA levels of FXR and CYP7A1, and the reason for this result is unclear. It is possible that some other factor(s) that counterbalances the suppressive effects of FXR may exist in the mevalonate/cholesterol pathway. Further investigations are needed to clarify this point.

It has been reported that overexpression of PPAR\textsuperscript{α} and the activation of PPAR\textsuperscript{α} by fibrates or Wy14643 decrease CYP7A1 mRNA levels and enzyme activity\textsuperscript{44–48} Our finding that pitavastatin treatment dose-dependently increased apo A-I mRNA levels in HepG2 cells agrees with that of Martin et al\textsuperscript{49} The in vitro data agree with the finding that pitavastatin treatment increases plasma apo A-I levels in patients with primary hypercholesterolemia\textsuperscript{6} Our finding that pitavastatin also increases the PPAR\textsuperscript{α} mRNA levels agrees with that of Martin et al who found that pitavastatin enhances PPAR\textsuperscript{α} activity in rabbit kidney RK13 cells\textsuperscript{49} Therefore, PPAR\textsuperscript{α} did not appear to have an inhibitory effect on the CYP7A1 gene under our experimental conditions, which supports the finding by other authors that PPAR\textsuperscript{α} is unlikely to be a major influence on CYP7A1.
activity under normal physiological conditions. In conclusion, the present study shows that pitavastatin increases the CYP7A1 mRNA levels in HepG2 cells, which suggests a novel mechanism for the potent LDL-C-lowering effects of pitavastatin (ie, increased conversion of cellular cholesterol into bile acids). However, the effects of pitavastatin on bile acid synthesis in human subjects warrant further investigation.

A low levels of high-density lipoprotein cholesterol (HDL-C) is a known risk-factor for CHD and the balance between HDL and LDL is important in predicting the risk of CHD. Therefore, approaches to raising high-density lipoprotein (HDL) have attracted much attention. Although the ability of statins to raise HDL is limited, the inhibition of cholesterol ester transfer protein (CETP) by small molecules, including JTT-705 and torcetrapib, has been shown to effectively increase HDL levels in humans with normal and low HDL-C levels. Therefore, pitavastatin could be a promising basal drug for complementary CETP inhibitors. In addition, the combination of a statin and a bile acid sequestrant, which indirectly depletes the intrahepatic C pool, a promising basal drug for complementary CETP inhibitors. Pitavastatin Increases CYP7A1 in HepG2 Cells molecules, including JTT-705 and torcetrapib, has been shown to increase HDL levels in humans with normal and low HDL-C levels. Therefore, pitavastatin could be a promising basal drug for complementary CETP inhibitors. In addition, the combination of a statin and a bile acid sequestrant, which indirectly depletes the intrahepatic C pool, is a promising basal drug for complementary CETP inhibitors. Therefore, our finding would be clinically important if confirmed in human subjects because a greater synergistic effect of bile acid sequestering resin or ezetimibe on LDL-C levels could be expected in subjects treated with pitavastatin, compared to those taking other statins.

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