Pitavastatin Increases ABCA1 Expression by Dual Mechanisms: SREBP2-Driven Transcriptional Activation and PPARα-Dependent Protein Stabilization but Without Activating LXR in Rat Hepatoma McARH7777 Cells

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Abstract. Hepatic ATP-binding cassette transporter A1 (ABCA1) plays a key role in high-density lipoprotein (HDL) production by apolipoprotein A-I (ApoA-I) lipidation. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, increase ABCA1 mRNA levels in hepatoma cell lines, but their mechanism of action is not yet clear. We investigated how statins increase ABCA1 in rat hepatoma McARH7777 cells. Pitavastatin, atorvastatin, and simvastatin increased total ABCA1 mRNA levels, whereas pravastatin had no effect. Pitavastatin also increased ABCA1 protein. Hepatic ABCA1 expression in rats is regulated by both liver X receptor (LXR) and sterol regulatory element–binding protein (SREBP2) pathways. Pitavastatin repressed peripheral type ABCA1 mRNA levels and its LXR-driven promoter, but activated the liver-type SREBP2-driven promoter, and eventually increased total ABCA1 mRNA expression. Furthermore, pitavastatin increased peroxisome proliferator–activated receptor α (PPARα) and its downstream gene expression. Knockdown of PPARα attenuated the increase in ABCA1 protein, indicating that pitavastatin increased ABCA1 protein via PPARα activation, although it repressed LXR activation. Furthermore, the degradation of ABCA1 protein was retarded in pitavastatin-treated cells. These data suggest that pitavastatin increases ABCA1 protein expression by dual mechanisms: SREBP2-mediated mRNA transcription and PPARα-mediated ABCA1 protein stabilization, but not by the PPAR–LXR–ABCA1 pathway.

Keywords: pitavastatin, ATP-binding cassette transporter A1 (ABCA1), sterol regulatory element–binding protein (SREBP2), peroxisome proliferator–activated receptor α (PPARα), McARH7777

Introduction

High-density lipoprotein (HDL) plays a central role in transporting cholesterol from extrahepatic tissues to the liver for its catabolism to bile acids (1, 2), and it is thought to contribute to removal of cholesterol from peripheral tissues and possibly decreasing its deposition in atherosclerotic lesions (3, 4). Apolipoprotein A-I (ApoA-I) is a major protein component of HDL. In adult organisms, ApoA-I is synthesized and secreted by the liver and small intestine (5, 6). ATP-binding cassette transporter A1 (ABCA1) is the major transporter for cholesterol ef-flux from peripheral tissues and lipidation of ApoA-I in the liver, and it is the key determinant of plasma HDL levels, as shown in Tangier disease and ABCA1-deficient mice (7–9). Recent studies have shown that hepatic ABCA1 has the most important role in maintaining plasma HDL. It has been reported that selective knock-
down of hepatic ABCA1 in mice induces low HDL level, and overexpression of hepatic ABCA1 in mice increases plasma HDL level (10). Therefore, it is assumed that hepatic ABCA1 is essential for the first step in the reverse cholesterol transport pathway.

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductases, the rate-limiting step in cholesterol synthesis (11). As a result of statin treatment, intracellular cholesterol concentrations are decreased, which leads to activation of the transcription factor, sterol responsive element–binding protein-2 (SREBP2), which regulates several genes controlling cholesterol homeostasis, including low-density lipoprotein (LDL) receptor (12, 13). Plasma LDL concentrations are decreased as a consequence of increased LDL clearance. In addition, statin treatment increases plasma HDL levels; however, the mechanism of this effect is still unclear (14, 15). Several lines of evidence have suggested that statins increase HDL by directly regulating genes related to HDL biogenesis. Statins up-regulated ABCA1 mRNA and ApoA-I mRNA in a hepatoma cell line, despite downregulated cholesterol ester transfer protein (CETP) mRNA (16 – 18). However, its detailed mechanism, especially ABCA1 regulation by statins, was not well elucidated. Hepatic ABCA1 expression is regulated by a dual promoter system driven by SREBP2 and liver X receptor (LXR), and this system is liver-specific (19). Statins increased ABCA1 mRNA levels in hepatoma cell lines, although ABCA1 mRNA levels were reduced in macrophage or fibroblast cell lines (16, 17, 20). Here, we investigated the mechanism of hepatic ABCA1 regulation by statins using the rat hepatoma cell line McARH7777 cells.

Materials and Methods

Compounds

Patavastatin was supplied by Nissan Chemical industries (Tokyo). Simvastatin, atorvastatin, and pravastatin were isolated from drug preparations obtained from local pharmacies.

Cells and reagents

The rat hepatoma McARH7777 cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum (FCS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37°C in 5% CO₂.

RNA isolation and analysis of gene expression by real-time quantitative PCR

McARH7777 cells were treated with statins for 24 h in DMEM containing 20% FCS. Total RNA was isolated from cells using Isogen reagent (Wako, Osaka) according to the manufacturer’s protocol. Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) using Taqman and SYBR green. The primer/probe sequences used were as follows: rat total ABCA1 forward (ccccggcggagtagaaagg), reverse (agggcagtcacaaaaagac), probe [6-carbonylfluorescein (FAM)–tctttgctatgttcctgccgce–6-carboxytetramethylrhodamine (TAMRA)]; rat peripheral type (type P) ABCA1 forward (tctgctccctgtcccccac), reverse (ccaacccctacacaaaccct), probe (FAM-ccctactttttctcctgcttgt-TAMRA); rat HMG-CoA synthase forward (ggtagatgctggaaagtataccattgg), reverse (tc ggcagtcttgccaga), probe (FAM-tgggccaggccaggatgggt-TAMRA); rat β-actin forward (agccatgtacgtagccatcca), reverse (tcaccggagtccatcacaatg), probe (VIC-tgtccctgtatcccctctgtcc-TAMRA); rat CPT1 forward (gtctgcgtggataacagtaa), reverse (tttggttcaggctggattg); rat peroxisome proliferator–activated receptor α (PPARα) forward (ccgggctgtgggctggctggggggggg), reverse (cccaagctctatcatcttcgctgttctg); rat ACO forward (ccgcaaggagggaggttggc), reverse (aagtcagaggatcaccag).

RNA interference

The duplexes of each Stealth select small interfering RNA (siRNA) targeting PPARα and negative control (nonsilencing siRNA) were purchased from Invitrogen. For transfection, McARH7777 cells were cultured overnight at a density of 1.5 × 10⁵ cells/well in 24-well culture plates and then siRNA transfection was performed using Lipofectamine 2000 RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. After 24 h, the medium was changed to medium containing statin. After another 24 h, the cells were washed with phosphate-buffered saline (PBS) and processed for total RNA isolation and whole-cell extracts.

Immunoblotting analysis

Protein samples were separated by 10% SDS-PAGE and transferred electrotherophoretically onto polyvinylidene fluoride membranes (ProBlott, Applied Biosystems). Membranes were blocked with 5% (wt/vol) nonfat milk in PBS containing 0.1% Tween for 1 h, incubated with antibodies for 1 h, and detected by chemiluminescence using West Dura extended duration substrate (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol.
**Transient transfection and enzymatic assay**

McARH7777 cells were cultured at a density of 1.5 × 10^5 cells/well in 24-well tissue culture plates overnight, and transfection was performed using FuGENE HD (Roche, Mannheim, Germany) according to the manufacturer’s protocol. The type P (−1040/+18) and liver type (type L) (−950/+123) ABCA1 promoter fragments were amplified by using rat tail genomic DNA as a template. The primers for the PCR amplifications were designed based on the nucleotide sequences type P forward (aaagcgtctcatctgccgaactgta), type P reverse (ctaatcttctgctctegaatata), type L forward (aaagcgctctatctcaagccac), and type L reverse (caagctcttaaagcagaccaagcatg). The amplicons were subcloned into the firefly luciferase expression vector pGL3-Basic (Promega, Madison, WI, USA) as described previously (19). The reporter construct (0.3 μg/well) and a pCMV β-galactosidase plasmid (0.01 μg/well) as an internal control were used to transfect cells in each well. After 24 h, the medium was changed to the medium containing PBS and assayed for luciferase activity using luciferase assay systems. Luciferase activities (relative light units) were normalized relative to β-galactosidase activity (optical density units).

**ABCA1 stabilization assay**

McARH7777 cells were cultured at a density of 3 × 10^5 cells/well in 12-well tissue culture plates for 3 days, and then the cells were treated with pitavastatin (10 μM) for 12 h prior to cycloheximide (100 μg/mL) treatment. After cycloheximide treatment for the indicated time, cells were washed with PBS and assayed for ABCA1 protein level. The detected signal was quantitated using Image J software from NIH Image.

**Antibodies**

Antibodies recognizing β-actin, ABCA1, and PPARα were obtained from Sigma, Novus Biologicals (Littleton, CO, USA), and Perseus Proteomics Inc. (Tokyo), respectively.

**Results**

**Statins increase ABCA1 mRNA levels and protein expression in McARH7777 cells**

McARH7777 cells were treated for 24 h with various concentrations of statin (0.1 – 10 μM). Statins modestly increased the level of ABCA1 (Fig. 1A) (Supplementary Fig. 1: available in the online version only). The order of potency of the induction by statins on expression was as follows: pitavastatin (1 μM) > simvastatin = atorvastatin (10 μM), but pravastatin had no effect on ABCA1 mRNA expression until 10 μM. In addition, pitavastatin markedly increased ABCA1 protein level in McARH7777 cells (Fig. 1B).

**Pitavastatin reduces type P ABCA1 mRNA levels and the corresponding promoter activity, but increases total ABCA1 mRNA levels via activating type L promoter**

It has been reported that rat hepatic ABCA1 expression is regulated by a dual-promoter system involving SREBP2 and LXR (19) (Fig. 2A). The type L ABCA1 mRNA regulated by SREBP2 is expressed exclusively in hepatocytes, whereas the type P ABCA1 mRNA regulated by LXR is widely expressed in both peripheral tissue and hepatocytes (19). Pitavastatin reduced type P ABCA1 mRNA expression, but total ABCA1 mRNA levels were increased (Fig. 2B).

Increased HMG-CoA synthase mRNA levels suggest that SREBP2 was activated by pitavastatin (Fig. 2B). However, as type L and type P ABCA1 mRNAs share the same sequences with the exception of exon 1, we were unable to measure the type L mRNA levels directly (19). Therefore, we investigated type L ABCA1 promoter activity by reporter assay in McARH7777 cells. Figure 2C shows that pitavastatin increased the type L ABCA1 promoter activities regulated by SREBP2, but this effect was abolished if the SREBP2-binding domain was mutated. Figure 2D shows that pitavastatin reduced the type P ABCA1–promoted activities regulated by LXR, but this effect was also abolished if the LXR-binding domain was mutated. These results show that pitavastatin reduced type P promoter activities and type P ABCA1 mRNA levels, but activated the type L ABCA1 promoter by activating SREBP2, leading to an increase in total ABCA1 mRNA expression.

Time course shows that increases in ABCA1 mRNA and protein levels were observed as early as 2 h after the addition of pitavastatin to the cell medium, which coincides with an increase in HMG-CoA synthase mRNA expression, while a decrease in type P ABCA1 mRNA expression peaked at 4 h (Supplementary Fig. 2: available in the online version only).

**Pitavastatin increases PPARα, ACOX and CPT1 mRNA levels in McARH7777 cells**

Pitavastatin was reported to activate PPARα in addition to SREBP2 (21). In McARH7777 cells, pitavastatin increased mRNA levels of PPARα, ACOX, and CPT1, which are downstream genes of PPARα (Fig. 3). These results showed that pitavastatin increased PPARα activities in this cell line.
Pitavastatin increases ABCA1 protein via PPARα activation

PPARα agonists have been reported to increase ABCA1 protein levels in hepatocytes and macrophages (22, 23). Therefore, we investigated whether PPARα activation contributes to pitavastatin-induced ABCA1 protein expression. As shown in Fig. 4A, knockdown of PPARα abolished the increase in ABCA1 protein by pitavastatin.

Notably, the PPARα agonist WY14643 increased ABCA1 protein level in McARH7777 cells, but ABCA1 mRNA expression was not induced (Fig. 4: B, C). Type P ABCA1 mRNA levels were significantly increased by WY14643, but total ABCA1 mRNA levels were unaffected. Although the LXR agonist TO901317 robustly increased type P ABCA1 mRNA levels (by 8-fold), total ABCA1 mRNA level was increased by only 1.5-fold. Because the type L promoter activity is unaffected by TO901317 (19), these data indicate that the type P ABCA1 mRNA level accounts for ca. 10% of total ABCA1 mRNA level. We also observed that fenofibric acid increased ABCA1 protein level, although ABCA1 mRNA levels were unchanged (data not shown). These results suggested that PPARα activation increases ABCA1 protein levels without inducing ABCA1 mRNA expression in this cell line.

Pitavastatin increases ABCA1 protein levels by retarding its degradation

To investigate whether pitavastatin increased ABCA1 protein levels by preventing its degradation, ABCA1 protein turnover was monitored. McARH7777 cells were treated with or without pitavastatin for 12 h, and then protein synthesis was blocked with cycloheximide. In control cells, ca. 25% of ABCA1 protein was degraded 6h after cycloheximide treatment, whereas degradation of ABCA1 protein in pitavastatin-treated cells was retarded (Fig. 5). These results suggest that pitavastatin protects ABCA1 protein from its degradation.

Taken together, the results of this study indicated that statins, especially pitavastatin, enhanced ABCA1 expression by dual mechanisms: SREBP2-mediated ABCA1 mRNA transcription and PPARα-mediated ABCA1 protein stabilization. However, the PPAR–LXR–ABCA1 pathway appears not to operate in this response because LXR-dependent transcription was repressed by pitavastatin.
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A) Schematic mechanism of rat hepatic ABCA1 regulation by LXR and SREBP2. Type P promoter: LXRE → ATG → Exon 1. Type L promoter: SRE → Exon 2 → ABCA1 gene. Total ABCA1 mRNA: Type P ABCA1 mRNA: Type L ABCA1 mRNA:

B) McARH7777 cells were treated for 24 h with various concentrations of pitavastatin (0 – 10 μM). Total RNA was isolated and determined by real-time quantitative PCR as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001, Dunnett’s test. C, D) McARH7777 cells transfected with type L and type P ABCA1 promoter luciferase plasmid together with β-galactosidase plasmid were treated for 24 h with various concentrations of pitavastatin (0 – 10 μM). Cell extracts were isolated and examined by the luciferase assay as described in Materials and Methods. Luciferase activities (relative light units) were normalized relative to β-galactosidase activity (optical density units). Vertical lines indicate the S.D. (n = 3).

Fig. 2. Pitavastatin reduces type P ABCA1 promoter activity and type P mRNA expression but increases type L ABCA1 promoter activity and total ABCA1 mRNA levels. A) Schematic mechanism of rat hepatic ABCA1 regulation by LXR and SREBP2. B) McARH7777 cells were treated for 24 h with various concentrations of pitavastatin (0 – 10 μM). Total RNA was isolated and determined by real-time quantitative PCR as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001, Dunnett’s test. C, D) McARH7777 cells transfected with type L and type P ABCA1 promoter luciferase plasmid together with β-galactosidase plasmid were treated for 24 h with various concentrations of pitavastatin (0 – 10 μM). Cell extracts were isolated and examined by the luciferase assay as described in Materials and Methods. Luciferase activities (relative light units) were normalized relative to β-galactosidase activity (optical density units). Vertical lines indicate the S.D. (n = 3).
Fig. 3. Pitavastatin increases PPARα, ACOX, and CPT1 mRNA levels in McARH7777 cells. McARH7777 cells were treated for 24 h with various concentrations of pitavastatin (0 – 10 μM). Total RNA was isolated and determined by real-time quantitative PCR as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001, Dunnett’s test.

Fig. 4. Pitavastatin increases ABCA1 protein via PPARα activation. A) McARH7777 cells were treated with PPARα siRNA for 24 h prior to pitavastatin (10 μM) treatment. Cells were exposed to pitavastatin for 24 h. Total protein was isolated and determined by Western blotting as described in Materials and Methods. B) McARH7777 cells were treated for 24 h with WY14643 (100 μM). Protein was isolated and examined by Western blotting as described in Materials and Methods. C) McARH7777 cells were treated for 24 h with WY14643 (100 μM). Total RNA was isolated and determined by real-time quantitative PCR as described in Materials and Methods. Vertical lines indicate the S.D. (n = 3), *P < 0.05, ***P < 0.001, Dunnett’s test.
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Discussion

Hepatic ABCA1 plays a major role in maintaining plasma HDL levels, and liver-specific ABCA1 knockout in mice causes 80% reduction in plasma HDL (10, 24). In this study, we showed that in rat hepatoma McARH7777 cells pitavastatin increased ABCA1 expression through dual mechanisms: SREBP2-mediated mRNA induction and PPARα-mediated enhancement of protein stabilization. However, the PPAR–LXR–ABCA1 pathway, a known mechanism for PPAR agonist–induced ABCA1 expression (22, 23, 25), did not contribute to the pitavastatin-mediated ABCA1 induction because pitavastatin repressed LXR activity in McARH7777 cells (Fig. 6).

Hepatic ABCA1 transcription is regulated by SREBP2 as well as LXR (19). In this study, pitavastatin was shown to activate the type L ABCA1 promoter in a manner dependent on SREBP2-binding element (SRE), thereby increasing type L ABCA1 mRNA expression, while LXR-regulated type P ABCA1 mRNA was diminished by pitavastatin (Fig. 2: B, C, D), eventually leading to an increase in total ABCA1 mRNA levels. The type P promoter activity was reduced in a LXR response element (LXRE)-dependent manner (Fig. 2D), indicating that LXR activity was repressed by pitavastatin. Pitavastatin is likely to diminish the LXR activity, probably by inhibiting synthesis of endogenous sterol LXR ligands, as reported previously (26).

Pitavastatin has been reported to increase PPARα activation by inhibiting PPARα phosphorylation (21). Indeed, we found that pitavastatin increased the mRNA levels of PPARα, ACOX, and CPT1, which are downstream of PPARα, in McARH7777 cells (Fig. 3). In addition, knockdown of PPARα diminished pitavastatin-induced ABCA1 expression (Fig. 4A), clearly indicating that PPARα activation is involved in the action of pitavastatin. Activation of PPARα is known to induce LXR expression, leading to augmentation of ABCA1 transcription. This PPAR–LXR–ABCA1 pathway has been shown to explain the mechanism of PPARγ- and PPARα-agonist–mediated ABCA1 induction (22, 23, 25). Indeed, the PPARα agonist fenofibrate was shown to increase ABCA1 mRNA via LXRα activation in peripheral cell lines such as THP1 and fibroblasts (23). However, our findings clearly indicated that this PPAR–LXR–ABCA1 pathway did not operate in pitavastatin-treated McARH7777 cells because pitavastatin repressed the ABCA1 promoter activity in an LXRE-dependent manner (Fig. 2: B, D).

We further investigated the mechanism by which pitavastatin-mediated PPARα activation increases ABCA1 protein levels. We found that PPARα agonists, WY14643 and fenofibrate, increased ABCA1 protein expression, although total ABCA1 mRNA levels were unaffected (Fig. 4: B, C; and data not shown), suggesting that
PPARα activation increases ABCA1 expression by a post-transcriptional mechanism. ABCA1 protein is unstable and rapidly degraded by calpain (27). Our experiments using cycloheximide indicate that ABCA1 degradation was apparently retarded in pitavastatin-treated cells (Fig. 5). Thus, stabilization of ABCA1 protein is involved in PPARα-mediated ABCA1 protein increase by pitavastatin. The mechanism underlying this stabilization is currently unknown. However, interaction of the ABCA1 C-terminus with the nuclear receptor LXRβ was shown to stabilize ABCA1 protein (28). ApoA-I is also known to stabilize ABCA1 protein and this signal is mediated by the interaction of the C-terminus of ABCA1 with the Rho guanine nucleotide exchange factor PDZ-RhoGEF (29).

Statins, HMG-CoA reductase inhibitors, are known to increase HDL levels in addition to reducing LDL, but the mechanism of this effect remains unclear (14, 15). Several lines of evidence indicated that the mechanisms underlying the increase in HDL by statins can be classified into two categories: increases in HDL production and decreases in HDL catabolism. Statins have been shown to increase ApoA-I protein and ABCA1 mRNA levels in hepatoma cell lines (16), but to reduce CETP activity (18). CETP mRNA was reported to be regulated by LXRα (30, 31), whereas hepatic ABCA1 mRNA expression is regulated by SREBP2 as well as LXRα (19). In this study, we showed that pitavastatin raised total ABCA1 mRNA levels by increasing SREBP2-regulated type L ABCA1 mRNA levels, while decreasing type P ABCA1 mRNA expression by repressing LXR activity. In addition, pitavastatin was shown to increase ABCA1 protein expression by enhancing PPARα-mediated ABCA1 protein stabilization. These findings may explain the mechanism by which statins increased ABCA1 expression in the hepatoma cell line, while LXR-target CETP expression was decreased (16 – 18).

Various statins have different abilities to increase ABCA1 mRNA expression. Pitavastatin, simvastatin, and atorvastatin increased total ABCA1 mRNA expression, but pravastatin and compactin did not (Fig. 1) (Supplementary Fig. 3: available in the online version only). Similarly, pravastatin was less effective in increasing HMG-CoA synthase mRNA expression than other statins (Supplementary Fig. 1). Higher concentration of compactin was required to increase HMG-CoA synthase mRNA expression, as well (Supplementary Fig. 3). Thus, the different ability of statins to increase ABCA1 mRNA expression may be due to the different inhibitory efficiency or permeable efficiency into the cell.

In summary, statins —especially pitavastatin— increased ABCA1 mRNA levels and protein expression by dual mechanisms: SREBP2-mediated mRNA transcription and PPARα-mediated ABCA1 protein stabilization, but not by the PPAR–LXR–ABCA1 pathway.

References
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