Fenofibric Acid, an Active Form of Fenofibrate, Increases Apolipoprotein A-I–Mediated High-Density Lipoprotein Biogenesis by Enhancing Transcription of ATP-Binding Cassette Transporter A1 Gene in a Liver X Receptor–Dependent Manner

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Objective—Fibrates are widely used drugs to reduce plasma triglyceride and increase high-density lipoprotein. Their active forms, fibric acids, are peroxisome proliferator-activated receptor-α activators, but no direct evidence has been demonstrated for their activation of ATP-binding cassette transporter A1 (ABCA1) in relation to clinically used fibrates. We investigated the reaction of fenofibric acid in this regard.

Methods and Results—Fenofibric acid was examined for the effect of increase of ABCA1 activity. It enhanced ABCA1 gene transcription and its protein level in macrophage cell line cells and fibroblasts and increased apolipoprotein A-I–mediated cellular lipid release, all in a dose-dependent manner. Enhancement of the gene transcription was examined by using a reporter assay system for liver X receptor responsive element (LXRE) and its inactive mutant. The results demonstrated that the effect of fenofibric acid is dependent on active LXRE.

Conclusions—Fenofibric acid increased transcription of ABCA1 gene in a liver X receptor–dependent manner. (Arterioscler Thromb Vasc Biol. 2005;25:1193-1197.)

Key Words: fenofibrate ■ fibrates ■ PPARα ■ ABCA1 ■ HDL ■ cholesterol ■ atherosclerosis

High-density lipoprotein (HDL) is a negative risk factor in coronary atherogenesis,1 and raising HDL is expected to protect us against atherosclerosis. Such an effect was demonstrated in experimental animals by specific gene expression2 or inhibition of cholesteryl ester transfer protein (CETP).3 Although no specific drug is available in clinical use for this purpose, a bile acid–sequestering resin and statins were shown to raise HDL by an unknown mechanism, besides lowering low-density lipoprotein, and subanalysis of these results indicated its independent effect of reducing the atherosclerosis risk.4–6 Fibric acids, active forms of fibrate drugs and activators of peroxisome proliferator-activated receptor-α (PPARα),7,8 are also known for an HDL-raising effect. This group of drugs has been widely used for a long time for the treatment of hyperlipoproteinemia, especially types IIb, III, and VI. Fibric acids enhance fatty acid catabolism and accordingly reduce plasma lipid level, predominantly triglyceride (TG). Increase of TG-rich lipoprotein results in increase of TG transfer to HDL in exchange with its cholesteryl ester by CETP, and therefore leads to production of small cholesterol-poor HDL as TG is hydrolyzed.9,10 Consequently, reduction of TG-rich lipoprotein by fibrates leads to the increase of HDL cholesterol by reversing this mechanism.11,12 Fibric acids were also shown to enhance transcription of the gene of apolipoprotein A-I (apoA-I) in the liver.13–15 A PPARα activator, Wy14643, was shown to upregulate the gene of ATP-binding cassette transporter A1 (ABCA1)16 that mediates and rate-limits biogenesis of HDL by the interaction of helical apolipoprotein and cells.17–20 ABCA1 expression is enhanced by loading cholesterol to cells via the liver X receptor (LXR),21,22 presumably because of the increase of oxysterol. The effect of Wy14643 was interpreted by the activation of the LXR pathway as it increased LXR.23 However, there has been no direct demonstration of the ABCA1 upregulation by fibric acids derived from fibrate drugs clinically used. In mouse atherosclerosis models, PPARα agonists did not appear to enhance ABCA1 expression in atherosclerotic lesion despite their effect of the regression.23,24 Here we report in vitro observation that fenofibric acid increases the expression of ABCA1 and apoA-I–mediated HDL production. The effect on ABCA1 expression was through the enhancement of the transcription of the ABCA1 gene being dependent on LXR.

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Materials and Methods

Cell Culture
RAW264 cells were maintained in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1) medium (IWAKI Glass) containing 2% TCM serum replacement (purchased from KN) at 37°C in 5% CO₂.25 Cells in 6-well plates at the concentration of 1.5x10⁶ cells per well were incubated 24 hours before the experiments.25 THP-1 cells (4.0x10⁶ cells per well) were differentiated with 3.2x10⁻⁷ M phorbol 12-myristate 13-acetate (PMA; Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 hours.26 BALB/3T3 clone A31 (obtained from RIKEN Cell Bank) was incubated in Eagle’s minimum essential medium (MEM) with 10% FCS. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO₂. PPARα activators, fenofibric acid, or Wy14643 (Calbiochem-Novabiochem) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.2% BSA (Sigma).

Cellular Lipid Release
RAW264 cells were washed with PBS and cultured an additional 48 hours in the presence of fenofibric acid or Wy14643 in DMEM/F-12 (1:1) medium containing 2% TCM and 0.2% BSA. During the last 24 hours of the drug treatment, 300 μmol/L of dibutyryl cAMP (dbcAMP; Wako) and apoA-I (10 μg/mL) were added to the medium.25 THP-1 cells and BALB/3T3 cells were also treated with the PPARα activators and apoA-I in 0.2% BSA-RPMI 1640 medium and 0.1% BSA-MEM, respectively. Cholesterol and choline-phospholipid released into the medium by apoA-I were determined enzymatically.25 Adherent cells were dissolved in 0.1 N NaOH for protein determination by bicinchoninic acid protein assay system (Pierce).

Reporter Gene Assay
The constructs of luciferase reporter genes were prepared as described previously.28 The 5'-flanking region of mouse ABCA1 gene (—1238/+57) was inserted into pGL3 vector (Promega) to generate ABCA1 promoter-luciferase reporter construct (pABCA1-Luc). The reporter plasmid with mutated and inactivated LXRE-responsive element (LXRE) (mutant LXRE) was generated by using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Mutations introduced were identical to those reported previously.21 Cells cultured in 24-well plates (3.0x10⁶ cells per well) were washed once with PBS or pABCA1-Luc vector or pABCA1-mutant LXRE-Luc vector were cotransfected with pRL-tk vector (Promega) by Superfect transfection reagent (Qiagen). Three hours after the transfection, cells were washed with PBS and cultured in the presence of fenofibric acid or Wy14643 for 24 hours. Cellular luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). Results were standardized by the Renilla luciferase activity derived from phRL-tk vector.

Immunoblotting of ABCA1
Cells incubated with fenofibrate or Wy14643 for 48 hours were harvested in cold PBS and pelleted by centrifugation. The cell pellet was suspended in 5 mmol/L Tris-HCl, pH 8.5, containing 1% protease inhibitor cocktails (Sigma) and placed on ice for 30 minutes. The cell suspension was centrifuged at 650xg for 5 minutes, and the supernatant was centrifuged at 105 000xg for 30 minutes to prepare the membrane fraction as a pellet. Immunoblotting of ABCA1 was performed according to the previous method.26

Results
Expression of ABCA1
The effects of fenofibric acid and Wy14643 on expression of ABCA1 are shown in Figures 1 and 2. The message of ABCA1 increased by fenofibric acid and Wy14643 in all types of cells examined: RAW264 cells treated with dbcAMP, PMA-differentiated THP-1 cells, and BALB/3T3 cells. ABCA1 protein also increased by the PPARα agonists being demonstrated by its immunoblotting analysis in all these cells (Figures 1 and 2).

ApoA-I–Mediated Cellular Lipid Release
ApoA-I induced release of cellular cholesterol and phospholipids into the medium from the cells examined. PPARα agonists fenofibric acid and Wy14643 increased the apoA-I–induced release of cholesterol and phospholipids in a dose-dependent manner (Figure 3). The increment of lipids released by the drugs was more prominent in cholesterol than phospholipid in RAW264 cells pretreated with dbcAMP. The maximum effect (102% increase in cholesterol release) was observed when the cells were treated with 25 μmol/L of fenofibric acid.

Reporter Gene Assay
Transcription of the ABCA1 gene was examined by using the reporter genes (pABCA1-Luc) in the dbcAMP-treated RAW264 cells (Figure 4). Fenofibric acid and Wy14643
Enhanced transcription of the ABCA1 reporter gene in a dose-dependent manner (Figure 4A and 4B). These effects were cancelled by substitute transfection of the mutant LXRE-containing reporter vector (pABCA1-mutant LXRE-Luc) to inactivate LXRE (Figure 4A and 4B), whereas 9-cis-retionic acid, a ligand for retinoid X receptor (RXR), and 22-oxysterol, a ligand for LXR, failed to increase the transcription of the mutant ABCA1 gene (Figure 4C).

Discussion

PPARs belong to the nuclear receptor superfamily group and act as ligand-activated transcription factors regulating the expression of certain target genes.29 The PPAR family contains 3 different subtypes, designated PPARα, PPARβ/δ, and PPARγ. PPAR subtypes display distinct expression patterns, different ligand specificities, and distinct biological functions.30–32 PPARs are activated by fatty acids and its metabolites and accordingly exert various effects in lipid homeostasis.33 Several subtype-specific synthetic compounds have been developed for clinical use, including fibric acids (PPARα agonist) and glitazones (PPARγ agonist).34 Fibrates are widely used drugs for hyperlipidemic patients because they significantly improve plasma lipid profiles by reducing TG-rich lipoprotein and raising HDL.35,36 The primary effects of fibric acids, active forms of fibrates, on plasma lipids have been attributed to their PPARα-mediated expression of the genes of various enzymes that regulate lipid metabolism.7,8 For HDL metabolism, the effects are partly explained by reduction of plasma TG itself and CETP reaction9,10 and by increased expression of the apoA-I gene.13–15 In addition, Wy14643, a nonclinical PPARα activator, was shown to enhance ABCA1 gene expression.16 Because LXR was also activated in the condition used in that work,16 and ABCA1 is known to be regulated by the LXR/RXR pathway, it was hypothesized that Wy14643 increases the transcription of the mutant ABCA1 gene via the LXR pathway.

We demonstrated the increase of ABCA1 by fenofibric acid, an active form of clinically used fibrate drug fenofibrate, in macrophage cell line cells and in mouse fibroblasts. These effects were also reproduced by a positive control Wy14643. To examine the mechanism, the reporter gene...
assay was used via a promoter of the ABCA1 gene by introducing a mutation in LXR response element. Inactivation of this element was verified by abolishment of its response to 9-cis retinoic acid and 22-oxysterol, and fenofibrate failed to enhance transcription of the mutant reporter gene. Therefore, PPARα in fact activates the ABCA1 gene by the LXR-dependent pathway. The results were inconsistent with the finding that PPARs form a heterodimeric complex with the RXR (not LXR) and bind to specific PPAR-response elements in the promoter region of target gene.37,38 However, a direct ligand of RXR, 9-cis retinoic acid, failed to activate the mutant gene, consistent with the established finding that dimerization of RXR with LXR is essential for enhancing ABCA1 gene transcription.21

Fenofibrate and Wy14643 reportedly have different affinity and distinct specificity to murine and human PPARs. However, both compounds showed equivalent capability in transactivation of the ABCA1 gene. Wy14643 activates not only PPARα but also PPARγ and PPARδ in cell-based transactivation assays39 at the concentration >30 μmol/L. Activation of PPARδ was suggested to affect the ABCA1-mediated HDL biogenesis on the basis that an agonist of PPARδ induced HDL synthesis in culture cells and in monkeys.40 Therefore, the effects of Wy14643 may include combined activation of various PPARs. In contrast, fenofibric acid is highly specific for activation of PPARα, at least up to 100 μmol/L.30 Because Cmax of fenofibrate is 30 μmol/L when it is orally administrated to human, it is most likely that the effect of this drug on the HDL biogenesis is based on the enhancement of ABCA1 expression by the mechanism shown in this article. Fenofibrate has been shown to retard progression of coronary atherosclerosis,41 consistent with the findings of reducing a risk of coronary heart disease by other fibrate drugs.42,43 The clinical effects of these drugs are attributed to improvement of plasma lipoprotein profile by reducing TG and raising HDL. Although decrease of TG and increase of HDL are linked in human by the action of CETP,10 the HDL are linked in human by the action of CETP,10 the effect of this drug on the HDL biogenesis is based on the finding that PPARs form a heterodimeric complex with the RXR (not LXR) and bind to specific PPAR-response elements in the promoter region of target gene.37,38 However, a direct ligand of RXR, 9-cis retinoic acid, failed to activate the mutant gene, consistent with the established finding that dimerization of RXR with LXR is essential for enhancing ABCA1 gene transcription.21

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