Statin-induced inhibition of the Rho-signaling pathway activates PPARα and induces HDL apoA-I

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Clinical benefits of cholesterol reduction have been established in large-scale primary and secondary intervention trials with statins, demonstrating that treatment with these drugs results in decreased morbidity and mortality related to coronary heart disease (CHD) (1, 2). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol synthesis, thus decreasing endogenous cholesterol synthesis. The lowering of intracellular cholesterol levels leads to the activation of sterol regulatory element-binding protein (SREBP) transcription factors, which activate the transcription of a number of genes involved in the clearance of LDL particles from plasma, such as the LDL receptor (3). Despite a significant reduction of LDL cholesterol, a number of patients still have clinical events likely due to the contribution of other factors to the atherogenic process (4). The new generation of more potent statins, such as atorvastatin, cerivastatin, and pitavastatin (NK-104), also lower plasma triglycerides due to a diminished VLDL production, probably as a consequence of less availability of cholesterol for VLDL assembly (5). In addition, they may lower triglycerides due to a strong induction of receptor-mediated remnant clearance (5). Furthermore, statin treatment also increases plasma concentrations of antiatherogenic HDL cholesterol and apoA-I (6), which play a crucial role in reverse-cholesterol transport (7). These observations are likely of clinical relevance, since high levels of HDL and apoA-I are considered to protect against development of CHD (8–10). Moreover, studies in transgenic animals have revealed that overexpression of human apoA-I in mice (11) and rabbits (12) increases plasma apoA-I and HDL cholesterol levels, leading to the inhibition of atherogenesis in dietary and genetically induced animal models of atherosclerosis.

In addition to their potent action on plasma lipid concentrations, statins exert pleiotropic properties and interfere with different vascular events leading to the formation of atherosclerotic lesions, effects that may contribute to their beneficial effects on CHD (13, 14). Statins may exert direct antiatherosclerotic effects by acting on smooth muscle cells (SMCs), endothelial cells, and macrophages (15, 16). Statins prevent SMC migration and proliferation (15) and suppress lipopolysaccharide-induced ICAM-1 expression in bovine aortic endothelial cells (17). Statins may also increase fibrinolytic capacity by inhibiting the expression of PAI-1 in SMCs and endothelial cells while increasing expression of tPA in endothelial cells (18) and suppressing tissue factor expression in cultured...
human macrophages (19). Therefore, statins may interfere with the formation and progression of the atherosclerotic plaque as well as with thrombotic events in hyperlipidemic patients, independently of their ability to reduce plasma cholesterol (20). Beneficial effects of statins against atherosclerosis could be attributed to their ability to suppress the synthesis of mevalonate (16) or downstream products of mevalonate such as isoprenoid intermediates. In vitro experiments demonstrated that the effects of statins on vascular cells were prevented by the addition of mevalonate, and isoprenoids such as farnesyl pyrophosphate (Fpp) or geranylgeranyl pyrophosphate (GGpp) have been implicated in this action (17, 21). In endothelial cells, the effects of statins were mimicked by C3 exoenzyme, an inhibitor of Rho activity (21). As such statins inhibit the prenylation of proteins, such as Ras and Rho that activate the mitogen-activated protein (MAP) kinase cascade or NF-kB pathway, leading to anti-inflammatory, antiproliferative and antiatherothrombotic effects.

PPARs belong to the superfamily of nuclear receptors that are ligand-activated transcription factors (22). Fatty acid derivatives and eicosanoids were identified as natural ligands for PPARs (23, 24). Following heterodimerization with the 9-cis retinoic acid receptor RXR, PPARs bind to specific PPAR response elements (PPREs) in the regulatory regions of target genes (25). Among the three different PPAR subtypes identified thus far, PPARα, PPARβ (NUC-1 or PPARδ), and PPARγ, PPARα mediates the lipid-lowering activity of the fibrate drugs (26). PPARα is considered a major regulator of intra- and extracellular fatty acid metabolism. In humans, fibrate activation of PPARα increases plasma levels of HDL, decreases VLDL synthesis and secretion, and reduces triglyceride levels. Furthermore, PPARα mediates the anti-inflammatory actions of fibrates, such as fenofibrate, at the level of the vascular wall (27, 28). PPARα has been shown to exert these effects by negatively interfering with NF-kB and activator protein-1 (AP-1) (25).

Since statins increase HDL and apoA-I plasma levels, the first goal of this study was to determine the molecular mechanism leading to increased apoA-I production under HMG-CoA reductase inhibition. Furthermore, because of the striking parallel between the effects of fibrate PPARα agonists and statins on lipoprotein metabolism and inflammation, we hypothesized the existence of a common mechanism via cross-talk of their pathways. Our results show that statins increase human apoA-I mRNA levels through activation of its promoter. Interestingly, statins activate PPARα by inhibiting the Rho A signal transduction pathway. These results provide a molecular basis for the increase in HDL levels in response to statins and establish a positive cross-talk between the statin and fibrate pathways.

Methods

Materials. Cerivastatin was kindly provided by Bayer AG (Wuppertal, Germany). Pitavastatin was provided by Nissan Chemical Industries Ltd. (Tokyo, Japan). Fenofibric acid was kindly provided by A. Edgar (Fournier, Dijon, France). Actinomycin D was from Roche Diagnostics (Meylan, France). Fpp and GGpp were purchased from Biomol Research Laboratories (Plymouth Meeting, Pennsylvania, USA). Mevalonate, norhydroxyauric acid (NDGA), farnesol, squalene, 22(R)- and 22(S)-hydroxycholesterol, cerulenin, and chenodeoxycholeic acid (CDCA) were purchased from Sigma-Aldrich (St. Quentin, France). Farnesyl transferase inhibitor and geranylgeranyl transferase inhibitor were from CN Biosciences Inc. (La Jolla, California, USA). Ketoprofen was from Ventris (Vitré, France). Arachidonyletrfluoromethyl ketone (AACOCF3) was from Euromedex (Souffelweyersheim, France). Etopoxir was a kind gift from R. Berge (University of Bergen, Bergen, Norway).

Cell culture and RNA analysis. Human hepatoma HepG2 cells (American Type Culture Collection, Rockville, Maryland, USA) were maintained in DMEM supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO2. Medium was changed every 2 days. At 80% confluence, the medium was replaced by a serum-free medium for 18 hours before treatment with statins to avoid interference of serum components. Cerivastatin was dissolved in water and pitavastatin in DMSO. Cells were subsequently incubated with the indicated compounds for the indicated period of time. At the end of the treatment period, cells were washed with ice-cold PBS and homogenized in 4 M guanidinium isothiocyanate. RNA preparation, Northern blot hybridizations, and quantifications were performed as described previously (29). Human apoA-I (30) and 36B4 cDNAs were used as probes.

Transient transfection assays. All the transfection experiments were performed in triplicate in human hepatoma HepG2 or in rabbit kidney RK13 cells. Cells were transfected in serum-free medium using the lipofectant method with a mixture of plasmids that contained, in addition to the firefly luciferase reporter and expression vectors, a Remila luciferase expression vector as a control for transfection efficiency. All samples were complemented with an equal total amount of DNA using pBluescript empty vector. After 2 hours, cells were incubated for another 40 hours with the indicated stimuli dissolved in their respective solvents and added at the indicated concentrations in medium supplemented with Ultraser serum, which is devoid of steroids and lipids (Biosepra SA, Cergy-Pontoise, France). The human apoA-I promoter (~2051, +91) and deletion (~256, +91) and (~128, +91), as well as wild-type A (Am) and mutant A (Am) sites in front of the minimal apoA-I promoter (P (min) ) constructs were described previously (31). The A-site mutated apoA-I promoter construct (~256, +91) was obtained using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California, USA) with the primer Am (5′-CTC CGG CCC CCA CTT TGG CCT CCC TGC AGC CCC CG-3′). The consensus DR1 PPRE containing reporter plasmids and the human PPARα expression vectors were
described previously (27, 32). The plasmids pGal5-TKpGL3, Gal4-hPPARα LBD, and pGal4-Ø have been described previously (33). The dominant negative constructs of Rho A, Cdc42, and Rac were kindly provided by P. Fort (Centre National de la Recherche Scientifique [CNRS], Unité Mixte de Recherche [UMR] 5535, Montpellier, France) and P. Chavrier (Institut Curie, CNRS-UMR144, Paris, France) (34).

**Kinase assay.** Purified PPARα protein was produced using the IMPACT-T7 system (New England Biolabs, Beverly, Massachusetts, USA). HepG2 cells were cultured in the presence of mevalonate (3 mM) or cerivastatin (5 µM) for 48 hours. Cells were lysed in kinase buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.25 mM EDTA, 0.1% Nonidet P-40 [NP40], and 10 mM NaF). Purified PPARα (2 µg) protein was incubated with cell extracts (5 µg) in the presence of γ[32P]ATP (5 µCi) for 30 minutes at 30°C in reaction buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM MnCl₂, 20 µM ATP, 500 µM EGTA, 25 mM β-glycerophosphate, and 1 mM orthovanadate). PPARα protein was immunoprecipitated using a polyclonal Ab raised against a peptide containing amino acids 10–56 of human PPARα (35) and consecutively washed with 1 ml RIPA (100 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, 0.5% deoxycholate, and 0.1% SDS), 1 ml RIPA-NaCl 1 M, 1 ml RIPA-TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA), and 1 ml TNE. Then samples were boiled in Laemmli buffer and loaded on 10% SDS-PAGE. After the run, the gel was treated for 30 minutes in neutral fixer (40% methanol, 3.5% paraformaldehyde), washed with 10% ethanol, 4% glycerol, and dried. The gel was stained with Ponceau S solution as control, and phosphorylated PPARα was visualized by autoradiography.

**Statistical analysis.** ANOVA, to evaluate global significance, followed by Scheffé test, to analyze for significant differences between groups, were performed.

**Results**

Statins induce human apoA-I mRNA in human hepatoma HepG2 cells. Since statin treatment results in increased

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**Figure 1**

Statins induce apoA-I gene expression at the transcriptional level in HepG2 cells by inhibition of HMG-CoA reductase. (a) Statins induce apoA-I mRNA in HepG2 cells in a dose-dependent manner. HepG2 cells were treated for 24 hours with the indicated doses of cerivastatin or pitavastatin or appropriate solvent in serum-free medium. RNA levels were quantified as described in Methods and expressed (means ± SD, n = 3/point) relative to the untreated control set at 1. Values without a common superscript are significantly different, P < 0.05. R.A.U., relative arbitrary units. (b) Statins induce apoA-I mRNA in HepG2 cells in a time-dependent manner. HepG2 cells were treated for 3, 6, 12, or 24 hours with 5 µM of cerivastatin, or pitavastatin, or appropriate solvent in serum-free medium. RNA levels were quantified as described in Methods and expressed relative to the untreated controls at each time point set at 1. (c) Mevalonate reverses the induction of apoA-I mRNA by statins. HepG2 cells were treated for 24 hours with cerivastatin (5 µM) and/or mevalonate (Mev.; 3 mM) or appropriate solvent in serum-free medium. (d) Statins regulate apoA-I gene expression in HepG2 cells at the transcriptional level. HepG2 cells were treated for 24 hours with cerivastatin (5 µM) or appropriate solvent in serum-free medium. Actinomycin D (Act. D; 5 µg/ml) was added to the medium 90 minutes before treatment. For all, total RNA (10 µg) was subjected to Northern blot analysis using human apoA-I and 36B4 cDNA probes.
plasma concentrations of apoA-I (6, 36), it was investigated whether statins regulate apoA-I at the level of gene expression. Therefore, human HepG2 hepatoma cells were treated with two different statins, and apoA-I mRNA levels were analyzed. Treatment with both cerivastatin and pitavastatin increased apoA-I mRNA in a dose-dependent manner, with significant increases already being observed at doses between 0.1 and 1 µM (Figure 1a). Furthermore, both statins induced apoA-I mRNA levels in a time-dependent manner. A significant increase was already observed at 6 hours, whereas a maximum was attained at 12 hours (Figure 1b).

To demonstrate that this induction of apoA-I is due to the pharmacological activity of statins on HMG-CoA reductase activity, it was determined whether addition of mevalonate, the product of HMG-CoA reductase, could reverse the induction of apoA-I mRNA by statins. Simultaneous incubation of cerivastatin with mevalonate completely reversed the effect of statins on apoA-I mRNA (Figure 1c). These results indicate that statins exert their effects on apoA-I gene expression by inhibition of HMG-CoA reductase and that downstream products of the mevalonate pathway must be responsible for the observed effects.

To study whether the statin induction of apoA-I mRNA levels occurs at the transcriptional level, the influence of the RNA polymerase II inhibitor actinomycin D on statin-induced apoA-I mRNA was studied in HepG2 cells. The addition of actinomycin D 90 minutes before statin treatment completely abolished the increase in apoA-I mRNA levels by cerivastatin (Figure 1d). These effects of statins on apoA-I gene expression were specific since 36B4 mRNA levels did not change. Thus, statins induce apoA-I mRNA levels by increasing apoA-I gene transcription.

**Statins induce apoA-I promoter activity.** Since statins induce apoA-I expression at the transcriptional level, it was next studied whether these drugs act by inducing apoA-I promoter activity. Transient transfection experiments in HepG2 cells carried out with a reporter driven by a 2-kb region of the human apoA-I promoter demonstrated that cerivastatin and pitavastatin significantly (3-fold and 3.5-fold) increased human apoA-I promoter activity (Figure 2a). Next, transfection experiments were performed testing the influence of statin treatment on different 5′-deletion constructs in order to map the statin-response element. The –256-bp proximal apoA-I promoter was induced to an extent similar to that of the 2-kb apoA-I promoter. However, further deletion to position –128 completely abolished the statin effect (Figure 2b). These results indicate that sequences within the –128-bp to –256-bp promoter region are mediating the statin induction of apoA-I promoter activity.

**Figure 1a**

Statins induce human apoA-I promoter activity in HepG2 cells. (a) The human apoA-I promoter (–2093, +91) containing firefly luciferase reporter vector (50 ng) was transfected in HepG2 cells in the presence of a Renilla luciferase reporter vector as internal control (2 ng). After 2 hours of transfection, cells were re-fed with DMEM supplemented with 2% Ultroser SF in the presence of cerivastatin (5 µM), pitavastatin (5 µM), or vehicle for 40 hours. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls. Statistically significantly differences between statin-treated and control groups are indicated (Scheffé: A P < 0.05; B P < 0.01). (b) The indicated human apoA-I promoter S′-deletion constructs (50 ng) were transfected together with the Renilla luciferase expression vector as internal control (2 ng) in HepG2 cells. HepG2 cells were subsequently treated with cerivastatin (5 µM) in DMEM supplemented with 2% Ultroser SF for 40 hours. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls.

**Figure 2a and b**

Statins induce human apoA-I promoter activity in HepG2 cells. (a) The human apoA-I promoter (–2093, +91) containing firefly luciferase reporter vector (50 ng) was transfected in HepG2 cells in the presence of a Renilla luciferase reporter vector as internal control (2 ng). After 2 hours of transfection, cells were re-fed with DMEM supplemented with 2% Ultroser SF in the presence of cerivastatin (5 µM), pitavastatin (5 µM), or vehicle for 40 hours. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls. Statistically significantly differences between statin-treated and control groups are indicated (Scheffé: A P < 0.05; B P < 0.01). (b) The indicated human apoA-I promoter S′-deletion constructs (50 ng) were transfected together with the Renilla luciferase expression vector as internal control (2 ng) in HepG2 cells. HepG2 cells were subsequently treated with cerivastatin (5 µM) in DMEM supplemented with 2% Ultroser SF for 40 hours. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls.
Inhibition of HMG-CoA reductase activity by statins enhances PPARα activity on a DR-1 site. RK13 cells were transfected with a reporter construct containing six copies of the direct repeat 1 (DR1) (DR1-TK-Luc) (10 ng) in the presence of cotransfected empty pSG5 or pSG5hPPARα expression vector (30 ng) and a Renilla luciferase reporter vector as internal control (2 ng). RK13 cells were incubated for an additional 40 hours with cerivastatin (5 µM), pitavastatin (5 µM), and/or mevalonate (3 mM) in appropriate solvent in DMEM supplemented with 2% Ultroser SF. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls.

**Figure 4**

Statins induce human apoA-I promoter A-site activity by activating PPARα. Previous studies have demonstrated that fibrates induce the expression of human apoA-I gene by PPARα, which interacts with a positive PPRE located in the A site (31). Since the statin-responsive element of the apoA-I gene is located in the same region, it was next determined whether statins may also act via the PPAR response element identified previously. Therefore, the influence of statins was analyzed on a reporter construct containing the apoA-I PPRE (–215, –195) cloned in front of the minimal apoA-I promoter (A wt P min) in RK13 cells, which are devoid of PPARα (33). Cerivastatin and pitavastatin did not affect the activity of this construct in the absence of cotransfected PPARα (Figure 3a). Cotransfection of PPARα significantly induced the activity of A wt P min, and statin treatment resulted in a further enhancement. As a control, the PPARα ligand fenofibric acid induced apoA-I A-site activity only in the presence of cotransfected PPARα (Figure 3a). By contrast, a construct containing the A site mutated in its DR2 PPRE sequence (A mut P min) was no longer activated by PPARα, nor by statin, nor fenofibric acid treatment. Similarly, neither PPARα nor statin treatment enhanced the activity of the minimal apoA-I promoter. These data indicate that the A site, which contains a positive PPAR-response element, is a target for statin induction in the presence of PPARα.

To determine whether statins activate human apoA-I promoter activity via the A site PPRE, the influence of statin treatment on the activity of wild-type and A-site mutated apoA-I promoter was tested next in HepG2 cells (Figure 3b). Again, statin treatment increased wild-type apoA-I promoter activity, but this effect was nearly abolished in the promoter mutated in the A site (Figure 3b).

**Figure 5**

Statins activate PPARα in a promoter-independent manner via its ligand-binding domain. To determine whether statins act via the PPARα ligand-binding domain (LBD), transfection experiments were performed using chimeric molecules in which the LBD of PPARα is fused to the DNA-binding domain of the yeast transcription factor Gal4. When RK13 cells were transfected with this construct, treatment with both cerivastatin and pitavastatin resulted in a marked increase of activity of the chimeric Gal4-hPPARα LBD/luciferase

<table>
<thead>
<tr>
<th>Control</th>
<th>Cerivastatin</th>
<th>Pitavastatin</th>
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<td>100%</td>
<td>120%</td>
<td>130%</td>
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**Figure 4**

Inhibition of HMG-CoA reductase activity by statins enhances PPARα activity on a DR-1 site. RK13 cells were transfected with a reporter construct containing six copies of the direct repeat 1 (DR1) (DR1-TK-Luc) (10 ng) in the presence of cotransfected empty pSG5 or pSG5hPPARα expression vector (30 ng) and a Renilla luciferase reporter vector as internal control (2 ng). RK13 cells were incubated for an additional 40 hours with cerivastatin (5 µM), pitavastatin (5 µM), and/or mevalonate (3 mM) in appropriate solvent in DMEM supplemented with 2% Ultroser SF. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls.
Figure 6
GGpp prevents the activation of PPARα on PPRE by statins. (a) RK13 cells were transfected with a consensus DR1-driven luciferase reporter vector (10 ng) in the presence of pSG5hPPARα expression vector (30 ng) and a Renilla luciferase reporter vector as internal control (2 ng). Cells were pretreated for 3 hours with ketoprofen (KTP; 50 µM), NDGA (10 µM), etomoxir (ETX; 50 µM), and AACOCF3 (10 µM). Medium was removed and replaced by fresh medium supplemented or not with cerivastatin (5 µM) for 40 hours. Cerulenin (5 µM) and cerivastatin were coincubated for 40 hours. (b) The same experiment as in a was performed with cerivastatin (5 µM) and/or CDCA (10 µM), squalene (10 µM), 22(S) and 22(R) HO-cholesterol (HO-Chol.; 10 µM), or vehicle in coincubation for 40 hours after transfection. (c) The same experiment as in a was performed with cerivastatin (5 µM) and/or Fpp (G224; 2 and 5 µM) or farnesol (FOH; 30 µM), or FTI (10 µM) in coincubation for 40 hours after transfection or vehicle. (d) The same experiment as in a was performed with cerivastatin (5 µM) and GGpp (G225; 2 and 5 µM), or GGTI (10 µM), or vehicle in coincubation for 40 hours after transfection. Firefly luciferase activities were normalized to Renilla control activities. Results represent normalized luciferase activity and are expressed as means ± SD (n = 3) relative to the PPARα-only controls (left bar) set as 1. Values without a common superscript are significantly different, Scheffé: P < 0.001.

Reporter system (Figure 5). By contrast, statins did not influence the activity of a construct containing only the Gal4 DNA-binding domain (DBD) pGal4-Ø (Figure 5). These data indicate that statins activate PPARα by its LBD in a promoter-independent manner.

Statins activate PPARα by inhibiting the GGpp/Rho signal transduction pathway. Since HMG-CoA reductase inhibition leads to activation of transcription factors of the SREBP family, which induce the expression of lipogenic enzymes (37–39) and as such may generate fatty acid or fatty acid–derived PPARα ligands (40), the influence of inhibitors of fatty acid synthesis or enzymes known to generate PPAR ligands on statin-induced PPARα activity was tested. Therefore, the influence of cerulenin, a potent inhibitor of fatty acid synthase (FAS) (41) that may generate long-chain fatty acid PPAR ligands (23, 24, 42); ketoprofen, an inhibitor of cyclooxygenase that may generate prostaglandin PPAR ligands such as 15-Δ12,14 prostaglandin J2 (43, 44); NDGA, an inhibitor of lipoygenase that may generate PPAR ligands such as 8(S)-hydroxyeicosatetraenoic acid (HETE) (45); and AACCOCF3, an inhibitor of phospholipase A2 (PLA2) that may generate phospholipid-derived PPAR activators (46) were tested. In addition, the influence of etomoxir, an inhibitor of CPT-1, was also analyzed. Incubation of statin led to a strong activation of the synthetic PPRE-reporter system (46) and GGpp (Figure 6d) in statin-induced PPARα activity was tested next. Interestingly, incubation with GGTI resulted in a significant activation of PPARα (P < 0.001) (Figure 6d). In addition, C3 exo-enzyme, which blocks Rho protein activity, enhanced statin induction of PPARα (twofold; data not shown). As expected, incubation with a farnesyl transferase inhibitor (FTI) did not influence basal nor statin-induced PPARα activity to an extent similar to that of mevalonate (Figure 6a). Since geranylgeranylation leads to activation of small G proteins, such as Rho and Rac, the influence of a specific geranylgeranyl transferase I inhibitor (GGTI) on PPARα activity was tested next. Interestingly, incubation with GGTI resulted in a significant activation of PPARα (P < 0.001) (Figure 6d).

Dominant negative Rho A activates PPARα. Geranylgeranylation of Rho GTP-binding protein family members induces their translocation from the cytosolic compartment to the membrane, leading to their activation. To identify which Rho family member is implicated in the regulation of PPARα, transient cotransfection experiments were performed on a synthetic PPRE in the presence of PPARα and dominant negative Rho A,
RK13 cells were transfected with the pGal5-TK-pGL3 reporter (10 ng) in the presence of pSG5hPPARα expression vector (30 ng), a Renilla luciferase reporter vector as internal control (2 ng), and expression vectors of dominant negative Rho A (T19N), Rac (T17N), Cdc42 (T17N), or empty vector (30 ng). After transfection, cells were further incubated for 40 hours with fenofibric acid (100 µM) or vehicle in DMEM supplemented with 2% Ultroser SF. Results represent normalized luciferase activity and are expressed as means ± SD (n = 3) relative to the PPARα only controls (left bar) set as 1. (b) RK13 cells were transfected with the pGal5-TK-pGL3 reporter (10 ng) and Gal4-PPARα LBD expression plasmids (100 ng), a Renilla luciferase reporter vector as internal control (2 ng), and expression vectors of dominant negative Rho A (T19N), Rac (T17N), Cdc42 (T17N), or empty vector (30 ng). After transfection, cells were further incubated for 40 hours with fenofibric acid (100 µM) or vehicle in DMEM supplemented with 2% Ultroser SF. Firefly luciferase activities were normalized to Renilla control activities. Values (means ± SD, n = 3) are expressed relative to controls.

Cdc42, and Rac1 expression vectors. Whereas cotransfection of dominant negative Cdc42 or Rac did not significantly influence PPARα activity, dominant negative Rho A induced PPARα, an effect that was enhanced by fibrate treatment (P < 0.001) (Figure 7a).

Since statins significantly enhance PPARα activity via its LBD (Figure 5), we further tested whether inhibition of Rho A also enhances Gal4-PPARα LBD activity. Whereas cotransfection of dominant negative Cdc42 or Rac did not significantly influence fenofibric acid–induced PPARα activity, cotransfection with dominant negative Rho A significantly induces this activity (P < 0.001) (Figure 7b).

Cerivastatin treatment decreases PPARα phosphorylation activity. Since Rho proteins activate protein kinase pathways, it was tested whether statin inhibition of Rho A leads to alterations in PPARα phosphorylation activity. To test this hypothesis, PPARα kinase assays were performed on cellular extracts of HepG2 cells treated with either mevalonate or cerivastatin. Most interestingly, cerivastatin treatment led to a marked decrease in PPARα phosphorylation activity in HepG2 cells (Figure 8a).

Statins and PPARα agonists activate PPARα synergistically. Since PPARα agonists and statins act by different pathways to activate PPARα, next it was tested whether combination treatment may result in a synergistic induction of PPARα activation. As expected, in the presence of cotransfected PPARα, cerivastatin, or fenofibric acid alone significantly induced PPARα activity (Figure 8b). Interestingly, coincubation of cerivastatin and fenofibric acid resulted in a synergistic effect on PPARα transactivation (P < 0.001).

Discussion
Statins are lipid-lowering drugs widely used in the treatment of hypercholesterolemia. Several intervention trials have demonstrated that statins decrease the incidence of cardiovascular events and improve survival rates both in secondary and primary prevention. In addition to lowering LDL cholesterol, statins reduce triglyceride levels and increase HDL cholesterol. HDL and its major protein constituent apolipoprotein A-I play a critical role in cholesterol metabolism due to their capacities to eliminate excessive amounts of cholesterol from peripheral arteries and return it back to the liver, the so-called reverse-cholesterol transport pathway. HDL cholesterol levels are inversely correlated with CHD (47), and statins increase HDL cholesterol and apolipoprotein A-I levels (6). In the present study we studied the molecular mechanism of statin action on apolipoprotein A-I production.

Statin treatment of human hepatoma cells resulted in a time- and dose-dependent increase of apolipoprotein A-I mRNA levels. A similar induction of hepatic apolipoprotein A-I mRNA after statin treatment has been demonstrated previously in vivo in rats (48). In vivo experiments in transgenic apolipoprotein A-I mice treated with statins revealed no effect on apolipoprotein A-I mRNA (data not shown), which is likely due to the rapid and almost complete catabolism of apoA-I mRNA after statin treatment has been demonstrated previously in vivo in rats (48). In vivo experiments in transgenic apolipoprotein A-I mice treated with statins revealed no effect on apolipoprotein A-I mRNA (data not shown), which is likely due to the rapid and almost complete catabolism of
Figure 9

Cross-talk between the statin and PPARα pathways. C3 T, C3 trans-
ferase; DN, dominant negative. The pathway in black is that implicat-
ed in PPARα regulation by statins. The other pathways of mevalonate
metabolism are depicted in gray. PP, pyrophosphate.

cerivastatin in this species (49). The effects of statins on
apoA-I expression occur at the transcriptional level
since actinomycin D pretreatment blocked the induc-
tion. Furthermore, the statin effect depends on down-
stream products of the mevalonate pathway since
mevalonate reversed the increase of apoA-I mRNA lev-
els. To determine the molecular mechanisms involved,
transient transfection experiments were performed,
and a direct effect on human apoA-I promoter activity
was demonstrated. Furthermore, a statin response ele-
ment was mapped between –256 bp and –128 bp. Inter-
estingly, a previous study in HepG2 cells demonstrat-
ed that cholesterol loading, either by LDL-uptake or
addition of free cholesterol, led to an increase in apoA-
I mRNA levels (50). Since high levels of exogenous cho-
lesterol lead to the downregulation of HMG-CoA
reductase, common mechanisms may be the basis of
the apoA-I induction by statins and cholesterol load-
ing. This induction of apoA-I gene expression by statins
extends the results from previous in vivo studies
demonstrating an increase in apoA-I production rate
(6, 36) after statin administration in humans. Increased
expression and production of apoA-I likely contribute,
therefore, to the increase of apoA-I and HDL plasma
levels observed after statin therapy.

A statin response element was mapped to the A site,
which also contains a functional PRE (31). The
induction of human apoA-I gene expression by
fibric acid, another class of hypolipidemic drugs, occurs
by activation of PPARα, which binds to this element.
Interestingly, fibrates and statins share a number of
pharmacological properties. Both classes of drugs
reduce triglyceride and increase HDL levels and exert
antithrombotic and anti-inflammatory actions in the
vascular wall (25, 51). These observations led us to test
whether statins could activate PPARα. Results from
cotransfection experiments with PPARα on HepG2
cells demonstrate that statins act directly on the apoA-
I A site via PPARα. In addition, in RK13 cells that are
devoid of PPARα, statins activate a synthetic PRE-
driven promoter only in the presence of cotransfected
PPARs. These results suggest a role for this transcrip-
tion factor in the statin regulation of apoA-I gene
expression and clearly establish a cross-talk between
the PPARα and statin-signaling pathways. Since
mevalonate addition reverses the PPARα activation by
statins, it can be concluded that statins are not direct
ligands, but rather that downstream products of the
mevalonate pathway inhibit PPARα activity. Transfec-
tion experiments using the LBD of PPARα fused to
the yeast transcription factor Gal4 DBD indicate that
statins induce the transactivation capacity of PPARα
in a general, promoter-independent manner and sug-
gest that statin treatment may result in the generation
of PPARα ligands or may increase the activity of the
DBD of PPARα.

Mevalonate is a key intermediate in the de novo syn-
thesis of both sterol and nonsterol isoprenoids. The
majority of mevalonate is converted to cholesterol,
which is a precursor of steroid hormones, bile acids,
vitamin D, and a wide variety of oxysterols. Further-
more, a variety of nonsteroidal isoprenoid products are
formed from mevalonate. Cholesterol depletion
induced by statins triggers the cleavage of the chole-
sterol-sensitive transcription factors, called SREBPs.
Studies in adipocytes revealed that ADD1/SREBP-1, a
transcription factor participating in adipose tissue dif-
ferentiation, leads to the production of endogenous
ligands for PPARγ (40). These intermediates are lipid
molecules that bind directly to PPARγ, since they dis-
placed the binding of synthetic ligands such as thiazo-
lidinediones. Interestingly, SREBP activation leads
to the induction of lipogenic enzymes such as FAS and
thus may lead to the production of fatty acids, which
are ligands of PPARα (23, 24, 42). In addition, PPAR lig-
ands may be produced by oxidation of endogenous
fatty acids by lipoxygenases (24, 42, 45, 52), or cyclooxy-
genases (43, 44), or by degradation of phospholipids by
phospholipase A2, which liberates fatty acids (46). This
raised the question of whether PPARα ligands might be
generated through activation of these pathways. How-
ever, experiments to reverse statin action on PPARα
activity using potent inhibitors of these enzymes were
without effect on statin-induced PPARα activity. Fur-
thermore, compounds derived from the cholesterol
biosynthetic pathway, including sterols, squalene, or
cholesterol metabolites such as bile acids or oxysterols,
did not modulate statin-induced PPARα activity. Thus
these data provide evidence that statins activate PPARα
via a pathway other than the sterol or SREBP pathways.

Mevalonate is not only a precursor for cholesterol
synthesis but also is a precursor of nonsterol iso-
prenoid compounds. Fpp and GGpp are substrates for
the posttranslational prenylation of proteins (53–55).
Farnesol is a Fpp-derived metabolite that has been
shown recently to induce PPARα activity and thereby
influence keratinocyte differentiation (56). However,
our results demonstrate that statin action on PPARα
was prevented by GGpp, but not Fpp nor farnesol, sug-
ggesting that geranylgeranyl-modified intermediates may antagonize PPARα. Fpp and GGpp are implicated in membrane translocation, leading to the activation of a variety of proteins, including Ras and Rho GTP-binding proteins, respectively (57, 58). Rho A, Rho B, Rac, and Cdc42 are the major substrates for posttranslational modification by geranylgeranylation, which leads to their activation and membrane translocation. This process has been shown to be inhibited by statins in SMCs indicating a direct effect of statins on the vascular wall via inhibition of Rho geranylgeranylation (59). After posttranslational modification by geranylgeranylation (18), the Rho family of small GTP-binding proteins can be inactivated by treatment with C3 exoenzyme, which selectively ADP-ribosylates low-molecular-weight G proteins of the Rho A and B subfamily, rendering them biologically inactive (60). C3 exoenzyme treatment enhanced statin-induction of PPARα transactivation (data not shown), further pointing to the implication of Rho proteins in the statin activation of PPARα. Since Rho A, but not Cdc42 or Rac dominant negative proteins, enhances PPARα activity and, more specifically, the PPARα LBD, it is likely that the effects of statins on PPARα are mediated by Rho A. Downstream targets of Rho family proteins have just begun to be identified, and the molecular mechanisms by which Rho proteins may regulate gene expression are not clearly understood. Posttranslationally modified Rho proteins control cytoskeletal reorganization, motility, and cell growth (61). Rho, Rac, and Cdc42 have been reported to regulate the ε-Jun NH2 terminal kinase (JNK), and the p38 MAP kinase (MAPK) cascades (62–64). PPARα activity is modulated by phosphorylation, resulting in either enhanced or lowered transcriptional activity (65). In this study, we show that statins decrease the phosphorylation of PPARα. Interestingly, a recent study demonstrated downregulation of PPARα activity after activation of the MAPK pathway (66). However, MAPK sites in PPARα were mapped in the NH2-terminal part of the protein (67). Since statins induce PPARα LBD by its activity, they likely act through a novel mechanism. Further studies are required to delineate the molecular mechanism of PPARα regulation by Rho A. The GGpp pathway has already been implicated in mediating the antithrombotic and anti-inflammatory effects of statins in SMCs and macrophages (16, 21). Our data provide evidence that the GGpp pathway is also implicated in the effects of statins acting on PPARα. Since PPARα exerts potent anti-inflammatory activities in vascular cells (27, 68), we speculate that the reported anti-inflammatory activities of statins are, at least in part, mediated by PPARα activation (Figure 9).

Previous studies have demonstrated induction of PPARγ activity by statins through the generation of ligands after SREBP activation (40). Furthermore, PPARγ transcription has been shown to be induced by statins through effects on the PPARγ promoter (69). However, this is the first time that a cross-talk of the PPARα and statin-signaling pathways is shown. Furthermore, we show that PPARα activation by statins occurs through a completely different molecular mechanism, implicating the GGpp pathway and prenylation of Rho family proteins. Moreover, we demonstrate that simultaneous treatment with statins and fibrate PPARα ligands results in a synergistic effect on PPARα transactivation. Thus PPARα is an important molecular target for the two major classes of hypolipidemic drugs. Together, these data provide a molecular rationale for combination therapy with statins and fibrates in the treatment of CHD.

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