Pitavastatin Downregulates Expression of the Macrophage Type B Scavenger Receptor, CD36

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Background—Pitavastatin (NK-104) is a novel inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol biosynthesis. In clinical trials, pitavastatin has been shown to significantly decrease serum LDL cholesterol and triglyceride levels and increase HDL cholesterol. Scavenger receptor–mediated accumulation of oxidized LDL (OxLDL)–derived cholesteryl ester is considered to be a critical step in the development of atherosclerotic foam cell formation. We studied the effect of pitavastatin on CD36 (a class B scavenger receptor) expression by murine macrophages.

Methods and Results—Treatment of J774 cells and murine peritoneal macrophages with pitavastatin decreased CD36 mRNA expression in a dose-dependent manner. Decreased CD36 mRNA was associated with decreased CD36 cell surface protein expression in human THP-1 cells and human monocyte-derived macrophages. Pitavastatin also reduced the increase in CD36 mRNA, cell surface protein, and binding/uptake of OxLDL induced by peroxisome proliferator–activated receptor-γ (PPARγ) ligands and/or OxLDL. Pitavastatin did not alter the half-life of CD36 mRNA, which suggests pitavastatin downregulates CD36 mRNA expression by reducing CD36 transcription. In addition, pitavastatin significantly decreased PPARγ mRNA and protein expression. Finally, pitavastatin increased p44/42 mitogen-activated protein kinase activity and PPARγ phosphorylation and increased the ratio of phosphorylated PPARγ to nonphosphorylated PPARγ.

Conclusions—The present data demonstrate that pitavastatin prevents OxLDL uptake by macrophages through PPARγ-dependent inhibition of CD36 expression and suggest that pitavastatin could modulate CD36-mediated atherosclerotic foam cell formation. (Circulation. 2004;109:790-796.)

Key Words: antigens, CD36 ■ macrophages ■ statins ■ cholesterol ■ lipoproteins

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins are potent inhibitors of cholesterol biosynthesis. Clinical trials have provided clear evidence that cholesterol-lowering therapy with this class of compounds decreases the incidence of coronary heart disease. However, the overall clinical benefits observed with statin therapy are greater than what might be expected from changes in lipid profile alone. This suggests that the beneficial effects of statins may extend beyond their effects on serum cholesterol levels. Experimental and clinical evidence indicates that some of the cholesterol-independent effects of statins involve (1) endothelial normalization of nitric oxide production, (2) antiinflammatory effects and inhibition of monocyte/endothelial cell adhesion, (3) inhibition of scavenger receptor expression, (4) strengthening of the fibrous cap, (5) inhibition of platelet thrombus formation/reduction of thrombotic response, and (6) inhibition of smooth muscle cell proliferation. Scavenger receptors are thought to play a significant role in atherosclerotic foam cell development because of their ability to bind and internalize modified lipids, such as oxidized LDL (OxLDL). Two major classes of human scavenger receptors, designated types A and B, have been identified. CD36, the defining member of type B scavenger receptors, binds OxLDL but not LDL. CD36 cDNA-transfected cells bind and internalize OxLDL, and binding of OxLDL to human macrophages was 50% blocked by antibodies to CD36. Both native and modified lipids upregulate expression of the class A and class B scavenger receptors. Inhibition of CD36 expression has been demonstrated to reduce the development of atherosclerosis in atherosclerosis-prone apolipoprotein E–null mice.

Recent studies have implicated peroxisome proliferator–activated receptor-γ (PPARγ) in the regulation of CD36 by oxidized lipoprotein. PPARγ is a member of the nuclear hormone receptor superfamily.
erodimerizes with the retinoid X receptor (RXR) and functions as a transcriptional regulator of genes that modulate lipid metabolism and adipocyte gene expression.²⁴ PPARγ is activated by such diverse agents as long-chain fatty acids, arachidonic and linoleic acid metabolites,²⁵ and the thiazolidinedione class of antidiabetic drugs.²⁶ The phosphorylation status of PPARγ has been shown to affect its transcription activity. PPARγ is a positive regulator for its target genes, whereas its phosphorylated form (PPARγ-Pi) is a negative regulator.²⁷

Pitavastatin, also known as NK-104 (CAS 147526-32-7; monocalcium bis [(3R, 5S, 6E)-7-(2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl)-3,5-dihydroxy-6-heptenoate]) is a recently developed HMG-CoA reductase inhibitor that significantly reduces serum total cholesterol, LDL cholesterol, and triglycerides while modestly raising HDL cholesterol.²⁸,²⁹ We evaluated the effects of pitavastatin on the expression of CD36 by murine macrophages and demonstrate a novel mechanism by which pitavastatin inhibits CD36 expression, through PPARγ-dependent inhibition of CD36 gene transcription.

**Methods**

**Cells and Reagents**

J774 cells (a murine macrophage cell line; ATCC, Rockville, Md) were cultured in RPMI 1640 medium containing 10% fetal calf serum, 50 µg/mL each of penicillin and streptomycin, and 2 mmol/L glutamine. Cells were switched to serum-free medium for 3 to 5 hours when the confluence was ~85%. Cells received treatments in serum-free medium.

THP1 cells (a human monocytic cell line; ATCC, Rockville, Md) were cultured in RPMI 1640 complete medium. Cells were adjusted to a density of 300×10⁶/cm² in dishes before addition of PMA to drive the differentiation into macrophages. After the completion of differentiation (~12 hours) and removal of PMA, cells were continued and treated in complete medium. These cells were used in fluorescence-activated cell sorter (FACS) studies because of the unavailability of antibodies to murine CD36.

Murine macrophages were obtained from C57BL/6 mice. Mice were injected intraperitoneally (3 mL per mouse) with 3% brewer yeast as a source of LPS and kept for 15 minutes at 37°C. After removal of peritoneal macrophages, they were washed once with PBS and cultured in 60-mm dishes with complete medium for 4 hours. Medium containing floating cells was aspirated, and adherent cells were washed once with PBS, then cultured in complete medium.

**Isolation of LDL and Preparation of OxLDL**

LDL (1.019 to 1.063 g/mL) was isolated from normal human plasma by sequential ultracentrifugation, dialyzed against PBS containing 0.3 mmol/L EDTA, sterilized by filtration through a 0.22-m filter, and stored under N₂ gas at 4°C. Protein content was determined by the method of Lowry.³⁰ LDL was iodinated by the method of Bilheimer et al as described by Goldstein et al using carrier-free [¹²⁵I]Na (Amersham Corp).

OxLDL was prepared as described previously.³⁰ The purity and charge of both LDL and OxLDL were evaluated by examining electrophoretic migration in agarose gels. The degree of oxidation of LDL and OxLDL was determined by measuring the amount of thiobarbituric acid reactive substances (TBAR). LDL had TBAR values of <1 mmol/mg. OxLDL had TBAR values of >10 and <30 mmol/mg. All lipoproteins were used for experiments within 3 weeks after preparation.

**Isolation of Total RNA, Purification of Poly(A⁺) RNA, and Northern Blotting**

Cells were lysed in RNAzol B (Tel-Test, Inc). Chloroform was extracted, and total cellular RNA was precipitated in isopropanol. After washing with 80% and 100% ethanol, the dried pellet of total RNA was dissolved in distilled water and quantified. The poly(A⁺) RNA was purified from ~80 µg of total RNA with the PolyAT tract mRNA Isolation System III (Promega).

Poly(A⁺) RNA was loaded on 1% formaldehyde agarose gel. After electrophoresis, poly(A⁺) RNA was transferred to a Zetabind GT genomic-tested blotting membrane (Bio-Rad Laboratories) in 10×SSC by capillary force overnight. The blot was UV crosslinked for 2 minutes and prehybridized with Hybrisol I (Oncor, Inc) for 30 minutes before the addition of ²³P randomly primed labeling probe for mouse CD36 or PPARγ or GAPDH. After overnight hybridization, the membrane was washed for 2×20 minutes with 2×SSC and 0.2% SDS and for 2×20 minutes with 0.2×SSC and 0.2% SDS at 55°C. The blot was autoradiographed by exposure to radiographic film (X-Omat AR, Kodak). Autoradiograms were assessed by densitometric scanning with a UMAX UC630 flatbed scanner attached to a Macintosh Hlci (Apple Computer) running NIH Image software. The probe for mouse CD36 is an XbaI-BglII digest (base pairs 193 to 805). The template DNA for PPARγ was generated by reverse transcription–polymerase chain reaction (RT-PCR) based on the published sequences. The sequences of 5’ and 3’ oligonucleotides used for PPARγ were TCGGGCTTGTCATGATCCTC (121–141) and GGTTGATAAAAGACGCGCTGG (551–571), respectively.

**Flow Cytometry**

After treatment, cells were lifted by addition of trypsin. Cells were washed 3 times with PBS. Approximately 1×10⁶ cells were suspended in 300 µL of PBS containing 5% mouse serum and incubated at 37°C for 30 minutes at room temperature with shaking. Cells then were added to 10 µL of mouse anti-human CD36 antibody conjugated to fluorescein isothiocyanate isomer 1 (FITC; Chemicon International Inc). After incubation with the antibody for 1 hour at room temperature, cells were washed 3 times with PBS. After suspension in PBS, cells were subjected to flow cytometric analysis with a Coulter FACScan.

**Determination of Binding/Uptake of [¹²⁵I]-Labeled OxLDL to Macrophages**

Binding/uptake of OxLDL was performed as described previously.¹⁸

**Analysis of Phospho-P42/44 Mitogen-Activated Protein Kinase by Western Blotting**

Analysis of phospho-P42/44 mitogen-activated protein (MAP) kinase was performed as described previously.³²

**Extraction of Nuclear Protein and Western Analysis of PPARγ and Phospho-PPARγ**

After treatment, cells were washed twice with PBS and then resuspended in 400 µL of cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF). After 15 minutes’ incubation on ice, 25 µL of 10% NP-40 was added to the cell suspension, which was subjected to a vortex for 10 seconds. The supernatant was removed after being spun for 30 seconds at 13 000 rpm. The pellet was resuspended in 100 µL of cold buffer C (20 mmol/L HEPES, pH 7.9, 400 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, freshly added 1 mmol/L DTT, 1 mmol/L PMSF, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, 0.1 mmol/L P-aminobenzamidine, and 10 µg/mL aprotinin) and kept for 15 minutes at 4°C. The mixture was spun for 5 minutes at 13 000 rpm, and the supernatant was collected as nuclear proteins.

Nuclear proteins (500 µg) from each sample were incubated with an antibody to mouse PPARγ antibody. Immunoblotted proteins were separated by SDS-PAGE and transferred onto nylono-enhanced nitrocellulose membrane, then analyzed by Western blot for...
phospho-PPARγ (PPARγ-Pi) by incubation with anti-phosphoserine antibodies. The nuclear proteins were also used to analyze PPARγ protein expression by SDS-PAGE/Western blot.

Results

Pitavastatin Downregulates CD36 Expression

To determine the effects of pitavastatin on macrophage expression of CD36, J774 cells were treated with various concentrations of pitavastatin for 24 hours. Total RNA was extracted and used to isolate Poly(A)⁺ RNA. Northern blotting was used to determine expression of CD36 mRNA. Pitavastatin decreased CD36 expression in a dose-dependent manner (Figure 1A). At a concentration of 10 μmol/L, pitavastatin inhibited expression of CD36 mRNA by more than half. To demonstrate that pitavastatin altered expression of CD36 in primary cells in a manner similar to its effect on macrophage cell lines, we evaluated the effect of pitavastatin on CD36 expression in murine peritoneal-derived macrophages. Pitavastatin had similar effects on expression of CD36 mRNA in murine peritoneal macrophages (Figure 1B). To demonstrate that the statin effect on CD36 expression was not limited to pitavastatin, J774 cells were treated with pravastatin and simvastatin. Both statins decreased expression of CD36 in a dose-dependent manner (Figure 1C).

We next determined the effect of pitavastatin on CD36 protein expression. PMA-differentiated THP-1 cells (a
human monocyte cell line) and human peripheral blood monocyte-derived macrophages were incubated with or without pitavastatin for 24 hours. CD36 surface protein expression was assessed by flow cytometry. Consistent with the effect of pitavastatin on CD36 mRNA, CD36 surface protein was substantially decreased (Figures 2A and 2B). To assess the functional significance of reduced expression of CD36 surface protein expression, we examined the binding/uptake of 125I-labeled OxLDL. Pitavastatin reduced both baseline binding/uptake of OxLDL and binding/uptake of OxLDL induced by a PPARγ ligand, prostaglandin J3 (PGJ3; data not shown).

**Pitavastatin Blocks Induction of CD36 Expression by PPARγ Agonists**

Ligand activation of PPARγ by OxLDL, PGJ3, or 15d-deoxy prostaglandin J3 (15d-PGJ3) induces CD36 gene transcription. To determine whether pitavastatin was able to abolish induction of CD36 mRNA by PPARγ ligands, macrophages were cotreated with OxLDL plus pitavastatin (Figure 3A) or PGJ3/15d-PGJ3 plus pitavastatin (Figure 3B). OxLDL, PGJ3, and 15d-PGJ3 each significantly increased CD36 expression. However, coinubation with pitavastatin decreased CD36 mRNA expression induced by PPARγ ligands (Figure 3). The inhibitory effect of pitavastatin on the induction of CD36 mRNA expression by PPARγ activation was also associated with decreased surface protein expression (Figure 4). Pitavastatin decreased CD36 surface protein expression induced by OxLDL in a concentration-dependent manner (Figure 4).

**Pitavastatin Decreases CD36 Expression at the Transcriptional Level and by Inactivating PPARγ**

To determine whether pitavastatin decreased CD36 mRNA expression by reducing CD36 transcription or by altering CD36 mRNA stability, cells were treated with or without pitavastatin (10 μmol/L) in the presence of the transcriptional inhibitor actinomycin D. The half-life of CD36 was similar in both the presence and absence of pitavastatin (data not shown). Because CD36 RNA stability was not altered by pitavastatin, it is likely that pitavastatin down-regulates CD36 mRNA at the transcriptional level.

To further investigate the mechanism by which pitavastatin regulates CD36 expression, we evaluated the effect of pitavastatin on PPARγ expression. Pitavastatin decreased expression of PPARγ mRNA (Figure 5A) and protein (Figure 5B) in a dose-dependent manner. The phosphorylation status of PPARγ has been shown to affect its transcriptional activity. PPARγ is a positive regulator for its target genes, whereas its phosphorylated form (PPARγ-Pi) is a negative regulator. Thus, the ratio of PPARγ-Pi to PPARγ is important for determining PPARγ-mediated transcriptional function. We evaluated PPARγ phosphorylation status in response to pitavastatin and found that expression of the nonphosphorylated form of PPARγ was decreased. Whereas the amount of PPARγ-Pi remained unchanged, the ratio of PPARγ-Pi to PPARγ was increased. This results in decreased PPARγ-dependent transcription and decreased expression of CD36. To determine the mechanism of PPARγ phosphorylation, we evaluated the effect of pitavastatin on p44/42 MAP kinase activity, which has been shown to phosphorylate serine residues in PPARγ.
Beginning 4 hours after treatment with pitavastatin (and lasting for 24 hours), we observed increased p44/42 MAP kinase activity, as indicated by p44/42 MAP kinase phosphorylation (Figure 5C).

Discussion

The present data demonstrate that pitavastatin inhibits expression of CD36 both by decreasing expression of PPARγ and through MAP kinase–mediated phosphorylation of PPARγ. This results in an increased ratio of phosphorylated PPARγ to nonphosphorylated PPARγ, which leads to decreased CD36 gene transcription.

PPARs become transcriptionally active when bound to ligand.24 Growth factors, such as epidermal growth factor and platelet-derived growth factor, have been shown to phosphorylate PPARγ via the MAP kinase signaling pathway and to decrease PPARγ transcriptional activity.25 The NH2-terminal domain of PPARγ contains a consensus MAP kinase site in a region conserved between PPARγ1 and PPARγ2 isoforms.33 PPARγ proteins migrate on immunoblots as closely spaced doublets, a pattern suggestive of phosphorylation.34,35 A putative MAP kinase site is phosphorylated by ERK2 and JNK.33 Phosphorylation significantly inhibits both ligand-independent and -dependent transcriptional activation by PPARγ.33 This repression is mediated by MAP kinase phosphorylation of Ser82 on PPARγ1.27 Mutation of the phosphorylated residue (Ser82) prevents PPARγ1 phosphorylation and the growth factor–mediated repression of PPARγ-dependent
transcription. This phosphorylation-mediated transcriptional repression results from an alteration in the ability of PPARγ to become transcriptionally activated by ligand and is not due to a reduced capacity of the PPARγ–RXR complex to heterodimerize or recognize its DNA binding site.37 We have previously shown that both transforming growth factor-β36 and HDL32 induce MAP kinase–mediated phosphorylation of PPARγ.

The effect of statins on scavenger receptor expression has been evaluated previously. Lovastatin inhibited expression of both the type A scavenger receptor and CD36. Lovastatin decreased type A scavenger receptor mRNA in PMA-treated THP-1 cells.8 This inhibition was reversed by the addition of exogenous mevalonate.9 Similarly, lovastatin inhibited CD36 expression in human monocytic U937 cells, as measured by quantitative RT-PCR and FACS.9 Mevalonate completely reversed the effects of lovastatin, whereas excess LDL was only partially effective. Lovastatin was also shown to decrease expression of CD36 and the type A scavenger receptor in human blood–derived monocytes in culture for 2 and 5 days but not in more mature macrophages.37 Finally, monocyte-derived macrophages had decreased expression of CD36 and the type A scavenger receptor in hypercholesterolemic patients treated with atorvastatin relative to monocyte-derived macrophages from untreated patients.38 However, in these studies, no molecular mechanisms were identified by which statins decreased scavenger receptor expression.

In conclusion, the present study demonstrates that pitavastatin modulates phosphorylation and activity of PPARγ, which leads to decreased expression of CD36, a major macrophage scavenger receptor for oxidized lipids. The effect of pitavastatin on inhibition of CD36 expression may have relevance to both atherosclerotic foam cell formation mediated by CD36 and PPARγ and expression of other PPARγ-responsive inflammatory mediators expressed within vascular lesions.

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References


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