Functional Interplay Between the Macrophage Scavenger Receptor Class B Type I and Pitavastatin (NK-104)

Jihong Han, PhD; Michael Parsons, MD; Xiaoye Zhou, MD; Andrew C. Nicholson, DVM, PhD; Antonio M. Gotto, Jr, MD, DPhil; David P. Hajjar, PhD

Background—Scavenger receptor class B type I (SR-BI), a receptor for high-density lipoprotein (HDL), plays an important role in the bidirectional cholesterol exchange between cells and HDL particles and the atherosclerotic lesion development. Enhancement of SR-BI expression significantly reduces, whereas lack of SR-BI expression accelerates, the atherosclerotic lesion development in proatherogenic mice. Statins, a class of inhibitors for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, significantly suppress de novo cholesterol synthesis and reduce the incidence of coronary heart disease. Statins also display multiple pleiotropic effects independently of cholesterol synthesis in the vascular cells. Here, we investigated the effects of pitavastatin (NK-104), a newly synthesized statin, on macrophage SR-BI expression.

Methods and Results—We found that pitavastatin significantly increased SR-BI mRNA and protein expression in a macrophage cell line in a concentration- and time-dependent manner. It also increased SR-BI expression in both mouse peritoneal and human monocyte-derived macrophages. Associated with increased SR-BI expression, pitavastatin enhanced macrophage HDL binding, uptake of \([14C]\)cholesteryl oleate/HDL, and efflux of \([3H]\)cholesterol to HDL. Pitavastatin abolished the inhibition of macrophage SR-BI expression by cholesterol biosynthetic intermediates. It also restored SR-BI expression inhibited by lipopolysaccharide and tumor necrosis factor-\(\alpha\) through its inactivation of the transcription factor nuclear factor-\(\kappa B\).

Conclusions—Our data demonstrate that pitavastatin can stimulate macrophage SR-BI expression by reduction of cholesterol biosynthetic intermediates and antiinflammatory action and suggest additional pleiotropic effects of statins by which they may reduce the incidence of coronary heart disease. (Circulation. 2004;110:3472-3479.)

Key Words: statins ■ macrophages ■ scavenger receptors ■ lipoproteins

High-density lipoprotein (HDL) performs various functions. In a pathway defined as reverse cholesterol transport, HDL provides an acceptor for the removal of cholesterol from peripheral tissues and carries the cholesterol to other lipoproteins for hepatic uptake and secretion. This process is an important mechanism in the removal of cholesterol from sites of lipid deposition. Conversely, HDL also provides steroidogenic and other tissues with a source of cholesterol.

The bidirectional flux of cholesterol across the plasma membrane requires the specific receptor for HDL binding. Scavenger receptor class B type I (SR-BI), a member of the family of class B scavenger receptors including CD36 and lysosomal integral membrane protein-II (LIMP-II), has been shown to have high affinity for HDL binding and moderate affinity for other lipoproteins, such as native LDL, acetylated LDL, oxidized LDL, and anionic phospholipid vesicles. In various cell types, SR-BI can bind HDL reversibly and mediate cholesterol efflux and cholesteryl ester uptake. A direct relationship has been observed between the flux rate of cholesterol through HDL and the levels of SR-BI expression. SR-BI is highly expressed in several tissues and cell types, including monocyte/macrophages. It has been thought to play an important role in the development of human atherosclerotic lesions, because high expression of SR-BI protects proatherogenic mice against lesion development, whereas disruption of SR-BI accelerates the onset of atherosclerosis. SR-BI expression can be affected by monocyte/macrophage differentiation and by several molecules, such as probucol, polyunsaturated fatty acids, cellular cholesterol levels, testosterone, lipopolysaccharide (LPS), oxidized LDL, and the cytokines tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interferon-\(\gamma\) (IFN-\(\gamma\)).

Statins, potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, significantly reduce total and LDL cholesterol levels in the blood and decrease the incidence of coronary heart disease in patients. Other pleiotropic effects of statins in the vessel wall include (1)
increasing endothelial NO synthase (eNOS) activity and tissue-type plasminogen activator (tPA); (2) decreasing plasminogen activator inhibitor-1 (PAI-1), endothelin-1, proinflammatory cytokine (interleukin [IL]-1β, IL-6) expression, and LDL oxidation; (3) reducing migration and proliferation of smooth muscle cells; and (4) reducing matrix metalloproteinases (MMPs), inducible NO synthetase (iNOS), and monocyte chemoattractant 1 (MCP-1).

Pitavastatin (NK-104, CAS 147526-32-7; monocalcium bis [(3R,5S,6E)-7-(2-cyclopentyl-4-(4-fluorophenyl)-3-quiolyl)-3,5-dihydroxy-6-heptenoate]) is a recently developed HMG-CoA reductase inhibitor. It significantly reduces serum total and LDL cholesterol and triglycerides, whereas it moderately increases HDL cholesterol. Recently, we reported that this statin significantly decreased the oxidized LDL receptor CD36. Downregulation of macrophage CD36 expression by pitavastatin was defined through a novel mechanism, because it can suppress peroxisome proliferator–activated receptor-γ (PPAR-γ) expression while increasing phosphorylation of PPAR-γ. Here, we have investigated the impact of pitavastatin on macrophage SR-BI expression in an attempt to more fully define its role in cholesterol metabolism. We demonstrate that pitavastatin can increase SR-BI expression through its influence on cholesterol biosynthetic intermediates and the inflammatory response.

Methods

Cell Lines and Reagents

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

To collect peritoneal macrophages, mice were injected with 3 mL of 4% thioglycolate and maintained with access to water and normal chow for 5 days. Mice were euthanized by decapitation, after which peritoneal macrophages were collected from the mouse abdomen by lavage with PBS. Cells were cultured in complete RPMI 1640 medium for 3 hours, and all floating cells were removed. Adhesive cells continued to be cultured with complete RPMI medium for 2 additional days and were treated as indicated.

To isolate human monocytes, blood was drawn from a healthy donor and then mixed (5:1) with dextran sedimentation mixture (15 g dextran, MW 500 000, 15 g d-glucose, and 4.5 g NaCl in 500 mL distilled water and filtered through a 0.2-µm filter). The mixture was incubated for 60 minutes at room temperature to sediment red cells. The upper plasma layer containing lymphocytes was collected and centrifuged for 10 minutes at 1000 rpm. The cell pellet was resuspended with serum-free RPMI medium and dispensed on top of Ficoll-Paque at room temperature. After centrifugation for 20 minutes at 2250 rpm, cells at the interface were carefully collected and washed twice with serum-free RPMI medium. Cells were then suspended in RPMI medium containing 20% FCS in FCS-prec coated dishes. After 4 hours in culture, floating cells were removed, and the remaining cells were allowed to complete the monocyte/macrophage differentiation process for 7 days more.

Rabbit polyclonal anti-human/mouse SR-BI antibody was purchased from Novus Biologicals Inc. Anti–nuclear factor (NF)-κB p65 and anti–IkB-α antibodies were purchased from Santa Cruz Biotechnology Inc. NF-κB DNA binding activity assay kits were purchased from Active-Motif, Inc. Chemicals were obtained from Sigma-Aldrich Co.

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

Cells were lysed in RNAzol B (Tel-Test, Inc), chloroform was extracted, and total RNA was precipitated in isopropanol. After it had been washed 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 100 µg of total RNA by use of the PolyATract mRNA Isolation System III (Promega).

Poly(A⁺) RNA was loaded on 1% formaldehyde agarose gel. After electrophoresis, Poly(A⁺) RNA was transferred to a Zeta-probe GT Genomic Tested Blotting Membrane (Bio-Rad Laboratories) in 10× SSC by capillary force overnight. The blot was UV-crosslinked for 2 minutes, then prehybridized with Hybrisol I (Oncor, Inc) for 30 minutes before the addition of a 32P-randomly primed labeling probe for mouse SR-BI 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 a x-ray film (X-Omat AR, Kodak). The semiquantitative assay of autoradiograms was assessed by densitometric scanning with a UMAX UC630 flatbed scanner attached to a Macintosh IIfci (Apple Computer) running NIH Image software. The probe for mouse SR-BI was generated by reverse transcription–polymerase chain reaction (RT-PCR) based on the published sequence. The sequences of 5’- and 3’-oligonucleotides used were TCGGGCTTGTCATGATCCTC (121-141) and GGGTCATAAAAAGCAGGCCTGG (551-571), respectively.

Analysis of HDL Binding, Efflux of Free Cholesterol, and Influx of Cholesteryl Ester

HDLs (1.063 to 1.210 g/mL) were isolated from normal human plasma by sequential ultracentrifugation, dialyzed against PBS containing 0.3 mmol/L EDTA, sterilized by filtration through a 0.22-µm filter, and stored under N2 gas at 4°C. Protein content was determined by the methods of Lowry. To perform the binding of HDL to macrophages, HDL was fluorescein-conjugated with a reactive succinimidyl-ester of carboxyl-fluorescein by use of a labeling kit purchased from Princeton Separations. After treatment, macrophages (~1×10⁶ cells/sample) were washed twice with cold PBS and then incubated with 20 µg/mL of labeled HDL in serum-free medium for 2 hours at 4°C. After washing twice with PBS, cells were subjected to fluorescence-activated cell sorting (FACS) (Coulter FACScan) to determine the binding of HDL to macrophages.

Efflux of free cholesterol from macrophages and influx of cholesteryl ester to macrophages after treatments were conducted according to a previous description.

Analysis of SR-BI, NF-κB p65, and IkB-α Expression by Western Blotting and SR-BI Surface Expression by FACS

After treatment, macrophages were washed twice with cold PBS, then scraped and lysed in ice-cold lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/mL NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L PMSF, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 50 µg/mL aprotinin, and 50 µg/mL leupeptin). Lysate was sonicated for 20 cycles, then microcentrifuged for 15 minutes at 4°C, and the supernatant was transferred to a new test tube. After the content had been determined, proteins were loaded and separated on 12% SDS-PAGE and transferred onto nylon-enhanced nitrocellulose membrane. The membranes were blocked with a solution of 0.1% Tween 20/PBS (PBS-T) containing 5% fat-free milk for 1 hour, then incubated with either rabbit polyclonal anti–SR-BI (1:2000), anti–NF-κB (1:2000), or anti–IkB-α (1:2000) antibody, respectively, for 2 hours at room temperature, followed by a washing for 3× 10 minutes with PBS-T buffer. The blot was reblocked with PBS-T containing 5% milk followed by incubation with horseradish peroxidase conjugated goat anti-rabbit IgG for another hour at room temperature. After washing 3×10 minutes with PBS-T, the membrane was incubated for 1 minute in a mixture of
was resuspended in 100 μL of cold buffer A (mmol/L: 10 HEPES, pH 7.9, 10 KCl, 0.1 EDTA, 0.1 EGTA, 1 DTT, and 0.5 PMSF). After 15 minutes’ incubation on ice, cell suspensions were added to 25 μL of 10% NP-40 and vortexed vigorously for 10 seconds. The supernatant was collected as nuclear proteins. Nuclear proteins were spun again for 15 minutes at 13,000 rpm with a microfuge, and the pellet was removed after spinning for 30 seconds at 13,000 rpm. The pellet containing nuclear proteins (8 μg/sample) and 32P-labeled oligo probe was end-labeled with [γ-32P]-ATP by T4 polynucleotide kinase and purified with a MicroSpin G-25 column. Reaction mixture was loaded on precooled, 5% polyacrylamide gel. The complex of nuclear protein–DNA probe was separated from the unbound probe by electrophoresis and detected by radiography after the gel was air-dried.

Data Analysis
All experiments were repeated at least 3 times, and representative results are presented. Data were analyzed by paired t test in an assay of cholesterol efflux, influx of cholesteryl oleate, and TNF-α secretion.

Results
To determine whether the novel statin pitavastatin (NK-104) can alter macrophage SR-BI expression, J774 cells were treated with pitavastatin at different concentrations overnight, and SR-BI expression was determined by Western blot (Figure 1A). Compared with control (cells treated with the solvent that dissolved pitavastatin, DMSO), we found that pitavastatin significantly induced SR-BI protein expression. The maximal induction was obtained with 10 μmol/L of pitavastatin (△3-fold). To determine whether the induction of SR-BI protein by pitavastatin also occurred at the transcriptional level, we analyzed the RNA transcript by Northern blot (Figure 1B). The maximal induction was obtained with 10 μmol/L of pitavastatin (△3-fold). To determine whether the induction of SR-BI mRNA by pitavastatin also occurred at the transcriptional level, we analyzed the RNA transcript by Northern blot (Figure 1B).

To study the dynamic changes of SR-BI expression in response to pitavastatin treatments, macrophages were treated with 10 μmol/L pitavastatin for various times and analyzed.
for SR-BI protein expression by Western blot (Figure 1C). The induction of SR-BI expression by pitavastatin was observed as early as 1 hour after treatment, suggesting a rapid response. Maximal induction occurred after 12 hours of treatment, and it was maintained for 24 hours after treatment.

We also determined whether other well-established statins could alter SR-BI protein expression in macrophages. Simvastatin, pravastatin, and rosuvastatin also significantly induced macrophage SR-BI expression (expression in response to pitavastatin were evaluated by determination of HDL, binding to macrophages, efflux of free cholesterol to HDL from macrophages, and influx of cholesteryl oleate/HDL to macrophages. Figure 2 demonstrates that pitavastatin significantly increased HDL binding to macrophages (Figure 2A), and it also enhanced the efflux of free cholesterol to HDL from macrophages and uptake of cholesteryl oleate/HDL by macrophages (Figure 2B).

To investigate the physiological relevance of statins on macrophage SR-BI expression, we isolated peritoneal macrophages from mice and treated them with pitavastatin overnight. SR-BI expression was determined by Western blot (Figure 1D). The functional consequences of increased SR-BI expression in response to pitavastatin were evaluated by determination of HDL, binding to macrophages, efflux of free cholesterol to HDL from macrophages, and influx of cholesteryl oleate/HDL to macrophages. Figure 2 demonstrates that pitavastatin significantly increased HDL binding to macrophages (Figure 2A), and it also enhanced the efflux of free cholesterol to HDL from macrophages and uptake of cholesteryl oleate/HDL by macrophages (Figure 2B).

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Both LPS and TNF-α suppressed more than 50% of SR-BI expression. Although NF-κB inhibitors alone did not show effects on SR-BI expression, they significantly blocked the inhibitory actions of LPS and TNF-α on SR-BI expression (Figure 5). These data indicate that LPS and TNF-α can decrease SR-BI expression through their activation of NF-κB. LPS more likely inhibits macrophage SR-BI expression through its stimulation of TNF-α production in macrophages, because the secretion of TNF-α in response to LPS treatment was rapid. After 2 hours of treatment with 200 ng/mL LPS, we detected that the concentration of TNF-α in serum-free cultured medium was 32.5 ± 2.3 ng/mL, compared with undetectable levels of TNF-α in control cells even after 10 hours of incubation.

To determine whether the induction of macrophage SR-BI expression by pitavastatin may have occurred as a result of some antiinflammatory action, we first determined whether pitavastatin could block the inhibitory actions of LPS and TNF-α on SR-BI expression. We found that LPS and TNF-α significantly decreased SR-BI expression; pitavastatin partially blocked LPS action but totally overwhelmed the TNF-α action on macrophage SR-BI expression (Figure 6).

Finally, to investigate this possibility further, we determined whether pitavastatin could alter NF-κB DNA binding activity using electrophoretic mobility shift assay. Figure 7A demonstrates that pitavastatin reduced more than 50% of NF-κB DNA binding activity in J774 macrophages. We further determined whether pitavastatin was able to regulate expression of NF-κB complex proteins. NF-κB p65 expression was moderately decreased, but IkB-α expression was significantly increased, by pitavastatin (Figure 7B). The opposite effects of pitavastatin on these 2 proteins could lead to the decreased NF-κB DNA binding activity.

Discussion

LDL cholesterol levels are correlated with the incidence of coronary heart disease. Both the reduction of dietary cholesterol and statin therapy to reduce de novo synthesis of cholesterol have had significant clinical benefits. Clinical benefits of statin therapy have been identified in addition to their lipid-lowering actions, suggesting that these pharmacological agents have numerous pleiotropic effects. In this study, we observed that statins can increase macrophage SR-BI expression through cholesterol biosynthetic intermediates and antiinflammatory action. Tsuruoka et al consistently demonstrated a similar observation in human keratinocytes by simvastatin.

The effects of pitavastatin on SR-BI expression through the influence on cholesterol biosynthesis are consistent with our previous report that lipid loading of macrophages can reduce SR-BI expression. In addition to macrophages, reducing
intracellular cholesterol levels by β-cyclodextran demonstrates a reverse relationship between SR-BI expression and the cellular cholesterol pool in an adrenal cell line (Y1-BS1). It is noteworthy that although macrophages express the cellular cholesterol pool in an adrenal cell line (Y1-BS1),18 it is noteworthy that although macrophages express the cellular cholesterol pool in an adrenal cell line (Y1-BS1).18 It is noteworthy that although macrophages express the cellular cholesterol pool in an adrenal cell line (Y1-BS1).18

Antinflammatory effects of statins include the inhibition of leukocyte-endothelium interactions, the reduction of the expression of adhesion molecules such as intercellular adhesion molecule-1, and the suppression of the production of proinflammatory cytokines (IL-1β, IL-6, and cyclooxygenase-2).10 The effects of statins on TNF-α are controversial. Lovastatin inhibits LPS-induced expression of TNF-α in rat primary astrocytes, microglia, and macrophages,20 but it synergizes antiproliferative activity in MmB16 melanoma cells and L1210 leukemia cells,21 antitumor effects against Ha-ras–transformed murine tumor,22 and induction of E-selectin, intercellular adhesion molecule-1, and vascular adhesion molecule-1 in endothelial cells with TNF-α.23 Although atorvastatin suppresses TNF-α production in Th1 cells,24 it works cooperatively with TNF-α to stimulate the generation of PAI-1 in PMA- or TGF-β1/1a,25 dihydroxyvitamin D3–differentiated HL-60 cells.25 Atorvastatin has no effect on TNF-α production in familial hypercholesterolemia patients.26 Rosuvastatin decreases TNF-α expression in apoE*3-Leiden transgenic female mice, whereas cerivastatin has no effect on human macrophage TNF-α production.27 In contrast, Kiener et al29 reported that atorvastatin, lovastatin, and simvastatin can stimulate the production of TNF-α in human monocytes. To determine whether pitavastatin increased macrophage SR-B1 expression by altering TNF-α levels, we first assessed the amount of TNF-α in culture medium after pitavastatin treatment. Consistent with some of the above-described observations, we did not see changes in TNF-α levels by pitavastatin in our cells up to 10 hours of treatment (data not shown); however, after 14 hours of treatment, we observed a moderate increase in TNF-α. For instance, TNF-α levels were 0.064 ± 0.051, 0.136 ± 0.054, and 3.153 ± 1.18 ng/mL in control and 1- and 5-μmol/L pitavastatin–treated samples, respectively (significantly different from control at P < 0.05). Greater increases in TNF-α levels were observed with longer treatments of cells, and the induction was concentration-dependent (data not shown). We also observed that pitavastatin failed to block LPS-induced TNF-α production (data not shown). We believe that these data suggest that induction of macrophage SR-B1 expression by pitavastatin is not through its effect on macrophage TNF-α production. Compared with the rapid increase in SR-B1 expression, pitavastatin increased TNF-α at a very slow rate. This action does not negatively affect SR-B1 induction, because we observed that pitavastatin significantly

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pitavastatin inhibited NF-κB DNA binding activity. Confluent J774 macrophages were treated with pitavastatin at indicated concentrations for 12 hours. Nuclear proteins were extracted to assess NF-κB DNA binding activity by electrophoretic mobility shift assay, and whole cellular proteins were used to assess NF-κB p65 and IκB-α expression by Western blot, respectively. A, Effect of pitavastatin on NF-κB DNA binding activity; 8 µg of nuclear proteins from each sample was used to conduct DNA-protein reactions as described in Methods. Samples a to c were PMA-treat Jurkat nuclear extract supplied in assay kits. In reaction of sample b, an ~100-fold excess of unlabeled probe was added to reaction system. In reaction of sample c, an ~100-fold excess of unlabeled mutant probe was added to reaction system. B, Effect of pitavastatin on NF-κB p65 and IκB-α expression: 40 µg of cellular proteins from each sample was loaded on 12% SDS-PAGE to separate and detect NF-κB p65 and IκB-α expression by Western blot.

Figure 7. Pitavastatin inhibited NF-κB expression. NF-κB inhibitors restore macrophage SR-BI expression that is initially inhibited by LPS or TNF-α (Figure 5) imply that NF-κB might be involved in the induction of SR-BI expression by pitavastatin. Indeed, we observed that pitavastatin functioned as an NF-κB inhibitor to restore SR-BI expression that was initially inhibited by LPS or TNF-α (Figure 6). Statins have been reported to reduce NF-κB activity in several cell types.31,32 We consistently detected that pitavastatin inhibited NF-κB activity in macrophages and that this inhibitory action was because of increased IκB-α expression and decreased NF-κB p65 expression (Figure 7). We interpret our results to indicate that the impact of pitavastatin on NF-κB activity may play a more important role in addition to its effects on cholesterol biosynthetic intermediates in its induction of macrophage SR-BI expression.

Finally, PPAR-γ, a transcriptional factor in the regulation of fatty acid metabolism, diabetes, inflammation, and atherosclerosis,31 plays a critical role in the regulation of CD36 expression. However, its role in the regulation of SR-BI expression is controversial. Although some PPAR-γ ligands have been reported to increase SR-BI expression34 and stimulate HMG-CoA reductase activity in macrophages,35 we have observed that most PPAR-γ ligands do not have any effect on macrophage SR-BI expression. In contrast, we observed that ciglitazone can have strong inhibitory effects on macrophage SR-BI expression (unpublished observations). Previously, we reported that pitavastatin decreased macrophage PPAR-γ expression at both the RNA and protein levels. This statin can increase mitogen-activated protein kinase activity and the level of phosphorylated PPAR-γ, a negative regulator for its target genes.12 Therefore, we believe that the induction of macrophage SR-BI expression by pitavastatin does not occur primarily through PPAR-γ but rather through cholesterol biosynthetic intermediates and NF-κB activity. These findings suggest additional pleiotropic effects of this drug during atherosclerotic foam cell development.

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References


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