Rosuvastatin Reduces Atherosclerosis Development Beyond and Independent of Its Plasma Cholesterol–Lowering Effect in APOE*3-Leiden Transgenic Mice

Evidence for Antiinflammatory Effects of Rosuvastatin

Robert Kleemann, PhD; Hans M.G. Princen, PhD; Jef J. Emeis, PhD; J. Wouter Jukema, MD, PhD; Ruud D. Fontijn, MSc; Anton J.G. Horrevoets, PhD; Teake Kooistra, PhD; Louis M. Havekes, PhD

Background—Statins can exert anti-inflammatory antithrombogenic effects through an anti-inflammatory action, independent of lowering cholesterol. We addressed the question whether the anti-inflammatory activities of statins can reduce atherosclerosis beyond the reduction achieved by cholesterol lowering per se.

Methods and Results—Two groups of 20 female APOE*3-Leiden mice received either a high-cholesterol diet (HC) or a high-cholesterol diet supplemented with 0.005% (wt/wt) rosuvastatin (HC+R). The HC diet alone resulted in a plasma cholesterol concentration of 18.9±1.4 mmol/L, and administration of rosuvastatin lowered plasma cholesterol to 14.1±0.7 mmol/L. In a separate low-cholesterol (LC) control group, the dietary cholesterol intake was reduced, which resulted in plasma cholesterol levels that were comparable to the HC+R group (13.4±0.8 mmol/L). Atherosclerosis in the aortic root area was quantified after 24 weeks. As compared with the HC group, the LC group had a 62% (P<0.001) reduction in cross-sectional lesion area. When compared with the LC group, the HC+R group showed a further decrease in cross-sectional lesion area (80%, P<0.001), size of individual lesions (63%, P<0.05), lesion number (58%, P<0.001), monocyte adherence (24%, P<0.05), and macrophage-containing area (60%, P<0.001). Furthermore, rosuvastatin specifically suppressed the expression of the inflammation parameters MCP-1 and TNF-α in the vessel wall and lowered plasma concentrations of serum amyloid A and fibrinogen, independent of its cholesterol-lowering effect.

Conclusions—Rosuvastatin reduces atherosclerosis beyond and independent of the reduction achieved by cholesterol lowering alone. This additional beneficial effect of rosuvastatin may be explained, at least partly, by its anti-inflammatory activity. (Circulation. 2003;108:1368-1374.)

Key Words: atherosclerosis • inflammation • inhibitors • cholesterol • cell adhesion molecules

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, statins, are lipid-lowering drugs that effectively lower LDL cholesterol levels and reduce the risk of cardiovascular events in hyperlipidemic and normolipidemic patients (reported in References 1 and 2, among others).2,4 These clinical studies also suggest that statins exert vascular protective effects that cannot be fully explained by their cholesterol-lowering properties.3,4 Other studies (for review, see Reference 5) indicate that atherosclerosis is not solely a lipid disorder but also an inflammatory disease. Statins have been found to exert anti-inflammatory effects in vascular cells both in vitro and in vivo, indicating that they may exert important protective effects in the arterial wall (summarized in Reference 6).6 Several in vivo studies demonstrated antiatherogenic effects of statins in the absence of lipid-lowering but with an anti-inflammatory effect as a common denominator.7–11 These studies do not reveal, however, whether the lipid-lowering effect and the anti-inflammatory effect contribute in an additive way to a reduction of atherosclerosis.

Rodent studies may help to discriminate between the cholesterol-lowering and the anti-inflammatory effects of statins. APOE*3-Leiden transgenic mice are an established model for hyperlipidemia and atherosclerosis.12,13 These mice have a lipoprotein profile that is very similar to the profile of patients with familial dysbetalipoproteinemia in which the elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction. Depending on their plasma cholesterol levels, APOE*3-Leiden mice have atherosclerotic lesions that are comparable to their...
human counterparts with respect to morphological, histological, and immunohistochemical characteristics, which allows analysis of the vasculoprotective effects of lipid-lowering compounds, including statins. Plasma cholesterol levels in APOE*3-Leiden transgenic mice can easily be titrated to a desired level by adjusting the dietary cholesterol intake. This enabled us to study additional effects of a statin on atherogenesis, independent of and additive to its cholesterol-lowering effect, and to compare the relative contribution of a potential additive effect to the effect achieved by cholesterol lowering alone.

The current study in APOE*3-Leiden mice addresses the question whether the HMG-CoA reductase inhibitor rosuvastatin, a compound with strong lipid-lowering qualities and anti-inflammatory properties, has antiatherogenic effects independent of but additive to its lipid-lowering effects and characterizes the nature of these additional effects.

Methods

Mice
Female heterozygous APOE*3-Leiden transgenic mice of generation F22 (13 to 14 weeks of age), characterized by an ELISA for human apoE, were used. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO). Animals were from TNO-Prevention and Health.

Diets
During a run-in period of 3 weeks, animals received a Western-type diet. Thereafter, animals were subdivided into 3 groups of 20 mice each on the basis of age and cholesterol level. After 1 week of adaptation, one group received a diet containing 1% (wt/wt) cholesterol and was termed the high-cholesterol control group (HC). The rosuvastatin-treated group (HC+R) received the same diet as the HC group but supplemented with 0.005% (wt/wt) rosuvastatin, equaling 5 mg/kg body wt per day. Rosuvastatin was provided by AstraZeneca UK Ltd. The low-cholesterol (LC) control group received a diet containing 0.15% (wt/wt) cholesterol at the start of the study and subsequently 0.25% (wt/wt) cholesterol beginning at 4 weeks, to reach the same plasma cholesterol level in these mice as in the HC+R group. Animals had free access to water and food. Food intake and body weight were monitored throughout the study.

Lipid and Lipoprotein Analysis
Total plasma cholesterol and triglyceride levels were measured after 4-hour fasting with kits No. 1489437 (Roche Diagnostics) and No. 337-B (Sigma Diagnostics), respectively. Lipoprotein profiles were obtained by density gradient ultracentrifugation. In isolated lipoproteins, analysis of lipids was performed with the use of high-performance thin-layer chromatography.

Quantification and Qualification of Atherosclerosis
After 24 weeks of diet feeding, mice were killed after anesthetization and dissected hearts were fixed and embedded in paraffin. Serial cross sections (5 μm) throughout the entire aortic valve area were prepared for histological analysis and stained with hematoxylin-phloxine-saffron (HPS). Atherosclerotic lesions were graded as described previously. Cross-sectional areas were quantified with the use of the morphometric software QWin (Leica). Four sections of each specimen, at intervals of 30 μm, were analyzed to determine the average lesion number, size, and area, respectively.

Monocytes and macrophages were immunostained with A1A31240 (1:3000, Accurate Chemical and Scientific). Adherent monocytes were counted in the same four sections used for quantification of atherosclerosis.

In Situ Hybridization
In situ mRNA hybridization was performed on eight 5-μm cross sections from the aortic root (spacing, 45 μm). Riboprobes were labeled with [35S]-UTP (Amersham Biosciences) by in vitro transcription of cloned cDNA fragments. These fragments were resequenced after subcloning from murine IMAGE-consortium cDNA clones obtained from the UK Human Genome Mapping Project Resource Centre (Cambridge, UK) and shown to correspond to the 767-bp full-length murine MCP-1 cDNA:33–800 (GenBank accession No. GI675429) and the 603bp EcoRI-BglII fragment of TNF-α cDNA:589 to 1192 (GenBank accession No. GI202084), respectively. The number of cells with positive in situ signal and the total number of nuclei, that is, cells in the intima and media, were determined, and data were presented as number of positive cells per 3×10² total cells.

Determination of von Willebrand Factor and Serum Amyloid A
Plasma von Willebrand factor (vWF), serum amyloid A (SAA), and fibrinogen were measured by ELISA specific for vWF, SAA (Biosource), and fibrinogen, respectively.

Statistical Methods
Data were not normally distributed, as verified by the Kolmogorov-Smirnov and the Shapiro-Wilk tests. Significance of differences was calculated by use of the nonparametric Mann-Whitney U test. Probability values of P < 0.05 were considered significant. All data are presented as mean ± SD.

Results

Effect of Rosuvastatin on Plasma Lipid and Lipoprotein Concentrations
The average food intake of the HC + R group (2.8 ± 0.1 g per mouse per day) was slightly reduced when compared with the HC group (3.0 ± 0.1 g) (P < 0.05) but not significantly different from the LC group (2.9 ± 0.1 g). After 24 weeks of treatment, the average body weight of the HC + R group (22.7 ± 1.3 g) was slightly reduced compared with the HC (24.3 ± 1.4 g) and LC (25.1 ± 2.0 g) control groups (P < 0.05).

Figure 1A shows the plasma total cholesterol concentrations over the whole intervention period. Between 7 and 24 weeks, the plasma cholesterol levels of the HC + R group were lowered by 25% (P < 0.05) compared with the HC group. The differences in cholesterol concentrations between the LC and the HC + R group during this period were not significant. Triglyceride levels in the HC + R group were lower than in the two control groups at all time points, except at 24 weeks (Figure 1B). Between 7 and 24 weeks of treatment, the average plasma triglyceride-lowering effect of rosuvastatin was 45% compared with the HC group (P < 0.05). The total cholesterol exposure over 24 weeks for the HC + R and the LC groups was comparable and, respectively, 27% (P < 0.001) and 32% (P < 0.001) lower than for the HC group (Figure 1C).

Figure 1D depicts the lipoprotein profiles for cholesterol at 13 weeks of treatment. Rosuvastatin mainly reduced VLDL and LDL cholesterol; LDL cholesterol was slightly increased, and LDL particles became smaller. HDL cholesterol was slightly reduced by rosuvastatin. No differences in lipid composition of lipoproteins, for example, free cholesterol, cholesterol esters, triglycerides, and phospholipids, were observed between the groups (data not shown).
Quantification of Atherosclerosis

After 24 weeks of treatment, atherosclerotic lesions and lesion area were quantified in cross sections of the aortic valve area. The total number of lesions per mouse did not differ significantly between the HC and LC control groups (Figure 2A), but treatment with rosuvastatin reduced the total number of lesions on average by 65% (P<0.001) and 58% (P<0.001) when compared with the HC and LC group, respectively.

Figure 2B shows the average size of individual lesions per mouse. The LC control group had a 56% (P<0.01) reduced size of individual lesions compared with the HC control group. However, treatment with rosuvastatin reduced the average size of individual lesions by 84% (P<0.001) when compared with the HC group and by 63% (P<0.005) when compared with the LC group. Determination of the cross-sectional lesion area revealed a similar effect (Figure 2C): The LC control group showed a 62% (P<0.001) reduced cross-sectional lesion area when compared with the HC group. Rosuvastatin-treatment resulted in a 92% (P<0.001) reduced cross-sectional lesion area as compared with the HC control group and an 81% (P<0.001) further reduction compared with the cholesterol-matched LC group. These data indicate that rosuvastatin treatment reduces atherosclerosis development by mechanisms dependent and independent of its cholesterol-lowering effect.

Effect of Rosuvastatin on Cellular Composition of Lesions

To evaluate the nature of the cholesterol-lowering independent effects of rosuvastatin, specific aspects relevant for the progression of atherosclerosis were analyzed in detail. Figure 3A shows the number of monocytes adhering to the endothelium. Attachment of monocytes to the endothelium was not significantly different between the HC and LC control groups. However, treatment with rosuvastatin significantly reduced the number of monocytes adhering to the endothelium when compared with the LC control, suggesting that diminished endothelial cell activation contributes to the antiatherosclerotic activity of rosuvastatin, independent of its cholesterol lowering. In addition, the macrophage-containing area in aortic plaques was markedly reduced by rosuvastatin when
compared with both control groups (Figure 3B). The area in the aortic root that contained necrotic cells and cholesterol clefts was reduced by 79% ($P < 0.05$) in the LC group and by 98% ($P < 0.005$) in the HC group when compared with the HC group (Figure 3C). No significant differences were found between the HC group and the LC group in this respect.

**Effect of Rosuvastatin on Expression of Proatherogenic Cytokines in the Vessel Wall**

The expression of cytokines that contribute to the progression of atherogenesis was evaluated in the aortic root by in situ mRNA hybridization (Figure 4A). The number of MCP-1–positive cells in the media and intima, normalized for the total number of cells in the vessel wall, was 57% ($P < 0.05$) reduced in the LC control group when compared with the HC group (Figure 4B). Treatment with rosuvastatin further reduced the proportion of MCP-1–positive cells by 77% ($P < 0.01$) when compared with the LC group and by 90% ($P < 0.01$) when compared with the HC group. A similar cholesterol-independent effect of rosuvastatin was observed for the proportion of TNF-α–positive cells (Figure 4C). Again, there was a trend toward a reduction in the number of TNF-α–positive cells by dietary cholesterol lowering. However, treatment with rosuvastatin reduced the number of TNF-α–positive cells in the vessel wall by 88% ($P < 0.05$) when compared with the LC group. Altogether, rosuvastatin treatment markedly suppressed the expression of MCP-1 and TNF-α in the lesions.

**Anti-Inflammatory Effects of Rosuvastatin**

To further assess the extent of the anti-inflammatory activity of rosuvastatin, we measured the plasma levels of vWF, a marker for the activation of vascular endothelial cells, and two general inflammation blood marker produced by the liver, serum amyloid A (SAA) and fibrinogen. No significant differences in plasma vWF concentrations were found between the groups (data not shown). Treatment with rosuvastatin reduced plasma SAA concentrations by 43% ($P < 0.005$) and 39% ($P < 0.05$) when compared with the HC and LC group, respectively (Figure 5A). Plasma fibrinogen concentrations of the LC group were 35% ($P < 0.001$) reduced when compared with the HC group. Rosuvastatin treatment further
reduced fibrinogen concentrations by an additional 19% (P<0.01), indicating an anti-inflammatory effect independent of plasma cholesterol lowering in the liver.

**Discussion**

In the absence of plasma lipid-lowering, statins have been shown to exert vasculoprotective effects, including anti-inflammatory activities.\(^6\)\(^{−}\)\(^11\) It is unknown however, whether these vasculoprotective effects of statins contribute in an additive way to the reduction of atherosclerosis as achieved by cholesterol lowering per se. In this report, we provide evidence that the reducing effect of rosuvastatin on atherosclerosis development exceeds the effect achieved by cholesterol lowering alone.

Comparison of ApoE\(^*\)3-Leiden mice on a high cholesterol diet (HC group) to ApoE\(^*\)3-Leiden mice on a low cholesterol diet (LC group) demonstrated that cholesterol lowering reduces the cross-sectional lesion area by 62%. The rosuvastatin-treated group (HC+R) displayed a further 81% reduction of the cross-sectional lesion area when compared
with the cholesterol-matched LC group. These results indicate additive beneficial effects of rosuvastatin on atherosclerosis that are independent of its plasma cholesterol-lowering effect and that contribute to a further reduction of disease progression. We characterized the nature of these effects and showed that anti-inflammatory activities of rosuvastatin in the vasculature and liver partly may explain the observed additional reduction of atherosclerosis.

Treatment of APOE*3-Leiden mice with rosuvastatin reduced plasma cholesterol levels by >25%. At the same time, lipoprotein composition remained unchanged. The cholesterol-lowering net effect of rosuvastatin in APOE*3-Leiden mice can be attributed primarily to the observed reduction of VLDL and IDL particles. Comparison of the HC and LC group allows estimation of the effect of a moderate decrease in plasma cholesterol concentrations (19 mmol/L to 14 mmol/L) on atherogenesis: Both the average size of individual lesions and the cross-sectional lesion area were strongly reduced. However, the average lesion number per mouse, for example, the incidence of lesions in the vessel wall, was comparable in both groups and thus independent of plasma cholesterol levels in this range. Importantly, treatment with rosuvastatin not only reduced the average size of individual lesions and the cross-sectional lesion area but also the average number of lesions compared with the LC group. This observation is novel and points to cholesterol-independent inhibitory effects of rosuvastatin on initiating events related to lesion formation.

Monocyte adhesion is an initiating event in lesion formation and involves MCP-1 as a central player in the recruitment of monocytes. Statins reduce monocyte-endothelial cell interactions in vitro. We observed a reduced number of monocytes adhering to the endothelium in the rosuvastatin-treated group and provide in vivo evidence for diminished monocyte-endothelial cell interactions in the aorta, independent of the plasma cholesterol-lowering effect. Our observation extends the reported attenuation of thrombin-induced leukocyte adherence to rat microvascular endothelial cells by rosuvastatin to the aorta.

In the absence of a statin effect on plasma cholesterol, statin-treated animals displayed a reduced macrophage accumulation in the intima and media, indicating a cholesterol-independent effect. Our observations support this notion, since cholesterol lowering alone had no effect on the macrophage-containing area and since rosuvastatin reduced the macrophage-containing area when compared with the cholesterol-matched LC group.

To address the nature of the activities of rosuvastatin that contribute to a further reduction of atherosclerosis independent of the plasma cholesterol-lowering effect, we analyzed the vascular expression of MCP-1 and TNF-α. Reduction of MCP-1 expression by other statins has been observed in vitro and in hypercholesterolemic patients. Comparison of the HC and LC groups revealed that lowering plasma cholesterol concentrations reduced the expression of both cytokines. However, an additional reduction was observed in the HC+R group, displaying a 77% (88%) reduced MCP-1 (TNF-α) expression compared with the LC group. These data may provide a first molecular explanation for the further reduction of atherosclerosis by rosuvastatin. Of note, rosuvastatin also reduced the plasma concentrations of liver-derived blood inflammation markers SAA and fibrinogen independent of its plasma cholesterol-lowering effect. SAA and fibrinogen are risk factors for cardiovascular disease, reflecting the overall inflammatory state. The anti-inflammatory activities of rosuvastatin are thus not restricted to the vasculature and, in addition, the reduction of liver-derived cardiovascular risk factors by rosuvastatin may contribute to the observed additional suppression of atherosclerosis development.

Elevated plasma triglyceride levels represent an independent risk factor for cardiovascular disease. Rosuvastatin treatment reduced plasma triglyceride concentrations by >45%. Our findings do not exclude the possibility that this decrease also contributes to the reduction of atherosclerosis. Lipoprotein profile analysis revealed a slight increase in LDL cholesterol and decrease in HDL cholesterol by rosuvastatin. Despite these effects rosuvastatin strongly reduced the development of atherosclerosis thereby underscoring the potency of its antiatherosclerotic effects independent of cholesterol lowering.

Together, our results provide convincing evidence that rosuvastatin exerts activities independent of cholesterol lowering that have an additional beneficial effect on atheroscle-
nosis development. The demonstrated anti-inflammatory activities of rosuvastatin in the vasculature and in the liver contribute to this additional effect. Although morphological and functional differences exist, murine and human lesions share many features. Our findings may be of relevance for the human situation and may lead to extended therapeutic indications based on the anti-inflammatory properties of statins.

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References

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