

Rosuvastatin Attenuates Monocyte-Endothelial Cell Interactions and Vascular Free Radical Production in Hypercholesterolemic Mice

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Received November 2, 2004; accepted January 20, 2005

ABSTRACT

One of the earliest observable events in atherogenesis is enhanced monocyte adhesion to the endothelium. In addition to reducing circulating levels of cholesterol, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) are thought to have direct salutary effects upon vascular cells. We hypothesized that the new statin, rosuvastatin, would have anti-inflammatory effects on the vessel wall. Eight-week-old apolipoprotein E-deficient mice were fed a normal chow diet for a period of 12 weeks. During this time mice were administered vehicle or rosuvastatin at a dose of 0, 1, 5, or 20 mg/kg by subcutaneous injection at the same time daily for a period of 2 or 6 weeks prior to sacrifice. At the end of the study, rosuvastatin-treated animals displayed lower plasma total cholesterol levels, whereas

showing little change in high-density lipoprotein cholesterol or triglycerides. Using a functional binding assay, we also demonstrated that endothelial adhesiveness for monocytes was significantly attenuated after 2 weeks of treatment with rosuvastatin. Quantitative real-time polymerase chain reaction determined that rosuvastatin reduced the expression of vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, and metalloproteinase-9 in the vessel wall. In addition, rosuvastatin inhibited vascular expression of p22^{phox} and superoxide production, as well as diminishing plasma 8-isoprostanes concentrations. Thus, treatment with rosuvastatin has acute anti-inflammatory actions that likely participate in its beneficial actions during atherogenesis.

The major cause of morbidity and mortality in Western civilization is atherosclerosis. The earliest observable abnormality of the vessel wall in animal models of atherogenesis is enhanced monocyte adherence to the endothelium (Ross et al., 1977). This event is mediated by the surface expression of endothelial adhesion molecules and chemotactic proteins induced by risk factors such as hypercholesterolemia. The exact mechanisms by which hypercholesterolemia accelerates monocyte adhesion (and, therefore, atherosclerotic disease) have not been fully delineated; however, recent evidence indicates that increased local oxidative stress may play a major role. In addition to inducing vascular smooth muscle cell proliferation (Griendling and Fitzgerald, 2003a), excess oxygen-derived free radicals can increase the expression of proinflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1) (Marui et al., 1993) and monocyte chemo-

tactic protein-1 (MCP-1) (Tsao et al., 1997), both of which are involved with the recruitment of monocytes.

One of the most important innovations in the treatment of atherosclerotic disease has been the development and use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). Several major clinical trials have demonstrated a clear benefit of statins upon cardiovascular endpoints as well as surrogate markers of coronary artery disease (Anonymous, 1994, 1998a,b; Heart Protection Study Collaborative Group, 2002). Yet, the mechanisms by which LDL cholesterol initiates atherogenesis are not completely delineated. Significant research has focused on how the cells within atherosclerotic lesions accumulate lipid. As such, simple lowering of cholesterol levels would reduce the rate of lipid accumulation and inhibit atherogenesis. However, closer examination of clinical trial data has led to the concept that the benefit of statins may go beyond their ability to lower circulating LDL levels (Anonymous, 1998a). In addition, recent preclinical studies have shown that statins can, indeed, directly affect intracellular processes. These actions

This work was supported by a grant from AstraZeneca.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.104.080002.

ABBREVIATIONS: VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoprotein; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; apoE, apolipoprotein E; HBSS, Hanks' balanced salt solution; HDL, high-density lipoprotein; PG, prostaglandin; RT-PCR, real-time polymerase chain reaction; MMP, metalloproteinase; ROS, reactive oxygen species; TA, 4-amino-2,2,6,6-tetramethylpiperidino-1-oxyl; CRP, C-reactive protein; NF- κ B, nuclear factor of the κ -enhancer in B cells; PPAR, peroxisome proliferator-activated receptor.

TABLE 1
Plasma lipid values

	ApoE(-/-) + Rosuvastatin				
	C57BL/6	0	1	5	20
	<i>mg/kg/day</i>				
2-Week treatment (mg/dl)					
TC	82 ± 3.7	675 ± 42	700 ± 39	481 ± 35*	371 ± 27**
HDL	32.2 ± 1.8	29.6 ± 1.8	31.1 ± 1.4	30.3 ± 1.9	29.6 ± 1.6
TG	95.7 ± 4.1	141 ± 5.3	135 ± 4.0	136 ± 4.3	143 ± 3.9
6-Week treatment (mg/dl)					
TC	88.8 ± 3.8	660 ± 44	451 ± 23*	400 ± 38**	305 ± 19**
HDL	30.7 ± 1.8	32.4 ± 1.4	31.0 ± 1.5	30.7 ± 1.5	28.3 ± 1.6
TG	94.2 ± 3.6	145 ± 4.4	147 ± 5.3	137 ± 4.1	137 ± 3.8

TC, total cholesterol; TG, triglycerides.
* $p < 0.05$ versus apoE(-/-) + vehicle.
** $p < 0.01$ versus apoE(-/-) + vehicle.

though having no changes in HDL cholesterol compared with C57BL/6 mice. Two-week treatment with rosuvastatin at 5 and 20 mg/kg reduced plasma total cholesterol levels. The lower dose of 1 mg/kg had no effect at this time point. However, following 6 weeks of treatment all doses reduced total cholesterol levels. None of the doses (at either time point) had any effect on HDL cholesterol or triglyceride levels.

Monocyte Binding. To monitor the effects of rosuvastatin on monocyte-endothelial cell interactions, thoracic aortae

were carefully harvested and functional binding assays performed using fluorescently labeled monocytoid cells (Fig. 1). Elevated cholesterol levels at 20 weeks of age were consistently associated with increased endothelial adhesiveness for monocytes compared with normocholesterolemic control animals (Fig. 2). Two-week treatment with rosuvastatin diminished monocyte binding at the highest dose tested (20 mg/kg). More robust effects were observed with 6 weeks of treatment in that both 5 and 20 mg/kg doses of rosuvastatin signifi-

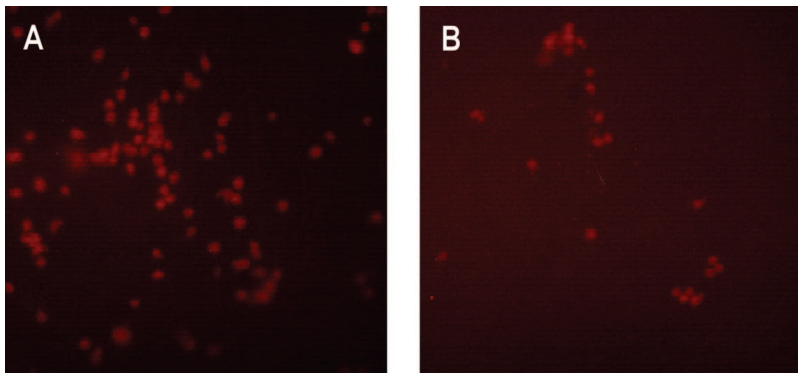


Fig. 1. Epifluorescent photomicrographs after monocyte adhesion to thoracic aortae derived from apoE-deficient mice treated with vehicle (A) or rosuvastatin (B) (20 mg/kg/day for 6 weeks).

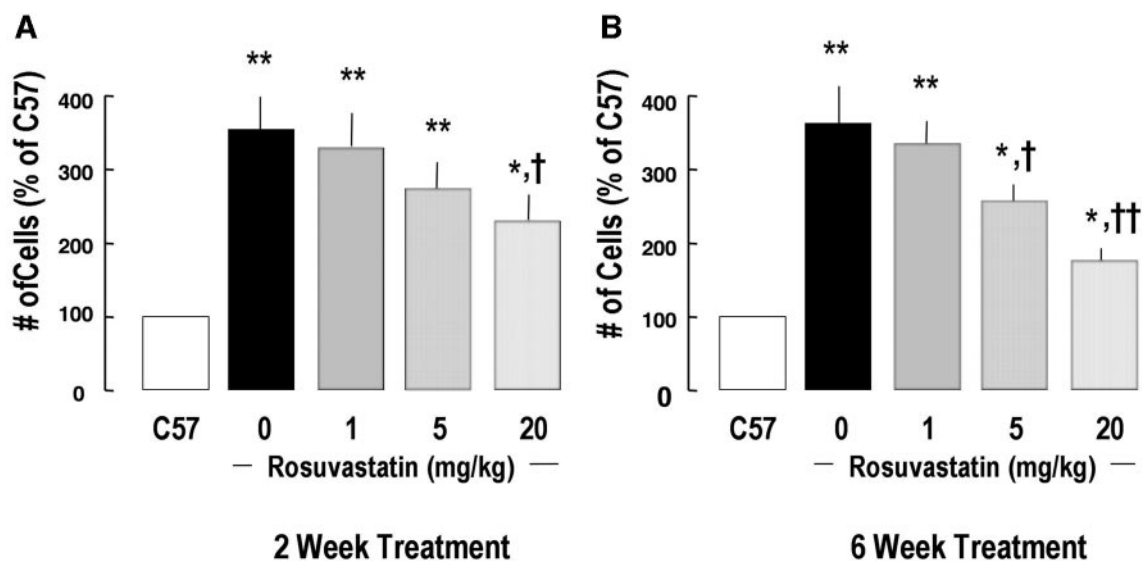


Fig. 2. Enhanced monocyte-endothelial cell interactions in hypercholesterolemia are inhibited by rosuvastatin treatment. Segments of thoracic aortae were harvested from control animals ($n = 10$), cholesterol animals ($n = 10$), and rosuvastatin treatment (1, 5, or 20 mg/kg/day; $n = 10$ each group) at 20 weeks of age. Rosuvastatin animals were treated for either 2 (A) or 6 (B) weeks prior to sacrifice. All values were expressed as a percentage of the normocholesterolemic C57BL/6 animal. *, $p < 0.05$ from C57BL/6; **, $p < 0.01$ from C57BL/6; †, $p < 0.05$ from 0 mg/kg; ††, $p < 0.01$ from 0 mg/kg.

cantly reduced endothelial adhesiveness by 33 and 55%, respectively.

Vascular Gene Expression and Oxidative Signaling.

To investigate potential underlying mechanisms that regulate endothelial adhesiveness, we determined the expression levels of known inflammatory mediators. Quantitative RT-PCR indicated that vascular mRNA levels of VCAM-1, MCP-1, and MMP-9 were higher in hypercholesterolemic animals (Fig. 3); rosuvastatin (20 mg/kg) potently inhibited this expression. Hypercholesterolemia is also known to induce an oxidative stress in several different cell types including vascular endothelial and smooth muscle cells. Indeed, it has been demonstrated that reactive oxygen species play an important role in the activation of several atherogenic genes as well as in the process of lipid peroxidation. Analysis of the mRNA levels of p22^{phox}, an important regulatory component of the vascular NAD(P)H oxidase, indicated elevated expression in the setting of hypercholesterolemia (Fig. 4A). To determine whether this was associated with enhanced oxidative stress, oxygen-derived free radical production from vascular segments was analyzed by EPR using TA. As shown in Fig. 4B, hypercholesterolemia induced superoxide production from the vessel wall. In addition, the plasma concentrations of the oxidative marker 8-epi-PGF_{2α} were also elevated (Fig. 5). Consistent with its role as an anti-inflammatory compound, rosuvastatin reduced both vascular oxidative stress as well as levels of 8-epi-PGF_{2α}. This response to rosuvastatin was observed after 2 weeks but was more evident with 6 weeks of treatment.

Discussion

The current results indicate that rosuvastatin attenuates endothelial adhesiveness for monocytes in hypercholesterolemic mice. This effect is associated with reduced vascular

oxidative stress as well as reduced expression of inflammatory molecules essential for monocyte accumulation and atherosclerosis.

The beneficial effects of statins on cardiovascular morbidity and mortality have been demonstrated in several primary and secondary prevention trials. These have included patients with a wide range of plasma cholesterol levels as well as concomitant risk factors. However, post hoc analysis of the West of Scotland Coronary Prevention Study (WOSCOPS) (Anonymous, 1998a), a study in which pravastatin was investigated in patients with moderate risk, indicated that some of the beneficial effects of statin treatment may go beyond its effects on cholesterol reduction. Specifically, patients in the treatment arm experienced greater benefit than would be predicted by the Framingham risk model. Furthermore, when pravastatin-treated patients were compared with placebo patients with similar LDL levels at the end of the study, the pravastatin cohort had a much lower risk of cardiovascular events.

These epidemiological findings are bolstered by preclinical animal studies with the same conclusion. One of the most convincing studies was one performed by Williams and colleagues

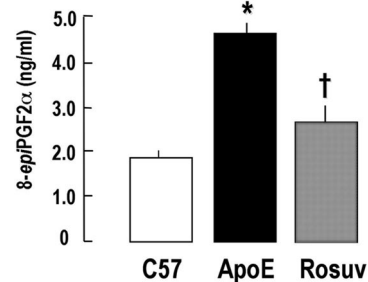


Fig. 5. Rosuvastatin attenuates the increase in plasma 8-epi-PGF_{2α} levels observed in hypercholesterolemia (20 mg/kg/day for 6 weeks; *n* = 8). *, *p* < 0.05 from C57BL/6; †, *p* < 0.05 from 0 mg/kg.

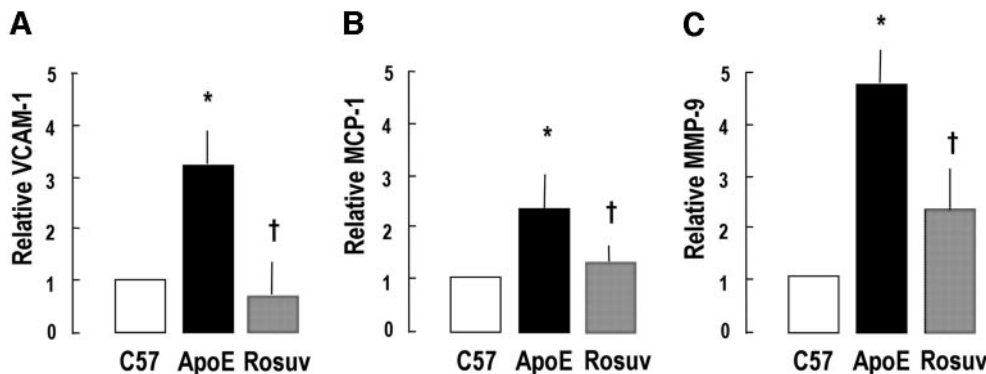


Fig. 3. Quantitative RT-PCR results indicate that the enhanced expression of vascular inflammatory genes induced by hypercholesterolemia is reduced by rosuvastatin (20 mg/kg/day; *n* = 7 per group). *, *p* < 0.05 from C57BL/6; †, *p* < 0.05 from 0 mg/kg. A, VCAM-1; B, MCP-1; C, MMP-9.

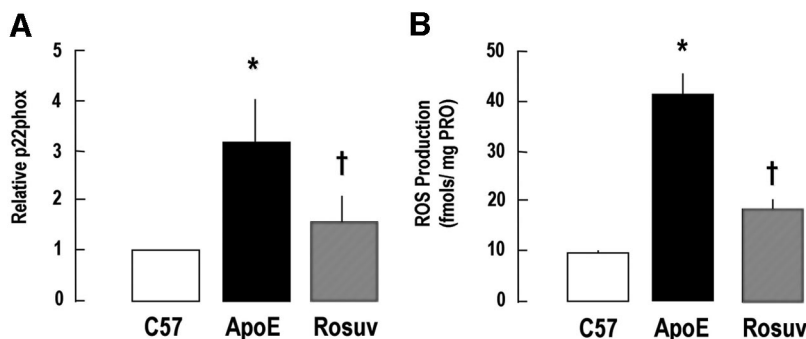


Fig. 4. A, expression of p22^{phox} is modulated by rosuvastatin (20 mg/kg/day; *n* = 7). B, EPR analysis of vascular tissue using TA indicates that hypercholesterolemia enhances superoxide production; this effect is inhibited by rosuvastatin treatment (20 mg/kg/day for 6 weeks; *n* = 7). *, *p* < 0.05 from C57BL/6; †, *p* < 0.05 from 0 mg/kg.

(Williams et al., 1998) in cynomologous monkeys placed on high-fat diets. After 2 years, the animals received either low-fat or high-fat diets with pravastatin for an additional 2 years. Animals were closely monitored and diets were modified to produce the same level of total cholesterol, LDL, and HDL in the plasma of both groups. At the end of the study, isolated vessels from the statin-treated animals displayed greater endothelium-dependent vasodilatation. Moreover, histological analysis of vessels indicated reduced atherosclerotic burden as well as less macrophage content in the lesions, suggesting a potential anti-inflammatory effect of statins.

Other animal models also displayed evidence that statins may modulate inflammatory processes. Pretreatment with simvastatin produced a beneficial effect similar to indomethacin in the carrageen-induced footpad model of local inflammation (Sparrow et al., 2001). Maggard et al. (1998) showed that pravastatin prevented the aggressive coronary vasculopathy in a rat model of heart transplantation. This effect was also associated with reduced inflammatory infiltrate into the lesional areas. In addition, statins can alter the expression and activity of immunomodulatory molecules such as nitric oxide (NO). Peng et al. (1995) demonstrated that statins enhance the expression and activity of eNOS, whereas Lefer and colleagues (Lefer et al., 1999) found that statins increased coronary flow and reduced neutrophil infiltrate in the isolated perfused rat heart due to enhanced bioactivity of NO. Kline and Scalia (2003) also showed that rosuvastatin effectively prevented microcirculation leukocyte-endothelium interactions in the diabetic (db/db) mouse independent of cholesterol or glucose lowering activity. As a radical species, NO is quickly neutralized by other free radicals such as superoxide anion. Thus, the effect of simvastatin upon NO bioavailability could be due to increased NO production or reduced inactivation by reactive oxygen species. Since we detected reduced free radical production from the vessel wall, bioactivity of NO should be increased. Coupled with the ability of rosuvastatin to up-regulate endothelial NOS levels (Laufs et al., 1998) the resultant increase in NO bioavailability could help explain the observed effects on endothelial adhesiveness.

Many of the direct effects of statins on inflammatory processes are dependent on their ability to inhibit intracellular HMG-CoA reductase and, thereby, reduce intracellular concentrations of isoprenoid compounds such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Laufs and Liao, 1998). These isoprenoids are essential for post-translational modification of proteins that allow anchoring to lipid membranes. Signaling molecules whose functions are dependent upon isoprenoid modification include the Rho family of GTPases. Indeed, the effect of statins on eNOS expression and activity is dependent upon inhibition of Rho GTPase geranylgeranylation and is reversible by mevalonate (Laufs and Liao, 1998).

Several markers of inflammation have also been used to investigate the potential anti-inflammatory effects of statins in humans. The best known inflammatory marker used in clinical trials is C-reactive protein (CRP). High circulating levels of CRP are indicative of acute as well as chronic inflammatory situations. Indeed, significantly elevated CRP levels have been associated with cardiovascular disease as well as most risk factors for coronary heart disease (Willerson and Ridker, 2004). A substudy of the Cholesterol and Recurrent Events (CARE) trial determined that baseline CRP, as well as another marker of

inflammation serum amyloid A, were both significantly higher in patients who later developed recurrent nonfatal myocardial infarction or fatal coronary event compared with matched controls who remained event free throughout the study (Ridker et al., 1999). Moreover, patients with elevated inflammatory markers had a 2-fold greater benefit from statin therapy than those without chronic inflammation, indicating an added effect of pravastatin.

Longer clinical studies have shown a small but consistent effect of statins to elevate HDL levels (Kjekshus and Pedersen, 1995; Downs et al., 1998). HDL is thought to have positive effects on atherogenesis by both reducing vascular cholesterol levels (i.e., reverse cholesterol trafficking) as well as by direct anti-inflammatory actions, presumably proteins found in the HDL moiety such as paraoxonase (Durrington et al., 2001). This indirect mechanism may not explain the effects of rosuvastatin in the current study since there were not any significant alterations in HDL levels observed. However, we cannot discount a direct effect of rosuvastatin on paraoxonase activity.

Our EPR results indicate for the first time that rosuvastatin can attenuate the enhanced superoxide anion production from vascular segments derived from apoE-deficient mice. Although several enzymatic sources have been implicated in vascular superoxide production, various lines of evidence indicate that the vascular NAD(P)H oxidase plays a significant role (Griendling and Fitzgerald, 2003a,b; Spiekermann et al., 2003). Overexpression of the p22^{phox} subunit of the NAD(P)H oxidase increases vascular smooth muscle cell production of reactive oxygen species (Fukui et al., 1997; Griendling and Fitzgerald, 2003a). Activation of NAD(P)H oxidase can then stimulate local inflammatory processes leading to leukocyte-endothelial cell interactions (Stokes et al., 2001). In the current study, rosuvastatin reduced expression of p22^{phox} and attenuated superoxide production, further implying a role of NAD(P)H oxidase. In support of this idea, Wassman et al. (2002) demonstrated that atorvastatin could inhibit angiotensin II-induced superoxide production as well as NAD(P)H oxidase subunit expression in cultured endothelial cells. Membrane translocation of the Rac1 GTPase, another NAD(P)H oxidase subunit required for enzyme activation, was also inhibited by atorvastatin. Reversal of these effects by the addition of mevalonate argues that atorvastatin decreases geranylgeranylation-dependent translocation of Rac1. These results, together with the findings of the current study, indicate that statins can simultaneously modulate several aspects of ROS production in vivo.

Inhibition of cellular oxidative stress is likely to play a pivotal role in rosuvastatin-mediated modulation of local inflammatory processes. Reactive oxygen species can activate nuclear factor- κ B (NF- κ B), a transcription factor that is essential for the transcription of several inflammatory molecules including VCAM-1, MCP-1, macrophage colony stimulating factor, MMP-9, and CRP (Marui et al., 1993; Tsao et al., 1997; Jovanovic et al., 2000; Yao et al., 2000; Agrawal et al., 2003). Thus, inactivation of ROS production and NF- κ B by rosuvastatin would modulate the expression of several molecules involved with leukocyte trafficking. Moreover, studies by Cammarano and Minden (2001) suggest that NF- κ B is activated Rho GTPase, offering another explanation for the anti-inflammatory actions of rosuvastatin.

Statins may also reduce inflammatory gene expression by

up-regulating members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors (Inoue et al., 2000; Zelvyte et al., 2002). Zelvyte and colleagues (Zelvyte et al., 2002) demonstrated an increase in PPAR- γ expression after incubation of cultured monocytes with pravastatin. Intriguingly, the promoters of several inflammatory molecules, including VCAM-1 and MMP-9, contain PPAR- γ binding sites. Likewise, Inoue et al. (2000) showed that reduced expression of p22^{phox} by statin was dependent upon enhanced expression of PPAR- α . These findings may have particular importance when statins are used in combination with PPAR agonists for the treatment of diabetes. Fibric acid derivatives (PPAR- α agonists) and thiazolidinediones (PPAR- γ agonists) are commonly used to reduce triglyceride levels and enhance insulin sensitivity, respectively, in patients with type 2 diabetes. Thus, the combinatorial effects of statins with PPAR agonists upon inflammatory gene transcription may help explain the added benefit of statins in diabetics observed in several large prevention trials (Pyorala et al., 1997; Goldberg et al., 1998).

In conclusion, we have demonstrated a potent anti-inflammatory effect of rosuvastatin in a murine model of hypercholesterolemia. This effect is associated with reduced vascular oxidative stress and local expression of inflammatory genes. Since many of these effects are directly related to the inhibition of HMG-CoA reductase and since rosuvastatin is considered one of the most effective statin compounds to date, it is presumed that these findings will translate into the clinical realm and have important implications for the role of rosuvastatin in the development of cardiovascular disease.

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