Pravastatin Has Cholesterol-Lowering Independent Effects on the Artery Wall of Atherosclerotic Monkeys

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Objectives. This study examined the direct effects of pravastatin on the artery wall of atherosclerotic monkeys after dietary lipid lowering.

Background. Clinical trials suggest that hepatic hydroxymethylglutaryl coenzyme A reductase inhibitors may reduce the risk of coronary heart disease out of proportion to their effect on angiographically assessed lumen stenosis.

Methods. Thirty-two cynomolgus monkeys were fed an atherogenic diet for 2 years (progression phase) and then fed a lipid-lowering diet either containing (n = 14) or not containing (n = 18) pravastatin in the diet for an additional 2 years (treatment phase). As designed, total plasma cholesterol and high density lipoprotein concentrations did not differ between groups at the beginning of or during the treatment phase of the experiment (p > 0.05).

Results. Quantitative angiography revealed that coronary arteries of the pravastatin-treated monkeys dilated 10 ± 3%, whereas those from untreated control monkeys constricted −2 ± 2% in response to acetylcholine (p < 0.05). There were no treatment effects on plaque size of coronary arteries measured at the end of the treatment phase of the study (0.110 ± 0.048 mm² [untreated] vs. 0.125 ± 0.051 mm² [pravastatin]; p > 0.05) or on the amount of reduction in plaque size in common iliac arteries during the treatment phase of the study (48 ± 5% [untreated] vs. 45 ± 6% [pravastatin]; p > 0.05). However, histochemical analysis of the atherosclerotic lesions indicated that the arteries from pravastatin-treated monkeys had significantly fewer macrophages in the intima and media, less calcification and less neovascularization in the intima (p < 0.05).

Conclusions. We conclude that compared with control monkeys, the arteries of pravastatin-treated monkeys had better dilator function and plaque characteristics more consistent with plaque stability than those of monkeys not receiving pravastatin. These beneficial arterial effects of pravastatin occurred independently of plasma lipoprotein concentrations and despite similar changes in plaque size between the groups.

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The traditional strategy for measuring the extent, severity and progression or regression of coronary artery atherosclerosis has been angiographic analysis of lumen narrowing or histomorphometric analysis of plaque size. However, serial angiographic studies indicate that acute ischemic syndromes often arise from thrombotic occlusion at sites that previously had only mild or insignificant narrowing (1–4). Furthermore, lipid-lowering studies have shown large reductions in clinical events despite only modest improvement in coronary stenoses (5–8). In monkeys, lipid-lowering studies have shown improved vasodilator function despite modest reductions in plaque size (9). These findings suggest that changes in plaque size or lumen encroachment alone do not predict coronary events.

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Abbreviations and Acronyms

ANOVA = analysis of variance  
HDL = high density lipoprotein  
HMG-CoA = hydroxymethylglutaryl coenzyme A  
LDL = low density lipoprotein  
QCA = quantitative coronary angiography  
TPC = total plasma cholesterol  
VLDL = very low density lipoprotein

CoA) reductase inhibitor that lowers plasma cholesterol concentrations in humans by 30% to 40% (25,26). This decrease in plasma cholesterol concentrations correlates with a reduction in coronary events (27). However, recent evidence indicates that HMG-CoA reductase inhibitors have beneficial effects on the incidence of transient myocardial ischemia (28), endothelium-mediated dilation (29–31) and smooth muscle cell proliferation (32). We designed a study to determine the effects of pravastatin on vasomotor function and plaque characteristics independent of any lipid-lowering effects.

Methods

Design. Experimental subjects included 32 adult (7 to 10 years of age) male cynomolgus monkeys (Macaca fascicularis) imported directly from Indonesia. They consumed an atherogenic diet containing 0.61 mg of cholesterol per kilocalorie of diet for 2 years (progression phase). The monkeys were divided into two groups so that groups had similar intimal areas in their iliac arteries (see later discussion) and similar total plasma cholesterol (TPC) and high density lipoprotein (HDL) cholesterol concentrations at the beginning of the treatment phase. Both groups then consumed a lipid-lowering diet containing 0.11 mg of cholesterol per kilocalorie of diet for an additional 2 years. The diets were adjusted slightly throughout the treatment phase to maintain equal changes in plasma lipoproteins. The monkeys’ lipid-lowering diets contained (n = 14) or did not contain (n = 18) pravastatin (20 mg/kg body weight per day). One iliac artery was surgically removed at the beginning of the treatment phase to serve as a baseline measure of plaque regression (see later details). Vascular reactivity was measured by coronary angiography at the end of the regression phase of the experiment, just before death and necropsy (see later details). Arterial tissue was removed and preserved in different manners for optimal determination of various plaque characteristics (see later details). All experimental procedures were done in compliance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the Bowman Gray School of Medicine.

Lipid and lipoprotein measurements. Venous blood samples were obtained from monkeys sedated with ketamine hydrochloride (10 to 15 mg/kg intramuscularly) during the progression phase of the experiment and at 3-month intervals during the regression phase of the experiment. All samples were collected from monkeys that had fasted overnight. Total plasma cholesterol was measured by the methods of Allain et al. (33) and HDL cholesterol by the methods described in the Manual of Laboratory Operations of the Lipid Research Clinics Program (34). Low density lipoprotein (LDL) cholesterol concentrations were determined indirectly by subtracting HDL cholesterol from TPC. Therefore, the term “LDL cholesterol” is used throughout this report, but actually represents LDL cholesterol plus very low density lipoprotein (VLDL) cholesterol. The details of the methods used to measure these components have been published previously (35).

Coronary artery reactivity. Vascular responses of large epicardial coronary arteries were measured just before death and necropsy. The monkeys were anesthetized with ketamine hydrochloride (15 mg/kg intramuscularly) and butorphanol (0.025 mg/kg intramuscularly). Supplemental doses of both agents were given as required to maintain light anesthesia. The animals were allowed to breathe spontaneously. These anesthetic agents were chosen because they do not interfere significantly with blood pressure, heart rate or respiration rate. The monkeys were warmed with a heating pad. A custom-designed 3F (tapered to 1.5F) catheter was inserted into the right femoral artery and advanced into the left main coronary artery under fluoroscopic guidance.

With an infusion pump (Harvard Apparatus), serial 2-min infusions were made in the following sequence: 1) 5% dextrose in water (control); 2) acetylcholine (10−8, 10−7 and 10−6 mol/liter) (estimated final concentration in the coronary artery); 3) another control; and 4) nitroglycerin (15 μg/min). Images were taken during hand injection of 2 ml of nonionic contrast solution (Omnipaque, Squibb) into the left main coronary artery. Quantitative coronary angiography (QCA) was done in the Wake Forest University Cardiology Image Analysis Laboratory. A single frame from baseline and after each infusion was selected for analysis on the basis of clarity of the image of the proximal 2 to 3 cm of the circumflex coronary artery. Criteria for clarity included maximal opacification, no overlapping structures and minimal motion artifact. In addition, images were considered acceptable only if the heart rate (monitored by electrocardiography) did not vary from baseline by >20% during infusion of agonists. Care was taken to select all frames from a single monkey at the same time in the cardiac cycle (end-diastole). Each frame was optimally magnified by use of a cine video projector (SME-3500, Sony Corporation of America) and digitized to a 480 × 384 × 10-bit gray-scale image by use of a frame grabber (4 megabytes, Epix Inc.) installed in a 486 personal computer. The mean diameters of the vessel segments of interest were measured by previously validated QCA methods (QCA Plus, Sanders Data Systems) (9). When possible, specific anatomic landmarks were used to ensure that the same portion of the vessel was analyzed after each infusion. Each film was analyzed identically on two separate occasions by an operator who was unaware of the initial results. For purposes of analysis, the average of the two measurements at
baseline and after each infusion were used. Estimates of the precision and reproducibility of the QCA methods, as applied to monkeys, have been published previously (9).

Evaluations of atherosclerotic plaque size in coronary arteries. Necropsies were done on the monkeys at the end of the treatment phase of the study. Monkeys were sedated with ketamine hydrochloride (15 mg/kg intramuscularly) for transport to the necropsy laboratory. At necropsy, sodium pentobarbital (13 mg/kg intravenously) was administered to attain surgical anesthesia. An infusion of Ringer’s solution was initiated through an 18-gauge needle inserted into the left ventricle. Death was effected with an intravenous injection of sodium pentobarbital (80 mg/kg). A 1-cm longitudinal incision was made in the abdominal aorta for drainage of blood from the cardiovascular system. When the drainage from the vena cava became clear, the heart was rapidly removed and the coronary arteries perfusion-fixed with 10% neutral buffered formalin for 1 h.

A total of 15 standardized blocks of coronary artery (five each from the left circumflex, left anterior descending and right coronary arteries) were cut perpendicular to the long axis of the arteries. The tissue blocks were dehydrated through increasing concentrations of ethanol and embedded in paraffin. Two 5-μm sections were cut from each block and stained with either hematoxylin-eosin or Verhoeff-van Gieson. Sections of arteries stained with Verhoeff-van Gieson were projected, using a microscope, onto a digitizer plate. Using a hand-held stylus and a computer-assisted digitizer, the component parts of the artery were traced. Intimal areas were determined by digitizing the area between the internal elastic lamina and the lumen surface of each artery section. The integration method was used to calculate intimal area. Lumen area was calculated by subtracting the intimal area from the area inside the internal elastic lamina.

Evaluation of plaque size in the common iliac arteries. Immediately before the treatment phase of the study, either a left or right common iliac artery was surgically removed from each monkey. Monkeys were tranquilized with ketamine hydrochloride (15 mg/kg intramuscularly) and then given 30 mg/kg of sodium pentobarbital intravenously to attain surgical anesthesia. The monkeys were intubated and placed on mechanical ventilation. The common iliac artery was removed through a midline abdominal incision. The artery was immediately cannulated and perfusion-fixed with 10% neutral buffered formalin at 100 mm Hg for 1 h. The contralateral common iliac artery was perfusion-fixed similarly in situ after removal of the heart. To study the extent and severity of iliac artery atherosclerosis, three standard blocks (each 3 mm long and representing the proximal, middle and distal portions of both arteries) were cut perpendicular to the long axis of the arteries. Histologic preparation and staining were the same as noted earlier. Intimal areas of the iliac artery removed before treatment were used to determine the change in intimal area, as measured at the end of the experiment. Extent of atherosclerosis in the iliac artery removed at baseline was used as a randomization variable to divide animals into treatment groups.

Evaluation of lesion characteristics in the carotid artery. Right common carotid arteries were collected from the monkeys at necropsy, embedded in OCT and snap-frozen in isopentane cooled in liquid nitrogen. Two sections of artery (each 5 mm long) were cut from the proximal common carotid artery. These sections were used to evaluate: 1) cell type—cell-specific antibodies: HHF35 anti-muscle actin for vascular smooth muscle cells (Enzo Diagnostics); CD68 (clone KP1) anti-human macrophage; 2) calcification—by hematoxylin-eosin stain; 3) neovascularization—CD31 anti-human endothelial cells, by hematoxylin background stain; and 4) necrosis—by hematoxylin-eosin stain. Serial sections were cut from each block and used for the hematoxylin-eosin staining and macrophage (smooth muscle) and endothelial cell determinations. Each tissue section was examined independently by two observers (G.K.S. and J.K.W.). Forty observations were made for each end point in each of the two groups. Data are presented as percent shift in frequency distribution of those observations between groups. The lesion characteristics were assigned numerical grades (1, 2 or 3) based on cell composition (macrophages and smooth muscle cells) and features of plaque complication such as calcification, necrosis or neovascularization.

Grade 1 lesions. In mild to uninvolved lesion sections, there was no plaque present (save for a minimal amount of fatty streak), few to no macrophages, no necrosis or calcification and only adventitial vasa vasmorum (Fig. 1, top).

Grade 2 lesions. In the moderately involved lesion sections, we generally observed mild to moderate intimal thickening, occasional nests of macrophages and lipid-laden smooth muscle cells in the intima, little to no necrosis and calcification and moderate adventitial neovascularization (Fig. 1, middle).

Grade 3 lesions. In the more advanced atheromas, there tended to be moderate to advanced atherosclerotic plaque development with numerous nests of macrophage and smooth muscle cells within the intima. Plaque necrosis and calcification were common, as well as neovascularization that extended well into the intima. Plaque necrosis was frequently associated with a thin fibrous cap (Fig. 1, bottom).

Statistics. Potential treatment effects on plasma lipoprotein concentrations were analyzed using repeated-measures analysis of variance (ANOVA). Potential treatment effects on extent of coronary artery atherosclerosis were analyzed using ANOVA. Potential treatment effects on plaque size reduction in the iliac arteries were analyzed using repeated-measures ANOVA. Potential treatment effects on frequency distributions of plaque characteristics were analyzed using the Mann-Whitney U test. Statistical significance was assigned at the 95% confidence level.

Results

Coronary heart disease risk factor measurements (plasma lipoproteins). Concentrations of TPC and LDL and HDL cholesterol were similar between groups at the beginning of the regression phase of the experiment. During the regression phase, there were similar decreases across time in TPC and
LDL and HDL cholesterol (Fig. 2) ($p > 0.05$ for all intergroup comparisons).

**Vascular reactivity.** There was an overall treatment effect of pravastatin on vascular response to acetylcholine ($p < 0.05$). There were no effects of treatment on dilator responses to nitroglycerin ($p > 0.05$ for all intergroup comparisons) (Fig. 3).

**Atherosclerosis extent.** Plaque size and lumen size measurements (intimal area) in the coronary arteries were similar between groups at the end of the lipid-lowering phase of the experiment ($p > 0.05$) (Table 1). There were similar reductions in plaque size of the common iliac arteries during the regression phase of the experiment in both groups ($p > 0.05$) (Table 1).

**Plaque characteristics.** Histologic grading of arteries indicated that there was a shift in frequency distribution from plaques with more macrophages in the intima and media to plaques with fewer macrophages in these areas when monkeys were treated with pravastatin ($p < 0.05$ vs. control) (Fig. 4). A similar shift in frequency distribution was seen when evaluating the extent of intima-media neovascularization ($p < 0.05$) (Fig. 5) and intimal calcification ($p < 0.05$) (Fig. 6).

**Discussion**

The major finding of this study was that pravastatin plus an aggressive lipid-lowering diet did not result in additional improvements in plasma lipids or coronary and iliac intimal area beyond what was achieved by the lipid-lowering diet alone. However, the addition of pravastatin was associated with improvements in endothelial function and a shift toward lesion characteristics associated with more plaque stability.

**Plasma lipoproteins.** Treatment of people with pravastatin and other HMG-CoA reductase inhibitors consistently reduces TPC and LDL cholesterol concentrations by 20% to 40% and increases HDL cholesterol concentrations by ~10% (26,28). These improvements in plasma lipoprotein concentrations have been associated with substantial reductions in coronary events (28). Results recently reported by the Regression Growth Evaluation Statin Study (REGRESS) group (28) indicate that addition of HMG-CoA reductase inhibitors to the conventional treatment of patients with angina pectoris reduces episodes of transient myocardial ischemia gauged by ambulatory ST segment monitoring. Furthermore, the effect of
pravastatin remained significant after adjustment for independent risk factors for the occurrence of ischemia. Treatment with lovastatin (31) or pravastatin (29) improves endothelium-mediated responses in patients with atherosclerosis. Collectively, these studies indicate that HMG-CoA reductase inhibitors have direct effects on the artery wall that may contribute to reducing coronary events.

These studies are intriguing, but it is difficult to determine if the observed results (e.g., on vascular reactivity) result from pravastatin’s plasma lipid-lowering properties or some lipoprotein-independent effects on the artery wall, or both. Thus, the current study isolated the plasma lipid effects of pravastatin by producing equal TPC lowering and HDL cholesterol raising in both pravastatin-treated monkeys and untreated control monkeys. This was done by feeding a plasma cholesterol-lowering diet to both groups of monkeys, while treating one group with pravastatin. As expected, there were similar reductions in TPC and increases in HDL cholesterol in both groups of animals throughout the regression phase of the experiment. Thus, it can be assumed that the arteries, liver and other cholesterol-sensitive tissues were exposed to similar amounts of cholesterol. We conclude that any observed effects of

| Table 1. Effects of Pravastatin on Coronary and Iliac Artery Atherosclerosis in Male Cynomolgus Monkeys |
|---|---|---|
| Coronary arteries | Control Group (n = 18) | Pravastatin Group (n = 14) |
| Intimal area (mm²) | | |
| Unadjusted | 0.469 ± 0.168 | 0.389 ± 0.139 |
| Adjusted* | 0.460 ± 0.061 | 0.401 ± 0.069 |
| Maximal intimal thickness (mm) | | |
| Unadjusted | 0.152 ± 0.037 | 0.119 ± 0.029 |
| Adjusted* | 0.150 ± 0.017 | 0.121 ± 0.019 |
| Lumen area (mm²) | | |
| Unadjusted | 1.254 ± 0.130 | 1.456 ± 0.251 |
| Adjusted* | 1.246 ± 0.118 | 1.467 ± 0.134 |
| Iliac arteries | | |
| Intimal area (mm²) | | |
| Baseline | 1.155 ± 0.542 | 1.088 ± 0.341 |
| Outcome | 0.832 ± 0.321 | 0.954 ± 0.379 |
| Change from baseline | −0.323 ± 0.240 | −0.133 ± 0.151 |

*Mean values are adjusted for baseline atherosclerotic extent in the iliac artery biopsy. Data are presented as mean values ± SEM.
pravastatin on measured experimental end points did not depend on its plasma lipoprotein-lowering properties.

**Vascular reactivity.** Dietary lowering of plasma cholesterol concentrations improves impaired endothelium-mediated dilation of coronary arteries among nonhuman primates (36). Two studies show that treatment with pravastatin improves or preserves endothelium-mediated dilation of arteries in patients with hypercholesterolemia and atherosclerosis (29,30). Results of the study by Kamata et al. (30) indicate that pravastatin preserves endothelium-mediated, nitric oxide–dependent dilation of arteries in cholesterol-fed mice. The investigators speculated that pravastatin’s effects on LDL oxidation may serve as a mechanism by which this agent preserves nitric oxide–mediated dilation. Results of our study indicate that pravastatin may have effects on acetylcholine-mediated dilation independent of its effects on plasma lipoproteins. We interpret this to indicate that there is a favorable effect of pravastatin directly at the level of the arterial wall affecting endothelial function, because we saw a beneficial effect of pravastatin on acetylcholine-mediated dilation (an index of nitric oxide release by the endothelium that has direct effects on smooth muscle cells), but not on nitroglycerin-mediated dilation (an index of smooth muscle cell responsiveness). We speculate, therefore, that pravastatin has direct effects on functional aspects of the artery wall (possibly the endothelium) that may explain, in part, its favorable effects on coronary events. However, the precise mechanism for the non-lipid-mediated effect on endothelial function remains unknown.

**Plaque extent.** Our results do not support an additional lipid-independent effect of pravastatin on reductions in plaque size. These results are not entirely unexpected. Treatment with HMG-CoA reductase inhibitors has been shown to reduce coronary events despite modest reductions in preexisting high grade stenoses (25,26). Cholesterol lowering with dietary manipulation results in modest reductions in coronary artery plaque size in monkeys (9,36) and inhibition of atherosclerotic progression in humans (37,38). However, the degree of plaque “shrinkage” greatly depends on the amount of cholesterol lowering produced. When plasma cholesterol concentrations in monkeys are reduced from 600 to 100 mg/dl, there is a reduction of ~50% in plaque size, mostly due to removal of foam cells (39). When TPC concentrations are lowered from 350 to 150 mg/dl, there is little reduction in plaque size (9,36). The second scenario is more consistent with the amount of cholesterol lowering produced in humans by dietary manipulations or drug treatment. Thus, it is unlikely that a reduction in plaque size per se is the major mechanism by which cholesterol lowering reduces the incidence of coronary events (40).
Plaque characteristics. The results of our study indicate that pravastatin has effects on certain characteristics of the plaque itself that are considered important in the pathogenesis of plaque disruption. Proliferation of vasa vasorum in the intima of atherosclerotic lesions is thought to be important in arterial wall metabolism and nutrition (41,42). These vessels are, however, thin-walled and fragile and may be prone to rupture (41). Atherosclerosis augments constricter responses of the intimal vasa vasorum (42), which may increase the chance of intraplaque rupture of vasa, hemorrhage and plaque rupture. Dietary lowering of plasma cholesterol causes regression of these intimal vasa in monkeys, possibly by attenuation of growth factor–stimulated angiogenesis (42). Treatment with pravastatin resulted in a shift toward less intimal vasa after dietary lowering of cholesterol. This may serve to stabilize the plaque by promoting regression of fragile, rupture-prone microvessels. However, if these vessels are stimulated to regress too quickly, thickened atherosclerotic arteries may not receive proper nutrition and may be more prone to necrosis and rupture. Therefore, one must be cautious about how to interpret regression or involution of plaque vasa vasorum.

Calcification is a frequent feature of complicated atherosclerotic plaque. Plaque rupture is commonly seen in association with areas of calcification. Why this is so remains uncertain. Calcification may represent fixed hard tissue within an artery that serves as a mechanical point of plaque disruption or production of tissue-degrading chemicals. Because we observed less calcification in the arterial intima of pravastatin-treated monkeys, pravastatin may stabilize atherosclerotic plaque by inhibiting the formation or promoting the removal of calcium deposits in atherosclerotic plaque.

Of the cells that are found in atherosclerotic plaque, macrophages and other inflammatory cells are most often implicated as being important to plaque disruption (15,16). Human autopsy studies of the characteristics of sites of fatal plaque disruption have consistently shown prominent accumulations of macrophages (16). These monocyteic cells can exhibit many functions that alter aspects of atheroma. Macrophages can degrade extracellular matrix by phagocytosis or by secreting proteolytic enzymes such as plasminogen activators and a family of metalloproteinases (collagenases, gelatinases and stromelysins) that may weaken the fibrous cap, predisposing it to rupture (10). It is thought that accumulations of macrophages at the “shoulder” region of the plaque may be especially dangerous for plaque disruption. In the present study, there were fewer intimal macrophages in the pravastatin-treated monkeys than in the untreated control monkeys. In a separate study, we observed increased proteolytic activity associated with macrophages found in the intima of atherosclerotic arteries (43,44). Thus, a reduction in macrophages by pravastatin may reduce intraplaque proteolysis and possibly the risk of plaque rupture.

We did not observe a lipid-independent effect of pravastatin on other plaque characteristics thought to be important in plaque disruption. There appeared to be little effect on the amount of necrosis in the intima or the thickness of the fibrous cap. Often, plaque disruption is found in areas of the plaque with extensive amounts of necrosis and associated with a thin, fibrous cap (12–14). Thus, improvements in necrotic core size and fibrous cap thickness may have occurred as a result of lipid lowering, but in this study there appeared to be no further effect of pravastatin on these plaque characteristics.

Potential study limitations and other considerations. Throughout this report, we have stated that pravastatin had (e.g., vascular reactivity) or did not have (e.g., atherosclerosis extent, fibrous cap thickness) an effect on the experimental end points. It should be emphasized that many of these end points (except plasma lipoproteins) were measured at one point in time (at the beginning or the end of the regression phase). Our statements hold for these time points but do not address what may have been happening throughout the regression period or what might have happened if the regression phase were extended. In addition, vascular reactivity studies were done only at the end of the study. We do not know if there were group differences at the beginning of the treatment phase. There were no effects of treatment on reduction of lesion size in the iliac arteries. However, it cannot be determined if the rate of plaque shrinkage was similar between groups. This may be important when one attempts to assign significance of involution of vasa vasorum as a factor in plaque nutrition.

Our evaluation of frequency distributions of plaque characteristics relied on subjective morphologic criteria ill suited for image analysis. The scores were, however, assigned by two separate investigators unaware of the other score or which treatment group was being evaluated. The observed differences in frequency distribution were consistent and easily discerned.

Several arterial sites were used in this study owing to limitations on what can be done with the small arteries of monkeys. Different arterial sites develop atherosclerosis at distinct rates and presumably regress at different rates. However, the basic underlying mechanisms of progression and regression are, most likely, similar. Use of nonhuman primate tissues in this study had advantages over those of humans because of the careful control for confounding variables such as drug treatment, diet, previous operation and cardiovascular risk factors. In addition, most human studies do not permit systematic analysis of optimally preserved transmural specimens. They are often difficult to interpret in this regard because of postmortem changes such as autolysis.

Conclusions. The results of this study indicate that pravastatin has effects on the artery wall independent of its effects on plasma lipoprotein concentrations. These include favorable effects on coronary artery vascular reactivity and a shift away from plaque characteristics associated with plaque rupture. Both of these arterial effects may explain, in part, the beneficial effect of pravastatin on reducing coronary events in humans.

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