Antiinflammatory and Antiarteriosclerotic Actions of HMG-CoA Reductase Inhibitors in a Rat Model of Chronic Inhibition of Nitric Oxide Synthesis

Weihua Ni, Kensuke Egashira, Chu Kataoka, Shiro Kitamoto, Masamichi Koyanagi, Shujiro Inoue, Akira Takeshita

Abstract—Recent studies suggest that some of the beneficial effects of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) may be due to their cholesterol-lowering independent effects on the blood vessels. Chronic inhibition of endothelial nitric oxide (NO) synthesis by oral administration of N\textsuperscript{\textsubscript{\textnoindent}}-nitro-L-arginine methyl ester (L-NAME) to rats induces early vascular inflammation as well as subsequent arteriosclerosis. The aim of the study is to test whether treatment with statins attenuates such arteriosclerotic changes through their cholesterol-lowering independent effects. We investigated the effect of statins (pravastatin and cerivastatin) on the arteriosclerotic changes in the rat model. We found that treatment with statins did not affect serum lipid levels but markedly inhibited the L-NAME–induced vascular inflammation and arteriosclerosis. Treatment with statins augmented endothelial NO synthase activity in L-NAME–treated rats. We also found the L-NAME induced increase in Rho membrane translocation in hearts and its prevention by statins. Such vasculoprotective effects of statins were suppressed by the higher dose of L-NAME. In summary, in this study, we found that statins such as pravastatin and cerivastatin inhibited vascular inflammation and arteriosclerosis through their lipid-lowering independent actions in this model. Such antiarteriosclerotic effects may involve the increase in endothelial NO synthase activity and the inhibition of Rho activity. (Circ Res. 2001;89:415-421.)

Key Words: arteriosclerosis ■ endothelium-derived factors ■ inflammation ■ monocyte chemoattractant protein-1 ■ nitric oxide synthase

Recent studies suggest that 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reduce the incidence of myocardial infarctions and ischemic strokes.\textsuperscript{1,2} The beneficial effects of statins are likely to result from stabilization of unstable atheroma prone to rupture, because cholesterol-lowering therapy with statins increases markers of plaque stability.\textsuperscript{3,4} Although such effects of statins are predominantly attributed to their lipid-lowering effects, subgroup analysis of the data from clinical trials suggests that there may be beneficial effects independent of their cholesterol-lowering effects.\textsuperscript{1,2,5} Endothelium-dependent nitric oxide (NO)-mediated vasodilatation has been demonstrated to improve at the early phase of statin treatment in humans.\textsuperscript{6–8} It is recently reported that addition of statins to cultured human endothelial cells increases endothelial-type NO synthase expression and activity.\textsuperscript{9} Thus, one may hypothesize that some of the beneficial effects of statins may be due to their cholesterol-lowering independent effects on endothelial cells. However, it is unclear whether statins attenuate cardiovascular inflammation and arteriosclerosis in vivo through their cholesterol-lowering independent effects.

Endothelium-derived NO has been recognized to be an antiinflammatory and antiarteriosclerotic molecule. Mice lacking endothelial-type NO synthase exhibit hypertension and an enhanced vascular remodeling in response to injury.\textsuperscript{10} We recently reported that chronic inhibition of NO synthesis by administration of N\textsuperscript{\textsubscript{\textnoindent}}-nitro-L-arginine methyl ester (L-NAME) induces early inflammation (monocyte infiltration into the coronary vessels associated with induction of monocyte chemoattractant protein-1 [MCP-1] expression) and subsequent arteriosclerosis (medial thickening and perivascular fibrosis) in the rats.\textsuperscript{11,12} The importance of our observation is supported by the well-recognized concept that the adhesion of mononuclear cells to, and their infiltration into, the blood vessel wall have been assumed to be crucial early events of arteriosclerosis.\textsuperscript{13} Such rat model may be useful to investigate lipid-lowering independent antiarteriosclerotic effects of statins, because administration of statins even at high doses to normal rats does not change serum cholesterol levels.\textsuperscript{14} Accordingly, in the present study, we used this rat model to investigate the antiarteriosclerotic actions of statins.
Materials and Methods

Animals and Experimental Protocol

The present study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines for Animal Experiments of Kyushu University Faculty of Medicine. Twenty-week-old male Wistar-Kyoto rats were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine.

Several groups of 20-week-old male Wistar-Kyoto rats were studied. The control group received untreated chow and drinking water. L group received L-NAME in drinking water (1 mg/mL). L1.0 group received L-NAME in drinking water (1.0 mg/mL) and pravastatin sodium (50, 100, or 250 mg/kg per day, by gavage). Lx group received L-NAME in drinking water (1.0 or 1.8 mg/mL) and ciminibrivastatin sodium intraperitoneally (1, 2, or 10 mg/kg per day). Treatment with statins was started 2 days before L-NAME administration was begun. On day 3 of L-NAME administration, systolic blood pressure (the tail-cuff method) was measured. Then the rats were anesthetized and euthanized for morphometric, immunohistochemical, and biological analyses. Furthermore, some rats in the control, L-NAME, L1.0+P100, and L1.0+P+2 groups were euthanized on day 28 for morphometric analysis. Pravastatin sodium and cerivastatin sodium were generously provided by Sankyo Co, Ltd (Tokyo) and Bayer Yakuhin, Ltd (Osaka), respectively.

Biochemical Analysis

Serum total cholesterol, triacylglycerol, and HDL cholesterol levels were determined with commercially available kits from Wako Pure Chemicals. Serum levels of L-NAME and N\(^-\)nitro-L-arginine (L-NAME) was measured by postcolumn fluorometric detection system for liquid chromatography. Serum NO\textsubscript{x} (NO\textsubscript{2} plus NO\textsubscript{3}) concentration was measured with use of a fluorometric assay kit (Wako). Serum statin concentrations were measured with use of high-performance liquid chromatography/mass spectrometry. The angiotensin-converting enzyme (ACE) activity was measured in cardiac tissues by fluorometric assay as described. Aortic NO\textsubscript{x}-generating capacity was measured by the Greiss method as described.

Histopathology and Immunohistochemistry

Histopathology and Immunohistochemistry were performed as described. Some sections were subjected to immunostaining using antibodies against rat macrophage/monocytes (ED-1, Serotec, Oxford, UK), proliferating cell nuclear antigen (PCNA) (Dako A/S, Glostrup, Denmark), endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) (Transduction Laboratories), or nonimmune IgG (Zymed Laboratory Inc). Cell enumeration was performed as described. Evaluation of the medial thickness and perivascular fibrosis of coronary arteries on day 28 was performed as previously described.

Northern Blot Analysis

Northern blot hybridization was performed as described. A rat MCP-1 cDNA probe, transforming growth factor-\(\beta\) (TGF-\(\beta\)) probe, and a mouse GAPDH cDNA (American Type Culture Collection, Manassas, Va) probe were used.

Western Blot Analysis

Proteins were prepared from the aorta and heart and separated on SDS-PAGE as described. Immunoblotting was performed using

### TABLE 1. Systolic Blood Pressure

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>134±4</td>
<td>132±3</td>
<td>130±6</td>
<td>133±6</td>
<td>128±4</td>
<td>138±8</td>
</tr>
<tr>
<td>L</td>
<td>138±5</td>
<td>167±3</td>
<td>186±6</td>
<td>191±9</td>
<td>188±5</td>
<td>180±4</td>
</tr>
<tr>
<td>L+P50</td>
<td>141±3</td>
<td>170±7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L+P100</td>
<td>128±4</td>
<td>163±12</td>
<td>135±8</td>
<td>140±8</td>
<td>145±7</td>
<td>143±9</td>
</tr>
<tr>
<td>L+P250</td>
<td>131±4</td>
<td>169±7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L+C1</td>
<td>142±5</td>
<td>167±12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L+C2</td>
<td>136±3</td>
<td>163±5</td>
<td>149±5</td>
<td>151±7</td>
<td>155±9</td>
<td>140±5</td>
</tr>
<tr>
<td>L+C10</td>
<td>127±5</td>
<td>171±7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed as mm Hg.

*\(P<0.05\) vs control group; †\(P<0.05\) vs day 0. ND indicates not determined.

### TABLE 2. Serum Lipid Levels, L-NNA Concentration, and Tissue ACE Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cholesterol, mmol/L</th>
<th>HDL Cholesterol, mmol/L</th>
<th>TG, mmol/L</th>
<th>L-NNA, (\mu)mol/L</th>
<th>Cardiac ACE Activity, nmol/mg per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 28</td>
<td>Day 3</td>
<td>Day 28</td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td>3.2±0.2</td>
<td>2.9±0.1</td>
<td>1.11±0.03</td>
<td>1.0±0.05</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td>L</td>
<td>2.9±0.2</td>
<td>2.8±0.2</td>
<td>1.13±0.04</td>
<td>1.1±0.02</td>
<td>0.39±0.05</td>
</tr>
<tr>
<td>L+P50</td>
<td>2.7±0.1</td>
<td>ND</td>
<td>0.99±0.05</td>
<td>ND</td>
<td>0.38±0.05</td>
</tr>
<tr>
<td>L+P100</td>
<td>3.1±0.2</td>
<td>2.8±0.2</td>
<td>1.07±0.12</td>
<td>1.2±0.01</td>
<td>0.38±0.06</td>
</tr>
<tr>
<td>L+P250</td>
<td>2.8±0.1</td>
<td>ND</td>
<td>1.01±0.04</td>
<td>ND</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>L+C1</td>
<td>2.6±0.2</td>
<td>ND</td>
<td>1.06±0.08</td>
<td>ND</td>
<td>0.37±0.05</td>
</tr>
<tr>
<td>L+C2</td>
<td>2.7±0.1</td>
<td>ND</td>
<td>0.96±0.04</td>
<td>1.1±0.01</td>
<td>0.39±0.04</td>
</tr>
<tr>
<td>L+C10</td>
<td>3.3±0.2</td>
<td>ND</td>
<td>1.07±0.12</td>
<td>ND</td>
<td>0.43±0.05</td>
</tr>
</tbody>
</table>

Data are mean±SE. \(n=7\) to 8. ND indicates not determined; (−), below the detection level (<0.02 \(\mu\)mol/L).

*\(P<0.05\) vs control group.
monoclonal antibodies to eNOS, iNOS (Transduction Laboratories), and to RhoA (Santa Cruz Biotechnology Inc). Immunodetection was accomplished using appropriate horseradish peroxidase-linked secondary antibody and the enhanced chemiluminescence (ECL) kit (Amersham Corp). Membrane and cytosolic proteins in cardiac tissue were isolated as described previously.20 Immunoblotting for RhoA in membrane and cytosolic fraction was performed as described above.

Statistical Analysis
Data are expressed as mean±SE. Statistical analysis of differences was compared by ANOVA and Bonferroni’s multiple comparison test. A level of *P*<0.05 was considered statistically significant.

Results

Systolic Arterial Pressure and Serum Lipids and L-NA
The L group showed a progressive rise in systolic arterial pressure throughout the study (Table 1). This increase in systolic blood pressure showed no significant change in all groups with statin treatment on day 3, whereas it was normalized on days 7, 14, 21, and 28 of treatment in the L1.0+P100 and L1.0+C2 groups.

Serum total, HDL cholesterol, and triacylglycerol concentrations in the statin-treated rats were not statistically different from those seen in control or L-NAME–treated rats (Table 2). Serum L-NAME and L-NNA concentrations were measured on day 3 of L-NAME treatment. L-NNA was not detected in the serum of control rats. In the L group, L-NNA concentration was 111±15 mmol/L, which was not significantly different from those in the L1.0+P100 or L1.0+C2 groups (Table 2). Serum L-NAME was below detection level (<0.02 μmol/L) in all groups (data not shown), indicating conversion of L-NAME to L-NNA after absorption.21

Effect of Statins on Local ACE Activity
Cardiac ACE activity was increased in the L group on day 3. This increased ACE activity was not influenced by treatment with statins (Table 2).

Effects of Statins on Inflammatory and Proliferative Changes
We found no evidence of inflammation in the control rats (Figure 1A). In contrast, on day 3, attachment of mononuclear leukocytes to the endothelium of coronary vessels and their infiltration into the vessel wall were noted in the L group (Figure 1A). A marked mononuclear leukocyte infiltration into the perivascular area and the myocardial interstitial area was also observed in this group. The majority of these cells were found to be ED-1–positive monocytes (Figure 1A).

Nuclear staining with PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, and myofibroblast-like cells (Figure 1A).

When ED-1–positive monocytes or PCNA-positive cells were counted by a single observer who was blind to the treatment protocols, the number of immunopositive cells per section was significantly greater in the L group than in the control group (Figure 2). The increases in ED-1–positive cells and PCNA positive cells were significantly reduced by treatment with statins in a dose-dependent manner (Figures 2A through 2D).

Effects of Statins on MCP-1 and TGF-β1 Gene Expression
When cardiac MCP-1 and TGF-β1 mRNA levels were examined by Northern blot analysis in the control, L-NAME, L1.0+P100, and L1.0+C2 groups on day 3, both MCP-1 and TGF-β1 mRNA levels were significantly increased in L group. Such increased gene expressions were significantly reduced in the L1.0+P100 and L1.0+C2 groups (Figure 3).

Effect of Statins on NO Synthase Expression and Activity
NOx-generating capacity from the aortic segments was examined in the control, L, L1.0+P100, and L1.0+C2 group. NOx-generating capacity in the thoracic aorta was markedly decreased in the L group. The blunted NOx-generating capacity by L-NAME was significantly restored by statin treatment (Figure 4A). To provide a direct evidence suggesting restoration of NO production in vivo, serum NOx was measured by a fluorometric assay. Serum NOx concentration
was significantly decreased in the L group, treatment of statins markedly restored serum NOx level in the L1.01P100 and L1.01C2 groups (Figure 4B).

**Figure 2.** Effect of statins on inflammatory and proliferative changes. A, Number of ED-1–positive monocytes infiltrated into the coronary vessels and myocardium in the control, L, and L+Py groups. B, Number of PCNA-positive cells appearing in the coronary vessels and myocardium in the control, L, and L+Py groups. C, Number of ED-1–positive monocytes infiltrated into the coronary vessels and myocardium in the control, L, and L+Cy groups. D, Number of PCNA-positive cells appearing in the coronary vessels and myocardium in the control, L, and L+Cy groups. E, Number of ED-1–positive cells in the control, L, L1.01P100, L1.01C2, L1.81P250, and L1.81C10 groups. F, Number of PCNA-positive cells in the control, L, L1.01P100, L1.01C2, L1.81P250, and L1.81C10 groups. *P<0.05 vs L group; †P<0.05 vs control group. Each bar represents n=5 to 9.

**Effects of High-Dose L-NAME on the Vasculoprotective Effects of Statins**

To examine if the beneficial effects of statins are due to restoration of eNOS activity, we determined if the higher dose of L-NAME could block the vasculoprotective effects of statins. The higher dose of L-NAME suppressed statin-induced restora-
tion of NO production (Figure 4B), statin-induced inhibition of vascular inflammatory/proliferative changes (Figures 2E and 2F), and statin-induced inhibition of RhoA translocation to membrane (data not shown) on day 3 of L-NAME administration. The high dose of L-NAME did not affect eNOS expression in both the heart and aorta (data not shown). Also, the high dose of L-NAME attenuated statin-induced inhibition of vascular remodeling on day 28 (data not shown).

Discussion
We have demonstrated herein that (1) treatment with statins such as pravastatin and cerivastatin inhibits early vascular inflammation and (2) statins also inhibit proliferative changes and the development of cardiovascular remodeling in a rat model of chronic inhibition of NO synthesis. Importantly, such antiinflammatory and antiarteriosclerotic effects of statins cannot be attributed to their lipid-lowering effects.

We have recently reported that MCP-1 mediates early vascular inflammation and subsequent vascular medial thickening and that TGF-β mediates perivascular and myocardial fibrosis in our model. Our present observations suggest that treatment with statins reduced such pathological disorders by inhibiting gene expression and biological activity of MCP-1 and TGF-β. Because restoration of endothelial NO-mediated vasodilation is an early event after cholesterol-lowering therapy with statins, we hypothesized that the beneficial effects of statins seen in this study involved the increase in NO synthase expression or activity. We found that treatment with statins augmented endothelial-type NO synthase protein expression and restored NO-producing capacity and serum NOx concentration. Such augmented endothelial NO synthase expression and activity are in agreement with prior in vitro studies that statins increased NO synthesis from the endothelial cells by increasing NO synthase mRNA stability. Therefore, statins are likely to restore the L-NAME–induced inhibition of endothelial NO synthase activity by augmenting NO synthase protein expression and thereby elicited antiinflammatory and antiarteriosclerotic actions in this model. Another several lines of evidence in our study may also support our hypothesis. We have demonstrated that the antiinflammatory and antiarteriosclerotic effects of statins in regular-dose L-NAME (1 mg/mL)–treated rats completely disappeared in rats treated with high-dose L-NAME (1.8 mg/mL) (Figures 2E and 2F). We have noticed that disappearance of the vasculoprotective effects of statins in high-dose L-NAME–treated rats corresponded to failure of restoration of NO production (Figure 4B), inhibition of vascular inflammatory/proliferative changes (Figures 2E and 2F), and inhibition of RhoA membrane translocation. These data substantially support the idea that the beneficial effects of statins observed in this model may be mediated at least in part by their effect on eNOS activity. Other possible mechanisms for vasculoprotective effects of statins, which were not investigated in the present study, include inhibition of
oxidative stress, inflammatory cytokine activity, or matrix metalloproteinase activity.

Our present finding is also consistent with the recently clarified role of Rho GTPase in mediating cardiovascular disease. For example, Rho promotes cell-cycle progression and proliferation in vascular smooth muscle cells, which are central events in the pathogenesis of several vascular lesions; Rho also mediates the activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB) in response to cytokines. Furthermore, Rho proteins may also be involved in mediating increases in oxidative stress.

We next hypothesized that the increased NO synthase activity by statins might result from inhibition of small GTPase protein Rho activity. We could demonstrate in the present study that statins prevented the L-NAME–induced increase in Rho activation. Since angiotensin II has been shown to induce Rho activation in several cell types in vitro, the L-NAME–induced increase in Rho activation may be mediated by increased angiotensin II activity. Evidence suggests that the Rho pathway controls multiple cell functions such as adhesion, proliferation, migration, and the calcium sensitivity of the contractile proteins, and that blockade of the Rho pathway with C3 toxin may increase NO synthase protein expression in vitro and in vivo. Accordingly, these results suggest that treatment with statins augments endothelial type NO synthase activity at least in part by inhibiting Rho activation.

It is reported that treatment with statins lowers blood pressure in hypertensive rats. Therefore, normalization of the angiotensin II–induced hypertension by statins at the late stage seen in the present study (Table 1) may not be an extremely surprising finding. Because we previously demonstrated that normalization of angiotensin II–induced hypertension by hydralazine treatment did not inhibit such pathological changes, it is unlikely that the antihypertensive effect of statins may contribute largely to the prevention of cardiovascular pathological changes. However, we cannot rule out the possibility that some of the beneficial effects of statins might have been due to normalization of blood pressure.

We previously demonstrated that the increased angiotensin II activity by overexpression of ACE mediates the cardiovascular pathological changes in this model. There was no significant difference in the enzyme activity between hearts from the L-NAME– and L-NAME plus statin groups. Thus, it is likely that statins did not affect cardiac angiotensin II activity but inhibited inflammatory and proliferative disorders induced by increased angiotensin II activity in our experiments.

Although very high doses of statins (pravastatin at 100 mg/kg per day and cerivastatin at 2 mg/kg per day) were needed to inhibit early inflammation and late atherosclerosis, serum concentrations of statins in our rat model were equivalent to those reported in humans. This discrepancy may be explained by more rapid metabolism of statins from circulation in rats. No serious side effects were observed by administration of such high doses of statins to rats. Thus, it is unlikely that vasculoprotective effects of statins seen in the present study resulted from clinically irrelevant serum concentrations.

In conclusion, this study has demonstrated direct antinflammatory and antiatherosclerotic effects of statins in a rat model of chronic inhibition of NO synthesis. Such vasculoprotective effects of statins are not likely to be mediated by their lipid-lowering actions but seem to be mediated by augmentation of endothelial-type NO synthase expression and activity by the inhibition of Rho activity. The observed vasculoprotective effects of statins are not related to hydrophilic versus lipophilic properties of statins, because both hydrophilic statin (pravastatin) and lipophilic statins (cerivastatin) exhibited similar effects. Recent clinical trials showed that reduction of ischemic cardiovascular events in patients after myocardial infarction by pravastatin therapy was related closely with the decrease in blood C-reactive protein levels. Pravastatin therapy reduced monocyte infiltration into atherosclerotic plaque and preserved endothelial functions in atherosclerotic monkeys. Such antiinflammatory effects of pravastatin are suggested to be a cholesterol-lowering independent action. The present study may provide a new aspect of how statins reduce cardiovascular ischemic events.

Acknowledgments

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References


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