Comparative effects of pitavastatin and probucol on oxidative stress, Cu/Zn superoxide dismutase, PPAR-γ, and aortic stiffness in hypercholesterolemia

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Umeji, Kyoko, Seiji Umemoto, Shinichi Itoh, Masakazu Tanaka, Shinji Kawahara, Tohru Fukai, and Masunori Matsuzaki. Comparative effects of pitavastatin and probucol on oxidative stress, Cu/Zn superoxide dismutase, PPAR-γ, and aortic stiffness in hypercholesterolemia. Am J Physiol Heart Circ Physiol 291: H2522–H2532, 2006. First published July 14, 2006; doi:10.1152/ajpheart.01198.2005.—Reactive oxygen species-scavenging enzyme Cu/Zn superoxide dismutase (SOD) regulated by peroxisome proliferator-activated receptors (PPARs) plays an important role in vascular responsiveness. However, it remains unknown whether statins restore vascular dysfunction through the activation of reactive oxygen species-scavenging enzymes in vivo. We hypothesized that pitavastatin restores vascular function by modulating oxidative stress through the activation of Cu/ZnSOD and PPAR-γ in hypercholesterolemia. New Zealand White male rabbits were fed either normal chow or a 1% cholesterol (CHO) diet for 14 wk. After the first 7 wk, the CHO-fed rabbits were further divided into three groups: those fed with CHO feed only (HC), those additionally given pitavastatin, and those additionally given an antioxidant, probucol. The extent of atherosclerosis was assessed by examining aortic stiffness. When compared with the HC group, both the pitavastatin and probucol groups showed improved aortic stiffness by reducing aortic levels of reactive oxidative stress, nitrotyrosine, and collagen, without affecting serum cholesterol or blood pressure levels. Pitavastatin restored both Cu/ZnSOD activity (P < 0.005) and PPAR-γ expression and activity (P < 0.01) and inhibited NAD(P)H oxidase activity (P < 0.0001) in the aorta, whereas probucol inhibited NAD(P)H oxidase activity more than did pitavastatin (P < 0.0005) without affecting Cu/ZnSOD activity or PPAR-γ expression and activity. Importantly, Cu/ZnSOD activity was positively correlated with the PPAR-γ activity in the aorta (P < 0.005), both of which were negatively correlated with aortic stiffness (P < 0.05). Vascular Cu/ZnSOD and PPAR-γ may play a crucial role in the antiatherogenic effects of pitavastatin in hypercholesterolemia in vivo. vascular dysfunction; atherosclerosis; pleiotropic effects; antioxidant; peroxisome proliferator-activated receptor; reactive oxygen species

Recent studies have suggested that increased vascular superoxide (O2−) production by vascular NAD(P)H oxidase may play a critical role in the progression of atherosclerosis (16). A primary cellular defense against O2− is superoxide dismutase (SOD) (12). Three SOD isoforms have been identified: the cytosolic, copper/zinc-containing SOD (Cu/ZnSOD); the mitochondrial manganese SOD (MnSOD); and the extracellular SOD (ecSOD) (12). The predominant activity of SOD in the vasculature is attributed to Cu/ZnSOD, which may play an important role in the pathogenesis of atherosclerosis (7, 9, 12). Peroxisome proliferator-activated receptors (PPARs), which include three members, α, γ, and β/δ, are ligand-activated transcription factors belonging to the nuclear receptor superfamily (2, 29). PPAR-α and PPAR-γ may have important anti-inflammatory, vasoprotective actions in addition to antiglycemic and/or antidyslipidemic activities, whereas PPAR-β/δ diminishes metabolic derangements by increasing lipid combustion in skeletal muscle (2, 29). PPAR-γ-activating ligands, such as insulin-sensitizing agents, display a number of potential antiatherogenic properties (11). On activation by their ligands, PPARs bind to specific PPAR response elements (PPREs) in the promoter region of their target genes (4). Importantly, the PPRE is located in the sequence of the Cu/ZnSOD gene and participates in the induction of the rat Cu/ZnSOD gene by means of peroxisome proliferators (4), which may contribute to the antiatherosclerotic effects of PPAR-γ within the vasculature (20).

The inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductases (statins) have favorable effects on the progression of atherosclerosis and plaque instability, independent of their lipid-lowering activity (26, 28, 36, 44). These “pleiotropic effects” of statins include improvement of endothelial function, antithrombotic actions, plaque stabilization, reduction of the vascular inflammatory process, and an antioxidant function through the inhibition of NAD(P)H oxidase or the increase in SOD activity (3, 6, 26, 32). Statins are also reported to improve insulin resistance (41) and antioxidant properties, including the restoration of Cu/ZnSOD activity (48), and prevent the development of diabetes in patients with hypercholesterolemia (13, 38). Taken together, these observations allow one to assume that statins may have antiatherogenic actions through PPAR and Cu/ZnSOD activation, in addition to antidyslipidemic actions. However, it is unknown whether or not statins themselves have PPAR and Cu/ZnSOD activation in hypercholesterolemia in vivo.

In the current study, we thus hypothesized that pitavastatin, a lipophilic statin (32), might inhibit atherosclerosis by regulating Cu/ZnSOD activity and PPAR-γ activity in hypercholesterolemia in vivo, and we examined the effects of pitavastatin on reactive oxygen species (ROS)-related enzymes, NAD(P)H oxidase and SODs, PPAR-γ, and vascular function.
assessed by aortic stiffness in cholesterol-fed rabbit aortas, compared with the effects of probucol.

MATERIALS AND METHODS

Materials. The pitavastatin was provided by Kowa (Tokyo, Japan), and the probucol was obtained from Daichi Pharmaceutical (Tokyo, Japan). The following were applied for immunohistochemistry or immunoblots: mouse monoclonal antibodies against human endothelial nitric oxide (NO) synthase (eNOS), mouse inducible NO synthase (iNOS) (BD Transduction Laboratories, Franklin Lakes, NJ), mouse nitrotyrosine (Zymed Laboratories), human von Willebrand factor (vWF, DBS), mouse PPAR-α (Affinity Bioreagents, Golden, CO), human PPAR-γ (Santa Cruz Biotechnology), mouse β-actin (as an internal standard; Cytoskeleton), and nonimmune goat or mouse IgG (as a negative control).

Experimental protocols. A total of 40 New Zealand White male rabbits (2.5–3 kg; Biotec, Fukuoka, Japan) were involved in this study. The control group (n = 7) was fed normal chow for 14 wk. The hypercholesterolemic group was fed a 1% cholesterol diet (n = 35) for the first 7 wk and was then further divided, randomly, into three groups for the last 7 wk: those fed cholesterol only (HC group; n = 11), those additionally given pitavastatin (0.05 mg/kg day−1; n = 12), and those additionally given probucol (1.3 g/day; n = 10). Fasting peripheral blood was collected for the measurement of plasma lipid to determine the total cholesterol, triglyceride, and high-density lipoprotein (HDL)-cholesterol concentrations. The Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine approved the experimental protocol used in this study.

Measurement of aortic stiffness. After intravenous pentobarbital sodium (50 mg/kg) anesthesia, the rabbits were strapped down in a supine position, and transabdominal M-mode echocardiography (Aloka SSD-1000, Aloka) was recorded with a transducer of 10 MHz just under the celiac artery of the abdominal aorta during several cardiac cycles on a strip-chart recorder at a sweep speed of 25 mm/s with simultaneous abdominal aortic pressure and heart rate measurements taken through the right femoral artery. We measured minimum aortic dimension (Dmin; in mm), maximum aortic dimension during the ejection period (Dmax; in mm), and the systolic amplitude of the internal dimension (ΔD = Dmax − Dmin). Stiffness parameter β was calculated using the equation β = ln(BP/BP0)/(ΔD/Dmin), where BP is the maximum systolic aortic pressure and BP0 is the minimum diastolic aortic pressure, as previously reported (22). This parameter β represents a physiological stiffness index of the vessel independent of the operating level of aortic pressure.

Tissue preparation. After the aortic stiffness was calculated, the rabbits were euthanized by the simultaneous administration of intravenous pentobarbital sodium (120 mg/kg) and heparin (50 U/kg) to prevent blood clotting. The aortas were quickly removed, and the whole aorta was harvested from each rabbit. The portion where aortic stiffness was to be measured was snap frozen with an optimum cutting temperature compound in liquid nitrogen to obtain fresh-frozen, 30-μm-thick sections for dihydroethidium (DHE) staining and was fixed for immunohistochemistry. The specimens fixed in 10% buffered formaldehyde were paraffin embedded, sectioned into 4-μm slices, and either stained with Sirius red and hematoxylin-eosin solutions for histological analysis or used for immunohistochemistry. The remaining aortic tissues were kept at −80°C for the other experiments.

Histological and immunohistochemical analyses. Selective and quantitative analyses for aortic morphology and protein expression were performed as described previously (22). The sections were quantified morphometrically with a camera control program system (ACT-1, version 2.51) with a digital camera (DXM1200F, Nikon) connected to an automation microscope (Eclipse E1000, Nikon). In each aorta, the cross-sectional area, total cell number in the aortic media, and intima-to-media ratio were obtained as previously reported (22). The total fractional fibrosis of the media was determined by Sirius red staining, and the collagen volume fraction, which was representative of collagen including types I and III, was identified by using birefringency under polarized light illumination as previously reported (22). All aortas were evaluated in a blind fashion with at least two slices for each rabbit aorta with the use of National Institutes of Health (NIH) Image 1.62, and the mean value of each aorta was used for statistical analysis.

Immunohistochemistry was performed using the avidin-biotinylated enzyme complex method (Vector Laboratories) (22). The percentages of the eNOS-, nitrotyrosine-, and iNOS-positive areas against the vWF-positive cell areas in the vessel wall were obtained with serial sections.

Immunoblotting. The rabbit aortic tissue was homogenized, and PPAR-α, PPAR-γ, and β-actin were separated by 15% sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. Immunoreactivity was next visualized with the enhanced chemiluminescence system (Amersham Biosciences) and then densitometrically analyzed using NIH Image 1.62 as described previously (23).

Determination of aortic SOD activity. The aortic tissues were homogenized in 50 mmol/l potassium phosphate (pH 7.4) containing 0.3 mol/l KBr and a cocktail of protease inhibitors. The homogenates were sonicated and extracted at 4°C for 30 min. The extracts were then centrifuged at 3,000 g for 15 min. SOD activity was measured using an SOD-525 reagent kit (Oxis) (49). Ethanol-chloroform extraction was used to inactivate MnSOD and to specifically measure Cu/ZnSOD activity. A specific analysis of the ecSOD in vessel extracts was performed as previously described (15).

Determination of aortic PPAR-γ activity. PPAR-γ activation was assayed using an ELISA-based transactivation TransAM PPAR-γ kit (Active Motif, Carlsbad, CA) following the manufacturer’s protocol. The PPAR-γ TransAM kit contains a 96-well plate with immobilized oligonucleotides containing a PPRE (5′-AACTAGGTCAAAG-GTCA-3′). PPARs contained in nuclear extracts bind specifically to this oligonucleotide and are detected through the use of an antibody directed against PPAR-γ. A horseradish peroxidase-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by spectrophotometry.

Detection of superoxide. Unfixed frozen aortic 30-μm-thick segments were prepared for in situ imaging of O2·− generation with the fluorescent DHE (Polysciences), as previously described (22, 30). In addition, isolated arteries from the HC group were incubated with or without a membrane-permeable superoxide scavenger Tempol (1 mmol/l) and were topically administered DHE (2 μmol/l) (39). The images were obtained with a laser scanning confocal microscope (LSM510, Zeiss). The cellular sites of O2·− production in both the intima and media, areas that were determined with hematoxylin-eosin-stained serial sections, were assessed in a blind fashion. These data are expressed as a percentage of the corresponding data for the control group.

Measurement of NAD(P)H oxidase activity and thiobarbituric acid-reactive substances. The NAD(P)H oxidase activity in aortic rings was measured according to the method of Miller et al. (31). Oxidative stress in the entire aorta was determined by measuring the thiobarbituric acid-reactive substances (TBARS) by the colorimetric method (43).

Statistical analysis. All values are expressed as means ± SE. The experimental groups were compared with ANOVA followed by Scheffé’s multiple comparison. Simple regression analyses were performed for the correlations. P < 0.05 was considered statistically significant.

RESULTS

Effect of pitavastatin and probucol on lipid profile, aortic stiffness, and vascular remodeling. The total serum cholesterol, triglyceride, and HDL-cholesterol levels were significantly
higher in the three cholesterol-fed groups than in the control group. Pitavastatin did not decrease serum cholesterol or triglyceride levels with the dose used in the study, and probucol decreased serum HDL-cholesterol levels by 24% in the cholesterol-fed rabbits, but no significant differences in those values were observed among the three cholesterol-fed groups (Table 1). The body weight, heart rate, and blood pressures were unaltered among the four groups (Table 2).

Figure 1 shows the representative M-mode echo tracing of the aortas in each group. The aortic stiffness index-β was significantly higher in the HC group than in the control group (Fig. 1 and Table 2). Among the three cholesterol-fed groups, both the pitavastatin and probucol groups showed a decrease in the aortic stiffness index-β to a level equal to that of the control group, reaching statistical significance, relative to values in the HC group, within 7 wk.

The total cell number, cross-sectional area in the media, and intima-to-media ratio were significantly increased in the HC group compared with those values in the control group (Table 2). When compared with the HC group, both drugs significantly reduced those values; pitavastatin reduced those values to the same levels as in the control group, whereas those values in the probucol group were significantly higher than those in the control group. The total fractional fibrosis and collagen volume fraction in the media was significantly increased in the HC group compared with the control group. Both drugs evenly reduced these values in cholesterol-fed rabbit aortas to the same levels as those found in the control group (Table 2).

Effect of pitavastatin and probucol on vascular oxidative stress. The oxidative stress of the abdominal aorta as assessed by TBARS showed that the HC group had significantly higher levels of TBARS than the control group (Table 2). Both pitavastatin and probucol significantly reduced the levels of TBARS to those equal to the control group, with no difference in these values being observed between the two drug-treated groups.

Figure 2 shows DHE staining to assess the O$_2^-$ content in the vessel wall. The control group showed minimal fluorescence in the endothelium and adventitia, and only a slight expression of O$_2^-$ was noticed in the aortic media. In contrast, the HC group showed a significant increase in DHE fluorescence, which reflected increased O$_2^-$ content throughout the vessel wall. In the presence of SOD mimetics Tempol, the fluorescence remarkably decreased throughout the aortic wall, suggesting that DHE staining mainly reflects an increase in O$_2^-$.

The quantitative analysis indicated that the O$_2^-$ content in both the intima and media from the HC group was significantly higher than that from the control group. Pitavastatin and probucol markedly suppressed the O$_2^-$ content not only in the intima but also in the media in comparison with the values in the HC group.

Effect of pitavastatin and probucol on NAD(P)H oxidase activity: correlation with aortic stiffness. As shown in Fig. 3, the HC group had 4.1-fold higher values of NAD(P)H oxidase activity in the aorta compared with those in the control group ($P < 0.0001$ vs. the control group). Probucol markedly reduced

Table 1. Serum lipid levels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HC</th>
<th>Probucol</th>
<th>Pitavastatin</th>
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<tbody>
<tr>
<td>$n$</td>
<td>7</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Serum total cholesterol, mmol/dl</td>
<td>1.12±0.21</td>
<td>37.63±4.45*</td>
<td>32.35±2.58*</td>
<td>32.32±2.13*</td>
</tr>
<tr>
<td>Serum triglycerides, mmol/dl</td>
<td>0.32±0.04</td>
<td>3.87±1.33*</td>
<td>4.71±1.22*</td>
<td>4.32±1.25*</td>
</tr>
<tr>
<td>Serum HDL cholesterol, mmol/dl</td>
<td>0.16±0.03</td>
<td>2.29±0.31*</td>
<td>1.73±0.17*</td>
<td>2.38±0.22*</td>
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</table>

Values are means ± SE of n rabbits. HC, hypercholesterolemic group; HDL, high-density lipoprotein. *$P < 0.0005$ vs. control group.

Table 2. Body weight, hemodynamic data, echographic data of abdominal aorta and aortic stiffness, aortic morphology, and oxidative stress

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HC</th>
<th>Probucol</th>
<th>Pitavastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>7</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.20±0.12</td>
<td>3.03±0.12</td>
<td>3.06±0.06</td>
<td>3.06±0.05</td>
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<tr>
<td>Hemodynamic data</td>
<td></td>
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<tr>
<td>Heart rate, beats/min</td>
<td>280±8</td>
<td>284±12</td>
<td>279±3</td>
<td>284±9</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>126±8</td>
<td>131±5</td>
<td>134±7</td>
<td>124±7</td>
</tr>
<tr>
<td>Diastolic pressure, mmHg</td>
<td>75±6</td>
<td>79±4</td>
<td>87±6</td>
<td>78±6</td>
</tr>
<tr>
<td>Echographic data of abdominal aorta</td>
<td></td>
<td></td>
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<tr>
<td>Minimum aortic dimension, mm</td>
<td>3.58±0.17</td>
<td>4.08±0.13</td>
<td>3.86±0.14</td>
<td>3.91±0.10</td>
</tr>
<tr>
<td>Maximum aortic dimension, mm</td>
<td>4.25±0.20</td>
<td>4.43±0.14</td>
<td>4.43±0.17</td>
<td>4.38±0.11</td>
</tr>
<tr>
<td>Aortic stiffness index-β</td>
<td>2.84±0.23</td>
<td>5.83±0.55*</td>
<td>3.14±0.51†</td>
<td>4.02±0.38†</td>
</tr>
<tr>
<td>Morphology of abdominal aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cross-sectional area, mm$^2$/kg</td>
<td>0.83±0.02</td>
<td>2.85±0.24†</td>
<td>1.83±0.31†</td>
<td>1.18±0.12‡</td>
</tr>
<tr>
<td>Intima-to-media ratio</td>
<td>0.03±0.02</td>
<td>0.61±0.18‡</td>
<td>0.37±0.21†</td>
<td>0.26±0.14‡</td>
</tr>
<tr>
<td>Total cell number/mm$^2$</td>
<td>1588±72</td>
<td>294±130‡</td>
<td>2091±167‡</td>
<td>1916±157‡</td>
</tr>
<tr>
<td>Total fractional fibrosis, %</td>
<td>20.4±0.01</td>
<td>31.3±0.02*</td>
<td>22.2±0.02‡</td>
<td>23.0±0.02‡</td>
</tr>
<tr>
<td>Collagen volume fraction, %</td>
<td>4.9±0.01</td>
<td>7.5±0.01*</td>
<td>5.3±0.01†</td>
<td>5.5±0.01†</td>
</tr>
<tr>
<td>Oxidative stress of abdominal aorta</td>
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<tr>
<td>TBARS, nmol/mg</td>
<td>8.97±3.99</td>
<td>30.67±5.38‡</td>
<td>13.27±1.04†</td>
<td>14.79±1.52‡</td>
</tr>
</tbody>
</table>

Values are means ± SE of n rabbits. TBARS, thiobarbituric acid-reacting substances. *$P < 0.05$ vs. control group; †$P < 0.05$ vs. HC group; ‡$P < 0.01$ vs. control group; §$P < 0.05$ vs. probucol group.
the NAD(P)H oxidase activity by 71% in the HC group, to the same levels as in the control group, whereas the NAD(P)H oxidase activity in the pitavastatin group was reduced significantly by 41% but did not reach the same level as the control group. There was also a significant positive correlation between the \( \beta \)-index and NAD(P)H oxidase activity.

Effect of pitavastatin and probucol on SOD activity: correlation with aortic stiffness. The total SOD activity in the aorta of the HC group was significantly reduced by 78% compared with that of the control group (Fig. 4). Pitavastatin significantly increased the total SOD activity, whereas probucol had no significant change on the total SOD activity. We next exam-
Fig. 3. Quantitative analysis of NAD(P)H oxidase activity in aortas (A) and relationship between percentages of NAD(P)H oxidase activity and β-index (B). A: bars indicate SE. *P < 0.0001, †P < 0.0005. Experiments, n = 4. RLU, relative light units. B: each point represents a different rabbit.

Fig. 4. Quantitative analyses of SOD isoform activities in aorta. Bars indicate SE. Experiments, n = 4–6. NS, not significant; ecSOD, extracellular SOD. *P < 0.05, †P < 0.005.
ined specific assays for each SOD isozyme. The Cu/ZnSOD activity in the HC group was 73% lower than that in the control group (P < 0.005 vs. the control group). Pitavastatin restored the Cu/ZnSOD activity to the same levels as in the control group. In contrast, probucol induced a modest increase in the Cu/ZnSOD activity, which did not reach the levels of the control group. The ecSOD activity in the HC group was 83% lower than that in the control group (P < 0.05 vs. the control group). Both pitavastatin and probucol had about a 3.3-fold increase in ecSOD activity compared with that in the HC group, though this increase did not reach statistical significance. The MnSOD activity was unchanged among the four groups.

There was a significant negative correlation between the β-index and the Cu/ZnSOD or ecSOD activity, whereas the MnSOD activity in rabbit aortas did not correlate with the β-index, as shown in Fig. 5 in the rabbit aortas.

Effects of pitavastatin and probucol on PPAR-α and PPAR-γ expression and on PPAR-γ activity: the correlation with SOD activity and aortic stiffness. We further evaluated the effects of two lipid-lowering drugs on the PPAR-α and PPAR-γ expression in the aorta, as shown in Fig. 6, A and B. PPAR-α in the HC group was significantly (70%) lower than that in the control group. Both drugs increased the PPAR-α expression to the same extent, i.e., by twofold, in both drug-treated groups but did not reach the level of statistical significance. PPAR-γ in the HC group was significantly reduced by 80% compared with that in the control group. Whereas probucol did not increase the PPAR-γ expression, pitavastatin increased the PPAR-γ expression in the aorta by a fourfold higher level than the levels in the HC group, and the PPAR-γ expression reached the same level as that of the control group. Furthermore, pitavastatin restored PPAR-γ activity to the same levels as in the control group (Fig. 6C), whereas probucol increased PPAR-γ activity but not to the levels found in the control group.

In addition, there was a significant negative correlation between the β-index and PPAR-γ activity in rabbit aortas, as shown in Fig. 6D. Furthermore, there was a significant positive correlation between the Cu/ZnSOD activity and PPAR-γ activity in the rabbit aortas, whereas the ecSOD or MnSOD activity did not correlate with the PPAR-γ activity, as shown in Fig. 6E.

Effect of pitavastatin and probucol on eNOS, nitrotyrosine, and iNOS expression. Figure 7A shows the representative serial immunohistochemical stainings of the vWF, eNOS, nitrotyrosine, and iNOS expression in the aorta. The endothelium was selectively stained with antibody against eNOS and vWF, whereas nitrotyrosine was stained only in the endothelium and intima but not in the media or adventitia (data not shown). In addition, iNOS was stained mainly in the endothelium and intima and slightly in the media and adventitia (data not shown).

Figure 7B shows the results of the quantitative analyses of each type of protein expression in the aorta, demonstrating that the HC group showed a significant 42% reduction in eNOS expression in comparison with the control group. In the HC group, pitavastatin and probucol restored eNOS in the endothelium to the same degree, to a level equal to that in the control group, and no difference was observed between the two drug-treated groups. Conversely, the nitrotyrosine expression in the HC group was significantly higher than in the control group. In the HC group, both drugs significantly suppressed nitrotyrosine expression in the endothelium and intima by 50%, with no difference being seen between the two drug-
treated groups. Although nitrotyrosine expression in the endothelium and intima was still twofold higher than in the control group, there were no significant differences seen among the drug-treated groups and the control group. Furthermore, the iNOS expression was significantly higher in the HC group than in the control group. In the HC group, pitavastatin and probucol significantly suppressed iNOS expression in the endothelium and intima by 84% and 70%, respectively, with no significant differences seen between the two drug-treated groups. Although iNOS expression in the endothelium and intima in both drug-treated groups was still twofold or 3.7-fold higher than in the control group, there were no significant differences among the drug-treated groups and the control group.

DISCUSSION

The present study demonstrated that both pitavastatin and probucol improve aortic stiffness by reducing vascular oxidative stress in 7-wk-cholesterol-fed rabbit aortas independent of.
lipid lowering. However, the mechanisms for the reduction of oxidative stress are entirely different between probucol and pitavastatin. We found that pitavastatin reduces ROS by selectively restoring Cu/ZnSOD activity in cholesterol-fed rabbit aortas via an increase in PPAR-γ expression and activity more effectively than probucol, whereas probucol reduces ROS by decreasing NAD(P)H oxidase activity more selectively and efficiently than pitavastatin. Furthermore, we found a significant positive correlation between PPAR-γ activity and Cu/ZnSOD activity in the rabbit aorta, indicating that pitavastatin improves aortic stiffness through the restoration of reduced Cu/ZnSOD activity via PPAR-γ activation independent of lipid lowering in hypercholesterolemia in vivo.

Aortic stiffness is critically associated with cardiovascular risk at the early stage of atherosclerosis, and oxidative stress is one of the important determinants for aortic stiffness (47, 50). We have recently reported that vascular oxidative stress is a critical determinant of aortic stiffness in hypercholesterolemia. Probucol, a lipophilic antioxidant, markedly improved aortic stiffness, whereas pravastatin, a hydrophilic statin, had no significant effect on aortic stiffness (22). In the current study, we demonstrated that the increase in aortic stiffness in hypercholesterolemia, where superoxide production is increased, is closely correlated with increased NAD(P)H oxidase activity and decreased activity of Cu/ZnSOD and ecSOD. Consistent with previous findings (5), pitavastatin restores Cu/ZnSOD activity in the hypercholesterolemic rabbit aorta independent of lipid lowering, which was associated with the decrease in vascular O_2^- content and the inhibition of the increase in aortic stiffness. In contrast, probucol had minor effects on Cu/ZnSOD activity. Cu/ZnSOD has been shown to attenuate vascular remodeling and alter vascular responsiveness (6, 9, 37). Thus pitavastatin may exert antiatherosclerotic effects, including the inhibition of an increase in aortic stiffness, by restoring Cu/ZnSOD activity, resulting in the reduction of ROS in the vascular wall independent of the lipid-lowering action.

Pitavastatin significantly restored the PPAR-γ expression and activity in the cholesterol-fed rabbit aorta. PPAR-γ activity was negatively correlated with aortic stiffness and was positively correlated only with Cu/ZnSOD activity in the vascular wall (Fig. 5). PPAR-α was not affected by the administration of pitavastatin in this study. This implies that pitavastatin restores vascular dysfunction mediated through the Cu/ZnSOD activation and the decrease in ROS in the vascular wall via PPAR-γ activation. It has been reported that the PPRE is located in the promoter of the Cu/ZnSOD gene and participates in the induction of the Cu/ZnSOD gene by the peroxisome proliferators (4). In contrast to our results, Han et al. (17) recently demonstrated in in vitro experiments using murine macrophages that pitavastatin inhibits the PPAR-γ-dependent CD36-mediated atherosclerotic foam cell formation. Although we did not examine which cells are important for restoring Cu/ZnSOD activity in cholesterol-fed rabbit aortas, and the precise mechanisms by which pitavastatin upregulates Cu/ZnSOD remain unknown, it has been reported that Cu/ZnSOD is expressed in the endothelium and smooth muscle cells and that PPAR-γ is expressed in a variety of cells in the vascular wall, such as the endothelium, vascular smooth muscle cells, and macrophages (24, 29). Importantly, we found that pitavastatin reduced ROS not only in the intima but also in the media. Taken together, PPAR-γ activation by pitavastatin may reduce ROS throughout the vascular wall and improve aortic stiffness through the restoration of reduced Cu/ZnSOD activity in hypercholesterolemia. PPAR-γ activators may promote the regression of fatty streaks by increasing the removal of cholesterol from macrophages (29), and they may also prevent vascular remodeling (19). Furthermore, Madamanchi et al. (27) reported that Cu/ZnSOD may be involved in vascular smooth muscle cell hyperplasia and hypertrophy. These data support our observation that pitavastatin may inhibit fatty streak formation and vascular remodeling more than probucol via the activation of PPAR-γ; it also appears that pitavastatin may
reduce ROS by restoring Cu/ZnSOD activity in the vascular wall in cholesterol-fed rabbits. Recent clinical trials demonstrated that PPAR-α or PPAR-γ agonist improved the outcome of atherosclerotic heart disease (10, 14), whereas dual-PPAR agonist did not (34). In addition, statins improved insulin resistance (41) and antioxidant activities, including the restoration of Cu/ZnSOD activity (48), prevented the development of diabetes, reduced lipid levels (13, 38), and improved clinical outcome in patients with hypercholesterolemia (1). These results, taken together with those of the present study, indicate that pitavastatin may selectively activate PPAR-γ and Cu/ZnSOD in hypercholesterolemia as pleiotropic effects.

Hypercholesterolemia is a central pathogenic factor of endothelial dysfunction and increases aortic stiffness in part by an impairment of endothelial NO produced by eNOS (45, 50). The activities of multiple oxidases, including Nox oxidases and NOS, can contribute to the generation of oxidant species in the vessel wall, and the activation of these enzymes induces cardiovascular dysfunction through multiple mechanisms. The NAD(P)H cellular redox systems have major roles in controlling oxidase activities, the metabolism of oxidant species, and signaling systems regulated by these species (46). Dysregulation of NO and increased oxidative and nitrosative stress are also implicated in the pathogenesis of cardiovascular dysfunc-

Fig. 7. A: immunohistochemical staining of von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), nitrotyrosine and inducible nitric oxide synthase (iNOS) in aorta using serial sections. Bar, 50 μm; e, endothelium; i, intima; m, media. B: quantitative analysis of eNOS, nitrotyrosine, and iNOS in aorta. Bars indicate SE. *P < 0.005 vs. control group; †P < 0.05 vs. HC group. Experiments, n = 4–8.
tion; peroxynitrite is a reactive oxidant that is produced from the reaction of NO with $\cdot$O$_2$ and iNOS also produces $\cdot$O$_2$ instead of NO when l-arginine is depleted or when there is a lack of the cofactor tetrahydrobiopterin (35). Probucol, a cholesterol-lowering lipophilic drug with potent antioxidant properties (8), preserves endothelium-derived NO action in association with limiting vascular oxidative stress, $\cdot$O$_2$ generation, and lipid peroxidation in vivo, without lipid lowering, in cholesterol-fed rabbits (21, 22). We found that pitavastatin and probucol decreased vascular $\cdot$O$_2$ production by either increasing SOD activity or decreasing NAD(P)H oxidase activity accompanied by the same increase in eNOS levels and decrease in nitrotyrosine and iNOS levels in cholesterol-fed rabbit aortas independent of lowering cholesterol, which will contribute to preserving NO bioavailability. Statins are reported to improve endothelial function by cholesterol-dependent and -independent mechanisms, such as the upregulation and activation of eNOS regulated by Rho GTPases (25). Consistent with our previous study (22), pitavastatin showed an inhibitory effect on NAD(P)H oxidase activity in hypercholesterolemia that was less than the effect of probucol. Our results agreed with those of Takayama et al. (42), demonstrating that pitavastatin only partly improves endothelial dysfunction, but not total vascular dysfunction, via the inhibition of NAD(P)H oxidase.

Sawayama et al. (40) demonstrated that probucol not only reduced the rate of the carotid intima-media thickness increment, but it also induced a lower incidence of cardiac events compared with pravastatin, independently of lipid lowering. In addition, when compared with pravastatin, atorvastatin, another lipophilic statin, showed a greater reduction in the progression of coronary atherosclerosis (33). Taken together with the results of our previous and current studies (22), these observations imply that the lipophilic statins may be more beneficial than the hydrophilic statins in preventing the progression of atherosclerosis through not only their inhibition of the ROS-generating system, NAD(P)H oxidase, but also through their restoration of the ROS-scavenging system, especially, Cu/ZnSOD, in hypercholesterolemia.

We previously examined vascular responsiveness to $\alpha$-receptor agonist assessed in vivo by the aortic pressure-diameter relationship at the early stage of atherosclerosis in hypercholesterolemic fat-fed rabbits and normal diet-fed rabbits, demonstrating that the aortic stiffness was unchanged by adrenergic stimulation in hypercholesterolemia (18). We also demonstrated that increased vascular oxidative stress may alter the phenotype of medial smooth muscle cells toward the synthetic type and consequently may increase aortic stiffness in hypercholesterolemia (22). These findings, taken together with the present results, lead to the conclusion that aortic stiffness may be influenced by the oxidative stress in the vascular wall more than by sympathetic nerve stimulation.

In this study, we did not examine the dose dependency of the effects of pitavastatin and probucol, the effects of the combination of both drugs in hypercholesterolemia, or the effects of each drug in rabbits with normal cholesterol levels. This study was designed not to investigate the antioxidant properties of the agents used, but rather to investigate whether Cu/ZnSOD, PPAR-γ, and oxidative stress might contribute to the increase in aortic stiffness induced by hypercholesterolemia in vivo and whether Cu/ZnSOD might be regulated by PPAR-γ but not PPAR-α. In addition, probucol reduced HDL-cholesterol levels by 24% compared with those in the control hypercholesterolemia group but did not reach statistical significance in the study. However, we cannot exclude the possibility that the reduction of HDL-cholesterol levels by probucol affects the oxidative stress. The precise mechanisms of the beneficial effects of pitavastatin on PPAR-γ in hypercholesterolemia in vivo also need to be clarified. Our findings provide information regarding one of the important mechanisms of the progression of atherosclerosis in hypercholesterolemia; both the Cu/ZnSOD activity and PPAR-γ expression and activity in the vascular wall may play a crucial role in the progression of atherosclerosis in hypercholesterolemia. In addition, strategies aimed at activating Cu/ZnSOD and PPAR-γ by pitavastatin as a PPAR-γ activator may have additional therapeutic potential against the progression of atherosclerosis in vivo.

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