Importance of NAD(P)H Oxidase–Mediated Oxidative Stress and Contractile Type Smooth Muscle Myosin Heavy Chain SM2 at the Early Stage of Atherosclerosis

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Background—Increased vascular oxidative stress induced by hyperlipidemia may alter the phenotype of vascular smooth muscle (SM) cells and play a crucial role in the progression of atherosclerosis. To clarify the mechanisms underlying vascular dysfunction and oxidative stress in hypercholesterolemia, we compared the effects of antioxidant probucol with those of pravastatin on aortic stiffness, phenotypic modulation, oxidative stress, and NAD(P)H oxidase essential subunit p22phox expression in aortic medial SM cells of cholesterol-fed rabbits by using color image analysis of immunostained sections.

Methods and Results—Japanese white male rabbits were fed either normal chow or 1% cholesterol diet for 14 weeks. After the first 7 weeks, cholesterol-fed rabbits were further divided into 3 groups: those fed with cholesterol feed only and those additionally given pravastatin (10 mg/d) or probucol (1.3 g/d) for the last 7 weeks. Within 7 weeks of treatment, probucol improved aortic stiffness more effectively than did pravastatin, inhibiting phenotypic modulation by selectively upregulating contractile-type SM myosin heavy chain isoform SM2 and by reducing both p22phox and superoxide content in medial SM cells of cholesterol-fed rabbit aorta. No significant differences in cholesterol levels, superoxide content, and endothelial NO synthase levels in the intima, aortic morphology and fibrosis, and synthetic-type myosin heavy chain in medial SM cells were observed between the 2 drug-treated groups.

Conclusions—These results suggest that oxidative stress and SM2 in medial SM cells might be important factors for vascular dysfunction, and strategies aimed at blocking NAD(P)H oxidase and upregulating SM2 may have therapeutic potential against the progression of atherosclerosis in hypercholesterolemia. (Circulation. 2002;105:2288-2295.)

Key Words: muscle, smooth ■ antioxidants ■ hypercholesterolemia ■ atherosclerosis
important role in reducing cardiovascular events. Statins have been seen to improve endothelial dysfunction by restoring endothelial NO synthase (eNOS) and reducing oxidative stress and NAD(P)H oxidase essential subunit p22phox in the vasculature, with or without lipid lowering.

On the other hand, several studies have demonstrated that the reduction in endothelial O2− levels or induction of NO in the endothelium is not sufficient to improve endothelium-dependent relaxation, suggesting that the media may contribute to vasomotor dysfunction in hyperlipidemia. Many studies have focused on the protective effects of lipid-lowering drugs on neointima and endothelial dysfunction. Little, however, is known about the direct effects of probucol or statins, especially on vascular function, oxidative stress, and/or phenotypic change of aortic medial SMCs at the early stage of atherosclerosis. In the present study, to clarify the mechanisms underlying vascular dysfunction and oxidative stress, we compared the effects of probucol with those of pravastatin on aortic stiffness, phenotypic modulation, oxidative stress, and p22phox expression in aortic medial SMCs of cholesterol-fed rabbits.

Methods

The Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine approved the experimental protocol used in this study, and the experiment was performed according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine, and law (No. 105) and notification (No. 6) of the Japanese government.

All chemicals were purchased from Sigma Chemical Co unless otherwise specified. The oxidative fluorescent dye hydroethidine (HE) was obtained from Polysciences Inc. Probucol was provided by Daiich Pharmaceutical Co Ltd, Tokyo, and pravastatin was a gift from Sankyo Co Ltd, Tokyo.

A total of 55 Japanese white male rabbits (2.5 to 3 kg; Kyudo Co Ltd, Kumamoto, Japan) were involved in this study. The control group (n=9) was fed normal chow for 14 weeks. The hypercholesterolemic group was fed 1% cholesterol diet (n=46) for the first 7 weeks, and then further divided, randomly, into 3 groups for the last 7 weeks: those fed cholesterol only (HC group; n=14); those additionally given pravastatin (10 mg/d; pravastatin group; n=16); and those additionally given probucol (1.3 g/d; probucol group; n=16). Fasting peripheral blood was collected to measure plasma cholesterol concentrations.

Aortic stiffness in terms of the β-index was obtained according to the method of Aikawa et al.15 Fixed specimens were paraffin-embedded by OCT compound in liquid nitrogen to obtain fresh-frozen sections for HE staining according to the method of Fukumoto et al.16 The percentage of total fractional fibrosis, cross-sectional area, total cell number in the aortic media, and intimal-to-medial ratio were determined according to the method of Fujii et al.17 Three points around the circumference of the area, without atheroma of the vessel sections, were examined in a blind fashion, using at least 2 slices for each rabbit aorta, and the mean value of each aorta was used for statistical analysis.

The experimental groups were compared using ANOVA followed by Scheffe’s multiple comparison. P<0.05 was considered significant.

Results

Body weight and total serum cholesterol levels were significantly higher in the 3 cholesterol-fed groups than in the control group, and no differences in those values were observed among the 3 cholesterol-fed groups (Table 1). Heart rate and blood pressures were unaltered among the 4 groups.

Aortic stiffness index-β was significantly higher in the HC group than in the control group (Table 1). Among the 3 cholesterol-fed groups, both the probucol and the pravastatin group showed a decrease in aortic stiffness index-β. Only in the probucol group did the decrease, to a level equal to that of the control group, reach statistical significance, relative to values in the HC group, within 7 weeks.

Total cell number and cross-sectional area of the media did not differ among the 4 groups (Table 2). In contrast, total fractional fibrosis and collagen volume fraction in the media were significantly increased in the HC group compared with the control group. Neither probucol nor pravastatin showed any effects on these values in cholesterol-fed rabbits. On the other hand, the intimal-to-medial ratio was significantly higher in the HC group than in the control group. Both probucol and pravastatin significantly and equally reduced those values compared with the values of the HC group.

Photographs in Figure 1A show that 3 of the MHC isoforms were specifically and heterogeneously stained, and α-SM actin was homogenously stained in medial SMC cytoplasm. Figure 1B shows the results of quantitative analysis for SM1, SM2, and NMHC-B/SMemb expression in medial SMCs. SM1 was unaltered among the 4 groups. In contrast, SM2 was downregulated by 64% in the HC group compared with the control group. The pravastatin and probucol groups, compared with the HC group, inhibited down-regulation of SM2 in medial SMCs. In addition, downregulation of SM2 in medial SMCs was more significantly suppressed in the probucol group than in the HC or pravastatin groups, with no difference seen in SM2 expression between the control and probucol groups. NMHC-B/SMemb in medial SMCs was significantly higher in the 3 cholesterol-fed groups than in the control group. In addition, 7 weeks of pravastatin or probucol treatment showed no effects on assisted color image analysis system using NIH Image version 1.62. The percentage of SM1-, SM2-, NMHC-B/SMemb-, and p22phox-positive cell areas against the α-SM actin–positive cell areas, and of the area positive for eNOS antibody in the vessel wall were obtained with serial sections, respectively. HE was used to evaluate in situ intracellular production of O2− and the cellular site of O2− production in both the intima and media, areas that were determined with hematoxylin-eosin–stained serial sections. These data were expressed as the fold increases against the corresponding data of the control group. The percentage of collagen volume fraction of the media was determined by Sirius red staining according to the method of Fukumoto et al.16 The percentage of total fractional fibrosis, cross-sectional area, total cell number in the aortic media, and intimal-to-medial ratio were determined according to the method of Fujii et al.17 Three points around the circumference of the area, without atheroma of the vessel sections, were examined in a blind fashion, using at least 2 slices for each rabbit aorta, and the mean value of each aorta was used for statistical analysis.

The experimental groups were compared using ANOVA followed by Scheffe’s multiple comparison. P<0.05 was considered significant.
NMHC-B/SMemb expression in the aortic medial SMCs of cholesterol-fed rabbits when serum cholesterol levels were unaltered. Photographs in Figure 2A show O$_2^-$ content in the vessel wall assessed by HE staining. The control group showed minimal fluorescence in the endothelium and adventitia, and only slight expression of O$_2^-$ was noticed in aortic medial SMCs. In contrast, the HC group showed significant increase in HE fluorescence, reflecting increased O$_2^-$ content throughout the vessel wall, especially in medial SMC cytoplasm. Figure 2B shows the results of quantitative analysis and indicates that the level of O$_2^-$ content in aortic media from the HC group was significantly higher than that from the control group. Among the 3 cholesterol-fed groups, 7 weeks of pravastatin treatment suppressed the level of O$_2^-$ content by 73%, especially in the aortic media, compared with values of the HC group. O$_2^-$ content in the probucol group maintained a level almost equivalent to that of the control group. On the other hand, 7 weeks of probucol treatment reduced the level of O$_2^-$ content by 54% in the aortic media, but in comparison with that of the HC group, the reduction did not reach statistical significance. Both pravastatin and probucol significantly reduced the level of O$_2^-$ content in the intima to a level equal to that of the control group, with no difference in O$_2^-$ content observed between the 2 drug-treated groups.

To clarify whether probucol affects NAD(P)H oxidase expression, we examined the respective effects of probucol and pravastatin on p22phox expression in aortic medial SMCs. Immunohistochemically, p22phox was expressed mainly in the endothelium and adventitia and slightly in the media of the control rabbit aorta (Figure 3A). Quantitative analysis showed that hypercholesterolemia induced a 5.4-fold increase in p22phox in the aortic media, endothelium, and adventitia compared with levels in the control group. Probucol significantly reduced p22phox in the aortic media by 44% compared with the level in the HC group, whereas the reduction of p22phox in the pravastatin group did not reach statistical significance within 7 weeks (Figure 3B).

To identify the localization and types of p22phox-expressing cells in the aortic media, immunohistochemistry of α-SM actin, SM2, NMHC-B/SMemb, and p22phox was performed on 14-week cholesterol-fed rabbits using serial sections (Figure 4A). Endothelium and adventitia were positively stained with antibody against p22phox. All of the SM2-, NMHC-B/SMemb-, and p22phox-expressing cells were specifically and heterogeneously stained in aortic medial SMCs from 14-week cholesterol-fed rabbits (brown against a pale blue background), respectively. All of the p22phox-expressing cells in the media were positive for α-SM actin and NMHC-B/SMemb. Interestingly, the positivity for p22phox varied relative to the grade of positive staining for SM2 but for NMHC-B/SMemb. Quantitative analysis showed a significant inverse correlation between p22phox and SM2 expression in the aortic media (Figure 4B), whereas no correlation between p22phox and NMHC-B/SMemb expression was observed in the aortic media ($r=0.31$; $P=NS$).

### Table 1. Body Weight, Serum Cholesterol Level, Hemodynamic Data, Echocardiographic Data, and Aortic Stiffness

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HC</th>
<th>Pravastatin</th>
<th>Probucol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rabbits</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>2.86±0.13</td>
<td>3.25±0.20*</td>
<td>3.20±0.13*</td>
<td>3.31±0.10*</td>
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<tr>
<td>Serum cholesterol, mmol/dL</td>
<td>0.59±0.36</td>
<td>23.53±1.71†</td>
<td>22.73±11.80†</td>
<td>20.35±3.13†</td>
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<tr>
<td>Hemodynamic data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>263±36</td>
<td>245±17</td>
<td>239±17</td>
<td>235±30</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>118±19</td>
<td>118±26</td>
<td>112±10</td>
<td>100±10</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>98±20</td>
<td>103±20</td>
<td>93±14</td>
<td>82±7</td>
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<tr>
<td>Echocardiographic data</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Minimum aortic dimension, mm</td>
<td>4.79±0.85</td>
<td>4.24±0.85</td>
<td>4.01±0.48</td>
<td>4.18±0.47</td>
</tr>
<tr>
<td>Maximum aortic dimension, mm</td>
<td>5.12±0.94</td>
<td>4.41±0.88</td>
<td>4.25±0.49</td>
<td>4.56±0.45</td>
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<tr>
<td>Aortic stiffness index β</td>
<td>2.42±0.21</td>
<td>4.55±0.86*</td>
<td>3.69±1.29</td>
<td>2.88±0.49‡</td>
</tr>
</tbody>
</table>

*Values are mean±SEM.
*†P<0.05 vs the control group; †P<0.001 vs the control group; and ‡P<0.05 vs the HC group.

### Table 2. Aortic Morphology, Cell Number, and Fibrosis in the Aortic Media

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HC</th>
<th>Pravastatin</th>
<th>Probucol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional area, mm$^2$/kg</td>
<td>0.63±0.12</td>
<td>0.67±0.71</td>
<td>0.58±0.81</td>
<td>0.57±0.83</td>
</tr>
<tr>
<td>Intimal-to-medial ratio</td>
<td>0.03±0.03</td>
<td>0.64±0.44*</td>
<td>0.21±0.16†</td>
<td>0.17±0.07†</td>
</tr>
<tr>
<td>Total cell number, mm$^2$</td>
<td>1561±82</td>
<td>1678±286</td>
<td>1557±128</td>
<td>1521±118</td>
</tr>
<tr>
<td>Total fractional fibrosis, %</td>
<td>21.6±8.3</td>
<td>30.1±11.0†</td>
<td>23.2±7.8</td>
<td>25.6±11.0</td>
</tr>
<tr>
<td>Collagen volume fraction, %</td>
<td>6.0±1.2</td>
<td>11.2±2.6‡</td>
<td>8.8±2.9</td>
<td>8.0±3.4</td>
</tr>
</tbody>
</table>

*Values are mean±SEM.
*†P<0.001 vs the control group; †P<0.05 vs the HC group; and ‡P<0.01 vs the control group.

Experiments, n=7 to 9.
Figure 5A shows the immunohistochemical localization of eNOS in rabbit aorta (brown color against a pale blue background). Endothelium was selectively stained with antibody against eNOS. Quantitative analysis demonstrated that the HC group showed a significant (90%) reduction of eNOS in comparison with the level in the control group. Pravastatin and probucol restored eNOS in the endothelium to the same degree, to a level equal to that of the control group, and no difference was observed between the 2 drug-treated groups (Figure 5B).

**Discussion**

We previously observed a significant increase in aortic stiffness and reduction of vascular responsiveness in 7-week cholesterol-fed rabbits at the early stage of atherosclerosis. In the present study, we showed no significant differences in hemodynamics, serum cholesterol levels, aortic morphology and fibrosis, or eNOS level and $O_2^-$/content in the intima between the probucol- and pravastatin-treated groups within 7 weeks of treatment, whereas probucol inhibited progression of aortic stiffness better than did pravastatin in cholesterol-fed rabbits over this period. These results indicate that the different effects of these drugs, at the doses used, on the direct action for SM function in the media might cause differences in the improvement of aortic stiffness.

Among the various SMC differentiation markers, myosin appears to be a powerful tool for studying the structural modifications and functional characteristics of vascular disease. We demonstrated that hypercholesterolemia induced downregulation of SM2 and upregulation of NMHC-B/SMemb in the media. Our findings are similar to results in the intima observed by Aikawa et al., indicating that the phenotype of medial SMCs appeared to change toward the
synthetic type and that this phenotypic modulation of medial SMCs might be responsible for the increased aortic stiffness in hypercholesterolemia. Moreover, both drugs inhibited phenotypic change of medial SMCs by selectively upregulating SM2 in the media. However, within 7 weeks and at the doses used, probucol might improve aortic stiffness by inducing greater changes than did pravastatin in the relative composition of SM-MHCs and NMHCs in aortic medial SMCs. Further, our data suggest that SM2 in the media, rather than NMHC-B/SMemb, might be one of the major determining factors for aortic stiffness in our experimental model.

It has been shown that hypercholesterolemia can induce vascular expression of NAD(P)H oxidase, which is an important source of $O_2^-$ production in animal models of hypercholesterolemia. Recent studies have demonstrated that p22phox-expressing SMCs in the neointima are positive for NMHC-B/SMemb but not for SM2, and have a greater capability to produce $O_2^-$ by NAD(P)H oxidase, suggesting that the redox state in the vasculature might affect phenotypic modulation, and that the phenotype of p22phox-expressing SMCs was the synthetic-type rather than the contractile-type. Our findings in the media are similar to results previously reported in the neointima, indicating that increased oxidative stress generated by upregulated NAD(P)H oxidase in the media might be responsible for inducing the phenotypic change of medial SMCs seen in hypercholesterolemia. Our results also suggest that NAD(P)H oxidase-mediated oxidative stress might regulate SM2 in medial SMCs, whereas NMHC/SMemb might be regulated by other factors, such as hypercholesterolemia itself rather than oxidative stress in vivo.

NO, a principal factor involved in the anti-atherosclerotic properties of the endothelium, is a powerful local vasodilator and is also involved in the regulation of SMC proliferation. Hypercholesterolemia is a central pathogenic factor of the endothelial dysfunction caused in part by an impairment of endothelial NO produced by eNOS. Our findings in the endothelium are consistent with results previously reported, suggesting...
gesting that both probucol and statins preserve NO by reducing \( \text{O}_2^- \) production and increasing in eNOS levels in experimental hypercholesterolemia, without lowering cholesterol. On the other hand, Miller et al\(^1\) demonstrated that reduction in endothelial \( \text{O}_2^- \) levels is not sufficient to improve endothelium-dependent relaxation, and that generation of reactive oxygen species within the media may contribute to vasomotor dysfunction in atherosclerosis. Further, Weisbrod et al\(^1\) also indicated that resistance of aortic SMCs to NO contributes to abnormal endothelium-dependent vasodilatation in cholesterol-fed rabbits. In our experiment, endothelial function was not examined, but probucol and pravastatin both inhibited intimal thickening, reduced \( \text{O}_2^- \) content in the intima and restored eNOS expression in the endothelium, and few differences were seen between the 2 drug-treated groups. In addition, 7-week treatment with probucol normalized \( \text{O}_2^- \) content and significantly reduced \( \text{p22}_{\text{phox}} \) in medial SMCs in comparison with levels in the HC group, whereas treatment with pravastatin over the same period did not. Taken together, these results suggest that an improvement of not only endothelial function but also medial SM function would be required for the restoration of vascular responsiveness in hypercholesterolemia.

There are several explanations for the different effects of the 2 drugs on \( \text{O}_2^- \) content, \( \text{p22}_{\text{phox}} \) level, and phenotypic modulation in medial SMCs. First, although the extent of lipid peroxidation, one of the important steps in the development of atherosclerosis, was not measured in this experiment, based on the \( \text{O}_2^- \) and \( \text{p22}_{\text{phox}} \) levels in the vasculature, the antioxidant property of probucol could be stronger than that of pravastatin at the doses used in this experiment.\(^7\) Second, probucol is a hydrophobic (lipid-soluble) compound and easily penetrates the aortic wall,\(^7\) whereas pravastatin is hydrophilic and cannot penetrate the vasculature as easily.\(^16\) The hydrophobic nature of probucol might contribute to its antioxidant property against lipid peroxidation, and thus result in effects different from those of pravastatin. Third, not only NAD(P)H oxidase but also other \( \text{O}_2^- \) generating systems, such as xanthine oxidase, generate \( \text{O}_2^- \) in the vasculature. However, the contribution of these enzymes would be much less than that of NAD(P)H oxidase.\(^8\) Finally, probucol might accelerate the scavenging system against \( \text{O}_2^- \);\(^21\) activation of the scavenging system against \( \text{O}_2^- \) in the vessel wall might be partly involved in the results of our experiment.

In conclusion, our findings suggest that the SM2 might be regulated by oxidative stress independent of cholesterol levels and that the upregulation of SM2 by reduced oxidative stress in the media obtained by antioxidants such as probucol might be sufficient to fully restore vascular dysfunction even in hypercholesterolemia. In addition, strategies aimed at reducing \( \text{O}_2^- \) content and/or blocking upregulated NAD(P)H oxidase expression not only in the endothelium and intima but also within the media may have therapeutic potential against the progression of atherosclerosis.

In this study, we did not examine the dose-dependency of the effects of probucol and pravastatin or measure the NAD(P)H oxidase activity. This study was designed not to
investigate the antioxidant property of the agents used but rather to investigate whether the phenotype of medial SMCs and oxidative stress might contribute to the increase in aortic stiffness induced by hypercholesterolemia in vivo, and to demonstrate a crucial role for medial SMCs and endothelial cells in vascular dysfunction in atherosclerosis. Although little is known about the mechanical properties of SM-MHCs and NMHCs of vascular SMCs in hyperlipidemia in vivo, and further experiments will be necessary to examine the antioxidant property of both agents, our findings provide a novel

**Figure 4.** A, Immunohistochemistry of α-SM actin, SM2, NMHC-B/SMemb, and p22phox in the thoracic descending aorta from 14-week cholesterol-fed rabbits using serial sections. The majority of p22phox-expressing SMCs are positive for NMHC-B/SMemb but not for SM2. Bar, 50 μm. B, Relationships between the percentages of p22phox- and SM2-positive cell areas relative to α-SM actin-positive cell areas in the aortic media using serial sections. Each point represents a different rabbit. Data were statistically analyzed by simple regression analysis.

**Figure 5.** A, Immunohistochemical staining of eNOS in an intact endothelial layer of the thoracic descending aorta. Bar, 100 μm. B, Quantitative analysis for eNOS expression in endothelium. Bars indicate SEM. *P<0.05 vs the control, pravastatin, and probucol groups. Experiments, n=4.
view of atherosclerosis and vascular dysfunction and offer important information for the development of more effective therapies for atherosclerosis.

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References
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