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Novel Mutation of Plakophilin-2 Associated With Arrhythmogenic Right Ventricular Cardiomyopathy

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Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disease characterized by dilatation and akinesis of the right ventricle, and causes life-threatening ventricular arrhythmia. Mutations of plakophilin-2 (PKP2) have recently been identified as one causative abnormality in ARVC. A case of ARVC with a mutation of PKP2 is reported here. Direct sequencing of the patient's DNA revealed an insertion mutation in exon 8 of PKP2 (1728_1729insGATG). The mutation caused the frameshift and the premature termination of translation (R577DfsX5). This is the first case report of PKP2 mutation found in Japanese ARVC patients. (*Circ J* 2006; **70:** 933–935)

Key Words: Arrhythmogenic right ventricular cardiomyopathy; Desmosome; Genetic analysis; Plakophilin-2

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disease characterized by dilatation and akinesis of the right ventricle that causes life-threatening ventricular arrhythmias. A characteristic pathological finding is a progressive fibro-fatty replacement of the right ventricular myocardium[1] About 30–50% of the cases of ARVC are inherited, and heterozygous mutations of ryanodine receptor-2 (RYR2)[2,3] and plakophilin-2 (PKP2) have been reported in familial ARVC[4] PKP2 has essential roles in the formation of desmosome and heart development[4,5] In this short report, we presents the first Japanese ARVC patient in whom a novel mutation of PKP2 was identified.

Case Report

A 30-year-old male was referred to hospital due to recurrent faintness. Physical examination revealed a heart rate of 50 beats/min and blood pressure of 114/60 mmHg. No heart murmur was heard and there were no signs of left or right ventricular failure. The chest X-ray revealed cardiomegaly with increased cardiothoracic ratio (58%). The blood chemistry showed no abnormalities. His resting electrocardiography (Fig 1A) showed T wave inversion in leads V_{1-4}, without right bundle branch block. Sustained ventricular tachycardia of the left bundle branch block morphology with an inferior axis was recorded during the period of faintness (Fig 1B). Signal-averaged ECG recordings showed positive late potentials according to the following criteria: filtered QRS duration 181 ms (>130 ms), duration of low amplitude signals <40 μV of the terminal QRS complex (LAS40) 102 ms (>40 ms), and root mean square voltage of the last 40 ms of the QRS complex (RMS40) 2.0 μV (<15 μV) (Fig 1C)[6] Echocardiography revealed an enlarged, hypokinetic right ventricle with a paper-thin free wall (Fig 2). Contrast-enhanced computed tomography demonstrated the dilated right ventricle and the presence of epicardial and intramyocardial fat deposits in the right ventricle (Fig 3). His aunt died suddenly in her fifties. Accordingly, the patient was diagnosed as ARVC and gave an informed consent for the genetic analysis.

Genetic Analysis

The patient's genomic DNAs were extracted from peripheral blood using standard methods after obtaining informed consent. The institutional review boards approved the protocols. All exons of PKP2[4] and some parts of RYR2 (exons 8–16, 44–49, 83, 84, 87–89, 91–105), were examined using denaturing high performance liquid chromatography (DHPLC; WAVE system, Transgenomic Inc, Omaha, USA)[3] A mixture of 15 μl of each DNA sample from the patient and from a normal control was heated for 5 min at 95°C, and then cooled down to various temperatures depending on the primer setting. The resultant chromatograms were compared for variation in shape or retention time. All variants identified by the DHPLC scanning were examined by direct sequencing using ABI PRISM 310 DNA Sequencer (Perkin Elmer, Foster City, USA).

We found no mutation in 35 exons of RYR2 gene, which are thought to be hot regions for mutations[3] In the exon 8 of PKP2 gene, however, the chromatogram of DHPLC showed a variant elution pattern in the patient's DNA (Fig 4). Direct sequencing showed overlapping figures due to an insertion mutation, causing the frameshift (1728_1729insGATG) (Fig 5A). This mutation caused a premature termination of translation at the codon 582 (R577DfsX5) (Fig 5B).

Discussion

Plakophilin (PKP) is an essential protein forming the desmosomal complex[4,5] Type 2 PKP (PKP2) encoded by *Pkp2* is the main isoform in cardiomyocytes. Grossmann et al reported that the ablation of mouse *Pkp2* resulted in the

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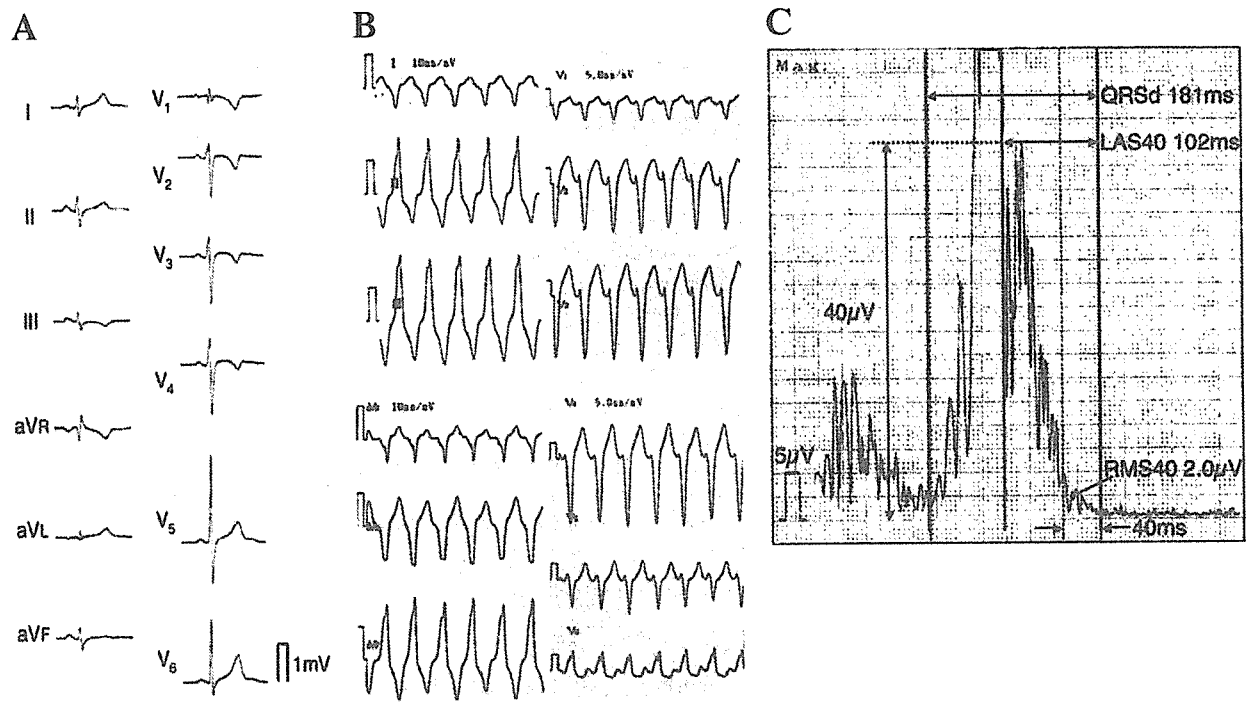

Fig 1. (A) Resting 12-lead electrocardiograms showing the T wave inversion in chest leads V_{1-4}, in the absence of right bundle branch block. (B) Sustained ventricular tachycardia of left bundle branch block morphology with an inferior axis was recorded during the period of faintness. (C) Signal-averaged ECG recordings.

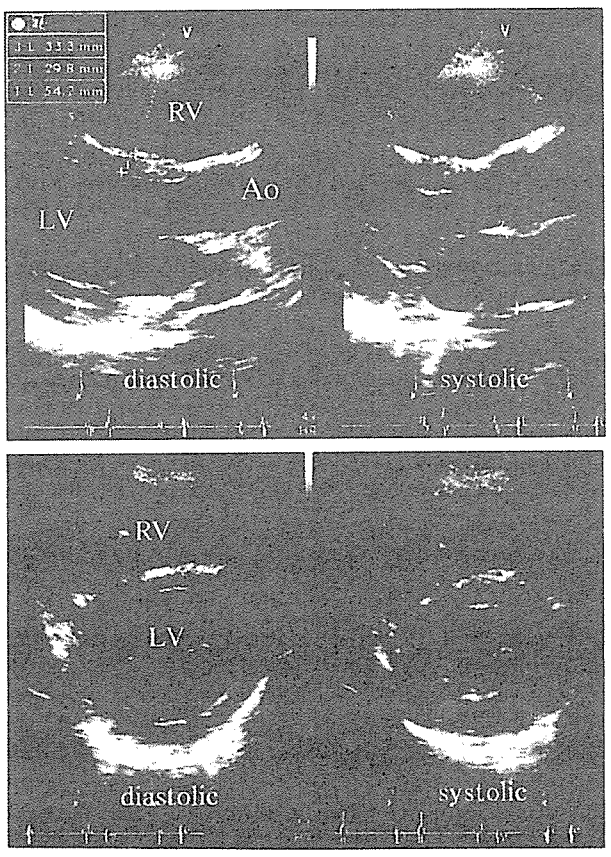

Fig 2. Echocardiography revealed an enlarged, hypokinetic right ventricle (RV) with a paper-thin free wall. LV, left ventricle; Ao, ascending aorta.

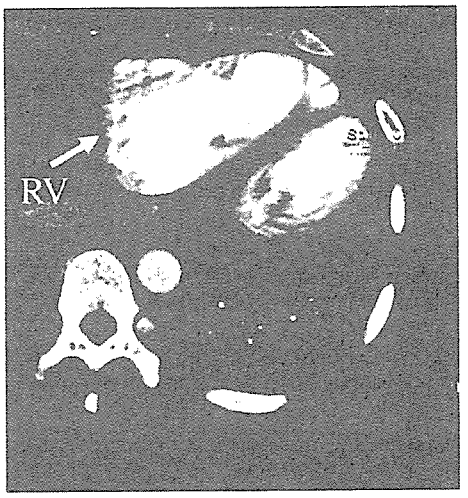

Fig 3. Computed tomography with contrast demonstrated the dilated right ventricle (RV) and the presence of epicardial and intramyocardial fat deposits in the RV.

lethal defect of heart morphogenesis at embryonic day 10.75[5] Genetically-engineered transgenic mice lacking *Pkp2* have been shown to disrupt the cell–cell contacts of adjacent cardiomyocytes, leading to right ventricular dilatation similar to that observed in human ARVC. Besides PKP2, the disruption of desmosomal proteins such as plakoglobin and desmoplakin has been identified in inherited forms of ARVC[7,8] Pashmforoush et al reported that disruption of the gene encoding α-actinin-associated LIM protein in mice caused dilatation and dysfunction of the right ventricle in utero[9] Therefore, Gerull et al stated that ARVC might be considered a desmosome disease[4] As shown in

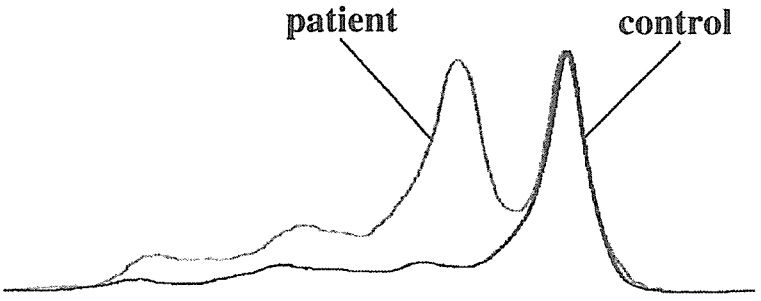

Fig 4. In exon 8 of the plakophilin-2 gene, denaturing high performance liquid chromatography chromatogram representing variant elution pattern of patient's DNA.

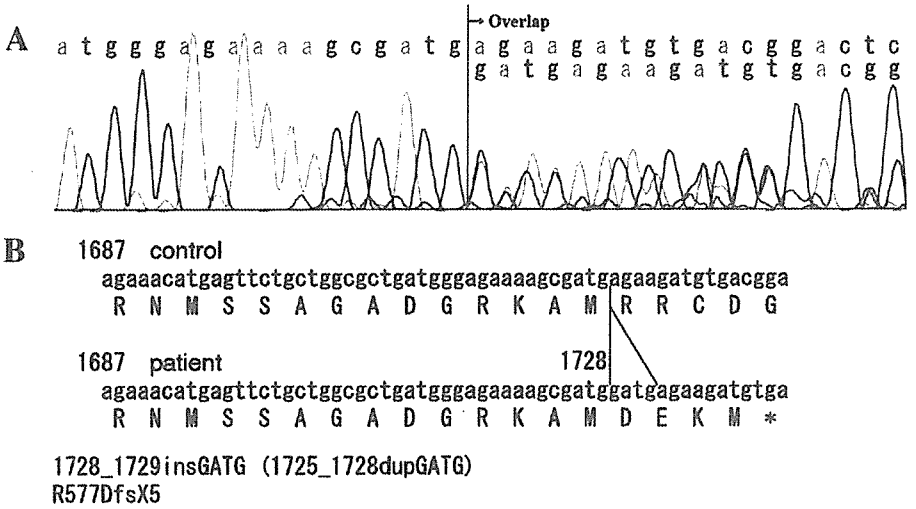

Fig 5. (A) Direct sequencing revealed insertion mutation 1728_1729ins GATG (1725_1728dupGATG). (B) Alignment of cDNA in the vicinity of codon 1728. The insertion mutation causes the frame shift and the premature termination of translation (R577DfsX5 indicated by asterisk).

Pkp2 knock-out mice, disruption of desmosome leads to the loss of cell to cell connection, which in turn causes replacement of the myocardium with fibro-fatty tissue and thereby causes a regional conduction delay.[5] Such histological changes may cause the positive late potential (Fig 1C).

In the present study, DHPLC enabled us to examine a number of DNA samples at the same time and save time detecting single base substitutions of DNA fragments. Yamanoshita et al reported that DHPLC is superior to single-strand conformational polymorphism (SSCP) in screening for mutations in terms of sensitivity.[10] However, DHPLC may not be 100% effective in the detection of mutations.[11] Moreover, we did not search for RYR2 mutations out of the regions known as hot sites.[3] Also, the possibility that there might be mutations within regulatory regions or intronic sequences important for splicing or transcription cannot be excluded.

In summary, we found a novel mutation of PKP2 associated with ARVC by using a screening technique of DHPLC and direct sequencing. This is the first case of PKP2 mutation found in a Japanese ARVC patient.

Acknowledgments

The authors are grateful to Dr Thierfelder (Max-Delbueck Center for Molecular Medicine, Berlin, Germany) for providing us with information on the primer sequences for Pkp2. Dr Horie was supported by Research Grants from the Japan Ministry of Education, Science, Sports and Culture and by Health Science Research Grants from Japan Ministry of Health, Labor, and Welfare.

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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 statin restores 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 ($O_2^-\cdot$) production by vascular NAD(P)H oxidase may play a critical role in the progression of atherosclerosis (16). A primary cellular defense against $O_2^-\cdot$ 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

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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 Daiichi 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 ($n = 33$) 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 $mg \cdot kg^{-1} \cdot day^{-1}$; $n = 12$), 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 (D_{min}; in mm), maximum aortic dimension during the ejection period (D_{max}; in mm), and the systolic amplitude of the internal dimension ($\Delta D = D_{max} - D_{min}$). Stiffness parameter β was calculated using the equation $\beta = \ln(BP_s/BP_d)/(\Delta D/D_{min})$, where BP_s is the maximum systolic aortic pressure and BP_d 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-biotinilated 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′-AACTAGGTCAAAGGTCA-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 $O_2^-\cdot$ 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 $O_2^-\cdot$ 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

Table 1. *Serum lipid levels*

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

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

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.

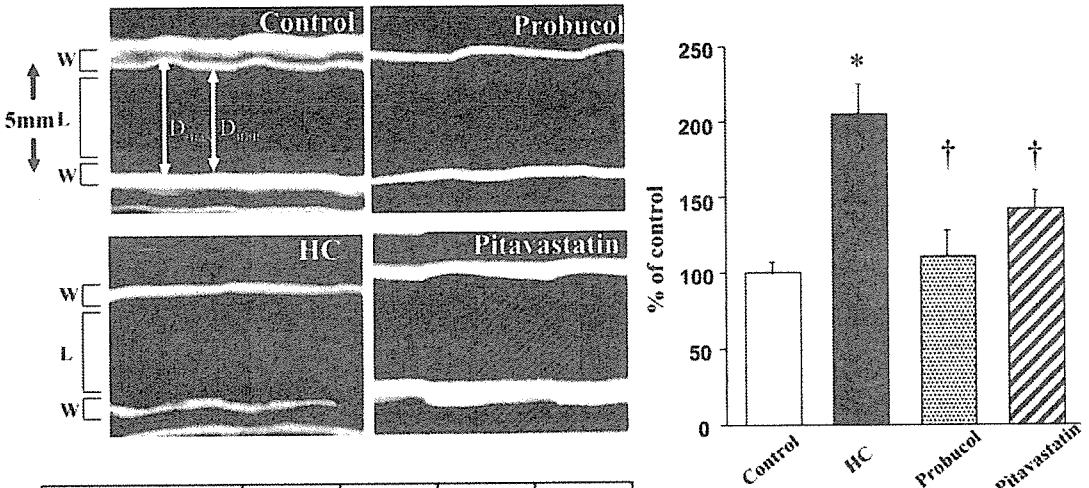
Figure 2 shows DHE staining to assess the $O_2^-\cdot$ content in the vessel wall. The control group showed minimal fluorescence in the endothelium and adventitia, and only a slight expression of $O_2^-\cdot$ was noticed in the aortic media. In contrast, the HC group showed a significant increase in DHE fluorescence, which reflected increased $O_2^-\cdot$ 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 $O_2^-\cdot$. The quantitative analysis indicated that the $O_2^-\cdot$ 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 $O_2^-\cdot$ 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 2. *Body weight, hemodynamic data, echographic data of abdominal aorta and aortic stiffness, aortic morphology, and oxidative stress*

	Control	HC	Probucol	Pitavastatin
n	7	11	10	12
Body weight, kg	3.20±0.12	3.03±0.12	3.06±0.06	3.06±0.05
Hemodynamic data				
Heart rate, beats/min	289±14	284±12	279±3	284±9
Systolic pressure, mmHg	126±8	131±5	134±7	124±7
Diastolic pressure, mmHg	75±6	79±4	87±6	78±6
Echographic data of abdominal aorta				
Minimum aortic dimension, mm	3.58±0.17	4.08±0.13	3.86±0.14	3.91±0.10
Maximum aortic dimension, mm	4.25±0.20	4.43±0.14	4.43±0.17	4.38±0.11
Aortic stiffness index-β	2.84±0.23	5.83±0.55*	3.14±0.51†	4.02±0.38†
Morphology of abdominal aorta				
Cross-sectional area, mm^2/kg	0.83±0.02	2.85±0.24‡	1.83±0.31*†	1.18±0.12†§
Intima-to-media ratio	0.03±0.02	0.61±0.18‡	0.37±0.21†‡	0.26±0.14†‡§
Total cell number/mm^2	1588±72	2944±130‡	2091±167†	1916±157†
Total fractional fibrosis, %	20.4±0.01	31.3±0.02*	22.2±0.02†	23.0±0.02†
Collagen volume fraction, %	4.9±0.01	7.5±0.01*	5.3±0.01†	5.5±0.01†
Oxidative stress of abdominal aorta				
TBARS, nmol/mg	8.97±3.99	30.67±5.38‡	13.27±1.04†	14.79±1.52†

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.



	Control	HC	Probucol	Pitavastatin
Aortic pressure, mmHg	130/82	130/84	133/80	124/82
Dmax, mm	4.4	4.5	4.5	4.1
Dmin, mm	3.7	4.2	3.9	3.7
β	2.37	5.95	3.3	3.82

Fig. 1. Representative M-mode echo tracing of aorta. D_{max}, maximum aortic dimension (in mm); D_{min}, minimum aortic dimension (in mm); W, aortic wall; L, aortic lumen; HC, hypercholesterolemic group. The β-indexes were obtained as described in MATERIALS AND METHODS.

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 β-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-

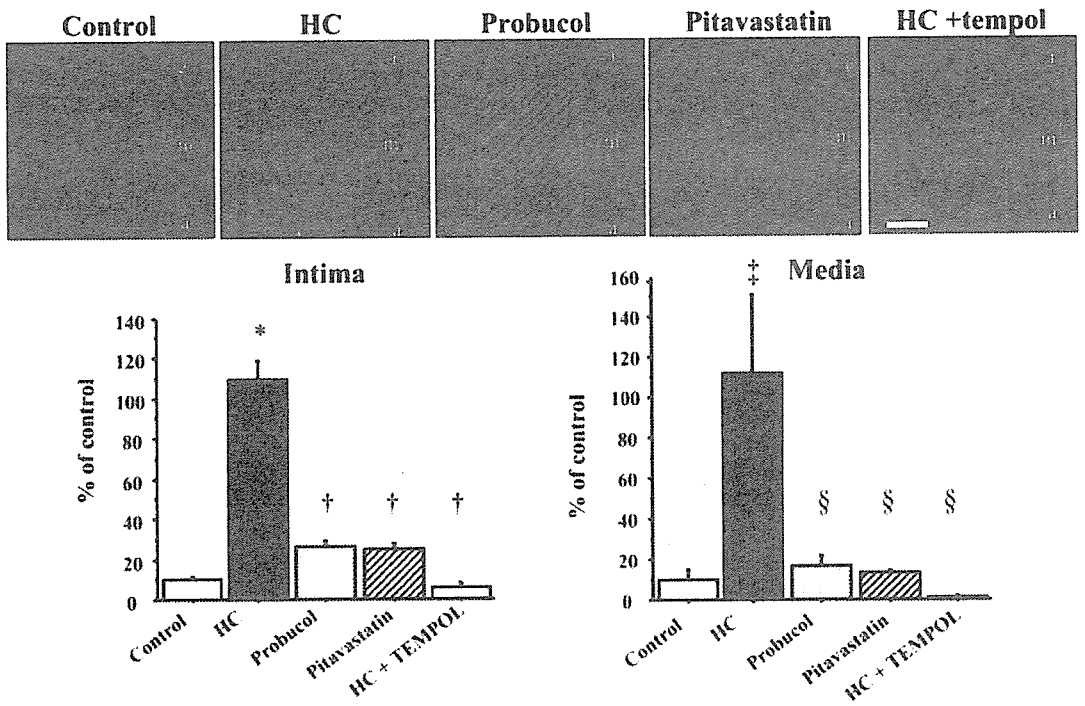


Fig. 2. Fluorescent photomicrographs showing in situ detection of superoxide in abdominal aorta labeled with the oxidative dyhydroethidine (DHE; red fluorescence) and the results of a quantitative analysis of ratio of fluorescence within intima and media. Note the increased fluorescence reflecting O_2^-· levels in the endothelium (e), intima (i), media (m), and adventitia (a) of HC group (bar, 50 μm). Bars indicate SE. Experiments, n = 3–9. *$P < 0.001$ vs. control group; †$P < 0.001$ vs. HC group; ‡$P < 0.05$ vs. control group; §$P < 0.05$ vs. HC group.

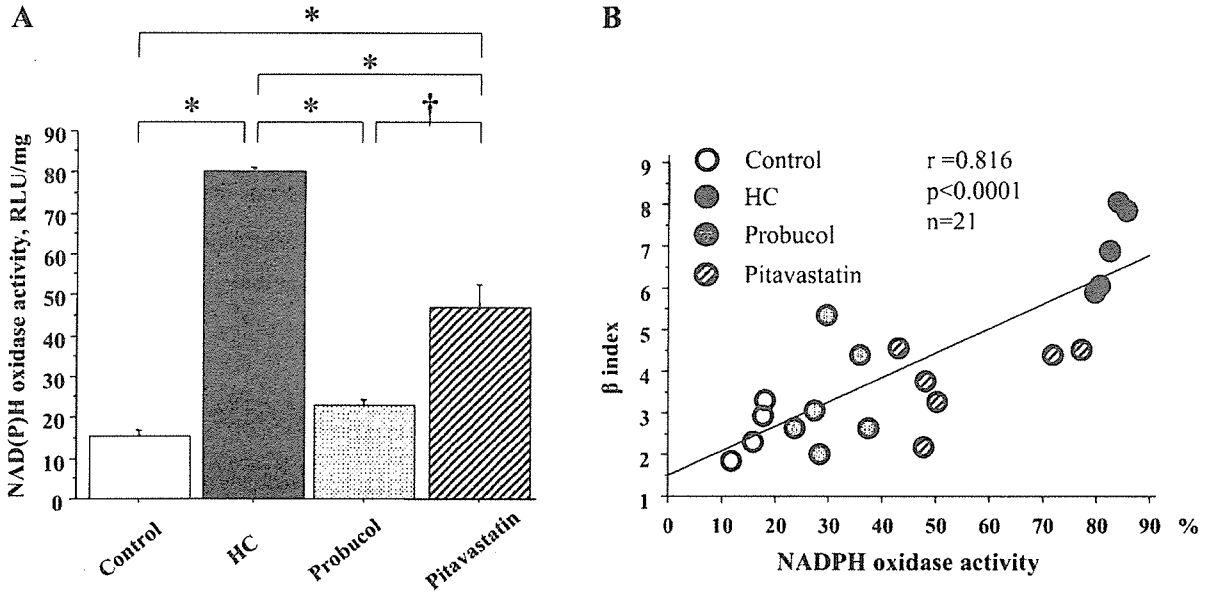


Fig. 3. Quantitative analysis of NAD(P)H oxidase activity in aortas (*A*) and relationship between percentages of NAD(P)H oxidase activity and β-index (*B*). *A*: bars indicate SE. $*P < 0.0001$, $\dagger P < 0.0005$. Experiments, $n = 4$. RLU, relative light units. *B*: each point represents a different rabbit.

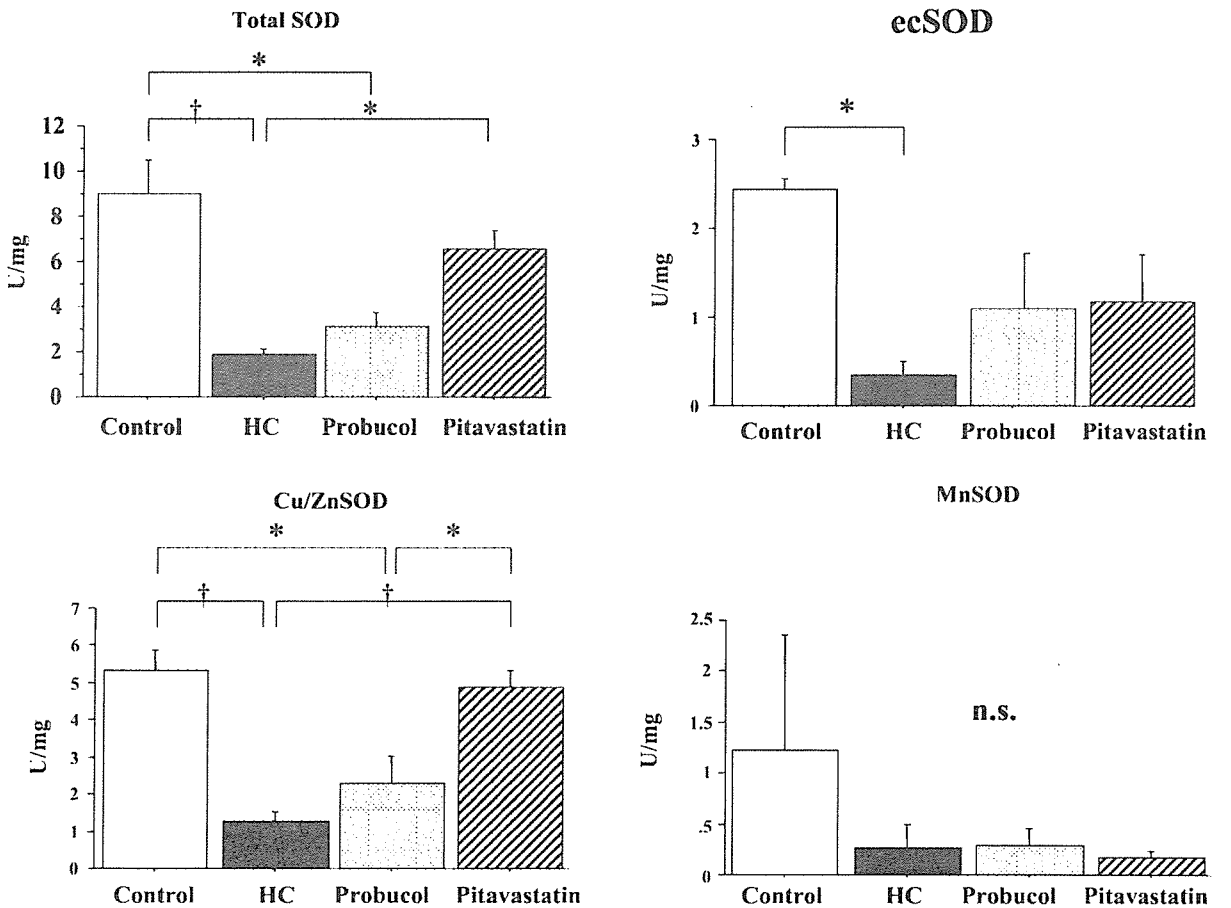


Fig. 4. Quantitative analyses of SOD isoform activities in aorta. Bars indicate SE. Experiments, $n = 4$–6. NS, not significant; ecSOD, extracellular SOD. $*P < 0.05$, $\dagger P < 0.005$.

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. 6*C*), 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. 6*D*. 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. 6*E*.

Effect of pitavastatin and probucol on eNOS, nitrotyrosine, and iNOS expression. Figure 7*A* 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 7*B* 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-

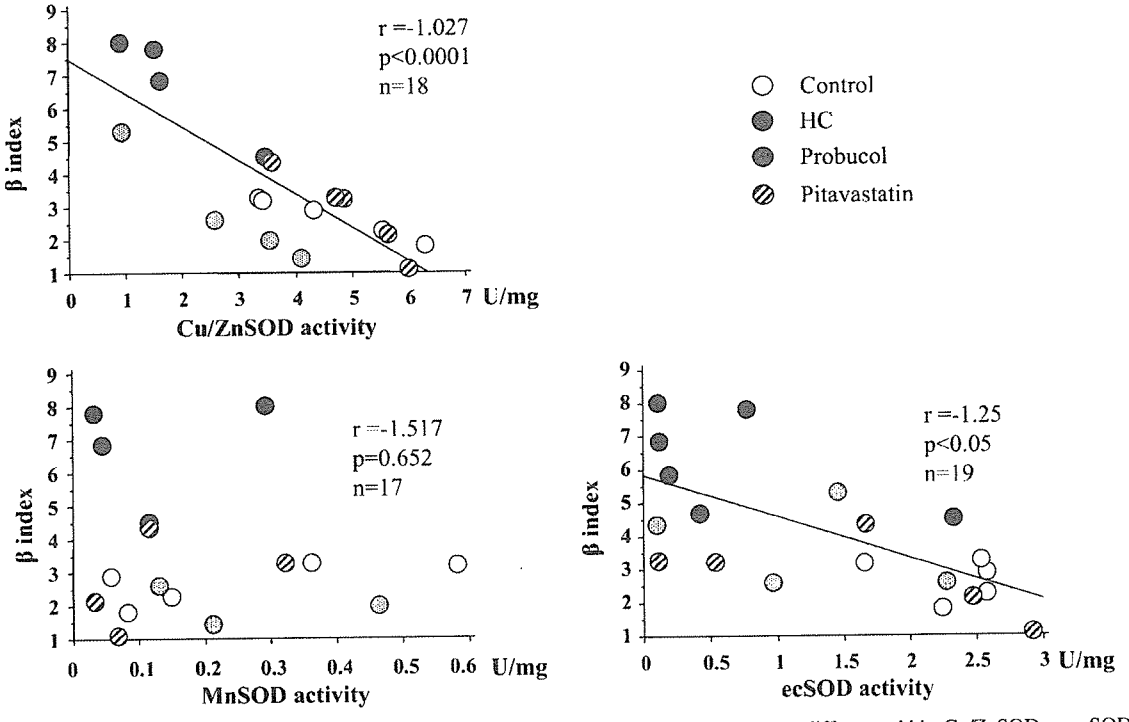


Fig. 5. Relationships between SOD isoform activities and aortic stiffness β-index. Each point represents a different rabbit. Cu/ZnSOD or ecSOD activity was negatively correlated with β-index.

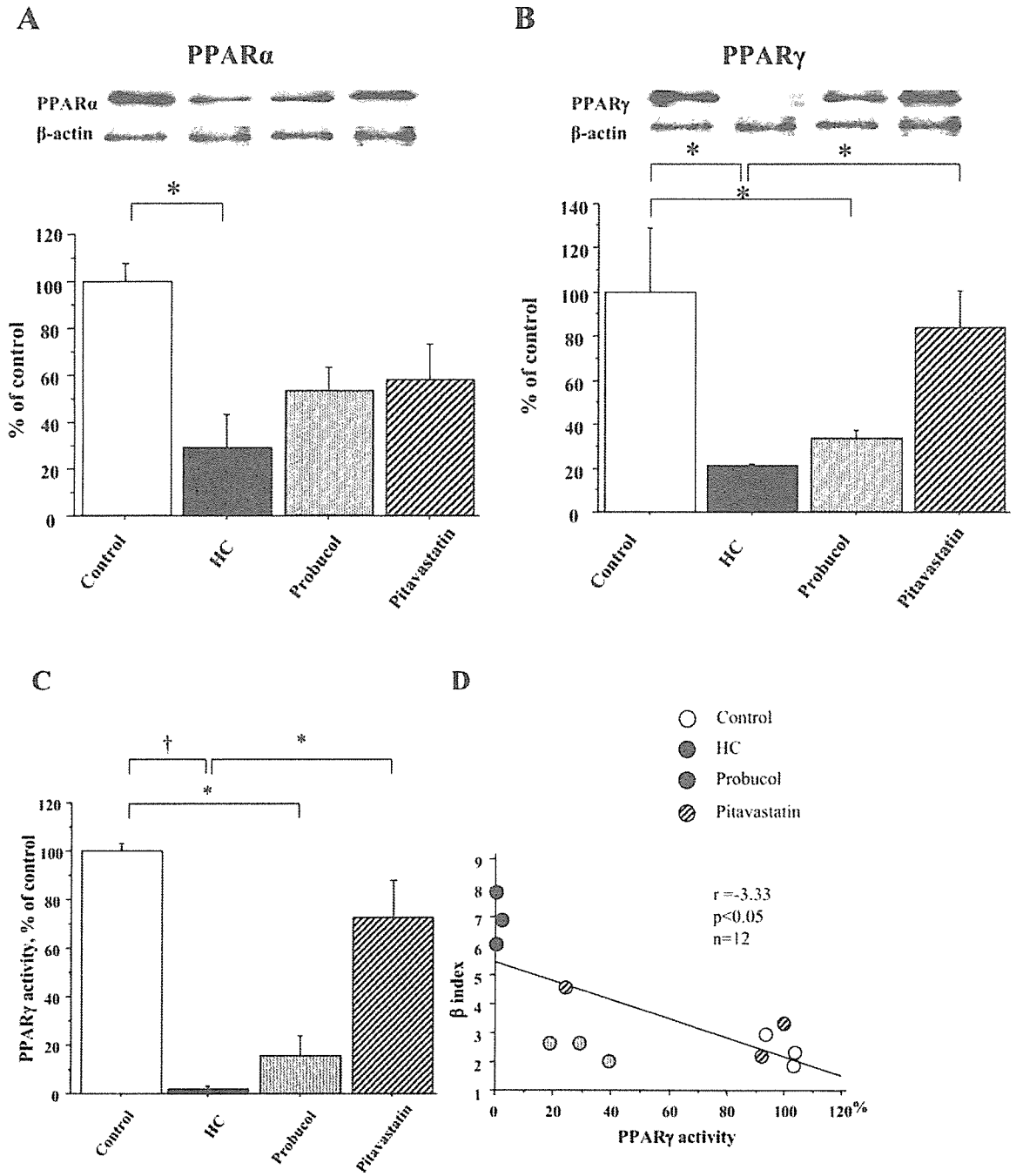


Fig. 6. Peroxisome proliferator-activated receptor (PPAR)-α and -γ expression in aortas assessed by immunoblotting (*A* and *B*) and quantitative analyses of PPAR-γ activities in aorta (*C*). Bars indicate SE. $*P < 0.05$, $\dagger P < 0.05$. Experiments, $n = 4-6$. Relationships between PPAR-γ activities and aortic stiffness β-index (*D*). Each point represents a different rabbit. PPAR-γ activities were negatively correlated with β-index.

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

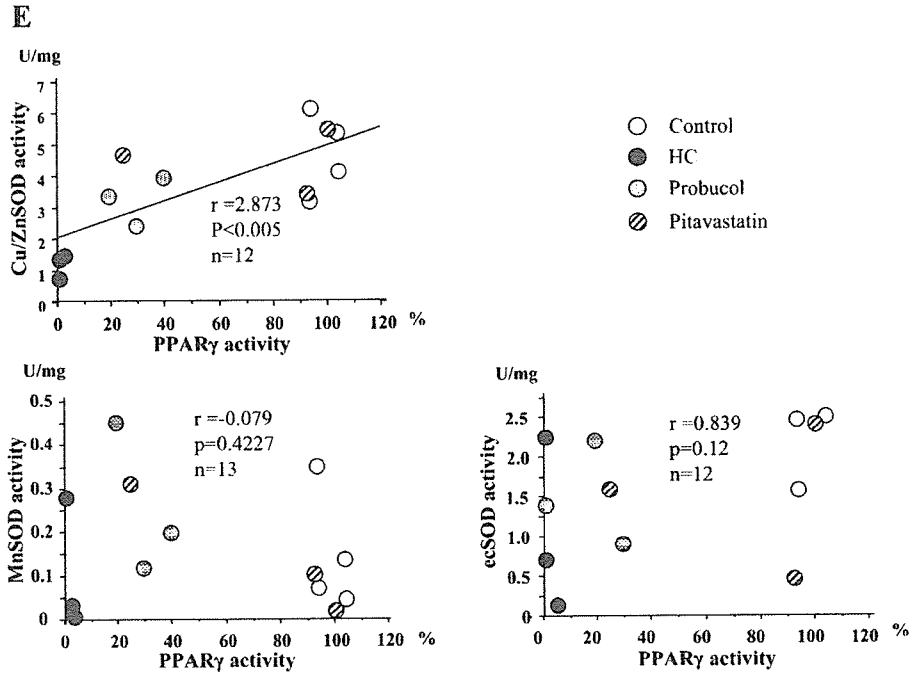


Fig. 6. *Continued*–Relationships between PPAR-γ activities and SOD isoform activities (*E*). Each point represents a different rabbit. Only Cu/Zn-SOD activity was positively correlated with PPAR-γ expression.

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

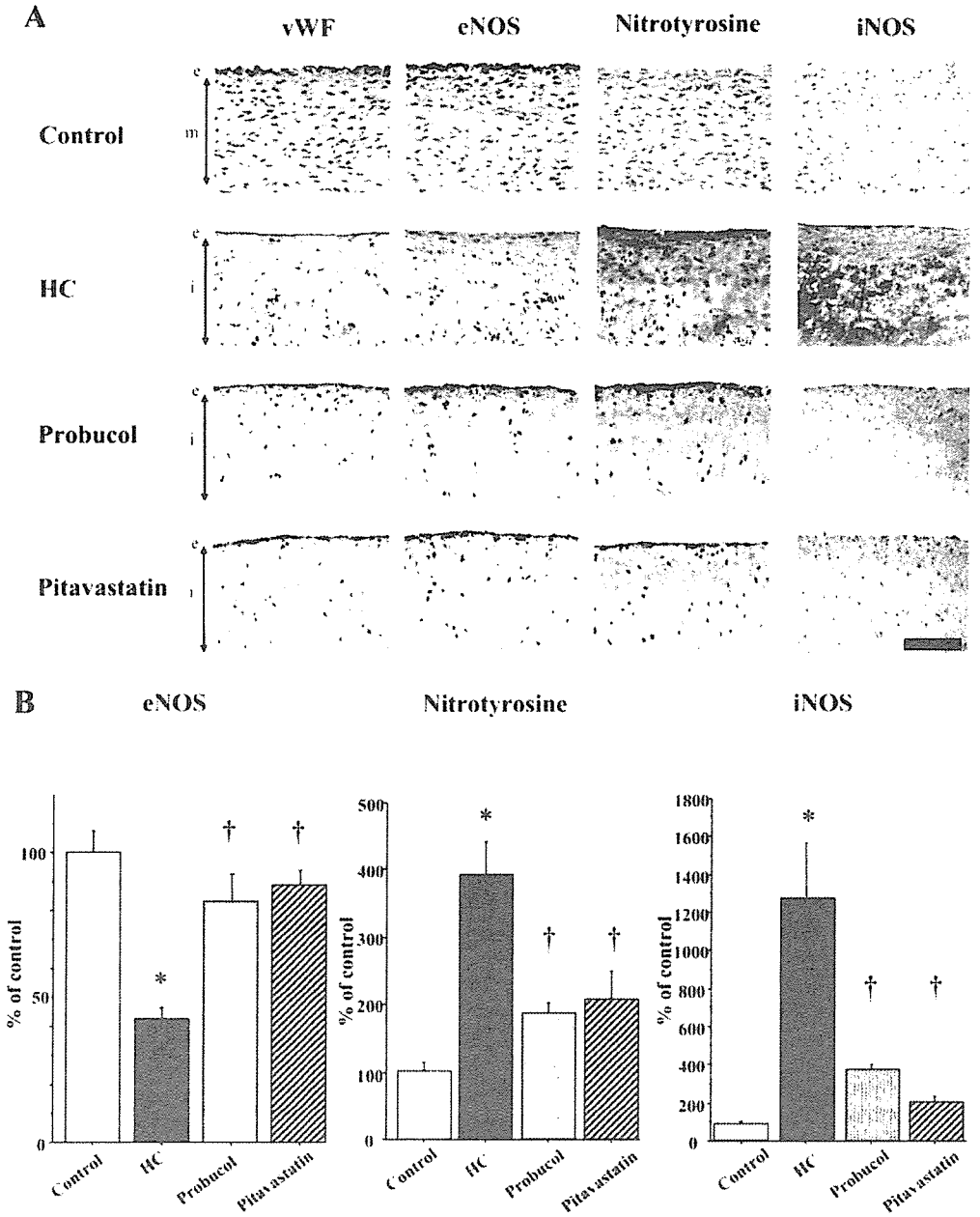


Fig. 7. *A*: immunohistochemical staining of von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), nitrotyrosine and inducible nitric oxide synthase (iNOS) in aorta using serial sections. Bar, 50 μm; e, endothelium; i, intima; m, media. *B*: quantitative analysis of eNOS, nitrotyrosine, and iNOS in aorta. Bars indicate SE. $*P < 0.005$ vs. control group; $\dagger P < 0.05$ vs. HC group. Experiments, $n = 4-8$.

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-

tion; peroxynitrite is a reactive oxidant that is produced from the reaction of NO with $O_2^-\cdot$, and iNOS also produces $O_2^-\cdot$ 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, $O_2^-\cdot$ generation, and lipid peroxidation in vivo, without lipid lowering, in cholesterol-fed rabbits (21, 22). We found that pitavastatin and probucol decreased vascular $O_2^-\cdot$ 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 increase, 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 α-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/Zn-SOD 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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Japan Assessment of Pitavastatin and Atorvastatin in Acute Coronary Syndrome (JAPAN-ACS)

— Rationale and Design —

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Background Many trials have shown that 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors reduce the incidence of cardiovascular events and mortality. One method of decreasing the incidence of cardiovascular events could be to reduce the progression of coronary atherosclerosis, and a recent study found that atorvastatin can cause coronary plaque to regress. To generalize this finding, using conventional HMG-CoA reductase inhibitors at many Japanese centers, randomized trials of pitavastatin and atorvastatin will be conducted with patients with acute coronary syndrome (ACS).

Methods and Results Patients with ACS who have undergone successful percutaneous coronary intervention under intravascular ultrasound guidance will be studied. They will be randomly allocated to pitavastatin or atorvastatin groups and followed up for 8–12 months. The primary endpoint will be the percent change in coronary plaque volume, and secondary endpoints will include absolute changes in coronary plaque volume, serum lipid levels and inflammatory markers. The safety profile will also be evaluated.

Conclusions This study will examine the ability of HMG-CoA reductase inhibitors to regress coronary plaque in Japanese patients with ACS and the findings should help to improve the prognosis of such patients and clarify the involved mechanisms. (*Circ J* 2006; **70:** 1624–1628)

Key Words: Acute coronary syndrome; Atherosclerosis; HMG-CoA reductase inhibitors; Intravascular ultrasound; Plaque

Many large-scale clinical trials have shown that 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors reduce the incidence of cardiovascular events.[1–8] The relationship between low-density lipoprotein (LDL)-cholesterol (C) level and cardiovascular event frequency is linear, and an alternative strategy is reducing LDL-C to lower the cardiovascular event rate.[7,8] Accordingly, current US guidelines (3rd Report of the U.S. National Cholesterol Education Program) suggest that the aim of treatment should be a LDL-C level <1.8 mmol/L (70 mg/dl) after acute coronary syndrome (ACS).[9] In the Japanese statin trial the incidence of coronary events in hypercholesterolemic patients was strongly correlated with the level of serum cholesterol[10] and the 2002 Guidelines for Diagnosis and Treatment of Atherosclerotic Diseases have established the goal of LDL-C management as <100 mg/dl in patients with coronary heart disease.[11] However, no clinical trials have supported the notion that LDL-C level <100 mg/dl reduces the risk of recurrent cardiovascular events in Japanese survivors of ACS episodes.

Although reducing cardiovascular morbidity and mortality are therapeutic goals for patients with hypercholesterolemia, clinical trials that can accurately detect treatment effects using an active comparator are essentially very large and require long-term follow-up. Surrogate endpoints might provide an alternative opportunity to demonstrate efficacy in a relatively small sample size with a short follow-up. Because progressive atherosclerosis is the underlying basis of cardiovascular disease, whether or not aggressive lipid-lowering therapies, including HMG-CoA reductase inhibitors, have substantial beneficial effects on this process should be determined.

Imaging studies of coronary plaque have contributed considerably to understanding the benefits of intensive lipid-lowering therapies. The Low Density Lipoprotein-Apheresis Coronary Morphology and Reserve Trial (LACMART) has shown that aggressive lipid-lowering therapy using LDL-apheresis induces the regression of coronary atherosclerotic plaque in patients with familial hypercholesterolemia, as evaluated by intravascular ultrasound (IVUS) imaging.[12] The Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial found that an intensive lipid-lowering strategy using atorvastatin (80 mg/day) reduced the progression of atherosclerosis compared with moderate treatment using pravastatin (40 mg/day).[13] However, 80 mg/day of atorvastatin does not facilitate plaque regression. An IVUS assessment in the setting of aggres-

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Table 1 Inclusion Criteria

- *Patients giving written consent by their own volition after being provided sufficient explanation for participation in this clinical trial.*
- *Patients 20 years or older at the time of their consent.*
- *Patients with hypercholesterolemia as defined by any of the following criteria: (1) TC ≥220 mg/dl; (2) LDL-C ≥140 mg/dl; (3) cholesterol-lowering treatment is necessary in accordance with the investigator's judgement when LDL-C ≥100 mg/dl or TC ≥180 mg/dl.*
- *Patients who have been diagnosed with acute coronary syndrome.*
- *Patients with successful PCI by IVUS guidance.*
- *Patients with coronary plaques (≥500 μm in thickness or 20% or more in % plaque) at ≥5 mm from the previously treated area in the same branch of coronary artery.*

TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; PCI, percutaneous coronary intervention; IVUS, intravascular ultrasound.

Table 2 Exclusion Criteria

- *Patients with bypass graft or in-stent restenosis at the site of PCI.*
- *Patients who have undergone previous PCI on the lesion site where the evaluation of coronary plaque volume is planned.*
- *Patients who have plaque in a non-culprit site on the PCI vessel and might undergo PCI during the treatment period.*
- *Patients receiving lipid-lowering drugs (statins, fibrates, probucol, nicotinic acid or cholesterol absorption inhibitors).*
- *Patients with familial hypercholesterolemia.*
- *Patients with cardiogenic shock.*
- *Patients receiving cyclosporine.*
- *Patients with any allergy to pitavastatin or atorvastatin.*
- *Patients with hepatobiliary disorders.*
- *Pregnant women, women suspected of being pregnant, or lactating women.*
- *Patients with renal disorders or undergoing dialysis.*
- *Patients who are ineligible in the opinion of the investigator.*

Abbreviation see in Table 1.

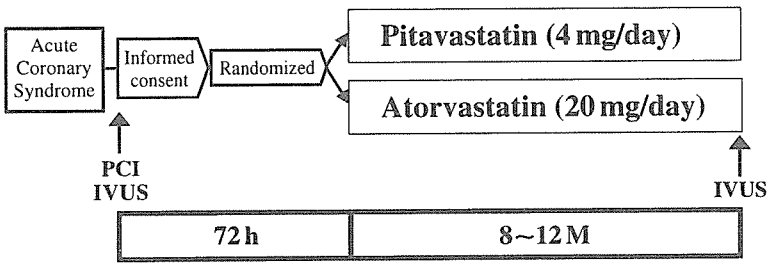

Fig 1. Protocol. PCI, percutaneous coronary intervention; IVUS, intravascular ultrasound.

sive lipid-lowering therapy with HMG-CoA reductase inhibitors was conducted in the Japanese Early Statin Treatment in Patients with Acute Coronary Syndrome (ESTABLISH) study, which demonstrated that 20 mg/day of atorvastatin reduces coronary plaque![14] This finding suggests that aggressive lipid-lowering therapy with HMG-CoA reductase inhibitors could reduce unstable coronary plaques in ACS, but this relatively small trial was conducted at a single center and its global impact remains unknown.

Pitavastatin is a HMG-CoA reductase inhibitor with powerful lipid-lowering effects that is commonly used in Japan. Its ability to lower LDL-C is comparable to that of atorvastatin and it also enhances high-density lipoprotein (HDL)-C![15,16] Moreover, pitavastatin is not metabolized by the cytochrome P450 3A4 pathway, which is the major metabolic pathway of atorvastatin![17] Recent reports have shown that pitavastatin has pleiotropic effects; it reduces the inflammatory response![18] and the generation of reactive oxygen species![19] improves endothelial function?[20] increases nitric oxide production?[21] inhibits cell adhesion?[22] attenuates smooth muscle cell contraction?[23] increases thrombomodulin expression?[24] enhances angiogenesis?[25] and promotes apolipoprotein (apo) A-I production?[26] However, its clinical effect on coronary plaque volume has not yet been investigated in Japan. Thus, the Japan Assessment of Pitavastatin and Atorvastatin in Acute Coronary Syndrome (JAPAN-ACS) trial will evaluate the effects of HMG-CoA reductase inhibitors with powerful lipid-lowering effects on coronary plaque regression in patients with ACS. The study will also assess whether or not the efficacy of pitavastatin is inferior to atorvastatin for plaque reduction.

Methods

Study Design

JAPAN-ACS will be a randomized non-blinded parallel group study. Patients who satisfy all criteria for inclusion will be enrolled after having undergone successful percutaneous coronary intervention (PCI) under IVUS guidance to treat an episode of ACS (Table 1). Patients who satisfy any of the exclusion criteria (Table 2) will not be enrolled. The included patients will give written informed consent and then be randomly allocated to receive either pitavastatin (4 mg) or atorvastatin (20 mg) daily (Fig 1). These doses were selected based on the results of the ESTABLISH study in which 20 mg/day of atorvastatin significantly reduced coronary plaque volume in patients with ACS![14]

The pitavastatin dosage of 4 mg/day causes the same LDL-C-lowering effect as 20 mg/day of atorvastatin[15,16] The randomization will be stratified by diabetes mellitus, gender and total cholesterol (TC) level. The supervising physician will administer the allocated drugs within 72 h after PCI. The participants will continue taking the allocated drugs until the end of study, or when certain endpoints are met, including death, any cardiovascular event, any adverse event or discontinued participation in the study. Investigators will follow up the participants for 8–12 months at 36 centers, and will conduct medical examinations, blood testing, IVUS and coronary angiography (CAG). Patient enrollment is planned for between November 1, 2005 and October 31, 2006 and the enrollment period may be extended if necessary. This study has been registered at clinicaltrials.gov (NCT00242944), according to the statement of the International Committee of Medical Journal Editors[27]

Endpoints

The primary endpoint will be the percent change in coronary plaque volume. Secondary endpoints include (1) absolute change from baseline in coronary plaque volume, (2) absolute and percent changes in minimal lumen diameter (MLD) and %stenosis at the site of lesion where the coronary plaque volume is evaluated, (3) absolute and percent changes in serum lipids and apolipoproteins (TC, LDL-C, triglyceride (TG), HDL-C, HDL_2-C, HDL_3-C, remnent lipoprotein-C, small dense LDL, non-HDL-C, LDL-C/HDL-C, apoA-I, apoB, apoE, apoB/apoA-I, malonyldialdehyde (MDA)-LDL, phospholipid and lipoprotein(a)), (4) absolute and percent changes in inflammatory markers (high sensitive C-reactive protein (hsCRP), pertussis toxin (PTX3)) and white blood cell count, (5) absolute and percent change in the coronary plaque area at the site of PCI, (6) absolute and percent changes in MLD and %stenosis at the site of PCI, (7) major adverse cardiovascular event (MACE; defined as cardiac death, Q or non-Q wave myocardial infarction, PCI or coronary artery bypass grafting), (8) death, and (9) any adverse incidents including changes in laboratory values.

Safety Monitoring

Safety will be evaluated by regular medical examination and laboratory tests at 1, 3, and 8–12 months after enrollment. The Event Assessment Committee will evaluate MACE and any other adverse events.

Sample Size Calculation

Because the effect of pitavastatin on coronary plaque volume has not been studied, we calculated the sample size based on the assumption that the effect of pitavastatin on the regression of coronary plaque volume is not inferior to that of atorvastatin. The % change in coronary plaque volume in patients with ACS determined by the ESTABLISH study was −13.1±12.8% (SD) in an atorvastatin group and 8.7±14.9% in a control group[14] We assumed that the mean and standard deviation of the % change in coronary plaque volume in patients receiving pitavastatin were equal to those of atorvastatin reported in the same study[14] Based on the standard deviation in the atorvastatin group, we established a non-inferiority margin of 5%. Accordingly, we calculated that groups of 150 participants with an α level of 5%, 80% power and a dropout rate of 30% would provide meaningful data.

Data Management

A data management center was established at the Research Institute for Production Development, which conducts patient enrollment, randomization and data follow-up in cooperation with the Department of Cardiovascular Medicine at Kyoto University Graduate School of Medicine. Patient information, blood samples, and IVUS images will be coded with a study identification number, and the key code for individual identification will remain blinded. Serum lipids, apolipoproteins and hsCRP will be measured at SRL Co, Ltd and PTX3 will be measured at Perseus Proteomix Co Ltd. IVUS images will be analyzed at the Division of Cardiology, Department of Medicine and Clinical Science at Yamaguchi University Graduate School of Medicine. CAG images will be analyzed at the Department of Cardiology, Juntendo University School of Medicine. An independent experienced investigator who is unaware of the patient groups will perform the quantitative IVUS analysis. Baseline and follow-up IVUS images will be reviewed together on a display, and target segments will be selected. One target segment will be determined at a non-PCI site (>5 mm proximal or distal to the PCI site) with a reproducible index side branch on the PCI vessel. The quantitative coronaty angiography analysis will also be performed by a single independent reviewer.

Statistical Analysis

An independent statistician with full access to the data will conduct all statistical analyses. The % change in coronary plaque volume after the 8–12 month study will be compared between groups by analysis of variance with adjustment for gender, presence of diabetes mellitus and TC level. The 2-sided 95% confidence interval will be calculated for the difference in drug effects ($\mu_P - \mu_A$) where μ_P and μ_A represent mean % change in coronary plaque volume in the pitavastatin and atorvastatin groups, respectively. Remaining within a 5% upper limit of confidence interval would confirm that pitavastatin is not inferior to atorvastatin. Similarly, not exceeding a 0% upper limit of confidence interval would indicate that pitavastatin is superior to atorvastatin.

General linear models will be used to assess relationships between the absolute change in coronary plaque volume and serum lipid level, and the % change in coronary plaque volume and serum lipid level at 8–12 months. Such models will also be used to evaluate relationships between inflammatory markers (hsCRP and PTX3) at 8–12 months, changes in coronary plaque volume and the effects of groups on changes in HDL-C.

One-sample t-tests will assess the absolute and % changes in serum lipid levels and inflammatory markers during the study period. General linear models will include the following covariates: drug, gender, age, history of coronary artery disease, hypertension, diabetes mellitus, family history of coronary artery disease, smoking status, LDL-C at baseline and HDL-C at baseline.

The number of adverse events will also be assessed to determine a safety profile. Subgroup and other analyses will also be conducted as necessary. Interim analyses have not been planned. The significance level will be 5% 2-sided (2.5% one-sided) and all statistical analyses will be performed using the SAS System Release 8.2 or SPLUS Version 7.0.

Ethical Considerations and the Role of Funding Source

This study will be conducted in accordance with the 'Declaration of Helsinki' established by the World Medical Association, the 'Ethical Principles in Clinical Studies' published by the Ministry of Health, Labor and Welfare of Japan and with the approval of the institutional review boards of each participating institution. The study will be explained to patients who meet the criteria for inclusion and written informed consent to participate will be obtained.

The Japan Heart Foundation supports the concept for this study and the funding source will not play any role in the design, performance, or reporting of the study, or in the decision to submit the findings for publication.

Conclusion

The ESTABLISH study has shown that aggressive lipid-lowering therapy with 20 mg/day of atorvastatin reduces coronary plaque![4] The JAPAN-ACS study will determine if the results from the ESTABLISH study are reproducible at multiple centers using other HMG-CoA reductase inhibitors and should also confirm the utility of aggressive lipid-lowering therapy in patients with ACS in Japan.

In 2005 Tani et al found that plaque regression correlated with a decrease in MDA-LDL and an increase in HDL-C levels in 75 patients with coronary artery disease patients who received pravastatin[28] In the same year Satoh et al indicated that fasting triglyceride is a significant risk factor for coronary artery disease among middle-aged Japanese men[29] and Hong et al demonstrated that the hsCRP level is associated with neointimal hyperplasia and restenosis after successful coronary artery stenting[30] Evaluation of the effect of changes in serum lipids, including LDL-C, HDL-C, TG and MDA-LDL, and inflammatory markers, including hsCRP, on coronary plaque will be valuable in the JAPAN-ACS study and should clarify the mechanisms of coronary plaque regression.

In conclusion, the JAPAN-ACS study will investigate a wide range of endpoints to determine the effect of aggressive lipid-lowering therapy, the utility of 2 HMG-CoA reductase inhibitors with powerful lipid-lowering effects, and the mechanisms of coronary plaque regression in Japanese patients with ACS.

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Appendix 1

Research Group Organization

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IVUS Core Laboratory: Division of Cardiology, Department of Medicine and Clinical Science, Yamaguchi University Graduate School of Medicine.

QCA Core Laboratory: Department of Cardiology, Juntendo University School of Medicine.

Data Center: Research Institute for Production Development, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine.

Study Statistician: Takeshi Morimoto (Center for Medical Education, Kyoto University Graduate School of Medicine).

Event Assessment Committee: Tetsu Yamaguchi (Cardiovascular Center, Toranomon Hospital); Satoshi Saito (Division of Cardiovascular Disease, Department of Medicine, Nihon University School of Medicine); Kazuo Kimura (Division of Cardiology, Yokohama City University Medical Center).

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ORIGINAL ARTICLE

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Aging and transmitral flow pattern in patients with systemic hypertension

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Abstract
Purpose. Currently, the transmitral flow (TMF) pattern is routinely recorded as the first step in the assessment of left ventricular diastolic function. In young, healthy subjects, it is known that the early diastolic flow (E wave) of TMF is larger than the late diastolic flow (A wave). The E/A ratio then gradually decreases with age. This change in the pattern of TMF can be expected to occur earlier in patients with systemic hypertension than in healthy subjects. However, data pertaining to this matter are limited for Japanese patients. The purpose of this study was to investigate the changing pattern of TMF with age in Japanese patients with systemic hypertension.
Methods. A database of echocardiographic examination reports was surveyed. A total of 553 patients with systemic hypertension (HT group) and 394 patients without hypertension or organic heart disease (control group) were included in this study. The patients were subdivided according to age, after which the E/A ratio was compared for different patient categories and age groups.
Results. The E/A ratio gradually decreased with age in the control group, and the mean value of E/A was <1 in the sixth decade. On the other hand, the E/A ratio rapidly decreased and was <1 in the fifth decade in the HT group.
Conclusion. In patients in the HT group, the E/A ratio decreased about a decade earlier compared with patients in the control group.

Keywords hypertension · Doppler echocardiography · transmitral flow · aging

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Introduction

Previous studies using Doppler echocardiography have demonstrated that transmitral flow (TMF) reflects left ventricular diastolic function.[1,2] In recent years, it has become widely accepted that not only systolic but also diastolic function is important when evaluating cardiac function. Given this, TMF is now routinely measured as the first step in the evaluation of left ventricular diastolic function using echocardiography during routine clinical practice.[3,4] When TMF is evaluated in young, healthy individuals, the E wave (E) is higher than the A wave (A), but the E/A ratio decreases with age, eventually leading to a situation where E is lower than A. In hypertensive patients, the contribution of atrial contraction to left ventricular filling is greater than in normotensive patients because of reduced left ventricular compliance and other factors. As a result, the decrease in E/A tends to begin at a younger age in hypertensive patients than in normotensive individuals. However, few data are available concerning this hypertension-associated change in E/A in Japanese patients. The present study was undertaken to investigate changes in the E/A ratio of TMF associated with aging in hypertensive Japanese patients.

Subjects and methods

Subjects in the present study were 553 patients diagnosed with hypertension who underwent echocardiography in the echocardiographic laboratory of our hospital (the hypertension [HT] group, composed of 290 men and 263 women, with a mean age of 66.0 ± 11.2 years). From the echocardiographic database at our hospital, we extracted data on basic parameters for these patients (left ventricular end-diastolic diameter, end-systolic diameter, aortic root