

Carvedilol reduces the severity of atherosclerosis in apolipoprotein E-deficient mice via reducing superoxide production

Kana Shimada¹, Emi Hirano², Takeshi Kimura¹, Masatoshi Fujita² and Chiharu Kishimoto¹

¹Department of Cardiovascular Medicine; ²Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Corresponding author: Chiharu Kishimoto. Email: kkishi@kuhp.kyoto-u.ac.jp

Abstract

It has been shown that oxidative stress may play an important role in the development of atherosclerosis, and carvedilol has the capacity of reducing oxidative stress. Accordingly, we assessed the hypothesis that carvedilol may reduce the severity of atherosclerosis in apolipoprotein E (apoE)-deficient mice in addition to its hemodynamic effects. Atherosclerosis was induced in apoE-deficient mice fed a high-fat diet containing 0.3% cholesterol. Mice were orally treated with propranolol (30 mg/kg/day), metoprolol (75 mg/kg/day) and carvedilol (10 mg/kg/day) over eight weeks (each group $n = 7-9$). Fatty streak plaque developed in apoE-deficient mice, and was suppressed in mice treated with all three drugs. The accumulation of macrophages and expression of CD4⁺ and CD8⁺ cells in the lesions were decreased by the treatment of the drugs, of which carvedilol was the most effective. In addition, carvedilol reduced superoxide production in aortic walls detected by ethidium staining. There were no significant changes in blood pressure among the study groups. The heart rates in the treated groups were decreased by 4%–12% compared with the control group, with carvedilol yielding the highest suppression of heart rate. The β -blocker treatment did not significantly modify the serum lipid profiles. Carvedilol may suppress atherosclerosis via reducing superoxide production, in addition to the hemodynamic modifications in this animal model.

Keywords: atherosclerosis, β -blocker, free radical, carvedilol, superoxide

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Introduction

Inflammation and many kind of stresses, especially oxidative stress and free radicals, may be considered to be key factors for the development of atherosclerosis.^{1,2} For example, angiotensin II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the type 1 receptor, which results in the production of superoxide anion.^{3,4} Thus, angiotensin II has deleterious effects on the vessel walls. In addition, the adhesion of mononuclear cells to vascular endothelial cells and oxidation of low density lipoprotein are important mechanisms in the initial step of atherosclerosis.¹⁻⁴

Carvedilol, a multiple-functional neurohormonal antagonist, has been shown to inhibit the above-mentioned phenomena in atherosclerosis and to provide a greater benefit than traditional β -blockers in chronic heart failure because of its antioxidant actions that synergize with its non-specific β - and α_1 -blocking effects.⁵⁻⁸ When carvedilol

and metoprolol were recently compared in clinical trials for heart failure, each showed beneficial β -blocking effects and decreased serum thiobarbituric acid reactive substance levels.⁶⁻⁹ However, it was demonstrated that the superior cardioprotection of carvedilol to metoprolol and propranolol is induced by its anti-inflammatory action in ischemia and reperfusion models.^{10,11} Nevertheless, it largely remains to be determined how carvedilol protects against experimental atherosclerosis.

In the present study using apolipoprotein E (apoE)-deficient mice, we have provided evidence for suppressive effects of atherosclerosis by carvedilol, focusing upon inhibitory effects for superoxide. In addition, to determine if the effects of carvedilol are attributable solely to the hemodynamic effect or if antioxidant property may also be involved, the effects of propranolol and metoprolol were also assessed in the same animal model.

Materials and methods

Experimental atherosclerosis

The apoE-deficient 129ola×C57BL/6 hybrid mice were generous gifts of Dr Edward M Rubin (University of California, Berkeley, CA, USA). These mice were mated with C57BL/6 mice to produce F₁ hybrids. The F₁ apoE^{+/-} mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apoE-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-h light–dark cycle with free access to food and water.

After being weaned at four weeks of age, mice were fed a normal chow diet (oriental yeast) until six weeks of age, when the animals were switched to a high-fat diet containing 20% fat and 0.3% cholesterol as previously described.^{12,13}

The experimental protocols were approved by the Institutional Ethics Committee for Animal Experiments of Kyoto University.

Treatment protocol

At six weeks of age, mice were orally treated via their drinking water with saline (control group, *n* = 9), 30 mg/kg/day of propranolol (propranolol group, *n* = 9), 75 mg/kg/day of metoprolol (metoprolol group, *n* = 8) and 10 mg/kg/day of carvedilol (carvedilol group, *n* = 7) for eight weeks. The dosage of the drug was determined from the previous reports and the preliminary study.⁷ The blood pressure and heart rate were periodically determined by the tail-cuff method using a photoelectric cuff detection system (Softron BP-98A, Tokyo, Japan) as previously described.⁷ At 14 weeks, the mice were killed by puncture of the ventricles under ether anesthesia. The organs were weighed, and the ratio of heart weight to body weight was calculated.

Tissue processing

Mice were killed by bleeding with puncture of the ventricles. The vasculature was perfused with sterile phosphate-buffered saline and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in optimal cutting temperature embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus eight slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin. The oil-red-O-stained sections were analyzed at a magnification of ×10, as previously described.^{9,10} The image was captured directly from the RGB camera attached to the light microscope and displayed on a microcomputer to quantify the cross-sectional surface area of the lesion and the cross-sectional surface area of the vessel. The fractional area of the lesion was calculated by dividing the whole vessel

area, including the lumen intima, media and adventitia, as previously described.^{12,13}

Superoxide production

To evaluate *in situ* superoxide production from the aorta, unfixed, frozen cross-sections of the specimens were stained with dihydroethidium (DHE; Molecular Probes, Tokyo, Japan) according to the previously validated method.^{14–16} In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10 μm thick sections, and incubated with 10 μmol/L DHE at 37°C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from aortas, fluorescence (intensity × area) was measured using a high-power image.

Immunohistochemistry

Anti-macrophage (anti-Mφ, M 3/84, 1:400; BD Biosciences–Pharmingen, San Diego, CA, USA), anti-CD4 (GK1.5; 1:50; BD Biosciences–Pharmingen) and anti-CD8 (53–6.7, 1:50; BD Biosciences–Pharmingen) antibodies were applied to acetone-fixed cryosections of aortic roots. After being washed, the sections were then exposed to second antibodies (horseradish peroxidase-conjugated or fluorescein isothiocyanate-conjugated antibodies), and the antibody binding was visualized with the so-called diaminobenzidine method or under a fluorescence microscope. Sections were counterstained with methyl green or Mayer's hematoxylin if necessary.

The positive-staining cells were counted in several fields at ×400 magnification (within a 1 mm² grid), and the percentage of the positive-staining cells/total infiltrating cells were calculated, as previously described.^{12,17}

Lipid measurement

Serum was separated by centrifugation and stored at –80°C. Serum total cholesterol and triglyceride levels were measured by the enzymatic method.¹³

Statistical analysis

Values were expressed as means ± standard deviation (SD). Statistical analysis of the data was performed by one-way analysis of variance, followed by the Fisher protected least-significant difference test. A value of *P* < 0.05 was considered statistically significant.

Results

Organ weights

Heart weight-to-body weight ratios were not significantly different among the study groups (Table 1).

Table 1 Hemodynamics

	<i>n</i>	HR (beats/min)	SBP (mmHg)	DBP (mmHg)	HW/BW (mg/g)
Control	9	644 $\pm$ 45	106 $\pm$ 8	67 $\pm$ 7	6.50 $\pm$ 0.35
Propranolol	9	581 $\pm$ 30*	106 $\pm$ 12	71 $\pm$ 11	5.88 $\pm$ 0.45
Metoprolol	8	616 $\pm$ 56	108 $\pm$ 13	65 $\pm$ 8	5.95 $\pm$ 0.82
Carvedilol	7	567 $\pm$ 41*	118 $\pm$ 11	70 $\pm$ 11	6.84 $\pm$ 0.60

Values are in mean $\pm$ SD

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; HW, heart weight; BW, body weight

There were no statistical differences among the three β -blockers for each parameter

* $P > 0.05$ versus control

Atherosclerotic lesions

ApoE-deficient mice were kept on a cholesterol-rich diet for eight weeks to induce fatty streak formation (Table 2, Figure 1). The surface areas covered by fatty streak lesions were quantified in oil-red-O-stained samples, and specimens from the control group were compared with those of β -blocker groups. Controls developed extensive lesions in the root of the aorta. In mice treated with β -blockers, the fraction area of lesions was reduced compared with the controls. Carvedilol suppressed the development of atherosclerosis the most of all the drugs.

Superoxide production

Ethidium fluorescence in the carvedilol group was significantly weaker than that in the control group as, specifically, the brightness of DHE-stained lesions from carvedilol treated mice was less than that from control mice (Table 2, Figure 1). Metoprolol and propranolol treatment failed to suppress the superoxide production significantly compared with the control. By consideration of a previous report¹⁵ and the current study, it may be that the origin of superoxide might be mainly from macrophages by their adjacent position, and partly from endothelial cells.

Macrophage and T-cell expression

The accumulation of macrophages and expression of CD4⁺ and CD8⁺ T-cells in the lesions were decreased by the treatment of the drugs compared with the control (Table 2,

Figure 2). There were no statistical differences among the three β -blockers for each parameter.

Hemodynamics

The heart rates in the treated groups were significantly decreased by 4–12% compared with the control group, with carvedilol producing the greatest suppression of heart rate (Table 1). There were no significant changes in blood pressure among the study groups. The results suggested that the three drugs have almost the same β -blocking property.

Lipid profiles

The drugs did not significantly modify the serum lipid profiles (Table 3).

Discussion

The present findings clearly demonstrated that the three β -blockers showed almost the same extent of negative chronotropic effects on apoE-deficient mice, that metoprolol and propranolol slightly reduced the severity of the disease, and that carvedilol markedly reduced the severity of atherosclerosis. The present results also showed that the superior cardioprotection of carvedilol over metoprolol and propranolol might be due to the suppression of tissue superoxide production.

There is increasing evidence to support the critical role of both free radicals and oxidative stress in the development of atherosclerosis.^{1–4,18–20} We have already demonstrated that MCI-186, a free radical scavenger and olmesartan, an angiotensin type 1 receptor antagonist, suppresses the severity of experimental atherosclerosis.¹³ Indeed, angiotensin stimulation has been reported to produce free radicals from various cells.²¹ Free radicals from vessel walls are thought to play critical roles in atherogenesis. It is considered that free radicals induce the expression of adhesion molecules and chemokines, accelerate atherosclerotic plaque formation, increase matrix metalloprotease production and finally cause vulnerable plaques.²² Superoxide anion is one of free radical members.

Table 2 Lesion area

	<i>n</i>	Lesion area, μm^2 (%)	Positive cells (%) [†]			Ethidium fluorescence (units) [‡]
			Macrophage	CD4	CD8	
Control	9	110.1 ± 59.5×10 ³ (14.1 ± 7.6)	25.3 ± 5.3	27.0 ± 9.6	12.6 ± 4.2	1.00 ± 0.09
Propranolol	9	65.2 ± 30.7×10 ³ (8.4 ± 3.9)*	15.5 ± 4.8*	20.4 ± 8.3	8.5 ± 4.4	0.90 ± 0.08
Metoprolol	8	55.8 ± 17.5×10 ³ (7.2 ± 2.2)**	16.2 ± 3.5*	18.4 ± 5.5	7.7 ± 3.9	0.79 ± 0.12
Carvedilol	7	44.9 ± 15.6×10 ³ (5.7 ± 2.0)**	4.2 ± 4.0**	8.8 ± 3.9**	5.7 ± 4.2**	0.64 ± 0.09*

Values are in mean $\pm$ SD

There were no statistical differences among the three β -blockers for each parameter

[†]Data were obtained by the number of positively-stained cells by all the counterstained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at $\times 400$

[‡]For quantification of ethidium fluorescence, fluorescence (intensity $\times$ area) was measured using a high-power-image. Three to five microscope fields were analyzed

* $P < 0.05$, ** $P < 0.01$ versus control

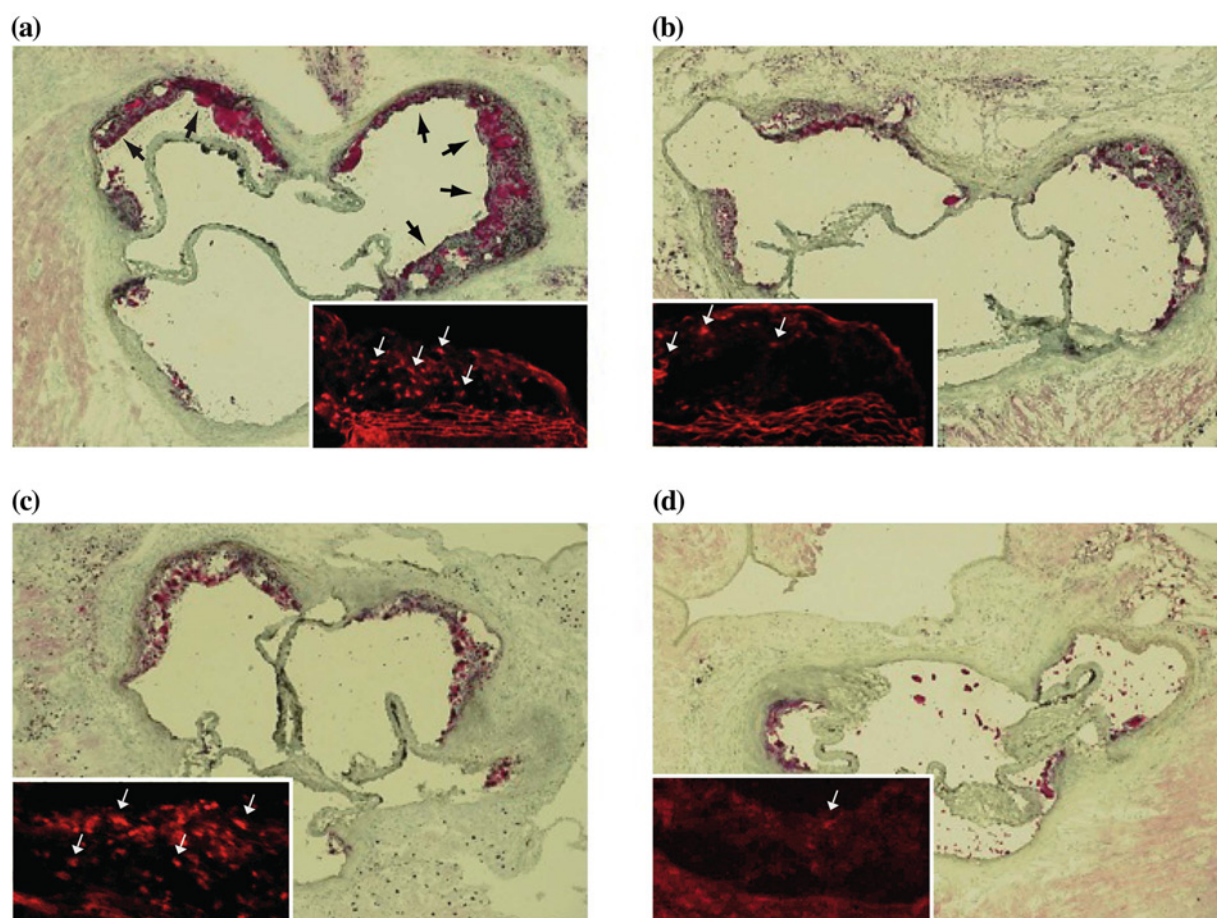


Figure 1 Effects of β -blockers on atherosclerotic lesions and superoxide production. The lesions in the drug-treated mice (b, c, d) were smaller and covered less inner circumference of the aortic root than those of the control mouse (arrows) (a). The intensity of ethidium fluorescence (white arrows) was less than that in the control group (a), suggesting less amount of superoxide production and the decrease of oxidative stress by the treatment. Carvedilol (d) was most effective. Inset boxes show magnified sample for dihydroethidium (DHE) staining. a, control; b, propranolol; c, metoprolol; d, carvedilol. Oil-red-O stain ($\times 40$), DHE stain ($\times 100$). (A color version of this figure is available in the online journal)

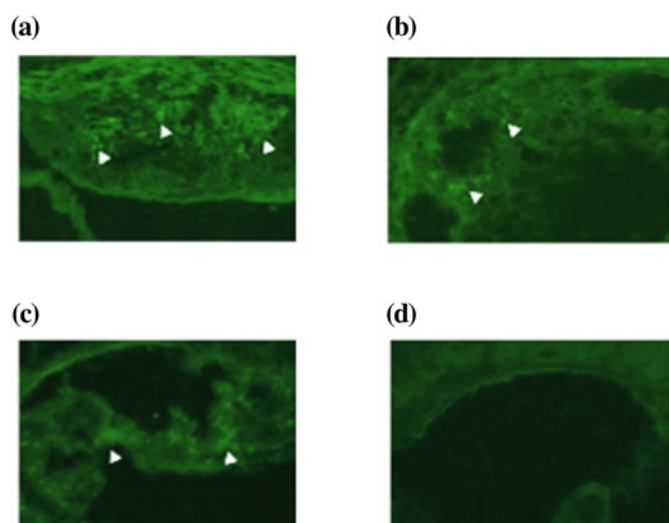


Figure 2 Effects of β -blockers on macrophage expression. The expression of macrophages (white arrowheads) in the drug-treated groups (b, c, d) was less than that in the control group (a). a, control; b, propranolol; c, metoprolol; d, carvedilol. (A color version of this figure is available in the online journal)

Table 3 Lipid profiles

	<i>n</i>	TC (mg/dL)	TG (mg/dL)
Control	9	1382 $\pm$ 232	40 $\pm$ 18
Propranolol	9	1504 $\pm$ 265	33 $\pm$ 14
Metoprolol	8	1260 $\pm$ 177	55 $\pm$ 50
Carvedilol	7	1405 $\pm$ 349	62 $\pm$ 35

Values are in mean $\pm$ SD

TC, total cholesterol; TG, triglyceride

Carvedilol blocks three (β_1 , β_2 and α_1) adrenergic receptors and therefore possesses a more comprehensive sympatholytic action than other β -blockers.^{8-11,23-27} It is unlikely that the atheroprotective effects of carvedilol demonstrated in this study are achieved by β -receptor blocking action *per se*, because of the presence of such a protection by the metoprolol and propranolol treatments. Although the present study did not precisely elucidate the role of α_1 -receptor blocking action *per se*, it is also unlikely that α_1 blockade is predominantly involved in the atheroprotection of carvedilol, because the vasodilatory effect of carvedilol is no longer prominent during chronic treatment.⁷

Recently, another new aspect of carvedilol regarding the inhibitory action against spontaneous Ca^{2+} release of cardiac ryanodine receptor in heart failure has been reported.²⁸ Another interesting finding was the concomitant antioxidant effect of carvedilol. From the previous reports, the antioxidant activity of the drug may be due to the carbazole moiety chemically, the direct inhibition for electron adduction, prevention of lipid peroxidation of cell membranes and inhibition of ROS generation.^{5–7}

It is well known that heart rate reduction may play a protective role against the development of atherosclerosis.^{29,30} Concerning the relative doses of the three β -blockers used in the present study, we compared the drug effects at similar blood pressure levels. Although the three β -blockers presented almost the same negative chronotropic action, carvedilol exhibited more potent suppressive effect against heart rate than metoprolol.³¹ The antiatherosclerotic effects of β -blockers in animal models were already reported in part, which were due to hemodynamic modification^{32,33} as well as antioxidative effects.^{24,34} In the present study, we clearly demonstrated that carvedilol suppressed not only the tissue superoxide production but macrophage accumulation with T-cell expression in aortic walls. Immunohistochemical study showed that macrophage accumulation and the intensity of DHE staining in the aortic wall were very close. It has already been established that the degree of instability of plaques correlates with the amount of macrophages and T-cell expression.²² Thus, the decrease of the intensity of macrophage and T-cell expression in aortic walls may reflect the decrease of instability of plaques by carvedilol treatment.

In conclusion, carvedilol treatment protects against experimental atherosclerosis in apoE-deficient mice by the suppression of superoxide production in the atherosclerotic lesions. The superior antiatherosclerotic effects of carvedilol to metoprolol and propranolol may be attributed to the suppression of tissue superoxide production in addition to the hemodynamic modifications.

Author contributions: KS and EH performed the experiments. TK and MF supervised the protocol. CK designated the studies and prepared the paper.

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