

**Carvedilol Reduces the Severity of Atherosclerosis in Apolipoprotein E-Deficient Mice
via Reducing Superoxide Production**

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Short title: β -blockers in apo E-deficient mice

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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 (apo) E-deficient mice in addition to its hemodynamic effects. Atherosclerosis was induced in apo E-deficient mice fed a high fat diet containing 0.3 % cholesterol. Mice were orally treated with propranolol (30mg/kg/day), metoprolol (75mg/kg/day), and carvedilol (10mg/kg/day) over 8 weeks (each group n=7~9). Fatty streak plaque developed in apo E-deficient mice, and was suppressed in mice treated with all the 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 % to 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 -blockers; free radicals; carvedilol; superoxide

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. Carvedilol, a multiple-functional neurohormonal antagonist, has been shown to provide a greater benefit than traditional β -blockers in chronic heart failure because of its antioxidant actions that synergize with its nonspecific β - and α_1 -blocking effects⁵. 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 levels⁶. 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^{7,8}. Nevertheless, it largely remains to be determined how carvedilol protects against experimental atherosclerosis.

In the present study using apolipoprotein E-deficient mice, we have provided evidence for suppressive effects of atherosclerosis by carvedilol, focusing upon inhibitory effects for superoxide. In addition, to determine whether the effects of carvedilol are attributable solely to the hemodynamic effect or whether 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 apolipoprotein E (apo E)-deficient 129ola $\times$ C57BL/6 hybrid mice were generous

gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F₁ hybrids. The F₁ apo E^{+/-} mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-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-hour light-dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when the animals were switched to a high fat diet containing 20% fat and 0.3% cholesterol as previously described.^{9, 10}

The experimental protocols were approved by the institutional ethics committee for animal experiments of Kyoto University.

Treatment Protocol

At 6 weeks of age, mice were orally treated via their drinking water with saline (control group, n=9), 30mg/kg/day of propranolol (propranolol group, n=9), 75mg/kg/day of metoprolol (metoprolol group, n=8), and 10 mg/kg/day of carvedilol (carvedilol group, n=7) for 8 weeks. The dosage of the drug was determined from the previous reports and the preliminary study.¹¹ 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.¹¹ At 14 weeks the mice were killed by puncture of the ventricle 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 ventricle. The vasculature was

perfused with sterile phosphate buffered saline (PBS) and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT 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 μ m thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections were immersed for 15 sec 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 x10, 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.^{9,10}

Superoxide Production

To evaluate in situ superoxide production from the aorta, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method.¹²⁻¹⁴ 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- μ m thick sections, and incubated with 10 μ M 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 $\times$ area)

was measured using a high-power image.

Immunohistochemistry

Anti-macrophage (anti-M ϕ , M 3/84, 1:400, PharMigen), anti-CD4(GK1.5, 1:50, PharMigen), and anti-CD8 (53-6.7, 1:50, PharMigen) 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 hemotoxylin if necessary.

The positive staining cells were counted in several fields at $\times 400$ magnification (within a 1-mm² grid), and the percentages of the positive-staining cells / total infiltrating cells were calculated, as previously described.^{9,15}

Lipid Measurement

Serum was separated by centrifugation and stored at -80°C . Serum total cholesterol (TC) and triglyceride (TG) levels were measured.

Statistical Analysis

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

RESULTS

Organ Weights (Table 1)

Heart weight to body weight ratios were not significantly different among the study

groups.

Atherosclerotic Lesions (Table 2, Figure 1)

Apo E-deficient mice were kept on a cholesterol-rich diet for 8 weeks to induce fatty streak formation. 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 (Table 2, Figure 1)

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. Metoprolol and propranolol treatment failed to suppress the superoxide production significantly compared with the control. By consideration with the previous report and the current study¹³, 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 (Table 2, Figure 2)

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.

Hemodynamics (Table 1)

The heart rates in the treated groups were significantly decreased by 4% to 12% compared with the control group with carvedilol producing the greatest suppression of heart rate. 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 (Table 3)

The drugs did not significantly modify the serum lipid profiles.

DISCUSSION

The present findings clearly demonstrated that the three β -blockers showed almost the same extent of negative chronotropic effects upon 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 atherosclerosis¹⁶⁻¹⁹. 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.¹⁰ Indeed, angiotensin stimulation has been reported to produce free radicals from various cells.²⁰ 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.²¹ Superoxide anion is one of free radical members.

Carvedilol blocks three (β_1 , β_2 , α_1) adrenergic receptors and therefore possesses a more comprehensive sympatholytic action than other β -blockers.^{22,23} It is unlikely that the atheroprotective effects of carvedilol demonstrated in this study is 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.^{11,24} 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.²⁵

It is well known that heart rate reduction may play a protective role against the development of atherosclerosis.^{26,27} Concerning the relative doses of the three β -blockers used in the present study, we compare the drug effects at similar blood pressure levels. The three β -blockers presented almost the same negative chronotropic action. The anti-atherosclerotic effects of β -blockers in animal models were already reported in part, which were due to hemodynamic modification^{28,29} as well as anti-oxidative effects.^{30,31} 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 unstablity of plaques correlates with the amount of macrophages and T cell expression.²¹ Thus, the decrease of the intensity of macrophage and T cell expression in aortic walls may reflect the decrease of unstablity of plaques by carvedilol treatment.

In conclusion, carvedilol treatment protects against experimental atherosclerosis in apo E-deficient mice by the suppression of superoxide production in the atherosclerotic lesions. The superior anti-atherosclerotic 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 experiments. TK and MF supervised the protocol. CK designated the studies and prepared the paper.

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FIGURE LEGENDS

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 control group (A), suggesting less amount of superoxide production and the decrease of oxidative stress by the treatment. Carvedilol (D) was most effective. Insets boxes show magnified sample for DHE staining.

A= Control

B= Propranolol

C= Metoprolol

D= Carvedilol

Oil-red-O stain($\times 40$)

DHE stain($\times 100$)

Figure 2. Effects of β -blockers on macrophage expression

The expression of macrophages(M ϕ) (white arrow-heads) in the drug-treated groups (B,C,D) was less than that in control group(A).

A= Control

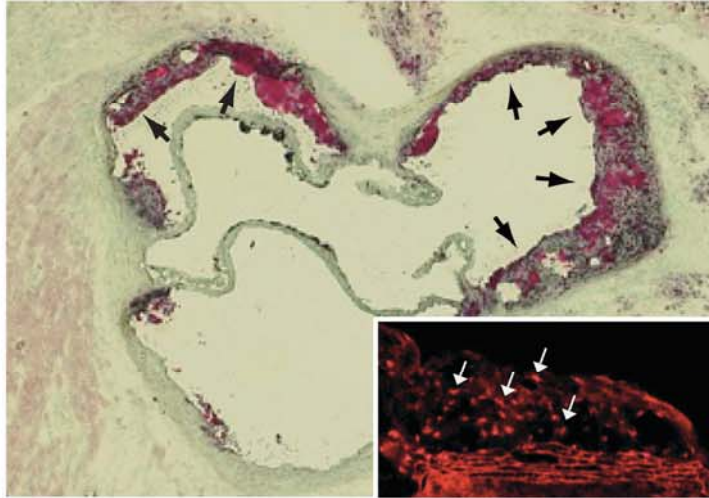
B= Propranolol

C= Metoprolol

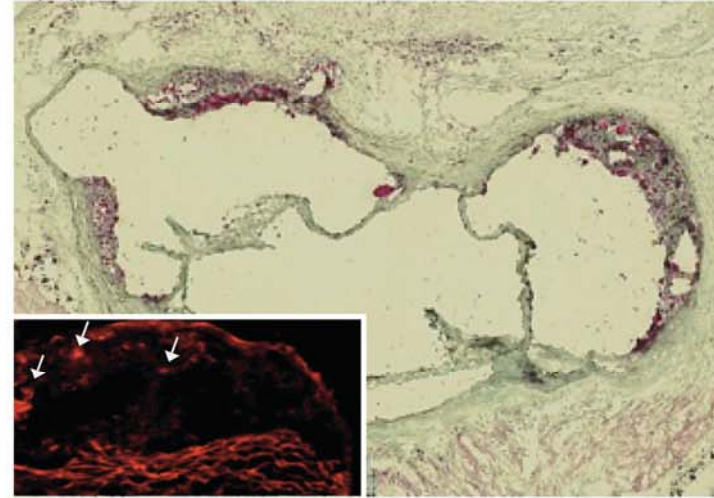
D= Carvedilol

Figure 1

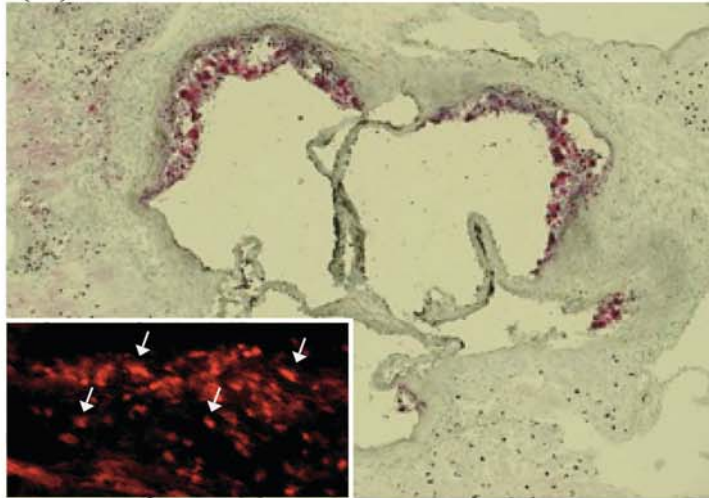
(A)



(B)



(C)



(D)

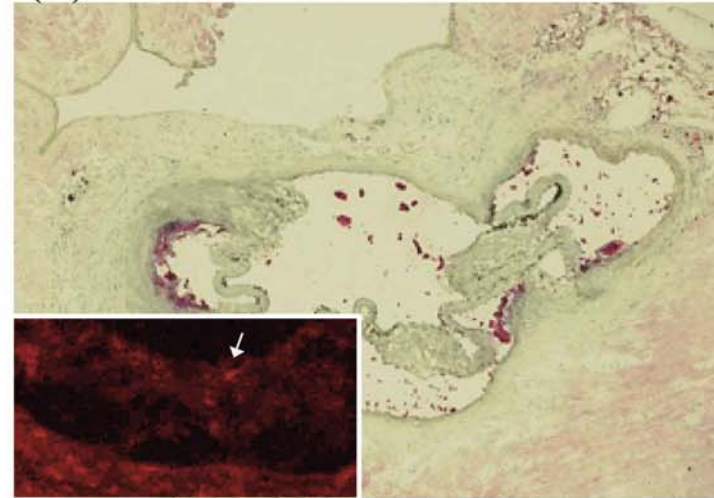


Figure 2

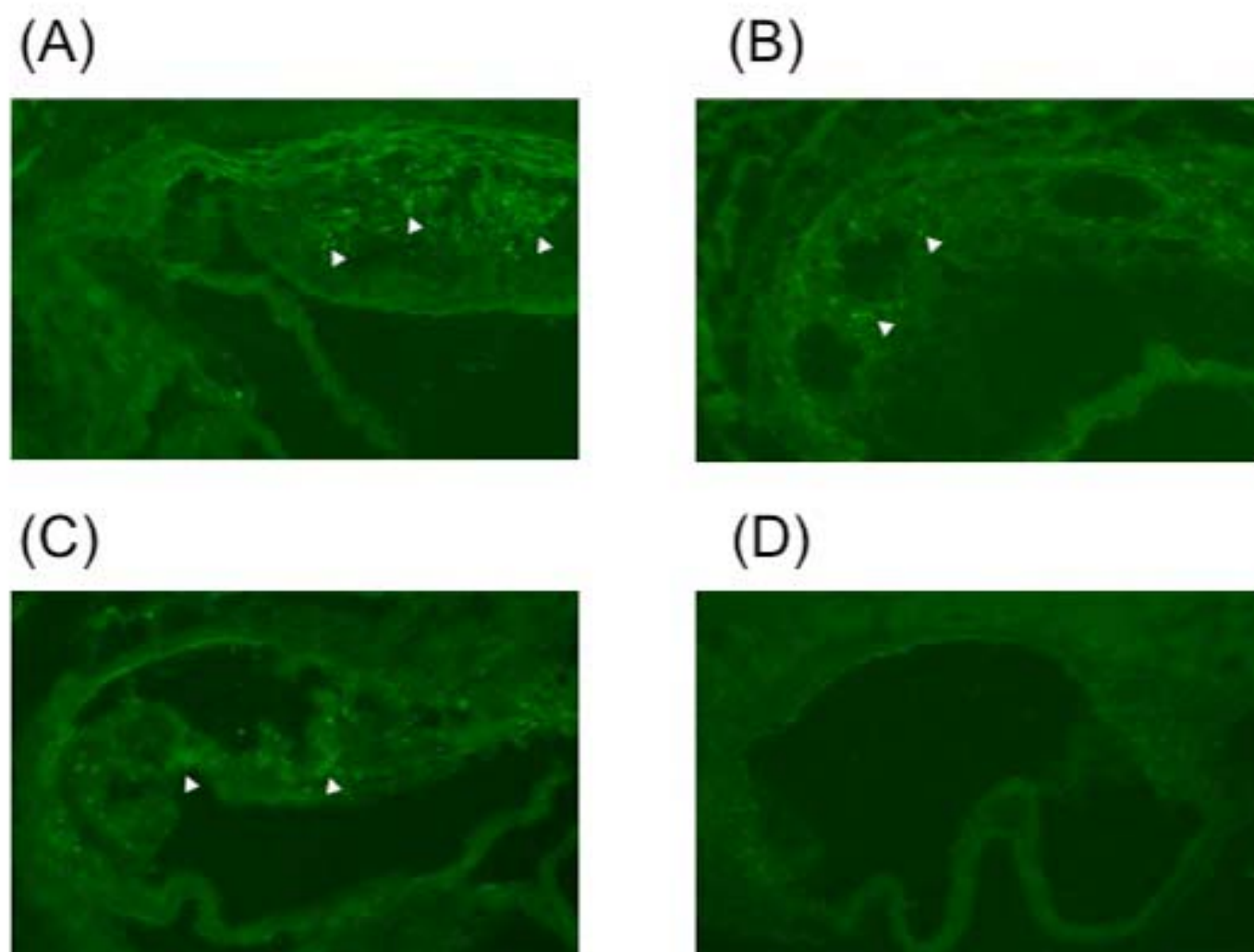


Table 1. Hemodynamics

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

(Mean±SD)

*P<0.05 vs Control.

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

There were no statistical differences among the 3 β -blockers for each parameter.

Table 2. Lesion Area

	n	Lesion area, μm^2 (%)	Positive cells (%) ⁺			Ethidium fluoescence (units) [§]
			M ϕ	CD4	CD8	
Control	9	110.1 $\pm$ 59.5 $\times 10^3$ (14.1 $\pm$ 7.6)	25.3 $\pm$ 5.3	27.0 $\pm$ 9.6	12.6 $\pm$ 4.2	1.00 $\pm$ 0.09
Propranolol	9	65.2 $\pm$ 30.7 $\times 10^3$ (8.4 $\pm$ 3.9)*	15.5 $\pm$ 4.8*	20.4 $\pm$ 8.3	8.5 $\pm$ 4.4	0.90 $\pm$ 0.08
Metoprolol	8	55.8 $\pm$ 17.5 $\times 10^3$ (7.2 $\pm$ 2.2)**	16.2 $\pm$ 3.5*	18.4 $\pm$ 5.5	7.7 $\pm$ 3.9	0.79 $\pm$ 0.12
Carvedilol	7	44.9 $\pm$ 15.6 $\times 10^3$ (5.7 $\pm$ 2.0)**	4.2 $\pm$ 4.0**	8.8 $\pm$ 3.9**	5.7 $\pm$ 4.2**	0.64 $\pm$ 0.09*

(Mean $\pm$ SD)

*P<0.05,**P<0.01 vs Control.

M ϕ ,Macrophage.

⁺Data were obtained by the number of positively stained cells by all the counterstained cells inside the internal elastic lamina. Three to 5 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 5 microscope fields were analyzed.

There were no statistical differences among the 3 β -blockers for each parameter.

Table 3. Lipid Profiles

	n	TC mg/dl	TG mg/dl
Control	9	1382±232	40±18
Propranolol	9	1504±265	33±14
Metoprolol	8	1260±177	55±50
Carvedilol	7	1405±349	62±35

(Mean±SD)

TC, total cholesterol;TG, triglyceride.