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Preventive Effects of Carvedilol on Nitrate Tolerance—A Randomized, Double-blind, Placebo-controlled Comparative Study Between Carvedilol and Arotinolol

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Objectives. **This study was designed to compare the preventive effect of nitrate tolerance between carvedilol with antioxidant properties and arotinolol without antioxidant properties.**

Background. **The attenuation of cyclic guanosine monophosphate (cGMP) production due to inactivation of guanylate cyclase by increased superoxide has been reported as a mechanism of nitrate tolerance. Carvedilol has been known to combine alpha- and beta-blockade with antioxidant properties.**

Methods. **To evaluate the preventive effect of nitrate tolerance, 24 patients with untreated hypertension were randomized to receive either carvedilol (10 mg twice a day [carvedilol group, n = 8]), arotinolol (10 mg twice a day [arotinolol group, n = 8]), or placebo (placebo group, n = 8). Vasodilatory response to nitroglycerin (NTG) was assessed with forearm plethysmography by measuring the change in forearm blood flow (FBF) before and 5 min after sublingual administration of 0.3 mg NTG, and at the same time blood samples were taken from veins on the opposite side to measure platelet cGMP. Plethysmography and blood sampling were obtained serially at baseline (day 0), 3 days after carvedilol, arotinolol or placebo administration (day 3) and 3 days after application of a 20 mg/24 h NTG tape concomitantly with carvedilol, arotinolol or placebo (day 6).**

Results. **There was no significant difference in the response of FBF (%FBF) and cGMP (%cGMP) to sublingual administration of NTG on days 0 and 3 among the three groups. On day 6, %FBF and %cGMP were significantly lower in the arotinolol group and the placebo group than days 0 and 3, but these parameters in the carvedilol group were maintained.**

Conclusions. **The results indicated that carvedilol with antioxidant properties may prevent the development of nitrate tolerance during continuous therapy with NTG compared with arotinolol without antioxidant properties.**

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Organic nitrates are widely used in cardiovascular medicine, but their continuous administration can result in the rapid development of tolerance (1–3). The underlying mechanisms responsible for nitrate tolerance probably are multifactorial (4) and may include neurohormonal counterregulatory mechanisms (5), intravascular volume expansion (6) or intrinsic abnormalities such as desensitization of the target enzyme guanylate cyclase (7) or a decrease in nitroglycerin (NTG) biotransformation (8). Recent experimental data have demonstrated that nitrate tolerance is associated with increased vascular superoxide anion production (9). Therefore, antioxidant may be effective in the prevention of the development of nitrate tolerance. Carvedilol has recently been reported as having a beneficial effect on the survival of patients with heart failure (10–12), and carvedilol has been known to combine alpha- and beta-blockade with antioxidant properties (13–16). On the other hand, arotinolol is an alpha- and beta-blocker without antioxidant properties which is available in Japan and the ratio of alpha to beta is similar to carvedilol (alpha:beta = 1:8) (17,18). Therefore, to investigate the effect of carvedilol on nitrate tolerance, we compared the vasodilatory response and the change of platelet cyclic guanosine monophosphate (cGMP) level during continuous administration of NTG between carvedilol with antioxidant properties and arotinolol without antioxidant properties in a randomized, double-blind, placebo-controlled study.

Methods

Patient population. The study population was composed of 24 patients with untreated hypertension (18 men and 6 women) ranging in age from 44 to 69 years, who had no history of renal or cardiac disease and who had normal findings on physical examination, serum electrolyte, cholesterol, blood urea nitrogen, creatinine levels and urine analysis. No subjects were taking medications at the time of the study. Patients' characteristics are shown in Table 1.

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Abbreviations and Acronyms

cGMP = cyclic guanosine monophosphate
FBF = forearm blood flow
NTG = nitroglycerin

Study protocol (Figure 1). We measured the vasodilator response to sublingual administration of NTG by plethysmography and platelet cGMP at baseline (day 0), after 3 days of administration of carvedilol, arotinolol or placebo (day 3) and after 3 days following the application of a 20 mg/24 h NTG tape concomitantly with carvedilol, arotinolol or placebo (day 6). An 18-gauge heparin lock was inserted in the contralateral forearm to allow venous blood sampling for measurements of the platelet cGMP level.

The baseline forearm blood flow (FBF) was recorded by plethysmography before and 5 min after administration of 0.3 mg of sublingual NTG on day 0. Subjects were then randomized by a double-blind parallel design to receive either carvedilol 10 mg twice a day (carvedilol group, n = 8), arotinolol 10 mg twice a day (arotinolol group, n = 8) or placebo twice a day (placebo group, n = 8). Subjects returned 3 days after treatment with carvedilol, arotinolol or placebo for measurements of FBF and platelet cGMP before and after administration of sublingual NTG. A 10-mg NTG tape (Millisrol Tape, Nippon Kayaku, Tokyo, Japan) was then applied twice a day. Final measurements were obtained 3 days after treatment with combined continuous transdermal NTG and carvedilol, arotinolol or placebo.

The study protocol was approved by the ethics committees of KINU Medical Association Hospital, Mitsukaido, Japan, and University of Tsukuba, Tsukuba, Japan, and written informed consent for participation in this study was obtained from all subjects.

Assessment of the vasodilator response to NTG. To evaluate the vasodilator response to NTG, the FBF was measured with a mercury-in-silastic strain gauge plethysmograph and the venous occlusion technique. The strain gauge was placed 5 cm below the antecubital crease and connected to a calibrated plethysmography. The FBF is expressed as the rate of change in the forearm volume (ml/min/100 ml forearm). The pressure in the venous occlusion or congesting cuff was 40 mm Hg.

Table 1. Patient Characteristics

	Carvedilol Group (n = 8)	Arotinolol Group (n = 8)	Placebo Group (n = 8)
Age (yrs)	57 ± 8	56 ± 9	57 ± 7
Male/female	6/2	7/1	5/3
Heart rate (beats/min)	68 ± 5	67 ± 5	68 ± 6
Systolic pressure (mm Hg)	178 ± 15	175 ± 14	174 ± 16
Diastolic pressure (mm Hg)	98 ± 11	101 ± 14	100 ± 13
Total cholesterol (mg/dl)	184 ± 24	182 ± 21	185 ± 25
Triglyceride (mg/dl)	124 ± 35	128 ± 26	126 ± 28
Creatinine (mg/dl)	0.8 ± 0.2	0.8 ± 0.3	0.8 ± 0.3

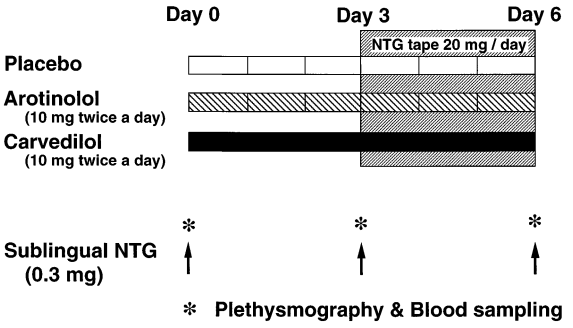


Figure 1. Study protocol. NTG = nitroglycerin.

Circulation to the hand was arrested during determinations of FBF by inflation of a cuff around the wrist to suprasystolic pressure. We used the average of three measurements made at 15-s intervals to represent the FBF.

Preparation of platelet cGMP. Blood samples were drawn into syringes containing 5 mM EDTA and a cGMP phosphodiesterase inhibitor (10^{-3} M 2-O-propoxyphenyl-8-azapurin-6-one dissolved in 1% triethanolamine). Platelet-rich plasma and platelet-poor plasma were prepared immediately after blood sampling by centrifugation at 200 g for 20 min. Platelet-rich plasma was further centrifuged at 2,500 g for 10 min, and the supernatant was discarded. The pellet was suspended in modified Tyrode's solution (containing 0.35% bovine serum albumin and 5 mM HEPES, pH 7.35) to obtain a final platelet count of 2 to 3 $\times$ 10^6 platelets/μl. The samples were stored frozen at −70°C until analysis (19).

Platelet cGMP assay. Trichloroacetic acid (0.5 ml in a final concentration of 6%) was added to 1 ml of the platelet preparation. After centrifugation at 2,500 g for 20 min, trichloroacetic acid was extracted four times from the supernatant with water-saturated ether. The aqueous phase was then assayed for cGMP using a commercially available radioimmunoassay kit (Yamasa Shoyu, Choshi, Japan) (20). The results are expressed in picomoles per 10^9 platelets. The coefficients of variation averaged 3.4% for intraassay error and 11.9% for interassay error.

Statistical analysis. Results are expressed as the mean ± SD for FBF and platelet cGMP level. Differences among the test days or differences before and after sublingual administration of NTG were analyzed by repeated measures of a one-way analysis of variance with Bonferroni's test, and differences among groups were analyzed by Student *t* test. A level $p < 0.05$ was accepted as statistically significant.

Results

Heart rate and blood pressure. Heart rates were not different among the three groups on day 0. Heart rates in the carvedilol group and the arotinolol group were significantly decreased on days 3 and 6, but heart rates in the placebo group did not change during study (Table 2).

Systolic blood pressure was not different among the three

Table 2. Heart Rate (beats/min) and Systolic Blood Pressure (mm Hg)

	Carvedilol Group	Arotinolol Group	Placebo Group
Heart rate			
Day 0			
Before	68 ± 5	67 ± 5	68 ± 6
After	67 ± 8	69 ± 8	67 ± 9
Day 3			
Before	59 ± 9†	60 ± 7†	68 ± 7
After	58 ± 8†	61 ± 9†	69 ± 8
Day 6			
Before	58 ± 6†	58 ± 8†	70 ± 9
After	59 ± 7†	57 ± 9†	71 ± 6
Systolic blood pressure			
Day 0			
Before	178 ± 15	175 ± 14	174 ± 16
After	154 ± 16*	156 ± 15*	155 ± 18*
Day 3			
Before	153 ± 15†	156 ± 13†	170 ± 18
After	140 ± 12*†	145 ± 15*†	157 ± 15*
Day 6			
Before	148 ± 18†‡	157 ± 15†	171 ± 15
After	134 ± 16*†‡	148 ± 14*†	164 ± 18*

Data are expressed as mean ± SD. Before = before sublingual administration of nitroglycerin; after = after sublingual administration of nitroglycerin. *$p < 0.05$ vs. before; †$p < 0.05$ vs. day 0; ‡$p < 0.05$ vs. day 3.

Table 3. Forearm Blood Flow (FBF, ml/min/100 ml arm) and Platelet cGMP Levels (pmol/10^9 PLT)

	Carvedilol Gruop	Arotinolol Group	Placebo Group
FBF			
Day 0			
Before	2.2 ± 0.7	2.4 ± 0.6	2.3 ± 0.8
After	2.9 ± 0.8*	3.1 ± 0.5*	3.0 ± 0.7*
Day 3			
Before	3.2 ± 0.6†	3.5 ± 0.6†	2.5 ± 0.6
After	4.2 ± 0.7*†	4.7 ± 0.7*†	3.3 ± 0.5*
Day 6			
Before	3.8 ± 0.6†‡	3.5 ± 0.8†	2.4 ± 0.8
After	4.8 ± 0.5*†‡	4.1 ± 0.7*†‡	2.6 ± 0.8*‡
cGMP			
Day 0			
Before	0.41 ± 0.17	0.40 ± 0.14	0.40 ± 0.14
After	0.50 ± 0.14*	0.55 ± 0.17*	0.56 ± 0.11*
Day 3			
Before	0.43 ± 0.14	0.45 ± 0.20	0.42 ± 0.14
After	0.60 ± 0.20*	0.63 ± 0.17*	0.58 ± 0.14*
Day 6			
Before	0.39 ± 0.20	0.32 ± 0.14†	0.28 ± 0.14†
After	0.51 ± 0.17*‡	0.36 ± 0.17*†	0.31 ± 0.17*†

Data are expressed as mean ± SD. Before = before sublingual administration of nitroglycerin; After = after sublingual administration of nitroglycerin. *$p < 0.05$ vs. before; †$p < 0.05$ vs. days 0 and 3; ‡$p < 0.05$ vs. the arotinolol and placebo groups.

groups on day 0 before and after sublingual administration of NTG. On day 3 systolic blood pressure before sublingual administration NTG was significantly decreased in the carvedilol group and the arotinolol group and significantly lower than the placebo group. Sublingual administration of NTG significantly decreased systolic blood pressure in the three groups. On day 6, systolic blood pressure in the carvedilol group before sublingual administration of NTG was significantly decreased compared with day 3. But systolic blood pressure in the arotinolol group and the placebo group before sublingual administration of NTG was not changed compared with day 3. After sublingual administration of NTG, systolic blood pressure was significantly decreased in the three groups. However, on day 6, systolic blood pressure after sublingual administration of NTG was lower in the carvedilol group than in the arotinolol group and the placebo group (Table 2).

Forearm blood flow. On day 0, there was no significant difference in the FBF before sublingual administration of NTG among the three groups. Sublingual administration of NTG increased the FBF in the three groups on day 0. On day 3, sublingual administration of NTG increased the FBF in the three groups, but the FBF before and after sublingual administration of NTG was higher in the carvedilol group and the arotinolol group than in the placebo group. There was no difference in FBF between the carvedilol group and the arotinolol group. On day 6, sublingual administration of NTG increased the FBF in the three groups, but the FBF after sublingual administration of NTG was significantly smaller in the arotinolol group and the placebo group than on day 3. In the carvedilol groups, the FBF was higher before and after sublingual administration of NTG than on day 3, and the FBF after sublingual administration of NTG was significantly greater in the carvedilol group than in the arotinolol group and the placebo group (Table 3).

There was no significant difference in the percent increase in the FBF on days 0 and 3 among the three groups (day 0: carvedilol group, 31 ± 12%; arotinolol group, 30 ± 11%; placebo group, 31 ± 9%; day 3: carvedilol group, 32 ± 9%; arotinolol group, 33 ± 12%; placebo group, 31 ± 13%). The percent increases in the arotinolol group and the placebo group were significantly lower on day 6 (arotinolol group, 18 ± 7%; placebo group, 15 ± 8%) than on days 0 and 3. In the carvedilol group, the percent increase in the FBF after sublingual administration of NTG was maintained on day 6 (27 ± 12%) and was significantly greater than that of the arotinolol group and the placebo group. (Fig. 2).

Platelet cGMP level. The platelet cGMP level was significantly increased after sublingual administration of NTG in the three groups on days 0 and 3. There was no significant difference among the three groups on days 0 and 3.

On day 6, the platelet cGMP levels in the arotinolol group and the placebo group were significantly lower than those on days 0 and 3, both before and after sublingual administration of NTG, whereas the platelet cGMP level in the carvedilol group on day 6 was significantly increased after sublingual administration of NTG, and was significantly higher than in the arotinolol group and the placebo group (Table 3).

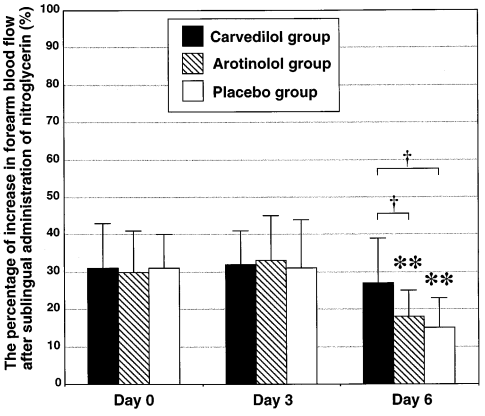


Figure 2. The percentage of increase in the FBF after sublingual administration of NTG. Data are expressed as the mean value ± SD. $^{**}p < 0.01$ vs. days 0 and 3; †$p < 0.05$ between the two groups.

There was no difference in the percent increase in the platelet cGMP level among the three groups on days 0 and 3 (day 0: carvedilol group, 39 ± 10%; arotinolol group, 38 ± 11%; placebo group, 39 ± 12%; day 3: carvedilol group, 40 ± 13%; arotinolol group, 39 ± 14%; placebo group, 38 ± 14%). The percent increase in cGMP level in the arotinolol group and the placebo group were significantly lower on day 6 (arotinolol group, 12 ± 9%; placebo group, 11 ± 8%) compared with that on days 0 and 3. The percent increase in the platelet cGMP level in the carvedilol group was maintained on day 6 (31 ± 11%) and was significantly greater than that of the arotinolol group and the placebo group (Fig. 3).

Discussion

This placebo-controlled, double-blind study demonstrated that carvedilol, during transdermal application of NTG, maintained the response of vasodilation and the intracellular production of cGMP after sublingual administration of NTG, but arotinolol did not have these effects. These findings suggest that carvedilol, an alpha- and beta-blocker with antioxidant properties, may prevent nitrate tolerance during continuous application of NTG.

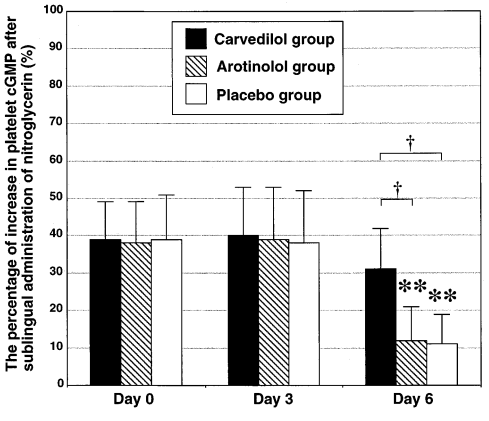
Mechanisms of nitrate tolerance. The mechanism of nitrate tolerance is multifactorial (4,21,22). Previous investigators proposed four possible mechanisms of nitrate tolerance after chronic exposure: 1) desensitization of the target enzyme guanylate cyclase (7); 2) an increase in phosphodiesterase activity (23); 3) intracellular sulfhydryl group depletion (24); and 4) impaired NTG biotransformation (25). However, on the basis of these mechanisms, attempts to prevent or reverse tolerance with sulfhydryl group donors (6,26,27) or angiotensin-converting enzyme inhibitors (28–30) have not been uniformly successful. Münzel et al. (9) recently demonstrated that enhanced angiotensin II activities resulted in increased production of oxygen-derived radicals that inhibit the dilator action of NTG-derived nitric oxide. An increase in oxidative stress due to an increase in free radicals may be one of the contributing factors in the mechanisms of nitrate tolerance. Therefore, we thought that an antioxidant may prevent nitrate tolerance.

Preventive effects of carvedilol on nitrate tolerance. Some investigations based on those proposed mechanisms have demonstrated preventive effects of antioxidants on nitrate tolerance. In an experimental animal study, Bassenge and Fink (31) demonstrated that vitamin C prevented nitrate tolerance in the dilatation of the coronary artery and the production of platelet cGMP. However, Münzel et al. (9) showed negative data by using superoxide dismutase in vitro. We cannot explain why superoxide dismutase did not prevent nitrate tolerance in the work by Münzel et al. In clinical studies we demonstrated that vitamins E and C could prevent nitrate tolerance (32–34). The effects of hydralazine on nitrate tolerance as a vasodilator with antioxidant properties have been also reported (35,36).

Carvedilol, in addition to being an adrenergic antagonist and a vasodilator, appears to be an antioxidant and free radical scavenger as well (13–16). On the other hand, arotinolol has been reported as an alpha- and beta-blocker without antioxidant properties (Fig. 4) (17,18). In the present study we observed nitrate tolerance in FBF and platelet cGMP levels and showed the attenuation of the change of FBF and platelet cGMP levels after sublingual administration of NTG in the placebo group. These phenomena were also observed in the arotinolol group. Therefore, we concluded that arotinolol without antioxidant properties might not prevent nitrate tolerance, and that carvedilol with antioxidant properties might prevent the attenuation of the response in the vasodilatory response and the intracellular production of cGMP after sublingual administration of NTG during continuous transdermal application of NTG. To our knowledge, the present study provides the first evidence in the clinical investigation that the development of nitrate tolerance may be prevented by the supplementation with carvedilol.

Study limitations. There are some limitations to the present study. First, we measured the platelet cGMP level to

Figure 3. The percentage of increase in platelet cGMP level after sublingual administration of NTG. Data are expressed as the mean value ± SD. $^{**}p < 0.01$ vs. days 0 and 3; †$p < 0.05$ between the two groups. cGMP = cyclic guanosine monophosphate.



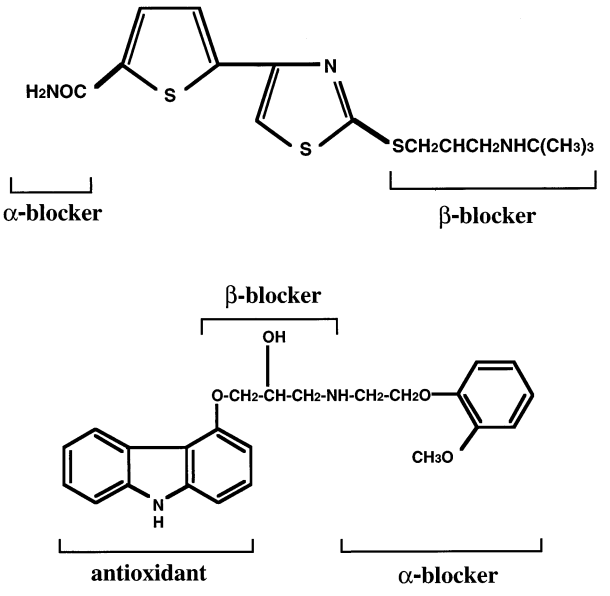


Figure 4. Chemical structures of arotinolol and carvedilol.

evaluate the intracellular production of cGMP. The in vivo effects of NTG on the intracellular production of cGMP in the vascular smooth muscle cells can only be evaluated in biopsies. Nitroglycerin activates soluble guanylate cyclase in platelets, and the increased level of platelet cGMP inhibits platelet adhesion (37,38). Platelets predominately contain the soluble guanylate cyclase (39,40). Therefore, platelets are an appropriate material for the clinical measurement of intracellular cGMP. In a previous study, we demonstrated that the platelet cGMP level can be used as an indicator of the effects of NTG and the development of nitrate tolerance (19). Second, we measured heart rate and blood pressure 5 min after sublingual administration of NTG at the same time of measurements of FBF and platelet cGMP levels. Despite about a 20 mm Hg fall in systolic blood pressure, the heart rate on day 0 did not change after sublingual administration of NTG in our results. We did observe an increase in heart rate 3 min after sublingual administration of NTG in electrocardiogram monitor, but the heart rate recovered until 5 min after sublingual administration of NTG. So we speculated that heart rate had been compensated 5 min after sublingual administration of NTG. We also observed the further decrease in blood pressure on day 6 compared with that on day 3 in the carvedilol group, but there was no difference in blood pressure between that on days 3 and 6 in the arotinolol group. We speculated further that the fall in blood pressure in the carvedilol groups was a result of maintained NTG effects without nitrate tolerance. However, we must note the possibility that the further fall in blood pressure in the carvedilol group on day 6 may represent not only an NTG effect but also continuous greater effect of carvedilol. Third, we demonstrated the preventive effects of carvedilol on nitrate tolerance in FBF and platelet cGMP, but we did not measure any indicator of peroxynitrate generation by oxygen free radicals (e.g., nitrotyrosine). Therefore, it is a speculation whether the mechanism of carvedilol on the prevention of nitrate tolerance is due to antioxidant properties. Fourth, we evaluated the effect of a single dose of carvedilol (10 mg twice a day) or arotinolol (10 mg twice a day) in this study. Because we can observe similar responses in heart rate and blood pressure on day 3, we thought that the effect of this dosage of carvedilol or arotinolol was similar as alpha- and beta-blockade in this study. We need further studies of adequate dosage of carvedilol to prevent nitrate tolerance in patients with ischemic heart disease or chronic heart failure. Finally, the present study demonstrated the preventive effect of carvedilol on nitrate tolerance in patients with untreated hypertension. Therefore, we cannot implicate our results directly to patients with coronary artery disease or chronic heart failure. We need further clinical studies in patients with ischemic heart disease or chronic heart disease.

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