Randomized, Double-Blind, Placebo-Controlled Study of the Preventive Effect of Supplemental Oral Vitamin C on Attenuation of Development of Nitrate Tolerance

HIDEKI WATANABE, MD, MASAAKI KAKIHANA, MD,* SADANORI OHTSUKA, MD,†
YASURO SUGISHITA, MD, FACC†

Ibaraki, Japan

Objectives. This study sought to evaluate the preventive effect of vitamin C, an antioxidant, on the development of nitrate tolerance.

Background. Decreased intracellular production of cyclic guanosine monophosphate (cGMP) is a mechanism of nitrate tolerance, and increased superoxide levels and reduced activation of guanylate cyclase have been observed in vitro.

Methods. In this double-blind, placebo-controlled study, 24 normal volunteers and 24 patients with ischemic heart disease (IHD) were randomized to receive either vitamin C (2 g three times daily [vitamin C group, n = 12]) or placebo (placebo group, n = 12). The vasodilator response to nitroglycerin was assessed with forearm plethysmography by measuring the change in FBF before and 5 min after sublingual administration of 0.3 mg of nitroglycerin. Blood samples were simultaneously obtained to measure platelet cGMP levels. FBF was measured, and blood sampling was performed serially at baseline (day 0), 3 days after administration of vitamin C or placebo (day 3) and 3 days after application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo (day 6).

Results. There were no differences between the vitamin C and placebo groups in percent increases in FBF (%FBF) or platelet cGMP levels (%cGMP) after administration of sublingual nitroglycerin on day 0 (%FBF: normal volunteers 31 ± 8 vs. 32 ± 10; patients with IHD 32 ± 9 vs. 32 ± 8; %cGMP: normal volunteers 37 ± 9 vs. 39 ± 10; patients with IHD 38 ± 10 vs. 39 ± 10 [vitamin C group vs. placebo group]) or day 3 (%FBF: normal volunteers 32 ± 9 vs. 33 ± 9; patients with IHD 31 ± 10 vs. 31 ± 10; %cGMP: normal volunteers 36 ± 8 vs. 37 ± 9; patients with IHD 39 ± 11 vs. 38 ± 10 [vitamin C group vs. placebo group]). The %FBF and %cGMP in the placebo group were significantly lower on day 6 than in the vitamin C group (%FBF: normal volunteers 30 ± 8 vs. 19 ± 4, p < 0.01; patients with IHD 29 ± 9 vs. 17 ± 6, p < 0.01; %cGMP: normal volunteers 36 ± 10 vs. 17 ± 6, p < 0.01; patients with IHD 37 ± 11 vs. 15 ± 5, p < 0.01 [vitamin C group vs. placebo group]).

Conclusions. These results indicate that combination therapy with vitamin C is potentially useful for preventing the development of nitrate tolerance.

(J Am Coll Cardiol 1998;31:1323–9)
©1998 by the American College of Cardiology

Organic nitrates are widely used in cardiovascular medicine, but their continuous administration can result in the rapid development of tolerance (1,2). The underlying mechanisms responsible for nitrate tolerance are probably multifactorial (3) and may include neurohormonal counterregulatory mechanisms (4), intravascular volume expansion (5) or intrinsic abnormalities, such as desensitization of the target enzyme guanylate cyclase (6) or a decrease in nitroglycerin biotransformation (7).

Recent experimental data (8) have demonstrated that nitrate tolerance is associated with increased vascular superoxide anion production. These radicals may inactivate the enzymes involved in the release of nitric oxide from nitroglycerin, leading to impaired cyclic guanosine monophosphate (cGMP) production and tolerance. A more recent study (9) showed that tolerance was associated with an enhanced propensity for vasoconstriction secondary to increased endothelin expression within the vascular smooth muscle. Vitamin C is known to be the most important endogenous water-soluble antioxidant, and the resistance of low density lipoprotein to oxidation is increased by oral intake of vitamin C (10). We recently reported (11) the preventive effect of intravenous administration of vitamin C on the development of nitrate tolerance in patients with congestive heart failure. We also reported (12) the preventive effect of oral supplemental vitamin E on the development of nitrate tolerance in normal subjects and patients with ischemic heart disease (IHD).

Therefore, the present study was designed to investigate the effect of oral supplemental vitamin C, an antioxidant, on

Figure 1. Study protocol. NTG = nitroglycerin; t.i.d. = three times daily. *Plethysmography and blood sampling.

nitrate tolerance during continuous administration of nitroglycerin in normal subjects and in patients with IHD.

Methods

Patients. The study included 24 normal volunteers (18 men, 6 women; 19 to 38 years old) and 24 patients with IHD (19 men, 5 women; 46 to 77 years old). The present study includes the same normal volunteers as those in our previous report (12). The normal volunteers had no history of renal or cardiac disease, and none had been exposed to any nitrate compounds or cardiac medications. Of the patients with IHD, 15 had received long-acting nitrates (isosorbide dinitrate retard [ISDN-Retard], 20 mg twice a day), and 12 had received calcium channel blocking agents. Long-acting nitrates were discontinued 72 h before the study. Other medications were continued throughout the study. The clinical characteristics of the study subjects are shown in Table 1.

Study protocol. We measured the vasodilator response to sublingual nitroglycerin by plethysmography and platelet cGMP at baseline (day 0), after 3 days of administration of vitamin C or placebo (day 3) and after 3 days of the application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo (day 6). An 18-gauge heparin lock was inserted in the contralateral forearm to allow venous blood sampling for measurements of platelet cGMP levels.

The protocol of the present study is shown in Figure 1 and is the same protocol used in our previous study (12). Baseline forearm blood flow (FBF) was recorded by plethysmography before and 5 min after administration of 0.3 mg of sublingual nitroglycerin on day 0. Normal volunteers and patients with IHD were then randomized by a double-blind, parallel-design to receive either 2 g three times daily of vitamin C (vitamin C group, n = 12) or placebo three times daily (placebo group,
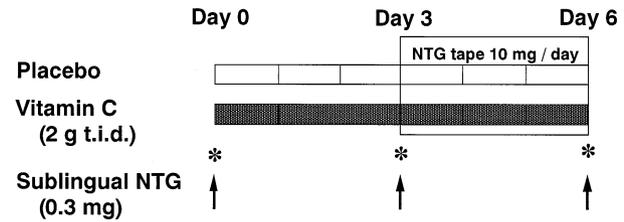
n = 12). Subjects returned 3 days after treatment with vitamin C or placebo for measurements of FBF and platelet cGMP levels before and after administration of sublingual nitroglycerin. A 5-mg nitroglycerin tape (Millisrol Tape, Nippon Kayaku, Tokyo, Japan) was then applied twice a day. Final measurements were obtained 3 days after treatment with combined continuous transdermal nitroglycerin and vitamin C or placebo.

The study protocol was approved by the ethics committee of Tsukuba University, Tsukuba, Japan, and written informed consent for participation in this study was obtained from all subjects.

Assessment of vasodilator response to nitroglycerin. To evaluate the vasodilator response to nitroglycerin, 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 plethysmograph. FBF is expressed as the rate of change in forearm volume (ml/min per 100 ml forearm). The pressure in the venous occlusion or congesting cuff was 40 mm Hg. Circulation to the hand was arrested during determinations of FBF by inflation of a cuff around the wrist to 10-mm Hg suprasystolic pressure. We used the average of three measurements made at 15-s intervals to represent FBF.

Preparation of platelet cGMP. Blood samples were drawn into syringes containing 5 mmol/liter EDTA and a cGMP phosphodiesterase inhibitor (10^{-3} mol/liter 2-O-propoxyphenyl-8-azapurin-6-one dissolved in 1% triethanolamine). Platelet-rich 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

Table 1. Clinical Characteristics of Study Subjects

	Normal Volunteers		Patients With IHD	
	Placebo Group (n = 12)	Vitamin C Group (n = 12)	Placebo Group (n = 12)	Vitamin C Group (n = 12)
Age (yr)	36 ± 8	32 ± 6	65 ± 10	64 ± 8
Men/women	9/3	9/3	10/2	9/3
Smokers	5 (42%)	4 (33%)	8 (67%)	9 (75%)
Mean blood pressure (mm Hg)	110 ± 15	114 ± 16	141 ± 12	138 ± 16
Pulse rate (beats/min)	72 ± 4	68 ± 4	77 ± 5	75 ± 7
Total cholesterol (mg/dl)	184 ± 16	183 ± 15	211 ± 16	208 ± 15

Data are expressed as number (%) of subjects or mean value ± SD. IHD = ischemic heart disease.
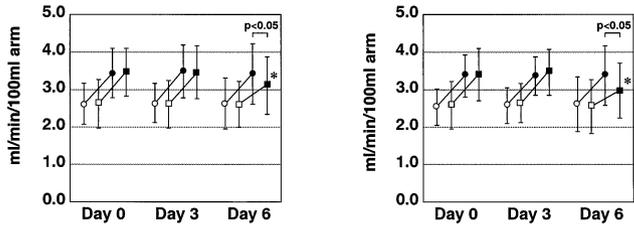
Figure 2. FBF before (**open circles** [vitamin C group] and **open squares** [placebo group]) and after sublingual nitroglycerin (**solid circles** [vitamin C group] and **solid squares** [placebo group]) in normal volunteers (**left**) and patients with IHD (**right**). Data shown are mean value ± SD. *p < 0.05 versus days 0 and 3.

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 3×10^6 platelets/μl. The samples were stored frozen at $-70°C$ until analysis (13).

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) (14). 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 mean value ± SD for FBF and as mean value ± SEM for platelet cGMP levels. Differences among the test days were analyzed by repeated measures analysis of variance with the Bonferroni test, and differences between before and after sublingual nitroglycerin were analyzed by the Student t test. A p level < 0.05 was accepted as statistically significant.

Results

FBF. Figure 2 shows the change in FBF before and after sublingual nitroglycerin in the normal volunteers and in patients with IHD. FBF (ml/min per 100 ml forearm) after sublingual nitroglycerin was increased both on day 0 (vitamin C group: normal volunteers 2.62 ± 0.55 to 3.44 ± 0.66; patients with IHD 2.54 ± 0.48 to 3.36 ± 0.56; placebo group: normal volunteers 2.63 ± 0.65 to 3.46 ± 0.64; patients with IHD

2.59 ± 0.64 to 3.4 ± 0.69) and on day 3 (vitamin C group: normal volunteers 2.64 ± 0.52 to 3.48 ± 0.71; patients with IHD 2.58 ± 0.48 to 3.37 ± 0.51; placebo group: normal volunteers 2.61 ± 0.63 to 3.46 ± 0.71; patients with IHD 2.64 ± 0.52 to 3.46 ± 0.61). There was no significant difference in FBF before and after nitroglycerin between the two groups in the normal volunteers and patients with IHD.

On day 6 (after 3 days of the application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo), FBF (ml/min per 100 ml forearm) in the placebo group was increased from 2.61 ± 0.61 to 3.11 ± 0.77 in normal volunteers and from 2.56 ± 0.72 to 2.98 ± 0.73 in patients with IHD after sublingual administration of nitroglycerin. However, the flow after sublingual nitroglycerin was significantly lower than that at days 0 and 3. By contrast, in the vitamin C group, the change in FBF after sublingual administration of nitroglycerin was similar to the change on days 0 and 3 (normal volunteers 2.63 ± 0.68 to 3.41 ± 0.81; patients with IHD 2.61 ± 0.72 to 3.38 ± 0.79). FBF after sublingual nitroglycerin in the vitamin C group was significantly greater than that in the placebo group in normal volunteers (p < 0.05) and in patients with IHD (p < 0.05).

The percent increase in FBF (%FBF) after sublingual administration of nitroglycerin is shown in Figure 3.

There was no significant difference on days 0 and 3 between the placebo group and the vitamin C group (day 0: vitamin C group, normal volunteers 31 ± 8%; patients with IHD 32 ± 9%; placebo group, normal volunteers 32 ± 10%; patients with IHD 32 ± 8% and day 3: vitamin C group, normal volunteers 32 ± 9%; patients with IHD 31 ± 10%; placebo group, normal volunteers 33 ± 9%; patients with IHD 31 ± 10%).

On day 6 (after 3 days of the application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo), the %FBF in the placebo group (normal volunteers 19 ± 4%; patients with IHD 17 ± 6%) was significantly reduced compared with that on days 0 and 3 (p < 0.01). By contrast, in the vitamin C group, %FBF after nitroglycerin (normal volunteers 30 ± 8%; patients with IHD 29 ± 9%) was maintained on day 6, and it was significantly greater than that in the placebo group (p < 0.01).

Platelet cGMP Level. Levels of platelet cGMP before and after sublingual administration of nitroglycerin are shown in Figure 4. In the present study, levels of platelet cGMP in the patients with IHD were significantly lower than those in the
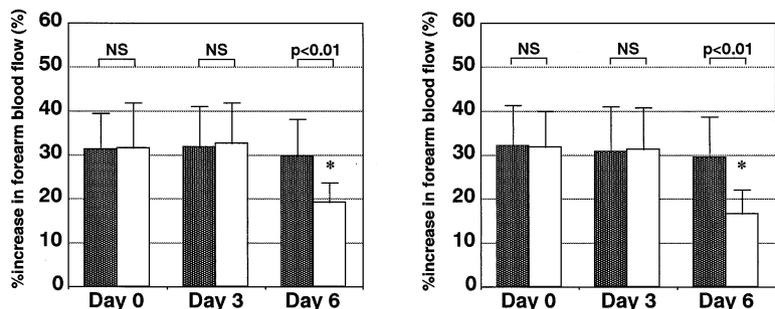
Figure 3. Percent increase in FBF after sublingual nitroglycerin in normal volunteers (**left**) and patients with IHD (**right**). **Open bars** = placebo group; **solid bars** = vitamin C group. Data shown are mean value ± SD. *p < 0.01 versus days 0 and 3.
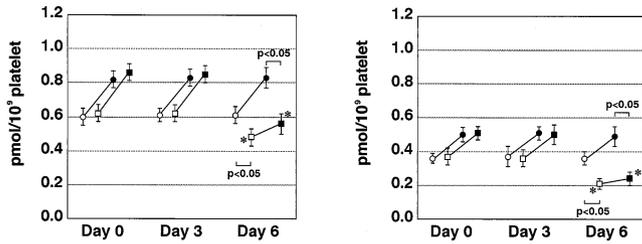
Figure 4. Platelet cGMP levels before and after sublingual nitroglycerin in normal volunteers **(left)** and patients with IHD **(right)**. Data shown are mean value ± SEM. Symbols as in Figure 1. *p < 0.05 versus days 0 and 3.

normal volunteers in the two groups on all testing days (p < 0.05).

Levels of platelet cGMP (pmol/10^9 platelets) in normal volunteers were significantly increased after sublingual administration of nitroglycerin on day 0 (vitamin C group 0.60 ± 0.05 to 0.82 ± 0.05; placebo group 0.62 ± 0.05 to 0.86 ± 0.05) and on day 3 (vitamin C group 0.61 ± 0.04 to 0.83 ± 0.05; placebo group 0.62 ± 0.05 to 0.83 ± 0.05). There was no significant difference between the two groups on days 0 and 3. Platelet cGMP levels in patients with IHD were also increased after sublingual administration of nitroglycerin on day 0 (vitamin C group 0.36 ± 0.03 to 0.50 ± 0.04; placebo group 0.37 ± 0.05 to 0.51 ± 0.04) and on day 3 (vitamin C group 0.37 ± 0.06 to 0.51 ± 0.04; placebo group 0.36 ± 0.05 to 0.50 ± 0.06). There was no significant difference between the two groups on days 0 and 3.

On day 6 (after 3 days of the application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo), platelet cGMP levels before nitroglycerin were significantly lower in the placebo group than those on days 0 and 3 (normal volunteers 0.48 ± 0.05, p < 0.05 vs. day 0 and day 3; patients with IHD 0.21 ± 0.03, p < 0.05 vs. day 0 and day 3). Platelet cGMP levels after sublingual nitroglycerin were also significantly lower (normal volunteers 0.56 ± 0.06, p < 0.05 vs. day 0 and day 3; patients with IHD 0.24 ± 0.04, p < 0.05 vs. day 0 and day 3). In the vitamin C group, platelet cGMP levels on day 6 were increased from 0.61 ± 0.05 to 0.83 ± 0.06 in normal volunteers and from 0.36 ± 0.04 to 0.49 ± 0.06 in patients with IHD after sublingual nitroglycerin. There was no significant

difference in the platelet cGMP levels in the vitamin C group among the testing days, and levels were significantly higher than those of the placebo group (p < 0.05).

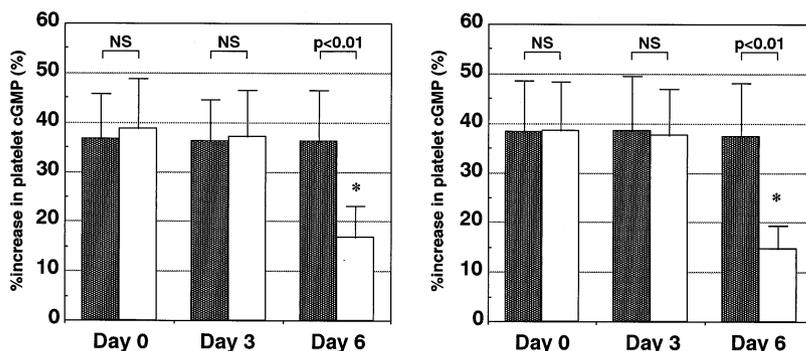The percent increase in platelet cGMP (%cGMP) after sublingual administration of nitroglycerin is shown in Figure 5.

On day 0 and day 3, there was no difference between the placebo group and the vitamin C group (day 0: vitamin C group, normal volunteers 37 ± 9%; patients with IHD 38 ± 10%; placebo group, normal volunteers 39 ± 10%; patients with IHD 39 ± 10% and day 3: vitamin C group, normal volunteers 36 ± 8%; patients with IHD 39 ± 11%; placebo group, normal volunteers 37 ± 9%; patients with IHD 38 ± 10%).

On day 6 (after 3 days of the application of a 10-mg/24-h nitroglycerin tape concomitantly with oral vitamin C or placebo), %cGMP in the placebo group (normal volunteers 17 ± 7%; patients with IHD 15 ± 5%) was significantly lower than that on day 0 and day 3 (p < 0.01). By contrast, in the vitamin C group, %cGMP was maintained on day 6 (normal volunteers 36 ± 10%; patients with IHD 37 ± 11%) and was significantly greater than that in the placebo group (p < 0.01).

Discussion

This placebo-controlled, double-blind study demonstrated that oral administration of 2 g of vitamin C three times daily, a water-soluble antioxidant, maintained the response of vasodilation and the intracellular production of cGMP after sublingual nitroglycerin during transdermal application of nitroglycerin in normal volunteers and in patients with IHD. These findings suggest that oral supplementation of vitamin C may prevent the development of nitrate tolerance during continuous nitrate therapy.

Mechanisms of nitrate tolerance. Although the phenomenon of nitrate tolerance was first described during the early part of this century (15), it was not considered clinically important (16) until later research demonstrated that nitrate tolerance limited the efficacy of these drugs in patients with IHD and congestive heart failure (17–19). The mechanism of nitrate tolerance is multifactorial (3,18,20). Nitrate tolerance is thought to be due to the inability of the vascular tissue to respond to nitroglycerin (21). Münzel et al. (19) have proposed

Figure 5. Percent increase in platelet cGMP levels after sublingual nitroglycerin in normal volunteers **(left)** and patients with IHD **(right)**. Data shown are mean value ± SD. Symbols as in Figure 3. *p < 0.01 versus days 0 and 3.

four possible mechanisms of nitrate tolerance after chronic exposure: 1) desensitization of the target enzyme guanylate cyclase (6); 2) an increase in phosphodiesterase activity (22); 3) intracellular sulfhydryl group depletion (23); and 4) impaired nitroglycerin biotransformation (24). They recently demonstrated (8) that enhanced angiotensin-II activities resulted in increased production of oxygen-derived radicals that inhibit the dilator action of nitroglycerin-derived nitric oxide. Although oxidative stress may inactivate the enzymes involved in nitric oxide production after organic nitrate administration, it is more likely that free radicals inactivate nitric oxide itself, resulting in the formation of peroxynitrite and leading to impaired cGMP production and nitrate tolerance.

Previous studies on prevention of nitrate tolerance. Because nitrate tolerance potentially limits the therapeutic efficacy of nitrates, there has been an extensive effort to develop effective strategies to prevent this phenomenon.

Some studies have found that the concomitant administration of angiotensin-converting enzyme inhibitors and nitroglycerin reversed or prevented nitrate tolerance (25–27), but other studies failed to confirm these results (28,29). Although it is difficult to explain the different efficacy of angiotensin-converting enzyme inhibitors in these studies, the use of higher doses of angiotensin-converting enzyme inhibitors may be needed to inhibit angiotensin-II formation. Münzel and Bassenge (30) reported that high dose enalapril reversed nitrate tolerance in vivo. These previous studies did not evaluate the intracellular production of cGMP. Therefore, more information is needed to determine the clinical usefulness of angiotensin-converting enzyme inhibitors and diuretic drugs in the prevention of nitrate tolerance.

In a recent study, Gogia et al. (31) demonstrated prevention of nitrate tolerance with the concomitant use of hydralazine in patients with chronic heart failure. Bauer and Fung (32) also demonstrated prevention of nitrate tolerance with concurrent administration of hydralazine in a rat model of congestive heart failure. Those studies suggested prevention of the nitrate-mediated decrease in renal blood flow and neurohormonal stimulation as a possible explanation for prevention of nitrate tolerance. Elkayam (33) more recently reported that in in vitro studies, hydralazine, by virtue of its antioxidant effect, may prevent the nitrate-mediated formation of vascular superoxide and thus may prevent nitrate tolerance.

The effect of vitamin C on nitrate tolerance. Ascorbate or vitamin C is the main water-soluble antioxidant in human plasma (34). It effectively scavenges superoxide and other reactive oxygen species and plays an important role in the regulation of intracellular redox state through its interaction with glutathione (35). Several large epidemiologic studies have suggested that dietary intake of vitamin C and plasma vitamin C concentration are inversely associated with the risk of IHD (36–38). Recently, vitamin C has been reported to reverse endothelial vasomotor dysfunction in the brachial circulation of patients with coronary artery disease (39) and to improve endothelial dysfunction in chronic smokers (40). We reported the preventive effects of intravenous administration of ascor-

bate on nitrate tolerance in hemodynamic function and platelet cGMP level in patients with congestive heart failure (11). In an experimental study, Bassenge and Fink (41) demonstrated that vitamin C prevented nitrate tolerance in the dilation of the coronary artery and the production of platelet cGMP. We demonstrated in the present study that oral supplementation of vitamin C, an antioxidant, prevented the attenuation of the vasodilatory response and the intracellular production of cGMP after administration of sublingual nitroglycerin during continuous transdermal application of nitroglycerin. The present study was performed using the same protocol as our previous study on vitamin E. In our previous study, we demonstrated the preventive effect of vitamin E on nitrate tolerance in normal volunteers and patients with IHD (12). These findings strongly support the theory that increased production and activity of oxygen-derived free radicals contribute to the development of nitrate tolerance in patients who receive long-term therapy with organic nitrate. On the basis of these findings, oral supplementation of antioxidants may be an effective treatment in patients with coronary artery disease, not only for the secondary prevention of coronary artery disease, but also for the prevention of nitrate tolerance in patients with coronary artery disease receiving continuous nitrate therapy.

Study limitations. There are some limitations in the present study:

1. We measured platelet cGMP levels to evaluate the intracellular production of cGMP. The in vivo effects of nitroglycerin on the intracellular production of cGMP in the vascular smooth muscle cells can be evaluated only in biopsy samples. In a previous study (13), we demonstrated that platelet cGMP levels can be used as an indicator of the effects of nitroglycerin and the development of nitrate tolerance. Nitroglycerin activates soluble guanylate cyclase in platelets, and the increased levels of platelet cGMP inhibit platelet adhesion (42). Platelets predominantly contain the soluble guanylate cyclase (43,44). Therefore, platelets are an appropriate material for the clinical measurement of intracellular cGMP. In the present study, we demonstrated lower platelet cGMP levels at baseline in patients with IHD than in normal volunteers. However, forearm blood flow at baseline or the responses of forearm blood flow and platelet cGMP to sublingual nitroglycerin were not different between normal volunteers and patients with IHD. We could not explain this discrepancy in the present study. More studies will be needed to elucidate this problem.

2. We did not compare the effects of other antioxidants such as vitamin E and beta-carotene in the present study. Vitamin C, vitamin E and beta-carotene may all favorably influence cardiovascular risk, but there are several important differences between these naturally occurring antioxidants. Vitamin C is water soluble and is present in most body fluids; vitamin E and beta-carotene are both lipid-soluble, and the concentrations of these compounds in plasma and specific cellular compartments differ. The primary antioxidant mechanisms of these antioxidants are also distinct. Thus, the beneficial effects observed in this study cannot necessarily be extrapolated to other antioxi-

dants. Recently, we reported the preventive effect of vitamin E on nitrate tolerance in our study performed using the same protocol (12). Therefore, both water- and lipid-soluble antioxidants may have a preventive effect on nitrate tolerance.

3. We did not measure plasma vitamin C levels or oxidative stress or hemodynamic function in the present study. The anti-ischemic efficacy and antianginal efficacy of vitamin C will require a separate study.

4. The dose of vitamin C given in this study, 2 g three times daily, is unusually high. In our preliminary study, low dose intake of vitamin C had no preventive effects on nitrate tolerance in this protocol (data not shown). In the present study, there was no adverse effect in all subjects. However, high dose vitamin C intake is known to induce an increase in the production of oxidized lipid because of depletion of vitamin E (45). Igarashi et al. (46) studied the effect of vitamin C on vitamin E levels in blood and found a statistically significant increase in the plasma vitamin E level in subjects receiving a vitamin E and vitamin C diet for 6 weeks. Thus, alpha-tocopherol may be regenerated by ascorbate not only from an alpha-tocopheroxyl radical but also from 8a-hydroperoxy alpha-tocopherones (47). Therefore, to prevent side effects, low dose intake of vitamin C concomitantly with vitamin E may be effective for the prevention of nitrate tolerance. We need further studies to establish an appropriate dose of vitamin C.

Conclusions. The present findings suggest that combination therapy with vitamin C is potentially useful for the prevention of nitrate tolerance during continuous nitrate therapy in patients with IHD. Further studies are needed to clarify the possible beneficial effects of antioxidant supplementation on the development of nitrate tolerance.

References

1. Abrams J. Tolerance to organic nitrates. Circulation 1986;74:1181–5.
2. Packer M, Lee W, Kessler P, Gottlieb S, Medina N, Yushak M. Prevention and reversal of nitrate tolerance in patients with congestive heart failure. N Engl J Med 1987;317:799–804.
3. Fung H. Solving the mystery of nitrate tolerance: a new scent on the trail? Circulation 1993;88:322–4.
4. Parker J, Farrel B, Fenton T, Cohanim M, Parker J. Counterregulatory responses to continuous and intermittent therapy with nitroglycerin. Circulation 1991;84:2336–45.
5. Dupuis J, Lalonde G, Lemieux R, Rouleau J. Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohumoral activation and lack of prevention with N-acetylcysteine. J Am Coll Cardiol 1990;16:922–31.
6. Waldman S, Rapoport R, Ginsburg R, Murad F. Desensitization to nitroglycerin in vascular smooth muscle from rat and human. Biochem Pharmacol 1986;35:3525–31.
7. Schröder H, Leitman D, Bennett B, Waldman S, Murad F. Glyceryl trinitrate-induced desensitization of guanylate cyclase in cultured rat lung fibroblasts. J Pharmacol Exp Ther 1988;245:413–8.
8. Münzel T, Sayegh H, Freeman B, Tarpey M, Harrison D. Evidence for enhanced vascular superoxide anion production in nitrate tolerance. J Clin Invest 1995;95:187–94.
9. Münzel T, Giaid A, Kurz S, Stewart D, Harrison D. Evidence for a role of endothelin-1 and protein kinase C in nitrate tolerance. Proc Natl Acad Sci USA 1995;92:5244–8.
10. Esterbauer H, Dieber-Rotheneder M, Striegl G, Waeg G. Role of vitamin E
11. in preventing the oxidation of low-density lipoprotein. Am J Clin Nutr 1991;53 Suppl:314S–21S.
11. Watanabe H, Kakihana M, Ohtsuka S, Sugishita Y. Randomized, double-blind, placebo-controlled study of ascorbate on the preventive effect of nitrate tolerance in patients with congestive heart failure. Circulation 1998;97:886–91.
12. Watanabe H, Kakihana M, Ohtsuka S, Sugishita Y. Randomized, double-blind, placebo-controlled study of supplemental vitamin E on an attenuation of the development of nitrate tolerance. Circulation 1997;96:2545–50.
13. Watanabe H, Kakihana M, Ohtsuka S, Enomoto T, Yasui K, Sugishita Y. Platelet cyclic GMP: a potentially useful indicator to evaluate the effects of nitroglycerin and nitrate tolerance. Circulation 1993;88:29–36.
14. Honma M, Satoh T, Takezawa J, Ui M. An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small volume samples from blood and tissue. Biochem Med 1977;18:257–73.
15. Crandall L, Leake C, Loevenhart A, Muehlberger C. Acquired tolerance to and cross-tolerance between the nitrous and nitric acid esters and sodium nitrite in man. J Pharmacol Exp Ther 1931;41:103.
16. Elkayam U, Aronow W. Glyceryl trinitrate (nitroglycerin) ointment and isosorbide dinitrate: a review of their pharmacological properties and therapeutic use. Drugs 1982;23:165–94.
17. Elkayam U. Tolerance to organic nitrates: evidence, mechanisms, clinical relevance, and strategies for prevention. Ann Intern Med 1991;114:667–77.
18. Mangione N, Glasser S. Phenomenon of nitrate tolerance. Am Heart J 1994;128:137–46.
19. Münzel T, Kurz S, Heitzer T, Harrison D. New insights into mechanisms underlying nitrate tolerance. Am J Cardiol 1996;77:24C–30C.
20. Packer M. What causes tolerance to nitroglycerin? The 100 year old mystery continues. J Am Coll Cardiol 1990;16:932–5.
21. Slack C, McLaughlin B, Brien J, Marks G, Nakatsu K. Biotransformation of glyceryl trinitrate and isosorbide dinitrate in vascular smooth muscle made tolerant to organic nitrates. Can J Physiol Pharmacol 1989;67:1381–5.
22. Axelsson K, Andersson R. Tolerance towards nitroglycerin, induced in vivo, is correlated to a reduced cGMP response and an alteration in cGMP turnover. Eur J Pharmacol 1983;88:71–9.
23. Needleman P, Johnson EM Jr. Mechanism of tolerance development to organic nitrates. J Pharmacol Exp Ther 1973;184:709–15.
24. Feelisch M, Nack EA. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol 1987;139:19–30.
25. Katz R, Levy W, Buff L, Wasserman A. Prevention of nitrate tolerance with angiotensin converting enzyme inhibitors. Circulation 1991;83:1271–7.
26. Muiesan M, Boni E, Castellano M, et al. Effects of transdermal nitroglycerin in combination with an ACE-inhibitor in patients with chronic stable angina pectoris. Eur Heart J 1993;14:1701–8.
27. Pizzulli L, Hagendorff A, Zirbes M, et al. Influence of captopril on nitroglycerin-mediated vasodilation and development of nitrate tolerance in arterial and venous circulation. Am Heart J 1996;131:342–9.
28. Dakak N, Makhoul N, Flugelman M, et al. Failure of captopril to prevent nitrate tolerance in congestive heart failure secondary to coronary artery disease. Am J Cardiol 1990;66:608–13.
29. Parker JD, Parker JO. Effect of therapy with an angiotensin-converting enzyme inhibitor on hemodynamic and counterregulatory response during continuous therapy with nitroglycerin. J Am Coll Cardiol 1993;21:1445–53.
30. Münzel T, Bassenge E. Long-term angiotensin-converting enzyme inhibition with high-dose enalapril retards nitrate tolerance in large epicardial arteries and prevents rebound coronary vasoconstriction in vivo. Circulation 1996;93:2052–8.
31. Gogia H, Mehra A, Parikh S, et al. Prevention of tolerance to hemodynamic effects of nitrates with concomitant use of hydralazine in patients with chronic heart failure. J Am Coll Cardiol 1995;26:1575–80.
32. Bauer J, Fung H. Concurrent hydralazine administration prevents nitroglycerin-induced hemodynamic tolerance in experimental heart failure. Circulation 1991;84:35–9.
33. Elkayam U. Prevention of nitrate tolerance with concomitant administration of hydralazine. Can J Cardiol 1996;12 Suppl C:17C–21C.
34. Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am J Med 1994;97:5S–13S.
35. Benedich A, Machlin I, Scandurra O, Burton G, Wayner D. The antioxidant role of vitamin C. Adv Free Radic Bio Med 1986;2:419–44.

36. Gey K, Stahelin H, Puska P, Evans A. Relationship of plasma level of vitamin C to mortality from ischemic heart disease. Ann NY Acad Sci 1987;498:110–23.

37. Riemersma R, Wood D, Macintyre C, Elton R, Gey K, Oliver M. Low plasma vitamin E and C and increased risk of angina in Scottish men. Ann NY Acad Sci 1989;570:291–5.

38. Singh R, Niaz M, Bishnoi I, et al. Diet, antioxidant vitamins, oxidative stress and risk of coronary artery disease: the Peerzada Prospective Study. Acta Cardiol 1994;49:453–67.

39. Levine G, Frei B, Koulouris S, Gerhard M, Keaney J, Vita J. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. Circulation 1996;93:1107–13.

40. Heitzer T, Just H, Münzel T. Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. Circulation 1996;94:6–9.

41. Bassenge E, Fink B. Tolerance to nitrates and simultaneous upregulation of platelet activity prevented by enhancing antioxidant state. Naunyn Schmiedebergs Arch Pharmacol 1996;353:363–7.

42. Pohl U, Busse R. EDRF increase cyclic GMP in platelets during passage through the coronary vascular bed. Circ Res 1989;65:1798–803.

43. Rapoport R, Murad F. Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. J Cyclic Nucl Pro Phosphoryl Res 1983;9:281–96.

44. Glass D, Frey W, Carr D, Goldberg N. Stimulation of human platelet guanylate cyclase by fatty acid. J Biol Chem 1977;252:1279–85.

45. Gerster H, Moser U. Is high-dose vitamin C intake associated with systemic conditioning? Nutr Res 1988;8:1327–32.

46. Igarashi O, Yonekawa Y, Fujiyama-Fujiyama Y. Synergistic action of vitamin E and vitamin C in vivo using a new mutant of Wistar-strain rats, ODS, unstable to synthesize vitamin C. J Nutr Sci Vitaminol 1991;37:359–69.

47. Yamauchi R, Kato K, Ueno Y. Reaction of 8a-hydroperoxy tocopherones with ascorbic acid. Agrc Biol Chem 1981;45:2855–61.