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

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Organic nitrates are widely used in cardiovascular medicine, but their continuous administration can result in the rapid development of tolerance (1,2). 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 biodetransformation (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 supplemental vitamin C, an antioxidant, on
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 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 (10⁻⁷ mol/liter 2-O-propoxypheynyl-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>
<thead>
<tr>
<th>Method</th>
<th>Placebo Group (n = 12)</th>
<th>Vitamin C Group (n = 12)</th>
<th>Placebo Group (n = 12)</th>
<th>Vitamin C Group (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>36 ± 8</td>
<td>32 ± 6</td>
<td>65 ± 10</td>
<td>64 ± 8</td>
</tr>
<tr>
<td>Men/women</td>
<td>9/3</td>
<td>9/3</td>
<td>10/2</td>
<td>9/3</td>
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<tr>
<td>Smokers</td>
<td>5 (42%)</td>
<td>433%</td>
<td>8 (67%)</td>
<td>9 (75%)</td>
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<tr>
<td>Mean blood pressure (mm Hg)</td>
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<td>114 ± 16</td>
<td>141 ± 12</td>
<td>138 ± 16</td>
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<tr>
<td>Pulse rate (beats/min)</td>
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<td>68 ± 4</td>
<td>77 ± 5</td>
<td>75 ± 7</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>184 ± 16</td>
<td>183 ± 15</td>
<td>211 ± 16</td>
<td>208 ± 15</td>
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</table>

Data are expressed as number (%) of subjects or mean value ± SD. IHD = ischemic heart disease.
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^{10} \text{ platelets/\mu l}. 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 \text{ 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 normal volunteers. The percent increase in platelet cGMP (%cGMP) after sublingual administration of nitroglycerin was significantly greater than that in the placebo group in normal volunteers (p < 0.05) and in patients with IHD (p < 0.05).
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; 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), %cGMP in the placebo group (normal volunteers 41% ± 10%; patients with IHD 37% ± 10%; placebo group, normal volunteers 40% ± 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%)

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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...
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 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 ascorbate 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 antioxidants.
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 antioxidant 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, alphatocopherol may be regenerated by ascorbate not only from an alpha-tocopheroxyl radical but also from 8a-hydropheroxy 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


