Attenuation of Nitrate Tolerance and Oxidative Stress by an Angiotensin II Receptor Blocker in Patients With Coronary Spastic Angina

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Background—Nitrites are widely used to treat coronary artery disease, but their therapeutic value is compromised by the rapid development of tolerance. Recently, the renin-angiotensin system has been suggested to play an important role in the development of nitrite tolerance.

Methods and Results—Sixty-four patients with coronary spastic angina were investigated to clarify the effect of angiotensin II type 1 receptor blocker (ARB) therapy on nitrite tolerance. Transdermal nitroglycerin (10 mg/d) and an ARB (candesartan, 8 mg/d) were administered to 21 patients (GTN+ARB group) for 3 days, whereas transdermal nitroglycerin and placebo were administered to 19 patients (GTN group). Another 18 patients were treated with placebo skin patches and placebo tablets for 3 days (control group). The brachial artery response to incremental doses of intravenous nitroglycerin (0.01, 0.1, and 1.0 μg/kg) was measured by ultrasound before and after transdermal nitroglycerin therapy. Before treatment, the arterial diameter was increased by nitroglycerin injection in each group. After treatment, the increase of arterial diameter was significantly suppressed in the GTN group but not in the control or GTN+ARB groups. The plasma level of thioredoxin (a marker of oxidative stress) was increased in the GTN group after treatment (P<0.01) but not in the control or GTN+ARB groups.

Conclusions—An ARB suppressed the development of nitrite tolerance during transdermal nitroglycerin therapy. These results suggest that increased oxidative stress induced by activation of angiotensin II may play an important role in the development of nitrite tolerance. (Circulation. 2003;108:1446-1450.)

Key Words: angiotensin • nitroglycerin • ultrasounds • vasodilation

Nitrites are widely used in the treatment of coronary artery disease and heart failure.1,2 Although these drugs are initially effective for such disorders, their therapeutic value is compromised by the rapid development of tolerance during sustained therapy.2,3 Nitrite tolerance has a multifactorial etiology that includes sulfhydryl depletion, activation of neurohormonal factors, expansion of plasma volume, and increased oxidative stress.2,3 Recently, Munzel and coworkers4,5 reported that the metabolism of nitroglycerin is associated with an increase of superoxide production during the development of nitrite tolerance. They postulated that activation of angiotensin II represents the first step toward nitrite tolerance and that increased superoxide flux is the final mediator, with the latter inactivating nitric oxide released either from the administered nitrate or from the vascular endothelium.5 However, there is still some controversy regarding the role of angiotensin II in the development of nitrite tolerance.

In the present study, we examined the effect of an angiotensin II type 1 receptor blocker (ARB), candesartan, on the development of nitrite tolerance and measured the plasma thioredoxin concentration as a marker of the cellular antioxidant status in patients with coronary spastic angina.

Methods

Subjects
Sixty-four patients with coronary spastic angina (mean age, 63.5±2.2 years) from our institution were enrolled in the study. They were all confirmed to have coronary vasospasm based on ST-segment changes after the intracoronary injection of acetylcholine,6 and none of them showed fixed coronary stenosis after administration of nitroglycerin during cardiac catheterization. None of the subjects had diabetes, a history of myocardial infarction, or evidence of impaired resting left ventricular function as assessed by echocardiography and cardiac catheterization. Written informed consent was obtained from all subjects before the study was commenced, and the

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study procedures were in accordance with the guidelines approved by the ethics committee of our institution.

**Study Design**

We divided the 64 study subjects into 3 groups. Twenty-three patients were randomized to receive transdermal nitroglycerin (10 mg/d, Nippon-kayaku) plus an ARB (candesartan 8 mg/d, Takeda; GTN + ARB group) for 3 days, and another 23 patients were randomized to receive 3 days of transdermal nitroglycerin plus an ARB placebo (GTN group). In addition, 18 subjects were treated with an ARB placebo and a placebo skin patch for 3 days (control group). Two subjects withdrew from the GTN group because of intolerable headache induced by nitrate therapy. No patient withdrew from the control group, so we studied a total of 58 subjects (control, 18; GTN, 19; GTN + ARB, 21). The clinical characteristics of these patients are shown in Table 1.

On the first day (day 0, 6:30 AM), we measured the brachial artery diameter of each subject using the method described below. We simultaneously obtained a blood sample to evaluate the lipid profile and the plasma level of thioredoxin. After these measurements were completed (day 0, 7:00 AM), treatment was started with transdermal nitroglycerin (or placebo) and candesartan (or placebo). Transdermal nitroglycerin was applied twice a day (7:00 AM and 7:00 PM) on days 0, 1, and 2 and was finally removed at 5:30 AM on day 3. Candesartan was administered orally in the morning (7:00 AM) on days 0, 1, and 2. On day 3, we remeasured the brachial artery diameter and obtained another blood sample at 6:30 AM. Measurement was performed 1 hour after the cessation of transdermal nitroglycerin/placebo therapy to minimize the influence of residual circulating nitrate. A detailed outline of the study protocol is shown in Figure 1.

**Blood Sampling and Assays**

Blood samples were obtained before and after 3 days of treatment. The fasting serum total cholesterol and triglyceride concentrations were measured by enzymatic methods, and the serum HDL cholesterol concentration was measured by heparin-Ca2+/Ni2+ precipitation. We also measured the plasma thioredoxin concentration as a marker of cellular antioxidant activity using an ELISA kit (Redox Bioscience, Inc). The detection limit of the assay was 2.0 ng/mL, and the intraassay and interassay coefficients of variation were 0.81% to 3.74% and 4.87% to 6.97%, respectively.

**Statistical Analysis**

Comparison of data among the 3 groups was performed using one-way ANOVA followed by the Bonferroni multiple-comparison test. The χ2 test was used to compare the prevalence of smokers. Changes of variables were assessed by ANOVA with repeated

### Table 1. Clinical Characteristics of the Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>GTN</th>
<th>GTN + ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>18</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Men/Women</td>
<td>8/10</td>
<td>11/8</td>
<td>9/12</td>
</tr>
<tr>
<td>Age, y</td>
<td>62.3±2.8</td>
<td>61.3±2.4</td>
<td>66.5±1.6</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.2±0.8</td>
<td>23.8±0.9</td>
<td>22.8±0.8</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>197.1±5.4</td>
<td>187.8±7.4</td>
<td>196.7±7.1</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>52.5±4.0</td>
<td>52.9±4.1</td>
<td>58.3±3.0</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>126.9±6.1</td>
<td>111.1±7.9</td>
<td>99.1±7.0</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>123.6±20.4</td>
<td>110.3±7.1</td>
<td>122.3±6.4</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7 (39)</td>
<td>6 (32)</td>
<td>6 (29)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.
measures followed by a post-hoc Scheffe test. In addition, differences between 2 means among the groups were assessed by the Student paired t test, as appropriate. Statistical significance was defined as \( P<0.05 \).

Results

There were no differences in age, body mass index, lipid profile, or smoking status among the 3 groups (Table 1). Hemodynamic parameters did not change after transdermal nitroglycerin (placebo) treatment in any group (Table 2).

There were no differences of the baseline arterial diameter between the 3 groups on day 0 (control, 3.71 \( \pm \) 0.14; GTN, 3.66 \( \pm \) 0.15; GTN + ARB, 3.66 \( \pm \) 0.11 mm; \( P=\text{NS by ANOVA} \)). There were also no differences in the baseline arterial diameter between the 3 groups after transdermal nitroglycerin treatment (control, 3.70 \( \pm \) 0.18; GTN, 3.86 \( \pm \) 0.15; GTN + ARB, 3.78 \( \pm \) 0.08 mm; \( P=\text{NS by ANOVA} \)).

On day 0, the brachial artery response to incremental doses of intravenous nitroglycerin was comparable among the 3 groups (control, 0.9 \( \pm \) 0.6% \( \times \) 9.3 \( \pm \) 1.2%; and 19.6 \( \pm \) 1.8%; GTN, 1.3 \( \pm \) 1.0%, 9.9 \( \pm \) 1.4%, and 18.6 \( \pm \) 1.6%; and GTN + ARB, 2.2 \( \pm \) 1.0%, 9.0 \( \pm \) 0.8%, and 17.5 \( \pm \) 2.0% at 0.01, 0.1, and 1.0 \( \mu \)g/kg; \( P=\text{NS by ANOVA} \) (Figure 3A). On day 3 of transdermal nitroglycerin (placebo) treatment, however, the response of the GTN group was decreased compared with that of the other 2 groups (control, 2.4 \( \pm \) 0.7%, 11.1 \( \pm \) 1.3%, and 19.7 \( \pm \) 1.1%; GTN, 0.1 \( \pm \) 0.7%, 4.6 \( \pm \) 1.1% \( \times \) and 11.9 \( \pm \) 1.5% \( \times \); GTN + ARB, 1.9 \( \pm \) 0.8%, 10.0 \( \pm \) 1.4%, and 19.2 \( \pm \) 1.9% at 0.01, 0.1, and 1.0 \( \mu \)g/kg; \( P<0.01 \) versus control, \( \dagger P<0.01 \) versus GTN + ARB by ANOVA at the same dose of intravenous nitroglycerin) (Figure 3B).

After 3 days of transdermal nitroglycerin therapy, the response to sublingual nitroglycerin was also blunted in the GTN group compared with that in the control and GTN + ARB groups (control, 20.5 \( \pm \) 1.8% [before] versus 21.0 \( \pm \) 2.1% [after]; GTN, 22.0 \( \pm \) 2.0% versus 17.7 \( \pm \) 2.7%, \( P<0.05 \); GTN + ARB, 21.6 \( \pm \) 2.2% versus 22.6 \( \pm \) 2.4%) (Figure 4).

The plasma thioredoxin level was increased after 3 days of nitroglycerin treatment in the GTN group, but it did not change in the control group or the GTN + ARB group (control, 32.2 \( \pm \) 5.4 versus 33.2 \( \pm \) 6.3; GTN, 36.9 \( \pm \) 7.8 versus 59.2 \( \pm \) 9.9 \( \times \); GTN + ARB, 30.9 \( \pm \) 3.9 versus 26.6 \( \pm \) 4.6 ng/mL, respectively; \( P<0.01 \) versus control, \( \dagger P<0.01 \) versus GTN + ARB by ANOVA) (Figure 5).

Discussion

The present study demonstrated that the response of brachial artery diameter to both intravenous and sublingual nitroglycerin was decreased in the GTN group by 3 days of transdermal nitroglycerin treatment, whereas the plasma concentration of thioredoxin was increased in this group. There were no such changes in the control and GTN + ARB groups. These findings suggest that concomitant administration of candesartan could suppress the development of nitrate tolerance during continuous nitroglycerin treatment in patients with coronary spastic angina. The present study is the first to show that an ARB suppressed both the development of nitrate tolerance and oxidative stress in humans.

The phenomenon of nitrate tolerance was first described in the early 20th century and was thought to be attributable to the inability of vascular tissues to respond to nitroglycerin. The

| Table 2. Hemodynamic Parameters Measured During the Study |
|-----------------|-----------------|-----------------|
|                 | Control         | GTN             | GTN + ARB       |
|                 | Day 0 | Day 3 | Day 0 | Day 3 | Day 0 | Day 3 |
| Heart rate, bpm | 67.3 ± 2.5 | 66.2 ± 2.2 | 64.7 ± 2.3 | 65.6 ± 1.8 | 69.4 ± 2.3 | 69.1 ± 2.6 |
| Systolic blood pressure, mm Hg | 115.3 ± 4.2 | 116.7 ± 3.7 | 114.1 ± 3.4 | 113.2 ± 4.0 | 118.2 ± 3.9 | 117.2 ± 3.7 |
| Diastolic blood pressure, mm Hg | 70.4 ± 2.2 | 68.2 ± 3.0 | 71.3 ± 1.8 | 71.9 ± 3.2 | 69.2 ± 1.8 | 66.9 ± 1.4 |

Values are expressed as mean ± SE.
clinical importance of this phenomenon is that it limits the efficacy of nitroglycerin when treating patients with coronary artery disease and heart failure.2–4 Although the mechanism of nitrate tolerance remains poorly understood, a beneficial effect of ACE inhibitors has already been reported by other investigators4,5,11,12 as well as by us.13 However, some studies did not reveal a beneficial effect of ACE inhibitors,14–16 Also, Parker and coworkers17 concluded that losartan had no impact on nitrate tolerance in healthy volunteers. The present findings are not supported by these negative studies, which investigated nitrate tolerance in patients with congestive heart failure,14,15 patients with coronary artery disease,15 and healthy volunteers.16 Although they investigated the effects of nitrate on hemodynamics14–17 or on the resistance vessels using plethysmography,17 we assessed the effect of an ARB on nitrate tolerance by measuring the diameter of a conduit artery in patients with coronary spastic angina. Jeserich et al18 reported that after 24 and 48 hours of continuous nitroglycerin administration was longer in our protocol. Second, they infused additional doses of nitroglycerin during continuous intravenous administration of the drug, whereas we observed the effect of nitroglycerin on brachial artery diameter after the cessation of continuous nitrate therapy. Therefore, the method of inducing nitrate tolerance was not the same. Jeserich et al also reported that the arterial diameter returned to baseline within 35 minutes after the withdrawal of continuous nitrate therapy,18 a finding that may support the timing of our measurement of arterial diameter.

Coronary vasospasm plays an important role in the pathogenesis of not only variant angina but also ischemic heart disease, including acute myocardial infarction and sudden death.19 Coronary spastic angina is evoked by spasm of large epicardial coronary arteries,19 and we previously showed that the coronary arteries of patients with coronary spastic angina have a supersensitive dilatory response to nitroglycerin.20 The present method can directly measure the effects on conduit arteries and is suitable for patients with coronary vasospasm.

Recently, it was revealed that activation of the angiotensin II type 1 receptor stimulates NADH/NADPH oxidase in the vascular endothelium and leads to production of reactive oxygen species.21 Munzel and coworkers4,5 reported that enhanced activation of the angiotensin II type 1 receptor by continuous nitrate therapy led to increased production of oxygen radicals by both endothelial cells and smooth muscle cells. These radicals can inactivate nitric oxide, leading to decreased cyclic GMP production, and thus cause nitrate tolerance. In fact, antioxidants like vitamin C and vitamin E have been reported to prevent the development of nitrate tolerance during continuous nitroglycerin treatment.22,23 An antioxidant effect of ARBs has also been recognized in recent years.24–27 ARBs decrease a marker of oxidative stress, thiobarbituric acid reactive substances, in an animal model26 and reduced the 8-isoprostane level in patients with hypercholesterolemia.27 Our results are supported by these observations.

In the present study, we measured plasma thioredoxin as a marker of oxidative stress. Thioredoxins is a small protein with a redox-active dithiol/disulfide at its active site, and it is critical for the redox regulation of protein function and for signaling via modulation of the thiol redox status.28 Cytosolic thioredoxin has numerous functions, including protection against oxidative stress, control of cell growth, and modulation of apoptosis.28–30 Thioredoxin is induced by oxidative stress and is secreted by cells. Previous studies have shown that the plasma/serum level of thioredoxin is elevated in oxidative stress–associated disorders, such as ischemia-reperfusion injury8 and viral infection.31 Thus, the plasma/serum level of thioredoxin is a good marker of the host response to oxidative stress.

Figure 4. Increase of brachial arterial diameter in response to sublingual nitroglycerin (0.3 mg) before (day 0) and after (day 3) treatment.

Figure 5. Changes in plasma thioredoxin level before (day 0) and after (day 3) treatment. †P<0.01 vs control group, ‡P<0.01 vs GTN+ARB group, *P<0.05 by ANOVA.
measurement. Nitroglycerin has a plasma half-life of approximately 1 to 4 minutes, but its biologically active dinitrate metabolites have a half-life of approximately 40 minutes, so we could not exclude an effect of dinitrate on vessel diameter.

Candesartan cilexetil is rapidly and completely hydrolyzed to the active compound candesartan during its absorption from the gastrointestinal tract. A single dose of candesartan cilexetil is known to reduce the arterial pressure at 4 hours after administration in patients with hypertension. The time until the maximum plasma concentration ($t_{max}$), plasma half-life ($t_{1/2}$), and mean residence time after candesartan cilexetil (8 mg) was administered to human volunteers were 4.3 to 4.4 hours, 9.05 to 9.4 hours, and 11.6 hours, respectively. The lack of hemodynamic changes in our subjects might have arisen because candesartan was administered for a short time (3 days) and because trough blood pressure was measured rather than the peak level (Figure 1).

The time required to induce tolerance was relatively short, but it may be that the short- and long-term mechanisms of nitrate tolerance are different. Munzel and coworkers mentioned the time course for the development of tolerance and pseudotolerance. They stated that pseudotolerance begins immediately after the initiation of nitrate treatment, whereas true tolerance requires at least 3 days of treatment. Additional investigation will be necessary to assess the long-term changes of brachial artery diameter and thioredoxin levels during nitrate therapy.

Also, the effects of ARB therapy on nitrate tolerance in patients with coronary spasm may not be the same as in patients with other types of coronary artery disease, so additional investigations are needed to determine the mechanisms of nitrate tolerance in patients with atherosclerotic disease.

In conclusion, ARB administration suppressed the development of nitrate tolerance during transdermal nitroglycerin therapy. This finding suggests that an increase of oxidative stress induced by angiotensin II activation may play an important role in the development of nitrate tolerance.

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References
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