Functional and Biochemical Analysis of Endothelial (Dys)function and NO/cGMP Signaling in Human Blood Vessels With and Without Nitroglycerin Pretreatment

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Background—In experimental animal models, long-term in vivo treatment with nitroglycerin (NTG) induces both endothelial dysfunction and tolerance to nitrates. However, it is still controversial whether nitrate tolerance in humans is associated with both endothelial dysfunction and impaired vascular response to nitrovasodilator-derived NO.

Methods and Results—Patients undergoing elective bypass surgery were randomized to receive 48 hours of continuous NTG infusion (NTG group) or no nitrate therapy (control group). Segments of surgically removed arteria mammaria, vena saphena, and arteria radialis not required for the bypass procedure were used to examine (1) the vascular responsiveness to NTG and the endothelium-dependent vasodilator acetylcholine; (2) the expression of the NO target, the soluble guanylyl cyclase; (3) the expression of the soluble guanylyl cyclase/cGMP effector target, the cGMP-dependent protein kinase (cGK); and (4) the cGK activity as assessed by the phosphorylation state of its vascular substrate, the vasodilator-stimulated phosphoprotein at serine239 (P-VASP). NTG treatment caused a marked degree of nitrate tolerance in all 3 vessel types studied and a significant cross-tolerance to the endothelium-dependent vasodilator acetylcholine in A. mammaria and A. radialis. Although soluble guanylyl cyclase, cGK-I, and VASP expression levels were not modified by NTG treatment, a marked decrease of P-VASP, a surrogate parameter for in-vivo cGK-I activity, was observed.

Conclusions—We conclude that long-term NTG treatment induces endothelial dysfunction and impaired vascular NO/cGMP signaling in humans, which can be monitored by measuring P-VASP levels. (Circulation. 2002;105:1170-1175.)

Key Words: nitroglycerin ■ endothelium ■ nitric oxide ■ bypass

Nitroglycerin are still widely used in the management of coronary artery disease (CAD), including in patients with stable and unstable angina, acute myocardial infarction, and congestive heart failure. Despite the beneficial hemodynamic and powerful anti-ischemic profile of nitroglycerin (NTG), the efficacy of this kind of treatment in patients with CAD remains disappointing. In fact, a recent meta-analysis even indicates that the long-term use of nitrates may be deleterious for patients with ischemic heart disease. At the cellular level, NTG is thought to cause vasorelaxation by releasing NO. NO, an endothelium-derived relaxing factor, activates the target enzyme soluble guanylyl cyclase (sGC) and increases tissue levels of the second messenger cGMP. cGMP in turn activates a cGMP-dependent protein kinase (cGK), which mediates vasorelaxation via phosphorylation of proteins that regulate intracellular Ca²⁺ levels and the cytoskeleton. Two major drawbacks of nitrate therapy were shown to be important, the rapid development of tolerance within 24 to 48 hours of continuous NTG treatment and the development of endothelial dysfunction during prolonged NTG treatment (so-called cross-tolerance). Although the precise mechanisms of tolerance and endothelial dysfunction in response to NTG therapy remain obscure, there is growing evidence that NTG-induced production of oxygen-derived free radicals such as superoxide plays an important role in mediating both phenomena. The oxidative stress concept of tolerance would well explain the tolerance and cross-tolerance phenomenon, because superoxide production stimulated by NTG therapy may inactivate the vasoactive compound released from NTG and may also inactivate NO released from the endothelium. Another important aspect of tolerance and cross-tolerance involves the effects of long-term nitrate therapy on the activity or expression of the cGK. Studies with cGK-I—
deficient mice and human cells demonstrated a complete disruption of the NO/cGMP-signaling pathway in vascular tissue.\textsuperscript{11} Therefore, the activity or expression of cGK-I may critically influence NTG-induced vasorelaxation. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at serine\textsuperscript{239} (P-VASP) has been shown to be a useful monitor for cGK-I activity in intact cells.\textsuperscript{12} In addition, we recently showed that the level of P-VASP in vascular tissue from hyperlipidemic and NTG-treated animals closely correlates with changes in endothelial function and vascular oxidative stress.\textsuperscript{13,14} Based on these observations, we hypothesized that in human tissue, P-VASP may also represent a novel biochemical marker for monitoring the NO-stimulated sGC/cGK-I pathway and endothelial integrity.

Based on these considerations, the present study was designed to (1) examine the consequences of in-vivo NTG treatment for tolerance and endothelial function in arteria mammaria, vena saphena, and arteria radialis from patients undergoing elective bypass surgery; (2) determine whether NTG-induced endothelial dysfunction is associated with changes in the activity and expression of the NO downstream targets sGC and cGK-I in these 3 vessels; and (3) test whether P-VASP can be assessed in human tissue such as A. mammaria.

## Methods

### Patient Selection

This investigation was a randomized study to determine whether long-term NTG therapy may cause tolerance and endothelial dysfunction in patients awaiting elective coronary bypass surgery for mild to moderate angina. Exclusion criteria were nitrate incompatibility, concomitant treatment with antioxidants such as vitamin E and C, and presence of significant valvular heart disease. The local ethics committee of Hamburg, Germany, approved this study. All patients involved in the study gave informed consent.

### Study Protocol

Patients were randomized to receive either NTG infusion (0.5 µg/kg per min) for 24 to 48 hours before bypass surgery (NTG group) or no nitrate therapy (control group). This NTG concentration was chosen because it is not only used in the treatment of acute coronary syndrome and acute myocardial infarction\textsuperscript{15} but also causes maximal dilation of large coronary arteries\textsuperscript{6} and induces tolerance within 2 to 3 days of continuous treatment.\textsuperscript{6} All other antianginal medication was continued unchanged, as summarized in the Table and Figures 1 through 3. Patients were withdrawn from the study when unstable angina developed, when significant hypotension (<90 mm Hg) or headache unresponsive to paracetamol occurred, or when surgery was postponed for other reasons. NTG treatment was continued until the beginning of surgery. During the operation, discarded segments of the A. mammaria, V. saphena, or A. radialis were collected.

### Vessel Preparation and Organ Chamber Studies

The vessels were cut into segments measuring 3 mm in length and placed in organ chambers (25 mL) filled with carbogen-equilibrated Krebs buffer and 10 µmol/L indomethacin. Resting tension was increased to optimize contractions to KCl (80 mmol/L), which was found to be 1 g for V. saphena, 2 g for A. mammaria, and 4 g for A. radialis. Vessels that did not respond to KCl with vasoconstriction were discarded.

To test endothelium-dependent and -independent vasodilators (acetylcholine [ACh] and NTG), vessels were preconstricted with phenylephrine to achieve 30% to 50% of maximal (KCl-induced) tone.

### Detection of cGK-I Protein Expression, cGK-I Activity, and P-VASP Protein Expression

A. mammaria segments from patients with and without NTG treatment were frozen and homogenized in liquid nitrogen. SDS-PAGE electrophoresis and electroblotting was performed as with sGC. Immunoblotting was performed with a polyclonal antibody

### Patient characteristics and vascular reactivity of A. mammaria without and with NTG-treatment

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>Control Group</th>
<th>NTG Group</th>
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<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>18</td>
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<td>10/5</td>
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<td>Cardiovascular risk factors</td>
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<td>age $\geq$ 65 y</td>
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<td>14</td>
</tr>
<tr>
<td>Hypertension</td>
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<td>2</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Smoking</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Extent of CAD, 1/2/3</td>
<td>1/5/33</td>
<td>0/5/27</td>
</tr>
<tr>
<td>Prior nitrate therapy</td>
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<td>5</td>
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<tr>
<td>Concomitant medication</td>
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<td></td>
</tr>
<tr>
<td>A.C.</td>
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<td>7</td>
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<tr>
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<td>7</td>
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<td>6</td>
</tr>
<tr>
<td>Statins</td>
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</table>

Values are number of patients or mean±SEM. ACE indicates angiotensin-converting enzyme.

![Figure 1](image)

Figure 1. Characteristics of patients with and without NTG treatment and relaxations of the A. mammaria tested in organ chambers in response to NTG and the endothelium-dependent vasodilator ACh. NTG treatment for 48 hours caused a marked degree of tolerance as well as endothelial dysfunction: *P<0.05 vs without NTG treatment. ACE indicates angiotensin-converting enzyme.
against cGK-I and a mouse monoclonal antibody (16C2) specific for P-VASP, as described recently. All P-VASP data are expressed as the P-VASP to VASP ratio, as previously described.

**Immunohistochemistry**

Briefly, excised tissue segments were incubated in Krebs buffer at 37°C for 10 minutes. The tissue segments were then embedded in OCT compound and frozen in a pentane/liquid nitrogen bath. Frozen tissues were cut at 6 μm and immediately fixed for 10 minutes in acetone at 20°C. Sections were allowed to air dry and were then rehydrated for 5 minutes in Tris buffer containing 0.05 mol/L Tris Base, 0.3 mol/L NaCl, and 0.1% Tween-20, pH 7.6. Nonspecific binding was blocked by incubating sections for 20 minutes with horse serum followed by either a 2-hour incubation at room temperature with the polyclonal antibody M4(1:100) to detect VASP or an overnight incubation at 4°C with the monoclonal antibody 16C2 (4 μg/mL) to detect P-VASP. Sections were stained using the Vectastain ABC kit (Vector Laboratories), coverslipped, and viewed under a light microscope.

**Oxidative Fluorescent Microtopography**

The oxidative fluorescent dye dihydroethidium (2 × 10^-6 mol/L) was used to evaluate the in situ concentration of superoxide in A. mammaria from patients with and without NTG treatment, as described recently.

**Statistical Analysis**

Results are expressed as mean±SEM. The ED_{50} value for each experiment was obtained by logit transformation. To compare P-VASP and VASP, cGK-I, and sGC expression in vessels with and without NTG treatment, one-way ANOVA was used. Comparisons of vascular responses were performed using multivariate ANOVA. A Scheffe’s post hoc test was used to examine differences between groups when significance was indicated. P<0.05 was considered significant.

**Results**

**Patient Characteristics**

The clinical characteristics of all patients participating in the study are summarized in the Table. There were no differences between the groups with respect to age, sex, prior nitrate therapy, extent of CAD, concomitant therapy, or presence of coronary risk factors (Table and Figures 1 through 4).

**Studies of Vascular Reactivity**

**NTG Responses**

In all 3 vessel types studied, NTG caused a dose-dependent relaxation. Most sensitive to NTG-induced relaxation was the A. radialis>A. mammaria>V. saphena (Figures 1 through 3). NTG treatment caused a rightward shift of the NTG dose-response relationship, compatible with nitrate tolerance. The degree of tolerance was most striking in A. radialis>A. mammaria, whereas the changes in endothelial function observed in V. saphena>A. mammaria (Figures 1 through 3).

**Acetylcholine Responses**

In all 3 vessel types studied, ACh caused a dose-dependent relaxation that was most potent in A. radialis>A. mammaria>V. saphena (Figures 1 through 3). Treatment with NTG caused a significant degree of endothelial dysfunction (as indicated by the rightward shift of the ACh dose-response relationship) in A. radialis>A. mammaria, whereas the changes in endothelial function observed in V. saphena did not reach a significant level.
Effects of NTG Treatment on sGC, cGK-I, and VASP Protein Expression

NTG treatment did not modify the protein expression of soluble sGC, cGK-I, and VASP in A. mammaria (Figures 4 and 5). VASP appears as a double band (between 45 and 50 kDa) because of the partial phosphorylation at serine 157, the cAMP-dependent protein kinase A (cAK) preferred site.

Influence of In Vivo NTG Treatment on P-VASP Levels

As previously demonstrated for animal tissues, we demonstrate here that endothelial removal as well as incubation with the NOS III inhibitor L-NNA drastically reduces P-VASP levels in human A. mammaria (data not shown). In contrast to its lack of effect on VASP protein expression, in vivo treatment of patients with NTG caused a significant drop in the level of P-VASP and therefore in the P-VASP/VASP ratio in A. mammaria (Figure 5).

Immunohistochemistry and Oxidative Fluorescent Microtopography

Immunohistochemical analysis of P-VASP in vessels from NTG-treated patients was markedly reduced compared with P-VASP in vessels from patients without NTG treatment (Figures 6A and 6B). Furthermore, VASP staining with the common VASP antibody M4 was similar in both control and NTG-treated A. mammaria groups (Figures 6C and 6D). Interestingly, reductions in P-VASP were closely related to increases in superoxide production in these vessels, as indicated by the strong ethidium staining (Figures 6E to 6H).

Discussion

The present study shows that continuous NTG treatment for 2 days of patients undergoing elective bypass surgery caused significant endothelial dysfunction in A. mammaria and A. radialis. This was accompanied by a marked reduction of the P-VASP/VASP ratio despite normal cGK-I and VASP expression reflecting inhibition of cGK-I activity. These findings indicate that the determination of P-VASP levels may be a novel indicator of both cGK-I activity and endothelium integrity under physiological and pathophysiological conditions in human tissue.

Phenomenon of Nitrate Tolerance

Nitrates are still widely used in the management of CAD and congestive heart failure. Despite potent anti-ischemic and vasodilator properties when given over a short duration, the long-term efficacy is questioned because of the rapid development of nitrate tolerance, which occurs within 1 to 3 days of continuous treatment and has been shown to occur in patients with acute myocardial infarction, chronic ischemic heart disease, and chronic congestive heart failure.

The present study confirms these observations. In patients without NTG pretreatment, NTG caused a dose-dependent relaxation in all 3 vessels types, being most potent in A. radialis compared with V. saphena. Continuous treatment with NTG for 2 days, however, caused a significant degree of tolerance in all 3 vessel types studied. Interestingly, the degree of tolerance was markedly higher in A. radialis compared with V. saphena and A. mammaria.
The NTG concentration for long-term treatment averaged ≈0.5 μg/kg per minute. This particular NTG dose was chosen because it is clinically used in patients with unstable angina and acute myocardial infarction,15 causes maximal dilation of large epicardial arteries,6 and induces tolerance in large epicardial arteries within 2 to 3 days6 of continuous treatment.

Do Nitrates Cause Endothelial Dysfunction?

Another side-effect related to long-term nitrate therapy is the induction of endothelial dysfunction. This has been observed most commonly in situations where NTG was administered chronically in vivo in experimental animal models.7,16,17 Whether nitrate therapy may adversely affect endothelial function in humans remains controversial. Recent studies performed by Parker and colleagues indicate that long-term transdermal NTG treatment causes endothelial dysfunction in patients with CAD9 as well as in healthy volunteers,18 whereas others failed to detect any effect of NTG therapy on endothelial function.19 The treatment period in the studies by Caramori et al9 and Gori et al18 was 5 and 6 days, respectively, and the NTG concentrations averaged 0.1 μg/kg per min. ACh-induced large coronary artery constriction as well as flow responses of the brachial artery to ACh were used as surrogate parameters for endothelial (dys)function. Continuous treatment with NTG patches for 5 to 6 days resulted in a marked inhibition of ACh-induced increases in forearm blood flow18 as well as an increased ACh-induced coronary constriction9 compared with the respective control group without NTG pretreatment. In addition, in the forearm study, the L-NMMA-induced reduction in forearm blood flow was significantly blunted in volunteers treated with NTG. On the basis of these findings, the authors concluded that NTG treatment has an inhibitory effect on basal as well as stimulated vascular NO bioavailability and that this is, at least in part, because of abnormalities in NOS III function.18

The present data clearly indicate that endothelial dysfunction is also encountered in both types of arteries studied, the A. mammaria and the A. radialis. Interestingly, the vessel that developed the highest degree of tolerance showed the highest degree of endothelial dysfunction.

Although the mechanisms underlying tolerance and NTG-induced endothelial dysfunction are still obscure, there is a growing body of evidence suggesting that long-term NTG therapy stimulates the production of oxygen-derived free radicals such as superoxide.7 This concept is supported by the demonstration that dihydrothidine staining (reflecting increased superoxide production) is drastically increased in vessels from patients treated with NTG (Figure 6). NO released from NTG may react with superoxide to form the highly reactive intermediate peroxynitrite.20 Peroxynitrite in turn may cause a reduction of vascular NO production by tyrosine nitration of NOS III or may even switch NOS III from a NO- to a superoxide-producing enzyme by oxidizing the critical NOS cofactor tetrahydrobiopterin.21 This concept has recently been confirmed by studies with chronically NTG-treated volunteers, whose endothelial dysfunction as well as nitrate tolerance was corrected by applying folic acid, a compound that restores NOS III function by increasing depleted intracellular BH₄ levels.22

The fact that NTG therapy indeed stimulates superoxide production in human tissue was recently demonstrated by Sage et al19 in patients undergoing bypass surgery. However, the authors failed to demonstrate any cross-tolerance to endothelium-dependent and -independent vasodilators. Also, in vitro modulation of vascular superoxide production did not modify the NTG dose-response relationship. In addition, they established a decreased tissue content of 1,2 glyceryl dinitrate in tolerant tissue, which was used as an argument to conclude that impaired NTG biotransformation specifically accounts for tolerance and that endothelial function is preserved.19 Although differences in the duration of NTG treatment (1 versus 2 days) and differences in the chosen NTG concentration for long-term infusion (0.15 versus 0.5 μg/kg per min) make it difficult to compare our study with the study from Sage et al,19 the recent demonstration of cross-tolerance to endothelium-dependent vasodilators in coronary arteries9,22 and the results of the present study as well as the studies by Gori et al18,22 challenge the concept that impaired NTG biotransformation is the sole reason for nitrate tolerance. In addition, animal experiments recently failed to demonstrate that NTG biotransformation is impaired in tolerant vascular tissue.23

P-VASP, a Novel Biochemical Surrogate Parameter of Endothelial (dys)function and NO/cGMP Signaling in Human Blood Vessels

It is now well established that the NO-cGMP pathway is a key regulator of vascular tone and that cGK-I mediates many of these NO/cGMP effects.4 Studies with cGK-I–deficient human cells and mice demonstrated that cGK-I ablation disrupts the NO/cGMP pathway in vascular cells and tissues.11,24 Gene-targeted loss of murine cGK-I abolished NO/cGMP-dependent relaxation of smooth muscle, resulting in severe vascular and intestinal dysfunctions, whereas cAMP-mediated smooth muscle relaxation was not impaired.11 These recent developments highlight the importance of assessing cGK expression or cGK activity in the setting of endothelial dysfunction. For this purpose, we studied the phosphorylation of VASP at serine239 to monitor cGK-I activity.12 VASP is a well-characterized substrate for cGK-I and cAK in platelets and endothelial and vascular smooth muscle cells.25 It is phosphorylated at 3 distinct sites (serine157, serine239, and threonine278) by both cGK-I and cAK with overlapping specificity and efficiency.12 Because cAK and cGK-I preferentially phosphorylate VASP at serine157 and serine239, respectively, the activation of cAK and cGK can be measured by specific monoclonal antibodies directed against differently phosphorylated VASP forms.12 Importantly, experiments with cGK-I–deficient vascular model systems have recently established that NO donor–induced VASP phosphorylation is primarily mediated by cGK-I.26

In the present study, we found significant levels of P-VASP in A. mammaria from patients without NTG treatment. Furthermore, there was a marked reduction in the P-VASP/VASP ratio but not of total VASP levels in A. mammaria from NTG-treated patients compared with con-
trols (Figure 5), as assessed with Western blotting technique and immunohistochemical studies (Figure 6). The presence of P-VASP in A. mammaria of untreated patients suggests that endogenous cGMP-elevating vasodilators (presumably NO) maintain a certain level of P-VASP in the vascular wall. Clearly, the decrease in P-VASP induced by long-term NTG treatment was not attributable to decreased availability of this cGK-I substrate, because total VASP expression at the protein level was not different in tolerant and nontolerant tissue. In addition, we found no changes in the expression of sGC and cGK-I. These findings indicate that the NO/cGMP pathway is functionally inhibited. Studies with the oxidative fluorescent dye dihydroethidine revealed that superoxide levels in tolerant tissue were markedly increased compared with controls, suggesting that NTG-induced increases in vascular superoxide production likely account for the observed decreases in P-VASP. This observation in human tissue is in complete agreement with results from previous experimental studies where tolerance was associated with a marked decrease in P-VASP whereas the expression of cGK-I, similar to the present study, was not modified at all. The concept that oxidative stress may, at least in part, be involved in the tolerance and cross-tolerance phenomenon is additionally strengthened by our previous observation that in vitro incubations with vitamin C as well as in vivo vitamin C treatment partially reversed tolerance, reduced vascular superoxide, and subsequently raised P-VASP levels.

Conclusions

The presented data show that NTG-induced tolerance and cross-tolerance and increases in oxidative stress were associated with a significant decrease in P-VASP. These findings indicate that endothelial (dys)function can be biochemically assessed by the determination of P-VASP levels in human arteries and veins. This analysis may be a useful tool in the future to monitor the functional integrity of the NO/cGMP effector pathway in human vascular tissue.

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References

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