Nitroglycerin Tolerance in Human Vessels
Evidence for Impaired Nitroglycerin Bioconversion

Peter R. Sage, MBBS, FRACP; Ivan S. de la Lande, DSc; Irene Stafford, BSc;
Catherine L. Bennett, BSc (Hons); George Phillipov, PhD;
John Stubberfield, MBBS, FRACS; John D. Horowitz, MBBS, PhD

Background—The basis for progressive attenuation of the effects of organic nitrates during long-term therapy (nitrate tolerance) remains controversial; proposed mechanisms include impaired nitrate bioconversion resulting in decreased release of nitric oxide (NO) from nitrates and/or increased NO clearance through a reaction with incrementally generated superoxide (O2−).

Methods and Results—Patients undergoing elective coronary artery bypass were randomized to receive 24 hours of intravenously infused nitroglycerin (NTG; nitrate group) or no nitrate therapy (control group). Discarded segments of the internal mammary artery and saphenous vein were used to examine (1) vascular responsiveness to NTG, sodium nitroprusside, and the calcium ionophore A23187; (2) bioconversion of NTG to 1,2- and 1,3-glyceryl dinitrate; and (3) the generation of O2−. Responses to NTG were reduced 3- to 5-fold in vessels from the nitrate group compared with control vessels (P<0.01 for both types of segments), whereas responses to sodium nitroprusside and A23187 were unchanged. Tissue content of 1,2-glyceryl dinitrate was lower (P<0.01) in the saphenous veins from the nitrate group than in those from the control group. O2− generation was greater (P<0.01) in internal mammary artery samples from the nitrate group than in those from the control group. However, incremental O2− generation induced by an inhibitor of superoxide dismutase did not affect NTG responses.

Conclusions—NTG tolerance in patients with coronary artery disease is nitrate-specific and is associated with evidence of impaired NTG bioconversion. Tolerance was associated with incremental O2− generation, but short-term elevation of O2− did not affect NTG responsiveness, suggesting increased NO clearance by O2− has a minimal contribution to tolerance. (Circulation. 2000;102:2810-2815.)

Key Words: nitroglycerin ■ nitrate tolerance ■ superoxide

Long-term therapy with most, if not all, organic nitrates is frequently associated with a progressive reduction of hemodynamic and antiaggregatory effects (a phenomenon termed nitrate tolerance).1,2 This imposes the major limitation of efficacy on nitrate therapy for stable angina pectoris, congestive heart failure, and acute myocardial infarction. This attenuation of hemodynamic effects may be due in part to neurohumoral mechanisms opposing nitric oxide (NO)-mediated vascular relaxation3 (pseudotolerance) and in part to a diminished NO effect4,5 (true tolerance). The mechanism(s) responsible for true tolerance remain controversial. Multiple theories have been proposed,6 but the major categories are (1) impaired nitrate bioconversion resulting in diminished NO release5,7 and (2) increased NO clearance mediated by the incremental generation of superoxide (O2−).8 The supporting evidence for these mechanisms has been derived almost entirely from animal studies; definitive evidence from studies in human subjects is lacking.

We now report an investigation of possible mechanisms of the induction of true tolerance to nitroglycerin (NTG) in human vessels, with an assessment of the following: (1) the extent of cross-tolerance between NTG and non-nitrate sources of NO, (2) changes in the nitrate bioconversion process associated with tolerance induction, and (3) the possible augmented generation of vascular O2− and its relationship with vascular responsiveness to NTG.

Methods

Patient Selection

The investigation was a randomized study of potential in vivo nitrate tolerance induction in patients awaiting elective coronary bypass surgery for mild to moderate angina. Exclusion criteria were con-
TABLE 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Nitrate Group</th>
</tr>
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<tbody>
<tr>
<td>(n=15)</td>
<td>(n=15)</td>
<td></td>
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<tr>
<td>Age, y</td>
<td>56±3</td>
<td>64±3</td>
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<tr>
<td>Sex, male/female</td>
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<td>10:5</td>
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<tr>
<td>Extent of CAD, 1:2:3</td>
<td>0:7:8</td>
<td>1:6:8</td>
</tr>
<tr>
<td>Conduit used, IMA:SV</td>
<td>12:11</td>
<td>13:9</td>
</tr>
<tr>
<td>Prior nitrate therapy</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Concomitant therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Adrenoceptor antagonist</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>L-Calcium channel blocker</td>
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<td>5</td>
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<tr>
<td>ACE inhibitor</td>
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<td>6</td>
</tr>
<tr>
<td>Statin</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Coronary risk factors</td>
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<tr>
<td>Hypercholesterolemia</td>
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<td>10</td>
</tr>
<tr>
<td>Hypertension</td>
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<td>8</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Diabetes</td>
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<tr>
<td>Family history</td>
<td>7</td>
<td>4</td>
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</table>

Values are no. of patients or mean±SEM. CAD indicates coronary artery disease.

Vascular Reactivity Studies

Segments were suspended under tension in 15-mL organ baths containing Krebs solution at 37°C. IMA segment resting tension was normalized for internal diameter, as previously described.9 Mean resting tension was 2.03 g and 2.04 g for control and tolerant IMA segments, respectively. SV segment resting tension was set at 1 g because this tension gave optimal contractions to KCl solution (120 mmol/L) in preliminary experiments. The segments were equilibrated for 60 minutes before exposure to KCl solution; segments contracting <1 g were discarded. After a further 30 minutes of washout, the segments were contracted with increasing concentrations of norepinephrine (0.01 to 10 μmol/L). Preliminary studies had revealed that blocking β-adrenoceptors and catecholamine uptake with 10 μmol/L propranolol and 30 nmol/L desipramine, respectively, potentiated responses to norepinephrine in SV but not IMA segments; these agents were routinely added to SV segments 15 minutes before exposure to norepinephrine. After a further 45 minutes of washout, the segments were preconstricted with norepinephrine to produce 70% of maximum tension in the IMA (1.1±0.1 μmol/L in both groups) and 50% of maximum tension in the SV (0.4±0.1 μmol/L in both groups). Once the contractile response had reached a plateau, each segment was exposed to increasing concentrations of NTG (0.001 to 10 μmol/L and 0.01 to 10 μmol/L for IMA and SV segments, respectively), sodium nitroprusside (SNP; 0.001 to 10 μmol/L and 0.01 to 10 μmol/L for IMA and SV segments, respectively), or the calcium ionophore A23187 (0.01 to 3 μmol/L for both IMA and SV). All segments were then assessed for endothelium-dependent relaxation; segments unresponsive to A23187 were not used for analysis. Time from harvest of vessels to assessment of relaxant responses was held constant at 3 hours and 15 minutes.

NTG Bioconversion Studies

NTG bioconversion studies were conducted only in SV segments because of limited tissue availability. Segments were weighed and equilibrated in Krebs at 37°C. At 3 hours after harvest, 1 segment was placed into 1 mL of Krebs at 37°C and incubated with 1 μmol/L NTG (final concentration) for 2 minutes. The segment was then rinsed for 5 s in ice-cold Krebs before snap-freezing in liquid nitrogen. A control segment was incubated with NTG vehicle but otherwise treated identically. SV segments were stored at −80°C until assay.

NTG and its dinitrate metabolites (1,2- and 1,3-glyceryl dinitrate [GDN]) were extracted from SV segments using a modification of the 2-step extraction procedure of Bennett et al.10 Frozen SV segments were placed in glass extraction tubes containing internal standard (1,4-dimethoxy-butan-2-ol) and extracted twice with 5 mL of hexane to selectively isolate the NTG; hexane extracts were then combined. The SV was then extracted with 5 mL of methyl tert-butyl ether containing the internal standard to isolate the 1,2- and 1,3-GDN. Organic phases were dried with anhydrous sodium sulfate, concentrated to ~200 μL under a stream of nitrogen, and stored at −20°C until assay. Standard curves were prepared by spiking 1 mL of Krebs with NTG (1 to 50 pmol), 1,2-GDN (0.4 to 10 pmol), and 1,3-GDN (0.1 to 5 pmol) and extracted as for SV.

NTG, 1,2-GDN, and 1,3-GDN were separated using a Varian 3300 gas chromatograph with an electron capture detector and a BP-1 (100% dimethyl polysiloxane) capillary column (25 m×0.53 m internal diameter; 1 μm of film) from SGE. Column temperature was programmed at 125°C; this was followed by thermal cleaning between each 1-μL injection. The injector temperature was 250°C. Hydrogen was the carrier gas (4 mL/minute), and nitrogen was the makeup gas (20 mL/minute). Under these conditions, the retention times for NTG, 1,2-GDN, 1,3-GDN, and the internal standard were 6.2, 6.5, 7.9, and 9.3 minutes, respectively. Peak height ratios for NTG, 1,2-GDN, and 1,3-GDN to internal standard were used for quantitation. The intra-assay coefficient of variation was ≤11% (n=5) for NTG, 1,2-GDN, and 1,3-GDN at concentrations of 1, 0.4, and 0.1 pmol, respectively, and the interassay assay coefficient of variation was <10% (n=9) at concentrations of 20, 4, and 2 pmol, respectively.

O$_2^-$ Generation

O$_2^-$ generation studies were performed only in IMA segments because preliminary investigations revealed a greater variability within control SV segments (mean±SEM, 48±10 counts·min$^{-1}$·mg$^{-1}$; n=10) than in IMA segments (mean±SEM, 32±4 counts·min$^{-1}$·mg$^{-1}$; n=10) for luminescence counts. Further patients (n=21) were randomized as described above, and IMA segments were obtained during the operation to determine O$_2^-$ generation via lucigenin-enhanced chemiluminescence, as described by Ohara et al.11 The segment was first equilibrated in Krebs solution at 37°C and then equilibrated in Krebs-HEPES buffer at 37°C for 30 minutes. It was then placed in 0.5 mL of Krebs-HEPES buffer containing 250 μmol/L lucigenin at 37°C in a Picolite luminometer (Packard), and luminescence counts were measured every minute for 15 minutes. The segments were then weighed. Background counts were determined for 15 minutes before adding the segment; they were then subtracted from the luminescence counts, and the results are ex-
pressed as count \cdot \text{min}^{-1} \cdot \text{mg}^{-1}. Results reported are the mean for 2 segments from each patient measured at 90 and 120 minutes after harvest. High concentrations of lucigenin were recently shown to contribute to \( O_2 \) generation (Figures 1B and 1C; Table 2).

### Materials

\((\pm)\)Arterenol bitartrate salt, the calcium ionophore A23187, DETCA, desipramine hydrochloride, HEPES sodium salt, bis-N-methylacridinium nitrate (lucigenin), dl-propranolol hydrochloride, TCA, desipramine hydrochloride, HEPES sodium salt, bis-N-

### Results

#### Patient Characteristics

The clinical characteristics of patients studied in the experiments on vascular reactivity and NTG bioconversion are summarized in Table 1. The groups were well balanced; similar numbers of patients in each group were on ACE inhibitors and nitrates before entry into the study and, at operation, similar numbers of each bypass conduit were used in each group.

Patient characteristics of the subgroup randomized to examine \( O_2 \) generation (n=21) were similar to those of the initial randomized cohort; again, no significant differences existed between control and nitrate groups. Specifically, no disparity existed regarding prior nitrate therapy, ACE inhibitor or statin therapy, or hypercholesterolemia.

#### Studies of Vascular Reactivity

##### Vasoconstrictor Responses

Vasoconstrictor responses to either KCl solution or norepinephrine did not differ between segments from the control and nitrate groups (data not shown).

##### Vasodilator Responses

In both IMA and SV segments, relaxations to NTG, SNP, and A23187 were characterized by sigmoid concentration-response curves.

#### Comparison of IMA and SV

SV segments were less sensitive than IMA segments to NTG. SV segments were also less responsive than IMA segments to

### Table 2. Reactivity of IMA and SV segments to NTG, SNP, and A23187

<table>
<thead>
<tr>
<th></th>
<th>No. of Subjects</th>
<th>log EC50, mol/L</th>
<th>( E_{max} ), %</th>
</tr>
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<tr>
<td></td>
<td>Control</td>
<td>Nitrates</td>
<td>Control</td>
</tr>
<tr>
<td>IMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG</td>
<td>9</td>
<td>10</td>
<td>−7.7±0.1</td>
</tr>
<tr>
<td>SNP</td>
<td>8</td>
<td>8</td>
<td>−7.0±0.1</td>
</tr>
<tr>
<td>A23187</td>
<td>8</td>
<td>8</td>
<td>−7.5±0.2</td>
</tr>
<tr>
<td>SV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG</td>
<td>9</td>
<td>8</td>
<td>−7.0±0.1</td>
</tr>
<tr>
<td>SNP</td>
<td>8</td>
<td>7</td>
<td>−6.8±0.2</td>
</tr>
<tr>
<td>A23187</td>
<td>7</td>
<td>6</td>
<td>−7.4±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*P<0.01 for control group vs nitrate group.
A23187; the maximum relaxation was 79% in IMA segments but only 42% in SV segments (Table 2).

**NTG Bioconversion Studies: SV**

NTG bioconversion was studied in SV segments from 8 patients in the control group and 7 patients in the nitrate group. Mean segment weight (36±7 versus 37±9 mg) and NTG content (0.54±0.06 versus 0.50±0.07 pmol/mg) were similar in both groups.

Tissue content of 1,2-GDN was much greater than that of 1,3-GDN in both groups (Figure 2). Tissue content of 1,2-GDN was significantly lower in segments from the nitrate group compared with control segments (0.10±0.01 versus 0.16±0.02 pmol/mg; P=0.012), but 1,3-GDN content was similar in both groups (Figure 2).

**O$_2^-$ Generation: IMA**

Chemiluminescence counts using 250 μmol/L lucigenin were ≈70% greater (P<0.01) in the segments from the nitrate group compared with control segments (Figure 3). With 10 μmol/L lucigenin, chemiluminescence counts were significantly (P<0.02) reduced relative to 250 μmol/L lucigenin, but the proportional difference between the nitrate and control groups remained similar (20±3 versus 9±1 count·min⁻¹·mg⁻¹; n=5 each group, P<0.01).

**Effects of Increased O$_2^-$ Generation**

Incubation of IMA segments with DETCA resulted in an ≈3-fold increase in chemiluminescence counts over control segments (P<0.01; Figure 4A). However, relaxation of IMA segments with NTG was unchanged (Figure 4B).

**Discussion**

The current study is the first investigation of the relative contribution(s) of impaired nitrate bioconversion and incremental O$_2^-$ generation to the induction of NTG tolerance in human vessels. The results can be summarized as follows. (1) Twenty-four hours of therapy with intravenously infused NTG at 10 μg/min in patients with stable angina induces a moderate degree of tolerance to NTG in both the IMA and SV (5-fold and 3-fold reductions in sensitivity, respectively), which is not accompanied by significant cross-tolerance to 2 non-nitrate sources of NO (SNP or A23187). (2) In the tolerant SV, NTG bioconversion to 1,2-GDN is impaired, but bioconversion to 1,3-GDN is unchanged. (3) Tolerance induction in the IMA is associated with a significant increase in O$_2^-$ generation, as measured by lucigenin-enhanced chemiluminescence. However, increasing O$_2^-$ generation by inhibiting endogenous superoxide dismutase with DETCA has no effect on the relaxation of the IMA by NTG.

Nitrate tolerance in the IMA was previously induced by Du et al after more prolonged prior nitrate therapy. Boesgaard et al demonstrated attenuation of the NTG effect on venous volume in humans after a 23-hour infusion of NTG at a rate slightly lower than that used in this study, although this may have included some component of pseudotolerance. Ex vivo tolerance to NTG in isolated human SVs has not previously
been reported. Thus, an important implication of the present observations is that some induction of true tolerance to NTG is inevitable within 24 hours, even at an infusion dose that is at the lower end of most therapeutic protocols.

The role of impaired bioconversion in tolerance induction has previously been investigated largely by 2 methods, which assess either (1) the extent of cross-tolerance to other NO-mediated vasodilators that do not use the nitrate bioconversion pathway\(^\text{15}\) or (2) NTG metabolism.\(^\text{7,16}\) The current study used both methods to determine the contribution of impaired NTG bioconversion to true tolerance in human vessels.

Cross-tolerance to non-nitrate sources of NO has been studied in many in vitro\(^\text{7,17–22}\) and in vivo\(^\text{5,8,23–26}\) animal models of tolerance induction. Most\(^\text{7,17–21}\) in vitro pharmacological studies on isolated vessels have shown minimal cross-tolerance to non-nitrate NO sources. However, the relevance of in vitro studies has been questioned because they exclude some factors that may be of importance to tolerance induction in vivo.\(^\text{3}\) The data from in vivo animal studies are conflicting. Some studies using isolated vessels\(^\text{21,23}\) or hemodynamic indices\(^\text{24,25}\) have demonstrated that tolerance induction is associated with no cross-tolerance to non-nitrate sources of NO. However, other\(^\text{8,26}\) have reported varying degrees of cross-tolerance. By comparison, cross-tolerance data in humans are limited. Studies examining cross-tolerance using in vivo tolerance induction cannot assess the relative contributions of true tolerance and pseudotolerance.\(^\text{27}\) To our knowledge, the only previous study on isolated vessels from tolerant subjects\(^\text{4}\) found no cross-tolerance to SNP or acetylcholine. Similarly, ex vivo studies of NTG tolerance induction at the level of platelet aggregation in human subjects showed no cross-tolerance between NTG and SNP.\(^\text{5}\) The lack of significant cross-tolerance to other NO-mediated vasodilators that was found in the current study is therefore in agreement with the few previous analogous human studies.

The current study demonstrates for the first time in humans that true NTG tolerance is associated with an impairment of the enzymatic bioconversion of NTG to 1,2-GDN. NTG is a prodrug that undergoes largely enzymatic bioconversion to yield dinitrates (1,2-GDN or 1,3-GDN) and NO, which is responsible for its pharmacological effects. Evidence from animal studies\(^\text{28,29}\) suggests that the conversion of NTG to 1,2-GDN and NO predominates in vascular tissue, thus representing the mechanism-based pathway responsible for the generation of NO. Consistent with this, we found that levels of 1,2-GDN were much higher than 1,3-GDN in both control and tolerant SV segments exposed to NTG. Animal studies have also demonstrated that NTG to 1,2-GDN bioconversion is impaired in association with tolerance induction in vitro\(^\text{29}\) or in vivo.\(^\text{7,16}\) Furthermore, nitrate tolerance in intact rabbits is associated with diminished NO generation from organic nitrates but not from 3-morpholino-sydnonimine, a direct NO donor.\(^\text{5}\) The results of the current study demonstrate that a similar process occurs in human vessels in vivo. Together, the lack of cross-tolerance to non-nitrate sources of NO and the impairment of the bioconversion of NTG to 1,2-GDN strongly support the primary role of impaired bioconversion in true tolerance to NTG in human vessels.

An alternative theory proposed recently\(^\text{8}\) is that NTG tolerance involves increased clearance of NO via increased O\(_2^-\), the latter being generated primarily as a result of angiotensin II-induced activation of reduced nicotinamide adenine dinucleotide oxidase.\(^\text{30}\) Furthermore, incremental O\(_2^-\)generation has recently been implicated in the pathogenesis of the de novo phenomenon nitrate resistance.\(^\text{31}\) For this reason, we explored the role of O\(_2^-\) with regard to tolerance induction in human vessels. The measurement of lucigenin-enhanced chemiluminescence with both 250 and 10 \(\mu\)mol/L lucigenin suggested that tolerant IMA segments had a greater potential for O\(_2^-\) generation than control segments; the difference was similar to that reported previously by Münzel et al\(^\text{8}\) in rabbit aortae. These experiments also confirmed the previous observation in some\(^\text{12}\) but not all\(^\text{32}\) studies that the higher concentration of lucigenin contributes to chemiluminescence via redox cycling.

The findings of intact responses to non-nitrate sources of NO, despite increased O\(_2^-\) generation in tolerant vessels, prompted the experiments using DETCA (1 mmol/L). Despite increasing O\(_2^-\) generation in the IMA \(\approx\) 3-fold, DETCA had no effect on NTG responses. This finding was in conflict with previous studies in bovine coronary arteries\(^\text{13}\) and rabbit aortae.\(^\text{33}\) However, both previous studies used a 10-fold higher concentration of DETCA (10 mmol/L). We found the latter concentration inhibited contractile responses, as did Omar et al,\(^\text{13}\) suggesting a more nonspecific impairment of cellular function. Furthermore, other investigators\(^\text{34,35}\) have also reported unchanged NTG responses after short-term...
elevation of O$_2^-$ concentrations. The results of the experiments with DETCA, together with the absence of cross-tolerance to non-nitrate sources of NO, suggest that increased NO inactivation by O$_2^-$ has a minimal effect.

This study has several limitations. First, we cannot exclude the possibility that mechanisms of tolerance may vary with the extent of tolerance induction. We examined only 1 NTG dosing regimen and induced only a moderate degree of tolerance. The previous animal studies that showed significant cross-tolerance to non-nitrate NO sources have, in general, used higher NTG regimens and reported greater degrees of tolerance. Moreover, in this model, we did not observe any evidence of increased sensitivity to vasococontractor agents in the tolerant segments; this phenomenon, which is related to pseudotolerance, may have become manifest with other tolerance-induction regimens. Critically, the results do not completely exclude a role for the observed increase in O$_2^-$ generation in tolerance induction; it is not possible only that more prolonged elevation of O$_2^-$ generation might affect NO clearance, but also that nitrate bioconversion might be redox-sensitive.

In summary, tolerance remains the major limitation to the clinical utility of organic nitrates. The results of this study should provide impetus to future investigations using nitrate bioconversion as an index of tolerance induction and should further stimulate attempts to identify the sulfhydryl-dependent enzyme system responsible for the bioconversion of NTG in vascular smooth muscle, with the expectation that the acceleration of bioconversion will provide a means to circumvent tolerance.

Acknowledgments

This work was supported by grants from the National Health and Medical Research Council of Australia. Dr Sage is a recipient of a National Heart Foundation of Australia Postgraduate Research Scholarship.

References

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_Circulation_. 2000;102:2810-2815
doi: 10.1161/01.CIR.102.23.2810
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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