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 (O$_2^-$).

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 O$_2^-$.

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 ($P<0.012$) in the saphenous veins from the nitrate group than in those from the control group. O$_2^-$ generation was greater ($P<0.01$) in internal mammary artery samples from the nitrate group than in those from the control group. However, incremental O$_2^-$ 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 O$_2^-$ generation, but short-term elevation of O$_2^-$ did not affect NTG responsiveness, suggesting increased NO clearance by O$_2^-$ 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). 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 relaxation (pseudotolerance) and in part to a diminished NO effect (true tolerance). The mechanism(s) responsible for true tolerance remain controversial. Multiple theories have been proposed, but the major categories are (1) impaired nitrate bioconversion resulting in diminished NO release and (2) increased NO clearance mediated by the incremental generation of superoxide (O$_2^-$). 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 O$_2^-$ 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 (n=15)</th>
<th>Nitrate Group (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56±3</td>
<td>64±3</td>
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<td>Sex, male/female</td>
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<td>10:5</td>
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<td>Extent of CAD, 1:2:3</td>
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<td>1:6:8</td>
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<tr>
<td>Conduit used, IMA:SV</td>
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<td>13:9</td>
</tr>
<tr>
<td>Prior nitrate therapy</td>
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<td>7</td>
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<tr>
<td>Concomitant therapy</td>
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</tr>
<tr>
<td>β-Adrenoceptor antagonist</td>
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<tr>
<td>L-Calcium channel blocker</td>
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<td>Statin</td>
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<td>4</td>
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<td>Coronary risk factors</td>
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<td>Hypercholesterolemia</td>
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<tr>
<td>Hypertension</td>
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<tr>
<td>Diabetes</td>
<td>2</td>
<td>3</td>
</tr>
<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.8 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 mMol/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.

O2− Generation O2− 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 O2− 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·min\(^{-1}\)·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 \(O_2^\cdot\) generation via redox cycling\(^{12}\); to exclude distortion of the results due to this artifactual generation, a limited number of additional experiments were also performed using 10 \(\mu\)mol/L lucigenin.

### Effects of Increased \(O_2^\cdot\) Generation

The effect of inhibiting endogenous superoxide dismutase with diethyldithiocarbamic acid (DETCA)\(^{13}\) on \(O_2^\cdot\) generation and relaxant responses to NTG was studied using IMA segments obtained from nonrandomized patients on no nitrate therapy (n=5). Segments were allocated to study either \(O_2^\cdot\) generation or NTG reactivity (performed in simultaneous experiments) in a manner identical to that described above. Before measuring \(O_2^\cdot\) generation or determining the relaxant response to NTG, some segments were exposed to 1 mmol/L DETCA for 30 minutes followed by 30 minutes of washout. Other segments were incubated with DETCA vehicle only.

### Statistics

Results are expressed as mean±SEM. Vascular relaxant responses were compared using the parameters concentration eliciting half the maximum response (EC\(_{50}\)) expressed in log units and maximum response (E\(_{\text{max}}\)) with an unpaired t test. NTG and dinitrate content are expressed in pmol/mg and were compared using an unpaired t test. Lucigenin-enhanced chemiluminescence is expressed as count·min\(^{-1}\)·mg\(^{-1}\) and was compared using an unpaired t test. DETCA studies were analyzed using a paired t test.

### Materials

(±)-)Arterenol bitartrate salt, the calcium ionophore A23187, DETCA, desipramine hydrochloride, HEPES sodium salt, bis-N-methylacridinium nitrate (lucigenin), dl-propranolol hydrochloride, and SNP were purchased from Sigma. NTG was purchased from NTG Chemicals. NaCl 118, KCl 4.69, CaCl\(_2\) 1.87, MgCl\(_2\) 1.20, K\(_2\)HPO\(_4\) 1.03, NaHCO\(_3\) 25, Na-HEPES 20, and glucose 11.1 for Krebs-HEPES (pH 7.4).

### 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 \(O_2^\cdot\) 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

#### Vasocostrictor Responses

Vasocostrictor 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.

#### IMA Segments

In the initial randomized cohort, no significant differences existed between control and nitrate groups. Specifically, no disparity existed regarding prior nitrate therapy, ACE inhibitor or statin therapy, or hypercholesterolemia. 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 \(O_2^\cdot\) 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.

#### SV Segments

Similarly, the SV segments from the nitrate group (1) were less responsive to NTG (Figure 1A), with an \(\approx\)5-fold increase in EC\(_{50}\) and a reduction in E\(_{\text{max}}\) from 97% to 85% (Table 2), and (2) did not differ in responsiveness to either SNP or A23187 (Figures 1B and 1C; Table 2).

#### Comparison of IMA and SV

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

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</tbody>
</table>

Values are mean±SEM. *P<0.01 for control group vs nitrate group.

Patient characteristics of the subgroup randomized to examine \(O_2^\cdot\) 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.
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 \( \mu \)mol/L lucigenin were \( \approx70\% \) greater (\( P<0.01 \)) in the segments from the nitrate group compared with control segments (Figure 3). With 10 \( \mu \)mol/L lucigenin, chemiluminescence counts were significantly (\( P<0.02 \)) reduced relative to 250 \( \mu \)mol/L lucigenin, but the proportional difference between the nitrate and control groups remained similar (20±3 versus 9±1 count \( \cdot \) \( \text{min} \) \(^{-1} \) \( \cdot \) \( \text{mg} \) \(^{-1} \); \( n=5 \) each group, \( P<0.01 \)).

**Effects of Increased O\(_2\)\(^-\) Generation**

Incubation of IMA segments with DETCA resulted in an \( \approx3\)-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 \( \mu \)g/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\(^6\) after more prolonged prior nitrate therapy. Boesgaard et al\(^14\) 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...
dynamic indices have demonstrated that tolerance induction is associated with no cross-tolerance to non-nitrate sources. However, the only previous study on isolated vessels from patients not exposed to nitrates. Open symbols indicate control segments, and closed symbols, DETCA-exposed segments. *P<0.01 for control vs DETCA-exposed segments.

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 or (2) NTG metabolism. 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 and in vivo animal models of tolerance induction. Most in vitro studies have shown minimal cross-tolerance to non-nitrate NO sources. However, the relevance of in vivo studies has been questioned because they exclude some factors that may be of importance to tolerance induction in vivo. The data from in vivo animal studies are conflicting. Some studies using isolated vessels or hemodynamic indices have demonstrated that tolerance induction is associated with no cross-tolerance to non-nitrate sources of NO. However, others 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. To our knowledge, the only previous study on isolated vessels from tolerant subjects 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. 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 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 or in vivo. 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. 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 is that NTG tolerance involves increased clearance of NO via increased O2- , the latter being generated primarily as a result of angiotensin II-induced activation of reduced nicotinamide adenine dinucleotide oxidase. Furthermore, incremental O2- generation has recently been implicated in the pathogenesis of the de novo phenomenon nitrate resistance. For this reason, we explored the role of O2- with regard to tolerance induction in human vessels. The measurement of lucigenin-enhanced chemiluminescence with both 250 and 10 μmol/L lucigenin suggested that tolerant IMA segments had a greater potential for O2- generation than control segments; the difference was similar to that reported previously by Münzel et al in rabbit aortae. These experiments also confirmed the previous observation in some but not all 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 O2- generation in tolerant vessels, prompted the experiments using DETCA (1 mmol/L). Despite increasing O2- generation in the IMA ≈3-fold, DETCA had no effect on NTG responses. This finding was in conflict with previous studies in bovine coronary arteries and rabbit aortae. 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, suggesting a more nonspecific impairment of cellular function. Furthermore, other investigators have also reported unchanged NTG responses after short-term

Figure 4. Effects of 1 mmol/L DETCA on lucigenin-enhanced chemiluminescence (A) and NTG responses (B) in IMA segments from patients not exposed to nitrates.
elevation of O\textsubscript{2}\textsuperscript{-} 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\textsubscript{2}\textsuperscript{-} 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.\textsuperscript{8,26} Moreover, in this model, we did not observe any evidence of increased sensitivity to vasoconstric- tor agents in the tolerant segments; this phenomenon, which is related to pseudotolerance, may have become manifest with other tolerance-induction regimens.\textsuperscript{9,27,28} Critically, the results do not completely exclude a role for the observed increase in O\textsubscript{2}\textsuperscript{-} generation in tolerance induction; it is possible not only that more prolonged elevation of O\textsubscript{2}\textsuperscript{-} 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,\textsuperscript{36} with the expectation that the acceleration of bioconversion will provide a means to circumvent tolerance.

Acknowledgments

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References

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