Therapy With Nitroglycerin Increases Coronary Vasoconstriction in Response to Acetylcholine

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Objectives. The purpose of this study was to investigate whether therapy with nitroglycerin (GTN) would lead to abnormal coronary artery responses to the endothelium-dependent vasodilator acetylcholine.

Background. Nitroglycerin therapy is associated with specific biochemical changes in the vasculature that may lead to increased vascular sensitivity to vasoconstrictors.

Methods. Patients were randomized to continuous transdermal GTN, 0.6 mg/h (n = 8), or no therapy (n = 7), for 5 days prior to a diagnostic catheterization. Patients had similar risk factors for endothelial dysfunction. Quantitative angiography was performed in the morning to measure the mean luminal diameter of the left anterior descending coronary artery (LAD) in response to intracoronary acetylcholine (peak concentration, 10\(^{-4}\) mol/liter). The transdermal preparation was removed from the GTN group, and 3 h later experimental procedures were repeated.

Results. In the morning, the GTN group experienced greater coronary constriction in response to acetylcholine infusion than those not receiving GTN (–19.6 ± 4.2% vs. –3.8 ± 3.0%; p = 0.01). Three hours later, the GTN group continued to display greater constriction to acetylcholine (–24.1 ± 5.9%) as compared to the non-GTN group (–1.8 ± 4.8%). When the morning and afternoon responses to acetylcholine were compared, the increase in coronary constriction in the GTN group was greater than the change observed in the non-GTN group (p < 0.05).

Conclusions. This study demonstrates that therapy with GTN causes abnormal coronary vasomotor responses to the endothelium-dependent vasodilator acetylcholine, changes that were persistent for up to 3 hours after GTN discontinuation. This nitrate-associated vasomotor dysfunction has implications with respect to the development of nitrate tolerance and the potential for adverse events during nitrate withdrawal.

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The organic nitrates are important agents in the therapy of coronary artery disease and congestive heart failure. Given acutely, they have potent hemodynamic and therapeutic effects that result from dilatation of venous and arterial vascular beds. A major limitation to their use is the development of tolerance, with loss of effectiveness during sustained therapy. Although a number of theories have developed concerning the mechanism of tolerance, the cause remains uncertain (1,2). In addition to this uncertainty, it is now recognized that therapy with nitrates is associated with increased vascular sensitivity to vasoconstriction (3,4). The mechanism of this increased sensitivity remains unknown, although animal models have demonstrated that exposure to nitroglycerin (GTN) is associated with increased vascular content of endothelin-1 as well as increased superoxide anion production by the endothelium (4,5). Importantly, Heitzer et al. (6) have demonstrated that treatment with GTN increases vasoconstrictor responses to both phenylephrine and angiotensin II in the human forearm.

These findings suggest that nitrate therapy may modify vascular responses in the coronary circulation and increase its sensitivity to vasoconstriction. Such responses may be involved in the genesis of tolerance and could provide an explanation for adverse events that have been reported following nitrate withdrawal (7–11). The present study tests the hypothesis that therapy with GTN would increase epicardial coronary sensitivity to the vasoconstrictor effects of acetylcholine and that this effect would be exaggerated following GTN withdrawal.

Methods

Study population. Patients between 35 and 75 years of age, referred for coronary angiography, were eligible for participation in the study. Clinical exclusion criteria were uncontrolled hypertension; symptomatic congestive heart failure or severe left ventricular dysfunction; history of coronary spasm; myocardial infarction or unstable angina within 30 days of randomization; second- or third-degree AV block; transluminal revas-
culation procedures in the previous 6 months; any previous transluminal revascularization of the left anterior descending coronary artery (LAD); coronary artery bypass grafting; type I diabetes mellitus; and clinically significant renal or hepatic disease. Thirty-seven patients were initially enrolled. One patient withdrew consent prior to the angiogram, and a second was withdrawn because of newly diagnosed anaemia. At the time of angiography, 20 patients were found to have more than a 25% diameter stenosis of the left main coronary or more than a 40% diameter stenosis in the LAD and were excluded because they were believed to be ineligible for intracoronary acetylcholine infusions based on the severity of their coronary artery disease. Therefore, 15 patients completed the protocol.

**Risk factors for endothelial dysfunction.** The total number of known risk factors for endothelial dysfunction was assessed for each patient. These factors included age >45 years, male sex, history of hypertension, noninsulin-dependent diabetes mellitus, current cigarette smoking, family history of premature coronary artery disease, and total cholesterol >5.3 mmol/liter. Serum lipid concentrations were evaluated in the fasting state at the time of angiography.

**Study protocol.** Placebo transdermal GTN preparations could not be obtained. Therefore, patients were randomly assigned to receive continuous transdermal GTN (Ciba-Geigy, Mississauga, Ontario), 0.6 mg/h, or no treatment in an investigator-blind fashion. The GTN patch was applied each morning and replaced every 24 h. Patients receiving long-acting nitrates prior to randomization had their nitrate withdrawn at least 1 week prior to the day of the study. Other vasodilators were discontinued for 48 h prior to randomization had their nitrate withdrawn at least 1 week prior to the day of the study. Other vasodilators were discontinued for 48 h prior to the angiogram, and beta-blockers were discontinued for 24 h. Patients were allowed to use sublingual GTN as required for angina, but no study was carried out within 6 h of GTN use. Vitamin C and E supplements were discontinued 7 days prior to study.

The angiogram was performed on the morning of the fifth day, at approximately 9:00 AM. In those randomized to GTN, the transdermal preparation was left in place during the morning study. Coronary angiography was performed with 7F diagnostic catheters using Judkin’s technique. Once angiographic suitability for the study had been confirmed, a single view best demonstrating the proximal and mid-portion of the LAD coronary artery was selected for each patient and used for the subsequent study angiograms. Heart rate, femoral arterial pressure, and electrocardiographic (ECG) leads I, II and V5 were continuously monitored.

Quantitative coronary angiograms were performed in the control state and after serial acetylcholine chloride infusions. Control and acetylcholine solutions were infused at 1.25 ml/min for 3 min into the left main coronary artery via the diagnostic catheter in the following sequence: 1) control: 5% dextrose in water, the vehicle for acetylcholine infusion; 2) three incremental acetylcholine infusions: concentrations of $10^{-6}$, $10^{-5}$, and $10^{-4}$, mol/liter to achieve intracoronary concentrations of $10^{-8}$, $10^{-7}$, and $10^{-6}$ mol/liter, assuming a left coronary blood flow of 125 ml/min; 3) recontrol: 5% dextrose in water. Angiography was repeated immediately after completion of each infusion. A 3-min time period was allowed to elapse between each angiogram and the following infusion, to compensate for contrast medium-induced changes in coronary tone.

Immediately following the completion of the morning study, the GTN patch was removed from patients in the GTN group. Three hours later, patients were returned to the catheterization laboratory and underwent identical intracoronary drug infusions using the same projection and radiographic technique as had been done in the morning.

The study protocol was approved by the Committee on Human Subjects Experimentation of the University of Toronto, and written informed consent was obtained from all patients.

**Quantitative measurements.** Left coronary artery angiograms were performed by power injection (Mederic, Pittsburgh, Pennsylvania) of 9 to 12 ml (3 to 4 ml/s) of nonionic contrast medium. Quantitation of coronary dimensions was performed using an automated edge detection system (CMS, Neuen, The Netherlands) and techniques previously reported by our laboratory (12). A single end-diastolic frame of each angiogram was selected by an investigator blinded to the study groups. The longest, clearly visualised segment of the proximal LAD was selected. In the GTN group, the mean length of the analyzed segment was 4.3 ± 0.6 cm; it was 4.4 ± 0.4 cm in the non-GTN group. The proximal and distal boundaries of the selected segments were referenced to precise anatomical landmarks to ensure replication of the analysis. The mean luminal diameter of the LAD segment was recorded from each study angiogram. An untapered portion of the contrast medium-filled 7F coronary catheter was filmed before each angiogram and used for calibration. All films were analyzed by a blinded technician. In our laboratory, coronary artery diameter measurements performed with this system have interobserver variability of 0.09 mm, and intraobserver variability of 0.01 mm on repeated analysis of the same frame.

**Statistical analysis.** Baseline characteristics were compared using unpaired t tests for continuous variables. A Fischer’s exact test was used to compare binary variables. Changes in mean luminal diameter from control values to each dose of acetylcholine were compared using a multivariate analysis of variance (MANOVA) within a general linear model procedure, with appropriate contrast statements (PROC GLM, MANOVA, SAS release 6.11, SAS Institute, Cary, North Carolina). This model allowed for analysis of between-group changes in the responses to acetylcholine for both the morning and afternoon angiograms. Furthermore, differences between groups in the overall response to acetylcholine during the morning, versus the afternoon, could also be compared.
Finally, the procedure also allowed for univariate comparisons at baseline and all subsequent time points between groups. Within-group changes of mean luminal diameter were compared using a paired $t$ test. All comparisons were made for changes in absolute values as well as percent changes from baseline. A two-sided $p$ value of $<0.05$ was considered to be significant. Data are expressed as mean ± SE.

### Results

**Baseline patient characteristics.** Eight patients were randomized to GTN therapy (five men; aged 56 ± 4 years) and seven to the non-GTN arm (five men; aged 62 ± 3 years). There were no significant differences between groups in terms of known risk factors for endothelial dysfunction (Table 1). All patients in the GTN group, and six patients in the non-GTN group, had atherosclerosis with intimal irregularities of the LAD. The average LAD stenosis was 8.8 ± 2.8% in the GTN group and 12.9 ± 6.0% in the non-GTN group. The groups were well matched in terms of concomitant medications at the time of randomization, with one patient in each group receiving angiotensin-converting enzyme inhibitors, vitamin supplementation and estrogen replacement therapy. Baseline heart rate and blood pressure at the time of both the morning and afternoon studies were similar in the two groups (not shown).

**Clinical response to acetylcholine infusion.** In the morning study, two patients in the GTN group developed angina associated with moderate coronary constriction during infusion of the maximal concentration of acetylcholine. In both cases, angina resolved with termination of the infusion, and no intracoronary GTN was given. In the afternoon study, the same two patients and one additional patient developed angina in response to the maximal acetylcholine infusion in the GTN group. In two of these patients the angina was severe, did not improve with discontinuation of the acetylcholine infusion, and required intracoronary GTN. In the non-GTN group, no significant chest pain was observed during any acetylcholine infusion.

**Coronary diameter responses.** The infusion of acetylcholine, $10^{-5}$ mol/liter, caused no significant change in mean luminal diameter of the LAD in either group, and this data is not presented. During the morning angiogram there was no difference between groups in control values for the diameter of the LAD (2.35 ± 0.14 vs. 2.21 ± 0.13 mm; $p = \text{NS}$). In the non-GTN group there was no significant change in the mean luminal diameter of the LAD during acetylcholine infusion. In contrast, in those receiving transdermal GTN, a highly significant decrease occurred in mean luminal diameter at the peak acetylcholine infusion rate ($p < 0.01$ vs. control) (Fig. 1). This decrease in mean luminal diameter of the LAD was significantly different from the change observed in the non-GTN group ($p < 0.01$). Similar results were observed when the responses were expressed as percent change (Fig. 2).

In the afternoon, 3 h after removal of transdermal GTN, the control diameter of the LAD was significantly smaller than was the control value for the morning angiogram in the GTN group (2.35 ± 0.14 vs. 2.03 ± 0.09 mm; $p < 0.01$). In the non-GTN group, there was no change in LAD diameter between the two control periods. In response to intracoronary acetylcholine in the afternoon study, there was no significant change in LAD diameter in the non-GTN group (Fig. 3). The GTN group, now 3 h after GTN patch removal, experienced highly significant decreases in LAD diameter in response to acetylcholine at both the $10^{-5}$ and $10^{-4}$ mol/liter concentrations ($p < 0.01$ vs. control). These decreases in LAD diameter were significantly different from those observed in the non-

### Table 1. Demographic, Clinical and Laboratory Characteristics of the Study Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>GTN Group (n = 8)</th>
<th>Non-GTN Group (n = 7)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56 ± 4</td>
<td>62 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
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<td>5/2</td>
<td>NS</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>2</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Non-insulin-dependent diabetes</td>
<td>3</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>2</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol &gt;5.3 mmol/liter</td>
<td>3</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>History of smoking</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Current</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>Former</td>
<td>3</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Total number of risk factors*</td>
<td>3.1 ± 0.4</td>
<td>3.3 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>History of MI</td>
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<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>History of coronary angioplasty</td>
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<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Serum cholesterol, mmol/liter</td>
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<td></td>
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<tr>
<td>Total</td>
<td>5.2 ± 2.5</td>
<td>5.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>HDL</td>
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<td>NS</td>
</tr>
<tr>
<td>LDL</td>
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<td>3.3 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/liter</td>
<td>1.2 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SE. *Risk factors: age >45 years, male sex, history of hypertension, non insulin-dependent diabetes, family history of CAD, total cholesterol >5.5 mmol/liter, current smoker, CAD, coronary artery disease; GTN, nitroglycerin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction.
GTN group (p < 0.01). Again, similar results were observed when the responses were expressed as percent change. In the GTN group, the LAD diameter at 10^{-4} mol/liter concentration was significantly smaller in the afternoon than in the morning (1.54 ± 0.13 vs. 1.89 ± 0.15 mm; p < 0.01).

When the morning and afternoon coronary responses to acetylcholine were compared, the between-group analysis revealed that the increase in coronary vasoconstriction in the GTN group was significantly different from the change observed in the non-GTN group. This difference was apparent when the data was analyzed using both absolute and percent changes in LAD diameter (p < 0.05 for both). In response to intracoronary GTN bolus, both groups presented similar LAD dilatation (14.8 ± 2.0 vs. 16.2 ± 5.4% for GTN and non-GTN groups, respectively; p = NS).

Figure 2. Percent change in mean luminal diameter of the left anterior descending artery (LAD) coronary artery from baseline, in response to maximal intracoronary acetylcholine infusion (10^{-4} mol/liter). *p < 0.01 versus morning control; †p < 0.01 versus afternoon control; ‡p < 0.05, response in GTN group versus non-GTN group. ▲ = Nonnitroglycerin group.

**Discussion**

We have demonstrated that therapy with transdermal GTN is associated with increased epicardial coronary artery constriction in response to acetylcholine and that this response was persistent 3 h after GTN discontinuation. The coronary responses to GTN demonstrated that endothelial-independent vasodilatation was preserved in both groups.

**Endothelial dysfunction.** Coronary vasoconstriction in response to acetylcholine is an accepted marker for endothelial dysfunction that occurs in the setting of a number of disease states (13–20). Abnormal endothelial function appears to be associated with the development of atherosclerosis and unstable coronary syndromes (20). The cause(s) of endothelial dysfunction remain unclear, but several independent risk factors have been identified (20,21). The finding that therapy with GTN is associated with increased vasoconstriction in response to an endothelium-dependent vasodilator is unique, and it has potentially important clinical implications.

**Mechanism of acetylcholine responses.** The mechanism of increased coronary constriction to acetylcholine during therapy with GTN is unknown. This response could result from an increase in the sensitivity of smooth muscle to the vasoconstrictor actions of acetylcholine, a change in nitric oxide metabolism that reduces the amount of nitric oxide available following exposure to acetylcholine, or some combination of these effects. Recently, Münzel et al. (5) demonstrated that therapy with GTN leads to an increase in superoxide anion production in the endothelium of rabbit aortae. They hypothesize that increased free radical production diminishes the effects of nitrates via inactivation of GTN-derived nitric oxide. Similarly, this increased free radical production associated with GTN therapy could lead to inactivation of endothelial nitric oxide released by acetylcholine. Other studies have demonstrated that nitrate exposure increases vascular sensitivity to a number of vasoconstrictors (3,4).

In humans, it has been reported that forearm resistance vessels are more sensitive to the vasoconstrictor effects of both phenylephrine and angiotensin II during therapy with intravenous GTN (6). Although the mechanism of this hypersensitivity is not known, Münzel et al. have provided evidence that it is mediated by increased autocrine production of endothelin-1, a potent vasoconstrictor peptide that may play a role in the abnormal endothelial-dependent vasomotor responses associated with atherosclerosis (4,22). Although it is not possible to draw conclusions concerning the mechanism of the abnormal coronary vasomotor responses to acetylcholine in the present study, available experimental data provides a clear rationale.

**GTN withdrawal.** Another important finding of this study is that GTN withdrawal may further increase the sensitivity of the coronary vasculature to the vasoconstrictor effects of acetylcholine. The smaller diameter of the LAD during maximal acetylcholine infusion in the afternoon was associated with occurrence of angina in the GTN group. Such abnormal endothelium-dependent coronary vasomotor responses during the nitrate-free period may be playing a role in previous
clinical observations concerning the effects of nitrate withdrawal. In the munitions industry (7,8), and some clinical trials (9–11), the withdrawal of nitrate therapy was associated with an increased incidence of acute coronary syndromes. Furthermore, in patients with stable angina it has now been clearly documented that withdrawal of GTN therapy has adverse effects on exercise tolerance that persist for up to 12 h during the nitrate-free period (9,23,24). The present study suggests that these clinical observations may be secondary to changes in coronary vasomotor function induced by GTN therapy. The mechanism by which GTN withdrawal further increased the vasoconstrictor effects of acetylcholine is not clear. It is probably related to persisting biochemical changes in the vasculature (induced at the time of the GTN administration) no longer opposed by the vasodilator effect of the nitrate.

In the GTN group, withdrawal of GTN therapy caused a significant decrease in coronary diameter between the morning and afternoon control measurements. The decrease in coronary diameter following removal of transdermal GTN could have resulted from rebound constriction of the coronary artery. Alternatively, it may have resulted from the reversal of continued GTN-induced coronary dilatation. In animal models (25–27), and in humans (28,29), there is evidence that initial increases in coronary diameter induced by GTN are lost during 72 h of continuous therapy. This would suggest that the GTN group had developed tolerance to the effects of transdermal GTN, and that the decrease in LAD diameter following nitrate removal represents a rebound effect.

**Potential weaknesses.** Potential weaknesses of this investigation should be considered. Because endothelium-dependent responses to acetylcholine are related to risk factors (21), it would be possible that the different responses of the GTN and non-GTN group were secondary to imbalances in baseline characteristics. Although any potential imbalance could have been avoided using a crossover design, this approach has its own methodologic problems (30), and would be associated with important practical and ethical limitations because of the need for repeat angiography. As randomization resulted in groups well matched in terms of risk factors, we do not believe that the different responses found were due to imbalanced baseline characteristics.

Furthermore, the coronary responses to acetylcholine in the non-GTN group were similar to the baseline responses of more than 100 patients with coronary artery disease receiving intracoronary acetylcholine in the TREND study (31). This suggests that the mild constriction observed in the non-GTN group was typical for this patient population, and that the greater constriction observed in the GTN group was the result of the nitrate. In the present investigation, continuous transdermal GTN was employed, and it is not known whether the observed responses to acetylcholine would have been different had an intermittent regimen been employed. Nevertheless, continuous nitrate therapy is used in many patients, particularly in the setting of unstable angina. Furthermore, because there is good documentation that intermittent regimens are associated with changes in exercise tolerance and rebound ischemic events

![Figure 3. Representative coronary angiograms from patients in the non-GTN group (left) and in the GTN group (right). Angiograms were obtained at the time of the morning control (control), during acetylcholine 10⁻⁴ mol/liter in the morning (AM Ach⁻⁴), and acetylcholine 10⁻⁴ mol/liter at the time of the afternoon study (PM Ach⁻⁴).](image-url)
(9,10,23,24), it seems likely that intermittent therapy may lead to similar changes in coronary vasomotor function.

Although short-term administration of low doses of GTN have shown to enhance coronary vasodilator response to acetylcholine (32), our findings indicate that long-term administration of clinically relevant doses of GTN is associated with a vasoconstrictive response to acetylcholine. A number of pharmacologic agents have been shown to improve endothelial vasomotor function (17,18,31,33–35). To our knowledge, the present study is the first report of a commonly used pharmaceutical causing an adverse effect on endothelium-dependent vasodilatation. We believe these observations have significant implications concerning the therapeutic application of long-acting nitrates. The finding that an organic nitrate increases vasoconstriction in response to an endothelium-dependent vasodilator serves to emphasize that there is yet much to be learned concerning a form of therapy that has been widely used for more than a century.

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References