The effects of organic nitrates on prostacyclin biosynthesis and platelet function in humans

DESMOND J. FITZGERALD, M.B., LOUIS ROY, M.D., ROSE MARIE ROBERTSON, M.D., AND GARRET A. FITZGERALD, M.D.

ABSTRACT The results of prior studies indicate that nitroglycerin stimulates prostacyclin release by cultured endothelium and by the coronary vasculature in vivo. However, the accuracy of these findings in coronary vasculature relies on plasma samples obtained from the circulation via cardiac catheters, a procedure we have shown to stimulate prostacyclin release, thereby confounding interpretation of drug action. We studied the effects of short-acting (nitroglycerin) and long-acting (isosorbide dinitrate) nitrates on a noninvasive index of prostacyclin synthesis, excretion of urinary 2,3-dinor-6-keto-PGF₁α. Nitroglycerin was infused into six subjects to either a maximum of 480 μg/min or until mean arterial pressure fell by 20 mm Hg. Urine was collected for negative ion chemical ionization gas chromatographic, mass spectrometric analysis before and during the nitroglycerin infusion and for two 2 hr periods after nitroglycerin. The peak nitroglycerin infusion rate was 387 ± 67 μg/min, which caused a fall in supine blood pressure (systolic/diastolic) of 11 ± 5/14 ± 4 mm Hg and a 12 ± 3 beats/min increase in heart rate. Excretion of 2,3-dinor-6-keto-PGF₁α (pg/mg creatinine) was unchanged from control infusion values (106 ± 19.5) either during (123 ± 21) or after (134 ± 14.6; 139 ± 36) nitroglycerin infusion. Platelet aggregation to arachidonic acid (0.33 to 1.33 μM) and epinephrine (1 to 10 μM) ex vivo was inhibited in only one subject in whom excretion of 2,3-dinor-6-keto-PGF₁α was unaltered. Serum thromboxane B₂ was not changed by nitroglycerin infusion. Similarly, oral administration of isosorbide dinitrate (10 and 40 mg four times per day) failed to alter 2,3-dinor-6-keto-PGF₁α excretion from placebo values in patients with angina pectoris. Noninvasive measurements indicate that nitrates failed to stimulate prostacyclin release in vivo; platelet inhibition during infusion of nitroglycerin was unrelated to altered prostacyclin synthesis in human beings.


NITROGLYCERIN is a potent vasodilator that is widely used in the treatment of angina pectoris. Despite its availability for over 100 years, the basis of its effect on vascular smooth muscle is poorly understood. Nitroglycerin also inhibits platelet function in vitro and has been reported to prolong the bleeding time in human beings. In view of these biological properties, it has been postulated that nitroglycerin mediates these effects by enhancing generation of the arachidonic acid metabolite prostacyclin in vivo. Formed predominantly by the cyclooxygenase enzyme of vascular endothelium, prostacyclin is a potent vasodilator and inhibitor of platelet function. Previous investigations have demonstrated that nitroglycerin enhances prostacyclin release (as measured by its stable degradation product, 6-keto-PGF₁α) from cultured endothelial cells, isolated bovine coronary artery, human saphenous vein, and rat aorta.

The present studies were designed to test the hypothesis that the hemodynamic and platelet inhibitory effects of organic nitrates are associated with an increase in excretion of a major urinary metabolite of prostacyclin, 2,3-dinor-6-keto-PGF₁α. We selected this approach because previous attempts to address this question have generally relied on pharmacologic inhibition of the cyclooxygenase enzyme to identify the relevance of prostacyclin formation to the action of nitroglycerin. However, these studies have generally yielded conflicting results and have been unaccompanied by reliable biochemical indexes of endogenous biosynthesis of prostacyclin.

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Methods

All clinical investigations were performed in the Elliot V. Newman Clinical Research Center after approval of the protocol by the Committee for the Protection of Human Subjects of Vanderbilt University.

Two studies were performed. In the first, seven healthy male volunteers received infusions of nitroglycerin at two different doses on separate occasions. These subjects were 22 to 45 years old (28 ± 3, mean ± SEM) and had abstained from all medications for at least 10 days before investigation. On the first study day, subjects lay supine and vehicle alone (5% dextrose in water) was infused at a constant rate (50 ml/min) for 2 hr via a peripheral arm vein. Blood pressure and heart rate were measured indirectly at 5 min intervals by an oscillometric technique (Dynamap; Applied Medical Research Corporation, Tampa, FL). After this stabilization period, the nitroglycerin infusion (Nitrobid; 50 mg in 250 ml 5% dextrose in water) was commenced. The solution was administered through a nonabsorbing plastic infusion set (Nitrostat, Parke Davis) from a glass bottle to prevent loss of nitroglycerin to the delivery set, which may occur with standard systems.18 The infusion rate was increased from an initial 5 μg/min by increments of not more than 100% at 5 min intervals to a maximum of 480 μg/min or until mean arterial pressure had decreased by 20 mm Hg, and continued for a total of 2 hr. After completion of the nitroglycerin infusions, the subjects remained supine and received a further 2 hr infusion of vehicle alone. To address the possible relationship between the dose of nitroglycerin administered and any alteration in prostacyclin biosynthesis, the nitroglycerin infusion was repeated on a separate occasion in four of the subjects to a maximum dose of 55 μg/min.

Urine was collected for the 2 hr before nitroglycerin administration, during the 2 hr nitroglycerin infusion period, and for two subsequent 2 hr periods to determine the excretion rate of 2,3-dinor-6-keto-PGF1α, a major urinary metabolite of prostacyclin.19 Blood samples (30 ml) were collected at the midpoint of each infusion period in 3.8% sodium citrate (9:1 vol:vol) for platelet aggregation studies. Blood (3 ml) was also collected into plain glass test tubes and incubated immediately at 37°C for at least 45 min after infusion. The serum was stored at −20°C for subsequent radioimmunoassay of thromboxane B2, the hydrolysis product of thromboxane A2.

The second investigation was performed in five patients with stable angina (60 to 69 years old, mean 64 ± 1.7) who had angiographically proven coronary artery disease or documented previous myocardial infarction. The patients abstained from anti-inflammatory drugs for at least 10 days before the study and from organic nitrates for at least 5 days before the study. Other antianginal therapy was continued unchanged throughout the study period. On days 3 and 4, isosorbide dinitrate was administered in doses of 10 and 40 mg four times daily, respectively. Urine was collected separately on each day for 24 hr for determination of 2,3-dinor-6-keto-PGF1α excretion.

Platelet aggregation studies. Platelet-rich plasma was prepared by centrifugation of citrated blood for 15 min at 900 rpm. After separation of the platelet-rich plasma, platelet-poor plasma was prepared by centrifugation of the remaining blood at 4000 rpm for 10 min. The platelet count of the platelet-rich plasma was adjusted to 300,000/ml with platelet-poor plasma. Platelet aggregation was determined in 0.5 ml aliquots of platelet-rich plasma by the light transmission method of Born20 with a Payton dual-channel aggregometer (Payton Associates, Buffalo, NY), with platelet-poor plasma representing 100% light transmission. Platelet aggregation was induced by arachidonic acid (0.26 to 1.33 mM), epinephrine (1 to 10 μM), and collagen (9.5 to 95 μg/ml). All studies were performed at 37°C and completed within 2 hr of blood withdrawal.

Biochemical analysis. 2,3-dinor-6-keto-PGF1α was measured by a stable-isotope dilution assay with negative ion chemical ionization gas chromatography–mass spectrometry as previously described.21 Briefly, 5 ng of a deuterated internal standard was added to a 5 ml aliquot of urine. After extraction and back extraction under alkaline and acidic conditions, the sample was derivatized as the methoxime, pentafluorobenzyl ester. After further purification by thin-layer chromatography, derivatization was completed by formation of the trimethylsilyl ether derivative. Quantitation was accomplished by stable-isotope dilution with a Hewlett Packard 5980 instrument operated in the negative-ion mode, monitoring m/z 566 for endogenous, 2,3-dinor-6-keto-PGF1α, and m/z 590 for the deuterium-labeled internal standard. Thromboxane B2 was determined by a modification of the method of Fitzpatrick et al.22

Statistical analysis. Multiple means were compared by two-way analysis of variance and paired data by Student’s paired t test.23 A two-tailed probability of 5% or less was considered significant.

Results

Studies in healthy volunteers

Hemodynamic response. Five of the seven normal volunteers received 480 μg/min iv nitroglycerin. In the two other subjects mean arterial blood pressure decreased by more than 20 mm Hg at 80 and 320 μg/min, respectively. The maximum change in blood pressure and heart rate during the nitroglycerin infusions are shown in figure 1. At the highest infusion rate, nitroglycerin induced a fall in systolic (18.3 ± 5.0 mm Hg; p < .05) and diastolic (19.3 ± 2.5 mm Hg; p < .001)

![Figure 1](http://circ.ahajournals.org/)

**FIGURE 1.** Hemodynamic response to high- (n = 7) and low-dose (n = 4) nitroglycerin (NTG). B.P. = blood pressure.
TABLE 1A
Platelet aggregation ex vivo during high-dose nitroglycerin infusion

<table>
<thead>
<tr>
<th>Subject (mM)</th>
<th>Arachidonic acid (lag time, min)</th>
<th>Epinephrine (% light transmission)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. NTG</td>
<td></td>
</tr>
<tr>
<td>J. O</td>
<td>0.33 1.12 1.4</td>
<td>93 95</td>
</tr>
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<td>S. W</td>
<td>0.33 1.32 1.28</td>
<td>96 87</td>
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<td>0.66</td>
<td>0.8 0.92 0.76</td>
<td>94 88</td>
</tr>
<tr>
<td>1.32</td>
<td>0.72 0.88 0.76</td>
<td>97 91</td>
</tr>
<tr>
<td>J. Z</td>
<td>0.66 1.44 1.24</td>
<td>86 74</td>
</tr>
<tr>
<td>B. C</td>
<td>0.66 1.34 1.04</td>
<td>85 79</td>
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<tr>
<td>0.99</td>
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<td>91 92</td>
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<tr>
<td>J. L</td>
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<td>36 95</td>
</tr>
<tr>
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<td>86 94</td>
</tr>
<tr>
<td>1.33</td>
<td>0.52 0.88 0.52</td>
<td>86 94</td>
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</table>

NTG = nitroglycerin.

blood pressures and an increase in heart rate (20 ± 3.6 beats/min; p < .01). Similarly, at the lower infusion rate (n = 4), nitroglycerin induced a significant decrease in systolic (24 ± 3.5 mm Hg; p < .01) and diastolic (15 ± 3.1 mm Hg; p < .01) blood pressures and an increase in heart rate (11 ± 2.1 beats/min; p < .05). There was no statistical difference in the blood pressure response to the two doses of intravenous nitroglycerin. However, the higher infusion rate appeared to cause a greater increase in heart rate. The effects of nitroglycerin on blood pressure and heart rate were sustained throughout the infusion.

*Platelet aggregation.* Platelet aggregation to arachidonic acid and epinephrine was studied with platelet-rich plasma in five subjects during high-dose nitroglycerin infusion and in all subjects during low-dose nitroglycerin infusion (tables 1A and 1B). Only one subject (J. Z.) demonstrated marked inhibition of platelet aggregation to both agonists during high-dose nitroglycerin infusion (figure 2). This subject also exhibited greater hemodynamic sensitivity to nitroglycerin than the other seven subjects, achieving the hemodynamic end point at 80 μg/min. During infusion at 55 μg/min in this subject, epinephrine-induced platelet aggregation was less markedly inhibited whereas platelet aggregation to arachidonic acid was unaltered (tables 1A and 1B).

*Biochemical analysis.* 2,3-dinor-6-keto-PGF_{1α} excretion (figure 3) did not alter significantly during either high- (n = 7) or low-dose (n = 4) nitroglycerin infusion. Serum thromboxane B_{2} generation (figure 3) was also unaltered during high- (n = 3) and low-dose (n = 4) nitroglycerin infusion.

*Studies in patients with coronary vascular disease.* In patients with angina pectoris, prostacyclin metabolite excretion was unchanged during administration of isosorbide dinitrate. One subject suffered an episode of chest pain on day 3 while receiving 10 mg of isosorbide dinitrate every 6 hr. In this subject, 2,3-dinor-6-keto-PGF_{1α} excretion increased on day 3 and returned to baseline on day 4. In the other four subjects, 2,3-dinor-6-keto-PGF_{1α} excretion was unchanged during administration of isosorbide dinitrate (figure 4).

**Discussion**

The hypothesis that organic nitrates mediate their hemodynamic and platelet effects via stimulation of prostacyclin biosynthesis in vivo was not supported by

**TABLE 1B**
Platelet aggregation ex vivo during low-dose nitroglycerin infusion

<table>
<thead>
<tr>
<th>Subject</th>
<th>Arachidonic acid (lag time, min)</th>
<th>Epinephrine (% light transmission)</th>
<th>Collagen (% light transmission)</th>
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<td>Conc. NTG</td>
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</tr>
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<td>1.33 0.8 0.6 0.52</td>
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<td>B. C.</td>
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</tr>
<tr>
<td>S. W.</td>
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<td>1 86 83 96</td>
<td></td>
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<tr>
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<td>0.76 0.78 0.52</td>
<td>5 99 90 95</td>
<td></td>
</tr>
<tr>
<td>0.66</td>
<td>0.68 0.6 0.48</td>
<td>10 90 92 100</td>
<td></td>
</tr>
<tr>
<td>1.33</td>
<td>0.56 0.48 0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J. L.</td>
<td>0.33 2.36 1.08 1.08</td>
<td>1 100 33.5 54</td>
<td></td>
</tr>
<tr>
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<td>0.8 0.8 0.8</td>
<td>5 100 99 100</td>
<td></td>
</tr>
<tr>
<td>1.33</td>
<td>0.72 0.76 0.76</td>
<td>10 100 100 100</td>
<td></td>
</tr>
</tbody>
</table>

NTG = nitroglycerin.
these results. Prostacyclin generation was assessed by measurement of a major urinary metabolite, 2,3-dinor-6-keto-PGF\textsubscript{1\alpha},\textsuperscript{19} with use of a highly specific and sensitive assay.\textsuperscript{21} Excretion of this metabolite is linearly related to the infusion rate of systemically administered prostacyclin over a 40-fold dose range and is an accurate predictor of the physiologic secretion rate of endogenous prostacyclin in human beings.\textsuperscript{22} Furthermore, measurement of a urinary metabolite is noninvasive, so that traumatic or chemical stimulation of vascular prostacyclin release associated with the sampling procedure\textsuperscript{25, 26} is avoided. We have previously shown that infusion of prostacyclin for a period of 2 hr, representing a mere doubling of the physiologic secretion rate of prostacyclin, is reflected by a marked increase in the rate of 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion.\textsuperscript{24} Such an increment would be well below the threshold dose necessary for prostacyclin to exert a systemic hemodynamic or platelet inhibitory effect.\textsuperscript{6} Thus even subthreshold increases in prostacyclin biosynthesis could be detected by this assay procedure.

In healthy volunteers, intravenous nitroglycerin at high and low infusion rates had little effect on 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion despite a marked decrease in systolic and diastolic blood pressures and an increase in heart rate. Importantly, the effects of nitroglycerin on blood pressure and heart rate persisted throughout the infusion period. Thus there was a clear dissociation between the hemodynamic response to intravenous nitroglycerin and an effect on prostacyclin biosynthesis. A similar dissociation was found between the platelet inhibitory effect of nitroglycerin and prostanoid formation. No change occurred in 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion or in serum thromboxane generation in the only subject in whom nitroglycerin markedly inhibited platelet aggregation ex vivo.

Similarly, in patients with angina pectoris, isosorbide dinitrate, an orally active organic nitrate, did not alter 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion. The increase in 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion seen in one subject on day 3 appears to be unrelated to administration of organic nitrate, since it was not dose-related and did not occur in the other patients. A possible explanation for this observation is that the episode of chest pain was associated with platelet activation.\textsuperscript{27} Increased generation of prostacyclin occurs in patients with severe atherosclerosis and platelet activation, perhaps reflecting an increased intensity and/or frequency of platelet-vascular interactions.\textsuperscript{28} Interestingly, we have recently observed a short-term increase in 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} excretion in patients who have suffered a myocardial infarction.

Previous studies of the effect of cyclooxygenase inhibitors on the response to nitroglycerin have given conflicting results. Cyclooxygenase inhibitors have been shown to inhibit the increase in canine coronary blood flow induced by nitroglycerin\textsuperscript{11} and the effect of nitroglycerin on forearm venous tone\textsuperscript{12} and bleeding time in human beings.\textsuperscript{4} However, cyclooxygenase inhibitors failed to alter the effect of nitroglycerin on blood pressure, heart rate,\textsuperscript{16, 17} or echocardiographic left ventricular dimensions\textsuperscript{17} in healthy volunteers.
Studies that have addressed the issue directly by measuring 6-keto-PGF$_{1\alpha}$ in plasma have also yielded conflicting results.$^{12,16}$ Analytical problems$^{25}$ and procedure-related artifacts$^{25,26}$ suggest cautious interpretation of these results. The baseline measurements of 6-keto-PGF$_{1\alpha}$ reported in such investigations have greatly exceeded the levels found in peripheral plasma by precise physicochemical methods.$^{30}$ It is difficult to address the relevance of previous studies in vitro to the effects of nitrates on prostacyclin synthesis in vivo. Such studies have produced conflicting results. This may be partially accounted for by variations in tissue preparation and assay methods for immunoreactive 6-keto-PGF$_{1\alpha}$. In some studies that suggest such a relationship, the nitrate concentrations used would be unlikely to be attained in vivo.

In conclusion, we found no evidence to support the hypothesis that the hemodynamic and platelet inhibitory effects of high- or low-dose nitrates are mediated by stimulation of prostacyclin biosynthesis in vivo.

### References

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