Platelet Cyclic GMP
A Potentially Useful Indicator to Evaluate the Effects of Nitroglycerin and Nitrate Tolerance

Hideki Watanabe, MD; Masaaki Kakihana, MD; Sadanori Ohtsuka, MD; Tsuyoshi Enomoto, MD; Kazuhiko Yasui, MD; and Yasuro Sugishita, MD

Background. The present study was designed to investigate the intracellular production of cyclic GMP (cGMP) in platelets in response to nitroglycerin and to determine the potential clinical value of platelet cGMP as an indicator of the effects of nitroglycerin and nitrate tolerance.

Methods and Results. Platelet cGMP levels and the diameters of the coronary arteries before and 2 minutes after intracoronary injection of 200 μg nitroglycerin were measured in 15 patients who had previously received nitrates (nitrates group) and in 16 who had not received any nitrates (no-nitrates group). Platelet cGMP levels increased significantly after nitroglycerin injection in the two groups, but plasma cGMP levels and plasma atrial natriuretic peptide levels did not change. The percent increase in platelet cGMP levels and the percent dilatation of the left anterior descending (LAD) and left circumflex (LCx) coronary arteries after nitroglycerin injection were higher in the no-nitrates group than in the nitrates group (platelet cGMP levels: artery, 74.2±18.3% versus 11.5±4.2%, P<.01; vein, 73.6±22.9% versus 9.0±3.1%, P<.01; coronary dilatation: LAD, 46.7±6.0% versus 9.9±2.5%, P<.01, LCx, 51.2±8.7% versus 6.1±3.0%, P<.01). The percent increase in platelet cGMP levels was significantly correlated with the percent dilatation of the coronary arteries (LAD: r=.90, P<.01; LCx: r=.92, P<.01) in the no-nitrates group and not in the nitrates group.

Conclusions. These results indicate that platelet cGMP can be used as an indicator for in situ evaluation of nitroglycerin effects and that patients who have received nitrates develop nitrate tolerance, which affects intracellular production of cGMP and vasodilation in the response to nitroglycerin. (Circulation 1993;88:29-36)

KEY WORDS • nitroglycerin • nitrates • natriuretic peptides • nitrovasodilators • angiography

Nitroglycerin are among the oldest and most widely used drugs in cardiovascular medicine. First synthesized in 1846, nitroglycerin was used clinically as early as 1879 in patients with angina pectoris. Its antianginal effects have mostly been attributed to its coronary and peripheral vasodilatory properties resulting in an improved oxygen supply-to-demand ratio. Unfortunately, when nitrates are given without interruption, a certain degree of tolerance to their beneficial effects appears within 24 hours. One theory advanced to explain how this tolerance develops is sulfhydryl group depletion. More recent studies have questioned this mechanism and proposed that tolerance is the result of a change in the affinity of the intracellular transformation site for organic nitrates. Pack and colleagues proposed a role for neurohumoral activation in the development of nitrate tolerance in their studies, and Dupuis and colleagues reported an increase of plasma renin activity after 1 and 6 hours of continuous intravenous administration of nitroglycerin. Packer suggests that multiple mechanisms interact to promote the development of tolerance to nitroglycerin. Nitrates stimulate soluble guanylate cyclase and elevate cyclic GMP (cGMP) levels. It is known that the conversion of GTP to cGMP is catalyzed by at least two isoenzyme forms of guanylate cyclase. The kinetic, physicochemical, and antigenic properties of the cytosolic and membrane-associated isoenzymes are quite different. Moreover, the relative abundance of the soluble and particulate forms is variable in different tissues and species. Although the intestinal mucosa and the retina predominantly express the particulate isoenzyme and platelets contain the soluble isoenzyme, most tissues such as vascular smooth muscle, have both isoenzymes. Furthermore, the regulation of these isoenzymes is quite different. The soluble enzyme appears to be unique in the sense that it can be activated by reactive free radicals such as nitric oxide and, probably, hydroxyl free radical. The particulate isoenzyme can be activated by agents such as the atrial natriuretic peptide and hemin. Plasma cGMP has been evaluated in patients with heart diseases and has been reported as clinical indicator of the effects for atrial natriuretic peptide but

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Received August 3, 1992; revision accepted March 15, 1993.
not for nitrates. Thus, up to now, there was no clinically useful biochemical indicator for the in vivo evaluation of nitrate effects or nitrate tolerance.

The aim of this study was to quantify the intracellular production of platelet cGMP in response to nitroglycerin and to determine its clinical correlation to vasodilation. We also investigated whether changes in platelet cGMP levels could serve as a biochemical indicator to evaluate the presence of nitrate tolerance.

**Methods**

**Study Population**

Thirty-one patients undergoing routine cardiac catheterization and coronary arteriography for the diagnosis and evaluation of ischemic heart disease or other heart diseases were included in this study. Of these 31 patients, 15 patients (nitrates group) had previously received nitrates (isosorbide dinitrate) and 16 patients (no-nitrates group) had not.

Table 1 shows the characteristics and hemodynamic variables of the patients. Age and sex were matched between the two groups (age: 55±8 versus 58±8 years, P=NS; male/female: 10/6 versus 9/6, no-nitrates group versus nitrates group, mean±SD). Five patients in the no-nitrates group and three in the nitrates group had a smoking history. Two patients (8 and 16) in the nitrates group had taken digitalis (0.25 mg digoxin) and diuretics (40 mg furosemide). Three patients in the nitrates group had taken β-blocker (25 mg atenolol) and 10 in the nitrates group had taken calcium channel blocker (40 mg nifedipine). Left ventricular function was normal in all patients except for two who had dilated cardiomyopathy (8 and 16 in the no-nitrates group). There was no difference in heart rate, mean

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<th>Other cardiac medication</th>
<th>Diagnosis</th>
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<th>Mean BP (mm Hg)</th>
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M, male; F, female; ISDN, isosorbide dinitrate; Dig, digoxin; Diu, diuretics; Ca, calcium channel blocker; β, β-blocker; AP, angina pectoris; ASD, atrial septal defect; CP, chest pain syndrome; DCM, dilated cardiomyopathy; OMI, old myocardial infarction; VSA, vasospastic angina; HR, heart rate; BP, blood pressure; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction.
### Table 2. Platelet cGMP and Coronary Diameters

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<th>No-nitrate group After (pmol/10⁶ PLT)</th>
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<th>LAD After (mm)</th>
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**PLT**, platelets; after, after nitroglycerin injection; before, before nitroglycerin injection; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery.

Blood pressure, left ventricular end-diastolic pressure, and left ventricular ejection fraction between the two groups (heart rate: 76±9 versus 73±10 beats per minute; mean blood pressure: 95±9 versus 95±11 mm Hg; left ventricular end-diastolic pressure: 10±3 versus 9±2 mm Hg; left ventricular ejection fraction: 69±10 versus 69±4; no-nitrates group versus nitrates group, mean±SD).

**Study Protocol**

Patients were studied after an overnight fast. All patients received 2000 to 3000 units heparin IV at the initiation of the study. In the nitrates group, nitrate treatment was continued through the study, and the dosage of isosorbide dinitrate was not changed. All cardiac medications except for isosorbide dinitrate were discontinued for at least 48 hours before this study. Written informed consent was obtained from all patients.

After routine cardiac catheterization was completed, control samples of blood (20 mL) were drawn from the femoral artery and vein to measure plasma cGMP, plasma atrial natriuretic peptide, and platelet cGMP. Control coronary angiography was per-
formed after blood sampling. Two minutes after IC injection of 200 μg nitroglycerin into the left coronary artery, blood samples were again drawn from the femoral artery and vein; then, coronary angiography was repeated.

**Preparation of Platelet and Plasma Samples for the cGMP Assay**

Blood samples from the femoral artery and vein were drawn into syringes containing 5 mM EDTA. Platelet-rich plasma and platelet-poor plasma were prepared by centrifugation at 200g for 20 minutes. Platelet-rich plasma was further centrifuged at 2500g for 10 minutes, and the supernatant was discarded. The pellet was suspended in modified Tyrode's solution (containing 0.35% bovine serum albumin and 5 mM HEPES, pH 7.35) to obtain a final platelet count of 2 to 3×10⁶ platelets/μL, and the samples were kept frozen at −70°C until analysis.⁹⁹

**Platelet cGMP Assay**

To 1 mL of platelet samples, 0.5 mL of trichloroacetic acid (final concentration, 6%) was added. After centrifugation at 2500g for 20 minutes, trichloroacetic acid was extracted four times from the supernatant with water-saturated ether. The aqueous phase was then assayed for cGMP using a commercially available radioimmunoassay kit (Yamasu Shoyu, Choshi, Japan),³⁰ and the results were expressed as picomoles per 10⁶ platelets. The coefficients of variation averaged 3.4% for intrassay error and 11.9% for interassay error.

**Plasma cGMP and ANP Assay**

Platelet-poor plasma was assayed for cGMP using a radioimmunoassay kit (Yamasu Shoyu, Choshi, Japan) and for atrial natriuretic peptide using a radioimmunoassay kit (Amersham). Plasma cGMP was expressed in picomoles per milliliter and plasma atrial natriuretic peptide in picograms per milliliter.

**Angiographic Analysis**

To assess the changes in vessel diameter, coronary angiograms were obtained from all patients before and 4 minutes after IC injection of 200 μg nitroglycerin. Coronary artery diameters were measured from the coronary angiograms by projecting magnified images of the angiograms on a screen. The diameters were measured of the proximal segments of the left anterior descending and the circumflex coronary arteries with no organic stenosis (segments 6 and 13 proposed by American Heart Association committee report).³¹

**Statistical Analysis**

Results are expressed as mean±SEM. Differences between the two groups were analyzed by the Mann-Whitney test and those between before and after nitroglycerin injection by the Wilcoxon matched-paired test. P<.05 was considered significant.

**Results**

**Plasma cGMP Levels and Plasma Atrial Natriuretic Peptide Levels**

Plasma cGMP levels did not change after nitroglycerin injection in either group (no-nitrates group: artery, 6.3±1.1 to 6.5±1.1 pmol/mL; vein, 7.1±1.2 to 6.9±1.0 pmol/mL; nitrates group: artery, 8.9±1.7 to 8.7±1.6 pmol/mL; vein, 9.0±1.8 to 8.7±1.7 pmol/mL). Plasma atrial natriuretic peptide also remained unchanged after nitroglycerin injection in both groups (no-nitrates group: artery, 79.6±14.9 to 79.6±15.3 pg/mL; vein, 70.8±9.4 to 70.5±8.8 pg/mL; nitrates group: artery, 61.6±9.6 to 63.2±10.6 pg/mL; vein, 59.0±9.0 to 59.5±8.6 pg/mL). Furthermore, there were no differences in plasma cGMP and plasma atrial natriuretic peptide levels between the two groups or between arterial and venous blood.

**cGMP Levels in Platelets**

Platelet cGMP levels are shown in Table 2. Nitroglycerin induced a significant increase in platelet cGMP levels in both groups (no-nitrates group: artery,
0.58±0.18 to 0.80±0.22 pmol/10⁹ platelets, P<.01; vein, 0.63±0.31 to 0.85±0.23 pmol/10⁹ platelets, P<.01; nitrates group: artery, 0.49±0.10 to 0.53±0.10 pmol/10⁹ platelets, P<.01; vein, 0.50±0.09 to 0.53±0.10 pmol/10⁹ platelets, P<.01. There was no difference in platelet cGMP levels before the injection of nitroglycerin between the two groups. The percent increase in platelet cGMP levels after nitroglycerin injection was significantly greater in the no-nitrates group than in the nitrates group (artery, 74.2±18.3% versus 11.5±4.2%, P<.01; vein, 73.6±22.9% versus 9.0±3.1%, P<.01, no-nitrates group versus nitrates group) (Fig 1). There were significant correlations between arterial and venous platelet cGMP levels from each individual before nitroglycerin dosing (r=.99, P<.01), after nitroglycerin dosing (r=.98, P<.01), and the percent increase due to nitroglycerin (r=.91, P<.01, Fig 2).

Moreover, there was no statistical difference in platelet cGMP levels and the percent increase in cGMP between patients with (n=8) and without (n=23) smoking history across the two groups (platelet cGMP before nitroglycerin: artery, 0.44±0.09 versus 0.57±0.14 pmol/10⁹ platelets; vein, 0.44±0.08 versus 0.61±0.14 pmol/10⁹ platelets; platelet cGMP after nitroglycerin: artery, 0.55±0.12 versus 0.71±0.16 pmol/10⁹ platelets; vein, 0.57±0.11 versus 0.74±0.17 pmol/10⁹ platelets; percent increase in cGMP: artery, 30.6±15.7% versus 48.5±14.0%; vein, 31.7±12.8% versus 46.0±17.2%; with smoking history versus without smoking history).

Response of Coronary Arteries to Nitroglycerin

The diameters and percent dilatation of the coronary arteries are shown in Table 2. In the no-nitrates group, the diameters of the proximal left anterior descending and left circumflex coronary arteries were 2.84±0.22 and 2.91±0.15 mm, respectively, before the injection of nitroglycerin, and 4.18±0.36 and 4.32±0.24 mm, respectively, after the injection. In the nitrates group, the diameters of the proximal left anterior descending and left circumflex coronary arteries were 2.83±0.12 and 3.03±0.18 mm, respectively, before the injection of nitroglycerin and 3.10±0.13 and 3.16±0.15 mm after the injection, respectively. There was no difference in the diameters of the two coronary arteries before the injection of nitroglycerin between the two groups.

Nitroglycerin caused diffuse coronary dilatation in all patients, and the percent dilatation was greater in the no-nitrates group than in the nitrates group (left anterior descending: 46.7±6.0% versus 10.0±2.5%, P<.01; left circumflex: 51.2±8.7% versus 6.1±3.0%, P<.01, no-nitrates group versus nitrates group) (Fig 3). Moreover, in the no-nitrates group, the percent increase in platelet cGMP levels was significantly correlated with the percent dilatation of the coronary arteries (platelet cGMP from artery and coronary diameter: left anterior descending, r=.90, P<.01; left circumflex, r=.92, P<.01) (Fig 4). Similar correlations were also observed between venous platelet cGMP and coronary diameter (left anterior descending, r=.81, P<.01; left circumflex, r=.71, P<.01). However, there was no significant correlation between them in the nitrates group.

Discussion

Nitrovasodilators are known to exert their relaxation effect on the vascular smooth muscle cells through activation of the intracellular soluble guanylate cyclase, leading to increased levels of cGMP. However, it is not possible to evaluate the effects of nitrovasodilators on cGMP levels in the vascular smooth muscle cells in vivo except by biopsy. Nitrovasodilators also activate soluble guanylate cyclase in platelets, and the increased level of platelet cGMP inhibits platelet function. Platelet is a convenient material for the clinical measurement of intracellular cGMP. Platelets are known to have predominantly the soluble guanylate cyclase. Therefore we investigated the effects of nitroglycerin and nitrate tolerance on platelet cGMP.

Effects of Nitroglycerin on Platelet cGMP and Plasma cGMP

The present results provided evidence for an action of nitroglycerin in platelets in vivo. Although plasma cGMP levels did not change after nitroglycerin injection, platelet cGMP levels were significantly increased. Previous studies have shown that increased plasma atrial natriuretic peptide activates particulate guanylate cyclase, and consequently, plasma cGMP increases. In the present study, plasma atrial natriuretic peptide levels did not change; nor did plasma cGMP. These results demonstrated that plasma cGMP was not elevated by activation of soluble guanylate cyclase. Thus, plasma cGMP cannot be used as a clinical indicator for the effects of nitroglycerin on the intracellular cGMP level. On the other hand, platelet cGMP increased significantly in the nitrates and no-nitrates groups after nitroglycerin injection. This finding suggested that platelet cGMP level may be a useful clinical indicator of the effects of nitrovasodilators on the intracellular cGMP. Control platelet cGMP levels
in the range of 0.03 to 0.4 pmol/10⁶ platelets have been reported by numerous authors.⁴²-⁴⁶ In the present study, control levels of platelet cGMP were within that range in most patients. There was no difference in the control levels of platelet cGMP between the two groups, even though patients in the nitrates group were treated with isosorbide dinitrate and continuously before this study. It was unclear from our data how control cGMP levels were maintained.

**Effects of Nitroglycerin on Epicardial Coronary Artery**

Intracoronary administration of nitroglycerin induced a consistent and pronounced vasodilation of the epicardial coronary arteries that was less pronounced in the nitrates group than in the no-nitrates group. This difference between the two groups was similar to the difference in the percent increase in platelet cGMP after nitroglycerin injection. In an in vitro experimental study, the relaxant effect of nitroglycerin has been significantly correlated with the increase in the cGMP levels in the vessels.⁴⁷ Thus, our results indicate that in patients administered nitrates, intracellular cGMP production in the response to nitroglycerin injection was impaired. However, there were some limitations in the relation between the coronary dilatation and platelet cGMP levels. As shown in Fig 4, the percent increase in platelet cGMP had good correlation with the percent dilatation of coronary arteries in the no-nitrates group, but no significant correlation was observed in the nitrates group.

**Nitrate Tolerance**

The phenomenon of tolerance to nitrovasodilators was recognized by the end of the 19th century in people working with nitroglycerin for the production of explosives.⁴⁸ However, despite intensive study during the past 100 years, the cause of nitrate tolerance remains controversial. Hemodynamic tolerance to nitroglycerin appears to be multifactorial.

Isolated vessel segments taken from animals treated for some days in vivo with, for example, nitroglycerin, show a very marked desensitization to its effects.⁴⁹-⁵² Incubation of isolated blood vessel segments in physiological solutions containing high amounts of nitroglycerin for as little as 1 hour also results in a very marked desensitization to nitroglycerin.⁴¹,⁴⁹,⁵²,⁵⁴ The biochemical aspects of vascular nitrate tolerance have been shown by Kukovetz and Holzmann, who found a very good correlation between the percent relaxation and the accumulation of cGMP in bovine coronary arteries in response to nitroglycerin, nitroprusside, and 3-morpholinosydnonimine (SIN-1) under different conditions and indicated that there was an impairment of glutathione–organic nitrate reductase activity due to cysteine deficiency. The extensive biochemical research of Ignarro and colleagues has indicated that the spasmylic effect of organic nitrates was mediated through a complex cascade of intracellular biochemical events involving denitration of the organic nitrates by reaction with a thiol-containing group, most probably cysteine, liberation of nitrite, and formation of nitric oxide, which activates guanylate cyclase. Interference at any step of chain of reactions could cause the development of tolerance to nitrovasodilators. It has repeatedly been demonstrated that tolerance is associated with an impaired increase in cGMP levels in response to nitrovasodilators.⁵³-⁵⁷ It has also been reported that guanylate cyclase prepared from nitroglycerin-tolerant vessels is less responsive to the effect of certain nitrovasodilators than that prepared from control vessels.⁵⁸,⁵⁹

Packer and colleagues proposed a role for neurohormonal activation in the development of nitrate tolerance in their study. Dupuis and colleagues reported that tolerance was accompanied by an increase of plasma renin activity after 1 hour and 6 hours of continuous intravenous nitroglycerin therapy. Neurohormonal activation has been known as a cause of hemodynamic tolerance.

Although the mechanisms of tolerance presented above have gained wide acceptance, they do not provide full explanation for all experimental findings, and it has been impossible to evaluate intracellular cGMP production in vivo. Platelets thus provide a useful material to investigate intracellular production of cGMP in response to nitrovasodilators. In the present study, the nitrates group was less responsive to nitroglycerin than the no-nitrates group with regard to platelet cGMP production and coronary artery dilatation. These results support those of previous reports that suggested that there was reduced cGMP production in nitrate tolerance. Platelet cGMP may be used as a clinical indicator of the effects of nitrovasodilators and a decreased response to nitroglycerin may serve as a indicator of nitrate tolerance. However, further studies will be necessary to establish the clinical value of platelet cGMP in predicting nitrate tolerance in patients who are receiving these drugs in more conventional dosing routes and regimens.
Study Limitations

There are several limitations to this study. First, we obtained our blood samples for measurements of platelet cGMP levels 2 minutes after intracoronary injection of 200 μg nitroglycerin. Armstrong and colleagues have shown that the half-life of nitroglycerin was 1.9 minutes after the cessation of intravenous infusion of nitroglycerin and 4.4 minutes after sublingual administration of 0.6 mg nitroglycerin. Wei and Reid have shown that plasma nitroglycerin levels were maximal at 2 minutes after sublingual administration of 0.6 mg nitroglycerin, when the changes in mean heart rate and systolic pressure were also maximal. In our preliminary studies (not published), we measured platelet cGMP in six patients with chest pain syndrome at 30 seconds and 1, 2, and 3 minutes after intracoronary injection of 200 μg nitroglycerin and found maximal levels at 2 and 3 minutes. Armstrong and coworkers also suggested injection of 200 μg nitroglycerin was both safe and sufficient to produce a minimum effective blood level. Böhme and coworkers have reported in their in vitro study that sodium nitroprusside increased cGMP in smooth muscle and platelet within 1 minute and that this effect was not significantly changed over 10 minutes. On the basis of these data, we chose 2 minutes after nitroglycerin injection as a timing of blood sampling and 200 μg as a dose of nitroglycerin.

Second, the possible contribution of cGMP degradation was not investigated in this study. A potential nitrate effect on cGMP degradation may be an important mechanism on nitrate tolerance. In our results, there was no difference in platelet cGMP levels before the injection of nitroglycerin between the nitrate and no-nitrate groups. Thus, it might be speculated that not only the production of platelet cGMP but also cGMP degradation increased in the nitrated group and that cGMP levels had kept their balance in platelet.

Third, in our study, 10 of the 15 patients in the nitrate group had been receiving calcium channel blockers until 48 hours before the study. Intracellular calcium increase is necessary for physiological platelet activity, particularly relating to aggregation. Calcium channel blockers have been known to inhibit platelet aggregation. Although the effect of nitroglycerin on the platelet aggregation was not investigated in this study, the results observed in the nitrate group might have been partially contaminated by a drug-drug interaction involving the calcium channel blockers.

Fourth, the issue of selection of patients for the present study relates to the final limitation. There were the substantial differences in the disease diagnosis between the nitrate and no-nitrate groups. This difference in patient composition might confound interpretation of the results.

Conclusion

In this study, we investigated platelet cGMP levels before and after the injection of nitroglycerin in 15 patients who had previously received nitrates and in 16 patients who had not. Platelet cGMP levels increased significantly after nitroglycerin injection in the two groups, but the percent increase in platelet cGMP levels was significantly higher in the no-nitrates group than in the nitrate group. The percent dilatation of coronary arteries after nitroglycerin injection was also significantly higher in the no-nitrates group than in the nitrate group. These results indicate that platelet cGMP can be a potentially useful indicator to evaluate the effects of nitroglycerin in vivo and that patients in the nitrated group had developed tolerance, which affects intracellular production of cGMP in response to nitroglycerin.

Acknowledgments

The authors are grateful to the staff of the angiography laboratory in blood sampling and catheterization. We thank Nippon Kayaku Co (Tokyo, Japan), especially Mr N. Todoki, Mr M. Hayami, Mr F. Mitani, and Mr T. Kumori, and BML Co (Tokyo, Japan), especially Mr Y. Shimamori, for their help in measurements of cGMP and atrial natriuretic peptide.

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Circulation. 1993;88:29-36
doi: 10.1161/01.CIR.88.1.29

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