Inhibition of Platelet Function by Organic Nitrate Vasodilators

By Andrew I. Schafer, R. Wayne Alexander, and Robert I. Handin

There is evidence that platelet activation in the coronary circulation may be important in the pathogenesis of myocardial ischemia. Since organic nitrate vasodilators are commonly used in coronary artery disease, we have studied the in vitro effects of these drugs on platelet function. Nitroglycerin, isosorbide dinitrate, and their biotransformation product, inorganic nitrite, inhibited platelet aggregation with collagen, epinephrine, arachidonate, and ionophore, and blocked both primary and secondary aggregation in response to ADP. Nitroglycerin was studied in more detail. Its inhibitory effect was reversible and not dependent on external calcium concentration. It inhibited arachidonic acid oxygenation as measured by the arachidonate-induced oxygen burst and malonaldehyde production. These effects were not due to an increase in intracellular cyclic AMP. This unusual generalized inhibition of platelet function by nitroglycerin possibly contributes to its beneficial effect in myocardial ischemia in part by attenuating platelet reactivity in the coronary circulation.

Platelet activation and aggregation in the coronary circulation may be important in the pathogenesis of myocardial ischemia.2 There is also evidence that vasoactive prostaglandin derivatives produced by activated platelets may constrict coronary arteries.2,3 Nitroglycerin (NTG) and other organic nitrate vasodilators have been used for many years to treat angina pectoris. More recently, these drugs have also been used to treat patients with congestive heart failure, to limit the size of myocardial infarcts, and for the long-term prophylaxis of coronary ischemia.4 Despite extensive experience with these coronary vasodilators, their mechanism of action remains undefined. We have studied the effect of organic nitrates on platelet function to determine if inhibition of platelet activation and aggregation might account for a part of their beneficial effect in coronary artery disease.

MATERIALS AND METHODS

Platelet Aggregation

Venous blood anticoagulated with 13 mM sodium citrate was centrifuged at 160 g for 10 min to obtain platelet-rich plasma (PRP). Aggregation was monitored using a standard nephelometric technique5 in which 0.4-ml aliquots of PRP were stirred at 37°C in a Parton dual-channel aggregometer (Payton Associates, Inc., Buffalo, N.Y.). Aggregation was initiated with freshly prepared 12 μM epinephrine, 0.125 mg/ml calfskin collagen, 2.9 μM and 29 μM ADP, 0.5 mM arachidonate, or 10 μM calcium ionophore A23187. Blood was obtained from volunteers who had not ingested aspirin for at least 10 days prior to donation. Platelet counts were determined in a Model F Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

Platelet Lysis

Platelet lysis was determined by 51Cr release.6 Platelet suspensions in plasma anticoagulated with EDTA saline were incubated with Na2[51CrlO4 (100 μCi/109 platelets) for 30 min at 37°C. The labeled platelets were washed twice with platelet-poor plasma, and then incubated for 5 min at 37°C with appropriate dilutions of NTG or lysed by the addition of distilled water and freeze-thawing. Released 51Cr in the separated supernatant was counted in an AUTOLOGIC series gamma counter (Abbott Laboratories, North Chicago, Ill.).

Gel Filtration

Platelets were separated from plasma by gel filtration by applying PRP to a 150 mm × 25 mm column of Sepharose 2B. The platelets were eluted with a modified Tyrode’s buffer containing 130 mM NaCl, 9 mM sodium bicarbonate, 6 mM dextrose, 10 mM sodium citrate, 10 mM Trizma base, 0.8 mM KH2PO4, 3 mM KCl, 0.9 mM MgCl2, 2 mM CaCl2, 0.5% albumin dialyzed overnight, and 0.2% fibrinogen, pH 7.3.

Oxygen Consumption

Polarographic measurements were made with a YSI Model 53 oxygen monitor equipped with a Model 5331 Clark type oxygen sensor (Yellow Springs Instrument Co., Yellow Springs, Ohio). The electrode was fitted to a Gilson water-jacketed cell (OX-15253, Gilson Medical Electronics, Middleton, Wisc.) connected to a Lauda circulator (K-2/RD) maintained at 37°C. Concurrent tracings of oxygen consumption and platelet aggregation could be made by connecting the outputs of both instruments to one dual-channel recorder.6 At 37°C, an 0.13 M NaCl solution was assumed to contain 400 nanoatoms O2/ml at equilibrium with room air. Full-scale deflection of the instrument was adjusted to correspond to this O2 content.10

Malonaldehyde (MDA) Formation

Protein was precipitated from platelet suspensions by the addition of an equal volume of 20% trichloroacetic acid (TCA) in 0.6 N HCl, followed by centrifugation at 8700 g for 15 min in a Beckman microfuge (Beckman, Palo Alto, Calif.). The protein-free supernatant was mixed with an equal volume of 0.53% thiobarbituric acid (TBA), heated at 70°C for 30 min, and cooled to room temperature. Fluorimetric measurements11 were made in a Perkin-Elmer MFP-44B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Conn.), with excitation and emission wavelengths of 510 nm and 550 nm, respectively.

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Fig. 2. Effect of NTG on platelet aggregation in response to 3

There were prepared by dilutions of malonaldehyde tetraethyl acetal in absolute ethanol with equal

553 nm, respectively. MDA standards were prepared by dilutions of malonaldehyde tetraethyl acetal in absolute ethanol with equal volumes of 0.2 N HCl, which were allowed to hydrolyze overnight.12

Assays of Adenylate Cyclase and Cyclic AMP

Platelet-rich plasma (PRP) was prepared from venous blood anticoagulated with 13.5% acid citrate dextrose (ACD). The PRP was adjusted to pH 6.5 with additional ACD, and a platelet pellet was prepared by centrifugation at 1500 g for 10 min. Platelet particulate fraction was prepared by freeze-thawing the platelet pellet in a dry-ice-acetone bath and suspending it in 2 ml ice-cold Tris saline (15 mM Tris-HCl, 138 mM NaCl, pH 7.6). Assay of adenylate cyclase activity in platelet particulate fractions was performed using a previously described modification13 of the method of Salomon et al.,14 using 32P-ATP as substrate and directly measuring the 32P cyclic AMP product. Determination of protein was by the method of Lowry et al.15 Measurement of cyclic AMP in intact platelets was performed by radioimmunoassay, using 125I cyclic AMP tracer (New England Nuclear, Boston, Mass.). TCA extracts of the treated platelets were purified by ion-exchange chromatography (Dowex 50W-X4 200–400 mesh hydrogen form, Bio-Rad Laboratories, Richmond, Calif.). 3H cyclic AMP was used to determine recovery during extraction and purification. Sensitivity of the assay was increased by acetylating the samples with acetic anhydride to give 2’-O-acetyl cyclic AMP.

Chemicals

Reagents were obtained from the following sources: epinephrine, bovine albumin (fraction V), TBA, and adenosine 5’-diphosphate from Sigma Chemical Company, St. Louis, Mo.; collagen from Worthington Biochemical, Freehold, N.J.; ionophore A23187 from Eli Lilly, Indianapolis, Ind.; sodium arachidonate from Nu Chek-Prep, Elysian, Minn.; MDA tetraethyl acetal from ICN K&K Laboratories, Plainview, N.Y.; Sepharose 2B from Pharmacia, Uppsala, Sweden. Dried fibrinogen (human) was obtained from the Massachusetts Red Cross. α-32P ATP, 3H cyclic AMP, and Na251CrO4 were obtained from New England Nuclear, Boston, Mass. PGE1 was kindly provided by Dr. John Pike (Upjohn, Kalamazoo, Mich.). 5,8,11,14-eicosatetraynoic acid (ETYA) was provided by Dr. W. E. Scott, Hoffmann-LaRoche, Nutley, N.J. Nitroglycerin (Nitrostat, Parke-Davis, Detroit, Mich.) and calcium ionophore A23187 (10 M), platelets were preincubated for 5 min with no addition (A) or with NTG at concentrations of 80 μM (B) and 800 μM (C). For aggregation with arachidonate (0.5 mM), concentrations of NTG were control (A), 15 μM (B), and 25 μM (C).

RESULTS

Platelet Aggregation Studies

Nitroglycerin (NTG) inhibited platelet aggregation in a time- and dose-dependent manner. Incubation with NTG for at least 5 min at 37°C was required to inhibit platelet aggregation. As shown in Fig. 1, partial inhibition of collagen-, epinephrine-, and ionophore-induced aggregation was observed with 80 μM NTG, and complete inhibition with 800 μM NTG. Arachidonate-induced aggregation was more easily inhibited, with 25 μM NTG completely abolishing platelet response. Primary and secondary aggregation to ADP were also inhibited by NTG. Complete inhibition of secondary aggregation to both 3 μM and 7 μM ADP was observed with 800 μM NTG (Fig. 2) A concentration-related decrease in primary aggregation was observed at both concentrations of ADP. In contrast, platelet agglutination in response to ristocetin (1.2 mg/ml) was not blocked by NTG. To exclude the possibility that an inert ingredient in Nitrostat
accounted for the antiplatelet effect of NTG, the
aggregation studies were repeated using NTG solution
for intravenous use. The same thresholds for inhibition
of aggregation were observed.

The effect of NTG on platelets was compared to
another organic nitrate vasodilator, isosorbide dinitrate. Inhibition of ADP-, epinephrine-, and arachidonic-acid-induced aggregation was seen but generally required higher concentrations of isosorbide dinitrate than NTG. Aggregation to collagen was not inhibited by the highest concentration of isosorbide dinitrate tested (8 mM) (Fig. 3). Inorganic nitrite (NaNO₂), which is the major biotransformation product of the organic nitrates, inhibited platelet aggregation in response to collagen, arachidonate, and ADP at a concentration of 60 μM (Fig. 4). Inorganic nitrate (NaNO₃), however, had no effect on aggregation at comparable concentrations (Fig. 5).

Reversibility of Nitroglycerin Effect

To exclude the possibility that the inhibition of platelet aggregation by organic nitrates reflected nonspecific platelet damage, we demonstrated that the inhibitory effect of NTG could be reversed by removing NTG. As shown in Fig. 6, platelet aggregation in response to epinephrine, ADP, collagen, and arachidonic acid (A) was inhibited by NTG (B). Gel filtration of the inhibited platelets resulted in prompt reestablishment of a response to the same aggregating stimuli (C). Finally, aggregation in the gel-filtered platelets could again be inhibited by NTG (D). Platelet lysis determined by ⁵¹Cr release was <1% after incubation of platelets with NTG at concentrations up to 800 μM.

Increasing the external calcium concentration up to 5 mM CaCl₂ in gel-filtered platelet suspensions failed to overcome the inhibitory effect of NTG on aggregation induced by collagen or 3 μM ADP (data not shown).

Arachidonic Acid Oxygenation

To examine the effect of NTG on platelet arachidonic acid oxygenation, we assayed the production of malonaldehyde (MDA) a breakdown product of prostaglandin endoperoxides, and the oxygen burst, a direct measure of arachidonic acid oxygenation. In intact platelets, the stimulus of 0.5 mM arachidonate produced a prompt burst of oxygen consumption, generated 0.38 nmole MDA/10⁹ platelets, and was accompanied by simultaneous aggregation. Preincubation with 25 μM NTG abolished the arachidonate-
induced oxygen burst and aggregation, and resulted in a 95% decrease in MDA formation (Fig. 7).

Similar studies using a platelet particulate fraction rather than intact cells produced only minimal inhibition of arachidonate-induced oxygen consumption and MDA generation (Fig. 8). Nitroglycerin suppressed MDA formation in platelet particulate fractions by 24%. In contrast, 0.5 mM aspirin, 10 μM indomethacin, and 30 μM ETYA, an inhibitor of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid oxygenation, inhibited MDA production by 77%, 81%, and 82% respectively, in the same preparations (data not shown).

**Assays of Platelet Adenylate Cyclase and Cyclic AMP**

To determine if the platelet inhibitory effect of organic nitrates was mediated by cyclic AMP, two sets of experiments were performed. In particulate fractions, up to 800 μM NTG did not stimulate platelet adenylate cyclase. At the same time, PGE1 produced a dose-dependent stimulation of cyclase activity with approximately a 23-fold increase over basal at 10^-6 M PGE1. By radioimmunoassay, up to 800 μM NTG did not increase the cyclic AMP content of intact platelets. In the same assay, PGE1 (10^-4 M) and the phosphodiesterase inhibitor theophylline (1 mM) raised cyclic AMP levels approximately 11-fold and 4-fold, respectively.

**DISCUSSION**

Aggregation of platelets within the myocardial microcirculation may play an important role in the development of ischemic heart disease.1,2 There is increasing evidence that, in patients who die suddenly with coronary artery disease, the critical ischemic event may be produced by intravascular platelet aggregates. Spontaneous platelet aggregation16 and elevated plasma levels of two platelet-specific proteins, beta-thromboglobulin17 and platelet factor 4,18 have been demonstrated in patients during acute coronary ischemic episodes. Furthermore, coronary vascular tone may be increased by the vasoactive arachidonic acid derivative thromboxane A2 produced by activated platelets.3,4 It was therefore of interest to investigate the effects of the commonly used organic nitrate coronary vasodilator NTG on various aspects of platelet function. Previous reports have shown inhibition of ADP,19 and epinephrine-induced20 platelet aggregation by NTG.
I. In vitro results in a aggregation (tracing B. lower panel). Preincubation for 5 min with were initiated simultaneously in PRP by the addition of 0.5 mM arachidonate (marked with arrows). Oxygen burst and MDA production (tracing A. upper panel) and aggregation (tracing A. lower panel) and with (B) 5 min preincubation with 25 \( \text{mM} \) NTG.

We have demonstrated that exposure of platelets to NTG in vitro results in a time- and dose-dependent inhibition of platelet aggregation. This effect was reversed by separating the inhibited platelets from NTG, precluding the possibility that the drug produces its inhibitory effect by generalized cytotoxicity. The long-acting organic nitrate, isosorbide dinitrate, was also shown to inhibit platelet aggregation. Nitroglycerin is largely degraded to inorganic nitrates in the liver by means of a glutathione-dependent organic nitrate reductase. It was therefore interesting to note that sodium nitrite, but not sodium nitrate, could also inhibit platelet aggregation.

The mechanism whereby nitroglycerin inhibits platelet function is unclear. Its ability to abolish the oxygen burst and to suppress MDA formation would suggest that it acts on the arachidonic-acid–thromboxane pathway. However, unlike aspirin, indomethacin, and ETYA, NTG does not exert these effects in broken-cell preparations, suggesting that intact cells are required for its action. Furthermore, the ability of NTG also to inhibit collagen-induced and primary ADP-induced aggregation implies a more generalized inhibitory effect on platelets.

Since these observations could be consistent with the generation of the intracellular cyclic AMP by NTG, the effects of this drug on cyclic nucleotides were examined. It has been shown that the inhibitory effect of NTG on smooth muscle tone is potentiated by phosphodiesterase inhibitors, suggesting that NTG may stimulate adenylate cyclase in that system. However, in smooth muscle preparations from several tissues, NTG has been shown not to alter cyclic AMP levels. In platelet particulate fractions in our studies, NTG did not stimulate membrane-associated adenylate cyclase, nor did it elevate the cyclic AMP level of intact platelets. This latter assay excludes the possibility of an inhibitory effect of NTG on platelet phosphodiesterase activity.

A new coronary vasodilator, diltiazem, has also been shown to inhibit platelet aggregation in vitro. This drug is a myocardial calcium antagonist, and its inhibitory action on platelets could be overcome by raising the extracellular calcium concentration. The failure of calcium to correct the inhibitory effect of NTG suggests a different mechanism of action but does not exclude the possibility that NTG inhibits platelets by interfering with internal calcium mobilization. The ability of NTG to inhibit platelet response to calcium ionophore in the absence of an effect on cyclic AMP would be consistent with this mode of action.

Another potent vasodilator, sodium nitroprusside, has also been reported to inhibit epinephrine-, collagen-, and primary ADP-induced platelet aggregation. It has been proposed that nitroprusside exerts its effect through direct inhibition of the platelet contractile proteins. Although nitroprusside has a nitroso moiety, it may be fundamentally dissimilar from the organic nitrates and nitrites. Nevertheless, organic
nitrates might also inhibit contractile protein function in the platelet.

The mechanism by which organic nitrates improve regional myocardial blood flow remains controversial. Although it would be intriguing to postulate that the inhibitory action of NTG on platelet function is important in its beneficial effect on coronary ischemia, this cannot be concluded solely on the basis of the in vitro studies described here. However, NTG could potentially attenuate platelet reactivity caused by various stimuli (e.g., catecholamines) in the coronary circulation. At present, this remains only a theoretical consideration. However, we have shown that the organic nitrates and their biotransformation product, inorganic nitrite, inhibit platelet function in a unique manner, since they inhibit primary aggregation in response to ADP and abolish thromboxane generation from arachidonic acid at a low concentration by a mechanism independent of cyclic nucleotides.

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