

Original Article

Pharmacological Effect of Nitroprusside on Platelet Aggregation

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ABSTRACT

Adequate platelet function and numbers are critical for postcardiopulmonary bypass patients. Endogenous and pharmacological sources of nitric oxide (NO) are known inhibitors of platelet aggregation. Sodium nitroprusside (SNP), used clinically to control blood pressure, is an inorganic source of NO. Our long-term goal is to determine if SNP infusion in the venous return line of the cardiopulmonary bypass system would preserve platelet numbers and function without affecting systemic vascular resistance. Our first requirement to accomplish this goal was to develop an assay that would detect the SNP effect on platelet aggregation. We, therefore, tested the hypothesis that clinical concentrations of SNP would inhibit platelet aggregation. We quantified platelet aggregation with the Medtronic Hepcon HMS whole blood aggregometer. Normal heparinized human blood was treated with 0.625 to 12.5 nM platelet activating factor (PAF), 0.25 to 5.0 μ M epinephrine, or 0.20 to 10 μ M adenosine 5'-diphosphate (ADP) to stimulate platelet aggregation. SNP was added at 10^{-5} M to determine its affect on PAF, epinephrine, and ADP stimulated platelet aggregation. The results demonstrated that PAF-stimulated platelet aggregation was significantly inhibited with SNP (10^{-5} M) to 82% ($p < .05$) of control and epinephrine and ADP mediated aggregation were not significantly affected. In conclusion, at clinically relevant concentrations SNP inhibits platelet aggregation by PAF but not with ADP or epinephrine.

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INTRODUCTION

Cardiopulmonary bypass (CPB) provides the patient with systemic circulation and oxygenated blood during open-heart surgery. The technique of bypass has made many advances in safety over the last 40 years; however, there are still many problems with the artificial circuit. Some of these problems include activation of blood elements from contact with an artificial surface, actual loss of blood elements, and nonphysiological blood flow patterns (1, 2). A portion of these problems can be corrected with engineering and mechanical techniques. The most serious problems, however, will be solved through pharmacological, immunological, and molecular means. Some major complications with patients on CPB are hemorrhage, thromboembolism, and inflammation, caused in part by impairment of platelet function resulting from CPB (3, 4). Furthermore, platelets are activated by exposure to CPB biosurfaces, resulting in significant structural and biochemical changes (5). Therefore, modulation of platelet function during CPB seems to be critical retention of adequate platelet function for post-CPB needs.

Medtronic, Inc. has developed a whole blood platelet aggregation assay utilizing the Hepcon HMS instrument.^a This instrument is currently used in many cardiac operating rooms to determine heparin doses required for anticoagulation during surgery, as well as protamine concentrations for reversal of heparin. This instrument can be modified to perform a whole blood platelet function test using the activating agent, platelet activating factor (PAF).

Platelet activating factor acts via specific receptors on the membranes of responsive cells. Platelet activating factor's stimulation of platelet aggregation has been well described. There is a rapid influx of extracellular calcium and an immediate mobilization of intracellular stores upon stimulation. There is a concomitant rapid second messenger turnover of phosphatidylinositol biphosphate, accompanied by production of diacylglycerol and inositol triphosphate (6). These events are associated with an increase in fibrinogen receptor GPIIb/IIIa and platelet aggregation (7).

Nitric oxide is normally synthesized by endothelial cells, macrophages, and other cells by oxidizing L-arginine through a family of enzymes called nitric oxide synthases. At high concentrations, NO is cytotoxic and it seems that NO plays a key role in the immune response of macrophages to bacteria and other pathogens (8). The vascular endothelium serves as a rich source of NO via endothelial nitric oxide synthase, termed as eNOS (9). This NO provides a means to inhibit platelet activation during normal conditions (10). In the absence of NO, the vasculature is not protected by this vasodilator and its antiplatelet actions (10). During cardiopulmonary bypass, the biosurfaces do not provide the NO to prevent platelet activa-

tion. Therefore, it follows that if an infusion of NO via sodium nitroprusside (SNP) were established into the CPB system, there might be a significant reduction in platelet activation and loss. Furthermore, at the termination of CPB, the SNP infusion could be stopped and the platelet function would be re-established to normal.

Based on the above logic, the aim of this study is to determine the effect of NO on selected platelet aggregation agonists. More specifically, this study is designed to quantify the effect of the NO donor SNP on PAF, adenosine diphosphate (ADP), and epinephrine-mediated platelet aggregation with an *in vitro* whole blood aggregometry system.

MATERIALS AND METHODS

The study was approved by the local University of Arizona Human Subjects Committee, and all subjects gave informed consent. Twelve normal donors between 20 and 50 years old, who were free of medications or substances, donated 25 ml of whole blood. Blood was obtained by venipuncture with a 21-gauge needle. The first 3 ml of blood drawn was discarded to prevent activation of the sample with contaminating tissue thromboplastin. The remaining blood sample was collected in a syringe containing heparin^a in a final concentration of 3 units per ml. The blood sample was then incubated at 37°C for 30 min to allow the platelets to stabilize before the experiment was begun. Following the 30-min incubation, the blood sample was divided for the platelet aggregation assay groups.

Platelet aggregation was quantified with the Medtronic HMS instrument using Platelet Function test cartridges^a designed to evaluate platelet function in fresh whole blood rapidly. This test is a modification of an activated clotting time (ACT) and was performed at 37°C. Each cartridge contained kaolin as a surface activator to initiate blood clotting. Platelet aggregation was evaluated by titration with 0.625 to 12.5 nM platelet activating factor (PAF), 0.25 to 5.0 μ M epinephrine,^b or 0.20 to 10 μ M adenosine 5'-diphosphate (ADP).^b The cartridges consisted of six channels each with a plunger assembly. The first two channels served as controls containing no activating agent. The agent was titrated in the remaining four channels. The sample blood was loaded into a 3-ml syringe with a 19-gauge blunt tipped needle attached and primed. The instrument mechanically dispensed 0.35 ml of blood into each channel. The end point of the test was the detection of clot formation in each channel of the test cartridge. The clot was detected by the fall rate of the plunger assembly through a photo-optical system in the instrument. Clotting times were measured in seconds.

Three repetitions of the assay to determine the optimal dose of SNP, were performed by treating whole blood samples for 5 min at 37°C with gentle rocking. The SNP concentrations ranged from 10^{-5} M to 10^{-8} M. Platelet function was then

a Medtronic Corporation, Parker CO 80134-9061

b Sigma-Aldrich Corporation, St. Louis, MO 63178

measured with the Hepcon HMS instrument with cartridges containing 12.5 nM PAF.

Once the optimal SNP dose was determined, dose response curves for platelet aggregation stimulators were obtained in three separate studies of each activator. Platelet aggregation was quantified using the Hepcon HMS instrument and the Platelet Function test cartridges. As described previously, the first two channels were controls, and the stimulating agent was titrated in the remaining four channels. Three clinically established platelet activators were used: PAF, ADP, and epinephrine. Table 1 shows final activator concentrations used. Each activator was prepared so that 20 μ l of each solution gave the required final concentration in the 0.35-ml blood sample. The appropriate stimulator added with and without SNP was placed in the test cartridges immediately before the blood being dispensed.

The SNP concentration response curves were carried out in the whole blood platelet aggregation assays. Data are expressed as clot ratios, which are given for cartridge channels 3 through 6. The clot ratio was calculated as 1 minus the ratio of the clotting time of the channel containing activator, as compared to the average of the reference clotting time of channels 1 and 2, which contained no platelet activator. This served to compare each blood sample continually to its own baseline clotting time. More specifically, as the concentration of the platelet activator increased, the clotting ratio increased.

All data are reported as mean $\pm$ standard deviation. Each point represents three subjects, and significant differences between the treatment groups (*) was at $p < .05$ analyzed by Student's *t*-test. The 50% inhibitory concentration (IC_{50}) was determined for SNP. The 50% effective concentration (EC_{50}) of each agonist was computed from the concentration curves.

RESULTS

Platelet Activator Dose Response Curves

It was found that the effects of SNP on whole blood platelet aggregation were within clinical concentrations of SNP; however, its effect was specific for only PAF-mediated aggregation. Three clinically established platelet activators were used to determine the inhibitory effect of SNP on whole blood platelet aggregation: PAF, ADP, and epinephrine. Each of these is utilized in clinical laboratories to determine platelet function. Each stimulator was placed in a test cartridge with and without 1×10^{-5} M nitroprusside immediately before the aggregation assay using the Hepcon HMS instrument.

Table 1: Platelet activators final blood concentrations

Activator	Channel 3	Channel 4	Channel 5	Channel 6
PAF	0.625 nM	1.25 nM	6.25 nM	12.5 nM
ADP	0.2 μ M	0.6 μ M	5.0 μ M	10 μ M
Epinephrine	0.25 μ M	1.0 μ M	2.0 μ M	5.0 μ M

Figure 1 shows that nitroprusside significantly inhibits PAF-stimulated whole blood platelet aggregation but does not inhibit ADP and epinephrine platelet aggregation. The PAF stimulated aggregation yielded an EC_{50} of 6.5 nM. SNP at a clinically relevant concentration of 10^{-5} M inhibited platelet aggregation by 59 and 82% at PAF concentrations of 1.25 and 6.25 nM, respectively ($p < .05$). As shown in Figure 2, the EC_{50} for epinephrine was 3.0 μ M, and SNP inhibited epinephrine stimulated platelet aggregation by 29, 31, and 29% at 1, 2, and 5 μ M, respectively ($p = NS$). Figure 3 shows the EC_{50} for ADP of 1.0 μ M and that SNP had no effect on ADP-stimulated platelet aggregation.

Nitroprusside Concentration Response

Concentrations of SNP at 10^{-8} to 10^{-5} M were assayed against the PAF (12.5 nM). Three repetitions of this experiment yielded IC_{50} of 10^{-8} M for nitroprusside. However, 10^{-5} M SNP was used in the remaining experiments, because 10^{-5} M is equivalent to the clinical blood level in a patient receiving an SNP infusion of 5 μ g/kg/min.

DISCUSSION

It was essential to this project to confirm that SNP would have an effect on platelet aggregation measured with the whole blood platelet aggregation assay. This study demonstrated that there is a differential effect on SNP, which was dependent upon the platelet activation agents. We characterized these SNP effects at clinically relevant concentrations of SNP of

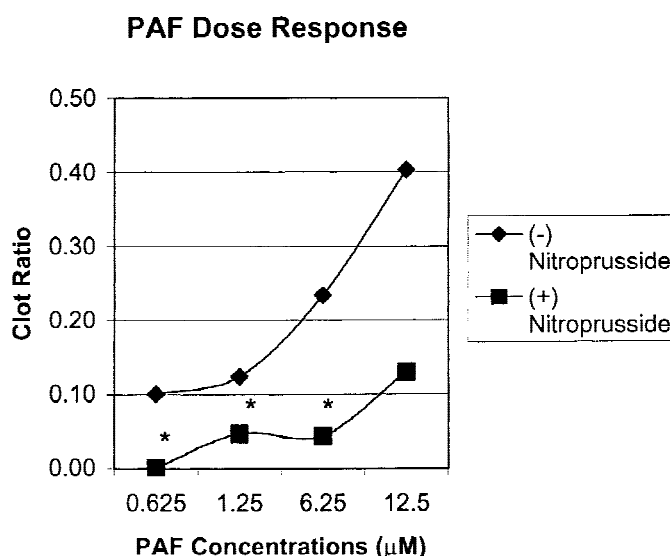


Figure 1: PAF Dose Response Curve: Whole blood platelet aggregation assay using varying concentrations of PAF; diamonds represent platelets alone, and squares represent blood treated with 1×10^{-5} M SNP; this figure represents the curves from three subjects (* $p < .05$)

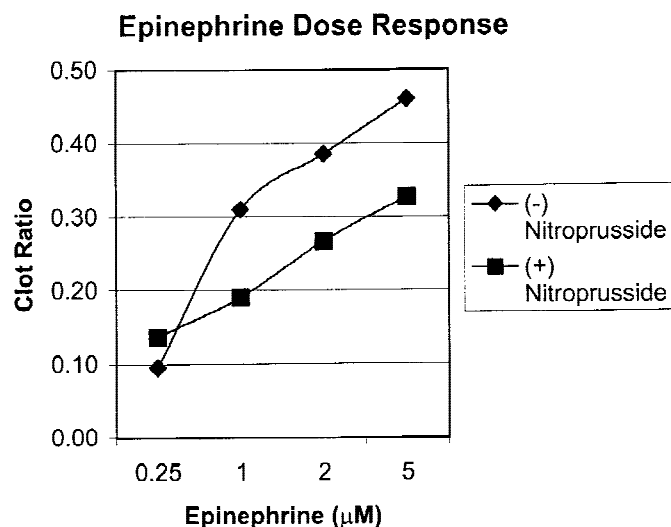


Figure 2: Epinephrine Dose Response Curve: Whole blood platelet aggregation assay using varying concentrations of epinephrine; this figure represents the curves from three subjects; there were no significant differences between the groups

10^{-5} M. The nitroprusside effect was inhibitory to PAF-stimulated aggregation and not cAMP and epinephrine. The latter two agonists stimulated platelet aggregation through a second messenger system that differs from PAF and, therefore, may not be susceptible to the NO effect. Finally, study data support the use of a whole blood platelet aggregation assay in a clinical setting for the measurement of platelet function.

Nitric oxide is a key modulator in vascular physiology; it mediates vasodilation, maintains endothelial homeostasis, and inhibits platelet aggregation, as well as inhibiting leukocyte-endothelial cell adhesion (11). Dysregulation of NO synthesis may play a role in the pathogenesis of several cardiovascular disorders, including essential hypertension, reperfusion injury, atherosclerosis, and the myocardial depression associated with septic shock (8, 12). The ability to inhibit platelet function with an exogenous source of NO serves as a powerful means to modulate platelet function. SNP seems to be a suitable NO source for this application.

Sodium nitroprusside is a potent vasodilator. It is used clinically to lower systemic vascular resistance with minimal effect on myocardial oxygen consumption and heart rate. It has been shown to inhibit platelet aggregation and increase bleeding time (13). SNP has a moderate increasing effect on the heart rate and cerebral blood flow, and moderately decreases the preload and markedly decreases the afterload. These effects begin after 1 min and last for 5 min (14). SNP decomposes rapidly with intravenous infusion, and releases NO (15). Free cyanide, which represents 44% of SNP by weight, is also released in the bloodstream, producing prussic acid. This is responsible for the acute toxicity; however, systemic cyanide toxicity is rare (16). This application of SNP as an infusion into the CPB circuit may preserve platelet function and numbers,

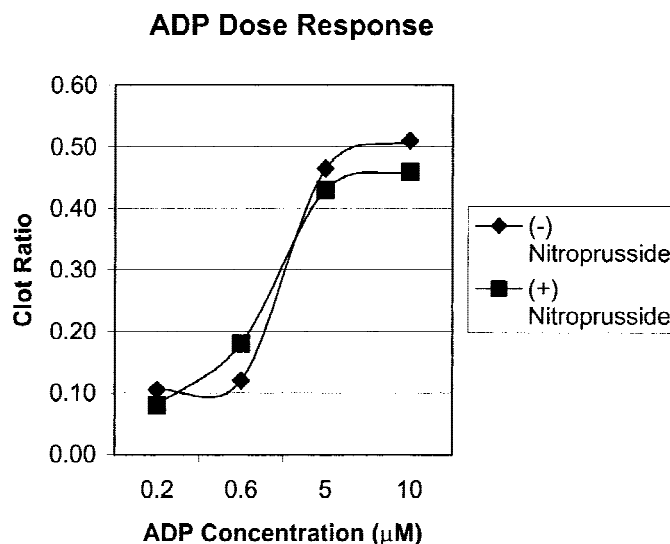


Figure 3: ADP Dose Response Curve: Whole blood platelet aggregation assay using varying concentrations of ADP; this figure represents the curves from three subjects; there were no significant differences between the groups

and once CPB is terminated, the normal platelet function should be restored.

All three agonists used in this study have specific and different receptors on the platelet membrane, and their intracellular molecular pathways lead to the up-regulation of the fibrinogen receptor GPIIb/IIIa (7, 17). Adenosine diphosphate causes conformational changes in its receptor, resulting in exposure of fibrinogen binding sites (17). Adenosine diphosphate is a primary aggregating agent and has different effects at different concentrations. At low concentrations of 0.1–0.5 μM, ADP induces platelet shape changes from discoid to spherical. Reversible aggregation occurs at concentrations of 0.5–1.5 μM; whereas, higher concentrations up to 5 μM causes a secondary phase of aggregation and release of dense, α -storage granules (17). Finally, the released ADP from the dense granules is responsible for the irreversible phase of aggregation *in vitro*.

This study attempted to show the effect of SNP on collagen-stimulated aggregation in the whole blood aggregation system. However, satisfactory aggregation curves could not be achieved. Therefore, this study does not report the SNP effect on collagen-mediated aggregation. Collagen is a pivotal platelet-activating agent. Collagen binds to two glycoprotein receptors on platelets. GP Ia-IIa complex, an integrin in the β_1 family, binds collagen types I and IV (7). The many distinct types of collagen have made it difficult to isolate and define the requirements of the collagen molecule for platelet aggregation. Different types of collagen seem to have different roles in initiating platelet aggregation or secretion of platelet granules. Collagen can induce the release of ADP, even in the absence of aggregation. In fact, it is thought that activation of platelets by

collagen requires ADP and the synthesis of prostaglandins (10). This intertwining makes it difficult to separate the pathways of platelet activation.

Epinephrine acts on the platelet membrane through α_2 -adrenergic receptors, which are predominant on the membrane (18). Platelet aggregation does not require a decrease in cyclic AMP. Catecholamines act on the α_2 -adrenergic receptors and inhibit adenylate cyclase via guanine nucleotide-binding regulatory proteins. Because epinephrine alone is essential for platelet aggregation, it must act in cooperation with other molecules to activate platelet aggregation. The ability of platelet agonists to act synergistically is generally accepted; however, the biochemical basis for these interactions is poorly understood. It has been shown that ADP and epinephrine can act together to expose the fibrinogen receptor (17).

Platelet activating factor stimulation of platelets is well characterized. It is known that there is a rapid influx of extracellular calcium and movement of intracellular calcium stores as a result of stimulation. This causes second messenger systems to begin their resultant cascades, thereby increasing fibrinogen receptors (GPIIb/IIIa) and platelet aggregation.

Platelet activating factor is a potent biological autocoid that affects most inflammatory cells and tissues. Platelet activating factor was discovered in the early 1970s independently by investigators as a soluble factor that activated platelets and lowered blood pressure (19). It has been found that PAF is part of a family of structurally related compounds. The historical name given to this phospholipid compound describes only one of its many physiological functions. Other pathological and physiological properties include purported roles in inflammation, asthma, pulmonary distress, hemostasis, ischemia, and shock (6, 20).

Platelet activating factor is rapidly synthesized by many cells after stimulation. Most immunological cells can produce PAF, as well as platelets, mesangial cells, and the vascular endothelium. Additionally, tissue sources of PAF have been found, including blood, skin, lung, myocardium, liver, brain, and kidney (6, 19). The use of PAF, therefore, as the primary platelet agent in the Medtronic HMS system is justified. Platelet activating factor is a ubiquitous, potent, and stable agent that seems to mediate platelet aggregation through a final common pathway of GPIIb/IIIa expression.

Platelet function is assessed clinically with the platelet aggregation test. This test requires that the platelets are isolated from whole blood, the concentration normalized, and functions assayed with specific activating agents. Activating agents are added to the sample cuvette. As platelets aggregate in the cuvette, light transmission increases because of clarification of the suspending medium, which allows more light transmission. This is recorded and printed out as an aggregation curve. Characteristic aggregation curves can be obtained with very specific activating agents such as ristocetin, collagen, ADP, epinephrine, thrombin, thromboxane, and PAF (21). Platelet function is

then reported as a subjective interpretation of aggregation curves over time as (+, ++, +++, +++++). The weaknesses of this assay are several: the number of platelets per unit of blood is not the same as the donor patient's, the reported parameters are very subjective, many of the blood elements are removed during the platelet preparation, and the assay requires technical expertise and time. The advantage of this assay is the ability to test platelet function with a number of activating agents to assess those pathways that are active and those that are inhibited. Platelet function testing during, or subsequent to, bypass is complex to assess with any assay system; however, the whole blood assay may provide a more clinically relevant assessment.

Platelets are activated via membrane receptors associated with biochemical signaling pathways within the platelet. Normally, endocrine and metabolic activating agents are derived from a site of vascular injury. These activating agonists may include: PAF (22), ADP (23), epinephrine (24), thrombin, and thromboxane A_2 . On a rank order of potency, thrombin and thromboxane A_2 are the most potent agonists. There are three levels of platelet activation: (1) platelet changes from a smooth disk to a spiny sphere; (2) the platelets' adhesion molecule expression and aggregation; and (3) degranulation of stored platelet α -granule products (platelet-derived growth factor, thrombospondin, platelet factors 1–7, coagulation proteins) and important factors in the dense granules (ADP, epinephrine, calcium, and serotonin) (25). These products have been measured during CPB to support the thesis that platelets are markedly activated during CPB (26).

Thrombocytopenia with CPB has been well documented, with some studies reporting as much as 30 to 50% decrease from baseline (24, 27). Platelets are sequestered in organs during bypass secondary to the CPB-induced stimulation (3). Other contributing factors may be physical removal of platelets, hemodilution, shear forces, exogenous drugs, and endogenous soluble mediators induce platelet numbers during bypass. Protamine causes a transient thrombocytopenia speculated to be mediated by the generation of complement (28). This is supported by the appearance of temporary platelet aggregates in the lungs and can be seen following protamine sulfate administration (29).

Endogenous soluble mediators released as a consequence of CPB cause platelet activation. These endogenous chemical mediators of thrombocytopenia include thrombin, complement, cytokines, and adrenaline (30). Thrombin, generated during bypass, stimulates platelet protein kinase-C activation, possibly through a GTP-binding protein-linked mechanism (31). Cardiopulmonary bypass causes elevated levels of adrenaline that is known to bind with the adrenoceptors on platelets and causing activation (24).

CONCLUSION

There are numerous pathways as a result of CPB that may

result in platelet activation, aggregation, sequestration, degranulation, and consumption. At the completion of CPB, a coagulopathic state may occur because of the platelet loss. The goal of this project was to test the hypothesis that SNP could inhibit platelet activation at pharmacological concentrations and assess whole blood aggregometry methodology to measure this inhibitory effect. This study demonstrated that the effects of SNP on whole blood platelet aggregation were within clinical concentrations of SNP, and its effects were significant only for PAF-mediated aggregation.

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