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Inhibitory effect of nitrite on coagulation processes demonstrated by thrombelastography

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ABSTRACT

Nitric oxide (NO) can be generated by two-step reduction pathway in which nitrate is converted first into nitrite and then into NO via several mechanisms, as well as from arginine by endogenous nitric oxide synthase (NOS). We have recently shown that nitrite ions in the presence of erythrocytes inhibit platelet aggregation and activation, as measured by aggregometry and flow cytometric analysis of P-selectin, through its reduction to NO under partially deoxygenated conditions. In the current study, we investigated how nitrite may affect overall clotting processes via modulating platelet function using thrombelastography (TEG). We measured three major TEG parameters, reaction time (*R*, time to initial fibrin formation), α angle (velocity of clot growth) and maximum amplitude (MA, maximum clot strength) using blood from healthy volunteers. An NO donor (DEANONOate) showed inhibitory effects on all TEG parameters in platelet rich plasma (PRP) and whole blood, resulting in delayed *R*, decreased angle, and reduced MA in a dose dependent manner. Nitrite ions also exhibited inhibitory effects in whole blood at 20% hematocrit, and this was greatly enhanced under hypoxic conditions, being demonstrable at 0.1 μM concentration. Neither compound changed any TEG parameters in plasma. Our results suggest that nitrite affects overall blood clotting and that TEG may be used to follow this process. Further the physiological effects of factors which determine NO bioavailability, such as endogenous levels of blood and tissue nitrite, may be useful as biomarkers for predicting hemostatic potential.

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1. Introduction

The physiological regulation of platelet reactivity in blood vessels is tightly controlled by balancing prothrombotic and antithrombotic signals through various mechanisms to maintain hemostasis. Nitric oxide (NO), which is a gaseous signaling molecule that can exert various physiological functions, is one of the potent inhibitors of platelet function. Numerous studies since the 1980's showed that NO inhibits platelet aggregation and adhesion to the endothelium via activation of soluble guanylyl cyclase (sGC) and subsequent cyclic guanosine monophosphate (cGMP) production resulting in mobilization of Ca^{2+} flux [1–3]. NO is generated from L-arginine and molecular oxygen by the action of nitric oxide synthase (NOS) [4]. In addition to this endogenous NO generation mechanism, another alternative pathway for NO production, which involves serial reductive pathways of nitrate, is now understood [5]. A diet rich in vegetables is a good source of inorganic nitrate. Once nitrate is absorbed from the diet, it can be reduced to nitrite mainly by bacterial nitrate reductases in the oral cavity [6] and nitrite can be further reduced to NO in blood and tissues by various mechanisms utilizing deoxyhemoglobin [7,8], deoxymyoglobin [9,10], xanthine oxidase [11,12], and non-enzymatic reduction in the presence of protons [13,14] or vitamin C [15]. These reductive pathways are prominently enhanced under hypoxic conditions making them a good backup system for the shutdown of oxygen-dependent NOS activity under hypoxic situations. Several animal studies showed the therapeutic effects of inorganic nitrite and nitrate in ischemic injury models, which provides compelling evidence of nitrite and nitrate contribution to NO bioavailability [5].

We recently demonstrated that nitrite ions could inhibit aggregation and activation of human platelets in the presence of erythrocytes through its reduction to NO and this effect was promoted at lower oxygenation where deoxyhemoglobin efficiently reduces nitrite to NO [16]. Moreover, we showed in mice that blood nitrite

Abbreviations: NO, nitric oxide; TEG, thrombelastography; ADP, adenosine diphosphate; AA, arachidonic acid.

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levels are positively correlated with tail bleeding time and inversely correlated with platelet aggregation and granule release upon membrane receptor activation by adenosine diphosphate (ADP) and collagen [17]. Taken together, these results suggest that nitrite might play a critical role in regulating platelet reactivity and overall blood clotting upon its reduction to NO in blood vessels especially under hypoxic conditions.

Regulation of platelet reactivity by NO in hemostatic processes is of importance for vascular homeostasis, however the potential effect of nitrite on clotting pathways has not been explored in detail. It is crucial to understand how nitrite may affect the dynamics of hemostasis in vessels and what is its physiological role in regulating platelet reactivity since it acts as a NO precursor in blood. In the current study, to explore the contribution of nitrite to regulating overall processes of coagulation, we employed thrombelastography (TEG) which is a method widely used in several clinical settings during surgery to monitor hemostasis. TEG measures viscoelastic changes of clotting blood with time assessing coagulation dynamics from the initiation of fibrin formation to clot firmness [18]. We compared DEANONOate (an NO donor) and nitrite effects on three major TEG parameters, reaction time (*R*, the time until initial fibrin formation), α angle (an indicator of clot growth velocity) and maximum amplitude (MA, clot strength) in platelet rich plasma or 20% hematocrit blood, to simulate blood found in the microcirculation, obtained from healthy volunteers. We observed an inhibition in coagulation by DEANONOate in both platelet rich plasma and whole blood, but nitrite effect was noticeable only in whole blood but this was accentuated in hypoxic conditions. These results suggest that nitrite might play a critical role in modulating hemostatic processes once it is converted to NO by deoxyhemoglobin in blood in the microvasculature.

2. Materials and methods

2.1. Ethics statement

This study was conducted with a clinical protocol reviewed by the NIDDK Institutional Review Board at NIH. Before inclusion in the study, informed written consent was obtained from each subject.

2.2. Reagents

Sodium nitrite, DEANONOate and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were purchased from Sigma (St Louis, MO). PlateletMapping® Assay kit and Biological QC Level I/II kit were purchased from Haemonetics (Braintree, MA).

2.3. Blood collection

Venipuncture was done in healthy volunteers (aged 23–49 years) using a 21-gauge butterfly needle into vacutainer tubes containing sodium citrate or sodium heparin (Becton Dickinson, Franklin Lakes, NJ). The first 3 mL of blood was discarded. Blood was kept in a polypropylene tube with a dual-position snap cap (Becton Dickinson, Franklin Lakes, NJ) for room air equilibration unless deoxygenation was performed. The pO_2 of blood was maintained at 100.5 ± 11.1 mmHg under room air condition and the oxygenation was measured using a blood gas analyzer (ABL80 FLEX CO-OX, Radiometer, Westlake, OH). All experiments were completed within 4 h after blood withdrawal.

2.4. Blood sample preparation

To obtain platelet-free plasma, whole blood was first centrifuged at 1600*g* for 15 min to get platelet-poor plasma. Then the platelet-poor plasma was filtered using a syringe filter unit (0.22 μm, Millipore, Billerica, MA) to remove remaining platelets. Platelet numbers were under the detection ranges with an analyzer (CELL-DYN 3700, Abbott, Abbott Park, IL) after filtration. For platelet-rich plasma, whole blood was centrifuged at 120*g* for 15 min and the upper phase was taken. To prepare blood with different hematocrit, whole blood was diluted with plasma (vol/vol) resulting in 5%, 10%, 20% and 30% hematocrit respectively. Whole blood of all volunteers participated in this study had an average hematocrit of 37.4 ± 4.6% and this was considered 40% hematocrit.

2.5. Preparation of deoxygenated blood

Whole blood in a glass Erlenmeyer flask with a stopper was deoxygenated by blowing helium gas into the flask with gentle stirring for 30 min at room temperature. The pO_2 was checked using a blood gas analyzer (ABL80 FLEX CO-OX, Radiometer, Westlake, OH) after deoxygenation and the average value was 37.4 ± 12.4 mmHg. The deoxygenated blood was kept in the flask and the stopper was opened only in a glove box for further manipulation.

2.6. Evaluation of coagulation by thrombelastography (TEG)

TEG experiments were performed at 37 °C using a Haemostasis Analyzer (TEG®5000, Haemonetics, Braintree, MA) according to the manufacturer's guidelines. The TEG analyzer was calibrated and evaluated daily by running quality control samples before experimental sample analysis. For platelet-free plasma samples, 1 mL of plasma was mixed with kaolin (Cat. No. 6300, Haemonetics, Braintree, MA). Then a 340 μL volume of kaolin-activated plasma was immediately added to the pre-warmed TEG cups which contained 20 μL of 0.2 M $CaCl_2$ to initiate coagulation. For heparinized blood or platelet-rich plasma, 360 μL of sample was added into the cups and mixed with activator and adenosine diphosphate (ADP) or arachidonic acid (AA) (2 μM and 1 mM respectively, PlateletMapping® assay kit, Cat. No. 07-014, Haemonetics, Braintree, MA) to induce platelet activation. DENONOate or nitrite was pre-incubated with plasma or blood at 37 °C for 5 min before induction of coagulation.

2.7. Statistics

Statistics were analyzed using ANOVA (*P*-value <0.05, statistically significant) with Origin 8 (Origin Lab Corporation, Northampton, MA). Data shown are presented as the mean ± standard deviation (SD).

3. Results

3.1. NO does not alter intrinsic coagulation pathway

To examine whether NO could directly influence the intrinsic coagulation pathway, we analyzed coagulation processes initiated by kaolin and calcium in platelet-free citrated-plasma using TEG (Fig. 1A). The NO donor, DEANONOate, did not change any of the three major TEG parameters, *R*, α angle and MA, from 0.01 to 1 μM suggesting that NO itself at physiological concentrations does not interfere with clotting factor pathways initiated through factor XII.