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# Platelet inhibition and increased phosphorylated vasodilator-stimulated phosphoprotein following sodium nitrite inhalation

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ABSTRACT

In the presence of red blood cells (RBCs), nitrite inhibits platelets through its conversion to nitric oxide (NO) by the reductase activity of partially deoxygenated hemoglobin. Inhaled sodium nitrite is being investigated as a therapy for pulmonary hypertension. Here, we measured platelet aggregation, P-selectin expression, platelet-leukocyte aggregates and phosphorylated vasodilator-stimulated phosphoprotein (P-VASP$^{Ser239}$) following sodium nitrite inhalation in healthy subjects. *In vitro* incubation of nitrite with deoxygenated whole blood showed an increase in P-VASP$^{Ser239}$, which was inhibited by ODQ, a soluble guanylyl cyclase (sGC) inhibitor. Immediately and 60 min after nitrite inhalation, P-VASP$^{Ser239}$ increased in platelets. Platelet aggregation, P-selectin expression, platelet-monocyte and platelet-lymphocyte aggregates decreased after inhalation. In conclusion, sodium nitrite administered to healthy subjects by inhalation can inhibit platelet activation and increase P-VASP$^{Ser239}$ in platelets. Platelet inhibition by nitrite administration may be useful in disorders associated with platelet hyperactivity.



## 1. Introduction

Nitrite anion ($NO_2^-$) is converted to nitric oxide (NO) by enzymatic and non-enzymatic processes [1]. Nitrite at physiologic concentration (0.1 μM) inhibits platelet aggregation in the presence of RBCs, and this effect is enhanced by deoxygenation [2,3]. Platelet inhibition by nitrite showed a positive correlation with levels of deoxygenated hemoglobin in blood [4]. Increased plasma nitrite levels following ingestion of nitrate either from beetroot juice or potassium nitrate decreased platelet aggregation and P-selectin expression, and increased cGMP levels in platelets [5,6]. Increased platelet aggregation induced by collagen was reported in mice ingesting a low nitrite/nitrate diet with reduced plasma nitrite levels [7]. Nitrite has been studied extensively in humans administered by intravenous [8,9], intra-coronary [10], and inhalation routes [11], and has entered phase 2 clinical trial for pulmonary hypertension [12–14].

Vasodilator-stimulated phosphoprotein (VASP) is an actin binding protein expressed in human platelets. The phosphorylated form of VASP displays a crucial role in platelet inhibition by inactivation of glycoprotein IIb/IIIa (GPIIb/IIIa) [15,16]. NO, administrated as NO-donor, increases P-VASP$^{Ser239}$ via cGMP/sGC/protein kinase G signaling [17,18]. Loss of GPIIb/IIIa regulation by VASP tends to cause platelet hyperactivation [15,19]. Increased platelet adhesion and decreased platelet inhibition by NO donors were reported in VASP knockout mice. In addition, anti-platelet drugs including cilostazole and dipyridomole induce P-VASP$^{Ser239}$ in

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platelets [20,21].

In this study, we aimed to investigate the effect of nitrite given by inhalation on platelet activity and P-VASP$^{Ser239}$, a marker of downstream signaling of NO/sGC/protein kinase G in platelets of healthy subjects. Platelet activity was determined by aggregometry and flow cytometry while P-VASP$^{Ser239}$ was detected by western blots.

## 2. Materials and methods

### 2.1. Subjects

This study was approved by the Ramathibodi Hospital Ethics Committee. The written informed consent was obtained in accordance with the Declaration of Helsinki. Healthy subjects were 8 females and 4 males, who were 27.00 ± 1.25 years of age.

### 2.2. In vitro experiments

Blood samples were collected in tube containing 3.8% sodium citrate. Whole blood samples (average % hematocrit = 44.23 ± 5.10) were partially deoxygenated by helium gas blown above cell suspension for 10 min with gentle stirring. The oxygen saturation was measured by blood gas analyzer (Nova Biomedical Critical Care Xpress, Waltham, MA). The deoxygenated blood contained partial pressure of oxygen ($PO_2$) 25 mmHg and oxygen saturation ($SO_2$) 65% while untreated blood at room air contained $PO_2$ 56 mmHg and $SO_2$ 90%. Nitrite (2 μM) was incubated in blood samples at 37 °C with or without 10 μM 1H- [1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ). Blood was collected at 5, 10 and 15 min after addition of nitrite and centrifuged at 120 × g for 3 min at room temperature to obtain platelet-rich plasma (PRP). Platelets were separated from PRP and collected with lysis buffer [50 mM Tris, 0.5% NP-40 and, 150 mM NaCl and a proteinase inhibitor cocktail III (1:1000 Calbiochem, La Jolla, CA).

### 2.3. Western blot analysis of P-VASP$^{Ser239}$

Protein (10 μg) was separated by 12% SDS-PAGE and transferred into nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk overnight, and incubated with anti-P-VASP$^{Ser239}$ (Millipore, Billerica, MA) or anti-VASP (Cell Signaling Technology, Danvers, MA) or GAPDH (Cell Signaling Technology, Danvers, MA). Anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was used as secondary antibody and followed by enhanced chemiluminescent detection (Biorad, Hercules, CA). The densitometry of bands was quantified by Image J software (National Institutes of Health).

### 2.4. Nitrite inhalation

Sterile sodium nitrite solution (50 mg/ml) for inhalation was prepared by the Faculty of Pharmaceutical Science, Chulalongkorn University. Sodium nitrite diluted with sterile saline (4 ml) was administered by nebulization using Beurer Nebulizer (IH 25/1, Beurer Medical, Ulm, Germany) for 15 min. Owing to the maximum tolerated dose of 90 mg [11], 40-mg sodium nitrite was initially administered to subjects. There was no adverse effect observed in any healthy volunteers.

Whole blood samples were collected at baseline, and time 0 and 60 min after the end of a 40-mg nitrite inhalation in tubes containing 3.8% sodium citrate. Whole blood was diluted 1:1 and 1:9 for aggregometry and flow cytometry, respectively. For western blots, platelets were separated from PRP and collected in lysis buffer containing proteinase inhibitors.

### 2.5. Nitrite measurement in whole blood

Blood samples were collected at baseline, and time 0 and 60 min after the end of 40-mg nitrite inhalation using heparin (143 units/10 ml) as an anticoagulant. Whole blood samples were mixed immediately with the nitrite-stabilizing solution containing 0.8 M ferricyanide, 10 mM N-ethylmaleimide, and 1% NP-40 in a 5:1 dilution [22], and stored at −80 °C. The nitrite levels were measured by tri-iodide based chemiluminescence (Eco Medics Analyzer CLD88, Duernten, Switzerland) [23].

### 2.6. Platelet aggregometry

Whole blood was diluted 1:1 with 0.9% NaCl. Platelet aggregation was induced by 20 μM ADP, and measured by an impedance aggregometer (model 500/560 CA, Chrono-Log Corporation, Howertown, PA) for 6 min at 37 °C.

### 2.7. Flow cytometry

Ten-fold diluted whole blood was stained with PE-labeled anti-CD62P (BD bioscience, San Jose, CA) and PE-Cy5-labeled anti-CD42b (BD bioscience, San Jose, CA), and stimulated with 20 μM ADP and U46619 (thromboxane $A_2$ agonist) for 10 min. Blood samples were fixed with 1% paraformaldehyde and analyzed by FACS Calibur (BD Bioscience, San Jose, CA). Percentages of P-selectin expression, a platelet degranulation marker, were calculated from 10,000 CD42b-positive events.

In addition, platelet-leukocyte aggregates were determined as markers of platelet activation. Ten-fold diluted whole blood samples were incubated with FITC-labeled CD42a (BD bioscience, San Jose, CA), and PerCP-labeled CD45 (BD bioscience, San Jose, CA) for 15 min in dark at room temperature. RBCs were lysed with FACS lysis buffer. Platelet-leukocyte aggregates were measured by FACS Calibur. Leukocyte subpopulations (neutrophils, monocytes and lymphocytes) were identified by the pattern of SSC and CD45 expression. CD42a (a platelet marker) positive events in each leukocyte subpopulation were identified [24].

### 2.8. Data and statistical analysis

All data are presented as means ± standard deviation unless indicated. Data processing and statistical analysis were analyzed by GraphPad Prism® version 4 (GraphPad software Inc., San Diego, CA). For the *in vitro* experiments, statistical significance was accepted at $P < 0.05$. For inhalation experiments, student's paired *t*-test was used to determine significant differences between values of baseline versus those of time 0 and 60 min after end of inhalation. We applied Bonferroni corrections as an adjustment for multiple testing and defined statistical significance at $P < 0.025$.

## 3. Results

### 3.1. In vitro incubation of whole blood with sodium nitrite

There was no change in platelet P-VASP$^{Ser239}$/VASP ratio when air equilibrated whole blood was incubated with 2 μM nitrite at room air (Fig. 1A and B). In deoxygenated whole blood, incubation of 2 μM nitrite increased P-VASP$^{Ser239}$/VASP ratio; the difference became statistically significant by 15-min after incubation. The increase in P-VASP $^{Ser239}$/VASP ratio was inhibited by sGC inhibitor ODQ (Fig. 1C and D).