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Effect of storage on levels of nitric oxide metabolites in platelet preparations

Ji Won Park¹, Barbora Piknova¹, James Kurtz², Shalini Seetharaman², Stephen J. Wagner², and Alan N. Schechter¹

¹Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892 USA

²Blood Components Department, American Red Cross Holland Laboratory, Rockville, MD 20855 USA

Abstract

Background—Nitric oxide (NO), a potent signaling molecule, is known to inhibit platelet function *in vivo*. We investigated how the levels of NO and its metabolites change during routine platelet storage. We also tested whether the material of platelet storage containers affects nitrite content since many plastic materials are known to contain and release nitrite.

Study design and methods—For nitrite and nitrate measurement, leukoreduced apheresis platelets (PLT) and concurrent plasma (CP) were collected from healthy donors using the Trima Accel. Sixty mL aliquots of PLT or CP were stored in CLX or PL120 Teflon containers at 20–24°C with agitation and daily samples were processed to yield PLT pellet and supernatant. In a separate experiment, PLT was stored in PL120 Teflon to measure NO generation using electron paramagnetic resonance (EPR).

Results—Nitrite level increased markedly in both PLT supernatant and CP stored in CLX containers at a rate of 58 nM/day and 31 nM/day respectively. However, there was a decrease in nitrite level in PLT stored in PL120 Teflon containers. Nitrite was found to leach from CLX containers and this appears to compensate for nitrite consumption in these preparations. Nitrate level did not significantly change during storage.

Conclusion—Platelets stored at 20–24°C maintain measurable levels of nitrite and nitrate. Nitrite decline in non-leachable Teflon containers in contrast to increases in CLX containers which leach nitrite, suggests that it is consumed by platelets, residual leukocytes or erythrocytes. These results suggest NO-related metabolic changes occur in platelet units during storage.

Keywords

nitric oxide; nitrite; platelet storage; transfusion

Author Correspondence: Alan N. Schechter, MD, Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Drive, 9N314, Bethesda, MD20892; telephone: 301-496-5408; fax: 301-402-0101; aschechter@niddk.nih.gov.

Reprint request to: Alan N. Schechter

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Introduction

Nitric Oxide (NO) is the endothelium-derived free radical that regulates vascular tone and other function by stimulating soluble guanylyl cyclase (sGC) in smooth muscle cells¹⁻³ and also plays an important role in the regulation of thrombosis and hemostasis by modulating platelet function^{4,5}. NO generated in the endothelium by endothelial nitric oxide synthase (eNOS) can diffuse into the lumen of the vessel and traverse the platelet membrane. NO binding to intracellular sGC leads to the generation of cyclic guanosine monophosphate (cGMP) in platelets and initiation of various downstream signals including reduction of Ca^{2+} mobilization⁶⁻⁸, shape change by cytoskeleton rearrangement^{9,10}, and decreased adhesion and aggregation^{10,11}. While the effect of endothelium-derived NO on platelet function is well established by numerous studies, the presence of an eNOS signaling pathway endogenous to platelets is still under debate. It was first reported in 1990 by Radomski *et al.* that platelets have a functional eNOS signaling pathway¹², however recent studies suggested that platelets do not express detectable amount of eNOS¹³⁻¹⁵. Gambaryan *et al.* suggested that previous studies might have used impure platelets which contained other cell types and/or inappropriate antibodies against eNOS¹⁴.

In addition to eNOS-derived NO production, an alternative pathway for NO generation was recently established showing that the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-), once considered inert end products from NO generation, can be reduced to NO by non-enzymatic as well as enzymatic pathways¹⁶⁻¹⁹. Nitrate from dietary or endogenous sources is first converted to nitrite by commensal bacteria in the oral cavity^{19,20}, nitrite then can be further reduced to NO by several pathways including deoxyhemoglobin^{21,22}, deoxymyoglobin^{23,24}, xanthine oxidoreductase²⁵⁻²⁷, or ascorbate²⁸. Recent studies on human subjects show that nitrate from dietary source inhibits platelet aggregation in response to platelet agonists such as collagen and ADP²⁹ and inorganic nitrite inhibits platelet aggregation in the presence of erythrocytes³⁰ suggesting a NOS-independent antiplatelet effect of nitrite metabolites.

Since there is increasing demand for platelet transfusion, it is of potential value to understand platelet physiology and biochemical changes which occur during storage to improve platelet quality. Platelet storage is currently restricted to 5 days because of the risk of bacterial contamination and because of well characterized decrements in PLT quality during storage, leading to a decline in platelet storage properties as well as apoptotic changes including externalization of phosphatidylserine and mitochondrial damage³¹⁻³⁶. There is no published study so far on the levels of NO and its metabolites as a function of platelet storage time. Considering the possibility that nitrite and nitrate can be converted back to NO and NO inhibits many aspects of platelet function, we studied NO metabolism in platelets by monitoring levels of nitrite, nitrate and NO during storage of apheresis platelet units and concurrent plasma.

Materials and Methods

Apheresis platelets collection and storage

Apheresis platelets were collected from healthy donors using a Trima Accel cell separator (Software version 5.1, CaridianBCT Inc., Lakewood, CO) with a targeted yield of 3.5×10^{11} platelets. Aliquots of 60 mL of apheresis PLT were transferred, using a sterile connecting device (TSCD, model SC-201A, Terumo Medical Corp., Elkton, MD), into CLX (Pall Corporation, East Hills, NY) or PermaLife PL120 Teflon (OriGen Biomedical, Austin TX) containers. Similarly, 60 mL aliquots of CP were sterilely transferred into CLX or PL120 Teflon containers. PLT and CP aliquots were stored in a PLT incubator (Helmer, Nobelsville, IN) at 20–24°C with constant reciprocal agitation.

Nitrite, nitrate and nitrosothiol analysis

Nitrite and nitrate levels in the apheresis platelet units were measured using a standard chemiluminescence method³⁷. For nitrite analysis, 1mL samples of PLT or CP were first centrifuged at 3,000 xg and the resulting supernatant and pellets were separately mixed with nitrite preserving solution ($K_3Fe(CN_6N$ -ethylmaleimide, water, Nondiet P-40) to maintain nitrite levels and kept frozen at $-70^{\circ}C$ until analysis. Samples were deproteinized by dilution with the same amount of methanol, then centrifuged for 5 min at 13,000 rpm (AccuSpinMicroR, Fisher Scientific, Pittsburgh, PA) and the supernatant was immediately injected into the chemiluminescent nitric oxide analyzer (NOA, Sievers, Model 280 NO analyzer, Boulder, CO) using helium as the carrier gas for chemiluminescence determination of NO. For nitrate analysis, 5% Triton X-100 in deionized water was added to PLT supernatant, pellets or CP instead of nitrite preserving solution at a 1:9 ratio between the sample and 5% Triton X-100. At the time of sample analysis, a 3:1 dilution of ethanol and thawed sample was centrifuged, and the supernatant was analyzed with NOA using the vanadium(III) chloride (VCl_3) and chemiluminescence assay. For nitrosothiol (SNO) analysis, 4 parts of a thiol-stabilization solution (NEM-DPTA; $K_3Fe(CN_6N$ -ethylmaleimide, Diethylenetriaminepenta acetic acid, Nondiet P-40, water) were mixed with one part of samples. A 9:1 dilution of sample and 5% acid sulfanilamide (AS) was incubated for 5 min; one half was injected into the NOA (I_3^- assay) to give combined SNO and iron-nitrosyl complexes. The other half was incubated with 50 mM $HgCl_2$ then incubated again with 5% AS, and injected into the NOA to give iron-nitrosyl complex levels³⁸.

NO measurement with EPR

Sixty mL of PLT or 45 mL of CP were stored in PL120 Teflon containers. Samples were taken on each day of storage and mixed with cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, 200 μ M final concentration) and frozen until EPR analysis. The cPTIO can be used as NO spin trap³⁹, as it specifically reacts with NO to yield a non-paramagnetic product. Therefore, the EPR line heights are inversely proportional to amount of NO in solution. In our study we used only the central peak height for NO detection purposes. No attempt was made to estimate the actual NO concentrations, as the stoichiometry of cPTIO reaction with NO is too complex⁴⁰. For the clarity of presentation, we plotted inverted central peak heights, so the values are directly proportional to the amount of NO present.

Aggregation measurement and analysis of CD62 positive cells

PLT aggregation was measured at $37^{\circ}C$ using a light transmittance instrument (Chronolog, Havertown, PA). PLT samples were diluted with CP to yield a PLT count of $3.0 \times 10^5/\mu L$. ADP and collagen were added together at final concentrations of 10 μ mol/L and 10 μ g/mL, respectively, to initiate aggregation. The extent of aggregation (percent amplitude) was measured to describe the aggregation process.

The percent of PLTs expressing membrane-bound CD62P (p-selectin) was measured by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). PLT samples were diluted to 1×10^6 PLTs/mL with PBS supplemented with 0.1% human albumin. Sample aliquots were incubated with fluorescein isothiocyanate (FITC)-conjugated CD61 monoclonal antibodies and with phycoerythrin (PE)-conjugated CD62P (eBioscience Inc., San Diego, CA) at saturating concentrations for 15 min at $22^{\circ}C$. Mouse IgG₁ PE and FITC PLT isotype control (BD Biosciences) were used at saturating concentrations as negative controls.

Results

Measurement of NO metabolites in PLT and CP

PLT and CP were stored in CLX or PL120 Teflon containers with continuous agitation and the levels of NO metabolites were analyzed. Nitrite, nitrate and nitrosothiol (SNO) contents were analyzed using a chemiluminescence method. We could not detect any noticeable amount of SNO (data not shown). Nitrite levels in both PLT supernatant and CP stored in CLX containers increased greatly during 4 days of storage at a rate of 31 nM/day and 58 nM/day, respectively, from an initial level of about 160 nM (Fig. 1A) and this increase was maintained until day 7 in a linear fashion (data not shown). In contrast, nitrite levels of the supernatant of PLT aliquots stored in PL120 Teflon containers slightly decreased from 285 nM to 230 nM while that of CP did not show any change (Fig. 1C). The levels of CD62-positive platelets stored in CLX and PL120 Teflon bags were very similar throughout storage period showing about 30% at day 4 (Fig. 1E) implying increased nitrite levels in PLTs stored in CLX did not affect platelet activation during storage.

Nitrate levels during storage of PLT aliquots in CLX containers were initially about 50 μ M and decreased slightly over 4 days to 41 μ M. No significant changes in nitrate levels were observed with the PLT stored in PL120 Teflon and CP aliquots stored in CLX or PL120 Teflon containers (Fig. 1B and D).

Analysis of nitrite leaching from platelet storage containers

To test whether platelet storage containers leach nitrite during storage, plasma was stored in CLX or PL120 Teflon containers and the nitrite content was measured. The CLX container, which is being used widely for platelet storage for transfusion, is made of polyvinyl chloride (PVC) plastics and triethylhexyl trimellitate (TEHTM) plasticizer; the PL120 Teflon container is made of polytetrafluoroethylene (PTFE) plastics and contains no plasticizer. Nitrite levels in plasma stored in CLX containers showed a linear increase at a rate of 45 nM/day, while plasma stored in PL120 Teflon container did not show any change in nitrite level over the 4 days (Fig. 2). There was no significant difference in nitrite leaching levels of CLX container between room air storage and anoxic argon chamber storage (data not shown), thus gas exchange with air does not seem related to nitrite leaching. Nitrate levels in plasma stored in CLX container did not show significant changes during the same period (data not shown).

Although we confirmed that nitrite leached from the CLX containers during storage, the fact that increase rate of nitrite is smaller in PLT supernatant than in CP (Fig. 1A) suggests that the platelets and/or other types of residual cells in the PLT utilized nitrite during the 4 days of storage.

Measurement of NO in PLT and CP during storage in PL120 Teflon

Using NO probe cPTIO, NO production was analyzed with EPR during storage in PL120 Teflon container (Fig. 3A). The amount of NO generated at each day in PLT was substantially greater than in CP; however NO production decreased gradually over time both in PLT aliquots and CP suggesting a decline in NO producing activity during storage. However this method was not amenable to quantitate analysis due to complicated kinetics of cPTIO reaction with NO.

In an additional series of experiments, L-Arginine (NOS substrate, 300 μ M) or L-NAME (N^G-nitro-L-arginine methyl ester, NOS inhibitor, 300 μ M) was added to the PLT at day 0 to determine if detected NO was enzymatically produced by NOS in platelet preparations during storage. There were no apparent changes in NO level after the addition of L-Arginine

or L-NAME compared to control (data not shown). The possible explanations are that: 1) NOS contribution to NO level change in apheresis platelets is trivial or absent since platelet might not even express any NOS as others suggested^{13–15}; or 2) changes in NO level were under the detection limit of EPR instrument we used. We also confirmed that addition of L-Arginine and L-NAME did not affect PLT aggregating property and the percentage of CD62 positive cells during storage (Fig. 3B and C). We then added nitrite (1 μ M) to the PLT at day 0 to check if additional nitrite would cause an increase in NO level during storage through its reduction to NO, however we did not observe any clear increase in NO level after the addition of nitrite (data not shown). It seems that endogenous level of nitrite in the PLT (~300nM) might be enough to create pseudo first order kinetics in NO generation reaction.

Discussion

The inhibitory role of endothelium-derived NO in platelet function has been known since the early 1980's^{41–43}. NO exerts its inhibitory role on platelets by stimulating cGMP production via sGC activation in platelets. cGMP then activates protein kinase G (PKG) which phosphorylates numerous proteins related to platelet function resulting in inhibition of platelet responses to its agonist. However, biological effects of NO generated from the serial reduction of nitrate or nitrite on platelet function are not well studied yet. Although nitrite and nitrate are the major oxidation products of NO metabolism, the reduction pathway of nitrate and nitrite to NO has been recognized only in the last decade. Our diet is a major source for nitrite and nitrate since one serving (~300g) of a nitrate-rich vegetable such as spinach or beetroot is known to contain more nitrate than what is produced endogenously over a day by all three NOS enzymes^{44,45}. Absorbed nitrate can be reduced to nitrite by bacterial nitrate reductases in the oral cavity and nitrite can be further reduced to NO in blood and tissues by several pathways⁴⁶ including deoxyhemoglobin, deoxymyoglobin, xanthine oxidase, mitochondrial respiratory chain enzymes, cytochrome P-450 and non-enzymatic reduction in the presence of protons or vitamin C. Webb *et al.* recently showed that oral intake of nitrate rich food such as beetroot juice decreased platelet aggregation²⁹ suggesting NO from exogenous sources might have a great effect on platelet function. Considering the potential effect of nitrite and nitrate on platelet function, it is important to study NO metabolism during platelet storage since changes in levels of nitrite and nitrate during storage could modulate platelet function and/or affect post-transfusion viability.

To better understand platelet physiology during storage, in this study, we examined the levels of NO derivatives, nitrite and nitrate, under routine storage conditions. We stored apheresis platelet aliquots in standard CLX containers, with the same surface to volume ratio and similar platelet counts as those in whole blood derived PLTs. CLX plastic is composed of PVC and the plasticizer triethylhexyl trimellitate⁴⁷. It has been known that plastic materials of storage containers are of great importance for regulation of gas exchange and maintenance of *in vitro* and *in vivo* platelet properties⁴⁸. Nitrite or other NO precursors are used in the processes of synthesizing polymeric matrices^{49–51} and are known to be slowly released from the final products. Therefore we examined whether nitrite is also released from platelet storage containers. As shown in Fig. 2, nitrite leaching was prominent in CLX, but absent in PL120 Teflon container. CLX and PL120 containers are made of different plastics, PVC and PTFE respectively, and PL120 does not contain any plasticizer. It seems that the chemical composition of each plastic material affects nitrite leaching levels.

There is no published information available on the levels of NO metabolites during platelet storage. In Fig. 1A, we observed an increase in nitrite level in both PLT supernatant and CP at a rate of 31 nM/day and 58 nM/day, respectively. We believe that this increase is caused by nitrite leaching from the material of the CLX containers during storage, based on our result in Fig. 2. However, the increase rate is much smaller in PLT supernatant (31 nM/day)

than in CP (58 nM/day). In addition, we observed that nitrite level slightly decreased at a rate of 12.5 nM/day in the supernatant of PLT aliquots stored in PL120 Teflon container which does not leach nitrite (Fig. 1C). These results imply that active platelets, residual leukocytes and/or contaminating erythrocytes in PLT utilized nitrite during storage. Although erythrocyte and leukocyte counts were significantly reduced in PLT preparations compared to those of whole blood, there were 1.3×10^6 erythrocytes and 2.0×10^4 leukocytes present in 60ml of 7.8×10^{10} PLT.

Srihirun *et al.* showed that nitrite could inhibit platelet aggregation and activation measured by changes in impedance and CD62-positive platelets, respectively, in the presence of 20% Hct erythrocyte through its reduction to NO and this was promoted by deoxygenation³⁰. In our study, it is not likely that deoxyhemoglobin in the residual erythrocytes of the PLT is responsible for the reduction of nitrite to NO because pO₂ was maintained constantly high throughout the entire storage period with values of 148 ± 11 mmHg at day 0 and 147 ± 26 mmHg at day 4. The reaction between nitrite and oxyhemoglobin which generates nitrate and methemoglobin is possible and this might have contributed to nitrite decrease in PLT during storage. Although Arora *et al.* reported that cytochrome P-450 reductase plays a role as a nitrite reductase in human platelets⁵², they used non-physiological concentrations of nitrite ($\sim 100 \mu\text{M}$) in order to observe NO generation and we observed no evidence that higher concentrations of nitrite than those normally present in plasma affected NO production in PLT. Therefore there is no substantial evidence so far that human platelets or leukocytes express specific nitrite reductases under physiological conditions. Further study should be performed to clarify the mechanism for the nitrite utilization in apheresis platelet units. The small amount of reduction of nitrate levels in PLT stored in CLX container is also at present unexplained but this may be a part of serial reduction pathways converting nitrate to other NO metabolites.

Although we could not quantitate the low levels of NO that appear to be produced by the stored PLT, our present study shows a clear difference between PLT and CP in NO levels during storage in PL120 Teflon containers (Fig. 3A). These likely reflect the above mentioned reductive pathways rather than an enzymatic production of NO by NOS. Based on previous reports on the absence of active NOS in platelets^{13–15} and our result suggesting no effect of L-Arginine and L-NAME on NO production (data not shown), contribution of endogenous NOS to NO production in PLT during storage seems to be very little.

Collectively, our current study shows that: 1) NO metabolite levels are measurable in apheresis platelet units as shown by nitrite consumption by PLT stored in Teflon containers where nitrite decreased over storage time 2) platelet preparations are unintentionally enriched by nitrite during routine storage in CLX containers by nitrite release from the plastic materials but this enrichment does not appear to be detrimental to PLT. However, NO which may be generated *in vivo* after platelet transfusion by the reduction of nitrite that was leached from the platelet container could modify the eventual function of transfused and endogenous platelets.

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Abbreviations

PLT(s)	apheresis platelet(s)
CP	concurrent plasma
NO	nitric oxide
NOS	nitric oxide synthase
EPR	electron paramagnetic resonance

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Figure 1. Changes in nitrite content during storage

Leukoreduced ($< 2 \times 10^4$ leukocytes/60 mL) apheresis platelets were collected from healthy donors using the Trima Accel. Sixty mL of PLT or CP was stored in CLX (A and B, $n=5$) or PL120 Teflon (C and D, $n=4$) containers at 20–24°C with agitation. Nitrite (A and C) and nitrate (B and D) were measured in the PLT supernatant, pellets and CP using a chemiluminescence method. The percentage of CD62 positive cell was measured by flow cytometry (E, $n=3$). Data are means $\pm$ SEM.

Figure 2. Nitrite leaching out of CLX container

Sixty ml of plasma was stored in CLX containers (n=4) or in PL120 Teflon containers (n=4) at 20–24°C and aliquots from each container were taken every day to measure nitrite content with triiodide (I_3^-) ozone-based chemiluminescence. Data are means $\pm$ SEM.

Figure 3. NO measurement during storage

PLT or CP aliquots were stored in PL120 Teflon containers at 20–24°C with agitation. Samples were taken every day and mixed with cPTIO (200 μ M) to analyze NO level with EPR. Inverted central peak heights were shown, so the values are directly proportional to the amount of NO present (A). Aggregation (B) was measured by PLT response to two agonists (ADP and collagen, 10 μ M and 10 μ g/ml, respectively) via changes in light transmittance and the percentage of CD62 positive PLT (C) was measured by flow cytometry. Data are means $\pm$ SEM.