**TITLE:**

**Platelet-based Detection of Nitric Oxide in Blood Using Measurement of VASP Phosphorylation.**

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**KEYWORDS:**

Platelets, red blood cells, nitrite, nitric oxide, blood clotting, deoxyhemoglobin, VASP protein

**SUMMARY:**

Here we present a protocol to address the potential use of platelets as a highly sensitive nitric oxide sensor in blood. It describes initial platelets preparation and use of nitrite and deoxygenated red blood cells as nitric oxide generators. Special attention is given to most common pitfalls to avoid.

**ABSTRACT:**

Platelets are blood components responsible for proper blood clotting. Their function is highly regulated by various pathways. One of the most potent vasoactive agents, nitric oxide (NO), can also act as a powerful inhibitor of platelet aggregation. Direct NO detection in blood is very challenging, due to its high reactivity with cell-free hemoglobin that limits NO half-life to the millisecond range. Currently, NO changes after interventions are only estimated based on measured changes of nitrite and nitrate (members of the nitrate-nitrite-NO metabolic pathway). However precise, these measurements are rather difficult to interpret *vis a vis* actual NO changes, due to the naturally high baseline nitrite and nitrate levels that are several orders of magnitude higher than expected changes of NO itself. Therefore, development of direct and simple methods that would allow one to detect NO directly is long overdue.

This protocol addresses a potential use of platelets as a highly sensitive NO sensor in blood. It describes initial platelet rich plasma (PRP) and washed platelet preparations and the use of nitrite and deoxygenated red blood cells as NO generators. Phosphorylation of VASP at serine 239 (P-VASPSer239) is used to detect the presence of NO. The fact that VASP protein is highly expressed in platelets and that it is rapidly phosphorylated when NO is present, leads to the unique opportunity to use this pathway to directly detect NO presence in blood.

**INTRODUCTION:**

Platelets, small disc-shaped cell fragments derived from megakaryocytes, are crucial for blood clotting. The clotting cascade is initiated by various bioactive molecules (such as collagen or ADP), released after the injury of vascular wall. The blood clotting process can be modified, among various effectors by nitric oxide (NO). NO, naturally produced by mammalian cells, is one of the most versatile physiological signals. It acts as a potent vasodilator, neurotransmitter and immune modulator, to name a few of its many functions. In the bloodstream, NO also helps to regulate the extent of blood clotting by inhibiting platelet aggregation. One of the most likely sources of NO in the bloodstream is nitrite, an inorganic ion that has been shown to serve as a precursor of NO. Reacting with red blood cells (RBCs), nitrite is reduced to NO and deoxyHb is oxidized to methemoglobin (metHb)1. NO released from RBCs is vasoactive and causes vasorelaxation2. This nitrite reduction pathway is an alternate NO generation pathway, acting together with and complementing the classical NO generation path by endothelial nitric oxide synthase at hypoxic conditions.

Platelets themselves are not able to reduce nitrite into NO but are very sensitive to its presence. In intact platelets, NO in the nanomolar range increases cGMP (EC50 = 10 nM) and phosphorylation of VASP (EC50 = 0.5 nM)3. Therefore, platelets may serve as an excellent sensor of nitrite reduction by RBCs and NO release into blood. There are several methods able to measure directly the extent of platelet activation – such as aggregometry and thromboelastography (TEG)4,5. However, these methods require expensive specialized instrumentation and rather large amounts of material. It is also possible to monitor events downstream, after NO is released from RBCs, using changes in platelet surface protein expression - such as P-selectin6. NO is also known to increase the amount of cGMP in the platelets7. Previously, we used cGMP to monitor NO release into blood after nitrite reduction by deoxygenated RBC8. This proved to be a very sensitive method, however cGMP is a short-lived molecule and its detection involves extensive labor. Another possibility, described in the presented protocol, uses phosphorylation of Vasodilator-stimulated phospho (VASP)-protein to detect presence of NO in blood. VASP is a substrate of protein kinase G activation, which is phosphorylated upon interaction with NO through the sGC/cGMP pathway9. Detectable VASP phosphorylation occurs at very low NO concentrations, which could make platelets a very sensitive detector of NO presence in blood. VASP is highly expressed in platelets, but not in other blood cells, which allows to follow selectively events involving platelets10.

Main goal of this protocol is to describe in details method for detection of NO release in whole blood using its interaction with platelets via monitoring VASP phosphorylation11,12. Described method allows early detection of low NO concentrations – in theory in nanomolar range which makes the present protocol more sensitive than cGMP determination and, due to the use of standard Western blot technique achievable in most laboratory settings.

**PROTOCOL:**

Blood samples were obtained from NIH blood bank (IRB approved protocol: 99-CC-0168).

1. **Blood sample preparation.**

**Note:** To avoid platelet activation, draw blood slowly and mix gently with citrate by inverting the tube several times.

**1.1. Platelet-rich plasma (PRP) preparation.**

1.1.1. Draw 30-50 ml of blood using 20G or larger diameter needle and add into a tube containing sodium citrate (3.8%) in a 1:9 ratio or acid citrate dextrose (ACD) (85 mM trisodium citrate, 66.6 mM citric acid, 111 mM glucose, pH 4.5) in a 1:6 ratio.

1.1.2. Aliquot 5 mL of freshly collected whole blood into empty 15-mL conical tubes. Centrifuge whole blood at 120 × g for 10 minutes at room temperature.

1.1.3. Carefully collect supernatant, containing platelets (platelet-rich plasma, PRP) from the upper portion by a plastic transfer pipette.

**Note:** PRP from step 1.1.3 is ready for use in experiments or for further processing to prepare washed platelets. The soft pellet obtained after centrifugation (step 1.1.2) contains red blood cells and white cells and can be further purified to obtain washed red blood cells (RBC) – see section 1.3**.** The experiment needs to be finished within 2 hours after blood drawing. When collecting PRP (supernatant), avoid collection of buffy coat-containing leukocytes accumulated on the PRP/RBC interface.

**1.2. Washed platelet preparation - optional.**

1.2.1. Centrifuge collected PRP at 400 × g for 10 minutes at room temperature. Discard supernatant and keep platelets pellet.

1.2.1. Gently wash the pellets by adding 5 mL of CGS buffer (120 mM NaCl, 12.9 mM trisodium citrate and 30 mM glucose, pH 6.5) and centrifuge at 400 × g for 10 minutes at room temperature.

1.2.3. Resuspend platelet pellets with 3 mL of modified Tyrode buffer (134 mM NaCl, 0.34 mM NaH2PO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose pH 7.4).

1.2.4. Dilute platelets (1:100 - as example, 10 µL of platelets in 990 µL Rees-Ecker solution) of Rees Ecker solution and count by hemocytometer.

1.2.5. Adjust number of platelets to 3×108 cells/mL by add Tyrode buffer.

1.2.6. Incubate platelet suspension at 37 °C for 1 hour before the start experiment.

**Note:** Washed platelets are stable for 5-8 hours at 37 °C.

**1.3. Preparation of washed red blood cells (RBC):**

1.3.1. After the collection. centrifuge the soft pellet obtained in step 1.1.3 at 2500 × g for 10 minutes at room temperature.

1.3.2. Discard the supernatant and wash the RBC pellet with 5 mL of PBS by centrifugation at 2500 × g for 10 minutes at room temperature. Do this for 3 times.

1.3.3. Discard PBS and use washed RBCs for further experiments.

**2. Deoxygenation**

2.1. Add 1 mL of the mixture of PRP (prepared in step 1.1.4. or 1.2.4.) and RBCs (prepared in step 1.3.) at desired hematocrit into a polypropylene bottle closed with a rubber stopper. For example, at 20% hematocrit, add 200 µL of RBCs in 800 µL of PRP.

**Note:** Suggested hematocrits are from 0 up to 40%.Do not use glass bottle or flask - platelets adhere to glass surface.

2.2. Insert needle connected to helium gas tank into closed bottle and make gas outlet by inserting a small syringe needle (26G) (**Figure 1**).

2.3. Slowly swirl the bottle at room temperature.

**Note:** Do not allow He gas to bubble through blood preparation! Slow gas flow is preferable for this procedure, excessively fast He gas flow leads to increased hemolysis. The most efficient gas flow needs to be determined by trial, as it highly depends on the geometry of the experimental setup.

2.4. Periodically follow deoxygenation process measuring partial oxygen pressure (pO2) using CO-oximeter.

**Note:** In our hands, 10 min deoxygenation decreases pO2 to 25 mmHg which is required for nitrite reduction by RBCs. Continuing deoxygenation did further reduce pO2 to 10 mmHg however, it led to increased hemolysis and increases in cell-free hemoglobin (**Figure 2**).

**3. Red blood cells nitrite reduction.**

3.1. Add 1 mL of PBS pH 7.4 into 0.0345 g of NaNO2- to make 500 mM stock solution of nitrite.

3.2. Dilute 500 mM NaNO2- to 250 µM NaNO2- (25x)by serial dilution in PBS pH 7.4.

3.2. Using microsyringe, inject nitrite (40 µL) through septum into deoxygenated sample of PRP and RBCs to achieve final concentrations of 10 µM in total 1 mL of sample.

**Note:** In this experiment, final concentrations of nitrite used is 10 µM.

3.3. Incubate the sample at 37 °C for 10 min or longer.

**Note:** Exact duration of RBC incubation with nitrite needed for maximal nitrite reduction might need adjustment for different type of experiments.

3.4. Remove the stopper and pipette 1 mL of sample to an Eppendorf tube.3.5. Centrifuge at 200 × g for 3 min at room temperature.

3.6. Pipette 300 µL of platelet suspension from the upper portion of supernatant and mix with ACD (pH 6.5) in a 1:9 ratio. Discard the RBC pellet.

3.7. Centrifuge the platelet suspension at 500 × g for 4 minutes at room temperature.

3.8. Add 80 µL of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% NP-40, pH 7.4) containing protease inhibitor cocktail III (1:500) to platelet pellets.

**4. Western blotting of VASP**

4.1. Load 15 µg protein of each sample in 2 separate 10% SDS-PAGE gels.

**Note:** Harsh stripping buffer, which contains β-mercaptoethanol cannot remove P-VASPSer239 and VASP antibodies from membrane, therefore P-VASP Ser239 and VASP should be run separately.

4.2. Run gels at 120 V for 1.30 hours and transfer to nitrocellulose membrane.

4.3. Block non-specific binding with 5% non-fat dry milk.

4.4. Incubate membrane with P-VASPSer239 (1:500), VASP (1:1000) and GAPDH (1:1000) for overnight at 4°C.

4.5. Incubate horseradish peroxidase (HRP) secondary antibody with membrane for 45 minutes at room temperature.

4.6. Expose membrane with imager machine and quantified band density using Image J software or similar.

**REPRESENTATIVE RESULTS:**

Venous blood samples have pO2 values between 50-80 mmHg. Deoxygenation by helium rapidly decreases pO2 to 25 mmHg within 10 minutes. Increased deoxygenation time slightly further decreases pO2. However, increased time of deoxygenation also leads to significantly increased levels of cell-free hemoglobin (determined by CO-Oximeter, visually seen on Figure 2 as increasingly red coloration of plasma) (Figure 2A-B). Increased hemolysis is not associated with stirring because stirring without deoxygenation does not induce hemolysis (Figure 2C).

We found that the presence of RBCs in lysed samples decreases P-VASPSer239 and VASP detected by Western blotting (**Figure 3**). Therefore, we first separate platelets and RBCs before running Western blots to detect P-VASPSer239 in platelets.

To show that there is enough time to separate RBCs out of platelets without affecting VASP phosphorylation, we used a short-lived NO donor. PROLI NONOate (NO donor with half-life of 1.8 seconds at 37 °C pH 7.4), rapidly increased P-VASPSer239 in platelets (within 10 seconds of incubation). P-VASPSer239 induced by PROLI NONOate is detected for 10 minutes after incubation with PRP (**Figure 4**).

Nitrite increases P-VASPSer239 in platelets in the presence of deoxygenated RBCs (pO2 25 mmHg). Nitrite reductase activity of deoxygenated RBCs depends on hematocrit and the maximum effect is observed at 20% hematocrit (**Figure 5**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Deoxygenation chamber.** Mixture of PRP and RBCs is added into a plastic bottle closed with a rubber septum. A closed bottle is connected to helium (He) line using a needle and He line. Oxygen is flushed out by a small syringe needle (26G) serving as a gas outlet.

**Figure 2. Partial oxygen pressure (pO2) and cell-free hemoglobin after deoxygenation.** (A) PRP and RBCs samples were deoxygenated for 3, 5, 10, 15, 30 and 50 minutes. pO2 of samples were measured by blood gas analyzer (n=3). Error bars are SEM. Representative pictures of supernatant of PRP+RBCs samples stirred with (B) or without helium (C).

**Figure 3. Representative VASP Western blot bands in samples of PRP/RBCs prepared at various hematocrit.** RBCs were added into PRP to 1, 5, 10, 15, 20, 40% hematocrit.Mixtures of PRP + RBCs were centrifugation at 500 × g for 5 minutes and lysed with lysis buffer.

**Figure 4. Representative Western blot bands of P-VASPSer239 and VASP in PRP after exposure to NO donor PROLI NONOate**. PROLI NONOate (10 μM) was added into PRP and incubated at 37 °C for 10 and 30 seconds (s) and 1, 2, 5, 10, 20 and 40 minutes (m).

**Figure 5. VASPSer239 phosphorylation depends on pO2 and hematocrit levels.** (A) PRP + RBCs (20% hematocrit) were deoxygenated to two different pO2 levels, 40 and 25 mmHg. P-VASPSer239 and VASP were measured after incubation of deoxygenated samples with 10 μM nitrite at 37 °C for 10 minutes. (B) Nitrite (10 μM) was added into deoxygenated PRP + RBCs at 0, 10, 20 and 40% hematocrit (pO2 = 25 mmHg). Fold increased (P-VASPSer239) was calculated relative to control (PRP+RBCs without nitrite) for each hematocrit value. Error bars are SEM.

**DISCUSSION:**

**Critical steps.**

Since platelets are easily activated, gentle handling of platelet-containing samples is required. Fast pipetting and vigorous shaking should be avoided. Platelet inhibitors such as prostacyclin (PGI2) can be used to prevent platelet activation; however, this may affect some signaling pathways inside platelets. For preparation of platelet pellets, we add ACD to platelet suspensions and use low speed centrifugation.

Fresh prepared platelets in PRP have only limited life span, up to 2 hours. To achieve high reproducibility, all experiments should be done only with fresh platelets and within 2 hours after blood collection. The presented data were obtained with platelets in PRP, which is considered more physiological than washed platelets. Although life span and purity increase for washed platelets, repeated centrifugation during preparation of washed platelets can lead to their spontaneous activation and this may result in inconsistent quality of platelet preparations. PRP is prefer over washed platelets since the procedure is faster and further handling could activate platelets. However, in specific cases, when the interference of plasma blood clotting factors could interfere with experimental setup, washing platelets represents solution to avoid these problems.

Oxygen contamination in deoxygenated samples is the most important critical step for this assay. RBCs can be pooled and deoxygenated before adding into PRP. However, transferring deoxygenated RBCs increases risk of oxygen contamination. In addition, deoxygenation of concentrated RBCs requires more time than deoxygenation of the full preparation at desired hematocrit, and longer exposure to He gas leads to increased levels of cell-free hemoglobin. Although cell-free hemoglobin can be washed out of deoxygenated RBCs, phosphate buffer saline or other buffers used for washing must be carefully deoxygenated to prevent oxygen contamination during centrifugation. To shorten the preparation process and avoid unnecessary oxygen contamination, each PRP+RBCs sample was prepared at desired hematocrit first and then deoxygenated before addition of nitrite.

To quantify P-VASPSer239 by Western blot, RBCs must be separated out of platelets. VASP is expressed in RBCs13, but levels are nonsignificant when compared to hemoglobin and much lower than in platelets. Since total protein concentration is used for loading controls in the Western blots, the presence of RBCs in lysate samples interferes with P-VASP and VASP measurement by Western blotting. Due to a slow rate of dephosphorylation by phosphatase enzymes in platelets14, P-VASP is sustained for10 minutes (Figure 4) and therefore it is possible to separate RBCs by centrifugation before collecting platelet cell lysates.

EC50 for VASP phosphorylation is almost an order of magnitude lower than EC50 for the peak cGMP increase, 0.5 nM and 9 nM, respectively3, which makes VASP phosphorylation one of the most sensitive methods to detect presence of low amounts of NO. However, VASP phosphorylation curve in response to NO is bell-shaped, phosphorylation decrease is observed after peaking at 3-30 nM of NO3. This nonlinear response to increasing NO concentrations makes VASP unsuitable for rigorous NO quantification.

**Limitations of the technique**

Since platelets in PRP have short life span, the assay must be done in fresh platelets within 2 hours after blood drawing. The inconsistence of pO2 levels after deoxygenation can be found since deoxygenation of samples is dependent on flow rate of helium gas and rate of swirling the bottle. Therefore, the time for deoxygenation need to be determined by trial. For the measurement of P-VASP by Western blots, antibody choice is critical and need to use direct NO donor as a positive control to check specificity. In addition to NO, P-VASPSer239 in platelets can be phosphorylated by the inhibition of phosphodiesterease enzymes and the activation of protein kinase A pathway.

**Significance of the method with respect to alternative method**

Measurement of P-VASPSer239 has some advantages over cGMP assay. cGMP in platelets has very short half-life (less than 10s) and the measurement of cGMP requires the addition of phosphodiesterase inhibitor3. Also, cGMP measurement uses rather expensive ELISA technique. VASP can be simply measured by an in-house Western blot protocol and therefore is more readily available.

**Future directions**

Increased P-VASPSer239 in platelets was successfully used as a sensor for NO generation by inhaled nitrite *in vivo6*. Therefore, we showed that it is possible to use P-VASPSer239 in platelets as a marker of NO generation *in vitro* and *in vivo* in the blood and possibly in various other situations. The unusually low EC50 makes this method an excellent candidate for detecting presence of very low NO concentrations in blood. Therefore, this protocol provides a possibility to study NO generation in blood at various physiological stimuli, such as during blood coagulation cascade, increased sheer stress or increased inflammation.

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**DISCLOSURES:**

Dr. Alan Schechter is listed as a co-inventor on several patents issued to the National Institutes of Health for the use of nitrite salts for the treatment of cardiovascular diseases. He receives royalties based on NIH licensing of these patents for clinical development but no other compensation.

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