



Platelet-based Detection of Nitric Oxide in Blood by Measuring VASP Phosphorylation

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Abstract

Platelets are the 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, the 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-VASP^{Ser239}) 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 a unique opportunity to use this pathway to directly detect NO presence in blood.

Keywords

Medicine; Issue 143; Platelets; red blood cells; nitrite; nitric oxide; blood clotting; deoxyhemoglobin; VASP protein

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Video Link

The video component of this article can be found at <https://www.jove.com/video/58647/>

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.

Introduction

Platelets are small disc-shaped cell fragments derived from megakaryocytes that 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 (methHb)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 ($EC_{50} = 10 \text{ nM}$) and phosphorylation of VASP ($EC_{50} = 0.5 \text{ nM}$)³. Therefore, platelets may serve as an excellent sensor of nitrite reduction by RBCs and NO release into blood. There are several methods that can directly measure 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 the 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 the vasodilator-stimulated phospho (VASP)-protein to detect the presence of NO in blood. VASP is a substrate of protein kinase G activation, which is phosphorylated upon the 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 the events involving platelets¹⁰.

The main goal of this protocol is to describe the method in detail for the detection of NO release in whole blood using its interaction with platelets by monitoring VASP phosphorylation^{11,12}. The described method allows early detection of low NO concentrations - theoretically in the nanomolar range which makes the present protocol more sensitive than cGMP determination, due to the use of standard Western blot techniques achievable in most laboratory settings.

Protocol

NOTE: 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. Platelet-rich plasma (PRP) preparation

1. Draw 30–50 mL of blood using a 20 G 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.
2. Aliquot 5 mL of freshly collected whole blood into empty 15-mL conical tubes. Centrifuge whole blood at $120 \times g$ for 10 min at room temperature.
3. Carefully collect the supernatant containing the 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 Step 1.3. The experiment needs to be finished within 2 h after drawing blood. When collecting PRP (supernatant), avoid the collection of buffy coat-containing leukocytes accumulated on the PRP/RBC interface.

2. Washed platelet preparation (optional)

1. Centrifuge collected PRP at $400 \times g$ for 10 min at room temperature. Discard the supernatant and keep the platelet pellets.
2. 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 $400 \times g$ for 10 min at room temperature.
3. Resuspend the platelet pellets with 3 mL of modified Tyrode buffer (134 mM NaCl, 0.34 mM NaH_2PO_4 , 2.9 mM KCl, 12 mM NaHCO_3 , 20 mM HEPES, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose pH 7.4).
4. Dilute the platelets (1:100 — as example, 10 μL of platelets in 990 μL of Rees-Ecker solution) with Rees Ecker solution and count by hemocytometer.
5. Adjust the density of platelets to 3×10^8 cells/mL by adding Tyrode buffer.

6. Incubate the platelet suspension at 37 °C for 1 h before starting the experiment.

NOTE: Washed platelets are stable for 5–8 h at 37 °C.

3. Preparation of washed red blood cells (RBC)

1. After the collection, centrifuge the soft pellet obtained in Step 1.1.3 at $2,500 \times g$ for 10 min at room temperature.
2. Discard the supernatant and wash the RBC pellet with 5 mL of PBS by centrifugation at $2,500 \times g$ for 10 min at room temperature. Repeat this step for 3 times.
3. Discard the PBS and use washed RBCs for further experiments.

2. Deoxygenation

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 the desired hematocrit into a polypropylene bottle closed with a rubber stopper. For example, at 20% hematocrit, add 200 μL of RBCs to 800 μL of PRP.

NOTE: Suggested hematocrits are from 0% up to 40%. Do not use a glass bottle or flask as platelets adhere to glass surface.

2. Insert the needle connected to helium gas tank into the closed bottle and make the gas outlet by inserting a 26 G needle (Figure 1).
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, and 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.

4. Periodically follow the deoxygenation process by measuring the partial oxygen pressure ($p\text{O}_2$) using a CO-oximeter.

NOTE: In our hands, 10 min of deoxygenation decreases $p\text{O}_2$ to 25 mmHg which is required for nitrite reduction by RBCs. Continuing deoxygenation did further reduce $p\text{O}_2$ to 10 mmHg; however, it led to increased hemolysis and increases in cell-free hemoglobin (Figure 2).

3. Red Blood Cell Nitrite Reduction

1. Add 1 mL of PBS (pH 7.4) into 0.0345 g of NaNO_2 to make 500 mM stock solution of nitrite. Dilute 500 mM NaNO_2 to 250 μM NaNO_2 (25x) by serial dilution in PBS (pH 7.4).
2. Using a microsyringe, inject nitrite (40 μL) through the septum into the deoxygenated sample of PRP and RBCs to achieve final concentrations of 10 μM in total 1 mL of sample.

NOTE: In this experiment, the final concentrations of nitrite used is 10 μM .

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

NOTE: Adjust the exact duration of RBC incubation with nitrite needed for maximal nitrite reduction for different type of experiments.

4. Remove the stopper and pipette 1 mL of the sample into a microcentrifuge tube.
5. Centrifuge at $200 \times g$ for 3 min at room temperature.
6. Pipette 300 μL of the platelet suspension from the upper portion of the supernatant and mix with ACD (pH 6.5) in a 1:9 ratio. Discard the RBC pellet.
7. Centrifuge the platelet suspension at $500 \times g$ for 4 min at room temperature.
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 the platelet pellets.

4. Western Blotting of VASP

1. Load 15 μg of the protein from each sample to 2 separate 10% SDS-PAGE gels.

NOTE: Harsh stripping buffer, which contains b-mercaptoethanol, cannot remove P-VASP^{Ser239} and VASP antibodies from the membrane; therefore, P-VASP^{Ser239} and VASP should be run separately.

2. Run the gels at 120 V for 1.30 h and transfer to the nitrocellulose membrane.
3. Block non-specific binding with 5% non-fat dry milk.
4. Incubate the membrane with P-VASP^{Ser239} (1:500), VASP (1:1,000) and GAPDH (1:1,000) for overnight at 4 °C.
5. Incubate horseradish peroxidase (HRP) secondary antibody with the membrane for 45 min at room temperature.
6. Expose the membrane with an imager machine and quantify the band density using ImageJ.

Representative Results

Venous blood samples have pO_2 values between 50–80 mmHg. Deoxygenation by helium rapidly decreases pO_2 to 25 mmHg within 10 min. Increased deoxygenation time slightly further decreases pO_2 . 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-VASP^{Ser239} and VASP detected by Western blotting (Figure 3). Therefore, we first separate platelets and RBCs before running Western blots to detect P-VASP^{Ser239} 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 s at 37 C pH 7.4), rapidly increased P-VASP^{Ser239} in platelets (within 10 s of incubation). P-VASP^{Ser239} induced by PROLI NONOate is detected for 10 min after the incubation with PRP (Figure 4).

Nitrite increases P-VASP^{Ser239} 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).

Discussion

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 the platelets. For the preparation of platelet pellets, we add ACD to the platelet suspensions and use low speed centrifugation.

Freshly prepared platelets in PRP have a limited life span, up to 2 h. To achieve high reproducibility, all experiments should be done only with fresh platelets and within 2 h after blood collection. The presented data were obtained with platelets in PRP, which is considered more physiological than washed platelets. Although the 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 preferred 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 being added to the PRP. However, transferring deoxygenated RBCs increases the risk of oxygen contamination. In addition, deoxygenation of concentrated RBCs requires more time than deoxygenation of the full preparation at the 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, the phosphate buffer saline or other buffers used for washing must be carefully deoxygenated to prevent oxygen contamination during the centrifugation. To shorten the preparation process and avoid unnecessary oxygen contamination, each PRP+RBCs sample was prepared at the desired hematocrit first and then deoxygenated before the addition of nitrite.

To quantify P-VASP^{Ser239} by Western blot, RBCs must be separated out of the platelets. VASP is expressed in RBCs¹³, but the 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 for 10 min (Figure 4) and therefore it is possible to separate RBCs by centrifugation before collecting platelet cell lysates.

The EC₅₀ for VASP phosphorylation is almost an order of magnitude lower than the 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 the presence of low amounts of NO. However, the VASP phosphorylation curve in response to NO is bell-shaped; a 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.

Since platelets in PRP have a short life span, the assay must be done in fresh platelets within 2 h after drawing blood. The inconsistency of pO₂ levels after the deoxygenation can be found since the deoxygenation of samples is dependent on the flow rate of helium gas and rate of swirling the bottle. Therefore, the time for deoxygenation needs to be determined by trial. For the measurement of P-VASP by Western blots, the antibody choice is critical, and one needs to use a direct NO donor as a positive control to check the specificity. In addition to NO, P-VASP^{Ser239} in platelets can be phosphorylated by the inhibition of phosphodiesterase enzymes and the activation of protein kinase A pathway.

The measurement of P-VASP^{Ser239} has some advantages over cGMP assay. cGMP in platelets has a very short half-life (less than 10 s) and the measurement of cGMP requires the addition of phosphodiesterase inhibitor³. Also, the cGMP measurement uses a rather expensive ELISA technique. VASP can be simply measured by an in-house Western blot protocol and therefore is more readily available.

Increased P-VASP^{Ser239} 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-VASP^{Ser239} 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 the 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 shear stress or increased inflammation.

Acknowledgements

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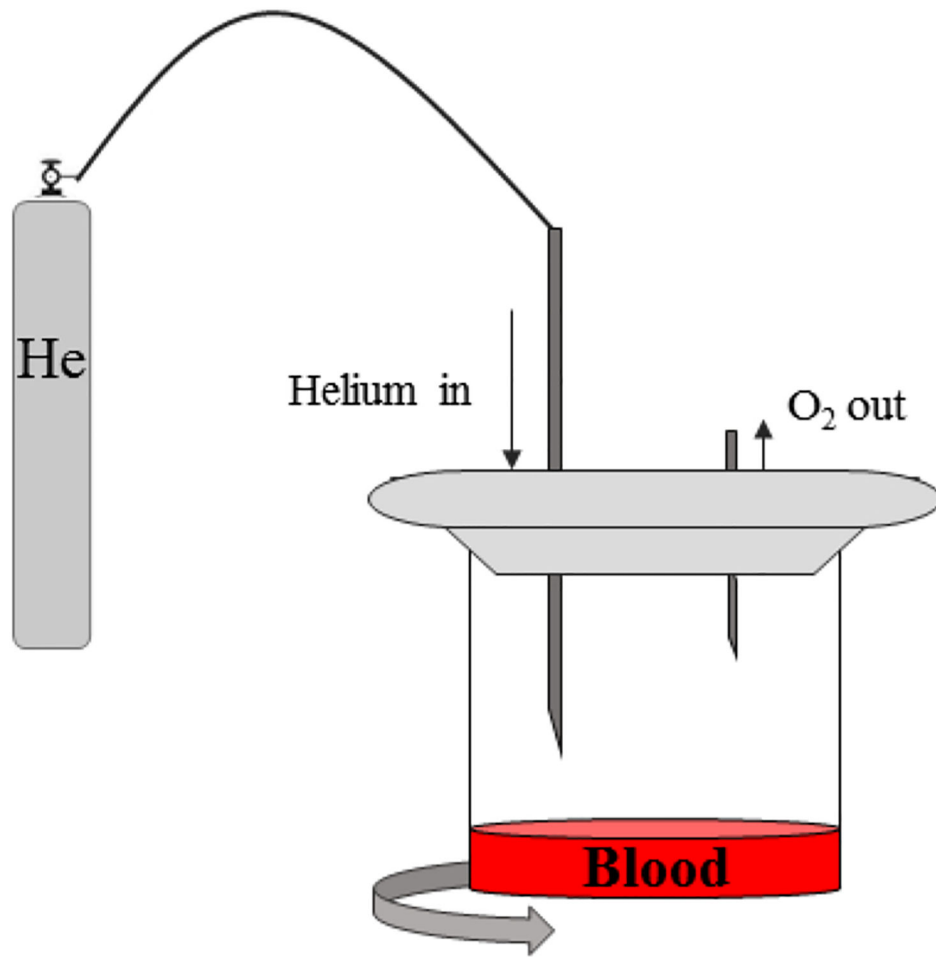
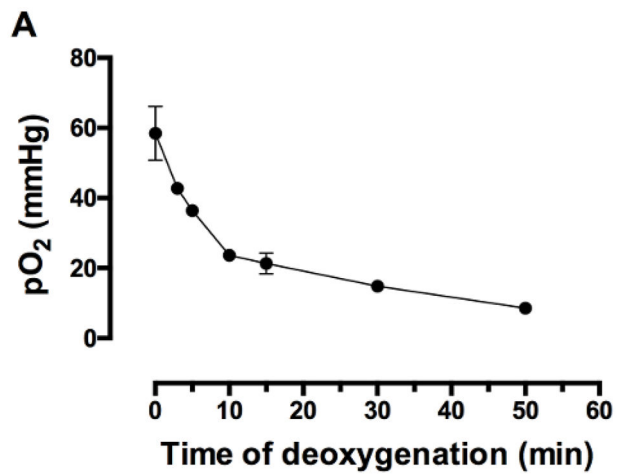
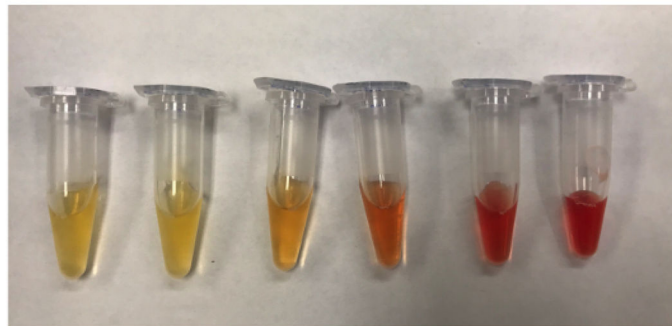


Figure 1: Deoxygenation chamber.

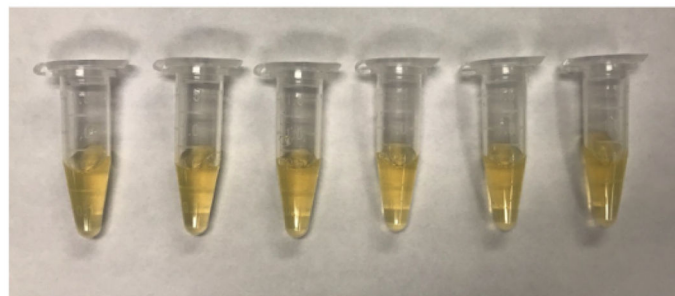
Mixture of PRP and RBCs is added into a plastic bottle closed with a rubber septum. The closed bottle is connected to helium (He) line using a needle. Oxygen is flushed out by a small syringe needle (26 G) serving as a gas outlet.



B Stirring with helium



C Stirring without helium



Time (min) **3** **5** **10** **15** **30** **50**

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 min. 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). Please click here to view a larger version of this figure.

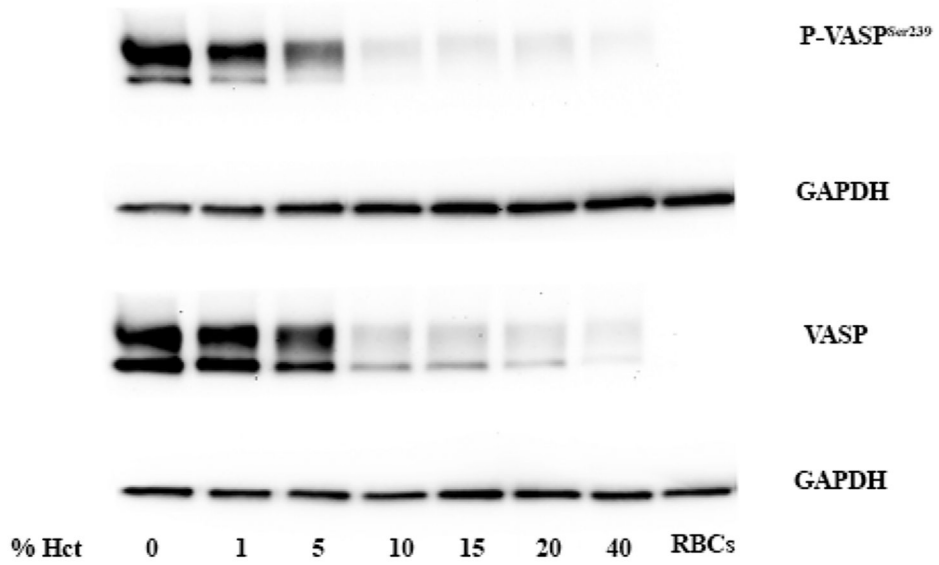


Figure 3: Representative VASP Western blot bands in samples of PRP/RBCs prepared at various hematocrit.

RBCs were added into PRP to 1, 5, 10, 15, 20, 40% hematocrit. Mixtures of PRP + RBCs were centrifuged at $500 \times g$ for 5 min and lysed with lysis buffer.

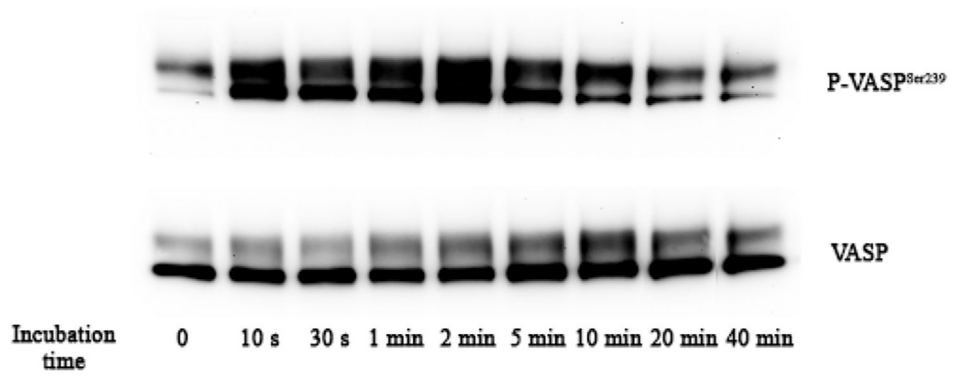


Figure 4: Representative Western blot bands of P-VASP^{Ser239} and VASP in PRP after exposure to NO donor PROLI NONOate. PROLI NONOate (10 M) was added into PRP and incubated at 37 °C for 10 and 30 s and 1, 2, 5, 10, 20 and 40 min.

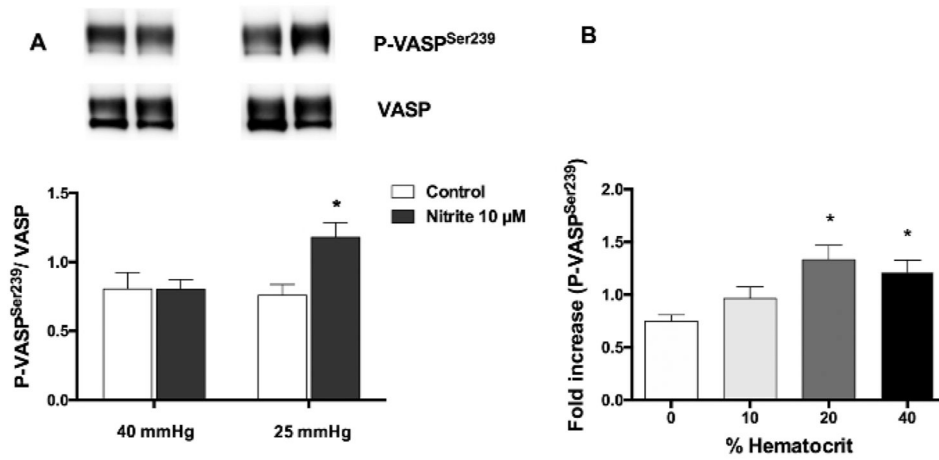


Figure 5: VASP^{Ser239} phosphorylation depends on pO₂ and hematocrit levels.

(A) PRP + RBCs (20% hematocrit) were deoxygenated to two different pO₂ levels, 40 and 25 mmHg. P-VASP^{Ser239} and VASP were measured after the incubation of deoxygenated samples with 10 M nitrite at 37 C for 10 min. (B) Nitrite (10 M) was added into deoxygenated PRP + RBCs at 0, 10, 20 and 40% hematocrit (pO₂ = 25 mmHg). Fold increased (P-VASP^{Ser239}) was calculated relative to control (PRP+RBCs without nitrite) for each hematocrit value. Error bars = SEM. Please click here to view a larger version of this figure.