Production of reactive species in neutrophils exposed to hyperoxia causes S-nitrosylation of β actin which increases formation of short actin filaments, leading to alterations in the cytoskeletal network that inhibit β₂ integrin-dependent adherence (J. Biol. Chem. 283: 10822, 2008). In this study we found that vasodilator stimulated protein (VASP) exhibits high affinity for S-nitrosylated short filamentous actin which increases actin polymerization. VASP bundles Rac 1, Rac 2, cyclic AMP-dependent and cyclic GMP-dependent protein kinases in close proximity to short actin filaments and subsequent Rac activation increases actin free barbed end formation. Using specific chemical inhibitors or reducing cell concentrations of any of these proteins with small inhibitory RNA abrogates enhanced free barbed end formation, increased actin polymerization and β₂ integrin inhibition by hyperoxia. Alternatively, incubating neutrophils with FMLP or 8-bromo-cyclic GMP activates respectively, either cyclic AMP-dependent or cyclic GMP-dependent protein kinase outside of the short F-actin pool and phosphorylates VASP on serine 135. Phosphorylated VASP abrogates the augmented polymerization normally observed with S-nitrosylated actin, VASP binding to actin, elevated Rac activity and elevated formation of actin free barbed ends; thus restoring normal β₂ integrin function.

Introduction: Reactive species generated when neutrophils are exposed to high oxygen partial pressures (hyperbaric oxygen or HBO₂) in vivo or in suspensions ex vivo inhibit β₂ integrin-dependent adherence in animals and humans (1-4). This effect ameliorates a variety of ischemia-reperfusion disorders in animal studies; yet HBO₂ does not impair neutrophil immune surveillance processes (5). The unexplained basis for these observations was the impetus for this project.

The reversible nature of HBO₂-mediated neutrophil β₂ integrin inhibition can be shown by incubating cells with chemoattractants such as FMLP or with membrane-permeable cyclic GMP (cGMP) analogs such as 8-bromo-cyclic GMP (8-br-cGMP) (1-4). The goal of this investigation was to evaluate the mechanism for reversal of HBO₂ effects by these agents and improve understanding of how hyperoxia disturbs the neutrophil cytoskeleton.

Neutrophils migrate by coordinating β₂ integrin adhesion with turnover of filamentous (F-) actin. Integrin adherence is controlled by conformational alterations in the extracellular structure to increase affinity and by clustering in the plane of the cell membrane to improve avidity. HBO₂ impedes avidity but not affinity changes by increasing production of reactive species derived from nitric oxide synthase (NOS) and myeloperoxidase (MPO), which cause S-nitrosylation of the four cysteine moieties closest to the carboxyl terminal.
This increases intracellular formation of short actin filaments that appears as a dense mass of intracellular F-actin by confocal microscopy. When actin from these cells is added to suspensions of monomeric G-actin it increases actin polymerization and solution viscosity (2).

Actin polymerization is regulated through generation of free high-affinity filament ends referred to as free barbed ends (FBEs). FBEs availability in neutrophils is modified three ways and all involve the Rac GTPase proteins (9). Rac 1 removes actin capping proteins to generate FBEs, Rac 2 as well as Cdc42 regulate de novo actin nucleation by the Arp 2/3 complex and also promote gelsolin dissociation from actin (9,10). Rac 2 also regulates activity of the coflin protein family which severs non-covalent bonds of existing FBEs (9). Rac proteins can regulate adhesion turnover either directly through downstream effectors and/or indirectly by antagonizing Rho (11,12). Rac, along with Cdc42, play central roles in regulating neutrophil \( \beta_2 \) integrin function and chemotaxis (13,14).

Vasodilator-stimulated phosphoprotein (VASP), a 46-kDa member of the Enabled family of proteins promotes actin filament nucleation, bundling and elongation by binding to monomeric, globular (G-) actin and to F-actin (15). VASP proteins are thought to regulate actin filament formation by facilitating recruitment of polymerization-competent multi-protein complexes. We had particular interest in exploring the potential role for VASP in HBO\(_2\)-exposed neutrophils. The driving force for HBO\(_2\)-mediated effects is an increased association of VASP with S-nitrosylated short F-actin, which initiates an actin polymerization process that includes PKA and PKG-dependent Rac 1 and 2 activation. Exposure to HBO\(_2\) does not increase VASP phosphorylation but VASP phosphorylation by protein kinases in cells treated with either FMLP or 8-br-cGMP decreases VASP binding and thus abrogates enhanced actin polymerization (16). While both protein kinases have a vast array of intracellular targets, they often have opposing effects on cell processes (17). VASP is among the intracellular targets where phosphorylation by either kinase has the same effect. VASP associates with actin by electrostatic interactions (15). Phosphorylation of VASP inhibits its binding to G-actin and its actin nucleation activity (18). There is conflicting information on the effect phosphorylation has on VASP binding to F-actin. In one report VASP phosphorylation was found to markedly decrease F-actin binding, but slightly increased binding was found under different experimental conditions in another (19,20).

FMLP-mediated effects depend on many proteins, including PKA and PKG; 8-bromo-cGMP will activate PKG, but in some systems appears to act predominantly via PKA (21,22). Whereas VASP can be phosphorylated by PKG or PKA, these kinases also bind to VASP which diverts their activity to alternative targets (23). Transient VASP phosphorylation by PKG in FMLP-activated neutrophils alters actin polymerization that augments \( \beta_2 \) integrin adherence (24,25). In endothelial cells, VASP is required for \( \beta_1 \) integrin function in a process that involves establishing a protein complex between actin and PKA, followed by PKA-mediated activation of Rac 1 (23,26). In fibroblasts, VASP appears to constrain Rac activity (27). In platelets, VASP phosphorylation reduces \( \beta_3 \) integrin function by modifying the protein complex linking the integrin cytoplasmic domain with actin fibers (28,29). VASP deficiency impedes both PKA and PKG dependent platelet aggregation (29).

In this study we show that VASP plays a key role in promoting actin polymerization within HBO\(_2\)-exposed neutrophils. The driving force for HBO\(_2\)-mediated effects is an increased association of VASP with S-nitrosylated short F-actin, which initiates an actin polymerization process that includes PKA and PKG-dependent Rac 1 and 2 activation. Exposure to HBO\(_2\) does not increase VASP phosphorylation but VASP phosphorylation by protein kinases in cells treated with either FMLP or 8-br-cGMP decreases VASP binding and thus abrogates enhanced actin polymerization.
which restores normal cytoskeletal control over β₂ integrin function.

**Experimental Procedures**

**Materials:** Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. N-[6-(biotinamido)hexyl]-3′-(2′-pyridylthio)propionamide (biotin-HPDP) and streptavidin-Sepharose were purchased from Pierce Chemical Co. (Rockford, IL). N-3-(aminomethyl) benzyl acetamine (NSC 23766), a Rac inhibitor, a PKG inhibitor, 8-Br-β-phenyl-1, N²-ethenoguanosine-3′, 5′-cyclic monophosphorothioate, Rp- iso mer [Rp-8br-PETcGMPS, and a PKA inhibitor, (R)-Adenosine, cyclic 3′,5′-(hydrogenphosphorothioate) triethylammonium [Rp-cAMPS] were purchased from Tocris Bioscience, Ellisville, MO. Purified mouse VASP and catalytic subunits of PKA and PKG were purchased from Promega. Ultrafree-MC filters, PVDF Immobilon-FL, and ZipTipC₁₈P₁₀ were from Millipore Corp. Rabbit skeletal muscle α-actin was from Cytoskeleton, Inc., Denver, CO. Antibodies were purchased from the following vendors: From Sigma-Aldrich, anti-biotin and anti-actin; from BD Biosciences (San Jose, CA), anti-PKA and PKG, anti-VASP, anti-phospho-serine 235 VASP; from Epitomics (Burlingame, CA) anti-phospho-serine 153 VASP. Small inhibitory RNA (siRNA) sequences were purchased from two sources. From Santa Cruz Biotechnology (Santa Cruz, CA) a control, scrambled sequence siRNA that will not cause specific degradation of any known cellular mRNA (UUCUCCGAAACGUGCACGU). VASP siRNA was a mixture of three sequences identified as strand A (GGGUGUCAAGUACAUCA), strand B (CCACUCCCAUCUUCAUCA) and strand C (GAGUGAACCUGAGAAGA). Rac 1 siRNA had the sequence (GUUCCUAUAUUUGCUUUUC) and Rac 2 siRNA was a mixture of three sequences, strand A (CAGUGUACUAAGUCACUA), strand B (CAGUGGCCCAAGGUAUUGA) and strand C (GAACCAAGGGAAGAGA). PKA siRNA was a pool of 3 different siRNA duplexes: Sense (GUUCAUGCUAGCUUACAAtt) and antisense (UUGUAAGCUAGCAUGGAACtt). PKG siRNA was also a pool of 3 different siRNA duplexes:

Sense (GAAGGUAGAUGCACAAAtt) and antisense (UUUGUGACUUAACCUCUCCtt); Sense (CCUUCCUAUCUACAGUAAtt) and antisense (UUACUGAUGAAGAAGGtt); and sense (CUCAUUCCCUCUACUAAAtt) and antisense (UUGAAGAAGGGAUGUAGtt). Sequences purchased from Thermo Scientific (Lafayette, CO) were a different control siRNA (UGGUUUAACUGUGACUAA) and a different VASP siRNA (UGCAUAUGGUAGGACCAAA).

**Animals:** Wild type mice (*Mus musculus*) and myeloperoxidase (MPO) knock-out mice were purchased (Jackson Laboratories, Bar Harbor, ME), fed a standard rodent diet and water ad libitum, and housed in the animal facility of the University of Pennsylvania. A colony of MPO knock-out mice was maintained from breading pairs purchased from Jackson Laboratories. Mice were exposed to O₂ at 2.8 atmospheres absolute (ATA) for 45 minutes following our published protocol (2). After anesthesia [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)] skin was prepared by swabbing with Betadine and blood was obtained into heparinized syringes by aortic puncture.

**Confocal microscopy:** Mice were exposed to air or to 2.8 ATA O₂ for 45 minutes, neutrophils isolated and placed on slides coated with fibrinogen following published methods (2). Cells were permeabilized by incubation for 1 hour at room temperature with PBS containing 0.1% (v/v) Triton X-100 and 5% (v/v) fetal bovine serum. Cells were then incubated overnight with 1:200 dilutions of Alexa 488-conjugated phalloidin plus primary antibodies to either VASP, Rac 1 or Rac 2. The next morning slides were rinsed three times with PBS and counterstained with a 1:500 dilution of RPE-conjugated secondary antibody. Images of neutrophils were acquired using a Zeiss Meta510 confocal microscope equipped with a Plan-Apochromat 63x/1.4NA oil objective. Fluorophore excitation was provided by 488 nm and 543 nm laser lines and resulting fluorescence was
separated using 500-530 nm and 560-615 nm band-pass filters.

**Isolation of Neutrophils and exposure to various agents:** Mice were anesthetized and neutrophils isolated from heparinized blood as previously described (2). In *ex vivo* studies, a concentration of 5 x 10^5 neutrophils/ml of PBS + 5.5 mM glucose were exposed to either air or 2.0 ATA O_2 for 45 minutes [we have shown that *ex vivo* exposures to 1 or 2 ATA O_2 are equivalent to *in vivo* exposures to 2.8 ATA (2)]. Cells were then used to evaluate β_2 integrin adherence function, pyrene actin polymerization or Rac activation. Before air/O_2 exposures inhibitors were added to some samples as described in Results. Where indicated, after air/O_2 exposures but prior to specific studies some cell suspensions were exposed for 5 minutes to UV light from a 200 watt mercury vapor lamp.

In studies using siRNA, prior to air/O_2 exposures cell suspensions were incubated for 20 hours at room temperature with siRNA following manufacturer’s instructions using control, scrambled sequence siRNA that will not lead to specific degradation of any known cellular mRNA or siRNA specific for mouse Rac 1, Rac 2, VASP, PKA or PKG. Pilot studies demonstrated that concentrations less than 0.04 nM did not reduce protein levels, 0.06 nM resulted in variable, intermediate effects and 0.08 nM achieved maximum decreases in protein levels. The magnitude of protein knock-down caused by 0.08 nM of the different siRNA species is shown in Supplemental Figure 1.

**Fibrinogen Coated Plate Adherence:** Preparation and use of fibrinogen coated plates to measure β_2 integrin specific neutrophil adherence in calcein AM-loaded cells was as previously described (2). Suspensions of 25,000 cells in 100 µl PBS were added to plate wells containing either PBS or solutions so that once added, cells would be exposed to 100 µM 8-br-cGMP or 100 nM FMLP. At the end of the 10 minute incubation wells were washed and adherence calculated as in (2).

**Actin polymerization in permeabilized cells:** Neutrophils suspensions were exposed to air or 2 ATA O_2 as outlined above, permeabilized using 0.2% n-octyl-b-glucopyranoside (OG) and actin polymerization assayed exactly as described in (30). Suspensions were incubated for 10 seconds by adding 0.1 volumes of OG buffer (60 mM PIPES, 25 mM Hepes (pH 6.9), 10 mM EGTA, 2 mM MgCl_2, 4% octyl glycoside, 10 µM phallacidin, 42 nM leupeptin, 10 mM benzamidine and 0.123 mM aprotonin). After the 10 second incubation 3 volumes of Buffer B (1 mM Tris [pH 7.0], 1 mM EGTA, 2 mM MgCl_2, 10 mM KCl, 5 mM β-mercaptoethanol and 5 mM ATP) was added. Actin polymerization was monitored for 5 minutes using a fluorescence spectrometer (355 nm excitation, 405 nm emission) when 1 µM pyrene-labeled rabbit skeletal muscle actin was added to PMN suspension.

**Rac activation:** Neutrophil suspensions prepared as described above were assayed for Rac activity using a commercial kit (Cytoskeleton, Inc., Denver, CO). Cells were centrifuged at 15,000 x g for 2 minutes and lysed for processing per the manufacturer’s instructions.

**Cytoskeletal protein analysis based on Triton solubility:** Neutrophil suspensions were exposed to air or HBO_2 for 45 minutes and then to 100 µM 8-br-cGMP or 100 nM FMLP for 10 minutes or to UV light for 5 minutes. Cell suspensions were then centrifuged at 200 x g for 10 minutes and resuspended in a solution of 0.5 mM DTSP [dithiobis (succinimidyl propionate)] to cross-link sulfhydryl-containing proteins within a proximity of ~ 12 Å following our published procedure (2,31). Cell lysates were partitioned into Triton-soluble G-actin and short F-actin and Triton-insoluble protein fractions and subjected to electrophoresis in gradient 4 - 15 % SDS-PAGE gels followed by Western blotting (2).

**Cell extract preparation and biotin-switch assay:** The biotin-switch assay was carried out on suspensions of rabbit skeletal muscle actin following published procedures (2). The kinetics of actin polymerization was measured using pyrene-labeled rabbit skeletal muscle α-actin following our published procedures (2). Polymerization was monitored in solutions containing 1 µM pyrene G-
actin plus 8 μM skeletal muscle G-actin in either 15, 25 or 50 mM KCl with 5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl$_2$, 2 mM MgCl$_2$, 1 mM ATP without or with 0.25 μM VASP. Studies were performed at room temperature and the linear change in fluorescence was recorded using an excitation wavelength of 365 nm and emission wavelength of 407 nm. Where indicated rather than using only skeletal muscle G-actin the solution contained 5 μM skeletal muscle G-actin and 3 μM SNO-actin.

S-nitrosylated G-actin (SNO-actin) was prepared by incubating 10 µM G-actin in 5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl$_2$ with 100 μM S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) in the dark at room temperature for 1 hour. Stock solutions of SNAP were prepared in 1 N HCl. Phosphorylated VASP was prepared using active subunits of PKA or PKG by diluting 50 µM VASP stock with an equal volume of 2X PKG binding buffer (80 mM TrisHCl, pH 7.4, 40 mM MgCl$_2$, 0.4 mM ATP, 600 nM cGMP, 40 units/ml aprotonin, and 2 μM pepstatin A) or PKA buffer (20 mM HEPES, pH 7.4 with 100 mM KCl, 10 mM MgCl$_2$, 0.4 mM ATP, 40 units/ml aprotonin, and 2 μM pepstatin A). When using PKG, the solution of 25 µM VASP in binding buffer was combined with an equal volume of 600 nM active catalytic subunit of protein kinase G prepared in 5 mM potassium phosphate buffer, pH 6.8 plus 1 mM EDTA and 0.5 ml/ml bovine serum albumin and the solution was incubated at 30°C for 2 hours. The phosphorylation reaction was then stopped by adding 1 μl of 1 mM PKG inhibitor protein (Calbiochem). When using PKA, 25 μM VASP in PKA was combined with 17 μM active catalytic subunit of protein kinase A (Promega) and incubate at 30°C for 60 minutes. The phosphorylation reaction was stopped by adding 1 μl of 1 mM PKA inhibitor protein (Promega).

Some studies were performed with polymerizing actin samples by incubating 1 mM ATP with 8 μM skeletal muscle G-actin or 5 μM skeletal muscle G-actin plus 3 μM SNO-actin at 37°C for 1 hour. The actin samples were diluted to 2 μM with 25 mM HEPES, pH 7.0, 15 mM KCl, 25 mM NaCl, 2 mM MgCl$_2$, 0.2 mM CaCl$_2$ and 1 μM ATP, and either 2 μM VASP or PKA-phosphorylated VASP was added. After 1 hour incubation at room temperature, samples were centrifuged at 12,000 x g for 1 hour. Pellets were resuspended in SDS buffer and the supernatants combined with 2 x SDS buffer for electrophoresis in gradient 4 - 15 % SDS-PAGE gels followed by Western blotting (2). Blots were probed for VASP and actin to assess the magnitude of actin sedimentation and ratio of VASP to actin.

**Statistical analysis:** Results are expressed as the mean±SE for three or more independent experiments. To compare data, we used a one-way analysis of variance (ANOVA) using SigmaStat (Jandel Scientific, San Jose, CA) and Newman-Keuls post-hoc test. Comparisons between two groups were done by t-test. The level of statistical significance was defined as p< 0.05.

**Results**

**Microscopic appearances of neutrophils:** Our prior investigation demonstrated that neutrophils taken from mice exposed to hyperoxia exhibited a dense pattern F-actin and there was no β2 integrin aggregation on the cell surface (2). To extend our exploration to proteins that control actin polymerization, cells from HBO$_2$-exposed mice were permeabilized to probe intracellular protein co-localizations with F-actin stained using Alexa488-conjugated phalloidin. The panel of images in Figure 1 show typical findings. The F-actin appearance in HBO$_2$-exposed cells is consistent with our previous report (2). Differences in the distributions of Rac 1, Rac 2, VASP, PKA and PKG in HBO$_2$-versus control, air-exposed cells are apparent. Co-localizations of proteins with F-actin were quantified by measuring yellow fluorescence in merged images and they were significantly different.

We have shown that HBO$_2$ effects can be reversed if cells are exposed to UV light [UV was shown to reverse S-nitrosylation of actin in HBO$_2$-exposed cells] or incubated with 8-br-cGMP or FMLP (2). The graphs in Figure 1 demonstrate that co-localization phenomena involving Rac 1, Rac 2, VASP, PKA and PKG are also reversed by these manipulations.
**β2 integrin-dependent adherence:** Adherence to fibrinogen coated plastic plates was used to screen β2 integrin function. As with prior studies, neutrophil adherence was inhibited whether cells were taken from mice exposed to hyperoxia (HBO2, 2.8 ATA O2 for 45 minutes) or if neutrophils were removed from normal, air-breathing mice and exposed to hyperoxia *ex vivo*. β2 integrin adherence of neutrophils from normal mice was 21.2 ± 2.9 (SE, n=10) % whereas neutrophils obtained after mice were exposed to HBO2 exhibited β2 integrin adherence of only 0.9 ± 1.0 (n=10, p<0.005) %. Adherence measured when neutrophils were exposed *ex vivo* to air or oxygen is shown in Table 1. Adherence inhibition by HBO2 did not occur if cells were exposed to UV light prior to placement on fibrinogen plates or if cells from myeloperoxidase (MPO) knock-out mice were use. Also consistent with prior studies, hyperoxia did not inhibit β2 integrin function if, while being exposed to hyperoxia, cell suspensions included NOS inhibitors (1400 W (N-3-(aminomethyl) benzyl acetamine) or L-NNAME). Additionally, the β2 integrin inhibitory effect did not occur if cells were exposed to 8-br-cGMP or FMLP after hyperoxia.

The inhibitory effect of hyperoxia on β2 integrin-specific adherence was also abrogated if during the hyperoxic exposure cells were incubated with inhibitors of Rac proteins (NSC 23766), PKG (br-PET cGMPs) or PKA (Rp-cAMPS). Incubation with Wortmannin, a general phosphoinositide 3 kinase inhibitor, had no significant effect (Table 1).

To more discreetly assess the roles for particular proteins, neutrophils were removed from air-breathing, control mice and incubated *ex vivo* with siRNA prior to exposure to hyperoxia. As outlined in Methods, the reduction in cell content of specific proteins caused by the various siRNA treatments was somewhat variable and ranged from 23 to 46 %. The transfection protocol caused a modest (NS) reduction in β2 integrin adherence as shown in Table 1 using non-specific siRNA. The various siRNA species had no significant effects on the adherence function of air-exposed, control cells. Mouse siRNA to Rac1, Rac2, VASP, PKA and PKG abrogated the inhibitory effect of hyperoxia on β2 integrin function. Note that because we lacked a small chemical inhibitor for VASP, we used two sources of siRNA. As shown, the two different control siRNA sequences as well as siRNA sequences to VASP exhibited virtually identical results. For all subsequent experiments described below, only siRNA from Santa Cruz was utilized.

In summary, these data indicate that a number of proteins are required for HBO2-mediated inhibition of β2 integrin adherence. As a next step in the study, we planned to monitor intracellular actin polymerization in neutrophils permeabilized with octyl-glucopyranoside (OG). Therefore, we wanted to assess whether OG treatment altered β2 integrin-specific adherence. Adherence in air-exposed cells subjected to OG-permeabilization did show a small, but significantly greater β2 integrin-specific adherence (26.6 ± 2.1 %, n=23) versus adherence shown by non-permeabilized cells (Table 1). OG-permeabilization did not abrogate the inhibitory effect of hyperoxia on β2 integrin-specific adherence. In OG-permeabilized cells exposed to HBO2, β2-integrin specific adherence was 1.8 ± 0.6 % (SE, n=23; p<0.001 versus air-exposed OG permeabilized cells).

**Actin polymerization in permeabilized neutrophils:** Because confocal imaging indicates that F-actin exhibits a markedly different appearance after neutrophils are exposed to HBO2, we wanted to examine the dynamics of intracellular actin polymerization. This was done with OG-permeabilized neutrophils incubated with pyrene-actin. The rate of polymerization was 3.6-fold higher in HBO2 exposed cells versus control, air-exposed neutrophils (Table 2). Fluorescence related to pyrene incorporation into filamentous actin rose linearly for 1 to 1.5 minutes followed by a plateau and if a second bolus of monomeric pyrene actin was added to cell suspensions, a second rise in fluorescence occurred at the same rate and duration (data not shown). If cells were stored overnight at 4°C and studied 24 hours later, the same actin polymerization dynamics were observed in control and HBO2 exposed cells. In all conditions, polymerization was inhibited by greater than 95% when 2 μM cytochalasin D was added to the suspension, indicating that the
response to hyperoxia was due to increased availability of actin FBEs.

The same agents and manipulations that abrogated the inhibitory effect of HBO$_2$ on $\beta_2$ integrin-dependent adherence also resolved the elevated availability of FBEs (Table 2). Generally, these manipulations had no significant effect on control, air-exposed cells. With FMLP exposure, however, control cells exhibited a significant increase in FBEs availability. In HBO$_2$ exposed cells incubated with FMLP, FBEs availability was significantly less than that observed with HBO$_2$ exposure alone (p<0.05) and the same magnitude as for control cells. The same siRNA interventions that abrogated the effect of hyperoxia on $\beta_2$ integrin adherence also restored the normal availability of FBEs seen in the control neutrophils (Table 2). Incubation with siRNA to PKA or PKG reduced FBEs availability in air-exposed, control cells and the same magnitude of actin polymerization was observed in HBO$_2$-exposed cells.

Before proceeding with further studies we wanted to directly address whether OG-permeabilization might influence the accelerated actin polymerization phenomenon observed following hyperoxia compared to cells left with intact cell membranes. To do this, isolated neutrophils with or without OG permeabilization were exposed to room air or HBO$_2$ for 45 minutes, formalin-fixed and then F-actin stained by incubating cells with TRITC-phalloidin. Fluorescence intensity of HBO$_2$-exposed cells not subjected to OG permeabilization was 2.1 $\pm$ 0.2 (SE, n=4) fold greater than in air-exposed cells. OG-permeabilized cells exhibited 2.2 $\pm$ 0.1 (n=4, NS) fold greater fluorescence after exposure to HBO$_2$. Therefore, OG-permeabilization did not appear to influence HBO$_2$-mediated augmentation of actin polymerization compared with that present in intact cells.

Rac activation: We next wanted to evaluate the activity of Rac proteins because, as was reviewed in Introduction, the three pathways that regulate FBEs availability in neutrophils involve the Rac GTPase proteins (9). Neutrophils were exposed to air (control) or HBO$_2$ and activity of Rac-1/2/3 was assayed as described in Methods using a commercial kit. Cells exposed to hyperoxia exhibited a marked elevation in Rac activity (Table 3). FMLP increased Rac activity in air-exposed cells. In HBO$_2$-exposed cells incubated with FMLP, Rac activity was at the same level as observed with control cells and significantly less compared to HBO$_2$-exposed cells not incubated with FMLP (p<0.05). Incubation with 8-br-cGMP after hyperoxia had no impact on Rac activation in air-exposed, control cells but it abrogated the HBO$_2$-mediated elevation of Rac activity. A role for S-nitrosylated proteins was shown because elevated Rac activity did not occur in cells exposed to UV light after hyperoxia. Similarly, cells from MPO knock-out mice did not exhibit elevated Rac activities after exposure to hyperoxia. Cells incubated with br-PET cGMPS (PKG inhibitor) or Rp-cAMPS (PKA inhibitor) during hyperoxia did not exhibit elevated Rac activity. When neutrophils from normal, wild-type mice were pre-incubated with siRNA to VASP, PKG or PKA, subsequent HBO$_2$ exposure did not augment Rac activation. We conclude that the same proteins required for HBO$_2$-mediated inhibition of $\beta_2$ integrin adherence and for accelerated FBEs formation are also required for activation of Rac proteins.

**Cytoskeletal regulatory proteins in cell actin fractions:** As was reviewed in Introduction, VASP is a multi-ligand protein that interacts with actin and a variety of microfilament proteins. We hypothesized that VASP might be the central element that unifies the roles for the five proteins found to influence HBO$_2$-mediated inhibition of $\beta_2$ integrin adherence. To evaluate whether protein associations may be altered in response to HBO$_2$, cells were incubated with a sulfhydryl cross-linking agent, partitioned into Triton-soluble G-actin, Triton-soluble short F-actin and Triton-insoluble F-actin pools and the fractions analyzed by Western blot. Figure 2 shows protein ratios as band density versus the density of the actin band with PBS. In evaluating these data sets it is
important to note that there were no significant differences in cell content of VASP, Rac 1, Rac 2, PKG or PKA in HBO₂-exposed versus control cells based on Western blots normalized to β-actin (data not shown).

There were significant elevations in the ratios for all proteins of interest in cells exposed to HBO₂ and incubated with PBS (Figure 2). Exposure to 8-br-cGMP or UV light had no significant effect on control neutrophils but these interventions abrogated the elevated protein ratios seen in HBO₂-exposed cells. When control neutrophils were incubated with FMLP, there were elevations in VASP and PKG protein ratios. HBO₂-exposed cells incubated with FMLP no longer exhibited significant elevations in protein ratios for VASP, Rac 1, Rac 2 or PKG but the elevation in PKA-to-actin persisted.

We found that the siRNA incubation protocol impeded the HBO₂-mediated increases in protein associations with actin, although they were still significantly elevated over air-exposed cells (Figure 3, non-specific siRNA, labeled as control). If cells were incubated with siRNA to PKA or PKG before exposure to HBO₂, protein elevations in the short F-actin pool were not observed. When cells were exposed to siRNA to VASP prior to HBO₂ exposure, hyperoxia no longer caused elevations in ratios for VASP-actin or PKA-actin. Elevations of Rac 1-actin and Rac 2-actin ratios were not only abrogated in the HBO₂-exposed cells, they were also significantly lower than observed in the air-exposed control cells. The elevated ratio of PKG-actin caused by hyperoxia persisted despite treatment with siRNA to VASP.

Results using the sulfhydryl cross linking agent with the G-actin and Triton insoluble F-actin pools are not shown because protein ratios were not significantly different between control and HBO₂-exposed cells. For example, in the G-actin pool the VASP-to-actin ratio in HBO₂-exposed cells was 1.32 ± 0.23 (n=11, NS) higher than in air-exposed, control cells and the ratio was 1.10 ± 0.18 fold (NS) higher in the Triton-insoluble F-actin pool.

**VASP phosphorylation:** Given that PKA and PKG can also phosphorylate VASP, it was important to evaluate whether VASP phosphorylation was altered by hyperoxia. No significant differences were identified in serine 235 phosphorylated VASP between air- and HBO₂-exposed cells whether they were incubated with PBS, FMLP, or 8-br-cGMP; but elevations of serine 153 phosphorylated VASP occurred in both control and HBO₂-exposed cells incubated with 8-br-cGMP or FMLP (Table 4).

**Ex vivo VASP linkage to actin:** Taken in total, results to this point suggest that VASP linkage to short F-actin in HBO₂-exposed cells may be a central element in controlling responses to hyperoxia. To more discretely evaluate the impact that actin S-nitrosylation has on VASP binding and actin polymerization, *ex vivo* pyrene actin polymerization was assayed using suspensions of standard G-actin or G-actin first subjected to S-nitrosylation by incubation with S-nitroso-N-acetyl-D,L-penicillamine (SNAP). S-nitrosylation of G-actin (SNO-actin) was verified by biotin-switch (data not shown).

Actin polymerization was accelerated when SNO-actin was included in a suspension of normal G-actin and the rate was markedly more rapid when VASP was included (Figure 4). If VASP (rather than G-actin) was incubated with SNAP and then used in suspensions of G-actin or SNO-actin, polymerization rates did not differ from rates seen with standard VASP shown in Figure 4 (data not shown). When VASP was first phosphorylated by incubation with catalytically active subunits of PKA or PKG, polymerization was significantly reduced versus with non-phosphorylated VASP. Figure 4 shows results with PKA incubation, PKG incubation gave identical results. Phosphorylation abrogated much of the enhancement in polymerization rate seen in VASP-containing suspensions. Phospho-VASP also abrogated virtually the entire enhanced rate caused by SNO-actin incubated without VASP.

Actin polymerization in buffer containing 25 or 50 mM KCl (versus 15 mM KCl as in Figure 4) is
shown in Supplemental Table 1. G-actin polymerization exhibited a nominal rate in 25 mM KCl solutions and a negligible polymerization rate in 50 mM KCl. SNO-actin exhibited significantly greater rates of polymerization versus G-actin with either KCl concentration. VASP increased G-actin polymerization significantly in 25 or 50 mM KCl solutions, but inclusion of VASP had no significant effect on SNO-actin polymerization rates. These results suggest that VASP associates with SNO-actin by electrostatic interactions as is true with normal actin (15).

We were interested in evaluating whether S-nitrosylation may alter VASP associations with F-actin. Samples of standard G-actin or SNO-actin were first polymerized, then incubated with VASP or phosphorylated VASP as described in Methods. After low-speed centrifugation we evaluated the amount actin that was sedimented and the ratio of VASP to actin in the pellets. Suspensions containing VASP exhibited more actin sedimentation than suspensions containing phosphorylated VASP and polymerized SNO actin sedimented more than polymerized standard G-actin (Table 5). The ratios of VASP/actin and phospho-VASP/actin did not differ significantly between standard G-actin and SNO-actin samples. We conclude from these results that when SNO actin polymerizes there are more filaments than with standard G-actin polymerization, as would be expected base on results in Figure 4; however, once actin filaments form, there is no difference in VASP association between standard actin filaments and SNO-actin filaments.

Roles for PKA and PKG with FMLP and 8-br-cGMP effects: The data indicate that PKA and PKG are required for all of the HBO2-mediated effects we have observed in neutrophils, yet as described in Introduction, these enzymes are also suspected to be involved with reversal of HBO2-mediated effects when cells are incubated with 8-br-cGMP or FMLP. To more discreetly evaluate the roles for these enzymes in reversal phenomena, cells were exposed to air or HBO2 and then incubated overnight with various siRNA species before assays were done. Note that this is a different paradigm than used in prior investigations when cells were incubated with siRNA species before exposure to HBO2. Figure 5 demonstrates β2 integrin specific adherence and Figure 6 shows FBEs availability measured as pyrene actin polymerization rates. The various manipulations had no significant effects on adherence by control, air-exposed cells. Incubation with FMLP caused a significant increase in pyrene actin polymerization in control cells exposed to control siRNA and siRNA to PKA, but actin polymerization was not significantly elevated when cells were incubated with siRNA to PKG before FMLP exposure.

The effects of hyperoxia remained intact when cells were incubated with control siRNA. That is, β2 integrin function was inhibited and elevations in actin FBEs were observed. Also, integrin function and actin polymerization rates in HBO2-exposed cells incubated with control siRNA were restored to the same levels seen in air-exposed cells by incubation with FMLP or 8-br-cGMP. This is entirely consistent with the data in Tables 1 and 2. After cells were incubated with siRNA to PKA, however, there was no reversal of the HBO2 effects by FMLP. This indicates that reversal of the HBO2-mediated effects by FMLP involves PKA. If cells were incubated with siRNA to PKG, 8-br-cGMP did not abrogate HBO2-mediated β2 integrin inhibition or elevation in FBEs. Thus, PKG plays a central role in reversal of hyperoxia effects initiated by 8-br-cGMP.

DISCUSSION:

Results from this study and prior work show that reactive species generated by hyperoxia cause actin S-nitrosylation in neutrophils which perturbs the actin cytoskeleton and inhibits β2 integrin-specific adherence (2). The effects of small chemical inhibitors and siRNA indicate that HBO2-mediated β2 integrin inhibition requires VASP, Rac1, Rac2, PKA and PKG (Table 1). Confocal microscope images demonstrate that there are elevations in co-localization of these proteins with F-actin (Figure 1).

Additions of pyrene actin to permeabilized HBO2-exposed neutrophils demonstrate that FBEs are increased versus air-exposed, control cells (Table 2). Repeated additions of pyrene-actin indicate
that this process goes on for at least 24 hours, suggesting that there must be a recycling of FBEs.

Rac GTPase proteins are responsible for FBE formation and actin polymerization in neutrophils (9). There are both unique as well as overlapping roles for Rac 1 and Rac 2 in neutrophils (32). The Rac inhibitor and siRNA experiments indicate that recycling of actin filaments with renewed formation of FBEs in HBO\textsubscript{2}-exposed cells involves activated Rac 1 and Rac 2 proteins (Table 2). HBO\textsubscript{2} exposure causes a marked increase in Rac activity that is abrogated by all of the interventions that resolve HBO\textsubscript{2}-mediated inhibition of $\beta_2$ integrins and elevations of FBEs (Table 3).

The \textit{ex vivo} experiments show that SNO-actin polymerizes more rapidly than normal G-actin and that VASP accelerates the process (Figure 4). An important aspect to the \textit{ex vivo} results is that accelerated actin polymerization due to S-nitrosylation does not require enzymatic actions of Rac proteins, PKG or PKA. Prompt self-association of SNO-actin will yield short F-actin and we previously reported that there is more Triton soluble short F-actin in HBO\textsubscript{2}-exposed versus control neutrophils (2). VASP has high affinity for this actin pool in HBO\textsubscript{2}-exposed cells (Figure 2) which will drive further actin polymerization (Figure 4). The Triton insoluble F-actin fraction did not exhibit elevations in protein ratios to actin (e.g. VASP-to-actin). This is consistent with finding that VASP affinity for polymerized SNO-actin does not differ from that observed with normal, non-nitrosylated actin (Table 5). We did not find significantly elevated protein ratios in the G-actin pool, which may occur simply because SNO-actin self associates rapidly to produce short F-actin.

Reducing cell content of VASP by siRNA species abrogated the additional Rac activation typically observed with HBO\textsubscript{2}-exposed cells (Table 3). VASP siRNA also prevented the elevations of associations between actin and Rac 1 or Rac 2 in HBO\textsubscript{2}-exposed neutrophils (Figure 3). PKA and PKG are also in close proximity to short F-actin in HBO\textsubscript{2}-exposed cells. Experiments using small chemical inhibitors as well as siRNA to PKA or PKG indicate that these two kinases are responsible for Rac 1 and Rac 2 activation. Whereas one might have anticipated that either PKA or PKG individually could cause Rac activation, responses to HBO\textsubscript{2} appear to require a concerted action by both kinases. The proximity between protein kinases and Rac 1 and 2 facilitated by VASP appears to be required for Rac activation, based on the inhibitory effect of siRNA to VASP (Table 3). There is precedence for this sort of interaction. VASP bundles actin with Rac 1 in endothelium and in so doing enhances Rac activation by PKA (23,26).

The efficiency of the various siRNA species for inhibiting HBO\textsubscript{2}-mediated events is remarkable given the limited degree to which they reduced cell protein contents (Supplemental Figure 1). If cells are incubated with siRNA to PKA or PKG before hyperoxia, the protein ratios are the same as control – but not reduced below the value seen with air-exposed, control cells exposed to control siRNA (Figure 3). This suggests that these protein associations are stable once formed and that associations with short F-actin involving newly synthesized PKG and PKA in the HBO\textsubscript{2}-exposed cells is required for on-going Rac activation. A slightly more complex pattern was observed with siRNA to VASP. Again, siRNA knock-down in HBO\textsubscript{2}-exposed neutrophils resulted in a VASP-actin ratio the same as control in the short F-actin pool, but not less than control. There were significantly lower amounts of Rac 1 and Rac 2 in close proximity to short F-actin in HBO\textsubscript{2}-exposed cells pre-incubated with siRNA to VASP. Values were actually lower than in the air-exposed, control cells suggesting that associations were rendered more labile in the HBO\textsubscript{2}-samples because of actin S-nitrosylation or a consequence of S-nitrosylation such as enhanced actin polymerization. The persistent elevation of the PKG-actin ratio in HBO\textsubscript{2}-exposed cells pre-incubated with siRNA to VASP implies that PKG proximity does not require elevation of VASP-actin. It is obvious that further work is needed to clarify the mechanism(s) for protein bundling.

Phosphorylation of VASP by PKA or PKG abrogates its ability to enhance \textit{ex vivo} actin polymerization (Figure 4) and reduces the affinity of VASP for filamentous actin (Table 5). This is consistent with work reported by others (15). We
found that including phosphorylated VASP in suspensions of SNO-actin actually inhibits its polymerization, indicating that phosphorylation is doing more than merely reducing VASP affinity for actin. The biochemical consequences of this interaction will require further study.

VASP is phosphorylated when neutrophils are exposed to FMLP or 8-br-cGMP (Table 4). We conclude from the data that reversal of HBO$_2$-mediated $\beta_2$ integrin-specific adherence by FMLP or 8-br-cGMP is due to VASP phosphorylation. This is based on the effects of siRNA to PKA or PKG in cells previously exposed to hyperoxia (Figures 5 and 6). FMLP acts through PKA whereas 8-br-cGMP acts through PKG. It is interesting that with either agent, serine 153 phosphorylated VASP is elevated but serine 235 phosphorylated VASP is not (Table 4). We recognize, however, that phosphorylation is a transient event and we may have missed elevations in phosphorylated proteins as we did not run time-course studies of cells incubated with FMLP or 8-br-cGMP. The protein kinases phosphorylate both sites but in ex vivo studies PKG preferentially phosphorylates serine 235 whereas PKA preferentially phosphorylates serine 153 (33).

The roles for PKG and PKA in neutrophil cytoskeletal control are obviously complex – they are required for HBO$_2$-mediated inhibition of $\beta_2$ integrins and they are also required for FMLP or 8-br-cGMP to abrogate the HBO$_2$ effects. We believe the findings from Table 3 and Figure 3 along with Figures 5 and 6 provide insight. Taken in total, it appears that proximity of PKG and PKA to Rac 1 and 2 facilitated by VASP in the short F-actin fraction is required for Rac activation, increased pyrene actin polymerization and $\beta_2$ integrin inhibition. There is precedence for considering that kinase substrates may differ based on whether PKA and PKG are linked to VASP and this offers an explanation for why the protein kinases act on Rac 1 and 2 in HBO$_2$-exposed cells versus phosphorylating VASP itself (23). As shown in Table 4, HBO$_2$ exposure did not significantly increase VASP phosphorylation. In that the PKG-actin and PKA-actin ratios are not reduced below the control level by siRNA incubations (Figure 3), yet siRNA incubations abrogate the reversal effects of FMLP and 8-br-cGMP (Figure 5 and 6), kinases outside the Triton-soluble short actin pool are needed for FMLP and 8-br-cGMP actions.

There is also a matter of temporal changes, which may have more functional significance such as when neutrophils are needed for immune surveillance. That is, if neutrophils are first activated by FMLP incubation, HBO$_2$ no longer will inhibit $\beta_2$ integrins (4). This can be explained by FMLP-mediated PKA activation and subsequent phosphorylation of VASP, which virtually eliminates actin polymerization augmentation caused by SNO-actin (Figure 4).

HBO$_2$ exposure increases the amount of VASP, Rac 1, Rac 2, PKG and PKA in close proximity to short F-actin (Figures 2 and 3). This is also apparent based on co-localization assessed by confocal microscopy (Figure 1). One cannot make a distinction between short F-actin and Triton-insoluble F-actin in the microscope images, however, which we believe is the reason why these proteins exhibit somewhat different patterns in Figure 1. That is, although the biochemical data indicate they all have increases in their proximity to short F-actin, the co-localization patterns in Figure 1 reflect relations to the total F-actin pool.

Overall, the data show that VASP plays a key role in promoting actin polymerization in neutrophils and it is the enhancement of this process within HBO$_2$-exposed neutrophils that leads to inhibition of $\beta_2$ integrin-specific adherence. The increased association of VASP with S-nitrosylated short F-actin increases FBEs formation and actin polymerization by a process that includes PKA and PKG-dependent Rac 1 and 2 activation. VASP phosphorylation by protein kinases in cells treated with either FMLP or 8-br-cGMP abrogates these processes by reducing VASP binding to SNO-actin, restoring more normal actin polymerization and thus reinstating $\beta_2$ integrin function.

This information offers important insights into neutrophil cell physiology and clinical use of HBO$_2$. Neutrophil $\beta_2$ integrin adhesion molecules participate in regulating neutrophil activation and endothelial adhesion (34). Whereas $\beta_2$ integrins are critically important in immune surveillance,
they are also central to tissue injury in processes such as ischemia-reperfusion. Inhibition of neutrophil adhesion ameliorates reperfusion injuries of brain, skeletal muscle and intestine, as well as smoke-induced lung injury, decompression sickness, and encephalopathy due to carbon monoxide poisoning in animal models (1,35-42). Inhibited β₂ integrin adhesion may be the basis for benefits of HBO₂ shown in human clinical trials involving coronary artery thrombolytic therapy, balloon angioplasty/stenting (43-46) and reductions of encephalopathy and cardiac compromise seen after cardiopulmonary bypass and carbon monoxide poisoning (47-49). The reversible nature of HBO₂-mediated β₂ integrin inhibition provides an explanation for why benefits are observed but hyperoxia does not lead to immunocompromise.

One may ask whether the complex HBO₂-mediated events bear a resemblance to any normal physiological process. That is, is there any natural scenario whereby SNO-actin is elevated in neutrophils to abrogate β₂ integrin function? While further work will be necessary to definitely answer this question, we speculate that these events may occur on a local microvascular level (versus the global or systemic level caused by HBO₂) due to endothelial production of nitric oxide. In previous studies we have shown that many of the same biochemical events that mediate HBO₂-mediated β₂ integrin inhibition also occur when neutrophils are exposed to a nitric oxide generating source (50).

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References:
1. Thom, S. R. (1993) *Toxicol Appl Pharmacol* **123**, 248-256
2. Thom, S. R., Bhopale, V. M., Mancini, J. D., and Milovanova, T. M. (2008) *J Biol Chem* **283**, 10822-10834
3. Thom, S. R., Mendiguren, I., Hardy, K., Bolotin, T., Fisher, D., Nebolon, M., and Kilpatrick, L. (1997) *Am J Physiol* **272**, C770-777
4. Chen, Q., Banick, P. D., and Thom, S. R. (1996) *J Pharmacol.Expt'l.Therap.* **276**, 929-933
5. Thom, S. R. (2009) *J Appl Physiol* **106**, 988-995
6. Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E., and Frame, M. C. (2007) *Nat Cell Biol* **9**, 1046-1056
7. Miranti, C. K., Leng, L., Maschberger, P., Brugge, J. S., and Shattil, S. J. (1998) *Curr Biol* **8**, 1289-1299
8. Anderson, S. I., Behrendt, B., Machesky, L. M., Insall, R. H., and Nash, G. B. (2003) *Cell Motil Cytoskeleton* **54**, 135-146
9. Sun, C. X., Magalhaes, M. A., and Glogauer, M. (2007) *J Cell Biol* **179**, 239-245
10. Arca, A. (1998) *J Biol Chem* **273**, 805-813
11. Webb, D. J., Parsons, J. T., and Horwitz, A. F. (2002) *Nat Cell Biol* **4**, E97-100
12. Rottner, K., Hall, A., and Small, J. V. (1999) *Curr Biol* **9**, 640-648
13. Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktoeries, K., Kalman, D., and Bourne, H. R. (2003) *J Cell Biol* **160**, 375-385
14. Dib, K., Melander, F., Axelsson, L., Dagher, M. C., Aspenstrom, P., and Andersson, T. (2003) *J Biol Chem* **278**, 24181-24188
15. Huttelmaier, S., Harbeck, B., Steffens, O., Messerschmidt, T., Illenberger, S., and Jockusch, B. M. (1999) *FEBS Lett* **451**, 68-74
16. Butt, E., Eigenthaler, M., and Genieser, H. G. (1994) *Eur J Pharmacol* **269**, 265-268
17. Goldberg, N. D., Haddox, M. K., Nicol, S. E., Glass, D. B., Sanford, C. H., Kuehl, F. A., and Estensen, R. D. (1975) *Adv Cyclic Nucleotide Res* **5**, 307-330
18. Kwiatkowski, A. V., Gertler, F. B., and Loureiro, J. J. (2003) *Trends Cell Biol* **13**, 386-392
19. Laurent, V., Loisel, T. P., Harbeck, B., Wehman, A., Grobe, L., Jockusch, B. M., Wehland, J., Gertler, F. B., and Carlier, M. F. (1999) *J Cell Biol* **144**, 1245-1258
20. Harbeck, B., Huttelmaier, S., Schluter, K., Jockusch, B. M., and Illenberger, S. (2000) J Biol Chem 275, 30817-30825
21. Selvatici, R., Falzarano, S., Mollica, A., and Spisani, S. (2006) Eur J Pharmacol 534, 1-11
22. Worner, R., Lukowski, R., Hofmann, F., and Wegener, J. W. (2007) Am J Physiol Heart Circ Physiol 292, H237-244
23. Schlegel, N., and Waschke, J. (2009) Am J Physiol Cell Physiol 296, C453-462
24. Eckert, R. E., and Jones, S. L. (2007) J Leukoc Biol 82, 1311-1321
25. Deevis, R. K., Koney-Dash, M., Kissenspennig, A., Johnston, J. A., Schuh, K., Walter, U., and Dib, K. (2010) J Immunol 184, 6575-6584
26. Schlegel, N., and Waschke, J. (2009) J Cell Physiol 220, 357-366
27. Garcia Arguinizonis, M. I., Galler, A. B., Walter, U., Reinhard, M., and Simm, A. (2002) J Biol Chem 277, 45604-45610
28. Horstrup, K., Jablonka, B., Honig-Liedl, P., Just, M., Kochsiek, K., and Walter, U. (1994) Eur J Biochem 225, 21-27
29. Aszodi, A., Pfeifer, A., Ahmad, M., Glauner, M., Zhou, X. H., Ny, L., Andersson, K. E., Kehrel, B., Offermanns, S., and Fassler, R. (1999) Embo J 18, 37-48
30. Glogauer, M., Hartwig, J., and Stossel, T. P. (2000) J Cell Biol 150, 785-796
31. Huttelmaier, S., Mayboroda, O., Harbeck, B., Jarchau, T., Jockusch, B. M., and Rudiger, M. (1998) Curr Biol 8, 479-488
32. Pai, S. Y., Kim, C., and Williams, D. A. Dis Markers 29, 177-187
33. Butt, E., Abel, K., Krieger, M., Palm, D., Hoppe, V., Hoppe, J., and Walter, U. (1994) J Biol Chem 269, 14509-14517
34. Brown, E., and Lindberg, F. (1996) Ann Med 28, 201-208
35. Zamboni, W. A., Roth, A. C., Russell, R. C., Graham, B., Suchy, H., and Kucan, J. O. (1993) Plast. Reconstr. Surg. 91, 1110-1123
36. Martin, J. D., and Thom, S. R. (2002) Aviat Space Environ Med 73, 565-569
37. Atochin, D., Fisher, D., Demchenko, I., and Thom, S. (2000) Undersea and Hyperbaric Med. 27, 185-190
38. Tahepold, P., Vaage, J., Starkopf, J., and Valen, G. (2003) J Thorac Cardiovasc Surg 125, 650-660
39. Tahepold, P., Valen, G., Starkopf, J., Kairane, C., Zilmer, M., and Vaage, J. (2001) Life Sci 68, 1629-1640
40. Ueno, S., Tanabe, G., Kihara, K., Aoki, D., Arikawa, K., Dogomori, H., and Aikou, T. (1999) Hepato. Gastroenterology 46, 1798-1799
41. Wong, H. P., Zamboni, W. A., and Stephenson, L. L. (1996) Surgical. Forum. 705-707
42. Yang, Z. J., Bosco, G., Montante, A., Ou, X. I., and Camporesi, E. M. (2001) Eur J Appl Physiol 85, 96-103
43. Sharifi, M., Fares, W., Abdel-Karim, I., Koch, J. M., Sopko, J., and Adler, D. (2004) Am J Cardiol 93, 1533-1535
44. Sharifi, M., Fares, W., Abdel-Karim, I., Petrea, D., Koch, J. M., Adler, D., and Sopko, J. (2002) Cardiovasc Radiat Med 3, 124-126
45. Shandling, A. H., Ellestad, M. H., Hart, G. B., Crump, R., Marlow, D., Van Natta, B., Messenger, J. C., Strauss, M., and Stavitsky, Y. (1997) Am.Heart J. 134, 544-550
46. Stavitsky, Y., Shandling, A. H., Ellestad, M. H., Hart, G. B., Van Natta, B., Messenger, J. C., Strauss, M., Dekleva, M. N., Alexander, J. M., Mattice, M., and Clarke, D. (1998) Cardiology 90, 131-136
47. Weaver, L. K., Hopkins, R. O., Chan, K. J., Churchill, S., Elliott, C. G., Clemmer, T. P., Orme, J. F., Jr., Thomas, F. O., and Morris, A. H. (2002) N Engl J Med 347, 1057-1067
48. Alex, J., Laden, G., Cale, A., Bennett, S., Flowers, K., Madden, L., Gardiner, E., McCallum, P., and Griffin, S. (2005) J Thorac Cardiovasc Surg 130, 1623-1630
49. Yogaratnam, J. Z., Laden, G., Guvendik, L., Cowen, M., Cale, A., and Griffin, S. (2010) *Cardiovasc Revasc Med* **11**, 8-19

50. Banick, P. D., Chen, Q., Xu, Y. A., and Thom, S. R. (1997) *J. Cell. Physiol.* **172**, 12-24
Figure 1. **Protein co-localizations with F-actin in neutrophil images shown by two-photon microscopy.** Neutrophils isolated from mice exposed to air or HBO\textsubscript{2} were placed on fibrinogen-coated slides, permeabilized and stained as described in Methods. Bar graphs show merge, yellow fluorescence intensity which reflects proteins co-localized with Alexa 488-conjugated phalloidin-stained F-actin. Bar graph data also show results when cells were co-incubated for 5 minutes before fixation with either 100 µM 8-br-cGMP or 100 nM FMLP or exposed to UV light. In each case, overlap was significantly different from PBS-incubated, air-exposed control neutrophils for only the HBO\textsubscript{2}-exposed cells incubated with PBS (* p<0.001, ANOVA). These data were obtained with cells from 3 or 4 mice in independent experiments by analyzing from 20 to 40 neutrophils in each trial.

Figure 2. **Protein associations in the Triton-soluble short F-actin fraction.** Neutrophil were isolated and exposed to air (control) or HBO\textsubscript{2} for 45 minutes followed by incubation with PBS or PBS containing 100 nM FMLP, 100 µM 8-br-cGMP or cells were exposed to UV light as outlined in Methods. Proteins were cross-linked by incubating cells with DTSP, and then fractioned based on Triton solubility (see Methods). After Western blotting, protein band densities were quantified and normalized to the β actin band in each lysate. The ratio of each protein relative to actin was compared to that calculated for the air-exposed, control neutrophils in each experiment. Therefore, data in the figure show the fold-increase in band density normalized to the ratio observed in control cells for the short F-actin fraction. Images at the top of each bar graph show blots from one experiment. The actin band for this experiment is also shown at the top of the figure. Sample size for each analysis are shown (n), data are mean ± SE, *p<0.05 versus air-exposed cells incubated with PBS.

Figure 3. **Protein associations in the Triton-soluble short F-actin fraction of cells treated with siRNA.** Neutrophil were isolated and incubated with siRNA as described in Methods. Samples labeled ‘control’ in the figure were incubated with control, scrambled sequence siRNA that will not lead to specific degradation of any known cellular mRNA. After 20 hours cells were exposed to air (control) or HBO\textsubscript{2} for 45 minutes, proteins were cross-linked by incubating cells with DTSP and then fractioned based on Triton solubility (see Methods). After Western blotting, protein band densities were quantified and normalized to the β actin band in each lysate. The ratio of each protein relative to actin was compared to that calculated for the air-exposed, control neutrophils in each experiment. Therefore, data in the figure show the fold-increase in band density normalized to the ratio observed in control cells for the short F-actin fraction. Images at the top of each bar graph show blots from one experiment. The actin band for this experiment is also shown at the top of the figure. Sample size for each analysis are shown (n), data are mean ± SE, *p<0.05 versus air-exposed cells incubated with PBS.

Figure 4. **Ex vivo actin polymerization rates.** Fluorescence was measured as described in Methods after 1 µM pyrene G-actin was added to solutions containing 15 mM KCl in 5 mM Tris-HCl (pH 8.0). Solutions labeled at the top of the figure as ‘actin’ contained 8 µM skeletal muscle G-actin, those labeled ‘SNO-actin’ contained 5 µM skeletal muscle G-actin and 3 µM SNO-actin (prepared by incubating G-actin with SNAP). Where indicated samples also contained 0.25 µM VASP or 0.25 µM VASP phosphorylated by prior incubation with the active subunit of PKA (see Methods). Although not shown, the results for phosphorylated VASP obtained by incubating samples with the active subunit of PKG were virtually identical to those with PKA-phosphorylated VASP. Data are mean ± SE, n is shown for each group. Rates for SNO-actin containing samples were all significantly different from comparable samples containing just G-actin (p<0.05). * p<0.05 for
VASP-containing sample versus other two samples in the same actin solution. † p<0.05 for phospho-VASP versus PBS sample in the same actin solution.

Figure 5. β₂ integrin specific neutrophil adherence in stimulated siRNA-exposed cells. Neutrophil were isolated, exposed to air or HBO₂ for 45 minutes and then incubated for 24 hours with siRNA as described in Methods. Samples labeled ‘control’ in the figure were incubated with control, scrambled sequence siRNA that will not lead to specific degradation of any known cellular mRNA. After 24 hours cells were loaded with calcein AM, and placed on fibrinogen coated plates containing either PBS or PBS with FMLP or 8-br-cGMP to achieve concentrations as shown in Table 2. After 10 minutes, fluid was removed and cell adherence calculated as described in Methods. Data are mean ± SE, n=4 for all studies, *p<0.05 versus air-exposed cells incubated with PBS after the indicated siRNA.

Figure 6. Effects of various siRNA incubations on pyrene actin polymerization in OG permeabilized and stimulated cells. Neutrophil were isolated, exposed to air or HBO₂ for 45 minutes and then incubated for 24 hours with siRNA as described in Methods. Samples labeled ‘control’ in the figure were incubated with control, scrambled sequence siRNA that will not lead to specific degradation of any known cellular mRNA. After 24 hours cells by permeabilization with 0.2% OG incubated with PBS or PBS with FMLP or 8-br-cGMP to achieve concentrations as shown in Table 4 and actin polymerization was monitored for 5 minutes after adding 1 µM pyrene-labeled rabbit skeletal muscle actin. Data are expressed as slopes, fluorescence increase per minute x 100. Values are mean ± SE, n =4 for all studies. *p<0.05 versus air-exposed cells incubated with PBS after the indicated siRNA.
Table 1. β2 integrin specific neutrophil adherence (%).

| Inhibitor/Modification | AIR         | HBO2        |
|------------------------|-------------|-------------|
| None (PBS control)     | 20.8 ± 1.5 (39) | 0.6 ± 0.2 (39) * |
| 0.1 µM L-NAME          | 21.1 ± 1.8 (5)    | 23.1 ± 2.2 (5)    |
| 0.1 µM 1400 W          | 19.2 ± 1.8 (3)    | 19.8 ± 1.7 (3)    |
| 100 nM FMLP            | 18.1 ± 2.5 (5)    | 18.6 ± 2.4 (5)    |
| 100 µM 8-br-cGMP       | 21.1 ± 2.2 (5)    | 19.0 ± 2.1 (5)    |
| 5 min UV               | 23.9 ± 2.7 (8)    | 26.2 ± 2.2 (8)    |
| MPO KO mice            | 22.4 ± 2.0 (5)    | 23.1 ± 2.2 (5)    |
| 50 µM NSC 23766        | 19.9 ± 2.2 (6)    | 20.4 ± 1.6 (6)    |
| 100 µM Rp-8br-PET cGMPS| 21.1 ± 2.2 (5)    | 19.0 ± 2.1 (5)    |
| 1 mM Rp-cAMPS          | 20.1 ± 2.1 (5)    | 19.9 ± 1.1 (5)    |
| 100 nM wortmannin      | 20.2 ± 1.2 (3)    | 0.8 ± 0.4 (3) *   |
| Control siRNA (SC)     | 17.7 ± 1.6 (18)   | 0.1 ± 0.1 (18) *  |
| Control siRNA (TS)     | 18.1 ± 0.9 (3)    | 0.08 ± 0.05 (3) * |
| Rac 1 siRNA (SC)       | 12.9 ± 2.5 (4)    | 12.5 ± 2.5 (4)    |
| Rac 2 siRNA (SC)       | 18.7 ± 1.6 (6)    | 13.4 ± 6.4 (6)    |
| VASP siRNA (SC)        | 16.0 ± 4.1 (4)    | 12.8 ± 3.4 (4)    |
| VASP siRNA (TS)        | 15.8 ± 2.2 (3)    | 15.5 ± 1.9 (3)    |
| PKA siRNA (SC)         | 19.6 ± 1.3 (5)    | 19.4 ± 1.1 (5)    |
| PKG siRNA (SC)         | 16.9 ± 2.7 (4)    | 12.5 ± 1.5 (4)    |

Adherence to fibrinogen-coated plates was measured using neutrophils obtained from wild type or MPO knock-out air-breathing mice. Cell suspensions were exposed for 45 minutes to air or HBO2 and then incubated with chemical agents or exposed to UV light as described in Methods. In studies with small inhibitory RNA (siRNA), neutrophil were isolated from normal mice and incubated with siRNA prior to air or HBO2 exposure as described in Methods. The bracketed letters (SC) indicates sequences obtained from Santa Cruz Biotechnology, (TS) obtained from Thermo Scientific. Samples labeled Control siRNA were incubated with control, scrambled sequence siRNA that will not lead to specific degradation of any known cellular mRNA, others were incubated with siRNA that will cause degradation of mRNA for Rac 1, Rac 2, VASP, PKA or PKG. Data are mean± SE, n=number of studies using neutrophils from different animals, *p<0.05 versus air-exposed control cells.
Table 2. Pyrene actin polymerization in OG permeabilized cells (rate/min X 10^2).

| Inhibitor/Modification          | AIR          | HBO₂        |
|---------------------------------|--------------|-------------|
| None (PBS control)              | 0.80 ± 0.09 (20) | 2.84 ± 0.15 (20) * |
| 0.1 µM L-NAME                   | 0.61 ± 0.08 (6)  | 0.54 ± 0.05 (6)  |
| 0.1 µM 1400 W                   | 0.67 ± 0.05 (5)  | 0.63 ± 0.08 (5)  |
| 100 nM FMLP                     | 1.64 ± 0.28 (10) * | 1.87 ± 0.81 (10) * |
| 100 µM 8-br-cGMP                 | 0.79 ± 0.04 (4)  | 0.78 ± 0.06 (4)  |
| 5 min UV                        | 0.98 ± 0.11 (4)  | 0.78 ± 0.14 (4)  |
| MPO KO mice                     | 0.63 ± 0.18 (3)  | 0.66 ± 0.17 (3)  |
| 50 µM NSC 23766 (Rac inhib)     | 0.66 ± 0.08 (4)  | 0.88 ± 0.08 (4)  |
| Control siRNA                   | 0.61 ± 0.16 (9)  | 2.38 ± 0.16 (9) * |
| Rac 1 siRNA                     | 1.10 ± 0.27 (4)  | 1.13 ± 0.28 (4)  |
| Rac 2 siRNA                     | 0.68 ± 0.20 (4)  | 0.54 ± 0.18 (4)  |
| VASP siRNA                      | 0.57 ± 0.37 (4)  | 0.58 ± 0.36 (4)  |
| PKA siRNA                       | 0.38 ± 0.04 (3) * | 0.38 ± 0.04 (3) * |
| PKG siRNA                       | 0.32 ± 0.04 (3) * | 0.40 ± 0.06 (3) * |

Neutrophil suspensions for studies with chemical agents or UV light were exposed for 45 minutes to either air or HBO₂ followed by permeabilization with 0.2% OG. For siRNA studies, neutrophils were isolated and incubated overnight with the siRNA sequences as described in Methods before air or HBO₂ exposure and then permeabilization with 0.2% OG. Actin polymerization was monitored for 5 minutes after adding 1 µM pyrene-labeled rabbit skeletal muscle actin. Data are expressed as slopes, fluorescence increase per minute x 100. Values are mean ± SE, n=number of studies using neutrophils from different animals, *p<0.05 versus air-exposed control cells.
Table 3. Rac activity.

| Inhibitor/Modification | AIR       | HBO₂      |
|------------------------|-----------|-----------|
| None (PBS control)     | 0.34 ± 0.01 (11) | 0.87 ± 0.05 (11) * |
| 100 µM 8-br-cGMP       | 0.36 ± 0.01 (3)  | 0.38 ± 0.01 (3)  |
| 100 nM FMLP            | 0.47 ± 0.01 (4) * | 0.46 ± 0.01 (4) * |
| 5 min UV               | 0.36 ± 0.01 (3)  | 0.38 ± 0.01 (3)  |
| 1 mM Rp-cAMPS          | 0.34 ± 0.01 (3)  | 0.34 ± 0.01 (3)  |
| (PKA inhibitor)        |           |           |
| 0.1 mM Rp-8br-PET cGMPS (PKG inhibitor) | 0.34 ± 0.01 (3) | 0.35 ± 0.01 (3) |
| MPO KO mice            | 0.34 ± 0.01 (4)  | 0.36 ± 0.01 (4)  |
| Control siRNA          | 0.36 ± 0.01 (5)  | 0.89 ± 0.02 (5) * |
| VASP siRNA             | 0.36 ± 0.01 (7)  | 0.38 ± 0.01 (7)  |
| PKG siRNA              | 0.37 ± 0.01 (3)  | 0.40 ± 0.04 (3)  |
| PKA siRNA              | 0.36 ± 0.01 (3)  | 0.44 ± 0.04 (3)  |

Neutrophil suspensions were exposed for 45 minutes to either air or HBO₂, lysed and Rac activity assayed. Where indicated cells were exposed to FMLP, 8-br-cGMP or UV light prior to lysis, whereas cells were incubated with PKA or PKG inhibitors for 1 hour before exposure to air or HBO₂. Cells were incubated with siRNA sequences overnight prior to air or HBO₂ exposure as described in Methods. KO = studies performed with neutrophils from MPO knock-out mice. Values are mean ± SE, n=number of studies using neutrophils from different animals, *p<0.05 versus air-exposed control cells.
Table 4. Ratio of phosphorylated VASP to total VASP in short F-actin fraction.

| Agent/intervention | AIR, Phospho-Ser 235 | Air, Phospho-Ser 153 | HBO₂, Phospho-Ser 235 | HBO₂, Phospho-Ser 153 |
|--------------------|----------------------|----------------------|----------------------|----------------------|
| PBS                | 1.00 ± 0.00          | 1.00 ± 0.00          | 1.35 ± 0.25          | 1.08 ± 0.14          |
| 100 nM FMLP        | 1.35 ± 0.26          | 1.58 ± 0.10 *       | 1.12 ± 0.07          | 2.22 ± 0.33 *       |
| 100 µM 8-br-cGMP   | 0.98 ± 0.06          | 1.70 ± 0.33 *       | 1.06 ± 0.04          | 1.92 ± 0.25 *       |
| 5 min UV           | 1.01 ± 0.03          | 1.20 ± 0.10          | 1.05 ± 0.03          | 1.09 ± 0.26          |

Neutrophil were isolated and exposed to air (control) or HBO₂ for 45 minutes followed by incubation with PBS or PBS containing 100 nM FMLP, 100 µM 8-br-cGMP or cells were exposed to UV light. Cells were fractioned based on Triton solubility (see Methods) and data show results for the short F-actin fraction. Western blotting was performed with phospho-specific VASP antibodies or an antibody that recognizes all forms of VASP. The ratio of each protein relative to total VASP was calculated and then normalized to the ratio calculated for the air-exposed, control neutrophils incubated with PBS in each experiment. Therefore, data show the fold-increase in band density normalized to the ratio observed in control cells for the short F-actin fraction. Sample size for each group was 3 to 5 replicates, data are mean ± SE, *p<0.05 versus air-exposed cells incubated with PBS.
Table 5. Actin and VASP in centrifuged pellets after *ex vivo* incubations using filamentous actin.

|                      | G-actin | SNO-actin |
|----------------------|---------|-----------|
|                      | + VASP  | + Phospho-VASP | + VASP | + Phospho-VASP |
| Actin band density   | 1.00 ± 0.00 | 0.33 ± 0.05 * | 1.75 ± 0.15 * | 0.67 ± 0.16 |
| VASP/actin ratio     | 0.87 ± 0.02 | 0.26 ± 0.05 | 0.84 ± 0.05 | 0.24 ± 0.05 |

G-actin or SNO-actin suspensions were polymerized as described in Methods and then incubated with either VASP or VASP that had been phosphorylated with the active subunit of PKA. After centrifugation at 12,000 x g the pellets were subjected to Western blot. Actin band density data were normalized to the actin band density of G-actin samples incubated with VASP; the * symbol indicates that a value is significantly different from the G-actin + VASP sample. The VASP/actin ratios were significantly different between suspensions incubated with phospho-VASP versus VASP within the G-actin and SNO-actin groups. The VASP/actin ratios were not different between the G-actin and SNO-actin groups for VASP or phospho-VASP samples. Data are mean ±SE, n=4 in all groups.
Figure 1.

Air

HBO₂

Phalloidan   VASP   MERGE

Air

HBO₂

Phalloidan   Rac 1   MERGE

Air

HBO₂

Phalloidan   Rac 2   MERGE

Air

HBO₂

Phalloidan   PKA   MERGE

Air

HBO₂

Phalloidan   PKG   MERGE

Air

HBO₂

HBO₂ VASP-actin

PBS 8br-cGMP fMLP UV

HBO₂ Rac 1-actin

PBS 8br-cGMP fMLP UV

HBO₂ Rac 2-actin

PBS 8br-cGMP fMLP UV

HBO₂ PKA-actin

PBS 8br-cGMP fMLP UV

HBO₂ PKG-actin

PBS 8br-cGMP fMLP UV
Figure 2.
Figure 3.

Band density ratio versus Air (control) + control siRNA

AIR (control) HBO

Cont. (11) Cont. (11) PKA (4) PKG (4) VASP (3) PKA (4) PKG (4) VASP (3)

siRNA type

VASP-actin

0.0 0.5 1.0 1.5 2.0

Rac 1-actin

0.0 0.5 1.0 1.5 2.0 2.5

Rac 2-actin

0.0 0.5 1.0 1.5 2.0 2.5

PKG-actin

0.0 0.5 1.0 1.5 2.0 2.5

PKA-actin

0.0 0.5 1.0 1.5 2.0 2.5

siRNA type

VASP

actin

54 kDa

43 kDa

Rac 1

22 kDa

Rac 2

25 kDa

PKG

75 kDa

PKA

40 kDa

AIR (control) HBO
Figure 4.

![Graph showing Pyrene-actin fluorescence](image-url)

- **Actin**
  - PBS (4)
  - +VASP (8)
  - +PO (4)

- **SNO-actin**
  - PBS (4)
  - +VASP (8)
  - +PO (4)

Fluorescence is measured in arbitrary units (a.u.) per minute (min). The data is represented as mean ± standard error of the mean (SEM).

* denotes statistical significance (p < 0.05) compared to control.
† denotes statistical significance (p < 0.05) between treatment groups.
Figure 5.
Figure 6.

- **Cont siRNA**
- **PKA siRNA**
- **PKG siRNA**

Actin polymerization rate × 10^2/min

- PBS
- FMLP
- cGMP

AIR (control) HBO₂

* denotes significant difference.
Neutrophil β2 integrin inhibition by enhanced interactions of vasodilator-stimulated phosphoprotein with S-nitrosylated actin
Stephen R. Thom, Veena M. Bhopale, Ming Yang, Marina Bogush, Shaohui Huang and Tatyana N. Milovanova

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