NITRIC OXIDE SYNTHASE-2 LINKAGE TO FOCAL ADHESION KINASE IN NEUTROPHILS INFLUENCES ENZYME ACTIVITY AND β₂ INTEGRIN FUNCTION

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Background: Reactive nitrogen species increase by an unclear mechanism with exposure to hyperbaric oxygen, which inhibits neutrophil β₂ integrin adherence.

Results: Nitric oxide synthase-2 activity increases when held in proximity to short-filamentous actin by focal adhesion kinase.

Conclusions: Protein associations are transient because of actin S-nitrosylation.

Significance: Nitric oxide synthase regulation in neutrophils depends on cytoskeletal protein associations.

This investigation was to elucidate the basis for augmentation of nitric oxide synthesis in neutrophils exposed to hyperbaric oxygen. Hyperoxia increases synthesis of reactive species leading to S-nitrosylation of β₂ actin which causes temporary inhibition of β₂ integrin adherence. Impaired β₂ integrin function and actin S-nitrosylation do not occur in neutrophils from mice lacking type-2 nitric oxide synthase (iNOS) or when incubated with 1400W, an iNOS inhibitor. Similarly, effects of hyperoxia were abrogated in cells depleted of focal adhesion kinase (FAK) by treatment with small inhibitory RNA and those exposed to a specific FAK inhibitor concurrent with hyperoxia. Nitric oxide production doubles within 10 minutes exposure to hyperoxia but declines to approximately half maximum production over an additional 10 minutes. Elevated nitric oxide production did not occur after FAK depletion or inhibition, or when filamentous actin formation was inhibited by cytochalasin D. Intracellular content of iNOS triples over the course of a 45 minute exposure to hyperoxia and iNOS dimers increase in a commensurate fashion. Confocal microscopy and immunoprecipitation demonstrated that co-localization/linkage of FAK, iNOS and filamentous actin increased within 15 minutes exposure to hyperoxia but then decreased below the control level. Using isolated enzymes in ex vivo preparations an association between iNOS and filamentous actin mediated by FAK could be demonstrated and complex formation was impeded when actin was S-nitrosylated or acetylated. We conclude that iNOS activity is increased by an FAK-mediated association with actin filaments but peak nitric oxide production is transient due to actin S-nitrosylation during exposure to hyperoxia.

Introduction: The goal of this study was to determine the mechanism for augmented nitric oxide (NO) production by neutrophils exposed to hyperbaric oxygen (HBO₂). The motivation...
for this research effort is to understanding how HBO₂ temporarily inhibits neutrophil β₂ integrin adhesion molecules, which participate in regulating neutrophil activation and endothelial adhesion (1). When animals or humans are exposed to HBO₂ at 2.8 to 3.0 atmospheres absolute (ATA), β₂ integrins on circulating neutrophils are temporarily inhibited (2-6). This effect offers a unique therapeutic opportunity because inhibition of neutrophil β₂ integrin adhesion by hyperoxia 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 (2,6-13). Inhibited β₂ integrin adhesion may be the basis for benefits of HBO₂ shown in clinical trials involving coronary artery thrombolytic therapy, balloon angioplasty/stenting (14-17) and the reduction of encephalopathy seen after cardiopulmonary bypass and carbon monoxide poisoning (18,19).

Previous studies have shown that whereas the function of β₂ integrins is temporarily inhibited [in humans the effect resolves within 24 hours (3)] neutrophil antibacterial responses remain intact (20,21). HBO₂ does not reduce neutrophil viability or cause immunocompromise, as functions such as degranulation and oxidative burst in response to chemoattractants are intact (2,3). In fact, HBO₂ causes a degree of cell activation based on elevation of β₂ integrin surface expression and oxidative burst without altering β₂ integrin affinity, as measured by binding beads coated with intercellular adhesion molecule-1 (22).

When neutrophils are exposed to hyperoxia, there is an increased production of reactive species derived from nitric oxide synthase (NOS) and myeloperoxidase (MPO), which cause S-nitrosylation (SNO-) of β actin (22). This increases actin polymerization because vasodilator stimulated protein (VASP) has higher affinity for the S-nitrosylated form of short filamentous actin (sF-actin) (23). 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. HBO₂-exposed cells exhibit greater actin filament formation and turnover which inhibits β₂ integrin clustering (also called avidity) and thus β₂ integrin adhesion (22,23).

Increased actin polymerization with exposure to hyperoxia causes an increase in linkage of focal adhesion kinase (FAK) to sF-actin (24). The scaffold protein FAK coordinates many integrin activities (25-28). Under normal conditions the antioxidant protein, NADPH-dependent thioredoxin reductase-1 (TrxR) is linked to actin filament-bound FAK (24). We found, however, that with exposure to hyperoxia this linkage is transient and FAK dissociates after 10 to 15 minutes. Therefore, denitrosylation of SNO-actin fails to occur spontaneously in HBO₂-exposed neutrophils because when FAK dissociates, the TrxR secondarily dissociates from FAK and thus becomes physically separated from the SNO-actin. If actin turnover is slowed by incubating cells with fMLP or 8-br-cGMP (which trigger phosphorylation of VASP causing its dissociation from sF-actin) then the FAK-TrxR complex with sF-actin is restored and actin S-nitrosylation is reversed (23,24).

Thus, SNO-actin impedes its own eradication because of the hastened actin filament turnover. HBO₂ fails to inhibit β₂ integrin function when cells are incubated in the presence of the non-specific NOS inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME) or with 1400 W (N-3-(aminomethyl) benzyl acetamine), which specifically inhibits NOS-2, inflammatory or iNOS (22). These findings suggest iNOS is the source for reactive nitrogen species that mediate SNO-actin formation. There are alternative sources, however, because neutrophils also contain small amounts of both NOS-1, neuronal or nNOS and NOS-3, endothelial or eNOS (29-31). Moreover, if nitrite were available from any source, myeloperoxidase can generate reactive nitrogen species (32-34).

There are a number of proteins that interact with NOS isoforms and either increase or decrease catalytic activity. All three NOS isoforms exhibit increased activity when associated with
actin filaments; some interactions are direct and some involve intermediate protein linkages (35,36). Specifically with regard to iNOS, activity in various cell types is heightened by F-actin linkage mediated by ezrin-radixin-moesin-binding phosphoprotein (EBP50) or α-actinin 4, and associations with either Rac1 or Rac2 enhance enzyme activity and redistribution to cytoskeletal structures (36).

FAK consists of an amino-terminal FREM (band four point one radixin, ezrin, moesin) homology domain, followed by a tyrosine kinase domain and a carboxyl-terminal focal adhesion targeting (FAT) domain. The FREM homology domain coordinates FAK activation by several growth factors (37,38). Links to the actin cytoskeleton and regulation of cell motility, proliferation and oncogenic transformation involve adaptor proteins such as paxillin or talin that bind integrin cytoplasmic tails and the carboxyl-terminal FAT region of FAK (39-41). There are no reports of a linkage between iNOS and FAK.

The purpose of this investigation was to verify iNOS as the source for reactive nitrogen species and elucidate the mechanism for enhanced enzyme activity in neutrophils exposed to hyperoxia. In the course of these studies, it became clear that iNOS-FAK linkage played a central role in modulating iNOS intracellular localization and activity.

Experimental Procedures:

Materials: Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP) and streptavidin-agarose were purchased from Prozyme (Hayward, CA). HisPur™ cobalt resin was purchased from Thermo Scientific/Pearce Inc. (Rockford, IL). Ultrafree-MC filters, PVDF Immobilon-FL, and ZipTipC18P10 were from Millipore Corp. Antibodies were purchased from the following vendors: anti-biotin and anti-actin (Sigma), anti-FAK (BD Biosciences, San Jose, CA), and anti-TrxR1 (Santa Cruz Biotechnology, Santa Cruz, CA). Small inhibitory RNA (siRNA) sequences were purchased from Santa Cruz Biotechnology. These included a control, scrambled sequence siRNA that will not cause specific degradation of any known cellular mRNA (UUCUCCGAACGUGUCACGU), and FAK siRNA that was a mixture of three sequences, strand A (GCAUCCUGAAAUUCUUUGA), strand B (CCAGUACUAAACAGUGAA) and strand C (CGACCAGGGAUUAUGAGAU).

Animals: Mice (Mus musculus) 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 iNOS knock-out mice was maintained from breading pairs purchased from Jackson Laboratories. 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.

Isolation of Neutrophils and exposure to various agents: Mice were anesthetized and neutrophils isolated from heparinized blood as previously described (22). A concentration of 5 x 10⁵ neutrophils/ml of PBS + 5.5 mM glucose was exposed to either air or 2.0 ATA O2 for 45 minutes [we have shown that ex vivo exposures to from 1 to 2.8 ATA O2 are equivalent to in vivo exposures to 2.8 ATA O2 (22)]. Where indicated, prior to air/HBO2 exposures some cell suspensions were exposed for 20 hours at room temperature to 0.08 nM siRNA following manufacturer’s instructions using control siRNA or siRNA specific for mouse FAK. These procedures are exactly as described in previous studies (23,24). Expressed as the ratio of FAK to actin in cell lysate Western blots, FAK siRNA cause a 52.5 ± 5.3 (SE, n=10) % reduction in FAK content. Note that this is a different paradigm for cell manipulation versus that used in one prior studies (24). In the former studies FAK depletion was performed after neutrophils had been exposed to hyperoxia.

Fibrinogen Coated Plate Adherence: Preparation and use of fibrinogen coated plates to measure β2 integrin specific neutrophil adherence in calcein AM-loaded cells was as previously described (22). Suspensions of
25,000 cells in 100 µl PBS were added to plate wells and at the end of the 10 minute incubation, wells were washed and adherence calculated as in (22).

**Microelectrodes:** Microelectrodes selective for NO were fabricated and mounted in a hyperbaric chamber as described in previous studies (42,43). Two-point calibrations for each electrode were made at 37°C in physiological buffer equilibrated with either 100% N₂ or 1,800 ppm NO (balance N₂). The electrodes were polarized at an oxidation potential of +850 mV relative to an Ag/AgCl reference electrode. Electrochemical oxidation currents were amplified with a sensitive electrometer (Keithley, model 610). The electrometer voltage output was low pass-filtered (analog circuit with 5-Hz cutoff) and digitized (1 sample/sec) by computer. Current sensitivities ranged between 0.5 and 5 pA/µM. Suspensions of 5 x 10⁵ neutrophils/0.5 ml PBS + 5.5 mM glucose in microwell plates containing a small stir bar were exposed to 2.0 ATA O₂ for 45 minutes while NO production was monitored.

**NOS activity assay in permeabilized neutrophils:** Isolated neutrophils were subjected to permeabilization using 0.2% n-octyl-b-glucopyranoside (OG) exactly as described in a previous publication (23). Cells were suspended in PBS (12.5 x 10⁵/ml) with 40 µM N-hydroxy-L-arginine to inhibit arginase. After 10 minutes, 20 mM [³H] L-arginine was added and 200 µl samples without or with 0.1 µM 1400 W were parceled for 10 minute exposures to air (control) or HBO₂. Immediately after the incubations 0.5 ml 1 M trichloroacetic acid was added to quench the reaction. Cells were pelleted by centrifugation at 5000g for 5 minutes, washed three times with ethyl ether and then passed through 2 ml Dowex 50WX8 resin. [³H] L-citrulline was eluted with two 0.5 ml washings with water and then analyzed using a scintillation counter.

**Cell extract preparation and biotin-switch assay:** Isolated neutrophils previously exposed to air (control) or HBO₂ were suspended in 2 ml HEN buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine), sonicated on ice for 30 seconds and then passed through a 28G needle five times. Lysates were centrifuged at 2000 X G for 10 minutes, supernatant recovered and made 0.4 % CHAPS using the 10% stock solution. The biotin-switch assay was carried out following published methods (22).

**Cytoskeletal protein analysis based on Triton solubility:** Neutrophil were processed following our published protocol (22). In brief, cells were suspended cytoskeleton stabilization buffer [CSK, 300 µl] (25 mM HEPES, pH 6.9, 0.2% Triton X-100, 1 M glycerol, 1 mM EGTA, 1 mM PMSF, 1 mM MgCl₂), incubated for 10 minutes at room temperature, then centrifuge at 15,000 X g for 5 minutes to obtain the Triton-insoluble pellets. Supernatant was centrifuged at 366,000 X g for 5 minutes and the supernatant Triton-soluble G-actin set aside. The Triton-soluble F-actin pellet was resuspended in CSK buffer and centrifuged at 300 x g for 10 minutes to remove debris. Where indicated both the Triton-soluble and Triton-insoluble proteins were electrophoresis in gradient 4 - 15 % SDS-PAGE gels and Western blotting (22) or subjected immunoprecipitation. Triton-insoluble proteins were dissolved with SDS buffer heated to 95°C and then electrophoresis followed by Western blotting.

**Immunoprecipitation of protein complexes:** Suspensions of G-actin or short F-actin containing 250 µg protein were pre-cleared and then incubated with 5 µg of antibodies on a shaker overnight at 4°C, then 30 µl 20% (w/v) protein G-Sepharose (pre-blocked with 2 % BSA) was added and incubated for 1.5 hours at 4°C. Samples were wash twice in CSK buffer, pelleted, suspended in 20 µl heated SDS buffer (62.5 mM Tris-HCl, 2 % SDS, 10% glycerol, 20 % β-mercaptoethanol) and incubate at 95°C for 15-20 minutes, electrophoresis and then analyzed by Western blotting.

**NOS Dimer/monomer differences:** Differences in presence of NOS dimers versus monomers were assayed following published methods (44,45). Neutrophils were lysed by suspension in buffer [100 mM NaCl, 40 mM Tris (pH 7.3), 4 mM tetrahydrobiopterin, 3 mM DTT, 2 mM L-
arginine, 0.1 % Triton X-100, 10 % glycerol], subject to freeze-thaw three times and then incubated at 37°C for 30 minutes. After centrifugation at 12,000 x g for 30 minutes at 4°C, samples were loaded on 5% SDS-PAGE gels followed by Western blotting (22). Blots were probed for iNOS and actin.

Confocal microscopy: Isolated neutrophils exposed to air or HBO2 were placed on slides coated with fibrinogen following published methods (22). 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 FAK or iNOS. The next morning slides were rinsed three times with PBS and counterstained with a 1:500 dilution of APC and RPE-conjugated secondary antibodies. Images of neutrophils were acquired using a Zeiss Meta510 confocal microscope equipped with a Plan-Apochromat 63×/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.

Ex vivo FAK-iNOS-F-actin interactions: Solutions of glutathione-S-transferase (GST)-tagged active human FAK (0.28 µM) were prepared from wash solution provided as a component of a GST protein interaction pull-down kit purchased from Pierce Biotechnology (Rockford, IL). His-tagged human kinase domain of FAK (3.6 µM) was prepared in modified C-buffer (40 µg/ 100 µl). For studies involving GST-FAK pull-down, columns were first loaded with 50 µl glutathione-agarose slurry plus 400 µl Pierce Corp. wash solution following the manufacturer instructions, then with GST-FAK (50 µl). Either 10 µl of 10 µM F-actin or G-actin was added to these preparations without or with 10 µl (4 µg) iNOS and incubated for 1 hour at room temperature. Columns were then washed three times with wash solution and proteins eluted with 250 µl manufacturer-supplied elution buffer. Samples were combined with 2 X SDS buffer for electrophoresis in gradient 4 - 15 % SDS-PAGE gels followed by Western blotting. Blots were probed for iNOS, FAK and actin.

For studies with His-FAK, a portion was first acetylated by incubation for 1 hour with 2 mM N-acetyllimidazole followed by incubation with Sephadex G-25, centrifuged and supernatant used in studies. Protein interactions were assessed by incubating 10 µl His-FAK solution with 10 µl iNOS and/or F-actin for 1 hour at room temperature with constant shaking. The mixture was then combined with 100 µl washed cobalt resin following our published method (24). Samples were centrifuged at 700g for 2 minutes, protein was eluted from the resin by incubation with elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole, pH 7.4) for 1 hour at room temperature and then centrifuged for 2 minutes at 700g. Samples were combined with 2 X SDS buffer for electrophoresis in gradient 4 - 15 % SDS-PAGE gels followed by Western blotting. Blots were probed for iNOS, FAK and actin.

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

Results:

Neutrophil β2 integrin dependent adherence: Neutrophil adherence and inhibition of β2 integrin function after cells were exposed to HBO2 is shown in Table 1. As with previous
studies, the effect of HBO\textsubscript{2} was abrogated if cells were incubated with NOS inhibitors L-NAME or 1400 W concurrent with exposure to hyperoxia (24). In this study we also found that cells obtained from iNOS knock-out mice (iNOS-KO) did not exhibit impaired function of $\beta_2$ integrins after exposure to HBO\textsubscript{2}. We conclude, therefore, that iNOS activation is required for the HBO\textsubscript{2}-mediated inhibition.

Interest in the role FAK may play in HBO\textsubscript{2} induced $\beta_2$ integrin inhibition was prompted by immunoprecipitation and imaging studies described below. The effect of HBO\textsubscript{2} was abrogated if neutrophils were depleted of FAK by siRNA treatment prior to hyperoxia, or if cells were incubated with PT 573228, a small chemical inhibitor of FAK kinase activity (46), during exposure to HBO\textsubscript{2}.

**NOS activity:** In a recent study we showed that exposure to hyperoxia for just 10 minutes will reduce $\beta_2$ integrin adherence by 50%, with complete inhibition occurring after 45 minutes exposure (24). Therefore, we were interested in examining production of NO by cells over a 45 minute incubation interval. The concentration of NO in suspensions of neutrophils exposed to HBO\textsubscript{2} was monitored with a NO-specific electrode as described in “Experimental Procedures”. The baseline NO concentration while cells were exposed to ambient air was 47.5 ± 21.9 nM and peak concentration of 97.0 ± 32 nM (p<0.05) occurred after 6.4 ± 0.6 minutes HBO\textsubscript{2} exposure.

The time-course for NO production by neutrophils exposed to hyperoxia is shown in Figure 1. Comparison across multiple experiments was aided by setting the ordinate scale as the relative elevation in NO concentration. Each experiment was normalized to the maximum concentration reached during exposure to HBO\textsubscript{2} and the figure shows mean ± SE for 7 experiments. As shown, the peak NO production was transient and dropped to 50% of the peak level at 20.5 ± 5.3 minutes of exposure.

Studies have shown that protein associations involving FAK are modified when cells are exposed to HBO\textsubscript{2} (24). The effect of hyperoxia on NO production was abrogated if FAK was depleted using siRNA, whereas incubation with control siRNA had no significant effect. After incubation with FAK siRNA the mean change in NO concentration was just 0.1 ± 0.2 % (n=3) over the baseline ambient air value. Similarly, when cells were incubated with the FAK inhibitor, PT 573228, concurrent with exposure to hyperoxia the value at 6.4 minutes was 5.6 ± 6.9% over the baseline (not significant, NS) and mean change in NO concentration across the 45 minute experiments was 7.4 ± 1.9 % (NS).

Incubation with cytochalasin D to inhibit actin filament formation prevents a number of HBO\textsubscript{2}-mediated effects (23,24). If cells were incubated with 2 $\mu$M cytochalasin D concurrent with exposure to HBO\textsubscript{2} the NO production value at 6.4 minutes was only 12.0 ± 4.3% over the baseline and mean change in NO concentration across the 45 minute experiments was only 9.0 ± 1.4 % (p<0.05 versus HBO\textsubscript{2} alone).

Activation of iNOS was corroborated by monitoring 1400W-inhibitable conversion of [\textsuperscript{3}H] L-arginine to [\textsuperscript{3}H] L-citrulline. Permeabilized air-exposed, control neutrophils (2.5 x 10\textsuperscript{5} cells/200 µl) generated 0.44 ± 0.05 (n=4) pmol L-citrulline over a 10 minute incubation period whereas those exposed to HBO\textsubscript{2} generated 4.2 ± 0.13 pmol (n=4, p<0.05).

**Cell iNOS content and dimer formation:** We next compared iNOS content between control and HBO\textsubscript{2}-exposed cells. Western analysis assessing the amount of iNOS in cells normalized to $\beta$-actin after 15 minutes exposure to HBO\textsubscript{2} was 1.33 ± 0.11 (NS, n=6) fold greater than control but after 45 minutes the ratio versus control was 2.46 ± 0.39 fold greater (n=8, p<0.01).

Activation of iNOS involves homodimer formation so we were interested in evaluating iNOS status in cells after exposure to HBO\textsubscript{2}. Compared to air-exposed control cells, the fraction of iNOS dimers to total iNOS present in cells exposed to HBO\textsubscript{2} did not differ. Following 15 minutes exposure to hyperoxia the mean value was 1.00 ± 0.18 (NS, n=6) fold and after 45 minutes of HBO\textsubscript{2} 1.08 ± 0.20 (NS, n=9) fold greater than control. However, due to greater
iNOS content the amount of iNOS dimer assessed as the 260 kDa band density versus actin was $3.98 \pm 0.6$ (p<0.05, n=12) fold greater than control in cells exposed to HBO2 for 45 minutes. The ratio after just 15 minutes exposure to HBO2 was $1.50 \pm 0.29$ (NS, n=9) fold greater than control.

Augmented iNOS protein synthesis by hyperoxia was inhibited when cells were incubated with cytochalasin D or 1400 W concurrent with 45 minutes HBO2. A representative experiment is shown in Figure 2. The iNOS/actin ratio versus control when cells were incubated with cytochalasin D was $1.15 \pm 0.03$ (n=3, NS) and with 1400 W $1.02 \pm 0.05$ (n=3, NS).

**Protein co-localization by confocal microscopy:** Because iNOS activity is augmented by association with F-actin, co-localization in neutrophils exposed to air (control) or 2.0 ATA O2 for 15 or 45 minutes was assessed by confocal microscopy. Our prime interest was associations among iNOS, FAK and F-actin. Figure 3 shows images as well as co-localization quantified by the magnitude of yellow fluorescence in stained cells. Fluorescence was significantly elevated in cells exposed to hyperoxia for 15 minutes but lower than control (air-exposed) cells when incubated for 45 minutes. This finding led us to question whether exposure to FMLP or 8-br-cGMP may reverse alterations in co-localization due to HBO2 because these agents inhibit actin turnover and other aspects of HBO2-mediated inhibition of $\beta_2$ integrin function (22,23). As shown, the elevation at 15 minutes and reduction in co-localization at 45 minutes were abrogated by these agents.

**Immunoprecipitation studies:** Quantitative evaluations of immunoprecipitated proteins were conducted on G and short F-actin fractions of air and HBO2-exposed samples. Using antibodies to iNOS, the amount of actin or FAK relative to the iNOS band density on Western blots of short F-actin fractions is shown in Figures 4A and 4B. Lysates of cells incubated with antibodies to iNOS after exposure to HBO2 for 15 minutes showed an elevated co-precipitation of actin and FAK and significantly less co-precipitation following exposure to HBO2 for 45 minutes compared to control (air-exposed) cells. Reciprocal immunoprecipitation studies examining iNOS precipitation with antibodies to actin or FAK resulted in similar patterns (Figure 4 A and B). These patterns were not found when cells were co-incubated with FMLP or 8-br-cGMP (Supplement – Table 1). That is, the significant elevation in protein associations seen at 15 minutes and reduction at 45 minutes were abrogated by cell activation with FMLP or 8-br-cGMP. We did not find significant differences in co-precipitated proteins when G-actin fractions were used versus the sF-actin fractions (data not shown).

**Actin S-nitrosylation in HBO2-exposed cells:** Prior work has shown that S-nitrosylated actin (SNO-actin) formation is the proximal event leading to HBO2-mediated inhibition of neutrophil $\beta_2$ integrin adherence (22,23). S-nitrosylation of neutrophil proteins was surveyed by the biotin switch assay, which covalently adds a disulfide-linked biotin to the labile S-nitrosylation sites on proteins. Western blots were probed with anti-actin antibodies, and in keeping with previous work, an actin band at 42 kDa was reliably visualized (Figure 5). For serial studies, the magnitude of biotin was normalized to actin loaded onto the gels. As described under “Experimental Procedures,” this required Western blotting using a separate sample from the cell lysate because the biotinylation procedure obscures protein recognition by anti-actin antibodies. As with previous studies, if the biotin switch analysis was performed on cell lysates treated with N-[6-(biotinamido) hexyl]-3’-(2’-pyridyldithio) propionamide or with ascorbate (but not both), with 1mM HgCl2, or if cells were exposed to UV light prior to cell lysis and biotin switch, the bands were not visualized (data not shown).

No elevation in SNO-actin occurred in HBO2-exposed cells from iNOS-KO mice, contrary to effects observed in wild type neutrophils. If cells from wild type mice were incubated with 1400 W or PT 573228 was included with the neutrophil suspension during exposure to hyperoxia, SNO-actin was not elevated. We also
examined SNO-actin formation when cells had been incubated with siRNA. When control siRNA-incubated cells were exposed to hyperoxia the biotin-actin ratio that was 2.2 ± 0.16 (n=4, p<0.05) fold-greater than found in cells exposed to just air, whereas cells incubated with siRNA to FAK then exposed to hyperoxia exhibited a ratio just 0.96 ± 0.05 fold (NS) that of air-exposed cells incubated with control siRNA and cells exposed to only air (no siRNA treatment) had 1.07 ± 0.06 fold biotin-actin ratio (NS).

FAK linkage to iNOS and F-actin ex vivo: The data suggested that FAK serves as a bridge linking iNOS and actin. To further examine this possibility, full-length human FAK was incubated with F-actin and iNOS followed by immunoprecipitation using anti-actin antibodies. Figure 6 (lane 1) shows all three proteins were precipitated, when FAK and F-actin are present both precipitate (lane 2), but if iNOS was incubated with F-actin no iNOS was precipitated and only the actin band appears on the Western blot (lane 3).

As an alternative approach glutathione transferase-tagged (GST-) FAK was incubated with purified iNOS and F-actin. As shown in Figure 7, resin linked GST-FAK could bind F-actin (lane 4) but if the F-actin had been previously incubated with SNAP to cause S-nitrosylation actin was not linked (lane 5). Lane 6 shows that GST-FAK can bind with both iNOS and F-actin. These studies were performed in triplicate and the same results always occurred.

An alternative set of experiments was performed using actin that was S-nitrosylated before filament formation, rather than after F-actin formation as in Figure 7. This allowed us to vary the amount of SNO-actin in filaments. Figure 8 shows linkage between FAK and F-actin (lane 5) and a marked reduction when GST-tagged FAK was incubated with F-actin made using mixtures of 25 or 50 % S-nitrosylated actin. In replicate experiments using 50 % S-nitrosylated actin, linkage to GST-FAK was reduced to 35 ± 9 (n=4, p<0.05) % of that resulting with F-actin containing no SNO-actin. Finally, we found that if GST-FAK was first incubated with 5 µM PT573228 no F-actin linkage occurred (lane 8).

To further explore linkages involving FAK and assess whether inhibition could be achieved with an alternative protein manipulation versus S-nitrosylation, we carried out a series of studies with a histidine-tagged (His-) fragment of human FAK that includes the kinase domain but not amino- or carboxyl-terminal segments (amino acids 393-698). The efficiency for pulling down iNOS and F-actin with this fragment was comparable to full length FAK. This comparison was achieved by taking the ratio of band densities for iNOS or actin in pull down studies and comparing them with band densities when just iNOS or actin was directly placed on the SDS gel at the same concentration as used in the pull-down samples. For example, in experiments using GST-FAK the actin band was 89.1 ± 5.9 (n=4) % as dense as with using only the actin solution and with His-FAK studies the actin band density was 95.0 ± 4.5% (NS versus GST-FAK studies).

There are 16 lysine residues rather evenly distributed in the His-FAK peptide and studies were conducted with un-manipulated protein and also with His-FAK that was first acetylated (see “Experimental Procedures”). Cobalt resin pull-down of His-FAK co-precipitated only 0.49 ± 0.2 % (n=3, p<0.001) as much iNOS and 26.1 ± 7.5 % (n=3, p<0.05) as much F-actin as did un-manipulated His-FAK. We conclude that associations involving iNOS and F-actin occur within the kinase domain of FAK and they can be abrogated by acetylating FAK.

Discussion:

Results from this study provide an explanation for why NO production is increased in HBO2-exposed neutrophils. There is strong precedence for cytoskeletal associations enhancing NOS activity (35,36). The novel aspect to neutrophil dynamics in response to hyperoxia is that the cytoskeletal linkage is mediated by FAK. Moreover, inhibition by cytochalasin D indicates that actin polymerization is also required for iNOS dimer formation and NOS activation. This adds further insight into the mechanism for
neutrophil β2 integrin inhibition by HBO2. The steps in the process based on current and previously published findings are as follows (22-24): (1) S-nitrosylated actin is formed when reactive species are generated by iNOS and MPO; (2) VASP exhibits enhanced affinity for S-nitrosylated sF-actin which (3) increases actin free barbed end formation because of subsequent cyclic AMP-dependent and cyclic GMP-dependent protein kinase mediated Rac 1 and 2 activation; (4) increased actin polymerization and filament turnover increases linkage of FAK to the short F-actin fraction; (5) iNOS becomes associated with F-actin via FAK and as dimers form it exhibits increasing activation; (6) enhanced actin turnover inhibits β2 integrin clustering and thus β2 integrin adhesion; but (7) S-nitrosylation of actin causes FAK to dissociate for sF-actin, decreasing iNOS activity; (8) thioredoxin reductase (TrxR) that is also linked to FAK and thus in proximity to short F-actin can affect de-nitrosylation, but when FAK dissociates the TrxR no longer remains linked to FAK and thus does not reduce SNO-actin.

There is a discrepancy in magnitude of iNOS activation during the first ~10 minutes exposure to hyperoxia such that rate of conversion of arginine to citrulline is some five-fold greater than the increase in rate assessed by observations made with the NO electrode (HBO2 doubles NO production based on electrode studies, but L-arginine-to-citrulline formation is increased almost 10-fold). A portion of this difference may arise because the electrode response is rapid whereas the imaging and biochemical investigations require cells to be decompressed from the hyperbaric chamber. Hence, chemical interactions will progress before the cell samples are fixed or processed. It is also likely that once NO forms, it undergoes a variety of reactions that will reduce the amount detected using the electrode.

An additional response to HBO2 is an elevation in iNOS content. As the response is inhibited when cells are incubated with 1400W or cytochalasin D we conclude that iNOS synthesis is triggered as a consequence of HBO2-induced increased enzyme activity. The mechanism for this response will require additional work. Given the rapidity of the elevation, within 45 minutes while cells are exposed to hyperoxia, we suspect it is due to enhanced translation of pre-formed mRNA or post-translational events such as alterations in protease activity.

The data in this and our prior studies indicate that iNOS activation is among the very first responses to hyperoxia and clearly actin S-nitrosylation drives ongoing events. Mention needs to be made of the time course for events, given that NO production peaks at about 6.5 minutes of HBO2. In this study we settled on examining cytoskeletal associations at 15 and 45 minutes. Preliminary studies were done at times as early as 6.5 minutes of HBO2 exposure, but we found an unacceptable degree of variability in the results. This was especially true with immunoprecipitation experiments that are relatively complex. Possible reasons may include a maturation of protein-protein associations within cells once reactive species are generated and it is also likely there is variation in the time course for responses across the cell populations.

In this study we found that cell activation with FMLP or 8-br-cGMP will abrogate the protein associations triggered by hyperoxia. This is consistent with our previous studies (22-24). Although there is a relatively complex series of biochemical events triggered by exposure to HBO2, the consequences all revolve around the actin cytoskeleton. We have shown that both FMLP and 8-br-cGMP activate cytosolic protein kinases that phosphorylate VASP and thus diminish its association with actin. This secondarily slows Rac activity which restores normal actin turnover, allowing FAK to re-associate with sF-actin. Once FAK re-associated, subsequent TrxR linkage drives SNO-removal which restores cytoskeletal control over β2 integrin function.

Data demonstrate that iNOS does not remain linked to F-actin in cells exposed to hyperoxia for 45 minutes. Peak enzyme activity at ~ 6.5 minutes generates 97.0 nM NO and by ~ 30 minutes of HBO2 the level drops to about 50% of the peak concentration. Thus, at 30 minutes the magnitude of NO production is almost
exactly the same as seen with the air-exposed, control cells. Therefore, although iNOS dimer content remains nearly 4-fold elevated over control, the enzyme no longer is active once dissociated from the actin cytoskeleton.

Findings in this study add to recognized roles for FAK. FAK functions predominantly as a scaffolding protein. In a previous study we found that the FAK kinase domain inhibitor (PT 573228) blocks TrxR linkage to FAK leading us to suggest the kinase domain played a role. We have now found that full length FAK and also a protein fragment that includes the kinase domain but not the amino- or carboxyl-terminal ends will bind iNOS and F-actin. Inhibition of the protein associations by PT 573228 is consistent with the kinase domain playing a role. Moreover, protein modifications via either S-nitrosylation or acetylation abrogate protein-protein associations.

In summary, this study adds to our knowledge of how β2 integrins are regulated in neutrophils. The results demonstrate further complexity to the role cytoskeleton plays in this important cell function. Further work is necessary to better define the mechanisms for protein-protein associations. In addition to a role for the FAK kinase domain, we can also say that the carboxyl-terminal portion of actin is involved. We have previously shown that S-nitrosylation occurs at the four cysteine moieties closest to the carboxyl-terminal end of actin (22).

Clinical utility for HBO2 in a variety of situations where neutrophil interactions mediate tissue injuries is suggested by multiple animal studies and several clinical trials. Obviously, further work is necessary along these lines and better elucidation of mechanisms should assist in this effort. Finally, given that NO plays such a complex role in cytoskeletal regulation and β2 integrin function, it is feasible that some events uncovered using HBO2 are aspects of normal physiology and perhaps, one or more NO-donor drug may function in a similar manner as HBO2.

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**Figure legends:**

**Figure 1.** Elevation in NO concentration of neutrophil suspensions normalized to maximum reached during exposures to 2 ATA O₂ for 45 minutes. Data show mean ± SE (shown in grey) for 7 experiments (n=7).

**Figure 2.** iNOS content in neutrophils. Neutrophil suspensions were exposed to air (control) or 2.0 ATA O₂ for 15 minutes (two samples shown) or 45 minutes. Western blots were probed for iNOS and β actin. The figure shows a representative set of experiments with cells exposed to air or hyperoxia and where indicated, neutrophil suspensions also included 2 µM cytochalasin D or 0.1 µM 1400W.
Figure 3. Protein co-localizations with F-actin in neutrophil images. Neutrophils were exposed to air or HBO2 for 15 or 45 minutes then placed on fibrinogen-coated slides with just PBS or with PBS plus 100 nM FMLP or 100 µM 8-br-cGMP. After a 15 minute incubation cells were fixed, permeabilized and stained as described in Methods. Images are shown for cells exposed to only PBS on slides. There were three groupings of studies: FAK and iNOS, phalloidin and iNOS, phalloidin and FAK. The bar graphs show merged fluorescence intensity which reflects protein co-localizations. These data were obtained with cells from mice in 4 to 8 independent experiments by analyzing from 30 to 65 neutrophils in each trial. Values in bar graphs are mean ± SE, * p<0.001 versus air-exposed cells.

Figure 4. Immunoprecipitation of short F-actin fraction neutrophil lysates. Neutrophils exposed to air (control) or 2.0 ATA O2 for 15 or 45 minutes were lysed, the short F-actin fraction was isolated and subjected to immunoprecipitation using antibodies to iNOS, FAK or actin. Western blots at the top show typical results. In 4 A, the left image shows immunoprecipitation using anti-iNOS and blots were also probed for actin and the right image shows lysates immunoprecipitated with anti-actin and blots were also probed for iNOS. In 4 (B) the left image shows immunoprecipitation using anti-FAK and blots were also probed for iNOS and the right image shows lysates immunoprecipitated with anti-iNOS and blots also probed for FAK. The ratios of band densities are shown and values are mean ± SE for 4 to 8 independent trials, *p<0.05 versus air, ANOVA.

Figure 5. S-nitrosylated β actin in neutrophil lysates. Neutrophil suspensions were exposed to air (control) or to 2.0 ATA O2 for 45 minutes, centrifuged, re-suspended in HEN buffer and processed for biotin-switch assay as described in Methods. Where shown neutrophils from wild type or iNOS KO mice were used. Some samples were concurrently incubated with 0.1 µM 1400 W or 5 µM PF 573228. The blots show a representative experiment, bar graphs show the fold-increase in actin band densities relative to control (air only) neutrophil lysates run on the same blots. Numbers are mean ± SE for 4–8 independent trials (*p<0.05 versus air, ANOVA).

Figure 6. Actin immunoprecipitation showing FAK-iNOS-F actin ex vivo attachment with isolated enzymes. Blot is representative of three independent experiments showing actin immunoprecipitation after (lane 1) co-incubation of purified iNOS with GST-tagged FAK and F-actin, (lane 2) co-incubation of just FAK and actin, or (lane 3) incubation of iNOS and actin as described in Methods.

Figure 7. FAK-iNOS-F actin ex vivo attachment with isolated enzymes. The pattern shown in the figure was identical in four replicate trials. The first three lanes show blots using only the single protein solutions (iNOS, F-actin or FAK). Solutions of GST-tagged FAK combined with either F-actin, F-actin first S-nitrosylated by incubation with SNAP, or F-actin plus iNOS were incubated for 1 hour and then eluted from glutathione-agarose slurry as described in Methods to assess protein-protein associations.

Figure 8. FAK-iNOS-F actin ex vivo attachment with isolated enzymes. The pattern shown in the figure was identical in three replicate trials. The first four lanes show blots using only F-actin, F-actin prepared using 25 or 50 % combinations of G-actin first S-nitrosylated by 1 hour incubations with SNAP, or GST-tagged FAK. The next lanes show eluted proteins from the glutathione-agarose slurry after GST-tagged FAK was incubated for 1 hour with standard F-actin (lane 5), with F-actin made using 25% S-nitrosylated G-actin (lane 6), with F-actin made with 50% S-nitrosylated G-actin (lane 7) or with standard F-actin plus 5 µM PF 573228.
Table 1. β2 integrin specific neutrophil adherence (%) – inhibitor effects.

| Cell type (+ agent)                      | AIR     | HBO2    |
|-----------------------------------------|---------|---------|
| Wild type (WT) PMN                      | 22.1 ± 0.9 | 0.35 ± 0.19 * |
| iNOS KO PMN                             | 19.2 ± 2.1 | 18.5 ± 2.2  |
| WT + 0.1 μM L-NAME                      | 26.5 ± 1.8 | 27.2 ± 4.1  |
| WT + 0.1 μM 1400W                       | 27.7 ± 4.5 | 25.8 ± 4.0  |
| WT PMN + control siRNA                  | 28.8 ± 0.9 | 0.50 ± 0.19 * |
| WT PMN + FAK siRNA                      | 24.2 ± 2.0 | 26.5 ± 1.3  |
| WT PMN + 10 μM PT573228                 | 28.9 ± 1.6 | 22.6 ± 1.6  |

Adherence to fibrinogen-coated plates was measured using neutrophils obtained from wild type or iNOS knock-out air-breathing mice. Cell suspensions were exposed for 45 minutes to air or HBO₂ and then incubated with chemical agents. Where indicated, cells were incubated with control siRNA or siRNA to FAK for 24 hours prior to adherence studies. Data are mean± SE, n=5-15 separate studies using neutrophils from different animals, *p<0.05 versus air-exposed cells incubated with just PBS.
Figure 2

| HBO₂   | AIR | 15 min | 15 min | 45 min | 45 min cytoD | 45 min 1400W |
|--------|-----|--------|--------|---------|--------------|--------------|
| Dimer  |     |        |        |         |              |              |
| Monomer|     |        |        |         |              |              |
| Actin  |     |        |        |         |              |              |

- Dimer: 260 kDa
- Monomer: 130 kDa
- Actin: 43 kDa
Figure 3.

The figure shows the effects of HBO2 on cell fluorescence under different conditions. The bar graphs illustrate the arbitrary fluorescence units for PBS, FMLP, and 8-br-cGMP treatments in AIR and HBO2 environments over 15 min and 45 min. The fluorescence images correspond to control and treated conditions, highlighting the changes in fluorescence intensity with time and treatment.
Figure 4A and B.

Figure 4A

Figure 4B
Figure 5.

[Image of Western blot analysis showing protein bands for anti-biotin and β-actin with different treatments: INOS KO, WT + PBS, WT + 1400W, WT + PT573228. The blot shows bands at 50 kDa, 36 kDa, and 42 kDa. A graph below the image shows the biotin/actin ratio for each condition.]
Figure 6

| MW (kDa) | 130 | 43 |
|----------|-----|----|
| iNOS     | X   | X  |
| FAK      | X   | X  |
| F-actin  | X   | X  | X  |
Figure 7.

Figure 7

| MW (kDa) | iNOS | F-actin | FAK | FAK+ SNO-F-actin | iNOS + FAK+ F-actin |
|----------|------|---------|-----|-----------------|---------------------|
| 130      |      |         |     |                 |                     |
| 43       |      |         |     |                 |                     |
Figure 8

MW (kDa) 130 43

F-actin F-actin F-actin FAK only FAK + F-actin FAK + F-actin FAK + F-actin FAK + F-actin

PT573228 +

SNO-actin

| 0 % | X | X | X |
|-----|---|---|---|
| 25 % | X | X | X |
| 50 % | X | X | X |
Nitric oxide synthase-2 Linkage to Focal Adhesion Kinase in Neutrophils Influences Enzyme Activity and β2 Integrin Function
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