Neutrophils Generate Microparticles During Exposure to Inert Gases Due to Cytoskeletal Oxidative Stress

Stephen R. Thom, Veena M. Bhopale, Ming Yang

Department of Emergency Medicine, University of Maryland, Baltimore, Maryland 21201

Running Title: Inert gas pressure causes microparticle formation by neutrophils

Key Words: singlet oxygen, S-nitrosylation, reactive nitrogen species, decompression, NADPH oxidase, focal adhesion kinase, filamentous actin

Address correspondence to: Stephen R. Thom, M.D., Ph.D., Department of Emergency Medicine, University of Maryland, 655 W. Baltimore St., Bressler Research Building Room 4-013, Baltimore, MD 21201, Telephone: 410-706-8294, Fax: 410-328-8028

E-mail: sthom@smail.umaryland.edu

Background: Microparticles are generated in vivo with exposures to high pressure gases by unclear mechanisms.

Results: High inert gas pressure causes singlet oxygen formation which initiates a cycle of actin S-nitrosylation, nitric oxide synthase-2 and NADPH oxidase activation leading to microparticle formation.

Conclusions: Inert gas-mediated oxidative stress causes microparticle production.

Significance: This mechanism may initiate events leading to decompression sickness.

This investigation was to elucidate the mechanism for microparticle (MPs) formation triggered by exposures to high pressure inert gases. Human neutrophils generate MPs at a threshold of ~ 186 kPa with exposures of 30 minutes or more. Murine cells are similar but MPs production occurs at a slower rate and continues for ~ 4 hours whether cells remain under pressure, or not. Neutrophils exposed to elevated gas but not hydrostatic pressure produce MPs according to the potency series: argon ~ nitrogen > helium. Following a similar pattern, gases activate type-2 nitric oxide synthase (NOS-2) and NADPH oxidase (NOX). MPs production does not occur with neutrophils exposed to a NOX inhibitor (Nox2ds), a NOS-2 inhibitor (1400W), or with cells from mice lacking NOS-2. Reactive species cause S-nitrosylation of cytosolic actin that enhances actin polymerization. Protein crosslinking and immunoprecipitation studies indicate that increased polymerization occurs because of associations involving vasodilator stimulated phosphoprotein, focal adhesion kinase, the H+/K+ ATPase β (flippase), the hematopoietic cell multidrug resistance protein ABC transporter (floppase) and protein disulfide isomerase in proximity to short actin filaments. Using chemical inhibitors or reducing cell concentrations of any of these proteins with small inhibitory RNA abrogates NOS-2 activation, reactive species generation, actin polymerization and MPs production. These effects were also inhibited in cells exposed to ultraviolet light which photo-reverses S-nitrosylated cysteine residues and by co-incubations with the antioxidant ebselen or cytochalasin D. The auto-catalytic cycle of protein activation is initiated by inert gas-mediated singlet O2 production.

Introduction: This investigation was aimed to improve understanding on the pathophysiology for decompression sickness (DCS). DCS is a systemic pathophysiological process that occurs after tissues become supersaturated with nitrogen or some alternative gas used to dilute...
O₂ in breathing mixtures during activities such as deep sea diving, high altitude aviation and space exploration. Circulating microparticles (MPs), membrane encapsulated cell fragments with diameters of 0.1 to 1 µm, are elevated in animals and humans after simulated or bona fide underwater diving (1-5). In a murine model, MPs were shown to initiate a systemic inflammatory process post-decompression that is related to neutrophil activation (6-9). Injuries identified in decompressed animals can be recapitulated by injecting decompression-induced MPs into naïve mice (7-9). Findings from these murine studies and also several trials involving human divers caused us to hypothesize that MPs production may actually occur because of high pressure exposures, rather than being a consequence of decompression per se (4,5).

There are three pathways for MPs generation: oxidative stress, apoptosis and cell activation/Ca²⁺ influx (10-12). It is widely accepted that MPs form when the normal asymmetric distribution of lipids between the inner and outer leaflets of the plasma membrane is lost (13). There are two enzyme classes that actively control plasma membrane lipid localization: the aminophospholipid translocases/‘flippases’, lipid-selective P-type ATPases that catalyze inward movement of aminophospholipids and ‘floppases’, a subgroup of ATP-dependent ABC lipid transporters that catalyze only outward movement of lipids. There are also bidirectional nonspecific ‘scramblases’, calcium-dependent and ATP-independent enzymes that catalyze bidirectional movement of lipids according to their concentration gradients. Proteolysis of the actin cytoskeleton occurs concurrent with changes in the activities of the phospholipid transport enzymes as MPs are generated (13). The precise role for cytoskeletal modifications impacting MPs dynamics is poorly understood, but cytoskeletal instability is known to trigger production (14).

Given the apparent central role of neutrophils in decompression pathophysiology, we chose to investigate MPs production by isolated neutrophils exposed to elevated partial pressures of various gases. Results suggested that cells were responding to an oxidative stress. Inert gases enhance the rate of reactive O₂ species (ROS) production by forming collision complexes (15-18). This occurs because of a transient shift of electron density from the collider molecular orbitals into the π orbitals of O₂ (15,16). These gases can elicit oxidative stress responses in unicellular organisms and enhance O₂ toxicity in whole animals under some conditions (19-23).

We found that human and murine neutrophils exhibited similar responses to high pressure gases, but murine cell MPs production persisted even after decompression. This offered an opportunity for more detailed analysis of mechanisms because post-exposure manipulations could be made and because we have previously found efficient methods to reduce intracellular proteins in murine cells using small inhibitory RNA (24-26).

Recent studies with mice have highlighted a role for inflammatory/inducible or type 2 nitric oxide synthase (NOS-2 or iNOS) in decompression-induced neutrophil activation (3,4,6-9). MPs elevations are lower and there are fewer manifestations of decompression stress in mice injected with a specific iNOS inhibitor (1400W, N-3-(aminomethyl) benzyl acetamine) and in iNOS knock-out (KO) mice (7). There is precedence for inert gases activating NOS isoforms. Helium at 71 kPa causes cardiac preconditioning in rabbits linked to activation of endothelial (type 3) NOS and exposure to 700 kPa activates iNOS in mice (27,28).

Enhanced actin turnover has been shown to cause iNOS activation in neutrophils (26). This is an oxidative stress response triggered when neutrophils are exposed to high O₂ pressures. Increased production of reactive species causes S-nitrosylation of cytosolic actin (SNO-actin) (29). Actin polymerization increases because the elongation factor, vasodilator stimulated phosphoprotein (VASP), has higher affinity for the SNO-actin (24). VASP also appears to bundle Rac 1, Rac 2, cyclic AMP-dependent and cyclic GMP-dependent protein kinases (PKA andPKG) in close proximity to short actin
filaments and subsequent Rac activation increases actin free barbed end formation. Increased actin turnover increases linkage of focal adhesion kinase (FAK) and iNOS activity is increased due to dimer formation by a FAK-mediated association with actin filaments (25).

The purpose of this investigation was to investigate MPs production by neutrophils exposed to high pressures of N₂ or noble gases. It is important in these studies to recognize that there was no elevation in O₂ partial pressure above that due to exposure to air. MPs production by human and murine neutrophils occurs while cells are exposed to elevated pressures of inert gas due to an auto-catalytic cycle of enzyme activation related to generation of reactive species and actin turnover.

Experimental Procedures:

Materials: Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Compressed gases were purchased from Air Products and Chemicals, Inc. (Allentown, PA). NADPH oxidase inhibitory peptide, Nox2ds that selectively inhibits the interaction between Nox2 and p47^{phox}, with the sequence NH-{3}-CSTRVRRQL-{CONH}{2}; and Scrmb-Nox2ds, a control scrambled amino acid peptide with sequence NH-{3}-CLRVTRQSR-{CONH}{2} were purchased from American Peptide Company (Sunnyvale, CA) (30). N-3-(aminomethyl)benzyl acetamine (NSC 23766), a Rac inhibitor; 3,4-Dihydro-6-[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-dimethylamino]-3-oxopropyl]-thio]methyl[thio]propanoic acid (MK571), an inhibitor of the multidrug resistance protein-1 ABC transporter (MRP-1, a ‘floppase’); 2-methyl-8-(phenylmethoxy)imidazo[1,2-alpyridine-3-acetonitrile (SCH28080), an inhibitor of the H⁺, K⁺-ATPase (a ‘floppase’) and quercetin-3-rutinoside, a protein disulfide isomerase (PDI) inhibitor, were purchased from Tocris Bioscience, Elllisville, MO. N-[6-(biotinamido)hexyl]-3’-(2’-pyridyl)dithio propionamide (biotin-HPDP) and streptavidin-agarose were purchased from Thermo/Fisher Scientific. Ultrafree-MC filters, PVDF Immobilon-FL, and ZipTipC_{18}P10 were from Millipore Corp. Antibodies to actin for general Western blotting and immunoprecipitation studies was purchased from Thermo/Fisher Scientific (catalogue # PA1-036) and for iNOS dimer assays from Santa Cruz Biotechnology, Santa Cruz, CA (catalogue # sc-650). Antibodies to biotin (catalogue # B3640) were purchased from Sigma. Anti-VASP (catalogue # 610448), anti-FAK (catalogue # 610087) and anti-PDI (catalogue # 610946) as well as Annexin V – conjugated APC (catalogue # 550474) and Annexin binding buffer solution were purchased from BD Pharmingen, San Jose, 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 (UUCUCCGAACGUGUCAGCU). FAK siRNA is a mixture of three sequences, strand A (GCAUCCUGAAAAUCUUUGA), strand B (CCAGUACUCAACAGUGAA) and strand C (CGACCAGGAUAUGAGAU), VASP siRNA is a mixture of three sequences, strand A (GGGGUGUCAAGUACAAUCA), strand B (CCACUCCCAUCUCCAUA) and strand C (GAGUGAACUGUGAGAAGA), MPRII (here after identified as floppase) siRNA is a pool of 3 different siRNA duplexes, strand A (sense: GGAAGCGACAUCUUUGAGAAtt and antisense: UUCUAAAGAUGUGCUUCC), strand B (sense: CUGAAGGGAUAUCUGAAAtt and antisense: UUUCAGAAUAUCUUCUCAAtt) and strand C (Sense: GCAUGAACUUGCACCUUUt and antisense AAAGGGUCAAGUCAUGCtt). PDI siRNA is a pool of 3 different siRNA duplexes, strand A (sense: GAACGCUAACUGAUUAAtt and antisense: UUGUUACUUGCACCUUCC), strand B (sense: GGAAGCGACAUCUACAGAAtt and antisense: UUUCGUAUCUGCACCUUCCtt) and strand C (sense: GCUACCACUUCGCAUUCAtt and antisense: UGAAUGCGGAUGUGAGCtt). The H⁺/K⁺ ATPase β (here after identified as
flippase) siRNA is a pool of 3 different siRNA duplexes, strand A (sense: CUGUACUACCGAGGUUCUtt and antisense: AGAAACCUGCGUAGUAGCtt), strand B (sense: GACAGCAUCAACUGUACAUtt and antisense: AUGUACAGUUGAUGCUGUCtt) and strand C (sense: CACUAAGGAAGGCCUAUCUtt and antisense: AGAUAGGCCUUCCUUAGUGtt).

Animals: Mice (Mus musculus) were purchased (Jackson Laboratories, Bar Harbor, ME), fed a standard rodent diet and water ad libitum, and housed in the university animal facility. A colony of iNOS knock-out mice was maintained from breeding 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: Heparin-anticoagulated blood (4 ml) was obtained from healthy human volunteers, centrifuged through a two-layer preparation of Histopaque 1077 and 1119 (Sigma) at 400 g for 30 min to isolate neutrophils, and cells were washed in PBS. Murine neutrophils were isolated from heparinized blood of anesthetized mice as previously described (29). Procedures for gas exposures were the same for human and murine cells. A concentration of 9 x 10⁵ neutrophils/ml of PBS + 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose was exposed at room temperature to either air at atmospheric pressure (~ 100 kPa) or air plus partial pressures of He, N₂ or Ar up to 690 kPa following published procedures (29). Where indicated, prior to gas exposures some murine 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 VASP, FAK, PDI, flippase, or floppase. The degree of protein reduction after these treatments was assessed by Western blotting. Expressed as the ratio of protein to actin in siRNA-incubated cell lysate Western blots versus protein concentrations in cells incubated with control siRNA, VASP band density after siRNA incubation was just 12.1 ± 2.5 (SE, n=6) % that of control, after FAK siRNA incubation 6.5 ± 1.4 (SE, n=6), after PDI siRNA 10.0 ± 2.8 (SE, n=6), after flippase siRNA 14.1 ± 1.1 (SE, n=6), and floppase siRNA 11.9 ± 1.6 (SE, n=6). Where indicated, inhibitors were present in cell suspensions during gas exposures as follows: 200 µM MK571 (floppase inhibitor), 200 µM SCH28080 (flippase inhibitor), 20 µM PT 573228 (FAK inhibitor), 50 µM NSC 23766 (Rac inhibitor), 30 µM quercetin-3-rutinoside (PDI inhibitor), 10 µM Nox2ds (NOX inhibitor) or 10 µM of the scrambled sequence control peptide to Nox2ds. In other studies after gas exposures but prior to specific studies cell suspensions were exposed for 5 minutes to UV light from a 200 watt mercury vapor lamp.

Actin polymerization in permeabilized cells: Neutrophils suspensions were permeabilized using 0.2% n-octyl-b-glucopyranoside (OG) then exposed to air or gas pressures as outlined above and actin polymerization assayed exactly as described in (31). 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 MgCl₂, 4% OG, 2 mM phallacidin, 42 nM leupeptin, 10 mM benzamidine and 0.123 mM aprotinin). After the 10 second incubation 3 volumes of Buffer B (1 mM Tris [pH 7.0], 1 mM EGTA, 2 mM MgCl₂, 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 the neutrophil suspension.

NOS activity assay in permeabilized neutrophils: Isolated neutrophils were subjected to permeabilization using 0.2% OG and NOS activity assessed exactly as described in (24). In brief, cells (9 x 10⁵/ml PBS + 5.5 mM glucose) were suspended with 40 µM N-hydroxy-L-arginine to inhibit arginase. After 10 minutes, 20 mM [³H] L-arginine was added without or with 0.1 µM 1400 W and at intervals of time up to 2
hours 0.7 M trichloroacetic acid added to quench the reaction. Samples were washed with ethyl ether, passed through Dowex 50WX8 resin and \([^{3}H] L\)-citrulline measured in the eluate. The same concentration of \([^{3}H] citrulline was generated if up to five-fold greater \([^{3}H] L\)-arginine concentration was used and no citrulline was detected if OG permeabilization was not performed (data not shown).

**NOS-2 Dimer/monomer differences:** Differences in presence of iNOS dimers versus monomers were assayed following published methods (26,32,33). 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 (29). Blots were probed for iNOS and actin.

**NADPH oxidase activity:** \(O_{2}\) utilization was monitored in suspensions containing 3.6 x 10^5 neutrophils in 400 µl PBS + 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose without or with 10 µM Nox2ds with a Model-5300 oxygen electrode assembly from Yellow Springs Instruments Co. (Yellow Springs, OH).

**Reactive species generation:** Neutrophil suspensions were prepared with 10 µM 2,7-dihydrodichlorofluorescein-diacetate DCF-diacetate (DCF-DA) and fluorescence was monitored (492 nm excitation, 530 nm emission) after incubations in air or air + 690 kPa He, N₂ or Ar following procedures described previously (7).

**Singlet \(O_{2}\) detection:** Singlet \(O_{2}\) was detected using the luminescent probe singlet oxygen sensor green (SOSG, Invitrogen Inc., Carlsbad, CA) following methods similar to those described by Gollmer, et al. (34). A solution of 1 mM SOSG was prepared in methanol, 2 µl added to 200 µl neutrophil suspensions that were incubated in the dark at room temperature for 30 minutes to allow uptake of the probe prior to inert gas exposures. After decompression cells were assayed (480 nm excitation, 535 nm emission) at intervals. Where indicated 1 mM ascorbic acid or 2 mM azide was added to suspensions 5 minutes prior to gas pressurization.

**Cytoskeletal protein associations based on Triton solubility:** Neutrophils were suspended in a solution of 0.5 mM dithiobis (succinimidyl propionate) (DTSP) to cross-link sulfhydryl-containing proteins within a proximity of ~ 12 Å following published procedures (29,35). 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 as described in (29).

**Immunoprecipitation of protein complexes:** Suspensions of short F-actin containing 250 µg protein were pre-cleared and then incubated with 5 µg anti-actin 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 processed, electrophoresed and analyzed by Western blotting as described in (29).

**Cell extract preparation and biotin-switch assay:** Isolated neutrophils previously exposed to air (control) or elevated pressures of inert gas were suspended in HEN buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine), lysed and subjected to the biotin-switch assay as previously described (29).

**MPs enumeration by flow cytometry:** Flow cytometry was performed with a 4-color dual laser analog FACSCalibur (Becton Dickinson, San Jose, CA) or an 8-color, triple laser MACSQuant (Miltenyi Biotec Corp., Auburn, CA) using the manufacturers’ acquisition software. At intervals of time following neutrophil incubation in air or high gas pressures cells were fixed using a commercial agent (100 µl/ml Caltag Reagent A fixation medium, Invitrogen Inc., Carlsbad, CA). Samples were centrifuged at 15,000g for 30 min to pellet neutrophils. EDTA was added to the supernatant.
to achieve 0.125 M to minimize MPs aggregation and tubes containing 50 μl of supernatant were mixed with 3 μl Annexin V – FITC in 100 μl of Invitrogen, Inc. Annexin binding buffer solution (1:10 v/v in distilled water). All reagents and solutions used for MPs analysis were sterile and filtered (0.1 μm filter). All tubes were incubated for 30 minutes in the dark prior to analysis. Analysis with both flow cytometer protocols involved establishing true-negative controls by a fluorescence-minus-one analysis. Both forward scatter and sideward scatter were set at logarithmic gain. Micro-beads of various diameters 0.3 μm (Sigma, Inc.), 1.0 μm and 3.0 μm (Spherotech, Inc., Lake Forest, IL) were used for initial settings and before each experiment, as an internal control. Annexin V-positive particles with diameters up to 1 μm were taken as MPs and the absolute number/ml was determined by counting the proportion of beads and the exact volume of solution from which MPs were analyzed.

**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:**

**Microparticle production by human neutrophils:** Isolated human neutrophils from healthy volunteers generate MPs while exposed to elevated partial pressures of N₂. Figure 1 shows the dose-dependency of the process expressed as number of MPs/neutrophil, which rises in a progressive fashion at a threshold pressure of ~ 186 kPa. As shown, air-exposed (control) human neutrophils generated just 0.001 ± 0.001 (SE, n=6) MPs/neutrophil over 4 hours. MPs/neutrophil in suspensions exposed to air + 186, 345 or 690 kPa N₂ were all significantly greater than the control sample when neutrophils were decompressed after 30 minutes gas exposure. Moreover, in suspensions assayed at up to 4 hours after decompression the values were virtually identical to MPs counts taken immediately after the 30 minute gas exposure. If cells were kept under pressure for 4 hours and fixed immediately on decompression the MPs/neutrophil counts were statistically insignificantly different from samples kept under pressure for just 30 minutes (data not shown). We conclude that MPs are generated within 30 minutes during exposures to high gas pressures and production does not continue after decompression.

Neutrophil viability when human cells were first isolated from blood was 90.2 ± 0.5 (n=12) %. Viability decreased slightly with incubation times of 30 minutes or more in air to 85.0 ± 1.1 (n=9) % but this was not significantly different from cells exposed to elevated gas pressures. For example, after 30 minute incubations with air + 690 kPa N₂ viability was 84.2 ± 0.9 (NS, n=7) %. There also was no loss of cells as might arise if gas exposure or decompression caused fragmentation. The suspensions all had 1.8 x 10⁵ neutrophils in 200 μl at the start of the studies. After incubations in ambient air the mean cell count was 1.77 (± 0.02) x 10⁵ and after 30 minute incubations with air + 690 kPa N₂ the cell count was 1.75 (± 0.03, NS) x 10⁵.

Because of information developed in the murine model of DCS we were interested in evaluating whether activities of iNOS and NADPH oxidase (NOX) were required for MPs formation (7,9). Whereas production by human cells exposed to air + 690 kPa N₂ for 30 minutes as shown in Figure 1 was 3.34 ± 0.17 (n=7) MPs/neutrophil, cells exposed to air + 690 kPa N₂ along with 1 mM 1400 W generated just 0.37 ± 0.27 MPs/neutrophil (n=3, NS versus control, p<0.05 versus air + N₂). Similarly, if cells were exposed to air + 690 kPa N₂ along with 10 µM Scrbm-Nox2ds, an inactive control peptide, production was 3.31 ± 0.21 MPs/neutrophil (n = 4, p<0.05 versus control, NS versus N₂ alone) but MPs production by cells exposed to air + 690 kPa N₂ along with 10 µM Nox2ds, a membrane-permeable NOX inhibitor (30), was 0.19 ± 0.19 MPs/neutrophil (n=3, NS versus control, p<0.05 versus N₂ alone). If cells were exposed to air + 690 kPa N₂ along with 1 mM ebselen, a non-specific antioxidant, production was 0.23 ± 0.23 MPs/neutrophil (n=3, NS versus control, p<0.05 versus N₂ alone).
**Microparticle generation by murine cells:** We were interested in evaluating murine neutrophils because of the body of work with mice exposed to decompression stress. We found that 30 minute incubations at high gas pressures were insufficient to trigger MPs production by murine cells, but if cells were exposed to gas pressures for 30 minutes and then monitored after decompression, MPs in the suspensions increased linearly for 4 hours. Data with exposures to air and air + 690 kPa N$_2$ are shown in Figure 2.

We also examined MPs production when cells were left at pressure for 4 hours and fixed immediately upon decompression. Table 1 shows MPs/neutrophil and cell viability for cells following exposures for 30 minutes and 4 hours. MPs production rates per hour were statistically insignificantly different when cells were exposed to an inert gas continuously for 4 hours or to gas pressure for just 30 minutes and then left at ambient pressure in air for the remainder of 4 hours. The data also show there were no significant differences in viability of cells as compared with air/control incubations.

Results of studies performed with cells exposed to He, N$_2$ or Ar at 690 kPa are shown in Table 1. Studies were also performed with cells exposed to 690 kPa hydrostatic pressure (no gas phase) which caused no significant MPs production versus that seen with air/control cells. As shown, He pressure increased MPs production, but not to the same magnitude as N$_2$ or Ar pressure.

There was no loss of cells due to the various incubations. For example, neutrophil cell counts at 4 hours in samples exposed to only air were 97.3 ± 5.1 (n=8) % of the counts at the start of the studies and for samples exposed to 690 kPa N$_2$ the cell counts were 96.2 ± 1.4 (n=4, NS) %. We conclude that as with the human neutrophil studies, the MPs production response was not due to apoptosis or to a physical perturbation such as gas bubble formation and cell lysis related to decompression.

A dose-response for MPs production was identified when murine cells were exposed to a range of N$_2$ pressures (Table 2, first column). Comparable with human neutrophil responses, MPs production was inhibited if cells were incubated with ebselen, 1400W or Nox2ds but not Scrmb-Nox2ds concurrent with gas pressure (see first two columns of Table 3).

An advantage with finding post-decompression MPs production in murine cells was that manipulations could be made after 30 minute gas exposures to further elucidate mechanisms. We found that MPs production was inhibited when cells were exposed to UV light (which photo-reverses S-nitrosylated cysteine residues) for 5 minutes after 30 minute exposures to 690 kPa N$_2$ (Table 3).

As was outlined in the Introduction, oxidative stress will modify neutrophil responses and some are mediated by cytoskeletal modifications. If cells were incubated with cytochalasin D, with small chemical inhibitors to Rac or FAK concurrent with N$_2$ exposure, or if cells were depleted of VASP or FAK by prior incubations with small inhibitory RNA (siRNA), MPs production by N$_2$ exposure was inhibited (Table 3).

Also as described in Introduction, MPs dynamics are controlled in part by enzymes that modify membrane lipids. N$_2$- mediated MPs production was abrogated by chemical inhibitors to the H+, K+-ATPase (flippase) or MRP-1 (floppase) and by depleting cells of these proteins with siRNA. Enhanced MPs production was also inhibited by a chemical inhibitor to PDI and depleting PDI with siRNA. These studies were prompted because N$_2$- mediated MPs production is inhibited by Nox2ds but not Scrmb-Nox2ds. PDI is known to facilitate NOX activation (36).

**NOS activation:** Activity of iNOS was monitored as $[^3]$H citrulline production inhibitable by co-incubation with 1400W in murine cells permeabilized with OG to remove the need for active $[^3]$H arginine transport. Figure 3 shows $[^3]$H citrulline production when $[^3]$H arginine was added to cells that were then
either left exposed to air at ambient pressure (control) or pressurized to 345 or 690 kPa N₂. At the indicated times cells were decompressed, TCA added immediately to stop the reaction and measurements taken (see Methods). Enzyme activity was significantly enhanced by gas pressure and activation with 345 kPa N₂ was roughly half that seen with 690 kPa. The dose-response pattern for iNOS activation after cells were exposed for 30 minutes to various pressures of N₂ is shown in Table 2, second column.

If OG-permeabilized murine neutrophils were first exposed for 30 minutes to air + 690 kPa N₂ and then [³H] arginine was added, the concentration of [³H] citrulline after incubation for 1 hour was 1.76 ± 0.08 (n=24) pmol, statistically insignificantly different from the amount produced if [³H] arginine was first added to cells that were then pressurized with 690 kPa N₂ for 1 hour (Figure 3). Moreover, if neutrophils were exposed for 30 minutes to air + 690 kPa N₂, decompressed and left in air at ambient pressure for the remainder of 4 hours before adding [³H] arginine, the concentration of [³H] citrulline after incubation for an additional 1 hour was 1.79 ± 0.18 (n=8) pmol, also insignificantly different from the 1 hour incubation shown in Figure 3. We conclude, therefore, that iNOS remains activated for at least 4 hours in neutrophils exposed for 30 minutes to 690 kPa N₂.

Enzyme activation with various inert gases followed a potency series similar to MPs production. Cells were exposed to various gases for 30 minutes, then [³H] arginine was added for a 1 hour incubation before quenching the reaction with TCA. The concentration of [³H] citrulline in cells that had been exposed to 690 kPa He was 0.66 ± 0.11 pmol (n=7, p<0.05 versus air/control and 690 kPa N₂). Similar studies using Ar resulted in 1.80 ± 0.21 pmol [³H] citrulline (p<0.05 versus air/control and He; NS versus 690 kPa N₂).

Activation of iNOS occurs with formation of dimers. Total intracellular content of iNOS normalized to actin content was not altered by exposures to inert gases (data not shown).

Analysis of iNOS dimers/actin in cell lysate Western blots exhibited a dose-response and gas potency series similar to the pattern of MPs generation and [³H] citrulline production. Compared to the ratio of iNOS dimers/actin in air-exposed control cell lysates, the ratio in cells studied immediately after 30 minute exposures to air + 690 kPa He exhibited dimer content 1.15 ± 0.01 fold higher (n=5, p<0.05 versus air). Exposures to air + 690 kPa N₂ increased dimers by 2.77 ± 0.22 fold (n=10, p<0.05 versus air and He) whereas exposures to half the pressure, 345 kPa N₂ increased dimers by 1.24 ± 0.06 fold (n=3, p<0.05 versus air and 690 kPa N₂). Cells that had been exposed to air + 690 kPa Ar had increased dimer content of 3.92 ± 0.35 fold (n=5, p<0.05 versus air and He, NS versus 690 kPa N₂).

Using 690 kPa N₂ as the representative inert gas, we next evaluated the impact of a variety of manipulations to determine whether augmentation of iNOS activity in murine neutrophils is inhibited in the same manner as MPs production. These data are shown in Table 3, second column. If cells were co-incubated with ebselen during N₂ exposure, iNOS activity was not enhanced. Importantly, in separate studies we found that ebselen had no inhibitory effect on the activity of purified iNOS and exposure to 690 kPa N₂ did not alter the activity of the purified enzyme (data not shown). Therefore, we conclude that intracellular inert gas-mediated events cause iNOS activation and they were prevented by the antioxidant.

If cells were incubated with the NOX inhibitor Nox2ds during the N₂ exposure, or exposed to UV light immediately following N₂ exposure, augmented iNOS activation was not identified (Table 3). Similarly, if cells were incubated with cytochalasin D, small chemical inhibitors to Rac or FAK concurrent with N₂ exposure, or if cells were depleted of VASP or FAK by siRNA incubations, iNOS activation was no longer increased by N₂ exposure. Enzyme activation was also abrogated by inhibiting or depleting flippase, floppase or PDI.

**NADPH oxidase activation:** The inhibitory effects of Nox2ds on gas pressure-induced MPs
production and iNOS activation strongly implicates a role for NOX. To examine this question more closely, cells were exposed to air + 690 kPa inert gas for 30 minutes, decompressed and then O$_2$ consumption was monitored. Figure 4 shows the effects of air + 690 kPa He, N$_2$ or Ar versus just ambient pressure air exposure and the impact of co-incubation with 10 µM Nox2ds during N$_2$ exposure. Analysis of the linear portion of consumption assays expressed as µmol O$_2$ consumed x 10$^{-3}$/min/1 x 10$^6$ neutrophils, the rate for air-exposed, control cells was 1.37 ± 0.14 (n=11). Hydrostatic pressure had no significant effect; the rate was 1.38 ± 0.15 (n=3). The rate following He exposure was 1.79 ± 0.13 (n=4, p<0.05 versus control). Rates for N$_2$- and Ar- exposed cells were, respectively, 5.00 ± 0.36 (n=10) and 5.83 ± 0.35 (n=5, for both p<0.05 versus air and He, no significant difference between the two gases). Cells exposed to air + 690 kPa N$_2$ concurrent with 10 µM Scrmb-Nox2ds was 4.98 ± 0.24 (n=3, p<0.05 versus air/control, NS versus N$_2$ alone) whereas when cells were co-incubated with air + 690 kPa N$_2$ concurrent 10 µM Nox2ds the rate was 1.30 ± 0.08 (n=3, NS versus air). Just as a dose-response was observed for MPs production and iNOS activation with progressively higher pressures of N$_2$, a comparable pattern was observed for NOX activation (Table 2, third column).

In that OG treatment was not required for O$_2$ consumption measurements, in contrast to iNOS measurements, we could examined whether OG treatment altered inert gas-mediated NOX activation. In four replicate studies with cells exposed to air or air + 690 kPa N$_2$ for 30 minutes, O$_2$ consumption was 98 ± 2% (n=4, NS) of that seen with cells that had not been subjected to OG permeabilization.

Figure 2 demonstrates that MPs production progresses for hours after exposure to high pressure gases. Similarly, iNOS remains active for at least 4 hours following gas exposure. Therefore, we examined NOX activity in cells at intervals of time after they had been exposed to high pressure inert gases for 30 minutes. We found that the enhanced activity persisted for 4 hours. For example, O$_2$ consumption by cells exposed to air + 690 kPa N$_2$ for 30 minutes and then left in air at ambient pressure for the remainder of 4 hours before performing the assay was 3.25 ± 0.24 x 10$^{-3}$/min/1 x 10$^6$ neutrophils (n=6, p<0.05 versus air/control), a decrement of ~35% from consumption by cells studied immediately after decompression.

NOX activity could not be evaluated while cells were under pressure because a single gas pressure exposure destroys the electrode on decompression. Therefore, an alternative approach was taken to further evaluate reactive species production.

**Reactive species generation:** Membrane permeable DCF-DA was added to murine neutrophil suspensions that were immediately pressurized for various times to 690 kPa N$_2$. Figure 5 shows fluorescence measurements on decompression. There was a rapid rise in fluorescence that reached a plateau of 12,126 ± 563 (n=12) fluorescence units at ~ 10 minutes. The N$_2$-mediated enhancement of DCF oxidation was decreased by 90 % if cells were co-incubation of Nox2ds (Table 3).

In comparison to the marked elevation of fluorescence seen with cells exposed to air + 690 kPa N$_2$, if DCF-DA was added to ambient air-exposed, control cell suspensions the fluorescence signal increased linearly but reached a value of only 7.2 ± 0.9 (n=11) fluorescence units at 10 minutes (see Figure 6, significantly different from the response to 690 kPa N$_2$). When DCF-DA was added to cell suspensions that were then pressurized for 10 minutes with 690 kPa He, DCF fluorescence was 3,647 ± 345 (n=3) units (p<0.05 versus air/control and 690 kPa N$_2$). Similar studies using Ar resulted in 14,238 ± 589 (n=3) fluorescence units (p<0.05 versus air/control and He; NS versus 690 kPa N$_2$).

We also found that when fluorescence was monitored for intervals of time after cells had been decompressed there were persistently enhanced rates of DCF fluorescence, albeit at lower magnitudes than during gas exposures (Figure 6). For all three gases, the rates of
increase were significantly greater (p<0.05) than for air-exposed, control cells. Rates for N\textsubscript{2} and Ar were significantly different from He, but not from each other.

Persistent production of reactive species could be demonstrated for hours after decompression. Thus, when cells were exposed to gas pressure for 30 minutes, decompressed and left in air at ambient pressure for 4 hours before adding DCF-DA the same rates of DCF fluorescence elevations were observed as in Figure 6. For example, as shown in Figure 6, cells assayed immediately after exposure to air + 690 kPa N\textsubscript{2} for 30 minutes exhibited a DCF fluorescence rate increase of 3.69 ± 0.18/min whereas those decompressed and left on the bench for the remainder of 4 hours exhibited an increase in fluorescence of 3.87 ± 0.46 fluorescence units/min (n=5, p<0.05 versus air, NS versus cells studied immediately after N\textsubscript{2} exposure).

The plateau in fluorescence beyond ~ 10 minutes incubation while cells were under pressure (see Figure 5) was not related to inadequate DCF-DA concentration because doubling concentration did not cause further change in fluorescence signal (data not shown). Moreover, there was excess non-oxidized DCF available in the air-exposed, control cells. If 0.5 mM H\textsubscript{2}O\textsubscript{2} was added to cells after the 10 minute incubation, fluorescence increased by 72.0 ± 2.2 % (n=4).

Because studies with DCF-DA did not require OG permeabilization we could also examine whether OG treatment altered inert gas-mediated reactive species production. Studies were done with cells subjected to the OG permeabilization procedure compared to those not permeabilized (we will refer to non-permeabilized cells as normal). Expressed as OG/normal cell DCF fluorescence, there were no significant differences in values seen after 10 minute incubations or in the post-decompression increased rates of triplicate trials. For example, the mean values after 10 minute incubations for air-exposed control cells was 108 ± 6%, after exposures to 690 kPa He the value was 98 ± 8%, after 690 kPa N\textsubscript{2} 99 ± 3% and after 690 kPa Ar 102 ± 6%.

We also evaluated the impact of a variety of manipulations on rate of DCF-DA fluorescence using 690 kPa N\textsubscript{2} as the representative inert gas exposure (Table 3). If cells were exposed to UV light immediately following the N\textsubscript{2} exposure enhanced DCF fluorescence was significantly diminished. Similarly, if cells were incubated concurrent with N\textsubscript{2} exposure with 1400 W, ebselen, cytochalasin D, small chemical inhibitors to Rac or FAK, or if cells were depleted of VASP or FAK by siRNA incubations prior to N\textsubscript{2} exposure, N\textsubscript{2}-mediated augmentation of reactive species production was decreased by ~ 90%. Enhanced production was also significantly diminished by inhibiting or depleting flippase, floppase or PDI.

**Actin S-nitrosylation in N\textsubscript{2}-exposed cells:**
Identifying a role for iNOS and NOX with MPs production suggests that reactive species produced by interactions between nitric oxide (NO) and ROS are required. Many of the more highly reactive species can cause protein S-nitrosylation. S-nitrosylation of murine neutrophil proteins was surveyed by the biotin switch assay, which covalently adds a disulfide-linked biotin to the labile S-nitrosylation sites on proteins. These studies were done with cells exposed to ambient air (control) and to air + 690 kPa N\textsubscript{2} for 30 minutes. A Western blot probing for biotin-containing proteins in air/control and N\textsubscript{2}-exposed cells is shown in Figure 7. In separate trials, the prominent band at ~ 42 kDa was cut from nitrocellulose paper, subjected to amino acid sequencing and identified as actin. If the biotin-switch analysis was performed on cell lysates treated with N-[6-(biotinamido) hexyl]-3’-(2’-pyridylidithio) propionamide -biotin or with ascorbate (but not both), or with 1mM HgCl\textsubscript{2}, the bands are not visualized (data not shown).

For serial studies, the magnitude of SNO-actin was evaluated by measuring the biotin band density normalized to blots assessing actin band density. As shown in Table 4, if cells were exposed to UV light prior to cell lysis and biotin-switch, or if during N\textsubscript{2} exposure cells were incubated with 1400W, Nox2ds, or
cytochalasin D no significant elevations of SNO-actin were detected.

**Actin Polymerization in Permeabilized Neutrophils:** The data generated thus far imply that inert gas-mediated MPs generation may involve a complex interaction that includes actin polymerization, given the inhibitory effects of cytochalasin D and perturbations of proteins such as VASP. Therefore, we next examined whether exposure to inert gases altered the dynamics of intracellular actin polymerization assessed as free barbed ends formation (FBEs). This was done with OG-permeabilized murine neutrophils incubated with pyrene-actin immediately following 30 minute incubations in air (control) or air + 690 kPa He, N\textsubscript{2} or Ar. Expressed as fluorescence elevation/minute versus that measured for air-exposed, control cells the values for He-exposed cells were 7.1 ± 1.2 fold higher (n=4, p<0.05 vs control), in N\textsubscript{2}-exposed cells 18.6 ± 2.4 fold higher (n=12, p<0.05 vs control and He) and for Ar-exposed cells 18.3 ± 2.7 fold higher (n=4, p<0.05 vs control and He, NS versus N\textsubscript{2}).

We then evaluated the impact of various manipulations on rate of actin polymerization comparing ambient air- and air + 690 kPa N\textsubscript{2} - exposed cells (Table 3). If cells were incubated with 1400W, ebselen, or Nox2ds during N\textsubscript{2} exposure, or exposed to UV light immediately after the N\textsubscript{2} incubation, actin turnover was restored to the control level. Similarly, if cells were incubated with small chemical inhibitors to Rac or FAK concurrent with N\textsubscript{2} exposure, or if cells were depleted of VASP or FAK by siRNA incubations prior to N\textsubscript{2} exposure, actin turnover was no longer hastened by N\textsubscript{2} exposure. Enhanced turnover was also abrogated by inhibiting or depleting flippase, floppase or PDI.

Data in Figure 2 indicate that MPs production continues for over 4 hours after N\textsubscript{2} exposure. If OG-permeabilized cells were exposed for 30 minutes to air + 690 kPa N\textsubscript{2}, decompressed and incubated in ambient air for the remainder of 4 hours before adding pyrene-actin the rate of fluorescence increase was 7.89 ± 0.66 (n=3) x 10\textsuperscript{3} units/min, comparable to the rate seen immediately after N\textsubscript{2} exposure (Table 3).

**Protein cross-linking and immunoprecipitation:** The small chemical inhibitors and siRNA-mediated protein depletion effects outlined in Table 3, the biotin switch assay that identified S-nitrosylated actin (Figure 7) and alterations in FBEs formation implicate actin and its turnover as essential for MPs production. Therefore, we were interested in evaluating associations of cytoplasmic actin with proteins exhibiting a role in MPs production. After cells were exposed for 30 minutes to either air (control) or air + 690 kPa N\textsubscript{2} they were treated with the membrane-permeable protein cross-linker DTSP and lysed 30 minutes later. Cell lysates were separated into Triton soluble G-, short filamentous (sF-) and Triton insoluble F-actin fractions. Blots were analyzed looking for differences in protein band densities relative to actin and whether changes seen in the N\textsubscript{2}-exposed cells were abrogated by exposure to UV light for 5 minutes before DTSP incubations. A representative Western blot is shown in Figure 8 and quantitative changes are outlined in Table 5. There were marked elevations in protein associations in the sF-actin fraction of N\textsubscript{2}-exposed cells. Rather than elevated ratios, we found reductions in the same protein associations of N\textsubscript{2}-exposed cells in G- and Triton insoluble F-actin fractions and these were, once again, abrogated by exposure to UV light (Table 5).

To further investigate potential protein associations the sF-actin fraction was subjected to immunoprecipitation using anti-actin antibodies. As shown in Table 6, N\textsubscript{2}-exposure triggered several of the same protein associations with cytoplasmic actin that had been identified with DTSP cross-linking.

**Singlet O\textsubscript{2} detection:** The inciting event that initiates oxidative stress associated with inert gas exposures of neutrophils remained unclear. Because collision complexes between inert gases and O\textsubscript{2} can generate singlet O\textsubscript{2} we sought evidence that this may occur in the neutrophil suspensions (15,16). Table 7 shows fluorescence associated with cells loaded with singlet oxygen sensor green (SOSG) prior to gas exposures. These data demonstrate the effects of gas...
pressure for 5 minutes. Exposures for just 5 minutes caused the same magnitude of fluorescence elevation as with cells exposed to gas for 30 minutes. No fluorescence was detected in cell suspensions exposed to air only for up to 30 minutes.

We found a dose-response pattern using a range of N\textsubscript{2} pressures (Table 7). The efficiency of singlet O\textsubscript{2} production by gas collision complexes depends on a number of variables (16). To discern differences in gas potency related more to characteristics such as gas van der Waals volume versus classical views on gas narcotic potency, studies were conducted with several different gases. Fluorescence followed the potency series Ar \(_{\sim}\) N\textsubscript{2} > He and nitrous oxide (N\textsubscript{2}O) and sulfur hexafluoride (SF\textsubscript{6}) generated scant singlet O\textsubscript{2}.

When cell suspensions were exposed to air plus 690 kPa N\textsubscript{2} in the presence of 2 mM azide or 1 mM ascorbate, agents that quench singlet O\textsubscript{2}, fluorescence was inhibited by, respectively, 93.8 \(\pm\) 2.2 % (n=3, p<0.05) and 91.1 \(\pm\) 2.8 % (n=6, p<0.05) \((37,38)\). A comparable magnitude of inhibition occurred with similar studies carried out using Ar (data not shown). Consistent with these inhibitory effects, when azide or ascorbate were added to murine neutrophil suspensions prior to pressurization with air plus 690 kPa N\textsubscript{2} for 30 minutes, MPs production after 4 hours incubation was not significantly different from control, air-only exposed samples. MPs in samples that included ascorbate were only 7.6 \(\pm\) 9.9% (n=3, NS) greater than control and azide-exposed samples 13.7 \(\pm\) 8.8 % (n=3, NS) greater than control. As may be expected, however, if ascorbate was added to suspensions after the 30 minute exposure to 690 kPa N\textsubscript{2} there was no inhibition of MPs production in suspensions incubated for 4 hours (data not shown).

Just as N\textsubscript{2}O and SF\textsubscript{6} generated scant singlet O\textsubscript{2}, these gases did not stimulate MPs production by neutrophils. At 4 hours after cells had been exposed to air + 690 kPa N\textsubscript{2}O for 30 minutes the MPs count was increased by only 0.8 \(\pm\) 2.3 % (n=8, NS) above control. MPs counts after similar experiments using 690 kPa SF\textsubscript{6} yielded values 17.8 \(\pm\) 6.3% (n=8, NS) lower than control, thus no elevations in MPs.

**Discussion:**
Study results demonstrate that MPs generation by human and murine neutrophils occurs during exposures to high pressures of N\textsubscript{2} or noble gases. A surprising difference with murine versus human neutrophils is the progressive generation of MPs over a 4 hour interval whether they remain at pressure beyond 30 minutes, or not. What limits ongoing activity in human cells is not known. This report clearly shows there are many components to MPs production, thus offering numerous opportunities for regulatory control.

MPs production is an oxidative stress response that requires iNOS and NOX activity. The dose-response using N\textsubscript{2} at various pressures is roughly linear for activation of both enzyme complexes (Table 2). The array of inhibitory interventions shown in Table 3 suggests an interdependency among cell processes. For example, exposure to 690 kPa N\textsubscript{2} increases actin polymerization and inhibiting polymerization with cytochalasin D also inhibits enhanced MPs formation, iNOS activation and increased DCF fluorescence that involves NOX activation. This pattern leads us to conclude that perpetuation of MPs production occurs due to an auto-catalytic cycle. Figure 9 illustrates the pathway supported by our observations.

The event that initiates the oxidative stress cycle is shown in the upper left of Figure 9. ROS are generated by inert gas-O\textsubscript{2} collision complexes. Collision-induced enhancement of singlet O\textsubscript{2} production is influenced by molecular size of the collider species and gas polarizability; efficiency follows the series Ar \(_{\sim}\) N\textsubscript{2} > He (15,16). Physical properties of gases used in this study are shown in Table 8. Collider molecules with van der Waals volumes above 35 ml/mol are less efficient, which led to inclusion of studies with SF\textsubscript{6} (39). The process also exhibits strong solvent dependence – anticipated to be greater in anhydrous intracellular spaces, as well as dependence on the mobility of electron density in the collider gas and its orbital overlay with O\textsubscript{2} (markedly different between N\textsubscript{2} and N\textsubscript{2}O) (15).
The role for singlet $O_2$ as the ‘spark’ initiating the cyclic process in Figure 9 is supported by the inhibitory effects of azide and ascorbate on $N_2$-mediated MPs generation.

Others have shown that singlet $O_2$ can cause amino acid oxidation as well as nitration/nitrosylation (with nitrite addition) which could initiate the Figure 9 cycle (40,41). While both azide and ascorbate are efficient singlet $O_2$ scavengers, at the concentrations used here they are poor scavengers for more highly reactive ROS, ‘NO and agents such as peroxynitrite (42,43). This is consistent with our finding that ascorbate was not an effective inhibitor of MPs production if added after murine neutrophils were exposed to high $N_2$ pressure.

The results indicate that neutrophils can be stimulated by compressed gases but not hydrostatic pressure to generate MPs. The potency series, $Ar \simeq N_2 > He$ also follows the well-established Meyer-Overton narcotic series. In this paradigm although $N_2$ is not a noble gas, its place in the narcotic series is based on molecular size and oil-gas partition coefficient. SF$_6$ exhibits a narcotic potency approximately 8.5-fold greater than $N_2$ whereas that for $N_2O$ is 39-fold (44). Our results clearly demonstrate that oxidative stress and MPs production by compressed gases are not due to a narcotic effect.

Figure 9 depicts a process which is centered on actin polymerization (depicted as FBEs in the Figure) similar to one we reported in other studies, where $S$-nitrosylation drives a series of steps leading to excessive actin FBEs turnover (24,29). Exposures to high pressure $N_2$ or noble gases trigger increased production of ‘NO from iNOS and ROS by NOX. Reactive nitrogen species such as NO$_2$ and peroxynitrite are produced which lead to SNO-actin formation, as documented by the biotin switch assay (Figure 7). We have reported that VASP exhibits higher affinity for $S$-nitrosylated short actin filaments which hastens actin polymerization (24,29). VASP also bundles Rac proteins, PKA and PKG in close proximity to short actin filaments, and subsequent Rac activation increases FBE formation. This process leads to increased linkage of FAK to short actin filaments. FAK mediates the association of iNOS with actin filaments and as dimers form, enzyme activity increases (26).

One paradigm for MPs formation involves exposure of phosphatidylserine on the cell surface as a consequence of translocation exerted by activated floppase(s) (45) with inhibition of flippase(s) activity (46) and activation of scramblase by calcium influx (47,48). Our results indicate that inhibition or depletion of flippase or floppase inhibits MPs generation. That is, perturbation of either enzyme impedes the process. Of even greater surprise was finding that inhibiting these enzymes will obstruct FBEs formation and the actin-based cycle involving reactive species production (Table 3). That is why a double-arrowed line was used in Figure 9. How these enzymes influence actin turnover will require additional work.

The data indicate that filamentous actin formation is required for NOX activation, consistent with findings by others (49). The persistence of NOX activity and production of reactive species reflected by DCF fluorescence prompted our investigation of a role for PDI in MPs formation. Data in Table 3 indicate an association between actin turnover (FBEs formation), FAK and reactive species production. How NOX could be activated in this on-going process was obscure. FAK is known to influence NOX activity based on several studies, but a direct linkage between FAK and NOX is tenuous (50-52). There is precedence, however, for cytoskeletal perturbations modifying PDI activity and oxidized PDI enhances NOX activity (53,54). Our data indicate that the NOX enzyme complex is activated, or repeatedly re-activated over the 4 hour interval post-decompression, by the cycle of events as shown in Figure 9. Others have also reported a link between PDI and floppase/flippase triggering phosphatidylserine turnover (55).

Pressurization with air followed by decompression has been shown to cause neutrophil activation assessed as $\beta_2$ integrin
expression and degranulation, but the effect is mild compared to traditional cell activators such as zymosan and phorbol ester (3,56,57).

Similarly, in this study NOX mediated O$_2$ consumption by cells exposed to high pressures of inert gas (Figure 4) was on the order of just 13% that reported with phorbol 12-myristate 13-acetate (57).

Whereas NOX activity is modest due to gas pressure, iNOS activity is quite robust. Moreover, iNOS activity was similar whether murine cells are under gas pressure (Figure 3) or for up to 4 hours after cells were exposed to N$_2$ pressure. This similarity in enzyme activation has implications for evaluating mechanisms of reactive species production in the DCF-DA assay.

The magnitude of DCF fluorescence change while cells were exposed to high pressures of inert gas (Figure 5) was several orders of magnitude higher than the rate of DCF fluorescence elevations seen after decompression (Figure 6). This discrepancy obviously cannot be explained by differences in iNOS activity. NOX activity could not be monitored while cells were pressurized, but a 100-fold higher rate than measured after cells were decompressed is not physiologically feasible. Hence, enhanced production of agents capable of oxidizing DCF while cells were exposed to high pressure inert gases is out of proportion to the rate of primary ROS and NO radical production. Augmentation of superoxide production could occur because of inert gas-O$_2$ collision complex formation in the presence of an electron donor, similar to reactions described in ex vivo studies (18). These so-called three-body reactions have a large rate constant for electron capture compared to that corresponding to O$_2$ itself due to van der Waals molecule formation (15,17).

NOX and iNOS activity and elevated DCF fluorescence in murine neutrophils all persist for 4 hours after 30 minute exposures to high pressures of inert gas. We believe this persistence occurs because of ongoing actin turnover, as shown by the pyrene free barbed end assay. However, this does not explain why measured iNOS activity plateaus at about 1 hour (Figure 3), NOX activity diminishes over ~ 10 minutes (Figure 4) and the post-decompression rate of DCF fluorescence appears to plateau after ~ 10 minutes (Figure 6).

We suspect that limitations in duration of these measurements is related to the dynamic nature of actin turnover. That is, it is likely that at any given time post-decompression only a fraction of the neutrophil population is generating reactive species at a rate that exceeds the antioxidant capacity of neutrophils to eradicate SNO-actin.

Using isolated proteins, an association between iNOS and filamentous actin mediated by FAK can be demonstrated but progressive complex formation is impeded when actin becomes S-nitrosylated (26). Activity of iNOS is increased by an FAK-mediated association with actin filaments in these studies but peak NO production was transient due to actin S-nitrosylation (26). Hence, we hypothesize that once cells have been exposed to N$_2$ or noble gases the cycle as in Figure 9 is perpetuated by vacillating levels SNO-actin and reactive species formation in neighboring cells. The diminution of NOX activity after ~ 10 minutes post-decompression could also be due to actin filament generation which contributes to enzyme complex disassembly (57).

DCF fluorescence is related both to iNOS and to NOX activation based on the various interventions/inhibitors described in Table 3. Results in Figure 5 require some added discussion, however. We believe that the 10 minutes required for fluorescence to reach a plateau when DCF-DA was added to cells occurs because this is the time required for probe uptake to reach a steady state. A similar profile with 10 minute plateau was reported when isolated cardiac myocytes were incubated with DCF-DA (58). In that study saponin permeabilization was shown not to cause DCF leakage from cells because of mitochondrial sequestration. A similar sequestration may explain why OG permeabilization had no significant effect on DCF fluorescence in our study.
The precise role for cytoskeletal modifications impacting MPs dynamics is poorly understood but as was mentioned in the Introduction, cytoskeletal instability is known to trigger platelet MPs production (14). Our data clearly demonstrate the requirement for actin polymerization and, as shown by the cross-linking studies in Figure 8 and Table 4, an array of proteins are in proximity to short actin filaments in response to gas exposures. Immunoprecipitation studies did identify statistically significant associations between short actin filaments, iNOS, FAK and VASP, but not with flippase, floppase and PDI. As associations were apparent based on the DTSP crosslinking studies [indicating that proteins were within a proximity of ~ 12 Å (35)], these interactions are either weak and tenuous or – more likely – there is no direct association of these proteins with actin filaments. The cyclical mechanism proposed in Figure 9 may be still more complex than shown and additional studies are needed. We have reported an association among FAK, actin turnover and thioredoxin reductase (TrxR) and TrxR is reported to enhance PDI activity (25,59). TrxR depletion did not have a consistent effect on inert gas-mediated MPs production, however (data not shown) so its role is not clear and it is possible that TrxR has greater impact on SNO-removal as in prior studies (25).

Identifying SNO-actin in N2-exposed neutrophils raises questions regarding immune responses. S-nitrosylation of multiple actin cysteine residues impairs β2 integrin adherence (24,29). Immune compromise is not known to occur with deep sea diving, leading us to believe that protein modifications differ from those associated with high pressure O2 exposures. This may be reflected by the rather nominal magnitude of NOX activation as discussed above and this issue, too, will require added study.

This study was prompted by observations associating MPs with DCS (3,4,6-9). DCS is assumed to occur because of gas bubbles arising from an excess of inert gas somewhere in the body, but little is known about the properties of gases under high pressure in tissues or about how bubbles develop. Murine studies suggest that MPs are nucleation sites for bubble formation and our new finding that gas pressure activates iNOS lends further support to this view (7). Human neutrophils exposed to ~ 186 kPa or more generate MPs. This is remarkable close to the threshold pressure of 135 kPa where endogenous bubbles are observed in humans after decompression (60). Also consistent with our findings, predictive response curves for DCS in rodents exhibit differences in potencies among the inert gases (Ar > N2 > He) (61).

Human cells produce MPs at a faster rate than murine neutrophils, at least for 30 minute exposures. Just as we find MPs generation rates differ, there are also species differences in propensity for DCS development. This has been suggested to arise because of differences in susceptibility rather than a fundamental difference in the nature of DCS pathophysiology (62). Humans are more sensitive to its development than are mice. The N2 ‘dose’ required to produce 50% incidence of DCS across seven species has been suggested to be related to body weight, with smaller animals better able to eliminate excess inert gas due to faster circulation times and higher metabolism (63). Alternatively, whether differences in DCS susceptibility occur because of MPs production rates is a testable hypothesis for future studies.

MPs are generated by neutrophils at inert gas pressures commonly experienced by human divers; 186 kPa is equivalent to the pressure of 18.6 meters of sea water. Hence, the biochemical responses we identified do have practical relevance. Whether cell types other than neutrophils exhibit similar responses to gas pressure needs to be investigated.

MPs dynamics in humans are influenced by exercise and the O2 partial pressure of the breathing mixture, which adds complexity to the interactions (3,4). While the relationship between MPs and DCS remains obscure, the mechanism described by this work offers an explanation for increases in neutrophil-derived MPs observed in mice and humans exposed to
high pressures (3,4,6). The cascade of inflammatory responses triggered by MPs and activation of iNOS in particular, may also offer some explanation for why onset of DCS symptoms can be delayed for many hours after decompression (7-9,64).

More broadly, there is poor understanding regarding the ability of noble gases to trigger an oxidative stress response that others have shown to improve ischemic tolerance (65,66). Generation of singlet O$_2$ offers a common initiation point for what may be several advanced mechanisms. Our findings also suggest that high pressure inert gases can provide a method for deep tissue singlet O$_2$ production which here-to-fore has only been achieved in superficial tissue cancer therapy using photosensitizers and visible light (67). Further work with this perspective is warranted.

Acknowledgment: Funding for this work was provided by a grant from the Office of Naval Research.

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Figure 1. MPs production by human neutrophils exposed to air (0 additional kPa N\textsubscript{2}) or air + 186, 345 or 690 kPa N\textsubscript{2} for 30 minutes. Data show MPs/neutrophil in suspensions immediately (open circles) after 1.8 x 10^5 cells had been exposed to air + various pressures of N\textsubscript{2} for 30 minutes or after suspensions were left in air at ambient pressure for 4 hours before counting (closed circles). Samples exposed to only ambient air were fixed immediately after suspensions had been prepared (open circle) or after 4 hours incubation (closed circle). Data are mean ± SE, n = 4 – 9 for each value. All values with elevated pressures of N\textsubscript{2} are significantly different from the 0 value (air only with no added pressure). There are no significant differences between samples at each pressure that were processed immediately (open circles) versus those left for 4 hours before MPs were counted (closed circles).

Figure 2. MPs production by murine neutrophils exposed to only air or those first exposed to air + 690 kPa N\textsubscript{2} for 30 minutes. Data show MPs/neutrophil in suspensions assayed at times indicated. All post-N\textsubscript{2} values other than time 0 are significantly different (p<0.05) from air-exposed control samples (open circles). Data are mean ± SE (n=4-12).

Figure 3. Activity of iNOS while murine neutrophils were exposed to air (control), or air plus 345 or 690 kPa N\textsubscript{2}. Data show[^3H] citrulline production as mean ± SE (n=3 for each 15, 30 and 120 minute measurement, n=5 for 60 minute values) when[^3H] arginine was added to 1.8 x 10^5 neutrophils that were then pressurized or left exposed to ambient air. At the indicated times cells were decompressed, TCA added and measurements taken. At each time N\textsubscript{2} values are statistically significantly different from air/control and the 60 and 120 minute 345 kPa N\textsubscript{2} values are statistically significantly different from 690 kPa N\textsubscript{2} values (p<0.05, ANOVA).

Figure 4. O\textsubscript{2} consumption by murine neutrophils after 30 minute exposures to air (control) or air plus 690 kPa He, N\textsubscript{2} or Ar. Data show changes in O\textsubscript{2} partial pressure over 20 minutes in buffer containing 1.8 x 10^5 cells studied immediately after cells had been exposed to the various inert gases as mean ± SE, with individual samples sizes as indicated (n). Also shown is the effect if cells were co-incubated with 10µM Nox-2ds while exposed to air + 690 kPa N\textsubscript{2}. Not shown, 10µM Nox-2ds had a similar effect of eliminating virtually all O\textsubscript{2} consumption whether cells had been exposed to just air, or air plus 690 kPa He or Ar.

Figure 5. Western blot showing biotinylated proteins. Lysates of murine neutrophils exposed only to air or to air + 690 kPa N\textsubscript{2} for 30 minutes were prepared according to the biotin switch assay. Entire gel is shown.

Figure 6. DCF fluorescence in murine neutrophils exposed to 690 kPa N\textsubscript{2}. 10 µM DCF-DA was added to suspensions containing 1.8 x 10^5 cells that were then pressurized to 690 kPa N\textsubscript{2} for the indicated times on the abscissa. Fluorescence was measured immediately after cells had been decompressed. Data are mean ± SE, n= 3 to 12 for each value.

Figure 7. DCF fluorescence in murine neutrophils previously exposed for 30 minutes to air or air plus 690 kPa He, N\textsubscript{2} or Ar. Suspensions containing 1.8 x 10^5 cells plus 10 µM DCF-DA were exposed to the indicated gas for 30 minutes, decompressed and fluorescence monitored for 10 minutes while cells were exposed to air at ambient pressure. Values show fluorescence subtracted from the value found when samples were first decompressed (e.g. as shown in Figure 6, each N\textsubscript{2} value were subtracted by ~12,126). Data are mean ± SE, individual samples sizes as indicated (n).

Figure 8. Protein associations in the Triton-soluble short F-actin fraction. Murine neutrophils were exposed to air (control) or air + 690 kPa N\textsubscript{2} for 30 minutes. Where indicated samples were then exposed to UV light for 5 minutes prior to addition of DTSP to cross-link proteins, then fractioned based on Triton
solubility (see Methods) and subjected to Western blotting. This is a representative blot among five replicate experiments. Data based on the blots are shown in Table 4.

**Figure 9. Schematic of proposed mechanism for gas-mediated MPs generation by neutrophils.** See Discussion for detailed explanation. Abbreviations used in the figure are as follows: O\textsubscript{2} indicates O\textsubscript{2} in air-saturated solutions; ROS, reactive oxygen species; NO\textsubscript{x} reflects higher order reactive nitrogen species such as nitrogen dioxide or peroxynitrite; SNO-actin, S-nitrosylated short filamentous actin; VASP, vasodilator stimulated phosphoprotein; Rac, Rac1 and 2 GTPases, FBEs, actin free barbed ends as reflecting actin turnover; FAK, focal adhesion kinase; PDI, protein disulfide isomerase; NOX, NADPH oxidase. Manipulations used to verify a role for each protein include neutrophils that had been incubated with siRNA to decrease intracellular content of the protein proximal to each arrow, KO mice (iNOS knock-out mice) and the agents in boxes are inhibitors used to impede the indicated step in the cycle (Cyto D indicates cytochalasin D).
Table 1. MPs production by murine neutrophils exposed to pressure:

| Exposure conditions       | MPs count                  | Viability at 4 hours |
|---------------------------|----------------------------|----------------------|
| 30 min Air (24)           | $0.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$/PMN/hr | 85.3 ± 1.9%          |
| 30 min Hydro. Press. (5)  | $0.2 \times 10^{-3} \pm 0.3 \times 10^{-3}$/PMN/hr | 88.3 ± 2.0%          |
| 30 min He (5)             | $1.01 \pm 0.12$/PMN/hr *  | 88.0 ± 0.6%          |
| 30 min N₂ (8)             | $3.84 \pm 0.21$/PMN/hr *† | 85.8 ± 1.7%          |
| 30 min Ar (5)             | $3.47 \pm 0.22$/PMN/hr *† | 82.7 ± 0.7%          |
| 4 hours Air (8)           | 0.0011 ± 0.0007/PMN@4hr   | 85.3 ± 1.9%          |
| 4 hours Hydro. Press (5)  | 0.0008 ± 0.002/PMN@4hr    | 86.7 ± 1.9%          |
| 4 hours He (4)            | $3.9 \pm 0.3$/PMN @ 4 hr *| 86.8 ± 2.0%          |
| 4 hours N₂ (8)            | $15.3 \pm 0.9$/PMN @ 4 hr *† | 85.7 ± 1.8%        |
| 4 hours Ar (4)            | $15.2 \pm 0.9$/PMN @ 4 hr *† | 82.5 ± 1.6%        |

MPs were counted in suspensions of neutrophils after exposures to 690 kPa hydrostatic pressure (Hydro. Press. = cells placed in closed syringes and pressurized with no gas phase), He, N₂ or Ar for 30 minutes or for 4 hours. The upper portion of the table shows production rates for suspensions studied at time intervals up to 4 hours after a 30 minute exposure to pressure and the lower portion of the table shows MPs/neutrophil in suspensions left at pressure continuously for 4 hours and fixed immediately on decompression. Cell viability was assessed as trypan blue exclusion in suspensions at 4 hours after the start of the exposure periods (no significant differences among samples). Data are mean ± SE, n= sample numbers, * = p<0.05 versus Air sample, † = p<0.05 versus He based on ANOVA.
Table 2. Dose responses for murine neutrophils following N\textsubscript{2} exposures.

| Air + kPa N\textsubscript{2} | MPs/PMN/hr | iNOS | NOX            |
|------------------------------|------------|------|----------------|
| 0                           | 0.0003±0.0002 (24) | 0.19 ± 0.02 (21) | 1.37 ± 0.14 (11) |
| 186                         | 0.08 ± 0.02 (4)* | 0.47 ± 0.12 (3)* | 1.95 ± 0.12 (4)* |
| 228                         | 0.72 ± 0.10 (4)* | 0.73 ± 0.13 (7)* | 2.42 ± 0.23 (4)* |
| 345                         | 1.14 ± 0.08 (8)* | 0.83 ± 0.22 (6)* | 3.57 ± 0.14 (4)* |
| 455                         | 2.08 ± 0.41 (4)* | 1.45 ± 0.15 (3)* | 4.09 ± 0.28 (4)* |
| 690                         | 3.84 ± 0.21 (8) *† | 1.76 ± 0.08(24) *† | 5.00 ± 0.36(10) *† |

Data show MPs production as particles/neutrophil/hour by cells first exposed to air + various additional pressure of N\textsubscript{2} for 30 minutes. iNOS indicates pmol of [\textsuperscript{3}H] citrulline produced in 1 hour after cells were exposed to N\textsubscript{2} pressure. NOX indicates rate of O\textsubscript{2} consumption as μmol O\textsubscript{2} x 10\textsuperscript{-3}/min/1 x 10\textsuperscript{6} neutrophils studied after 30 minute exposures to N\textsubscript{2}. Values were taken from the linear portion of consumption assays as shown in Figure 4. *p<0.05 versus 0 kPa N\textsubscript{2} (air-exposed, control) in each column; † indicates significantly different from 186 and 228 kPa values by ANOVA.
Table 3. Impact of various agents on N$_2$-mediated enhancement of microparticle (MPs) production, iNOS activation, DCF-DA fluorescence and free barbed ends (FBEs) formation.

| Agent       | MPs/PMN/hr x 10$^2$ | iNOS (pmol [H$^+$] citrulline/hour) | DCF (fluor @ 10 minutes x 10$^4$) | FBEs (fluor/min) X 10$^3$ |
|-------------|----------------------|------------------------------------|----------------------------------|---------------------------|
|             | Air                  | N$_2$                              | Air                              | N$_2$                      | Air                        | N$_2$                      |
| PBS         | 0.03±0.02 (24)       | 384±21 (8) *                       | 0.19±0.01 (21)                   | 1.76±0.08 (24) *           | 0.72±0.09 (11)             | 1213±56.3 (12) *           |
| KO          | 0.00±0.00 (5)        | 0.00±0.00 (5)                      | 0±0 (8)                          | 0.01±0.03 (5)              | ND                        | ND                        |
| 1400W       | 0.00±0.00 (4)        | 0.13±0.06 (5)                      | 0±0 (8)                          | 0±0 (8)                    | 0.93±0.07 (3)              | 74±13.0 (3) *              |
| Ebselen     | 0.06±0.02 (3)        | 0.06±0.04 (3)                      | 0.20±0.001 (3)                   | 0.14±0.04 (3)              | 0±0.0 (4)                 | 63.0±48.1 (4)              |
| UV          | 0.02±0.003 (4)       | 0.05±0.03 (4)                      | 0.20±0.003 (5)                   | 0.10±0.001 (5)             | 0.95±0.18 (3)              | 100.4±56.5 (3) *           |
| Cyto D      | 0.09±0.003 (3)       | 0.13±0.06 (3)                      | 0.20±0.008 (3)                   | 0.14±0.05 (6)              | 0.92±0.19 (3)              | 141.2±30.3 (3) *           |
| Nox2ds      | 0.04±0.02 (3)        | 0.05±0.03 (3)                      | 0.20±0.001 (3)                   | 0.04±0.01 (4)              | 0.92±0.19 (3)              | 117.0±48.4 (3) *           |
| Scrm-Nox2ds | 0.02±0.003 (3)       | 496±12 (3)*                        | 0.18±0.01 (3)                    | 1.57±0.08 (5) *            | 0.91±0.22 (3)              | 1139±82.8 (4) *            |
| Rac-i       | 0.08±0.08 (3)        | 0.16±0.12 (3)                      | 0.21±0.003 (4)                   | 0.31±0.003 (4)             | 0±0.0 (4)                 | 76.0±76.0 (4)              |
| Cont-si     | 0.03±0.02 (4)        | 405±42 (4) *                       | 0.20±0.001 (4)                   | 1.71±0.04 (4) *            | 0.81±0.19 (4)              | 1214±51.1 (4) *            |
| VASP-si     | 0.09±0.17 (4)        | 0.15±0.07 (4)                      | 0.20±0.001 (4)                   | 0.03±0.001 (4)             | 0.97±0.22 (3)              | 281.1±81.4 (3) *           |
| FAK-i       | 0.04±0.02 (3)        | 0.07±0.03 (3)                      | 0.20±0.001 (3)                   | 0.18±0.14 (3)              | 1.00±0.25 (3)             | 236.8±98.3 (3) *           |
| FAK-si      | 0.05±0.02 (4)        | 0.08±0.04 (3)                      | 0.19±0.002 (4)                   | 0.09±0.002 (4)             | 0.72±0.12 (3)              | 205.1±58.3 (3) *           |
| Flip-i      | 0.07±0.02 (3)        | 0.16±0.12 (3)                      | 0.20±0.001 (4)                   | 0.19±0.14 (4)              | 4.2±4.2 (4)               | 45.8±45.8 (4)              |
| Flip-si     | 0.02±0.02 (4)        | 0.06±0.04 (4)                      | 0.20±0.002 (4)                   | 0.16±0.11 (4)              | 0.98±0.13 (3)              | 222.4±95.9 (3) *           |
| Flop-i      | 0.00±0.01 (3)        | 0.05±0.02 (3)                      | 0.20±0.001 (4)                   | 0.16±0.11 (4)              | 0.0±0.0 (4)                | 12.7±33.9 (4)              |
| Flop-si     | 0.04±0.04 (4)        | 0.05±0.04 (3)                      | 0.20±0.001 (4)                   | 0.32±0.16 (4)              | 0.95±0.10 (3)              | 235.8±97.3 (3) *           |
| PDI-i       | 0.02±0.02 (3)        | 0.01±0.003 (3)                     | 0.19±0.003 (4)                   | 0.24±0.003 (4)             | 0.57±0.22 (3)              | 146.0±55.8 (3) *           |
| PDI-si      | 0.04±0.03 (4)        | 0.06±0.06 (4)                      | 0.20±0.003 (4)                   | 0.12±0.10 (4)              | 0.85±0.14 (4)              | 133.8±17.8 (4) *           |

Isolated murine neutrophils were exposed to air or air + 690 kPa N$_2$. MPs/PMN reflects MPs counts in suspensions first exposed to air versus 690 kPa N$_2$ for 30 minutes and then left in air at ambient pressure for 4 hour incubations. NOS activity reflects pmol [H$^+$] citrulline produced when [H$^+$] arginine was added immediately after 30 minute air/N$_2$ exposures and cells incubated for 1 hour at ambient pressure in air. DCF fluorescence was assessed when DCF-DA was added to cell suspensions that were then exposed to
air or 690 kPa N\textsubscript{2} for 10 minutes. Inhibitor values in the N\textsubscript{2} column are all significantly different from N\textsubscript{2} + PBS (p<0.05). FBEs reflect pyrene actin fluorescence in arbitrary units/min in cells studied immediately after 30 minute air or 690 kPa N\textsubscript{2} exposures. All values are mean ± SE (n=number of independent trials), *p<0.05 versus air value. Abbreviations and manipulations are as follows: PBS, phosphate buffered saline; KO, neutrophils from NOS-2 knock-out mice; 1400W, incubation with 1 mM 1400W during air/N\textsubscript{2} exposure; Ebselen, incubation with 1mM ebselen during the air/N\textsubscript{2} exposure; UV, cells exposed to UV light for 5 minutes after the 30 minutes air/N\textsubscript{2} exposure before readings taken; Cyto D, incubation with 5µM cytochalasin D during the air/N\textsubscript{2} exposure; Nox2ds incubation with 10µM Nox-2ds during the air/N\textsubscript{2} exposure; Scrmb-Nox2ds, incubations performed with 10 µM control, scrambled sequence peptide to Nox-2ds; Rac-i incubation with 50 µM NSC 23766 during the air/N\textsubscript{2} exposure; Cont-si cells incubated with control, scrambled sequence small inhibitory RNA for 24 hours prior to the experiment; VASP-si cells incubated with small inhibitory RNA to vasodilator stimulated phosphoprotein for 24 hours prior to the experiment; FAK-i incubated with 20 µM PT 573228 during the air/N\textsubscript{2} exposure; FAK-si, cells incubated with small inhibitory RNA to focal adhesion kinase for 24 hours prior to the experiment; Flip-i cells incubated with 200 µM SCH 28080 during the air/N\textsubscript{2} exposure; Flip-si cells incubated with small inhibitory RNA to flippase for 24 hours prior to the experiment; Flop-i cells incubated with 200 µM MK 571 during the air/N\textsubscript{2} exposure; Flop-si cells incubated with small inhibitory RNA to floppase for 24 hours prior to the experiment; PDI-i cells incubated with 30 µM quercetin-3-rutinoside during the air/N\textsubscript{2} exposure, PDI-si cells incubated with small inhibitory RNA to protein disulfide isomerase for 24 hours prior to the experiment; ND = no experiments performed with KO mice.
Table 4: SNO actin/β-actin band density ratios from air and 690 kPa N₂ exposed neutrophils.

| Incubation/intervention | AIR       | N₂        |
|-------------------------|-----------|-----------|
| PBS                     | 1.00 ± 0.00 (5) | 2.50 ± 0.34 (5) * |
| UV                      | 1.03 ± 0.11 (3) | 1.07 ± 0.17 (3) |
| Cytochalasin D          | 1.22 ± 0.04 (3) | 1.10 ± 0.05 (3) |
| Nox 2ds                 | 0.99 ± 0.09 (3) | 1.08 ± 0.14 (3) |
| 1400 W                  | 1.04 ± 0.04 (3) | 1.08 ± 0.09 (3) |

Data reflect band densities on Western blots of lysates from cells exposed to air or air + 690 kPa N₂ for 30 minutes that had been subjected to the biotin switch procedure. PBS indicates cells incubated with buffer only during air/N₂ exposure; UV indicates cells exposed to UV light for 5 minutes after air/N₂ exposure; other interventions involved incubation with 5µM cytochalasin D, 1 mM 1400W or 10µM Nox-2ds during air/N₂ exposure. All values are mean ± SE (n=number of independent trials), *p<0.05 versus control.
Table 5. Protein associations assessed by DTSP cross-linking in sF-actin, G-actin and Triton-insoluble actin fractions of neutrophils exposed to air (control) or for 30 minutes to 690 kPa N₂.

|        | VASP/actin | FAK/actin | iNOS/actin | Flip/actin | Flop/actin | PDI/actin |
|--------|------------|-----------|------------|------------|------------|------------|
| **sF-actin** |            |           |            |            |            |            |
| 690 kPa N₂ | 4.68 ± 1.21* | 2.91 ± 0.39* | 2.79 ± 0.29* | 2.48 ± 0.45* | 3.28 ± 0.68* | 4.03 ± 0.43* |
| Air + UV  | 1.10 ± 0.11 | 1.09 ± 0.08 | 1.22 ± 0.08 | 1.02 ± 0.07 | 1.19 ± 0.15 | 1.11 ± 0.04 |
| N₂ +UV    | 0.86 ± 0.11 | 0.96 ± 0.08 | 1.38 ± 0.37 | 1.01 ± 0.15 | 1.17 ± 0.26 | 0.88 ± 0.11 |

Values are ratios calculated based on the band densities of the identified protein relative to the actin band density in each sample. In each table the values are normalized to air-exposed control protein band ratios of individual experiments, thus values greater than 1.0 reflect increases in protein associations with actin, whereas values less than 1.0 indicate lower protein associations. Where indicated cells were exposed to UV light for 5 minutes prior to addition of DTSP to cross-link proteins, then fractioned based on Triton solubility (see Methods). Data are mean ± SE (n=5 for all samples), * reflects values significantly different from normalized air-exposed control values (p<0.05, ANOVA).
Table 6. Immunoprecipitation of short F-actin fraction from lysates of murine neutrophils exposed to 690 kPa N\textsubscript{2} for 30 minutes versus those incubated in air (set as a protein/actin ratio = 1 for each experiment).

|                | VASP/actin  | FAK/actin  | iNOS/actin | Flip/actin | Flop/actin | PDI/actin |
|----------------|-------------|------------|------------|------------|------------|-----------|
| **690 kPa N\textsubscript{2}** | 1.61 ± 0.24* | 2.15 ± 0.20* | 1.57 ± 0.34* | 1.38 ± 0.21 | 1.36 ± 0.41 | 0.99 ± 0.11 |
| **N\textsubscript{2} + UV**   | 0.88 ± 0.20 | 1.11 ± 0.14 | 1.19 ± 0.24 | 0.85 ± 0.20 | 1.49 ± 0.43 | 0.84 ± 0.09 |
| **Control/UV**   | 1.17 ± 0.17 | 1.21 ± 0.19 | 1.13 ± 0.27 | 1.02 ± 0.11 | 0.96 ± 0.24 | 1.26 ± 0.11 |

The short F-actin fraction was isolated from neutrophils and subjected to immunoprecipitation using antibodies to actin. Where indicated cells were exposed to UV light for 5 minutes after air/N\textsubscript{2} incubation and prior to lysis. Western blots were carried out and data expressed as the ratio of protein band densities versus actin band density for each lysate normalized to the ratio found with air-exposed (control) cells in each experiment, thus values are normalized to 1.0 for air-exposed cells without UV exposure. Data are mean ± SE (n=6), *p<0.05 ANOVA.
Table 7. Singlet oxygen sensor green (SOSG) fluorescence in neutrophil suspensions after gas exposures.

|                | Air  | Air +            |
|----------------|------|------------------|
|                | 0 ± 0 (26) | 441 ± 5 (3)* | 4101 ± 260 (3)* |
|                | 186 kPa N₂ | 345 kPa N₂ | 690 kPa N₂ |
|                | 690 kPa Ar | 690 kPa He | 690 kPa SF₆ |
|                | 690 kPa N₂O |        |             |
| Cells were incubated with SOSG for 30 minutes and then exposed to air + indicated gas for 5 minutes. Data show fluorescence (arbitrary units) after gas exposure. Data are mean ± SE, sample sizes are as shown. *p<0.05 versus Air (ANOVA); 690 kPa N₂ and Ar values are significantly different from 690 kPa He, SF₆ and N₂O; and all N₂ values are significantly different from each other (ANOVA).
Table 8. Physical properties of gases used in this study.

| Gas   | MW (g/mol) | Van der Waals volume (ml/mol) | Polarizability ($10^{-24}$ cm$^3$) | Oil:gas partition coefficient |
|-------|------------|-------------------------------|---------------------------------|-------------------------------|
| He    | 4          | 11.9                          | 0.21                            | 0.016                         |
| N$_2$ | 28         | 15.8                          | 1.74                            | 0.069                         |
| Ar    | 40         | 16.8                          | 1.64                            | 0.13                          |
| N$_2$O| 44         | 18.9                          | 3.03                            | 1.4                           |
| SF$_6$| 146        | 46.8                          | 6.54                            | 0.293                         |

Data are from (39,68,69), MW = molecular weight.
Figure 1

- Immediate processing
- 4 hour post-decompression

MPs/PMN

Additional kPa N₂ for 30 min
Figure 2

![Graph showing the relationship between hours and MPs/PMN over time.](image)

- **Post – 30 min**
- **690 kPa N₂**
- **Air exposure only**

**Y-axis:** MPs/PMN
**X-axis:** Hours (0 to 6)

**Key Points:**
- Initial value at 0 hours.
- Linear increase up to 2 hours.
- Plateau from 2 to 4 hours.
- Slight increase from 4 to 6 hours.
Figure 4

- Air (9)
- Nox 2ds + 690 kPa N₂ (3)
- 690 kPa He (4)
- 690 kPa N₂ (9)
- 690 kPa Ar (5)

mmHg O₂ vs. Minutes
Figure 5

![Graph showing DCF fluorescence (arbitrary units) over time (minutes).](image)

**DCF fluorescence (arbitrary units)**

**Minutes**
Figure 6

![Image of a graph showing DCF fluorescence (arbitrary units) versus minutes for different gases: Air (14), He (6), Ar (6), N₂ (14). The graph indicates a linear increase in fluorescence over time for each gas type.]
Figure 8

|    | Air | N<sub>2</sub> | Air-UV | N<sub>2</sub>-UV |
|----|-----|--------------|--------|-----------------|
| 190 kDa | ![Image] | ![Image] | ![Image] | ![Image] |
| 130 kDa | ![Image] | ![Image] | ![Image] | ![Image] |
| 110 kDa | ![Image] | ![Image] | ![Image] | ![Image] |
| 55 kDa  | ![Image] | ![Image] | ![Image] | ![Image] |
| 46 kDa  | ![Image] | ![Image] | ![Image] | ![Image] |
| 33 kDa  | ![Image] | ![Image] | ![Image] | ![Image] |
| 43 kDa  | ![Image] | ![Image] | ![Image] | ![Image] |

**Proteins:**
- Floppase
- iNOS
- FAK
- PDI
- VASP
- Flippase
- Actin

**Conditions:**
- Air
- N<sub>2</sub>
- Air-UV
- N<sub>2</sub>-UV
Figure 9

Inert gases + O₂ → ROS

**NO** → iNOS → NOx

- Ebselen
- KO mice 1400W
- siRNA
- PT573228
- Cytochalasin D

**NO** → Nox → Floppase/Flippase

- siRNA Quercetin-3-rutinoside
- MK571
- SCH28080
- siRNA

**NO** → FBEs

- Cyto D

**NO** → VASP/Rac

MPs Production

Production

KO mice

NSC23766 siRNA

Cyto D

UV
Neutrophils Generate Microparticles During Exposure to Inert Gases Due to Cytoskeletal Oxidative Stress
Stephen R. Thom, Veena M. Bhopale and Ming Yang

J. Biol. Chem. published online May 27, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M113.543702

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