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# Hyperbaric oxygen treatment induces platelet aggregation and protein release, without altering expression of activation molecules

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## Abstract

**Objectives:** To investigate the effect of hyperbaric oxygen (HBO) on platelet physiology.

**Design and methods:** Human platelets were exposed to HBO (97.7% $O_2$, balance $CO_2$ at 2.2 ata) or control (CON; 5% $CO_2$, balance air at 1 ata) for 90 min, and analyzed for aggregation, protein release, $^{\cdot}NO$ production, and activation.

**Results:** HBO induced 29.8±3.0% of platelets to aggregate compared with CON (5.5±0.9%). Proteins observed to be released in greater abundance from HBO- compared with CON-treated platelets included 14-3-3 zeta and α-2-macroglobulin. Release of $^{\cdot}NO$ by platelets was unaffected following exposure to HBO, as was platelet activation as measured by surface expression of PECAM-1, CD62P and the activated form of $\alpha_{IIB}\beta_{IIIa}$.

**Conclusions:** Exposure to HBO induces both platelet aggregation and protein release. Further study will better define the precise mechanisms and effects of HBO on platelet activation.


*Keywords:* Hyperbaric oxygen; Platelets; Aggregation; Pressure; 2-D PAGE; Flow cytometry; Nitric oxide; Mass spectrometry

## Introduction

Hyperbaric oxygen (HBO) therapy (the intermittent inhalation of 100% oxygen at a pressure greater than that at sea level, or 1 atmosphere absolute (ata)), is a recognized treatment for decompression illness, carbon monoxide poisoning and chronic wounds. Hyperbaric oxygen therapy has been suggested to improve the symptoms of such conditions through both an increase in pressure and an increase in the partial pressure of oxygen ($pO_2$) of the blood. An increased $pO_2$ induces the dissociation of CO from cytochrome *c* oxidase [1] and inhibits lipid peroxidation [2], effects which have both been suggested to improve the symptoms of CO poisoning. In decompression illness, increased pressure causes a reduction in gas bubble size, and an increase in $pO_2$ removes nitrogen from the bubble [3]. The processes by which HBO aids chronic wound healing are less well-understood, but potentially include the promotion of angiogenesis and fibroblast collagen synthesis [4], and an increase in oxidative killing [5], since oxidative bacterial killing is directly proportional to local oxygen tension [6].

The therapeutic effects of HBO are anti-inflammatory and immunosuppressive in nature, and many investigations have focused on the effect of the therapy on cells of the immune system such as leukocytes, and their interactions with cells such as vascular endothelial cells. For example, HBO has been shown to decrease the surface expression of intracellular adhesion molecule-1 (ICAM-1) by endothelial cells following exposure to hypoxia and hypoglycaemia [7]. Conversely, the administration of HBO, with no prior stimulus, has been shown

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to increase ICAM-1 expression by pulmonary vascular endothelial cells [8].

Platelets are another important cell type involved in immune system functions. Platelets influence the tethering and rolling of leukocytes along the endothelium prior to their diapedesis [9], and aid leukocyte adhesion during situations of high shear stress [10]. Depletion of platelets via antiplatelet antiserum has been shown to reduce leukocyte accumulation in bronchoalveolar fluid and airway tissue following allergen exposure [9]; this reduction in leukocyte recruitment was restored upon transfusion of platelets. The effect of HBO on platelet function has generally been limited to the effects of the therapy on platelet count and aggregation. For example, an increase in platelet count following exposure to HBO has been observed in both rats [11] and humans [12], whereas exposure to 100% oxygen at 2.4 ata has been shown to decrease ADP- and collagen-induced platelet aggregation in rabbits [13]. Thom et al. [14] reported no effect on platelet aggregation in humans following exposure to 100% oxygen at 2 ata. Further studies have also focused on the effect of pressure on platelet aggregation. Price et al. [15] demonstrated that exposure of PRP to 273 ata inhibited ADP-induced aggregation and that this inhibitory effect was reversible upon decompression. Klausen et al. [16] drew blood from deep-sea divers at 37 ata directly into glutaraldehyde, and demonstrated the occurrence of platelet activation based cell morphology. Conversely, Murayama [17] reported that exposure to reduced barometric pressure (0.5 ata) induced platelet aggregation, and suggested that this could account for reported decreases in platelet count following exposure to altitude, or decompression during diving.

During both physiological and inflammatory processes, platelets also secrete proteins and peptides. Exposure to HBO has been shown to both decrease and increase protein secretion in various cell types and animal models. A decrease in pro-inflammatory cytokine secretion in response to exposure to HBO has been observed in blood cultures [18], stimulated monocyte-macrophage cultures [19], and in a model of traumatic brain injury [20]. Such decreases may lower inflammatory responses at the area of injury. Hyperbaric oxygen has also been shown to increase the expression of angiopoietin 2 (Ang2) in human umbilical vein endothelial cells [21], a protein which has been suggested to enhance wound healing by remodelling blood and lymphatic vessels at the margins of healing wounds [22]. Additionally, an increase in the secretion of the angiogenic-promoting factor basic fibroblast growth factor by fibroblasts occurs following exposure to HBO [23]. The effect of HBO on release of platelet proteins has not, to our knowledge, been thoroughly investigated.

Nitric oxide ($^{\cdot}$NO) is a gaseous biological mediator which is known to play a role in inflammatory processes. Exposure to HBO has been demonstrated to both increase and decrease $^{\cdot}$NO synthesis in different experimental systems: Thom et al. [24] demonstrated perivascular $^{\cdot}$NO production to increase following exposure to 100% oxygen at 2.8 ata, whereas Huang et al. [25] found that exposure to HBO decreased $^{\cdot}$NO synthesis during LPS-induced lung injury, and hence attenuated the injury. When platelets are activated, they release $^{\cdot}$NO [26]. The inhibitory effects of $^{\cdot}$NO on platelet function are well known [27–29], and this release of $^{\cdot}$NO is believed to prevent excessive recruitment of platelets to the thrombus [30]. However, low concentrations of $^{\cdot}$NO have also been demonstrated to activate platelets due to the biphasic response of cGMP which consists of an initial transient stimulatory response, followed by an inhibitory effect [31]. The same group later demonstrated that protein secretion by platelets can be decreased by inhibiting cGMP-dependent protein kinase [32].

In light of the contradiction surrounding the effects of HBO on platelet function, the aim was to investigate whether the administration of HBO causes activation in the form of platelet aggregation, protein release and $^{\cdot}$NO production, without necessarily leading to overt changes in surface expression of protein activation markers.

## Methods

Iso-electric pH gradient strips, molecular mass markers, sample/rehydration buffer, agarose, ampholytes and DC protein assay kit were all purchased from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). Thiourea, protease inhibitor cocktail, iodoacetamide, Sigmacote®, L-arginine, N-nitro-L-arginine methyl ester (L-NAME) and acetylsalicylic acid (ASA) were obtained from Sigma-Aldrich (Poole, Dorset, UK). S-nitrosoglutathione (GSNO) and manganese (III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) were purchased from Axxora (UK) Ltd. (Nottingham, UK). Collagen (type I) was purchased from Labmedics Ltd. (Manchester, UK). Dithiothreitol (DTT), urea, sodium dodecyl sulphate (SDS), tris hydroxymethylaminoethane, G250 colloidal Coomassie, Amicon centrifugal concentrators (MWCO 5000 kDa), cell scrapers and 60 mm glass dishes were all purchased from Fisher Scientific (Loughborough, Leicestershire, UK), and 0.45 μm syringe-driven filters were purchased from Millipore (Watford, Hertfordshire, UK).

### *Platelet isolation and treatment protocol*

Ethics approval to take blood from healthy volunteers was granted by the North and East Devon Research Ethics Committee (reference: 04/Q2102/97). All individuals gave written informed consent before participating. All subjects abstained from aspirin for 14 days prior to the donation, and all other drugs for 2 days prior to the donation. Human platelets were isolated from freshly-drawn blood samples using prostacyclin as described by Radomski and Moncada [33], and either resuspended in modified Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM $Na_2HPO_4$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4), or prepared as platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was retained to act as a control in the aggregation experiments. The platelet suspension was stored for 15 min at room temperature (RT) prior to experimentation. Platelets were resuspended at final concentrations of $2.07\pm0.05\times10^8$ cells $mL^{-1}$ and $1.04\pm0.07\times10^8$ cells $mL^{-1}$ ($n=3$; mean ± SD)

for the protein release, and protein expression experiments, respectively. Platelet-rich plasma was prepared at physiological concentrations for the aggregation experiments, in that the cell count was not adjusted. Platelet preparations were contaminated with less than 0.4% red blood cells ($0.80 \pm 0.53 \times 10^6$ cells $mL^{-1}$) in the protein release experiments, less than 0.5% ($0.50 \pm 0.18 \times 10^6$ cells $mL^{-1}$) in the protein expression studies, and less than 0.4% ($0.51 \pm 0.47 \times 10^6$ cells $mL^{-1}$) in the aggregation experiments. No obvious mononuclear cell contamination was observed. L-arginine (100 μM) was added to the isolated platelet preparations.

Aliquots (6 mL) of either isolated platelet suspensions or PRP, were pipetted into separate glass dishes, siliconized using Sigmacote®. One dish was placed in the CON chamber, and the other in the HBO chamber. Both chambers were flushed through with their respective gas mixes (97.7% oxygen, balance carbon dioxide (HBO), and 5% carbon dioxide, balance air (CON)) for 5 min at a rate of approximately 2–3 $L \cdot min^{-1}$. The HBO chamber was then compressed to 2.2 ata, whereas the CON chamber was retained at 1 ata. Both treatments lasted for 90 min, and upon completion of the treatment, the HBO chamber was slowly decompressed over approximately 2 min, and the platelet samples were analyzed via aggregation, 2-D PAGE and flow cytometry as described below. In a separate experiment to elucidate the individual effects of pressure and oxygen on platelet aggregation, platelets were exposed to a gas mix of either 9.3% oxygen, 2.3% carbon dioxide, balance nitrogen at 2.2 ata (pressure control), or 5% carbon dioxide, balance oxygen at 1 ata (oxygen control), for 90 min.

### *Platelet aggregation*

Prior to placing the dishes in the chamber, 100 μM L-arginine, L-NAME, ASA, or GSNO, or the equivalent volume of ultrapure water (control) was added to both the PRP and the PPP. The effect of MnTBAP on platelet aggregation was also determined. Since MnTBAP is only water soluble at high pH, it was prepared using 200 mM NaOH. Therefore a corresponding solution was made without the addition of MnTBAP to act as a vehicle control. To ensure that the vehicle had no effect on aggregation, 100 μM of MnTBAP was added to one dish of PRP and one dish of PPP, and the corresponding volume of vehicle was added to one dish of PRP and one dish of PPP, and all four dishes were exposed to HBO.

Platelet aggregation was measured in PRP using a PAP-4 platelet aggregometer (BioData, Alpha Laboratories, UK) with constant stirring at 800 rpm at 37 °C, according to the manufacturer's instructions. Baseline aggregation was recorded for 2 min; during this period, type I collagen (CI) was suspended in PPP to result in a final concentration, upon addition of 50 μL to each cuvette, of 5 $\mu g \cdot mL^{-1}$. Upon the addition of collagen, aggregation was recorded for 7 min.

The remaining PRP that was not utilized in the aggregation experiments was centrifuged at 1000 $g_{av.}$ for 10 min at RT, and the PPP was transferred to a 15-mL tube and stored at −80 °C for subsequent nitrite and nitrate quantification as described below.

### *Nitrite and nitrate quantification*

The nitrite and nitrate content of the plasma stored at −80 °C was quantified using a modified version of the Griess assay as described in detail elsewhere [34]. Briefly, samples were deproteinized using a modified version of an earlier method [35]. A 100-μL aliquot of sample was incubated with 200 μL of water and 300 μL of 0.3 M NaOH for 5 min at RT. Three hundred μL of 5% zinc sulphate were added, the tube vortexed, and the solution was incubated at RT for 10 min. The samples were centrifuged at 10,000 $g_{av.}$ for 15 min, and the supernatant gently pipetted off and stored on ice until use.

The modified Griess nitrite assay was carried out at RT (20–23 °C). Fifty μL of nitrite standard or sample solution were pipetted in triplicate into a 96-well microplate, followed by 50 μL of 50 mM sodium phosphate buffer, pH 7.4 (stored at RT during use). The diazotization reaction was then initiated by addition of 100 μL of sulfanilamide (SAN) in 1M HCl; between 30 and 90 s later the coupling reagent (100 μL of 0.1% naphthylethylenediamine dihydrochloride (NED)) was added. The absorbance at 540 nm ($A_{540}$) was measured 15 min after the NED addition in a Dynatech Laboratories MRX plate reader (Billingshurst, UK).

The total nitrate plus nitrite assay was also performed at RT. An assay mix consisting of 0.72 mL of 50 mM sodium phosphate buffer (pH 7.4), 800 μL of nitrate reductase (NR; 1.6 U $mL^{-1}$), and 200 μL of 1 mM NADPH, was made and kept on ice during use. Briefly, 50 μL of standard or sample were pipetted in triplicate into a 96-well plate. Next, 50 μL of assay mix were then added, and the well contents were mixed thoroughly and incubated at RT for 30–40 min. The diazotization and coupling reactions were then carried out by adding 100 μL each of 1% SAN in HCl and 0.1% NED, in succession, with a delay of about 1 min between the additions. The $A_{540}$ was measured as above.

To ensure that any potential differences in the MnTBAP samples could not be attributed to the colouration of MnTBAP (dark brown), the $A_{540}$ of the samples was measured. Fifty μL of the sample were mixed with 250 μL of 50 mM sodium phosphate buffer (pH 7.4), and the $A_{540}$ was measured in triplicate for each sample.

### *2-D PAGE and liquid chromatography–mass spectrometry (LC–MS/MS)*

#### *Sample preparation*

Platelet suspensions were gently pipetted from each dish into two 15-mL centrifuge tubes (Alpha Laboratories, UK), and centrifuged at 1000 $g_{av.}$ for 10 min at RT. In the meantime, 50 μL of modified Tyrode's buffer were added to each dish, and the remaining platelets were scraped off using a cell scraper. Following centrifugation, the supernatant was gently pipetted off and filtered through a 0.45 μm filter to eliminate any platelet contamination, and the platelet pellet was stored on ice. Twenty μL of protease inhibitor cocktail consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin, were added to the filtered supernatant,

which was then stored at −80 °C until analysis. The scraped platelets from the dish were pipetted into the tube containing the platelet pellet, and 20 μL of protease inhibitor cocktail were added. The pellet was also stored at −80 °C until analysis.

When the supernatant was required, it was defrosted on ice, and then concentrated and desalted to approximately 100 μL using an Amicon centrifugal concentrator and then freeze-dried using a Heto Drywinner (Allerod, Denmark). The freeze-dried samples were reconstituted in 25 μL of water, and a Lowry protein assay was conducted. In order to assess relative protein changes between the HBO and CON treatments by 2D PAGE, equal amounts of total protein were loaded on three separate occasions.

*Rehydration and iso-electric focusing*

Platelet protein samples were resuspended in sample/rehydration buffer (7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 2% Bio-Lyte 3/10 ampholytes, 0.001% bromophenol blue and 1.6% DTT), and absorbed onto an 11 cm p*I* 4–7 immobilised pH gradient (IPG) strip according to the manufacturer's instructions. Strips were rehydrated for 1 h on the bench, then approximately 2 mL of mineral oil were pipetted onto the strip to prevent evaporation during rehydration, and the strip was actively rehydrated at 50 V/strip for a further 16 h using a Protean IEF cell (Bio-Rad Laboratories, UK), according to the manufacturer's instructions.

On completion of rehydration, paper wicks were placed over the electrodes in a clean iso-electric focussing tray; 8 μL of 3.5% (w/v) DTT were pipetted onto the wick at the positive electrode, and 8 μL of water onto the wick at the negative electrode. The IPG strip was then transferred to the clean focusing tray, maintaining the gel side down. The strip was overlaid with 2 mL of mineral oil, and the focusing tray placed in the iso-electric focusing cell (Protean IEF cell; Bio-Rad Laboratories, UK). The cell was programmed according to the manufacturer's instructions for electrophoresis in three stages. During stage 1, the voltage was set to reach 250 V using a linear gradient over 20-minute period; during stage 2, the voltage was set to reach 8000 V, again using a linear gradient, but over 2.5 h; in stage 3, the cell was set to reach 80,000 V h using a rapid gradient.

*Gel electrophoresis and staining*

Upon completion of iso-electric focusing, the IPG strip was removed from the focusing tray and the strip transferred gel side up to a channel in a rehydration/equilibration tray. The strip was equilibrated initially in 2 mL of equilibration buffer (6 M urea, 2% (w/v) SDS, 0.375 M Tris buffer (pH 8.8), 20% (v/v) glycerol) with 20 $mg \cdot mL^{-1}$ DTT for 10 min, and then with the same equilibration buffer but with 25 $mg \cdot mL^{-1}$ iodoacetamide for a further 10 min. The strip was then dipped briefly into XT MOPS running buffer (Bio-Rad Laboratories, UK) and placed, gel side up, onto an 11 cm Criterion XT Bis–Tris 4–12% pre-cast gel (Bio-Rad Laboratories, UK), mounted into a mini-Protean gel tank per the manufacturer's instructions (Bio-Rad Laboratories, UK), and the reservoirs filled with XT MOPS running buffer. Electrophoresis was performed at 200 V (constant voltage) for approximately 60 min.

On completion of electrophoresis, the gel was gently removed from the plates and washed briefly in ultrapure water (gels were continually agitated on a Luckham R100 Rotatest shaker (Burgesshill, Suffolk, UK)). The gel was then incubated in 50% ethanol, 2% phosphoric acid overnight. After this, the gels were washed in ultrapure water for an hour, with two changes of water during this time. Gels were then incubated for 1 h in approximately 100 mL per gel of 34% methanol, 2% phosphoric acid, 17% ammonium sulfate. At the end of this hour, approximately 100 mg of colloidal Coomassie blue were sprinkled onto each gel and the gel was left shaking for 3 days. At the end of this period, the gel was rinsed well with ultrapure water and damp tissue was used to very gently blot any specks of the stain from the gel, and from the gel-staining box. The gel was left to shake in ultrapure water until adequate de-staining was obtained.

*Image and statistical analysis*

The Coomassie blue-stained 2-D gels were scanned and digitized under UV light with flat fielding using the Chemidoc XRS Gel Documentation system (Bio-Rad Laboratories, UK). The same scanning conditions were used for all 2-D gels in a matched set. Image data were analyzed using the image analysis software, PDQuest (Version 8, Bio-Rad Laboratories, UK). The images were warped, to improve spot matching accuracy, and normalized using a local linear regression model. The analysis set manager was used to analyze spot differences between gels. A spot was required to change in at least two of the three gels in a treatment in order to be deemed to have changed. A spot was deemed to have increased or decreased if there was more than a two-fold difference in intensity. Quantitative spot comparisons across gels were made using *t*-tests, with statistics being performed on replicate groups.

*LC–MS/MS analysis*

The spots that were deemed to have changed, according to the criteria above, either quantitatively or qualitatively, were excised manually and placed in a microfuge tube with approximately 50 μL of water. The samples were kindly analyzed by Dr. Garry Rucklidge and Mr. Gary Duncan of the Rowett Research Institute (Aberdeen, UK). The proteins were enzymatically digested with trypsin, desalted and partially separated using a nano LC system with a C18 PepMap 100 column (LC Packings, Surrey, UK). The mass spectrometry was performed using a Q-Trap (triple quadrupole/linear ion trap mass spectrometer; Applied Biosystems/MDS Sciex, Warrington, UK) fitted with a nanospray ion source.

*Mascot analysis*

Database mining was performed using the MS/MS ion module in Mascot (www.matrixscience.com), based on searches within human sequences only. The search parameters allowed for one missing cleavage. Trypsin was selected to indicate the digestion enzyme. Oxidation of methionine was selected as a variable modification, and carbamidomethyl as a fixed modification. Mass values were classified as mono-isotopic. A peptide mass tolerance of ±1.2 Da, and a fragment

mass tolerance of ±0.8 Da were chosen. The threshold used by Mascot is that an event is significant if it is expected to occur at random with a frequency less than 5% ($P<0.05$) (www.matrixscience.com).

*Flow cytometry*

Platelet suspensions (100 μL containing $1\times10^7$ cells; CON, HBO, resting and thrombin-activated) were pipetted into microfuge tubes. The thrombin-activated controls were incubated with 0.1 U·mL$^{-1}$ thrombin at 37 °C in a heating block for 3 min. An aliquot (10 μL) of a 200 μg·mL$^{-1}$ stock of human-derived IgG was then added to all tubes to block non-specific binding via Fc receptors, and the contents of the tube were gently mixed and incubated for 10 min at RT. A 10-μL aliquot of either the antibody (anti-PECAM-1, anti-CD62P, or PAC-1), or the appropriate isotype control (IgG or IgM) at the same concentration as the corresponding antibody were then pipetted into the appropriate tubes. The tubes were gently mixed and incubated for 30 min in the dark at RT. Following this incubation, 500 μL of diluent buffer were added to each tube. Then, 500 μL of 2% paraformaldehyde in Tyrode's buffer was added to each tube to fix the platelets. Samples were stored at 4 °C in the dark until analysis by flow cytometry.

Platelets exposed to both HBO and CON were also stimulated with maximal (0.1 U·mL$^{-1}$), and sub-threshold (0.01 U·mL$^{-1}$) concentrations of thrombin, to establish a possible priming effect of the treatment. Thus, post-exposure, platelets were stimulated with both 0.1 U·mL$^{-1}$ and 0.01 U·mL$^{-1}$ of thrombin at 37 °C for 3 min. Samples were prepared for flow cytometry as described above.

A Coulter Epics XL MCL (Beckman Coulter, High Wycombe, Buckinghamshire, UK) was used to quantify cell surface markers of activation. The instrument parameters were set as follows: forward scatter voltage, 100; integral, 5; discriminator, 100; side scatter voltage, 500; integral, 50; FITC voltage, 917; and PE voltage, 965. The fluidics was set at a flow rate of approximately 50–100 cells min$^{-1}$ to prevent platelet activation, and to optimize resolution. A total of 10,000 events were recorded, with the exception of the priming experiments, when a total of 5000 events were recorded.

Flow cytometric data were analyzed by determining mean fluorescence intensity (MFI; arbitrary units) of the gated platelet population. Isotype MFIs were subtracted from the antibody MFIs to remove non-specific binding. Two distinct populations of cells were apparent in the resting, HBO and CON samples, and in the samples activated with 0.01 U·mL$^{-1}$ thrombin, and separate gates were placed around these populations [1,2]. Analysis was performed as above.

*Statistical analysis*

The HBO platelet aggregation data were normalised to CON in order to analyze the effect of the addition of different compounds. Data were analyzed using Kruskal–Wallis followed by Mann–Whitney *U* tests as post hoc tests. A probability level of $P<0.05$ was chosen as the threshold for acceptance of statistical significance. All data are expressed as the mean value±SD, and are for $n=3$ or 4 as stated.

## Results

*Effect of HBO on platelet aggregation*

Exposure to the HBO treatment (pre-collagen) induced aggregation compared with those exposed to CON ($P=0.021$; Fig. 1A). The percentage of platelet aggregation in the PRP exposed to HBO was 29.8±3.0 compared with 5.5±0.9 in the PRP exposed to CON. There was also an increase in collagen-induced aggregation in the PRP exposed to HBO, compared with PRP exposed to CON ($P=0.018$; Fig. 1A). The maximum aggregation post-collagen was 90.5±1.0% in the PRP exposed to HBO compared with 82.5±2.9% in the PRP exposed to CON. The addition of L-arginine, L-NAME, acetylsalicylic acid (ASA), or S-nitrosoglutathione (GSNO), had no effect on HBO-induced aggregation compared with the vehicle control ($P>0.05$; data not shown).

The addition of 100 μM MnTBAP to the PRP prior to exposure to HBO had no effect on HBO-induced aggregation ($P=0.773$; Fig. 1B). The percentage of platelet aggregation in the PRP containing MnTBAP was 26.5±8.0 pre-collagen, compared with 24.9±7.2 in the PRP containing the buffer vehicle control. The percentage of platelet aggregation post-collagen was 43.6±10.4% in the PRP containing MnTBAP, and 84.4±8.3% in the PRP containing the buffer vehicle control, and platelet aggregation was significantly decreased by the addition of MnTBAP post-collagen, compared with the buffer vehicle control ($P=0.021$).

Platelet aggregation was significantly greater in the pressure control compared with the oxygen control, pre-collagen ($P=0.019$; Fig. 1C). Platelet aggregation in the pressure control was 19.3±1, compared with 3.9±0.6 in the oxygen control. The percentage of aggregation post-collagen was 83.6±3.5 in the pressure control compared with 75.0±4.6 in the oxygen control, and this post-collagen aggregation was significantly greater in the pressure control than the oxygen control ($P=0.020$).

*Effect of HBO on nitrite and nitrate levels*

There was no difference in either nitrite or nitrate content between HBO and CON in any of the treatments, or between the pressure and oxygen controls ($P>0.05$; Table 1). However, the nitrite concentration corresponding to the MnTBAP-treated platelets was significantly higher compared with that of the vehicle control ($P=0.043$). The $A_{540}$ values of the MnTBAP samples were measured, to ensure that any possible differences could not be attributed to the dark colour of the MnTBAP interfering with the assay. The results demonstrated that there was no difference in $A_{540}$ between the samples containing the MnTBAP, compared with those containing the vehicle control ($P=0.309$; data not shown). The nitrite concentrations of both the HBO and CON samples which contained GSNO were significantly greater than their corresponding control samples, as expected ($P=0.021$).

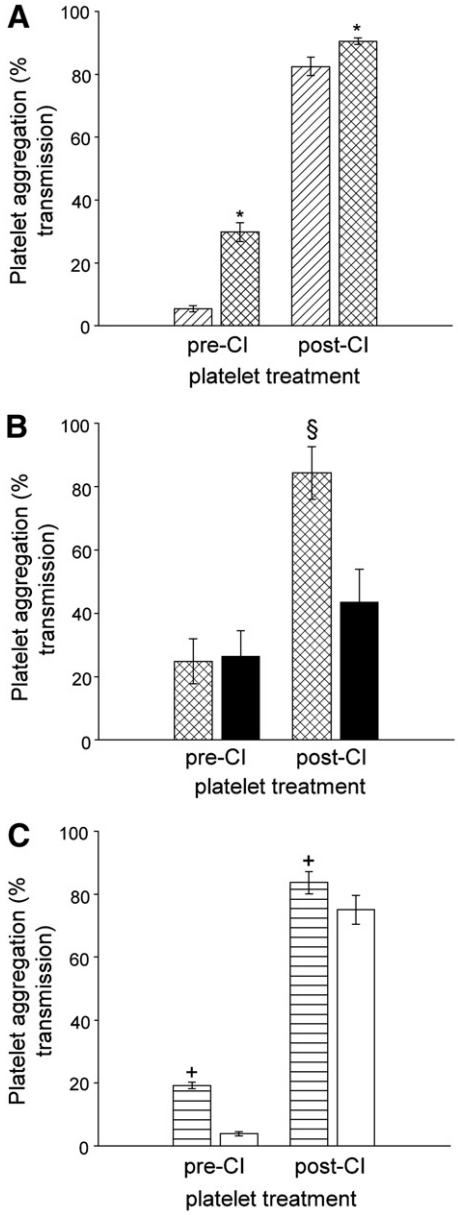


Fig. 1. (A) Platelet aggregation following a 90-minute exposure of platelet-rich plasma to either CON (diagonal bars) or HBO (hatched bars), pre and post addition of collagen (5 μg·mL$^{-1}$; pre-CI and post-CI, respectively). Platelet suspensions were spiked with water as control prior to exposure to either HBO or CON. (B) In a separate experiment, either 100 μM MnTBAP (closed bars) or the appropriate vehicle control (hatched bars) was added prior to exposure to HBO. (C) Platelet aggregation was also quantified following a 90-minute exposure of PRP to either a pressure control (horizontal bars) or oxygen control (open bars), pre and post addition of collagen. Platelet aggregation was significantly increased following exposure to HBO. *indicates a significant difference between CON and HBO ($P<0.05$). §indicates a significant difference compared with MnTBAP vehicle control ($P<0.05$). +indicates a significant difference between pressure and oxygen controls ($P<0.05$). All data are represented by mean±SD, $n=4$).

### *Effect of HBO on platelet protein release*

Three independent experiments for the analysis of released proteins were performed. Spots on the 2-D PAGE gels were selected for nano LC–MS/MS on the basis of absence or presence, and also on the basis of quantitative changes (Fig. 2 and Tables 2 and 3). The criteria for spot selection were that the same spot must have changed in at least two of the gels in one treatment (HBO or CON), but in no more than one gel of the other treatment. Four qualitatively-altered spots and 3

Table 1
Nitrite and nitrate contents (nmol/10$^8$ cells) of plasma from platelets exposed to HBO, CON, pressure or oxygen controls ($n=3$ or 4; * denotes statistical significance compared with appropriate control)

| Sample | Nitrite (nmol/10$^8$ cells; mean±SD) | Nitrate (nmol/10$^8$ cells; mean±SD) |
|---|---|---|
| Control CON | 1.85±0.22 | 12.69±5.58 |
| Control HBO | 1.64±0.37 | 11.72±5.77 |
| L-arginine CON | 2.18±1.09 | 12.78±4.16 |
| L-arginine HBO | 1.28±1.27 | 11.97±5.45 |
| L-NAME CON | 2.00±0.32 | 12.37±4.52 |
| L-NAME HBO | 1.87±1.15 | 12.49±7.99 |
| GSNO CON | 10.40±3.61* | 18.07±9.29 |
| GSNO HBO | 10.45±3.45* | 16.94±7.16 |
| MnTBAP control | 0.91±0.60 | 15.50±7.72 |
| MnTBAP | 1.92±0.74* | 15.12±10.88 |
| ASA CON | 1.54±0.72 | 14.86±10.01 |
| ASA HBO | 1.36±0.52 | 15.95±9.83 |
| Pressure control | 2.44±1.72 | 13.56±9.85 |
| $O_2$ control | 1.02±0.54 | 12.23±8.59 |

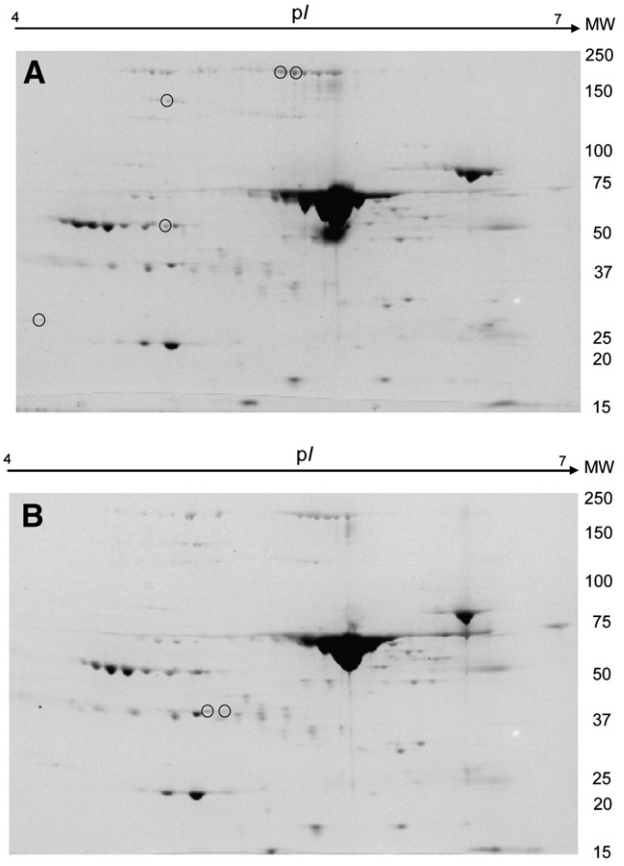


Fig. 2. Representative 2-D electrophoresis profiles of Coomassie Blue-stained images of released proteins from platelets exposed to HBO (A) and CON (B). The proteins that were determined to be either qualitatively (presence or absence) or quantitatively (>2-fold change) different as determined using PDQuest, are circled. These spots were excised and analysed by LC–MS/MS.

Table 2
Densities of spots that were identified, via *t* tests using PDQuest software, as being significantly increased in the platelets exposed to HBO compared with CON

| | | | | Mean density |
|---|---|---|---|---|
| Spot ID | CON 1 | CON 2 | CON 3 | |
| 1201 | 43584.0 | 16004.6 | 27258.1 | 28948.9 |
| 5901 | 21644.8 | 35450.0 | 77228.5 | 44774.43 |
| 5903 | 26075.5 | 22231.8 | 72272.7 | 40193.33 |
| | HBO 1 | HBO 2 | HBO 3 | |
| 1201 | 60889.3 | 58700.8 | Not present | 59795.1 |
| 5901 | 192874.6 | 402987.4 | 93821.1 | 229894.4 |
| 5903 | 140890.2 | 31966.0 | 133111.0 | 101989.1 |

quantitatively-altered spots were identified. Table 2 demonstrates the spot densities of the quantitative matches that were found to be statistically significant. The seven spots of interest were excised and subjected to nano LC–MS/MS. The resulting ions were analyzed using the MS/MS ion module in Mascot. Table 3 demonstrates the proteins that were identified using the MS/MS ion search. Statistically significant matches were made for all seven proteins.

*Expression of surface proteins*

There was no difference in expression of any of the markers investigated in the platelets exposed to HBO compared with CON (Fig. 3). For example, PECAM-1 expression was 3.23±0.85 in the HBO-treated platelets compared with 2.53±1.81 in the CON-treated platelets ($P=0.827$). CD62P expression in the HBO-treated platelets was 1.68±0.65 compared with 1.22±0.19 in the CON-treated platelets ($P=0.275$). PAC-1 expression was 1.05±0.48 in the HBO-treated platelets compared with 1.13±0.17 in the CON-treated platelets ($P=0.513$).

There was no difference in expression of any of the markers investigated in the platelets exposed to HBO compared with CON and stimulated with either 0.1 U·mL$^{-1}$ or 0.01 U·mL$^{-1}$ of thrombin (data not shown; $P>0.05$)). There were also no differences when the HBO and CON results were compared with the corresponding thrombin results ($P>0.05$).

Table 3
Identification of the proteins analyzed by LC–MS/MS that changed either quantitatively or qualitatively after exposure to either HBO or CON, obtained using the Mascot module, MS/MS ion search

| Spot ID | MOWSE score | Number of peptides matched | Molecular mass (Da) | Accession number in SwissProt | Identified protein |
|---|---|---|---|---|---|
| *Qualitative changes* | | | | | |
| 3302 | 574 | 12 | 42108 | ATHUG | Actin gamma 1 |
| 3304 | 599 | 12 | 42108 | ATHUG | Actin gamma 1 |
| 3503 | 561 | 12 | 53025 | XHHU3 | Anti-thrombin III precursor |
| 3503 | 550 | 12 | 54612 | AAA52173 | Vitamin D-binding protein precursor |
| 3702 | 761 | 17 | 123429 | KUHU | Caeruloplasmin |
| *Quantitative changes* | | | | | |
| 1201 | 614 | 13 | 27899 | PSHUAM | 14-3-3 protein zeta |
| 5901 | 1789 | 37 | 164600 | MAHU | α-2-macroglobulin precursor |
| 5903 | 109 | 3 | 67690 | 1AO6A | Serum albumin, chain A |

Significant matches were provided for all of the indicated proteins.

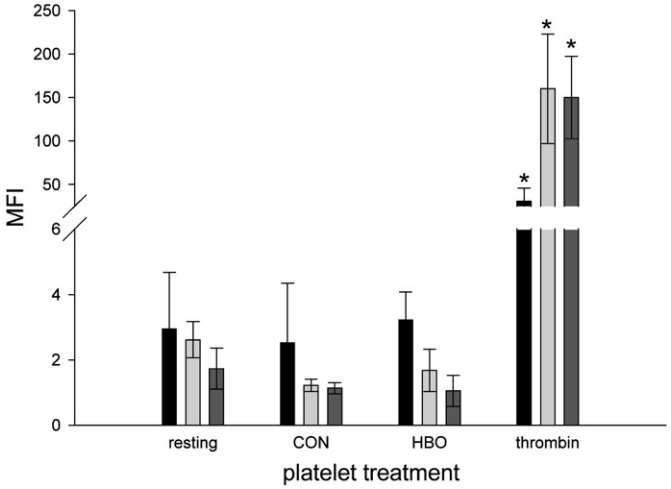


Fig. 3. Flow cytometric analysis of the effect of exposure to HBO or CON on PECAM-1 (black bars), CD62P (light grey bars), and PAC-1 (dark grey bars) expression in platelets. Results are expressed as mean fluorescence intensity (MFI)±SD ($n=3$). Resting and thrombin-activated (0.1 U·mL$^{-1}$) platelets act as negative and positive controls, respectively. * denotes statistical significance compared with resting platelets ($P<0.05$).

Two distinct populations of cells were observed in the resting, HBO and CON samples (Fig 4A), and in the samples activated with 0.01 U·mL$^{-1}$ of thrombin. The populations [1,2] were gated on resting unstained platelets, and analysis was performed on these gated regions. There were no differences in the percentages of cells present within these two populations (Table 4). There were no differences in protein expression, in either population 1 (data not shown) or 2 (Fig. 4B), and in HBO-treated platelets compared with CON ($P>0.05$). However, there was a suggestion of an increase in CD62P expression in HBO compared with CON in population 2 (Fig. 4B).

With regards to the priming experiments, there were no differences in protein expression for any of the markers investigated in population 1, in HBO plus 0.01 U·mL$^{-1}$ thrombin-stimulated platelets compared with CON plus 0.01 U·mL$^{-1}$ thrombin-stimulated platelets ($P>0.05$; data not shown). However, there was a suggestion of a decrease in both CD62P (Fig. 4Ci) and PAC-1 (Fig. 4Cii) expression after stimulation with 0.01 U·mL$^{-1}$ thrombin in HBO compared with CON in population 1. There were no differences in protein expression for any of the markers investigated in population 2, in HBO plus 0.01 U·mL$^{-1}$ thrombin-stimulated platelets compared with CON plus 0.01 U·mL$^{-1}$ thrombin-stimulated platelets ($P>0.05$; data not shown). Neither the HBO- nor the CON-treated platelets differed compared with resting values in either of the populations ($P>0.05$).

## Discussion

The results of our study show that exposure to HBO induces aggregation in platelets, and altered protein release by them, but

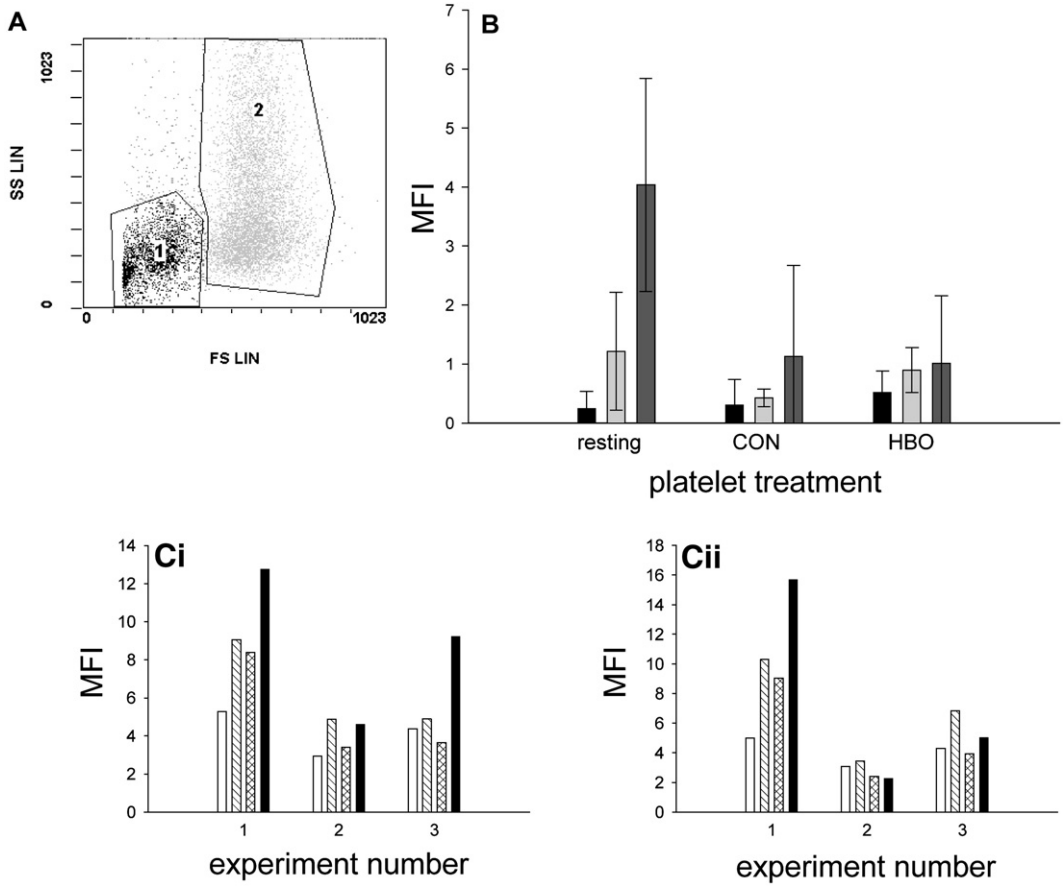


Fig. 4. (A) Flow cytometric analysis revealed two distinct populations of cells in the resting, HBO and CON samples, and in the samples activated with 0.01 U·mL$^{-1}$ of thrombin. The populations (1 and 2) were gated on resting unstained platelets and analysis was performed on these gated regions. (B) The effect of exposure to HBO or CON on PECAM-1 (black bars), CD62P (light grey bars), and PAC-1 (dark grey bars) expression in platelets, in population 2. Results are expressed as mean fluorescence intensity (MFI)±SD ($n$=3). (C) Individual data demonstrating MFI of CD62P (i), and PAC-1 (ii) expression in population 1 in the three separate replicate experiments in resting (open bars), CON plus 0.01 U·mL$^{-1}$ thrombin (diagonal bars), HBO plus 0.01 U·mL$^{-1}$ thrombin (hatched bars), and thrombin (0.01 U·mL$^{-1}$; closed bars).

does not affect the expression of surface markers of activation, or the release of ·NO. It is of interest that the majority of the identified proteins have been implicated in inflammation, and that HBO therapy has previously been suggested to have an anti-inflammatory mode of action.

Such HBO-induced aggregation *in vitro* has not to our knowledge been previously reported; indeed *ex vivo* investigations have demonstrated no effect of HBO on platelet aggregation [14]. Klausen et al. [16] reported a change in the morphology of platelets from deep-sea divers at 37 ata. Blood was drawn directly into glutaraldehyde at depth for immediate fixation, in order to prevent any decompression effects. Platelets obtained at depth were examined by transmission electron microscopy, and observed to be spherical with multiple projections extending from their surface, with centrally-located organelles. Such changes are indicative of a change in the activation status of the platelets. The authors reported that these effects generally diminished upon decompression to the surface. These results are in agreement with the present study which demonstrated that the observed increase in aggregation was in part due to the increase in pressure. The possibility that exposure to HBO may induce platelet aggregation might initially be of concern considering the health status of the patients who normally require HBO therapy. However, to our knowledge there is no evidence in the literature that thrombotic events occur during exposure to HBO. It is possible that HBO may result in the formation of micro-aggregates, but that these aggregates do not adhere to the endothelium. Indeed, there was no evidence of an up-regulation of surface markers of activation in the present study, including markers of activation that would facilitate platelet interaction with the endothelium. This is in keeping with Thom et al. [14] who recently demonstrated that exposure to 100% oxygen at 2 ata did not result in activation of $\alpha_{IIb}\beta_{IIIa}$.

Table 4
The percentage of cells present within the two distinct populations (mean±SD; $n$=3)

| Treatment | Population 1 | Population 2 |
|---|---|---|
| Resting | 35.2±8.8 | 51.9±6.6 |
| CON+0.01 U·mL$^{-1}$ thrombin | 33.9±9.3 | 54.7±8.1 |
| HBO+0.01 U·mL$^{-1}$ thrombin | 34.6±9.3 | 53.8±8.1 |
| 0.01 U·mL$^{-1}$ thrombin | 35.8±9.5 | 51.1±7.6 |

With regards to the two distinct populations of platelets that were observed during flow cytometric analysis, population 1 was smaller and less granular, compared with population 2. Light transmission, as was used to measure platelet aggregation, may also be affected by cell volume, because an increase in cell volume increases light transmission [36]. If the effect of HBO-induced aggregation were due to increased cell volume, then the percentage of cells in population 2 may have been greater in the platelets exposed to HBO compared with CON. This was not the case, however, and the percentage of cells in both populations did not differ between conditions.

The effect of HBO exposure on protein release by platelets has never been thoroughly investigated. However, HBO has been shown to have an effect on protein expression and secretion by other cell types. For example, HUVECs were exposed to 98% oxygen, 2% carbon dioxide at either 2.5 ata, or under normobaric conditions for 90 min, and Ang2 expression was demonstrated to increase in both the conditions compared with normal cell culture conditions [21]. Interestingly, this increase in expression appeared to be via a ·NO-dependent mechanism, as demonstrated by the use of L-NAME, which inhibited the increased expression due to HBO. Exposure of fibroblasts to HBO at 2 ata has been shown to increase their secretion of bFGF [23]. An increase in platelet protein content has previously been demonstrated to occur in response to exposure to HBO at 2.2 ata [37], although the source of this increase was not identified.

The preliminary results reported in the present paper demonstrate that the majority of the proteins that were found to be up-regulated by exposure to HBO have been shown to play a role in inflammation. Spot 1201 was identified as 14-3-3 zeta, a PKC inhibitor that regulates arachidonic acid metabolism in platelets, and has previously been reported to be released by thrombin-activated platelets [38]. PKC is known to negatively regulate collagen-induced platelet aggregation by inhibiting filopodia generation [39]; presumably inhibition of this process may thus result in enhanced aggregation. Interestingly, one of the first proteins that was shown to bind to 14-3-3 protein zeta was the GPIb-IX complex of platelets [40]. Indeed, 14-3-3 zeta has been shown to regulate vWF binding to GPIb-IX [41]; the authors of this study proposed that inhibition of the interaction of 14-3-3 zeta with GPIb-IX may be a potential therapeutic target in thrombosis [41]. 14-3-3 protein zeta may therefore be involved in mediating the HBO-induced aggregation that was observed in the present study. Spot 5901 was identified as α-2-macroglobulin precursor. An up-regulation in α-2-macroglobulin is associated with increased secretion. This is a protease (elastase inhibitor) which is present in the α granules of platelets [42]. There is an up-regulation of proteases such as elastase in the aspirate of chronic wounds compared with that of acute wounds, and fibronectin degradation has been demonstrated to be correlated with cleavage of α-2-macroglobulin in such chronic wounds [43]. Although elastase activity is required for both remodeling of the wound, and facilitation of wound cell migration, dysregulated activity has been demonstrated to result in extracellular matrix degradation and tissue breakdown [44]. Therefore, suppression of this activity would presumably enhance scaffold development, and the healing of chronic wounds. Interestingly, α-2-macroglobulin is present at a lower concentration in the wound aspirate of chronic wounds, compared with both plasma and the aspirate of acute wounds [45]. These results are in agreement with the recent work of Alleva et al. [46] who have shown that alpha lipoic acid supplementation in combination with HBO therapy can affect protease expression in wounds, thereby promoting the healing process. A protein that was present in the releasate from platelets exposed to HBO, but not CON was the ferroxidase caeruloplasmin. This is usually regarded as a plasma protein, and it has not been previously identified in platelets. Caeruloplasmin has long been known to have antioxidant properties [47], and has been demonstrated to inhibit lipid peroxidation induced by UV irradiation and thrombin in platelets [48]. Caeruloplasmin has also been shown to catalyse both the release of .NO from RSNOs [49], and the formation of RSNOs [50]. There was no evidence of an increase in ·NO production by platelets following exposure to HBO in the present study.

In conclusion, our results demonstrate that exposure to HBO induces aggregation and protein release by platelets, but does not cause platelet activation as evidenced by cell surface expression of marker proteins, or release of ·NO. The role of these released proteins in the physiology of different therapeutic uses of HBO requires further study.

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