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Bubbles, microparticles, and neutrophil activation: changes with exercise level and breathing gas during open-water SCUBA diving

Stephen R. Thom,[1,2] Tatyana N. Milovanova,[1] Marina Bogush,[1] Ming Yang,[1] Veena M. Bhopale,[1] Neal W. Pollock,[4] Marko Ljubkovic,[5] Petar Denoble,[4] Dennis Madden,[5] Mislav Lozo,[5] and Zeljko Dujic[5]

[1]*Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania;* [2]*Department of Emergency Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania;* [3]*Divers Alert Network, Durham, North Carolina; and* [4]*Department of Integrative Physiology, University of Split School of Medicine, Split, Croatia*

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Thom SR, Milovanova TN, Bogush M, Yang M, Bhopale VM, Pollock NW, Ljubkovic M, Denoble P, Madden D, Lozo M, Dujic Z. Bubbles, microparticles, and neutrophil activation: changes with exercise level and breathing gas during open-water SCUBA diving. *J Appl Physiol* 114: 1396–1405, 2013. First published March 14, 2013; doi:10.1152/japplphysiol.00106.2013.—The study goal was to evaluate responses in humans following decompression from open-water SCUBA diving with the hypothesis that exertion underwater and use of a breathing mixture containing more oxygen and less nitrogen (enriched air nitrox) would alter annexin V-positive microparticle (MP) production and size changes and neutrophil activation, as well as their relationships to intravascular bubble formation. Twenty-four divers followed a uniform dive profile to 18 m of sea water breathing air or 22.5 m breathing 32% oxygen/68% nitrogen for 47 min, either swimming with moderately heavy exertion underwater or remaining stationary at depth. Blood was obtained pre- and at 15 and 120 min postdive. Intravascular bubbles were quantified by transthoracic echocardiography postdive at 20-min intervals for 2 h. There were no significant differences in maximum bubble scores among the dives. MP number increased 2.7-fold, on average, within 15 min after each dive; only the air-exertion dive resulted in a significant further increase to 5-fold over baseline at 2 h postdive. Neutrophil activation occurred after all dives. For the enriched air nitrox stationary at depth dive, but not for other conditions, the numbers of postdive annexin V-positive particles above 1 μm in diameter were correlated with intravascular bubble scores (correlation coefficients ~0.9, $P < 0.05$). We conclude that postdecompression relationships among bubbles, MPs, platelet-neutrophil interactions, and neutrophil activation appear to exist, but more study is required to improve confidence in the associations.

decompression sickness; intravascular bubble; leukocytes; platelets; antigen sharing; CD41; integrins; ultrasound

THE FOCUS OF THIS INVESTIGATION was to evaluate whether correlations could be identified for intravascular gas bubbles and changes in several blood-borne formed elements in humans performing open-water SCUBA diving. Our ultimate goal is to improve understanding of the pathophysiology of decompression sickness (DCS). DCS is a systemic pathophysiological process that occurs after tissues become supersaturated with gas. Inert gases inhaled while breathing are taken up by tissues in proportion to the ambient pressure, and, when pressure is reduced, some of the gas released from tissues may form bubbles due to the presence of gas cavitation nuclei (15, 43, 44).

Address for reprint requests and other correspondence: S. R. Thom, Institute for Environmental Medicine, Univ. of Pennsylvania, 1 John Morgan Bldg, 3620 Hamilton Walk, Philadelphia, PA 19104-6068 (e-mail: sthom@mail.med.upenn.edu).

Decompression-induced elevations in circulating microparticles (MPs) occur in animals and humans after simulated or bona fide underwater diving (27, 38, 41). MPs are defined as membrane lipid bilayer-enclosed vesicles with a diameter of 0.1–1.0 μm. They are generated when cells undergo oxidative stress, apoptosis, or cell activation/calcium influx (13, 14, 22, 32). MPs are present in peripheral blood of healthy individuals, increase with traumatic and inflammatory disorders, and may serve as intercellular messengers because they can contain cytokines or other signaling proteins, mRNA, and micro-RNA (28). They are characterized by surface expression of antigenic markers from parent cells and many also have surface-bound annexin V because, as MPs are formed, negatively charged phosphatidylserine residues become exposed.

We have hypothesized that MP elevations postdive occur because of bubble-mediated shear stress and stimulation of calcium-activated big conductance potassium channels, as well as oxidative stress from activated neutrophils (39, 42). In a recent study, however, our laboratory found that intra-MP nitrogen dioxide is a bubble nucleation site. Particles enlarge beyond a diameter of 1 μm due to influx of inert gas on decompression, and this physical change or subsequent alterations in surface antigens lead to decompression-induced neutrophil activation and vascular injury (40, 42). Thus the cause-effect relationships among MPs, bubbles, and decompression stress appear more complex than initially envisioned. Unfortunately, gas-containing MPs are too small to be detected by ultrasound, which, depending on the instrument and several technical variables, may be able to detect structures with diameters as small as ~24–160 μm (16, 18, 20).

Air is the most common breathing gas used in underwater diving, but use of so-called "enriched air nitrox" (EAN), a combination of nitrogen with O_2 content above 21%, has grown rapidly over the past 20 yr (25). The merit to EAN depends on the actual dive being performed. EAN use reduces the uptake of inert gas and, ultimately the risk of DCS for the same dive profile relative to breathing air, with a practical benefit increasing for exposures greater than 12 m of sea water (msw) (25). The maximum effective depth of EAN is limited by the higher partial pressure of O_2, as one reaches the limits of O_2 toxicity more quickly than with air as the breathing gas. Using EAN while following air decompression limits will improve decompression safety; however, it is common to use EAN to extend the time at depth by calculating allowable exposure time based on the actual nitrogen partial pressure. This derivation is commonly termed the "equivalent air depth" (1). The physiological ramifications of EAN have not been well documented by experimental studies. Anecdotal reports of

EAN use have included claims of less fatigue (which may be a subclinical manifestation of DCS) compared with air breathing (5). This subjective benefit was not corroborated under simulated diving conditions in a hyperbaric chamber (19).

Exertion while undergoing decompression stress at altitude has been documented to increase the rate of development of venous gas bubbles and also the incidence of DCS (23, 24). A similar association with exertion while diving underwater is thought to occur, although it is not rigorously documented. Others have reported that moderate or strenuous physical exercise will increase some subsets of MPs (4, 30, 34, 35). Therefore, comparing responses between diving with exertion vs. undergoing the same decompression stress with minimal exertion offers an alternative approach to examine MPs in diving.

In a previous study with volunteers undergoing repetitive SCUBA diving, our laboratory described elevations in MPs and neutrophil activation consistent with the animal studies, but a significant negative correlation between intravascular bubble scores and circulating MPs (38). Blood was collected at 80 min postdive, and we speculated that the single sampling relatively late after diving may have contributed to this result. Therefore, this study was planned to assess associations between MP number, intravascular bubbles, and neutrophil activation at an earlier, as well as later, time point than in the previous study, while comparing two breathing gases and two levels of exertion.

METHODS

Subjects. Twenty-four divers, four women, were recruited for this investigation. All experimental procedures in the study were completed in accordance with the Declaration of Helsinki and approved by Ethical Committees of the University of Split School of Medicine, Divers Alert Network, and the University of Pennsylvania. All subjects gave informed written consent. Baseline screening of all divers, including assessments of body fat and maximum aerobic capacity, was according to our published protocol (38) and was performed because there is evidence to suggest that some anthropomorphic features and physical fitness may alter the risk of DCS (9, 29). Health status and previous diving experience was self-reported. Anthropometric characteristics are shown in Supplemental Table S1. (The online version of this article contains supplemental data.)

Study protocol. All subjects were certified divers with diving experience ranging from 4 to 25 yr. Divers provided their own diving equipment; all wore wetsuits between 5 and 8 mm in thickness for thermal comfort. The initial series of dives, performed near Split, Croatia, occurred in April 2012, with a second series completed in late November 2012 to increase samplings for dives involving moderate exertion. The dives were conducted in the same location as described in a previous study (38). A 2- to 3-min surface swim through calm water was required for divers to reach the descent point and again to return to the exit point. The water temperature for all dives was ~16°C with minimal current. Divers had refrained from any diving and swimming activities for at least 7 days before the first dive in the experimental series. Diving profiles were monitored electronically (Sensus, Mississauga, Ontario, Canada).

Each dive was conducted with a dive leader to ensure compliance with standardized exertion or remaining stationary at depth. All air dive profiles (21% O_2 in breathing gas) were a direct descent to 18 m for an actual bottom time of 47 min, then 2-min ascent to the surface. EAN dives (breathing gas with 32% O_2 and the balance nitrogen) were a direct descent to 22.5 m and bottom time of 47 min to provide an equivalent air depth. The goal was to match the partial pressure of nitrogen used in the air dives. Each diver was equipped with a Galileo dive computer (Johnson Outdoors); the resolution of this device is ±0.01 m. Depth and heart rate data were logged at 4-s intervals throughout the dive. Subjects either swam continuously while at depth at a pace intended to represent a sustained moderate to moderately heavy work rate (Ex dive) or remained at depth with minimal movement, maintaining their position in the water column using their buoyancy compensators (Rest dive). The four dive groups are summarized as Air-Rest, Air-Ex, EAN-Rest, and EAN-Ex.

Mean exercise intensity was estimated as oxygen extraction ($\dot{V}O_2$, ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) from mean heart rate data recorded during each dive using a regression equation developed by Dwyer (10) from open-water swimming diver trials conducted at 20 msw in similar water temperature ($\dot{V}O_2 = 0.0177 \times$ heart rate + 0.150). The estimates were converted to metabolic equivalents (MET = $\dot{V}O_2/3.5$ ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) for intuitive clarity. Heart rate could not be used to estimate $\dot{V}O_2$ during the resting dives because of the cold-induced tachycardia experienced by subjects during these trials.

The number of subjects involved with each condition differed as emphasis was focused on Air-Ex and EAN-Ex conditions. The experimental dives were conducted once per day with at least a 4-day break before the next dive in the series. In the April series, 18 divers were split into two groups and either started with Air-Ex or EAN-Ex, and then switched their gas supply for the alternative exercise dive 4 days later. Following a 4-day period when no SCUBA diving was done, divers performed one "Rest" dive breathing either air or EAN. One diver who had missed the EAN-Ex dive performed this condition 6 days after his Rest dive. The November series was performed with six divers, different from those in the April series, who performed the Air-Ex dive followed 4 days later by the EAN-Ex dive. One diver was unable to complete the Air-Ex condition in April, so this group totaled just 23 vs. 24 in the EAN-Ex group. Also, one diver did not participate in a dive involving remaining stationary at depth. Hence the Air-Rest condition involved nine divers, and EAN-Rest involved eight divers.

Transthoracic echocardiography (TTE) imaging was performed with a phase array probe (1.5–3.3 MHz) using a Vivid q scanner (General Electric, Waukesha, WI) on each subject postdive every 20 min for 2 h in a beach-side room ~50 steps from the dive site. These studies were exactly as described in our laboratory's previous report (38). TTE monitoring of all four cardiac chambers was conducted by groups of two technicians trained in cardiac imaging. Individual subjects were scanned by the same technician team throughout the study, and bubble signals were graded in real time through consensus of the two evaluators. Grading employed a modified Brubakk scale that has been used in several studies (26, 38). The grading system is as follows: 0 = no bubbles; I = occasional bubbles; II = at least one bubble every four cardiac cycles; III = at least one bubble every cardiac cycle; IV = continuous bubbling with modifiers (a = at least one bubble per cm^2 in all frames, b = at least three bubbles per cm^2 in all frames, or c = almost complete whiteout but individual bubbles can still be discerned); and V = "whiteout", where individual bubbles cannot be discerned. Resting scores were recorded as the lowest stable score maintained during the observation period. Movement scores reflected the highest score achieved following the movement series for each of the two limbs tested, as described previously (38).

Venous blood was collected by a trained phlebotomist before and at 15 and 120 min after the dives. Blood was drawn into Cyto-Chex BCT test tubes that contain a proprietary preservative (Streck, Medimark Europe, Grenoble, France). The volume drawn per sample (two tubes) was ~5 ml.

Materials. Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Annexin binding buffer and the following antibodies were purchased from BD Pharmingen (San Jose, CA): fluorescein isothiocyanate (FITC) conjugated anti-annexin V, FITC-conjugated anti-human myeloperoxidase (MPO), R-phycoerythrin (PE) conjugated anti-human CD142, PerCP/Cy5.5 conjugated anti-human CD41, PerCP/Cy5.5 conjugated anti-human CD14,

allophycocyanin conjugated anti-human glycophorin A (CD235), allophycocyanin conjugated anti-human von Willebrand factor (vWF). R-PE-conjugated anti-human CD41 was purchased from e-Biosciences (San Diego, CA), PerCP Cy5.5-conjugated anti-human CD66b from Biolegend (San Diego, CA), and Alexa 647-conjugated anti-human CD18 from Serotec (Raleigh, NC).

Standard procedures for MPs and neutrophil acquisition and processing. Blood samples in tubes containing preservative were sent by express mail to the University of Pennsylvania, where all analyses were performed within 24 h after arrival, ~2–6 days from time of collection. As described previously, MPs and neutrophil characteristics remain unchanged when samples stored at either 4°C or at room temperature are processed in a time span of 3 wk from time of collection (38). All reagents and solutions used for MP analysis were sterile and filtered (0.2-μm filter). In brief, a small sample of the whole fixed blood was set aside for evaluation of neutrophils, to count leukocytes and platelets, and to calculate hematocrit by micro-hematocrit centrifuge. The remaining blood was centrifuged for 5 min at 1,500 *g*; the supernatant was made 0.2 M EDTA and then centrifuged at 15,000 *g* for 30 min. Aliquots of the 15,000 *g* supernatant were stained with antibodies for analysis by flow cytometry and confocal microscopy.

Flow cytometry. Flow cytometry was performed with a 10-color FACSCanto (Becton Dickinson, San Jose, CA) using standard acquisition software. Gates were set to include 0.3- to 1.0-μm particles, with exclusion of background corresponding to debris usually present in buffers. MPs were stained with annexin V antibody and analyzed as previously described, including microbeads with diameters of 0.3 μm (Sigma,), 1.0 μm, and 3.0 μm (Spherotech, Lake Forest, IL) to assess the size of particles. Analysis of neutrophils was performed on fixed blood samples, as previously described (39).

Confocal microscopy. The sizes of annexin V-positive particles were measured following published procedures (38). In brief, images were acquired using a Zeiss Meta510 confocal microscope equipped with a Plan-Apochromat 63×/1.4 numerical aperture oil objective. Particle suspensions stained with R-PE-conjugated anti-annexin V antibody were combined with a small number of FITC-containing 0.86 μm beads (Sigma) to provide comparison and were visually inspected to be sure no particle aggregates were present. The mean number of annexin V-positive particles evaluated among the 192 measurements (different dive groups, 8–24 divers/group × 3 samples/dive) was 797 ± 42 (SE, range 462–1,336). Digital images were obtained and analyzed using Image J software. The annexin V-positive particles were categorized based on diameter as <1 μm, 1–2 μm, or >2 μm and the proportion used to calculate the populations of particles of various sizes in each diver. That is, flow cytometer data are not sufficiently precise to use directly for determining particle sizes, so the gated data showing MP number (0.3–1 μm) was taken as the proportion <1 μm, and other size populations were calculated from this measurement.

Statistical analysis. Parametric data are expressed as means ± SE; nonparametric data are expressed as median and 25th and 75th percentile values. We used Sigmastat software (Systat, Point Richmond, CA) for the statistical analysis. MP numbers, neutrophil activation, and size of annexin V-positive particle populations, all parametric data, were analyzed by repeated-measures ANOVA followed by the Holm-Sidak test. Bubble scores (nonparametric data) were analyzed using repeated-measures ANOVA on ranks, and correlations involving the bubble data sets, MPs, and assays of neutrophil activation were evaluated by the Spearman rank order test with the Bonferroni correction for multiple comparisons. Significance was accepted at $P < 0.05$.

RESULTS

Diving and intravascular bubbles. Divers reported no adverse effects from any of the SCUBA activities. Hematocrit, leukocyte, and platelet counts were not significantly different pre-/postdive for any of the dive conditions (data not shown). There were no discernible differences among the 4 women vs. 20 men in this study, so data for all divers were grouped according to each dive type. The mean exercise intensity was 5.7 ± 0.2 MET while at depth in both the Air-Ex and EAN-Ex trials. As discussed in METHODS, heart rate could not be used to estimate metabolic rate during the Rest dives.

Circulating bubbles quantified by TTE were documented in all subjects with no significant differences in maximum bubble scores among the four dive conditions. Maximum bubble scores are shown in Fig. 1; the median maximum score for all

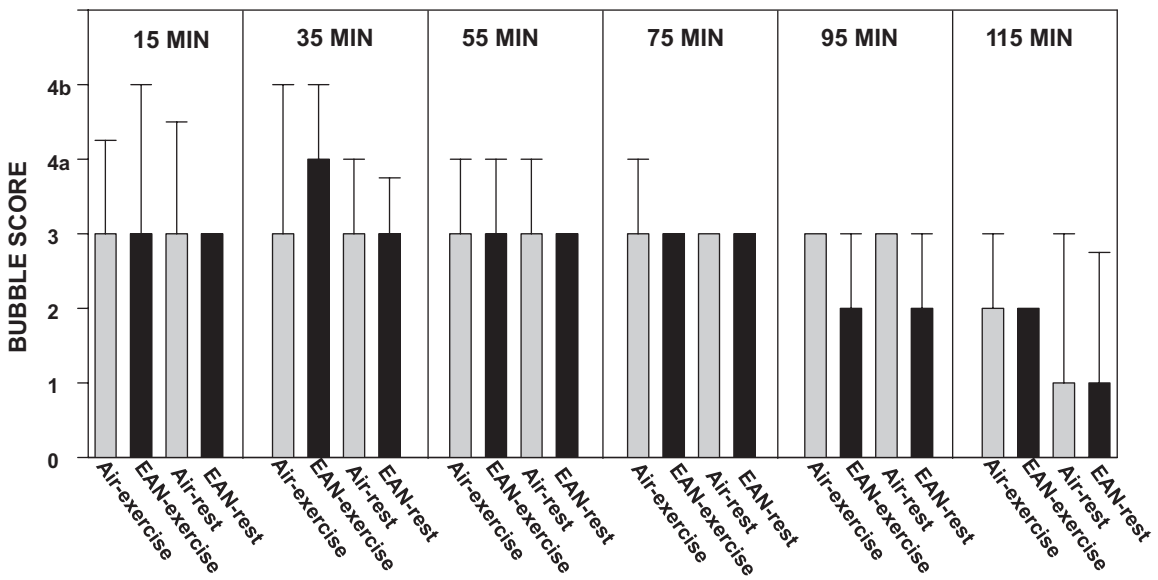

Fig. 1. Bubble scores following each dive. Data show median and 75th percentile values for these nonparametric data. Measurements were taken at 20-min intervals for 2 h and included data while at rest and also after prescribed arm or leg movements. The maximum bubble score almost always occurred after limb movements, and the maximum score is what was plotted. There were no significant differences among the four dive groups. EAN, enriched air nitrox.

groups was III. Similarly, the median values using scores at all ultrasound sampling times, rather than focusing on the maximum score, remained at III, with no significant differences among the groups.

MP elevations. Circulating MP counts related to each dive profile are shown in Table 1. There were no significant differences in annexin V-positive particle size distributions among the four groups before diving. Mean values 15 min after all dives were significantly greater than predive values for all four dive conditions, and there were no significant differences among the groups. That is, at 15 min, the postdive particle numbers and size distributions did not appear to be influenced in a statistically significant fashion by breathing gas (air vs. EAN) or level of exercise (exertion vs. stationary at depth).

The level of activity and breathing gas during the dive had a significant impact on particle counts measured 2 h postdive in some conditions (Table 1). In the Air-Ex condition, particle counts for all sizes were significantly greater than at 15 min and also greater than counts in the three other dive conditions. The 2-h sampling value in the EAN-Rest and EAN-Ex conditions for particles with diameters between 1 and 2 μm and >2 μm were also significantly greater than the values at 15 min postdive. Particle counts in the Air-Rest dive group at 2 h were not significantly different from values at 15 min postdive.

Particle surface protein expression patterns. MP subtypes were characterized by surface markers for vascular cell proteins. Figure 2 shows the percentage of annexin V-positive MPs coexpressing each surface protein marker. There were significant differences vs. predive values for all subtypes at 15 and 120 min postdive, but no significant differences between the 15- and 120-min measurements. Comparisons among the four dive conditions (vertical comparison in Fig. 2) identified significant differences for MPs expressing CD142, vWF, and CD14 between Air-Ex and several other conditions.

The populations of annexin V-positive MPs exhibiting surface markers for two vascular cell types were evaluated because interactions and thus antigen sharing has been described in previous animal and human studies (38, 39, 42). A complex pattern was identified, as shown in Fig. 3. When comparing changes in MPs within the four dive conditions based on time of sampling (look horizontally across the figure), significant differences from predive values are indicated by "P", and values significantly different at 15 and 120 min postdive are indicated by "15". The greatest numbers of significantly different values for multiple positive MPs were found in the Air-Ex condition. When comparing numbers of specific MP types among the four conditions (look vertically down at each MPs subtype), significantly fewer CD66b-vWF dual-positive MPs were found with EAN-Rest at both 15 and 120 min postdive vs. the other three conditions (identified with a asterisk). A number of significant differences were also found, with greater numbers present in the Air-Ex group than the other three conditions. In one instance, at 15 min postdive, there were significantly more CD66b-CD235 dual-positive MPs with EAN-Ex than in the Rest conditions.

Leukocyte activation in divers. Table 2 shows measurements of neutrophils obtained pre- and postdive. The neutrophil population was identified by surface expression of CD66b, and activation was assessed by measuring the presence of several proteins on the cell surface quantified as geometric mean fluorescence intensity, and also the percentage of the total CD66b-positive cell populations with mean fluorescence above 10 arbitrary fluorescence units (indicated as percentage in Table 2). Measurements were made for CD18 (a component of β_2-integrins) and MPO. Surface expression of CD41, the platelet-specific integrin α_{2b}-protein, was also assessed as an index of platelet-neutrophil interactions. Several significant differences were identified before diving among the four conditions. Consistent with our laboratory's previous report (38), neutrophils exhibited evidence of activation based on significant elevations in all measurements at 15 and 120 min postdive (horizontal assessment across Table 2). In a few cases, significant differences were noted between 15- and 120-min measurements (indicated by the dagger symbol in Table 2). There were a number of significant differences when the four diving conditions were compared (vertical assessment). These are shown in the lowest row in Table 2, as numbers that indicate each dive condition, as described in the table caption.

Correlations among variables. Correlations were sought among the various measurements made in each diving condition. A correlation was found to be statistically significant after adjustment for multiple measurements between divers' maximum bubble scores and postdive annexin V-positive particle numbers in the EAN-Rest condition. For 15-min postdive readings, the correlation coefficient (CC) between maximum bubble score and number of particles with 1- to 2-μm diameters was 0.884 ($P = 0.046$), and for those >2 μm in diameter, it was 0.885 ($P = 0.046$). At 120 min, the CC between maximum bubble score and particles between 1 and 2 μm was 0.902 ($P = 0.00005$). Correlations were not significantly different for total number of particles of all sizes or those <1 μm at either sampling time. For example, at 120 min, the CC between maximum bubble score and total number of annexin V-positive particles of all sizes was 0.652 ($P = 0.072$); for MPs <1 μm, 0.652 ($P = 0.072$). If analyses were done using bubble score for the first reading only (which often was the

Table 1. *Annexin V-positive particle numbers before and after each dive*

		Predive				15 min Postdive				2 h Postdive			
Group	*n*	Total	<1	1–2	>2	Total	<1	1–2	>2	Total	<1	1–2	>2
EAN-Rest	8	1,448 ± 146	1,422 ± 143	26 ± 2	0.5 ± 0.2	3,050 ± 329	2,902 ± 317	132 ± 14	16 ± 3	3,858 ± 622	3,465 ± 555	323 ± 63†	70 ± 11†
Air-Rest	9	1,182 ± 128	1,147 ± 123	34 ± 6	0.5 ± 0.3	3,741 ± 552	3,579 ± 529	143 ± 20	19 ± 6	4,692 ± 873	4,270 ± 783	355 ± 82	67 ± 16
EAN-Exercise	24	1,352 ± 104	1,314 ± 101	37 ± 3	0.8 ± 0.2	3,915 ± 670	3,709 ± 636	181 ± 32	25 ± 4	4,540 ± 751	4,092 ± 676	366 ± 62†	82 ± 15†
Air-Exercise	23	1,609 ± 147	1,569 ± 144	39 ± 4	0.8 ± 0.3	3,793 ± 453	3,592 ± 428	173 ± 24	28 ± 4	8,270 ± 1,109*†	7,492 ± 1,012*†	638 ± 85*†	140 ± 17*†

Values show particle numbers as means ± SE; *n*, no. of subjects in each group. As described in METHODS, the proportion of particles with different sizes; those <1-μm diameter and thus microparticles (MPs) by definition, those 1–2 μm, and those >2 μm were evaluated by confocal microscope. All 15-min and 2-h postdive values are significantly different from the comparable predive values. EAN, enriched air nitrox. *Values significantly different ($P < 0.05$) vs. all others in the column (vertical analysis). †Significantly different from the 15-min value in same row (horizontal analysis).

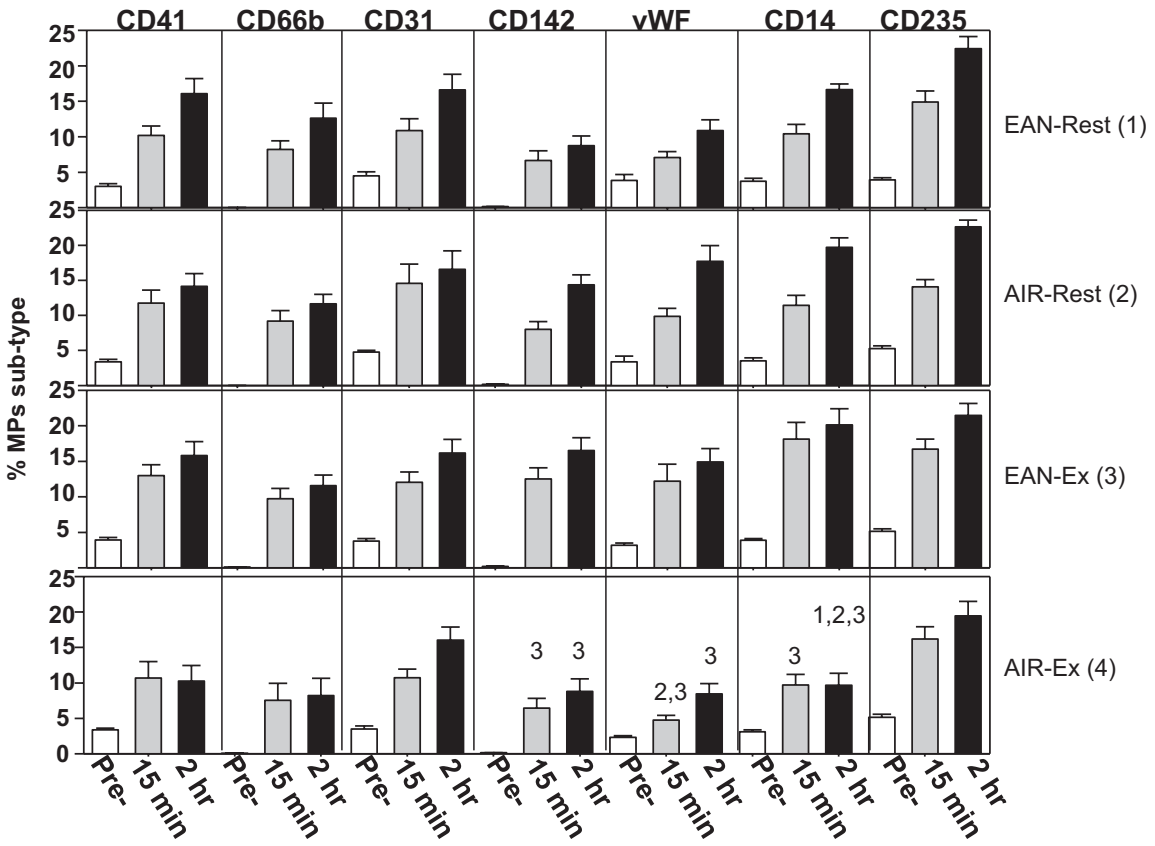

Fig. 2. Microparticles (MPs) expressing surface markers for various antigens. MPs expressing the platelet-specific CD41 are shown in *column 1*, those expressing the neutrophil protein CD66b in *column 2*, CD31 (platelet endothelial cell adhesion molecule) in *column 3*, CD142 or tissue factor in *column 4*, von Willebrand factor (vWF) in the *column 5*, the common leukocyte antigen CD14 in the *column 6*, and the erythrocyte-specific protein glycophorin A (CD235) in *column 7*. Values are means ± SE. The postdive (Post) values for all MP subtypes are significantly different from the predive (Pre) values, with no significant difference between the 15-min and 2-h values. A few subtypes exhibited significant differences among the four dive groups. These are shown as numbers above the bars: [1] significant difference compared with EAN-Rest; [2] significant difference compared with Air-Rest; [3] significant difference compared with EAN-exercise ($P < 0.05$, ANOVA).

maximum bubble score, but not always), values for CCs and their P values were virtually the same as with use of the maximum bubble score (data not shown). There were no significant positive or negative correlations between bubble scores and MP numbers for any of the other dive conditions.

Because we found several predive neutrophil activation parameters to be significantly different among the four dive conditions, we also examined whether correlations were present between pre- and postdive measurements. For the Air-Ex condition, the predive CD41 percent greater than 10 units correlated with 2-h postdive CD41 percent greater than 10 units (CC = 0.646, $P = 0.038$). In the EAN-Ex group, the predive CD41 percent greater than 10 units correlated with the 15-min postdive CD41 percent (CC = 0.569, $P = 0.044$).

DISCUSSION

Results from this study offer a number of new observations regarding human responses to decompression. It is important to first mention that we previously carried out a series of open-water control dives at just 5 msw depth (thus nominal decompression), which demonstrated that stresses due to swimming the same underwater course as used in the present study in 16°C sea water while breathing from SCUBA apparatus generated scant intravascular bubbles (the median scores at all reading times were zero) and no significant changes in annexin V-positive particle number or sizes. Several of the changes associated with swimming at 5 msw were similar to the Air-Ex findings in the previous and present studies. There was an increase in one particle subtype, the CD41-positive MPs, and increases in two neutrophil activation parameters (~3-fold elevations in percentage of CD66b-positive cells expressing surface CD41 and MPO) (38).

Similar to the previous study, there was consistent evidence of bubble generation following the SCUBA dives in the present series (Fig. 1). New data show there is little variation among the bubble score values based on activity level (stationary at depth or sustained moderately heavy exercise) or breathing gas (EAN or air) for these dives that were matched based on nitrogen partial pressure. Similarly, at 15 min postdive, the elevations in annexin-V positive particles, those that are by definition MPs (<1 μm diameter) as well as larger particles, were essentially the same in all dive groups (Table 2). In the prior study (38), our laboratory reported that MPs increased 2.4-fold at 80 min following a dive; the same elevation was observed 15 min postdive in our present study Air-Ex group (the same activity and breathing gas as in the prior investigation).

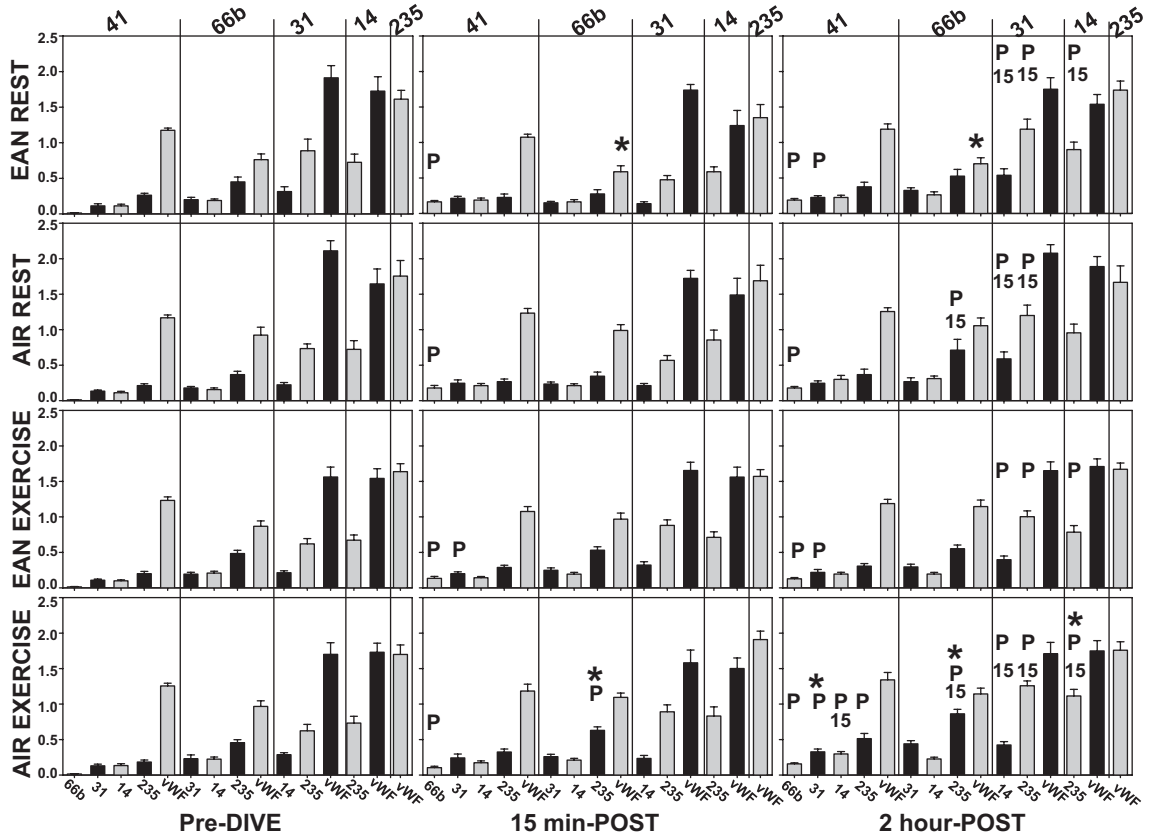

Fig. 3. MPs display multiple cell markers. MPs were stained to detect proteins from vascular cell sources. Data are the percentage of MPs expressing three markers: annexin V, the cell surface antigen shown at the *top*, and the antigen shown at the *bottom*. Values are means ± SE. [P] Value is significantly different from the Pre value ($P < 0.05$, ANOVA). [15] Significantly different from the 15-min value. *Value significantly different from other dive groups (vertical comparison).

Not only absolute number, but also the proportions of annexin V-positive particles over 1 μm in diameter increased from predive to 15 min postdive in all four diving conditions (Table 1). For example, in the EAN-Rest group at 15 min postdive, particles between 1- and 2-μm diameter represented 4.3% of all particles, and those >2 μm represented 0.5%. Although absolute numbers of particles were not significantly elevated in all groups at 2 h postdive vs. at 15 min, proportions increased. Again, using EAN-Rest as the example, particles between 1- and 2-μm diameter represented 8.4% of all particles, and those >2 μm represented 1.8%. For the other three dive conditions, proportions at each time point were similar to those for the EAN-Rest group. Following dives under all conditions except Air-Rest, statistically significant elevations were found for the number of annexin V-positive particles between 1- and 2-μm and >2-μm diameter at 2 h vs. the 15-min values. Particle size expansion on decompression is consistent with prior investigations (38–40, 42).

Clearly, diving with different exercise intensities and different breathing gases has complex effects on development of annexin V-positive particles by 2 h postdive. The Air-Ex group was the only one showing marked elevations in total particles at 2 h, making this dive condition distinctly different from the other three types (Table 1). The influence of exercise on MP production has some similarities with reports from others. Moderate or strenuous physical exercise was shown to increase MPs from leukocytes and platelets, but not from endothelium or erythrocytes, and, in some studies, changes were concurrent with an elevation in leukocyte and platelet counts (4, 30, 34, 35). In these reports, MP elevations were found immediately after exercise and at 2 h postexercise; one group has shown different rates of elevations up to 2 h between trained and untrained subjects (34). Mean $\dot{V}O_2$ values could be estimated for two of these studies, which we believe to be a better assessment of exertion than description of percentage of aerobic capacity or peak $\dot{V}O_2$ values (30, 34). Exercise intensity in these reports ranged between 10.9 and 15.6 MET. Hence, they involved levels of exertion from 1.9 to 2.7 times greater than occurred in Air-Ex and EAN-Ex dives. The exertion in these studies compared with what occurred in the Rest dives is expected to be even greater, although it is possible that effort associated with shivering/cold stress could have influenced responses. Therefore, SCUBA diving appears to cause a magnitude of MP generation out of proportion to the level of exertion.

It is possible that some of the differences observed among the four diving conditions in our study were influenced by particle clearance vs. production. Surface phosphatidylserine, as is present on most MPs, constitutes a recognition signal that enables clearance/phagocytosis of particles (2). The major MP clearance pathway is thought to involve lactadherin binding to surface-expressed phosphatidylserine and subsequent clearance via splenic macrophages (6), but an alternative pathway involves MP binding by Del-1 on endothelial cells, suggesting that there can be clearance from most vascular beds (7). In the

Table 2. *Neutrophil activation*

		MPO						CD18						CD41					
		%			GM			%			GM			%			GM		
Group	*n*	Pre	15 m	2 h	Pre	15 m	2 h	Pre	15 m	2 h	Pre	15 m	2 h	Pre	15 m	2 h	Pre	15 m	2 h
1. EAN-Rest	8	4.0 ± 0.4	10.8 ± 1.1	13.8 ± 1.0*	10.5 ± 0.8	27.8 ± 1.0	30.2 ± 2.1	3.7 ± 0.2	10.1 ± 1.7	12.5 ± 1.4	15.5 ± 0.7	27.0 ± 0.3	28.0 ± 1.6	8.3 ± 0.5	12.2 ± 0.5	14.5 ± 1.2	25.4 ± 0.9	105.8 ± 3.7	117.0 ± 4.4*
2. Air-Rest	9	4.6 ± 0.4	10.9 ± 1.4	15.3 ± 0.9*	12.1 ± 0.6	26.1 ± 1.3	30.2 ± 1.1*	4.3 ± 0.4	10.1 ± 1.2	13.6 ± 0.8*	16.2 ± 0.5	30.0 ± 0.3	28.0 ± 0.4	6.6 ± 0.7	13.3 ± 1.0	12.1 ± 0.7	24.7 ± 1.0	114.2 ± 4.5	118.6 ± 4.7
3. EAN-Exercise	24	3.2 ± 0.3	10.9 ± 0.6	13.8 ± 1.1	12.6 ± 0.5	22.0 ± 0.6	23.1 ± 0.7	3.0 ± 0.2	15.5 ± 1.3	17.7 ± 1.0*	15.1 ± 0.8	25.0 ± 0.5	25.9 ± 1.2	8.9 ± 0.8	15.5 ± 0.8	18.6 ± 1.1	22.6 ± 0.5	123.3 ± 2.8	135.5 ± 3.7*
4. Air-Exercise	23	3.3 ± 0.4	10.7 ± 0.7	16.4 ± 1.3*	10.1 ± 0.5	23.5 ± 1.5	30.0 ± 1.3*	3.9 ± 0.3	15.8 ± 0.5	20.5 ± 1.2	10.7 ± 0.4	25.4 ± 1.3	30.0 ± 1.4	8.7 ± 0.7	16.2 ± 1.2	21.2* ± 1.5	19.7 ± 0.7	120.0 ± 3.2	139.6 ± 7.1
Groups significantly different		1 vs. 3 2 vs. 3 2 vs. 4	NS	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4	1 vs. 3 3 vs. 4	1 vs. 3	1 vs. 3 2 vs. 3 3 vs. 4	NS	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4	1 vs. 4 2 vs. 4 2 vs. 3 2 vs. 4	NS	NS	2 vs. 3 2 vs. 4	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4	1 vs. 4 2 vs. 4 3 vs. 4	1 vs. 3 1 vs. 4 2 vs. 3	1 vs. 3 1 vs. 4 2 vs. 3 2 vs. 4

Values are means ± SE; *n*, no. of subjects in each group. Neutrophils were identified by CD66b staining and coexpression of CD18, myeloperoxidase (MPO), or platelet-specific CD41 assessed by flow cytometry. The *left-side* columns show the percentage (%) of CD66b-positive cells expressing a mean fluorescence value ≥10 arbitrary units for each surface marker; *right-side* columns show CD66b-positive cell geometric mean fluorescence (GM) for each marker. All 15-min and 2-h postdive values were significantly greater than the predive (Pre) values ($P < 0.05$). *Significant differences between the 15-min and 2-h values occurred within groups (horizontal chart analysis). Comparisons among the groups (vertical analysis) demonstrated several as significantly different as shown in the *bottom* row. For example, for the first column indicating values for %MPO Pre, the notation "2 vs. 3" indicates *group 2* (Air-Rest group) value is significantly different from the value for *group 3* (EAN-Exercise). NS indicates no significant differences among the dive conditions.

murine model, we have found differences in clearance rates among MPs with different surface markers (42). There are insufficient data to assess the rate of particle clearance after diving in humans. In our laboratory's previous study (38), most postdive changes present after the first day of diving in a repetitive series had resolved before diving on the fourth day. Hence, if similar changes occur after each dive, they resolve within 24 h, before the start of the next day of diving (38).

Postdecompression there were elevations of MPs with surface markers from numerous vascular cell types (Fig. 2). There are few differences among the dive conditions. Lower numbers of particles expressing vWF, CD14, and CD142 in the Air-Ex condition may reflect production or clearance differences, and the physiological implications for these changes are unclear. A few differences were notable in terms of particles sharing antigens from several cell types (Fig. 3). Once again, the Air-Ex group stands out among the others as exhibiting the greatest number of differences by 2 h postdive. MP interactions leading to sharing or blending of surface markers, as depicted by data in Fig. 3, have been described ex vivo, and our laboratory reported their in vivo occurrences in mice and humans postdive (38–40, 42).

Given that inert gas bubbles are thought to precipitate DCS, and bubble grades over III have a stronger association with DCS than lower grades, we were particularly interested in looking for a correlation between bubble scores and annexin V-positive particles in this study (12). The only group in which a positive correlation was found between particles and bubble scores was the EAN-Rest group. Significant correlations occurred at both 15 and 120 min postdive. There are several possible reasons why this group is unique among the four that will be discussed below. We should first acknowledge that this group represents the smallest number of divers, diminishing overall confidence in the apparent association. However, this dive group also shows the lowest total number of annexin V-positive particles postdive (Table 1) and among the fewest changes for dual-positive MPs (Fig. 3). These findings suggest the dive may have been less provocative in a physiological sense than the other conditions, knowing that elevations in numbers of annexin V-positive particles and antigen sharing increase in relation to decompression depth in the murine model (39, 42).

We acknowledge that some correlations may be mere random events, but it is also important to remember that statistically significant correlations among variables can fail to be demonstrated if an interaction exists but is nonlinear. Oxidative stress, annexin V-positive particle number, particle size, and particle surface protein patterns all appear to be important for neutrophil activation and postdecompression vascular injuries, and vascular shear stresses are likely more complex with Ex vs. Rest diving. These may be reasons for the failure to show significant correlations between bubble scores and annexin V-positive particles with Ex diving. Why there is a difference between Air-Rest and EAN-Rest may relate to differences in MP generation due to differences in O_2 partial pressures at depth or during the decompression (ascent) phase of the dive.

Intra-MP nitrogen dioxide gas (which appears to be the basis for bubble nucleation within MPs) is synthesized by inducible/inflammatory nitric oxide synthase (NOS-2 or iNOS) (40). If iNOS activity influences MP dynamics in humans, as was shown in mice, then alterations in gas partial pressures may impact intravascular bubbles. Oxygen partial pressure in the

Air dives was ~59 kPa, whereas in the EAN dives it was 105 kPa, 78% higher. Alterations in iNOS activity by this level of hyperoxia has not been examined specifically, but at a pressure of 202-kPa O_2, iNOS activity in neutrophils increases fourfold over that observed with 20.5-kPa O_2, due to an enhanced association with filamentous actin (37). Liberation of nitric oxide from cells into the extracellular space is a crude measure of NOS activity. Production by neutrophils exposed to 101-kPa O_2 is significantly elevated over that generated by cells exposed to ~20.5 kPa, and about one-third as much as cells exposed to 202-kPa O_2 (36). Hence, it is quite likely that iNOS activity can be increased by the O_2 partial pressure experienced during EAN vs. Air dives. Nitrogen partial pressure in all the dives was ~223 kPa. Although no data have been reported for nitrogen, exposure to just 71-kPa helium causes cardiac preconditioning in rabbits, linked to activation of endothelial NOS, and exposure to 700 kPa activates iNOS in mice (lower pressures were not investigated) (17, 31). This biochemical effect of inert gas may be the reason why nominal decompression from as little as 135 kPa can result in intravascular bubble formation in 50% of humans (11).

We believe mention should also be made that some data in the present study differ from that in our laboratory's previous report (38). Divers in the previous study exactly matched the present study Air-Ex group, but, whereas median bubble scores for all dives in the current series were III, the median scores for the four repetitive dives performed in our laboratory's previous human study were IVb ($P < 0.05$, ANOVA on ranks). As the same technicians performed echocardiography using the same equipment, we do not think the difference is due to bubble detection skills or apparatus. Variability in bubble scores among divers following the same profile is well recognized, although poorly explained (3, 12).

While the 2.4-fold elevation in MP number 15 min postdive exactly matches the elevation found following the Air-Ex diving in our laboratory's previous study (38), the magnitude of circulating MPs before diving differed considerably. In divers studied many months apart in the prior investigation, we saw predive MP counts differ over threefold (~2,500 to ~8,000/μl), and, in the present investigation, the predive mean number was ~1,600/μl (38). Although the analysis techniques were the same in both studies, variations in flow cytometry measurements can still occur due to subtle alterations in laser intensity and gating, as well as differences in antibodies among commercial vendors. There can also be inherent differences among the human subjects, such as predive exertion or health status. Many of these variables can only be speculated upon; hence, other than acknowledging different "baseline" numbers, further study is required to ascertain reasons for variability in numbers for circulating MPs.

In our laboratory's previous investigation, where blood was only collected at 80 min postdive, there were significant negative correlations between bubble scores and MPs after each dive (38). Thus the data suggested that a lower MP count, at least at 80 min postdive, portends higher bubble scores. Expansion and thus rupture of MPs could explain the negative correlations found in our laboratory's previous study. Negative correlations were not observed between bubble scores and MPs in our present investigation, which obviously diminishes the veracity of the observations. Possible reasons for these differences, in addition to the difference in maximum bubble scores as mentioned (III vs. IVb), are discussed below.

In the present investigation, we were surprised to find some differences among the groups in terms of predive variables pertaining to neutrophil activation (Table 2). As outlined in METHODS, the Ex dives were conducted before the Rest dives, and, when significant differences occurred predive, often the higher values were in the Rest dive groups. This was the case for CD41 and CD18 geometric mean and MPO percent evaluations. It is possible that waiting only 4 days between dives was too short an interval to allow all neutrophil activation parameters to return to baseline. We suspect, however, that the pathways for cell activation as well as clearance of activated cells from the blood may be complicated by variables such as repeated diving and/or exertion. In our laboratory's previous study involving daily dives for 4 days, we did not observe an alteration in predive neutrophil activation on the fourth vs. first day (38).

Some predive indexes of platelet (CD41)-neutrophil interactions were correlated with CD41 presence on postdive neutrophils in Air-Ex and EAN-Ex groups. Contact with platelets will trigger neutrophil oxidative burst and degranulation, which could exacerbate postdive interactions, but data are insufficient to warrant discussion on why the relationship was not observed in the Rest dives (21, 33). We had anticipated that correlations might occur between MPs, bubble score, and various postdive measurements of neutrophil activation, but this did not occur. The measurements in Table 2 all relate to neutrophil activation, but they monitor slightly different aspects of the cell populations. Gradations to neutrophil activation have been described by others, and decompression as in this study leads to a lower degree of neutrophil activation than many chemical agonists (8, 38). Progressive elevations in neutrophil activation occur in the murine model with more provocative decompression (39). Further study is required to assess whether greater activation may occur in humans with varying degrees of decompression stresses and, in the extreme, if it will contribute to the DCS syndrome. While no symptoms were reported in our study, the dives produce substantial bubble scores, consistent with a meaningful decompression stress. It is possible that symptomatic exposures could be associated with sharply amplified activation patterns.

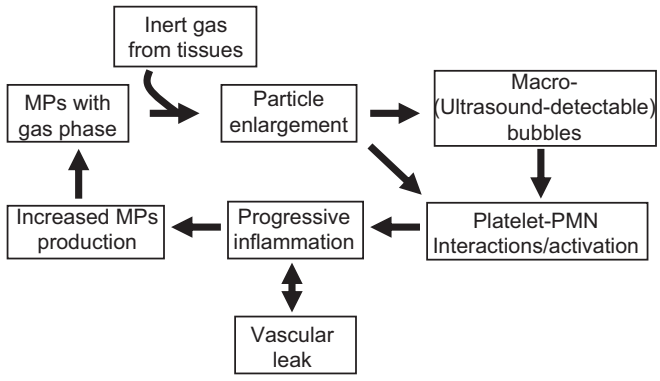

Fig. 4. Schematic showing hypothetical links between MPs and decompression stress. The decompression-induced cycle begins with inert gases from supersaturated tissues entering the gas phase within circulating MPs. Particles enlarge and interact with platelets and neutrophils to cause progression of the inflammatory process, and some particles enlarge to the point where ultrasonic detection is possible. The remainder of the cycle is as described in the text. PMN, polymorphonuclear neutrophil.

The findings in this study can be put into a context with those obtained using the murine model. Figure 4 summarizes these and outlines our hypothesis for the mechanism of decompression stress, which we define as MP enlargement and new particle generation, neutrophil activation, and, in the murine model, tissue damage manifested by a vascular leak. We have shown that MP enlargement occurs due to uptake of inert gas from super-saturated tissues postdive because, before any exposure to elevated pressure, a fraction of MPs contain a gas phase of nitrogen dioxide (40). Nitrogen dioxide is generated by iNOS, thus showing there is a metabolic link to annexin V-positive particle dynamics. Decompression-induced enlargement of annexin V-positive particles and/or concurrent changes in their surface marker protein pattern mediate increases in neutrophil interactions with platelets or platelet-derived membranes, neutrophil activation documented as elevations in surface expression of β_2-integrins, measured as CD18 staining, and also degranulation, documented by cell-surface MPO and subsequent vascular damage. If MP enlargement is prevented (interventions or manipulations used in prior studies include surfactant infusion to lyse MPs, hydrostatic recompression, and prevention of MPs gas phase by inhibiting iNOS or use of iNOS knockout mice), decompression stress is ameliorated. These interactions are even more complex, however, because we have found that neutrophil activation contributes to postdecompression elevations in MP number, establishing the possibility of a positive-feedback scenario. In the mouse model, if neutrophil activation is diminished (with use of thrombocytopenic mice and MPO knockout mice) or if intra-MP gas production is reduced (iNOS inhibitor treatment or with iNOS knockout mice), the postdecompression increases in MP number are lower or entirely prevented.

We speculate that progressive elevations in MPs at 2 h postdive were only observed in the Air-Ex group, because this dive poses the most stressful condition among the four studied. The role that O_2 partial pressure plays in differences between Air-Ex and EAN-Ex dives will require additional study because of the complex influence of exertion. The Rest dive is anticipated to pose fewer concurrent or complex cause-effect relationships. If indeed gas-phase MPs are the origin for Doppler-detectable bubbles, why was a correlation between MPs and bubbles only observed in the EAN-Rest group? The obvious difference between the two Rest dives is O_2 partial pressure, and, if 105-kPa O_2 increases iNOS activity, more internal gas synthesis will increase particle radius. The radius of these particles will increase further during ascent from depth. Expansion of the MPs will increase particle surface area and probably reduce wall thickness, reducing surface tension. Both of these effects will enhance inert gas diffusion into MPs, and all three processes (increased iNOS activity/gas production, increased particle surface area, and reduced wall thickness) are expected to improve the probability that Doppler-detectable bubbles will form. The MP-bubble correlation in the EAN-Rest group may occur because there are fewer confounding events between MP expansion and production of Doppler-detectable bubbles than with the other three dive conditions. Note that there is no particular reason to suspect that directly activating iNOS should lead to more MPs. An aspect of the hypothesis that instigated our study of MPs, that bubble-mediated shear stress may stimulate MPs generation, appears to remain a viable mechanism and perhaps explains why SCUBA diving results in generation of MPs out of proportion to the level of exertion. Carrying this notion forward, one might predict that EAN use at an equivalent air depth would increase the number of Doppler-detectable bubbles relative to Air diving. As this did not occur, further work is required to better evaluate the role for metabolism and specifically iNOS activity either to support our hypothesis, or to sort out alternative mechanisms.

In conclusion, our results provide additional insight into decompression pathophysiology, and, therefore, they are relevant for SCUBA diving, high-altitude aviation, and space exploration. MP production and enlargement postdecompression appear to be linked to intravascular bubbles for at least some diving activities, although this needs to be validated in larger numbers of divers. The pathophysiological associations among MPs, platelet-neutrophil interactions, neutrophil activation, and vascular injury have been established in the murine model. That MPs can serve as a nucleation site for intravascular bubble formation also poses the possibility that MPs actually precipitate macro- or Doppler-detectable bubbles. Further work is needed to determine whether breathing gas and physical activity level truly influence bubbles and physiological responses correlated with MPs. None of the divers in our studies manifested evidence of DCS, so further work is needed to support the notion of a link between MPs and the absolute risk of DCS.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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