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Presence of dipalmitoylphosphatidylcholine from the lungs at the active hydrophobic spots in the vasculature where bubbles are formed on decompression

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Arieli R, Khatib S, Vaya J. Presence of dipalmitoylphosphatidylcholine from the lungs at the active hydrophobic spots in the vasculature where bubbles are formed on decompression. *J Appl Physiol* 121: 811–815, 2016. First published August 11, 2016; doi:10.1152/japplphysiol.00649.2016.—Most severe cases of decompression illness are caused by vascular bubbles. We showed that there are active hydrophobic spots (AHS) on the luminal aspect of ovine blood vessels where bubbles are produced after decompression. It has been suggested that AHS may be composed of lung surfactant. Dipalmitoylphosphatidylcholine (DPPC) is the main component of lung surfactants. Blood samples and four blood vessels, the aorta, superior vena cava, pulmonary vein, and pulmonary artery, were obtained from 11 slaughtered sheep. Following exposure to 1,013 kPa for 20.4 h, we started photographing the blood vessels 15 min after the end of decompression for a period of 30 min to determine AHS by observing bubble formation. Phospholipids were extracted from AHS and from control tissue and plasma for determination of DPPC. DPPC was found in all blood vessel samples and all samples of plasma. The concentration of DPPC in the plasma samples ($n = 8$) was 2.04 ± 0.90 μg/ml. The amount of DPPC in the AHS which produced four or more bubbles ($n = 16$) was 1.59 ± 0.92 μg. This was significantly higher than the value obtained for AHS producing less than four bubbles and for control samples ($n = 19$) (0.97 ± 0.61 μg, $P = 0.027$). DPPC leaks from the lungs into the blood, settling on the luminal aspect of the vasculature to create AHS. Determining the constituents of the AHS might pave the way for their removal, resulting in a dramatic improvement in diver safety.

lung surfactant; nanobubbles; blood vessel; luminal aspect

THE MAIN LIMITATION ON DIVING is decompression sickness, in which bubbles evolve from gas that has been dissolved in the tissues and blood to become supersaturated at high pressure. Most severe cases of decompression illness (DCI) are caused by vascular bubbles, in particular on the arterial side. The various signs and symptoms of inflammation, neutrophil activation, and platelet aggregation observed in DCI are due to microparticles, which are composed of cell membranes stripped from the parent cell by bubbles (2, 12, 17). The main focus of research should therefore be directed at the vascular bubbles.

Tyrrell and Attard (16), among others, have shown that tiny, flat gas nanobubbles measuring 5–100 nm form spontaneously when a smooth hydrophobic surface is submerged in water containing dissolved gas. We recently showed that these nanobubbles are the seeds of the decompression bubbles which cause DCI (3, 4). We have also shown that there are active hydrophobic spots (AHS) on the luminal aspect of ovine blood vessels where bubbles are produced after decompression (2, 5, 6, 7). Thus permanent nanobubbles at the AHS are the seeds of decompression bubbles, and we have also suggested that they may be a cause of autoimmune disease (1). AHS are also to be found in the arterial circulation, where bubble production represents a high risk of DCI because of the possibility of direct blockage by bubbles of arteries in the central nervous system.

Hills (9) described a hydrophobic oligolamellar lining on the luminal aspect of ovine blood vessels. He also suggested, based on flushing of the hydrophobic layer with chloroform and an electron microscope examination, that it was composed of phospholipids whose source may be in the lung surfactant.

Dipalmitoylphosphatidylcholine (DPPC) is the main component (40%) of the lung surfactants. DPPC also has a higher compaction capacity than the other phospholipids, because its apolar tail is less bent (10). Thus it can be compacted into a dense homogeneous layer. The purpose of the present study was to determine whether DPPC is present at the AHS. In addition, although DPPC should not normally be found in blood vessels, if we assume a possible leak of DPPC from the lung into the blood, it should be identifiable within the systemic circulation. We therefore wished to investigate whether DPPC may be found in the plasma. Finally, there is high variability in bubble production between sheep and within the AHS. Some sheep have large numbers of active spots, whereas others have only a few. This is similar to the difference between bubblers and nonbubblers in diving (2). There are AHS which produce only a few bubbles, whereas others produce bubbles in greater quantities. We wished to establish a correlation between the activity of AHS (bubbling level) and the amount of DPPC that was found there. If we can establish the nature of the AHS, it may be possible to develop an appropriate treatment for their removal and thus prevent the formation of bubbles in the vascular system.

METHODS

The methods were described in detail in our previous report (2) and will be presented briefly here.

Tissue Preparation

The complete heart and lungs from 11 slaughtered sheep (taken on separate days) were obtained at the abattoir. A blood sample was also taken from the throat incision by using a heparinized syringe. In the laboratory, under saline and without any exposure to air, samples from four blood vessels, the aorta, superior vena cava, pulmonary vein, and pulmonary artery, were gently stretched with metal clips on micro-

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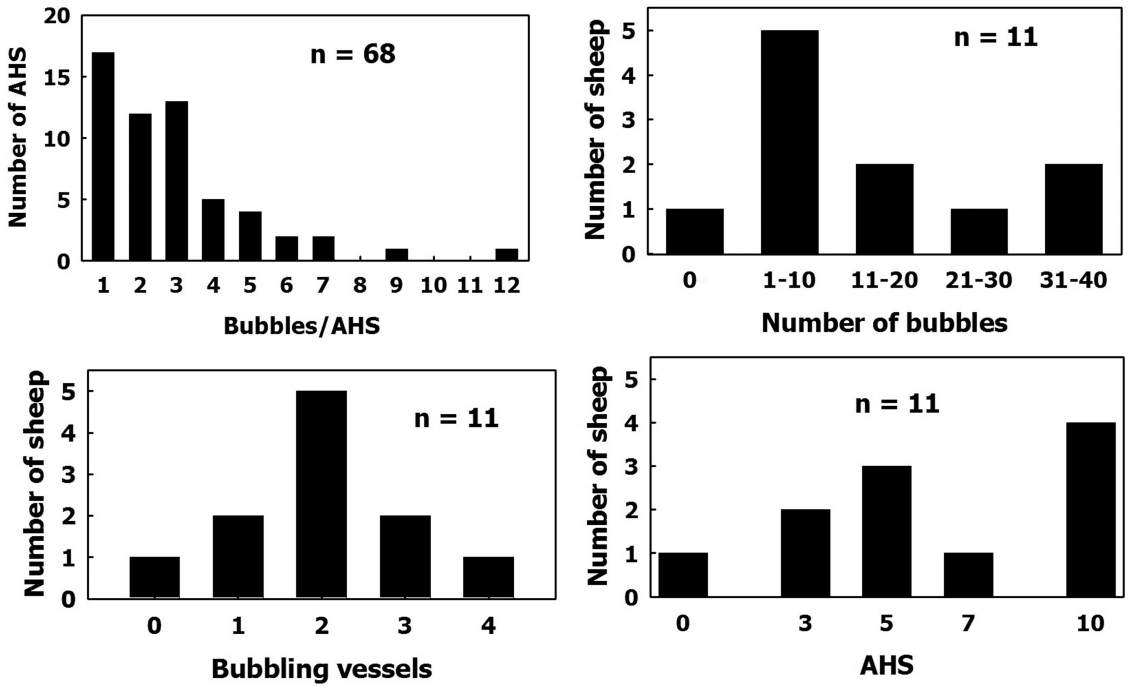

Fig. 1. Distribution of the four bubble-producing blood vessels: aorta, superior vena cava, pulmonary artery, and pulmonary vein (panel at *bottom left*); number of active hydrophobic spots (AHS) (panel at *bottom right*); and bubble production over 30 min (panel at *top right*) presented for 11 sheep. The total number of AHS as a function of the number of bubbles produced is shown in panel at *top left*.

scope slides, with the luminal aspect exposed. Four slides were placed anaerobically on the bottom of two Pyrex bowls (diameter 26 cm, height 5 cm), two in each bowl under 2.5 cm saline. One bowl was placed in the high-pressure chamber and the second was kept in a refrigerator at 6°C until the following day. Blood samples were centrifuged in a cooled centrifuge (4°C) for 15 min at 1,200 *g* for plasma separation. The plasma was kept in a freezer at −20°C until extraction of the phospholipids.

Protocol

AHS can be determined by observing the formation of bubbles after decompression. The bowl containing the two blood vessels was transferred to a 150-liter hyperbaric chamber and placed on a pair of welded aluminum plates, with cooled water circulating in the space between the two plates. Cooling during the hyperbaric exposure was necessary for tissue preservation. The temperature of the circulating water for the samples from the first two sheep was 12°C, and this was later reduced to 10, 9, and finally to 6°C. Saline temperature was 1°C above that of the circulating water. Pressure was elevated at a rate of 200 kPa/min to 1,013 kPa, 90 m of sea water, and remained at that pressure overnight (20.4 ± 1.0 h). In the morning, the chamber was decompressed at a rate of 200 kPa/min. The bowl was placed carefully on a nearby table for photography, with a flow of saline delivered over the blood vessel at a flow rate of 100 ml/min by using a peristaltic pump. It was shown in our previous study (2) that circulating saline enhanced bubble development by replenishing gas-saturated saline in the vicinity of the AHS. We began automated photographing at 1-s intervals 15 min after the end of decompression for a period of 30 min, which was found in previous studies to be the most bubble-productive period. At the end of this photographic session, the slide was photographed against scaled paper and was transferred to a freezer (−20°C). The same protocol was followed for the second bowl the next day.

The photographs of each sample were examined in sequence for the appearance of bubbles. Each bubble was observed until detachment. Bubbles produced near the edges of the tissue were not taken into consideration, and therefore the last 1 mm to the edge of the tissue was not included in the analysis. At the end of the observation period, the location of AHS and the number of bubbles produced at each were noted on the photograph that had been prepared for use during tissue sampling. The area of the blood vessel samples was measured with the image processing program Image-Pro-Plus (Media Cybernetics, Bethesda, MD). The mean sampled area of all the blood vessels was 10.3 ± 0.9 cm^2.

Phospholipid Extraction

Internal standard solution of 1 mg of 1,2-diheptadecanoyl-PC in 1 ml methanol was prepared.

Tissue sampling. While viewing the photograph of a blood vessel with its AHS, a section of the blood vessel containing the AHS was dissected out, weighed (0.028 ± 0.018 g), and placed in a test tube.

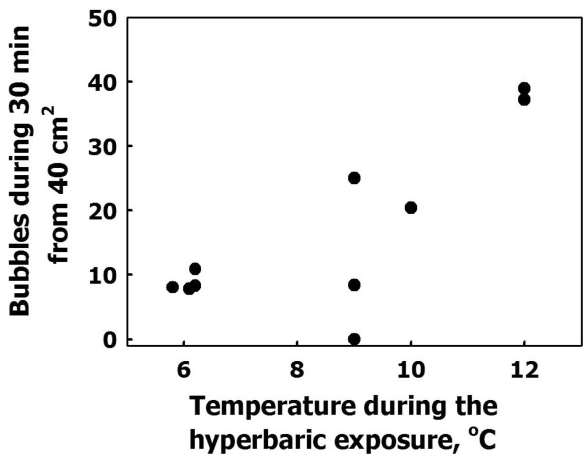

Fig. 2. Total bubble production in the four sampled vessels of a sheep as a function of the temperature during the hyperbaric exposure.

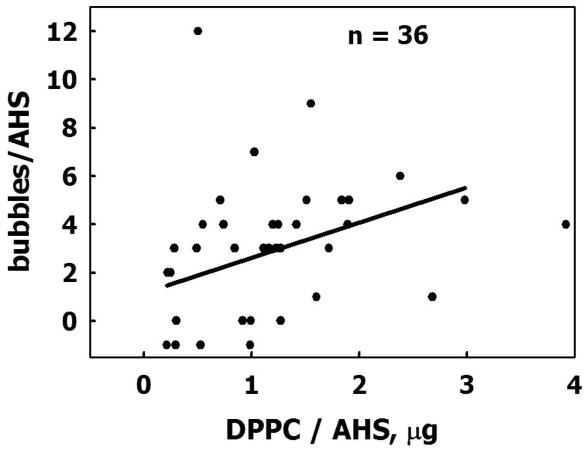

Fig. 3. The number of bubbles produced at AHS plotted against their DPPC content. Samples from a sheep in which none of the four vessels produced bubbles were given the value −1 for bubble production. The regression line is shown as derived from all the data excluding the two extremes.

For comparison, similar control samples were taken from blood vessels which did not produce bubbles. Two microliters of internal standard solution, 1 ml of chloroform, and 2 ml methanol were added. After homogenization for 2 min, another 1 ml of chloroform was added. Following another 30 s homogenization, 1 ml of double distilled water was added and a 30-s homogenization was performed. The homogenate was centrifuged (4°C, 2,000 rpm, 10 min), and the lower chloroform phase was collected.

Plasma sampling. Two microliters of internal standard solution, 2 ml of chloroform, and 1 ml methanol were added to 0.5 ml of plasma and vortexed for 2 min. The solution was centrifuged at 2,000 rpm for 10 min at 4°C. The lower chloroform phase was collected. Another 1 ml of chloroform and 0.5 ml methanol were added to the upper phase, vortexed for 2 min, centrifuged (4°C, 2,000 rpm, 10 min), and the chloroform phase obtained was added to the previous phase.

Common procedure. The following procedure was common to both the tissue and plasma chloroform phase. After the addition of ~250 mg sodium sulfate and vortexing for 30 s, the test tube was kept still for 10 min and then filtered through cotton. The solvent was dried by a flow of nitrogen, and the test tube containing phosphatidylcholine was stored in a freezer at −20°C for further analysis. Forty tissue extraction samples and 10 plasma samples were prepared for determination of DPPC.

Determination of DPPC

Samples were redissolved in 0.5 ml of methanol, vortexed and, centrifuged at 2,000 *g* for 10 min at 4°C. The supernatant was then collected and injected into a QTOF LC/MS (Agilent Technologies, Santa Clara, CA) for the analysis of DPPC and the internal standard (IS) 1,2-diheptadecanoyl-PC. Ten microliters of the supernatant was injected into a 1290 infinity LC system (Agilent Technologies, Santa Clara, CA) connected to a C-18 reverse-phase column, XTerra C18 3.5 μm, 4.6 × 20 mm (Waters, Milford, MA). The solvents used for separation of DPPC and the IS were solvent A (DDW with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). Solvent B remained at 93% for 17 min, then increased from 93% at 17 min to 98% at 20 min, remaining at 98% for another 5 min, with a flow rate of 0.5 ml/min. The LC eluent was introduced directly into the electrospray ionization (ESI^+) source connected to a ultra high definition accurate-mass Q-TOF LC/MS 6540 (Agilent Technologies, Santa Clara, CA). The ESI capillary voltage was set at 3,500 V, fragmentor 150 V, gas temperature 350°C, gas flow 8 ml/min, and nebulizer 35 psi. The mass spectra (m/z 100–1,700) were acquired in a positive-ion mode. The calibration curve of DPPC and IS was prepared in methanol at a concentration range of 0.05 to 5 ppm and injected under identical conditions. A linear curve was obtained with $R^2 = 0.9971$ and 0.9955 for DPPC and the IS, respectively.

Statistical Analysis

Normality test (Shapiro-Wilk) denied normality for the amount of DPPC in AHS and, therefore, Mann-Whitney rank sum test was used to compare the amount of DPPC in AHS which produced four or more bubbles, with the amount of DPPC in AHS producing less than four bubbles within 30 min and in control tissue samples.

RESULTS

Data for the number of blood vessels in each animal that produced bubbles, the number of AHS per animal, and bubble production per animal and per active hydrophobic spot are presented in Fig. 1.

There was only one sheep (no. 35 in our series) in which none of the four vessels produced bubbles (Fig. 1, panel at *bottom left*). Most of the sheep had two vessels that bubbled; all four vessels bubbled in only one animal.

Data for the total number of AHS on the four vessels for each animal (Fig. 1, panel at *bottom right*) show that only one (again no. 35) had no AHS on any of the four vessels. The median was 5 AHS per sheep, and four animals had 10 AHS.

Most of the bubbling vessels produced 1–10 bubbles over 30 min (Fig. 1, panel at *top right*). In one sheep there were no bubbles (no. 35); in two animals the bubbling vessels produced 31–40 bubbles.

AHS activity is represented by the number of bubbles produced at AHS over 30 min (Fig. 1, panel at *top left*). Most of the AHS produced 1–3 bubbles, whereas there were less AHS which produced larger numbers of up to 12 bubbles. Bubble production was thought to represent the activity of the AHS. However, it was most probably related to the temperature of the saline during exposure to hyperbaric pressure, as may be seen from Fig. 2. It seems that bubble production dropped when the exposure temperature was reduced. This may be related to the rate of diffusion during bubble growth after decompression, due to the temperature of the saline in the bowl and lower gas loading during hyperbaric exposure at a lower temperature. Thus bubble production may be an expres-

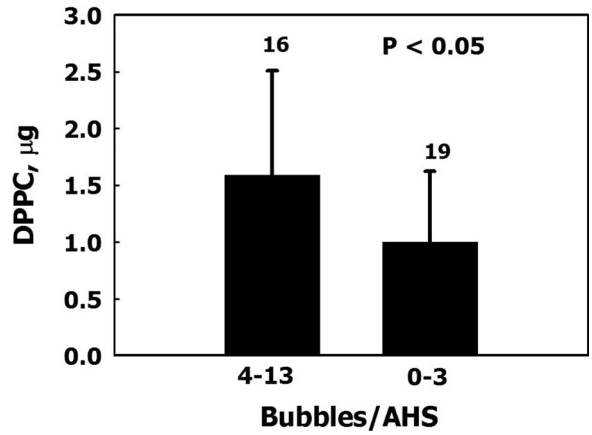

Fig. 4. The amount of DPPC (means ± SD) in AHS which produced more than four bubbles over 30 min compared with AHS, which produced less than four bubbles, and control samples.

sion not only of the activity of the AHS but also of the exposure temperature.

The blood sample from one of the sheep was inadequate for phospholipid extraction because of clotting. Dipalmitoylphosphatidylcholine was found in all the plasma samples ($n = 10$) and all the blood vessel samples ($n = 40$). There was a sampling error (erroneous addition of IS) in two plasma samples and five blood vessel samples, and these data were disregarded. The concentration of DPPC in plasma was 2.10 ± 0.80 μg/ml ($n = 8$). The amount of DPPC in each sampled tissue is shown on the *x*-axis of Fig. 3, with the number of bubbles produced by the sample shown on the *y*-axis. For tissue samples from an animal in which no bubbles were produced by any of the four vessels, bubble production was given the value −1. For the regression line, the two extreme points (shown in Fig. 3) were removed to present the possible trend. The amount of DPPC in AHS which produced four or more bubbles was 1.59 ± 0.92 μg ($n = 16$), which was significantly higher than in AHS which produced less than four bubbles and control samples (0.97 ± 0.61 μg, $n = 19$; $P = 0.027$; Fig. 4). Although bubble production was affected by the exposure temperature, a high bubble count was related to the large amount of DPPC at the AHS.

DISCUSSION

The presence of the lung surfactant dipalmitoylphosphatidylcholine in the systemic circulation and at AHS on the luminal aspect of various blood vessels, and its relation to bubble production on decompression, is a new finding. This brings to a crescendo and perhaps a finale the debate that has been going on for the past half century regarding the origin of gas micronuclei from which vascular bubbles form on decompression, and the finding 25 years ago of an oligolamellar hydrophobic lining on the luminal aspect of blood vessels (9). DPPC leaks from the lung into the blood. Because surfactants tend to settle close to each other from a dilute solution to create aggregates (14), the DPPC settles and creates AHS. When these reach a critical area, nanobubbles are formed and serve as gas micronuclei for decompression bubbles.

Reduction of the exposure temperature in the present series of experiments from 12 to 6°C interfered with possible correlations between the number of bubbles as a parameter indicative of AHS activity, the amount of DPPC in the AHS, the concentration of DPPC in plasma, and the distribution of bubblers and nonbubblers among the sheep. However, there were significantly greater quantities of DPPC in AHS which produced four or more bubbles than in less productive tissues, which agrees with the trend seen in Fig. 3 for increased bubble formation with a rise in DPPC. Further research under standardized conditions is required to establish these relationships. Our previous study (2) and others have shown that a detached bubble carries with it pieces of the underlying substrate, so reducing the content of DPPC at the AHS. Therefore the dependence of bubble formation on the amount of DPPC in the AHS could be greater than was established at the end of our observations in the present study.

Continuous deposition of DPPC at the ADH throughout life may provide an explanation for the increased risk in decompression sickness with age (8, 15). Removal of DPPC from the AHS by detached bubbles, which carry away pieces of the substrate, may provide an explanation for adaptation to diving by experienced active divers (2, 11, 13, 18).

The present findings warrant further research. The finding that DPPC is present in the systemic circulation may point to a possible relationship between the level of DPPC in plasma, the bubble activity of the AHS, and a definition of susceptibility to decompression sickness in divers. Determining the constituents of the AHS could pave the way for exploring methods of removing them. Eliminating the AHS may result in a dramatic improvement in diver safety.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

R.A. conception and design of research; R.A., S.K., and J.V. performed experiments; R.A., S.K., and J.V. analyzed data; R.A. and J.V. interpreted results of experiments; R.A. prepared figures; R.A. drafted manuscript; R.A., S.K., and J.V. edited and revised manuscript; R.A. approved final version of manuscript.

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