Endothelial damage by bubbles in the pulmonary artery of the pig

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Nossum V, Koteng S, Brubakk, AO. Endothelial damage by bubbles in the pulmonary artery of the pig. Undersea Hyper Med 1999; 26(1)1–8.—A method for measuring endothelial damage caused by decompression was developed for vessels with a large radius. Segments of the pulmonary artery from pigs (8–12 wk old) were tested for endothelium damage using a system for recording changes in the tension in the vessel wall. Substance P (SP) was used as an endothelial-dependent dilation agonist. A significant decrease was found in the total response (T_{max}) for SP as a result of endothelium damage, and the reduction in response was related to the number of bubbles. Furthermore, the sensitivity of the vessels to the agonist was significantly reduced after exposure to bubbles. Staining the endothelium with silver nitrate and light microscopy confirmed mechanical endothelium damage.

endothelial damage, tension measurements, pulmonary artery, bubbles

In addition to the important role the lungs have in gas exchange, they participate in generation, activation, and inactivation of biological active compounds (1). These compounds influence the tone of vascular smooth muscle cells in the airways and circulatory system; they can also trigger inflammatory responses. Damage to the pulmonary circulatory system can lead to serious changes in the lungs' functions and responses (2).

The endothelial regulation of cerebral vessels has received increasing attention in recent years (3). Acetylcholine, adenosine 5-triphosphate, 5-hydroxytryptamine (5-HT), substance P (SP), vasopressin, angiotensin II, histamine, and bradykinin all produce endothelium-dependent responses. These agonists, of which SP is the strongest acting, act by releasing endothelium-derived relaxing factor (EDRF) with vasodilating capabilities from the endothelial cells. EDRF is considered to be nitric oxide (NO) (4). Endothelial damage in blood vessels is associated with total or partial loss of relaxation response in the vascular smooth muscle cells as a response to an endothelial-dependent dilator (5).

Studies have shown that gas bubbles are formed in the venous system in nearly all decompressions (6), and the risk of developing decompression illness (DCI) increases with the number of gas bubbles (7). These gas bubbles can cause mechanical damages as cracks and wounds in the endothelial layer of the vessel wall. Neutrophils and blood platelets will be degraded in these cracks. Fibrin can also attach itself, and the endothelial cells in bronchial venules will swell and burst. Edema can also occur in the peribronchiolar part of the pulmonary artery (8). The damage is not necessarily caused mechanically but by interaction between the bubble surface and components of the blood (9). The endothelial cells can be torn from their basal layer and the endothelial nuclei protrude into the lumen. This leads to increased permeability for proteins in the endothelial layer. If the gas bubbles have the same effect in the brain vasculature, it might cause a decreased function in the blood–brain barrier (10).

The exact mechanism for endothelial damage is not clear, but is probably related to leukocyte activation (11), leading to the release of oxygen radicals (12) in addition to the mechanical changes in the endothelial cells. Vasoactive substances such as bradykinine (13) and smooth muscle activating factor (SMAF) (14) have been found in the lungs after decompression. These vasoactive substances induce separation of intracellular junctions in the endothelium (15). By studying fluid transport over the vessel wall, endothelial damage can be confirmed (16).

Although it is well documented that gas bubbles can damage the endothelium, there is a need to develop methods that can quantify this damage. Previous studies have shown that the relaxation and contraction of the muscular layer of the arteries is dependent on an intact endothelial layer and that this can be studied in vitro by studying the relaxation response (17). In the present study, a modification of this method was used for studying endothelial damage caused by bubbles from decompression.

MATERIAL AND METHODS

Subjects and compression profile: Two groups of pigs were studied: four controls and eight experimental animals, aged 8–11 wk and weighing 24 ± 2.0 kg, were fasted for 40 h with free access to water. Thirty minutes before induction of anesthesia, the pigs received premedication: 7–9 mg · kg^{-1} azaperonum (Sedaperone, Janssen) was injected intramuscularly; atropine sulphate (1 mg, atropine, Hydro Pharma) was thereafter given intravenously via an ear vein. Anesthesia was induced by thiopental sodium (5 mg · kg^{-1} thiopenton natrium, Nycomed Pharma) and ketamine (20 mg · kg^{-1}, Ketalar, Parke Davis) and maintained throughout the experiment by a continuous i.v. infusion of ketamine in 0.9% NaCl (30 mg · kg^{-1} · h^{-1}) together with bolus doses of α-chloralose in 0.9% NaCl (10–15 mg · kg^{-1}, 0.25% solution). The four control animals were then given a lethal dose of pentobarbital or potassium chloride. The eight experimental animals were placed in a pressure chamber, lying on their back, breathing spontaneously. They were compressed down to 500 kPa (40 m) in 4 min. They stayed at this level for 3 h, followed by linear decompression at a rate of 200 kPa · h^{-1}. At 500 kPa the animals were breathing a heliox mixture. The O_2 tension of the breathing gas was 35 kPa, and the O_2 tension was increased to 100 kPa during decompression. One hour after decompression was ended, the animals were given a lethal dose of pentobarbital or potassium chloride intravenously. The experimental protocol was reviewed and approved by the Norwegian Committee for Animal Experiments.

Bubble detection: The number of gas bubbles in the pulmonary artery were detected using a 5 MHz transesophageal transducer connected to an ultrasonic scanner (CFM 750, Vingmed Sound, Horten, Norway). The images were transferred to a computer and counted continuously using a program previously described (18). The results are given as number of bubbles per square centimeter.

Equipment for tension measurements: The recording equipment was custom made for this study, and specially developed for measuring precise tension changes in the vessel wall from segments with large diameter. The design can be seen in Figs. 1 and 2. The metal prongs are parallel with their ends pointing in the same direction. This makes it easy to mount the cylindrical vessel segment. One prong was connected to a force displacement transducer attached to a vessel tension measuring instrument (Fig. 1) for continuous recording of the isometric tension, and the other to a displacement device. The position of the holder could be changed by means of a mobile unit allowing fine adjustments of the vascular tension by varying the distance between the metal prongs. The vessel tension measuring instrument has four buffer containers. The measuring units (called channels) are mounted next to each other, one for each buffer container, with a shared display for reading temperature and tension in millinewton (Fig. 1). The instrument has a button for switching between temperature and tension for each channel. Adjustment for drift caused

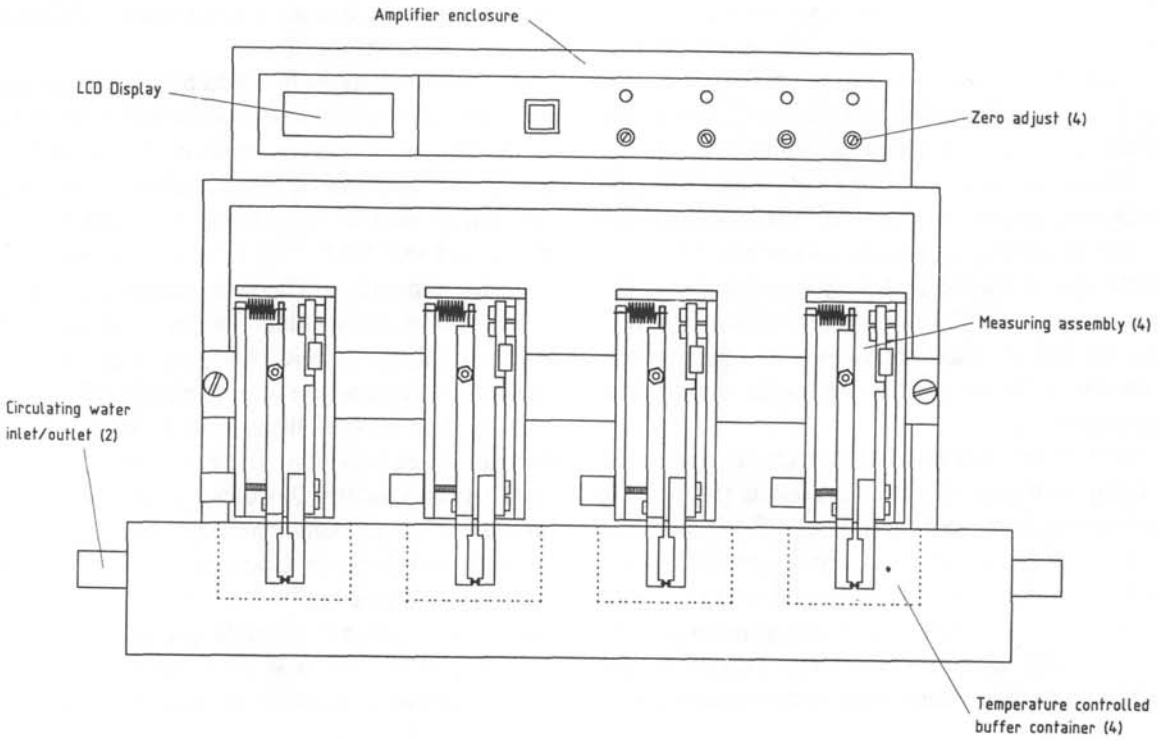

FIG. 1—Vessel tension measuring instrument, front view (IFBT, NUST, Norway).

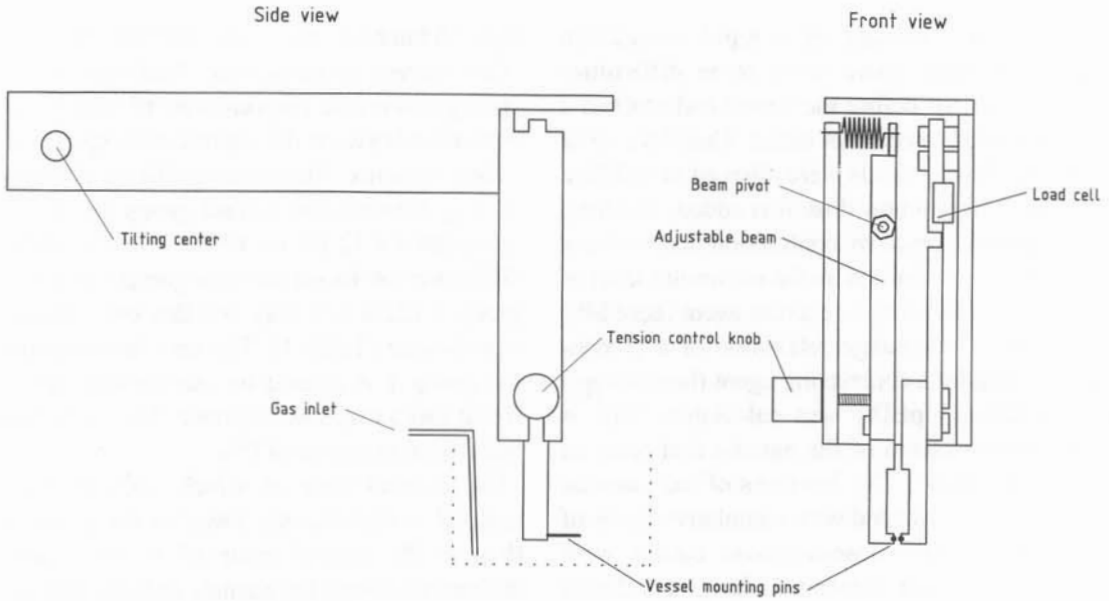

FIG. 2—Vessel tension measuring instrument, measuring unit (IFBT, NUST, Norway).

by temperature changes, and calibration is also possible for each channel. The instrument frame is made of 8-mm Plexiglas with polystyrene buffer containers. The channels are also connected to this frame, and each one can be tilted to make the mounting of the vessel segments easier (Fig. 2). Each channel has a thin film cantilever beam transducer S-100-0N5 (Strain Measurement Devices Ltd.) for recording of mechanical activity. The signals from the transducers go through an amplifier which is connected to a Macintosh computer where a specially designed LabVIEW program processes the results (Edvinsson, Lund University).

From a separate thermostat-controlled water reservoir, water with the correct temperature is pumped into the polystyrene frame to ensure that the buffer temperature is correct at all times. There were no significant differences in the buffer temperature among the four buffer containers. Five percent CO_2 gas was pumped into the buffer for control of the pH, and the amount of gas injected could be controlled by a valve for each buffer container. Two Vessel Tension Measuring Instruments, each containing four channels and buffer containers, were connected in series with a common water reservoir. A total of eight vessel segments could therefore be tested at the same time.

Experimental procedure: The first branch of the pulmonary artery was carefully dissected from the right lung. The vessel was then rapidly placed in cold oxygenated (5% CO_2:95% O_2) Na-Krebs buffer solution of the following composition in millimolars: 119 NaCl, 10 $NaHCO_3$, 1.2 $MgCl_2$, 4.6 KCl, 1 NaH_2PO_4, 1.5 $CaCl_2$, and 11 glucose, and stored cold for a maximum of 48 h. Before each experiment the vessels were cut into cylindrical segments (1.5–2.5 mm), with a diameter between 2 and 3 mm. Each cylindrical segment was mounted on two parallel L-shaped metal prongs. Special care was taken to ensure that the endothelial layer was not damaged. The control specimens was checked for endothelial damage by a staining method using silver nitrate to ensure that the technique itself did not cause significant endothelial damage. If the endothelial layer was intact it would appear as a brick-like pattern in a light microscope, and it was easy to see if it was disrupted. There were no signs of damage caused by the dissection. The mounted specimens were immersed in temperature-controlled (37°C) tissue baths containing Na-Krebs buffer. Five percent CO_2 in O_2 was continuously bubbled through the solution to keep the pH at 7.4.

Method: This method is based, with some modifications, on the method described by Edvinsson et al. (17,19), and the vasomotor reactivity was analyzed using a modification of a tissue-bath technique originally described by Högestätt (20).

A tension of 8–10 mN was applied to the segments and they were allowed to stabilize at this level of tension for 1–1.5 h. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 m*M*) K-Krebs buffer solution that had the same composition as the Na-Krebs buffer solution except that some of the NaCl was replaced by an equimolar concentration of KCl.

The vessels were precontracted with cumulative doses of norepinephrine until they had reached a stable level (50–75% of the response to potassium). Thirty minutes after stabilization, cumulative doses of SP were added in dose steps (10^{-12}–10^{-7} *M*). The response that followed depended on how much of the endothelial layer was dam-

aged by the bubbles. Because of a rapid contraction following the relaxation, there were some difficulties adding a new dose of SP before the vessel had reached a stable level and started to contract again. Therefore, after each new dose of SP the vessels were allowed to stabilize for a few minutes before a new dose was added. The total response, relaxation followed by contraction, led to a new term called T_{max}, which is defined as the maximum level of stabilized relaxation induced by a relaxing agent (here SP). T_{max} is expressed as percentage relaxation of a precontracted level, induced by a contracting agent (here norepinephrine). In addition $-pED_{50}$ was calculated. This is defined as the concentration of the agonist that leads to 50% of the total response. The functions of the vascular smooth muscle cells were tested with cumulative doses of sodium nitroprusside, and dose-response curves were calculated. Because sodium nitroprusside is endothelial independent, those vessel segments that did not respond had a functional failure not only in the endothelial layer, and these segments were rejected.

To evaluate the ability of the method to measure endothelial damage, some control segments with the endothelial layer rubbed off mechanically by a wooden stick were also tested and stained. All values are the mean value from three or four segments from the same vessel.

Staining: After the experiment, each segment was cut open in strip form and laid down on an object glass with the vessel lumen-side up. Then it was covered with 1% $AgNO_3$ for 2 min, followed by a mix (1:1) of 3% BrH_4N and 3% Br_2Co for 2 min more. The vessel segment was not fixed in any way, and had to be photographed in a light microscope immediately. Magnification was ×250 and ×500.

Drugs: (±) Norepinephrine[+]-hydrogen-tartrate, substance P, sodium nitroprusside-dihydrate (all Sigma, St. Louis, MO) and silver chloride (staining) were dissolved in saline or small amounts of distilled water. All concentrations given are final molar concentration in the tissue bath during the experiments.

Statistics: The data were analyzed using Student's *t* test for unpaired data. $P < 0.05$ was accepted as significant. The results shown in tables, figures, and the text are expressed as the mean value ± standard deviation or standard error of mean.

RESULTS

Pulmonary artery bubbles: Gas bubbles were detected during a 3-h observation period after decompression in all animals in the experimental group. They were divided into two groups according to the amount of bubbles. Animals scoring up to 1.5 bubbles · cm^{-2} maximum observed during a 3-h period were classified as group 1 ($n = 4$), those with more than 1.5 bubbles · cm^{-2} were classified as group 2 ($n = 4$).

Contraction by potassium: Potassium (60 m*M*) induced strong contractile responses in all vessels. There was no difference between the control and experimental animals.

Total response: There is a significant difference, $P < 0.05$, in T_{max} between the control group and the experimental group (84.4 ± 12.1% vs. 43.7 ± 35.3%). Moreover, a large difference was found between group 1 (64.6 ± 40.5%) and group 2 (22.8 ± 9.9%), but this did not reach statistical significance (Table 1). The very large standard deviation for group 1 is caused by one animal with few bubbles which had a very low response. The rubbed control vessel had a total response of 0%.

The concentration at which 50% of the response is reached is significantly lower in the experimental group than in the control group ($P < 0.01$), and there is no difference between the animals with few and many bubbles. Concentration-response relaxation curves were obtained by adding SP cumulatively. Figure 3 *top* shows the response (mean ± SEM) for all concentrations in the control group and the experimental group; whereas Fig. 3 *bottom* shows the same response in groups 1 and 2.

Total response related to the number of bubbles: The number of vascular gas bubbles and exposure time differed between the experimental animals. Most of the animals with high levels of vascular gas bubbles had low T_{max} for SP. Figure 4 shows total response for each experimental animal related to their maximum amount of bubbles per square centimeter during a 3-h observation period. This indicates how the total response (T_{max}) in the vessel decreases with increasing number of bubbles.

Figure 5 shows the relationship between the number of bubbles integrated over a 3-h observation and the reduction in T_{max}. The integrated bubble number can be considered a measure of the total decompression stress. This shows essentially the same picture as that in Fig.4, indicating that

Table 1: Total Response Values for Each Group Values Represent Mean ± SM[a]

	Substance P	
Group	$-pED_{50}$	T_{MAX}, %
Control group	11.6 ± 1.3	84.4 ± 12.1
Experimental group	10.01 ● 0.48**	43.7 ± 35.3*
Group 1	10.04 ± 0.44	64.6 ± 40.5
Group 2	9.98 ± 0.58^{NS}	22.8 ± 9.9^{NS}
Rubbed control	–	0

[a]Statistics: Student's *t* test for unpaired data. Level of significance, *$P < 0.05$, **$P < 0.01$, ^{NS} = not significant.

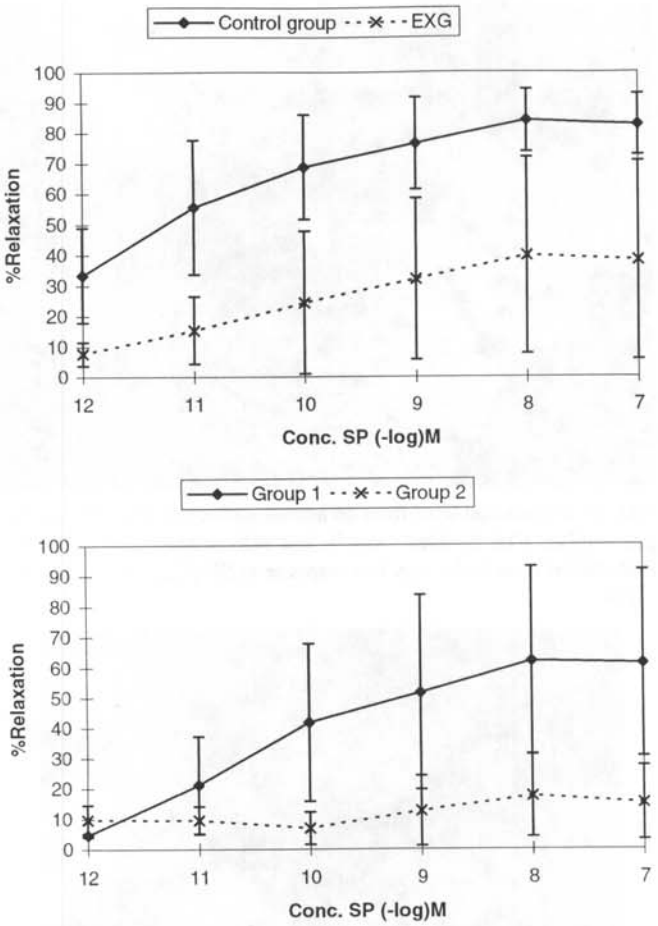

FIG. 3—*Top*, Concentration-response relaxation curves obtained by adding SP cumulatively (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} *M*) to isolated pulmonary artery for the control group (◆) and the experimental group (×). Each dot symbolizes mean ± SEM. *Bottom*, concentration-response relaxation curves obtained by adding SP cumulatively (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} *M*) to isolated pulmonary artery for group 1 (◆, less or equal to 1.5 bubbles · cm^{-2}) and group 2 (×, more than 1.5 bubbles · cm^{-2}). Each dot symbolizes mean ± SEM.

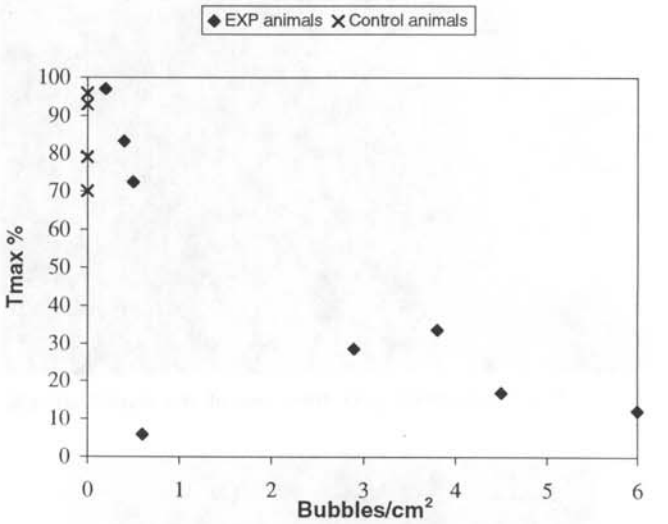

FIG. 4—Total response for each experimental animal related to its maximum amount of bubbles · cm^{-2} during a 3-h observation period. ◆ = experimental animals; × = control animals.

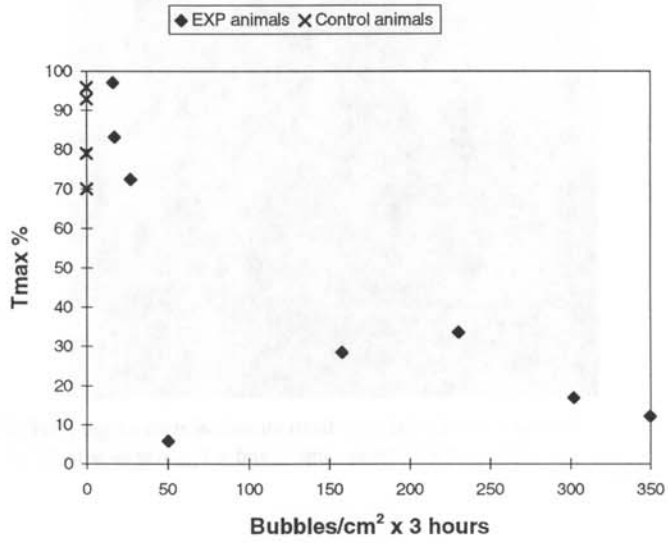

FIG. 5—Endothelial response (expressed as T_{max}) for the experimental group related to total bubble stress during a 3-h observation period (integral), expressed as bubbles · cm^2 × 3 h. ◆ = experimental animals; × = control animals.

an injury mainly is related to the maximum number of bubbles and not to the duration of the exposure.

Microscopic analysis: Observations in the light microscope confirmed the damage to the endothelial layer found by measuring the changes in the tension in the vessel wall. The observations were photographed and we made a comparison between the pictures and the number of gas bubbles. No damage was seen in the endothelium in the control animals. Figure 6 shows an example of an intact endothelial layer. When comparing the pictures in the experimental animals to the reduction in T_{max}, we could see that pigs with high levels of vascular bubbles all showed serious damage in the endothelial layer compared to the controls when studied in the light microscope. One of the pigs with few vascular gas bubbles also showed large disruptions in the endothelial layer, and all of these segments had very low T_{max} responses. The amount of endothelial damage seems to be connected to T_{max} response to SP. Figure 7 shows the damage in the endothelial layer (*black spots*) of the pulmonary artery from an animal with a high level of vascular gas bubbles (6.0 bubbles · cm^{-2}) and a T_{max} response to SP of 12.1%. Figure 8 shows an example from an animal with a low level of vascular gas bubbles (0.6 bubbles · cm^{-2}), but with significant damage to the endothelial layer and a very low response to SP (T_{max}: 5.8%). Figure 9 shows the rubbed control vessel with totally disrupted endothelial layer and no response to SP (T_{max}: 0.0%).

It is not possible to make an accurate quantification of the endothelial damage observed under the microscope.

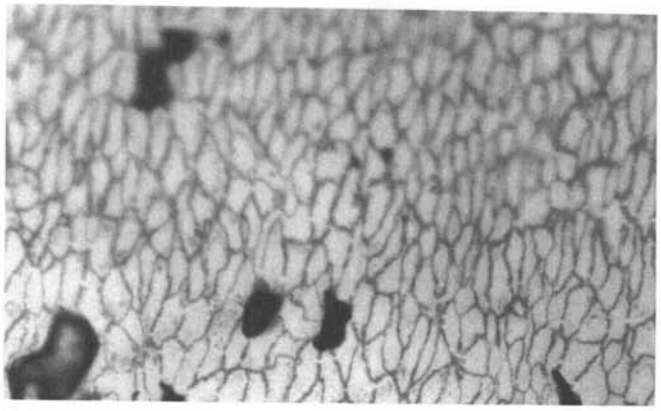

FIG. 6—Intact endothelial layer from one of the control animals. Original ×500.

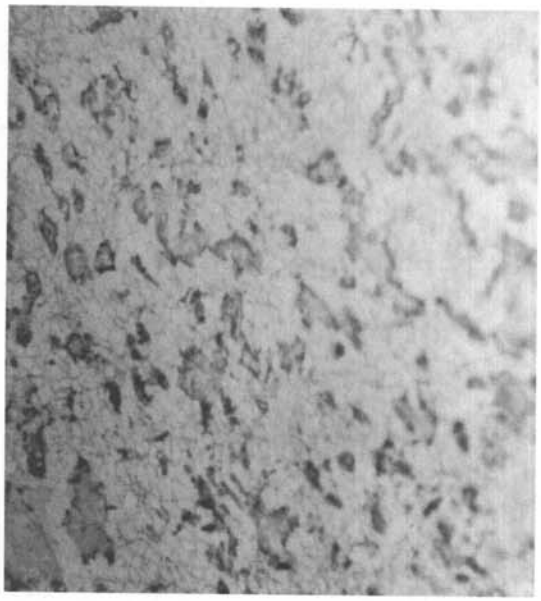

FIG. 7—Damaged endothelial layer from an animal with a high level of vascular gas bubbles (6.0 bubbles · cm^{-2}) and a T_{max} response to SP of 12.1%. ×250.

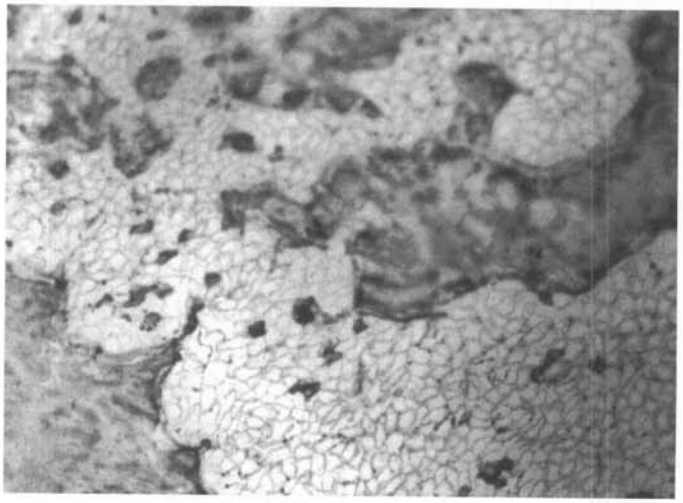

FIG. 8—Endothelial layer from an animal with a low level of vascular gas bubbles (0.6 bubbles · cm^{-2}), but with serious damage to the endothelial layer and a very low response to SP (T_{max} 5.8%). Original ×250.

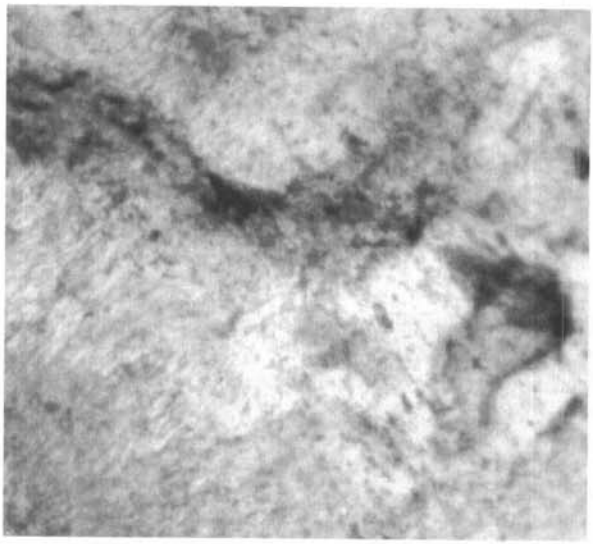

FIG. 9—Totally damaged endothelial layer in the rubbed control. ×250.

DISCUSSION

Intravascular gas bubbles may be formed during decompression, and there is great individual variability in the tendency to form gas bubbles (13). This study showed that gas bubbles from decompression can lead to a reduced response to SP measured with our technique, and that the reduced response seems to be related to the maximum number of bubbles observed during a 3-h period.

All previous studies have measured the reduction in tension of the vessel wall caused by total mechanical disruption of the endothelial layer, whereas we wanted to study the effect of partial endothelial damage caused by bubbles. Our vessels are also of a larger radius than those used in previous studies. In this study the vessels where allowed to stabilize after adding each new concentration of dilating agonist. Initial studies showed that the vessels reacted with an immediate contraction following the relaxation caused by SP, thus making it impossible to reliably add another dose at the time of the maximum relaxation response. What causes this contraction and whether it is endothelial dependent is uncertain. Yang et al. (21) has suggested that cyclooxygenase-dependent, endothelium-derived contracting factors (EDCF) are released together with NO, and that this limits the effect of the vasodilatation. This would be in accordance with earlier studies by Vincent et al. (22) who showed that in the eye artery in the pig, the relaxation caused by SP ($10^{-8}M$) reaches a maximum after 1 min (70% relaxation), and then starts to fade until it reaches the precontracting level after 7 min. Another study also showed that the response to SP is dependent on an intact endothelium, but the relaxation is rather

short and is followed by a contraction (23). The relaxation by SP is mediated through the release of acetylcholine whereas contraction is via prostaglandin action. If the endothelial layer is removed, the relaxation is abolished (23).

Substance P can probably also work as a vasoconstrictor directly on vascular smooth muscle cells if the endothelium is partly or totally lost (24). This is also in accordance with our studies where lower concentrations of SP gave contractions in the rubbed control. In the four control animals, the total response (T_{max}) varied between 96 and 70% of precontraction, and this response consisted of both a relaxation and a contraction phase, with a relaxation phase approximately double that of the contraction. When the endothelium was mechanically removed, the vessel responded to SP with an initial contraction but ended up with a total response of 0%. Previous studies have shown that the relaxation response to SP is quite sensitive to endothelial damage, a light rubbing is sufficient to abolish the response (25).

From the above, it seems that SP has vasoconstricting as well as vasodilating effects on the vessel wall. These are endothelium dependent, and when the endothelium is intact the vasodilating effects predominate. Destruction of the endothelium allows SP to reach the subendothelial smooth muscles, causing a contraction and leading to a reduction in the total response (T_{max}) to the drug.

The lowest values for T_{max} were found in group 2, the animals that had been exposed to more than 1.5 bubbles · cm^{-2}. This is a number of bubbles that roughly corresponds to grades III and IV on the Spencer scale (26). In fact, except for one animal with few bubbles and a low response, the animals in the group with few bubbles had no reduction in T_{max}. It is perhaps dangerous to draw too many conclusions from few data, but Sawatzky and Nishi (27) found that at least one single instance of grades III and IV bubbles was seen in 95% of all divers with clinical symptoms of DCI, and there is a clear statistical relationship between the occurrence of grade III and IV bubbles and DCI (6). However, the observation that one animal with few bubbles had considerable damage to the endothelium and a large reduction in T_{max} indicates that the individual's biological reaction to the bubbles is of importance.

Mean $-pED_{50}$ for SP in the experimental group was lower than in the control animals. This indicates a decreased sensitivity if gas bubbles are present. The experimental group needed a higher concentration of SP (16 times more) to give a 50% reduction of T_{max}. There seems to be a connection between T_{max} and $-pED_{50}$ when the experimental group is compared to the control group. There is, however, no difference in $-pED_{50}$ between the two groups with different bubble numbers. This indicates that even small amounts of gas are able to achieve an increase in sensitivity, maybe by destroying receptors for SP. In the segments where the endothelium was destroyed mechanically, the sensitivity was reduced even further (Table 1).

Considering observations made under the light microscope, it seems that as the number of gas bubbles increases, the possibility for endothelium damage also increases. Observations under the microscope confirm the results from measuring the changes in tension in the vessel wall.

Several previous studies have shown that gas bubbles can lead to endothelial damage (10,28). This study supports this and further shows that this damage is related to the amount of gas present. It is not unlikely that the bubble size could play a factor and that bubbles with a large diameter make more damage than bubbles with a small diameter. It is not possible to measure the absolute size of the bubbles with our method. We do believe, however, that the majority of the bubbles are the same size, due to the effect of mixing the blood in the right heart, achieving the same effect as breaking waves in seawater (29). This is further confirmed as all bubbles observed have a similar intensity, the reflected intensity is proportional to the geometric cross section of the bubble (30).

More studies are needed to document this further and to show the mechanism for such damage.

This study was supported by Philips Petroleum Norway as part of the HADES project. The help of Ingunn Gjeving Holmen, Astrid Hjelde, and Anne-Lise Ustad in performing the pig experiments, and Arnfinn Sira for the developing of the Vessel Tension Measuring Instrument is gratefully acknowledged. The pig experiments have been supported by Statoil, Norsk Hydro, Saga and Norwegian Petroleum Directorate as part of the OMEGA project.—*Manuscript received April 1998; accepted November 1998.*

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