Eur J Appl Physiol 2002) 86: 209–214
DOI 10.1007/s00421-001-0531-y

ORIGINAL ARTICLE

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Small amounts of venous gas embolism cause delayed impairment of endothelial function and increase polymorphonuclear neutrophil infiltration

Accepted: 14 September 2001 / Published online: 10 November 2001

Abstract Gas bubbles from decompression and gas embolization lead to endothelial dysfunction and mechanical injury in the pig, rabbit and lamb. In the study presented here, 0.01 ml air/min/kg was infused through a catheter into the jugular vein in 12 rabbits for 60 min. The endothelial response was measured using tension measurements in the blood vessel wall, and morphological changes where quantified using light microscopy and image processing. Percent lung water content was calculated and used to estimate the severity of pulmonary oedema. The infusion led to a significant decrease in the acetylcholine-mediated endothelial-dependent vasodilatation in the pulmonary artery 6 h after the infusion 26-h group, $n=6$). A decrease in substance-P-mediated endothelial-dependent vasodilatation was also detected. No changes where seen in a group of rabbits examined 1 h after infusion 21-h group, $n=6$). The impaired endothelial-dependent vasodilatation caused by the bubbles is probably biochemical in origin, since no visible changes were seen in the endothelial layer. A significant increase in polymorphonuclear neutrophils was observed in the 6-h group compared to the 1-h group. This study demonstrates that small numbers of bubbles, corresponding to ''silent bubbles'', lead to an impairment of the endothelial-dependent vasoactive response.

Keywords Pulmonary artery · Neutrophils · Endothelial cells · Vascular bubbles · Lung oedema

Introduction

Intravascular gas bubbles occur in the venous system during most decompressions 2Brubakk et al. 1986; Eckenhoff et al. 1990). Venous gas bubbles may also develop following laparoscopy, by accidental injection or in cardiopulmonary bypass surgery 2Hoka et al. 1997; Johnston et al. 1993; Webb et al. 1997), and they lead to endothelial damage 2Nossum et al. 1999; Philp 1974; Warren et al. 1973). As the number of gas bubbles increases, the likelihood of endothelium damage also increases, and such damage seems to be related to the amount of gas present 2Nossum et al. 1999).

The amount of endothelial damage resulting from gaseous microemboli may be important because endothelial cells are the source of many vasoactive factors, including nitric oxide 2NO). NO regulates the diameter of blood vessels and blood flow and it is an important mediator of pulmonary vascular tone 2Busse et al. 1993; Stewart and Baffour 1990). After increased shear stress or binding of vasodilators, such as endothelium-dependent vasodilators, to surface receptors, vascular endothelial cells synthesize NO via activation of the enzyme endothelial NO synthase 2eNOS; Mülsch et al. 1989; Rubanyi et al. 1986). NO then diffuses into the underlying vascular smooth muscle cells, activates soluble guanylate cyclase and initiates a cascade resulting in smooth muscle relaxation 2Fiscus 1988). If the endothelial lining becomes disrupted or damaged by gas emboli, endothelium-dependent vasodilatation could be depressed. This may cause a reduction in regional perfusion 2Helps et al. 1990) and an exaggerated response to vasoconstrictor agents 2Busse et al. 1993; Ku 1987; Stewart and Baffour 1990). A reduced production of NO, a decrease in the density or number of receptors for acetylcholine and substance P, or an altered function of these receptors, can lead to loss of endothelium-dependent pulmonary vasodilatation. Alternatively, there may be an increased degradation of NO or inhibition of eNOS activity 2Fineman et al. 1999).

To determine whether infusion of air through the heart and into the pulmonary artery impairs the endothelium-dependent regulation of pulmonary vascular tone, we studied the response to endothelium-dependent vasodilators using an in vitro method for isolated

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vessels. We compared the changes in endothelial-dependent vasodilatation to acetylcholine and substance P, and endothelial-independent vasodilatation to sodium nitroprusside after 60 min of air infusion in two groups of animals, 1 and 6 h after infusion. The endothelial layer was examined using light microscopy to evaluate possible mechanical damage to the endothelial lining.

Methods

Twelve locally bred New Zealand Black rabbits of both genders, weighing 2.8–3.4 kg, were used. No signs of illness were detected in these animals during the study period. The experiments were performed in accordance with the Principles of laboratory animal care 2NIH Publication NO 85–23 revised 1985). The experimental protocol was approved by the Norwegian Committee for Animal Experiments.

Anaesthesia

The rabbits were tranquillized with an intramuscular injection of midazolam 25 mg; Dormicum, Hoffmann-La Roche, Basel, Switzerland) and fluanisone 27 mg) + fentanyl 20.22 mg; Hypnorm, Janssen-Cilag, Saunderton, Buckinhamshire, UK). Within 60 min the rabbits received an additional intramuscular injection of half of the above dose of both midazolam and fentanyl/fluanisone. Thirty minutes before the observation period, the rabbits were given an intramuscular injection of Buprenorfin 20.02–0.05 mg/kg; Temgesic, Reckitt and Colman). Body temperature was measured with a rectal probe and kept at 39.0 20.5)°C by means of a heating blanket. The animals were allowed to breathe spontaneously throughout the experiment.

Infusion

A catheter 20.36 mm inner diameter) was placed into the jugular vein and moved centrally. Air was infused for 60 min by a syringe in a special build pump. The amount of air infused was 0.01 ml/kg/min, corresponding to 8–10 bubbles/min. Blood-gases 2oxygen and carbon dioxide tension, PO_2 and PCO_2, respectively) and pH were monitored before, during and 1 and 6 h after air infusion 21-h group and 6-h group, respectively).

Bubble detection

Gas bubbles were detected in the heart using a 5-MHz transducer connected to an ultrasonic scanner 2750 Vingmed, Horten, Norway). The number of gas bubbles was evaluated using a grading system from 0 to 5 2Eftedal et al. 1994): grade 0 is no bubbles, 1 represents an occasional bubble, 2 represents at least one bubble every fourth heart cycle, 3 is at last one bubble every heart cycle, 4 is continuous bubbling, and 5 is massive bubbling. This scoring system is approximately exponential compared with the number of bubbles in the right ventricle 2Eftedal et al. 1994). The grades observed were converted to bubbles/cm^2 using the conversion table given by Eftedal et al. 21998).

Observation period

The rabbits were divided into two groups of six animals, the 1-h group was observed for 1 h after air infusion, and the 6-h group was observed for 6 h after air infusion. After the observation period, the animals were given a lethal intravenous dose of potassium chloride, under anaesthesia 2midazolam 5 mg and fluanisone 7 mg + fentanyl 0.22 mg). The lungs were immediately harvested.

Wet-dry weight of the lungs

The dry weight of the lung tissue was determined from a less than 1-g section of the left lung. The tissue was weighed 2wet weight), incubated at 120°C for 7 days, and then weighed again 2dry weight). Percent lung water content [2wet weight – dry weight)/wet weight×100] was used to estimate the severity of pulmonary oedema.

Lung histology

From all rabbits selected samples from the right and left upper and lower lung were fixed in a solution consisting of 70% ethanol, 4% formaldehyde and 5% acetic acid. Four samples per animal were taken. On the next day the specimens were transferred to 80% ethanol, before dehydration and embedding in paraffin for histopathology. Sections were cut at 5 μm and stained with haematoxylin-eosin-safran. An investigator who was blinded to treatment estimated the accumulation of polymorphonuclear neutrophils 2PMNs) present in the tissue. Four fields from each lung section were examined at ×400 with the aid of a Nikon YS2-H light microscope. This microscope was equipped with an eyepiece containing a 10×10 graticule grid 20.5×0.5 cm). The number of grid points falling on the tissue determined the field area. By counting the total number of PMNs in that field divided by the number of lung tissue grid points, the number of PMNs per unit lung tissue was calculated. Each rabbit was represented by a mean value of eight data points from both the left and right lung. The results are expressed as mean number of PMNs per unit lung tissue, and one unit lung tissue = 0.25 mm^2.

Tension measurements of isolated vessels

A modified tissue-bath technique 2Edvinsson et al. 1974; Högestätt et al. 1983) was used, as described previously by 2Nossum et al. 1999, 2000). The pulmonary artery was carefully dissected from the right lung with the aid of a dissection microscope. The vessels were cut into cylindrical segments with length ranging from 1.0–1.5 mm and with a diameter between 1 and 2 mm. Each cylindrical segment was mounted on two parallel L-shaped metal prongs and immersed in temperature-controlled 237°C) tissue baths containing a sodium-Krebs buffer of the following composition: 119 mM NaCl, 10 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 4.6 mM KCl, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, and 11 mM glucose. Air comprising 5% CO_2 in O_2 was bubbled continuously through the sodium-Krebs buffer to keep it at pH 7.4. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich 260 mM) Krebs buffer solution. The vessels were pre-contracted with cumulative doses of noradrenaline and the relaxation response was tested with cumulative doses of acetylcholine 2$10^{-9}$–10^{-4} M) and substance P 2$10^{-12}$–10^{-7} M). The response depended upon how much of the endothelial layer was damaged by the bubbles. The maximum relaxation response 2I_{max}) was defined as the maximal dilatory response regardless of the concentration induced by an agonist, and is expressed as a percentage of the pre-contraction induced by a pre-contracting agent. The performance of the vascular smooth muscle cells was evaluated with cumulative doses of sodium nitroprusside 2$10^{-9}$–10^{-5} M). In addition, dose-response curves for all agonists were calculated.

Silver nitrate staining

The segments were cut open in strip form and mounted carefully with needles on a Parafilm-covered cork plate with the vessel lumen-side 2endothelial layer) up. The mounted segments were stained with silver nitrate using a method described by 2Abrol et al. 1984). The stained segments were transferred to an object glass and mounted using an aqueous mounting medium.

Microscopy and photography

Each segment was examined by light microscopy 2Nikon Microphoto-FXA fluorescence microscopy) and photographed 2Nikon FX-35DX) at ×250 2Fujifilm ISO 100). Since each photograph only partly covered the segment, several photographs of each segment were taken. In order to claim reproducible and reliable results, the photographs were taken in the same manner for each segment 2three or five pictures). The photographs were also taken in the same pattern in order to be as representative as possible, usually three pictures from each segment.

Quantification of endothelial damage

Endothelial damage was evaluated using an image-processing program 2Adobe Photoshop 5.0). All photographs from each segment were scanned into the computer. The area containing damaged endothelial layer was coloured using the paint bucket function to increase the contrast and to simplify the quantification. The level of damage was calculated from the pixel dimensions of the marked and stained area 2reflecting the endothelial rupture) and compared to the pixel dimension of the whole picture 2expressed as a percentage). This procedure was followed for all of the photographs from each segment. The mean value for each animal was calculated from the mean value of every segment. The value is a result of at least 12 photographs and represents the final percentage of endothelial damage for this vessel.

Drugs

2±)Noradrenaline[+]-hydrogen-tartrate, substance P, acetylcholine and sodium nitroprusside-dihydrate 2all Sigma) were dissolved in saline or small amounts of distilled water. All concentrations given are the final molar concentration in the tissue bath during the experiments.

Statistics

Data were subjected to analysis using the Mann-Whitney U and Wilcoxon signed-rank tests for unpaired and paired data, as appropriate. The level of statistical significance was set at $P < 0.05$. The results are expressed as mean 2SD).

Results

Pulmonary artery bubbles

Eight to 10 bubbles/min were infused into the superior caval vein and corresponded to grade 1–2 when detected with a ultrasonic scanner in the pulmonary artery. There was no significant difference in this parameter between the 1-h and 6-h groups: 0.08 20.03) bubbles/cm^2 21-h group) and 0.09 20.02) bubbles/cm^2 26-h group). All rabbits survived the infusion and observation period. The measurement of blood-gases showed no change in PCO_2 for the animals during the air infusion compared to that observed prior to and after infusion.

Relaxation response

The 6-h group showed a lower 2$P = 0.04$) I_{max} response [48.3 222.4)%] to acetylcholine compared to the 1-h group [74.5 215.5)%; Table 1]. From the dose-response curves, it appears that the dose-related relaxation response was lower at 10^{-7} M for the 6-h group compared to the 1-h group 2$P = 0.003$; Fig. 1). Although the difference was not significant, a lower response was seen for all other concentrations of acetylcholine in the 6-h group compared to the 1-h group.

There was a lower I_{max} in the 6-h group compared to the 1-h group for substance P 2Table 1). However, the difference between the 6-h group [42.9 216.8)%] and the 1-h group [60.4 210.)7%] was not statistically significant. Dose-response curves showed lower relaxation responses for the 6-h group compared to the 1-h group at two

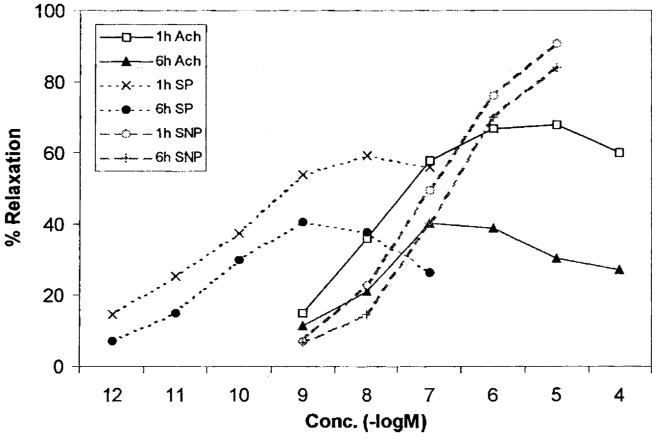

Fig. 1 Dose-response curves for the responses to acetylcholine 2*ACh*, *solid lines*), substance P 2*SP*, *dotted lines*) and sodium nitroprusside 2*SNP*, *dashed lines*) in the animals observed 1 h after the infusion of air into the jugular vein 2the 1-h group, $n = 6$) and in those observed 6 h after the infusion 2the 6-h group, $n = 6$)

Table 1 Comparison of values for animals observed 1 h after the infusion of air into the jugular vein 2*1-h group*) and for those observed 6 h after the infusion 2*6-h group*). Maximum %-relaxation values 2I_{max}) for acetylcholine, substance P and sodium nitroprusside, wet weight, polymorphonuclear leucocyte 2*PMN*) infiltration and mechanical damage for the 1-h and 6-h groups. Values are presented as the mean 2SD) in each group

Experimental group	I_{max} 2%)			Wet weight 2%)	PMN/unit lung tissue	Mechanical damage 2%)
	Acetylcholine	Substance P	Sodium nitroprusside			
1-h group 2$n = 6$)	74.5 215.5)	60.4 210.7)	90.7 25.9)	80.55 22.65)	0.0597	1.9 21.2)
6-h group 2$n = 6$)	48.3 222.4)*	42.9 216.8)	83.9 29.5)	79.87 21.00)	0.1202**	1.6 20.5)

*$P = 0.04$
**$P = 0.0004$

concentrations (P = 0.003; Fig. 1). Lower responses were seen for every concentration in the 6-h group compared to the 1-h group.

Application of the endothelial-independent agonist, sodium nitroprusside resulted in no significant differences in I_{max} between the two groups. The dose-response curves did not show any differences between the two groups (Fig. 1).

Wet weight and PMN infiltration

The pulmonary water content was 80.6 (2.7)% in the 1-h group and 79.9 (1.0)% in the 6-h group (Table 1). The difference was not significant.

The results of the histological examination (left and right lung) from the 1-h and the 6-h group are given in Fig. 2. No significant difference was found between the left and right lung, for both the 1-h and the 6-h group. However, an increase in PMNs was observed in the 6-h group compared to the 1-h group (P = 0.004; Fig. 2).

Mechanical damage

Evaluation of the endothelial layer by light microscopy did not reveal any mechanical damage for the two groups (Fig. 3). The percent damage for the 1-h group was 1.9 (1.2)%, and did not differ significantly from that of the 6-h group [1.6 (0.5)% mechanical damage; Table 1].

Discussion

The results of this study show that the endothelium-dependent response to vasoactive substances in the pulmonary artery would change 6 h after the infusion of small amounts of air bubbles. Furthermore, there was an increase in PMN infiltration at that time. However, no difference in oedema formation (wet weight) was found between the 1-h and 6-h groups. There were no signs of mechanical damage in the pulmonary endothelial layer, as assessed by light microscopy, indicating a biochemical disruption to the endothelial layer.

The change in the vasoactive response occurred 6 h after the infusion, while the response seems to have been unaffected after 1 h. The loss of endothelium-dependent vasoactivity was reduced for the I_{max} to acetylcholine, while the response to substance P was reduced at some concentrations (dose-response). The endothelium-independent response to sodium nitroprusside seems to have been unaffected by air bubbles in both groups, and confirms that the change in vasoactive response is only related to the endothelial function and not to function in the vascular smooth muscle layer. Albertine et al. (1984) and Berner et al. (1989) showed that with air embolization, the pulmonary vascular endothelium is the site of injury.

The water content of the lungs was not difference between the groups, while there was a greater infiltration of leucocytes in the animals that survived for 6 h after the infusion of air. Hjelde et al. (1999) found a connection between the duration of observation and PMN accumulation in animals exposed to many bubbles. Thus, it seems that PMN infiltration increases with time. The number of PMNs observed after 1 h was similar to the number of PMNs seen in control animals observed for 2 h without exposure to bubbles (Hjelde et al. 1999).

From the light microscope analysis, it appears that in the present study the infusion of air did not result in damage to the endothelial layer. However, following exposure to more bubbles (Nossum et al. 1999, 2000), mechanical injury to the endothelium does occur, in addition to a decreased response to endothelium-dependent vasodilators. Yet any mechanical damage would probably have an acute effect and would also be observed in the 1-h group.

The dose-response curves obtained for both acetylcholine and substance P revealed a large difference between the 6-h group and the 1-h group. Normally,

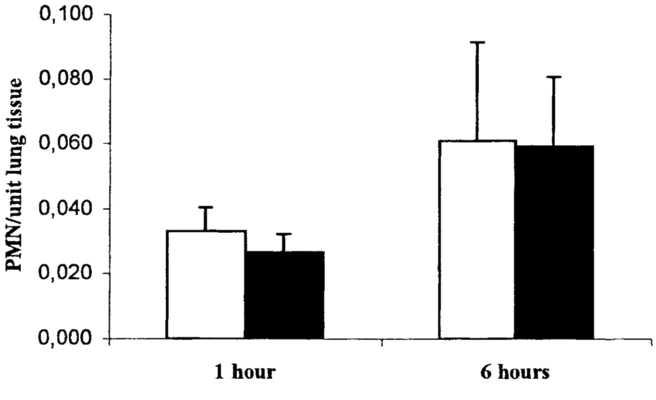

Fig. 2 Number of polymorphonuclear leucocytes (*PMN*) per unit of lung tissue for the left (*open bars*) and right lungs (*solid bars*) from animals in the 1-h ($n = 6$) and 6-h ($n = 6$) groups. Values are mean ± SD

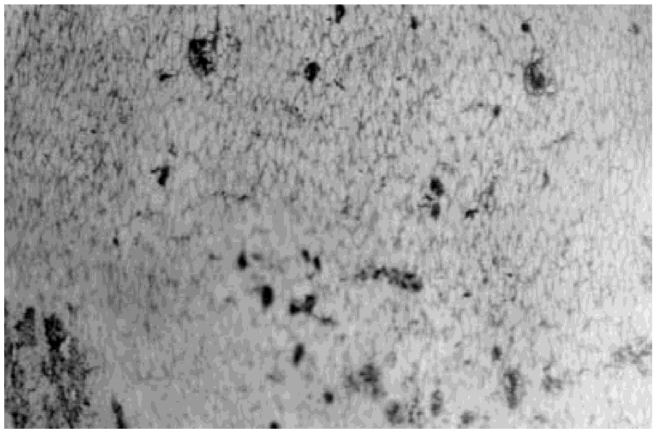

Fig. 3 Photomicrograph of an intact endothelial layer from an animal in the 6-h group that exhibited a decreased relaxation response (magnification × 250)

PMNs circulate within the vasculature as unstimulated cells and do no damage to the vascular endothelium. However, these cells can become activated and dramatically increase oxygen uptake, resulting in the production of oxygen metabolites, lysosomal enzyme release and subsequent endothelial damage 2Fantone and Ward 1982; Roberts 1988). The surface of the bubbles acts as a foreign substance and is capable of activating the alternative complement pathway in vitro 2Hjelde et al. 1995; Ward et al. 1986, 1987). During activation of the complement pathway, three anaphylactic peptides are released into the fluid phase, with C5a being the most important. Intravascular complement activation leads to acute lung injury, and PMNs play a key role in this development 2Czermak et al. 1998; Till et al. 1982). Complement-activated PMNs, when in close contact with lung vascular endothelium, may release toxic oxygen metabolites that can destroy the endothelium 2Sacks et al. 1978; Tofukuji et al. 1998). Gaseous microemboli can cause direct vascular injury as a result of transient capillary obstruction 2Feinstein et al. 1984).

Complement-activated PMNs are associated with the production and release of highly reactive oxygen species such as superoxide anion 2O_2^-•), hydrogen peroxide 2H_2O_2) and hydroxyl radical 2OH•; Fantone and Ward 1982; Roberts 1988) which, when in close contact with lung vascular endothelium, can destroy the endothelium 2Sacks et al. 1978; Varani et al. 1985). Hjelde et al. 21999) demonstrated an increase in pulmonary neutrophil accumulation over 2 h in decompressed rabbits 2Hjelde et al. 1999). The activation of PMNs leads, through a cascade of events, to the formation of $ONOO^-$, which reduces NO. A decrease in NO will subsequently increase the expression of the surface adhesion molecules that are responsible for adhesion between stimulated PMNs and the endothelium, and activate more PMNs. This explains the decrease in endothelial response and the accumulation of PMNs that occurs after 6 h.

The difference in endothelium response was not significant for all concentrations of acetylcholine and substance P. Two animals in the 6-h group demonstrated an I_{max} response above 60% for both substances. There are individual differences in the endothelial response to high amounts of gas bubbles from decompression 2Nossum et al. 1999, 2000). Hjelde et al. 21995) demonstrated an inter-individual difference in complement activation when sera from divers were incubated in the absence or presence of air bubbles in vitro 2Hjelde et al. 1995). Bergh et al. 21993) investigated complement activation by air bubbles in vitro and found that the responsiveness of the complement system to air bubbles in both rabbits and humans varies considerably.

Gas bubbles may enter the pulmonary circulation either as a result of pressure reduction or following accidents or medical procedures. If few bubbles are present, no clinical symptoms will be evident, and such bubbles have been termed ''silent'' bubbles. The present study, together with that of Hjelde et al. 21999) demonstrates that small numbers of gas bubbles would affect the endothelium and lead to increased PMN infiltration in the lungs. Contrary to findings by 2Nossum et al. 1999, 2000), the small number of gas bubbles did not lead to mechanical disruptions in the endothelial layer, as evaluated by light microscopy, suggesting that the changes in endothelial function is biochemical in origin.

Acknowledgements This work was supported by the Norwegian Petroleum Directorate, Norsk Hydro, Esso Norge and Statoil under the ''Dive contingency contract'' 2No 4600002328) with Norwegian Underwater Intervention 2NUI). The help of Anne-Lise Ustad, Arnfinn Sira and Tove Svartkjønnli is gratefully acknowledged.

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