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The effect of vascular bubbles on endothelial function

Dr.philos.-thesis 227

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CONTENTS

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LIST OF PAPERS

I. Nossum V, Koteng S and Brubakk AO
Endothelial damage by bubbles in the pulmonary artery of the pig
Undersea Hyper Med 1999; 26(1) 1-8

II. Hjelde A, Nossum V, Steinsvik M, Bagstevold JI and Brubakk AO
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Scand J Clin Lab Invest 2002; 62 263-270

III. Nossum V, Hjelde A and Brubakk AO
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IV. Nossum V, Hjelde A, Bergh K and Brubakk AO
Lack of effect of anti-C5a monoclonal antibody on endothelial injury by gas bubbles in the rabbit after decompression
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V. Nossum V, Hjelde A, Bergh K, Ustad A-L and Brubakk AO
Anti-C5a monoclonal antibodies and pulmonary polymorphonuclear leukocyte infiltration – endothelial dysfunction by venous gas embolism
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ABBREVIATIONS AND DEFINITIONS

Ach	Acetylcholine
DCS	Decompression sickness
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
ET	Endothelin
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
Mab	Monoclonal antibody
MAC	Membrane attack complex
mN	Milli newton
NO	Nitric Oxide
NOS	NO synthase
PGI_2	Prostacycline
PMN	Polymorphonuclear neutrohpile
SNP	Sodium nitroprusside
SP	Substance P
VCAM	Vascular cell adhesion molecule
VGE	Vascular gas embolism

I_{MAX}: The maximal dilatory response induced by an agonist expressed as a percentage of the precontraction induced by a precontracting agent.

T_{MAX}: The maximum level of stabilised relaxation response induced by an agonist expressed as a percentage of the precontraction induced by a precontracting agent.

-pED_{50}: The concentration of the agonist that leads to 50% of the relaxation response (I_{MAX} or T_{MAX}).

INTRODUCTION

Aims of the study

The present study has been carried out at the Department of Circulation and Medical Imaging, Faculty of Medicine, Norwegian University of Science and Technology during the years 1998-2003. The purpose of the study was to:

- Study the effect of vascular gas bubbles on the brain and the lung
- Study changes in the endothelial function caused by gas bubbles
- Study the preventive effects of monoclonal anti-C5a antibody on functional changes caused by gas bubbles

It is important to reveal any changes in the functions of the endothelium caused by gas bubbles, as the endothelium probably plays an important role in the development of decompression sickness (DCS). Furthermore, we followed up previous studies using monoclonal anti-C5a antibody trying to prevent damages caused by gas bubbles. In order to prevent damages caused by gas bubbles and maybe prevent DCS, the mechanisms behind have to be revealed. This thesis is part of an ongoing project that for several years has tried to bring to light the “secrets” of DCS.

Endothelial Morphology

The arterial vessel wall consists of three layers. *Tunica intima* covers the inside of the blood vessel and includes the endothelial cells, connective tissue and an elastic membrane. In the middle is *tunica media* made of smooth muscle cells and elastin fibres, and on the outside is *tunica adventitia* consisting of loose connective tissue with collagen and elastin fibres, Figure1.

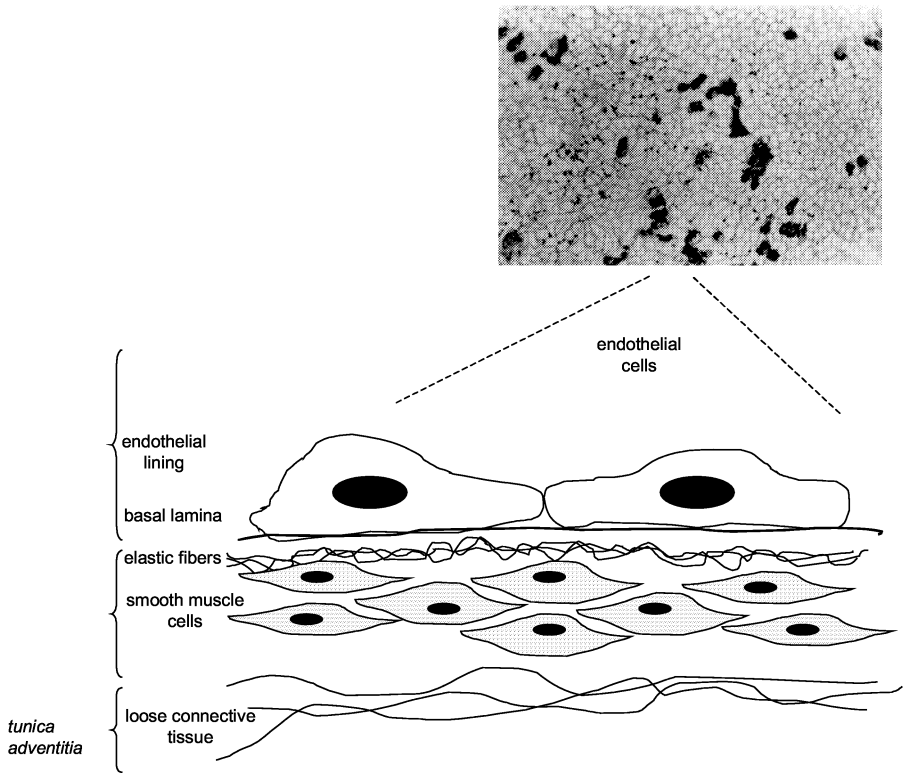

Figure 1. A schematic diagram of the structure of the blood vessel wall with the three layers of *tunica intima*, *tunica media* and *tunica adventitia*. A microphoto illustrates the pattern of the endothelial cell as they are lined in an artery (x250).

The amounts of connective tissue and smooth muscle in the vessel wall vary according to the vessel's diameter and function, but the endothelial lining is always present. The density of endothelial cells lining the vessel wall differs throughout the circulation depending on the need of their presence, vessel diameter, function and localisation. The greatest density of endothelial cells in the blood vessels is in the brain and constitutes the blood-brain barrier that is impermeable to fluid. The endothelium is considered to be one of the most important and extensive organs in the body participating in cardiovascular

hemostasis, and the amount of endothelial cells in an adult person is almost equivalent with an organ with the size of a liver.

The endothelial cell surface is covered by glycocalyx, functioning as a molecular sieve that enmeshes enzymes anchored in the plasma membranes. The glycocalyx is thought to play an important role in maintaining the permeability characteristics of endothelium [142]. The endothelial glycocalyx seems to facilitate (or limit) access of circulating substances to endothelial enzyme sites [127-129]. It may also act as a size, shape and charge barrier and aid discrimination of small solutes, and some of its functions are possibly related to interactions with blood cells and other circulating components of blood. Thus a damaged glycocalyx would enhance the tendency for immune complexes to bind to endothelium [130] and create conditions favouring complement activation and intravascular coagulation with loss of gradients between blood and parenchyma [129].

Endothelial Function

The endothelium plays an important role in vascular regulation by integrating diverse mechanical and biochemical signals and by responding to them through the release of various vasoactive substances. It serves and participates in highly active metabolic and regulatory functions including control of primary hemostasis, blood coagulation and fibrinolysis, platelet and leukocyte interactions with the vessel wall, interaction with lipoprotein metabolism, presentation of histocompatibility antigens, regulation of vascular tone and growth. Many important vasoactive endogenous substances like prostacyclin, thromboxane, nitric oxide, endothelin, angiotensin, endothelium derived hyperpolarizing factor, other free radicals and bradykinin are formed in the endothelial cells and control the functions of vascular smooth muscle cells and of circulating blood cells. The balances may be disturbed by numerous endogenous and exogenous factors including psychological and physical stress, vasospasm, inflammation, leukocyte and platelet adhesion and aggregation, thrombosis, abnormal vascular proliferation, atherosclerosis and hypertension.

Endothelial cells synthesise specific proteins and these cells have receptors and enzymes capable of reacting with certain hormones and other excitatory substances as they pass in the circulating blood. The endothelium is anti-thrombogenic unless injured, but once it is injured, endothelium becomes thrombogenic and then thrombolytic. The endothelium may sometimes restrain the development of inflammation and at other times may promote it. In addition to provide sites for exchange of nutrients and metabolites, the endothelium interacts with pro-hormones and hormones to determine the composition of blood moving downstream.

The endothelial surface has peptidase enzymes, some of which may act on the kinins and angiotensins and some of which may act on other substrates. Among these other enzymes are dipeptidyl aminopeptidase IV, aminopeptidase A and carboxypeptidase N (CPN) [32]. CPN may be of physiologic significance in the processing of circulating anaphylatoxines, and is present on the surface of endothelial cells with direct access to circulating substrates [132].
For a more detailed description of endothelial function see ref. [5;130;131].

Endothelium-derived Vasodilators

Nitrogen oxide (NO)

Furchogott and Zawadzki were the first to demonstrate the vital role of vascular endothelium in mediating acetylcholine-induced vasodilataion (endothelium-derived relaxing factor, EDRF) [50]. Several years later, Rubanyi et al. showed that EDRF is NO [75;124]. NO is a free radical gas generated by NO synthase (NOS) through the oxygenation of one of the guanidino nitrogen atoms of L-Arginine and co-factors situated in the blood [88]. There are three main isoforms of NOS, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), which differ in their dependence on free intracellular Ca^{2+} levels, as well as in their expressional regulation and distribution [16;47;118]. It is revealed that eNOS expression is restricted to the endothelium and the submucosal nerves [85]. The three isoforms of NOS (nNOS, iNOS and eNOS) is further described elsewhere [76].

The target tissue effects of NO depend on its quantity. At lower concentrations, NO serves regulatory roles via activation of soluble guanylate cyclase, resulting in increased cGMP levels in target cells. In vascular smooth muscle, cGMP causes relaxation by reducing intracellular Ca^{2+} and by down regulating the contractile apparatus, mostly mediated by cGMP-dependent protein kinase [68]. NO is considered to be the one of the most important regulators of vascular tone. It shows diverse biological actions and also serves as a neurotransmitter. High levels of NO and products of its interaction with other oxygen free radicals are toxic, a fact that is utilised by cells of the immune system to kill invading bacteria or tumour cells [60]. Interactions of nitric oxide and other oxygen radicals released in tissue injury are multi-faceted, and NO may oppose or enhance the oxidant tissue damage depending on relative quantities of NO and oxygen radicals produced and on the activity of antioxidants defence mechanisms [60]. Endothelium-derived NO suppresses platelet functions including aggregation and secretion of vasoactive substances into the vascular lumen. Because of its rapid adsorptive loss to hemoglobin, the NO action appears to be limited to a small area [119].

Prostacyclines (PGI_2)

Prostacyclines (PGI_2) belong to the eicosanoids, a group of fatty acids that affect a wide variety of physiological processes and play an important part in pain, fever, and inflammation. Eicosanoids are continuously synthesized in membranes from fatty acid chains, and there are four major classes – prostaglandins, prostacyclins, thromboxanes and leukotrienes. Prostacyclins are derived from arachidonate catalysed by the enzyme cyclooxygenase. PGI_2 influence the contraction of smooth muscle and the aggregation of platelets, and when released to the cell exterior they are rapidly degraded by enzymes in the extracellular fluid [4]. The vasodilatory activity of PGI_2 is determined by the expression of specific receptors in vascular smooth muscle [59].

Prostacyclin-receptors are coupled to adenylate cyclase to elevate cyclic AMP levels in vascular smooth muscle [87]. This in turn stimulates ATP-sensitive K^+ channels to cause hyperpolarization of the cell membrane and

inhibit the development of contraction. Prostacyclin facilitates the release of NO by endothelial cells [145] and the action of prostacyclin in vascular smooth muscle is potentiated by NO [33]. Endothelial cells release PGI_2 and NO in a coupled manner [55], but interactions between the system synthesising PGI_2 and NO remain unclear.

Endothelium-derived hyperpolarizing factor (EDHF)

Endothelium-dependent relaxation has been demonstrated in pulmonary arteries of animals [24] and humans [54]. Since the vasodilatation induced by acetylcholine is partly inhibited by pre-treatment with NO inhibitors [156], whereas an NO agonist can cause relaxation in pre-constricted pulmonary arteries [90], it is likely that NO significantly contributes to the endothelium-dependent relaxation of the pulmonary vessels. The endothelium-dependent vasodilatation is not always completely NO dependent. Other factors such as yet unidentified endothelium-derived hyperpolarizing factor may participate significantly in different species and vascular beds [158]. Studies have established that endothelium-dependent hyperpolarization of vascular smooth muscle is resistant to the combined inhibition of both NOS and cyclooxygenases [44]. Accordingly, a substance different from NO and prostacyclin mediate the endothelium-dependent relaxation in these arteries. This substance of endothelium-dependent vasodilatation has been ascribed as endothelium-derived hyperpolarizing factor (EDHF). The molecular formula or identity of EDHF is yet not known [43;44], Figure 2.

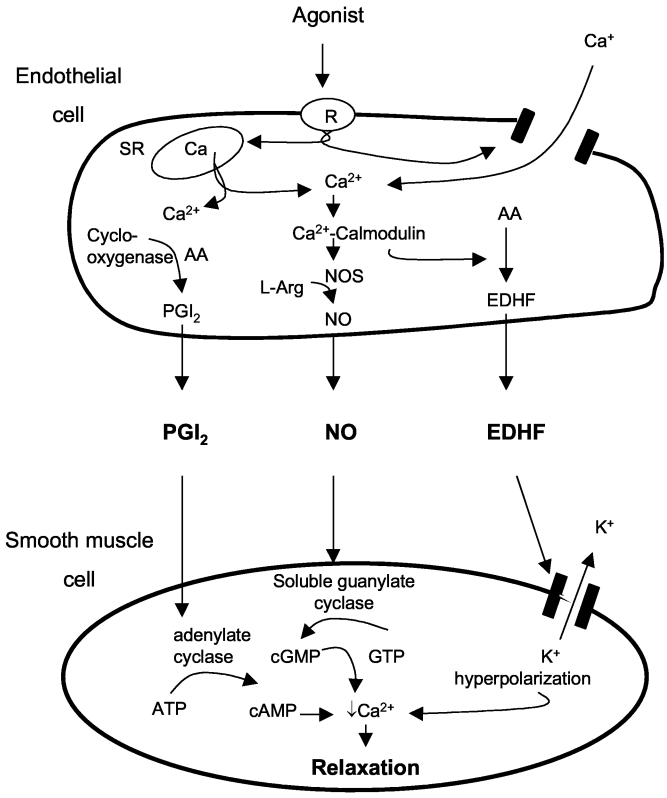

Figure 2. The release of relaxing factors from endothelial cells and their effect on vascular smooth muscle cells. Binding of an agonist to the receptor (R) at the endothelial cell leads to release of Ca^{2+} from sarcoplasmatic reticulum (SR) and influx of Ca^{2+}. This leads to higher intracellular concentration of Ca^{2+} which again stimulates production of nitric oxide (NO) from L-Arginine, and prostacyclin (PGI_2) and endothelium-derived hyperpolarization factor (EDHF) from arachidon acid (AA). NO stimulates soluble guanylate cyclase which increase formation of cyclic GMP (cGMP) which increases extrusion of Ca^{2+} in vascular smooth muscle cell, and to inhibition of contraction. PGI_2 stimulates adenylate cyclase to elevate cyclic AMP (cAMP) levels in smooth muscles which stimulates hyperpolarization through K^+ channels and inhibits contraction. EDHF acts by opening K^+ channels in vascular smooth muscle leading to hyperpolarization which again inhibits vasoconstriction by closing voltage-sensitive Ca^{2+} channels. (Modified [98]).

Endothelium-derived Contracting Factors

Endothelins (ET)

ETs are a family of potent and long-acting vasopressor peptides (ET-1, ET-2, ET-3) made of a peptide of 21 amino acids with two disulphide bridges. Althoug ETs are produced in various tissues, vascular endothelial cells produce ET-1, but not ET-2 or ET-3. Vascular endothelial cells produces ET-1 by the *de novo* synthesis because the endothelial cells do not contain any storage vesicles for ET-1. It is produced by the *endothelin converting enzyme* and the synthesis and release is regulated at the transcriptional level rather than during secretory processes [76;173]. The vascular action of the ET peptides are considered to be mediated through two types of receptors, ET_A [8] and ET_B receptors [139]. In the vascular system, the endothelin ET_A receptor is mainly situated on the smooth muscle cells mediating contraction. The endothelin ET_B receptor is situated on the endothelial cells mediating vasodilatation and in some vascular regions on the smooth muscle cells mediating contraction [138]. ET-1 and ET-2 have strong affinity for the ET_A receptor, whereas all three isopeptides have similar affinity for the ET_B receptors [139].

Ageing and regenerated endothelium

Endothelium-dependent vasodilatation to acetylcholine fades with ageing [152;153]. It is also showed that ET-1 induced endothelium-dependent release of NO and EDHF disappears with ageing [106]. A study by Haas et al. showed that there was an age-related decrease in relaxation of corporal tissue in response to acetylcholine, but with NO supplementation there was no difference in relaxation [57]. This suggests that the synthesis of NO may be impaired with ageing and they found upregulation of eNOS, but the endothelium was anatomically intact. However, Chinellato et al. concluded that the age-induced changes in vascular response in male New Zealand white rabbits are related to an impaired mechanism at smooth muscle level [25].

In vivo removal of the endothelium results in an increased number of endothelial cells in the previously denuded area. The regenerated endothelial cells loose their ability to release NO when challenged with aggregating platelets, thrombin, serotonin or α_2-adrenergic receptor agonists [144;146-148].

Vasoactive agonists

Some vasoactive substances contract vascular smooth muscle while others have a dilatory effect. These two reactions do not only differ in their mechanisms of action, but also in their dependence to intact endothelium, anatomical distribution and physiological halftime. Co-activation, synergism and modulation may be important when several agonists or neurotransmitters are released simultaneously. Some of these agonists or neurotransmitters are discussed below.

Acetylcholine is synthesized by the transfer of an acetyl group from acetyl CoA to choline and this reaction is catalyzed by choline acetyltransferase. Acetylcholine triggers two classes of membrane receptors, the nicotinic acetylcholine receptor and the muscarinic acetylcholine receptor. The muscarinic receptor is localised in the endothelial cells and works through a G protein that triggers a second messenger that again leads to the generation of NO and relaxation of vascular smooth muscle cells (described earlier) [75;123;173]. Acetylcholine can induce different responses in different target cells, depending on which receptor protein it binds to and the intracellular pathway [4;50]. Both concentration and vascular muscle tone affect whether acetylcholine induces contraction or relaxation in the vascular bed. Whether the response is dependent of an intact endothelial layer is still not clear [6;7;49;50].

Substance P (SP) is a neuropeptide and one of the most potent vasodilators known when the endothelium is intact [6;7;30]. Substance P works through a second messenger cascade activating phospholipase C and the production of inositol 1,4,5-triphosphate (IP_3). This leads to the release of Ca^{2+} from IP_3 sensitive Ca^{2+} stores [42]. Increase in the intracellular Ca^{2+} concentration stimulates the release of NO and subsequent vasodilatation in the underlying vascular muscle layer [14;163]. Studies have shown that SP

participates in some inflammatory processes such as vascular leakage [83], migration and activation of leukocytes [111] .

Noradrenaline belongs to the catecholamines and work as a transmitter at smooth-muscle junctions. Noradrenaline works through two different adrenergic receptors, alfa-adrenergic and beta-adrenergic [151]. Alfa-adrenerge receptors are located in the vascular smooth muscle cells, leading to vasocontraction once noradrenaline binds to the receptor. The beta-adrenerge receptor is situated in the heart muscle.

Endothelial dysfunction

Endothelial dysfunction exists in many arterial diseases in addition to damages resulting from gaseous microemboli. Endothelial dysfunction is characterised by deterioration of endothelial vasodilator function. It can manifest either by decreased secretion of vasodilator mediators, increased production of vasoconstrictors, increased sensitivity to vasoconstrictors, increased production of vasoconstrictors and/or resistance of vascular smooth muscle to endothelial vasodilators. Endothelial injury can result from mechanical or (bio)chemical damaging factors or stimulation of endothelial cell layer to inappropriate or abnormal physiological secretion [159]. However, hypertension, atherosclerosis and hyperlipidemia can also be the cause of endothelial dysfunction [31] but will not be further discussed here.

Potent releasers of endothelial vasodilator mediators possess specific receptors at the surface of endothelial cells. The distribution of these receptors and the signal transduction pathways to which they are coupled, may vary from one arterial segment to another or be modulated by the physiological status of a given artery. Hence, endothelial dysfunction will differential affect shear stress- or hormone-induced endothelium-dependent vasodilatation in the vascular bed. Endothelial cells release small amounts of vasoconstrictor prostaglandins but their influence are under normal circumstances masked by the production of prostacyclin, NO and EDHF. Reactive oxygen species secreted in response to shear stress may inactivate NO or facilitate the mobilization of cytosolic Ca^{2+} in vascular smooth muscle cells [122] and hereby promote contraction.

Few cell types are attacked more directly than endothelial cells in the early stages of inflammation. Disappearance of endothelium is among the early changes of necrotizing vasculitis. It appears that the endothelium is not only a "victim" or target of inflammation but also an active participant. This will be further discussed later.

Gas Embolism / Bubbles

Venous Gas Embolism (VGE)

There are two broad categories of gas embolism, venous and arterial, which are distinguished by the mechanism of gas entry and the site where the emboli ultimately lodge. VGE occurs when gas enters the systemic venous system [112]. The gas is transported to the lungs through the pulmonary arteries, and can cause interference with gas exchange, cardiac arrhythmia, pulmonary hypertension, right ventricular strain, and eventually cardiac failure. VGE is an important issue of concerns both in the accomplishment of several medical procedures, and occur in the venous system during most decompressions [17;34]. Venous gas bubbles may develop following laparoscopy, by accidental injection [97], neurosurgery in the sitting position [96], trauma, or in cardiopulmonary bypass surgery [69;80;105;168].

Arterial Gas Embolism

The lung is considered to be a good filter for gas bubbles, but the lung may be damaged, resulting in bubbles travelling through the pulmonary vein and the left atrium. Gas bubbles may break through the lung filter if the lung is overloaded [160] and enter the arterial circulation. In addition, venous gas bubbles may pass through a patent foramen ovale (PFO) or other extraordinary connections in the heart to reach the arterial circulation. The foramen ovale is functionally closed in the majority of the population except in 20-34 % of humans where the foramen ovale is patent after foetal life [58]. Arterial gas emboli may also occur after direct injection into the arterial circulation, or finally, at least theoretically, by *de novo* formation during decompression.

Pathological and physiological effects of bubbles

During resting conditions, the endothelial cell lining of blood vessels is a relatively inert surface that regulates and secures unhindered flow of cellular elements through the capillary beds. In response to an inflammatory signal initiated by the bubbles, endothelial cells may be converted from an inactivated to an activated state resulting in cellular function changes (described later).

Rapid entry or large volumes of gas put strain on the right ventricle because of the migration of the emboli to the pulmonary circulation. The pulmonary arterial pressure increases, and the increased resistance to right ventricular outflow causes diminished pulmonary venous return. The alteration in the resistance of the lung vessels and the mismatch between ventilation and perfusion cause intrapulmonary right-to-left shunting and increased alveolar dead space, leading to arterial hypoxia and hypercapnia [105]. Elevation of pulmonary artery pressure caused by bubbles [161;162] may be accompanied by a decrease in cardiac output [20]. The first microvessels encountered by venous bubbles are the pulmonary capillaries, and it has been demonstrated *in vivo* that bubbles generated by decompression or directly infused to the venous circulation become trapped there [18-21].

Gas embolism may thus mediate bubble-induced increased permeability in the blood-brain barrier (BBB), blood-lung barrier (BLB) [27] and tissue injury. Breakdown of the BBB and BLB may allow proteins and other substances to translocate into the extravascular brain tissue, with subsequent edema formation. Gas bubbles following decompression increased the permeability of the lung vasculature to Trypan blue and revealed endothelial damage [27;150]. Obstruction of pulmonary vessels by bubbles may be accompanied by damage to the endothelium, accumulation of leukocytes, release of thromboxanes and leukotrienes [27], and the release of vasoactive substances [95]. The degree of endothelial damage can be evaluated by studying transport of fluid over the vessel wall. Ohkuda et al. found a dose dependent increase in lymph flow and lymph protein clearance with a negative lymph to plasma protein ratio after infusion of low doses of air bubbles into the pulmonary artery. However, they

found a positive ratio at higher doses of air bubbles indicating a possible mechanical damage at high levels of air bubbles. This study indicate that the pulmonary capillaries retain some ability to sieve proteins at lower infusion levels [110].

Studies by Albertine et al. revealed gaps in the endothelium of arterioles following air embolisation [3]. The gap formation occurs where embolic impacts and degranulated platelets and neutrophils are localised at the gap. Deposition of fibrin and swelling of endothelial cells were observed in addition to gap formation without damage to the membrane [99].

The surface of the bubbles acts as a foreign substance and is capable of activating the alternative complement pathway *in vitro* [65;166;167]. The degree of activation is related to the amount of gas bubbles introduced [10]. Bubbles also interact with formed elements of blood and plasma proteins. They may stimulate platelet aggregation [115-117], denature lipoproteins [91], activate and aggregate leukocytes [117], increase release of cytokines [41], kinin and coagulation systems [117], and by means of these proinflammatory events, cause both capillary leakiness and hemoconcentration [15].

Complement system

The complement system complements and amplifies the action of antibody. It is one of the principal means by which antibodies defend vertebrates against most bacterial infections and is the initial barrier against the spread of infectious agents. The complement system is a multiprotein cascade and is composed of at least 30 plasma and membrane bound proteins formed by the liver and macrophages. Approximately half the proteins are directly involved in the pathways constituting the system, while the remainder proteins function as essential regulators [51;63;107]. Activation of the system initiates a sequence of biochemical reactions where each component activates the next reaction allowing considerable amplification to occur in the system. A small initiating signal stimulate the formation of large quantities of active products strictly controlled at every stage by multiple inhibitory and control proteins. The

complement system is initiated through three different pathways: classical, alternative and lectine pathways.

The classical pathway are initiating by antigen-antibody complexes and consists of plasma proteins [89;93;121]. Although binding to immunoglobulin remains the primary mechanism, the complement system can be activated by viruses, nucleic acids, proteins, lipids, polysaccharides and polyanionic compounds [52]. The classical complement cascade ends up with the C5 convertase which cleaves C5 into C5a and C5b, the latter becoming part of the membrane attack complex (MAC) [100].

The alternative pathway of complement activation provides a nonspesific natural defence system against microorgansims and other pathogens which operates independently of specific antibody [56;113]. It is active against a wide range of targets, including pathogenic microorganisms, virus-infected cells, neoplastic cells and erythrocytes from certain species. It has also been demonstrated that gas bubbles *in vitro* activate the complement system along this pathway [65;166;167].

The lectine complement pathway (LPC) is an antibody-independent cascade initiated by binding of mannose-binding lectine to cell surface carbohydrates. It differs from the classical pathway only in this initial step [157].

The cleavage of the protein C5 by the C5 convertase of either pathway produces C5a and C5b. This is the final enzymatic step in the complement cascade [102;103]. The rest of the cascade (C5-9) is activated in the same way independent whether the initial activation was classical, lectine or alternative. The chain reaction from C5b to C9 is characterised by continuous binding of each component to each other creating a macromolecule. The completion of the complement activation leads to the formation of the terminal complement complex that exists in two forms. One form exists in a fluid phase that is non-lytic and can be detected in plasma. The other form is the membrane attack complex (MAC), which can cause cell-lysis by penetrating lipid membranes such as the endothelium.

The anaphylatoxine C5a

The anaphylatoxine C5a is the most potent of the anaphylactic peptides (C3a, C4a and C5a) [73] which are characterised by their ability to enhance vascular permeability [81;155;170], release histamine from mast cells and to cause smooth muscle contractions. C5a can also cause aggregation and trapping of PMNs in the pulmonary capillaries [28]. C5a activates platelets and monocytes, resulting in the release of cytokines and other inflammatory mediators that amplify neutrophil-endothelial cell adhesion.

C5a activates neutrophils to express adhesive properties such as L-selectin and CD11/CD18, which can bind with intercellular adhesion molecule (ICAM-1) on activated endothelial cells [45;46;120]. Once expressed on the lumenal surface, P-selectin is available to recruit neutrophils from the passing circulation. This rapid response result in an almost immediate adhesion of neutrophils and they are free to release proteases and oxygen-derived free radicals that directly lead to diffuse capillary leak and impaired oxygenation. This contribute to endothelial cell barrier dysfunction and fluid extravasation. P-selectin is unique compared to other adhesion molecules, because it is the most ready available of the adhesion molecules [164]. In order to mediate PMN extravasation at the site of inflammation, these actions are important for cellular interaction between endothelial cells and PMNs.

Complement activation of endothelial cells

Normally, endothelial cells maintain a barrier between cells and molecules of the vascular lumen and those of the organ parenchyma. Resting endothelial cells actively resist thrombosis, and heparan sulfate on the surface binds endothelial cell superoxide dismutase thus promoting antioxidants activities of the endothelium [1;140]. Injuries from inflammation and ischemia-reperfusion may lead to major, often irreversible, cell and tissue injury initiated via complement-mediated mechanisms. Activation of complement in turn activates the endothelium, which causes disruption of endothelial junctions resulting in

edema and upregulation of adhesion molecules, leading to exacerbate inflammatory processes, Figure 3.

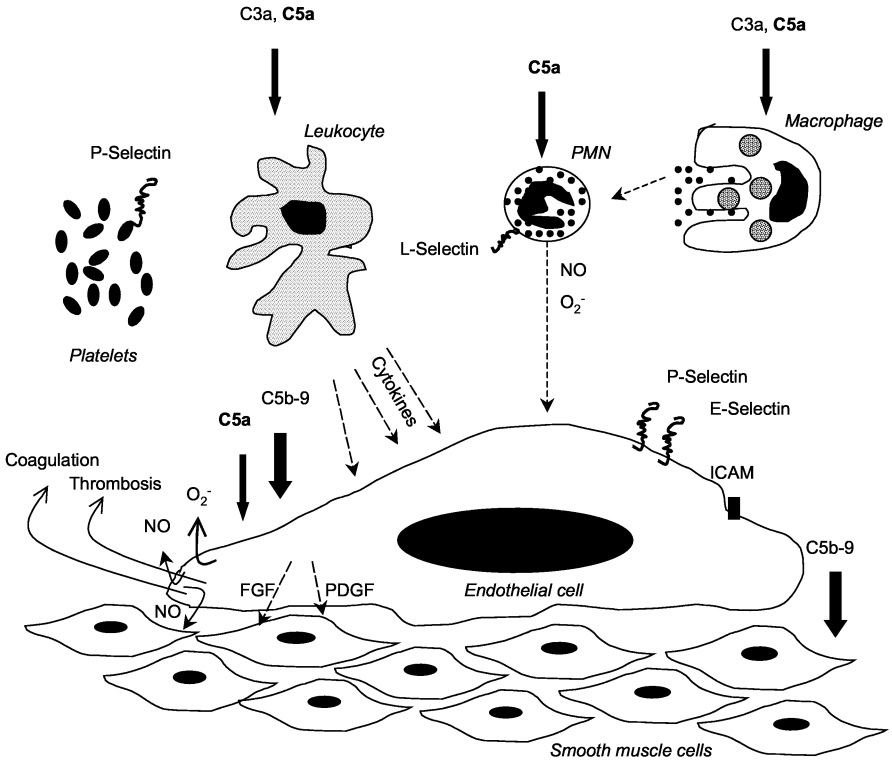

Figure 3. Active fragments released during complement activation, such as anaphylatoxines C3a and C5a, lead to the release of cytokines and other inflammatory mediators (dashed lines) from leukocytes and endothelial cells, as well as upregulation of adhesion molecules (P-selectin, E-selectin). The terminal complex (C5b-9) also has prelytic, agonist-like, effects on endothelial cells leading to procoagulant and prothrombotic states and downregulation or destruction of nitric oxide. C5b-9-stimulated release of growth factors, such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). All these effects of prolonged or inappropriate complement activation are injurious. (Modified [126]).

Once infectious agents have invaded a tissue where concentration of complement is low and neutrophils are scarce, neutrophils must be sequestered or walled off. In tissues complement initiates the walling off of infected sites by interrupting the blood-tissue barrier of endothelium. Within minutes of complement activation, terminal complement complexes increase small blood vessel permeability by disrupting endothelium integrity [135]. After exposure to infectious agents, endothelial cells undergo a series of changes, also known as activation:

- Increased expression of tissue factor and plasminogen activator inhibitor type 1 (PAI-1) and loss of thrombomodulin makes endothelial cells procoagulant
- Increased production of ET-1 and thromboxane A2 caused by increased vasoconstriction
- Expression of adhesion molecules and chemokines makes the endothelial cells proinflammatory

[136]

Complement mediates activation of endothelial cells by different pathways. Complement interaction with endothelial cell receptors up-regulates mRNA for adhesion molecules like E-selectin, ICAM-1 and VCAM-1 (vascular cell adhesion molecule), increasing endothelial adhesiveness for leukocytes [136]. Insertion of the membrane attack complex in endothelial cells induces expression of IL-1α [134]. IL-1α in turn acts in an autocrine manner, up-regulating E-selectin, ICAM-1, and VCAM-1 on endothelial cells [133;154]. Membrane attack complex stimulates endothelial cells to produce chemokines, and expression of adhesion molecules and chemokines promote local activation and influx of leukocytes to the site of injury in addition to coagulation. Coagulation and vasoconstriction may lead to slowing of blood flow and associated pathophysiologic conditions.

METHODS

Animal research guidelines

All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). The local ethics committee for animal research approved the experimental protocol.

Tissue preparation

Rabbits (Chinchilla, New Zealand Black and New Zealand White) and pigs (Sus Scrofa Domestica) that had undergone decompression or air infusion, were either sacrificed under anaesthesia with potassium chloride or they died as a result of injuries due to massive bubbling after decompression. The lungs were harvested immediately for subsequent evaluation of tissue injury. Branches from the pulmonary artery were carefully dissected from the right lung in pigs and rabbits. All vessel preparations were immediately immersed in cold oxygenated (5% CO_2, 95% O_2) Na-Krebs buffer and stored in a refrigerator for a maximum of 48 hours before tension measurements. The vessels had a resting diameter between 1-2 mm for rabbits and 2-3 mm for pigs. They were dissected free from all adherent tissue under a microscope, and cut into circular segments with length ranging from 1-1.5 mm. The vessels were also investigated for changes in endothelial layer morphology and measurement of apoptosis in endothelial cells. Selected samples from the right and left upper and lower lung from the rabbits were harvested for histologic examination (PMN accumulation). Finally, less than 1 g was taken from the right lung tissue from the rabbits to estimate pulmonary edema.

Brains from rats (Sprague Dawley) that had undergone decompression were harvested for specific gravity examinations. The rats (300-370 g) were decapitated and the brain was submerged directly in kerosene to avoid evaporation.

Pulmonary Endothelium

Endothelial dysfunction in Arteria Pulmonalis

The method described in Paper I, III, IV and V is based on the method described by Edvinsson with colleges [35;36] and the vasomotor reactivity was analysed using a modification of a tissue-bath technique originally described by Högestätt [74].

A tension of 5.5-7.5 mN for rabbit artery or 8.0-10.0 mN for pig artery was applied to the segments and they were allowed to stabilise at this level of tension for 1-1.5 hour. The contractile capacity of each vessel segment was examined by exposure to a potassium-rich K-Krebs buffer solution.
The vessels were precontracted with cumulative doses of norepinephrine until they had reached a stable level. The relaxation response where tested with cumulative doses of acetylcholine (10^{-9}-10^{-4}M) and substance P (10^{-12}–10^{-7}M). The response that followed depended on how much of the endothelial layer that was damaged by the bubbles. The relaxation response I_{MAX} is defined as the maximal dilatory response induced by an agonist expressed as a percentage of the precontraction induced by a precontracting agent. T_{MAX} is defined as the maximum level of stabilised relaxation response (Paper I). In addition, -pED_{50} (Paper I and IV) is defined as the concentration of the agonist that leads to 50% of the relaxation response (I_{MAX} or T_{MAX}). The functionality of the vascular smooth muscle cells was tested with sodium nitroprusside or bradykinine and dose-response curves were calculated. Because sodium nitroprusside and bradykinine is endothelial independent, those vessels that did not respond had a functional failure in the vascular smooth muscle cells, and these segments were rejected.

Tension measurements where performed in paper I, III, IV and V and described in detail there.

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. Two

Vessel Tension Measuring Instruments each containing four channels with four separate buffer containers were connected in series. A total of eight vessel segments could therefor be tested at the same time. The equipment is further described in Paper I and used to perform tension measurements in paper I, III, IV and V.

Detection of mechanical endothelial injury

In order to visualise the mechanical injury in the endothelial layer two different procedures for staining the endothelial layer with silver nitrate were used. In Paper I and III the vessel strips where covered with 1% silver nitrate ($AgNO_3$) for 2 min, followed by a mix (1:1) of 3% BrH_4N and 3% cobalt bromide (Br_2Co) for 2 min more. The vessel segment was not fixed in any way, and was photographed immediately in a microscope. In paper IV the strips were kept in 5% glucose for 2 min and then stained for 20 sec in 0.25% $AgNO_3$. They were then immersed rapidly six times in 5% glucose to remove excess silver nitrate and placed in 3% Br_2Co solution for 3 min to improve contrast, after which they were washed six times in 5% glucose as described. At last exposure to ultraviolet fluorescent light at a distance of 15 cm for 30 min gave optimum visualisation of the endothelial borders. This technique is modified from Abrol et al. 1984 [2]. After the staining procedures, the stained segments were transferred to an object glass and mounted using an aqueous mounting medium. The method described in Paper I and III gave no opportunity to store the objects and the segments had to be photographed in a light microscope immediately. Abrol et al.'s method was an improvement since the objects could be stored in a freezer and photographed later. Magnification was x250 and x500.

Apoptosis in pulmonary endothelium

Immunohistochemical detection of apoptosis in pulmonary endothelial cells where performed in Paper V. Staining procedures followed those of an ApopTaq in situ apoptosis kit (Intergen).

Pulmonary edema and histology of the lung

In Paper IV and V the percent lung water was used to estimate severity of pulmonary edema. The dry weight of the lung tissue was determined from a less than 1g section of the left lung. The tissue was weighted (wet weight), incubated at 120 °C for 7 days, and then weighted again (dry weight). Percent lung water content was used to estimate severity of pulmonary edema. The lung water content was calculated from the following relation:

$$\% \text{ water} = \frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} * 100$$

This method is earlier described by Gillinov et al. [53] and has previously been applied in our laboratory [67].

Pulmonary tissue injury was evaluated by histological evidence of PMN accumulation of the lung (Paper III and V). From all rabbits four selected samples from the right and left upper and lower lung were taken for histological examination. An investigator who was blinded to the treatment given to the rabbits estimated the accumulation of polymorphonuclear neutrophils (PMN). Each rabbit was represented by a mean value of 16 data points from the lungs and the results are expressed as the average number of PMNs per unit lung tissue.

Cerebral edema

The use of specific gravity in the measurement of cerebral edema is well documented in the literature [48;67;108]. A decrease in specific gravity indicates increased water content of the brain tissue and changes in the permeability of the blood-brain barrier. High sensitivity, ease of measurements, and the use of small samples make this method suitable for the measurement of cerebral edema. However, when gas bubbles are present, the method may be

influenced by the presence of accumulated gas, which also leads to a reduction in specific gravity. The method used in Paper II distinguish between gas bubbles and edema causing the change in specific gravity following decompression.

Complement

Anti-C5a antibody and control monoclonal antibodies (Mab)

The production of murine neutralizing anti-rabbit C5a Mab has been described in detail elsewhere [11] and developed at the Department of Microbiology, NTNU, Trondheim. The capacity of the IgG given will be sufficient to bind every C5a molecule generated in plasma *in vivo*. As control antibody was used an IgG1 Mab (Mab 3A3D10) raised in our laboratory against a psoriasis-associated dermal antigen, cultivated and prepared similarly and administered at equivalent amounts. Thus, both groups were given each an IgG1, thus implying an identical physical and biological behaviour from the two immunoglobulines. The specificity to the ligands, C5a versus the skin antigen, however, differs between the two antibodies.

Using a randomized, blinded protocol, the rabbits in Paper IV and V were divided into two groups. One group received anti-C5a murine Mab 4B1C11 whereas a sham murine Mab 3A3D10 was administrated to another group of animals 30 minutes before air infusion (Paper V) or 30 minutes prior to pressure exposure (Paper IV).

Quantification of C5a

The quantification of rabbit C5a des-Arg in EDTA plasma was performed by a C5a-specific sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies, described in detail by Bergh et al. [11]. Much of the attention concerning the role of complement activation in decompression sickness (DCS) has been focused on C5a. In particular, C5a-induced leukocyte activation is a critical mediator of vascular injury. Assays for direct quantification of C5a based on neoepitope-specific monoclonal antibodies have been developed for both rabbits and humans [11;12]. There was an attempt to

develop monoclonal antibody for pig at our laboratory, but we did not succeed. This is why we decided to use rabbits in our studies. Because of the reactivity toward a neoepitope on C5a, an epitope that is concealed in the native complement component C5a and exposed on the anaphylatoxin fragment only after the enzymatic cleavage during complement activation, C5a des-Arg may be directly quantified in the presence of native C5. This enzyme-linked immunosorbent assay is extremely sensitive (detection limit 20 pg/ml). It is superior to the commercially available RIAs (radioimmunoassay) due to specificity and sensitivity [84]. In the text C5a will be used synonymously with plasma analogue C5a des-Arg.

Blood sampling and preparation

Blood samples were collected from the central ear artery prior to the dive (*predive*) and after the dive (after one hour observation (*postdive 1hr*) and two hours observation time (*postdive 2hr*)). Samples (1.5 ml) for analysis of C5a-desArg were drawn into EDTA (final concentration 13 mmol/l) tubes (Beckton Dickinson, Meylan Cedex, France) and immediately placed on ice. The plasma was separated by centrifuging at 600 g for 10 minutes within 30 minutes and was stored at -80 °C until analyzed.

Bubble detection

In Paper I the number of bubbles in the pigs' pulmonary artery were detected using a 5MHz transesophageal transducer connected to an ultrasonic scanner (CFM 750, Vingmed, Horten, Norway). The images were transferred to a computer and counted continuously using a program previously described [40]. In the rats (Paper II) the right pulmonary artery was monitored for gas bubbles using a 10 MHz transducer. Bubbles could be seen in the right ventricle and the pulmonary artery and evaluated using a grading system from 0 to 5. Grade 0 is no bubbles, 1 is occasional bubbles, 2 is at least one bubble every fourth heart cycle, 3 is at last one bubble every heart cycle, 4 is continuos bubbling, and 5 is massive bubbling. This scoring system is approximately exponential compared with the number of bubbles in the right ventricle [39]. Gas bubbles in the rabbits

(Paper III and IV) were detected using a 5MHz transducer and evaluated using the same grading system as in Paper II. The grades observed were converted to bubbles/cm^2 using the conversion table given by Eftedal et al. [38].

Statistical analysis

The data in Paper I were analysed using Student's *t* test for unpaired data. Owing to the small number of animals, non-parametric methods were used in the other papers (Paper II, III, IV and V). The Kruskal-Wallis test was performed to assess significance between the groups. Correlation between bubble grade and cerebral gas volume (PaperII) was calculated using the Spearman's rho. The data were subjected to analysis using Mann-Whitney and the Wilcoxon signed-rank test for unpaired data and paired data as appropriate. Parametric methods were used in the methodology part of the study (Paper II). Furthermore, the data in the five papers have been presented as mean, standard error of the mean (SEM) or standard deviation (SD) to show individual variability. The level of statistical significance was set at $P<0.05$.

SUMMARY OF PAPERS

Paper I

Endothelial damage by bubbles in the pulmonary artery of the pig

In this paper a method for measuring endothelial damage in the pulmonary artery was developed and established at our laboratory. Endothelial dysfunction was tested using a system for recording changes in the vessel wall tension as a response to endothelium-dependent vasoactive substances. Different amount of gas bubbles were detected in pigs that had undergone decompression. Significant decrease in endothelial response and sensitivity were found in the pigs that had undergone decompression compared to the control group. There was a difference in response depending on bubble amount within the experimental group, but the difference was not significant. The results from the tension measurements were confirmed by analysis using the light microscope. Microscopy studies revealed that pigs with high level of vascular bubbles all showed serious damage to the endothelial layer. No damage was seen in the endothelium in the control animals. A comparison between total decompression stress and endothelium response revealed a decrease in total response in the vessel with increasing number of vascular gas bubbles.

Paper II

Evaluation of cerebral gas retention and oedema formation in decompressed rats by using a simple gravimetric method

A specific gravity method for distinguishing between bubbles and edema following decompression was developed. The brombenzene/kerozene gradient column was found to be a sensitive method for distinguishing between gas retention and edema formation in decompressed animals. Factors such as simplicity, ease of measurement and high sensitivity make this a reliable method. Evaluation of the extent to which the change in specific gravity is due to retained gas in cerebral tissue showed that rats with a high bubble score had higher gas retention compared to rats with a low bubble score. However, the

major contribution to the change in specific gravity in decompressed animals is due to edema formation.

Paper III

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

Endothelial function was studied after infusion of 0.01 ml air/min/kg into the jugular vein in twelve rabbits for 60 minutes. The infusion led to a significant decrease in the acetylcholine-mediated endothelial-dependent vasodilatation in the pulmonary artery 6 hours after the infusion. There were no signs of change in a group of rabbits examined 1 hour after infusion. The 6 hour group had a significant increase in polymorphonuclear neutrophils in the lungs, however no visible changes were seen in the endothelial layer in any of the animals when examined in the light microscope. No pulmonary edema was revealed. The study demonstrates that small number of bubbles, corresponding to “silent bubbles”, lead to a delayed impairment of the endothelial-dependent vasoactive response.

Paper IV

Lack of effect of anti-C5a monoclonal antibody on endothelial injury by gas bubbles in the rabbit after decompression

The effect of pre-treatment with anti-C5a monoclonal antibody trying to prevent endothelial damage caused by gas bubbles from decompression was examined. Animals with many bubbles showed significantly more vascular damage than animals with fewer bubbles. Tension measurements developed in Paper I were used and demonstrated that gas bubbles from decompression cause endothelial damage. As expected, the high bubble count decreased the endothelial response, however anti-C5a antibody could not prevent endothelial damage caused by this large amount of gas bubbles. Light microscopy studies confirmed mechanical disruption in the endothelial layer. The amount of gas bubbles present is of great importance whether endothelial damage will occur or not. The role of the complement system and a presumed beneficial effect of

anti-C5a antibody can not be excluded, particularly at a lower rate of bubble formation.

Paper V

Anti-C5a monoclonal antibodies and pulmonary polymorphonuclear leukocyte infiltration – endothelial dysfunction by venous gas embolism

In this study a possible effect of monoclonal anti-C5a antibody preventing pulmonary endothelial dysfunction and polymorphonuclear leukocyte infiltration caused by low-grade VGE were studied. Same infusion protocol was used as in Paper IV and antibody treatment was performed as in Paper III with a 6 hours observation period. The monoclonal anti-C5a antibody treatment reduced PMN infiltration significantly compared to the control group and apparently improved endothelial function. However, the sham-antibody treatment also had a protective effect on PMN infiltration and apparently improved endothelial function. There were no major signs of apoptosis in pulmonary endothelial cells in any of the examined animals.

GENERAL DISCUSSION

The influence of gas bubbles on endothelial function

The risk of developing DCS increases with the amount of gas bubbles observed in the pulmonary artery [109]. DCS can occur even with small bubble amounts. The term “silent bubbles” has been used to describe bubbles observed in the vascular system without clinical symptoms of DCS [9]. Since all venous blood normally goes through the lung any effect of vascular bubbles, either short or long term should be observed here. Gas bubbles generated either by decompression or directly infused to the venous circulation are trapped in the pulmonary capillaries [18-21]. Albertine et al. and Berner et al. showed that with air embolization, the pulmonary vascular endothelium is the site of injury [3;13]. Our hypothesis that gas bubbles in the blood vessels could influence endothelial function was thus confirmed. Animals with large amount of gas bubbles in the pulmonary artery from decompression showed a decrease in endothelial derived vasoactivity compared to unexposed controls. This was also demonstrated in earlier studies in rabbits [14;30]. The number and exposure times of bubbles varied among the animals and were related to endothelial and pulmonal dysfunction (Paper I and IV). It seems that the maximum bubble number is more important than duration of exposure considering damage to the endothelial layer (Paper I). Observations in the light microscope (Paper I and IV) were compared to the vasoactive response and confirmed mechanical endothelial damage. There are individual differences, but it seems to be a relationship between high amount of gas bubbles and mechanical endothelial damage. The mechanical injuries were observed immediately after bubble exposure, which implies an acute endothelial injury.

Infusion of a smaller and controlled amount of gas bubbles did not cause any mechanical disruptions in the endothelium (Paper III). However, vasoactive response and measurement of polymorphonuclear neutrophil (PMN) infiltration revealed a dysfunction in the endothelial layer (Paper III and V). The change in vasoactive response and PMN infiltration appeared between one to six hours

after bubble exposure, which indicate a delay in disruption of the endothelium and PMN infiltration. The fact that no dysfunction of the endothelial derived vasoactive response was observed in the infused animals after one hour implies no acute mechanical endothelial disruption. It is rather a biochemical or immunological response that develops with time. Earlier findings by Hjelde et al. showed a connection between the observation period 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 hour was similar to the number of PMNs seen in unexposed, anaesthetised rabbits observed for 2 hours [67].

Gas bubbles that enter the brain circulation may pass through the arterioles and capillary beds without obstructing the blood flow. Nevertheless, these bubbles may alternate the blood-brain barrier (BBB) and disrupt brain function. Alteration of the BBB results in transport of proteins across the barrier and subsequent formation of edema in the brain tissue [26;64]. Considering our findings in vasoactivity in the pulmonary artery and PMN infiltration related to the bubble score, the relationship between bubble score and damage to the BBB correlates. We found that increasing bubble score altered the BBB and that the change in specific gravity was in accordance with the bubble score. There was a higher cerebral gas retention in the animals with a high bubble score compared to animals with a low bubble score. A study by Hjelde et al. [66] indicates a threshold value of infused bubble concentration resulting in cerebral edema. Increasing the number of bubbles infused had no further impact on the development of cerebral edema.

Studies by Huang and Lin showed that air embolism increased vascular permeability as demonstrated by increased pulmonary water content, suggesting a mechanism linking air bubbles, activated complement and PMN in this injury [70;71]. This is in contrast to our findings were the water content of the lungs remained unchanged. Considering the alterations we found in the brain, this is somewhat unexpected. The explanation is most likely the use of different methods measuring the water content in the lung. Huang and Lin used

a perfusing system for isolated lungs while our method is less sensitive, and probably failed to measure possible changes.

Anti-C5a monoclonal antibody and endothelial injury

Our hypothesis was that anti-C5a antibody would reduce tissue damage in rabbits caused by intravascular gas bubbles by eliminating circulating C5a and presumably thereby reducing the effects of C5a on endothelial dysfunction and PMN. Anti-C5a antibody represents a therapeutic approach for blocking inflammatory effects of complement activation. Studies have revealed several beneficial effects using anti-C5a antibody as pre-treatment, such as blockage of zymosan-activated human PMN chemotaxis [61] and lysosomal enzyme release from PMNs [62]. Pre-treatment with anti-C5a antibodies reduced pulmonary damage in different models of injured rats [29;104;141]. The protective effects were associated with diminished vascular permeability and reduced PMN accumulation in the lung. In a study on pigs by Park et al. attenuated relaxation to substance P i.a. after cardiopulmonary bypass (CPB) was prevented by the previous administration of monoclonal anti-C5a antibody [114]. In the same study constitutive NOS was decreased after CPB, and this decrease was prevented by anti-C5a monoclonal antibody.

In Paper IV anti-C5a monoclonal antibody failed to prevent endothelial damage due to a high amount of intravascular gas bubbles from decompression. The lack of effect from anti-C5a antibody was somewhat unexpected since earlier studies at our laboratory [10;65] clearly demonstrated that gas bubbles are capable of activating the complement system along the alternative pathway. The degree of activation was related to the number of gas bubbles introduced [10], and resulted in a series of events leading to activation and sequestration of PMNs in the pulmonary capillaries with subsequent vascular permeability changes and damage of endothelial cells, as previously described [81;137;143;149;155;170]. The lack of effect from anti-C5a antibody is due to an extensive mechanical injury of the endothelial layer caused by the large amounts of bubbles. The mechanical damages may thus mask the presumed effect from anti-C5a antibody. A previous study at our laboratory

showed no difference in PMN accumulation in the lung tissue between rabbits treated with anti-C5a antibody compared to rabbits given control antibody [67]. That study also concludes with a mechanical damage which masks any beneficial effect from anti-C5a antibody.

Based on our findings in Paper III and IV the next step was to investigate whether anti-C5a antibody could prevent endothelial injury and subsequent PMN infiltration caused by small amounts of intravascular gas bubbles (Paper V). Six rabbits were pre-treated with the anti-C5a antibody whereas a sham monoclonal antibody was administered to six other animals. A third group of rabbits were subjected to an identical protocol except antibody treatment and were used for control. Contrary to our earlier findings (Paper IV) the anti-C5a antibody treatment reduced PMN infiltration and improved slightly the endothelial-derived vasoactivity compared to the control group. Few intravascular gas bubbles do not disrupt the endothelium mechanically and a protective effect of anti-C5a antibody can be demonstrated. However, the reduced PMN infiltration and the slight improvement of endothelium-derived vasoactivity in the sham antibody group was surprising. We are reluctant to ascribe the reduced PMN lung infiltration to the effect of blocking anaphylatoxin C5a alone. In addition to neutralising C5a effectively there might be an additional effect elicited by injecting murine immunoglobulins in the rabbit. A trend was also noted in the group treated with a sham antibody of identical isotype. A study by Kayar et al. showed that decompression sickness in the rat is reduced substantially by pre-dive injection of bovine serum albumin but not by rat albumin [82]. They suggest that pre-dive injections of foreign protein cause varying numbers of platelets or leukocytes to adhere to the foreign protein molecules, thus leaving substantially fewer blood cells free to interact with bubbles and interfere with tissue perfusion during the subsequent decompression. A different activation of leukocytes in presence of foreign protein is also a possibility according to Kayar et al. They further proposed that pre-dive activation of complement in response to the injection of various foreign proteins may have left the animals partially decomplemented during the subsequent dive and therefore less able to respond to intravascular bubbles.

This theory is in accordance with our findings in Paper V where both anti-C5a antibody and anti-sham antibody had a protective effect on PMN infiltration and apparently improved endothelial function. We speculate that the protective effect is due to an immune response caused by foreign Ig in addition to the effect of blocking C5a.

Another approach in explaining the protective effect from antibodies or foreign proteins preventing intravascular damage caused by gas bubbles, is the so called Heat-Shock Proteins (HSP). These proteins occurs in all organisms and there is substantial evidence that HSPs play important physiological roles in normal conditions and situations involving both systemic and cellular stress [86;92]. Moderate stress can have a beneficial effect on subsequent injury to the organism and exposure to one stressor can induce changes that prevent injury to other stressors. The HSP seem to be central to this. Several physiological signals such as hypoxia/hyperoxia [77], ischemia-reperfusion [22], acidosis [169], viral infection, reactive oxygen species [165] and reactive nitrogen species may activate the expression of HSP. Huang et al. found that heat shock induced by increasing the core temperature to 41°C for 15 minutes four hours before air infusion, prevent the animals from acute lung injury induced by pulmonary air embolism [72]. It has become evident that HSPs serve as modulating signals for immune and inflammatory responses, and contributes to both intracellular and extracellular responses to physiological stress [101]. It seems that injection of a monoclonal antibody (Paper V) or a foreign protein [82] works as a stressor to the immune system and hereby activation and expression of HSP. The protective effect from HSP may prevent endothelium injury initiated by gas bubbles from decompression or infusion. A period of no-stress is necessary between the application of the stressor (antibody or foreign protein) and the main event, VGE. A prolongation of the period between pre-treatment with the antibody and the VGE exposure might have improved the endothelial function and decreased the PMN infiltration further.

Long term effects of pulmonary endothelial injury

There is little doubt that endothelial damage has occurred in the pulmonary artery in both pigs and rabbits as a consequence of different amounts of VGE from decompression and infusion. The results reveal acute effects caused by intravascular gas bubbles, but it is difficult to assume any long term effects without some speculations. Direct evidence for endothelial injury and dysfunction remain relatively limited in a clinical situation.

The pulmonary vascular bed receives the entire cardiac output and delivers its venous blood directly into the systemic arterial circulation. Reactions occurring between blood solutes and pulmonary endothelium may influence specific functions of remote target tissues and homeostasis [125]. The homeostatic barrier function of the endothelium is ultimately maintained by the dynamic regulation of the endothelial cell shape, endothelial cell-to-cell adherence, and endothelial – extracellular matrix adherence [94]. One theory might be that dysfunction observed locally in the pulmonary artery could affect the entire organism or influence some biochemical reactions.

One should anticipate that whether the endothelial injury is mechanical (Paper I and IV) or biochemical (Paper III and V) is of importance for possible long term effects. Shimokawa et al. showed that regenerated endothelium after mechanical removal had a significant reduction in endothelium-dependent relaxation to platelets 4 weeks after denudation, while there was no significant alteration 8 days after denudation [144]. Studies in the light microscope revealed that the mean medial thickness was significantly increased 4 weeks after denudation. Electron microscopy studies revealed that regenerated endothelial cells were morphologically different from native ones. The number of endothelial cells had increased from the control level, and they were elongated and irregularly oriented. Further studies by Shimokawa et al. revealed that G protein-dependent endothelium-dependent relaxation were reduced 10 weeks after endothelial regeneration [148]. Our studies also revealed changes in endothelial-dependent relaxation without mechanical rupture, and it is unknown how repeatedly exposures to “silent bubbles” will affect the endothelial lining

after regeneration. It is likely that the sum of acute effects would give a lasting effect, reversible or irreversible.

Considering the findings in Paper V it is natural to conclude that an intact immune system would affect the outcome using foreign antibody or protein preventing endothelial dysfunction. It is also possible that an enervated immune system before exposure to VGE leads to a higher degree of injury and/or decreased response to the preventive effect using a foreign antibody. There are individual differences in the amount of developed bubbles from decompression (Paper I, II and IV), the degree of endothelial injury (both mechanical and biochemical) caused by the same amount of bubbles and the individual response to antibodies (Paper IV and V). Hjelde et al. demonstrated inter-individual differences in complement activation when sera from divers were incubated in the absence or presence of air bubbles in vitro [65]. Bergh et al. 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 varied considerably over time [10].

Methodological considerations

The method for tension measurements is widely used in several research laboratories. Previous studies have measured the reduction in vessel wall tension caused by total mechanical disruption of the endothelial layer, but this method had never been used for studying endothelial function following bubble exposure. The aim of this study was to determine the effect of partial endothelial damage caused by bubbles. In addition to building and developing the equipment (Paper I), the procedures had to be established. Our research group had considerable knowledge and experience concerning bubble formation, detection of intravascular bubbles, injuries from bubble exposure following decompression and different animal models.

For quantifying vasomotor reactivity two terms were used: Tmax defined as the total relaxation response after stabilisation of tension (Paper I) and Imax, defined as the maximum relaxation response (Paper III, IV and V). The Tmax used in Paper I included a contraction phase and a subsequent stronger

relaxation. What causes this contraction is uncertain. Yang et al. has suggested that cyclooxygenase-dependent endothelium-derived contracting factors (EDCF) are released together with NO, and that this limits the effect of the vasodilatation [174]. Studies have shown that both acetylcholine [50] and substance P [23] work as a vasoconstrictor directly on vascular smooth muscle cells if the endothelium is partly or totally lost. Because of the complex vasoactive response when the endothelial layer is damaged, the Imax term was used in Paper III, Paper IV and V as a measure of damage or dysfunction. Using Imax the next concentration in the dose-response series is introduced while the vessel still relaxes and thereby avoids the contraction that follows when using Tmax. Both terms quantify a possible endothelial damage or dysfunction, but Imax is the term most used in the literature [37;78;79]. It is possible to block the contraction phase of the vasoactive response to different agonists and thereby examine only the vasodilatory response after endothelial injury. When the endothelium is intact the vasodilating effects predominate and that is not the case if the endothelium is injured. The endothelium-derived vasoactive response is measured as a relative value independent of terms (Tmax or Imax).

Tension measurements of vasoactivity in the blood vessel is a reliable method for detecting possible endothelial dysfunction. The results are relative relaxation using the basal tension and the precontraction as reference points. Individual differences between each animal and each blood vessel segment do not affect the total outcome. The possibility for mechanical damage with large amount of gas bubbles is obvious and to confirm this, a simple but accurate staining method was used. Silver nitrate staining of the blood vessel lumen visualises the endothelial borders and mechanical ruptures in the endothelial layer appear by light microscopy. A possibility considered but not used in this thesis is combining silver nitrate staining with computer processing to measure mechanical disruption with accuracy. Furthermore it is possible to study the endothelial cells and gap junctions through an electron microscope. This however, demands much time and effort preparing the objectives and was not considered in this study.

Future perspectives

Based on these findings we believe that endothelial damage by bubbles can occur in man as well as in the pig, the rabbit and the rat. Pre-treatment with anti-C5a antibody neutralised C5a and prevented tissue injury when small amounts of bubbles were present (Paper V), but did not prevent tissue injury with large amounts of bubbles (Paper IV). It seems that a model with a large amount of bubbles probably is not a good model to study a possible therapeutic and protective effect of anti-C5a antibody. The possible protective effect from anti-C5a or a foreign protein should therefore be further investigated in models using small amounts of bubbles. A foreign protein can easily be tested on other animals such as the pig since it is commercial available in contrast to pig anti-C5a antibody which we failed to develop after several attempts.

The hypothesis using anti-C5a antibody or a foreign protein as a stressor prior to a longer period of no-stress with subsequent VGE is another interesting approach trying to prevent tissue damages observed both in the pulmonary endothelium and the brain. Considering bubbles developed during decompression, a study by Wisløff et al. indicate that biochemical processes are involved in bubble formation. This is of importance for future search for methods for prevention and treatment of DCS. They also report that exercise can act as a preconditioner protecting against injury, and that NO production plays a central role [171;172]. The goal for future studies would be trying to prevent both bubble formation (decompression) and to prevent the complex immune cascade leading to tissue injury initiated by intravascular gas bubbles. To reach this, further studies are needed trying to reveal the mechanisms behind endothelial dysfunction, the delay in endothelial dysfunction with small amounts of bubbles, the role of heat shock proteins – if any and the role of the complement cascade and C5a or other complex inflammatory cascades.

CONCLUSIONS

- Intravascular gas bubbles lead to endothelial dysfunction with subsequent PMN infiltration and cerebral edema
- The amount of gas bubbles are of importance whether the endothelial dysfunction is mechanical or biochemical
- Gas retention in the rat brain increase with higher bubble score, but edema is still the major contributor to the change in specific gravity
- Pre-treatment with monoclonal anti-C5a antibody had no effect reducing PMN infiltration and endothelial dysfunction caused by high degree of vascular gas bubbles and mechanical injury in the pulmonary endothelial lining.
- Pre-treatment with monoclonal anti-C5a antibody reduced PMN infiltration and endothelial dysfunction caused by small amounts of bubbles, also known as “silent bubbles”
- A possible beneficial effect of other foreign antibodies can not be excluded

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