Microvessel damage in acute respiratory distress syndrome: the answer may not be NO²

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Keywords: complications, acute respiratory distress syndrome; complications, vascular inflammation

Acute lung injury and acute respiratory distress syndrome (ARDS) arise from a wide variety of causes, but the common clinical manifestations are refractory hypoxaemia and increased pulmonary vascular resistance. A major pathological feature of acute lung injury and ARDS is damage to the pulmonary vascular endothelium, secondary to the activation and adhesion of platelets, neutrophils and monocytes. This may result in loss of endothelium-derived vasodilators, such as nitric oxide and prostacyclin. Loss of these factors may contribute to the widespread and indiscriminate pulmonary vasoconstriction seen in ARDS, detected clinically as an increase in pulmonary vascular resistance. The clinical consequence is hypoxaemia, since blood flow to ventilated alveoli is limited by vasoconstriction and local thrombus formation. As well as inducing vasodilation, nitric oxide inhibits the release of cytokines and prevents the expression of adhesion molecules in endothelial cells, smooth muscle cells, leucocytes and platelets. Thus a simple paradigm might be that damage to the pulmonary vascular endothelium enhances basal tone and the tendency towards vasoconstriction, abolishes endothelium-dependent relaxation and promotes adhesion of platelets and leucocytes, leading to inflammation and pulmonary hypertension.

The vascular endothelium is a complex piece of anatomical and biochemical machinery, especially in the lung, where large and small vessels have a completely different embryonic origin. In vitro studies of the pulmonary circulation, much confusion has arisen because of the use of different generations of vessels, which have different functions, i.e. conduit as opposed to resistance arteries. The true seat of pulmonary vascular resistance in large animals is the pulmonary microvasculature (vessels of ≤400 μm in diameter), yet the endothelial responses of these small vessels have hardly been studied. In general terms, in the systemic circulation at least, nitric oxide is a feature of large vessels, whereas a different mediator, endothelium-derived hyperpolarizing factor (EDHF), characterizes microvessels. It would not be unreasonable to speculate that a similar situation occurs in the pulmonary circulation. This article reviews the pathophysiology of ARDS and how the various endothelium-derived relaxing factors (EDRFs) may be affected by the condition. It is suggested that, if the pulmonary microcirculation is the principal source of pulmonary vascular resistance, pulmonary hypertension in ARDS may be linked to loss of EDHF activity, not nitric oxide. If so, inhaled nitric oxide may not be the most appropriate therapy.

Pathophysiology of ARDS

ARDS is a severe and aggressive acute inflammation which is characterized by distinct phases (Fig. 1). In the first phase, invading and dividing bacteria activate polymorphonuclear leucocytes (PMNs; neutrophils) whose primary function is to destroy the bacterial cells. This occurs via the respiratory burst, a surge of superoxide release from the PMNs that kills and fragments bacteria which are then phagocytosed. On destruction, bacteria shed the lipopolysaccharides that make up the bacterial cell wall (endotoxins). Bacterial endotoxins promote further aggressive adhesion of PMNs, monocytes and platelets to the endothelium as well as to each other. In the bone marrow, endotoxins cause an indiscriminate release of mature and immature PMNs. These newly released PMNs are less deformable than normal leucocytes, and this feature, coupled with reduced transit times through the diseased

²This review is accompanied by Editorial I.
pulmonary circulation, makes PMNs more likely to adhere to the pulmonary microvascular endothelium.

Neutrophils directly damage endothelial cells, in the same way as they damage bacteria, through oxidative bursts. Activated leucocytes release cytokines, including tumour necrosis factor alpha (TNFα) and interleukin-1β. These substances stimulate the expression of adhesion molecules in endothelial and vascular smooth muscle cells, thus promoting further adhesion of neutrophils, monocytes and platelets. Proteases, thrombogens and vasoconstrictors are also released.

Several hours after the onset of infection, wide gaps appear in the endothelial cell layer, allowing free passage of PMNs and monocytes to the underlying smooth muscle to promulgate the ongoing cascade. Microvascular fluid leakage results in pulmonary oedema. Since fluid-filled alveoli are not available for gaseous exchange, local hypoxic vasoconstriction occurs, inducing pulmonary hypertension. Hypoxia-induced microvascular constriction reduces flow, further increasing the likelihood of leucocyte adhesion and increasing the likelihood of endothelial damage. In this environment, alteration of the relationship between EDRFs and the underlying vascular smooth muscle is inevitable.

EDRFs and their fate in ARDS

Nitric oxide

The demonstration by Furchgott and Zawadski that endothelial cells could alter the function of vascular smooth muscle introduced a new paradigm in vascular pharmacology. These workers demonstrated that acetylcholine applied to blood vessels with intact endothelium caused vascular smooth muscle to relax. When the endothelium was rubbed off (as was common practice in those days to achieve a ‘pure’ smooth muscle preparation), acetylcholine caused a small contraction. Over the next few years it was determined by experimental deduction that this EDRF was a diffusible, short-lived highly reactive species which induced relaxation by activating smooth muscle cyclic GMP. By 1986 it was becoming clear that a nitrogen-containing molecule could fit these criteria; the most likely such molecule was a simple, unstable molecule: nitric oxide. Late in 1986, Louis Ignarro’s group published evidence suggesting that the pharmacological profiles of nitric oxide and EDRF were identical. Shortly thereafter, the group of Salvador Moncada isolated nitrite products (oxidized forms of nitric oxide) from a blood vessel perfused with acetylcholine. EDRF had been conclusively demonstrated to be nitric oxide.

Nitric oxide is released by a number of dilators, including acetylcholine, bradykinin and substance P. Shear stress is also a potent stimulator of nitric oxide release from endothelial cells. All these mediators stimulate release of nitric oxide from the endothelial cell by increasing intracellular concentrations of inositol trisphosphate. This results in the release of Ca²⁺ from intracellular stores in conjunction with calmodulin, to form a calcium–calmodulin complex which activates endothelial nitric oxide synthase.
L-Arginine is converted to L-citrulline, producing nitric oxide. Nitric oxide diffuses to the vascular smooth muscle, where it activates guanylnyl cyclase and causes an increase in cyclic GMP concentration. Cyclic GMP probably elicits vascular relaxation by causing re-uptake of calcium into the sarcoplasmic reticulum.

**Nitric oxide as a therapeutic strategy in ARDS**

Before the discovery of nitric oxide, the pulmonary hypertension associated with respiratory failure was notoriously difficult to treat, because the available pulmonary vasodilators, such as sodium nitroprusside, prostacyclin, isoprenaline and tolazoline, had to be given intravenously. Although these agents reduced pulmonary arterial pressure, they did so by inducing global pulmonary vasodilation, thereby increasing perfusion to underventilated areas, and so worsening the degree of shunt. In addition, these drugs caused systemic hypotension. Inhaled nitric oxide is delivered to the ventilated alveoli, where it crosses the alveolar membrane to effect vasodilation of the underlying vascular smooth muscle, detected clinically as a reduction in pulmonary vascular resistance. The first evidence for the beneficial effect of inhaled nitric oxide in pulmonary hypertension was published in 1991, and a large volume of literature has been published since, demonstrating that inhaled nitric oxide can reduce pulmonary artery pressures without worsening shunt or inducing systemic hypotension. Consequently, inhaled nitric oxide has been hailed as a major step forward in the treatment of pulmonary hypertension associated with ARDS.

This clinical response to inhaled nitric oxide is variable and fraught with problems. First, some adult intensive care patients with type 1 respiratory failure do not respond to inhaled nitric oxide therapy: 30–40% of patients with increased pulmonary artery pressure and hypoxaemia do not respond to nitric oxide. Second, the effect of nitric oxide on pulmonary hypertension is transient, being most effective in the first 24 h after onset of symptoms. Third, the effect of nitric oxide is dose dependent, but not in the way that might be expected. Very low dose nitric oxide is beneficial in patients with ARDS, but at high doses of nitric oxide, systemic oxygenation is, paradoxically, worsened, even though pulmonary vascular resistance continues to decrease. All of these factors make the response of individual patients to nitric oxide unpredictable, and may contribute to the observed lack of effect of nitric oxide therapy on outcome in the intensive care unit. To understand how such a situation might come about, it is necessary to examine how nitric oxide affects the pathology of ARDS.

**Nitric oxide and the pathology of ARDS**

Cytokines enhance the expression of inducible nitric oxide synthase, principally from vascular smooth muscle cells (Fig. 1). Cytokine activity also causes both endothelial cells and and neutrophils to increase production of superoxide. Some superoxide production occurs normally in all cells, as a result of metabolic activity. In non-diseased cells, the enzyme superoxide dismutase traps superoxide, preventing mitochondrial damage. In diseased cells, overproduction of superoxide has two consequences. First, the increased volume of superoxide production overwhelms the capacity of superoxide dismutase, even though the amount of this enzyme is increased. Second, and most importantly, superoxide reacts three times more efficiently with nitric oxide than superoxide dismutase. The combination of enhanced nitric oxide production by the vascular smooth muscle cells and superoxide from the endothelial cells results in the formation of the free radical peroxynitrite, a cell poison.

The introduction of inhaled nitric oxide to this environment has several effects. The first is pulmonary vasodilation in ventilated areas, secondary to the stimulation of vascular smooth muscle cyclic GMP. This is the beneficial effect seen in the first 24 h. Continued administration of nitric oxide will have the following effects: (1) inhaled nitric oxide stimulates lung macrophages to produce superoxide radicals and stimulates inducible nitric oxide synthase in these cells; (2) nitric oxide inhibits mitochondrial metabolic activity. This further contributes to superoxide formation (by inhibition of the electron transport system). Delivery of a high inspired oxygen concentration to the patient will also result in an increased delivery of superoxide radicals to the lung. Superoxide and nitric oxide from all these sources will interact to form high levels of peroxynitrite, a pulmonary irritant and an irreversible inhibitor of mitochondrial function. Peroxynitrite is found in abundance in the lungs of patients with ARDS. Peroxynitrite (and nitric oxide at therapeutic concentrations) destroy surfactant by lipid peroxidation and prevent further surfactant production by mitochondrial poisoning of alveolar type II cells. It may be speculated that this would enhance the tendency to alveolar collapse. Nitric oxide may also exacerbate the alveolar epithelial permeability induced by inflammatory cytokines, an effect most likely mediated by peroxynitrite. Peroxynitrite damages pulmonary arterial endothelium, which will profoundly affect the production of EDRFs. This substance also inhibits the potent pulmonary vasodilator prostacyclin by a direct action, and encourages the formation of the vasoconstrictor, prostaglandin H2. Peroxynitrite produces a slow relaxation of the pulmonary artery, possibly due to lack of ATP for smooth muscle cell contraction. This may contribute to the initial decrease in pulmonary vascular resistance during the first 24 h of inhaled nitric oxide administration. Tachyphylaxis to peroxynitrite develops and contributes to the general loss of vascular relaxant activity seen in ARDS. Therefore, while nitric oxide initially reduces pulmonary vascular resistance, accumulating toxic metabolites predispose the pulmonary arterial circulation to constriction through the various mechanisms described above. It is
proposed that this may be partly responsible for the rebound hypertension seen in a sub-group of patients where inhaled nitric oxide is withdrawn, even when the gas no longer appears to have a therapeutic effect, i.e. nitric oxide may no longer induce active relaxation, but in some patients, may nevertheless protect against vasoconstriction, in spite of its toxic effects. When the nitric oxide is withdrawn, rebound hypertension may occur.

**Eicosanoids (products of cyclo-oxygenase)**

Prostaglandins, in particular prostacyclin and prostaglandin E2, are produced by vascular endothelia and elicit vascular smooth muscle relaxation. Like nitric oxide, they also inhibit adhesion of platelets, neutrophils and monocytes and as such prevent thrombus formation and protect against leucocyte-induced damage to the endothelium. Inhaled prostacyclin has been used with some success in primary pulmonary hypertension and does improve arterial oxygenation in ARDS, although the effect of this agent on outcome has not been defined. By contrast, other cyclo-oxygenase products have deleterious effects in ARDS. Cytokines promote the expression of cyclo-oxygenase-2 in vascular smooth muscle cells, resulting in increased local formation of thromboxane A2. A study performed in patients after oesophagogastrectomy has shown that those who develop ARDS have significantly greater pulmonary thromboxane production than those without respiratory complications. Both endotoxin and TNFα increase the release of prostaglandin E2 from pulmonary vascular endothelial cells, particularly in the microvasculature. Although a vasodilator, prostaglandin E2 is a classic inflammogen in that it promotes capillary leak and thus may contribute to pulmonary oedema in ARDS.

**Endothelium-derived hyperpolarizing factor**

Even before the discovery of nitric oxide, some workers were able to elicit endothelium-dependent relaxations in various blood vessels which did not fit the accepted view of EDRF. By the mid-to-late 1980s, it was clear that some component of EDRF could hyperpolarize vascular smooth muscle, and the term endothelium-derived hyperpolarizing factor (EDHF) was coined. Although endothelium-dependent hyperpolarization of vascular smooth muscle is elicited by the action of acetylcholine on endothelial cells, it is not affected by known inhibitors of nitric oxide synthase and so cannot involve the L-arginine pathway. It is not a prostaglandin, as inhibition of cyclo-oxygenase enzymes has no effect. While EDHF always causes hyperpolarization, it is not always associated with vascular smooth muscle relaxation. This property has not been explained.

**What is EDHF?**

In spite of considerable effort, the identity of EDHF remains a mystery. Originally the sequence of events was thought to be diffusion of EDHF from the endothelium to the vascular smooth muscle cell, where opening of potassium channels in the smooth muscle membrane initiated outflow of potassium and hence hyperpolarization of the cell. Whereas the diffusion of nitric oxide was easily demonstrated, diffusion of EDHF from endothelial cell to smooth muscle cell has never been shown convincingly, in spite of some early successes. Close apposition of the two cell types is probably required for transmission of EDHF. This probably explains why EDHF is more prominent in the microvessels, where the endothelial layer is related to a single layer of vascular smooth muscle cells, unlike conduit arteries, which are characterized by multiple smooth muscle layers.

**Mechanism of hyperpolarization**

Another complex issue is the type of potassium channels involved in the hyperpolarization. Inhibition of endothelium-dependent hyperpolarization by the combination of two potassium channel blockers, charybotoxin and apamin, is the only commonly agreed characteristic of EDHF activity. Charybotoxin inhibits large conductance calcium-dependent potassium channels, whereas apamin inhibits small conductance calcium-dependent potassium channels. They do not block EDHF when administered separately. This unique property of EDHF has not been explained. Either two separate potassium channels need to be inhibited to block the effect of EDHF, or a single unidentified potassium channel (for example, a voltage-dependent calcium channel) is involved.

This issue is crucial to understanding the role of a particular group of arachidonic acid metabolites, the epoxyeicosatrienoic acids (EETs), in EDHF function. This family is derived by the action of cytochrome P450-dependent enzymes, the epoxygenases, on arachidonic acid, a component of the cell membrane. Epoxygenases are abundant in endothelial cells and show some sequence homology with nitric oxide synthases. When applied topically to vascular smooth muscle cells, EETs cause hyperpolarization and relaxation by opening of smooth muscle potassium channels, so they are candidates for the EDHF response. Unfortunately, direct application of EETs to vascular smooth muscle does not cause relaxation in some vessels where the EDHF phenomenon can clearly be demonstrated. With regard to smooth muscle hyperpolarization, EETs operate large conductance potassium channels exclusively, and so are blocked by charybotoxin alone. Further, inhibition of epoxygenase enzymes seems to block endothelium-dependent hyperpolarization of smooth muscle cells in some arteries but not others. Overall, the evidence suggests that EDHF cannot be explained simply by secretion of EETs from the endothelium and a subsequent direct action of these substances on the vascular smooth muscle cell.
Recently, an alternative and more likely sequence of events has been investigated (Fig. 2). It has been shown that receptor-mediated breakdown of endothelial cell membrane arachidonic acid causes an increase in endothelial EET levels. The EETs stimulate a rise in intracellular calcium, which in turn may be responsible for activation of calcium-sensitive potassium channels on the endothelial cell membrane. These may be the charybdotoxin- and apamin-sensitive potassium channels that are classically associated with the EDHF phenomenon. Elegant electrophysiological experiments by several groups have now shown that these channels are on endothelial cells rather than on vascular smooth muscle cells. Thus, receptor-mediated breakdown of endothelial cell membrane arachidonic acid leads to the intracellular accumulation of EETs, followed by opening of cell membrane potassium channels, outward movement of potassium and hyperpolarization of the endothelial cell. Hyperpolarization of the smooth muscle cell follows. The transmission of EDHF is thus a series of electrical events carrying a signal from the endothelial cell to the smooth muscle cell. This explains the apparent requirement for close apposition of the endothelium to the smooth muscle cell in order to observe the EDHF response.

Transmission of the EDHF signal

How is the signal transmitted across the intracellular space? One prominent group publishing in Nature has proposed that, during hyperpolarization of endothelial cells, there is a small net outward movement of potassium ions, which cross the intercellular space to open barium-sensitive potassium channels on the smooth muscle cell, precipitating hyperpolarization of the smooth muscle cell. This group considered that potassium ions could be described as an EDHF. The concentration of potassium ions in the intercellular space is crucial to this argument. If the potassium ion concentration remains below 5 mM, only the potassium channels will be activated and hyperpolarization occurs. Above this level, the direct action of potassium on the cell membrane will initiate depolarization of smooth muscle and so initiate contraction. Therefore, this initially attractive theory has a difficult conceptual problem: the number of potassium ions crossing the intercellular gap and negotiating the basal lamina between the two cell types has to be sufficient to initiate hyperpolarization, but still low enough to avoid depolarization of the smooth muscle cell membrane. Such a fine degree of regulation would be unprecedented. The majority of groups who have subsequently tested this theory have had limited success in reproducing the original findings, although the rat hepatic artery investigated in the Nature paper may be a special case. Generally, it is accepted that while potassium is crucial to the EDHF phenomenon, the exact role of this ion remains undefined.

How, then, is the electrical signal transmitted? Many recent investigations have implicated electrical conduits between the endothelial and smooth muscle cell; so-called myo-endothelial gap junctions. Gap junctions are formed by a number of membrane proteins, the connexins. Many connexins have been described and are named according to their molecular weight; connexins 37, 40 and 43, for example, are common in blood vessels. Six connexin proteins unite to form a connexon, and several connexons make up a gap junction plaque. Gap junctions are channels linking one cell with another. The channels are large enough to transmit ions and small molecules. The fluid within the junction has an electrical conductance which depends on the connexins present in the channel. Gap junctions are attractive as mediators of vascular smooth muscle function as their connexin composition changes rapidly in response to local changes in shear stress and flow. These areas of close communication are far more common in the thinner-walled microvessels than in large vessels. Pharmacological disruption of gap junctions blocks the action of EDHF, suggesting that these structures are conduits for the hyperpolarizing signal. When endothelial cell membrane potential and smooth muscle membrane potential are measured simultaneously in an intact vessel, application of acetylcholine causes both cells to hyperpolarize. After administration of the gap junction inhibitor, 18α-glycyrrhetinic acid, hyperpolarization of endothelial cells still occurs, but not that of smooth muscle, indicating that the EDHF signal has been blocked. These data represent good evidence for a role for gap junctions in EDHF. Unfortunately, as with the EETs, the predominance
of the gap junction in EDHF responses varies among different blood vessels. Most authors now believe that both are involved but that the relative importance of EETs and gap junctions is highly variable, making a unifying hypothesis of EDHF action impossible.

**EDHF and smooth muscle relaxation**

It is simply not clear how EDHF initiates vascular smooth muscle relaxation. Hyperpolarization of the smooth muscle cell membrane by EDHF may close voltage-operated calcium channels, preventing calcium entry (Fig. 2). An alternative and radically different explanation is that stimulation of EETs in the endothelial cells may increase endothelial cyclic AMP concentrations, and that the cyclic AMP may subsequently pass through the gap junction to initiate relaxation. This interesting idea may account for smooth muscle relaxation but not smooth muscle hyperpolarization.

**EDHF in the context of the pulmonary microcirculation**

Since the pulmonary microvasculature is the source of pulmonary vascular resistance, an understanding of the effects of inflammation on EDHF function is imperative. Such studies are currently in their infancy. Cytokines and lipopolysaccharide increase the production of inducible nitric oxide synthase in pulmonary vascular smooth muscle cells. It has been suggested that the resulting nitric oxide diffuses to the endothelial cells, where it decreases the expression of cytochrome P450 enzymes, reducing EET production and thereby inhibiting EDHF-mediated relaxation.

The effect of inflammation on gap junction activity in the pulmonary circulation is unknown, but there are two highly speculative possibilities. First, alterations in blood flow and shear stress caused by the onset of pulmonary hypertension might lead to a reactive change in myo-endothelial gap junction composition or number, resulting in decreased EDHF activity. Second, gap junctions can be damaged directly by the inflammatory process. Pro-inflammatory mediators specifically disrupt myo-endothelial junctions in the systemic circulation, possibly by altering connexin expression. Connexin proteins are manufactured in the endoplasmic reticulum and reach the cell surface after passing through the Golgi apparatus. These manufacturing elements are initially enhanced in pulmonary endothelial cells which are exposed to inflammatory processes, but they are lost as cell injury progresses. Since the turnover rate of connexin proteins in gap junctions is high, loss of cellular manufacturing capacity would inevitably lead to failure of myo-endothelial communication, i.e. loss of EDHF expression.

**Conclusion**

The discovery of endothelium-derived nitric oxide was only the start of a very complex and exciting story. Vascular smooth muscle function in disease is largely determined by the fate of the endothelium. There are probably many EDRFs, and their character and function are likely to depend on vessel size, location and the effect of environmental factors. In terms of therapy, it initially seemed reasonable to replace lost endothelial factors, as in the use of inhaled nitric oxide and prostacyclin in ARDS. The variable efficacy of these interventions probably reflects the high degree of complexity of the underlying disease process. Inhaled nitric oxide, in combination with high levels of oxygen delivery, may exacerbate the formation of toxic radicals, further damaging the endothelium. The role of a vitally important component, EDHF, has not yet been considered, but its dominant role in the microcirculation means that understanding this complex phenomenon is imperative.

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