Differential Regulation of Lung Endothelial Permeability *in Vitro* and *in Situ*

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**Key Words**

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**Abstract**

In the lungs, increased vascular permeability can lead to acute lung injury. Because vascular permeability is regulated primarily by endothelial cells, many researchers have studied endothelial cell monolayers in culture, in order to understand the pathomechanisms of pulmonary edema. Such studies are based on the assumption that endothelial cells in culture behave like endothelial cells *in situ*. Here we show that this assumption is largely unfounded. Cultured endothelial cells show profound differences compared to their physiological counterparts, including a dysregulated calcium homeostasis. They fail to reproduce the pulmonary responses to agents such as platelet-activating factor. In contrast, they respond in a Rho-kinase dependent fashion to thrombin, LPS or TNF. This is a striking finding for three reasons: (i) in the lungs, none of these agents increases vascular permeability by a direct interaction with endothelial cells; (ii) The endothelial Rho-kinase pathway seems to play little role in the development of pulmonary edema; (iii) This response pattern is similar for many endothelial cells in culture irrespective of their origin, which is in contrast to the stark heterogeneity of endothelial cells *in situ*. It appears that most endothelial in culture tend to develop a similar phenotype that is not representative of any of the known endothelial cells of the lungs. We conclude that present cultured endothelial cells are not useful to study the pathomechanisms of pulmonary edema.
Introduction

Vascular permeability is the physiological basis for one of the five classic hallmarks of inflammation: swelling (lat. tumör). Edema, the modern word for swelling due to an excessive accumulation of fluid in tissue, results from an increased fluid flux from the plasma into the interstitial space across the vascular wall. As initially described by Starling in what has become known as the Starling equation [1], the pathomechanism of edema formation can be differentiated into a hydrostatic type, which results from changes in the hydrostatic or colloid osmotic pressure gradient across the vascular wall, and a permeability type, which is caused by changes in the permeability of vascular endothelial cells for water (hydraulic conductivity, $L_p$) and/or protein (sieving coefficient = 1/reflection coefficient). Such increases in endothelial permeability may occur in order to dilute a toxin, to bring anti-microbial mediators and effectors to interstitial sites of pathogen invasion, or as an untoward side effect when leukocytes leave the vasculature and head towards the site of injury. Widespread increases in vascular permeability, however, bear serious risks such as loss of organ function and systemic hypovolemia. Therefore, edema formation is generally tightly controlled in terms of who, when and where.

Who: As we will discuss, only a few selected mediators such as platelet-activating factor (PAF), leukotrienes, bradykinin and histamine can directly trigger an increase in endothelial permeability. When: The opening of paracellular pores stimulated by these mediators is only transient, usually for merely a couple of minutes. Where: These mediators cause edema preferably in the veins and venules, which are the most permeable vessels of the pulmonary vasculature [2], and to some extent in the capillaries, possibly because that is where the hydrostatic pressure is lower (thus allowing for a better control of fluid extravasation) and the vascular wall thinner as compared to arterioles and arteries.

Because in several organs such as the brain, the intestine or the lungs, swelling can become highly detrimental and potentially fatal, the mechanisms of increased endothelial permeability are of great therapeutic interest. In the lung, increased vascular permeability causes first interstitial and subsequently alveolar edema, resulting in impairments of gas exchange and lung mechanics. Although mild-to-moderate increases in vascular permeability are also present in hydrostatic-type lung edema [3, 4], overt vascular barrier failure is a characteristic hallmark of acute lung injury and its most severe form in patients, the acute respiratory distress syndrome (ARDS)[5]. Given the high incidence and fatality rate of ARDS, it is not surprising that regulation, failure and reconstitution of lung vascular permeability has become a focus of intense research over the past decade. Owing to the considerable difficulties of comprehensively studying molecular mechanisms in endothelial cells in situ, endothelial cells in culture have been studied intensively in vitro under the premise that they represent relevant properties of the vascular bed in question. Here we will examine this premise for pulmonary endothelial cell monolayers that have been extensively studied in order to gain insight in the pathomechanisms of pulmonary edema.

The heterogeneity of pulmonary endothelial cells

Since increased endothelial permeability can occur in all organs, one might wonder to what extent the underlying mechanisms may be identical in all vascular beds. There exist some general principles in all endothelial cells such as the critical role of the adherens junction protein VE-cadherin for establishing the endothelial barrier [6]. However, given the heterogeneity of endothelial cells, stark differences can be expected from organ to organ as well as between arteries, veins and capillaries within an organ [7-9]. There can even be mosaics of different endothelial cells in the same vascular bed; e.g. endothelial pacemaker cells that generate calcium waves in the endothelium are directly juxtaposed to endothelial non-pacemaker cells [7, 8]. This great cellular heterogeneity of endothelial cells is reflected by widely different values for hydraulic conductivity in various vascular beds [10], a
phenomenon that is likely related to morphologically heterogeneous endothelial junctions [9]. One vascular bed that clearly stands out, is the pulmonary circulation that differs from other organs by its low pressure, the reversed partial pressures of blood gases in its arteries and veins, the phenomenon of hypoxic vasoconstriction and the mechanism by which PAF enhances vascular permeability [11-13].

Therefore, when studying the molecular mechanisms of vascular permeability in endothelial cells in culture, it is imperative to establish which particular anatomical location any such model does reflect. While in vitro studies on endothelial permeability were initially frequently performed in human umbilical vein endothelial cells (HUVECs) due to their abundant availability, the recognition of the high degree of heterogeneity and local specialization of the vascular endothelium has led researchers to focus more specifically on pulmonary microvascular, pulmonary artery, and – in some cases – pulmonary venous endothelial cells. However, the degree to which these seemingly site- and organ-specific cell types truly reflect the properties of their corresponding counterparts in vivo is rarely taken into consideration or discussed. Furthermore, the gold standard for the regulation of endothelial permeability in the intact lung is not straightforward. Because most of the pro-inflammatory mediators and mechanisms that alter pulmonary vascular permeability can also regulate leukocyte activation and function, results from in vivo animal experiments need to be interpreted with caution. For instance, the Rho-dependent protein kinase (ROCK), which has frequently been implicated in vascular permeability in endothelial cells in culture [14], seems also to be involved in neutrophil migration [15] and granule exocytosis [16]. The latter effects may contribute critically to ROCK-dependent changes in endothelial permeability in vivo, in particular because pulmonary edema triggered e.g. by bacterial lipopolysaccharides (LPS), complement or transfusion of MHC I mAbs is reduced in neutropenic animals [17-19]. Thus, protective effects of Rho kinase-inhibitors in vivo could potentially relate to neutrophils rather than endothelial cells [20, 21].

Possible solutions to this problem are endothelial cell-specific knockout mice or isolated perfused lungs. Isolated lungs possess several distinct advantages, that in our view make this model particularly well suited to validate cell culture experiments dedicated to understanding the mechanisms of pulmonary edema formation [22-24]:
- continuous monitoring of edema formation by weight measurement (very sensitive because 1 µL fluid accumulation = 1 mg weight gain, high signal-to-noise ratio);
- quantification of vascular permeability to water and proteins by determination of the filtration coefficient, the reflection coefficient and the total vascular surface area;
- quantification of microvascular pressure by the double occlusion method;
- elimination of blood-borne leukocytes by perfusion with the buffer of choice;
- simplified pharmacokinetics of applied drugs and substances due to limited compartmentalization and metabolism;
- exclusion of hydrostatic edema by constant pressure perfusion;
- monitoring of molecular responses in arteriolar, capillary, and venular endothelial cells in situ by real-time fluorescence microscopy.

Comparison of studies on vascular permeability in isolated perfused lungs and in endothelial cell monolayers

When comparing endothelial barrier function in vitro and in the isolated perfused lung, the resulting correlation is remarkably poor. Figure 1 shows the effects of various agents that have been tested for their effects on vascular permeability in calf pulmonary artery cells (CPAE) and in human microvascular endothelial cells of the lung (HMVEC-L) in comparison to their effects on weight gain in isolated perfused rat lungs.

Thrombin, H₂O₂ and rh-VEGF (in CPAE cells) induce strong responses in pulmonary aortic or microvascular endothelial cell monolayers, but have almost no acute effect in isolated lungs. Conversely, PAF, the PGE₂-derivative misoprostol and bradykinin trigger
edema in the isolated perfused lung, but not in the two cultured endothelial cell types. Only leukotriene C4 and ceramide cause permeability changes both in perfused lungs and in cell culture. The differences between the results in isolated lungs versus cells in vitro are most likely not explained by the fact that the concentrations differed for some of the agents tested, (i) because those agents that failed to cause edema in intact lungs were clearly active as shown by the strong vasoconstriction induced by thrombin [26] and by the toxicity of H2O2 after longer perfusion times (>30 min; data not shown), and (ii) because those agents that failed to increase permeability in vitro were ineffective even at higher concentrations (lower concentrations showed also no effect, data not shown). Another relevant observation is that the responses in both endothelial cell types were nearly identical, hinting at the possibility that in culture endothelial cells may adopt similar phenotypes (see below).

These findings raise the question to what extent studies with endothelial cells in culture reflect vascular permeability in the intact lung. In the next section we will examine this question in more detail with respect to three agents that are frequently used to study the mechanisms of vascular permeability in endothelial cells in culture, namely thrombin, tumor necrosis factor-α (TNF-α), and endotoxin (bacterial lipopolysaccharides; LPS) and that all cause actin stress fiber formation in cultured endothelial cells [27]. In addition, we will discuss the effects of PAF, a molecule that has been studied in depth in the IPL model, and of sphingosine-1-phosphohate, which has been proposed to have barrier-protective properties on lung endothelial cells.

**Platelet-activating factor (PAF)**

PAF is a lipid mediator that has been implicated in clinical ARDS and its blockade is protective in most experimental models of ARDS [28]. In vivo, PAF causes edema due to leakage from capillaries and even more so from veins [29]. In isolated lungs PAF increases vascular permeability within minutes by a complex mechanism that starts with activation of the acid sphingomyelinase with subsequent ceramide formation [30] that leads to the recruitment of caveolin-1 into the caveolae of pulmonary endothelial cells. The recruited caveolin-1 engages TRPC6 channels that become disinhibited in part by the dramatic decrease in endothelial NO levels through the silencing of eNOS by its interaction with caveolin-1 [31, 32]. We have recently reviewed this mechanism in detail [11, 33].
In pulmonary endothelial cells in culture it has been difficult to reproduce this effect of PAF, in that even PAF concentrations as high as 100 nM do commonly not result in a decrease of trans-endothelial electrical resistance as a measure of monolayer permeability in vitro (Fig. 2) [26, 35]. This failure is not explained by lack of PAF-receptors that are present (Fig. 2) and functional as was shown by the PAF-dependent production of prostacyclin [35]. Some groups were successful in increasing vascular permeability in endothelial cell monolayers by PAF in HUVEC cells, in murine pulmonary artery and in murine pulmonary microvascular endothelial cells [29, 36, 37]. The reasons why PAF is active in terms of increasing the permeability of cultured endothelial cells in some labs but not in others are not clear. Importantly, however, the mechanisms between the PAF-induced edema formation in isolated lungs and in endothelial cells in culture show principal differences, as recently reviewed by us in detail [11]: in isolated lungs PAF causes edema by a decrease in NO-levels [31], whereas in cultured cells PAF causes edema by an increase in NO-levels [37]. This is an important difference, because the induction of high NO levels by PAF is a response typical for non-pulmonary vascular beds [11]. Another notable discrepancy relates to the relevance of the myosin light chain kinase that seems to play no critical role in endothelial cell monolayers [29], whereas ML-7, an inhibitor of myosin light chain kinase, attenuated the PAF-induced increase in vascular permeability in intact lungs [38]. Again, this behavior of the cultured cells is reminiscent of extra-pulmonary endothelial cells in which ML-7 did also have no effect on the PAF-induced increase in vascular permeability [39]. Thus, it appears as though pulmonary endothelial cells take on a phenotype in cell culture that is akin to that of extra-pulmonary endothelia.

**Thrombin**

Thrombin is a peptide that increases endothelial cell permeability in most cell culture systems that have been tested so far. Thrombin causes formation of intercellular gaps and a drop in trans-endothelial electrical resistance within minutes (Fig. 2). Similar to TNF-α and LPS (see below), stimulation of cultured endothelial cells with thrombin increases the cytosolic Ca²⁺ concentration ([Ca²⁺]ᵢ) and activates myosin light chain kinase (MLCK), as well as the small GTPases RhoA, Rac1 and Cdc42 [27].
In isolated lungs, thrombin causes vasoconstriction and increases vascular resistance [25, 40, 41]. As a consequence, lungs perfused with thrombin are prone to hydrostatic edema as a result of the ensuing increase in vascular pressures. Once this pressure increase is eliminated, either by preventing the pressor response [40, 41] or by constant pressure perfusion (as was utilized in Fig. 1) [26, 42], thrombin, however, no longer causes edema in the isolated perfused lung. These findings are in line with observations in mesenteric vessels, where thrombin – in contrast to PAF – also failed to increase vascular permeability [43]. Interestingly, when mesenteric venules were taken into culture for at least 6 hours, they started to respond to thrombin in a Rho-kinase-dependent manner [43]. Similar observations were made in the skin [44]. Thus, it appears that under culture conditions the vascular endothelium may become responsive to the permeability-increasing properties of thrombin. In the intact lung, however, thrombin does not seem to play a critical role in lung vascular barrier regulation, as shown e.g. for LPS-induced pulmonary edema [45]. To our knowledge, there is as yet no study that shows that blocking thrombin or its receptors has beneficial effects on vascular permeability in models of ARDS independent of thrombin’s role in coagulation and the regulation of vascular tone.

There have been attempts to measure changes in filtration coefficient in isolated lungs in response to thrombin perfusion [46-48]. However, the $K_{fc}$ is expressed in relation to capillary pressure which therefore must stay constant during the measurement [49, 50]. Unfortunately, as noted above, thrombin increases vascular resistance, so capillary pressure is unlikely to stay constant during perfusion with thrombin, and measured changes in $K_{fc}$ in response to thrombin are thus difficult to interpret. Notably, the failure of thrombin to induce edema in the isolated perfused lung is - similarly to the lack of effectiveness of PAF in cultured endothelial cells - not attributable to a lack of its protease-activated receptors on the vascular endothelium of the intact lung, as thrombin still elicits an endothelial $[Ca^{2+}]_i$ response in the isolated perfused lung preparation (Fig. 3).

**TNF-α**

Analogous to potentially confounding vasoactive effects of thrombin which may mimic changes in vascular permeability, *in vitro* experiments with TNF-α are notoriously prone to false-positive increases in permeability due to the well-recognized property of TNF-α to induce apoptosis and cell death [51, 52]. Several studies in endothelial cells in culture have shown that TNF-α increases monolayer permeability within 1h to 5h [52-56], although
sometimes permeability is studied for up to 24h [57] at which time the mechanisms leading to increased permeability may differ [58]. In the first hours, TNF-α causes MLC- and Rho-kinase-dependent actin stress fiber formation [51] in cultured endothelial cells. This response is mediated by Rho-kinase which has consequently been linked to the ensuing increase in permeability [54, 59], although this mechanism was questioned in one study [52]. Besides Rho-kinase, the following signaling molecules have been implicated in the TNF-α induced permeability increase in vitro: P-Rex1/Rac1 [55, 56], PKCa [60], p38 [54, 61], NO derived from endothelial NOS [58, 62] leading to formation of peroxynitrite [63], and tyrosine kinase-mediated phosphorylation of VE-cadherin [55, 64]. Interestingly, despite the fact that monolayer permeability usually starts to increase earliest 1h after stimulation with TNF-α, many of the signalling events that have been linked to the increase in permeability reach their maximum within 5-20 minutes, such as the activation of RhoA [59], rac1 [55], MAP kinases [54], src kinase [64], the phosphorylation of VE-cadherin [55], and the inactivation of myosin phosphatase target subunit 1 [59]. It is as yet unclear why seemingly similar responses would lead to an immediate permeability increase in the case of thrombin (see above), but not in the case of TNF-α. Another remarkable observation is the fact that TNF-α does not increase endothelial [Ca2+]i in vitro, and may even suppress the endothelial [Ca2+]i response to e.g. thrombin [65, 66], while it elicits a distinct endothelial [Ca2+]i increase in the intact lung [67, 68].

In early in vivo studies injection of TNF-α caused pulmonary edema with wet-to-dry ratios between 6 and 7 [51, 69, 70], whereas in later studies TNF-α failed to cause edema when administered intravenously [71, 72]. It seems possible that these differences are explained by the known synergistic effects between TNF-α and LPS [73], as the early TNF-α preparations were always contaminated with LPS. And contrary to a widely held belief TNF-α does not seem to mediate LPS- or bacteremia-induced lung edema [74, 75]. Of note, if TNF-α is administered into the airways rather than intravenously, it elicits profound edema [76, 77], presumably through direct activation of alveolar macrophages and alveolar epithelial cells, yet not endothelial cells, as TNF-α itself is too big to cross the alveolar barrier [67].

In line with the notion that TNF-α cannot cause edema by itself, TNF-α failed to cause pulmonary edema in isolated perfused lungs within 1 hour [78, 79] or 3 hours (own unpublished data). These findings are supported by the observation that in TNF-α perfused lungs antibodies in the perfusate did not reach the alveolar space and vice versa, indicating that the lung vascular barrier was intact [67]. In those studies where edematogenic properties of TNF-α were described in isolated lungs, the experiments had always been performed in the presence of leukocytes [79-82] raising the possibility that TNF-α acted primarily on immune rather than endothelial cells. Taken together, it appears likely that TNF-α itself does not directly increase vascular permeability in the intact lung.

**LPS**

Recently, there is growing interest in studying the effect of LPS on pulmonary endothelial cell monolayers in culture. In these models, LPS increases permeability sometimes almost immediately [83], but generally within 2h [84-90]. Similar to TNF-α, LPS-induced edema formation has been linked to the formation of actin stress fibers that appears to depend on Rho-kinase [84, 91], phosphorylation of MLC [84] and a loss of cadherin expression [88].

In general, the extent of edema formation inducible by administering LPS i.p. or i.v is surprisingly mild [92]. This kind of edema is dependent on neutrophils [17, 45, 93-96] and possibly also the liver [97]. In line with this view, high doses of LPS failed to cause pulmonary edema within 2.5h in isolated perfused lungs when perfused with blood- and cell-free buffer [98, 99], and caused edema only when leukocytes were present in the perfusate [81, 100, 101]. Analogous to the effects of thrombin and TNF-α, these findings indicate that LPS alone is not able to cause pulmonary edema.

One possibility worth considering is that LPS may alter vascular permeability by causing apoptosis [52, 102]. However, there is yet no convincing link between endothelial apoptosis and pulmonary edema in intact lungs. Instead we have shown in a model of LPS-induced
lung injury that treatment with a pan-caspase inhibitor sufficient to prevent apoptosis [103] had no effect on pulmonary edema [104].

Taken together, we conclude that the physiological significance of the permeability changes of endothelial monolayers in response to TNF-α, thrombin and LPS is unclear.

**Sphingosine-1-Phosphate**

Sphingosine-1-phosphate is an agent that stabilizes the barrier properties of endothelial monolayers by binding to S1P₁-receptors and subsequent activation of the Rho family of small GTPases, cytoskeletal reorganization, adherens junction and tight junction assembly, and focal adhesion formation [105]. In perfused lungs, 1 µM S1P – a concentration that does not activate the S1P₂- or S1P₄-mediated vasopressor response [106, 107] – reduces the PAF-induced weight gain by one third (Fig. 4). However, at the same concentration S1P alone slightly increased weight gain, possibly by activation of S1P₂-receptors [108]. Unfortunately, the mechanisms of the S1P₁-mediated barrier-enhancing effects have not yet been studied in detail in isolated lungs, so that at present it is uncertain whether the same mechanisms are operative in vitro and in the intact organ. Experiments in vivo have replicated the barrier-protective effect of S1P, but are difficult to interpret, not only because S1P₁-receptors are rapidly internalized [109], but also because in addition to stabilizing barrier properties S1P receptors also affect chemotaxis and apoptosis of neutrophils as well as pulmonary epithelial permeability [104]. As conditional, cell-specific ko mouse models for the five different S1P receptors are currently not available, it remains to be shown to which extent the barrier-protective effects of S1P in in vivo experiments are indeed attributable to a direct effect on the vascular endothelium.

**The role intracellular calcium signalling**

Increases in endothelial [Ca²⁺], resulting either from the entry of extracellular Ca²⁺ via store-operated, receptor-operated, or voltage-dependent Ca²⁺ channels, constitutes one of the primary mechanisms of endothelial barrier regulation, in that it regulates cytoskeletal reorganization, activates myosin light chain kinase, and decreases the formation of barrier-stabilizing cAMP [111, 112]. Analogously, inhibition of Ca²⁺ entry or release has proven effective to attenuate vascular permeability increases in response to inflammatory mediators such as PAF [32, 38] or mechanical stimulation such as endothelial cell stretch [3, 4, 113] in isolated lungs. Yet, the finding that pro-inflammatory stimuli such as TNF-α or thrombin are able to elicit distinct endothelial [Ca²⁺], responses, yet fail to induce marked increases in endothelial permeability in isolated perfused lung preparations (see above) suggests that an increase in endothelial [Ca²⁺] in response to a physiological stimulus rather than a pharmacological agonist may by itself not be sufficient to trigger an endothelial barrier leak.

The fact that the same mediators effectively increase permeability in cultured pulmonary endothelial cells raises the interesting possibility that the intact lung endothelium may have developed a set of barrier-protective mechanisms that are lost once endothelial cells are cultured in vitro. This notion is supported by a body of data demonstrating that basal

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**Fig. 4.** Weight gain in isolated rat lungs that were perfused and treated with a bolus injection of 5 nMol PAF into the pulmonary artery as described [31, 32, 110]. One µM S1P was added 10 min prior to administration of PAF. Data are mean ± SEM of 3 independent experiments each.
[Ca\(^{2+}\)]\(_i\) homeostasis as well as time profile and amplitude of stimulated [Ca\(^{2+}\)]\(_i\) responses differ markedly between pulmonary endothelial cells in culture and in the intact lung. In 1996, Ying and Bhattacharya discovered that endothelial cells in the intact pulmonary vasculature display spontaneous [Ca\(^{2+}\)]\(_i\) oscillations that originate from a specific subset of endothelial cells located at the vascular branch points, which they accordingly termed pacemaker cells, and from where these oscillations were propagated as an intercellular Ca\(^{2+}\) wave via intercellular gap junctions to adjacent endothelial cells [114]. These pacemaker-generated [Ca\(^{2+}\)]\(_i\) oscillations were shown to be critical for the endothelial [Ca\(^{2+}\)]\(_i\) response to pharmacological or mechanical stimulation by histamine or stretch, respectively [114, 115]; yet this pacemaker activity is universally absent in cultured endothelial cells, suggesting that endothelial cells in vitro lose the ability to spontaneously generate [Ca\(^{2+}\)]\(_i\) oscillations [114] which may be regarded as an indicator of their inability to regulate their [Ca\(^{2+}\)]\(_i\) homeostasis in a physiological manner.

Along these lines, it has become evident from a series of real-time imaging studies in the isolated perfused lung preparation that endothelial [Ca\(^{2+}\)]\(_i\) responses to various stimuli differ markedly between the intact lung and cultured endothelial cells with respect to the time profile and the amplitude of the response. While pharmacological stimulation of cultured endothelial cells by Ca\(^{2+}\)-mobilizing agents such as PAF or mechanical stretch typically trigger [Ca\(^{2+}\)]\(_i\) responses within seconds and frequently of amplitudes in the range of 500-1000 nMol/L [116-119], the characteristic [Ca\(^{2+}\)]\(_i\) response in the pulmonary endothelium of the intact lung is characteristically much slower within minutes, and rarely exceeds amplitudes >200-250 nMol/L [32, 115]. This discrepancy may point toward the existence of mechanisms which enable the endothelial cell in situ to blunt and attenuate potentially harmful, excessive increases in [Ca\(^{2+}\)]\(_i\); yet such mechanisms may become lost under conditions of cell culture. One such putative mechanism is the above mentioned communication between endothelial cells via gap junctions. In a highly elegant study, Parthasarathi and coworkers showed that uncaging of Ca\(^{2+}\) in an individual endothelial cell of the isolated lung results in a rapid spread of Ca\(^{2+}\) to adjacent endothelial cells via gap junctions [48]. Such a mechanisms may help to protect the primarily targeted cell from an excessive overload in Ca\(^{2+}\), yet would fail in a cell culture system where the gap junction-forming connexins are characteristically lost in most cell types [120, 121].

**The role of the Rho-kinase pathways**

As discussed above, cell culture experiments with thrombin, LPS and TNF-\(\alpha\) have led to the conclusion that the Rho-kinase pathway with subsequent stress fiber formation constitutes an important mechanism in pulmonary endothelial vascular permeability. This hypothesis, however, has yet to be confirmed in intact lungs. To the contrary, stress fibers have never been shown in pulmonary endothelial cells in situ, whereas non-edematogenic agents such as thromboxane or angiotensin can induce actin stress fibers in culture [122]. Notably, the structural arrangement of the endothelial actin cytoskeleton differs markedly between cultured cells and endothelial cells of the intact lung, in that the former align in the direction of flow [123], whereas the latter are arranged perpendicular to the flow axis in a circular or spiral arrangement [124]. Further, intratracheal LPS challenge does not result in activation of RhoA [125] and RhoA-mediated contractile mechanisms are not involved in bradykinin-, PAF- or leukotriene C4-induced hyperpermeability in various vascular beds in situ (see Fig. 5 and refs [38, 126]). When overexpressed, RhoA may possibly play a role in vascular permeability as was suggested by studies in RhoGDI-1-deficient mice [127]. These and other findings led Curry and Adamson to state: “Although the RhoA-dependent pathways activated by thrombin are well understood, the reason why they are so robust in some cultured endothelial cell monolayers is not understood.” [43].

Importantly, in culture the response pattern of the thrombin/Rho-kinase/stress fiber axis is dependent on the stiffness of the substrate: the stiffer the substrate the more pronounced the role of the Rho-kinase pathway becomes [128-130]. Starting at a substrate stiffness of about 5 kPa, thrombin is able to increase vascular permeability by a Rho-kinase-
dependent mechanism [129]. However, at this stiffness the response to thrombin is weak [129] and reversible [130]. The estimates of the stiffness of normal lungs range from 0.5 kPa as determined by atomic force microscopy [131] to 5 kPa based on uniaxial tension tests or model-based calculations [132-134]. However, none of these estimates refers to the stiffness of pulmonary vessels, which remains unknown. If the observation that HUVEC cells grow better at 0.3 kPa than at 5 kPa [135] gave a hint as to the stiffness of pulmonary capillaries and veins in situ, this would help to explain why thrombin fails to cause edema in these vessels. Further, the finding that fibrotic lungs become up to ten times stiffer during progression of the disease [131] gives rise to the interesting hypothesis that in such lungs thrombin may, in fact, cause edema by Rho-kinase dependent mechanisms. This discussion also sheds a new light on experiments with endothelial cells grown on conventional cell culture material, the stiffness of which is in the range of GPa, conditions that seem to greatly favor Rho-kinase dependent mechanisms.

The phenotypes of endothelial cells

Phenotypic differences between endothelial cells in culture and in situ

The discussion so far shows that endothelial cells in culture compare rather poorly to those in intact isolated lungs with respect to their responses to mediators involved in the regulation of vascular permeability. In addition, endothelial cells in culture differ from endothelial in situ by further relevant properties:

- In general endothelial permeability to albumin is 10-100 times greater in cell culture as compared to the intact vasculature [136]. Similar differences have been derived for the hydraulic conductivity of endothelial cells isolated from rat pulmonary arteries or the microcirculation [137].
- Endothelial cells in culture generate higher cytosolic NO-concentrations than endothelial cells in situ [138].
- Many endothelial cells in culture lack the typical glycocalyx that is an important regulator of vascular permeability [139].
- Plasma membrane protein expression in cultured rat pulmonary microvascular endothelial cells differs markedly from the pattern observed in in situ. Forty-one percent of proteins expressed in vivo were not detected in vitro [140].
- It has been stated that endothelial cells in culture may express ten-fold less caveolae than in situ [140, 141].
- Endothelial cells in culture are usually kept under static conditions. However, it is clear that the endothelial phenotype is also determined by the shear stress originating from blood flow [142], by the hydrostatic pressure [143], and by the oscillatory profile of both parameters [144].
- The interaction with pericytes that may control important endothelial cell functions are lost.

The programming of endothelial cells

The discussion above shows that under the currently prevailing culturing conditions endothelial cell in vitro display elementary differences in comparison to their in situ counterparts. To some extent, it appears as though with respect to endothelial cell permeability all endothelial cells – irrespective of their origin – develop a similar phenotype in culture. This phenotype seems to be more representative of systemic than of pulmonary endothelial cells, because these cells respond to PAF (if they respond at all) with increased NO production [37] which is typical for extrapulmonary endothelial cells rather than with decreased NO-production which is characteristic of pulmonary venous endothelial cells in situ [11, 31]. However, these cells do also show properties that are not observed by their counterparts in vivo, in particular the increased permeability in response to LPS, TNF-α and thrombin and the preponderance of Rho-kinase-dependent mechanisms.

That endothelial cells of different origin can converge on a uniform phenotype in culture has been demonstrated before for microvascular endothelial cells from the lung, the skin and the intestine, that do all develop a very similar phenotype, based on gene expression analysis, when taken into culture [8]. Thus, it appears possible that the heterogeneity of endothelial cells with respect to vascular permeability is more determined by their microenvironment (nurture) than by their genetic programming (nature).

Recently, Aird has introduced the concept of multistability, originally derived from systems theory, to explain the heterogeneity of endothelial cells [8]. According to this theory, the factors that determine the fate of endothelial cells are robustness, homeostasis, memory and plasticity. Following this concept, we would argue that e.g. with respect to their permeability response to thrombin endothelial cells in culture show robustness (not changed by random perturbations) and homeostasis (not changed by subtle intracellular noise and small variation in the extracellular environment). While this phenotype is stable, the plasticity of endothelial cells allows them to enter another stable state, for instance when the cells grow on materials softer than 5 kPa (see above). However, it is not at all clear whether the Rho-kinase-dependent response to thrombin is retained (e.g. an original feature of these cells) or whether it is gained in response to stiff substrates. Clearly, the use of cultured endothelial cell monolayers for studying the mechanisms of permeability assumes that the cultured cells have a memory of their original behavior, such as the expression of Notch in systemic arterial and EphB4 in venous endothelial cells [146] (of note, human pulmonary endothelial venous cells do not express EphB4 [147]). At present, the extent of this memory, its prevalence over plasticity in response to the specific conditions of cell culture, and the effects that changes in this balance will have on the endothelial permeability response to agonists such as PAF is unclear, but – based on above considerations and the obvious discrepancies between in vitro and in situ systems – the effect of plasticity must be considered substantial.

Aird furthermore makes the important point that "This framework requires us not to ask whether a property is plastic or robust, but how plastic and robust it is to particular environmental changes" [8]. Thus, we need to ask how plastic and robust the response
of pulmonary endothelial cells is when it comes to vascular permeability. The discussion above suggests that endothelial cells in culture assume a phenotype that is different from most – if not all – endothelial cells in situ. The phenotype of endothelial cells is determined by mechanical forces (e.g. substrate stiffness, shear stress, hydrostatic pressure) [148, 149], by matrix components [150], by the geometry [151], and by cells and mediators from the environment [152, 153]. It appears as though the plasticity of cells is relatively high when they are removed from their original environment. They are rapidly reprogrammed (in terms of gene expression) as has been shown for instance for post-capillary venous endothelial cells [154], arterial and venous ECs from human umbilical cord [155] and pulmonary epithelial cells [156]. As noted above, this reprogramming has been shown to result in very similar phenotypes when microvascular endothelial cells from originally very different vascular beds are taken in culture [8].

Conclusions

Vascular permeability is regulated primarily by endothelial cells. The marked heterogeneity of endothelial cell phenotypes is reflected in the equally heterogeneous regulation of vascular permeability. Examples are the tropism of thapsigargin for pulmonary artery [157] and of PAF for pulmonary vein endothelial cells [29] as well as the different roles of NO in PAF-induced edema in pulmonary versus systemic vessels [11]. Therefore, when studying vascular permeability responses in vitro one should start with culturing the relevant endothelial cells. It seems for instance not justified to use umbilical vein endothelial cells for studies of pulmonary edema formation, unless the relevance of the model is clearly demonstrated.

Beyond this organ- and vasculature-specific heterogeneity, however, it appears that all vascular endothelial cells when taken into culture undergo rapid reprogramming under the presently used static two-dimensional cell culture conditions. The assumption that this reprogramming should not affect the mechanisms relevant to pulmonary vascular permeability does not seem based on any solid data or concepts other than wishful thinking. On the contrary, it seems that – speaking in terms of systems theory – the robust Rho-kinase-dependent phenotype is an attractor that makes different endothelial cells behave similar in culture.

Taking primary cells into culture has proved a monumental task, which – in our opinion – has not yet convincingly been accomplished for any of the lungs’ many cell types. Here we have discussed the many shortcomings of cultured pulmonary endothelial cells when it comes to studying the mechanisms of increased vascular permeability. This leads us to conclude that at present, key mechanisms of increased vascular permeability in the lungs cannot be adequately reproduced by endothelial cell monolayers in culture. With a better understanding of the factors that regulate endothelial cell plasticity, it may be possible to identify crucial cues such as typical mechanical forces, mediators and geometry that trigger the endothelial phenotype specific for the pulmonary circulation. However, one may even wonder whether there is a typical pulmonary endothelial cell at all. Could it be that pulmonary endothelial cells – similar to the pulmonary epithelial cells – instead form a sort of phenotype gradient along the vascular tree? And could it be that different types of endothelial cells exist as mosaics in direct proximity to each other including the endothelial pacemaker cells [7] that act conjointly to generate the full picture of vascular permeability in vivo? These notions describe the dimension of the problem that we need to solve.

However, as difficult as it has been to reproduce the specific mechanisms of increased vascular permeability, it seems much easier to reproduce mechanisms of barrier protection as is illustrated by S1P or cAMP-raising compounds that are effective in vitro and in vivo [158, 159]. To vary a line of Tolstoy: All happy endothelial cells are alike; each unhappy endothelial cell is unhappy in its own way.
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Disclosure Statement

None of the authors has any disclosures to make.

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