Review

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

Stefan Uhlig^a Yang Yang^a Josephine Waade^a Claudia Wittenberg^b
Aaron Babendreyer^a Wolfgang M. Kuebler^{b,c}

^aInstitute of Pharmacology and Toxicology, RWTH Aachen University, Aachen, ^bInstitute of Physiology, Charité-Universitätsmedizin Berlin, Berlin, Germany, ^cDepartments of Surgery and Physiology, University of Toronto, Ontario, Canada

Key Words

Endothelial cells • Pulmonary endothelial cells • Pulmonary edema • Vascular permeability • Platelet-activating factor • Calcium • Rho-kinase • Thrombin • LPS • TNF • Sphingosine-1-phosphate

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 depend 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 phenotyp that is not representative of any of the known endothelial cells of the lungs. We conclude that at present cultured endothelial cells are not useful to study the pathomechanisms of pulmonary edema.

Copyright © 2014 S. Karger AG, Basel

Introduction

Vascular permeability is the physiological basis for one of the five classic hallmarks of inflammation: swelling (lat. *tumor*). 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 = $1/\text{reflection coefficient}$). 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 μ L 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

Fig. 1. Correlation of permeability promoting properties of various agents in isolated perfused rat lungs (IPL) and endothelial cell layers. The weight gain in g (X-axis) and the transendothelial electrical resistance (EVOMX Epithelial Tissue Volt/Ohmmeter, World Precision Instruments) in CPAE cells (A) and HMVEC-L cells (B) (Y-axis) were followed for 30 min in the IPL and up to 180 min in the cell culture system. The dotted line represents an ideal regression line and was not calculated. The r^2 values were 0.018, $p=0.70$ for A and 0.0018, $p=0.90$ for B. Taken from Ref. [25] with permission. The correlation between the responses in CPAE and HMVEC-L cells was not different from unity.

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-phosphate, 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].

Fig. 2. Human microvascular endothelial cells of the lung (HMVEC-L) in culture. A. 4000 HMVEC-L cells (Lonza; 7th-8th passage) per well were cultured according to the manufacturer's instructions on 96-well E-plates. Confluency was reached after about 50 hours as determined by impedance spectroscopy (xCelligence, Roche). 10 U/mL thrombin (■), 10 nM PAF (●) or 100 nM PAF (▲) or medium (○) were added to the plates within 2.5 min, whereupon impedance measurements continued as described [34]. B. PAF-receptor expression (PAFR) probed by RT-PCR was comparable to the expression of eNOS, endothelin-1 and VE-cadherin. The gene expression data were normalized to the TATA box protein (TBP). All data are $\pm$ SD from 3 independent experiments.

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 Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and activates myosin light chain kinase (MLCK), as well as the small GTPases RhoA, Rac1 and Cdc42 [27].

Fig. 3. Endothelial cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) response to thrombin in the isolated perfused lung as determined by real-time fluorescence imaging [24]. Representative images (left) show fura-2 loaded endothelial cells color-coded for $[\text{Ca}^{2+}]_i$ in a venular capillary of the isolated perfused rat lung. Images were obtained at baseline (top) and after thrombin infusion (50 U/mL; bottom). Vessel margins are depicted by dotted lines, arrows indicate the direction of perfusate flow. Group data (right) show mean endothelial $[\text{Ca}^{2+}]_i$ before (baseline) and after thrombin infusion. Data from $n=5$ each, $*p<0.05$ vs. baseline.

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 $[\text{Ca}^{2+}]_i$ 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 $\text{TNF-}\alpha$ are notoriously prone to false-positive increases in permeability due to the well-recognized property of $\text{TNF-}\alpha$ to induce apoptosis and cell death [51, 52]. Several studies in endothelial cells in culture have shown that $\text{TNF-}\alpha$ 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], PKC α [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 $[Ca^{2+}]_i$ *in vitro*, and may even suppress the endothelial $[Ca^{2+}]_i$ response to e.g. thrombin [65, 66], while it elicits a distinct endothelial $[Ca^{2+}]_i$ 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

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 μ M S1P was added 10 min prior to administration of PAF. Data are mean $\pm$ SEM of 3 independent experiments each.

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 μ M 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 $[Ca^{2+}]_i$, resulting either from the entry of extracellular Ca^{2+} via store-operated, receptor-operated, or voltage-dependent Ca^{2+} 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 Ca^{2+} 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 $[Ca^{2+}]_i$ responses, yet fail to induce marked increases in endothelial permeability in isolated perfused lung preparations (see above) suggests that an increase in endothelial $[Ca^{2+}]_i$ 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

$[Ca^{2+}]_i$ 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 mechanism 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- α 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-

Fig. 5. Role of Rho-kinase in bronchoconstriction and edema formation. Isolated perfused rat lungs were perfused with Krebs-Henseleit buffer supplemented with albumin as described [31, 32, 110]. Airway resistance and lung weight were measured continuously. At time point 0 a bolus of 5 nMol PAF (A,B), 1 μ Mol bradykinin (C,D) or 100 nMol leukotriene C4 (LTC₄; E,F) was infused into the pulmonary artery. The Rho-kinase inhibitor Y27632 (10 μ M, filled circles) was added 10 min before addition of the mediators. The total volume of recirculating buffer was 100 mL. Data are mean $\pm$ SD of 3 independent experiments each.

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 [145] 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.

Acknowledgement

This study was supported by the Deutsche Forschungsgemeinschaft within the SPP1267 “Sphingolipids - Signal and Disease”.

Disclosure Statement

None of the authors has any disclosures to make.

References

- 1 Staverman AJ: Non-equilibrium thermodynamics of membrane processes. *Trans Faraday Soc* 1952;48:176-185.
- 2 Qiao RL, Bhattacharya J: Segmental barrier properties of the pulmonary microvascular bed. *J Appl Physiol* 1991;71:2152-2159.
- 3 Yin J, Hoffmann J, Kaestle SM, Neye N, Wang L, Baeurle J, Liedtke W, Wu S, Kuppe H, Pries AR, Kuebler WM: Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of transient receptor potential vanilloid 4. *Circ Res* 2008;102:966-974.
- 4 Jian MY, King JA, Al-Mehdi AB, Liedtke W, Townsley MI: High vascular pressure-induced lung injury requires P450 epoxide-dependent activation of TRPV4. *Am J Respir Cell Mol Biol* 2008;38:386-392.
- 5 Ware LB, Matthay MA: The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334-1349.
- 6 Vestweber D: VE-cadherin: The major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol* 2008;28:223-232.
- 7 Pries AR, Kuebler WM: Normal endothelium. *Handb Exper Pharmacol* 2006:1-40.
- 8 Regan ER, Aird WC: Dynamical systems approach to endothelial heterogeneity. *Circ Res* 2012;111:110-130.
- 9 Aird WC: Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* 2007;100:158-173.
- 10 Michel CC: Capillary permeability and how it may change. *J Physiol* 1988;404:1-29.
- 11 Kuebler WM, Yang Y, Samapati R, Uhlig S: Vascular barrier regulation by PAF, ceramide, caveolae, and NO - an intricate signaling network with discrepant effects in the pulmonary and systemic vasculature. *Cell Physiol Biochem* 2010;26:29-40.
- 12 Maron MB: Differential effects of histamine on protein permeability in dog lung and forelimb. *Am J Physiol* 1982;242:H565-572.
- 13 Farhi LE, Sheehan DW: Pulmonary circulation and systemic circulation: Similar problems, different solutions. *Adv Exp Med Biol* 1990;277:579-586.
- 14 Birukova AA, Smurova K, Birukov KG, Kaibuchi K, Garcia JG, Verin AD: Role of Rho GTPases in thrombin-induced lung vascular endothelial cells barrier dysfunction. *Microvasc Res* 2004;67:64-77.
- 15 Rajagopalan LE, Davies MS, Kahn LE, Kornmeier CM, Shimada H, Steiner TA, Zweifel BS, Wendling JM, Payne MA, Loeffler RF, Case BL, Norton MB, Parikh MD, Nemirovskiy OV, Mourey RJ, Masferrer JL, Misko TP, Kolodziej SA: Biochemical, cellular, and anti-inflammatory properties of a potent, selective, orally bioavailable benzamide inhibitor of Rho kinase activity. *J Pharmacol Exp Ther* 2010;333:707-716.
- 16 Kasper B, Brandt E, Ernst M, Petersen F: Neutrophil adhesion to endothelial cells induced by platelet factor 4 requires sequential activation of ras, syk, and JNK MAP kinases. *Blood* 2006;107:1768-1775.
- 17 Abraham E, Carmody A, Shenkar R, Arcaroli J: Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1137-L1145.
- 18 Till GO, Johnson KJ, Kunkel R, Ward PA: Intravascular activation of complement and acute lung injury. *J Clin Invest* 1982;62:1126-1135.
- 19 Looney MR, Su X, Van Ziffle JA, Lowell CA, Matthay MA: Neutrophils and their FC gamma receptors are essential in a mouse model of transfusion-related acute lung injury. *J Clin Invest* 2006;116:1615-1623.
- 20 Koksel O, Yildirim C, Tiftik RN, Kubat H, Tamer L, Cinel L, Kaplan MB, Degirmenci U, Ozdulger A, Buyukfisar K: Rho-kinase (Rock-1 and Rock-2) upregulation in oleic acid-induced lung injury and its restoration by Y-27632. *Eur J Pharmacol* 2005;510:135-142.
- 21 Tasaka S, Koh H, Yamada W, Shimizu M, Ogawa Y, Hasegawa N, Yamaguchi K, Ishii Y, Richer SE, Doerschuk CM, Ishizaka A: Attenuation of endotoxin-induced acute lung injury by the Rho-associated kinase inhibitor, Y-27632. *Am J Respir Cell Mol Biol* 2005;32:504-510.

- 22 Uhlig S, von Bethmann AN: Determination of vascular compliance, interstitial compliance, and capillary filtration coefficient in rat isolated perfused lungs. *J Pharmacol Tox Methods* 1997;37:119-127.
- 23 Uhlig S: The isolated perfused lung; in Uhlig S, Taylor AE (eds): *Methods in pulmonary pharmacology*. Basel, Birkhäuser, 1998, pp 29-55.
- 24 Kuebler WM, Parthasarathi K, Lindert J, Bhattacharya J: Real-time lung microscopy. *J Appl Physiol* 2007;102:1255-1264.
- 25 Lindner K: Regulation of vascular permeability in the lung by ceramide: Research Center Borstel, University of Lübeck, 2005, Ph.D. thesis.
- 26 Uhlig S, Lindner K: Novel mechanisms of endothelial cell permeability in the lung. Lessons from studies with intact lungs and animals.; in Tooke J, Shore A, Whatmore J (eds): *The microcirculation and vascular biology*. Bologna, Monduzi Editore, 2002, pp 221-234.
- 27 Vandenbroucke E, Mehta D, Minshall R, Malik AB: Regulation of endothelial junctional permeability. *Ann N Y Acad Sci* 2008;1123:134-145.
- 28 Uhlig S, Göggel R, Engel S: Mechanisms of platelet-activating factor (PAF)-mediated responses in the lung. *PharmacolRep* 2005;57:S206-221.
- 29 Knezevic II, Predescu SA, Neamu RF, Gorovoy MS, Knezevic NM, Easington C, Malik AB, Predescu DN: Tiam1 and rac1 are required for platelet-activating factor-induced endothelial junctional disassembly and increase in vascular permeability. *J Biol Chem* 2009;284:5381-5394.
- 30 Göggel R, Winoto-Morbach S, Vielhaber G, Imai Y, Lindner K, Brade L, Brade H, Ehlers S, Slutsky AS, Schütze S, Gulbins E, Uhlig S: PAF-mediated pulmonary edema: A new role for acid sphingomyelinase and ceramide. *Nat Med* 2004;10:155-160.
- 31 Yang Y, Yin J, Baumgartner W, Samapati R, Solymosi EA, Reppien E, Kuebler WM, Uhlig S: Platelet-activating factor reduces endothelial NO production - role of acid sphingomyelinase. *Eur Respir J* 2010;36:417-427.
- 32 Samapati R, Yang Y, Yin J, Stoerger C, Arenz C, Dietrich A, Gudermann T, Adam D, Wu S, Freichel M, Flockerzi V, Uhlig S, Kuebler WM: Lung endothelial Ca^{2+} and permeability response to platelet-activating factor is mediated by acid sphingomyelinase and transient receptor potential classical 6. *Am J Respir Crit Care Med* 2012;185:160-170.
- 33 Yang Y, Uhlig S: The role of sphingolipids in respiratory disease. *Ther Adv Respir Dis* 2011;5:325-344.
- 34 Ludwig A, Sommer A, Uhlig S: Assessment of endothelial permeability and leukocyte transmigration in human endothelial cell monolayers. *Methods Mol Biol* 2011;763:319-332.
- 35 Dodam JR, Olson NC, Friedman M: Differential effects of tumor necrosis factor- α and platelet-activating factor on bovine pulmonary artery endothelial cells *in vitro*. *ExpLung Res* 1994;20:131-141.
- 36 Bussolino F, Camussi G, Aglietta M, Braquet P, Bosia A, Pescarmona G, Sanavio F, D'Urso N, Marchisio PC: Human endothelial cells are target for platelet-activating factor. I. Platelet-activating factor induces changes in cytoskeleton structures. *J Immunol* 1987;139:2439-2446.
- 37 Predescu S, Knezevic I, Bardita C, Neamu RF, Brovcovich V, Predescu D: Platelet activating factor-induced ceramide micro-domains drive endothelial NOS activation and contribute to barrier dysfunction. *PloS One* 2013;8:e75846.
- 38 Göggel R, Uhlig S: The inositol trisphosphate pathway mediates platelet-activating-factor-induced pulmonary oedema. *Eur Respir J* 2005;25:849-857.
- 39 Adamson RH, Zeng M, Adamson GN, Lenz JE, Curry FE: PAF- and bradykinin-induced hyperpermeability of rat venules is independent of actin-myosin contraction. *Am J Physiol Heart Circ Physiol* 2003;285:H406-417.
- 40 Waypa GB, Vincent PA, Morton CA, Minnear FL: Thrombin increases fluid flux in isolated rat lungs by a hemodynamic and not a permeability mechanism. *J Appl Physiol* 1996;80:1197-1204.
- 41 Horgan MJ, Fenton JW, 2nd, Malik AB: Alpha-thrombin-induced pulmonary vasoconstriction. *J Appl Physiol* 1987;63:1993-2000.
- 42 Lindner K, Uhlig U, Uhlig S: Ceramide alters endothelial cell permeability by a nonapoptotic mechanism. *Br J Pharmacol* 2005;145:132-140.
- 43 Curry FR, Adamson RH: Vascular permeability modulation at the cell, microvessel, or whole organ level: Towards closing gaps in our knowledge. *Cardiovasc Res* 2010;87:218-229.
- 44 Kim MH, Curry FR, Simon SI: Dynamics of neutrophil extravasation and vascular permeability are uncoupled during aseptic cutaneous wounding. *Am J Physiol Cell Physiol* 2009;296:C848-856.
- 45 Uchiba M, Okajima K, Murakami K, Okabe H, Takatsuki K: Endotoxin-induced pulmonary vascular injury is mainly mediated by activated neutrophils in rats. *Thromb Res* 1995;78:117-125.

- 46 Vogel SM, Gao X, Mehta D, Ye RD, John TA, Andrade-Gordon P, Tiruppathi C, Malik AB: Abrogation of thrombin-induced increase in pulmonary microvascular permeability in PAR-1 knockout mice. *Physiol Genomics* 2000;4:137-145.
- 47 Troyanovsky B, Alvarez DF, King JA, Schaphorst KL: Thrombin enhances the barrier function of rat microvascular endothelium in a PAR-1-dependent manner. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L266-275.
- 48 Parthasarathi K, Ichimura H, Monma E, Lindert J, Quadri S, Issekutz A, Bhattacharya J: Connexin 43 mediates spread of Ca^{2+} -dependent proinflammatory responses in lung capillaries. *J Clin Invest* 2006;116:2193-2200.
- 49 Parker JC, Townsley MI: Evaluation of lung injury in rats and mice. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L231-246.
- 50 Uhlig S, von Bethmann AN: Determination of vascular compliance, interstitial compliance and capillary filtration coefficient in isolated perfused rat lungs. *J Pharm Tox Meth* 1997;32:119-127.
- 51 Goldblum SE, Hennig B, Jay M, Yoneda K, McClain CJ: Tumor necrosis factor- α -induced pulmonary vascular endothelial injury. *Infect Immun* 1989;57:1218-1226.
- 52 Petrache I, Verin AD, Crow MT, Birukova A, Liu F, Garcia JG: Differential effect of MLC kinase in TNF- α -induced endothelial cell apoptosis and barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1168-1178.
- 53 Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D: Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J Exp Med* 1989;169:1977-1991.
- 54 Nwariaku FE, Rothenbach P, Liu Z, Zhu X, Turnage RH, Terada LS: Rho inhibition decreases TNF-induced endothelial mapk activation and monolayer permeability. *J Appl Physiol* 2003;95:1889-1895.
- 55 Naikawadi RP, Cheng N, Vogel SM, Qian F, Wu D, Malik AB, Ye RD: A critical role for phosphatidylinositol (3,4,5)-trisphosphate-dependent rac exchanger 1 in endothelial junction disruption and vascular hyperpermeability. *Circ Res* 2012;111:1517-1527.
- 56 Shao M, Yue Y, Sun GY, You QH, Wang N, Zhang D: Caveolin-1 regulates rac1 activation and rat pulmonary microvascular endothelial hyperpermeability induced by TNF- α . *PloS One* 2013;8:e55213.
- 57 Partridge CA, Jeffrey JJ, Malik AB: A 96-kda gelatinase induced by TNF- α contributes to increased microvascular endothelial permeability. *Am J Physiol* 1993;265:L438-447.
- 58 Bove K, Neumann P, Gertzberg N, Johnson A: Role of eNOS-derived NO in mediating TNF-induced endothelial barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L914-922.
- 59 Mong PY, Petruccio C, Kaufman HL, Wang Q: Activation of Rho kinase by TNF- α is required for JNK activation in human pulmonary microvascular endothelial cells. *J Immunol* 2008;180:550-558.
- 60 Ferro T, Neumann P, Gertzberg N, Clements R, Johnson A: Protein kinase C- α mediates endothelial barrier dysfunction induced by TNF α . *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1107-L1117.
- 61 Petrache I, Birukova A, Ramirez SI, Garcia JG, Verin AD: The role of the microtubules in tumor necrosis factor- α -induced endothelial cell permeability. *Am J Respir Cell Mol Biol* 2003;28:574-581.
- 62 Ferro TJ, Gertzberg N, Selden L, Neumann P, Johnson A: Endothelial barrier dysfunction and p42 oxidation induced by TNF- α are mediated by nitric oxide. *Am J Physiol* 1997;272:L979-988.
- 63 Neumann P, Gertzberg N, Vaughan E, Weisbrot J, Woodburn R, Lambert W, Johnson A: Peroxynitrite mediates TNF- α -induced endothelial barrier dysfunction and nitration of actin. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L674-L684.
- 64 Angelini DJ, Hyun SW, Grigoryev DN, Garg P, Gong P, Singh IS, Passaniti A, Hasday JD, Goldblum SE: TNF- α increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1232-1245.
- 65 Watanabe K, Tanaka H, Wen FQ, Yoshida M: Effect of cytokines on thrombin-stimulated increases in intracellular calcium and PGI2 production by cultured human umbilical vein endothelial cells. *Cellular Signall* 1996;8:247-251.
- 66 Yan W, Tiruppathi C, Qiao R, Lum H, Malik AB: Tumor necrosis factor decreases thrombin receptor expression in endothelial cells. *J Cell Physiol* 1996;166:561-567.
- 67 Kuebler WM, Parthasarathi K, Wang PM, Bhattacharya J: A novel signaling mechanism between gas and blood compartments of the lung. *J Clin Invest* 2000;105:905-913.

- 68 Parthasarathi K, Ichimura H, Quadri S, Issekutz A, Bhattacharya J: Mitochondrial reactive oxygen species regulate spatial profile of proinflammatory responses in lung venular capillaries. *J Immunol* 2002;169:7078-7086.
- 69 Stephens KE, Ishizaka A, Larrik JW, Raffin TA: Tumor necrosis factor causes increased pulmonary permeability and edema. *Am Rev Respir Dis* 1988;137:1364-1370.
- 70 Johnson J, Brigham KL, Jesmok G, Meyrick B: Morphologic changes in lungs of anesthetized sheep following intravenous infusion of recombinant tumor necrosis factor alpha. *Am Rev Respir Dis* 1991;144:179-186.
- 71 Bonsignore MR, Valenti A, Spatafora M: Tumor necrosis factor-alpha does not cause lung edema in rabbits. *J Appl Physiol* 1992;73:173-178.
- 72 Reiss LK, Adam D, Uhlig S: Acid sphingomyelinase-deficient mice are protected from the lethal cardiovascular effects in TNF-induced septic shock. *NS Arch Pharmacol* 2012;385:74-75.
- 73 Rothstein JL, Schreiber H: Synergy between tumor necrosis factor and bacterial products cause hemorrhagic necrosis and lethal shock in normal mice. *Proc Natl Acad Sci* 1988;85:607-611.
- 74 Gatti S, Faggioni R, Echtenacher B, Ghezzi P: Role of tumour necrosis factor and reactive oxygen intermediates in lipopolysaccharide-induced pulmonary oedema and lethality. *Clin Exp Immunol* 1993;91:456-461.
- 75 Lechner AJ, Johannis CA, Matushak GM: A recombinant tumor necrosis factor- α p80 receptor:Fc fusion protein decreases circulating bioactive tumor necrosis factor- α but not lung injury or mortality during immunosuppression-related gram-negative bacteremia. *J Crit Care* 1997;12:28-38.
- 76 Ryan AJ, McCoy DM, McGowan SE, Salome RG, Mallampalli RK: Alveolar sphingolipids generated in response to TNF- α modifies surfactant biophysical activity. *J Appl Physiol* 2003;94:253-258.
- 77 Drey Mueller D, Martin C, Kogel T, Pruessmeyer J, Hess FM, Horiuchi K, Uhlig S, Ludwig A: Lung endothelial ADAM17 regulates the acute inflammatory response to lipopolysaccharide. *EMBO Mol Med* 2012;4:412-423.
- 78 Schulman CI, Wright JK, Nwariaku F, Sarosi G, Turnage RH: The effect of tumor necrosis factor-alpha on microvascular permeability in an isolated, perfused lung. *Shock* 2002;18:75-81.
- 79 Hocking DC, Phillips PG, Ferro TJ, Johnson A: Mechanisms of pulmonary edema induced by tumor necrosis factor- α . *Circ Res* 1990;67:68-77.
- 80 Lo SK, Everitt J, Gu J, Malik AB: Tumor necrosis factor mediates experimental pulmonary edema by ICAM-1 and CD18-dependent mechanisms. *J Clin Invest* 1992;89:981-988.
- 81 Horgan MJ, Palace GP, Everitt JE, Malik AB: TNF-alpha release in endotoxemia contributes to neutrophil-dependent pulmonary edema. *Am J Physiol* 1993;264:H1161-H1165.
- 82 Hattar K, Oppermann S, Ankele C, Weissmann N, Schermuly RT, Bohle RM, Moritz R, Krogel B, Seeger W, Grimminger F, Sibelius U, Grandel U: C-ANCA-induced neutrophil-mediated lung injury: A model of acute Wegener's granulomatosis. *Eur Respir J* 2010;36:187-195.
- 83 Fu P, Birukova AA, Xing J, Sammani S, Murley JS, Garcia JG, Grdina DJ, Birukov KG: Amifostine reduces lung vascular permeability via suppression of inflammatory signalling. *Eur Respir J* 2009;33:612-624.
- 84 Joshi AD, Dimitropoulou C, Thangjam G, Snead C, Feldman S, Barabutis N, Fulton D, Hou Y, Kumar S, Patel V, Gorshkov B, Verin AD, Black SM, Catravas JD: Heat shock protein 90 inhibitors prevent LPS-induced endothelial barrier dysfunction by disrupting RhoA signaling. *Am J Respir Cell Mol Biol* 2014;50:170-179.
- 85 Xing J, Wang Q, Coughlan K, Viollet B, Moriasi C, Zou MH: Inhibition of AMP-activated protein kinase accentuates lipopolysaccharide-induced lung endothelial barrier dysfunction and lung injury in vivo. *Am J Pathol* 2013;182:1021-1030.
- 86 Xing J, Birukova AA: Anp attenuates inflammatory signaling and Rho pathway of lung endothelial permeability induced by LPS and TNFalpha. *Microvasc Res* 2010;79:56-62.
- 87 Umapathy NS, Zemskov EA, Gonzales J, Gorshkov BA, Sridhar S, Chakraborty T, Lucas R, Verin AD: Extracellular beta-nicotinamide adenine dinucleotide (beta-NAD) promotes the endothelial cell barrier integrity via PKA- and EPAC1/Rac1-dependent actin cytoskeleton rearrangement. *J Cell Physiol* 2010;223:215-223.
- 88 Chatterjee A, Snead C, Yetik-Anacak G, Antonova G, Zeng J, Catravas JD: Heat shock protein 90 inhibitors attenuate LPS-induced endothelial hyperpermeability. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L755-763.
- 89 Birukova AA, Xing J, Fu P, Yakubov B, Dubrovskiy O, Fortune JA, Klivanov AM, Birukov KG: Atrial natriuretic peptide attenuates LPS-induced lung vascular leak: Role of PAK1. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L652-663.

- 90 Dudek SM, Munoz NM, Desai A, Osan CM, Meliton AY, Leff AR: Group V phospholipase A2 mediates barrier disruption of human pulmonary endothelial cells caused by LPS in vitro. *Am J Respir Cell Mol Biol* 2011;44:361-368.
- 91 Chen W, Pendyala S, Natarajan V, Garcia JG, Jacobson JR: Endothelial cell barrier protection by simvastatin: GTPase regulation and NADPH oxidase inhibition. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L575-583.
- 92 Matute-Bello G, Frevert CW, Martin TR: Animal models of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L379-399.
- 93 Heflin AC, Brigham KL: Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. *J Clin Invest* 1981;68:1253-1260.
- 94 Kopaniak MM, Movat HZ: Kinetics of acute inflammation induced by escherichia coli in rabbits. II. The effect of hyperimmunization, complement depletion, and depletion of leuokocytes. *Am J Pathol* 1983;110:13-29.
- 95 Chang SW, Feddersen CO, Henson PM, Voelkel NF: Platelet-activating factor mediates hemodynamic changes and lung injury in endotoxin-treated rats. *J Clin Invest* 1987;79:1498-1509.
- 96 Chignard M, Balloy V: Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1083-L1090.
- 97 Siore AM, Parker RE, Stecenko AA, Cuppels C, McKean M, Christman BW, Cruz-Gervis R, Brigham KL: Endotoxin-induced acute lung injury requires interaction with the liver. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L769-L776.
- 98 Uhlig S, Brasch F, Wollin L, Fehrenbach H, Richter J, Wendel A: Functional and fine structural changes in isolated rat lungs challenged with endotoxin ex vivo and in vitro. *Am J Pathol* 1995;146:1235-1247.
- 99 Winn R, Nickelson S, Rice CL: Fluid filtration coefficient of isolated goat lungs was unchanged by endotoxin. *J Appl Physiol* 1988;64:2463-2467.
- 100 Chian CF, Chiang CH, Yuan-Jung C, Chuang CH, Liu SL, Yi-Han J, Zhang H, Ryu JH: Apocynin attenuates lipopolysaccharide-induced lung injury in an isolated and perfused rat lung model. *Shock* 2012;38:196-202.
- 101 Welsh CH, Lien DC, Worthen GS, Henson PM, Weil JV: Endotoxin-pretrated neutrophils increase pulmonary vascular permeability in dogs. *J Appl Physiol* 1989;66:112-119.
- 102 Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards CK, Schuchman EH, Fuks Z, Kolesnick R: Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med* 1997;189:1831-1841.
- 103 Künstle G, Leist M, Uhlig S, Revesz L, Feifel R, MacKenzie A, Wendel A: ICE-protease inhibitors block murine liver injury and apoptosis caused by CD95 or by TNF-alpha. *Immunol Lett* 1997;55:5-10.
- 104 Uhlig S, Yang Y: Sphingolipids in acute lung injury. *Handbook Exper Pharmacol* 2013:227-246.
- 105 Wang L, Dudek SM: Regulation of vascular permeability by sphingosine 1-phosphate. *Microvasc Res* 2009;77:39-45.
- 106 Szczepaniak WS, Pitt BR, McVerry BJ: S1P2 receptor-dependent Rho-kinase activation mediates vasoconstriction in the murine pulmonary circulation induced by sphingosine 1-phosphate. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L137-145.
- 107 Ota H, Beutz MA, Ito M, Abe K, Oka M, McMurtry IF: S1P(4) receptor mediates S1P-induced vasoconstriction in normotensive and hypertensive rat lungs. *Pulm Circul* 2011;1:399-404.
- 108 Sanchez T, Skoura A, Wu MT, Casserly B, Harrington EO, Hla T: Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN. *Arterioscler Thromb Vasc Biol* 2007;27:1312-1318.
- 109 Shea BS, Brooks SF, Fontaine BA, Chun J, Luster AD, Tager AM: Prolonged exposure to sphingosine 1-phosphate receptor-1 agonists exacerbates vascular leak, fibrosis, and mortality after lung injury. *Am J Respir Cell Mol Biol* 2010;43:662-673.
- 110 Uhlig S, Wollin L, Wendel A: Contributions of thromboxane and leukotrienes to PAF-induced impairment of lung function in the rat. *J Appl Physiol* 1994;77:262-269.
- 111 Wu S, Jian MY, Xu YC, Zhou C, Al-Mehdi AB, Liedtke W, Shin HS, Townsley MI: Ca²⁺ entry via alpha1G and TRPV4 channels differentially regulates surface expression of P-selectin and barrier integrity in pulmonary capillary endothelium. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L650-657.
- 112 Alvarez DF, King JA, Weber D, Addison E, Liedtke W, Townsley MI: Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: A novel mechanism of acute lung injury. *Circ Res* 2006;99:988-995.
- 113 Hamanaka K, Jian MY, Weber DS, Alvarez DF, Townsley MI, Al-Mehdi AB, King JA, Liedtke W, Parker JC: TRPV4 initiates the acute calcium-dependent permeability increase during ventilator-induced lung injury

- in isolated mouse lungs. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L923-L932.
- 114 Ying X, Minamiya Y, Fu C, Bhattacharya J: Ca^{2+} waves in lung capillary endothelium. *Circ Res* 1996;79:898-908.
 - 115 Kuebler WM, Ying X, Bhattacharya J: Pressure-induced endothelial Ca^{2+} oscillations in lung capillaries. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L917-L923.
 - 116 Naruse K, Sokabe M: Involvement of stretch-activated ion channels in Ca^{2+} mobilization to mechanical stretch in endothelial cells. *Am J Physiol* 1993;264:C1037-1044.
 - 117 Lin AY, Rui YC: Platelet-activating factor induced calcium mobilization and phosphoinositide metabolism in cultured bovine cerebral microvascular endothelial cells. *Biochim Biophys Acta* 1994;1224:323-328.
 - 118 Ito S, Suki B, Kume H, Numaguchi Y, Ishii M, Iwaki M, Kondo M, Naruse K, Hasegawa Y, Sokabe M: Actin cytoskeleton regulates stretch-activated Ca^{2+} influx in human pulmonary microvascular endothelial cells. *Am J Respir Cell Mol Biol* 2010;43:26-34.
 - 119 Hirafuji M, Maeyama K, Watanabe T, Ogura Y: Transient increase of cytosolic free calcium in cultured human vascular endothelial cells by platelet-activating factor. *Biochem Biophys Res Commun* 1988;154:910-917.
 - 120 Sutovsky P, Flechon JE, Flechon B, Motlik J, Peynot N, Chesne P, Heyman Y: Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. *Biol Reprod* 1993;49:1277-1287.
 - 121 Revel JP, Yee AG, Hudspeth AJ: Gap junctions between electrotonically coupled cells in tissue culture and in brown fat. *Proc Natl Acad Sci U S A* 1971;68:2924-2927.
 - 122 Morel NML, Petruzzo PP, Hechtman HB, Shepro: Inflammatory agonists that increase microvascular permeability in vivo stimulate pulmonary microvessel endothelial cell contraction. *Inflammation* 1990;14:571-583.
 - 123 Birukov KG, Birukova AA, Dudek SM, Verin AD, Crow MT, Zhan X, DePaola N, Garcia JG: Shear stress-mediated cytoskeletal remodeling and cortactin translocation in pulmonary endothelial cells. *Am J Respir Cell Mol Biol* 2002;26:453-464.
 - 124 Kerem A, Yin J, Kaestle SM, Hoffmann J, Schoene AM, Singh B, Kuppe H, Borst MM, Kuebler WM: Lung endothelial dysfunction in congestive heart failure: Role of impaired Ca^{2+} signaling and cytoskeletal reorganization. *Circ Res* 2010;106:1103-1116.
 - 125 Yao HY, Chen L, Xu C, Wang J, Chen J, Xie QM, Wu X, Yan XF: Inhibition of rac activity alleviates lipopolysaccharide-induced acute pulmonary injury in mice. *Biochim Biophys Acta* 2011;1810:666-674.
 - 126 Adamson RH, Curry FE, Adamson G, Liu B, Jiang Y, Aktories K, Barth H, Daigeler A, Golenhofen N, Ness W, Drenckhahn D: Rho and Rho kinase modulation of barrier properties: Cultured endothelial cells and intact microvessels of rats and mice. *J Physiol* 2002;539:295-308.
 - 127 Gorovoy M, Neamu R, Niu J, Vogel S, Predescu D, Miyoshi J, Takai Y, Kini V, Mehta D, Malik AB, Voyno-Yasenetskaya T: RhoGDI-1 modulation of the activity of monomeric RhoGTPase RhoA regulates endothelial barrier function in mouse lungs. *Circ Res* 2007;101:50-58.
 - 128 Krishnan R, Klumpers DD, Park CY, Rajendran K, Treppe X, van Bezu J, van Hinsbergh VW, Carman CV, Brain JD, Fredberg JJ, Butler JP, van Nieuw Amerongen GP: Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *Am J Physiol Cell Physiol* 2011;300:C146-154.
 - 129 Huynh J, Nishimura N, Rana K, Peloquin JM, Califano JP, Montague CR, King MR, Schaffer CB, Reinhart-King CA: Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. *Sci Transl Med* 2011;3:112ra122.
 - 130 Birukova AA, Tian X, Cokic I, Beckham Y, Gardel ML, Birukov KG: Endothelial barrier disruption and recovery is controlled by substrate stiffness. *Microvasc Res* 2013;87:50-57.
 - 131 Liu F, Mih JD, Shea BS, Kho AT, Sharif AS, Tager AM, Tschumperlin DJ: Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression. *J Cell Biol* 2010;190:693-706.
 - 132 Suki B, Ito S, Stamenovic D, Lutchen KR, Ingenito EP: Biomechanics of the lung parenchyma: Critical roles of collagen and mechanical forces. *J Appl Physiol* 2005;98:1892-1899.
 - 133 Rausch SM, Martin C, Bornemann PB, Uhlig S, Wall WA: Material model of lung parenchyma based on living precision-cut lung slice testing. *J Mech Behav Biomed Mater* 2011;4:583-592.
 - 134 Bel-Brunon A, Kehl S, Martin C, Uhlig S, Wall WA: Numerical identification method for the non-linear viscoelastic compressible behavior of soft tissue using uniaxial tensile tests and image registration - application to rat lung parenchyma. *J Mech Behav Biomed Mater* 2014;29:360-374.
 - 135 Robinson KG, Nie T, Baldwin AD, Yang EC, Kiick KL, Akins RE Jr: Differential effects of substrate modulus on human vascular endothelial, smooth muscle, and fibroblastic cells. *J Biomater Res* 2012;100:1356-1367.

- 136 Michel CC, Curry FE: Microvascular permeability. *Physiol Rev* 1999;79:703-761.
- 137 Parker JC, Stevens T, Randall J, Weber DS, King JA: Hydraulic conductance of pulmonary microvascular and macrovascular endothelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L30-37.
- 138 Hall CN, Garthwaite J: What is the real physiological NO concentration in vivo? *Nitric oxide* 2009;21:92-103.
- 139 Yang Y, Schmidt EP: The endothelial glycocalyx: An important regulator of the pulmonary vascular barrier. *Tissue Barriers* 2013;1: pii: 23494.
- 140 Durr E, Yu J, Krasinska KM, Carver LA, Yates JR, Testa JE, Oh P, Schnitzer JE: Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. *Nature Biotechnol* 2004;22:985-992.
- 141 D'Alessio A, Al-Lamki RS, Bradley JR, Pober JS: Caveolae participate in tumor necrosis factor receptor 1 signaling and internalization in a human endothelial cell line. *Am J Pathol* 2005;166:1273-1282.
- 142 Seebach J, Dieterich P, Luo F, Schillers H, Vestweber D, Oberleithner H, Galla HJ, Schnittler HJ: Endothelial barrier function under laminar fluid shear stress. *Lab Invest* 2000;80:1819-1831.
- 143 Muller-Marschhausen K, Waschke J, Drenckhahn D: Physiological hydrostatic pressure protects endothelial monolayer integrity. *Am J Physiol Cell Physiol* 2008;294:C324-332.
- 144 Qiu Y, Tarbell JM: Interaction between wall shear stress and circumferential strain affects endothelial cell biochemical production. *J Vasc Res* 2000;37:147-157.
- 145 Benjamin LE, Hemo I, Keshet E: A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated byPDGF-B and VEGF. *Development* 1998;125:1591-1598.
- 146 Kim YH, Hu H, Guevara-Gallardo S, Lam MT, Fong SY, Wang RA: Artery and vein size is balanced by notch and ephrin B2/EphB4 during angiogenesis. *Development* 2008;135:3755-3764.
- 147 Hall SM, Hislop AA, Haworth SG: Origin, differentiation, and maturation of human pulmonary veins. *Am J Respir Cell Mol Biol* 2002;26:333-340.
- 148 Hoefer IE, den Adel B, Daemen MJ: Biomechanical factors as triggers of vascular growth. *Cardiovasc Res* 2013;99:276-283.
- 149 Liu HB, Zhang J, Xin SY, Liu C, Wang CY, Zhao D, Zhang ZR: Mechanosensitive properties in the endothelium and their roles in the regulation of endothelial function. *J Cardiovasc Pharmacol* 2013;61:461-470.
- 150 Madri JA, Williams SK: Capillary endothelial cell cultures: Phenotypic modulation by matrix components. *J Cell Biol* 1983;97:153-165.
- 151 Mammoto A, Mammoto T, Ingber DE: Mechanosensitive mechanisms in transcriptional regulation. *J Cell Sci* 2012;125:3061-3073.
- 152 Goerdt S, Steckel F, Schulze-Osthoff K, Hagemeyer HH, Macher E, Sorg C: Characterization and differential expression of an endothelial cell-specific surface antigen in continuous and sinusoidal endothelial, in skin vascular lesions and in vitro. *Exp Cell Biol* 1989;57:185-192.
- 153 Aird WC, Edelberg JM, Weiler-Guettler H, Simmons WW, Smith TW, Rosenberg RD: Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. *J Cell Biol* 1997;138:1117-1124.
- 154 Lacorre DA, Baekkevold ES, Garrido I, Brandtzaeg P, Haraldsen G, Amalric F, Girard JP: Plasticity of endothelial cells: Rapid dedifferentiation of freshly isolated high endothelial venule endothelial cells outside the lymphoid tissue microenvironment. *Blood* 2004;103:4164-4172.
- 155 Aranguren XL, Agirre X, Beerens M, Coppiello G, Uriz M, Vandersmissen I, Benkheil M, Panadero J, Aguado N, Pascual-Montano A, Segura V, Prosper F, Luttun A: Unraveling a novel transcription factor code determining the human arterial-specific endothelial cell signature. *Blood* 2013;122:3982-3992.
- 156 Gonzalez R, Yang YH, Griffin C, Allen L, Tighe Z, Dobbs L: Freshly isolated rat alveolar type I cells, type II cells, and cultured type II cells have distinct molecular phenotypes. *AmJPhysiol Lung Cell MolPhysiol* 2005;288:L179-L189.
- 157 Stevens T: Functional and molecular heterogeneity of pulmonary endothelial cells. *Proc of the Am Thorac Soc* 2011;8:453-457.
- 158 Birukova AA, Wu T, Tian Y, Meliton A, Sarich N, Tian X, Leff A, Birukov KG: Iloprost improves endothelial barrier function in lipopolysaccharide-induced lung injury. *Eur Respir J* 2013;41:165-176.
- 159 Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, Pearse D, Tudor RM, Garcia JG: Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med* 2004;169:1245-1251.