Negative-Feedback Loop Attenuates Hydrostatic Lung Edema via a cGMP-Dependent Regulation of Transient Receptor Potential Vanilloid 4

Jun Yin, Julia Hoffmann, Stephanie M. Kaestle, Nils Neye, Liming Wang, Joerg Baeurle, Wolfgang Liedtke, Songwei Wu, Hermann Kuppe, Axel R. Pries, Wolfgang M. Kuebler

Abstract—Although the formation of hydrostatic lung edema is generally attributed to imbalanced Starling forces, recent data show that lung endothelial cells respond to increased vascular pressure and may thus regulate vascular permeability and edema formation. In combining real-time optical imaging of the endothelial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and NO production with filtration coefficient (K\(_f\)) measurements in the isolated perfused lungs, we identified a series of endothelial responses that constitute a negative-feedback loop to protect the microvascular barrier. Elevation of lung microvascular pressure was shown to increase endothelial [Ca\(^{2+}\)], via activation of transient receptor potential vanilloid 4 (TRPV4) channels. The endothelial [Ca\(^{2+}\)], transient increased K\(_f\), via activation of myosin light-chain kinase and simultaneously stimulated NO synthesis. In TRPV4 deficient mice, pressure-induced increases in endothelial [Ca\(^{2+}\)], NO synthesis, and lung wet/dry weight ratio were largely blocked. Endothelial NO formation limited the permeability increase by a cGMP-dependent attenuation of the pressure-induced [Ca\(^{2+}\)] response. Inactivation of TRPV4 channels by cGMP was confirmed by whole-cell patch-clamp of pulmonary microvascular endothelial cells and intravital imaging of endothelial [Ca\(^{2+}\)]. Hence, pressure-induced endothelial Ca\(^{2+}\) influx via TRPV4 channels increases lung vascular permeability yet concomitantly activates an NO-mediated negative-feedback loop that protects the vascular barrier by a cGMP-dependent attenuation of the endothelial [Ca\(^{2+}\)] response. The identification of this novel regulatory pathway gives rise to new treatment strategies, as demonstrated in vivo in rats with acute myocardial infarction in which inhibition of cGMP degradation by the phosphodiesterase 5 inhibitor sildenafil reduced hydrostatic lung edema. (Circ Res. 2008;102:0-0.)

Key Words: pulmonary edema • vascular permeability • vascular endothelium • phosphodiesterase type 5 inhibitor • nitric oxide

The pathogenesis of hydrostatic lung edema has been attributed predominantly to an imbalance in Starling forces, ie, fluid extravasation attributable to an increased hydrostatic or reduced oncotic pressure gradient across the microvascular barrier. This classic view has been challenged by the findings of Parker and Ivey in isolated perfused rat lungs, which demonstrated an increase in lung filtration coefficient (K\(_f\)) following elevation of left atrial pressure (P\(_{LA}\)).

This increase was attenuated by the β-adrenergic agonist isoproterenol, indicating that the K\(_f\) increase was not only caused by an enlarged vascular surface area but also resulted from an increase in vascular permeability that could be counteracted via the cAMP signaling pathway. The latter finding suggests that active endothelial responses may contribute critically to the formation of hydrostatic lung edema.

By use of real-time fluorescence imaging techniques, we recently identified such endothelial responses to an acute elevation in hydrostatic pressure in intact lung microvessels.

In isolated perfused rat lungs, P\(_{LA}\) elevation increases the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in lung microvascular endothelial cells by Ca\(^{2+}\) influx via gadolinium-inhibitable cation channels and stimulates NO formation by activation of endothelial NO synthase. However, the role of these endothelial responses in the pathogenesis of hydrostatic lung edema is yet unclear.

Endothelial Ca\(^{2+}\) entry is a potential cause for endothelial retraction by activation of myosin light chain kinase (MLCK) and, thus, may increase endothelial permeability and promote edema formation. The mechanosensitive cation channel mediating the endothelial Ca\(^{2+}\) response to hydrostatic pres-
sure remains to be identified. A potential candidate is the transient receptor potential vanilloid 4 (TRPV4), which has been characterized as a cation channel functioning in transduction of membrane stretch, shear stress, and direct mechanical activation (reviewed elsewhere). Of note, TRPV4 is expressed in both micro- and macrovascular lung endothelial cells, and its pharmacological activation was recently shown to cause lung edema.

The role of endothelial NO synthesis in the formation of lung edema has been a matter of controversy. NO may increase or alternatively reduce microvascular permeability, the outcome being determined by the specific conditions and vascular beds under investigation as well as the activity of interrelated signaling pathways and the presence of scavenger molecules.

To address the regulation of vascular barrier function by endothelial responses in intact lungs, we combined real-time fluorescence imaging techniques with measurements of lung vascular permeability and edema formation. Here, we demonstrate that hydrostatic stress induces Ca\(^{2+}\) influx into endothelial cells via TRPV4 and increases lung vascular permeability by a Ca\(^{2+}\)-dependent activation of MLCK. We describe a novel negative-feedback loop in this scenario that effectively limits progressive barrier deterioration via an intrinsic NO/cGMP-signaling pathway that directly regulates TRPV4.

**Materials and Methods**

Methodological details are provided in the online data supplement at http://circres.ahajournals.org. In brief, isolated lungs of rats or mice were prepared as previously described. At baseline, lungs were perfused with constant flow of 14 mL/min (rats) or 1 mL/min (mice) at P\(_{L\text{A}}\) of 5 cmH\(_2\)O (rats) or 3 cmH\(_2\)O (mice) and pulmonary arterial pressure (P\(_{P\text{A}}\)) of 10±1 cmH\(_2\)O (both), respectively. In situ real-time fluorescence microscopy was performed as previously described. In isolated rat or mouse lungs, endothelial [Ca\(^{2+}\)] was determined by fluorescence imaging of the [Ca\(^{2+}\)]-sensitive dye Fura-2. Calcium concentration in endoplasmic stores (ER [Ca\(^{2+}\)]) was estimated from the fluorescence intensity of Fura-2FF, which localizes to the endoplasmic reticulum. NO production was measured by fluorescence imaging of the NO-sensitive dye DAF-FM. The ratio of fluorescence intensity of DAF-FM relative to its individual baseline (DAF-FM\(^0\)) reflects cumulative NO production over time, whereas its first derivative \(\Delta F/F_0\) determined in 5 minutes intervals reflects actual NO production. The lung vascular filtration coefficient (K\(_f\)) was determined as measure of pulmonary vascular endothelial permeability by dividing the rate of lung weight gain after a P\(_{L\text{A}}\) increment by the resultant elevation in capillary pressure. Conventional whole-cell voltage clamp configuration was performed to measure transmembrane currents in single rat pulmonary arterial endothelial cells as described previously.

Acute hydrostatic pulmonary edema was induced in vivo in a rat model of myocardial infarction by ligation of the left anterior descending coronary artery (LAD). Phosphodiesterase (PDE) inhibitors were added to the perfusate 10 minutes before \(P_{L\text{A}}\) elevation, respectively. B, GSNO (250 \(µ\)mol/L) or the NO synthase inhibitor L-NAME (250 \(µ\)mol/L) were added to the perfusate 10 minutes before \(P_{L\text{A}}\) elevation, respectively. \(P<0.05\) vs control (n=5 each).

**Results**

**Regulation of Lung Vascular Permeability**

Elevation of P\(_{L\text{A}}\) from 5 to 15 cmH\(_2\)O increased lung filtration coefficient (K\(_f\)), indicating deterioration of the vascular barrier. This effect was attenuated by both Gd\(^{3+}\), which blocks the endothelial Ca\(^{2+}\) influx\(^3\) and the MLCK inhibitor ML-7 (Figure 1A). The fact that neither Gd\(^{3+}\) nor ML-7 altered lung perfusion pressures (data not shown) indicates that their effects on K\(_f\) did not result from changes in hemodynamics or vascular surface area. To analyze the role of endothelial NO synthesis in the regulation of vascular permeability, we blocked NO synthases by N\(^\text{G}\) nitro-L-arginine methyl ester (L-NAME) or administered the exogenous NO donor S-nitrosoglutathione (GSNO). GSNO attenuated the pressure-induced increase in K\(_f\), whereas L-NAME amplified it, indicating a barrier-protective role for NO under hydrostatic stress (Figure 1B).

**Regulation of Endothelial [Ca\(^{2+}\)], Response by NO**

Because NO attenuated the Ca\(^{2+}\)-dependent K\(_f\) increase, we tested whether NO may directly interfere with the endothelial [Ca\(^{2+}\)] response. P\(_{L\text{A}}\) elevation for 30 minutes induced a progressive [Ca\(^{2+}\)] increase in lung capillary endothelial cells as demonstrated by real-time fluorescence imaging (Figure 2A). Inhibition of NO synthase by L-NAME enhanced the endothelial [Ca\(^{2+}\)] response, whereas it was attenuated by
addition of GSNO (Figure 2B). The pressure-induced \([Ca^{2+}]_i\) increase was likewise intensified by the soluble guanylate cyclase (sGC) inhibitor oxadiazolo quinoxalin-1-one (ODQ), whereas the sGC stimulator Bay 41-2272 or the cell-permeable cGMP analog 8Br-cGMP largely abrogated it (Figure 2C). These data demonstrate that NO limits the endothelial \([Ca^{2+}]_i\) response to hydrostatic stress via activation of its downstream target sGC and subsequent formation of cGMP.

Next, we tested whether cGMP formation also reduces lung vascular permeability and edema formation. Whereas ODQ amplified the \(K_f\) increase following PLA elevation, Bay 41-2272 and 8Br-cGMP attenuated the response to an almost similar extent as the \(Ca^{2+}\) influx blocker Gd\(^{3+}\) (Figure 2D). Both 8Br-cGMP and Gd\(^{3+}\) reduced hydrostatic edema formation at \(P_{L,\alpha}\) of 15 cmH\(_2\)O, as revealed by lung wet/dry weight ratio analysis (Figure 2E). These findings indicate that NO limits hydrostatic edema formation by a cGMP-mediated attenuation of the endothelial \([Ca^{2+}]_i\) response.

The attenuation of the endothelial \([Ca^{2+}]_i\) increase by NO may establish a negative-feedback loop which limits not only the increase in \(K_f\) but also pressure-induced endothelial NO production itself. Imaging of the NO-sensitive dye DAF-FM in lung endothelial cells in situ revealed enhanced NO production at elevated \(P_{L,\alpha}\) (Figure 3A), as previously reported.\(^4\) This effect was blocked when lungs were perfused with Ca\(^{2+}\) free buffer (Figure 3B) or in the presence of Gd\(^{3+}\) (data not shown). Consistent with the notion that cGMP attenuates the pressure-induced \([Ca^{2+}]_i\) response, both Bay 41-2272 and 8Br-cGMP markedly diminished NO production in response to \(P_{L,\alpha}\) elevation (Figure 3C). Thus, \(Ca^{2+}\)-dependent NO production and cGMP-dependent attenuation of the \([Ca^{2+}]_i\) response establish a negative regulatory feedback loop in lung vascular endothelial cells.

**Figure 2.** Regulation of endothelial \([Ca^{2+}]_i\) and lung edema formation by NO and cGMP. A, Fura-2–loaded endothelial cells of lung venular capillaries were imaged in situ by real-time fluorescence microscopy. Representative images of the 340/380 ratio color coded for \([Ca^{2+}]_i\) were obtained at \(P_{L,\alpha}=5\) cmH\(_2\)O (left) and 30 minutes after \(P_{L,\alpha}\) elevation to 15 cmH\(_2\)O (right). Vessel margins are depicted by dotted lines. Replicated in \(n=5\). B and C, Group data of EC \([Ca^{2+}]_i\) are shown as 5-minute averages at baseline (\(P_{L,\alpha}=5\) cmH\(_2\)O) and over 30 minutes of \(P_{L,\alpha}\) elevation to 15 cmH\(_2\)O. GSNO (250 \(\mu\)mol/L), the NO synthase inhibitor L-NAME (250 \(\mu\)mol/L), the sGC inhibitor ODQ (10 \(\mu\)mol/L), the sGC stimulator Bay 41-2272 (10 \(\mu\)mol/L), or the cGMP analog 8Br-cGMP (100 \(\mu\)mol/L) was added to the perfusate 10 minutes before \(P_{L,\alpha}\) elevation. *\(P<0.05\) vs control (\(n=5\) each). D, \(K_f\) was determined in isolated perfused rat lungs at baseline (\(P_{L,\alpha}=5\) cmH\(_2\)O) and after 30 minutes of pressure elevation (\(P_{L,\alpha}=15\) cmH\(_2\)O) (right). The sGC inhibitor ODQ (10 \(\mu\)mol/L), the sGC stimulator Bay 41-2272 (10 \(\mu\)mol/L), or the cGMP analog 8Br-cGMP (100 \(\mu\)mol/L) was added to the perfusate 10 minutes before \(K_f\) measurements or \(P_{L,\alpha}\) elevation, respectively. *\(P<0.05\) vs control (\(n=5\) each). E, Group data of wet/dry weight ratio from isolated lungs perfused for 30 minutes at \(P_{L,\alpha}\) of 5 cmH\(_2\)O (left) or 15 cmH\(_2\)O (right), respectively. Gd\(^{3+}\) (10 \(\mu\)mol/L) or 8Br-cGMP (100 \(\mu\)mol/L) was added at the beginning of the experiment. *\(P<0.05\) vs control at \(P_{L,\alpha}=15\) cmH\(_2\)O (\(n=5\) each).
alveolar septa (data not shown; findings in keeping with previously published results). Application of ruthenium red (RuR) blocked the increase of endothelial 
$[Ca^{2+}]_i$ and attenuated the increase in $K_f$ following PLA elevation (Figure 4A and 4B). Of note, RuR inhibited the $K_f$ increase to a similar extent as the Ca$^{2+}$-influx blocker Gd$^{3+}$, suggesting that the pressure-induced endothelial 
$[Ca^{2+}]_i$ response and the subsequent Ca$^{2+}$-dependent $K_f$ increase are mediated by TRPV4. Consistent with this notion, pharmacological activation of TRPV4 by 4PDD increased $K_f$ at baseline $P_{LA}$ of 5 cmH$_2$O. Yet, following stimulation with 4oPDD, $P_{LA}$ elevation did not elicit a further increase in $K_f$, suggesting that the responsible mechanosensitive channels had already been activated (Figure 4B). To solidify this concept in a genetic loss-of-function model using TRPV4 gene-targeted mice, we assessed lung endothelial responses to acute hydrostatic stress in TRPV4-deficient (TRPV4$^{-/-}$) mice versus wild-type littermates (TRPV4$^{+/+}$). Pressure-induced endothelial 
$[Ca^{2+}]_i$ increase (data not shown) and NO production (Figure 4C) were preserved in wild-type, but absent in TRPV4$^{-/-}$ mice, solidifying the functional role of TRPV4 in lung microvascular mechanotransduction. Consistent with this notion, hydrostatic edema formation was drastically reduced in TRPV4$^{-/-}$ mice as compared with wild type (Figure 4D).

Next, we determined whether cGMP attenuates the endothelial 
$[Ca^{2+}]_i$, response by preventing Ca$^{2+}$ influx via TRPV4 or by stimulating Ca$^{2+}$ uptake into endosomal stores. The cGMP analog 8Br-cGMP failed to increase ER 
$[Ca^{2+}]_i$, as determined by Fura-2FF fluorescence at baseline, as well as at elevated $P_{LA}$ (Figure 5A). Yet, 8Br-cGMP blocked the 4oPDD-induced endothelial 
$[Ca^{2+}]_i$, increase to a similar degree as RuR (Figure 5B). The notion that cGMP inhibits endothelial TRPV4 is further supported by whole-cell patch-clamp recordings in pulmonary microvascular endothelial cells. As compared with untreated control cells, 4oPDD activated an inwardly rectifying current that reversed near $-20 \text{ mV}$ and was blocked by pretreatment with 8Br-cGMP (Figure 5C and 5D).

PDE5 Inhibition Attenuates Hydrostatic Lung Edema

Our finding that cGMP limits endothelial Ca$^{2+}$ influx via TRPV4 and, thus, attenuates hydrostatic lung edema may give rise to new therapeutic strategies. Because PDE5 which rapidly degrades cGMP to GMP has recently been identified in pulmonary arterial endothelial cells, it may present a new target for lung barrier protection. PDE5 expression in lung vascular endothelial cells was confirmed by Western blotting and immunohistochemistry (data not shown). Sildenafil attenuated the increases of both endothelial 
$[Ca^{2+}]_i$, and $K_f$ in response to hydrostatic stress (Figure 6A and 6B), suggesting...
that PDE5 activity may regulate TRPV4-mediated endothelial Ca\(^{2+}\) influx in the lung. Sildenafil did not alter lung perfusion pressures consistent with the notion that the pulmonary vascular bed is already fully dilated under physiological conditions and lacks a myogenic response.\(^{16,17}\) Thus, sildenafil-induced changes in \(K_f\) were not attributable to the vasoactive but exclusively to the barrier-protective properties of the PDE5 inhibitor.

We tested the hypothesis that PDE5 inhibition may counteract lung vascular barrier deterioration and lung edema formation in vivo in a model of acute myocardial infarction. Within 90 minutes, LAD ligation resulted in considerable lung edema formation and protein leakage as evidenced by increased wet/dry lung weight ratio and Evans blue extravasation, respectively (Figure 6C and 6D), and decreased arterial oxygen tension (Figure 6E). Strikingly, administration of intravenous sildenafil at the time of LAD ligation effectively inhibited lung edema formation and protein leakage and increased arterial oxygenation (Figure 6C through 6E).

**Discussion**

The present study identifies signaling cascades within endothelial cells that regulate the lung vascular barrier response to hydrostatic stress. Mechanosensitive TRPV4 channels mediate a pressure-induced Ca\(^{2+}\) influx that increases vascular permeability yet, at the same time, activates a negative-feedback mechanism that limits the endothelial [Ca\(^{2+}\)]\(_i\) response and, thus, protects the vascular barrier. This intrinsic feedback loop involves the pressure-induced synthesis of NO and cGMP, which in turn blocks Ca\(^{2+}\) entry via TRPV4. Hence, pharmacological elevation of endothelial cGMP may provide a new strategy in treatment of hydrostatic lung edema.

**Methodological Considerations**

Identification of this signaling cascade was facilitated by a combination of real-time imaging with \(K_f\) measurements in the isolated perfused lung. Real-time imaging has recently developed into a powerful technique to reveal intra- and intercellular signaling pathways at the alveolocapillary barrier.\(^2\) \(K_f\) determination stands a robust assessment of lung microvascular barrier properties, provided that nonspecific factors that may affect the weight gain can be effectively excluded. In the present study, potential influences of lymphatic drainage, vascular compliance, and epithelial barrier properties were accounted for by the specific setup and protocol for \(K_f\) measurements, as outlined in the online data supplement. Similarly, vasoactive responses did not contribute to our findings, because the pulmonary vasculature is fully dilated at...
rest independent of endogenous NO synthesis and lacks a myogenic response.17,18 Accordingly, none of the applied pharmacological agents caused a change in perfusion pressures in the isolated lung preparation.

Ca2+-Dependent Regulation of Lung Vascular Permeability

The endothelial [Ca2+]i response and the increase in lung K+ were inhibited by RuR, whereas the TRPV4 activator 4aPDD mimicked the response to pressure stress, therefore suggesting a role for TRPV4 in the endothelial mechanotransduction and subsequent edema formation. Yet, it has to be considered that RuR blocks several members of the TRPV channel subfamily, as well as L-type Ca2+ channels19 and the mitochondrial Ca2+ uniporter.20 Similarly, 4aPDD is not absolutely specific for TRPV4 because it may, eg, enhance the biological activity of active phorbol esters such as phorbol 12-myristate 13-acetate.21 To rule out these nonspecific effects, we robustly confirmed the functional role of TRPV4 in mechanotransduction and permeability regulation by demonstrating the absence of a pressure-induced endothelial [Ca2+]i response and the attenuation of hydrostatic lung edema in TRPV4-deficient mice. Because TRPV4 activation increases endothelial permeability preferentially in lung capillaries,8 changes in K+ and imaged endothelial [Ca2+]i responses likely occurred in the same vascular compartment. Hydrostatic stress induced a sustained elevation of lung endothelial [Ca2+]i via TRPV4, and inhibition of Ca2+ influx or MLCK attenuated the concomitant K+ increase, consistent with the notion of a Ca2+-dependent contraction of endothelial myofibrils.

NO-Dependent Negative-Feedback Loop

NO has been proposed to either increase or decrease microvascular permeability depending on the specific experimental conditions, species, and vascular bed studied.11 In the lung, the microvascular barrier is more permeable than in systemic vascular beds, as exemplified by the fact that hydraulic conductivity is 10 times smaller and the reflection coefficient is considerably higher in skeletal muscle as compared with lung.22 In the lung hydrostatic stress, both endogenous NO formation and addition of an exogenous NO donor proved barrier protective in that L-NAME amplified the permeability increase, whereas GSNO attenuated it. Real-time fluoro-
ence imaging demonstrated that the barrier-protective effect of NO was attributable to an attenuation of the endothelial \([\text{Ca}^{2+}]_{i}\) response and mediated by cGMP. Administration of a cGMP analog blocked the better part of the pressure-induced increase in wet/dry lung weight ratio and, thus, had similar effects as the Ca^{2+} channel inhibitor Gd^{3+}. Taken together, these findings demonstrate that NO can reduce hydrostatic lung edema via a cGMP-dependent attenuation of the endothelial \([\text{Ca}^{2+}]_{i}\) increase.

NO and cGMP may attenuate endothelial \([\text{Ca}^{2+}]_{i}\) signaling by inhibition of extracellular Ca^{2+} entry or by regulation of Ca^{2+} uptake into and release from endosomal stores.\(^{23,24}\) Measurements of ER \([\text{Ca}^{2+}]_{i}\) and studies on the regulation of TRPV4 indicate that cGMP impaired the endothelial \([\text{Ca}^{2+}]_{i}\) response primarily by inhibition of Ca^{2+} influx. The obvious interpretation is that endothelial signal transduction cascades established a negative-feedback loop in which hydrostatic pressure triggers TRPV4-mediated endothelial Ca^{2+} influx, which stimulates NO formation by endothelial NO synthase. NO activates sGC to form cGMP, which in turn limits the endothelial Ca^{2+} influx. The notion of a closed-loop negative control was confirmed by the fact that sGC stimulation or 8Br-cGMP both attenuated endothelial NO formation.

Negative-feedback control of NO biosynthesis has been reported previously, in that NO and NO-donor agents can directly inhibit endothelial NO synthase in a noncompetitive fashion.\(^{25}\) NO may also downregulate endothelial NO synthase expression via a cGMP-mediated pathway.\(^{26}\) Hence, negative feedback regulation of NO synthesis can occur at the level of direct enzyme inhibition, protein expression, or, as demonstrated in the present study, posttranslational regulation.

**Role of TRPV4 in the Regulation of Vascular Permeability**

TRPV4 channels are expressed in pulmonary arterial and lung microvascular endothelial cells, and TRPV4 activation by 4aPDD has been shown to increase endothelial permeability in the presence of extracellular Ca^{2+}.\(^{8}\) Our data are in agreement with these findings in that 4aPDD induced a marked rise in endothelial \([\text{Ca}^{2+}]_{i}\), concomitant with an increase in vascular permeability. After stimulation with 4aPDD, hydrostatic stress had no additional effect on endothelial permeability, suggesting that underlying sensory signaling pathways were already activated.

The present study identified cGMP as a critical regulator of TRPV4 in lung microvascular endothelial cells. As shown by patch-clamp recordings in pulmonary microvascular endothelial cells and by real-time imaging in intact lung microvessels, the 4aPDD-induced inward current and \([\text{Ca}^{2+}]_{i}\), increase were both blocked by 8Br-cGMP. Although regulation of the canonical transient receptor po-
tential isoform 3 (TRPC3) by cGMP has been described. The present findings are the first to report regulation of a TRPV channel by cyclic nucleotides.

**PDE5 Inhibition As Therapeutic Strategy in Hydrostatic Lung Edema**

The identification of a cGMP-dependent feedback loop in the regulation of endothelial permeability may give rise to new therapeutic interventions for the prevention or treatment of hydrostatic lung edema. Endogenous activators or pharmacological stimulators of sGC such as NO or Bay 41-2272 may strengthen the vascular barrier by attenuating the endothelial \([Ca^{2+}]\), response to hydrostatic stress yet, at the same time, may promote lung edema because cGMP blocks fluid absorption by alveolar epithelial cells. Inhibition of PDE5 may be a more advantageous concept in this context, because PDE5 is expressed in lung endothelium but not in alveolar epithelial cells.

In an in vivo model of acute myocardial infarction, LAD occlusion caused hydrostatic lung edema and protein extravasation as previously described. PDE5 inhibition by sildenafil reduced lung fluid accumulation by approximately 80% and reconstituted arterial oxygenation to physiological levels. In parallel, PDE5 inhibition decreased protein leakage as determined by Evans blue extravasation, suggesting that the protective effect of sildenafil was primarily attributable to a reduction in endothelial permeability. This notion is supported by our findings in isolated perfused rat lungs in which sildenafil attenuated the pressure-induced increase in endothelial \([Ca^{2+}]\), and permeability. Vasoactive effects of sildenafil may potentially have been conducive to its antiinflammator properties, yet this contribution can be considered small because sildenafil did neither affect mean arterial pressure in vivo nor alter pulmonary hemodynamics in isolated lungs. Similarly, the recently described positive inotropic effects of sildenafil are expected to play a minor role in this setting, because expression of PDE5 in cardiomyocytes is negligible at baseline and only increases in chronic heart failure. Sildenafil may also potentially alter lymphatic tone in vivo, yet, again, this would only have a minor impact on hydrostatic stress, because lymphatic drainage from the lung is primarily driven by respiratory mechanics rather than lymphatic contractions. PDE5 inhibition may present a promising new strategy to attenuate lung edema in acute hydrostatic stress. This approach is even more attractive because sildenafil has also been demonstrated to ameliorate ischemia/reperfusion injury in acute myocardial infarction.

**Acknowledgments**

We thank Ursula Hilse for technical assistance.

**Sources of Funding**

This study received financial support from the Deutsche Forschungsgemeinschaft (Ka1218/4-1); the European Commission under the 6th Framework Programme (contract no. LSHM-CT-2005-018725, PULMOTENSION); Pfizer GmbH, Karlsruhe, Germany; and the Kaiserin-Friedrich Foundation, Berlin, Germany.

**Disclosures**

None.

**References**

Negative-Feedback Loop Attenuates Hydrostatic Lung Edema via a cGMP-Dependent Regulation of Transient Receptor Potential Vanilloid 4

Jun Yin, Julia Hoffmann, Stephanie M. Kaestle, Nils Neye, Liming Wang, Joerg Baeurle, Wolfgang Liedtke, Songwei Wu, Hermann Kuppe, Axel R. Pries and Wolfgang M. Kuebler

Circ Res. published online March 6, 2008;

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/early/2008/03/06/CIRCRESAHA.107.168724.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/03/10/CIRCRESAHA.107.168724.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
A negative feedback loop protects the lung from excessive hydrostatic edema via a cGMP-dependent regulation of TRPV4

Jun Yin, Julia Hoffmann, Stephanie M. Kaestle, Nils Neye, Liming Wang, Joerg Baeurle, Wolfgang Liedtke, Songwei Wu, Hermann Kuppe, Axel R. Pries, and Wolfgang M. Kuebler

Online data supplement
Materials and Methods

Animals

Male Sprague-Dawley rats (385 ± 22 g, bw) were obtained from Charles River Laboratories (Sulzfeld, Germany). Male TRPV4-deficient (TRPV4−/−) mice and wild type littermates (TRPV4+/+) (25±3 g bw) were a generous gift from R. Köhler (Dept. of Internal Medicine, University of Marburg, Germany). All animals received care in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press, Washington, DC 1996). The study was approved by the animal care and use committee of the local government authorities.

Materials

The TRPV4 activator 4-α-phorbol-12,13-didecanoate (4αPDD), S-Nitrosoglutathione (GSNO), and the inhibitors of TRPV4, endothelial NO synthase (eNOS), and soluble guanylate cyclase (sGC) ruthenium red, Nω-nitro-L-arginine methyl ester (L-NAME), and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), respectively, were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Human albumin (20%) was from Octa Pharma GmbH (Langenfeld, Germany), the soluble guanylate cyclase stimulator Bay 41-2272 from Biotrend GmbH (Cologne, Germany), the cGMP analog 8Br-cGMP from Calbiochem (Darmstadt, Germany). Sildenafil™ was a gift from Pfizer GmbH (Karlsruhe, Germany).

The Ca²⁺-sensitive dye fura-2 AM and the NO sensitive fluorophor 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) were obtained from Molecular Probes (Eugene, OR). Polyclonal anti-rabbit phosphodiesterase (PDE) 5 antibody was from Cell Signaling Technology (Danvers, MA), anti-rabbit TRPV4 antibody from Alomone Labs (Jerusalem, Israel), and horseradish-peroxidase conjugated secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolated perfused lung models

Isolated rat or mouse lungs were constantly inflated with a gas mixture of 21% O₂, 5% CO₂, and balance N₂ at a positive airway pressure of 5 (rats) or 3 (mice) cmH₂O. For real-time fluorescence microscopy and determination of lung filtration coefficient (Kf), lungs were perfused with autologous heparinized blood or Krebs-Henseleit buffer containing 3% bovine
serum albumin, respectively. At baseline, left atrial pressure (P<sub>LA</sub>) was adjusted to 5 (rats) or 3 (mice) cmH<sub>2</sub>O, yielding pulmonary arterial pressures (P<sub>PA</sub>) of 10±1 cmH<sub>2</sub>O at a constant perfusion rate of 14 (rats) or 1 (mice) mL/min at 37°C, respectively. P<sub>PA</sub> and P<sub>LA</sub> were continuously monitored and digitally recorded (DASYlab 32; Datalog GmbH, Moenchengladbach, Germany).

In situ real-time fluorescence microscopy and image analysis

In situ real-time fluorescence microscopy experiments were performed as previously described<sup>1</sup>. Isolated perfused rat or mouse lungs were positioned on a vibration-free table and superfused with saline at 37°C to prevent drying. For local delivery of fluorescent probes, a microcatheter was wedged in a pulmonary vein draining a capillary area on the lung surface<sup>2,3</sup>. Endothelial fluorescence was excited by a near monochromatic beam from a digitally controled galvanometric scanner (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany). Fluorescence emission was collected through an upright intravital microscope (Axiovert<sup>Varion</sup> 100 HD; Zeiss, Jena, Germany) equipped with an apochromat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany) and appropriate dichroic and emission filters (FT 425 and BP 505-530 for Fura-2 and Fura-2FF, FT 510 and LP 520 for DAF-FM; all Zeiss, Jena, Germany) by a CCD camera (Sensicam; PCO, Kelheim, Germany) and subjected to digital image analysis (TILLvision 4.01; T.I.L.L. Photonics). Single venular capillaries were viewed at a focal plane corresponding to maximum vessel diameter (15-27 µm). All fluorescence images were obtained in 10 s intervals and background corrected, with background determined in images captured before fluorophore loading. Fluorescence was quantified in 4 µm² areas along the microvascular wall, representing single lung endothelial cells.

For quantification of endothelial [Ca<sup>2+</sup>]<sub>i</sub>, membrane-permeant Fura-2 AM (5 µmol/L) which de-esterifies intracellularly to impermeant Fura-2, was infused into lung microvessels for 20 min. Fluorescence images were recorded at excitation wavelengths of 340, 360 and 380 nm, and endothelial [Ca<sup>2+</sup>]<sub>i</sub> was determined from the 340/380 ratio based on a K<sub>d</sub> of 224 nmol/L and appropriate calibration parameters<sup>4,5</sup>. ER [Ca<sup>2+</sup>]<sub>i</sub> was determined by Fura-2FF using a protocol similar to that used for Fura-2<sup>6</sup>. For measurement of NO production, we infused cell-permeant DAF-FM diacetate (5 µmol/L) which de-esterifies to DAF-FM. Intracellular DAF-FM is converted via an NO dependent mechanism to an intensely fluorescent benzotriazole derivative with fluorescence intensity linearly reflecting NO concentration<sup>1</sup>. DAF-FM fluorescence was excited at 480 nm and fluorescence intensity (F) was expressed relative to
its individual baseline \( (F_0) \). Because the NO-dependent conversion of DAF-FM into a fluorescent benzotriazole derivative is irreversible, the ratio \( F/F_0 \) reflects cumulative NO production over time, while its first derivative \( \Delta F/F_0 \) determined in 5 s intervals reflects actual NO production.

*Evaluation of pulmonary endothelial permeability*

Lung vascular filtration coefficient \( (K_f) \) was measured in isolated perfused rat lungs by a gravimetric technique (PRS 320-3, Kern, East Sussex, UK). Lung weight changes, pulmonary arterial \( (P_{PA}) \) and left atrial pressure \( (P_{LA}) \) were continuously monitored (HSE Type 705/1; Hugo Sachs Elektronik, March-Hugstetten, Germany) and digitally recorded (DASYlab 32; Datalog GmbH, Moenchengladbach, Germany).

After ensuring that lungs were under isogravimetric conditions for \( >5 \) min, \( P_{LA} \) was increased by 4 cmH2O and \( K_f \) was determined from the resulting weight gain \( (\Delta W) \) by dividing the rate of weight gain \( \Delta W/\Delta t \) measured between 18 and 20 min after the \( P_{LA} \) increment by the resultant elevation in capillary pressure\(^7\). Results were normalized to 100 grams initial lung weight, assuming the density of the filtered fluid to be 1 g/mL. Pulmonary capillary pressure \( (P_c) \) was calculated from \( P_{PA} \) and \( P_{LA} \) as previously suggested by Gaar and colleagues\(^8\). Next, \( P_{LA} \) was reset to baseline and deviations from the isogravimetric state were corrected for by subtracting the linear regression of \( \Delta W/\Delta t \) at baseline \( P_{LA} \) from the actual \( \Delta W/\Delta t \)\(^9\).

Particular consideration was given to the potential influence of non-specific factors that may affect lung weight gain, such as vasoactive responses, lymphatic drainage, vascular compliance, and changes in epithelial barrier characteristics and alveolar fluid reabsorption.

*Vasoactive responses:* The pulmonary vasculature is fully dilated under baseline conditions\(^{10}\) and lacks a myogenic response\(^{11}\). Importantly, the low resting tone in the lung is independent from NO synthesis\(^{12}\). Accordingly, vasodilatory drugs or NOS inhibitors have no vasoactive effects in the isolated lung. This notion was confirmed by the fact that none of the applied pharmacological interventions altered perfusion pressures in the perfused lung preparation.

*Lymphatic drainage:* In the present setup, lymphatic effluent and transudate from the lung were constantly collected and added to the lung weight gain. Consistent with the notion that lymphatic drainage is low in isolated and continuously inflated lung preparations due to the lack of respiratory movements\(^{13}\), extrapulmonary fluid collection accounted for less than 3.6% of the total lung weight gain and did not differ with pharmacological interventions. *Vascular compliance:* After a pressure increment, lung weight gain follows a biexponential function
with the fast and slow component reflecting the vascular and interstitial filling phase, respectively\textsuperscript{14}. Mathematical adaptation as well as functional data demonstrate that vascular filling is completed within < 5 min\textsuperscript{14, 15}. Accordingly, weight gain measurements between 18 and 20 min after the P\textsubscript{LA} increment can be considered as independent from vascular compliance. *Epithelial conductance and alveolar fluid reabsorption:* To warrant that changes in transepithelial conductance or alveolar fluid reabsorption do not affect lung weight change, \( K_f \) analysis was confined to lungs in which \( \Delta W \) returned to less than 10% of the peak weight gain after reversal of the P\textsubscript{LA} increment.

Given adequate consideration of these non-specific factors, the \( K_f \) measurement yields a robust assessment of endothelial permeability in the intact lung preparation\textsuperscript{16}.

*Western blot analysis*

For protein analyses from whole lung tissue, aliquots of frozen rat lungs were powdered, lysed and homogenized in extraction buffer (20 mmol/L Na\textsubscript{3}PO\textsubscript{4} (pH 7.8), 1% Triton-100, 2.5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L phenylmethanesulphonylfluoride (PMSF), 10 \( \mu \)g/mL aprotinin, 10 \( \mu \)g/mL leupeptin, and 10 \( \mu \)g/mL pepstatin). Samples were shock frozen in liquid nitrogen and thawed on ice for three times. Lysates were collected by pelleting the cellular debris for 15 min at 19,800 g and total protein concentration was determined by the Bradford protein assay (Bio-rad Protein Assay; Bio-Rad, Munich, Germany).

For protein analyses from fresh lung endothelial cells (FLEC), endothelial cells were separated by a magnetic bead immunosorting technique. Briefly, lungs were excised and digested with collagenase for 30 min. The reaction was stopped with FCS, the lungs were homogenized, sieved through a 100 \( \mu \)M sieve and centrifuged (1000 g, 5 min). Cell pellets were resuspended and incubated for 5 min in lysis buffer (0.829% NH\textsubscript{4}Cl, 0.1% KHCO\textsubscript{3}), then centrifuged, washed with PBS + 1% albumin and resuspended in RPMI-1640 medium. For immunosorting, \( 4 \cdot 10^8 \) human-anti mouse DNA-linked IgG-coated magnetic beads (4.5 \( \mu \)m; Cellektion\textsuperscript{TM} Pan Mouse IgG; Dynal, Karlsruhe, Germany) were incubated with 50 \( \mu \)L mouse anti-human von Willebrand factor antibody (Chemicon, Boronia Victoria, Australia) for 2 h at room temperature. Beads were washed to remove excess antibody and incubated with the lung cell suspensions for 1 h at 4\textdegree C.

FLEC expressing von Willebrand factor attached to the antibody-labeled beads, and were magnetically isolated (magnetic particle concentrator; Dynal), collected, washed, and resuspended in RPMI-1640 medium. Beads were detached by DNase releasing buffer and...
FLEC were quantified in a Neubauer chamber. FACS analyses and trypan blue exclusion assay revealed an endothelial cell fraction of > 98% as determined by positive staining with an R-phycoerythrin-conjugated mouse anti-rat CD31 monoclonal antibody (BD Biosciences, Heidelberg, Germany) and a cell viability of > 96% (n = 3 each). FLEC protein was extracted and protein concentration was determined as described above.

Sample proteins (50 µg/slot for homogenate and 30 µg/slot for FLEC, respectively) and a prestained protein-weight marker (Bio-Rad, Munich, Germany) were size-fractionated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany) with a Protean II wet blotter (Bio-Rad). Equal loading was confirmed by staining nitrocellulose membranes with ponceau dye (Sigma). The membranes were blocked at room temperature in 5% dry milk powder (Töpfer, Dietmannsried, Germany), soluted in phosphate buffer saline containing 1% Tween (PBST) for 1.5 h, incubated with matching primary rabbit anti-TRPV4 or rabbit anti-PDE 5 antibody (1:1000 in 5% dry milk powder PBST over night at 4°C) and washed three times with PBST (5 min each). Subsequently, NC membranes were incubated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology Inc, Heidelberg, Germany), washed 5 times, and protein bands were visualized by enhanced chemiluminescence (ECL; Perkin Elmer GmbH, Freiburg, Germany).

Patch-clamp electrophysiology

Conventional whole-cell voltage-clamp configuration was performed to measure transmembrane currents in single pulmonary microvascular endothelial cells (PMVECs) by the standard giga seal patch-clamp technique as previously described \textsuperscript{16}. Confluent PMVECs were trypsin dispersed, seeded onto 35 mm plastic culture dishes, and allowed to reattach 24 h before patch-clamp experiments were performed. Patch-clamp recordings were obtained using single PMVECs. Recording pipettes were heat polished to produce a tip resistance in the range of 3 to 5 MΩ in our internal solution. The pipette solution contained (in mmol/L) 130 N-methyl-D-glucamine, 10 HEPES, 2 EGTA, 1 Ca(OH)₂, 2 Na⁺-ATP, 1 NPA, 0.1 NPPB and pH was adjusted to 7.2 with methane sulfonic acid. The external bath solution contained (in mmol/L) 120 aspartic acid, 5 Ca(OH)₂, 5 CaCl₂, 10 HEPES, 0.5 3,4-diaminopyridine and pH was adjusted to 7.4 with tetraethylammonium hydroxide. Both solutions were adjusted to 290–300 mOsm/L with sucrose. Currents were recorded with a computer-controlled EPC9 patch-clamp amplifier (HEKA; Lambrecht, Germany). Cell capacitance and series resistance
were calculated with the software supported internal routines of the EPC9 and compensated before each experiment. Voltage pulses were applied from -100 to +60 mV in 20 mV increments after the whole-cell configuration was achieved, with 200-ms duration during each voltage step and a 3-s interval between steps. The holding potential between each step was 0 mV. Data acquisition and analysis were performed with Pulse/PulseFit software (HEKA) and filtered at 2.9 kHz.

In vivo model of acute hydrostatic pulmonary edema

Male Sprague-Dawley rats weighing 350-400g were anesthetized intraperitoneally with a combination of medetomidine (0.5 mg/kg bw, Domitor®, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg bw, JanssenCilag, Neuss, Germany), and midazolam (5 mg/kg bw, Dormicum®, Roche, Basel, Switzerland) as previously described 17. Rats were placed in the supine position on a thermostatically controlled electric heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, March-Hugstetten, Germany) to maintain body temperature at 37.0-37.5 °C. A tracheostomy was performed and rats were ventilated with room air at 70 breaths/min. The left carotid artery and the right jugular vein were cannulated for measurement of systemic hemodynamics. A thoractomy was performed, the pericardium was opened and the left anterior descending coronary artery (LAD) was ligated 2-3 mm from its origin by a 7-0 prolene suture. Sixty minutes after LAD occlusion, an arterial blood sample was taken for blood gas analysis and Evans blue dye (20 mg/kg bw) was injected subsequently via the right jugular vein. Thirty minutes later, animals were sacrificed by exsanguination and lungs were excised. Lung edema was determined from right lungs as wet/dry weight ratio by use of the microwave drying technique 18. Capillary leakage was determined from left lungs by the Evans blue extravasation technique 19. In brief, lungs were perfused free of blood with phosphate-buffered saline (PBS), snap frozen in liquid nitrogen, homogenized in PBS (1 ml/100 µg tissue) at 4°C, and incubated with 2 volumes of formamide for 18 h at 60°C. Samples were centrifuged at 5000 g for 30 min, and the optical density of the supernatant was determined spectrophotometrically at 620 nm.

Immunohistochemistry

Rat lungs were perfusion-fixed with 4% paraformaldehyde in 0.1 M PO₄ buffer at pH 7.4 for 30 min. Paraffin sections (5 µm) were deparaffinized in xylene for 10 min, rehydrated and blocked (10% methanol, 3% H₂O₂, 0.2% Triton X-100 in PBS) for 15 min. After washing and
trypsination (0.1% trypsin in 0.1 M TBS), lung sections were immersed into 0.3% Na-dodecyl-sulfate (SDS) for 10 min. Next, sections were incubated with rabbit anti-TRPV4 (1:200; Alomone) or rabbit anti-PDE 5 (1:100; CellSignaling Technology) antibodies, diluted in a solution containing 4% normal donkey serum, 0.3% Triton X-100, and 0.05% Tween 20 at 4°C in a humid chamber overnight. Sections were re-incubated with biotinylated secondary donkey anti-rabbit (1:200; Alomone) antibody, diluted in the same solution as the primary antibody, for 1 hour at room temperature and overnight at 4°C. Sections were then immersed into extravidin-peroxidase (1:100, Sigma) for 1 hour, and into diaminobenzidine (DAB) solution (0.05% DAB, 0.03% H₂O₂, 0.1% (NH₄)₂Ni(SO₄)₂ in PBS) for 20 min. Sections which were immunoprocessed as described above but without incubation in the primary antisera served as controls for the assessment of background staining. After dehydration and cover slipping, stained and control sections were viewed for microvascular expression of TRPV4 and PDE 5.

Statistical analysis

Statistical analysis was performed by use of SigmaStat software (SigmaStat 3.0, Jandel Scientific, San Rafael, CA). Data are presented as mean ± SEM. Statistical analyses within groups were performed by Wilcoxon matched pairs signed rank test and repeated measures ANOVA on ranks (Friedman test). Different groups were compared by Mann-Whitney U-test and ANOVA on ranks (Kruskal-Wallis test). Statistical significance was assumed at p<0.05.
References


