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DISSERTATION

Functional relevance and regulation of Transient Receptor Potential Vanilloid 4 channel in hydrostatic lung edema

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Funktionelle Relevanz und Regulation des TRPV4-Kanäle im hydrostatischem Lungenödem

ZUSAMMENFASSUNG

Traditionell wurde die Entstehung des hydrostatischen Lungenödems ausschließlich einem Ungleichgewicht der Starling´schen Kräfte, d.h. einer vermehrten Flüssigkeitsfiltration aufgrund eines Ungleichgewichts der hydrostatischen und onkotischen Druckgradienten über die Wand der pulmonalen Mikrogefäße, zugeschrieben. Diese klassische Sichtweise wurde durch die Ergebnisse jüngerer Untersuchungen in Frage gestellt, in denen eine aktive Regulation der mikrovaskulären Permeabilität bei hydrostatischem Stress nachgewiesen wurde, was auf eine wesentliche Rolle endothelialer Regulationsmechanismen in der Pathogenese des hydrostatischen Lungenödems hinweist. In unseren Untersuchungen haben wir optische Bildgebungsverfahren zur Visualisierung endothelialer Reaktionen, insbesondere von Änderungen der endothelialen Ca^{2+} Konzentration ($[Ca^{2+}]_i$) und der Synthese von Stickstoffmonoxid (NO), in der intakten Lunge mit Messungen des vaskulären Filtrationskoeffizienten (K_f) als Maß der pulmonalvaskulären Flüssigkeitspermeabilität kombiniert. Mit Hilfe dieses Ansatzes konnten wir eine endotheliale Reaktionen auf hydrostatischen Stress identifizieren, die in Form eines negativen Rückkopplungsmechanismus die pulmonale Gefäßbarriere schützen. Unsere Untersuchungen zeigen, dass hydrostatischer Stress den endothelialen Einstrom von Ca^{2+} durch Aktivierung mechanosensitiver Kationenkanäle stimuliert. Der resultierende endotheliale $[Ca^{2+}]_i$ Anstieg führt durch Aktivierung der Myosin-Leichtkettenkinase zu einem Anstieg von K_f und stimuliert zugleich die endotheliale NO Produktion durch Aktivierung der endothelialen NO Synthase. In nachfolgenden Experimenten konnten wir den *transient receptor potential vanilloid 4* (TRPV4) Kanal als mechanosensitiven Kationenkanal, der den endothelialen $[Ca^{2+}]_i$ Anstieg bei hydrostatischem Stress vermittelt, identifizieren. Entsprechend wiesen $TRPV4^{-/-}$ Mäuse eine deutliche Abschwächung des endothelialen $[Ca^{2+}]_i$ Anstiegs, der NO Produktion und des Lungenödems bei hydrostatischem Stress auf. Endothelial-gebildetes NO minderte den Anstieg der vaskulären Permeabilität über eine cGMP-abhängige Abschwächung der endothelialen $[Ca^{2+}]_i$ Antwort. Nachfolgende Patch-clamp Untersuchungen pulmonaler mikrovaskulärer Endothelzellen sowie intravitales Imaging des endothelialen $[Ca^{2+}]_i$ bestätigten die Inaktivierung von TRPV4 durch cGMP. Demzufolge steigert der Druck-induzierte Ca^{2+} Einstrom über TRPV4 Kanäle die pulmonalvaskuläre Permeabilität, aktiviert aber zugleich einen NO-vermittelten Rückkopplungsmechanismus, der

die Gefäßbarriere durch eine cGMP-abhängige Hemmung der endothelialen $[Ca^{2+}]_i$ Antwort schützt. Die Identifizierung dieses neuen regulatorischen Signalweges bietet Ansätze für neue Behandlungsstrategien. So konnten wir in der vorliegenden Arbeit in einem Modell des akuten Myokardinfarkts eine deutliche Abnahme des Lungenödems durch Hemmung der cGMP Hydrolyse mittels des Phosphodiesterase 5 Hemmers Sildenafil erzielen. In einem Modell chronischen hydrostatischen Stresses infolge einer Herzinsuffizienz konnte durch Inhalation von NO ebenfalls eine Abnahme des Lungenödems erzielt werden. Desgleichen verbesserte eine Dauertherapie mit Sildenafil in diesem Modell die mikrovaskuläre Barrierefunktion und reduzierte das Lungenödem. Die Regulation des mechanosensitiven TRPV4 Kanals durch einen negativen Rückkopplungsmechanismus über NO und cGMP stellt folglich einen neuen intrazellulären Regulationsweg und eine vielversprechende therapeutische Strategie für die Behandlung des hydrostatischen Lungenödems dar.

ABSTRACT

Traditionally, hydrostatic lung edema has been attributed exclusively to an imbalance in Starling forces, i.e. increased fluid filtration due to an imbalance of hydrostatic and oncotic pressure gradients across the lung microvascular barrier. This classical view has been challenged by recent findings demonstrating an active regulation of lung microvascular permeability in hydrostatic stress, suggesting that endothelial responses may contribute critically to formation of hydrostatic lung edema. Here, we combined real-time optical imaging of endothelial responses, in particular changes in the endothelial Ca^{2+} concentration ($[Ca^{2+}]_i$) and nitric oxide (NO) synthesis, in intact lungs with measurements of lung vascular filtration coefficient (K_f) as a measure of lung microvascular water permeability. By these approaches, we identified a series of endothelial responses to hydrostatic stress that constitute a negative feedback loop which protects the pulmonary vascular barrier. We demonstrate that hydrostatic stress stimulates endothelial Ca^{2+} entry by activation of mechanosensitive cation channels. The resulting increase in endothelial $[Ca^{2+}]_i$ increases K_f by activation of myosin light-chain kinase and simultaneously stimulates endothelial NO production by activation of endothelial NO synthase. In subsequent experiments, we could identify the transient receptor potential vanilloid 4 (TRPV4) channel as mechanosensitive cation channel which mediates the endothelial $[Ca^{2+}]_i$ response to hydrostatic stress. Consistent with this notion, we found pressure-induced increases in endothelial $[Ca^{2+}]_i$, NO synthesis, as well as lung wet/dry weight ratio to be markedly attenuated in $TRPV4^{-/-}$ mice. Notably, endothelial NO formation was shown to limit the permeability increase via a cGMP-dependent attenuation of the pressure-induced $[Ca^{2+}]_i$ response. Inactivation of TRPV4 channels by cGMP was confirmed by whole-cell patch-clamp of pulmonary microvascular endothelial cells and intravital imaging of endothelial $[Ca^{2+}]_i$. 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 endothelial $[Ca^{2+}]_i$ response. Identification of this novel regulatory pathway gives rise to new treatment strategies, as demonstrated *in vivo* in rats with acute myocardial infarction that inhibition of cGMP degradation by the phosphodiesterase 5 inhibitor sildenafil reduced hydrostatic lung edema. In a model of chronic pulmonary hydrostatic stress due to congestive heart failure, inhalation of NO was shown to markedly reduce lung wet/dry weight ratios. Similarly, long-term application of sildenafil protected lung vascular barrier function and reduced lung edema. Hence, negative feedback regulation of TRPV4 via NO/cGMP signaling

in lung endothelial cells presents a novel signaling cascade and a promising therapeutic strategy for the treatment of hydrostatic lung edema.

INTRODUCTION

Traditionally, the pathogenesis of hydrostatic lung edema has been predominantly attributed to an imbalance in Starling forces, i.e. fluid extravasates into the interstitial or alveolar compartment when the outward directed hydrostatic pressure gradient exceeds the inward directed oncotic pressure gradient across the vascular wall. Yet, via determining lung filtration coefficient (K_f) as a measure of vascular permeability in isolated rat lungs, recent studies have challenged this view by demonstrating that a K_f increase during hydrostatic stress was blunted by the β-adrenergic agonist isoproterenol.[1] These findings suggest that vascular permeability increases in hydrostatic stress, and thus incorporate active endothelial responses into the pathophysiology of hydrostatic lung edema. Extracellular Ca^{2+} entry into endothelial cells has been recognized as an indispensable requirement of K_f elevation in many conditions.[2] In accordance with this notion, marked increases in the endothelial cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and nitric oxide (NO) formation have been detected in lung microvessels at hydrostatic stress.[3, 4] Although these findings support the notion of an active endothelial participation in hydrostatic lung edema regulation, our understanding is hampered by the lack of insights into underlying endothelial signaling cascades and regulatory mechanisms. An additional complexity in this scenario consists in the fact that the mechanosensitive cation channel mediating the endothelial Ca^{2+} response to hydrostatic stress has not been identified yet. The transient receptor potential vanilloid 4 (TRPV4) channel which has been described as sensitive to both shear stress and membrane stretch[5] is an attractive candidate for a mechanosensor in the pulmonary microvasculature.

By combining real-time *in situ* fluorescence imaging with lung vascular filtration coefficient measurements in both acute and chronic *in vivo* and *ex vivo* lung models, we aimed to address the regulation of lung vascular barrier function by endothelial cells in hydrostatic stress, characterize the underlying endothelial signaling cascade and identify the mechanosensitive cation channel involved. Results from these experiments are expected to advance our basic understanding of hydrostatic lung edema formation and therefore, to yield new therapeutic strategies for the treatment of this frequent and critical condition.

MATERIAL AND METHODS

➢ ***Animals*** Male Sprague-Dawley rats and C57BL/6 mice were obtained from Charles River Laboratories. Male TRPV4 deficient ($TRPV4^{-/-}$) mice were a generous gift from Dr. R Köhler (Dept. of Internal Medicine, University of Marburg, Germany). All experiments were approved by the local government authorities.

➢ ***In vivo model of acute hydrostatic stress*** Rats were anesthetized and ventilated (tidal volume 6 ml/kg bw) as described.[3] After thoracotomy, the pericardium was opened and the left anterior descending coronary artery (LAD) was ligated 2-3 mm from its proximal origin by a 7-0 prolene suture. 60 min after LAD occlusion, Evans blue dye (20 mg/kg bw) was injected via the right jugular vein. After additional 30 min, animals were sacrificed by exsanguinations and lungs were excised. Lung edema was determined from right lungs as wet/dry weight ratio. Capillary leakage was determined from left lungs by the Evans blue extravasation technique: lungs were perfused free of blood, snap frozen in liquid nitrogen, homogenized at 4°C, and incubated with 2 volumes of 4% 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.[6]

➢ ***In vivo model of chronic hydrostatic stress*** Congestive heart failure was induced in male juvenile rats (93±7 g body weight) by supracoronary aortic banding as described.[7, 8] Aortic stenosis was induced by implantation of a titanium clip with a pre-defined internal diameter of 0.8 mm around the ascending aorta. Sham animals underwent thoracotomy with implantation of clips in the peri-aortal connective tissue under otherwise identical circumstances. Nine weeks after aortic banding, rats had developed congestive heart failure (CHF) and moderate hydrostatic lung edema.[7, 8] To test the effects of inhaled nitric oxide (NO) on hydrostatic lung edema and cyclic nucleotide levels in plasma, sham or CHF rats were anesthetized and NO was inhaled via a regulated flowmeter to yield inspiratory concentrations of 10-50 ppm. Blood samples were drawn before and after inhalation of NO, collected in EDTA tubes and plasma phosphodiesterases were blocked by 3-isobutyl-1-methylxanthine (IBMX; 167 µg/mL blood; Sigma- Aldrich, Taufkirchen, Germany). Concentrations of cyclic guanosine 3',5'-monophosphate (cGMP) were measured using a commercially available low-pH enzyme-linked immunosorbent assay (R&D Systems, Minneapolis). Lung edema was determined as wet/dry weight ratio.

➢ ***Isolated perfused lung model*** Lungs were excised *en bloc* from anesthetized rats or mice, continuously inflated and pump-perfused at 14 ml/min (rats) or 1 ml/min (mice) and 37ºC as

reported.[9] For real-time fluorescence microscopy and determination of lung filtration coefficient (K_f), lungs were perfused with autologous heparinized blood or Krebs-Henseleit buffer containing 3% bovine serum albumin, respectively. At baseline, left atrial pressure (LAP) was adjusted to 5 cmH_2O, yielding pulmonary artery pressures (PAP) of 10±1.5 cmH_2O. PAP and LAP were continuously monitored and recorded (DasyLab®32). Acute hydrostatic stress in isolated perfused lungs was applied by elevation of the venous outflow reservoir.

➢ ***In situ real-time fluorescence microscopy*** Real-time *in situ* fluorescence microscopy was performed in isolated rat or mouse lungs as described.[3, 4, 10] Endothelial cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was determined by the fura-2 ratiometric imaging technique.[4, 10] Fura-2AM (5 μmol/L), which de-esterifies intracellularly into membrane-impermeant fura-2, was loaded to lung capillary endothelial cells, and fura-2 fluorescence at excitations of 340, 360, and 380 nm was recorded. $[Ca^{2+}]_i$ was determined from the 340/380 ratio based on a K_d of 224 nmol/L and appropriate calibration parameters. Endosomal Ca^{2+} levels in lung endothelial cells were determined by fura-2FF using a similar protocol as for fura-2.[10] DAF-FM diacetate (5 μmol/L), which converts irreversibly to an intensely fluorescent benzotriazole derivative in an NO dependent mechanism, was used for imaging endothelial nitric oxide (NO) production.[3] When excited at 480 nm, DAF-FM fluorescence intensity (F) expressed relative to its individual baseline (F_0) reflects cumulative endothelial NO production.[3]

➢ ***Measurement of lung filtration coefficient*** In isolated perfused rat lungs, lung weight changes, LAP and PAP were continuously monitored and digitally recorded (DasyLab®32). After a 5-min isogravimetric baseline, lung vascular filtration coefficient (K_f) as a measure of endothelial permeability was determined by dividing the rate of weight gain ($\Delta W/\Delta t$) recorded between 18 and 20 min after an LAP increment (4 cmH_2O) by the resultant increase in capillary pressure (Pc).[11] Pc was calculated from PAP and LAP as described.[12] At elevated LAP, deviations from the isogravimetric state were corrected for by subtracting the linear regression of $\Delta W/\Delta t$ at baseline LAP from the actual $\Delta W/\Delta t$.[13]

➢ ***Isolation of lung endothelial cells*** Fresh lung endothelial cells (FLECs) were separated by a magnetic bead immunosorting technique.[14] Human-anti mouse DNA-linked IgG-coated magnetic beads (4.5 μm; Cellektion™ Mouse IgG; Dynal) were incubated with mouse anti-human von Willebrand factor antibody (Chemicon) and FLECs. FLECs bound to beads were magnetically isolated (magnetic particle concentrator; Dynal), and flow cytometry analyses

and trypan blue exclusion assay revealed an endothelial cell fraction of > 98% and a cell viability of > 96%.

➢ ***Western blot analyses*** After protein extraction from lung homogenate and FLEC, total protein concentration was determined by a protein assay (Bio-Rad). Equal loading of protein was confirmed by staining nitrocellulose membranes with ponceau dye. After blocking, the membrane was probed with matching primary rabbit anti-TRPV4 or anti-PDE5 antibody (1:1000 in 5% dry milk powder PBST over night at 4°C), followed by horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology Inc.), and visualized by enhanced chemiluminescence (ECL; Perkin Elmer).

➢ ***Immunohistochemistry*** After deparaffinization and rehydration, 5 µm thick rat lung sections were incubated with rabbit anti-TRPV4 (1:200; Alomone) or rabbit anti-phosphodiesterase 5 (PDE5) (1:100; CellSignaling Technology) antibodies at 4°C in a humid chamber overnight, and then re-incubated with biotinylated secondary donkey anti-rabbit (1:200; Alomone) antibody. Sections without incubation in primary antisera served as controls.

➢ ***Patch clamp electrophysiology*** Confluent lung vascular endothelial cells were trypsin dispersed, seeded onto culture dishes, and allowed to reattach 24 hours before patch-clamp experiments. Currents were recorded with a computer-controlled EPC9 patch-clamp amplifier (HEKA; Lambrecht). Cell capacitance and series resistance were calculated with the software supported internal routines and compensated before each experiment. Data acquisition and analysis were performed with Pulse/PulseFit software (HEKA) and filtered at 2.9 kHz.

➢ ***Data and statistical analysis*** Data are presented as means±SEM. Statistical analyses within groups were performed by Wilcoxon matched pairs signed rank test and repeated measures ANOVA on ranks (Friedman test). Differences between groups were determined by Mann-Whitney *U* test and ANOVA on ranks (Kruskal-Wallis test). A value of $p<0.05$ was considered statistically significant.

RESULTS

➢ ***Regulation of lung vascular permeability*** To address the effects of hydrostatic stress on lung vascular permeability and identify underlying signalling pathways, we measured K_f in the isolated perfused rat lung model. Elevation of LAP increased K_f, but this effect was attenuated by both Gd^{3+}, which blocks the endothelial Ca^{2+} influx in response to mechanical

stimulation,[4] or the myosin light chain kinase (MLCK) inhibitor ML-7. These findings suggest that hydrostatic stress increases lung vascular permeability via a Ca^{2+} dependent, MLCK-mediated mechanism. Based on the notion that endothelial cells generate NO upon mechanical stimulation,[3] we next tested the role of NO in the regulation of lung vascular permeability. The inhibition of NO synthesis by *N*ω-nitro-L-arginine methyl ester (L-NAME) attenuated the pressure-induced K_f increase. By contrast, the exogenous NO donor *S*-nitrosoglutathione (GSNO) amplified the K_f increase, indicating a barrier-protective effect of NO in hydrostatic stress.

➢ ***Regulation of endothelial $[Ca^{2+}]_i$ and K_f response by NO*** Since NO attenuated the pressure-induced K_f increase, and based on the notion that this K_f increase is dependent on endothelial $[Ca^{2+}]_i$ signalling, we hypothesized NO could modulate the endothelial $[Ca^{2+}]_i$ response to hydrostatic stress. During LAP elevation over 30 min, we detected a progressive increase in endothelial $[Ca^{2+}]_i$ that was significantly reduced by GSNO, but enhanced by L-NAME. Likewise, either the soluble guanlylate cyclase (sGC) stimulator Bay 41-2272 or the cell-permeable cGMP analog 8Br-cGMP markedly abrogated the pressure-evoked $[Ca^{2+}]_i$ response, which on the other hand was intensified by pretreatment with the sGC inhibitor [1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-l-one] (ODQ). These findings indicate that NO attenuates the endothelial $[Ca^{2+}]_i$ response to hydrostatic stress via activation of its downstream target sGC and subsequent formation of cGMP. In line with this notion, subsequent measurements of K_f and lung wet/dry weight ratio showed that cGMP analogues also reduced lung vascular permeability and edema formation in hydrostatic stress. Whereas ODQ amplified the K_f increase, nearly the same extent of attenuation as Gd^{3+} could be found in the presence of Bay 41-2272 or 8Br-cGMP, indicating that NO reduced lung vascular permeability via a cGMP-mediated attenuation of the endothelial $[Ca^{2+}]_i$ response to hydrostatic stress.

Consistent with the well-documented regulation of endothelial NO synthase by Ca^{2+}, we observed that the increase in fluorescence intensity of the NO-sensitive dye DAF-FM at hydrostatic stress was totally absent when lungs were perfused with Ca^{2+}-free buffer or with Gd^{3+}. Similarly, pressure-induced NO formation was largely attenuated in the presence of either Bay 41-2272 or 8Br-cGMP, demonstrating that NO production is negatively regulated by its downstream product cGMP. Hence, the identified NO-cGMP mediated regulation of endothelial $[Ca^{2+}]_i$ constitutes a negative-feedback loop which serves to protect the lung from barrier deterioration and excessive edema formation in hydrostatic stress.

Endothelial mechanosensing by TRPV4 To better understand the cellular mechanisms underlying this signalling cascade, we aimed to identify the mechano-sensitive ion channel which mediates the pressure-induced endothelial $[Ca^{2+}]_i$ increase. Due to its sensitivity to both shear stress and stretch [5] and its described ability of induce lung edema upon activation,[2, 15] TRPV4 channel was considered as a putative candidate. Consistent with findings by Alvarez et al.,[2] we identified TRPV4 expression in both lung homogenate and fresh lung vascular endothelial cells by Western Blot and immunohistochemistry analyses (Fig.1). The TRPV4 inhibitor ruthenium red (RuR) (1 µmol/L) blocked the endothelial $[Ca^{2+}]_i$ response and attenuated K_f increase following LAP elevation. On the contrary, specific TRPV4 activator 4α-Phorbol 12-13-dicaprinate (4αPDD) induced a marked $[Ca^{2+}]_i$ increase at baseline LAP.

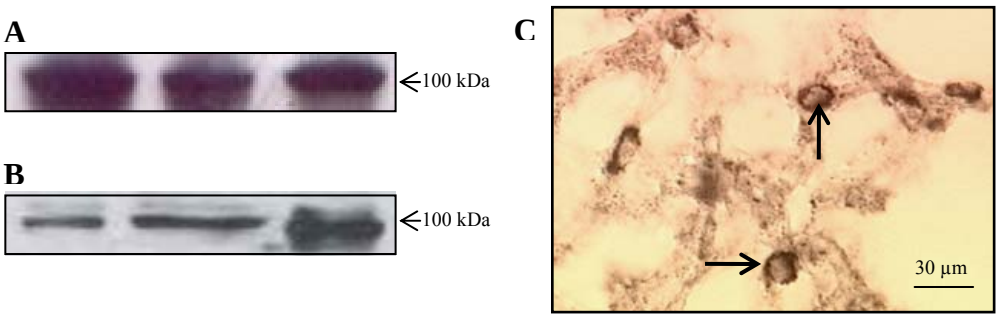

Figure 1: TRPV4 expressions in lung microvascular endothelial cells. Representative images are from western blot in whole lung homogenate (A), fresh isolated lung vascular endothelial cells (B) and TRPV4 immunostaining of rat lungs (C). Arrows indicate TRPV4 positive endothelial cells in lung septal capillaries. Replicated in n=3 each.

Notably, LAP elevation after TRPV4 stimulation by 4αPDD did not elicit a further increase in K_f, indicating that the responsible mechanosensitive ion channel had already been stimulated by the TRPV4 activator in this scenario. We solidified the notion of a critical role of TRPV4 as mechanosensor in hydrostatic lung edema in a genetic loss-of-function model using TRPV4 gene-targeted mice.[16] Endothelial $[Ca^{2+}]_i$ increase (Fig.2) and NO production in response to hydrostatic stress were completely absent in TRPV4 deficient mice ($TRPV4^{-/-}$) in contrast with wild-type littermates ($TRPV^{+/+}$), verifying the functional relevance of TRPV4 in lung vascular mechanotransduction and subsequent lung edema formation. Consistent with this view, we found lung edema was drastically reduced in $TRPV4^{-/-}$ mice at elevated hydrostatic stress as compared with wild type.

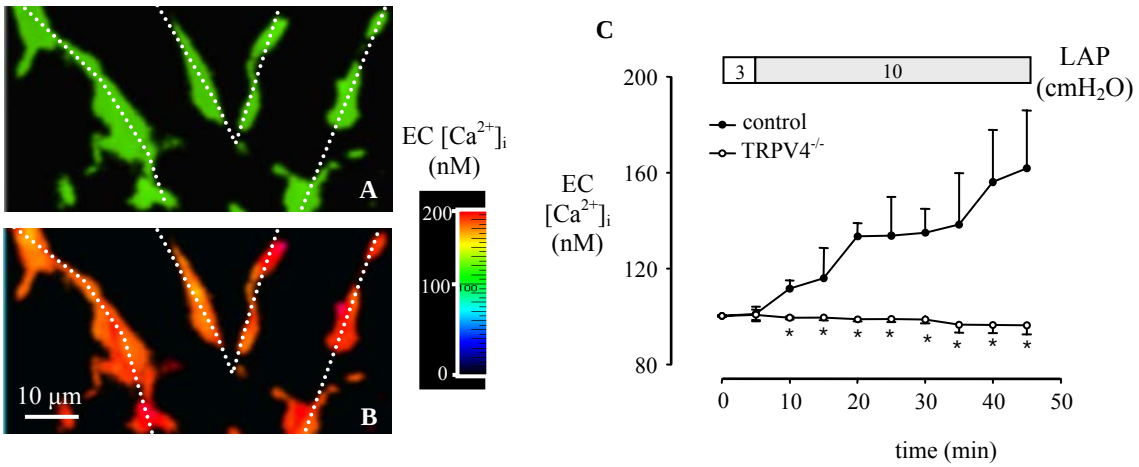

Figure 2: Endothelial $[Ca^{2+}]_i$ increase in response to elevated pressure is absent in TRPV4$^{-/-}$ mice. Representative images are from fura-2 loaded lung microvascular endothelial cells of wild type mice, pseudo color coded for endothelial $[Ca^{2+}]_i$ at baseline (3 cmH_2O) (A) and elevated pressure (10 cmH_2O) (B). Vessel margins are depicted by white lines. (C) Temporal profile of endothelial $[Ca^{2+}]_i$ response to pressure in wild type TRPV4$^{+/+}$ (filled circle) and TRPV4$^{-/-}$ (open circle) mouse lungs. Data are mean± SEM, n=3 each.

Next, we investigated whether the attenuation of the pressure-induced $[Ca^{2+}]_i$ response by cGMP was caused by reduced Ca^{2+} influx or enhanced Ca^{2+} uptake into endosomal stores. The endoplasmic reticulum (ER) Ca^{2+} level, analyzed by fluorescence imaging of fura-2FF, did not differ between control lungs and lungs treated with a cGMP analogue at either baseline or elevated pressure, thus ruling out a critical role of endosomal Ca^{2+} uptake in the cGMP-dependent regulatory pathway. The notion that cGMP inhibits endothelial Ca^{2+} influx via TRPV4 was further substantiated by whole-cell patch-clamp recordings in pulmonary microvascular endothelial cells. TRPV4 activation induced an inwardly rectifying current that reversed at +20 mV and could be blocked by pretreatment with 8Br-cGMP. In analogy, fluorescence imaging of fura-2 loaded endothelial cells in isolated rat lungs revealed a marked endothelial Ca^{2+} response to 4αPDD that was completely blocked by 8Br-cGMP.

➢ ***PDE5 inhibition attenuates lung edema in acute hydrostatic stress*** We then extended our study to apply the notion of a cGMP-regulated lung barrier function in a pre-clinical model of hydrostatic lung edema. First, we identified expression of PDE5, which rapidly hydrolyzes cGMP to GMP, in pulmonary vascular endothelial cells by Western blot analysis and immunohistochemistry. Second, we showed in isolated rat lungs that the PDE5 inhibitor sildenafil (0.4 µmol/L) markedly attenuated both the increases in endothelial $[Ca^{2+}]_i$ and K_f following LAP elevation. Third, in an *in vivo* model of acute hydrostatic lung edema following myocardial infarction in the rat, we could demonstrate that lung vascular

permeability and lung edema were significantly attenuated by an intravenous bolus injection of sildenafil. Collectively, these findings identify PDE5 inhibition as a promising therapeutic strategy for the treatment and/or prevention of hydrostatic lung edema in the clinical situation.

➢ ***NO inhalation attenuates lung edema in chronic hydrostatic stress*** Based on the notion that activation of the NO/cGMP signaling pathway in lung endothelial cells confers protection from acute hydrostatic lung edema, we next tested the hypothesis whether this regulatory pathway may similarly present a therapeutic strategy in lung edema following chronic hydrostatic stress. We therefore assessed the effects of inhalation of NO in a rat model of CHF induced by supracoronary aortic banding. CHF rats exhibited endothelial dysfunction characterized by a lack of endogenous NO synthesis [17] and moderate lung edema.[7, 8] Exogenous supplementation of NO by inhalation significantly reduced lung edema, which is in line with the notion that the NO/cGMP pathway has a barrier-protective, anti-edematous effect in lung hydrostatic stress. Importantly, the barrier-protective effects of endothelial second messenger signaling via cyclic nucleotides may expand to inflammatory types of lung edema and involve cAMP as well as cGMP signaling, as indicated by our recent finding that inhalation of the PDE3 inhibitor milrinone attenuates lung injury and barrier failure in a cAMP-dependent fashion in an experimental model of acute lung injury induced by oleic acid infusion.[18]

Most of the presented results above have been published in several peer-reviewed international Journals.[8, 9]

➢ ***Long term inhibition of PDE5 attenuates lung edema in chronic hydrostatic stress*** Recently, we explored the effects of endothelial cGMP elevation by long term oral application of sildenafil over a period of 8 weeks in the CHF rat model of chronic hydrostatic stress. As demonstrated in Fig. 3, lung vascular permeability at elevated hydrostatic stress was markedly reduced in rats treated with sildenafil for 8 weeks. These data are in line with the finding that sildenafil treatment also attenuated lung edema in this model. In conjunction with the fact that lung vascular remodeling and pulmonary hypertension were similarly reduced by sildenafil (data not shown), these findings suggest PDE5 inhibition as a new therapeutic strategy in lung edema and pulmonary hypertension following chronic heart failure (manuscript in preparation).

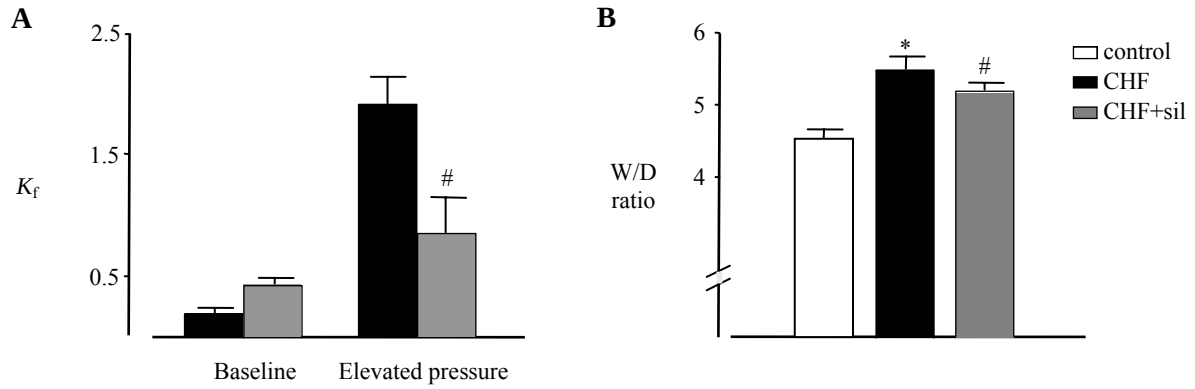

Figure 3: Chronic administration of the PDE5 inhibitor sildenafil (60 mg/kg BW/day) over 8 weeks attenuated lung vascular filtration coefficient (K_f, ml * min^{-1} * cm H_2O^{-1} * 100 g^{-1}) and hydrostatic edema in rats with congestive heart failure (CHF). (A) K_f was determined in isolated perfused rat lungs from vehicle (dark bar) and sildenafil (grey bar) treated CHF rats at baseline pressure (5 cmH_2O) and elevated pressure (15 cmH_2O), respectively. (B) Group data of wet/dry lung weight ratio of controls, vehicle (dark bar) and sildenafil (grey bar) treated CHF rats. Data are mean±SEM, n=5 each, * $p<0.05$ vs. control, # $p<0.05$ vs. CHF.

DISCUSSION

In the present study, we identified a novel signalling cascade within endothelial cells that regulates lung barrier function and edema formation at hydrostatic stress. Mechanosensitive TRPV4 channels mediate pressure-evoked Ca^{2+} influx that increases vascular permeability, yet simultaneously, activates a negative-feedback loop that attenuates the endothelial $[Ca^{2+}]_i$ response, and thus protects vascular barrier function and regulates lung edema. This endothelial intrinsic feedback loop comprises NO and cGMP synthesis upon pressure-induced Ca^{2+} influx, which reversely inhibits Ca^{2+} entry via TRPV4. We have tested our finding that NO/cGMP dependent negative feedback loop regulates lung vascular permeability in both acute and chronic animal models of lung hydrostatic stress, and demonstrated that lung edema could be attenuated by either inhalation of exogenous NO or pharmacological inhibition of cGMP hydrolysis by the PDE5 inhibitor sildenafil.

➢ ***Methodological considerations*** Details and limitations of the applied animal models and imaging techniques have previously been discussed.[10, 19] K_f measurement yields a robust assessment of lung microvascular barrier properties, provided that an isogravimetric baseline, an appropriately small pressure increment and defined time constants are considered to eliminate potential influences of vascular compliance, epithelial barrier properties and alveolar fluid clearance in the microgravimetric measurements. The detected differences in K_f between groups were not attributable to differences in hemodynamic parameters at the time of measurement, but rather reflect changes in barrier properties, since vasoactive effects of the

applied drugs and agents are negligible in the isolated perfused lung preparation which lacks a myogenic response and is fully dilated independent of NO under resting conditions. [20]

- ***NO/cGMP dependent negative feedback loop*** Previous studies have yielded conflicting data on the effects of NO on vascular permeability, presumably due to the fact that the effects of NO depend on the specific experimental conditions, the vascular bed studied, as well as the abundant availability of NO versus NO scavengers.[21] In the present study, we demonstrate that in the lung, where the microvascular barrier has a markedly higher permeability as compared with the systemic vasculature,[22] both endogenous and exogenous NO inhibited the increase in lung vascular permeability during hydrostatic stress, while inhibition of endogenous NO synthesis by L-NAME increased fluid extravasation. This regulatory effect is based on the inhibition of mechanical Ca^{2+} influx rather than stimulation of endosomal stores, and is interpreted as a negative feedback loop in that endothelial NO/cGMP are generated upon Ca^{2+} entry, but reversely inhibit Ca^{2+} influx by regulation of TRPV4. This concept was further verified by the fact that sGC stimulator and 8Br-cGMP both attenuated endothelial NO formation.
- ***Role of TRPV4 in the regulation of lung vascular permeability*** Compelling evidence provided by us and others[2] demonstrated that TRPV4 channels are expressed in pulmonary microvascular endothelial cells and, upon activation, increase lung vascular permeability in a Ca^{2+}-dependent mode. Notably, hydrostatic stress failed to elicit an additional K_f increase in response to LAP elevation in lungs pretreated with the TRPV4 activator 4αPDD, indicating that both pressure and 4αPDD increase K_f via a common pathway. The present study identified a critical regulation of TRPV4 by cGMP by use of patch-clamp recordings in pulmonary microvascular endothelial cells and by real-time in situ fluorescence microscopy in isolated lung. Although cGMP has been described to regulate transient receptor potential canonical channel isoform 3 (TRPC3),[23] our findings cast new insights into the regulation of the vanilloid subfamily of TRP channels. Yet the molecular mechanisms involved in the activation of TRPV4 by hydrostatic stress and the inhibitory regulation of this channel by cGMP are still obscure, and remain to be elucidated in subsequent studies.
- ***cGMP-dependent therapeutic strategies in acute and chronic hydrostatic lung edema*** The identification of an NO/cGMP dependent negative feedback loop in the regulation of lung vascular permeability may give rise to new interventions for the prevention or treatment of hydrostatic and potentially also other forms of lung edema. In a model of acute myocardial infarction, hydrostatic lung edema was evident in parallel with prominent vascular leakage. Bolus injection of the PDE5 inhibitor sildenafil reconstituted vascular barrier function in the

lung and attenuated lung edema, which was primarily attributable to a reduced vascular permeability and attenuated endothelial $[Ca^{2+}]_i$ response to hydrostatic stress as shown in the isolated rat lung model. In rats with chronic heart failure, we tested the therapeutic potential of a long term oral application of sildenafil to treat hydrostatic lung edema. Potential inotropic effects of sildenafil were excluded by deliberately choosing a heart failure model caused by mechanical banding of the ascendant aorta, in which the resultant increase in left atrial pressure is largely independent of left ventricular contractility. In another series of experiments we could demonstrate a reduction of hydrostatic lung edema in chronic heart failure by inhalation of NO. However, it should be noted that due to the complexity of this pathological condition, the effects of NO cannot be attributed unequivocally to vascular barrier regulation alone, but may equally involve effects on pulmonary hemodynamics and alveolar fluid clearance. While the current development and preclinical testing of specific TRPV4 inhibitors may provide new pharmacological strategies for the treatment of hydrostatic lung edema, the identification of the lung endothelial NO/cGMP feedback loop provides an alternative and/or additional therapeutic strategy for this critical disease.

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Curriculum Vitae

CV is not available online for private reason.

Publication list

- **J. Yin**, J. Hoffmann, S.M. Kaestle, N. Neye, L. Wang, J. Baeurle, S. Wu, H. Kuppe, A. R. Pries, W. M. Kuebler
  Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of TRPV4.
  Circulation Research 2008 Apr 25; 102(8):966-74

- M. Bueltmann, X. Kong, M. Mertens, N. Yin, **J. Yin**, Z. Liu, A. Koster, H. Kuppe, W. M. Kuebler
  Inhaled milrinone attenuates experimental acute lung injury
  Intensive Care Medicine 2009 Jan;35(1):171-8.

- N.Yin, S.M.Kaestle, **J. Yin**, A.R.Pries, H.Kuppe, W.M.Kuebler
  Inhaled NO versus aerosolized iloprost for the treatment of pulmonary hypertension with left heart disease
  Critical Care Medicine 2009 Mar;37(3):980-6

Anteilserklärung

Re: Publications Contributions

Datum: 16.03.2009

To whom it may concern,

By signing this letter, it is confirmed that:

1. **J. Yin**, J. Hoffmann, S.M. Kaestle, N. Neye, L. Wang, J. Baeurle, S. Wu, H. Kuppe, A. R. Pries, W. M. Kuebler
   Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of TRPV4.
   Circulation Research 2008 Apr 25; 102(8):966-74
   Jun Yin performed 85% of all experiments alone (as real time fluorescence imaging, lung microvascular filtration coefficient measurement, lung immunohistology analysis, Evans blue extravasation measurement, lung wet/dry ratio measurement). He acquired and analyzed the data, and drafted text and figures of the manuscript.

2. M. Bueltmann, X. Kong, M. Mertens, N. Yin, **J. Yin**, Z. Liu, A. Koster, H. Kuppe, W. M. Kuebler
   Inhaled milrinone attenuates experimental acute lung injury
   Intensive Care Medicine 2009 Jan;35(1):171-8.
   Jun Yin contributed 20% of all work for this paper (hemodynamic measurement). He acquired and analyzed part of the data, drafted part of text of the manuscript.

3. N. Yin, S. M. Kaestle, **J. Yin**, A. R. Pries, H. Kuppe, W. M. Kuebler
   Inhaled NO versus aerosolized iloprost for the treatment of pulmonary hypertension with left heart disease
   Critical Care Medicine 2009 Mar;37(3):980-6.
   Jun Yin contributed 30% of all work for this paper (as induction of congestive heart failure animal model, plasma cAMP and cGMP measurement). He acquired and analyzed part of the data, and drafted part of the text and figures of the manuscript.

Jun Yin

Prof. Dr. med. Axel R. Pries

Erklärung

"Ich, YIN, Jun, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: "Functional relevance and regulation of Transient Receptor Potential Vanilloid 4 channel in hydrostatic lung edema" selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe."

Datum Unterschrift

16. MRZ.2009