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DISSERTATION

Hypoxic pulmonary vasoconstriction in intact mouse lungs: new insights into cellular mechanisms and therapeutic targets

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German abstract

Die hypoxische pulmonale Vasokonstriktion (HPV) ist eine physiologische Reaktion auf alveoläre Hypoxie, die den pulmonalen Blutfluss in alveoläre Bereiche mit hohen Sauerstoffpartialdruck lenkt, und dadurch während einer lokalen alveolären Hypoxie das Ventilations-/Perfusions-Verhältnis optimiert. Obwohl das Prinzip der HPV bereits vor Jahrzehnten erkannt wurde, sind die zugrundeliegenden genauen Mechanismen noch immer unbekannt. Weder der Prozess der Sauerstoffrezeption, noch der genaue Signalweg, welcher der HPV zugrunde liegt, sind vollständig entschlüsselt.

Hier stellen wir die Hypothese auf, dass die HPV ihren Ursprung auf der alveolokapillären Ebene nimmt, von wo aus das hypoxische Signal in einer Connexin 40 (Cx40)-abhängigen Weise in Form einer endothelialen Membrandepolarisation zu vorgeschalteten Arteriolen weitergeleitet wird. In einer Reihe von *in situ* und *in vivo* Experimenten konnten wir zeigen, dass durch Hemmung oder Defizienz von Cx40 die HPV deutlich abgeschwächt wird. Durch Real-time Fluoreszenz-Bildgebung konnten wir zudem zeigen, dass Hypoxie in $Cx40^{+/+}$ Mäusen zu einer endothelialen Membrandepolarisation in den alveolären Kapillaren führt, die zu den vorgelagerten Arteriolen weitergeleitet wird; in $Cx40^{-/-}$ Mäusen konnte eine solche Weiterleitung hingegen nicht beobachtet werden. An der anschließenden Umwandlung der endothelialen Depolarisation in eine Vasokonstriktion sind endotheliale spannungsabhängige α_{1G} Ca^{2+}-Kanäle, die zytosolische Phospholipase A_2 und Epoxyeicosatriensäuren beteiligt.

In weiteren Studien konnten wir eine zuvor unbekannte Rolle für den cystic fibrosis transmembrane conductance regulator (CFTR) und den transient receptor potential Vanilloid 4 (TRPV4) Kationen-Kanal in der HPV zeigen. Die HPV wurde durch die Hemmung oder den Mangel jeweils beider Kanäle blockiert. Darüber hinaus konnten wir auch die transient receptor potential channel 6 (TRPC6) Inhibitoren 2910-0498 und 8009-5364 sowie das vasoactive intestinal peptide (VIP) Analog RO251553 als neue Inhibitoren in der HPV in der Mauslunge identifizieren.

Zusammenfassend werden in dieser Arbeit Cx40, CFTR und TRP-Kanäle als neue mechanistische Komponenten und therapeutische Ziele in den zellulären Signalwegen der HPV beschrieben.

English Abstract

Hypoxic pulmonary vasoconstriction (HPV) is a physiological response to alveolar hypoxia which distributes pulmonary blood flow to alveolar areas of high oxygen partial pressure, thereby optimizing the ventilation/perfusion ratio during localized alveolar hypoxia. Although the principle of HPV was recognized decades ago, its exact pathway still remains elusive. Neither the oxygen sensing process nor the exact pathways underlying HPV are fully deciphered yet.

Here, we speculated that HPV originates at the alveolo-capillary level, from where the hypoxic signal is propagated as endothelial membrane depolarization in a connexin 40 (Cx40)-dependent manner to upstream arterioles. In a series of experiments we showed HPV to be largely attenuated by Cx40 inhibition or deficiency *in situ* and *in vivo.* Real-time fluorescence imaging revealed that hypoxia causes endothelial membrane depolarization in alveolar capillaries that propagates to upstream arterioles in $Cx40^{+/+}$ but not in $Cx40^{-/-}$ mice. The subsequent transformation of the endothelial depolarization into a vasoconstriction involves endothelial voltage-dependent α_{1G} subtype Ca^{2+} channels, cytosolic phospholipase A_2 and epoxyeicosatrienoic acids.

In further studies we identified previously unrecognized roles for cystic fibrosis transmembrane conductance regulator (CFTR) and the transient receptor potential vanilloid 4 (TRPV4) cation channel in HPV, as HPV was largely blocked by inhibition or deficiency of either of the two channels, respectively. We further report the newly identified canonical transit receptor potential channel 6 (TRPC6) inhibitors 2910-0498 and 8009-5364 and vasoactive intestinal peptide (VIP) analog RO251553 as novel blockers of HPV in isolated mouse lungs.

In summary, our studies identify Cx40, CFTR, and TRP channels as novel mechanistic components and therapeutic targets in the cellular signaling pathway that mediates HPV.

Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a fundamental physiological response of the pulmonary vasculature, which optimizes lung ventilation/perfusion (V/Q) matching by redirecting blood flow from poorly to better ventilated areas[1]. Despite extensive physiological and cell biological studies since the original landmark discovery of HPV by Ulf von Euler and Göran Liljestrand, the underlying oxygen sensing and signal transduction processes remain a topic of intense research and controversy. Current concepts of HPV are largely based on the notion that pulmonary arterial smooth muscle cells (PASMCs) constitute both the sensor and transducer of the hypoxic signal as well as its contractile effector[1]. However, from a conceptual point, the ideal site for an oxygen sensor in HPV should be located in the actual area of pulmonary gas exchange, i.e. in the alveolar capillaries[2], while the presence of vascular smooth muscle cells and a detectable HPV response is confined to pulmonary arterioles. These considerations suggest a spatial dissociation between oxygen sensor and effector in HPV, which would necessitate the existence of a retrograde signaling mechanism from the alveolar gas exchange unit to medium and large PASMC-bearing arterioles and arteries. In the systemic circulation, intercellular gap junctions composed of connexins (Cxs) mediate such a conducted response. Among the major connexins expressed in the vascular bed, Cx40 is of particular interest as it is exclusively expressed in endothelial cells, the only vascular cell type present at the alveolar gas exchange unit. Cx40 has been shown to mediate endothelium-dependent conducted responses in the systemic vasculature[3]. The major subject of my dissertation was to test the hypothesis that Cx40 is required for the propagation of a conducted response in the pulmonary microvasculature that mediates HPV in the intact lung.

Ca^{2+} entry is a critical step in HPV, and the transient receptor potential (TRP) channel family has recently been implicated to mediate Ca^{2+} entry in response to hypoxia in pulmonary artery smooth muscle cells. In particular, canonical TRP channel 6 (TRPC6) has been demonstrated to be essential for the acute HPV response[4]. However, no selective TRPC6 blockers are available so far. From a large library of drug-like compounds, the group of Prof. Michael Schaefer (Rudolf-Boehm-Institute for Pharmacology and Toxicology, University of Leipzig) recently identified compounds 2910-0498 and 8009-5364 as selective

blockers of TRPC6 channels. In collaboration with Prof. Schaefer, we tested these novel inhibitors for their potential to block HPV in the isolated mouse lung, and found they could attenuate HPV response largely. Another member of the TRP family, the transient receptor potential vanilloid 4 (TRPV4), which is expressed in both, pulmonary vascular endothelial and smooth muscle cells, has been characterized as a polymodal Ca^{2+} channel with mechanosensitive properties. Our group therefore studied a potential direct contribution of TRPV4 to stretch-induced, Ca^{2+}-dependent changes in endothelial permeability in hydrostatic lung edema, and I contributed lung edema measurements in isolated perfused mouse lungs to these studies. More recently, and in line with a proposed role for TRPV4 in chronic hypoxic pulmonary hypertension[5], I started to analyze the role of TRPV4 in HPV in isolated mouse lungs.

Furthermore, we tested for a functional role of cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel and member of the adenine nucleotide-binding cassette (ABC) transporter family, in HPV. Mutations in the CFTR gene cause cystic fibrosis (CF), the most frequent genetic disease in the Caucasian population. As CF patients suffer from V/Q mismatching[6], we tested whether CFTR may play a functional role in HPV, and analyzed underlying mechanisms.

Lastly, we tested for the ability of the stable vasoactive intestinal peptide (VIP) analog RO251553 to antagonize pulmonary vasoconstriction in response by hypoxia. VIP analogs have been proposed as a promising therapy in chronic obstructive pulmonary disease (COPD) based on their airway dilatory and anti-inflammatory potential. In addition, we tested for a potential vasodilatory effect of RO251553, which may provide additional benefit in patients with COPD and concomitant pulmonary hypertension.

Material and Methods

- ***Animals*** Experiments were performed in male C57BL/6 mice, in mice deficient in Cx40(*Cx40*$^{-/-}$), the α_{1G} subtype T type Ca^{2+} channel (*Cacna1g*$^{-/-}$), TRPV4 (*Trpv4*$^{-/-}$) or CFTR (*CFTR*^{tm1HGU}) and their corresponding wild type littermates.

- ***Isolated perfused mouse lung model*** Isolated perfused mouse lungs were prepared as previously described[7]. Lungs were perfused by Hanks' Balanced Salt Solution containing 5% bovine serum albumin and 5% dextran (pH 7.35 –7.45.). Pulmonary arterial pressure (PAP) and left atrial pressure (LAP) were measured continuously. To assess the response to hypoxia, inspiratory oxygen fraction (FIO_2) was switched from 0.21 to 0.01, and changes in PAP were recorded and intrinsic pulmonary vascular resistance calculated from four point pressure-flow curves as previously described[7].
- ***Oxygenation during regional and global hypoxia in vivo*** In anesthetized mice, ventilation-perfusion mismatch was induced by intratracheal instillation of 25 µl saline causing partial occlusion of the larger airways as previously described[4]. To address the effects of global hypoxia *in vivo*, arterial oxygenation in anesthetized and ventilated mice was monitored continuously while FIO_2 was decreased stepwise from 0.21 in 0.01 increments.
- ***Ventilation-perfusion matching in vivo*** Regional lung perfusion was quantified by the fluorescent microsphere technique in mice subjected to either two- or one-lung ventilation[8].
- ***Real-time fluorescence imaging*** Pulmonary endothelial membrane potential (E_m) and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined by real-time *in situ* fluorescence microscopy as previously reported[9,10].
- ***cPLA$_2$ translocation*** Hypoxia-induced translocation of cytosolic phospholipase A_2 ($cPLA_2$) in endothelial cells of the isolated perfused mouse lung and in cultured human pulmonary artery endothelial cells (PAECs) was assessed by use of an indirect immunofluorescence assay as described[11,12].
- ***EETs concentration in isolated perfused lungs*** The concentrations of 8,9-EET, 11,12-EET, and 14,15-EET were determined as described[13] in isolated mouse lungs that had been ventilated with normoxic (21% O_2) or hypoxic (1% O_2) gas for 10 min.

- ***Western Blot analyses*** For $K_v1.5$, $K_v2.1$, Cx43, or Ser-368 phospho-Cx43, Western blot analyses were performed from whole lung homogenate, freshly isolated lung endothelial cells, or cultured pulmonary artery endothelial cells.
- ***Immunofluorescence histology*** In mouse lungs, the expressions of Cx40, Cx43 and von Willebrand factor (vWF), which served as endothelial marker, were imaged by immunofluorescence confocal microscopy.
- ***Statistical analyses*** Due to the cumulative nature of this Dissertation, results are reported in prose style rather than as data values and figure graphs. Accordingly, no statistical analyses are given here, but are reported in detail in the attached manuscripts. All statistical analyses were performed by use of SigmaStat software. Data were presented as means±SEMs, and statistical significance was determined by Student *t*-test or one-way ANOVA as appropriate. Non-linear regression analyses were performed by use of SigmaPlot software. Statistical significance was accepted at $P<0.05$.

Results and Discussion

Cx40 expression in mouse lungs is confined to the vascular endothelium By double fluorescence immunohistology, we determined the distribution of Cx40 in lungs of *Cx40*$^{+/+}$ mice. Cx40 expression was evident in pulmonary arteries and capillaries where it colocalized with vWF staining attesting to its endothelial specific expression. No Cx40 staining was detectable in lungs of *Cx40*$^{-/-}$ mice, or in extravascular lung parenchyma of *Cx40*$^{+/+}$ mice (Fig. 1 in the attached manuscript by Wang *et al*., *J Clin Invest* 2012).

Cx40 is critical for HPV in intact lungs , Hypoxic ventilation (1%O_2) increased PAP markedly in isolated perfused lungs of *Cx40*$^{+/+}$ mice, yet this response was substantially reduced in *Cx40*$^{-/-}$ lungs. A similar attenuation of HPV was detected in *Cx40*$^{+/+}$ lungs perfused with the nonspecific gap junction inhibitor 18α-glycyrrhetinic acid or the Cx40 specific inhibitory mimetic peptide gap27^{40}. Neither Cx40 deficiency nor pharmacological inhibition of gap junctions by 18α-glycyrrhetinic acid or gap27^{40} blocked pulmonary vasoconstriction in response to angiotensin II demonstrating that Cx40 is not required for pulmonary vasoconstriction *per se* (Fig. 2 in Wang *et al*.).

We considered the possibility that connexin 43 (Cx43) may be downregulated or inactivated in endothelial cells of *Cx40*$^{-/-}$ mice. By double fluorescence immunohistology and western blot, neither Cx43 expression nor Ser-368 phosphorylation of Cx43 were found to differ between lungs of *Cx40*$^{+/+}$ and *Cx40*$^{-/-}$ mice. Notably, inhibition of Cx43 by gap27^{43} also attenuated the HPV response, albeit without reaching statistical significance. Importantly, combination of both, gap27^{40} and gap27^{43} blocked HPV to a significantly greater extent than each mimetic peptide alone (Fig. 3 in Wang *et al*.). While these data do not rule out a potential role of Cx43 in HPV, they clearly demonstrate that the role of Cx40 described herein does not relate to secondary changes in Cx43-mediated gap junctional communication.

To test the relevance of Cx40 for pulmonary V/Q matching *in vivo*, we induced V/Q mismatches in anesthetized mice by tracheal instillation of saline droplets resulting in regional occlusion of alveolar ventilation. While *Cx40*$^{+/+}$ responded with only a modest decline in arterial partial pressure of O_2 (PaO_2), the same maneuver resulted in considerable hypoxemia in *Cx40*$^{-/-}$ mice. Stepwise reductions in the inspiratory FIO_2 from

0.21 to 0.19 were well tolerated in *Cx40*$^{+/+}$, but resulted in a dramatic drop in SaO_2 in *Cx40*$^{-/-}$ mice. To directly demonstrate the inability of *Cx40*$^{-/-}$ mice to redistribute blood flow from hypoxic to normoxic lung areas, we quantified regional lung perfusion by the fluorescent microsphere technique in mice subjected to one-lung ventilation. In line with an intact HPV response, blood flow to the ventilated lung was approximately twice as high as perfusion of the non-ventilated lung in *Cx40*$^{+/+}$ mice. Yet in *Cx40*$^{-/-}$ mice, the perfusion difference was significantly reduced, demonstrating V/Q mismatching (Fig. 4 in Wang *et al*.).

Cx40 propagates hypoxia-induced endothelial membrane depolarization In the systemic circulation, conducted responses are realized by the rapid propagation of membrane potential (E_m) changes via intercellular gap junctions[3]. To test whether Cx40 may mediate a similar signal propagation in HPV, we monitored the effects of hypoxia on endothelial E_m in isolated lungs of *Cx40*$^{+/+}$ and *Cx40*$^{-/-}$ mice. A switch from normoxic to hypoxic ventilation caused endothelial depolarization both, in alveolar capillaries and upstream arterioles of *Cx40*$^{+/+}$ lungs. In isolated lungs of *Cx40*$^{-/-}$ mice, hypoxia caused endothelial depolarization only in alveolar capillaries, while it was largely abrogated in upstream arterioles. These findings identify a critical role for Cx40 in the propagation of the hypoxia-induced endothelial depolarization from the level of the alveolar capillaries to the upstream arterioles.

As voltage-gated K^+ (K_v) channels, in particular $K_v1.5$ and $K_v2.1$, are considered critical in both, hypoxia sensing and HPV[14], we next demonstrated their expression in fresh lung endothelial cells by western blot analysis. Inhibition of K_v channels by 4-aminopyridine caused endothelial membrane depolarizations in pulmonary microvessels of both *Cx40*$^{+/+}$ and *Cx40*$^{-/-}$ mice to a similar extent as did hypoxia in pulmonary arterioles of *Cx40*$^{+/+}$ mice, thus attesting to the potential involvement of these channels in the hypoxic response of lung microvascular endothelial cells (Fig. 6 in Wang *et al*.).

Endothelial $[Ca^{2+}]_i$ signaling is required for hypoxic pulmonary vasoconstriction As lung microvascular endothelial cells express voltage-dependent Ca^{2+} channels (VDCCs) of the α_{1G} subtype T-type, we considered that membrane depolarization may trigger endothelial $[Ca^{2+}]_i$ signaling, a notion that we tested by real-time imaging of the endothelial

$[Ca^{2+}]_i$ response to hypoxia. In lungs from *Cx40*$^{+/+}$ mice, hypoxia induced a distinct increase in endothelial $[Ca^{2+}]_i$ in both lung capillaries and arterioles. In lungs from *Cx40*$^{-/-}$ mice, this endothelial $[Ca^{2+}]_i$ response to hypoxia was conserved in alveolar capillaries, but largely absent in corresponding upstream arterioles. Inhibition of the endothelial $[Ca^{2+}]_i$ response to hypoxia in both lung capillaries and arterioles of *Cx40*$^{+/+}$ mice by the VDCC blocker mibefradil identified VDCCs as functional link between membrane depolarization and endothelial $[Ca^{2+}]_i$ signaling. This notion was substantiated by the subsequent finding that the endothelial $[Ca^{2+}]_i$ increase to hypoxia in both, capillaries and arterioles is lost in lungs of α_{1G}-deficient (*Cacna1g*$^{-/-}$) mice, while the depolarization response is unaffected. Chelation of endothelial Ca^{2+} blocked HPV in lungs of *Cx40*$^{+/+}$mice, yet did not attenuate endothelial depolarization in response to hypoxia, substantiating the notion that in the endothelial response to hypoxia, E_m changes occur upstream of the $[Ca^{2+}]_i$ increase (Fig. 7 in Wang *et al*.).

cPLA$_2$ regulates hypoxic pulmonary vasoconstriction Recent studies suggest a functional relevance for the arachidonic acid (AA) metabolites epoxyeicosatrienoic acids (EETs) in HPV[13]. As the generation of AA metabolites in endothelial cells is mediated via the Ca^{2+}-dependent activation of cPLA$_2$[15], we applied indirect immunofluorescence imaging to test whether cPLA$_2$ translocates in response to hypoxia in endothelial cells of the isolated perfused lung and in cultured PAECs. While translocated cPLA$_2$ was hardly detectable under normoxic conditions, hypoxia induced a marked and rapid translocation of cPLA$_2$ in endothelial cells of intact mouse lungs and in PAECs, respectively. HPV was largely blocked by pretreatment of isolated lungs from *Cx40*$^{+/+}$ mice with a specific inhibitor of cPLA$_2$, yet not in lungs treated with an inhibitor of the Ca^{2+}-independent phospholipase A$_2$, thus highlighting the functional relevance of cPLA$_2$ activation in HPV. We next quantified the levels of 8,9-EET, 11,12-EET and 14,15-EET in isolated lungs perfused at either normoxia and hypoxia. In line with their proposed role as mediators of HPV[13], pulmonary levels of 11,12-EET and 14,15-EET increased during hypoxia in *Cx40*$^{+/+}$ mice, while the concentration of 8,9-EET decreased. In contrast in *Cx40*$^{-/-}$ mice, no hypoxia-induced stimulation of 11,12-EET and 14,15-EET production was evident. Perfusion with exogenous 11,12-EET caused a similar increase in PAP in both, *Cx40*$^{+/+}$ and *Cx40*$^{-/-}$mouse

lungs, confirming that EETs act as vasoconstrictive mediators in the pulmonary circulation and that this response itself does not depend on Cx40 (Figs. 8 & 9 in Wang *et al*.). These results have been published in "***The Journal of Clinical Investigation***" *122(11):4218-30, 2012*, with myself listed as first author.

Novel pharmacological TRPC inhibitors block hypoxia-induced vasoconstriction To identify novel and specific pharmacological blockers for TRPC6, Prof. Michael Schaefer's research group (Rudolf-Boehm-Institute of Pharmacology and Toxicology, University of Leipzig) screened the Chembionet collection of chemically diverse drug-like molecules, and identified and subsequently characterized compounds 2910-0498 and 8009-5364 as novel selective blockers of TRPC6 activation. In collaboration with Prof. Schaefer, we tested the efficacy of these two compounds to block HPV in isolated perfused mouse lungs based on the documented relevance of TRPC6 for HPV[4]. Both compounds effectively inhibited hypoxia induced pulmonary vasoconstriction, verifying the effectiveness of these blockers in an intact organ setting.
These results have been published in "***Cell Calcium"****51:194-206,* 2012 with myself listed as a co-author.

Physiological and pathophysiological relevance of TRPV4 channel mediated Ca^{2+} influx in lung endothelial and smooth muscle cells Based on the identification of TRPV4 as a mechanosensitive ion channel with high expression in the pulmonary endothelium, our group studied the role of TRPV4 in hydrostatic lung edema formation. In a series of physiological and real-time imaging experiments in isolated lungs, we could show that TRPV4 mediates a pressure-induced Ca^{2+} influx into endothelial cells that increases lung vascular permeability, and thus contributes to edema formation. Yet, at the same time, TRPV4-mediated Ca^{2+} entry was shown to stimulate the synthesis of NO and cGMP, which in turn blocks Ca^{2+} entry via TRPV4 in a negative feedback loop that limits the endothelial Ca^{2+} response and protects the vascular barrier.
These results have been published in "***Circulation Research****" 102: 966-974, 2008* with myself listed as a co-author.

Based on the notion that TRPV4 i) is also highly expressed in pulmonary artery smooth muscle cells, ii) regulates vascular tone by mediating Ca^{2+} influx, iii) is activated by EETs which have been implied in HPV[13], and iv) has recently been implicated in lung vascular responses to chronic hypoxia[5], we tested for a potential role of TRPV4 in HPV. Preliminary data from ongoing experiments show that the HPV response in isolated lungs is largely reduced in lungs of *Trpv4*$^{-/-}$ as compared to *Trpv4*$^{-/-}$ mice, and in lungs of *Trpv4*$^{+/+}$ in the presence of a specific TRPV4 inhibitor. Conversely, HPV is enhanced in lungs of *Trpv4*$^{+/+}$ mice in the presence of pharmacological TRPV4 openers.
A manuscript reporting these data and subsequent mechanistic *in vitro* experiments is currently in preparation with myself listed as an equally contributing first author.

Other molecular mechanism and therapeutic targets in HPV

As CF patients suffer from V/Q mismatching, we tested for a potential role of CFTR in HPV. In isolated mouse lungs, we show that HPV is attenuated by CFTR inhibition or deficiency, respectively. By fluorescence microscopy, we could further show that CFTR inhibition blocked the hypoxia induced Ca^{2+} influx into pulmonary artery smooth muscle cells. Subsequent Western blot analyses of pulmonary artery smooth muscle cell fractions showed that hypoxia induced a rapid translocation of TRPC6 to caveolae that was blocked in the presence of a CFTR inhibitor. A manuscript reporting these data, which provide novel evidence that CFTR is required for the translocation of TRPC6, and the subsequent Ca^{2+} entry and smooth muscle contraction in HPV, is currently in preparation with myself listed as an equally contributing first author.

Finally, we tested for the ability of the stable VIP analog RO251553 to attenuate lung vasoconstrictive responses to hypoxia. In isolated mouse lungs, RO251553 inhibited HPV in a concentration-dependent manner. Furthermore, inhalation of RO251553 provided sustained inhibition of the PAP increase in response to hypoxia in rats *in vivo* in the absence of detectable adverse effects on systemic hemodynamics. A manuscript describing these findings is currently in revision at PLOS One with myself listed as an equally contributing first author.

Summary

The present studies report fundamentally novel mechanistic concepts and signaling pathways that underlie HPV in intact lungs. In contrast to prevailing paradigms, our data revealed a pivotal role for the pulmonary endothelium in HPV, which conducts a hypoxia induced endothelial depolarization retrogradely via Cx40-containing gap junctions from the alveolar area of gas exchange to the upstream arteriolar resistance vessels, where the depolarization elicits an endothelial Ca^{2+} entry via α_{1G}-subtype T-type Ca^{2+} channels, thus causing the generation of vasoconstrictive AA metabolites such as EETs via activation of $cPLA_2$. Our data further identify novel roles for TRPV4 and CFTR in HPV and the pulmonary artery smooth muscle cell response to hypoxia, and validate the efficacy of new pharmacological compounds including the recently identified TRPC6 blockers 2910-0498 and 8009-5364 and the stable VIP analog RO251553 to inhibit lung vasoconstrictive responses to hypoxia *in situ* and *in vivo*, respectively.

References

1. Ward JP, McMurtry IF. Mechanisms of hypoxic pulmonary vasoconstriction and their roles in pulmonary hypertension: New findings for an old problem. *Current opinion in pharmacology*. 2009;9:287-296
2. Hislop A, Reid L. Normal structure and dimensions of the pulmonary arteries in the rat. *Journal of anatomy*. 1978;125:71-83
3. de Wit C, Roos F, Bolz SS, et al. Impaired conduction of vasodilation along arterioles in connexin40-deficient mice. *Circulation research*. 2000;86:649-655
4. Weissmann N, Dietrich A, Fuchs B, et al. Classical transient receptor potential channel 6 (*Trpc6*) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103:19093-19098
5. Yang XR, Lin AH, Hughes JM, et al. Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol.* 2012;302(6):L555-68
6. Soni R, Dobbin CJ, Milross MA, et al. Gas exchange in stable patients with moderate-to-severe lung disease from cystic fibrosis. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*. 2008;7:285-291
7. Spohr F, Busch CJ, Reich C, et al. 4-aminopyridine restores impaired hypoxic pulmonary vasoconstriction in endotoxemic mice. *Anesthesiology*. 2007;107:597-604
8. Glenny RW, Bernard S, Brinkley M. Validation of fluorescent-labeled microspheres for measurement of regional organ perfusion. *J Appl Physiol*. 1993;74:2585-2597
9. Kuebler WM, Parthasarathi K, Lindert J, et al. Real-time lung microscopy. *J Appl Physiol*. 2007;102:1255-1264
10. Song C, Al-Mehdi AB, Fisher AB. An immediate endothelial cell signaling response to lung ischemia. *American journal of physiology. Lung cellular and molecular physiology*. 2001;281:L993-1000
11. Kuebler WM, Parthasarathi K, Wang PM, et al. A novel signaling mechanism between gas and blood compartments of the lung. *The Journal of clinical*

investigation. 2000;105:905-913

12. Schievella AR, Regier MK, Smith WL, et al. Calcium-mediated translocation of cytosolic phospholipase a_2 to the nuclear envelope and endoplasmic reticulum. *The Journal of biological chemistry*. 1995;270:30749-30754
13. Keseru B, Barbosa-Sicard E, Popp R, et al. Epoxyeicosatrienoic acids and the soluble epoxide hydrolase are determinants of pulmonary artery pressure and the acute hypoxic pulmonary vasoconstrictor response. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2008;22:4306-4315
14. Archer SL, Souil E, Dinh-Xuan AT, et al. Molecular identification of the role of voltage-gated K^+ channels, $Kv_{1.5}$ and $Kv_{2.1}$, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. *The Journal of clinical investigation*. 1998;101:2319-2330
15. Chakraborti S, Michael JR, Chakraborti T. Role of an aprotinin-sensitive protease in protein kinase calpha-mediated activation of cytosolic phospholipase a_2 by calcium ionophore (a23187) in pulmonary endothelium. *Cellular signalling*. 2004;16:751-762

Affidavit

"I, Liming Wang certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Hypoxic pulmonary vasoconstriction in intact mouse lungs: new insights into cellular mechanisms and therapeutic targets". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

Liming Wang had the following share in the following publications:

Publication 1: **Wang L**, Yin J, Nickles HT, Ranke H, Tabuchi A, Hoffmann J, Tabeling C, Barbosa-Sicard E, Chanson M, Kwak BR, Shin HS, Wu S, Isakson BE, Witzenrath M, de Wit C, Fleming I, Kuppe H, Kuebler WM. Hypoxic pulmonary vasoconstriction requires connexin 40 mediated endothelial signal conduction. J Clin Invest, 2012.
Contribution in detail: Liming Wang performed 85% of all experiments alone, acquired and analyzed the data, and drafted text and figures of the manuscript.

Publication 2: Urban N, Hill K, **Wang L**, Kuebler WM, Schaefer M. Novel pharmacological TRPC inhibitors block hypoxia-induced vasoconstriction. Cell Calcium, 2012.
Contribution in detail: Liming Wang performed 30% of all work for this paper (as isolated mouse lung experiment), acquired and analyzed part of the data, and drafted part of the text and figures of the manuscript.

Publication 3: Yin J, Hoffmann J, Kaestle SM, Neye N, **Wang L**, Baeurle J, Wu S, Kuppe H, Pries AR, Kuebler WM. Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of TRPV4. Circulation Research, 2008.
Contribution in detail: Liming Wang performed 10% of all work for this paper (as mouse lung image), acquired and analyzed part of the data, and drafted part of the text and figures of the manuscript.

Signature, date and stamp of the supervising University teacher

Signature of the doctoral candidate

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List of Publications

- Yin J, Hoffmann J, Kaestle SM, Neye N, **Wang L**, Baeurle J, Wu S, Kuppe H, Pries AR, Kuebler WM. Negative-feedback loop attenuates hydrostatic lung edema via a cGMP-dependent regulation of TRPV4. *Circulation Research*， 2008 Apr 25; 102(8):966-74 (Impact Factor: 9.489)

- Urban N, Hill K, **Wang L**, Kuebler WM, Schaefer M. Novel pharmacological TRPC inhibitors block hypoxia-induced vasoconstriction. *Cell Calcium,* 2012 Feb; 51(2):194-206. (Impact Factor: 3.766)

- **Wang L**, Yin J, Nickles HT, Ranke H, Tabuchi A, Hoffmann J, Tabeling C, Barbosa-Sicard E, Chanson M, Kwak BR, Shin HS, Wu S, Isakson BE, Witzenrath M, de Wit C, Fleming I, Kuppe H, Kuebler WM**.** Hypoxic pulmonary vasoconstriction requires connexin 40 mediated endothelial signal conduction. *J Clin Invest, 2012 Nov 1;122(11):4218-30.* (Impact Factor:13.069)

- Solymosi EA, Kaestle-Gembardt SM, Vadász I, **Wang L**, Neye N, Chupin CJ, Rozowsky S, Ruehl R, Tabuchi A, Schulz H, Kapus A, Morty RE, Kuebler WM. Chloride transport-driven alveolar fluid secretion is a major contributor to cardiogenic lung edema. *Proc Natl Acad Sci U S A*. 2013 May 3. [Epub ahead of print] (Impact Factor: 9.681)

- **Wang L**, Isakson BE, de Wit C, Kuppe H, Kuebler WM. Connexin 40 is essential for hypoxic pulmonary vasoconstriction, Experimental Biology, New Orleans, 2009, *The FASEB Journal* 2009 (Abstract)

- **Wang L**, Yin J, Tabuchi A, Isakson BE, de Wit C, Kuppe H, Kuebler WM. A critical role for connexin 40 in hypoxia induced pulmonary vasoconstriction, Experimental Biology, Anaheim, California, 2010, *The FASEB Journal* 2010 (Abstract)

- **Wang L**, Yin J, Tabuchi A, Isakson BE, de Wit C, Kuppe H, Kuebler WM. A critical role of connexin 40 in hypoxia induced pulmonary vasoconstriction, American Thoracic Society, Denver, Colorado, 2011, *Am J Respir Crit Care Med* (Abstract)
- **Wang L**, Yin J, Yin N, Wolff G, Kuebler WM. Vasodilatory Effect Of The Vasoactive Intestinal Peptide Analog RO 25-1553 In The Pulmonary Circulation: A New Therapy For COPD? American Thoracic Society, San Francisco, California, 2012, *Am J Respir Crit Care Med* (Abstract)

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