Endothelial hyperpermeability in severe pulmonary arterial hypertension:

Role of store operated calcium entry

Chun Zhou¹,³, Mary I. Townsley¹,²,³, Mikhail Alexeyev¹,³, Norbert F. Voelkel⁴, and Troy Stevens¹,²,³

Departments of Physiology and Cell Biology¹ and Internal Medicine², Center for Lung Biology³,
University of South Alabama, Mobile AL 36688; Division of Pulmonary Disease and Critical Care Medicine, Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA 23284

Address Correspondence To:
Troy Stevens, Ph.D.
Lenoire Locke Professor and Chair
Department of Physiology and Cell Biology
Director, Center for Lung Biology
College of Medicine
University of South Alabama
Mobile, AL 36688
Phone: 251-460-6056
Fax: 251-460-7452
Email: tstevens@southalabama.edu
Abstract

Here, we tested the hypothesis that animals with severe pulmonary arterial hypertension (PAH) display increased sensitivity to vascular permeability induced by activation of store operated calcium entry. To test this hypothesis, wild type and TRPC4 knockout Fischer 344 rats were given a single injection of Semaxanib (SU5416; 20 mg/kg) followed by three weeks of exposure to hypoxia (10% oxygen) and a return to normoxia (21% oxygen) for an additional two to three weeks. This Semaxanib/hypoxia/normoxia (i.e. SU5416/hypoxia/normoxia) treatment caused PAH, as evidenced by development of right ventricular hypertrophy, pulmonary artery medial hypertrophy, and occlusive lesions within precapillary arterioles. Pulmonary artery pressure was increased five-fold in Semaxanib/hypoxia/normoxia-treated animals compared to untreated, Semaxanib-treated, and hypoxia-treated controls, determined by isolated perfused lung studies. Thapsigargin induced a dose-dependent increase in permeability that was dependent upon TRPC4 in the normotensive perfused lung. This increase in permeability was accentuated in PAH lungs, but not in Semaxanib- or hypoxia-treated lungs. Fluid accumulated in large perivascular cuffs, and although alveolar fluid accumulation was not seen in histological sections, Evan’s blue dye conjugated to albumin was present in bronchoalveolar lavage fluid of hypertensive, but not normotensive lungs. Thus, PAH is accompanied by a TRPC4-dependent increase in the sensitivity to edemagenic agents that activate store operated calcium entry.

Index Terms:

1. Edema
2. Canonical transient receptor potential 4 (TRPC4)
3. Calcium channels
4. Semaxanib (SUGEN 5416)
5. Filtration coefficient

Running Title: Endothelial hyperpermeability in PAH
Introduction

Pulmonary arterial hypertension (PAH) is a progressive vasculopathy characterized by medial hypertrophy and hyperplasia, distal extension of smooth muscle into typically non-muscularized arterioles, and complex occlusive lesion formation in small precapillary arterioles and supernumerary vessels (1, 19, 20, 22, 40, 49, 53, 57, 58, 63-66, 71). Endothelial dysfunction contributes to this vasculopathy in at least two ways. First, in small precapillary arterioles endothelial cell apoptosis is thought to lead to exuberant overgrowth of apoptosis resistant cells that display disordered angiogenesis (19, 20, 22, 49, 63-65). In this case, hyperproliferation causes endothelial accumulation within the blood vessel, where in the most severe form of remodeling the cells form multiple luminal slits rather than open vascular channels, e.g. plexogenic arteriopathy. Second, pulmonary artery endothelial cells become disordered with apparent structural abnormalities (28, 52). The functional consequence(s) of these changes is less clear, although an imbalance in the normal production of endothelial vasodilators and vasoconstrictors accompanies PAH (37, 39, 41, 55).

In addition to the abnormal production of vasoactive mediators, conduit and resistance artery endothelial cells may possess an abnormally hyperpermeable barrier. While the contribution of endothelial hyperpermeability to remodeling in PAH has not received considerable attention, its potential importance has been recognized for decades (59). This issue has been revisited in recent studies of group I PAH (i.e. heritable PAH). Mutations in bone morphogenetic protein receptor II (BMPR-II) constitute a principal genetic cause of the heritable disease (56). Animals harboring endothelial BMPR-II mutations spontaneously develop PAH (67), and their endothelium is hyperpermeable and pro-inflammatory (32, 34, 47). The mechanisms responsible for such increased susceptibility to vascular leak remain incompletely explored, although Prewitt and colleagues have recently suggested that BMPR-II deficiency impairs normal caveolae function (47). In their studies, BMPR-II deficiency increased paracellular and transcellular protein flux, dependent upon Src kinase activation; inhibition of Src kinase reduced the hyperpermeability response. The pathophysiological significance of pulmonary arterial hyperpermeability in PAH is unknown. Nonetheless, investigators have speculated that disrupted
endothelium may promote growth factor access to underlying smooth muscle, allow delivery of inflammatory mediators and immune cells to the vascular wall, and decrease vascular and potentially airway compliance.

A hyperpermeable endothelial barrier may display increased sensitivity to circulating inflammatory mediators. Many inflammatory mediators activate store operated calcium entry, and the resulting calcium influx increases paracellular transport leading to pulmonary edema, especially in extra-alveolar blood vessels (for review see 13-15, 18). TRPC4 contributes to the molecular anatomy of store operated calcium entry channels (6, 16, 17), and it has recently been incriminated in the pathogenesis of PAH (5). Fischer rats (F344) subjected to a single Semaxanib (i.e. Sugen5416) injection followed by three weeks of hypoxia (10%) and a return to normoxia for 2-5 weeks (Semaxanib/hypoxia/normoxia) develop severe PAH that culminates in right heart failure and death (5, 31). TRPC4 deficiency confers a survival benefit in these animals (5). Whereas TRPC4 deficiency does not reduce either the pulmonary arterial pressure or the Fulton Index, it decreases the extent of occlusive lesion formation and appears to preserve cardiac output (5). Fischer rats with severe PAH display an exaggerated permeability response to thapsigargin that appears to require TRPC4 (23). Based upon these studies, we tested the hypothesis that animals with severe PAH display increased sensitivity to permeability induced by activation of store operated calcium entry.
Materials and Methods

Animals. All experimental procedures were performed in accordance with current provisions of the U.S. Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. Male TRPC4 wild type (TRPC4⁺/⁺), heterozygous (TRPC4⁺/⁻), and null littermate (TRPC4⁻/⁻) F344 rats were anesthetized with Nembutal (65 mg/kg body weight). TRPC4⁻/⁻ F344 rats were generated by Transposagen Biopharmaceuticals (Lexington, KY), as part of the Knockout Rat Consortium (Trpc4tm1Bni, targeted mutation 1, Bernd Nilius), and were bred and genotyped both at Transposagen and at the University of South Alabama as previously described (5).

Genotyping of the TRPC4-KO Rats. Rat tail snips were collected according to the guidelines of the University of South Alabama Animal Care and Use Committee. DNA was extracted from tail snips, as described previously (12), and 2 µL of the resulting DNA solution was subjected to PCR analysis using three primers (primer A, 5’-GTGTTGGTCTCCATTACTTCAGCT-3’; primer B, 5’-ATTCTTCCCTTTGAGCCCACT-3’; and transposon primer, 5’-CTGACCTAAGACAGGGAATT-3’) in a total volume of 20 µL containing 1x GoTaq Green PCR master mix (Promega, Madison, WI) and 1 mmol/L of each primer. The cycling parameters were denaturation at 94°C for 5 minutes; then 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute; and extension at 72°C for 7 minutes.

Isolated Lung and Assessment of Endothelial Permeability. Animals were anesthetized using Nembutal (65 mg/kg body weight). Once a surgical plane was achieved, as defined by the absence of a withdrawal reflex following toe and tail pinch, animals were intubated and ventilated, a sternotomy was performed, and pulmonary artery and left ventricular catheters were placed. Heart and lungs were removed en bloc and suspended in a humidified chamber, where mechanical ventilation and flow were established. Rat lungs were perfused at constant flow (40 mL/min/kg of body weight) with either buffer (in mmol/L: 119.0 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.0 Na₂HPO₄, 1.18 KH₂PO₄, 23 NaHCO₃, 5.5 glucose) containing 4% BSA.
and physiological (2.2 mmol/L) CaCl$_2$, adjusted to pH 7.4 at 38°C, or with buffer and BSA plus 6% autologous whole blood.

Hemodynamic measurements and the filtration coefficient ($K_f$) were measured as previously described (44, 45), using zone 3 conditions. Baseline $K_f$ was calculated as the rate of weight gain obtained 13 to 15 minutes after a 10 cmH$_2$O increase in pulmonary venous pressure, normalized per 100 g predicted wet lung weight. $K_f$, the product of specific endothelial hydraulic permeability and surface area for exchange, is a sensitive measure of lung endothelial permeability when surface area is fully recruited. To determine the optimal dose of thapsigargin in subsequent experimental protocols, dose-response studies were conducted in the isolated lung preparation. Thapsigargin was added to the perfusate reservoir over a range of concentrations (0-300 nM), and a second $K_f$ value was determined 15 min after later.

Evan’s blue dye albumin measurements in the bronchoalveolar lavage fluid. To evaluate albumin extravasation, Evan’s blue dye was added to a 5% (w/v) solution of bovine serum albumin (BSA) to give a final concentration of 1.5 mg/ml, the mixture was dialysed against excessive 5% BSA overnight at 4°C in a Slide-A-Lyzer® Dialysis Cassettes (Pierce). During the isolated lung perfusion, after lungs were challenged with 150 nM thapsigargin for 15 minutes, 2.5 ml albumin-bound Evan’s blue was added to the circulating perfusate 5 minutes after a 10 cmH$_2$O-raise of venous pressure, and kept circulating at this higher venous pressure for 10 more minutes. After double occlusion, whole-lung bronchoalveolar lavage was performed by instilling 2 mL of PBS. Evan’s blue dye albumin in bronchoalveolar lavage fluid was determined by measuring absorption at 620 nM, and extrapolated against the standard curve of Evan’s blue-conjugated albumin.

Rat Pulmonary Arterial Hypertension Model. PAH was induced by a single subcutaneous injection of Sugen 5416 (20 mg/kg; Semaxanib; Cayman Chemical, Ann Arbor, MI) on day 1, followed by exposure to three weeks of normobaric hypoxia (Hx; 10% O$_2$) and then re-exposure to normoxia (Nx; 21% O$_2$) for two to three additional weeks (1, 60). The hemodynamic and histopathological parameters of PAH were
compared among three experimental groups of age- and weight-matched rats (n = 5-10 each): male Fischer 344 (F344) TRPC4+/+, male F344 TRPC4-/-, and male F344 TRPC4+/-.

Each experimental group included a set of normoxia time control rats.

Lung Histology. The trachea was ligated and lungs were submersion fixed with 10% formalin, paraffin embedded and prepared for light microscopy. The left lungs were cut in a horizontal plane in the middle of lung, and 5-µm slices were stained with hematoxylin and eosin for examination.

Statistical Analysis. Quantitative data are presented as mean±SEM. Group means were compared using one- or two-way ANOVA with Bonferroni (Sidak) post hoc test as appropriate. P values < 0.05 were considered statistically significant.
Results

**Thapsigargin induces a TRPC4-dependent increase in lung permeability.** Previous studies in Sprague Dawley (11) and Fischer 344 (23) rats have established that thapsigargin increases endothelial cell permeability, in part dependent upon TRPC4. In addition, Tiruppathi and colleagues (61) demonstrated that TRPC4 plays an important role in lung endothelial cell permeability, using TRPC4 knockout mice. Here, we sought to determine sensitivity to the thapsigargin-induced increase in permeability in the normotensive F344 rat, and evaluate the importance of TRPC4 to this response.

To test this idea, thapsigargin was tested over a range of concentrations, from 10-300 nM (**Figure 1A**). In the normotensive TRPC4+/+ F344 rat, thapsigargin induced a dose-dependent increase in $K_f$. 75 nM represented the threshold concentration where $K_f$ first became significantly increased ($p < 0.05$). Near maximal responses were observed at 150 nM thapsigargin. When compared to previous studies using Sprague-Dawley rats (11), baseline $K_f$ values were higher and the sensitivity to thapsigargin was lower in Fischer rats. Baseline $K_f$ values were similar between normotensive TRPC4+/+ and TRPC4-/- rats ($p = 0.99$). However, the normotensive TRPC4-/- rats were relatively insensitive to thapsigargin, as $K_f$ did not significantly increase ($p < 0.05$) until the 300 nM concentration (**Figures 1A and 1B**). These data support the idea that TRPC4 channels contribute to the store operated calcium influx that increases endothelial cell permeability.

In wild type F344 rats, the threshold concentration for thapsigargin to increase $K_f$ was 75 nM as previously reported (23), and 150 nM yielded a near-maximal effect, given the range of concentrations examined. Therefore, we studied the permeability response to thapsigargin at 150 nM in additional experiments. Baseline perfusion pressures in normotensive isolated lungs were approximately 10 cm H$_2$O in both wild type and TRPC4-/- rats, and Fulton indices for these animals were less than 0.30, indicating baseline hemodynamic values were similar and largely unaffected by loss of TRPC4 expression (**Figure 2A**). Baseline $K_f$ values were slightly lower in TRPC4+/+ ($0.12 \pm 0.03$) than in TRPC4-/- ($0.21 \pm 0.03$) rats ($p < 0.05$). Thapsigargin (150 nM) increased $K_f$ by approximately 2-fold in normotensive wild type...
controls (Figure 2B; p < 0.05). This permeability evoking effect of thapsigargin was abolished altogether in TRPC4−/− rat lungs (p < 0.05 vs wild type).

Thapsigargin promotes fluid accumulation in perivascular cuffs, but does not cause alveolar flooding (4, 11, 35, 36, 70). We examined lung histology following thapsigargin treatment (75 nM) in normotensive wild type and TRPC4+/− rat lungs. As seen in Figure 2C, consistent with previous reports (4, 11, 35, 36, 70), fluid accumulation was extensive and limited to perivascular cuffs around arteries and veins of all sizes. Dilated lymphatic channels were visible. In all cases, fluid was retained in the interstitial spaces and could not be seen in the alveoli. Fluid accumulation did not appear to be as extensive in lungs from TRPC4−/− rats, although histology was not quantified because images were assessed in parallel with physiological parameters, e.g. Kf. Thus, thapsigargin increases endothelial cell permeability, especially in extra-alveolar arteries and veins, dependent upon the expression of TRPC4.

**Pulmonary arterial hypertension reveals a greater sensitivity to the thapsigargin-induced increase in permeability.** We next examined endothelial cell barrier integrity, and its sensitivity to thapsigargin, in animals with severe PAH; experimental PAH was induced using the Semaxanib/hypoxia/normoxia model (see Materials and Methods). The baseline pulmonary artery perfusion pressure in isolated TRPC4+/+ lungs was approximately 50 cm H2O (≈ 38 mm Hg; Figure 3A), which is approximately 5-fold higher than normotensive values. This is a large elevation in pulmonary artery pressure, especially considering the perfusion rate in these isolated lung studies was roughly 20% of the baseline cardiac output in vivo. There was no difference in pulmonary artery perfusion pressures among TRPC4+/+, TRPC4+/− and TRPC4−/− rat groups (p = 0.99). The Fulton index was 3-fold higher in all three hypertensive groups when compared with normotensive controls (p < 0.05), although there was no difference in the Fulton index among TRPC4+/+, TRPC4+/− and TRPC4−/− groups (p = 0.99 between TRPC4+/+ and TRPC4+/− and p = 0.24 between TRPC4+/+ and TRPC4−/−). Thapsigargin (150 nM) increased Kf nearly 10-fold in hypertensive TRPC4+/+ lungs (Figure 3B), which represents a tremendous potentiation above normotensive controls (see Figure 2B; p < 0.05), and in this case, TRPC4 deletion was unable to rescue the hyperpermeability response. We previously reported that at lower thapsigargin concentrations (75 nM), TRPC4 deletion is
protective against the increase in permeability (23). Thus, this higher thapsigargin concentration appears to recruit additional store operated calcium entry mechanisms that are not dependent upon TRPC4 in the hypertensive circulation.

We have previously quantified the histological changes seen in the lungs of F344 rats treated with Semaxanib/hypoxia/normoxia (5). Consistent with these previous results, severe precapillary pulmonary vascular remodeling was verified in the hypertensive animals (Figure 3C). Medial hypertrophy with apparent distal extension of smooth muscle was seen in pulmonary arteries and arterioles. Occlusive lesions were prominent in small precapillary segments. Following the thapsgargin exposure, extensive fluid accumulation was observed in perivascular cuffs, especially surrounding arteries and veins. However, we noted that in no circumstance were cuffs seen in or around occlusive lesions; fluid did not accumulate in the attenuated interstitial compartment of these remodeled vessels. A similar degree of vascular remodeling was observed in TRPC4+/− and TRPC4−/− lungs. As we have previously quantified and reported (5), TRPC4 deletion reduced the extent of remodeling within occlusive lesions.

We noticed that the permeability defect determined by $K_f$ was considerably more severe than what could be predicted by the accompanying histology sections. Therefore, we generated a movie documenting the thapsigargin-induced hyperpermeability response in PAH lungs from TRPC4+/+ rats (Supplemental movie 1). Following thapsigargin application (150 nM), fluid is seen dripping from the lung, due to its clearance through open lymphatics. As filtration due to the permeability defect exceeds apparent lymphatic clearance, the lung collects water to the point that it can no longer inflate under the established ventilatory parameters. Fluid can be seen moving through the trachea coincident with ventilation. The lung surface appearance is suggestive of alveolar flooding, although as seen in representative histological sections (Figure 3C), prominent alveolar flooding was not seen.

To further address this issue, we repeated a series of experiments in both normotensive and PAH animals. In these studies, after baseline $K_f$ was assessed and thapsigargin (150 nM) was applied, Evan’s blue dye conjugated to albumin was recirculated and a second $K_f$ was measured. Upon completion of the experiment, Evan’s blue dye was measured in the bronchoalveolar lavage fluid. As is seen in Figure 3D,
PAH lungs displayed an increased sensitivity to thapsigargin. Whereas Evan’s blue dye could not be detected in the airways of normotensive lungs, it did accumulate in the airspaces of PAH lungs. These data therefore indicate that in this model of PAH, endothelium displays a hypersensitivity to activation of store operated calcium entry. Perhaps most importantly, these data indicate that the endothelial barrier is sufficiently fragile in PAH to enable activation of store operated calcium entry to cause water and protein access to the airways.

Three-week hypoxia exposure is not sufficient to sustain either pulmonary arterial hypertension or the increased sensitivity to thapsigargin. As a control for the Semaxanib/hypoxia/normoxia treatment, animals were exposed to chronic hypoxia (10% $O_2$) for three weeks followed by two to three additional weeks of normoxia (i.e. room air, barometric pressure approximately 760 mm Hg), at which time the experiment was terminated and lungs were isolated and perfused. Baseline pulmonary artery perfusion pressure of these hypoxia-exposed TRPC$^{+/+}$ lungs was approximately 10 cm H$_2$O (Figure 4A), similar to normoxia controls (see Figure 2A), and the Fulton index in these animals was 0.32 ± 0.01, versus 0.26 ± 0.01 in the controls (p < 0.05) and 0.74 ± 0.02 in Semaxanib/Hypoxia/Normoxia-exposed animals (p < 0.05). Pulmonary artery perfusion pressures and the Fulton index were not different among the hypoxia-exposed TRPC$^{+/+}$, TRPC$^{+/−}$, and TRPC$^{−/−}$ lungs (p = 0.73 and 0.41, respectively). Therefore, whereas pulmonary perfusion pressures were similar to untreated control animals, hypoxia-exposed animals retained some evidence of right ventricular remodeling two weeks after their hypoxia exposure.

Seventy-five and 150 nM thapsigargin concentrations increased $K_f$ approximately 2-fold in TRPC$^{+/+}$ lungs (Figure 4B). The magnitude of this increase was similar to what we observed in normotensive controls (see Figures 1 and 2), and less than that in lungs from Semaxanib/hypoxia/normoxia-treated animals (Figure 3; p < 0.05). This permeability response was abolished in TRPC$^{−/−}$ lungs in response to 75 nM, but not 150 nM, thapsigargin. Histology revealed prominent perivascular fluid cuffs around arteries and veins, but again, alveolar flooding was not seen.
Despite a normal pulmonary artery perfusion pressure, small arterioles and large arteries appeared muscularized. These findings suggest that the three-week hypoxic exposure had caused elevated pulmonary artery pressure with an associated pulmonary vascular remodeling, and further, that upon return to normoxia the pressure had normalized whereas the remodeling in blood vessels and the right ventricle had not fully regressed.

Semaxanib is not sufficient to increase either pulmonary arterial pressure or the sensitivity to thapsigargin. As an additional control for the Semaxanib/hypoxia/normoxia treatment, animals were administered Semaxanib and then maintained under normoxia control conditions for 5-6 weeks, at which time lungs were isolated and perfused. The baseline perfusion pressure was slightly elevated compared to untreated controls (control = 7.6 ± 0.4 versus Semaxanib = 10.4 ± 0.4 cm H₂O), although this difference achieved statistical significance (p < 0.05). The Fulton index in this treatment group was not different from the untreated time controls (Figure 5A; p = 0.53), and the results in wild type and TRPC4⁻/⁻ animals were similar (p = 0.99). Thapsigargin (75 and 150 nM) increased Kᵢ in lungs from both wild type and TRPC4⁻/⁻ rats approximately 2-fold (Figure 5B), similar to the responses seen in untreated control lungs (Figures 1 and 2). Large perivascular fluid cuffs were once again prominent in lungs from wild type and TRPC4⁻/⁻ rats, but alveolar flooding was absent (Figure 5C).
PAH is a vasculopathy characterized by endothelial dysfunction, in both small precapillary arterioles and resistance and conduit arteries. Thapsigargin activates store operated calcium entry channels, which induce extra-alveolar endothelial permeability, targeting a vascular location, e.g. pulmonary artery, most prominently impacted in PAH. We recently reported that thapsigargin induces TRPC4-dependent high frequency endothelial cell cytosolic calcium transients that are coupled to a permeability defect in severe PAH (23). Our present studies extend these observations, as we now resolve that neither the hypoxia nor Semaxanib treatments alone are sufficient to recapitulate the hyperpermeability sensitivity of PAH. Moreover, the endothelial cell barrier in PAH is fragile; thapsigargin increases protein and fluid accumulation in the air spaces, an uncharacteristic physiological response following activation of store operated calcium entry channels.

In the normoxia time control lungs, thapsigargin induced a dose-dependent increase in permeability that was dependent upon TRPC4. This was not a surprising result. The importance of TRPC4 in the molecular anatomy of store operated calcium entry channels that increase endothelial cell permeability has been established (for review see 13-15, 18), and TRPC4 knockout mice are resistant to calcium agonists that evoke store operated calcium entry and increase permeability (61). However, the sensitivity to thapsigargin had not been established in F344 rats. When compared to results obtained previously using Sprague-Dawley rat lungs (11), the thapsigargin-induced increase in permeability was modest, yet the half maximal concentration was nearly identical among strains. Lungs from TRPC4 knockout rats were largely insensitive to thapsigargin; a significant increase in permeability was only seen with a 300 nM concentration of thapsigargin. These findings continue to support an important role for TRPC4 in disorders of lung permeability. They also bring into question whether high concentrations of thapsigargin can activate TRPC4-independent store operated calcium entry channels, or perhaps other non-store operated calcium entry channels, to disrupt the endothelial cell barrier in PAH. This issue will have to be addressed in future studies.
PAH lungs displayed enhanced sensitivity to thapsigargin; the hyperpermeability of PAH was due to TRPC4, especially at lower thapsigargin concentrations (23). Although pulmonary artery, vein and double occlusion pressures were increased in PAH lungs compared to normotensive lungs, they were not different in the wild type and TRPC4 knockout studies. Therefore, increased hydrostatic pressure is not responsible for the observed increase in thapsigargin sensitivity. Moreover, the increased sensitivity cannot be attributed to either Semaxanib treatment or hypoxia exposure alone, since typical thapsigargin permeability responses were observed following these treatments. Endothelial dysfunction that accompanies PAH, especially in extra-alveolar segments, renders that barrier more susceptible to permeability edema, at least following activation of store operated calcium entry channels. This finding is similar to the hyperpermeability phenotype in mice harboring a BMPR-II mutation (32, 34, 47).

Vascular permeability in chronic forms of pulmonary hypertension has not been extensively studied, although indices of endothelial dysfunction have been periodically reported for more than 50 years (collated in Table 1, except for citations specifically discussed below). This issue has recently been tested in heritable PAH. Mice engineered to possess a heterozygous knock-in of the BMPRIIR899X mutation develop mild PAH by approximately 6-weeks of age (34). Coincident with PAH development, lungs in these animals exhibit a basal and lipopolysaccharide-induced hyperpermeability when compared to controls. Pulmonary artery endothelial cells obtained from mice and blood outgrowth endothelial cells obtained from humans expressing dysfunctional BMPRII are similarly hyperpermeable, suggesting increased permeability may be an important factor in the pathophysiology of heritable disease. Consistent with these findings, endothelial cells isolated from heterozygous mice carrying the BMPR2\textsuperscript{Δex4-5/+} mutation displayed a basal macromolecular hyperpermeability (47). Monocrotaline-induced PAH is a nongenetic cause of PAH associated with decreased BMPRII expression (33, 51). In this case, exposure to the injurious pyrrole causes cellular apoptosis and increased permeability, and is used as a model for drug-induced endothelial injury and PAH (Table 1). These studies suggest that endothelial cell barrier dysfunction is a critical determinant of vascular remodeling in PAH.
Yet hyperpermeability is not a consistent finding among all forms of chronic pulmonary hypertension. In the few examples studied, store operated calcium entry-dependent permeability responses were ablated in pulmonary hypertension due to both chronic hypoxia and congestive heart failure. In the former example, one-week hypoxia (10%) exposure led to increased pulmonary artery pressure and right ventricular hypertrophy, but the thapsigargin-induced increase in permeability was abolished (62). Note that this experimental design is different than the studies reported here. In our work, animals were exposed to hypoxia (10%) for three weeks and allowed to return to normoxia for two weeks, as a control for the Semaxanib/hypoxia/normoxia exposure. The discrepancy among these results suggests that hypoxic hypertension is accompanied by loss of thapsigargin sensitivity, which is rescued upon the return to normoxia. Alternatively, our current studies may reflect the relative insensitivity of Fischer rats to hypoxia (26).

Development of heart failure was also accompanied by loss of the thapsigargin-induced increase in permeability, both in the dog pacing (30) and in the rat aortocaval fistula (3) models, due at least in part to a down-regulation of TRPC1, TRPC3 and TRPC4 (3). Whereas store operated calcium entry channel expression and function in endothelium was lost in heart failure, TRPV4 channel expression and activation remained intact. This is an interesting contrast since the TRPC4-induced, and not the TRPV4-induced, increase in permeability occurs in extra-alveolar vessels, which is the vascular location most relevant to pulmonary arterial hypertension (2, 4, 35). Endothelial cell barrier dysfunction may be one of the discriminating features among the various forms (e.g. classifications) of pulmonary hypertension.

The extensive permeability response seen in PAH reveals important and unanswered questions regarding how fluid accumulates within interstitial spaces, airways and alveoli. We observed large increases in $K_f$ and extensive perivascular fluid cuff formation, but alveolar flooding was not seen by histology. **Supplemental movie 1** illustrates fluid dripping from the lung due to its clearance through open lymphatics, and the appearance of fluid within the trachea. Evan’s blue dye was detected in bronchoalveolar lavage fluid. What remains undetermined is how, using histological approaches that have previously detected alveolar edema fluid, can the alveoli appear devoid of fluid and protein by
histology while Evan’s blue dye is detected by bronchoalveolar lavage. Future studies will be required to identify possible vascular leaks sites in order to track the origin of fluid recovered by bronchoalveolar lavage.

In summary, we demonstrate that TRPC4 controls endothelial cell barrier integrity in both normotensive and PAH circulations. The PAH circulation, in particular, is susceptible to permeability induced by agents that activate store operated calcium entry. While the importance of endothelial hyperpermeability in evolution of PAH remains uncertain, we must now consider its role in promoting the vascular remodeling that increases pulmonary vascular resistance, and we must evaluate whether the fragile nature of the endothelium in this disease increases the risk of certain PAH patients to development of pulmonary edema.
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References


20. Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, and Tuder RM. Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-
specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell


Figure Legends

Figure 1. Thapsigargin induces a dose-dependent increase in $K_f$ that requires TRPC4 expression. [A] After establishing an isogravimetric state, a baseline $K_f$ was measured and thapsigargin at the indicated concentration was added to the reservoir and allowed to recirculate for 15 minutes before a second $K_f$ was measured. Thapsigargin induced a dose-dependent increase in permeability in lungs from wild type (TRPC$^{+/+}$) and TRPC4 knockout (TRPC4$^{-/-}$) rats, estimated by $K_f$. Data are expressed as mean ± SEM. n = 6-10 per concentration and * denotes a significant difference, where $p < 0.05$ using one-way ANOVA with Bonferroni’s post hoc test. [B] Concentration response curves generated from data obtained in Figure 1A reveal the half maximal concentration for increased permeability in TRPC4$^{+/+}$ lungs is approximately 49 nM, similar to that previously reported in Sprague Dawley rat lungs (11). Within the range of concentrations tested, the apparent half-maximal concentration is right shifted to at least 220 nM in TRPC4$^{-/-}$ lungs.

Figure 2. The TRPC4-dependent increase in permeability corresponds with formation of perivascular fluid cuffs, and not with alveolar flooding. [A] Lungs were isolated and perfused as described in Materials and Methods. Baseline perfusion pressures were less than 10 cm H$_2$O and the Fulton index (RV/LV+S) was less than 0.30 in TRPC4$^{+/+}$ and TRPC4$^{-/-}$ lungs, characteristic features of a normotensive circulation. [B] Thapsigargin increased $K_f$ approximately 2-fold in TRPC4$^{+/+}$ lungs, and the increase in permeability was significantly reduced in TRPC4$^{-/-}$ lungs. * denotes $p < 0.05$ using unpaired t test. [C] Thapsigargin promoted fluid accumulation in perivascular cuffs, especially in TRPC4$^{+/+}$ lungs, but did not cause alveolar flooding. Arrows identify perivascular cuffs and L denotes a dilated lymphatic channel.
Figure 3. Hyperpermeability response to thapsigargin is revealed in animals with severe pulmonary arterial hypertension. [A] Lungs from Semaxanib/hypoxia/normoxia-treated animals were isolated and perfused as described in the Materials and Methods. Baseline pulmonary perfusion pressure was approximately 50 cm H₂O and the Fulton index was approximately 0.75 in TRPC4⁺/⁺, TRPC4⁺/-, and TRPC4⁻/⁻ lungs; there were no differences among groups in either of measurements. The dashed line reflects average values reported in normotensive TRPC4⁺/⁺ lungs from Figure 1 for comparison. [B] Thapsigargin (150 nM) increased Kᵣ approximately 10-fold in TRPC4⁺/⁺ lungs. This hyperpermeability response to thapsigargin was not abolished in TRPC4⁻/⁻ lungs at the 150 nM concentration. [C] Thapsigargin induced extensive perivascular fluid cuffs, especially in TRPC4⁺/⁺ lungs (arrows). Arteries and arterioles were remodeled, with evidence for medial hypertrophy, shown by arrowheads. Complex obliterator lesions were resolved in small precapillary arterioles (*), consistent with previous reports in the F344 rat (5). Dilated lymphatics were seen (L). As previously reported (5), complex lesions were less severe in TRPC4⁻/⁻ than in TRPC4⁺/⁺ lungs. [D] Thapsigargin (150 nM) promotes accumulation of Evan’s blue dye conjugated to albumin in the bronchoalveolar lavage of PAH but not normotensive lungs. PAH increases sensitivity to the thapsigargin (150 nM)-induced increase in permeability (Left Panel), consistent with the data shown in Figures 2 and 3. This hyperpermeability response corresponds with the appearance of Evan’s blue dye in the bronchoalveolar lavage fluid, an effect that is not seen in normotensive lungs (Right Panel). Two representative lung images are shown from the normotensive (Left) and PAH (Right) animals. The left PAH lung represents the low responder reported in the accompanying graph, whereas the right PAH lung represents one of the high responders. * denotes p < 0.05 using unpaired t test. BL refers to baseline and TG refers to thapsigargin.

Figure 4. Three-week hypoxia exposure is not sufficient to sustain either the pulmonary arterial hypertension or the hyperpermeability response to thapsigargin. [A] Animals were exposed to
hypoxia (10% oxygen) for three weeks, and then returned to normoxia for an additional two weeks. Pulmonary perfusion pressures were approximately 10 cm H$_2$O and Fulton indexes were approximately 0.30 in both TRPC4$^{+/+}$ and TRPC4$^{-/-}$ lungs. The dashed line reflects average responses reported in normotensive TRPC4$^{+/+}$ lungs from Figure 1 for comparison. P = ns using unpaired t test. [B] Thapsigargin (75 and 150 nM) increased $K_f$ approximately 2-fold in TRPC4$^{+/+}$ lungs. This effect was abolished in TRPC4$^{-/-}$ lungs at 75 nM (p < 0.05 using unpaired t test), but not at 150 nM, thapsigargin. [C] Thapsigargin induced perivascular cuffing without evidence of alveolar flooding, especially apparent in lungs from TRPC4$^{+/+}$ rats (arrows). Despite the normal pulmonary artery pressures seen in [A], pulmonary artery and arteriole media was remodeled, characteristic of the hypertrophy and hyperplasia that accompanies chronic hypoxia exposure (arrowhead).

Figure 5. Semaxanib inoculation is not sufficient to induce pulmonary arterial hypertension or cause a hyperpermeability response to thapsigargin. [A] F344 rats received a single subcutaneous Semaxanib injection, and were then maintained under normoxia for an additional five weeks. Lungs were isolated and perfused as described in the Materials and Methods. Pulmonary perfusion pressures were approximately 10 cm H$_2$O, and the Fulton index was approximately 0.30 in both TRPC4$^{+/+}$ and TRPC4$^{-/-}$ rats. The dashed line reflects average responses reported in normotensive TRPC4$^{+/+}$ lungs from Figure 1 for comparison. P = ns using unpaired t test. [B] Thapsigargin increased $K_f$ approximately 2-fold at 75 and 150 nM concentrations in both TRPC4$^{+/+}$ and TRPC4$^{-/-}$ lungs. P = ns using unpaired t test. [C] Extensive perivascular cuffs were noted in both TRPC4$^{+/+}$ and TRPC4$^{-/-}$ lungs (arrows), while fluid was not seen in alveoli.
Table Legend

Table 1. Examples of studies documenting increased lung permeability in commonly used models of pulmonary hypertension. These additional papers are not specifically highlighted in the discussion, but they provide supportive evidence for an endothelial cell permeability defect in pulmonary arterial hypertension. Endpoints assessing endothelial barrier integrity and/or permeability are highlighted. SD refers to Sprague-Dawley rat, F344 refers to Fischer rat and R26R refers to ROSA26 reporter mouse.
Table 1.

<table>
<thead>
<tr>
<th>Model of Pulmonary Hypertension</th>
<th>Species</th>
<th>Permeability Measurement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monocrotaline</strong></td>
<td>Albino Rat</td>
<td>Endothelial and epithelial cell injury, and interstitial edema</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>Albino Rat</td>
<td>Increased lung weight-to-body weight ratio, extravascular albumin, and interstitial edema</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>Wistar Rat</td>
<td>Increased albumin in bronchoalveolar lavage fluid</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Higher wet lung weight and increased albumin in bronchoalveolar lavage fluid</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased albumin in lung tissue</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased lung wet weight-to-dry weight ratio and lung extravascular albumin content</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Endothelial injury and associated subendothelial edema</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Lung perivascular edema and increased extravascular albumin</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased lung wet-to-dry ratio</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>F344 Rat</td>
<td>Lung alveolar septal edema</td>
<td>(43)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased bronchoalveolar lavage fluid protein and lung wet weight</td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased bronchoalveolar lavage fluid protein and lung wet weight</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td>F344 Rat</td>
<td>Increased bronchoalveolar lavage fluid protein and lung wet weight</td>
<td>(68)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased bronchoalveolar lavage fluid protein and lung wet weight</td>
<td>(69)</td>
</tr>
<tr>
<td></td>
<td>SD Rat</td>
<td>Increased bronchoalveolar lavage fluid protein and lung wet weight</td>
<td>(25)</td>
</tr>
<tr>
<td><strong>Pneumonectomy and Monocrotaline</strong></td>
<td>Wistar Rat</td>
<td>Increased lung water content</td>
<td>(38)</td>
</tr>
<tr>
<td><strong>BMPR II deletion</strong></td>
<td>R26R Mouse</td>
<td>Increased lung tissue albumin</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>R26R Mouse</td>
<td>Increased lung tissue albumin</td>
<td>(8)</td>
</tr>
</tbody>
</table>
Supplemental Movie

Supplemental Movie 1. Thapsigargin induces severe edema in PAH, which impairs lung inflation during ventilation. The lung was isolated from a Semaxanib/hypoxia/normoxia-treated rat, and perfused under conditions described in the Materials and Methods. After establishing an isogravimetric state, thapsigargin (75 nM) was applied to the pulmonary artery and allowed to recirculate for 10 minutes, at which time venous pressure was elevated and a $K_f$ measurement was made for 15 minutes. The movie represents 30 minutes of this experiment compressed into approximately 30 seconds. Following addition of thapsigargin and elevation of venous pressure, fluid drips are seen from the lung due to increased flow through the lymphatics. The filtration rate ultimately exceeds the rate of fluid loss, resulting in severe tissue edema. This tissue edema becomes sufficient to impair lung expansion during ventilation, consistent with decreased lung compliance. While this treatment causes increased Evan’s blue dye accumulation in the bronchoalveolar lavage fluid (see Figure 3D), as seen in the matched histology (see Figure 3C), fluid was not detected in the alveoli. Note that this experiment was not a part of the experimental group summary data.
Figure 1
Zhou et al.
$K_f (\Delta/\Delta_{\text{max}} \%)$ vs. Thapsigargin Log (Dose)

- **TRPC4^{+/+}**
- **TRPC4^{-/-}**
Figure 2
Zhou et al.
Thapsigargin (150 nM)

K_f (fold increase)

TRPC4^{+/+}  TRPC4^{-/-}

*
Normoxia Time Controls

TRPC4 +/-: Thapsigargin (75 nM)
Normotension

TRPC4 −/−: Thapsigargin (75 nM)

Normoxia Time Controls
Figure 3
Zhou et al.
Thapsigargin (150 nM)

[B] $K_f$ (fold increase)

<table>
<thead>
<tr>
<th>TRPC4+/+</th>
<th>TRPC4+/−</th>
<th>TRPC4−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thapsigargin (150 nM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Semaxanib/Hypoxia/Normoxia
TRPC4+/+: Thapsigargin (150 nM)
Semaxanib/Hypoxia/Normoxia
TRPC4⁻/⁻: Thapsigargin (150 nM)
Figure 4
Zhou et al.
Thapsigargin (150 nM)

Thapsigargin (75 nM)

[B]

$K_f$ (fold increase)

TRPC4$^{+/+}$  TRPC4$^{-/-}$

TRPC4$^{+/+}$  TRPC4$^{-/-}$

Thapsigargin (75 nM)

Thapsigargin (150 nM)
Hypoxia/Normoxia
TRPC4^{+/+}: Thapsigargin (75 nM)
[C] Hypoxia/Normoxia
TRPC4⁻/⁻: Thapsigargin (75 nM)
Figure 5
Zhou et al.
Thapsigargin (75 nM)

- TRPC4+/+
- TRPC4−/−

Thapsigargin (150 nM)

- TRPC4+/+
- TRPC4−/−

$K_f$ (fold increase)
Semaxanib/Normoxia
TRPC4 +/-: Thapsigargin (75 nM)
Semaxanib/Normoxia
TRPC4^{-/-}: Thapsigargin (75 nM)