Abstract
The canonical transient receptor potential 4 (TRPC4) protein contributes to the molecular make-up of endothelial store-operated calcium entry channels. Store-operated calcium entry is a prominent mode of calcium influx in endothelium. Store-operated calcium entry channels are activated by inflammatory mediators and growth factors, and in endothelium, this process induces inter-endothelial cell gaps that increase permeability. Pulmonary endothelium within extra-alveolar segments, including pulmonary arteries, is especially sensitive to the activation of store-operated calcium entry. Pulmonary arterial hypertension (PAH) is characterized by endothelial cell dysfunction in arteries. As one of the topics for the 2017 Grover Conference Series, we examined whether an endothelial cell permeability defect accompanies PAH and, if so, whether TRPC4 contributes to this defect. Through a series of studies conducted over the past five years, we find endothelial cell barrier dysfunction occurs early in the progression of experimental PAH. Endothelium within the arterial segment, and perhaps in other vascular segments, is highly susceptible to disruption secondary to both activation of store-operated calcium entry channels and high flow. This phenomenon partly depends upon TRPC4 channels. We discuss whether endothelial cell hyperpermeability is relevant to human disease, and more specifically, whether it is relevant to all groups of pulmonary hypertension.

Keywords
edema, plexiform lesions, store-operated calcium entry, TRPC channels, semaxanib (Sugen 5416)

Introduction
Pulmonary arterial hypertension (PAH) remains a significant clinical problem. Although remarkable strides have been made toward understanding the pathophysiology of this disease, and new therapeutic options continue to be introduced, no available therapy has effectively eliminated disease progression (for reviews, see Chin and Rubin and Lai et al. and references therein). Excessive vasoconstriction, rarefaction, and fixed, obstructive lesions interplay to increase pulmonary artery pressure (PAP) and the workload of the right ventricle, ultimately leading to right heart failure. Most available therapies target mechanisms responsible for excessive vasoconstriction, whereas emerging treatments seek to prevent the progression of vascular obstruction. Ion channels have represented potential targets for treatment of PAH. Inhibitors of voltage-gated (L type) calcium channels have been used for many years. More recently, receptor-operated calcium entry and calcium-sensing receptor channels have been implicated in the pathogenesis of PAH, and these channels may represent translatable targets for therapy. In this 2017 Grover Conference Series, we explore a potential role for canonical transient receptor potential 4 (TRPC4) channels in PAH. TRPC4 channels contribute to store-operated calcium entry in endothelial cells. Activation of these channels increases endothelial cell permeability. Through the study of TRPC4 channels in PAH, we identify...
an endothelial cell permeability defect that accompanies disease progression. In this Grover Conference seminar, we review our lab’s work regarding the contribution of TRPC4 channels to endothelial cell permeability in both the normotensive and hypertensive circulations and consider whether or how increased endothelial cell permeability contributes to this progressive vasculopathy.

**Store-operated calcium entry and endothelial cell permeability: a role for TRPC4 ion channels**

Store-operated calcium entry is the principal mode of calcium influx in non-excitable cells, including endothelium. However, the molecular basis of ion channels responsible for endothelial cell store-operated calcium entry remains incompletely understood. TRPC channels were among the first putative molecular candidates for store-operated channels. These proteins possess intracellular amino- and carboxy-termini with six intermediate transmembrane spanning domains. Functional channels do not comprise monocarboxy-termini with six intermediate transmembrane spanning domains. Functional channels do not comprise monomeric TRPC proteins but, rather, heterotetrameric TRPC proteins. Endogenously expressed endothelial cell store-operated calcium entry channels in vivo and in vitro contain a combination of TRPC1 and TRPC4 subunits. Protein 4.1 is also a part of this larger complex. It binds to the carboxy-terminus near the channel pore and serves as a gate for opening the channel. Studies addressing the complete channel stoichiometry, and how and where the channel complex is organized, are still ongoing.

TRPC1/TRPC4 is activated by endoplasmic reticulum store depletion. This store depletion occurs transiently in response to inflammatory agonists that activate Gq-coupled receptors and it occurs more stably in response to the plant alkaloid thapsigargin. In either case, calcium store depletion promotes a TRPC1/TRPC4-dependent increase in calcium influx that increases cytosolic calcium. In whole cell electrophysiology experiments, calcium store depletion elicits activation of membrane current, the so-called I_{SOC}. This I_{SOC} is a small inwardly rectifying current with a reversal potential of \(\approx +40\) mV, suggesting it has some calcium selectivity. It appears that calcium selectivity of I_{SOC} while being dependent upon TRPC4, also requires the presence of Orai1. The nature of interaction between the endothelial cell TRPC1/TRPC4 channel and Orai1 is not fully resolved. Orai1 may fulfill the role of a subunit for this larger channel complex or it may be an associated, yet independent, ion channel.

Hyperpermeability in the arterial segment

Although activation of store-operated calcium entry channels promotes permeability in the pulmonary artery, the physiological importance of increased permeability in this segment is not clear. Fluid accumulation in the peri-arterial cuff reduces lung compliance, perhaps relevant to asthma and high-altitude pulmonary edema. We also wondered whether arterial hyperpermeability contributes to PAH, since endothelial cell dysfunction is a cardinal feature of this disease.

PAH is characterized by medial hypertrophy and adventitial thickening of arteries, and rarefaction and occlusive neointimal lesions in small precapillary arterioles. Endothelial cell dysfunction has been described in both these vascular segments; however, the type of dysfunction reported is clearly different. In the former case, endothelial cell dysfunction includes an imbalance of the production of vasodilators and vasoconstrictors, whereas in the latter case, endothelial cell dysfunction includes an imbalance of the production of vasodilators and vasoconstrictors, whereas in the latter case, endothelial cell dysfunction includes apoptosis and overgrowth of apoptosis-resistant cells that occlude the lumen. Here, we consider evidence that pulmonary arteries also have a permeability defect.

Evidence for an endothelial cell permeability defect has been consistently reported in animal models of PAH, usually in monocrotaline-induced PAH (see Zhou et al. and references therein). Most reports of endothelial cell dysfunction in the arterial segment highlight increased wet to dry weights with accumulation of interstitial edema. This issue has more recently been addressed in heritable PAH. Mice harboring a genetic knock-in with the bone morphogenetic protein receptor 2-R899X, or BMPR2R899X, mutation develop mild PAH, and they have increased basal and LPS-stimulated permeability responses. Pulmonary artery endothelial cells obtained from these mice, and blood outgrowth endothelial cells obtained from humans
with this dysfunctional BMPR2 mutation, are also hyperpermeable.\textsuperscript{38} Similarly, mice harboring the $\text{BMPR2}\Delta\text{ex5-6}/+\text{mutation}$ exhibit a permeability defect.\textsuperscript{39} Thus, pulmonary artery endothelial cell hyperpermeability is a characteristic of both monocrotaline-induced and heritable forms of PAH.

The Voelkel and Tuder labs discovered that rats pretreated with the vascular endothelial growth factor receptor 2 inhibitor SUGEN5416 (e.g. Semaxinib) and exposed to three weeks of hypoxia (e.g. $\approx 10\%$ oxygen) develop PAH that is progressive when the animals are returned to normoxia.\textsuperscript{40} Hypertension worsens through at least 13 weeks, and at this later time point small precapillary arterioles possess occlusive neointimal lesions, including plexiform lesions, that resemble the human condition.\textsuperscript{41} Thus, this SUGEN/Hypoxia/Normoxia model has become a widely used animal model for PAH research. Sprague-Dawley rats represent the most widely used animal in this model. One of the caveats to this model is that Sprague-Dawley rats do not progress to heart failure, bringing into question how the pulmonary vascular remodeling is functionally related to right heart failure.

Our group tested the SUGEN/Hypoxia/Normoxia model in Fischer 344 rats.\textsuperscript{42} These animals developed severe PAH at the eight-week time point (Fig. 1a). By eight weeks, extensive occlusive neointimal lesions were seen. Lumens were $> 50\%$ occluded in nearly $70\%$ of the lesions quantified. Using the Heath–Edwards classifications, $5–10\%$ of the vessels showed evidence of grade VI lesions, with fibrinoid necrosis and perivascular inflammation (e.g. necrotizing arteritis). Approximately $20\%$ of the arterioles were

![Fig. 1. SUGEN/Hypoxia/Normoxia elicits similar increases in PAH in Sprague-Dawley and Fischer 344 rat (F344) strains, although F344 animals progress to death. (a) Animals were exposed to eight weeks of the SUGEN/Hypoxia/Normoxia protocol and hemodynamic status tested. Right ventricular systolic pressure (RVSP) and the Fulton Index (RV/LV + S) were increased, whereas the cardiac index (CI) was reduced, in both rat strains. Normotensive values from historic data are shown in the dashed line. (b) SUGEN/Hypoxia/Normoxia-exposed F344 animals developed severe occlusive lesions, as determined by assessment of vascular occlusion density and the Heath-Edwards classification. Lesion density was less pronounced in TRPC4 KO rats. (c) Whereas SUGEN/Hypoxia/Normoxia treatment does not cause PAH that progresses to death in Sprague-Dawley rats, death is prominent in F344 animals. However, TRPC4 KO animals have a survival benefit. *, Significance differences from baseline values. This figure is adapted from Alzoubi et al.\textsuperscript{42}](image)
determined to have grade IV, or plexiform, lesions. In total, 90% of the vessels displayed some abnormality (Fig. 1b). However, what was particularly unique about these animals is that the PAH progressed to acute death due to apparent right heart failure (Fig. 1c). We also noted in these studies that Fischer rats with a TRPC4 knockout (KO) had a survival benefit, with less pronounced vascular remodeling. Our study in the Fischer rat was corroborated by Jiang et al., who reported that 78% of the Fischer rats exposed to SUGEN/Hypoxia/Normoxia die, with evidence of dilated right ventricular failure. Thus, SUGEN/Hypoxia/Normoxia elicits a form of PAH in Fischer rats that progresses to death, similar to the human condition.

The work of Jiang et al. was also notable because they identified a strain of Sprague-Dawley rats that progressed to death. These animals displayed some sensitivity to SUGEN alone, with an increase in the Fulton Index and roughly 20% mortality. Exposure to both SUGEN/Hypoxia similarly increased the Fulton Index, but death rates increased to nearly 50% in these animals. Genetic analysis comparing the animals that do not progress to right ventricular failure versus those who do may shed novel insight into mechanisms coupling pulmonary vascular remodeling and right heart dysfunction.

To determine whether an endothelial cell permeability defect accompanies development of PAH in the Fischer rat, we evaluated the filtration coefficient in isolated and perfused lungs at the five-week time point (e.g. SUGEN/3-weeks hypoxia/2-weeks normoxia). Lungs from these hypertensive animals were extremely sensitive to activation of store-operated calcium entry channels using thapsigargin. Whereas thapsigargin modestly increased permeability in the Fischer normotensive lungs, it increased permeability several-fold in the Fischer hypertensive lungs (Fig. 2). Extensive edema fluid was seen coursing through the interstitial spaces of these lungs. Thus, the SUGEN/Hypoxia/Normoxia experimental model in the Fischer rat is characterized by a significant permeability defect, including endothelial dysfunction within the arterial segment.

### Studies on TRPC4 in PAH

We examined whether TRPC4 contributes to the permeability defect in PAH. To test this idea, we used a TRPC4 KO rat that was generated on a Fischer background. Filtration coefficient was tested over a range of thapsigargin concentrations in both normotensive and hypertensive lungs. Thapsigargin induced a dose-dependent increase in permeability in the normotensive wild-type (WT) animals, an
TRPC4 ion channel contributes to endothelial cell calcium oscillations in normotensive and hypertensive pulmonary arteries. Pulmonary arteries were isolated from WT littermate controls and TRPC4 KO normotensive and hypertensive Fischer rats. The vessels were cut longitudinally, pinned flat in a customized viewing chamber, and loaded with the Fluo-4 calcium indicator. Cytosolic calcium was imaged for 3 min under baseline conditions, thapsigargin (75 μM) was added, and then cytosolic calcium was imaged for another 3-min period. Images were captured at 9.8 frames/s. (a) Endothelial cells elicit infrequent cytosolic calcium oscillations under baseline conditions. The occurrence of these basal oscillations was similar in both WT controls and TRPC4 KO vessels but was significantly increased in hypertensive arteries. The occurrence of cytosolic calcium oscillations was reduced in hypertensive arteries from TRPC4 KO animals. (b) Thapsigargin increased the occurrence of cytosolic calcium oscillations in the hypertensive arteries, but these events were less prevalent in the TRPC4 KO arteries. This figure is adapted from Francis et al.44
effect that was impaired in TRPC4 KO lungs (Fig. 3a). The hypertensive lungs displayed high sensitivity to thapsigargin, as described above, and this effect was also significantly reduced in lungs from the KO animals (Fig. 3b). It is notable that there was no difference in either the Fulton Index or the PAP among WT and KO animals. However, TRPC4 KO animals displayed fewer and/or less severe occlusive neointimal lesions than the WT animals, and as previously shown in Fig. 1c, they had a survival benefit. Thus, TRPC4 impacts endothelial cell function in the SUGEN/Hypoxia/Normoxia model of PAH by controlling endothelial hyperproliferation, barrier integrity, and survival.

**Calcium oscillations and pulmonary artery endothelial cell permeability**

We wondered how TRPC4 contributes to the hyperpermeable endothelial cell phenotype in experimental PAH. To address this issue, conduit pulmonary artery segments were isolated from normotensive and hypertensive animals, the endothelium was loaded with the Fluo-4 calcium dye, and calcium transients were measured. Whereas most studies examine endothelial cell cytosolic calcium with low temporal resolution, in these studies, we measured the calcium signal at 9.8 frames/s. This faster resolution enabled measurement of fast calcium oscillations, or transients, which would be missed when measuring at slower time constants. We noted that normotensive pulmonary artery endothelial cells had infrequent calcium oscillations under resting conditions (Fig. 4a). The number of these “spontaneous” calcium oscillations was increased significantly in the hypertensive artery endothelium. TRPC4 KO had little impact on the number or duration of calcium oscillations in normotensive vessels but decreased the endothelial cell oscillations significantly in hypertensive arteries. Thus, experimental PAH is accompanied by an increase in cytosolic calcium oscillations at baseline, and the number of these events is TRPC4-dependent.

Thapsigargin elicits a large, sustained increase in endothelial cell cytosolic calcium. However, by measuring calcium signals at high frequency, we found that cytosolic calcium oscillations are superimposed on the global rise in cytosolic calcium. The number of these calcium oscillations expanded greatly in hypertensive pulmonary arteries and they were significantly reduced in pulmonary artery endothelium from TRPC4 KO animals (Fig. 4b).

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**Fig. 5.** The pulmonary arterial hypertensive circulation does not accommodate high perfusion rates. PAP and lung weight were measured in response to increased perfusion rates in normotensive and hypertensive Fischer rat lungs. Hypertension was induced using the five-week SUGEN/Hypoxia/Normoxia protocol. (a) Normotensive lungs accommodate large increases in perfusion without a large increase in either PAP or permeability. (b) PAP increases at low perfusion rates in hypertensive lungs, leading to increased permeability. Two populations of responders were identified based upon their flow-pressure response, including high and low responders. Dashed lines represent the flow-pressure response in normotensive lungs. *Significantly different from baseline values. This figure is adapted from Zhou et al.**
The permeability defect in experimental PAH was closely aligned with the prominence of endothelial cell calcium oscillations, more so than with the global rise in cytosolic calcium. Moving forward it will be important to assess how these transient changes in cytosolic calcium couple to protein complexes that regulate barrier integrity, such as VE-cadherin and other elements of the contractile cytoskeleton.

**Flow-dependent hyperpermeability in PAH**

As discussed below (see “Summary: the clinical translation of experimental PAH”), PAH is not a primary permeability disorder, yet endothelial cell dysfunction is a well-recognized component of the vasculopathy. We therefore wondered how well the endothelium in the hypertensive circulation accommodates increased blood flow, which would be relevant to the complex blood flow patterns in this disease and increased cardiac output during exercise. To test this idea, normotensive and hypertensive rat lungs were isolated and perfused with a salt solution containing 6% autologous whole blood. Perfusion was initiated at just 8 mL/min and increased every 5 min until the circulation could no longer accommodate the flow (Fig. 5a). Flow was increased in the normotensive circulation to 96 mL/min with only modest increases in PAP and lung weight. In contrast, in the hypertensive circulation (e.g. five weeks of SUGEN/Hypoxia/Normoxia), flow could only be increased to 56–72 mL/min before PAP and lung weight were grossly increased (Fig. 5b). Thus, flow (e.g. increased cardiac output) elicits an increase in permeability in PAH.

**Intolerance to retrograde perfusion in PAH**

The normotensive pulmonary circulation tolerates blood flow in both forward (e.g. anterograde) and reverse (e.g. retrograde) orientations. We wondered whether the hypertensive pulmonary circulation could also accommodate retrograde perfusion. To test this idea, flow-pressure responses were tested in isolated perfused lungs from normotensive and hypertensive animals. Retrograde perfusion was supported in the normotensive circulation, similar to forward flow experiments. However, retrograde perfusion was not tolerated in the hypertensive pulmonary circulation. In these experiments, the perfusate failed to return through the arteries to the reservoir. Within minutes, the airspace was filled with fluid and frothy edema was cleared through the trachea. Retrograde perfusion was not rescued by either the vasodilators Fasudil (10 μM) and SKF-96365 (50 μM) or...
increasing flow rates. However, retrograde perfusion was tolerated in lungs isolated from animals with hypoxic (10% oxygen for three weeks) hypertension, and in normotensive lungs subjected to high outflow pressures. Therefore, Fischer rats exposed to the SUGEN/Hypoxia/Normoxia protocol for five weeks develop a form of vascular remodeling that does not allow for retrograde perfusion, resulting in abrupt alveolar flooding.

We tested the time course necessary for the hypertensive circulation to acquire an intolerance to retrograde perfusion. The pulmonary circulation was evaluated after one and three weeks of the SUGEN/Hypoxia protocol. PAH was progressive from one to five weeks, as both the Fulton Index and the severity of vascular remodeling worsened (Fig. 6). Retrograde perfusion was tolerated at both the one-week and three-week time points. However, at the three-week time point, only lower flow rates could be retroperfused. Thus, as vascular remodeling becomes more severe, the circulation does not accommodate retrograde perfusion and it becomes susceptible to endothelial cell barrier disruption. Retrograde perfusion represents a novel way to assess the severity of vascular remodeling in PAH.

Summary: the clinical translation of experimental PAH

Endothelial cell dysfunction contributes to PAH. Endothelial cell abnormalities are commonly noted in this disease, including: (1) an imbalance in the arteriolar production of vasoconstrictors and vasodilators; (2) endothelial cell apoptosis possibly contributing to rarefaction; and (3) the overgrowth of apoptosis resistant cells that lose the “law of the monolayer” and contribute to occlusive neointimal lesion formation. In this series of experiments, we highlight an additional endothelial cell defect in severe experimental PAH, where arterial (and perhaps other segments) endothelial cells become hyperpermeable. This defect is evident when the endothelium is exposed to stimuli that activate store-operated calcium entry channels and when the circulation is exposed to increased flow rates. TRPC4 channels contribute to this phenomenon, especially when using stimuli that activate store operated calcium entry.

The clinical relevance of this finding in experimental PAH remains unknown. However, two independent labs recently reported that BMPR2 mutations elicit increased endothelial cell permeability. It may be that patients with these, or other related, mutations are susceptible to increased endothelial permeability. Clearly not all patients with pulmonary hypertension have a hyperpermeable endothelial cell defect. Hypoxic hypertension, for example, decreases endothelial cell permeability. Left heart failure also leads to venule and capillary remodeling that reduces endothelial cell permeability. It is therefore most likely that endothelial cell hyperpermeability contributes to a subset of Group I PAH patients and not to other causes of this disease. This idea will need to be tested in future experiments.

It is not clear how an endothelial cell permeability defect, especially in the arterial segment, would impact disease progression in group 1 PAH. A hyperpermeable surface may allow inflammatory mediators and growth factors to access the underlying smooth muscle and promote cell growth and differentiation. It may also promote interstitial edema and contribute to dyspnea and exercise limitation. In addition, it may increase the risk of developing pulmonary edema during infection and other inflammatory or immune challenges. For now, these issues have not been resolved and represent important areas of future investigation.

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Conflict of interest

The author(s) declare that there is no conflict of interest.

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