Protective role of FKBP51 in calcium entry-induced endothelial barrier disruption

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Abstract

Pulmonary artery endothelial cells (PAECs) express a cation current, \( I_{\text{SOC}} \) (store-operated calcium entry current), which when activated permits calcium entry leading to inter-endothelial cell gap formation. The large molecular weight immunophilin FKBP51 inhibits \( I_{\text{SOC}} \) but not other calcium entry pathways in PAECs. However, it is unknown whether FKBP51-mediated inhibition of \( I_{\text{SOC}} \) is sufficient to protect the endothelial barrier from calcium entry-induced disruption. The major objective of this study was to determine whether FKBP51-mediated inhibition of \( I_{\text{SOC}} \) leads to decreased calcium entry-induced inter-endothelial gap formation and thus preservation of the endothelial barrier. Here, we measured the effects of thapsigargin-induced \( I_{\text{SOC}} \) on the endothelial barrier in control and FKBP51 overexpressing PAECs. FKBP51 overexpression decreased actin stress fiber and inter-endothelial cell gap formation in addition to attenuating the decrease in resistance observed with control cells using electric cell-substrate impedance sensing. Finally, the thapsigargin-induced increase in dextran flux was abolished in FKBP51 overexpressing PAECs. We then measured endothelial permeability in perfused lungs of FKBP51 knockout (FKBP51−/−) mice and observed increased calcium entry-induced permeability compared to wild-type mice. To begin to dissect the mechanism underlying the FKBP51-mediated inhibition of \( I_{\text{SOC}} \), a second goal of this study was to determine the role of the microtubule network. We observed that FKBP51 overexpressing PAECs exhibited increased microtubule polymerization that is critical for inhibition of \( I_{\text{SOC}} \) by FKBP51. Overall, we have identified FKBP51 as a novel regulator of endothelial barrier integrity, and these findings are significant as they reveal a protective mechanism for endothelium against calcium entry-induced disruption.

Keywords
FKBP51, \( I_{\text{SOC}} \), calcium, endothelial barrier, cytoskeleton

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Endothelial cells line the inside of blood vessels forming a semi-permeable barrier that is critical for blood and tissue homeostasis. Disruption of the endothelial barrier due to formation of inter-endothelial cell gap formation leads to increased permeability.1 In pulmonary endothelial cells, calcium entry through a store-operated calcium (SOC) channel, ISOC, leads to inter-endothelial cell gap formation and endothelial barrier disruption.2–4 ISOC is a small, inwardly rectifying current that can be activated by the Ca\(^{2+}\)-ATPase blocker thapsigargin.2–7 While ISOC conducts both calcium and sodium ions, it exhibits anomalous mole fraction...
behavior reflecting moderate selectivity for calcium. Indeed, it is calcium permeation which is important for inter-endothelial cell gap formation, as incubation of cells in the absence of extracellular calcium but in the presence of sodium does not lead to thapsigargin-induced gap formation. As such, it would be expected that inhibition of calcium entry through ISOC will preserve the endothelial barrier. However, it has not yet been determined whether inhibition of ISOC is endothelial barrier protective and this was one focus of the current study.

In order to study ISOC inhibition, it is important to first identify interacting proteins that play a role in ISOC channel function. The ISOC channel is tethered to the cytoskeleton and its activation depends upon an intact spectrin–protein 4.1–TRPC4 interaction. TRPC4 is a member of the transient receptor potential canonical (TRPC) protein subfamily of the transient receptor potential (TRP) protein superfamily and is likely a pore-forming subunit of the ISOC channel. TRPC4 interacts with TRPC1 as well as with orai1 which influences channel activity and calcium selectivity. More recently, we demonstrated that the large molecular weight immunophilins FKBP51 and FKBP52 are also part of the ISOC channel heterocomplex.

FKBP51 and FKBP52 are members of the FK506-binding protein immunophilin family that are best known for their roles in steroid receptor signaling, where they have opposing effects on receptor function. Whereas FKBP52 potentiates steroid receptor activation, FKBP51 inhibits activation. We have shown similar opposing roles of FKBP52 and FKBP51 in calcium signaling. More specifically, FKBP52 promotes thapsigargin-induced calcium entry in rat pulmonary artery endothelial cells (PAECs) whereas FKBP51 inhibits calcium entry through ISOC. As ISOC inhibition leads to the formation of inter-endothelial cell gaps, the FKBP51-mediated inhibition of ISOC may be of pharmacological value. However, it is unknown whether alteration of FKBP51 levels in PAECs directly affects inter-endothelial cell gap formation and barrier integrity. Addressing this question was the major focus of the current study. To study the relationship between FKBP51 and endothelial barrier function, we generated a PAEC cell line that stably overexpresses FKBP51. Overexpression of FKBP51 led to inhibition of thapsigargin-induced ISOC and preservation of the endothelial barrier as assessed by decreased actin stress fiber formation and decreased inter-endothelial cell gap size and quantity. Additionally, resistance changes measured via electric cell-substrate impedance sensing (ECIS) were negligible and there was no increase in dextran flux in FKBP51 overexpressing cells following thapsigargin treatment. In situ, Evans Blue dye-conjugated albumin tissue permeation was greater in lungs from FKBP51 knockout (FKBP51−/−) mice following calcium entry compared to wild-type (WT) mice. Overall, these data suggest that the physiological role of FKBP51 is to limit calcium entry-induced endothelial barrier disruption.

Mechanisms underlying the effects of FKBP51 on thapsigargin-induced ISOC in PAECs are not yet understood. As FKBP51 has been implicated in regulating microtubule polymerization, and ISOC channel function is sensitive to changes in microtubule polymerization, we investigated the role of the microtubule network in the FKBP51-mediated inhibition of ISOC. FKBP51 overexpressing PAECs exhibited a more extensive microtubule network compared to control cells. Cells were then treated with nocodazole at different concentrations to achieve a range of partial to almost complete microtubule depolymerization. At low nocodazole concentrations, which affected only limited microtubule depolymerization, FKBP51 was unable to inhibit ISOC. Thus, FKBP51 regulates microtubule polymerization that is critical for the inhibition of ISOC. Collectively, our observations reveal that FKBP51 is a regulator of endothelial barrier integrity, and by inhibiting ISOC it serves to protect endothelial cells from actin rearrangement and endothelial barrier disruption.

Methods

Reagents

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Cell culture medium was obtained from Thermo Fisher Scientific (Waltham, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA) and penicillin/streptomycin was obtained from Thermo Fisher Scientific. EquaFETAL® bovine serum (EBS) and fetal bovine serum (FBS) were purchased from Atlas Biologicals, Inc. (Fort Collins, CO, USA) and Thermo Fisher Scientific, respectively. Hank’s Balanced Salt Solution (HBSS) was purchased from Thermo Fisher Scientific. Monoclonal antibodies to FKBP51 and expression plasmids for human FKBP51 have been described previously. β-actin antibody was purchased from Santa Cruz Biotechnology. Doxycycline was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA).

Cell culture

PAECs were isolated from Sprague-Dawley rats and characterized as described. Cells are grown in high glucose DMEM supplemented with 10% FBS or EBS, penicillin G (50 U/mL), and streptomycin (0.05 mg/mL). Animal work was approved by the University of South Alabama Institutional Animal Care and Use Committee and was performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

FKBP51 overexpressing cells were generated by lentiviral transduction. Production of lentiviral supernatants and infection of target cells was performed as described previously. Briefly, lentivirus containing supernatants were produced by CaPO4-mediated transfection of HEK293FT cell lines using established protocols.
functions for lentiviral constructs were provided in trans by cotransfection of the vector plasmid with two helper plasmids, psPAX2 and pMD2.G (both gifts from Didier Trono [Ecole Polytechnique Federale de Lausanne; Addgene (Cambridge, MA, USA)] plasmid #12260 and #12259, respectively). Transduction of PAECs with the lentivirus encoding human FKBP51 (lV3899) was performed in 35-mm dishes at 50% confluence by incubating them overnight with corresponding supernatant in the presence of 10 μg/mL polybrene. The next day, supernatant was removed and cells were allowed to recover for 24 h in fresh DMEM after which they were selected with puromycin (1 μg/mL) for three days.

**Quantitative polymerase chain reaction (qPCR)**

Messenger RNA was prepared from PAECs using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and reverse transcribed to complementary DNA (cDNA) using an iScript cDNA preparation kit (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed by SYBR Green incorporation using a CFX Connect (Bio-Rad) thermocycler with the following primers:

- rHPRT1qPCR Forward 5'-GACCTCTCAGMTGGGATAC-3'
- rHPRT1qPCR Reverse 5'-TCAAATCCCTGAAGTGCTCAT-3'
- hFKBP51qPCR Forward 5'-GCTGAGCAGGGAGAGGATAT-3'
- hFKBP51qPCR Reverse 5'-TCTCCAATCATCGGCGTTTC-3'

**Western blotting**

Cells were lysed by scraping and sonification for 10 s (Branson Digital Sonifer model S-450D; Branson, Danbury, CT, USA) at 12% output in RIPA buffer (Boston Bioproducts; Ashland, MA, USA) with 1% protease inhibitor cocktail followed by centrifugation at 12,000 × g for 20 min. Whole cell lysates were electrophoresed on 4–12% bis-Tris gels (Thermo Fisher Scientific). Proteins were transferred to nitrocellulose membranes at 100 V. Membranes were blocked with milk (5% non-fat dry milk/0.2% BSA in PBS supplemented with 0.1% Tween-20) for 1 h and then rocked with primary antibodies overnight at 4°C and with secondary antibodies for 1 h at room temperature. Supersignal West Pico or West Femto chemiluminescent substrates (Thermo Fisher Scientific) were used to record the current evoked by step depolarization for 20 ms at room temperature. Currents were measured with an Axopatch 200B Amplifier (Molecular Devices; Sunnyvale, CA, USA). PClamp10 software was used to record the current evoked by step depolarization in 20 mV increments from −100 to +80 mV. Currents were measured as the mean value of the current amplitude during the last 20 ms of each step. The standard pipette solution contained (in mmol/L) 130 N-methyl-D-glucamine, 10 Hepes, 2 Mg²⁺-ATP, 1 N-phenylanthranilic acid,
FKBP51 Protects the Endothelial Barrier

Male and female mice aged 10–12-weeks (20–25 g) were used in this study. To perform the in-chest lung perfusion protocol, anesthetized mice were weighed and tracheostomized. Lungs were exposed by removal of the anterior chest wall and cannulas inserted in the pulmonary artery and left ventricle. Lungs were then perfused in situ under constant flow (2 mL/min) without recirculation with 4% bovine serum albumin (BSA) in HBSS buffer containing 2 mM Ca^{2+} at 37°C for 15 min after which they were challenged with 30 mM thapsigargin for 15 min. Finally, 2.5 mL (0.1 g dye/5% BSA) albumin-bound Evans Blue were added to 45 mL perfusate for an additional 15 min. Lungs were then excised, blotted dry, weighed, and homogenized in PBS (1 mL·100 μg⁻¹ tissue) and incubated with two volumes...
of formamide (18 h, 60°C) followed by centrifugation at 12,000 × g for 20 min. Optical density of the supernatant was determined by spectrophotometry at 620 nm.

**Statistical analysis**

Statistical analysis was performed using GraphPad version 5.0 software (San Diego, CA, USA). Data are presented as mean ± SEM. Comparisons between multiple groups were performed using a two-way ANOVA and the Bonferroni post hoc test. Student’s t-tests were used elsewhere. Values were considered significantly different when P < 0.05. Sample size for in vitro experiments is the number of independent experiments using independently passaged cell cultures. For in situ experiments, we used the minimum number of animals required to achieve statistical significance.

**Results**

**Characterization of \( I_{SOC} \) and actin stress fiber formation in PAECs**

Thapsigargin, a plant alkaloid, causes emptying of the endoplasmic reticulum calcium store by blocking the SERCA \( Ca^{2+}/ATPase \) pump.\(^{24}\) In PAECs, thapsigargin treatment leads to global calcium entry that is sensitive to 2-aminoethoxydiphenyl borate (2-APB) and YM-58483.\(^{25}\) While both 2-APB and YM-58483 are SOC entry inhibitors, 2-APB is considered to be relatively non-specific,\(^{26}\) whereas YM-58483 is a potent inhibitor of the \( I_{CRAC} \) (calcium release activated calcium current).\(^{27}\) We were particularly interested in 2-APB because it blocks calcium entry through some TRPC channels,\(^{28–30}\) and the endothelial ISOC channel is composed of at least TRPC1 and TRPC4 subunits.\(^{5,31}\) Xu et al.\(^{25}\) showed that treatment of PAECs with 2-APB resulted in a \( \sim 50\% \) decrease in thapsigargin-induced global calcium entry. Similarly, YM-58483 decreased global calcium entry by \( \sim 50\% \), overall suggesting that thapsigargin activates multiple calcium entry pathways in PAECs. Indeed, one of these pathways is \( I_{SOC};^{2,3,5–7} \) however, it is unknown whether \( I_{SOC} \) is sensitive to 2-APB or YM-58483. We thus performed patch-clamp electrophysiology to measure \( I_{SOC} \) in the presence of 2-APB or YM-58483. Thapsigargin treatment activated \( I_{SOC} \), a small current of –22.8 ± 2.5 pA at –40 mV that has a reversal potential of \( \sim +32 \) mV (Fig. 1a), and lanthanum reduced the current to noise level, consistent with that previously reported.\(^{4}\) 2-APB reduced \( I_{SOC} \) by \( \sim 46\% \) (average

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**Fig. 1.** \( I_{SOC} \) inhibition is associated with decreased actin stress fiber formation. (a) (Top) Current tracings of \( I_{SOC} \) activated by thapsigargin (1 μM) at testing potentials -100 mV to +80 mV. (Bottom) Corresponding I/V curves. In control PAECs or PAECs treated with the \( I_{CRAC} \) blocker YM-58483 (10 μM), thapsigargin activates a normal \( I_{SOC} \). In PAECs treated with La\(^{3+}\) (50 μM) or the store-operated channel blocker 2-APB (75 μM), \( I_{SOC} \) was significantly decreased. \(*P < 0.05\) for 2-APB vs. control and La\(^{3+}\) vs. control; n = 3. (b) Cytochemistry staining for actin stress fibers reveals stress fiber formation in PAECs only when \( I_{SOC} \) is activated. (Top) Maximum \( I_{SOC} \) currents are shown at each testing potential under different conditions. (Bottom: top panel) Whole field view of PAECs treated with thapsigargin and stained for actin stress fibers. Cells were bathed in the same solution (\( I_{SOC} \) solution) used in patch-clamp electrophysiology so that upon thapsigargin application cation current was reflective of calcium entry. PAECs were also treated with YM-58483 when in non-selective solution, HBSS + \text{Ca}^{2+} (1.3 mM). (Bottom: bottom panel) Expanded view of inset from top panel. Thapsigargin-induced \( I_{SOC} \) resulted in actin stress fiber formation (arrowheads) in control PAECs and in PAECs treated with \( I_{CRAC} \) blocker YM-58483. However, stress fiber formation was prevented and cortical actin maintained (arrows) when cells were treated with La\(^{3+}\) or 2-APB, which block \( I_{SOC} \). Images were acquired at 40X magnification. Images shown are representative of n = 3.
differences at all testing potentials) whereas YM-58483 had no appreciable effect. The observation that $I_{\text{SOC}}$ is sensitive to 2-APB but not YM-58483 is consistent with TRPCs 1 and 4 contributing to the ISOC channel make-up.

We next wanted to determine whether inhibition of $I_{\text{SOC}}$ leads to decreased thapsigargin-induced stress fiber formation. It was previously shown that thapsigargin treatment of PAECs leads to downstream actin stress fiber and inter-endothelial cell gap formation. As thapsigargin activates multiple calcium entry pathways, it was not clear whether the $I_{\text{SOC}}$ pathway in particular is important for actin stress fiber formation. To address this question, we performed cytochemistry of thapsigargin-treated PAEC monolayers using Rhodamine-phalloidin to visualize actin filaments. To rule out contributions from other ionic currents, e.g. Cl$^{-}$, K$^{+}$ and Na$^{+}$, the cells were bathed in the same solution (which we refer to as $I_{\text{SOC}}$ solution) that is used for patch-clamp electrophysiology. In this way, any stress fiber formation that occurs will be the result of calcium current. Additionally, cells were treated with the $I_{\text{SOC}}$ inhibitors, La$^{3+}$ and 2-APB, or with the $I_{\text{CRAC}}$ inhibitor YM-58483.

In PAECs treated with thapsigargin, $I_{\text{SOC}}$ was activated and actin stress fibers formed throughout the monolayer (Fig. 1b). In the presence of La$^{3+}$ or 2-APB, both of which inhibited $I_{\text{SOC}}$, thapsigargin-induced actin stress fiber formation was abolished. In the presence of YM-58483, which did not inhibit $I_{\text{SOC}}$, actin stress fibers still formed, although at decreased levels compared to control PAECs. We also tested YM-58483 when the cells were bathed in HBSS with calcium instead of the $I_{\text{SOC}}$ solution. Here in the presence of YM-58483, again we observed actin stress fiber formation. Overall, these observations suggest that calcium entry must occur through specific channels, one in particular being the ISOC channel, for actin stress fibers to form. As $I_{\text{SOC}}$ activation leads to actin stress fiber and inter-endothelial cell gap formation, inhibition of $I_{\text{SOC}}$ should be protective to the endothelial barrier. Indeed, here we show that inhibition of $I_{\text{SOC}}$ correlates with decreased stress fiber formation. A caveat however is that both La$^{3+}$ and 2-APB can block other channels in addition to the ISOC channel. We have identified another $I_{\text{SOC}}$ inhibitor, the immunophilin FKBP51, although it has not yet been determined whether inhibition of $I_{\text{SOC}}$ by FKBP51 is protective to the endothelial barrier.

**Increased FKBP51 expression inhibits $I_{\text{SOC}}$ in PAECs**

We have previously demonstrated that treatment of PAECs with dexamethasone upregulates FKBP51 expression leading to decreased thapsigargin-induced $I_{\text{SOC}}$. In that study we also demonstrated FKBP51-mediated inhibition of $I_{\text{SOC}}$ in doxycycline-inducible FKBP51 HEK293 cells. However, to be certain that the dexamethasone effect on $I_{\text{SOC}}$ in PAECs was specifically due to FKBP51 upregulation, in the current study we generated via lentiviral construct a PAEC cell line that constitutively overexpresses FKBP51 (cFKBP51). This cell line also gave us a tool to study the effect of FKBP51 on endothelial barrier function. qPCR and western blot analysis confirmed upregulation of FKBP51 (Fig. 2a and b). At the protein level, FKBP51 expression was increased by $3.1\pm0.5$-fold compared to control cells. Using whole cell patch-clamp electrophysiology, $I_{\text{SOC}}$ was decreased by $\sim40\%$ in the FKBP51 overexpressing cells compared to control cells (Fig. 2c).

We then measured global calcium entry following thapsigargin treatment using the ratiometric dye Fura 2/AM. Using a calcium add-back approach, we observed that cFKBP51 PAECs exhibited calcium entry that was not statistically different from control cells (Fig. 2d and e). Whereas increased FKBP51 expression in PAECs does not change the global calcium entry, it does inhibit $I_{\text{SOC}}$. Taking into consideration that measurement of global calcium reflects calcium entry through all thapsigargin-activated calcium channels, these observations suggest that FKBP51 is principally acting on the ISOC channel and not other calcium entry channels.

**Increased FKBP51 expression inhibits calcium entry-induced stress fiber formation**

As we have shown that FKBP51 is principally inhibiting calcium entry through the ISOC channel in PAECs, we next went on to determine whether inhibition of $I_{\text{SOC}}$ by FKBP51 decreases thapsigargin-induced stress fiber formation. Here, WT and cFKBP51 PAECs were grown to confluence, treated with thapsigargin to activate calcium entry, fixed, permeabilized, and stained with AlexaFluor 568 phalloidin to visualize filamentous actin. In control PAECs without thapsigargin treatment, actin was principally localized to the cell periphery as the cortical actin rim (Fig. 3a, top left). Following thapsigargin treatment, actin stress fibers appeared throughout the monolayer (Fig. 3a, bottom left). In cFKBP51 PAECs, there was very little thapsigargin-induced actin stress fiber formation (Fig. 3a, compare top and bottom right). Quantitation of filamentous actin (F-actin) using phalloidin revealed a $21\pm7\%$ increase in F-actin in control PAECs following thapsigargin treatment, reflective of stress fiber formation (Fig. 3b). On the other hand, thapsigargin had no effect on F-actin in cFKBP51 cells. Overall, these data indicate that inhibition of $I_{\text{SOC}}$ by FKBP51 decreases calcium entry-induced actin stress fiber formation.

**Increased FKBP51 expression leads to increased microtubule polymerization in PAECs**

We have shown that FKBP51 inhibits $I_{\text{SOC}}$ and downstream actin stress fiber formation. In endothelial cells, there is cross-talk between the actin and microtubule networks where microtubule depolymerization precedes actin stress fiber formation and microtubule stabilization decreases actin stress fiber formation. Further, FKBP51 was
shown to promote microtubule polymerization in a Xenopus oocyte system. Thus, we questioned whether microtubules are playing a role in FKBP51's effect on I_{SOC}. We first wanted to determine whether increased FKBP51 expression promotes microtubule polymerization in PAECs. Immunoblotting was performed for total and tyrosinated tubulin in control and cFKBP51 PAECs. Tyrosination is a post-translational modification that occurs on α-tubulin, and detyrosinated microtubules exhibit enhanced stability. While total tubulin levels did not differ between the two cell types, cFKBP51 cells exhibited decreased tyrosinated tubulin (46 ± 7%), suggesting more detyrosinated tubulin and increased microtubule stability (Fig. 4a).

Immunocytochemistry was then performed to visualize the microtubule cytoskeletal network. In contrast to WT PAECs which showed microtubules extending from the centrosome partially into the cytosol, the cFKBP51 cells exhibited a much more extensive microtubule network throughout the entire cytosol, suggesting increased microtubule polymerization (Fig. 4b). To quantitatively confirm the more extensive microtubule network in cFKBP51 cells, the number of the microtubule plus-ends and their spatial orientation relative to the nucleus and the plasma membrane were measured. Here, microtubule plus-ends were identified using EB-1 antibody, and we observed that microtubules in cFKBP51 cells were more numerous and extended farther away from the nucleus as compared to control cells, overall revealing increased microtubule polymerization in the cFKBP51 cells (Fig. 4c and d). Further, following a 20-min thapsigargin treatment, the number and localization of microtubule plus-ends were not different than the respective vehicle treatment in both WT and cFKBP51 PAECs. Overall, we have determined that FKBP51 impacts microtubule dynamics in PAECs by increasing microtubule polymerization.

**FKBP51 inhibits I_{SOC} through microtubule polymerization**

We next wanted to determine whether microtubule polymerization by FKBP51 leads to inhibition of I_{SOC}. To address this question, we treated WT or cFKBP51 PAECs with nocodazole over a concentration range that effected limited to complete dissolution of the microtubule network. In the absence of nocodazole, cFKBP51 PAECs again exhibited a more extensive microtubule network than WT cells (Fig. 5a). With a 10-μM nocodazole treatment,
the microtubule network in WT PAECs was dramatically reduced, and at 100 μM nocodazole, the network was completely disrupted. In cFKBP51 cells, at 10 μM nocodazole, the extended microtubule network was slightly retracted from the cell periphery, but was largely still intact. At 100 μM nocodazole, the microtubule network was mostly condensed to the perinuclear region. We then measured I_{SOC} under these same conditions to determine whether extended microtubules are required for the FKBP51-mediated inhibition of I_{SOC}.

In the WT PAECs, in the absence of nocodazole, a normal I_{SOC} was recorded (Fig. 5b). In the presence of 1 and 10 μM nocodazole, I_{SOC} was decreased in a concentration-dependent manner. At 100 μM nocodazole, with complete dissolution of the microtubule network, the cells could not be patched because a gigaseal could not be attained. When WT PAECs were treated with taxol (10 μM) to stabilize microtubules, I_{SOC} was also decreased. Thus, we observed that I_{SOC} function is dependent on microtubules, which is in agreement with previous observation.\(^3\) If the extended microtubules in cFKBP51 cells are required for inhibition of I_{SOC}, then we would expect that when microtubules are partially depolymerized, inhibition of I_{SOC} will be prevented. Indeed, we observed that when cFKBP51 cells were treated with 1 μM nocodazole, I_{SOC} inhibition was decreased, as evidenced by an increased current, and at 10 μM nocodazole I_{SOC} inhibition was completely prevented (Fig. 5c). To better visualize this, currents recorded at –60 mV holding potential under the conditions of 0 μM nocodazole and 10 μM nocodazole were plotted (Fig. 5d). At 0 μM nocodazole, the current in cFKBP51 PAECs was decreased compared to control cells revealing the FKBP51-mediated inhibition of I_{SOC}. However, at 10 μM nocodazole, FKBP51 was not able to inhibit I_{SOC}, as the current recorded in cFKBP51 cells in 10 μM nocodazole was not significantly different from the current recorded in WT cells with no nocodazole treatment. These data demonstrate that I_{SOC} in PAECs is dependent upon a delicate balance of microtubule polymerization, and that increased microtubule polymerization observed in cFKBP51 PAECs is required for the FKBP51-mediated inhibition of I_{SOC}.

**Increased FKBP51 expression reduces calcium entry-induced endothelial permeability**

Since we observed that increased FKBP51 expression promotes microtubule polymerization and decreases calcium
entry-induced actin stress fiber formation, we next questioned whether inter-endothelial cell gap formation is also reduced. Here, confluent monolayers of control and cFKBP51 PAECs were treated with thapsigargin and inter-endothelial cell gap formation was monitored via videomicroscopy over 45 min (Fig. 6a). Time 0 min represents the cells under resting conditions, 5 min before the addition of thapsigargin. Quantitation of inter-endothelial cell gaps revealed that in the basal state, cFKBP51 cells exhibited fewer and smaller gaps compared to control PAECs, suggesting a tighter basal barrier in the cFKBP51 cells (Fig. 6b). Following thapsigargin treatment, there was an increase in inter-endothelial cell gaps in both cell types. However, the cFKBP51 PAECs exhibited fewer and smaller gaps than control cells, revealing a protective effect of FKBP51 on the endothelial barrier. We also performed ECIS® as a measure of endothelial barrier integrity. Following thapsigargin treatment, control PAECs exhibited a decrease in resistance of $17 \pm 2\%$ whereas cFKBP51 cells exhibited $7 \pm 3\%$ decrease in resistance (Fig. 7a–c), indicating less endothelial barrier disruption as compared to control cells. Finally, we performed dextran flux assays using 40 kD dextran to measure macromolecular permeability across the endothelial barrier. Treatment with thapsigargin resulted in increased permeability in control PAECs whereas there was no permeability increase in cFKBP51 cells (Fig. 7d). Overall, these data reveal that FKBP51 protects the endothelial barrier from calcium entry-induced disruption.

Knockout of FKBP51 exacerbates calcium entry-induced endothelial permeability in situ

As we have shown that overexpression of FKBP51 reduces calcium entry-induced endothelial barrier disruption, we wanted to determine whether knockout of FKBP51 leads to increased endothelial barrier disruption. For this we used FKBP51$^{-/-}$ mice and measured lung endothelial
permeability in situ. Here, we performed an in-chest lung perfusion protocol in WT and FKBP51−/− mice. Thapsigargin was perfused to activate calcium entry and was followed by perfusion of Evans Blue dye-conjugated albumin. Tissue permeation of Evans Blue dye-conjugated albumin was measured as an indicator of endothelial permeability. Lungs from FKBP51−/− mice exhibited 3 times greater permeability following thapsigargin treatment compared to WT mice (Fig. 8), suggesting that the physiological role of the FKBP51-mediated inhibition of I_{SOC} is to limit calcium entry-induced endothelial barrier disruption.

**Discussion**

Activation of I_{SOC} in pulmonary endothelium leads to the formation of inter-endothelial cell gaps and endothelial barrier disruption.7 One paradigm holds that calcium entry causes actin stress fiber formation which then leads to inter-endothelial cell gap formation. Indeed, Moore et al.8 demonstrated that thapsigargin-induced calcium entry is upstream of actin stress fiber formation and inter-endothelial cell gap formation in PAECs. Similarly, Murphy et al.38 showed that thrombin-mediated actin...
stress fiber and inter-endothelial cell gap formation are calcium-dependent. An interesting observation in this study was that despite causing large increases in cytosolic calcium, ionomycin did not induce actin stress fiber formation, overall suggesting that calcium must enter through specific channels to initiate actin stress fiber formation. However, several studies have called into question whether actin stress fiber formation is required for calcium-mediated endothelial barrier disruption. In the ionomycin experiment in Murphy et al.’s study, the endothelial barrier was disrupted in the absence of stress fiber formation. In another study using human umbilical vein endothelial cells, Sandoval et al. observed thapsigargin-induced calcium entry and barrier disruption without the formation of actin stress fibers, while thrombin-induced calcium entry did result in the formation of actin stress fibers. More recently, Stolwijk et al. treated human dermal microvascular endothelial cells with thrombin, histamine, and sphingosine-1-phosphate (S1P), all of which activate Gq-linked G protein coupled receptors (GPCRs). Actin stress fibers were observed within 2–5 min following thrombin treatment, when the endothelial barrier was disrupted, but the stress fibers became more prominent after 10 min, which coincided with the endothelial barrier recovery phase. In histamine-treated cells, actin stress fibers were not observed until 20 min post treatment, which was after barrier disruption occurred. On the other hand, calcium entry following S1P treatment did not lead to actin stress fiber formation or barrier disruption. Collectively, these studies support the idea that not all increases in cytosolic calcium lead to actin stress fiber formation and further, that endothelial barrier disruption can occur independently of actin stress fiber formation. In the current study, we demonstrate that 

FKBP51 overexpression inhibits inter-endothelial cell gap formation in PAECs. (a) Control and cFKBP51 PAECs were treated with thapsigargin (1 μM) and inter-endothelial cell gap formation monitored via videomicroscopy. Time 0 min represents the cells under resting conditions, 5 min before the addition of thapsigargin. Arrows indicate areas of inter-endothelial cell gaps. Images were acquired at 40X magnification. (b) Quantitation of size and number of inter-endothelial cell gaps. Under resting conditions, control cells exhibited more gaps than cFKBP51 cells, perhaps suggesting a tighter basal barrier. After thapsigargin treatment, control cells exhibited more and larger gaps than cFKBP51 cells. *p < 0.05; n = 3. 

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cells, calcium entry through the ISOC channel leads to actin stress fiber formation which contributes to endothelial barrier disruption.

We began our study by characterizing \( I_{\text{SOC}} \) in terms of its sensitivity to the SOC entry inhibitors 2-APB and YM-58483. 2-APB is considered to be relatively non-specific and can block calcium entry through some TRPC channels. On the other hand, YM-58483 is a potent inhibitor of \( I_{\text{CRAC}} \). While the endothelial ISOC channel is composed of at least TRPC1 and TRPC4 subunits, the CRAC (calcium release activated calcium) channel is composed of orai1 subunits. \( I_{\text{CRAC}} \) is similar to \( I_{\text{SOC}} \) in that it is a small, non-voltage activated, calcium-selective current. However, the CRAC channel is distinct from the ISOC channel in that it is very highly calcium selective, with an estimated \( \text{Ca}^{2+}:\text{Na}^+ \) permeability ratio of 1000:1 whereas the \( \text{Ca}^{2+}:\text{Na}^+ \) permeability ratio of ISOC is estimated at \( >10:1 \). Further, while the CRAC channel is the gold standard of SOC entry channels, it has been questioned as to whether the ISOC channel truly represents a SOC entry channel. For the purpose of our study, it is irrelevant as to whether ISOC is, or is not, store-operated. What is important is that calcium entry through the ISOC channel leads to endothelial barrier disruption and that it is inhibited by FKBP51. Here we showed that \( I_{\text{SOC}} \) is inhibited by 2-APB but not YM-58483, which adds further evidence that the endothelial ISOC channel makeup consists of TRPC1/ TRPC4 and not orai1.

**Fig. 7.** FKBP51 overexpression protects endothelial barrier integrity and decreases permeability. (a, b) ECIS was performed to measure changes in endothelial barrier integrity upon activation of calcium entry with thapsigargin. Baseline resistance values were obtained for \( \sim 30 \text{ min} \), at which time calcium entry was activated with thapsigargin (1 \( \mu \text{M} \), arrow). (a) Absolute resistance values and (b) normalized, to baseline, resistance values. (c) Bar graph showing maximum change in resistance relative to baseline (0.1 s before addition of thapsigargin) for each cell type. The thapsigargin-induced decrease in resistance was inhibited in cFKBP51 cells. \( ^{*}P < 0.05 \) as determined by Student’s \( t \)-test; \( n = 11 \). (d) Endothelial permeability was measured using the Transwell dextran flux protocol. Calcium entry was activated with thapsigargin (1 \( \mu \text{M} \)). While control PAECs exhibited increased permeability following thapsigargin treatment, there was no increase in permeability in cFKBP51 cells. \( ^{*}P < 0.05 \) for thapsigargin-treated control cells vs. untreated control cells; \( n = 3–6 \).
Lungs from FKBP51–/– (KO) mice exhibited measuring tissue permeation of Evans Blue dye-conjugated albumin. Activation calcium entry and was followed by perfusion of Evans Blue was performed in which thapsigargin (30 nM) was first perfused to activate calcium entry compared to WT mice. Data were analyzed via Student’s t-test. *P < 0.05; n = 3 per group.

Channel contributes only a small fraction to the total thapsigargin-induced calcium entry. Second, this observation is consistent with our earlier observations in PAECs which were treated with dexamethasone to upregulate FKBP51 expression. While dexamethasone treatment inhibited SOC, the global calcium measurement was not significantly decreased compared to control cells. In the same study, overexpression of human FKBP51 in HEK293 cells only modestly decreased the global calcium entry while still inhibiting SOC. In our experiments, we used thapsigargin to activate SOC, which activates multiple calcium entry pathways including both store-dependent and store-independent channels. Thus, our observations that FKBP51 inhibits SOC but not other thapsigargin-activated pathways suggest that FKBP51 is acting specifically on the SOC channel.

Two major observations in our study were: (1) FKBP51 promotes microtubule polymerization that is critical for the inhibition of SOC; and (2) FKBP51 inhibits calcium entry-induced actin stress fiber formation. As both microtubule and actin cytoskeletons are influenced by FKBP51, it is worth considering the idea of cross-talk between the actin and microtubule networks in endothelial cells. In human pulmonary artery endothelial cells treated with thrombin, microtubule depolymerization preceded actin stress fiber formation and microtubule stabilization decreased actin stress fiber formation. The same effect of microtubule stabilization was also observed in cells treated with TNF-α. An early study by Verin et al. showed that inhibitors of microtubule polymerization increased myosin light chain (MLC) phosphorylation and actin stress fiber formation. They further showed that the increase in MLC phosphorylation was mainly due to decreased myosin-specific phosphatase activity not increased MLC kinase activity, suggesting that microtubules signal to stress fiber formation in a calcium-independent manner. In support of this idea, Birukova et al. subsequently demonstrated a role for Gz12/13 subunits in thrombin-mediated disassembly of microtubules and downstream endothelial barrier disruption. As actin stress fibers can also form in a calcium-dependent manner, the question arises as to whether calcium-mediated actin stress fiber formation occurs via cross-talk with microtubules. In the current study, we did not observe a change in microtubules in WT cells following thapsigargin-induced calcium entry, suggesting that calcium-induced actin stress fibers form independent of microtubule disassembly. However, we cannot discount the possibility that microtubule disassembly did actually occur but had recovered by the time of our measurement, which was at 20 min post thapsigargin treatment. Indeed, Alieva et al. showed microtubule decrease as early as 5 min post thrombin treatment and complete recovery by 60 min. If microtubule disassembly does indeed precede thapsigargin-induced actin stress fiber formation, then the question arises as to whether the FKBP51-mediated increase in microtubule polymerization prevents this disassembly and thereby contributes to the inhibition of actin stress fiber formation.

Another major observation in this study is that FKBP51 plays a protective role against calcium entry-induced endothelial barrier disruption. We observed that overexpression of FKBP51 in PAECs decreased inter-endothelial cell gap formation and permeability compared to WT cells. On the other hand, in FKBP51–/– mice endothelial permeability was increased compared to WT mice, suggesting that the physiological role of FKBP51 is to limit calcium entry-induced endothelial barrier disruption. A potential physiological role of FKBP51 is in acute inflammation where the endothelium transiently and limitedly increases its permeability to allow leukocytes to reach the site of inflammation. If the limited permeability increases becomes dysregulated, excessive...
movement of macromolecules and fluid occurs across the endothelial barrier leading to edema. Indeed, disruption of the FKBP51-mediated inhibition of $I_{\text{SOC}}$ may occur in pathophysiological settings such as sepsis or acute illness where patients can experience critical illness related corticosteroid insufficiency (CIRCI).$^{52-54}$ As FKBP51 is potently regulated by corticosteroids,$^{55}$ it is expected that CIRCI will lead to decreased levels of FKBP51, and decreased FKBP51 expression would allow persistent activation of $I_{\text{SOC}}$ and exacerbated endothelial barrier disruption. While the current study provides biochemical evidence for the FKBP51-mediated inhibition of $I_{\text{SOC}}$ and its protection of the endothelial barrier, our future studies will study the role of this pathway in health and disease.

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

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