Materials and Methods

Materials: Human aortic endothelial cells (HAEC, Cell Applications, San Diego, CA) were maintained in endothelial cell growth medium (Cell Applications) and passages 2 and 8 were used for all experiments. For all experiments, endothelial cells were washed twice with phosphate buffered solution after each application of reagents for the indicated times. HAEC were treated with sirolimus (0 – 500 nmol/L, Cat. No: 53123, EMD Milipore, Billerica, MA) diluted in DMSO in addition to full serum growth media at 37 degrees and 5% carbon dioxide. Control cells received DMSO. The maximum dose of sirolimus was chosen based on average arterial wall concentration of sirolimus immediately after sirolimus-eluting stent placement in an *in vivo* model[1]. Ryanodine (50 μmol/L, Cat. No: ab10083, Abcam, Cambridge, UK), an alkaloid and stabilizer of the ryanodine release channel (RyR)[2], treatment was performed for 60 minutes prior to sirolimus treatment or 24 hours after transfection of siRNA. Immunoblotting was performed with commercially available antibodies against phospho-PKCα (Ser657) (Millipore, Billerica, MA), PKCα, VE-Cadherin, p120-catenin, FKBP12.6, phospho-p70S6K (Thr 389), p70S6K, phospho-4EBP (Ser 65), phospho-Myosin Light Chain 2 (Thr 18/Ser 19) and beta-actin (Cell Signaling, Danvers, MA).

In Vitro and *Ex Vivo* Pharmacologic Treatment: HAEC were treated with sirolimus (0 – 500 nmol/L) or Torin2 (0-1000 nmol/L, Cat. No: 4248, Tocris, Bristol, UK) in endothelial cell growth media. A maximum dose of 500 nmol/L was chosen for SRL because initial arterial wall tissue levels after SRL eluting stent placement have been determined *in vivo* previous studies [1, 3]. Torin dose (25 nmol/L) was chosen based on its EC50 and reduction in cell viability at that dose[4]. Ryanodine (50 μmol/L) treatment was performed for 60 minutes prior to SRL treatment or 24 hours after transfection of siRNA. Ryanodine dose was chosen based on previous studies showing inhibition of the ryanodine release channels (RyR2)[2].

VE-Cadherin and p120 dual immunoflourescent staining on human coronary artery from a 33 year-old female with mitral valve prolapse collected 4 hour post-mortem. Bisected ice cooled fresh vessel into four pieces of vessel rings, about 0.5 cm length (2 for control group and 2 for SRL group). Incubate in M199 media (Invitrogen, Carlsbad, CA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), endothelial cell growth factor and heparin (Cell Sciences, Canton, MA) at 37 degrees and 5% CO_2 for 24 hrs. For treatment sample, 2.75 mmol/L sirolimus stock solution (LC Laboratories, Woburn, MA) was diluted to 0.5 mmol/L with DMSO, and added to above growth media at 1:1000.

siRNA Mediated Inhibition of PKCα and FKBP12.6 in HAECs: HAECs were plated in 50% confluence for transfection the next day. Target proteins for siRNA were confirmed with western blots (Figure S1A).

Table S1. Target siRNA sequences

siRNA	Sequence (sense, antisense)
PKCα	5'UUAUGAAGAAAGAACAAUCCGAUGG3', 5'CCAUCGGAUUGUUCUUUCUUCAUAA3'
FKBP12.6	5'CCCUCCCAAUGCCACCCUCAUCUUU3', 5'AAAGAUGAGGGUGGCAUUGGGAGGG3'

Target siRNA sequences were constructed and validated (Invitrogen). Negative, non-targeting siRNA sequences (Scr) were obtained from Invitrogen with similar GC content as targeting siRNA sequences. Transfection into adherent endothelial cells was done with the HiPerfect®

reagent system (Qiagen, Valencia, CA). TEER measurements, immunohistochemistry, immunoblotting and immunoprecipitation were performed at 24 hours after transfection.

Immunoblotting: Protein from HAECs were processed and separated on 7.5% polyacrylamide gel as previously reported[3]. Blot membranes were incubated with commercially available antibodies against phospho-PKCα (Ser657) (Millipore), PKCα, VE-Cadherin, p120-catenin, FKBP12.6, phospho-p70S6K (Thr 389), p70S6K, phospho-4EBP (Ser 65), phospho-Myosin Light Chain 2 (Thr18/Ser19), Myosin Light Chain 2 and beta-actin (Cell Signaling). Reactive bands were detected by chemiluminesence and quantified using area x density analysis with Quantity One 4.5.2 1-D Analysis Software (Bio-Rad, Hercules, CA). For each experimental group, a ratio of phosphorylated protein to total protein was calculated and normalized to control. Experiments were repeated three to four times with HAECs (n = 3-4 per group) unless otherwise stated.

Immunoprecipitation: HAECs were lysed in a modified lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, 0.1 mM PMSF) (Cell Signaling) and a total of 500 µg protein was isolated and incubated with 10 µg of antibodies at 4°C (p120, VE cadherin, phospho-PKCα) at the indicated times. Precipitates were collected with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Reactive bands were detected by chemiluminesence and quantified using area x density analysis with Quantity One 4.5.2 1-D Analysis Software (Bio-Rad). For each experimental group, a ratio of phosphorylated protein to total protein was calculated and normalized to control. Experiments were repeated three times (n = 3 per group).

Transendothelial electrical resistance: HAECs (both treated and transfected cells) were seeded at 5.0×10^4 cells/cm^2 on a pre-constructed 8-well gelatin-coated gold electrode (Applied Biophysics, Troy, NJ) and grown to confluence. The small electrode and the larger counter-electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 µA was supplied by a 1-V, 4000-Hz AC signal connected serially to a 1-MΩ resistor between the small electrode and larger counter-electrode. The voltage was monitored by a lock-in amplifier, stored, and processed by a personal computer. The same computer controlled the amplifier output and switched the measurement to different electrodes in the course of an experiment. Before each experiment, endothelial monolayers were washed with endothelial growth medium and used for measuring changes in TEER. TEER was measured in real-time using ECIS software (Applied Biophysics, Troy, NJ) and is expressed as specific electrical resistance (Ω cm^2). Data are presented as the change in resistive portions of the resistance normalized to its value at baseline for each treatment groups and statistical comparison was made between treatment groups to exclude variation in baseline monolayer characteristics (n = 3 wells for each treatment group).

Endothelial Permeability Assay: HAECs were seeded at $5.0\text{x}10^5$ cells/ml on collagen-coated hanging transwell insert with 1 µm pores (Cat. No. ECM644, EMD Milipore) with 200 µl for endothelial growth media in the transwell insert and 500 µl in the receiver well. Before each experiment, endothelial monolayers were washed with endothelial growth medium. After treatment period, FITC-Dextran at a 1:40 dilution was added into the transwell insert and FITC-Dextran permeation was measured in the receiver well with florescent plate reader at 485 nm and 535 nm excitation and emission spectra after 4 hour equilibration period in 37 degrees Celsius. Data are presented as the percent change of FITC-Dextran content in the receiver well compared with control (n = 4 wells for each experiment).

Immunohistochemistry: Confluent HAEC, mice aortic endothelium, mouse myocardium and human coronary endothelium were fixed in 4% paraformaldehyde and permeabilized with 0.05% Triton-X. Mice were perfused with saline and abdominal aortas and hearts were collected prior to fixing. Tissues were incubated in primary antibodies (VE Cadherin 1:250 rabbit Cat. No: 2158S, Cell Signaling and p120, 1:100 goat, Cat. No: SC1730, Santa Cruz) or FITC-conjugated primary antibody (CD31, Cat. No: 561813, BD Bioscience, Franklin Lakes, NJ) for 24 hours and fluorescently labeled secondary antibody if required (donkey anti-rabbit IgG 488 nm, Cat. No: 821206 and donkey anti-goat IgG 555 nm Cat. No: A21432, Invitrogen). In addition prior to FITC-labeled CD31 antibody incubation, mice were given Evans Blue (excitation 550 nm, emission 620 nm) per protocol above ("*Mouse Vascular Permeability Assay" section*). HAEC, mice aortic endothelium, mouse myocardium and human coronary endothelium were mounted slides with mounting media (Vector Labs, Burlingame, CA). DAPI was used as a nuclear couterstain (Invitrogen). Tissue was visualized using a 20x objective on a Nikon Eclipse TE-2000U microscope (Nikon, Tokyo, Japan) with HeNe laser, and driven by EZ-C1 Viewer v3.5 software (Nikon). For a negative control, secondary antibodies were used alone without primary antibody incubation (supplemental figure V).

Live Cell Imaging and Intracellular Calcium Measurements: For live cells imaging for intracellular calcium measurement, HAECs were treated per protocol (see "Materials" section) after plated on glass cover slips. Groups were subsequently loaded with Fluo-3 AM (Invitrogen; ex: 488 nm em: 520 nm; loading concentration - 10 μmol/L) and FuraRed (Invitrogen; ex: 488 nm, em: 660 nm; loading concentration - 20 μmol/L) and serum starved in phenol-free media (Hanks balanced buffered solution supplemented with 10mM of HEPES (Sigma-Aldrich) for 1 hour prior to imaging and were maintained at 37°C with a stage heater during imaging. Images were acquired every 10 seconds for each group using a Nikon A1R-A1 microscope with Argon Ion Laser (λ excitation = 488 nm) and ratio of Fluo-3/FuraRed (F/F_o) excitation ratio monitored for > 10 HAECs with NIS-elements software (Nikon) and intensity modulated display was acquired for qualitative assessment of intracellular calcium flux.

Image Analysis: Interendothelial gap area and relative membrane to cytosol fluorescence intensity of p120 and VE-cadherin was quantified using NIS-elements v3.0 (Nikon). Projection images were generated by collecting the maximum pixel intensity from each image of the Z stack and by projecting pixel intensity onto the single (projection) image. The area of intercellular endothelial gaps was quantified using by manually outlining cells with contiguous VE cadherin staining and selecting for gaps. The values are expressed as a percentage of the total surface area. Colocalization was quantified using ImarisColoc Algorithm which created a channel of only pixels that overlapped using the Costes Method[5] and expressed in both in intensity scatter plots and Pearson correlation coefficients of the region of interest (Bitplane AG, Zurich, Switzerland). At least 4 high powered fields of > 10 cells for HAEC and mice aortic endothelium (from at least 3 animals) were measured for each analysis. For human coronary endothelium, given the lack of tissue, at least 4 high powered fields of > 5 cells were used for analysis.

Quantification of HAEC Proliferation: HAEC proliferation with torin2 and sirolimus treatments was quantified by the BrdU Cell Proliferation Assay (Merck KGaA, Darmstadt, Germany) at 24 hours as previously described[6]. This assay was conducted in 96 well culture plates and proliferation was quantified by measuring absorbance at dual wavelengths of 450-540 nm. Results were normalized to HAEC controls.

Mouse Vascular Permeability Assay: Adult wild type (8-10 weeks) C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were given sirolimus (1 mg/kg/day) for 3 days intraperitoneally in

vehicle (0.25% PEG and 0.25% Tween 20) in addition to vehicle alone in accordance to the Emory University Institutional Animal Care and Use Committee. Animals were perfused with saline and abdominal aortas and hearts were collected for immunohistochemistry analysis after sacrifice. Sirolimus dose used has been previously shown to inhibit mTOR signaling in murine models and correspons to therapeutic trough levels in humans[7-10]. For vascular permeability studies, under isoflurane inhaled anesthetic, Evans' blue albumin (EBA) 0.3% was introduced via intraorbital injection and allowed to perfuse for 20 minutes. Animals were sacrificed and perfused with 10 ml with PBS through the left ventricle over 1 minute to remove residual EBA. Abdominal aortas, lungs and heart were isolated and desiccated at 60 degrees Celsius for 24 hours followed by Evans' blue elution in formamide overnight. Absorbance was measured at 620 nm and 720 nm, correcting for contaminating heme pigments[11]. Six mice were used in each group (n = 6). Results were expressed in μg of Evans' blue per mg of tissue.

Statistical Analysis: Statistical analysis was performed with JMP Pro v10 (SAS, Cary, North Carolina). All data was expressed as mean $\pm$ SD. Comparison among groups was made using unpaired Student's *t* test. A p-value of < 0.05 was considered statistically significant. Sample size calculations was performed by G*Power v3 (Dusseldorf, Germany). For *in vivo* experimental groups, a large effect size (≥ 2) was determined based on data the derived *in vitro* data for endothelial permeability (supplemental table I-II). With the criterion of significance set at 0.05, a sample size of 3 - 4 per groups was determined for each experiment to achieve a power of 0.8.

Supplemental References

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