Recapitulating Physiological and Pathological Shear Stress and Oxygen to Model Vasculature in Health and Disease

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Supplementary Information

Supplementary Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were maintained at 5% CO_2 , 21 %O_2 and 37°C in humidified incubators. Cells (passages 2–5; PromoCell, Heidelberg, Germany) were cultured in endothelial growth media (EGM; Promocell), had media changed every other day and passaged every 3–4 days with 0.05% trypsin (Invitrogen, Carlsbad, CA). For control conventional experiments, HUVECs were seeded and allowed to attach to a petri-dish in atmospheric (21% O2) conditions for 24 hours, and then subjected to hypoxia (1% O2), or 5-FU (7 mM) with or without Resveratrol (100 uM) for additional 24 hours. 1% oxygen concentration was achieved by flushing a hermetically sealed chamber with a 1% O_2-5%CO_2-N_2 balance for 3 min at 3 psi and three times every thirty minutes. Humidity was maintained in the chamber by inclusion of sterile water in a petri dish at the bottom of the chamber.

Fabrication of μVM

The microfluidic system was developed as described previously with slight modifications [14]. Briefly, we created microchannels of 300 μm width, 250 μm in height, and 2.5 cm in length using standard photo lithography techniques. We then spun coat PDMS at 500 rpm for 30 seconds to control the thickness of the layer above the cell culture channel. The PDMS and the glass slide were then bonded together by mild O_2 plasma exposure of both surfaces. At each end of the channel, we punched a 3 mm open circle to accommodate the O_2 sensor spots (OXY-4 mini; PreSens GmbH, Regensburg, Germany) to continuously monitor the dissolved oxygen (DO) level in the cell microenvironment as demonstrated in our previous work [14]. The system is composed of 3 mm sensor patches, fiber-optic guides (i.e. reader), and a transmitter device that is connected to a computer. A second and thicker layer of PDMS was then bonded to both ends of the channel. We made slight modifications on the previous design in order to prevent any air bubbles from entering the system. A small chamber, which serves as a bubble trap, was incorporated into the inlet port by curing the PDMS in a custom-made poly(methyl methacrylate) (PMMA) mold. We punched an additional hole in the inlet port to manually remove air bubbles accumulated in the bubble trap. For the low O_2 experiments, this microfluidic device was housed inside an O_2 enclosure consisting of two PMMA plates. The plates with vacuum grease in between were screwed together to make the chamber airtight.

Cell Maintenance in μVM

All components of the system were autoclaved separately and further sterilized with ethanol for 10 minutes after assembly. The glass surface of the device was coated with Fibronectin (10 μg/ml) prior to cell seeding. Cell seeding was achieved by injecting a cell suspension with a density of 5 million cells/ml. After 3 hrs of attachment period, growth media was supplied at a flow rate of 0.01 ml/hr overnight using a syringe pump (Chemyx, Stafford, TX). In static control experiments, the flow rate was maintained at 0.01 ml/hr, which is sufficient to provide nutrients and exerts a negligible shear stress of 0.007 dyn/cm^2 [26]. In high shear and physiological condition experiments, media was

circulated at a flow rate of 20 ml/hr between the microbioreactor and a media reservoir using a peristaltic pump (Ismatec, Wertheim, Germany). Physiological O_2 was generated by continuously flushing both the microbioreactor and the media reservoir with a medical grade gas mixture (5% CO_2, 1% O_2 and balance N_2). Ischemic conditions were created by lowering the shear stress and O_2 tension simultaneously to 0.01 dyn/cm^2 and 1% O_2 (5% CO_2, 5% O_2 and balance N_2). Drug treatment was performed by injecting 5-FU alone (Sigma-Aldrich, St. Louis, MO) or 5-FU and Resveratrol (Sigma-Aldrich) together directly into the media reservoir to give a clinically relevant concentration of 7 mM [20, 27] and 100 μM [28], respectively.

Immunofluorescent Staining and Imaging

We analyzed the cellular morphology and protein localization using fluorescent imaging of fixed cells as previously [10, 14, 26]. All solutions used in fixation and staining steps in the μVM were injected at a flow rate of 1 ml/hr for a volume of 300 μl. Cells were first fixed with 3.7% paraformaldehyde solution for 2 hours at room temperature, permeabilized with 0.1% Triton X-100 for 15 minutes and then stained with Phalloidin (1:40) and 4=6-diamidino-2-phenylindole (DAPI) (1:1,000) to visualize the cytoskeleton and nuclei, respectively. For immunofluorescent labeling, cells were incubated for 2 hours with anti-human PECAM1 (1:100; Sigma-Aldrich) or VECAD (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and rinsed with phosphate buffer saline (PBS) followed by incubating with anti-mouse IgG Cy3 conjugate (1:50; Sigma Aldrich) for 1 hour. The fluorescently labeled cells were examined using fluorescence microscopy (Olympus BX60; Olympus, Center Valley, PA).

We performed fluorescent staining of *in vivo* tissue samples for analyses of cellular morphology. Mouse arteries were fixed immediately in ice cold 3.7% paraformaldehyde for 2 hours. Connective and adipose tissue around artery segments were removed and the artery was cut longitudinally using micro scissors under the dissecting microscope. Samples were then permeabilized with 0.1% Triton X-100 for 30 minutes. For immunofluorescent labeling, cells were incubated overnight with anti-mouse VECAD (1:200; Sigma-Aldrich), rinsed with PBS, and incubated with anti-goat IgG Cy3 conjugate (1:50; Sigma-Aldrich) for 2 hours. For *en face* imaging, samples were mounted on a glass slide using mounting medium (Dako, Glostrup, Denmark) ECs facing up. The fluorescently labeled arteries were examined using confocal microscopy (LSM 510 Meta; Carl Zeiss).

Quantification of Proliferation, Protein Localization, Cell Elongation and Intercellular Gaps

To identify the protein localization, the cellular membrane and nucleus were manually distinguished using the software CellProfiler (Broad Institute, Cambridge, MA). The cytoplasm was identified subtracting the region of the nucleus from the region surrounded by the cellular membrane. Protein localization was determined by calculating the ratio of the mean fluorescence intensities of the adherens junction proteins located at the cellular membrane edge and cytoplasm. The detailed image analyses using CellProfiler software used as previously described [29]. Changes in morphological elongation were assessed through comparing the eccentricity, measure of the deviation of a conic section from a circle, using Cell Profiler. Eccentricity is equal to zero for a circle and one for a parabola.

Intercellular gaps were identified using VECAD fluorescence at the magnification of 100X, as extracellular regions absent of VECAD fluorescence and surrounded on all

sides by cellular membranes of two or more adjacent cells. Gap area and number were measured highlighting the individual gaps manually in ImageJ and normalized to the number of cells and averaged across all images. The number of proliferating cells was determined by counting the Ki-67 positive cells in the immunofluorescent images.

Real time quantitative RT-PCR

We performed two-step RT-PCR using the Taqman Gene Expression Cells-to-CT kit (Ambion, Grand Island, NY) on the μVM under different conditions. Cold PBS was flushed through the microbioreactor for 15 minutes. The PDMS layer and excess PBS were removed without disturbing the monolayer of cells. RNA was collected by adding lysis solution directly on top of the channel. The lysed cells were collected and incubated at room temperature within the stop solution. RNA was then converted to cDNA with reverse transcriptase according to manufacturer's instructions. PCR step was performed using TaqMan Universal PCR Master Mix and Gene Expression Assay (Applied Biosystems, Foster City, CA) as previously described [10]. The triplicate values for fold change in expression were averaged and graphed with standard deviations.

Isolation of RNA from mouse aorta was performed using the method previously described by Nam et al [30]. Briefly, an incision was made in the right atrium and PBS was flushed through the left atrium until clear. The descending aorta was dissected and PBS was flushed through. The aorta was excised and 200 μl of TRIzol (Invitrogen) was injected through the aorta to collect the RNA of ECs for RT-PCR. The relative expression of *eNOS* was normalized to the amount of *PECAM1* to avoid any mRNA contamination from smooth muscle cells.

TUNEL Assay

The fraction of cells undergoing apoptosis was determined by using TUNEL assay (*Roche* Diagnostics Corp.; Indianapolis, IN) according to manufacturer's instructions. Briefly, cells in the microbioreactor were fixed with 3.7% paraformaldehyde for 1 hour, permeabilized in 0.1% Triton-X (prepared in 0.1% Sodium Citrate) on ice and incubated in label solution at 37 ^{o}C for 1 hour. Cells were then stained with DAPI before imaging with fluorescent microscope (Olympus BX60). For positive control, DNA of cells was defragmented by incubating in DNAse (3000 U/ml) for 1 hour at 37 ^{o}C prior to staining.

Mouse Drug Treatment

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins Medical School. 8-10 week old BALB/c mice (Charles River, Wilmington, MA) received three consecutive intravenous injections of 5-FU (60 mg/kg dissolved in 1% DMSO) or 5-FU and Resveratrol (6.5 mg/kg dissolved in 1% DMSO) every other day for mRNA extraction studies and single injection for the other analyses. Control animals were treated with equal amounts of PBS or 1% DMSO in PBS (vehicle control). The drug doses were determined to achieve the same clinically relevant drug concentrations used in the *in vitro* experiments. The average drug concentration in blood stream was calculated based on the first order clearance rates of 5-FU (16.4 μg ml^{-1} hour^{-1}) and Resveratrol (6.84 μg ml^{-1} hour^{-1}) from the blood [31, 32]. Animals were then sacrificed the day after the final injection. Prior to harvesting the tissue of interest for analyses, *vena cava* was cut and intra-cardiac injection of ice-cold PBS was performed to remove the blood.

Vascular Permeability in Mice

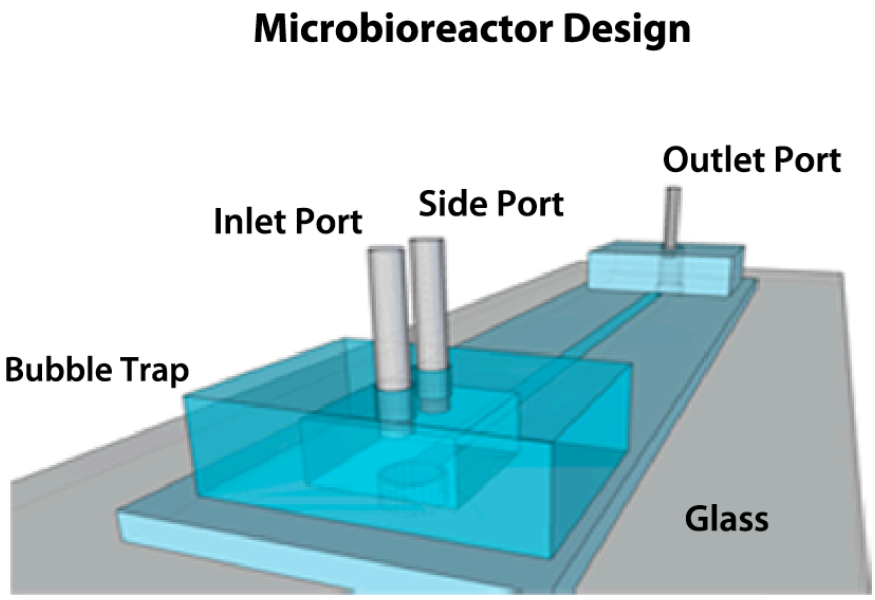
Permeability of mouse microvasculature was analyzed by injecting Evans blue dye (30 mg/kg in 150 in PBS; Sigma Chemical Co.) intravenously into mice one day after injection of a single dose of 5-FU alone or 5-FU and Resveratrol. Ten minutes after the injection of the dye, mustard oil (Sigma Chemical Co.) diluted to 5% in mineral oil was applied to the surfaces of the ear. The pictures of the ears were taken after 20 minutes. Mice were sacrificed 30 minutes after the injection of the dye. The Evans blue dye was extracted from the intestines, lungs and liver by incubating the organs in 1 ml of Formamide (99.5%, Sigma Aldrich) overnight at 60 ^{o}C and measured using spectrometer at 620 nm. The amount of extravasated dye per weight of tissue was calculated for comparison between samples. Water accumulation in different organs was examined by measuring wet-to-dry weight ratio. The wet weights of lung, intestines and liver were measured immediately after removal of the organs. The dry weight of the organs was measured after evaporation of the water in the organs at ^{o}60 C for 48 hours.

Concavalin A binding was used to assess the endothelial barrier function of descending aorta in a similar manner shown by others [33]. The aorta segments were first fixed in 3.7% paraformaldehyde, cut longitudinally, and mounted as flat on a glass slide with the lumen side of the vessels facing up. The sample was then blocked by 5% BSA solution for 1 hour and 10 µl drop of CON A solution was added only on the luminal side. After an hour of incubation at room temperature, CON A localization was analyzed by taking Z-stack images using confocal microscope (LSM 510 Meta; Carl Zeiss).

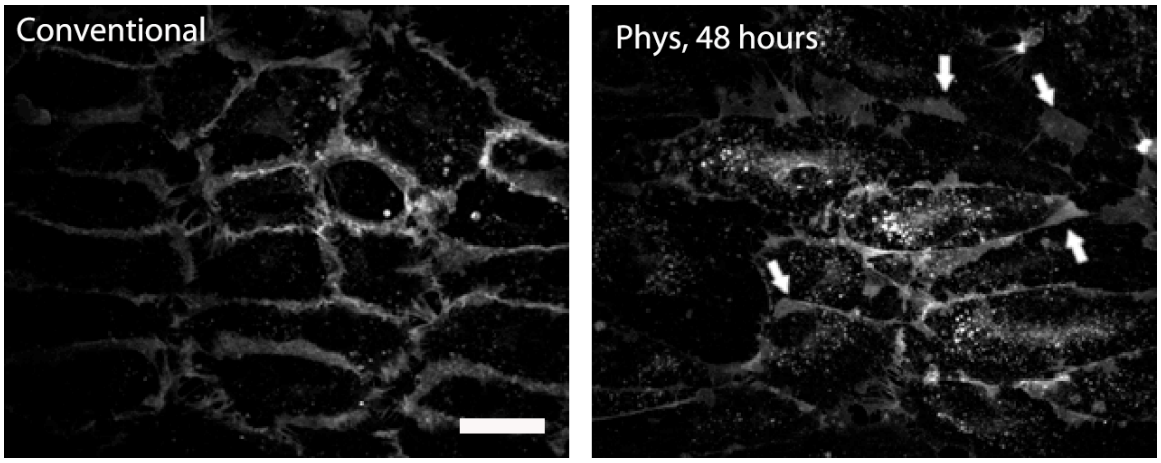
Statistics

All *in vitro* experiments using µVM and chambers were performed in triplicates. Real-time RT-PCR was also performed on triplicate samples with triplicate readings. *In vivo* study was conducted using at least five independent mice experiments. Graphs were plotted with standard deviation (SD). Significance levels were determined using unpaired two-tailed t-tests where appropriate (GraphPad Prism 4.02) and set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$ as indicated.

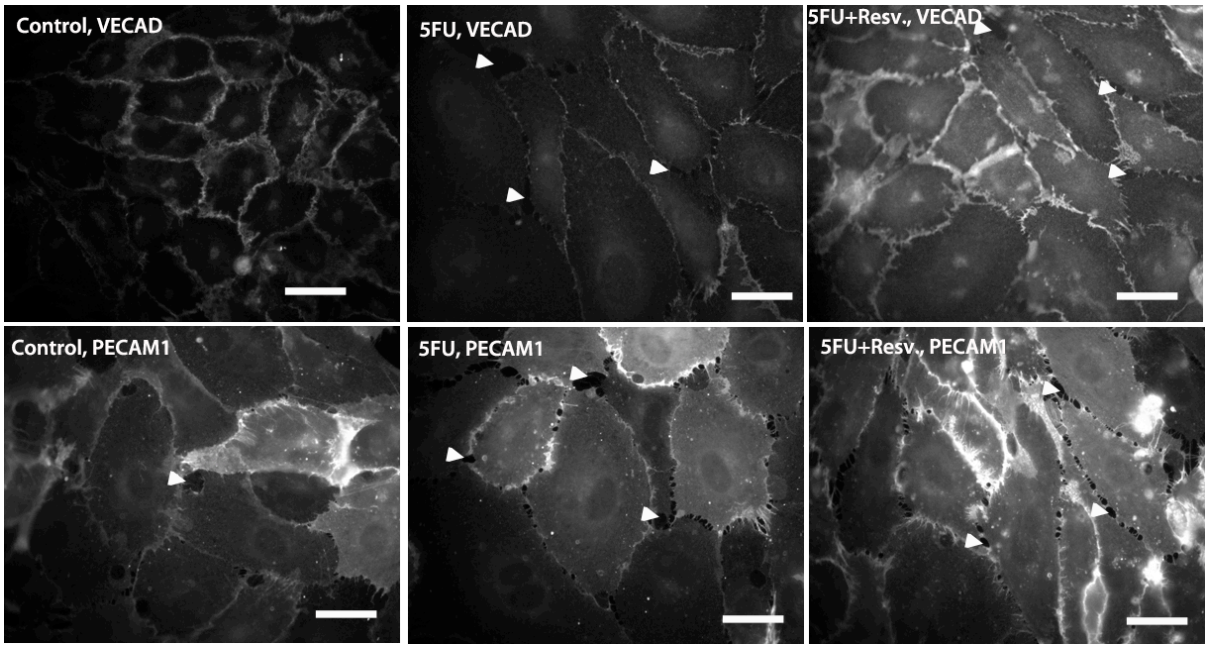
Supplementary Figures



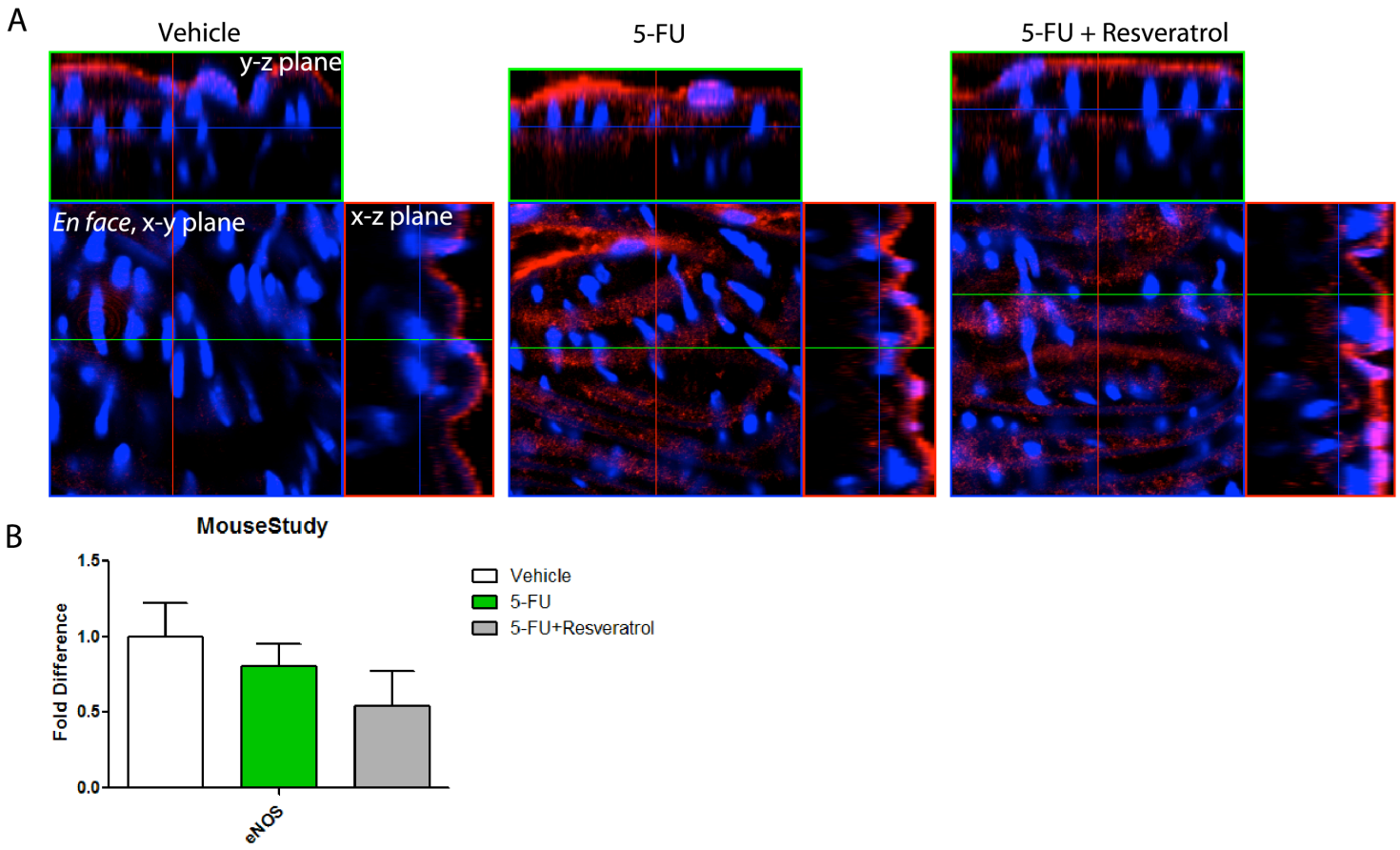
Supplementary Figure 1. Illustration of the improved microfluidic design with expanded inlet bubble trap and additional side port for bubble removal. The system is composed of a microchannel for adherent cell culture and medium flow, placed in an O_2-impermeable encasing made of poly(methyl methacrylate) that provides an accurately controlled O_2 microenvironment. The top wall of the microchannel was designed to be sufficiently thin to permit O_2 exchange between the culture channel and the encasing (14). Here we expanded the volume of the inlet port from 9 mm^3 to 130 mm^3 to accommodate for bubbles entering the system as well as added a side port to enable the removal of any accumulated bubbles For low oxygen experiments, the microbioreactor was inserted in an O_2-impermeable encasing made of poly(methyl methacrylate) as previously described (14).



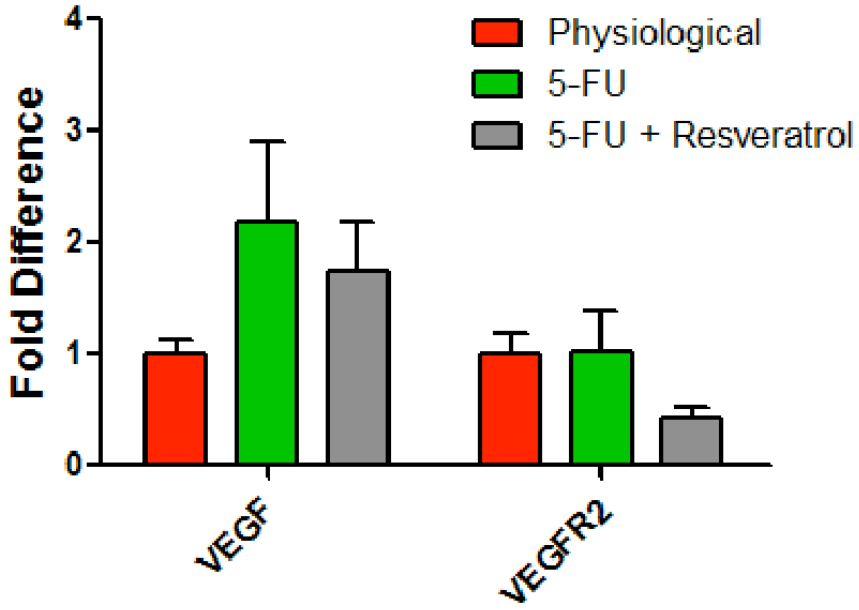
Supplementary Figure 2. PECAM1 localized as thick structures at some certain regions of cell-cell contact. Immunofluorescent images of PECAM1 of ECs cultured at conventional or physiological conditions for 48 hours. Arrows indicate the thick PECAM1 structures. Scale bar: 20 μm



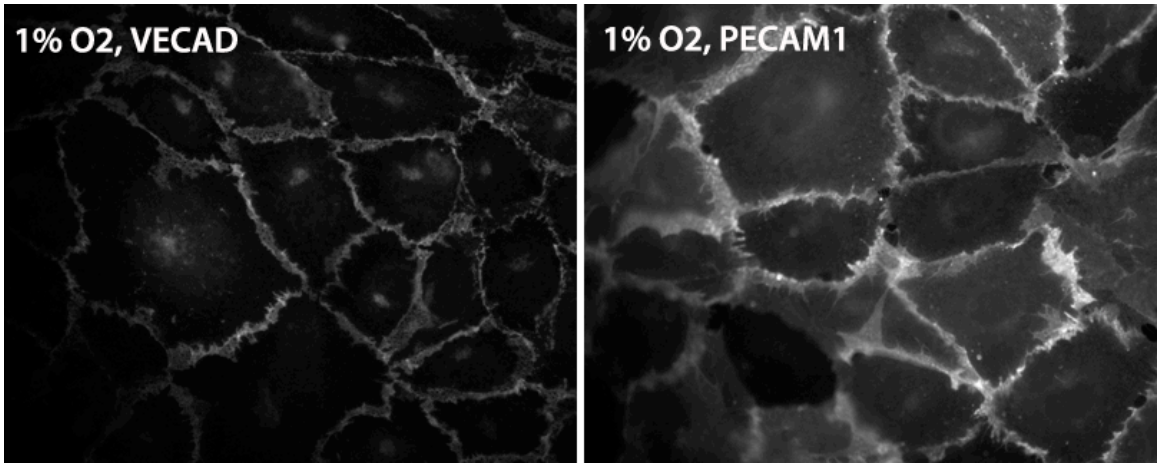
Supplementary Figure 3. 5-FU treatment induced intercellular gaps formation that is not overturned by Resveratrol in petri-dish control experiments. Immunostaining images of VECAD (upper panel) and PECAM-1 (lower panel) of ECs treated with 5FU only or 5FU+Resveratrol in comparison with untreated control**.** Some regions with intercellular gaps are indicated with arrowheads. Scale bar: 20 μm



Supplementary Figure 4. 5-FU induces hyperpermeability in mouse aorta in an *eNOS*-independent manner (A) *En face* imaging of Con A binding throughout the layers of the descending aorta revealed that 5-FU causes a slight increase in the permeability of the EC monolayer. Scale Bar: 20 μm **(B)** mRNA levels of *eNOS* in the ECs collected from the descending aorta of mice. (*p<0.05)



Supplementary Figure 5. Drug treatment does not affect mRNA levels of *VEGF* and *VEGFR2*. Gene expression levels of VEGF and VEGFR2 do not change in 5-FU treated or 5-FU and Resveratrol treated ECs compared to ECs cultured at physiological conditions. (*$p<0.05$)



Supplementary Figure 6. Hypoxia treatment did not induce intercellular gaps formation in petri-dish control experiments. Immunostaining images of VECAD (left) and PECAM-1 (right) of ECs treated in 1% O_2 for 24 hours in petri-dish. Scale bar: 20 μm