Supplementary Information for

Discordance between eNOS phosphorylation and activation revealed by multispectral imaging and chemogenetic methods

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www.pnas.org/cgi/doi/10.1073/pnas.1910942116

Supplementary Materials

Methods

Cell culture, plasmid transfection, and adenoviral infection

EA.hy926 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v) containing 1% HAT (5 mM hypoxanthine, 20 µM aminopterin, 0.8 mM thymidine), 4 mM glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. BAEC were maintained in culture in Bovine Endothelial Cell Growth Medium (Cell Applications, San Diego USA). Cells were plated onto culture dishes or glass coverslips coated with Attachment Factor Protein (Gibco-BRL) and studied between passages 3 to 6. Cells at ~80% confluency were transfected in cell culture medium using the transfection reagent PolyJet (SignaGen Laboratories) with 1 µg plasmid DNA following the manufacturer's protocols. Cells were incubated for 5 h, and then the culture media was replaced. All imaging experiments were performed 16-24 h after transfection. AV5-DAAO-HyPer expression in endothelial cells was achieved by adenoviral transduction at a multiplicity of infection of 1000 for both EA.hy926 and BAEC cell studies.

Measurement of nitrate levels

Levels of nitrate, an oxidized product of NO, were measured in culture supernatants by a fluorometric assay using the 2,3-diaminonaphthalene (DAN) reagent using a nitrate/nitrite fluorometric assay kit (Cayman Chemical, 780051). EA.hy926 cells were seeded in equal cell numbers and incubated for 48 h to reach confluence. The cells were starved for 3 hours prior to the experiment, washed with PBS, and then phenol red-, FBS- and antibiotic-free DMEM culture medium was added. The cells were treated with VEGF (10 ng/ml), insulin (100 nM), ATP (30 µM) or histamine (10 µM) in the culture medium. Cell supernatant samples (20 µl) were collected, and the assay was performed according to the manufacturer's instructions. Fluorescence was measured with a microplate reader (Varioskan Flash, Thermo Fisher Scientific) with excitation at 360 nm and emission at 430 nm. Nitrate concentrations in the medium samples were calculated using a standard curve analyzed in parallel with the experimental samples.

Cell preparation for live-cell imaging of H_2O_2, NO, and Ca^{2+}

Prior to the imaging experiments, cells were washed with PBS and incubated for two hours in a HEPES-buffered solution containing 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM D-glucose, 0.1% vitamins, 0.2% essential amino acids and 1% penicillin-streptomycin, pH 7.4. Imaging experiments were performed using a custom perfusion system with a peristaltic pump to maintain stable superfusion conditions. Superfusion solution consists of a physiological buffer with the following composition: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose and 1 mM HEPES, pH 7.4.

NO imaging experiments

Cells transfected with geNOp constructs were incubated immediately before the imaging experiment with a 1 mM iron(II) fumarate solution containing 1 mM ascorbic acid prepared in HEPES physiological salt solution. Cells were incubated in the iron(II) containing buffer for 20

minutes. After washing with PBS, cells were incubated in Imaging Buffer (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM D-glucose, 0.1% vitamins, 0.2% essential amino acids and 1% penicillin-streptomycin, pH 7.4) for two hours.

Fura-2 imaging experiments

Cells were washed with PBS and incubated in Imaging Buffer for approximately 30 minutes, and 3.3 µM Fura-2/AM solution was added for an additional 30 min. After washing the cells twice with PBS, cells were incubated in Imaging Buffer for 30 minutes before the imaging experiment.

Real-time fluorescent imaging

For live-cell imaging, the intensiometric C-geNOp was excited at 420, and emission was analyzed at 480 nm. The intensiometric R-GECO and O-geNOp biosensors were excited at 542-582 nm, and emission was collected at 604-644 nm. The ratiometric HyPer was excited both at 420 nm and 490 nm, and emission was collected at 530 nm. All cells were visualized using a ×40 oil immersion objective (Olympus). Images were acquired with a CCD camera from Hamamatsu. Real-time signals were acquired using Metafluor Software (Molecular Devices). For each experiment, an additional region for the background signal was acquired. Background subtraction was calculated in Microsoft Excel. All intensiometric biosensors were normalized to 100% to the average signal in the corresponding cell population. geNOps signals are calculated using the formula $\Delta F = 1-(F/F0)x100$. Ratiometric signals or HyPer were calculated by first subtracting the background in each wavelength and calculating the ratio by dividing the intensity of the emission signals excited by 490nm/420nm.

Cell treatment and preparation of cellular lysates

EA.hy926 and BAECs were cultured in complete growth media and studied at the same passages that were used for the live cell imaging experiments. 3 hours prior to the experiment, the cells were washed with PBS followed by adding either FBS- and antibiotic-free media or the iron(II) fumarate-containing Ca^{2+} buffer used for fluorescence imaging (1mM iron(II) fumarate solution including 1 mM ascorbic acid in HEPES-buffered solution containing 2 mM $CaCl_2$, 138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM D-glucose, 0.1% vitamins, 0.2% essential amino acids and 1% penicillin–streptomycin, pH 7.4). The cells were incubated until treatments with the specific concentration of the agonists (30 µM ATP, 10 µM histamine, 10 ng ml^{-1} VEGF, and 100 nM insulin) for indicated times. Additionally, both types of endothelial cells were infected with adenovirus 5-HyPerDAAO-NES for 24-48 h at the same MOI (1000) as was utilized for the imaging experiments ,and analyzed 24-48 h after the adenoviral transduction under fluorescence microscopy to establish virus expression. The cells were then incubated with D-alanine (10 mM) for the times indicated.

Immunoblot analysis

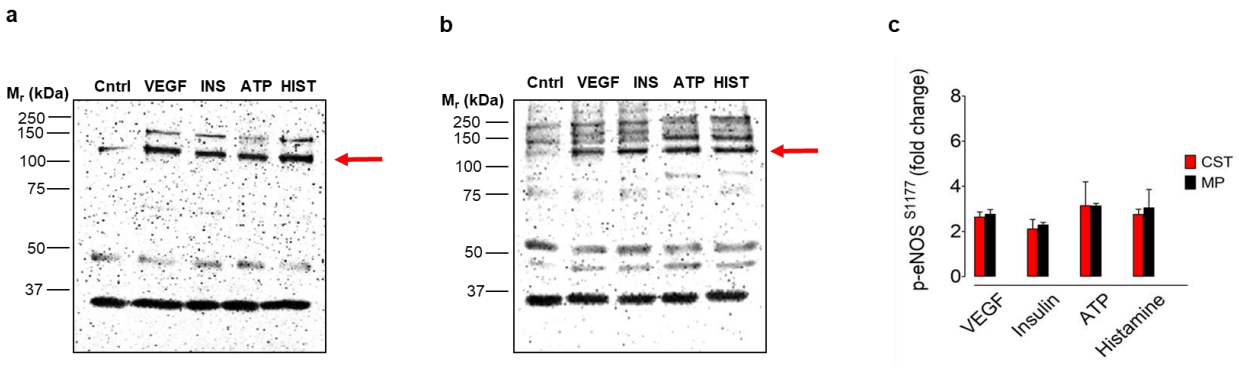
After drug treatments, the cells were washed with ice-cold PBS and then radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF, 2 µg/ml leupeptin, 2 µg/ml antipain, 2 µg/ml soybean trypsin inhibitor, and 2 µg/ml lima trypsin inhibitor). Cells were harvested by scraping and centrifuged at 13,500 × *g* for 5 min at 4 °C. Cell lysate were prepared by boiling in Laemmli sample buffer to elute bound immune complexes, loaded (30 µg) on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking

with 2% bovine serum albumin (BSA; Sigma Aldrich) in Tris-buffered saline with 0.5% (v/v) Tween 20 (TBST; Boston BioProducts), the membranes were incubated overnight at 4 °C with phosphospecific primary antibodies (1:1000) directed against phospho-eNOS at Ser^{1177} (anti-$pSer^{1177}$ antibodies were either from Cell Signaling Technology or EMD-Millipore, as indicated); at Thr^{495} (Cell Signaling Technology); or at Ser^{114} (EMD-Millipore). The membranes were washed with TBST three times and incubated with a horseradish peroxidase-labeled anti-rabbit immunoglobulin G secondary antibody (Cell Signaling Technology) at a 1:2000 dilution in TBST containing 2% BSA for 1 h. The membranes were then stripped, blocked and re-blotted with total eNOS antibody (1:1000; Cell Signaling Technology) as loading control. Immunoblots were analyzed by chemiluminescence using a ChemiImager 4000 (Alpha-Innotech) and quantitative densitometric analyses were performed using ImageJ (NIH, Bethesda).

Statistical analysis

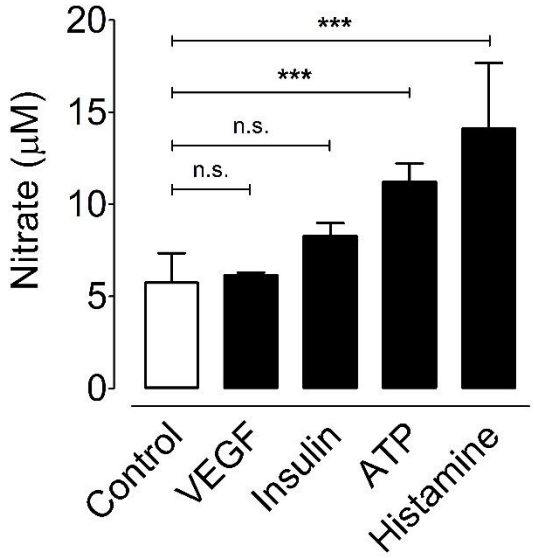
Data are presented as mean values ± SEM. Statistical analysis was performed using GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA). Analysis of variance and t-tests were used for evaluation of the statistical significance for paired groups. $P<0.05$ was defined as significant. Analysis of variance (ANOVA) with post-test Tukey's multiple comparison test was used for comparison of results with each other within a treatment. At least three different experiments have been performed for each experimental set-up. For live-cell imaging, the number of experiments is indicated as "n=X/x" where X indicates the number of independent experiments and x indicates the total number of individual cells analyzed.

Supplementary Figure 1: Agonist-mediated eNOS ser1177 phosphorylation probed with two different phosphospecific antibodies.



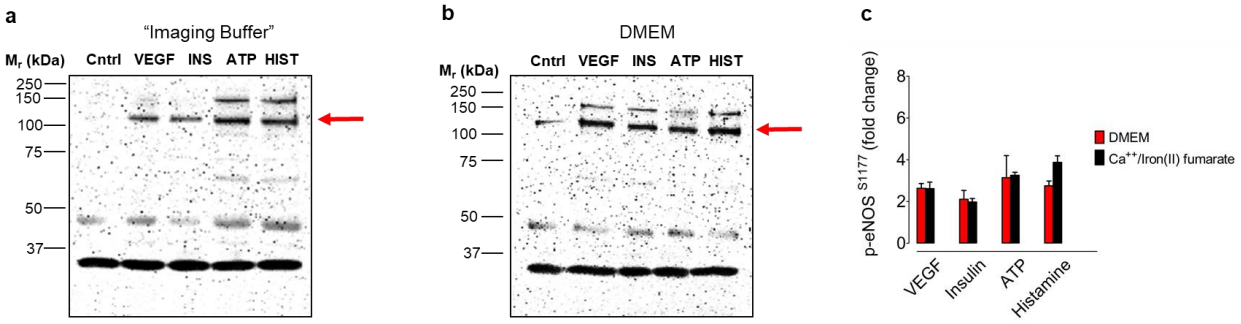
(**a**) Shown are immunoblots of EA.hy926 cell lysates probed with eNOS phosphoserine-1177 phosphospecific antibodies from Cell Signaling Technology or (**b**) EMD-Millipore following treatment of cells with VEGF (10 ng/ml), insulin (INS, 100 nM), ATP (30 μM) or histamine (HIST, 10 μM) for 15 minutes. The red arrow denotes eNOS (136 kDa). Panel **c** shows pooled data from the three independent experiments in immunoblots probed with the Cell Signaling Technology (CST) or Millipore (MP) phosphospecific antibodies. The values are presented as mean ± S.D. All data were analyzed by two-way ANOVA, and no significant differences in agonist-mediated eNOS ser1177 phosphorylation responses were found between the two different antibodies.

Supplementary Figure 2: Agonist-promoted nitrate generation analyzed by the Griess assay.



Nitrate levels were determined using a commercial Griess Assay kit (Cayman Chemicals) to analyze culture supernatants of EA.hy926 endothelial cells treated with VEGF (10 ng/ml), insulin (100 nM), ATP (30 µM) or histamine (10 µM) for 20 minutes. The data are derived from three independent experiments, each performed in duplicate. The values are presented as mean ± S.D. ***P<0.001 versus control group using one-way ANOVA and Tukey's multiple comparison test.

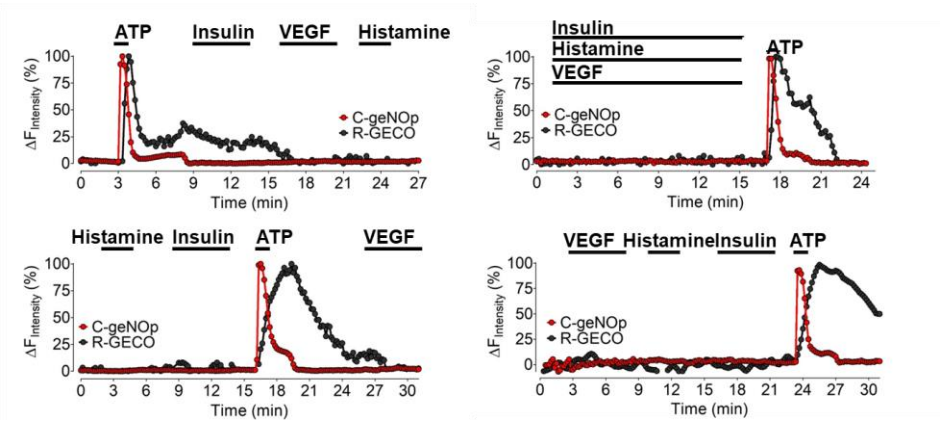
Supplementary Figure 3: Agonist-mediated eNOS phosphorylation in DMEM vs. "Imaging" Buffer".



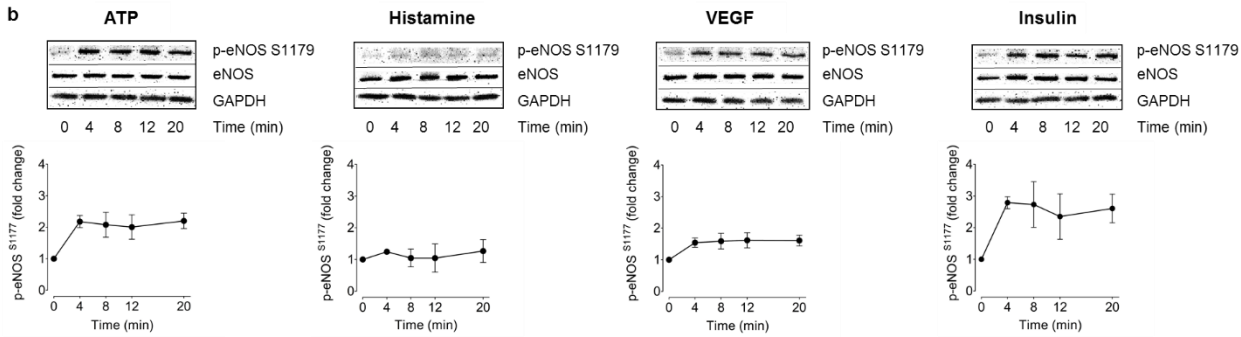
Panels **a** and **b** show representative immunoblots of eNOS serine 1177 phosphorylation in EA.hy926 endothelial cells in response to stimulation by VEGF, insulin (INS), ATP or histamine (HIS) at 10 ng/ml, 100 nM, 30 µM and 10 µM, respectively for 15 minutes. The cells were treated in: Panel **a-** "Imaging Buffer" (HEPES-buffered Ca^{2+}-containing Fe(II) fumarate/ascorbic acid medium, as used for the pretreatment of cell prior to imaging experiments); or Panel **b**- DMEM culture medium. Panel **c** shows pooled data from three separate experiments in cells incubated with DMEM (in red) or with "Imaging Buffer" in black. Values are presented as mean ± S.D. Data were analyzed by two-way ANOVA followed by Bonferroni post-tests. For each agonist, there were no significant difference between the phosphorylation responses seen with the two different incubation buffers.

Supplementary Figure 4: Multispectral imaging of intracellular Ca^{2+} and NO in BAEC

a

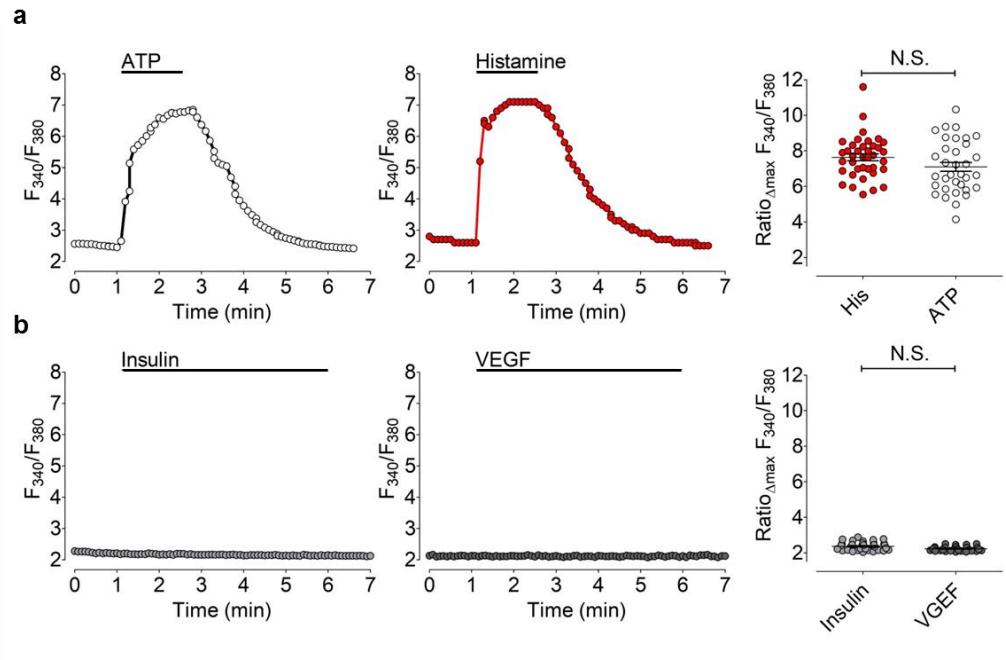


b



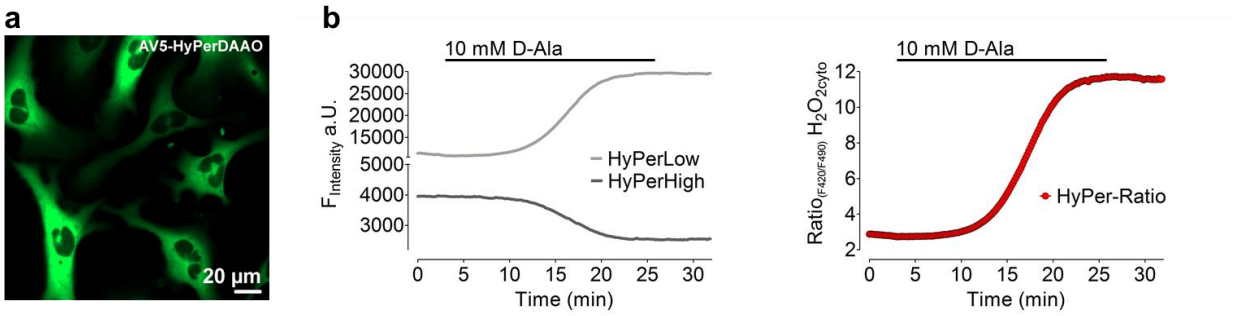
(**a**) These figures show representative multispectral imaging experiments analyzing BAEC co-transfected with R-GECO and C-geNOp. Cells were sequentially treated with 30 µM ATP, 10 µM histamine, 10 ng/ml VEGF, or 100 nM insulin as indicated. (n=3/16). (**b**) The upper panels demonstrate representative immunoblots from time course experiments in BAEC in response to agonists for different times as shown. Cell lysates were resolved by SDS-PAGE and probed with antibodies directed against phospho-eNOS Ser^{1177}. Equal loading was confirmed by probing immunoblotting with an antibody directed against total eNOS. The pooled data shown in the lower panels represent the quantitative analysis of intensities corresponding to eNOS phosphorylation at S1177. The values are presented as mean ± S.D. **P<0.05, **P<0.01* versus control using ANOVA and Tukey's multiple comparison test.

Supplementary Figure 5: Ca^{2+} imaging of EA.hy926 cells in response to agonists.



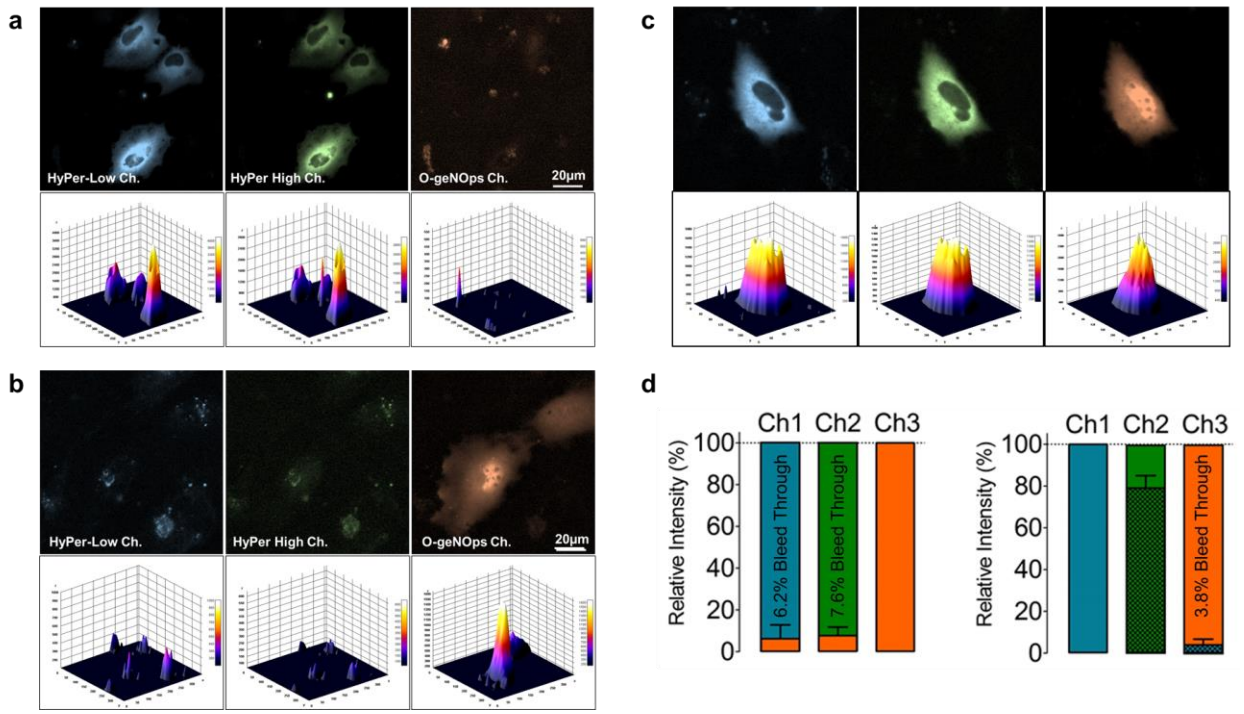
(**a**) Representative Fura2/am Ca^{2+} traces of EA.hy926 cells in response to 30 µM ATP (white dots) and 10 µM histamine (red dots). Scatter dot blot represents maximum agonist-mediated responses for ATP (red dots, n=3/34) and histamine (white dots, n=3/38). (**b**) Representative Ca^{2+} traces of EA.hy926 cells in response to 100 nM insulin and 10 ng/ml VEGF as indicated. Scatter dot blot represents maximum agonist-mediated responses for insulin (n=3/38) and VEGF (n=3/34). **P<0.05* versus control using the unpaired t-test.

Supplementary Figure 6: Functionality test of Ad5 HyPerDAAO in EA.hy926 cells.



(**a**) Representative widefield image of EA.hy926 cells infected with adenovirus encoding for HyPerDAAO. Cells were imaged 16 hours after infection. (**b**) Left panel represents the single wavelength of the HyPer biosensor DAAO fusion chimera in response to 10 mM D-alanine. The right panel shows the respective ratiometric signal of the left panel.

Supplementary Figure 7: Spectral differences of HyPer and O-geNOp biosensors permit multispectral imaging.



Upper panels show representative widefield images of HyPer biosensor (**a**) in the excitation/emission wavelengths 420/480 nm (left panels), 480/515 nm (middle panels) and 550/565 nm (right panels), O-geNOp biosensors (**b**), and co-expression of HyPer and O-geNOp biosensors (**c**). Lower panels in figures a-c show relative fluorescence intensities of the indicated biosensor in the respective channels. (**d**) Bars represent average fluorescence intensities of the bleed through into the respective other channel for O-geNOp (left panel, n=3/8), and HyPer (right panel n=3/10). Error bars are shown in ±SD.

Supplementary Movie 1: Ratiometric real-time imaging of DAAO-mediated H_2O_2 in a single endothelial cell.

The movie shows an original measurement of dynamic changes of HyPer-DAAO fluorescence over time upon cell treatment with 10 mM D-alanine. D-alanine was added via a peristaltic pump driven perfusion system.