SUPPLEMENTAL DATA

FUNCTIONAL SIGNIFICANCE OF CYTOSOLIC eNOS: REGULATION OF HYPERPERMEABILITY.

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SUPPLEMENTAL MATERIAL AND METHODS

Indirect Immunofluorescence Microscopy to assess colocalization of GFP-eNOS-G2A and sGC. ECV-GFP-eNOS-G2A permanently transfected cells (in which eNOS is targeted to the cytosol) were grown on glass coverslips to form a confluent monolayer. After washing with ice-cold PBS, they were fixed with methanol at -20°C for 10 minutes. After two washes with PBS, cells were permeabilized with 0.5% Triton X-100 at room temperature for 5 minutes, washed twice again with PBS, and blocked with 1% BSA-PBS (bovine serum albumin; phosphate buffer solution) at room temperature for 30 minutes. After washing twice with 0.1% BSA-PBS, cells were incubated with rabbit antibody against the alpha-1 unit of soluble guanylyl cyclase, sGC (Sigma Chemicals, St. Louis, MO) at 1:500 dilution in 0.1% BSA-PBS at 37C using a humid chamber. After two 5-minute washes in 0.1% BSA-PBS, cells were incubated with the secondary anti-mouse IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 555 Conjugate, red) antibody at 1:250 dilution in 0.1% BSA-PBS and incubated for 30 minutes in 37C in a humid chamber. Subsequently, the coverslips were mounted on a glass slide using mounting media containing 4',6-diamidino-2-phenylindole (DAPI) for identification of nuclei. The slides were kept in dark until observed under a Zeiss Axiovert 100 microscope to assess for colocalization of eNOS (GFP) and sGC).

Calibration of NO electrode. Nitrogen-equilibrated saline in 35 ml gas tonometer was used as a zero reference for NO, and, 400 and, 800 parts per million (ppm) NO in nitrogen were used to establish an 0-600-1,200 nM calibration range. According to the National Institute for Occupational Safety and Health Manual of Analytical Methods (1), 400 ppm and 800 ppm NO are equivalent to 600 nM and 1,200 nM NO, respectively, in the tonometer.

1. National Institute for Occupational Safety and Health Manual of Analytical Methods (NMAM), 4th Edition, Issue 1, Method 6014, U.S. Department of Health, Education, and Welfare.

SUPPLEMENTAL RESULTS

Determination of Monolayer Permeability. Figure S1 illustrates the measurement of monolayer permeability to FITC-dextran70 in ECV-eNOS-GFP cells.

PAF does not induce translocation of eNOS-CAAX. We applied 100 nM PAF to ECV-GFPeNOS-CAAX for 1 minute, 3 and 5 minutes to test whether or not PAF would cause the movement of eNOS-CAAX to intracellular compartments. There was no difference among the results obtained at the tested times. The results (Fig. S2) confirm that the CAXX modification targets and anchors eNOS firmly to the plasma membrane at least for the duration of our experiments. PAF did not stimulate translocation of GFPeNOS-CAAX away from the plasma membrane. The GFPeNOS-CAAX modification does not alter the ability of eNOS to produce NO, and therefore, the results support the concept that eNOS translocation to cytosol is necessary for the onset of hyperpermeability.

Cytosolic colocalization of GFP-eNOS-G2A and sGC. In an effort to define the cytosolic binding partners of eNOS, using fluorescence microscopy, we tested for the possible interaction

of eNOS with sGC, a recognized receptor of NO. Our results (Figure S3) show that GFP-eNOS-G2A and sGC colocalize in the cytosol.

SUPPLEMENTAL FIGURES

Figure S1

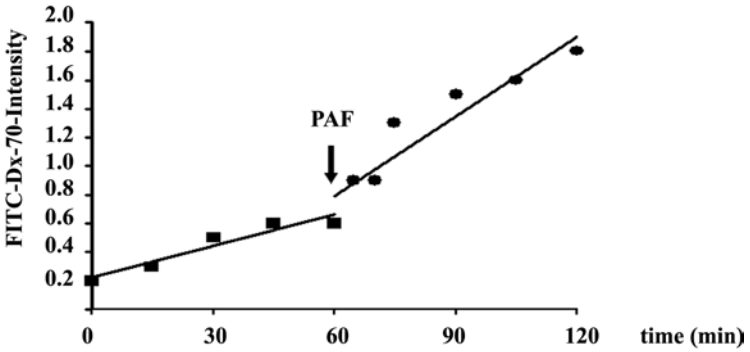

Figure S1. Measurement of monolayer permeability. The graph displays the time course of a typical experiment in ECV-eNOS-GFP cells. The fluorescence intensity of FITC-dextran 70 (in arbitrary units) reflects the concentration of the labeled dextran in the abluminal diffusion chamber. The quick change in slope upon application of PAF (at arrow) indicates the change in permeability. Solid squares = baseline; solid circles = 100 nM PAF.

Figure S2

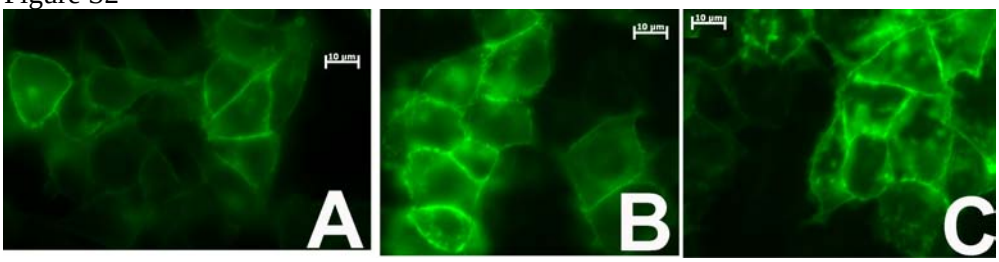

Figure S2. Location of GFP-eNOS-CAAX by fluorescence microscopy. GFP-eNOS-CAAX is seen clearly targeted to the plasma membrane. Application of 100 nM PAF fails to translocate GFP-eNOS-CAAX away from the plasma membrane. Panel A: Control image. Panel B: Image after 1-minute exposure to 100 nM PAF. Panel C: Image after 3-minute exposure to 100 nM PAF.

Figure S3

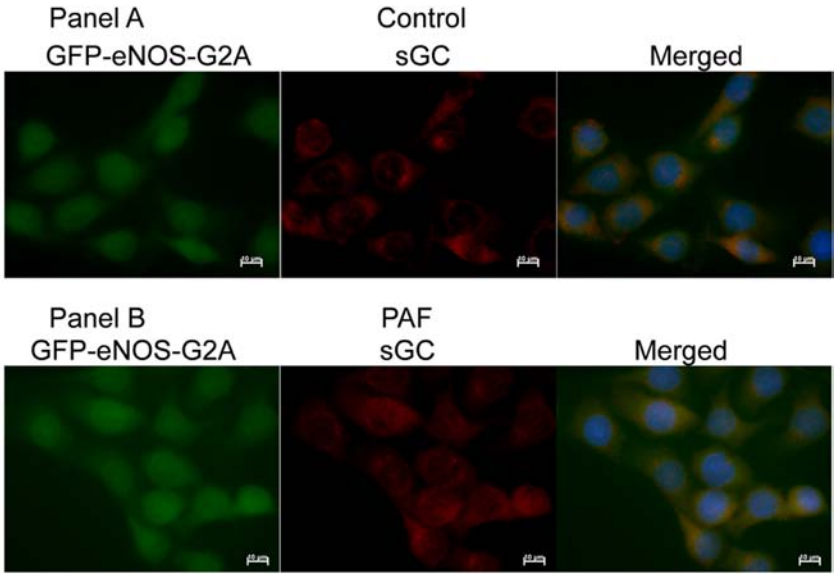

Figure S3. Cytosolic colocalization of GFP-eNOS-G2A and sGC. Indirect immunofluorescence demonstrates colocalization of eNOS G2A and sGC in the cytosol of ECV-304 cells transfected with GFP-eNOS-G2A, which targets eNOS to the cytosol. The nuclei are stained with DAPI. Panel A: control images of GFP-eNOS-G2A and sGC. Panel B: Images of GFP-eNOS-G2A and sGC after application of 100 nM PAF. Colocalization of eNOS and sGC is indicated by the yellow color.