MATERIALS AND METHODS

Cells, Antibodies, and Reagents. Human umbilical vein endothelial cells (HUVECs, Cascade Biologic) were cultured according to the manufacturer's protocols on plates coated with collagen (Attachment Factor, Gibco). Brain microvascular endothelial cells (BMVEC, CellScience) and coronary artery endothelial cells (CAEC, Invitrogen) were grown on plates coated with collagen and fibronectin (CellScience) and cultured according to the manufacturer's protocol. HUVEC and CAEC were cultured in the presence of gentamycin (10 μg/ml) and amphotericin B (0.25 μg/ml). BMVEC were cultured in the presence of penicillin (50 U/ml), streptomycin (50 μg/ml), and amphotericin B (0.25 μg/ml). Endothelial cultures at 90–100% confluence from passage 2 to 4 were used in all experiments. Primary antibodies used were anti-VWF (Dako A0082, Abcam #ab6994), anti-VWF-HRP (DAKO P0026), anti-GPIb antibody AK2 (RDI, CBL166C), anti-P-selectin-FITC (BD 555523), anti-αvβ3 (Millipore MAB1978), and anti-ADAMTS13 (gift from Dr. Jing-fei Dong). Secondary antibodies used were anti-rabbit AlexaFlour 488 (Invitrogen), anti-mouse AlexaFluor488 (Invitrogen A21202), anti-goat AlexaFluor 594 (Invitrogen), anti-rabbit Texas Red (Abcam ab6800), and anti-rabbit FITC (Invitrogen). Cocaine and metabolites benzoylecgonine, cocaethylene, ecgonine methylester, and norcocaine were purchased from Sigma-Aldrich according to U.S. Drug Enforcement Agency requirements. Cocaine, cocaine metabolites, and endothelial agonists histamine (Sigma), 1-deamino-8-D-arginine vasopressin (Teva), and epinephrine (Helena Labs) were dissolved in PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) before use. Forskolin (Sigma D3658) was used in designated experiments.

Parallel-Plate Flow Chamber. A parallel-plate flow chamber system was used as previously described.[1] Microscopy and imaging were performed using an inverted-stage microscope (Olympus IX81) equipped with a high-speed digital camera (Hamamatsu C4743-80-12AG). Images were acquired at 40× magnification and analyzed using SlideBook software (Olympus). Experiments were performed at a calculated shear stress of 2.5 dyne/cm^2. All solutions and cell chambers were kept at 37°C. Endothelial cells were treated with agonist or control agent for 8 min at 37°C before assembly in the parallel-plate flow chamber. Fresh, washed human platelets from healthy human donors were obtained as previously described[1] as per a protocol approved by the Institutional Review Board of the University of Washington School of Medicine. Acquired images were subsequently analyzed for the number of platelet strings formed in 10 random view fields per plate. For experiments using the GPIb blocking antibody AK2, platelets were incubated for 10 min with 4 μg/ml AK2 antibody before being perfused over stimulated or unstimulated endothelial cells as above.

VWF Dot Blot. Culture dishes containing confluent endothelial cells were washed twice with serum-free medium and incubated for 5–30 min at 37°C in 500 μl serum-free medium containing indicated agonists or controls. Supernatants were collected from each well and cleared by centrifugation at

2,000*g* for 10 min. The VWF content of the supernatants was analyzed using a standard dot blot apparatus (100 µl supernatant/well). Western blotting of the PVDF membranes was then performed using a polyclonal rabbit anti-VWF antibody (Abcam #ab6994) followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Pierce) and binding detected using a chemiluminescent substrate (ECL Plus, GE Healthcare). Images were analyzed and signals were quantified using an ImageQuant 350 (GE Healthcare), with a dilution series of normal human reference plasma (CryoCheck, Precision BioLogic) used for normalization. Results were also normalized based on endothelial cell number. Data shown represent three independent experiments for each type of endothelial cell, run in 6 serial dilutions in duplicate for each sample, generating 12 data points per condition for each endothelial cell supernatant and 36 data points overall.

Immunofluorescence. Endothelial cells were grown to confluence on coated coverslips in 6-well plates. The wells were washed with serum-free medium and cells were fixed in 4% paraformaldehyde in serum-free medium with or without 0.1% Triton X-100 to permeabilize the cell membrane and allow intracellular staining. The coverslips were blocked with 5% BSA in PBS, and stained with the indicated primary and secondary antibodies. The cells were imaged using the same microscope and camera described above. Fluorescence images were acquired at 40× or 60× magnification in 0.1 µM z-axis sections, followed by deconvolution to a 2D image using Slidebook 5.5 software (Intelligent Imaging Innovations, Inc.). Total VWF immunofluorescence was then calculated per field as a function of the number of DAPI-stained nuclei (cell number) present. Data shown are from 3–6 fields for each of three replicate experiments per endothelial cell type.

VWF Multimer Analysis. Endothelial cell supernatants were collected as above. Samples were subjected to non-reducing gel electrophoresis as previously described.[2]

cAMP Assay. Intracellular cAMP was assayed using a commercially available kit (R&D Systems KGE002B). Endothelial cells were incubated with agonist or control, washed, and lysed per manufacturer's protocol. Cell lysates were then assayed by ELISA as per the manufacturer's recommendations.

Statistical Methods. VWF responses from endothelial cells treated with different agonists were compared using Tukey's HSD (honestly significant difference) method.[3] The cell type experiment-wise Type I error rate was controlled at the 0.05 level. All statistical computations were done using the R language and programming environment.

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