Cocaine and Specific Cocaine Metabolites Induce von Willebrand Factor Release From Endothelial Cells in a Tissue-Specific Manner

William E. Hobbs, Emily E. Moore, Rebecca A. Penkala, Doug D. Bolgiano, José A. López

Objective—Cocaine use is associated with arterial thrombosis, including myocardial infarction and stroke. Cocaine use results in increased plasma von Willebrand factor (VWF), accelerated atherosclerosis, and platelet-rich arterial thrombi, suggesting that cocaine activates the endothelium, promoting platelet–VWF interactions.

Approach and Results—Human umbilical vein endothelial cells, brain microvasculature endothelial cells, or coronary artery endothelial cells were treated with cocaine or metabolites benzoylecgonine, cocaethylene, norcocaine, or ecgonine methylester. Supernatant VWF concentration and multimer structure were measured, and platelet–VWF strings formed on the endothelial surface under flow were quantified. Cocaine, benzoylecgonine, and cocaethylene induced endothelial VWF release, with the 2 metabolites being more potent than the parent molecule. Brain microvasculature endothelial cells were more sensitive to cocaine and metabolites than were human umbilical vein endothelial cells or coronary artery endothelial cells. Coronary artery endothelial cells released VWF into the supernatant but did not form VWF–platelet strings. Intracellular cAMP concentration was not increased after treatment with cocaine or its metabolites.

Conclusions—Both cocaine and metabolites benzoylecgonine and cocaethylene induced endothelial VWF secretion, possibly explaining thrombotic risk after cocaine ingestion. VWF secretion is likely to vary between vascular beds, with brain endothelial cells being particularly sensitive. These results suggest that clinical management of cocaine-induced ischemia may benefit from therapies aimed at disrupting the VWF–platelet interaction. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: cocaine ■ endothelium ■ platelets ■ thrombosis ■ von Willebrand factor

Cocaine use is associated with an increased risk of a variety of thrombotic events, including stroke and myocardial infarction (MI). The risk of MI is estimated to increase 6-fold with cocaine use. Although cocaine is rapidly metabolized by liver and plasma enzymes, the risk of thrombosis remains elevated for many days after ingestion, possibly an effect of cocaine metabolites, some of which retain adrenergic activity and can persist in blood for 1 to 2 weeks. It is unknown how these metabolites, which include benzoylecgonine, ecgonine methylester, and norcocaine, affect platelet or endothelial functions. Another metabolite, cocaethylene, which is produced when cocaine is consumed with ethanol, is known to retain potent neuronal stimulation ability and is associated with an extremely high risk of sudden death. Its relationship to thrombotic risk is also unknown.

Although one likely cause of tissue ischemia is cocaine-induced vasospasm in the setting of increased tissue oxygen demand, platelet-rich arterial thrombi have also been observed in coronary and cerebral vessels of up to 14% of cocaine users who died suddenly after ingesting cocaine. Furthermore, a 10-fold increased incidence of acute coronary stent thrombosis, a platelet-dependent process, was observed in cocaine users, which was not explained by noncompliance with antiplatelet therapy. Thus, in addition to the sympathomimetic effects of cocaine leading to increased tissue oxygen demand (increased heart rate, blood pressure, contractility, and metabolism) and decreased tissue oxygen delivery (vasoconstriction), cocaine likely contributes to ischemic events by promoting platelet aggregation on endovascular surfaces.

The primary endothelial determinant of platelet–endothelial attachment is von Willebrand factor (VWF), a multimeric protein secreted from endothelial cells. On endothelial cell activation, VWF is rapidly secreted into plasma from storage granules called Weibel-Palade bodies. VWF is secreted as a high molecular weight form referred to as ultralarge VWF (ULVWF). ULVWF can be retained on the vascular surface via P-selectin and integrin αβ3 with long ULVWF strands extending up to several millimeters into the bulk flow. VWF binds platelets via the platelet glycoprotein Ib-IX-V complex, with ULVWF, unlike normal plasma forms of VWF, being able to spontaneously bind this receptor. The binding of platelets to VWF is the first step in thrombus formation, promoting subsequent platelet activation and aggregation. Under normal

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circumstances, ULVWF is processed proteolytically by the plasma metalloproteinase ADAMTS13 into the smaller, less-reactive VWF forms normally found in plasma.

Cocaine use is associated with a 40% increase in plasma VWF concentration, suggesting that cocaine induces endothelial secretion of VWF and supporting a role for platelet adhesion to the endothelium in cocaine-associated arterial thrombosis. In addition, because platelet binding to processed plasma VWF is increased under high shear stress, cocaine-induced vasocstriction would be expected to promote thrombus formation. Histopathologic studies support this scenario, demonstrating numerous endovascular abnormalities in cocaine abusers, who tend to be young (>35 years of age) with few, if any, additional cardiovascular risk factors. These findings include accelerated atherosclerosis, intimal hyperplasia, and increased adventitial inflammation. Endothelial dysfunction induced by cocaine is also suggested by the findings of upregulated endothelial adhesion molecules, increased vascular permeability, and increased circulating endothelial cells after cocaine exposure.

We hypothesize that cocaine acts as an endothelial agonist and VWF secretagogue, promoting platelet-mediated thrombosis by inducing exposure of ULVWF on endothelial surfaces. Given the prolonged thrombotic risk after cocaine ingestion, we also hypothesize that cocaine metabolites induce ULVWF release from endothelial cells. We therefore assessed the ability of cocaine and its metabolites to induce VWF release from cultured human vascular endothelial cells from a variety of tissue beds, including umbilical vein, brain, and coronary artery.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

VWF is secreted from endothelial cells in response to a variety of agonists, including histamine, epinephrine, dDAVP, and hypoxia. After secretion, VWF can either remain tethered to the endothelial surface or be released into the supernatant. We assessed the ability of cocaine and cocaine metabolites to directly induce VWF secretion from human endothelial cells of various tissues.

Cocaine Induces VWF Release From Endothelial Cells

We treated endothelial cells from 3 different sources (human umbilical vein endothelial cells [HUVEC], brain microvasculature endothelial cells [BMVEC], and coronary artery endothelial cells [CAEC]) with cocaine at several concentrations and assessed their secretion of VWF. Cocaine induced VWF secretion in a concentration-dependent manner from the 3 endothelial cell types (Figure 1A). In all 3 cell types, cocaine at a concentration of 1 μg/mL induced a similar extent of VWF secretion as 50 μM histamine or 4 μg/mL dDAVP, both known potent VWF secretagogues. This cocaine concentration, which is within the range detected in the plasma of cocaine users, increased VWF secretion >300% above the unstimulated baseline. Lower concentrations of cocaine (0.5 μg/mL) also induced endothelial VWF release to twice that of unstimulated HUVEC and BMVEC (Figure 1A–1C). In contrast, VWF release from CAEC treated with 0.5 μg/mL cocaine was not different from that of untreated cells (P=0.99; Figure 1D). For all concentrations of cocaine tested, secretion from BMVEC was greater than that from HUVEC and CAEC.

Cocaine Metabolites, Benzoylecgonine and Cocaethylene, Induce VWF Release From Human Endothelial Cells

The risk of cardiovascular complications after cocaine ingestion persists long after cocaine levels fall below the minimum cocaine concentration able to stimulate endothelial VWF in our assay. Therefore, we also studied 4 of the major cocaine metabolites for their ability to induce VWF release from endothelial cells. Only benzoylecgonine and cocaethylene induced VWF release, and both induced it in all 3 cell types (Figure 1B–1D). The concentrations of cocaine metabolites used here have been reported in the plasma of cocaine users. In general, BMVEC released larger quantities of VWF than HUVEC or CAEC in response to the 2 metabolites. Benzoylecgonine and cocaethylene at both 1 μg/mL and 0.1 μg/mL induced significant increases in VWF secretion in both HUVEC (Figure 1B) and BMVEC (Figure 1C) compared with untreated cells (P<0.05). However, CAEC responded robustly only at the higher concentration (Figure 1D). Both benzoylecgonine and cocaethylene were more potent than cocaine in inducing endothelial VWF release, because cocaine at 0.1 μg/mL failed to induce VWF release from any of the cell types.

ULVWF Released From HUVEC and BMVEC Remains Tethered to the Endothelial Surface and Binds Platelets Under Flow

On initial secretion, a subset of ULVWF molecules remains tethered to the endothelial surface, forming fibers under flow capable of reaching several millimeters in length. Such incredibly long ULVWF strings can spontaneously bind hundreds of platelets and can be easily visualized as beads on a string by phase-contrast microscopy (Figure 2A). This platelet–VWF interaction is dependent on the platelet VWF receptor, GPIb. Using a parallel-plate flow chamber, we assessed ULVWF–platelet string formation in cocaine-treated HUVECs (Figure 2B) and BMVECs (Figure 2C) exposed to a constant shear stress of 2.5 dyne/cm². Mock-treated HUVECs and BMVECs failed to form strings (Figure 2B), whereas cocaine (1 μg/mL) induced numerous VWF–platelet strings in both HUVEC and BMVEC (Figure 2B and 2C). Benzoylecgonine and cocaethylene also induced VWF–platelet string formation on HUVEC and BMVEC at concentrations reported to occur in plasma, but norcocaine and ecygone methylester did not (Figure 2B and 2C). In HUVEC, the lowest concentration of cocaethylene (0.1 μg/mL) induced a similar number of VWF–platelet strings as 50 μM histamine, whereas 1 μg/mL cocaine produced fewer platelet–VWF strings than histamine. In BMVEC, benzoylecgonine and cocaethylene at both concentrations and cocaine at 1 μg/mL induced similar numbers of VWF–platelet strings as 50 μM histamine. This indicates that BMVEC are more sensitive than HUVEC to cocaine and the longer-lived cocaine metabolites. As has been reported, CAEC did not produce VWF–platelet strings after treatment with histamine.
We obtained a similar result in CAEC treated with cocaine or cocaine metabolites (data not shown). As expected, formation of VWF–platelet strings in cocaine- and cocaine metabolite–treated HUVEC was prevented by pretreating the platelets with a GPIb-blocking antibody (Figure 2D).

**Endothelial Cell VWF Content Varies Between Tissue Sources**

We investigated whether BMVEC secreted more VWF than CAEC when treated with cocaine or its active metabolites because the former cell line contained more intracellular VWF. Quantification of the immunofluorescence signals from anti–VWF-labeled permeabilized cells indeed revealed that BMVEC contained more intracellular VWF than either HUVEC or CAEC (Figure 3).

**CAEC Secrete ULVWF in Response to Cocaine and Cocaine Metabolites and Express P-selectin, αvβ3, and ADAMTS13**

One potential explanation for the inability of CAEC to produce platelet–VWF strings after stimulation by agonists is that they do not secrete VWF in the ULVWF form. We therefore assessed the multimer pattern in VWF secreted from CAEC treated with histamine, cocaine, benzoylecgonine, or cocaethylene (Figure 4). As compared with VWF in normal human plasma, stimulated CAEC supernatants contained abundant ULVWF but apparently less than HUVEC or BMVEC supernatants and of lower maximum size (Figure 4).

We also investigated whether the inability of CAEC to form platelet strings was because these cells are deficient in P-selectin or the integrin αvβ3, loss of either of which could prevent tethering of secreted ULVWF to the endothelial surface. By immunofluorescence of unstimulated endothelial cells, P-selectin was present in endothelial cells from each tissue type, colocalizing with VWF in a distribution consistent with Weibel-Palade bodies (Figure 3). After agonist stimulation of CAEC, P-selectin appeared on the cell surface as it did in HUVEC (Figure 5A). In addition, integrin αvβ3 was present on the surfaces of the 3 types of endothelial cells (Figure 5B). This indicates that CAEC express the appropriate cell surface proteins to retain secreted ULVWF on the cell surface.
Because ADAMTS13 normally cleaves ULVWF, and could remove VWF from the endothelial surface after secretion from endothelial cells, we evaluated the possibility that CAEC express more ADAMTS13 than do BMVEC. ADAMTS13 distribution within each endothelial cell type was similar (Figure 5B).

VWF Secretion Induced by Cocaine and Its Metabolites Does Not Involve Increased Intracellular cAMP

The mechanism by which cocaine and its metabolites induce endothelial VWF release is unknown. Increased intracellular cAMP precedes Weibel-Palade body exocytosis and VWF secretion in response to several agonists, including dDAVP but not histamine. We therefore examined intracellular cAMP concentrations in lysates of endothelial cells treated with cocaine or its metabolites. The intracellular cAMP concentration did not change relative to untreated cells after incubation with cocaine and cocaine metabolites at concentrations shown to induce VWF secretion in HUVEC (Figure 6). Similar results were obtained in BMVEC and CAEC (data not shown).

Discussion

Cocaine abuse remains a major public health problem, contributing to >400,000 emergency room visits per year. In addition to the problem of drug addiction and its attendant social and medical issues, cocaine ingestion is associated with both acute and chronic cardiovascular disease, including MI and stroke. The thrombotic effects of cocaine include increased rates of both arterial and venous thrombosis. These thrombotic events can be delayed after cocaine use, sometimes occurring when cocaine is no longer detectable in plasma. Our results indicate that this prolonged effect of cocaine on thrombotic risk is related to the continued prothrombotic activity of the long-lived cocaine metabolites benzoylecgonine and cocaethylene.
Our results indicate that endothelial cells in culture release VWF in response to agonism by cocaine, benzoylecgonine, and cocaethylene, but not by cocaine metabolites, norcocaine, or ecgonine methyl ester. Cocaine, benzoylecgonine, and cocaethylene are extremely potent VWF secretagogues, with similar ability to induce VWF release as 50 μg/mL dDAVP, both known strong VWF secretagogues. Our results also indicate that the metabolites benzoylecgonine and cocaethylene are at least as potent at inducing endothelial VWF as cocaine, and in some endothelial cell types are more potent agonists than cocaine and able to induce significant VWF release from endothelial cells at very low concentrations. Particularly significant is the effect of cocaethylene, which is only formed when cocaine is ingested simultaneously with ethanol. This finding may explain the particularly elevated risk for cardiovascular events seen accompanying combined cocaine and ethanol use. Brain microvascular endothelial cells are especially sensitive to cocaine, benzoylecgonine, and cocaethylene as compared with umbilical vein or coronary artery–derived endothelial cells. This was true both for VWF released into the culture supernatant in ULVWF form as well as for secreted VWF that remains tethered to the endothelial surface. Platelet binding to endothelial surface–bound VWF was found to be dependent on the platelet receptor, GPIb. This supports a model in which cocaine-associated thrombosis involves direct action of cocaine and cocaine metabolites on endothelial cells to secrete VWF, followed by platelet binding and subsequent activation.

Several years ago, it became clear that platelets were a target of cocaine, the mechanisms of this effect have yet to be fully elucidated. In response to cocaine, platelets become activated and release constituents of their alpha granules, promoting thrombosis. Cocaine also decreases nitric oxide levels that, in addition to inhibiting vasodilatation, can lower the threshold for platelet activation. Further evidence that platelets are involved in the cardiovascular pathophysiology associated with cocaine is the high incidence of acute coronary stent thrombosis in cocaine users, even in those compliant with antiplatelet therapy. It is not known whether benzoylecgonine, or cocaethylene, which we found induce endothelial VWF release, can also activate platelets. Platelets bind ULVWF spontaneously via an interaction of the VWF A1 domain with the platelet receptor GPIb, initiating platelet aggregation and thrombosis. When VWF is cleaved to lower–molecular-weight VWF by ADAMTS13, the A1 domain is no longer able to spontaneously bind platelets but retains its ability to promote platelet aggregation and thrombosis. Accumulation of ULVWF is associated with the devastating thrombotic disease, thrombotic thrombocytopenic purpura. Consistent with a similar mechanism for cocaine–associated thrombosis, a thrombotic thrombocytopenic purpura–like syndrome has been reported in cocaine users.
VWF has been detected in chronic cocaine abusers. Whether to induce ULVWF secretion. Indeed, increased circulating lin-1,35 stromal cell–derived factor-1,24 monocyte chemotactic VWF concentrations. The ability of all forms of VWF to bind be expected to potentiate the prothrombotic effect of increased Vasoconstriction induced by cocaine and its metabolites would lium are also mechanistically important and likely additive. effects of cocaine and its metabolites on the vascular endothe-

\[ \kappa_B \] activity,42 and activation of endothelial cell nuclear factor nitric oxide synthase activity and nitric oxide production,35

Figure 5. Coronary artery endothelial cells (CAEC) translocate P-selectin to the cell surface and express \( \alpha v \beta 3 \) and ADAMTS13. A, Endothelial cells were incubated with serum-free media (unstimulated) or cocaine 1 \( \mu g/mL \) (stimulated). Cells were then fixed without permeabilization and stained with an anti-P-selectin antibody (green). B, Endothelial cells fixed without permeabilization (\( \alpha v \beta 3 \)) or with permeabilization (ADAMTS13). Cell surface \( \alpha v \beta 3 \) (green) and intracellular ADAMTS13 (red) were detected for each indicated endothelial cell type. In all panels, nuclei are stained with 4',6-diamino-2-phenylindole (blue). BMVEC indicates brain microvasculature endothelial cells; and HUVEC, human umbilical vein endothelial cells.

suggesting that in at least a subset of this group there is, in fact, an accumulation of ULVWF. Whether increased ULVWF can be detected in cocaine abusers is unknown.

Previous studies have demonstrated probable endothelial activation after cocaine use, as documented by increased endothelial release of platelet-derived growth factor,21 endothelin-1,35 stromal cell–derived factor-1,24 monocyte chemotactic protein-1,24 and soluble intercellular adhesion molecule-1.24 In addition, cocaine is associated with decreased endothelial nitric oxide synthase activity and nitric oxide production,55 activation of endothelial cell nuclear factor \( \kappa B \) activity,19 and increased numbers of circulating endothelial cells.24 Signals that induce such endothelial activation would also be expected to induce ULVWF secretion. Indeed, increased circulating VWF has been detected in chronic cocaine abusers.19 Whether the increased VWF concentration is attributable to increased production or delayed clearance cannot be discerned from measurements of the plasma concentration. Our studies suggest that the elevated VWF concentration in the blood of cocaine users is largely attributable to increased endothelial secretion in response to cocaine and 2 of its major metabolites.

The cardiovascular complications of cocaine use had been presumed to be primarily caused by the effects of cocaine on the sympathetic nervous system, causing vasoconstriction of coronary vessels while simultaneously increasing myocardial oxygen demand by increasing heart rate, contractility, blood pressure, and cellular metabolism. Cocaine metabolites have also been shown to induce cerebral and coronary vasoconstriction, particularly benzoylecgonine and cocaethylene.43,44 Although these mechanisms likely contribute to cocaine-induced myocardial ischemia, our results demonstrate that the effects of cocaine and its metabolites on the vascular endothelium are also mechanistically important and likely additive. Vasoconstriction induced by cocaine and its metabolites would be expected to potentiate the prothrombotic effect of increased VWF concentrations. The ability of all forms of VWF to bind and activate platelets is enhanced by high-fluid shear stress.45 This physical force is increased in proportion to the velocity of blood flow and in an inverse relationship to the radius of the blood vessel. Flow velocity increases and vessel diameter decreases during vasoconstriction, increasing the likelihood of VWF-dependent shear-induced platelet aggregation. The threshold for aggregation will be further decreased and the extent increased in the presence of elevated VWF concentrations. Furthermore, the propensity to develop a flow-restricting thrombus would be further increased by additional effects of cocaine and cocaine metabolites on platelet activation.

The anesthetic properties of cocaine relate to its ability to slow nerve conduction by blocking voltage-gated sodium channels.46 It produces addiction by blocking presynaptic dopamine and serotonin reuptake receptors, potentiating the effect of these neurotransmitters in the synaptic cleft in reward centers of the brain.7 Similarly, the sympathomimetic effects of cocaine are a consequence of its ability to block presynaptic reuptake of catecholamines at sympathetic nerve terminals and stimulate central sympathetic outflow.47,48 It is unknown how cocaine and its metabolites activate endothelial cells to secrete VWF, whether by engaging a cell surface receptor or an intracellular target. Also unknown are the signaling pathways involved. We found that endothelial cAMP concentrations do not increase in endothelial cells treated with cocaine or its metabolites.

It is noteworthy that MI is a more common thrombotic consequence of cocaine abuse than is stroke. Our studies in a cell culture system suggest that brain-derived endothelial cells contain more VWF and are more sensitive to cocaine and cocaine metabolites than coronary artery–derived endothelial cells. The inability of CAEC to produce platelet–VWF strings is of unclear significance in light of the clinical and pathological data supporting a role for platelet–VWF interaction in cocaine-associated MI. Although we have shown that CAEC contain similar intracellular ADAMTS13 quantities as the 2 other endothelial types studied, it is possible that ADAMTS13 on
the surface of CAEC is more active than in other cell types, leading to increased clearance of VWF from the surface of CAEC in the static assays used in our studies. In addition, unlike other tissue beds, coronary arterial flow is subjected to compressive forces exerted by cardiac muscle and differs from flow in other arteries in being highest during diastole instead of systole. It remains possible as well that cocaine- and cocaine metabolite–activated coronary artery endothelium is able to capture circulating VWF and platelet–VWF aggregates, a process that would be promoted by increased VWF and ULVWF abundance in the blood, platelet activation, and vasoconstriction-induced shear stress. It is possible that in vivo models may be more revealing than in vitro studies.

Interestingly, evidence for cocaine-induced thrombosis has been reported after low-dose intranasal cocaine delivery to mice.33 It is interesting to speculate that the ability of cocaine to arrest epistaxis and other bleeding events is not simply attributable to local vasospasm, as has been postulated, but rather to platelet-mediated thrombosis within bleeding vessels. The septal necrosis reported in chronic cocaine abusers whose preferred route of administration is snorting may thus represent thrombosis as opposed to sequelae of vasoconstriction.

In summary, we report here that cocaine and 2 of its metabolites, benzoyl-leucine and cocaethylene, potently stimulate release of hyperadhesive forms of VWF from endothelium, providing at least 1 mechanism by which this commonly used recreational drug can cause potentially catastrophic thrombotic events. The likelihood of developing occlusive coronary thrombi is increased even further by the vasoconstrictive effect of these drugs. These data also suggest that therapy for these events should be tailored toward disrupting this mechanism of thrombosis.

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Disclosures

None.

References


**Significance**

Individuals who abuse cocaine are at high risk of thrombosis, particularly stroke and myocardial infarction. More than 240,000 individuals are seen each year in US emergency rooms with cocaine-induced chest pain, of which an estimated 15,000 suffer myocardial infarction. The risk period for cocaine-associated thrombosis extends days after cocaine is no longer detectable in plasma, including a 10-fold increased risk of acute coronary stent thrombosis after angioplasty. We hypothesized that longer-lived cocaine metabolites participate in increased thrombotic risk by directly activating endothelial cells to release von Willebrand factor. We found that 2 cocaine metabolites, benzoylecgonine and cocaethylene, are potent inducers of von Willebrand factor release from brain and coronary-artery–derived endothelial cells, and in some cases more potent than cocaine itself. This suggests that a common mechanism of cocaine-induced thrombosis is von Willebrand factor–dependent and could be treated by disrupting the platelet–von Willebrand factor interaction.
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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. 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/cm². 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 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
2,000g 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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