Platelets subjected to elevated levels of fluid shear stress in the absence of exogenous agonists will aggregate. Shear stress-induced aggregation requires von Willebrand factor (vWF) multimers, extracellular calcium (Ca\(^2+\)), adenosine diphosphate (ADP), and platelet membrane glycoprotein (GP)Ib and GPIIb-IIIa. The sequence of interaction of vWF multimers with platelet surface receptors and the effect of these interactions on platelet activation have not been determined. To elucidate the mechanism of shear stress-induced platelet aggregation, suspensions of washed platelets were subjected to different levels of uniform shear stress (15 to 120 dyne/cm\(^2\)) in an optically modified cone and plate viscometer. Cytoplasmic ionized calcium ([Ca\(^2+\)]) and aggregation of platelets were monitored simultaneously during the application of shear stress; [Ca\(^2+\)] was measured using indo-1 loaded platelets and aggregation was measured as changes in light transmission. Basal [Ca\(^2+\)] was approximately 60 to 100 nm/L. An increase of [Ca\(^2+\)] (up to >1,000 nm/L) was accompanied by synchronous aggregation, and both responses were dependent on the shear force and the presence of vWF multimers. EGTA chelation of extracellular Ca\(^2+\) completely inhibited vWF-mediated [Ca\(^2+\)] and aggregation responses to shear stress. Aurin tricarboxylic acid, which blocks the GPIb recognition site on the vWF monomer, and 6D1, a monoclonal antibody to GPIb, also completely inhibited platelet responses to shear stress. The tetrapeptide RGDS and the monoclonal antibody 10E5, which inhibit vWF binding to GPIIb-IIIa, partially inhibited shear stress-induced [Ca\(^2+\)] and aggregation responses. The combination of creatine phosphate/creatine phosphokinase, which converts ADP to adenosine triphosphate and blocks the effect of ADP released from stimulated platelets, inhibited shear stress-induced platelet aggregation without affecting the increase of [Ca\(^2+\)]. Neither the [Ca\(^2+\)] nor aggregation response to shear stress was inhibited by blocking platelet cyclooxygenase metabolism with acetylsalicylic acid. These results indicate that GPIb and extracellular Ca\(^2+\) are absolutely required for vWF-mediated [Ca\(^2+\)], and aggregation responses to imposed shear stress, and that the interaction of vWF multimers with GPIIb-IIIa potentiates these responses. Shear stress-induced elevation of platelet [Ca\(^2+\)], but not aggregation, is independent of the effects of released ADP, and both responses occur independently of platelet cyclooxygenase metabolism. These results suggest that shear stress induces the binding of vWF multimers to platelet GPIb and this vWF-GPIb interaction causes an increase of [Ca\(^2+\)] and platelet aggregation, both of which are potentiated by vWF binding to the platelet GPIIb-IIIa complex.

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MATERIALS AND METHODS

Washed platelet preparation. Blood was obtained from healthy individuals who had not ingested any medications for 2 weeks before donation. The blood was drawn into 15% (vol/vol) acid-citrate-dextrose (ACD). Platelet-rich plasma (PRP) was obtained by centrifugation at 150g for 15 minutes. pH of the PRP was adjusted to 6.5 with ACD, and the platelets were pelleted by centrifugation at 1,900g for 15 minutes. The platelets were resus-
pended and washed in a HEPES buffer solution (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na2HPO4, 1 mmol/L MgSO4, 5.5 mmol/L glucose, and 3.5 g/L bovine serum albumin [Sigma Chemical Co, St Louis, MO]), pH 7.0, containing 0.1 mmol/L CaCl2, 50 U/mL sodium heparin (from porcine intestine; Elkins-Sinn, Inc, Cherry Hill, NJ), and 2.5 U/mL apyrase (Grade V, Sigma). The washed platelets were then either loaded with indo-1/AM (described below), or pelletted and washed in a HEPES buffer solution (10 mmollL HEPES, 145 mmollL NaCl, 5 mmollL KCl, and 3.5 g/L bovine serum albumin [Sigma Chemical Co, St Louis, MO]), pH 7.0, containing 0.1 mmollL CaCl2, 50 U/mL sodium heparin (from porcine intestine; Elkins-Sinn, Inc, Cherry Hill, NJ), and 2.5 U/mL apyrase (Grade V, Sigma). The washed platelets were then either loaded with indo-1/AM (described below), or pelletted and washed in a HEPES buffer, pH 7.4, containing 1 mmollL CaCl2. The platelet concentration was adjusted to 300,000/μL using an electronic particle counter (Model ZBI; Coulter Electronics, Inc, Hialeah, FL). Before each viscometer experiment, it was demonstrated that these platelets aggregated normally in response to collagen (2 μg/mL; Hormon-Chemie, Munich, Germany) and ADP (5 μmol/L; Sigma) in the presence of fibrinogen (1 mg/mL; Helena Lab, Beaumont, TX).

vWF preparation. For experiments with vWF, purified vWF multimers were added to the washed platelet suspensions. The vWF multimeric forms were purified and fractionated from normal human cryoprecipitate as described previously, and the vWF antigen levels of the purified fractions were quantified by solid-phase immunoradiometric assay (IRMA). vWF multimers were separated by sodium dodecyl sulfate (SDS)-1% agarose gel electrophoresis, overlaid with rabbit anti-human vWF polyclonal antibody, and analyzed by autoradiography. The purified vWF forms used in this study were enriched in the largest multimers found in plasma. Unless otherwise specified, a 300% antigen level (100 U/dL) was added to the washed platelet suspensions.

Cone and plate viscometer. Washed platelet suspensions were subjected to fluid shear stress in a cone and plate viscometer (Ferranti Electric, Inc, Commmack, NY). The viscometer was modified optically to simultaneously measure platelet aggregation and [Ca2+]i (Fig 1). Aggregation was measured by monitoring light transmission through the sample using a collimated beam of light at 690 nm incident on a photomultiplier tube interfaced with a computer. Aggregation was calculated using the following formula: percent aggregation (%T) = 100 × (T - T0/Tb - T0) where Tb is the initial light transmission of the washed platelet suspension; T0 is the light transmission of buffer; and T is the light transmission of the platelet sample during shear. Platelet particle number was measured using a Coulter ZBI with a channelizer interfaced with a computer as previously described. [Ca2+]i was measured by the ratio (R) of fluorescence emission at 400 nm and 480 nm of the calcium fluorophore, indo-1, after excitation at 340 nm. Indo-1 was loaded into the platelet cytoplasm by adding 1 μmol/L of its acetoxymethyl ester (indo-1/AM, dissolved in 0.1% vol/vol dimethyl sulfoxide [Molecular Probes, Eugene, OR]) to the platelets suspended in HEPES buffer, and incubating for 90 minutes at room temperature. The platelets were then centrifuged at 1,000g and resuspended in HEPES buffer (with 1 mmol/L CaCl2, unless otherwise stated) for the viscometer experiments. The indo-1/AM treatment did not affect the platelet aggregation responses to ADP and collagen. [Ca2+]i was determined by measuring the minimum ratio (Rmin) with lysed indo-1 loaded platelets under calcium-free conditions (200 mmol/L EGTA) and the Rmax under calcium-saturated condition (200 mmol/L CaCl2) and using the following equation: [Ca2+]i = Kd(F0/Fx) (R - Rmin)/(Rmax - R). For experiments with Mn2+, the loaded platelets were resuspended in buffer with added Ca2+. Just before the viscometer experiments, either 1 mmol/L EGTA or 1 mmol/L CaCl2 was added. For experiments with Mn2+, the loaded platelets were resuspended in buffer with 2 mmol/L CaCl2. Just before the viscometer experiments, varying concentrations of MnCl2 (0 to 4 mmol/L) were added.

EGTA and Mn2+ experiments. Indo-1 loaded platelets were resuspended in buffer without added Ca2+. Just before the viscometer experiments, either 1 mmol/L EGTA or 1 mmol/L CaCl2 was added. For experiments with Mn2+, the loaded platelets were resuspended in buffer with 2 mmol/L CaCl2. Just before the viscometer experiments, varying concentrations of MnCl2 (0 to 4 mmol/L) were added.

Aurin tricarboxylic acid (ATA) and RGDS experiments. Dialyzed fractions of ATA polymers (Sigma) greater than 2,500 daltons were prepared as previously described. Purified vWF multimers were incubated with ATA (6 μg/mL final concentration), or its vehicle,
for 1 minute at room temperature, and then the mixture was added to washed platelet suspensions.

The tetrapeptide RGDS (arginine-glycine-aspartic acid-serine) was obtained from Calbiochem (La Jolla, CA). Washed platelet suspensions were incubated with RGDS (200 μmol/L), or its vehicle, for 1 minute. This concentration completely inhibits platelet aggregation caused by ligand binding to GPIb-IIIa.14,15

*Monoclonal antibody (MoAb) experiments.* Purified murine MoAbs 6D1 (against GPIb) and 10E5 (against GPIb-IIIa) were generously provided by Dr Barry S. Coller (State University of New York Health Science Center at Stony Brook). Washed platelets were incubated for 5 minutes at room temperature with either 6 μg/mL of 6D1 or 10 μg/mL of 10E5. These concentrations completely inhibit aggregation associated with ligand binding to GPIb or GPIb-IIIa, respectively.16,17

*CP/CPK and acetylsalicylic acid (ASA) experiments.* Creatine phosphate (CP, Sigma), 5 mmol/L, and 200 U/mL creatine phosphokinase (CPK, Sigma) were added to platelet suspensions immediately before viscometer experiments. ASA-treated platelets were prepared by either the administration of 640 mg of ASA to normal donors an hour before venipuncture or by incubating PRP for 30 minutes at room temperature with 55 μg/mL ASA. ASA (Sigma) solution was prepared by dissolving 10 mg of ASA in 1 mL of absolute ethanol and then adding 10 mL of 140 mmol/L NaCl. One hundred microliters of this solution was added to 10 mL of PRP for the 30-minute incubation. Control platelet samples were treated identically except that ASA was omitted from the incubation solution.

RESULTS

In the presence of purified vWF multimers (100% antigen level), platelet [Ca2+]i and aggregation increased in response to increasing levels of fluid shear stress. Figure 2 shows platelet [Ca2+]i responses to shear stresses of 30, 60, 90, and 120 dynes/cm². The greatest elevation of [Ca2+]i occurred at 90 and 120 dynes/cm²: [Ca2+]i increased from a basal level of 60 to 100 nmol/L to a maximal level of >1,000 nmol/L 100 seconds after the initiation of shear stress. Higher shear stresses induced a considerable amount of platelet aggregation, as shown in Fig 2.

To corroborate the validity of the aggregation data measured optically in real-time, washed platelets were subjected to different shear stresses and the platelet number determined by an electronic particle counter. Figure 3 demonstrates that shear stress-induced aggregation measured as the percent reduction of the initial particle number correlates well with the extent of aggregation determined in the optically modified cone and plate viscometer; however, electronic particle counting is more sensitive for measuring the small aggregates that develop in the absence of exogenous vWF or at the lower shear. Figure 3 also shows no significant decrease of the platelet particle number at a shear stress of 15 dynes/cm². Similarly, no [Ca2+]i response or optical evidence of aggregation was observed at

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**Fig 2.** Simultaneous measurement of [Ca²⁺], and platelet aggregation of platelets subjected to varying levels of shear stress. Washed human platelet suspensions with (vWF) or without (control) purified vWF multimers (100% antigen level) were subjected to shear stresses of 30, 60, 90, and 120 dynes/cm². The [Ca²⁺]i and aggregation data (representative of eight separate experiments) were obtained simultaneously for each sample.
a shear stress of 15 dynes/cm² in the cone and plate viscometer (data not shown). In contrast, the addition of was associated with aggregation thrombin addition). 

increase of [Ca²⁺]i (to 1,200 nmol/L within 15 seconds) that cm² shear stress in the cone and plate viscometer caused an increase of [Ca²⁺]i, (to 1,200 nmol/L within 15 seconds) that was associated with aggregation (> 80% at 1 minute after thrombin addition). 

In the absence of added vWF multimers, platelets demonstrated little increase of [Ca²⁺]i in response to shear stress (Fig 2). However, aggregation at the higher shear stresses did increase significantly despite the absence of added purified vWF multimers (Figs 2 and 3). This is the result of aggregation supported by shear stress-induced release of vWF from the platelet α-granules.⁶ 

To determine if the quantity of exogenous vWF affects shear stress-induced responses, platelet [Ca²⁺]i in response to varying amounts of vWF multimers at a constant shear stress of 120 dynes/cm² was examined. Shear stress-induced changes of [Ca²⁺]i increased within a narrow range of vWF antigen levels, with an absent [Ca²⁺]i response at antigen levels less than 10% and the maximal [Ca²⁺]i increase occurring at antigen levels between 50% and 50% (data not shown). 

Chelation of extracellular Ca²⁺ with 1 mmol/L EGTA completely inhibited changes in [Ca²⁺]i, and aggregation of platelets subjected to a shear stress of 120 dynes/cm² in the presence of exogenous vWF (Fig 4). These results suggest that shear stress-induced increases of [Ca²⁺]i occur as a consequence of transmembranous influx of Ca²⁺. To evaluate this directly, the influx of extracellular Mn²⁺, which occurs through the putative divalent cation channel responsible for Ca²⁺ transport, was examined. Washed indo-1 loaded platelet suspensions were mixed with purified vWF multimers (100% antigen level) after pre-incubation with ATA. To examine further the role of GPIb in shear stress-induced changes of [Ca²⁺]i, experiments were performed using the anti-GPIb MoAb 6D1. Both [Ca²⁺]i, and aggregation responses to purified vWF multimers (100% antigen level) of platelet suspensions subjected to a shear stress of 120 dynes/cm² were inhibited completely by 6 µg/mL 6D1 (Fig 6, C and D). 

We next examined the effect of the MoAb 10E5 (which binds to GPIb-IIIa and inhibits vWF interaction with this GP complex) on platelet [Ca²⁺]i, and aggregation re-
Fig 4. Effect of chelation of extracellular Ca\(^{2+}\) on the simultaneous measurement of [Ca\(^{2+}\)]\(_i\) (A) and aggregation (B) of platelets subjected to shear stress. Platelet suspensions containing purified vWF multimers (100\% antigen level) were subjected to a shear stress of 120 dynes/cm\(^2\). The samples had either 1 mmol/L CaCl\(_2\) or 1 mmol/L EGTA added to the suspending buffer just before the application of shear stress. These data are representative of six experiments.

Responses to purified vWF multimers (100\% antigen level) at a shear stress of 120 dynes/cm\(^2\). Figure 6, C and D, shows that 10 \(\mu\)g/mL 10E5 did not completely inhibit changes of [Ca\(^{2+}\)]\(_i\) and platelet aggregation under these conditions. To corroborate this, platelets were pretreated with the tetrapeptide RGDS, which also binds to GPIIb-IIIa and blocks its interaction with vWF.\(^{14,15}\) Figure 6, E and F, shows that 200 \(\mu\)mol/L RDGS also did not completely inhibit platelet [Ca\(^{2+}\)]\(_i\) and aggregation responses to vWF at a shear stress of 120 dynes/cm\(^2\). The extent of the inhibition with RGDS was comparable to that observed with 10E5.

To evaluate the role of intact platelet cyclooxygenase activity and ADP released from the dense granules of activated platelets in vWF-mediated responses to shear stress, we measured the [Ca\(^{2+}\)]\(_i\) and aggregation responses to shear stress of platelet suspensions pretreated with ASA or CP/CPK, respectively. Platelets treated with ASA (both in vivo or in vitro) did not differ from non-aspirinized platelets in either the [Ca\(^{2+}\)]\(_i\) increase or the extent of platelet aggregation during the application of 120 dynes/cm\(^2\) shear stress in the presence of purified vWF (data not shown). In contrast, CP/CPK inhibited platelet aggregation without inhibiting shear stress-induced changes of [Ca\(^{2+}\)]\(_i\) (Fig 7).

DISCUSSION

Experiments described in this report show that platelets respond to pathologic levels of shear stress (>30 dynes/cm\(^2\)) with both an increase of [Ca\(^{2+}\)]\(_i\) and aggregation. Shear stress-induced platelet responses require that exogenous vWF multimers (or vWF multimers released from platelets) bind to their platelet surface GP receptors.
The change of platelet \([Ca^{2+}]\) is due entirely to the transmembranous influx of extracellular \(Ca^{2+}\). This conclusion is based on observations that EGTA, or excess extracellular \(Mn^{2+}\), completely inhibits the \([Ca^{2+}]\) and aggregation responses. The \([Ca^{2+}]\) response of platelets to shear stress is different from that observed with ADP- and thrombin-induced platelet activation in stirred systems. Using ADP or thrombin, the majority of the platelet \([Ca^{2+}]\) increase is the result of release from intracellular \(Ca^{2+}\) stores and the kinetics of the response are faster. However, one important aspect of the \([Ca^{2+}]\) response of platelets subjected to shear stress in the presence of vWF is identical to the response of platelets treated with soluble agonists: in both cases inhibition of the increase in platelet \([Ca^{2+}]\) is associated with an inhibition of aggregation.

Both vWF binding sites on the platelet membrane, GPIb and GPIb-IIIa, have been shown to be involved in shear stress-induced platelet aggregation. Data from experiments described here allow us to suggest the relative importance of GPIb and GPIb-IIIa in platelet responses to shear stress. When vWF binding to GPIb is inhibited by either ATA or the MoAb 6D1, both \([Ca^{2+}]\) and aggregation responses are completely inhibited. In contrast, the inhibition of vWF binding to GPIb-IIIa only partially inhibits platelet \([Ca^{2+}]\) and aggregation responses to shear stress. These results show that vWF binding to GPIb is absolutely essential for shear stress-induced platelet \([Ca^{2+}]\) and aggregation responses. vWF binding to GPIb-IIIa potentiates platelet responses to shear stress, but, in the absence of vWF binding to GPIb, the vWF/GPIb-IIIa interaction is insufficient for the initiation of shear stress-induced \([Ca^{2+}]\) and aggregation responses.

Platelet \([Ca^{2+}]\) responses to vWF under shear stress are not affected by the inhibition of cyclooxygenase by ASA. This result is consistent with the previously demonstrated lack of effect of ASA on shear stress-induced aggregation over the initial 30 seconds of shear. Experiments in which CP/CPK was used to remove the effect of ADP released by platelets (or contaminating erythrocytes) subjected to shear stress demonstrate that, as has been reported previously, ADP is essential for shear stress-induced platelet aggregation. In contrast, CP/CPK had little effect on the platelet \([Ca^{2+}]\) response to shear stress in the presence of purified vWF multimers. This shows that the \([Ca^{2+}]\) response to shear stress is not a consequence of feedback platelet activation by released ADP, and suggests that the shear stress-induced vWF/GPIb interaction stimulates an increase of platelet \([Ca^{2+}]\), that precedes ADP release.

The molecular mechanisms of shear stress-induced vWF/GPIb binding are not yet known. Previous studies suggest that shear stress does not affect the structure of plasma vWF. Therefore, shear stress may be the physiologic (or pathophysiologic) equivalent of ristocetin: shear stress may alter some characteristic of platelet surface GPIb and permit ligand binding to occur. Once bound to vWF, platelet GPIb appears to function as a signal molecule,
GPIIb-IIIa may be a \( Ca^{2+} \) channel or may be adjacent to a \( Ca^{2+} \) channel. Additional evidence in support of this hypothesis comes from a recent report demonstrating that voltage-independent calcium currents in thrombin-stimulated platelet membranes are decreased when ligand binding to GPIIb-IIIa is blocked by an MoAb or the synthetic RGDS peptide. Our data showing that blocking the vWF binding site on GPIIb-IIIa of intact platelets suppresses (but does not eliminate) platelet \( [Ca^{2+}]_i \) responses to vWF in the shear field (Fig 6) are consistent with the observations cited. However, it should be emphasized that the mechanism by which GPIIb-IIIa potentiates shear stress-induced platelet \( [Ca^{2+}]_i \) responses is not defined. GPIIb-IIIa is a binding site for vWF (that contributes to shear stress-induced aggregation) and may be a divalent cation channel, but the precise relationship between ligand binding GPIIb-IIIa and the platelet \( [Ca^{2+}]_i \) response to shear stress is unknown.

Results of experiments presented here suggest that the activity of a platelet \( Ca^{2+} \) transporter is important when pathologic levels of arterial wall shear stress are generated, such as those occurring with acute vascular occlusion in areas of vasospasm, atherosclerotic constriction, or a ruptured atherosclerotic plaque. There are also data indicating that shear stress-regulated platelet \( Ca^{2+} \) channels may affect the development of chronic arterial occlusive disease. It has been reported that changes of basal platelet \( [Ca^{2+}]_i \) correlate with levels of shear stress in the brachial artery of hypertensive humans, suggesting that the platelet \( [Ca^{2+}]_i \) response to shear stress may contribute to the pathogenesis of chronic, as well as acute, arterial disease. Results of experiments presented here using the cone-plate viscometer suggest a common mechanism by which arterial wall shear stress influences the pathogenesis of both acute and chronic arterial vasoocclusive disease: shear stress modulates platelet aggregation by directly affecting the level of platelet \( [Ca^{2+}]_i \).

In summary, experiments presented here indicate that high levels of arterial wall shear stress (\( >30 \) dynes/cm²) induce plasma vWF to bind to platelet GPIIb, and that this initiates the transmembranous influx of \( Ca^{2+} \) associated with platelet aggregation. This platelet response, which is not inhibited by ASA and is potentiated by a functional platelet GPIIb-IIIa complex, may mediate platelet aggregation at sites of arterial vasoocclusion. Elucidation of the molecular interactions regulating this pathway should provide the foundation for the development of new therapies for acute and chronic atherothrombotic diseases.

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