Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s)

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Pathological arterial blood flow generates fluid shear stresses that directly cause platelet aggregation. The mechanism of shear-induced platelet aggregation is incompletely understood, but involves von Willebrand factor (vWF) binding to platelet glycoprotein (GP) Ib and GP IIb–IIIa, leading to the transmembrane influx of Ca^{2+} and the activation of protein kinase C. To investigate this further, shear-stress-induced protein tyrosine phosphorylation (PTP) of washed platelets was studied in a cone-plate viscometer. A time- and shear-stress-dependent tyrosine phosphorylation of substrates with approx. M_r 29000–31000, 36000, 50000, 58000, 64000, 76000, 85000 and 105000 was observed. PTP in response to a threshold shear stress of 0.3 mN/cm^2 (30 dyn/cm^2) was enhanced in most cases by exogenous

purified human vWF, and PTP in response to a pathological shear stress of 0.9 mN/cm^2 (90 dyn/cm^2) was inhibited in some cases by inhibiting vWF binding to GP Ib or GP IIb–IIIa, or by inhibiting Ca^{2+} responses with extracellular EGTA. Shear-induced PTP of a substrate of $M_r \approx 31000$ appeared to be independent of GP Ib, and PTP of a substrate(s) of $M_r \approx 29000$ was shear-stress-dependent but independent of extracellular Ca^{2+} . Cytochalasin D, which inhibits GP Ib–cytoskeleton interactions, inhibits the PTP of a substrate of $M_r \approx 76000$. These results suggest that tyrosine phosphorylation may be involved in transmembrane signalling that mediates platelet adhesion and aggregation in response to pathological shear stresses generated at sites of arterial vaso-occlusion.

INTRODUCTION

Platelets are essential for normal haemostasis and contribute to the pathogenesis of arterial thrombosis [1]. In response to blood-vessel injury, platelets adhere to sites of exposed sub-endothelium, become activated, and aggregate to form a stable platelet thrombus [2]. This sequence of responses is triggered by von Willebrand factor (vWF) binding to the platelet surface glycoprotein (GP) Ib/IX complex [3]. This binding appears to require pathological arterial flow [4]. It is hypothesized that a conformational change in GP Ib/IX and/or vWF develops at shear stresses $> 0.3 \text{ mN}/\text{cm}^2$ (30 dyn/cm^2) (the time-averaged limit of normal wall shear stress in large and medium-sized arteries; equivalent to a shear rate in whole blood of 750 s^{-1}), thus permitting binding to occur [5]. Recent data indicate that shear-stress-induced platelet adhesion, activation and aggregation are coupled through the vWF–GP Ib/IX interaction, which has been shown to signal directly platelet Ca^{2+} responses [6] and the activation of protein kinase C [7]. The mechanisms by which shear stress permits vWF–GP Ib/IX binding to develop and signal are unknown.

Platelets contain a large amount of protein tyrosine phosphate, indicating the presence of tyrosine kinases and tyrosine phosphatases that may have signalling functions [8–10]. It is established that platelets contain large amounts of the protein tyrosine kinase $\text{pp60}^{\text{c-src}}$ and other 'src-related' tyrosine kinases such as *fyn*, *lyn*, *yes* and *src* [11–14]. These become activated and phosphorylate many substrates after platelet stimulation induced by thrombin, collagen, prostaglandin endoperoxides, platelet-activating factor, ADP, adrenaline, IgG Fc binding and possibly CD36 [11–20]. Of note is that some tyrosine phosphorylation depends on aggregation mediated by fibrinogen binding, such

that there are GpIIb–IIIa-dependent substrates [e.g. p125^{Fak} (focal adhesion kinase), and unidentified proteins of M_r 84000, 95000, 100000, 108000 and 140000] and GP IIb–IIIa-independent substrates [e.g. p21^{ras} GAP (GTPase-activating protein) and unidentified proteins of M_r 27000, 34000, 68000 and 85000] [14–16]. The effects of tyrosine phosphorylation on platelet activation are poorly understood, but, as in dividing cells, one consequence of protein tyrosine phosphorylation in platelets is the generation of D-3 phosphoinositides. These are synthesized after agonist-induced platelet activation, possibly in association with protein kinase C activity or fibrinogen binding to GP IIb–IIIa [19–21]. The function of tyrosine kinase-directed synthesis of D-3 phosphoinositides in platelets is not known, but they associate with the cytoskeleton and may be involved in cytoskeletal reorganization leading to shape changes and secretion [22]. There is recent evidence that $\text{pp60}^{\text{c-src}}$ becomes activated and associates with the cytoskeleton after platelet stimulation [22–24].

Protein tyrosine phosphorylation (PTP) is a phylogenetically conserved mechanism of effecting cytoskeletal interactions needed for changes in cell shape [25,26]. PTP is also an established mechanism of signalling cellular activation in response to a ligand binding its cell-surface receptor [27]. Because shear stresses cause deformation of the platelet plasma membrane, which could potentially affect the conformation of the GP Ib/IX complex tethered to the cytoskeleton, and because there may be a link between cell deformation, cytoskeletal reorganization and the activation of tyrosine kinases, we examined whole platelet lysates for shear-induced PTP under conditions in which the flow of signals between extracellular mechanical shear stresses, ligand binding and cytoskeletal reorganization is controlled.

Abbreviations used: PTP, protein tyrosine phosphorylation; GP, glycoprotein; vWF, von Willebrand factor; PMA, 4 β -phorbol 12-myristate 13-acetate; ATA, aurointricarboxylic acid; Fak, focal adhesion kinase.

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MATERIALS AND METHODS

Materials

Monoclonal antibody 4G10 (directed against phosphotyrosine [17,18]) was purchased from Upstate Biotechnology Inc. (UBI; Lake Placid, NY, U.S.A.). Monoclonal antibodies 6D1 (directed against GPIb [28]) and 10E5 (directed against GpIIb-IIIa [29]) were obtained from Dr. Barry Coller (SUNY Health Sciences Center, Stony Brook, NY, U.S.A.). Fatty-acid-free BSA, Sepharose 2B-300, phosphocreatine, creatine kinase, phosphotyrosine and 4 β -phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO, U.S.A.). Human α -thrombin was obtained from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Cytochalasin D was obtained from Calbiochem (San Diego, CA, U.S.A.). Fractionated aurointricarboxylic acid (ATA) polymers of average M_r 2500 were provided by Dr. Mark Weinstein (U.S. Food and Drug Administration).

Platelet preparation

Venous blood from healthy volunteer donors who had not taken medications affecting platelet function for at least 10 days was collected in 15% (v/v) acid-citrate-dextrose (ACD; NIH Formula A). Blood was centrifuged at 220 g for 14 min, and the platelet-rich plasma was acidified to pH 6.5 with ACD and treated with phosphocreatine (5 mM) and creatine kinase (25 units/ml). This preparation was layered over a gradient of fatty-acid-free BSA and centrifuged at 1700 g for 15 min, as previously described [3]. Interface platelets were collected and subjected to repeat BSA-density-gradient separation. Removal of extracellular vWF by this washing procedure has been previously demonstrated [3]. Platelets isolated in this manner were then suspended in buffer containing 6 mM glucose, 130 mM NaCl, 9 mM NaHCO₃, 10 mM sodium citrate, 10 mM Tris base, 3 mM KCl, 2 mM Hepes and 0.9 mM MgCl₂, pH 7.35. Washed platelets were gel-filtered through Sepharose 2B-300 and suspended in buffer with 1 mM CaCl₂ at 2.5×10^8 platelets/ml.

vWF preparation

vWF was purified from normal human cryoprecipitate and quantified by solid-phase immunoradiometric assay, as previously described [3]. The multimeric composition of the vWF preparations was determined by SDS/agarose-gel electrophoresis using 1% agarose and a continuous buffer system, followed by gel overlay with rabbit ¹²⁵I-labelled anti-(human vWF) IgG and autoradiography.

Immunoblotting

Washed platelet suspensions were subjected to fluid shear stress in a cone-plate viscometer (Ferranti Electric, Commack, NY, U.S.A.) at 22 °C as previously described [6,7]. Reactions were stopped by addition of a solution of 50% glycerol, 10% dithiothreitol, 8% SDS and trace Bromophenol Blue, followed by immediate boiling for 10 min. SDS/PAGE was performed on 6–16% gradient slab gels as described by Laemmli [30]. The proteins were transferred to polyvinylidene difluoride membranes by standard methods described by Towbin et al. [31]. Substrates containing protein phosphotyrosine were identified by binding of monoclonal antibody 4G10 and reported by an alkaline-phosphatase-conjugated secondary antibody, by using a kit from UBI according to the manufacturer's instructions.

RESULTS

Washed human platelets were subjected to 0.9 mN/cm² (90 dyn/cm²) shear stress for 2 min on the cone-plate viscometer, and lysates from 5×10^8 platelets/ml were immunoblotted with the anti-phosphotyrosine monoclonal antibody 4G10. Figure 1 shows results from the first experiment, including a comparison of the protein band pattern between control unstimulated platelets

Figure 1 Platelets subjected to shear stress on the cone-plate viscometer demonstrate PTP

PTP of unstimulated platelets (C), platelets stimulated for 2 min by 0.9 mN/cm² (90 dyn/cm²) shear stress (S), and platelets stimulated for 1 min by 100 nM PMA (P) or 1 unit/ml thrombin (T). In all Figures, M_r values on the left refer to marker proteins.

Figure 2 Time course of PTP of platelets subjected to 0.9 mN/cm² (90 dyn/cm²) shear stress

This shear stress causes a time-dependent tyrosine phosphorylation of substrates with M_r approx. 29 000, 31 000, 36 000, 50 000, 58 000, 64 000, 76 000, 85 000 and 105 000. This immunoblot is representative of four experiments.

Figure 3 Increasing shear stress causes increasing platelet tyrosine phosphorylation at 1 min

There appear to be at least two distinct bands at M_r 29 000 and 31 000, and these bands, as well as the bands at M_r 50 000 and 76 000, are prominently phosphorylated in a shear-stress-dependent manner. This immunoblot is representative of three experiments. (1 dyn = 0.01 mN.)

Figure 4 Effect of exogenous vWF (100 units/dl = 100% antigen level) on the time course of PTP induced by 0.3 mN/cm² (30 dyn/cm²) shear stress

Exogenous vWF results in enhanced PTP of substrates with M_r approx. 29 000, 31 000, 36 000, 50 000, 64 000 and 76 000. This immunoblot is representative of three experiments.

(C), sheared platelets (S), platelets treated for 1 min with the direct protein kinase C activator PMA (P; 100 nM), and platelets treated for 1 min with human α -thrombin (T; 1 unit/ml). When 5 mM phosphotyrosine was added to the primary antibody

Figure 5 Effect of preincubating platelets for 5 min with the anti-GP Ib monoclonal antibody 10E5 (10 μ g/ml) and the anti-GP IIb-IIIa monoclonal antibody 6D1 (10 μ g/ml) on tyrosine phosphorylation in response to 1 min of 0.9 mN/cm² (90 dyn/cm²) shear stress

These antibodies inhibit some of the tyrosine phosphorylation events developing under shear, but a band of M_r approx. 31 000 remains tyrosine-phosphorylated when GP Ib is blocked by monoclonal antibodies (MAb). The prominent reporting of bands at M_r approx. 28 000–29 000 is due to non-specific binding. This immunoblot is representative of five experiments.

incubation solution, all bands present in Figure 1 were inhibited (results not shown).

A time course of platelet PTP in response to 0.9 mN/cm² (90 dyn/cm²) shear is shown in Figure 2, which confirms that such shear stress causes a time-dependent tyrosine phosphorylation of substrates of approx. M_r 29 000, 31 000, 36 000, 50 000, 58 000, 64 000, 76 000, 85 000 and 105 000. In comparison with Figure 1, bands at M_r approx. 29 000 and 31 000 are much more apparent in sheared platelets. All the other bands are seen with shear, thrombin and PMA, although the intensity of the bands at M_r approx. 36 000, 50 000, 58 000, 64 000 and 76 000 appears to be greater with shear.

Figure 3 shows that PTP at 1 min is dependent on the level of shear stress applied to washed platelets. It also shows that there appears to be at least two distinct bands at M_r 29 000, and that these bands, as well as the bands at M_r 58 000 and 76 000, are prominently phosphorylated in a shear-stress-dependent manner. Figure 3 also demonstrates that the PTP response to shear varies among experiments using different donors; in this case the phosphorylation of the M_r 31 000 and 36 000 substrates is not appreciable.

Figure 6 Effect of preincubating platelets for 1 min with RGDS peptide (200 µg/ml) or M_r -2500 ATA on 0.9 mN/cm² (90 dyn/cm²)-shear-stress-induced tyrosine phosphorylation at 1 min.

The RGDS peptide, which inhibits vWF binding to GP IIb-IIIa, inhibits the tyrosine phosphorylation of most protein substrates. ATA enhances the phosphorylation of most bands, probably due to non-specific effects [32]. The RGDS immunoblot is representative of three experiments, and the ATA immunoblot is representative of eight experiments.

To begin to determine the ligand specificity of shear-stress-induced platelet PTP, physiological quantities of purified human vWF having a normal multimer size distribution were added to washed platelets sheared at 0.3 mN/cm² (30 dyn/cm²). This shear stress is associated with exogenous vWF-dependent platelet activation, thus minimizing the effects of vWF secreted by platelets as higher shear stresses are applied, and allowing for a better analysis of vWF-dependent and vWF-independent phosphorylations under shear [6,7]. Figure 4 shows that exogenous vWF (100% antigen level $\approx$ 100 units/dl) results in enhanced PTP of substrates with M_r approx. 29000, 31000, 36000, 50000, 64000 and 76000 when platelets are sheared at 0.3 mN/cm² (30 dyn/cm²).

The receptor specificity of platelet tyrosine phosphorylation in response to 0.9 mN/cm² (90 dyn/cm²) shear stress for 1 min was next investigated by preincubating platelets for 5 min with monoclonal antibodies 6D1 (10 µg/ml) and 10E5 (10 µg/ml), specific for platelet GP Ib and GP IIb-IIIa respectively. These antibodies have been previously shown to inhibit platelet Ca²⁺ and protein kinase C responses to shear stress [6,7]. Figure 5 shows that these antibodies inhibit some, but not all, of the tyrosine phosphorylation events developing under shear. Of note is that a band of M_r approx 31000 remains tyrosine-phosphorylated when GP Ib is blocked by monoclonal anti-

Figure 7 Effect of preincubating platelets for 10 min at room temperature with 5 mM EGTA on 0.9 mN/cm² (90 dyn/cm²)-shear-stress-induced tyrosine phosphorylation at 1 min

EGTA, which under these conditions inhibits the transmembrane influx of Ca²⁺ without affecting GP IIb-IIIa function, has very little effect on tyrosine phosphorylation of protein bands of M_r approx 29000, whereas it decreases most other shear-induced PTPs. This immunoblot is representative of three experiments.

bodies. Figure 5 also shows that both antibodies are associated with prominent reporting of bands at M_r approx. 28000–29000. This is due to non-specific binding (results not shown) and confounds the analysis of the receptor-dependency of platelet PTP of substrates with these relative molecular masses. To circumvent this problem and investigate further the receptor specificity of shear-induced tyrosine phosphorylation, platelets were preincubated for 1 min with either 200 µg/ml RGDS peptide (which inhibits vWF binding to GP IIb-IIIa [6]) or 64 µg/ml M_r -2500 aurintricarboxylic acid (ATA, which inhibits vWF binding to GP Ib [32]). Figure 6 shows that RGDS peptide inhibits, and ATA enhances, most of the shear-induced PTP. The enhanced PTP of M_r -2500 ATA probably reflects a direct stimulatory effect of ATA on platelets, as has been previously observed with larger-sized ATA fractions [32].

Platelet aggregation and Ca²⁺ responses to shear stress are completely dependent on extracellular Ca²⁺ [6], platelet protein kinase C activation in response to shear is partially dependent on both extra- and intra-cellular Ca²⁺ [7], and platelet p72^{src} activity in response to chemical agonists is negatively regulated by Ca²⁺ [12]. To determine if platelet Ca²⁺ responses to shear stress affect PTP, platelets were preincubated for 10 min at room temperature with 5 mM EGTA before being subjected to 0.9 mN/cm²

Figure 8 Effect of preincubating platelets for 15 min with cytochalasin D (20 μ M) on 0.9 mN/cm² (90 dyn/cm²)-shear-stress-induced tyrosine phosphorylation at 1 min

Cytochalasin D inhibits the PTP of a band at M_r approx. 76 000, whereas the remaining tyrosine-phosphorylated substrates are relatively unaffected. Platelet aggregation is not inhibited under these conditions.

(90 dyn/cm²) shear stress for 1 min. This preincubation inhibits the transmembrane influx of Ca²⁺ without affecting the ligand-binding capacity of GP IIB-IIIa [33,34]. Figure 7 shows that EGTA has very little effect on tyrosine phosphorylation of protein bands of M_r approx. 29 000, although it decreases most other shear-induced PTPs.

Platelet activation in response to shear stress depends on vWF binding to GP Ib. It is known that GP Ib has non-covalent structural interactions with the platelet cytoskeleton, specifically actin-binding protein [35,36], which may affect platelet responses to some chemical agonists [37]. To determine if GP Ib binding to the platelet cytoskeleton affects PTP in response to shear, cytochalasin D (20 μ M) was added to washed platelets for 15 min before being subjected to 0.9 mN/cm² (90 dyn/cm²) for 1 min. Under these conditions, GP Ib becomes untethered from its cytoskeletal connections and actin polymerization is inhibited [38]. Figure 8 shows that cytochalasin D inhibits the PTP of a band at M_r approx. 76 000, while the remaining tyrosine-phosphorylated substrates are unaffected. Shear-induced platelet aggregation is not inhibited under these conditions (results not shown).

DISCUSSION

Experiments reported here demonstrate that platelets exposed to elevated arterial-wall shear stresses in a cone-plate viscometer develop tyrosine phosphorylations of a number of protein substrates, with M_r approx. 29 000, 31 000, 36 000, 50 000, 58 000, 64 000, 76 000, 85 000 and 105 000. Some of these phosphorylations are also observed after platelet stimulation with α -thrombin or PMA, and some are observed to occur predominantly or exclusively with shear.

Increasing shear stress causes increasing tyrosine phosphorylation of many of these substrates, and shear-stress-induced tyrosine phosphorylation depends, in most cases, on vWF binding to platelet GP Ib or GP IIB-IIIa. Shear-stress-induced platelet PTP is, in some cases, also dependent on extracellular Ca²⁺, suggesting that the transmembrane influx of Ca²⁺ regulates

tyrosine kinase or phosphatase activity. Cytochalasin D, which disrupts the GP Ib-actin-binding-protein interaction and inhibits actin polymerization, inhibits tyrosine phosphorylation of a single protein band of M_r approx. 76 000.

Many platelet protein substrates have variable degrees of tyrosine phosphorylation in response to shear stress, and this variability appears to be donor-dependent. The most apparent is the M_r -36 000 protein, whose phosphorylation also appears to be dependent on vWF binding to GP Ib and GP IIB-IIIa. This raises the possibility that the variable GP Ib-dependent phosphorylation of a platelet M_r -36 000 protein may relate to a polymorphism in the gene coding for GP Ib α [39]. This polymorphism, which alters the length of the GP Ib α -chain, has been hypothesized by Lopez et al. [39] perhaps to affect platelet shear responsiveness: GP Ib α -chains of greater length may make platelets more susceptible to the activating effects of shear stress.

The functional effect of platelet tyrosine phosphorylation in response to shear stress is difficult to study, because of the lack of reagents that specifically inhibit tyrosine kinases or tyrosine phosphatases in intact platelets. Genistein has been used as a membrane-permeant inhibitor of platelet tyrosine kinases [40], but many of its effects on platelet function are directly attributable to its competitive antagonism of prostaglandin endoperoxide binding [41]. We have observed that 500 μ M genistein inhibits aggregation and PTP in response to shear (K. Razdan, J. D. Hellums and M. H. Kroll, unpublished work; $n = 3$). These data are susceptible to misinterpretation, however, because of the direct inhibitory effect of genistein on platelet activation in response to prostaglandin endoperoxides, although previous studies suggest that prostaglandin endoperoxides are relatively unimportant for platelet aggregation in response to shear [42]. The inhibitory effect of genistein on platelet aggregation in response to shear is, however, consistent with results of experiments using vanadate to inhibit protein tyrosine phosphatases in permeabilized platelets, where vanadate-enhanced PTP was associated with the direct stimulation of platelet aggregation and secretion [10,43]. It is therefore plausible, but unproven, that tyrosine phosphorylation regulates platelet aggregation in response to mechanical shear stresses.

Tyrosine kinase activity could potentially affect as many different molecular switches involved in platelet responses to shear as there are tyrosine kinases, tyrosine phosphatases or substrates for tyrosine phosphorylation. To date, some seven platelet tyrosine kinases have been identified, of which at least three appear to be activated when platelets are stimulated by chemical agonists: p72^{src} [12], p125^{Fak} [13] and pp60^{c-src} [43a]. The identity of substrates of tyrosine phosphorylation in platelets is in most cases unknown. The functional importance of these PTP events is also unknown, although putative function can be ascribed on the basis of an understanding of the function of identified substrates in other cell types.

Tyrosine phosphorylation occurs at 8 s, the earliest time point that can be captured in the cone-plate viscometer. This suggests the possibility that tyrosine phosphorylation regulates early, and perhaps initial, platelet responses to shear. Consistent with this interpretation are data suggesting the presence of GP Ib-independent tyrosine phosphorylation in response to shear. From experiments presented in Figure 5, it appears that a band at M_r approx. 31 000 is relatively unaffected by vWF binding to GP Ib. This suggests the possibility of specific GP Ib-independent tyrosine kinase activity developing before vWF binding. Figures 3 and 7 show that a doublet of M_r approx. 29 000 is clearly phosphorylated in a shear-stress-dependent manner, but its phosphorylation is independent of extracellular Ca²⁺. Since shear

stress induces vWF binding to GP Ib and GP IIb-IIIa, and this binding results in the transmembrane influx of Ca^{2+} , observations that there are substrates of tyrosine phosphorylation independent of Ca^{2+} suggest that the phosphorylation of these protein substrates is another early platelet response that could occur as a direct result of shear-mediated cell deformation and perhaps precede vWF binding.

The mechanisms by which a mechanical shear stress signals a morphological or functional cellular response are unknown. It is theorized that a 'mechanotransducer' converts the physical force into a biochemical response, but the structural characteristics of a prototypical mechanotransducer are at present unknown [44]. It is generally hypothesized that the cytoskeleton is involved in transducing mechanical stimuli, and recent data indicate that an integrin (related to platelet GP IIb-IIIa) transduces a mechanical force across the plasma membrane through its interaction with the cytoskeletal proteins actin and actin-binding protein [45]. This would represent an important mechanism of cellular adhesion-activating coupling, which we [3] and others [46] have shown to occur in human platelets. Of note is that Fak, a protein tyrosine kinase that may be activated in human platelets [13], colocalizes in focal adhesions with the cytoskeletal proteins actin, actin-binding protein and talin, and this kinase may be involved in regulating signalling initiated by cellular interactions with extracellular-matrix proteins in other cell types [47].

In summary, results of experiments presented here indicate that PTP develops in platelets subjected to mechanical shear stresses. This indicates that shear stress affects the balance between platelet tyrosine kinase and tyrosine phosphatase activity, possibly by activating tyrosine kinases that are of uncertain functional importance. Most platelet PTP depends on vWF binding to GP Ib or GP IIb-IIIa, although preliminary data suggest that there is a shear-stress-dependent phosphorylation of a substrate of M_r approx. 31 000 that may occur independent of vWF binding to GP Ib. Inhibiting GP Ib/cytoskeletal interactions with cytochalasin D prevents shear-stress-induced tyrosine phosphorylation of a substrate of M_r approx. 76 000. The identity of substrates of shear-stress-induced platelet PTP is at present unknown. Elucidating the structure of these substrates may help to clarify our understanding of the mechanisms by which physical forces activate platelets and affect the pathogenesis of acute and chronic atherosclerotic arterial diseases.

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