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Purinergic $P2Y_{12}$ Receptor Blockade Inhibits Shear-Induced Platelet Phosphatidylinositol 3-Kinase Activation

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ABSTRACT

Pathologically elevated shear stress triggers aspirin-insensitive platelet thrombosis. Signaling mechanisms involved in shear-induced platelet thrombosis are not well understood. To investigate these, we examined the hypothesis that functionally important platelet phosphatidylinositol 3-kinase (PI3-K) activity is stimulated by an in vitro shear stress of 120 dynes/cm^2 (shear rate of 6000 sec^{-1}). Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) production was examined in washed human platelets subjected to pathological shear stress in a cone-plate viscometer. PIP_3 production peaks 30 s after shear begins and is initiated by von Willebrand factor (VWF) binding to the glycoprotein (Gp) Ib-IX-V complex. Inhibiting PI3-K with wortmannin or 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) results in the inhibition of shear-induced platelet aggregation. In resting platelets, class IA PI3-K associates with the tyrosine kinase Syk. Within 30 s of beginning shear, PI3-K-associated Syk becomes tyrosine phosphorylated. Inhibiting Syk activation with piceatannol results in the inhibition of PIP_3 production and aggregation. Selective blockade of the $P2Y_{12}$ receptor results in the inhibition of Syk phosphorylation, PIP_3 production, and aggregation. These results indicate that shear-induced VWF binding to platelet GpIb-IX-V stimulates functionally important PI3-K activity. PI3-K activation is signaled by rapid feedback amplification that involves $P2Y_{12}$ receptor-mediated activation of Syk.

High levels of wall shear stress are generated at sites of arterial injury, such as a ruptured atherosclerotic plaque, where laminar blood flow is forced through a narrowed luminal diameter (Berndt et al., 2001). Such pathological shear stress causes platelet-dependent thrombosis (Kroll et al., 1996). The trigger for shear-dependent platelet adhesion is von Willebrand factor (VWF) in the plasma and vessel wall binding to platelet glycoprotein (Gp) Ib-IX-V, which causes a transient tethering of platelets to the damaged vessel wall and signals the activation of αIIbβ3. Further adhesion and αIIbβ3 activation develop via platelet GpVI and α2β1 binding exposed collagen fibrils (Moroi et al., 1997; Savage et al., 1998). Shear-dependent platelet thrombus formation is ADP-dependent (Turner et al., 2001), but it is not affected by inhibiting platelet cyclooxygenase with aspirin or other agents (Maalej and Folts, 1996).

Mechanisms by which shear-induced VWF binding to GpIb-IX-V activates αIIbβ3 have not been established. There is direct evidence that pathological shear causes VWF-dependent platelet calcium, protein kinase C, and tyrosine kinase signaling responses, and each of these signals may contribute to αIIbβ3 activation (Kroll et al., 1993; Razdan et al., 1994; Shattil et al., 1998; Kuwahara et al., 1999). There is also pharmacological evidence that shear-induced VWF binding to platelet GpIb-IX-V causes phosphatidylinositol 3-kinase (PI3-K)-dependent αIIbβ3 activation (Yap et al., 2002), although shear-induced D3-phosphorylation of platelet polyphosphoinositides has not been reported. Such shear-induced phosphorylations are likely to occur, however, because PI3-K-mediated synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) is known to be an important signal for the up-regulation of αIIbβ3 function under low shear conditions (Kovacsovics et al., 1995).

There are several types (or classes) of platelet PI3-K (Rittenhouse, 1996). The type IA PI3-Ks encompass several isoforms varying in their 110-kDa catalytic (designated p110 α or β) and 85-kDa regulatory subunits (designated p85 α or β) (Fruman et al., 1998; Wymann and Pirola, 1998). Minimal requirements for the activation of type IA PI 3-kinases are: 1)

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ABBREVIATIONS: VWF, von Willebrand factor; PI3-K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphatidylinositol; AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP; A3P5P, adenosine 3′,5′ diphosphate; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PRP, platelet-rich plasma; Gp, glycoprotein.

binding to the small GTP-binding protein ras or Rho, 2) binding to phosphotyrosine-containing proteins, and 3) colocalization with their preferred substrate PIP_2 (Stephens et al., 1994; Fruman et al., 1998). Both ras and phospholipid binding are mediated by domains in the p110 α or β catalytic subunit, whereas phosphotyrosine binding is mediated by two SH2 domains in the p85 α or β regulatory subunit. P85 α and β also possess one SH3 domain that recognizes polyproline sequences. Type IA PI3-Ks SH3 domain mediates its binding to src and src-family tyrosine kinases, and these interactions serve to activate its catalytic domain. In platelets, type IA PI3-K is activated downstream of G-protein-coupled receptor-induced phospholipase Cβ stimulation (such as is triggered by thrombin binding to the protease-activated receptor 1 receptor) and by agonists that directly activate src or src-family kinases (such as collagen binding to GpVI) (Kroll and Reséndiz, 2002).

Platelets contain a type IB PI3-K (designated PI3-Kγ), which is a 110-kDa protein with an N terminus possessing a G protein $\beta\gamma$ binding domain and a pleckstrin homology phospholipid binding domain. Type IB PI3-K seems to be regulated primarily by binding to a G protein $\beta\gamma$ subunit and, like type IA PI3-K, it prefers PIP_2 as substrate. Type IB PI3-K may be the major stimulatory response initiated by ADP binding to its $P2Y_{12}$ receptor, thereby mediating feed-forward amplification of prothrombotic platelet responses triggered by several different agonists through secreted ADP activating the $P2Y_{12}$/Gi pathway (Stephens et al., 1994; Trumel et al., 1999; Selheim et al., 2000; Hirsch et al., 2001).

Platelets also express a type II PI3-K (Zhang et al., 1998). In contrast to the type IA and IB PI3-K, type II PI3-K prefers phosphatidylinositol (PI) as substrate. It is a larger protein (up to 210 kDa) that binds to PI through a C2 domain and catalyzes the synthesis of phosphatidylinositol 3-phosphate, synthesis of which by type II PI3-K is calcium-dependent and occurs downstream of ligand binding to activated αIIbβ3 (Zhang et al., 1998).

Because there is good evidence that the activation of type I PI3-K affects αIIbβ3 function in static or stirring platelets, we examined the hypothesis that type I PI3-K generates proaggregatory signals after shear-induced VWF binding to GpIb-IX-V in washed platelet suspensions exposed to 120 dynes/cm^2 in a cone-plate viscometer. We also investigated the molecular mechanism of shear-induced PI3-K activation.

Materials and Methods

Chemicals and Reagents. The specific $P2Y_{12}$ antagonist AR-C69931MX was kindly provided by AstraZeneca (Loughborough, UK) (Turner et al., 2001). Methanol, chloroform, HCl, silica gel plates, scintillation fluid, dimethyl sulfoxide, paraformaldehyde, Triton X-100, aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, the $P2Y_1$ antagonist adenosine 3′,5′ diphosphate (A3P5P), the synthetic Arg-Gly-Asp-Ser peptide, ADP, purified human fibrinogen, and sodium orthovanate were purchased from Sigma, (St. Louis, MO). Piceatannol was purchased from Roche Molecular Biochemicals (Indianapolis, IN). [^{32}P]Orthophosphate, protein Sepharose A and G, enhanced chemiluminescence reagents, and film were from Amersham Biosciences (Piscataway, NJ). The PI3-K inhibitors LY294002 and wortmannin, and purified human VWF, were from Calbiochem (San Diego, CA). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA).

Platelet Preparation. Venous blood was obtained from healthy volunteer donors with a 19G needle and collected in 15% acid-citrate-dextrose. Blood was centrifuged at 270*g* at 24°C for 15 min. Platelet-rich plasma (PRP) was collected, the pH was adjusted to 6.5 with acid-citrate-dextrose, and PRP was treated with phosphocreatine (5 mM) and creatine phosphokinase (25 U/ml). Platelets were then separated from the PRP by a second centrifugation at 1600*g* at 24°C for 15 min. Platelets were suspended in Tyrode's buffer (138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, and 5.5 mM glucose, pH 6.5) containing phosphocreatine and creatine phosphokinase, and then centrifuged at 1200*g* at 24°C for 10 min. Platelets were resuspended in JNL buffer (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 $MgCl_2$, pH 7.35, to which 1 mM $CaCl_2$ and 5 μg/ml purified VWF were added). For labeled analyses, platelets were incubated with 2 mCi/ml of [^{32}P]orthophosphate at 37°C for 1 h, washed, and resuspended in JNL buffer. The platelet count was adjusted to 2.5×10^8 platelets/ml. ADP-induced Syk phosphorylation was measured in stirring platelets at 37°C in a Chrono-Log aggregometer (Havertown, PA). For aggregometer experiments, 1 mg/ml purified fibrinogen was added to the washed platelet suspension.

Shear system. Washed human platelets were subjected to fluid shear stress (120 dynes/cm^2) in a cone-plate viscometer at 24°C as described previously (Kroll et al., 1996). Five microliters of the sheared platelet suspension was fixed in 500 μl of 1% paraformaldehyde, and aggregation was measured by flow cytometry as described previously (Feng et al., 2002). AR-C69931MX and/or A3P5P were added to platelet suspensions 20 min before shearing. LY294002, wortmannin, the RGDS peptide, the monoclonal antibody 5D2 (which blocks the GpIbα recognition domain of VWF and was produced by the Baker Medical Research Institute, Victoria, Australia), and the monoclonal antibody AK2 (which blocks the VWF recognition domain of GpIbα and was purchased from RDI Inc., Flanders, NJ) were incubated with platelets for 15 min before shearing. When appropriate, equivalent volumes of DMSO (vehicle for LY294002 and wortmannin) or mouse IgG were added 15 min before positive control reactions.

Analysis of lipids. Sheared ^{32}P-labeled platelets were immediately mixed with an ice-cold mixture of methanol/chloroform/HCl (8:8:1, v/v/v). Lipids were extracted using the method of Bligh and Dyer and dried under a stream of N_2 as described previously (Kroll et al., 1993). Dried lipids were reconstituted in 50 μl of chloroform and spotted on Silica Gel G thin-layer chromatography plates pretreated with 1% potassium oxalate. Plates were developed in a solution of chloroform/methanol/NaOH/H_20 (60:47:11:2) and dried. Bands were visualized by autoradiography, and individual lipids were scraped from the thin-layer chromatography plate and quantified by liquid scintillation counting. Paired statistical analyses of these data were performed using Microsoft Excel (Microsoft Corp., Redmond, WA). PIP_2 and PIP_3 lipid standards (Matreya Inc., Pleasant Gap, PA) were visualized by spraying the plates with molybdenum blue.

Immunoprecipitation and Western Blotting. Resting and sheared platelets were lysed in an equal volume of ice-cold radioimmunoprecipitation assay buffer (2% Triton X-100, 2 mM Na_3VO_4, 2 mM NaF, 20 mM EDTA, 2 mM PMSF, 2 mg/ml deoxycholic acid, and 20 μg/ml aprotinin, leupeptin, and pepstatin A) and briefly sonicated. Platelet lysates were either subjected directly to 7.5% SDS-polyacrylamide gel electrophoresis followed by immunoblotting, or they were centrifuged at 15,000*g* at 4°C for 15 min in preparation for immunoprecipitation. PI3-K was immunoprecipitated using the mouse monoclonal anti-p85 antibody AB6 (Upstate Biotechnology, Lake Placid, NY); we confirmed that this antibody does not cross-react with type IB PI3-Kγ (data not shown). Mouse IgG was used as a control. Lysates were incubated with the immunoprecipitating antibody at 4°C overnight, followed by a 1-h incubation with 40 μl of protein G at 4°C. Samples were washed four times with ice-cold phosphate-buffered saline, resuspended in 50 μl of 2× sample buffer, and boiled for 4 min. Proteins were separated in 7.5% SDS-polyacryl-

amide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk for 1 h and incubated with the primary antibody at 4°C overnight. The blotting antibodies used were anti-phosphotyrosine-4G10 (Upstate Biotechnology), anti-Syk-4D10 (Santa Cruz Labs, Santa Cruz, CA), and anti-PI3-K-AB6. Tyrosine phosphorylated Syk was identified by stripping and reprobing anti-phosphotyrosine-4G10–blotted membranes of AB6-immunoprecipitates (which showed only one discrete tyrosine phosphorylated band migrating at ~72 kDa) with anti-Syk-4D10. Immunoreactive bands were reported by enhanced chemiluminescence.

Results

Pathological Shear Stress Causes VWF/GpIb-IX-V–Dependent PIP$_3$ Synthesis That Signals Aggregation. The activation of type I PI3-K results in the conversion of PIP$_2$ to PIP$_3$. Figure 1 shows that washed platelets synthesize PIP$_3$ within 30 s of beginning shear at 120 dynes/cm^2. When all type I PI3-Ks are completely inhibited with either 100 nM wortmannin or 10 μM LY294002, shear-induced PIP$_3$ production is eliminated. Despite the complete inhibition of PI3-K, shear-induced aggregation is only partially inhibited (Fig. 1, bottom). Platelet aggregation in response to 120 dynes/cm^2 shear stress is not inhibited by 1 or 10 nM wortmannin, concentrations considered by some (Rittenhouse, 1996) but not others (Stephens et al., 1994; Fruman et al., 1998) to separate functional responses caused by type IA PI3-K (inhibited by ≥1 nM) from those caused by type IB PI3-K (inhibited by ≥10 nM). The IC$_{50}$ of LY294002 for shear-induced aggregation is ~25 μM, which is a concentration higher than that required to eliminate shear-induced PIP$_3$ production (as shown in Fig. 1). These data indicate that LY294002 inhibits other non–PI3-K pathways of shear induced platelet aggregation, and implicate another LY294002-sensitive kinase (such as casein kinase) as a component of one such pathway (Davies et al., 2000).

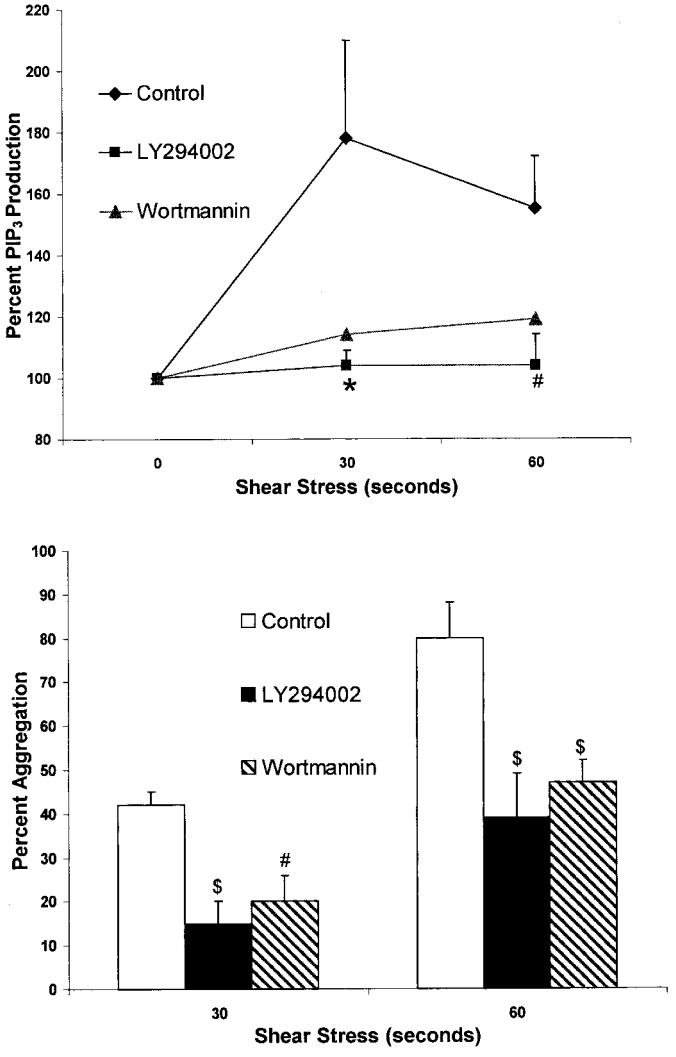

Fig. 1. Platelets sheared at 120 dynes/cm^2 synthesize PIP$_3$. The PI3-K inhibitor LY294002 (10 μM) or wortmannin (100 nM) inhibits both PIP$_3$ synthesis and aggregation in response to shear stress. (n = 3 for untreated and LY294002-treated platelet PIP$_3$ measurements and for all aggregation measurements; the effect of wortmannin on shear-induced PIP$_3$ is the average of duplicate measurements; values represent means and bars represent S.D.; *, $p < 0.05$; #, $p < 0.02$; $, $p < 0.01$; compared with controls using Student's t test).

Because most platelet responses to shear stress are triggered by VWF binding to GpIb-IX-V, we tested the effect of inhibitors of shear-induced VWF binding to GpIbα on PIP$_3$ synthesis and platelet aggregation. Both are abolished by blocking VWF binding to GpIbα with the monoclonal antibody AK2 (6 μg/ml) or the monoclonal antibody 5D2 (25 μg/ml) (data not shown). PIP$_3$ synthesis is not inhibited by 0.5 mM RGDS, which inhibits shear-induced VWF binding to αIIbβ3 and platelet aggregation (data not shown).

Shear-Induced PIP$_3$ Production Depends on Tyrosine Kinase Activity Signaling Syk Associated with PI3-K. The activation of type IA PI3-K depends on it binding to tyrosine phosphorylated proteins (Stephens et al., 1994; Fruman et al., 1998). To determine whether shear-induced PI3-K activity results from the phosphotyrosine-dependent activation of a type IA PI3-K, we measured PIP$_3$ production in platelets pretreated with piceatannol, a tyrosine kinase inhibitor that is selective for src, Syk, and Fak (Law et al., 1999). Figure 2, top, shows that piceatannol (25 μg/ml) inhibits shear-induced PIP$_3$ synthesis. This concentration of piceatannol has been previously shown by us to inhibit shear-induced platelet aggregation (Feng et al., 2002).

To understand the mechanism by which type IA PI3-K is activated after shear-induced VWF binding to GpIb-IX-V, we tested the hypothesis that the tyrosine kinase Syk is involved upstream of PI3-K activation. There are two reasons why this hypothesis was developed. First, there is evidence from blocking experiments using intact sheared platelets and from experiments using genetically engineered heterologous system (in which GpIb-IX-FKBP chimeras are cross-linked) that VWF induces GpIb-IX-V–dependent platelet Syk phosphorylation (Razdan et al., 1994; Kasirer-Friede et al., 2002). Second, there is evidence that Syk activates type IA PI3-K in B lymphocytes (Beitz et al., 1999) and NK cells (Jiang et al., 2002).

The bottom of Fig. 2 shows that Syk coimmunoprecipitates with type IA PI3-K in resting platelets. Pathological shear stress stimulates the tyrosine phosphorylation of Syk associated with PI3-K. Piceatannol inhibits the tyrosine phosphorylation of Syk coimmunoprecipitated with PI3-K but does not affect the association between Syk and PI3-K.

Shear-Induced PIP$_3$ Synthesis Is Inhibited When the P2Y$_{12}$ Receptor Is Blocked. Shear-dependent platelet aggregation depends on released ADP binding to both P2Y$_1$ and P2Y$_{12}$ receptors (Turner et al., 2001). To determine whether

shear-induced PI3-K activation is caused by a rapid feedback mechanism involving secreted ADP binding to one or both of its receptors, shear-induced PIP_3 synthesis was measured after washed platelets were pretreated with antagonists against $P2Y_1$ (100 μM A3P5P) or $P2Y_{12}$ (0.5 μM AR-C69931MX). Figure 3 shows that shear-induced PIP_3 synthesis peaks at 30 s, and returns to baseline values at 60 s. The $P2Y_{12}$-specific inhibitor AR-C69931MX, but not the $P2Y_1$-specific inhibitor A3P5P, inhibits shear-induced PIP_3 synthesis at 30 s. Figure 3 also shows that only AR-C69931MX, but not A3P5P, has a significant inhibitory effect on shear-induced washed platelet aggregation at 30 and 60 s. The inhibitory effect on shear-induced aggregation of AR-C69931MX + A3P5P combined is not significantly greater than AR-C69931MX alone.

$P2Y_{12}$ Signals the Tyrosine Phosphorylation of Syk Associated with PI3-K. Because only $P2Y_{12}$ receptor blockade inhibits shear-induced platelet PIP_3 synthesis, we were faced with an apparent conundrum: how does the $P2Y_{12}$ receptor signal the activation of a phosphotyrosine-dependent type IA PI3-K? This conundrum is based on the generally accepted idea that $P2Y_{12}$ activates only type IB PI3-K through the disassociation of Giα from Gi$\beta\gamma$, with the $\beta\gamma$ subunit stimulating the type IB PI3-K (Trumel et al., 1999; Dangelmaier et al., 2001). In fact, this concept is not completely correct, in that there are published data demonstrating that type IA PI3-K is activated by both phosphotyrosine and G protein $\beta\gamma$ subunits and that the interaction of a type IA PI3-K with phosphotyrosine-containing proteins *and* $\beta\gamma$ subunits is one mechanism for coordinating multiple signals for PIP_3 production (Thomason et al., 1994; Tang and Downes, 1997; Fruman et al., 1998). With this in mind, we sought to determine whether shear-induced type IA PI3-K activation is directed by separate signals converging from GpIb-IX-V, $P2Y_1$, and the $P2Y_{12}$ receptor. To test this hypothesis, we examined for phosphorylated Syk associated with type IA PI3-K immunoprecipitated from sheared platelets pretreated with monoclonal antibody AK2 (which inhibits its VWF binding to GpIb-IX-V), AR-C69931MX, or A3P5P. As expected, AK2 inhibits the phosphorylation of Syk associated with PI3-K (data not shown). Figure 4 shows that AR-C69931MX, but not A3P5P, inhibits the tyrosine phosphorylation of Syk associated with type IA PI3-K. These data suggest that Syk phosphorylation in platelets stimulated by shear-dependent VWF binding to GpIb-IX-V results from released ADP feedback activating the $P2Y_{12}$ receptor.

If pathological shear stress causes Syk phosphorylation through released ADP binding to the $P2Y_{12}$ receptor, then one should observe that Syk phosphorylation in response to ADP alone is blocked by AR-C69931MX. To test this hypothesis, we examined the effect of 10 μM ADP on Syk phosphorylation in washed platelets stirring in an aggregometer at 37°C in the presence of 1 mg/ml purified human fibrinogen and 1 mM $CaCl_2$. Figure 5 shows that 10 μM ADP causes the tyrosine phosphorylation of Syk at 15 s and that this phosphorylation gradually decreases over the course of the next

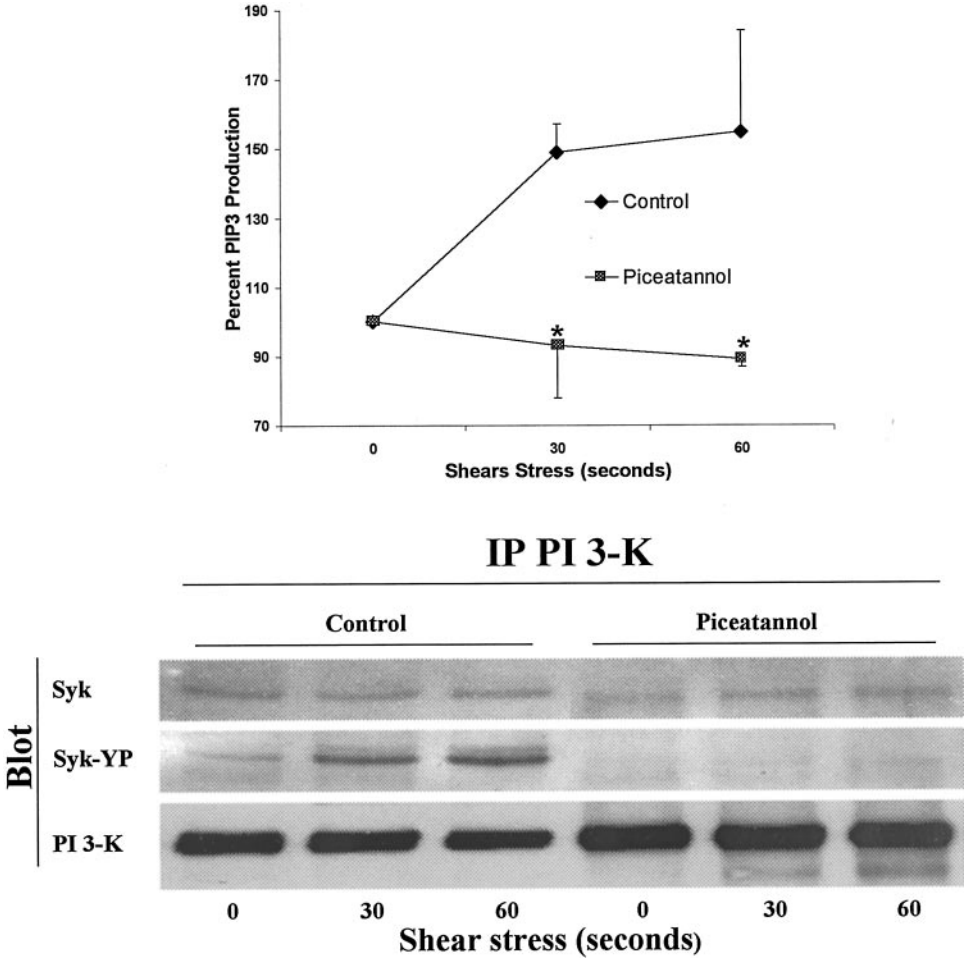

Fig. 2. Shear-induced PIP_3 synthesis is inhibited by blocking tyrosine kinase activity with 25 μg/ml piceatannol ($n = 3$; values represent means and bars represent S.D.; *, $p < 0.03$ compared with controls using Student's t test). Piceatannol inhibits the tyrosine phosphorylation of PI3-K-associated Syk (IP, immunoprecipitated; Syk-YP, tyrosine phosphorylated Syk; the data are representative of three separate experiments).

45 s. We next investigated how ADP-induced Syk phosphorylation is affected by either $P2Y_{12}$ or $P2Y_1$ receptor blockade. Consistent with our observations under high-shear conditions that Syk phosphorylation is $P2Y_{12}$- dependent, we find that AR-C69931MX, but not A3P5P, inhibits ADP-induced Syk phosphorylation in stirring washed platelets (Fig. 5). A3P5P (1 mM), which in some cases is required to block ADP-induced platelet aggregation, only partially inhibits ADP-induced Syk phosphorylation (data not shown), even though this concentration is 5-fold greater than the maximal A3P5P concentration required to abolish ADP-induced platelet calcium signaling (Jin et al., 1998).

Because Syk phosphorylation could be caused by postaggregation αIIbβ3-mediated "outside-inside" signaling, we also examined ADP-induced Syk phosphorylation under nonstirring conditions. Figure 5 shows that Syk phosphorylation occurs in unstirred nonaggregated platelet suspensions, suggesting that ADP-induced Syk phosphorylation, like shear-induced PIP_3 synthesis, is not downstream of ligand binding to αIIbβ3. In support of this conclusion, we also observe that ADP-induced Syk tyrosine phosphorylation is not inhibited even when αIIbβ3 activation is eliminated by preincubating washed platelet suspensions with 4 mM EGTA (data not shown).

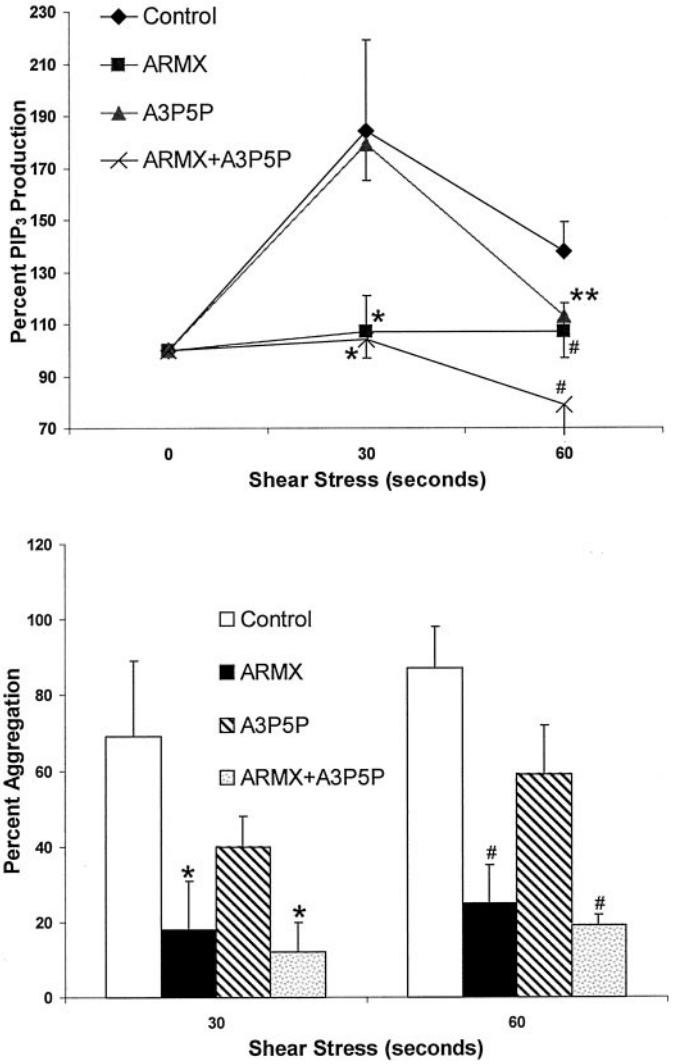

Fig. 3. $P2Y_{12}$ receptor blockade with AR-C69931MX (ARMX; 0.5 μM), but not $P2Y_1$ receptor blockade with A3P5P (100 μM), inhibits shear-induced PIP_3 synthesis at 30 s. ARMX also inhibits shear-induced aggregation of washed platelets. (n = 3; values represent means and bars represent S.D.; *, $p < 0.05$; #, $p < 0.02$; **, $p = 0.135$ compared with controls using Student's t test).

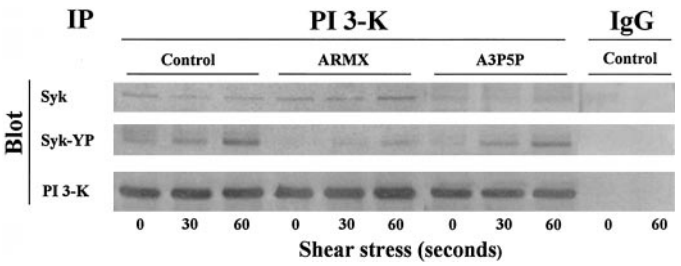

Fig. 4. Shear causes the tyrosine phosphorylation of Syk (Syk-YP) that is associated with immunoprecipitated (IP) PI3-K. This is inhibited by $P2Y_{12}$ receptor blockade with AR-C69931MX (ARMX; 0.5 μM), but not $P2Y_1$ receptor blockade with A3P5P (100 μM). Data are representative of three separate experiments.

Discussion

Experiments presented in this report reveal several data that begin to untangle a nexus of signaling responses developing in platelets activated by shear-induced VWF/GpIb-IX-V interactions. We have shown for the first time that shear-induced VWF binding to GpIb-IX-V stimulates platelet PIP_3 synthesis, and that inhibiting PIP_3 synthesis partially inhibits shear-induced aggregation. We have shown that functionally important shear-induced PI3-K activation is dependent on tyrosine kinase activity and that the major route of PIP_3 synthesis is via a $P2Y_{12}$-dependent pathway. We have also shown data in support of the hypothesis that shear-induced PI3-K activation is related to the tyrosine phosphorylation of a molecular complex comprising class IA PI3-K and Syk, thus presenting mechanistic evidence for a novel $P2Y_{12}$-induced response.

There are long-standing data that pathological shear-induced secretion of stored platelet ADP is an important early response that contributes greatly to the magnitude of platelet

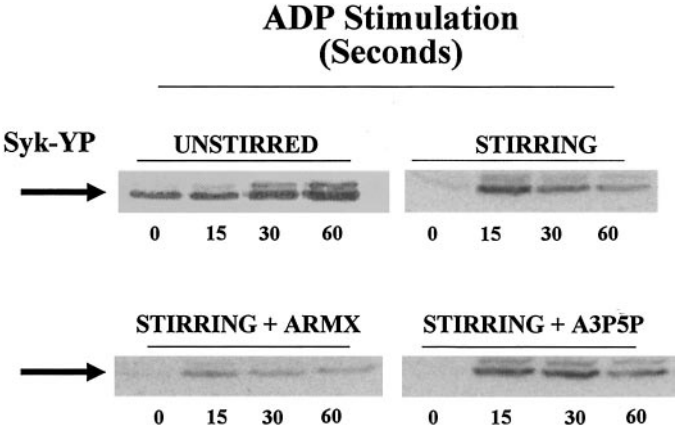

Fig. 5. 10 μM ADP causes Syk phosphorylation in washed platelet suspensions containing 1 mg/ml purified human fibrinogen and 1 mM $CaCl_2$ under both stirring and unstirred conditions (different donors assayed on different days). $P2Y_{12}$ receptor blockade with AR-C69931MX (ARMX; 0.5 μM), but not $P2Y_1$ receptor blockade with A3P5P (100 μM), inhibits ADP - induced Syk phosphorylation (same donor assayed on same day). Syk-YP, tyrosine phosphorylated Syk. Data are representative of two to three separate experiments.

aggregation when washed platelets, platelet-rich plasma, or whole blood is subjected to elevated shear stress in a cone-plate viscometer (Turner et al., 2001). The in vivo relevancy of such observations is unlikely, however, and there is no evidence that blood platelets are subjected to elevated shearing stress for time periods greater than 1 s, even under conditions of severe native or prosthetic heart valve stenosis. Nonetheless, it is certain that the cone-plate viscometer is a model useful for studying platelet adhesion-activation coupling: by providing precise control over rheological forces and accommodating sample volumes adequate for biochemical analyses, it is an excellent tool for investigating mechanisms by which shear-induced VWF binding to GpIb-IX-V leads to platelet aggregation. In fact, it is clear that data obtained in the cone-plate viscometer foreshadowed observations using flow chambers, animal models, and clinical specimens showing that secreted ADP contributes substantially to the accrual of platelets onto thrombogenic surfaces (Weiss et al., 1986; Moake et al., 1998; Fredrickson et al., 2000; Turner et al., 2001; Sakariassen et al., 2001). Cone-plate viscometer experiments also predicted accurately that the pharmacological inhibition of ADP binding to platelets would result in the inhibition of shear-induced thrombus formation (Sakariassen et al., 2001; Turner et al., 2001).

The precise mechanism by which the $P2Y_{12}$ receptor functions in vivo is not known (Hollopeter et al., 2001). Although there is irrefutable evidence that ADP binding to $P2Y_{12}$ abrogates platelet adenylyl cyclase activity stimulated through G_s-coupled heptahelical prostacylin or prostaglandin E_2 receptors, there is no evidence that ADP binding influences basal levels of the inhibitory second messenger cyclic adenosine 3′,5′ monophosphate in platelets (Pflieger and Herbert, 1996), and it is obvious that platelet $P2Y_{12}$ is coupled to other proaggregatory signal pathway(s). There is evidence that one of these pathways involves G protein $\beta\gamma$-activated type IB PI3-K (Stephens et al., 1994; Fruman et al., 1998; Trumel et al., 1999; Selheim et al., 2000; Hirsch et al., 2001). Data presented in this report provide unexpected evidence that $P2Y_{12}$ is also coupled to a proaggregatory signal pathway involving the sequential activation of Syk followed by type IA PI3-K, and indicate that $P2Y_{12}$ may be coupled to both types IA and IB PI3-K. Mechanisms by which $P2Y_{12}$ signals the activation of platelet tyrosine kinases are currently unknown, although there is evidence for $\beta\gamma$-responsive tyrosine kinases in other types of cells (Langhans-Rajasekaran et al., 1995).

These results provide a molecular basis for clinical observations that thienopyridine-mediated platelet $P2Y_{12}$ receptor blockade is beneficial in diverse syndromes of arterial thrombosis triggered by shear-dependent platelet activation, including ischemia of the coronary, carotid and peripheral arteries (CAPRIE, 1996; Yusuf et al., 2001). The results also elucidate mechanisms of shear-induced platelet aggregation, and suggest that understanding signal pathways coupling shear-induced VWF/GpIb-IX-V interactions to ADP secretion might provide important new molecular targets for the development of novel antithrombotic agents that have a therapeutic index superior to ticlopidine or clopidogrel.

Whereas the thienopyridine drugs have already had a major favorable impact on the natural history of atherothrombotic vascular diseases, they are not without limitations (Quinn and Fitzgerald, 1999). One such limitation to their potential efficacy is the magnitude of their inhibitory effect in whole blood. In contrast with data in Fig. 3, in which AR-C69931MX inhibits shear-induced washed platelet aggregation significantly more than A3P5P, in vitro studies of shear-induced platelet aggregation in whole blood (or even platelet-rich plasma) demonstrate a relatively lesser effect from $P2Y_{12}$ receptor blockade and a relatively greater effect of $P2Y_1$ receptor blockade (Turner et al., 2001). These differences may be related to the quantity of red cells in the platelet preparation. Red cells are vulnerable to sublytic mechanical damage over a large range of shear stresses. Red cell damage results in the release of ADP, and the contribution of red cell-derived ADP to shear-induced aggregation is very important (Goldsmith et al., 1995). Red cell-derived ADP, because of its large quantity, is less inhibitable than the small amount of ADP released by shear-activated washed platelets, probably because the higher concentrations of exogenous ADP are less susceptible to competitive antagonism. There are some in vivo data to support this conclusion: when ticlopidine is ingested 12 to 36 h before angioplasty, there is very little inhibition of ex vivo shear-induced whole blood platelet aggregation for up to 7 days after an acute coronary intervention, despite the establishment of steady-state $P2Y_{12}$ receptor blockade (Fredrickson et al., 2000).

In addition to the therapeutic limitations of the thienopyridine drugs, there is also a significant risk of bleeding, including severe bleeding (when clopidogrel is combined with aspirin, life threatening bleeding occurs in ~2%) (Yusuf et al., 2001). These facts underscore the potential clinical benefits of improving upon the pharmacology of $P2Y_{12}$ receptor blockade. They also point to the importance of beginning to examine costimulatory shear-induced activation pathways that bypass the effects of released ADP and/or PI3-K activation. Such pathways are certainly present; data presented in Figs. 1 and 2 clearly show that there is significant residual aggregation even when ADP receptors or PI3-K is blocked.

Results presented here also suggest that the inhibition of platelet protein tyrosine kinases and phosphatidylinositol 3-kinases may not result in an antithrombotic effect very different from that of ticlopidine or clopidogrel. Rather, efforts to improve the standard of care for persons with acute arterial ischemia might focus on the regulation of secretory pathways stimulated by shear-induced VWF binding to GpIb-IX-V. Little is known about signals regulating the rapid release of ADP and other prothrombotic molecules from platelets subjected to pathological shear stress. It may be important to try to identify such signals and determine their role in both physiological hemostasis and pathological thrombosis. This will help the process of testing the hypothesis that prothrombotic signals initiated by shear-induced VWF binding to GpIb-IX-V differ from those maintaining microvascular hemostasis under low shear stress conditions, and that the perturbation of shear-induced signaling to the secretory apparatus may inhibit thrombosis without compromising hemostasis.

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