Involvement of the Mitogen-activated Protein Kinase c-Jun NH₂-terminal Kinase 1 in Thrombus Formation*

Received for publication, February 23, 2007, and in revised form, September 4, 2007 Published, JBC Papers in Press, September 4, 2007, DOI 10.1074/jbc.M701596200

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The involvement of the mitogen-activated protein kinase c-Jun NH₂-terminal kinase-1 (JNK1) has never been investigated in hemostasis and thrombosis. Using two JNK inhibitors (SP600125 and 60), we have demonstrated that JNK1 is involved in collagen-induced platelet aggregation dependent on ADP. In these conditions, JNK1 activation requires the coordinated signaling pathways of collagen receptors (α2β1 and glycoprotein (GP) VI) and ADP. In contrast, JNK1 is not required for platelet adhesion on a collagen matrix in static or blood flow conditions (300–1500 s⁻¹) involving collagen receptors (α2β1 and GPVI). Importantly, at 1500 s⁻¹, JNK1 acts on thrombus formation on a collagen matrix dependent on GPIb-von Willebrand factor (vWF) interaction but not ADP receptor activation. This is confirmed by the involvement of JNK1 in shear-induced platelet aggregation at 4000 s⁻¹. We also provide evidence during rolling and adhesion of platelets to vWF that platelet GPIb-vWF interaction triggers αIIbβ3 activation in a JNK1-dependent manner. This was confirmed with a Glanzmann thrombasthenic patient lacking αIIbβ3. Finally, in vivo, JNK1 is involved in arterial but not in venular thrombosis in mice. Overall, our in vitro studies define a new role of JNK1 in thrombus formation in flowing blood that is relevant to thrombus development in vivo.

Blood platelets play a key role in hemostasis and thrombosis. At the site of vascular injury, platelets roll and adhere to various components of the subendothelial matrix through a number of adhesive receptors present on the platelet surface. Several mechanisms support the initial adhesion of platelets and their subsequent activation and aggregation into a thrombus (1). Collagen is the most thrombogenic component of the subendothelial matrix. It supports firm platelet adhesion mediated by its two major surface receptors, the integrin α2β1 and glycoprotein (GP) VI, and induces aggregation, secretion, and procoagulant activity (1). At higher shear rates found in small arteries, arterioles, and diseased vessels (2–4), initial platelet arrest depends on the interaction of von Willebrand factor (vWF) itself bound to collagen fibers (5, 6). This interaction is unstable, but firm platelet adhesion and platelet recruitment occur via the interaction of the collagen with its receptors (α2β1 and GPVI) and the interaction of vWF with the integrin αIIbβ3 (6).

Mitogen-activated protein (MAP) kinases are a family of serine/threonine protein kinases that comprises three subgroups: extracellular signal-regulated kinases (ERKs), p38, and c-Jun NH₂-terminal kinases (JNKs) (7). Mammalian JNKs were found to be activated by a variety of environmental stresses, inflammatory cytokines, and growth factors (8, 9). JNKs have also been characterized by their ability to phosphorylate regulatory sites in the NH₂ terminus of the transcription factor c-Jun (10) and a variety of additional transcription factors such as Elk-1 and ATF-2 (11, 12). JNK substrates are not limited to transcription factors or nuclear proteins such as the apoptotic proteins Bcl-X(L) or the protein cytoskeleton paxillin (13, 14). In platelets, ERK2, p38, and JNK1 are present and activated by various agonists. The involvement of ERK2 and p38 in hemostasis and thrombosis is still a matter of debate. For example, Marshall et al. (15) demonstrated that αIIbβ3 activation was dependent on the Src kinase pathway but not the ERK2 pathway. By contrast, Li et al. (16, 17) showed that activation of integrin αIIbβ3 after vWF binding to GPIb was dependent on protein kinase G and ERK2. Recently, our group demonstrated a critical role for ERK2 in platelet adhesion, in blood flow conditions, and in platelet spreading (18, 19). With other agonists, such as low doses of collagen (20) or thrombin (21), ERK2 is involved in platelet aggregation. In these conditions, ERK2 acts on platelet secretion and activation of myosin light chain kinase (20, 22).

The other MAP kinase, p38, is involved in collagen-induced platelet aggregation (23), vWF-induced platelet activation (24), and procoagulant activity (25). In vivo, the involvement of MAP kinases is largely unexplored. Recently, in a model of thrombotic occlusion of injured arterioles and venules, P2X1 and...
ERK2 were reported to be involved in shear stress-controlled thrombosis (26). Moreover, in a model of artery carotid injury, p56 was also involved in thrombosis (27).

Our group found that JNK1 is present in platelets and down-regulated by αIIbβ3 engagement after thrombin induction (28). Moreover, JNK1 is activated by porcine vWF (29) and by thrombin (28), but its role in platelet activation and thrombosis has never been investigated. In this study, we examined the involvement of JNK1 in platelet aggregation induced by collagen and vWF and platelet adhesion in blood flow conditions and in a mouse model of thrombosis. We found that JNK1 was involved in collagen-induced platelet aggregation dependent on ADP. In blood flow conditions, JNK1 was required for thrombus formation but not platelet adhesion over a collagen matrix. Moreover, JNK1 was partially involved in the αIIbβ3-activated pathway required for shear-induced platelet aggregation, for platelet rolling, and for firm adhesion to vWF. Finally, in vivo, JNK1 was found to be involved in a mouse model of arterial thrombosis. To our knowledge, this is the first report showing the involvement of JNK1 in thrombus formation.

**EXPERIMENTAL PROCEDURES**

*Materials—* Equine type I collagen was obtained from Kordia (Leiden, The Netherlands). Purified von Willebrand Factor was purchased from C.A.F.-D.F.C. (Brussels, Belgium). Leupeptin, aprotinin, aprotinin grade VII, prostaglandin E1, Me2SO, MRS2179, monoclonal antibody (mAb) directed against β-tubulin, and polyclonal antibody against vWF were from Sigma. Indomethacin was obtained from Cayman Chemical Company (Ann Arbor, MI). JNK inhibitors SP600125 and N-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide (6o) (30), d-Phe-Pro-Arg chloromethylketone dihydrochloride (PPACK), and phenylarsine oxide were from Calbiochem-VWR (Fontenay-sous-Bois, France). Calcein-AM and Alexa Fluor® 488-phalloidin were purchased from Molecular Probes (Eugene, OR). The stress-activated protein kinase/JNK assay kit was from Cell Signaling (Beverly, MA). Rose Bengal was purchased from Acros Organics (Halluin, France). Antibody to human αIIbβ3/αvβ3 (ReoPro®) was from Lilly (Leiden, The Netherlands). The mAb 6F1 directed against the α2β1 integrin was generously provided by Prof. B. S. Coller (Rockefeller University, New York, NY). The mAb G19H10 that blocks the binding of vWF to GPIb was produced as previously described (31). AR-C69931 MX was generously provided by Dr. B. Humphries (Astra Zeneca, UK). Polyclonal antibody directed against the phosphorylated form of JNK1 (JNK1-P) was purchased from Promega (Madison, WI). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Preparation and Fluorescence Labeling of Human Platelets—Venous blood was collected from healthy donors and from a patient with Glanzmann thrombasthenia (GT) characterized by an absence of αIIbβ3 (32). Written informed consent was obtained from all the donors. Platelet-rich plasma was obtained by centrifugation (120 × g for 15 min at 20°C), and platelets were isolated by differential centrifugation as previously described (20). The platelet pellet was resuspended in Tyrode's buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 3 mM KCl, 5 mM NaHCO3, 0.5 mM MgCl2, 1 mM CaCl2, and 10 mM glucose).

To study platelet adhesion to vWF, platelets in platelet-rich plasma were labeled with 2.5 μM calcein-AM and resuspended at 3 × 10^7/μl in autologous plasma, whereas erythrocytes were maintained as a packed cell suspension. Reconstituted blood was obtained by gently mixing labeled platelets and erythrocytes at 45% hematocrit just before perfusion.

Platelet Aggregation—Platelet aggregations were monitored by measuring light transmission through the stirred suspension of washed platelets at 37°C using a Chronolog dual-beam aggregometer (Coultronics, Margency, France). The platelets (4 × 10^5/μl) were preincubated with SP600125 (10 min, 2–10 μM) or with 6o (30 min, 2–10 μM) or Me2SO at 37°C without stirring. The quantity of Me2SO never exceeded 0.2% (v/v). Platelet aggregation was initiated by adding type I collagen (0.2–10 μg/ml). Aggregation was measured and expressed as the percentage of change in light transmission, with the value for the blank sample (buffer without platelet) set at 100%.

Immunoblotting—The samples were subjected to immunoblotting as previously described (33). The platelets were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 μM phenylarsine oxide, 1% SDS, 5 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4). The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with various primary antibodies: anti-JNK1-P (1/5000), anti-c-Jun-P (1/1000), and anti-tubulin (1/4000). Immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (Perbio Science, Brebières, France).

JNK1 Activity—JNK1 activity was examined after collagen stimulation in the presence or absence of various concentrations of SP600125 or 6o (5 and 10 μM) or Me2SO. JNK1 activity was measured according to the manufacturer’s instructions (Cell Signaling). Briefly, the platelets (4 × 10^5/μl) were lysed with cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM/ml leupeptin, 10 μg/ml aprotinin) and centrifuged at 14,000 × g for 10 min at 4°C. Immobilized c-Jun fusion protein beads (20 μl) were added in the supernatant and incubated with gentle rocking overnight at 4°C to pull down JNK. After centrifugation (14,000 × g; 30 s; 4°C), the pellet was washed twice with cell lysis buffer and twice with kinase buffer on ice (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The pellet was suspended in 50 μl of kinase buffer supplemented with 200 μM ATP and incubated 30 min at 30°C. The proteins were separated by SDS-PAGE according to standard protocols and c-Jun-GST substrate, phosphorylated by JNK1 (c-Jun-P), was detected by Western blotting using specific antibody.

Platelet Deposition—Platelet suspension (2 × 10^5) was preincubated with SP600125 or 6o (10 μM) or Me2SO in the presence or absence of ReoPro® (10 μg/ml) and then plated on type I collagen (50 μg/ml) for 60 min at 20°C. After three washes, platelet deposition was quantified by an acid phosphatase assay, as previously described (34).
Platelet Perfusion Studies—Platelet adhesion to type I collagen under flow was evaluated using a parallel plate perfusion chamber. Glass coverslips were coated overnight at 4 °C with type I collagen (50 μg/ml). Whole blood, collected into 40 μM PPACK, was perfused for 4 min, using a KD Scientific syringe pump (Fisher) at shear rates of 300 or 1500 s⁻¹ followed by washing in the same conditions. After fixation (4% paraformaldehyde) and permeabilization (0.2% Triton X-100), F-actin of adherent platelets was detected using Alexa Fluor® 488-phalloidin (1/200).

Platelet adhesion to vWF under flow was evaluated using glass microcapillary tubes (Fiber Optic Center, New Bedford, MA). Microcapillary tubes were coated overnight at 4 °C with purified vWF (20 μg/ml). Reconstituted blood was drawn through the coated glass microcapillary at a shear rate of 1500 s⁻¹ for 4 min and then rinsed in the same conditions.

Adherent platelets were recorded using an inverted epifluorescent microscope (Nikon Eclipse TE2000-U; Champigny sur Marne, France), coupled to Metamorph 7.0r1 software (Universal Imaging Corporation). The surface covered by platelets was expressed as the relative surface covered to facilitate the comparison of different experimental conditions.

Quantification of Platelet Surface Velocity—To calculate the velocity of platelets interacting with the surface of vWF-coated microcapillary, reconstituted blood containing labeled platelets (3 × 10⁹/μl) was perfused at a shear rate of 1500 s⁻¹. Real time platelet adhesion and platelet rolling were recorded at 2 frames/s. Platelet velocity was determined as previously described (35).

Shear-induced Platelet Aggregation (SIPA)—SIPA was measured by means of a coaxial cylinder shearing device, as previously described (36). Briefly, washed platelets (1.8 × 10⁹/μl) were preincubated with SP600125 (10 μM) or Me₂SO and were exposed to a shear rate of 200 or 4000 s⁻¹ for 2 min at 20 °C with vWF (40 μg/ml). The samples were fixed with 1.25% paraformaldehyde, and the number of single platelets was measured by flow cytometry (Coulter Epics XL, Beckman Coulter, Roissy, France) before and after exposure to shear. SIPA was expressed as the percentage of disappearance of single platelets.

Animals—All of the experimental procedures on animals were performed according to French legislation on the protection of animals. FVB mice were obtained from Janvier Laboratories (Le Genest-St-Isle, France) and kept in animal facilities. Eight- to twelve-week-old male mice were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg). Eight- to twelve-week-old male mice were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg/kg), and 5 min at 150 °C. Upon the addition of 0.2 volume of ACD, 1 μM prostaglandin E₁, the platelets were isolated by centrifugation at 850 × g for 5 min. The platelets were resuspended at 0.5 × 10⁶/μl and labeled with 10 μM calcein-AM for 20 min at 37 °C.

Photochemically Induced Thrombosis Model—Vascular injury of caecum arteries and venules was induced by the phototoxic dye rose bengal as previously described (37). Anesthetized mice were catheterized via the jugular vein. Fluorescently labeled murine platelets (200 μl; 0.5 × 10⁶ platelets/μl) were injected through the catheter, followed by an intravenous bolus of rose bengal (30 mg/kg), which was infused (30 mg/kg/h) for 10 min, the time required for the injected platelets to reach steady-state, prior to induction of the vascular injury and then throughout the experiment. To evaluate the involvement of JNK1 in thrombus formation, a bolus plus perfusion of the inhibitor SP600125 or Me₂SO was applied through the catheterized jugular vein 10 min prior to vessel wall injury (bolus of 0.91 μmol/kg = 200 μg/kg followed by an intravenous infusion at 200 μg/kg/h). The mouse intestines were then spread on a microscopic glass, and the arterioles and venules lying on the caecum were visualized with the epifluorescent microscope. Injury was induced by exposing the selected arterioles and venules at 540 nm for 30 s. Recruitment of fluorescent platelets to the lesion site was registered in real time and recorded in the memory of an attached computer for analysis with the Metamorph program. We analyzed the vessel occlusion time, defined as blood flow arrest for at least 1 min.

Statistics—The results are expressed as the means ± S.E. for at least three independent experiments. Statistical significance was assessed with a one-way ANOVA with Dunnett’s post-test or the Mann-Whitney test, depending on the case.

RESULTS

JNK1 Is Involved in Collagen-induced Platelet Aggregation—We have previously reported that JNK1 was present in platelets (28), but its involvement in hemostasis and thrombosis remains to be established. Therefore, we first investigated the phosphorylation level of JNK1 in collagen-induced platelet aggregation. Platelet suspension was incubated with various doses of collagen (0.2–10 μg/ml) under stirring conditions. Whereas JNK1 was not phosphorylated in resting platelets, phosphorylated JNK1 (JNK1-P) was detected at a low collagen concentration (0.2 μg/ml) (Fig. 1) and reached a maximum at 2 μg/ml collagen after 2 min. These results indicate that JNK1 is activated during platelet aggregation induced by collagen.

To further investigate the role of JNK1 in platelets, we used two JNK inhibitors, SP600125 and 6o. We first assessed the inhibitory effect of SP600125 and 6o in conditions of collagen-induced platelet aggregation by testing JNK1 activity. This
activity was quantified by the phosphorylation level of the specific substrate, c-Jun (10). SP600125 and 6o inhibited JNK1 activity in a dose-dependent manner (Fig. 2).

Next, the role of JNK1 was investigated in platelet aggregation induced by collagen in conditions where platelets were pretreated with SP600125, 6o, or Me₂SO. At low collagen concentrations (0.2 and 0.5 μg/ml), platelet aggregation was totally or partially impaired by both inhibitors (10 μM), whereas at a high collagen concentration (5 μg/ml), these inhibitors had no effect (Fig. 3, A and B). We next examined the effect of various concentrations of JNK inhibitors (2–10 μM) on platelet aggregation induced by a low collagen concentration (0.2 μg/ml). Two μM of SP600125 or 6o impaired platelet aggregation by 28 ± 3 and 14 ± 3%, respectively, compared with control, whereas 10 μM of SP600125 or 6o impaired platelet aggregation by 98 ± 1 and 98 ± 4%, respectively (p < 0.01) (Fig. 3C).

In these conditions, at a low collagen concentration (0.2 μg/ml), platelet aggregation and JNK1 phosphorylation were mostly impaired by ADP/ATP scavenger apyrase (2 units/ml) and the antagonist of ADP receptor P2Y12 (AR-C69931MX; 10 μM), the P2Y1 antagonist (MRS2179) having a weak inhibitory effect on JNK1 phosphorylation (Fig. 4). Taken together, these results indicate that JNK1 is involved in platelet aggregation induced by low collagen concentrations dependent on ADP.

**Collagen-induced Platelet Deposition Does Not Require JNK1 Activation**—First, we examined the state of JNK1 phosphorylation in collagen-induced platelet deposition. Platelet suspensions were plated on type I collagen for various times. JNK1-P was detected after 15 min of adhesion, followed by a decrease in the JNK1 phosphorylation level (Fig. 5A). To investigate the role of JNK1 in platelet deposition on collagen, a static adhesion assay was performed in conditions where only platelet adhesion occurred (2 × 10⁵ platelets/μl), which was confirmed by the identical adhesion observed in the presence or absence of anti-αIIbβ3, ReoPro® (10 μg/ml) (Fig. 5, B and C). Neither of the two JNK inhibitors (10 μM) affected platelet adhesion. Our results show that JNK1 is not required for collagen-induced platelet deposition.

**JNK1 Is Required in Thrombus Formation on Collagen under Blood Flow Conditions at High Shear Rates**—We next investigated the role of JNK1 in platelet adhesion to collagen at different shear rates. Whole blood was perfused over immobilized type I collagen at low (300 s⁻¹) and high (1500 s⁻¹) shear rates. After 4 min of perfusion, thrombi were smaller at 300 s⁻¹ compared with 1500 s⁻¹, and the covered surface reached 22.5 ± 0.5 and 40.6 ± 7.4%, respectively (Fig. 6A). At 300 and 1500 s⁻¹, only 15 ± 8% of platelet surface coverage and aggregate size were dependent on ADP/ATP (data not shown). The addition
JNK1 and Thrombus Formation

![Figure 4](image)

**FIGURE 4. Role of ADP in JNK1 activation.** The figure illustrates the effect of ADP on JNK1 phosphorylation and platelet aggregation induced by various concentrations of type I collagen (0 to 5 μg/ml) in the presence or absence of apyrase (2 units/ml), P2Y12 antagonist AR-C69931 MX (10 μM), or P2Y1 antagonist MRS2179 (10 μM). JNK1-P was detected by Western blotting using a specific polyclonal antibody against JNK1-P. The data are representative of at least three independent experiments.

of SP600125 or 6O, at 300 s⁻¹, did not modify surface coverage (adhesion/aggregation) on immobilized collagen (Fig. 6). In contrast, at 1500 s⁻¹, surface coverage was strongly impaired by SP600125 (71 ± 5%; p < 0.01) (Fig. 6A) and 6O (52 ± 2%; p < 0.01) (Fig. 6B). In addition, the size of thrombi measured by confocal laser scanning microscopy was impaired by 77% in the presence of SP600125 (10 μM) at 1500 s⁻¹ (data not shown). These results show that JNK1 engagement in thrombus formation is dependent on shear conditions. To better understand the apparent discrepancy observed between the low and high shear rates, we next examined the different receptors involved.

Blood perfusion assays were performed in the presence or absence of mAb 6F1, which blocks α2β1, and mAb G19H10, which blocks GPIb. At 300 s⁻¹, 6F1 inhibited surface coverage by platelets by 99 ± 2% (p < 0.01), whereas G19H10 had no effect (Fig. 7A). VWF deposition on the collagen surface visualized by immunofluorescence microscopy after perfusion at 300 s⁻¹ confirmed that small amounts of VWF were deposited (Fig. 7B) and that thrombus formation was only dependent on collagen receptors. In contrast, at 1500 s⁻¹, 6F1 and G19H10 impaired surface coverage (aggregation and/or adhesion) by 50 ± 3 and 87 ± 7%, respectively (p < 0.01) (Fig. 7A). Larger amounts of VWF were deposited on immobilized type I collagen (Fig. 7B), showing that in conditions where JNK1 is involved, thrombus formation is mostly dependent on GPIb-vWF interaction. Finally, to further investigate the role of JNK1 at 1500 s⁻¹, adhesion without thrombi was examined in control platelets from GT patient characterized by an absence of αIIbβ3 (ReoPro®) and in platelets from GT patient characterized by an absence of αIIbβ3. When thrombi were inhibited in the presence of ReoPro®, only platelet adhesion was observed, and the addition of SP600125 did not affect adhesion (Fig. 8A). Importantly, in the absence of thrombi, platelet adhesion was inhibited only by 6F1 (98 ± 1%) and not by G19H10 (8 ± 5%), indicating that platelet adhesion was dependent on collagen receptors (α2β1 and GPVI) but not on GPIb-vWF interaction (Fig. 8B) as previously described (37). Our results show that, at a high shear rate, thrombus formation dependent on GPIb-vWF interaction requires JNK1 activation.

**FIGURE 5. Role of JNK1 in static platelet adhesion to type I collagen.** A shows the phosphorylation kinetics of JNK1 (JNK1-P) in adherent platelets immobilized on type I collagen. Platelet adhesion (2 × 10⁵/μl) was added to wells coated with collagen (50 μg/ml) for 15–60 min. JNK1-P of adherent platelets was detected by Western blotting using a specific polyclonal antibody against JNK1-P. The data are expressed as the means ± S.E. of three independent experiments.

**JNK1 Is Involved in Shear-induced Platelet Aggregation**—To further study the involvement of JNK1 in thrombus formation in relation to GPIb-vWF interaction, we examined the effect of SP600125 on SIPA by measuring the disappearance of single platelets in the presence of soluble VWF (40 μg/ml). SIPA is dependent on vWF-GPIb interaction. At a low shear rate (200 s⁻¹), no significant disappearance of single platelets was
observed. In contrast, at a higher shear rate (4000 s$^{-1}$), the disappearance of single platelets reached 41.0 ± 4.2% with control platelets and 18.9 ± 2.3% in the presence of JNK1 inhibitor, corresponding to an inhibitory effect of 52 ± 11% ($p < 0.01$). These results indicate that JNK1 is involved in SIPA.

Platelet Rolling and Adhesion to vWF Are Dependent on JNK1—
To further investigate the role of JNK1 in platelet interactions between vWF and its receptors, GPIb and αIIbβ3, we examined platelet rolling and platelet adhesion on a vWF matrix. Platelet translocation velocity was examined after 60 s of perfusion. The average velocity of platelets translocating on immobilized vWF (1.36 ± 0.08 μm/s) increased to 1.95 ± 0.07 μm/s ($p < 0.01$) in the presence of ReoPro® (Fig. 9A). Moreover, an elevated velocity was observed in the presence of SP600125 alone (1.91 ± 0.08 μm/s) ($p < 0.01$), which did not increase after the addition of ReoPro®. These data strongly suggest that JNK1 is involved in platelet rolling and in αIIbβ3 engagement.

To confirm the involvement of JNK1 in the αIIbβ3 engagement, we examined the effect of SP600125 on firm platelet adhesion to immobilized vWF at 1500 s$^{-1}$. SP600125 treatment partially impaired platelet adhesion (37 ± 2%; $p < 0.001$) after 4 min of perfusion (Fig. 9B). In parallel, ReoPro® inhibited platelet adhesion by 60 ± 1% ($p < 0.001$). This was confirmed by using platelets from the GT patient where platelet adhesion was impaired by 58%. In both conditions (ReoPro® and GT) adhesion was not modified by the addition of SP600125 (55 ± 1 and 59%, respectively). Moreover, the addition of the ATP/ADP scavenger apyrase or indo- methacin did not affect platelet adhesion on vWF (Fig. 9C).

By contrast, the addition of prostaglandin E1, which inhibits platelet activation, impaired surface coverage by 56 ± 4%. To confirm the inhibitory effect of SP600125 on platelet adhesion to vWF at high (1500 s$^{-1}$) shear rate, the second JNK inhibitor, 6o, was assessed. In the presence of 6o (10 μM), platelet adhesion was inhibited but in a lesser potency than SP600125 (data not shown), confirming the involvement of JNK1. Our results show that αIIbβ3 activation, which is
required for firm platelet adhesion to vWF, is partially dependent on JNK1.

**JNK1 Is Involved in Arteriolar Thrombosis**—The role of JNK1 was investigated in a model of arteriolar and venular thrombosis in mice. The development of the thrombus, and vessel occlusion was monitored in real time under a microscope. Fig. 10 shows a chronological series of images of developing thrombosis in the caecum arterioles and venules of mice treated with Me2SO or SP600125. The mean venous occlusion time was not impaired in mice treated with SP600125 (36.7 ± 2.6 min) compared with controls treated with Me2SO (27.5 ± 3.5 min). By contrast, in arterioles, occlusion times were significantly longer in mice treated with SP600125, reaching 50.2 ± 2.0 min compared with 26.8 ± 3.9 min in controls (p < 0.001). Taken together, these results show that JNK1 is primarily involved in arteriolar thrombosis but not in venular thrombosis.

**FIGURE 8. Role of JNK1 in platelet adhesion to collagen matrix under blood flow conditions.** Blood was perfused at a high (1500 s⁻¹) shear rate in a parallel plate chamber over glass coverslips coated with 50 μg/ml of type I collagen. A shows the effect of SP600125 (10 μM) on adhesion of control platelets with ReoPro® (10 μg/ml) and platelets lacking vWF (GT patient). B represents the effect of 6F1 (20 μg/ml) and G19H10 (30 μg/ml) on the surface covered by adherent platelets. The data are expressed as the means ± S.E. of four independent experiments. Statistical significance of difference was analyzed by one-way ANOVA (**, p < 0.01).

**FIGURE 9. Role of JNK1 in firm platelet adhesion to immobilized vWF under blood flow conditions.** Reconstituted blood containing calcein-labeled platelets was perfused at a high (1500 s⁻¹) shear rate through vWF-coated microcapillaries. The effects of SP600125 (10 μM) and ReoPro® (10 μg/ml) on platelet velocity (A) were investigated after 1 min of perfusion. B shows the effect of SP600125 (10 μM) on platelet adhesion to immobilized vWF after 4 min of perfusion in control platelets with or without ReoPro® (10 μg/ml) and in platelets from the GT patient. C shows the effect of prostaglandin E₁ (PGE₁, 1 μM), apyrase (7.5 units/ml) and indomethacin (5 μM) on platelet adhesion. The covered surface and platelet velocity are quantified as described under “Experimental Procedures.” The data are expressed as the means ± S.E. of at least three independent experiments. Statistical significance of difference was analyzed by one-way ANOVA (**, p < 0.01; ***, p < 0.001).
Moreover, platelet rolling and firm platelet adhesion on the vWF matrix at a shear rate of 1500 s\(^{-1}\) strongly suggest that JNK1 acts on αIIbβ3 engagement after GP Ib-vWF interaction. Finally, in a model of arteriolar and venular thrombosis in mice, only the mean arterial occlusion time is significantly longer in mice treated with JNK1 inhibitor compared with control mice.

In recent years, a lot of progress has been made concerning the role of MAP kinases ERK2 and p38 in platelet aggregation and secretion induced by collagen (20, 23, 38). We found, with others, that ERK2 was involved in low concentration collagen-induced platelet aggregation, which was dependent on ADP (20) and/or ATP via the P2X1 ion channel, previously reported as contributing to the amplification of platelet granule release (38). Here, we have shown for the first time that JNK1 was activated in conditions of collagen-induced platelet aggregation. The level of phosphorylated JNK1 argues in favor of different pathways involved at low and high concentrations of collagen. We found that platelet aggregation induced by only low doses of collagen were dependent on the JNK1 pathway. In these conditions, identical inhibition of JNK1 phosphorylation was observed with the ADP/ATP scavenger apyrase and the antagonist of P2Y12 receptor of ADP. Surprisingly, ADP alone, in conditions of platelet aggregation, was not sufficient to induce full JNK1 activation (data not shown). This provides strong evidence that in conditions of collagen activation, JNK1 phosphorylation like ERK2 phosphorylation (20) requires the P2Y12 receptor of ADP and an additional pathway working in combination with ADP. The relationship between ERK and JNK remains to be established. Moreover, the link between ADP via its P2Y12/P2Y1 receptors and JNK1 is still unknown. Phosphatidylinositol 3-kinase is a potential candidate for the role of signaling intermediate. In proliferative cells, JNK activation has been reported to be dependent on phosphatidylinositol 3-kinase (39). If this is the case, different pathways are involved in ERK2 and JNK1 activation in platelets because inhibitors of phosphatidylinositol 3-kinase did not significantly affect collagen-induced ERK2 activation (results not shown). The Ras family GTP-binding protein Rap1b and/or Rap2b reported to require a G\(_i\)-dependent pathway in platelets (40–42) could be other candidates. Indeed, Rap2, sharing 60% homology with Rap1, was described as enhancing MAPK kinase kinase isofrom (MAP4K4)-induced activation of JNK (43). Further studies are required to answer to these questions.

We show here, for the first time, that in blood flow conditions on collagen, JNK1 is involved in thrombus formation but not in adhesion and is dependent on shear stress. In static adhesion or at a low shear rate (300 s\(^{-1}\)) involving collagen receptors only (GPVI and α2β1) and supporting small thrombi, JNK1 was not involved. In these conditions, small thrombi were independent of ADP (data not shown). The fact that JNK1 was involved in collagen-induced aggregation but not in small thrombi in blood flow could be explained by the involvement of ADP at the low concentration of collagen in conditions of platelet aggregation.

In contrast, at a high shear rate (1500 s\(^{-1}\)), the vWF was readily detected bound to the collagen matrix. In these conditions, vWF-GP Ib interaction that can secondarily activate α2β1 (44) was essential to form large thrombi, strongly suggesting that much of the effect of JNK1 on thrombus forma-
**JNK1 and Thrombus Formation**

...tion depended on vWF. These data are in agreement with the inhibitory effect of JNK inhibitor observed in SIPA and with previous reports showing a crucial role for GPIb-vWF interaction in platelet recruitment in arterial thrombus (45, 46). In contrast, platelet adhesion, observed in the presence of ReoPro® or with platelets from the GT patient, was dependent on collagen receptors but not GPIb-vWF interaction as previously described (6) and independent of JNK1.

How does JNK1 function in thrombus formation under blood flow conditions? The role of JNK1 in relation to GPIb-vWF-mediated platelet activation in thrombus formation is in accordance with previous work (47) showing that, at high shear rates, vWF is predominantly distributed at the outer areas of thrombi, whereas fibrinogen is present in the inner area. In contrast, at low shear rates, only fibrinogen appears in thrombi. Also, in blood flow (1500 s⁻¹) over a collagen matrix, platelet surface coverage and aggregate size were largely independent of ADP (15%) as previously described (48). This confirms that JNK1 is involved in collagen-induced formation of packed large thrombi under physiological conditions and supports the hypothesis that JNK1 acts via an independent ADP/ATP pathway but a dependent GPIb-vWF pathway that participates in thrombi formation.

Furthermore, platelet adhesion in blood flow conditions (1500 s⁻¹) on a vWF matrix was independent of ADP and thromboxane A2. In these conditions, JNK1, involved in the “inside-out” pathway, could directly activate αIIbβ3 required for firm adhesion. In this case, greater inhibition of adhesion observed with an anti-αIIbβ3 (ReoPro®) or with GT platelets suggests that JNK1 is partially involved in the activation of αIIbβ3. The other possibility is that JNK1, involved in the inside-out pathway, acts downstream of αIIbβ3 activation on integrin clustering or on interaction of αIIbβ3 with the cytoskeleton. Staining of the αIIbβ3 antibody PAC1, which recognized only the active form of integrin, did not provide an answer to this question (results not shown). The mechanism of αIIbβ3 activation by GPIb-IX-V is still controversial. Li et al. (17) suggested that αIIbβ3 activation is mediated through activation of cyclic guanosine monophosphate (cGMP)-dependent protein kinase, protein kinase G, and ERK2 pathway. In contrast, Marshall et al. (15) confirmed a crucial role for Src and showed an inhibitory effect of PKG. Src could be a candidate upstream of JNK1 because in proliferative cells Src has been reported to be involved in JNK1 activation (49, 50). Further studies are required to elucidate the signaling pathway of JNK1 activation. The fact that JNK1 participates in platelet rolling and adhesion in blood flow conditions (1500 s⁻¹) on vWF supports the hypothesis that JNK1 plays a major role in platelet adhesion to the arterial subendothelium, which was confirmed by in vivo studies.

Finally, in arterial and venular murine thrombosis, we found that JNK1 is required for arterial but not venular occlusion in relation to shear stress controlled signaling processes. The relatively smaller prolongation of occlusion times by the inhibitor of JNK in venules can be explained by a weaker effect of vWF in venular thrombosis than in arterial thrombosis. In fact, inhibition of GPIb-vWF interaction by the recombinant murine vWF-A1 domain increased the time of arterial thrombosis but not the time of venular thrombosis (26). Our results show for the first time that JNK1 contributes to thrombus formation and thrombosis in a shear-controlled manner coupled to the interaction of vWF-GPIb-mediated signaling pathway.

**Acknowledgments—**We are grateful to A. Bruel (Video microscopy Platform, IFR 105, Institut d’Hématologie, Université Paris VII Denis Diderot, Paris) for technical assistance with the video microscopy facilities and Y. Baudouin, V. Morin, C. Clément, and E. Sulpice for technical assistance.

**REFERENCES**

25. Siljander, P., Farndale, R. W., Feige, M. A., Comfurius, P., Kos, S., Bevers,

JNK1 and Thrombus Formation

NOVEMBER 2, 2007 • VOLUME 282 • NUMBER 44
JOURNAL OF BIOLOGICAL CHEMISTRY 31999

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Involvement of the Mitogen-activated Protein Kinase c-Jun NH$_2$-terminal Kinase 1 in Thrombus Formation

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