A role for p38 MAP kinase in platelet activation by von Willebrand Factor

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Summary

Platelet activation induced by von Willebrand factor (VWF) binding to the membrane GPIb-IX-V receptor involves multiple signal transduction pathways. Among these, recruitment and activation of the FcγRIIA and stimulation of phospholipase A_2 represent independent events equally essential to support a complete platelet response. Phospholipase A_2 is activated by calcium and by phosphorylation through MAP kinases. In this work, we found that VWF stimulated the rapid and sustained phosphorylation of p38 MAP kinase (p38MAPK). In vitro kinase assay revealed that VWF-stimulated phosphorylation of p38MAPK was associated with increased kinase activity. Binding of VWF to GPIb-IX-V, but not to integrin $\alpha_{IIb}\beta_3$, was required to support phosphorylation of p38MAPK. Neither the blockade of the membrane FcγRIIA by a specific monoclonal antibody or the prevention of thromboxane A_2 synthesis by cyclooxygenase inhibitors affected VWF-induced p38MAPK activation. However, phosphorylation of p38MAPK was prevented by the tyrosine kinase Syk inhibitor piceatannol. Treatment of platelets with the p38MAPK inhibitor SB203580 totally prevented VWF-stimulated platelet aggregation. Moreover, release of arachidonic acid induced by VWF was strongly impaired by inhibition of p38MAPK. We also found that VWF induced phosphorylation of cytosolic phospholipase A_2, and that this process was prevented by the p38MAPK inhibitor SB203580. These results demonstrate that p38MAPK is a key element in the FcγRIIA-independent pathway for VWF-induced platelet activation, and is involved in the stimulation of phospholipase A_2 and arachidonic acid release.

Keywords

p38 MAP kinase, von Willebrand Factor, tyrosine phosphorylation, phospholipase A_2, arachidonic acid

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Introduction

Mitogen-activated protein kinases (MAP kinases) are a family of serine-threonine kinases activated by a dual phosphorylation on threonine and tyrosine residues separated by a single amino acid (1). Three major groups of MAP kinases have been described: the extracellular signal-regulated kinases (ERKs), the c-Jun-NH_2-terminal kinases (JNKs), and the p38 MAP kinases (2). The ERKs, that include ERK1 and ERK2, are activated by a number of growth factors and mitogens, and are mainly involved in cell proliferation and differentiation (3). ERKs are phosphorylated by specific MAP kinase kinases (MEK1/2), that are in turn regulated by a Ras-Raf pathway (3-5). The JNKs (JNK1 and JNK2) are activated in response to different physical and chemical stresses and cytokines, and are implicated in apoptosis (6, 7). The p38 MAP kinases include

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several members (p38α, p38β, p38γ, and p38δ) activated by many stimuli, including hormones, stresses, and cytokines, and are involved in inflammation and apoptosis (8). A role for MEK3/6 and the small GTPases Rac and cdc42 in the activation of p38 MAP kinases has been documented (8, 9).

Different members of the MAP kinase family of proteins have been identified in human platelets, including ERK1, ERK2, p38α MAP kinase (or simply p38MAPK) and JNK1 (10-12). ERK2 is the predominant ERK in platelets, and is activated in response to thrombin, vasopressin and collagen, as well as upon clustering of the low affinity receptor for IgG, FcγRIIA (10, 13-16). Moreover, activation of ERK2 has been recently described in platelets activated by von Willebrand factor (VWF) (17). Activation of ERK2 has been shown to be down-regulated by integrin $\alpha_{IIb}\beta_3$ engagement during platelet aggregation (18). JNK1 seems to be regulated in human platelets in a manner very similar to ERK2, as it is activated in response to thrombin and down-regulated by integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregation (12). Platelet p38MAPK has been found to be phosphorylated in response to a number of stimuli, including thrombin, collagen, the thromboxane A_2 analogue U46619, and low density lipoproteins (11, 15, 19, 20). The role of ERKs in platelets is still unclear, since pharmacological inhibition of these kinases does not impair primary activation induced by extracellular agonists (21). However, it has been recently proposed that ERK2 may mediate integrin $\alpha_{IIb}\beta_3$ activation promoted by platelet interaction with VWF (17). By contrast, inhibition of p38MAPK by SB203580 has been reported to reduce platelet aggregation in response to low concentrations of collagen and U46619, but not thrombin (19). A possible target for p38MAPK in platelets is the cytosolic phospholipase A_2 ($cPLA_2$). In fact p38MAPK, but not ERK2, has been shown to phosphorylate $cPLA_2$ on Ser^{505}, an event associated with the activation of the enzyme (16, 22, 23). However, the physiological relevance of $cPLA_2$ phosphorylation by p38MAPK in intact platelets is still controversial. Prevention of $cPLA_2$ phosphorylation by inhibition of p38MAPK inhibits arachidonic acid release induced by low concentration of collagen, but not by thrombin (16, 22). However, since $cPLA_2$ can also be activated by calcium (24, 25), it is possible that an adequate increase of the intracellular concentration of this ion may compensate the prevention of Ser^{505} phosphorylation of $cPLA_2$ by p38MAPK.

Although p38MAPK has been widely investigated in thrombin- or collagen-stimulated platelets, very little is known about its possible involvement in platelet activation by VWF. VWF is a multimeric adhesive protein able to trigger platelet adhesion and activation associated with stimulation of phospholipase C (PLC) and PLA_2, as well as protein tyrosine kinases (26-29). The main platelet receptor for VWF, glycoprotein (GP) Ib-IX-V is physically associated with FcγRIIA (30, 31), and a recent work has demonstrated that this association occurs within the membrane lipid rafts (32). We have previously shown that FcγRIIA is actually activated through tyrosine phosphorylation upon VWF binding to GPIb-IX-V, and that this event initiates a signalling cascade that is required for PLC activation and cytoskeleton reorganisation (27, 33). However, we have also shown that binding of VWF to GPIb-IX-V leads to the FcγRIIA-independent activation of $cPLA_2$, and that the consequent production of TxA_2 is absolutely required to complement the FcγRIIA-mediated signalling pathway for platelet aggregation (32).

In order to further characterise details and players in the VWF-activated signalling pathways, we have examined in this study the role of MAP kinases. We have found that p38MAPK is activated upon platelet stimulation by VWF by a mechanism independent of FcγRIIA engagement. Moreover, we have provided evidence that p38MAPK may be involved in the phosphorylation and activation of $cPLA_2$ promoted by VWF.

Materials and methods

Materials

Sepharose CL-2B and protein A-Sepharose were from Amersham Pharmacia Biotech. Thrombin, ristocetin, acetylsalicylic acid, indomethacin, piceatannol and mouse IgG were from Sigma. von Willebrand Factor (Haemate P) was obtained from Behringwerke. Purified VWF was a gift from Dr. M. Berndt (Baker Medical Research Institute, Melbourne, Australia). Polyclonal antibodies against p38MAPK and $cPLA_2$ were from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody 4G10 was purchased from UBI. Polyclonal anti-phospho-p38MAPK (Thr180/Tyr182) and the p38MAPK assay kit were from Cell Signalling. The monoclonal antibody IV.3 against FcγRIIA was obtained from Medarex. The anti-glycoprotein Ib antibody AK2 was a gift of Dr. Patrizia Noris (IRCCS, Policlinico San Matteo, Pavia, Italy). Arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(N)] was from PerkinElmer Life Science.

Platelet preparation and stimulation

Blood from healthy volunteers was taken into tri-sodium citrate (3.2% w/v) for platelet-rich plasma (PRP) studies, and into citric acid/citrate/dextrose (ACD; 130 mM citric acid; 152 mM sodium citrate; 112 mM glucose) for gel-filtered platelet studies. In both cases, a ratio of anticoagulant to whole blood of 1:9 was used. PRP was isolated from whole blood by centrifugation at 200 × g for 10 mins, and directly used for aggregation studies. Alternatively, platelets were isolated from PRP by gel-filtration on Sepharose CL-2B and eluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4) as previously described (34). Platelet samples (0.1-0.4 ml, 10^9 platelets/ml) were incubated at 37°C under constant stirring, and then stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin or with 0.6 U/ml thrombin. In some experiments platelets were preincubated with 1 mM RGDS, 20 μg/ml IV.3 monoclo-

nal antibody or 20 μg/ml control IgG for 2 mins before the addition of the agonists. Preincubation with 5 or 10 μg/ml piceatannol, or with 2 or 10 μM SB203580 was performed for 10 mins, while preincubation with 1 mM acetylsalicylic acid or 10 μM indomethacin was for 30 mins. Except for the time course experiments, the reactions were blocked after 1 min.

Analysis of p38MAPK phosphorylation

Platelet stimulation was stopped by addition of 0.5 volumes of SDS-sample buffer 3X (37.5 mM Tris, 288 mM glycine, pH 8.3, 6% SDS, 1.5% DTT, 30% glycerol, 0.03% bromophenol blue), and samples were heated at 95°C for 5 mins. Platelet proteins were separated by SDS-PAGE on 12% acrylamide gels and transferred to nitrocellulose. Nitrocellulose membranes were blocked with 6% BSA in Tris buffer saline (20 mM Tris/HCl, pH 7.5, 0.2 M NaCl) for 3 hours at room temperature, and then incubated overnight at 4°C with anti-phospho-p38MAPK (1:1.000 dilution). Membranes were washed with 50 mM Tris/HCl, pH 7.4, 0.2 M NaCl, 1 mg/ml polyethylene glycol 20000, 1% BSA, 0.05% Tween 20, and incubated with peroxidase-conjugated secondary antibody (1:4000 dilution) for 45 mins. Reactive proteins were visualised with a chemiluminescence reaction. Nitrocellulose filters where then stripped by incubation with 62.5 mM Tris/HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol for 30 mins at 50°C, and reprobed with antibodies against p38MAPK (4 μg/ml). Phosphorylation of p38MAPK was also investigated by immunoprecipitation and immunoblotting. Samples of resting and stimulated platelets were lysed with an equal volume of ice-cold immunoprecipitation buffer 2X (20 mM Tris/HCl, pH 7.2, 300 mM NaCl, 2 mM EGTA, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM PMSF, 4 mM Na_3VO_4, 2 mM NaF, 2% Triton X-100, 0.2% SDS, 2% sodium deoxycholate). Insoluble material was removed by centrifugation, and lysates were precleared for 1 hour at 4°C with 90 μl of protein A-Sepharose (50 mg/ml stock solution). The cleared supernatants were incubated with 2 μg of anti-p38MAPK antibody for 2 hours at 4°C, and the immunocomplexes were precipitated by the addition of 90 μl of protein A-Sepharose for 45 mins. After brief centrifugation, immunoprecipitates were washed three times with 1 ml of immunoprecipitation buffer 1X, resuspended in 25 μl of SDS-sample buffer, and heated at 95°C for 5 mins. Precipitated proteins were separated by SDS-PAGE on 12% acrylamide gels and transferred to nitrocellulose. Tyrosine phosphorylation of p38MAPK was detected by immunoblotting with anti-phosphotyrosine antibody (1 μg/ml) followed by a second staining with antibodies against p38MAPK. All the reported figures are representative of at least three different experiments giving similar results.

Analysis of $cPLA_2$ phosphorylation

Platelet stimulation was stopped by addition of 0.5 volumes of SDS-sample buffer 3X, and samples were heated at 95°C for 5 mins. Platelet proteins were separated by SDS-PAGE on 7.5% acrylamide gels and transferred to nitrocellulose. Phosphorylation of $cPLA_2$ was evaluated based on the typical mobility shift on SDS-PAGE, revealed through immunoblotting with a specific antibody.

p38MAPK activity assay

p38MAPK activation was evaluated using a p38MAPK assay kit, exploiting the ability of activated kinase to phosphorylate the transcription factor ATF2. Briefly, resting or stimulated platelets were lysed by adding an equal volume of lysis buffer 2X (40 mM Tris/HCl pH 7.5, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2% Triton X-100, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM Na_3VO_4, 2 μg/ml leupeptin, 2 mM PMSF). p38MAPK was immunoprecipitated from cell lysates with 20 μl of immobilised anti-phospho-p38MAPK monoclonal antibody for 2 hours at 4°C. After brief centrifugation, immunocomplexes were washed twice with lysis buffer, and twice with kinase buffer 1X (25 mM Tris, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). Immunocomplexes were then resuspended in 50 μl of kinase buffer, and incubated for 10 min at 30°C in the presence of 50 μM ATP and 1 μg of the substrate ATF2. The reaction was stopped by adding 25 μl of SDS-sample buffer 3X, and ATF-2 phosphorylation was detected by immunoblotting using an anti-phospho-ATF2 antibody.

Release of [^{3}H]arachidonic acid

Gel-filtered platelets (0.5×10^9 platelets/ml) were labelled with [^{3}H]arachidonic acid (1 μCi/ml) for 90 mins at 37°C. Platelets were then recovered by centrifugation in the presence of 5 mM EDTA and resuspended in the original volume of HEPES buffer. Platelet samples (0.2 ml) were stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin or with 0.6 U/ml thrombin for the indicated times, and reaction was stopped by the addition of 0.8 ml of 10 mM EDTA and 10 μM indomethacin. After centrifugation at 10000 x g for 3 mins, the [^{3}H]arachidonic acid released in the supernatant was measured by liquid scintillation counting.

Results

Phosphorylation of p38MAPK in VWF-stimulated platelets

We investigated the ability of VWF to stimulate the phosphorylation of p38MAPK expressed in human platelets using a double experimental approach. Samples of gel-filtered platelets were stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin or with 0.6 U/ml thrombin at 37°C for 1 min under constant stirring. Tyrosine phosphorylation of p38MAPK was initially evaluated by immunoprecipitation of the protein and immunoblotting with anti-phosphotyrosine antibody. Figure 1A shows that

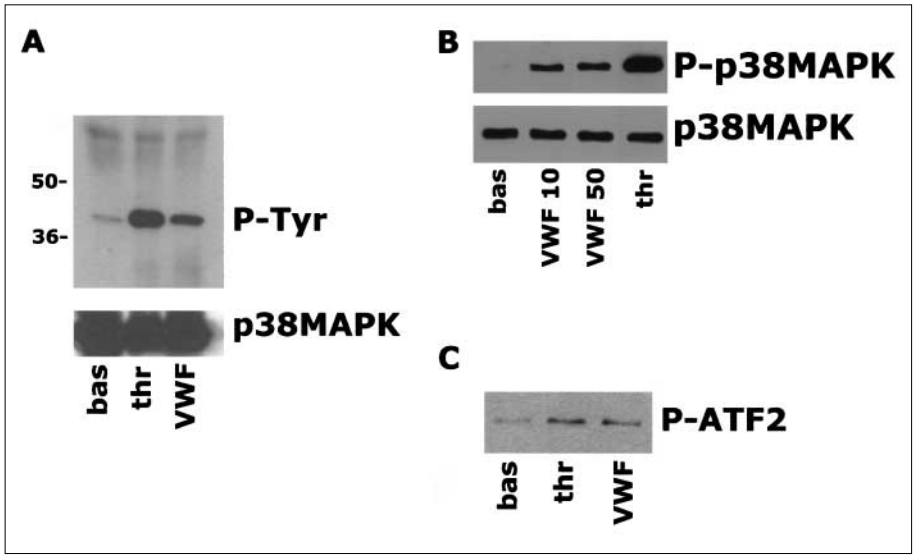

Figure 1: Phosphorylation of p38MAPK in VWF-stimulated platelets. Gel-filtered platelets were treated with buffer (bas) or stimulated with 10 μg/ml (or 50μg/ml, when indicated) VWF and 0.5 mg/ml ristocetin (VWF) or with 0.6 U/ml thrombin (thr) for 1 min at 37°C under constant stirring. (A) Reaction was stopped by lysis with immunoprecipitation buffer. p38MAPK was immunoprecipitated with a specific antibody, and analysed by immunoblotting with anti-phosphotyrosine antibody (P-Tyr). Blots was then reprobed with the same anti-p38MAPK antibody used for immunoprecipitation (p38MAPK), as indicated on the right. (B) Reaction was stopped by addition of 0.5 volumes of SDS-sample buffer 3X, and 25 μl aliquots of total platelet lysates were subjected to SDS-PAGE on 12% acrylamide gels. Proteins were transferred to nitrocellulose and probed with an antibody specific for phosphorylated form of p38MAPK (P-p38MAPK) and subsequently with an anti-p38MAPK antibody. (C) p38MAPK kinase activity was measured on the immunoprecipitated protein using a kinase assay kit, exploiting the ability of the active kinase to phosphorylate the transcription factor ATF2. The reaction products were separated by SDS-PAGE on 12% acrylamide gel, and analysed by immunoblotting with an antibody specific for the phosphorylated form of ATF2 (P-ATF2).

p38MAPK was only weakly tyrosine phosphorylated when immunoprecipitated from resting platelets. Stimulation of platelets with either thrombin or VWF significantly increased the reactivity of immunoprecipitated p38MAPK to anti-phosphotyrosine antibody. VWF-induced tyrosine phosphorylation of p38MAPK was weaker than that induced by thrombin (about 30%, as evaluated by densitometric analysis). By reprobing the same nitrocellulose filter with the anti-p38MAPK antibody used for the immunoprecipitation, we confirmed that similar amount of protein was immunoprecipitated from each sample.

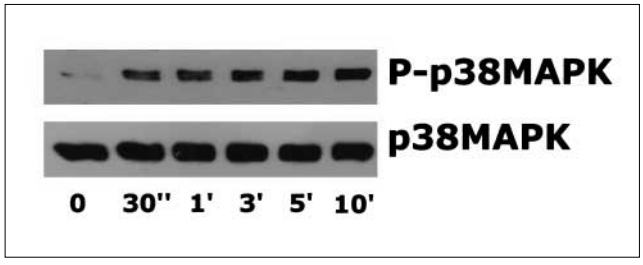

Figure 2: Time course of VWF-induced p38MAPK phosphorylation. Platelet samples were stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin (VWF) for the indicated times, where 0 represents unstimulated platelets. Upon platelet lysis, phosphorylated p38MAPK was detected by immunoblotting with a phospho-specific antibody (P-p38MAPK). Control for equal loading was performed by reprobing the same nitrocellulose with an anti-p38MAPK antibody, as indicated on the right.

Similar results were also obtained when whole platelet lysates were directly immunoblotted with an antibody recognising the phosphorylated form of p38MAPK (Thr180/Tyr182). Figure 1B shows that even this approach was able to detect a VWF-induced phosphorylation of p38MAPK, and confirms that this event was weaker to that detected in thrombin-stimulated platelets. Moreover, Figure 1B also shows that increasing the concentration of VWF to 50 μg/ml did not result in a further increase in the level of phosphorylation of p38MAPK, indicating that maximal response was already triggered by 10 μg/ml VWF. Similar results were obtained using a highly purified VWF from a different source, thus excluding a contribution of possible contaminating molecules (data not shown).

We next analysed whether VWF-induced phosphorylation of p38MAPK was associated with activation of the enzyme. Using a non radioactive kinase assay kit we found that the catalytic activity of p38MAPK, measured as the ability to promote the phosphorylation of the transcription factor ATF2, was increased in both thrombin- and VWF-stimulated platelets (Fig. 1C). This indicates that VWF actually induces both phosphorylation and activation of p38MAPK.

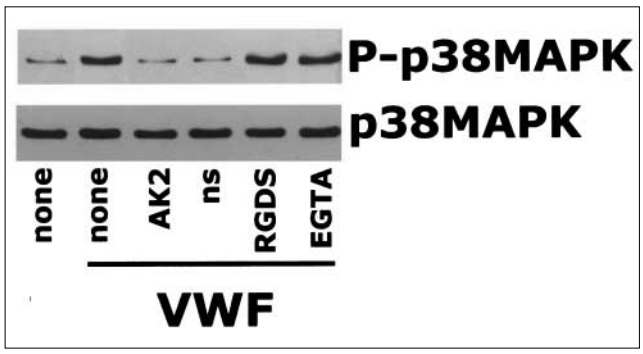

Figure 3: Role of GPIb-IX-V and integrin $\alpha_{IIb}\beta_3$ on VWF-induced phosphorylation of p38MAPK. Platelet samples were incubated with buffer (none), or stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin (VWF) for 1 min under constant stirring in the absence (none) or presence of 1 μl of AK2 ascitic fluid, 1 mM RGDS, or 1 mM EGTA. Alternatively, stimulation with VWF was performed without stirring (ns). Phosphorylation of p38MAPK was evaluated by immunoblotting analysis with anti-phospho-p38MAPK, and anti-p38MAPK antibodies, as indicated on the right.

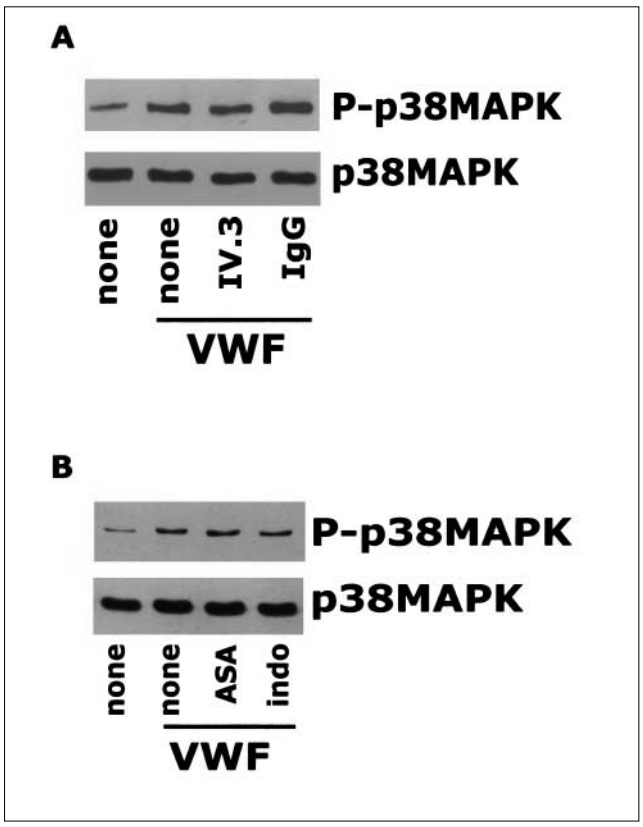

Figure 4: Phosphorylation of p38MAPK is independent of FcγRIIA activation and TxA_2 production. (A) Platelet samples were incubated with buffer (none), with 20 μg/ml IV.3 (IV.3) or 20 μg/ml unrelated IgG (IgG) for 2 mins and then stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin for 1 min (VWF). Phosphorylation of p38MAPK was analysed by immunoblotting with an antibody recognising the phosphorylated form of p38MAPK, and an antibody recognising the total p38MAPK, as indicated on the right. (B) Platelet samples were incubated with buffer (none), with 1 mM acetylsalicylic acid (ASA) or with 10 μM indomethacin (indo) for 30 mins and stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin for 1 min (VWF). Phosphorylation of p38MAPK was analysed by immunoblotting with anti-phospho-p38MAPK, and anti-p38MAPK antibodies.

Phosphorylation of p38MAPK in VWF-stimulated platelets requires agonist binding to GPIb-IX-V, is independent of both FcγRIIA and TxA_2, and involves the tyrosine kinase Syk

The kinetics of VWF-induced phosphorylation of p38MAPK was investigated in platelets stimulated with VWF for increasing times. Figure 2 shows that phosphorylation of p38MAPK was very rapid and sustained, as it was clearly detected as early as 30 seconds after platelet stimulation. This indicates that this event occurs in an early phase of VWF-induced platelet activation.

We next investigated the role of GPIb-IX-V, integrin $\alpha_{IIb}\beta_3$, and platelet agglutination on VWF-induced phosphorylation of p38MAPK. Figure 3 shows that preincubation of platelets with AK2, a monoclonal antibody against the membrane glycoprotein GPIbα subunit, totally prevented phosphorylation of p38MAPK induced by VWF. Moreover, the omission of platelet stirring, that allows VWF binding but prevents the cell-cell contact leading to platelet agglutination, also blocked phosphorylation of p38MAPK. By contrast, phosphorylation of p38MAPK was independent of ligand binding to integrin $\alpha_{IIb}\beta_3$, as it was not altered in the presence of the RGDS peptide or EGTA (Fig. 3).

We have previously demonstrated that VWF binding to GPIb-IX-V activates platelets through the coordinated action of FcγRIIA and TxA_2 (33). To investigate the role of FcγRIIA on p38MAPK phosphorylation, platelets were preincubated with the anti-FcγRIIA antibody IV.3, or control IgG, and then stimulated with VWF. Interestingly, the blockade of FcγRIIA had no effect on phosphorylation of p38MAPK (Fig. 4A). Therefore, VWF-induced p38MAPK phosphorylation occurs independently of FcγRIIA engagement. To evaluate the role of TxA_2, platelets were preincubated with two different cyclooxygenase inhibitors, acetylsalicylic acid and indomethacin. Figure 4B shows that neither inhibitors affected VWF-induced phosphorylation of p38MAPK, demonstrating that this event is also independent of TxA_2 production. Identical results were also obtained by analysing the tyrosine phosphorylation of immunoprecipitated p38MAPK (data not shown).

VWF binding to its receptor induces the activation of the tyrosine kinase Syk, by a mechanism that is only partially dependent on FcγRIIA recruitment (29, 33). We found that the kinetics of Syk activation in VWF-stimulated platelets was very similar to that of p38MAPK phosphorylation, being rapid and sustained (data not shown). Therefore, we investigated whether

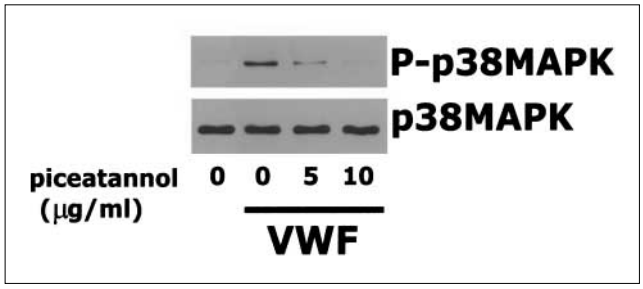

Figure 5: p38MAPK phosphorylation lies downstream Syk activation. Platelet samples were treated with 5 or 10 μg/ml piceatannol (corresponding to about 20 and 40 μM, respectively) or with an equivalent volume of dimethylsulfoxide, and stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin for 1 min (VWF). Phosphorylation of p38MAPK was analysed by immunoblotting with anti-phospho-p38MAPK, and anti-p38MAPK antibodies.

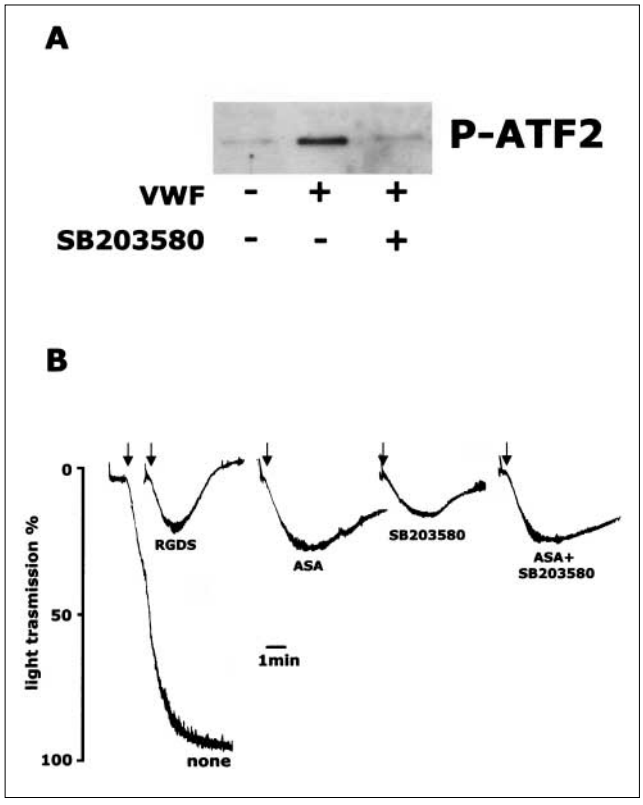

Figure 6: Effect of p38MAPK inhibition on ristocetin-induced platelet aggregation. (A) Gel-filtered platelets were incubated with 10 μM SB203580 for 10 mins and then stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin (VWF). p38MAPK activity was measured on the immunoprecipitated protein, by evaluating the phosphorylation of the substrate ATF2 revealed by immunoblotting with a specific anti-phospho-ATF2 antibody. (B) Platelet aggregation was measured using PRP treated with 0.75 mg/ml ristocetin (added as indicated by the arrows), in the absence of inhibitors (none), in the presence of 1 mM RGDS, upon incubation with 10 μM SB203580 for 10 mins, upon incubation with 1 mM acetylsalicylic acid (ASA) for 30 mins, or upon incubation with both acetylsalicylic acid and SB203580, as indicated.

phosphorylation of p38MAPK could be regulated by Syk. Gel-filtered platelets were preincubated with 5 μg/ml or 10 μg/ml piceatannol, a selective Syk inhibitor, and then stimulated with VWF. Figure 5 shows that piceatannol completely blocked phosphorylation of p38MAPK induced by VWF.

Role of p38MAPK in VWF-induced platelet activation

We next investigated the physiological role of p38MAPK in VWF-induced platelet activation by analysing the effects of a widely used selective inhibitor of p38MAPK, SB203580. Using a p38MAPK assay kit, we found that preincubation of platelets with 10 μM SB203580 for 10 mins completely inhibited the kinase activity of p38MAPK, as demonstrated by the lack of phosphorylation of the transcription factor ATF2 (Fig. 6A). Incidentally, these results also indicate that, at the concentration used, SB203580 does not dissociate from the enzyme during the washing steps of immunoprecipitated p38MAPK. The effect of SB203580 on VWF-induced platelet aggregation was then analysed using platelets in PRP. Addition of ristocetin to PRP caused a biphasic platelet aggregation (Fig. 6B). The first phase was probably due to platelet agglutination by the multimeric nature of plasma VWF, while the second phase was a real integrin $\alpha_{IIb}\beta_3$-dependent aggregation, as demonstrated by the finding that it was prevented by the integrin $\alpha_{IIb}\beta_3$ antagonist peptide RGDS (Fig. 6B). We found that the integrin-dependent platelet aggregation was totally prevented by SB203580, and that a very similar effect was also observed in aspirin-treated platelets (Fig. 6B). Moreover, the combined addition of aspirin and SB203580 to platelets did not result in a greater inhibitory effect.

To better clarify the relationship between p38MAPK and TxA_2 production in VWF-stimulated platelets, we analysed the effect of SB203580 on arachidonic acid release from [^{3}H]arachidonic acid labelled platelets. Figure 7A shows the time course of arachidonic acid release in VWF- and thrombin-stimulated platelets. VWF was able to promote a significant release of arachidonic acid, although less efficiently than thrombin. The maximal amount of arachidonic acid released in VWF-stimulated platelets accounted for about 4% of the total incorporated arachidonic acid, while the amount released by thrombin was about three times higher. This is in agreement with previous finding showing that a relatively small amount of TxA_2 is produced upon platelet stimulation with VWF (26, 33). However, figure 7B shows that release of arachidonic acid induced by VWF was strongly and significantly inhibited by preincubation of platelets with either 2 μM or 10 μM p38MAPK inhibitor SB203580 ($p<0.001$). By contrast, a less remarkable effect was observed in thrombin-stimulated platelets. The inhibitory effect of SB203580 on VWF-induced release of arachidonic acid was not due to the reported ability of this compound to inhibit cyclooxygenase. In fact, figure 7B also shows that aspirin only mini-

mally affected arachidonic acid release in VWF-stimulated platelets. Moreover, preincubation of aspirin-treated platelets with 2 μM SB203580 still resulted in a strong inhibition of arachidonic acid release comparable to that observed in control cells.

Arachidonic acid is released by the action of $cPLA_2$, and a role for p38MAPK in agonist-induced phosphorylation and activation of $cPLA_2$ has been proposed (16, 22). Therefore, we investigated the phosphorylation of $cPLA_2$ in VWF-stimulated platelets. Figure 7C shows that treatment of platelets with VWF resulted in a typical band shift of $cPLA_2$, revealed by immunoblotting with a specific anti-$cPLA_2$ antibody and supposed to be related to the phosphorylation of the enzyme. Pretreatment of platelets with 10 μM SB203580 totally prevented $cPLA_2$ phosphorylation and suppressed the VWF-induced shift in electrophoretic mobility. Moreover, figure 7C also shows that VWF-induced band shift of $cPLA_2$ still occurred in aspirin-treated platelets, and that it was still prevented by SB203580 even at 2 μM. This indicates that the observed effect of SB203580 was not due to the high concentration of the inhibitor or to its ability to inhibit cyclooxygenase These results suggest that p38MAPK may be responsible for the phosphorylation of $cPLA_2$ promoted by VWF.

Discussion

We have previously shown that platelet activation by VWF requires the concomitant activation of two independent signalling pathways, involving the recruitment of FcγRIIA, that leads to the Syk-dependent phosphorylation of PLCγ2, and the activation of PLA_2, that leads to the production of TxA_2, respectively (33). In this work we have identified p38MAPK as an important element in the FcγRIIA-independent, but TxA_2-dependent pathway for platelet activation induced by VWF, and we have provided evidence that p38MAPK activation lies upstream TxA_2 production and may regulate $cPLA_2$.

By using a double experimental approach, we found that p38MAPK is actually phosphorylated upon platelet stimulation by VWF. Phosphorylation of p38MAPK was an early event upon VWF binding to GPIb-IX-V, being already evident 30 seconds after agonist addition. Moreover, as expected, phosphorylation of p38MAPK was associated with an increase of its enzymatic activity as detected by an "in vitro" kinase assay using the transcription factor ATF2 as exogenous substrate. Previous works have reported phosphorylation and activation of p38MAPK in thrombin or collagen stimulated platelets (11, 15, 19). Moreover, p38MAPK activation has been shown to be also triggered by LDL binding to platelets (20). p38MAPK phosphorylation induced by thrombin was also confirmed in our study, and found to be stronger than that promoted by VWF.

We have also characterised the modality of p38MAPK activation induced by VWF. We have found that phosphorylation of

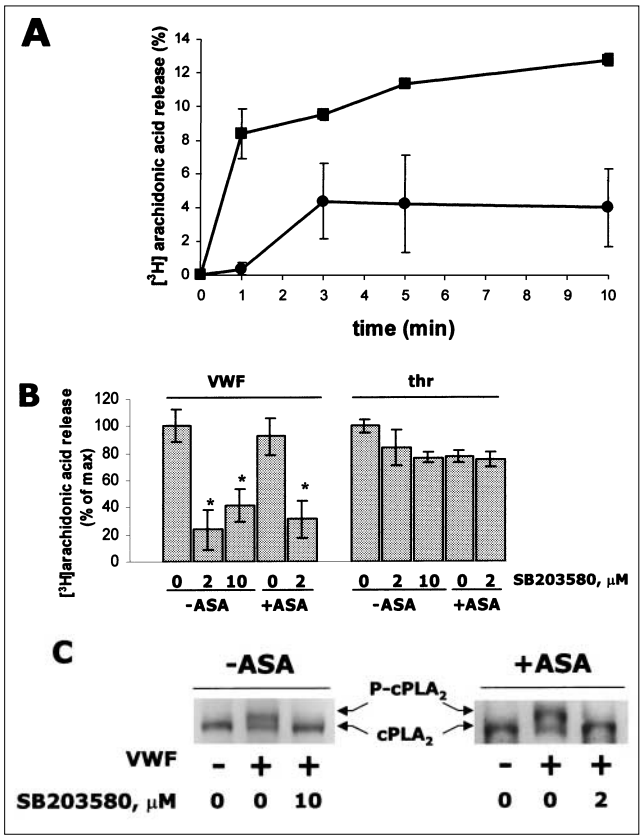

Figure 7: Role of p38MAPK on VWF-induced arachidonic acid release and phosphorylation of $cPLA_2$. (A) Time course of arachidonic acid release in thrombin- and VWF-stimulated platelets. [^{3}H]arachidonic acid labelled-platelets were stimulated with 0.6 U/ml thrombin (black squares) or with 10 μg/ml VWF and 0.5 mg/ml ristocetin (black circles) for increasing times. Upon centrifugation, [^{3}H]arachidonic acid released in the supernatant was measured by liquid scintillation counting. The data represent the percentage of total [^{3}H]arachidonic acid incorporated, after subtraction of the radioactivity measured in the supernatant from unstimulated platelets, and are the mean ± SD of three different experiments. Typical measured radioactivity in the supernatant from resting platelets was 808 ± 92 cpm (n = 6), representing 1.62 ± 0.18% of total [^{3}H]arachidonic acid incorporated. (B) Effect of SB203580 on arachidonic acid release. [^{3}H]arachidonic acid labelled-platelets were preincubated at 37 °C in the absence or presence of 1 mM acetylsalicylic acid (ASA) for 30 mins, and then treated with vehicle or with SB203580 (2 μM or 10 μM, as indicated) for 10 mins. Stimulation was performed with 0.6 U/ml thrombin (thr) or with 10 μg/ml VWF and 0.5 mg/ml ristocetin (VWF) for 5 mins. Released [^{3}H]arachidonic acid in the supernatant was evaluated by liquid scintillation counting. Data are presented as % of maximal arachidonic acid released by each agonist in the absence of p38MAPK inhibitor, and represent the mean ± SD of four different experiments; * $p<0.001$ (C) Phosphorylation of $cPLA_2$ in VWF-stimulated platelets. Gel-filtered platelets and aspirin-treated gel-filtered platelets were incubated with vehicle or with 2 μM or 10 μM SB203580 for 10 mins, and then stimulated with 10 μg/ml VWF and 0.5 mg/ml ristocetin (VWF). Aliquots of total platelet proteins were separated by SDS-PAGE on 7.5% acrylamide gel and analysed by immunoblotting with an anti-$cPLA_2$ antibody. Phosphorylation of $cPLA_2$ is evidenced by a typical shift in the electrophoretic mobility of the protein.

p38MAPK was dependent on VWF binding to GPIb-IX-V, but not to integrin $\alpha_{IIb}\beta_3$. Moreover, although many intracellular events elicited by VWF have been shown to be directly triggered by the recruitment of FcγRIIA, we found that VWF-induced phosphorylation of p38MAPK was not altered in the presence of the FcγRIIA blocking antibody IV.3. This indicates that phosphorylation of p38MAPK is part of the previously identified FcγRIIA-independent pathway for VWF-induced platelet activation, that also includes the production of TxA_2 and the activation of the tyrosine kinase Pyk2 (33, 35). We have also found that VWF-induced phosphorylation of p38MAPK is prevented by the Syk inhibitor piceatannol, suggesting that Syk lies upstream p38MAPK. Since the specificity of piceatannol has been recently questioned (36), the involvement of other kinases in the phosphorylation of p38MAPK cannot be confidently excluded. However, we have previously shown some selective effects of piceatannol on platelet activation by different agonists, and the crucial role of Syk in the early phase of platelet activation by VWF has been well documented (29, 33). We have previously shown that activation of Syk is partially mediated by the recruitment of FcγRIIA (33). The possibility that the FcγRIIA-independent phosphorylation of p38MAPK is mediated by Syk indicates that the pool of Syk activated by a FcγRIIA-independent mechanism is sufficient to fully support p38MAPK phosphorylation. In this regards, it is interesting to note that a role of a different ITAM containing receptor, FcR γ-chain in GPIb-IX-V-triggered activation of Syk has been reported (37). Although several mediators, such as MAPKK 3 and 6, and the small GTPases Rac and cdc42, have been implicated in p38MAPK activation in nucleated cells, the mechanism regulating this kinase in platelets has been poorly investigated. The possible role of Syk suggested in the present study is most likely to be indirect, and to be mediated by other effectors. Previous findings have implicated the tyrosine kinase Pyk2 in p38MAPK activation in a number of nucleated cells (38, 39). We have recently shown that Pyk2 is rapidly activated in VWF-stimulated platelets by a FcγRIIA-independent mechanism that involves the kinase Syk (34). Therefore, it is possible that Pyk2 represents a link between Syk and p38MAPK activation. This hypothesis, however, awaits specific inhibitors of Pyk2 to be experimentally verified.

A key element of the FcγRIIA-independent pathway for VWF-induced platelet activation is TxA_2. Since inhibition of cyclooxygenase does not affect VWF-induced activation of p38MAPK, it is reasonable to conclude that p38MAPK lies upstream TxA_2 production. This would be in agreement with previously reported evidence that p38MAPK can mediate phosphorylation and activation of $cPLA_2$ in platelets stimulated with thrombin or collagen (16, 22). To investigate the role of p38MAPK in platelet activation by VWF we exploited the widely used cell-permeable inhibitor SB203580. We found that SB203580 was able to abolish the integrin $\alpha_{IIb}\beta_3$-dependent platelet aggregation induced by VWF. A very similar effect was also elicited by inhibition of cyclooxygenase. This is consistent with the hypothesis that p38MAPK and TxA_2 are actually consequential elements within the same signalling pathway. Since SB203580 has been reported to also inhibit cyclooxygenases and thromboxane synthase (40), the correlation between p38MAPK and TxA_2 was analysed at the level of PLA_2 activation and arachidonic acid release in control and aspirin-treated platelets. We have found that, in VWF-stimulated platelets, the 85 kDa $cPLA_2$ underwent a shift in the electrophoretic mobility typically associated with phosphorylation of the enzyme on Ser^{505}. The prevention of VWF-induced shift in the electrophoretic mobility of $cPLA_2$ by SB203580, under conditions in which the kinase activity of p38MAPK is totally suppressed, indicates that p38MAPK mediates phosphorylation of $cPLA_2$. Previous studies with platelets stimulated with thrombin or collagen suggested that the contribution of proline-directed phosphorylation on activation of $cPLA_2$ may not always be relevant, especially under conditions in which the increase of the intracellular concentration of calcium is sufficient to compensate the lack of $cPLA_2$ phosphorylation (16, 22, 24, 25). However, by measuring arachidonic acid release from [^{3}H]arachidonic acid-labelled platelets, we have demonstrated that phosphorylation by p38MAPK significantly contributes to $cPLA_2$ activation by VWF. In fact the agonist-induced release of arachidonic acid was decreased by about 70%. By contrast, the impact of inhibition of p38MAPK on arachidonic acid release induced by thrombin was more limited, as expected.

In conclusion, this work provides new information to more precisely delineate the FcγRIIA-independent mechanism of platelet activation induced by VWF, and reveals a central role for activated p38MAPK in the phosphorylation and activation of $cPLA_2$ and release of arachidonic acid.

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