Restrained glycoprotein VI-induced platelet signaling by tyrosine protein phosphatases independent of phospholipase Cγ2

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ABSTRACT

The platelet collagen receptor glycoprotein VI (GPVI) signals to activation of phospholipase Cγ2 (PLCγ2) and phosphoinositide 3-kinases (PI3K), causing platelet activation and aggregation. The non-receptor Src homology tyrosine phosphatases Shp1/2 modulate GPVI signaling in partly opposite ways, both of which are targeted by the potential drug NSC87877. Effect measurements of the Shp1/2 inhibitor NSC87877 on platelet activation via GPVI using light transmission aggregometry, Ca2+ flux assay, western blotting and flow cytometry. Effect measurements of selective PI3K inhibitor TGX221. Inhibition of Shp1/2 with NSC87877 enhanced platelet aggregation induced by the GPVI agonist, collagen-related peptide (CRP). Furthermore, NSC87877 antagonized the effects of PI3Kb inhibition, but not of Btk inhibition. Both NSC87877 and TGX221 suppressed the CRP-induced phosphorylation of PLCγ2 at activation site Tyr759. These findings indicate that drug interference of the two phosphatases Shp1/2 subtly enhances GPVI-induced platelet responses via a mechanism not involving PLCγ2 activation, even upon PI3K inhibition.

Introduction

Platelet stimulation by the collagen receptor, glycoprotein VI (GPVI), induces granule secretion and platelet aggregation through a tyrosine kinase signaling cascade, involving Src-family kinases (SKK), Syk and Btk kinases. Commonly, two GPVI-induced signaling pathways are distinguished, one via phospholipase Cγ2 (PLCγ2) triggering a rise in cytosolic [Ca2+]i, and one via phosphoinositide 3-kinases (PI3K) that is more directly linked to integrin αIIbβ3 activation. Collagen and collagen-related peptide (CRP) are bona fide ligands of GPVI, as platelets from patients with a GPVI deficiency are greatly impaired in activation and aggregation with these agonists.

Platelet GPVI signals with its co-receptor FcR g-chain, containing an immunoreceptor tyrosine-based activation motif (ITAM). In platelets and other blood cells, the ITAM-linked receptors are modified in signaling action by tyrosine phosphatases containing an immunoreceptor tyrosine-based inhibition motifs. Two such phosphatases are Shp1 and Shp2 (short for SH2 domain-containing protein tyrosine phosphatases), both of which dephosphorylate signaling proteins upon GPVI activation.

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In spite of their structural similarities, the roles of Shp1 and Shp2 are known to be partly different. In mouse platelets, the isoform Shp2 suppresses GPVI- and integrin αIIbβ3-induced responses. On the other hand, mouse platelet Shp1 can act as a
positive regulator of GPVI-induced signaling. Both isoforms are inactivated by the compound NSC87877, which binds to the catalytic cleft of either phosphatase. This compound is of current interest for tumor-targeted therapies. When applied as such a drug, NSC87877 will likely affect platelet functions, although to which extent and in which way is unclear.

In the present study with human platelets, we studied the effects of NSC87877 on signaling and activation responses induced by the GPVI agonist CRP. This allowed us to check for off-target effects of the drug, and simultaneously to define the combined roles of both tyrosine phosphatases Shp1 and Shp2.

Materials and Methods

Materials

Cross-linked CRP was purchased from CambCol Laboratories (Cambridge, UK). NSC87877 came from Cayman Chemical (Ann Arbor, MI, USA). TGX221 was from sources described before, while ibrutinib was from Toronto Research Chemicals (North York, Canada). Used as fluorescent stains were Alexa Fluor (AF) 647-conjugated anti-human CD62P mAb (Biolegend, London, UK) and FITC-PAC1 mAb (BD Bioscience, Franklin Lakes, NJ, USA). Other materials for functional assays and for Western blotting are as described elsewhere.

Preparation of blood and platelets

Blood was drawn into 3.2% trisodium citrate from healthy volunteers through venipuncture. Donors had not received anti-platelet medication for at least two weeks and gave full informed consent according to the declaration of Helsinki. Studies were approved by the local Medical Ethics Committees.

Platelet-rich plasma (PRP) and washed platelet preparations were isolated following an established procedure to remove other blood cells. In brief, PRP was obtained by centrifugation at 240 g for 15 min. After the addition of 10 vol% acid citrate dextrose (ACD) (80 mM trisodium citrate, 183 mM glucose, 52 mM citric acid), platelets were isolated by centrifugation at 5,500 g for 2 min. The pelleted platelets were resuspended into Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5.5 mM glucose, and 0.1% bovine serum albumin). After the addition of apyrase (1 U/mL), the cells were resuspended into Hepes buffer pH 7.4. Measurements of platelet aggregation were performed using a Chronolog aggregometer (Havertown, PA, USA). Platelet samples were stimulated with CRP (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 5.5 mM glucose, and 0.1% bovine serum albumin). After the addition of apyrase (1 U/mL), the cells were resuspended into Hepes-Tyrode’s buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM Hepes, 5 mM glucose, and 1 mM MgCl₂, pH 7.3) at requested platelet count.

Light transmission aggregometry

Aggregation of washed platelets (250×10⁶/L) was measured at 37°C under stirring with a Chronolog aggregometer (Havertown, PA, USA). No fibrinogen was added, implicating that the aggregation response relied on fibrinogen secretion. Samples of 300 µL were pre-incubated with either dimethylsulfoxide (DMSO), NSC87877 (10-20 µM) and/or TGX221 (1-3 µM) for 5 min at room temperature, followed by a temperature adjustment to 37°C.

Cytosolic Ca²⁺ measurements

Washed platelets (200×10⁶/L) were loaded with a mixture of Fura-2 acetoxyethyl ester (3 µM) and pluronic (0.4 µg/mL) for 40 min at room temperature. After centrifugation in the presence of 1:10 ACD and apyrase (1 U/mL), the dye-loaded platelets were resuspended at the same concentration into Hepes buffer pH 7.4. Samples of 200 µL in 96-wells plate were pre-incubated with DMSO vehicle or inhibitor for 10 min, after which 1 mM CaCl₂ was added, and the temperature was adapted to 37°C. Agonist-induced ratiometric changes in fluorescence were measured per well with a FlexStation 3 (Molecular Devices, San Jose, CA, USA). Calibrated changes in cytosolic [Ca²⁺] were calculated from ratiometric values. Measurements were performed in triplicate wells.

Western blotting

For western blotting according to established procedures, washed platelets (800×10⁶/L) were preincubated with DMSO vehicle, TGX221 and/or NSC87877. Also added was 9 µM integrin to prevent aggregation. Platelet samples (300 µL) were stimulated with 0.5 µg/mL CRP for different times, after which reactions were stopped by addition of 300 µL 2× lysis buffer. The processing of lysed samples by reduction, boiling, and loading onto PAGE 4-12% Bis-Tris gels was as described elsewhere.

Statistics and data processing

Statistical analysis was performed with GraphPad Prism 8 software (San Diego, CA, USA). A paired Student t-test was used with significance set at p<0.05.

Results

Shp1/2 inhibition by NSC87877 enhances glycoprotein VI-induced platelet aggregation

We first established how inhibition of Shp1/2 by NSC87877 affected GPVI-induced platelet aggregation, as a standard
platelet function assay. The compound was used at a concentrations of 10 μM, which was previously shown to block the catalytic activity of both Shp1 and Shp2. In response to sub-maximal doses of CRP (0.2-1.0 μg/mL), we found a significant enhancing effect of NSC87877 on the aggregation response, when comparison to the vehicle control condition (Figure 1A and B). This agreed with the GPVI-restraining role of Shp2, previously observed in mouse platelets. At the highest CRP dose of 5.0 μg/mL, the aggregation was already maximal, precluding an effect of NSC87877 (Figure 1B).

For assessment of the contribution of GPVI-induced PI3K activation, we used the PI3K inhibitor, TGX221, which was previously shown to nearly completely block GPVI-induced thrombus formation. Initial experiments using 1 or 3 μM TGX221 indicated a consistent, donor-independent aggregation response at 3 μM (data not shown), which concentration was then chosen. In TGX221-treated platelets, the CRP-induced aggregation was reduced by ~50% (Figure 1C). Importantly, the reduction was rescued by the simultaneous treatment with 10 μM NSC87877 (Figure 1D). Together, these findings suggest that NSC87877, likely by antagonizing the negative regulation by Shp2, enhances the CRP/GPVI-induced signaling bypassing PI3K activation.

**Figure 1.** Enhanced collagen-related peptide-induced platelet aggregation by inhibition of Shp1/2. (A-B) Washed platelets (250×10⁹/L) were incubated with vehicle (Veh.) or Shp1/2 inhibitor NSC87877 (NSC, 10 μM) for 10 min. Platelet aggregation was monitored in response to indicated doses of collagen-related peptide (CRP, 0.2-5 μg/mL). A) Representative light transmission (%T) changes (axes 480 s, 100%T) of platelet aggregation induced by 0.5 μg/mL CRP. B) Effect of NSC87877 on maximal aggregation induced by CRP (0.2-5 μg/mL); C-D) Platelets (250×10⁹/L) were incubated with vehicle control (Veh.), NSC87877 (NSC, 20 μM), TGX221 (TGX, 3 μM) or a combination for 10 min; C) Representative light transmission changes (axes 480 s, 100%T) on maximal aggregation induced by 0.5 μg/mL CRP; D) Effect of NSC87877 and TGX221 on maximal aggregation, expressed as % of vehicle control. Mean values ± SD (n=3 donors); *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001 vs. vehicle (paired Student’s t-test).

Shp1/2 inhibition by NSC87877 reduces glycoprotein VI-induced platelet PLCγ2 tyrosine phosphorylation and Ca²⁺ responses

Given the ability of the Shp1/2 isoforms to alter the GPVI-induced tyrosine phosphorylation of signalosome components Syk, Src and PLCγ2, we assessed the phosphorylation of critical tyrosine residues in these proteins. Western blot analysis of platelets stimulated with sub-maximal CRP (0.5 μg/mL), using the broad anti-phospho-tyrosine mAb 4G10, indicated a high near-optimal phosphorylation of most protein bands after 45 s and 90 s of stimulation (Figure 2A). Using selective phospho-tyrosine mAbs, we confirmed this for phospho-Syk Tyr525-526, phospho-Src Tyr419 and phospho-PLCγ2 Tyr759 (Figure 2B). Quantitative blot analysis of 90-s samples indicated that preincubation with NSC87877 did not influence the three tyrosine phosphorylation sites (Figure 2C-E). By itself, TGX221 slightly reduced only the Tyr759 phosphorylation of PLCγ2. Strikingly, the combination of NSC87877 and TGX221 caused a significant reduction in phospho-Tyr419 of Src and in phospho-Tyr759 of PLCγ2, when compared to NSC87877 alone (Figure 2C-E). In contrast, the phosphorylation extent of Syk was not affected by the combination of inhibitors. This pointed to a Shp1-like (positive regulatory) action mechanism on tyrosine phosphorylation events, downstream of Syk, which becomes apparent under condition of PI3Kb inhibition.

The Tyr759 PLCγ2 phosphorylation is a known regulatory site for phospholipase activity, thus implicating a role in downstream platelet Ca²⁺ responses. To investigate this further, we measured in Fura-2-loaded platelets the rises in cytosolic [Ca²⁺] induced by GPVI agonist CRP. However, NSC87877 treatment did not significantly affect the [Ca²⁺] traces, neither in the absence nor presence of TGX221 (Figure 3A and B). On the other hand, in agreement with our aggregation data, the treatment with TGX221 (with or without NSC87877) halved the rises in [Ca²⁺] induced by CRP. Collectively, these data point to a suppressive effect of Shp1/2 inhibition on CRP-induced PLCγ2 tyrosine phosphorylation and ensuing Ca²⁺ responses in platelets.

**Contribution of glycoprotein VI-mediated Btk activation**

Downstream of GPVI, the tyrosine kinase Btk is a known regulator of PLCγ2 activation. In particular, Btk catalyzes the phosphorylation of PLCγ2 at Tyr759, thereby enhancing its activity in evoking [Ca²⁺] responses. In order to check for a role of Btk in the signaling modulation by Shp1/2, we used the selective, irreversible inhibitor ibrutinib. Strikingly, we found that the CRP-induced Ca²⁺ response was almost completely abrogated by ibrutinib alone, an effect that could not be reverted by the additional presence of NSC87877 (Figure 3C and D). In another approach, we examined the effect of ibrutinib alone or in combination with NSC87877 on CRP-induced integrin αIIbβ3 expression (Figure 4A) and P-selectin expression (Figure 4B), using flow cytometry. In none of these responses, the inhibitory effect of ibrutinib was overcome by NSC87877, regardless of the CRP dose that was used (Figure 4). Together, this pointed to absence of Shp1/2 regulation of GPVI-induced responses acting via Btk.
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Discussion

Prior work has indicated that the tyrosine phosphatases Shp1 and Shp2 play nonredundant, partly contrary roles in ITAM-mediated platelet activation.9,13,14 Both Shp isoforms are known to co-regulate the GPVI signaling pathway, by differently affecting the activity of SFK, Syk and the effector protein PLCγ2.10 In the present paper, we show that platelet treatment with the pan-Shp1/2 inhibitor NSC87877, a potential anti-cancer drug, enhanced GPVI-induced platelet aggregation responses, thus pointing to a net antagonistic effect of the combined tyrosine phosphatases on ITAM signaling by GPVI. Interestingly, the blockage of Shp1/2 with NSC87877 enhanced platelet aggregation even under conditions of PI3Kβ blockage. Given the relative importance of the PI3Kβ isoform,17 this indicated interference with a non-PI3K pathway.

In contrast to the results of platelet aggregation, we noticed that Shp1/2 inhibition with NSC87877 suppressed CRP-induced PLCγ2 tyrosine phosphorylation rather than enhancing it. Also, the Ca2+ signal downstream of PLCγ2 was not enhanced. We found that the combination of NSC87877 plus TGX221 markedly suppressed the phosphorylation of PLCγ2 at Tyr759, and to a lesser degree of Src at Tyr419 after CRP stimulation. Furthermore, inhibition of the upstream tyrosine kinase Btk - a major regulator of Tyr759 PLCγ2 phosphorylation - caused a prominent reduction in the Ca2+ signal, which was not reversed by PI3K inhibition. Altogether, these findings suggest that the ability of tyrosine phosphatase Shp2 to dampen the GPVI-induced signaling in platelets concerns a pathway aside from PLCγ2 and PI3K.

In mice, megakaryocytic deletion of Shp1 resulted in platelets being less responsive to CRP, whereas deletion of Shp2 caused hyper-responsiveness.9,13,14 Accordingly the present inhibitory effect of NSC87877 on GPVI PLCγ2 phosphorylation needs to be attributed to the Shp1 isoform. In contrast, the enhancing effect of the drug on platelet aggregation points to a role of the Shp2 isoform. Given the role of SFK in platelet aggregation and the

Figure 2. Unchanged collagen-related peptide-induced tyrosine phosphorylation by inhibition of Shp1/2 in contrast to inhibition of phosphoinositide 3-kinase β. Washed platelets (800×10⁹/L) were incubated with vehicle medium, NSC87877 (NSC, 20 μM), TGX221 (TGX, 3 μM) or combination of both for 10 min. The cells were then stimulated with collagen-related peptide (CRP, 0.5 μg/mL) for 45-90 s. Changes of protein tyrosine phosphorylation were measured by western blotting using anti-Tyr 4G10 mAb. A) Representative western blot of collagen-related peptide-induced tyrosine phosphorylation changes in platelets. Molecular size of separated kDa markers is indicated on the left side; B) Blots showing collagen-related peptide-induced increased phosphorylation (45 s) of phospho-phospholipase Cγ2 Tyr759, phospho-Src Tyr419 and phospho-Syk Tyr525+526, using specific antibodies; C-E) Densitometric analysis of tyrosine phosphorylation changes after 45 s of Syk p-Tyr525+526 (C), Src p-Tyr419 (D), and phospholipase Cγ2 p-Tyr759 (E). Data are presented as arbitrary staining units (AU) versus sample-size control (anti-nicotinamide adenine dinucleotide phosphate mAb), normalized for the vehicle control condition. Mean values ± standard deviation (n=3 donors). *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001 vs. vehicle control (paired Student’s t-test).
Src-dependent action mechanism of Shp, the molecular pathway is likely Src-dependent. However, this hypothesis needs further confirmation.

Conclusions

Considering the current interest of Shp2 inhibitors in tumor-targeted therapies, we conclude that the drug NSC87877 moderately enhances platelet aggregation upon GPVI stimulation through the inhibition of Shp2. The drug hence may help to normalize platelet plug formation in hemostatic-impaired patients.

**Figure 3.** Impaired collagen-related peptide-induced platelet Ca\(^{2+}\) responses by inhibition of phosphoinositide 3-kinase \(\beta\) or Btk, but not of Shp1/2. A-B) Samples of Fura-2-loaded platelets (200×10⁶/L) were pre-incubated with vehicle control (Veh.), NSC87877 (NSC, 20 μM), TGX221 (TGX, 3 μM) or combination for 10 min, before adding to a 96-wells plate. After addition of 1 mM CaCl₂, the loaded platelets were stimulated with collagen-related peptide (CRP, 10 μg/mL), while 340/380 nm fluorescence ratio changes were recorded per well; A) Representative traces of calibrated nM changes in [Ca\(^{2+}\)]i per condition; B) Effects of NSC87877 and TGX221 on integrated [Ca\(^{2+}\)]i rises (area-under-curve over 10 min). Data are normalized to the control condition per experiment; C-D) Fura-2-loaded platelets were pre-incubated with vehicle control, NSC (20 μM), ibrutinib (Ibru., 1 μM) or combination for 10 min. Stimulation of the loaded platelets was again with 1 mM CaCl₂ and 10 μg/mL CRP; C) Representative nM [Ca\(^{2+}\)]i traces per condition; D) Effects of NSC87877 and ibrutinib on integrated [Ca\(^{2+}\)]i rises (area-under-curve over 10 min). Data are normalized to the control condition per experiment; B, D) Mean values ± SD (n=3 donors); *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001 vs. vehicle (paired Student’s t-test).

**Figure 4.** Additional effects Shp1/2 and Btk inhibition on collagen-related peptide-induced platelet activation markers by flow cytometry. Washed platelets (50×10⁹/L) were incubated for 10 min with vehicle control (Veh.), NSC87877 (NSC, 20 μM), ibrutinib (Ibru., 1 μM) or combination, and subsequently activated with collagen-related peptide (0.1-5.0 μg/mL). A, B) Extent of integrin αIIbβ3 activation and P-selectin expression after 10 min, as determined by flow cytometry. Shown are representative heatmaps of inhibitor effects, assessed as percentage responses of positive platelets, with values indicated. Color code gives scaled values between 0% (blue) to 100% (red) in comparison to vehicle control per agonist dose. Means of duplicate runs per blood sample for 3 donors; *p<0.05 vs. vehicle (paired Student’s t-test).
References