Signal Transducer and Activator of Transcription 3 (STAT3) Regulates Collagen-Induced Platelet Aggregation Independently of Its Transcription Factor Activity

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Background—Platelet hyperactivity induced by inflammation is a known risk factor for atherosclerosis and thrombosis, but its underlying mechanisms remain poorly understood.

Methods and Results—The signal transducer and activator of transcription 3 (STAT3) was activated in collagen-stimulated platelets. Activated STAT3 served as a protein scaffold to facilitate the catalytic interaction between the kinase Syk (spleen tyrosine kinase) and the substrate PLCγ2 to enhance collagen-induced calcium mobilization and platelet activation. The same interaction of STAT3 with Syk and PLCγ2 was detected in HEK293 cells transfected with cDNAs for Syk and PLCγ2 and stimulated with interleukin-6. Pharmacological inhibition of STAT3 blocked ≈50% of collagen- and a collagen-related peptide–induced but not thrombin receptor–activating peptide– or ADP-induced aggregation and ≈80% of thrombus formation of human platelets on a collagen matrix. This in vitro phenotype was reproduced in mice infused with STAT3 inhibitors and mice with platelet-specific STAT3 deficiency. By forming a complex with its soluble receptor, the proinflammatory cytokine interleukin-6 enhanced the collagen-induced STAT3 activation in human platelets.

Conclusions—These data demonstrate a nontranscriptional activity of STAT3 that facilitates a crosstalk between proinflammatory cytokine and hemostasis/thrombosis signals in platelets. This crosstalk may be responsible for the platelet hyperactivity found in conditions of inflammation. (Circulation. 2013;127:476-485.)

Key Words: collagen ■ hemostasis ■ platelets ■ STAT3 ■ thrombosis ■ transcription factors

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor activated by cytokine-induced intracellular signals.1,2 This signal pathway plays a critical role in inflammation and megakaryopoiesis.3 For the latter, thrombopoietin binds its receptor (a product of the proto-oncogene c-Mpl) on megakaryocytes to activate the receptor-associated Janus kinase (JAK),3,4 resulting in the recruitment and tyrosine phosphorylation of STAT3. Activated STAT3 changes conformation, dimerizes through its SH2 domain, and translocates into the nucleus, where it regulates the transcription of multiple genes required for platelet production.2,4

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As the offspring of megakaryocytes, platelets maintain much of the megakaryopoiesis signaling machinery, including JAK and STAT3.5,6 However, platelets are anucleated cells with a limited capacity for protein synthesis. Therefore, it is unclear whether platelet STAT3 is merely a “leftover” with no specific function, remains as an active transcription factor, or participates in intracellular signaling independently of its transcriptional activity. It has been reported that the transcription inhibitor actinomycin D blocks the thrombopoietin-dependent potentiation of platelet reactivity and that thrombopoietin induces the receptor-dependent tyrosine phosphorylation and dimerization of STAT3 in platelets.7,8 Activated STAT3 binds to the regulatory D-loop region of platelet mitochondrial DNA to regulate its transcription.4 This suggests that STAT3 remains active as a transcriptional factor in platelets. However, several lines of evidence also suggest a nontranscriptional role for STAT3 in platelets. First, thrombopoietin does not directly activate human platelets5,6,9 but primes them for activation and...
aggregation induced by ADP, epinephrine, and collagen. This thrombopoietin effect is detected within minutes after stimulation, too short to be explained by transcriptional and translational activities in activated platelets. Second, thrombin has been reported to induce STAT3 activation in platelets through a JAK3-dependent pathway that is mediated through a G protein–coupled receptor. Consistent with this report, the JAK3 inhibitor WHI-P131 prevents thrombin-induced platelet shape changes, granule secretion, and aggregation.

These reports led us to hypothesize that STAT3 has a non-transcriptional activity that regulates agonist-mediated platelet activation. We tested this hypothesis in collagen-stimulated platelets because spleen tyrosine kinase (Syk), which is essential for collagen-mediated signaling in platelets, also participates in cytokine-induced signaling pathways. Here, we present supportive data from in vitro experiments on human and mouse platelets and in vivo experiments on mice with megakaryocytes/platelet-specific STAT3 deficiency.

Methods

Materials

Human blood was obtained from healthy donors under a protocol approved by the Institutional Review Board of Baylor College of Medicine and the Puget Sound Blood Center. For platelet aggregation, a 15% citrated human platelet-rich plasma (PRP) was drawn into 10% acid-citrate-dextrose buffer (85 mmol/L sodium citrate, 111 mmol/L glucose, and 71 mmol/L citric acid, pH 6.5). The whole blood was centrifuged at 150g for 15 minutes at 24°C to obtain platelet-rich plasma, which was then centrifuged at 900g for 10 minutes to obtain platelets. Platelets were washed with a CGS buffer (13 mmol/L sodium citrate, 50 mmol/L glucose, and 120 mmol/L sodium chloride, pH 7.2) and suspended in a buffer containing Ca2+ (0.2 U/mL). Platelets were recalcificated immediately before being stimulated with type I fibrillar collagen, cross-linked CRP, fibrinogen (0.2 U/mL). Platelets were recalcificated immediately before being stimulated with type I fibrillar collagen, cross-linked CRP, fibrinogen (0.2 U/mL).

For mouse experiments, C57BL/6 mice (18–22 weeks old) were infused with STA21 or vehicle control daily for 3 days. They were then anesthetized with 3% isoflurane by inhalation to draw blood from the inferior vena cava (0.38% sodium citrate, final concentration). Blood samples were diluted with an equal volume of Ca2+- and Mg2+-free Tyrode buffer (138 mmol/L sodium chloride, 5.5 mmol/L glucose, 12 mmol/L sodium bicarbonate, 2.9 mmol/L potassium chloride, and 0.36 mmol/L sodium phosphate dibasic, pH 7.4). To measure thrombus formation under flow conditions, blood was collected in 0.32% sodium citrate solution and centrifuged at 900g for 15 minutes to obtain platelet-rich plasma. Platelets were washed with a CGS buffer and resuspended in a buffer containing Ca2+, Mg2+, and a final concentration of 0.3% sodium citrate.

Antibodies against total STAT3, Tyr705-phospho STAT3, Ser727-phospho STAT3, Tyr525/S526-phospho Syk, Tyr1217-phospho PLCγ2, total and phospho-STAT1, and phospho-STAT5 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against total Syk and PLCγ2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total and phosphorylated STAT3, Tyr705-phospho STAT3, and Ser727-phospho STAT3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against total Syk and PLCγ2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total and phosphorylated STAT3, Tyr705-phospho STAT3, and Ser727-phospho STAT3 were purchased from Cell Signaling Technology (Danvers, MA).

The STAT3 inhibitor STA21 [(S)-ochromycinone deoxytetrangomycin] was synthesized (Midland Certified Reagent Co, Midland, TX) and purified by anion-exchange high-pressure liquid chromatography on Q Sepharose followed by pressure filtration in H2O. Immediately before use, STA21 was made by diluting the stock solution 300-fold in PBS to a final DMSO concentration of 0.1% immediately before use. The vehicle control solution was PBS containing 0.1% DMSO.

The STAT3 inhibitor T40214 is a guanine-rich oligonucleotide (GCGGCGGCAGCGCGGCG). It is in a linear structure in low extracellular [K+] (≤5 mmol/L) but forms a symmetrical G-quartet structure in high intracellular [K+] (≥140 mmol/L) when it is delivered into cells with polyethyleneamine nanobeads (molecular weight, ≥200 kDa). The Sh2 domain of STAT3 to inhibit the tyrosine phosphorylation and dimerization of STAT3 and a scrambled control oligonucleotide (CGGGCGGCAGCGCGGCG) were commercially synthesized (Midland Certified Reagent Co, Midland, TX) and purified by anion-exchange high-pressure liquid chromatography on Q Sepharose followed by pressure filtration in H2O. Immediately before use, T40214 and the control oligonucleotide (1 mg/mL) were coupled to polyethyleneamine nanobeads in an oligo/polyethyleneamine bead ratio of 1 to 2 (wt/wt) with a coupling efficiency of 60% to 70%. The coupled beads were extensively washed with PBS before use.

Platelet Granule Release

A whole-blood aggregometer was also used to measure ATP released per the manufacturer’s instructions (Chrono-log, Haverton, PA). A commercial ELISA was used to measure serotonin released from platelets according to the manufacturer’s instructions (Immuno-Medical Laboratories, Minneapolis, MN). Briefly, washed platelets before and after treatment with 20 μmol/L STA21 were stimulated with 5 μg/mL collagen for 10 minutes and centrifuged at 900g for 10 minutes. The supernatants from each sample were collected and diluted 50 times in PBS for serotonin measurements.

Flow Cytometry

Expression of the platelet adhesion receptors integrin αIIbβ3, glycoprotein (GP) Ibα, CD62P, and CD45 were purchased from BD Biosciences (San Jose, CA). Mouse CD41-APC, mouse CD45-PE-Cy7, and mouse TER119-APC-Cy7 antibodies were purchased from eBioscience (San Diego, CA).

A protease inhibitor cocktail (10 mmol/L AEBSF, 8 mmol/L aprotonin, 0.2 mmol/L leupeptin, 0.4 mmol/L bestatin, 0.15 mmol/L pepstatin A, and 0.14 mmol/L E-64) was purchased from Sigma-Aldrich (St. Louis, MO). Fura-2/AM was purchased from Molecular Probes (Eugene, OR). Human fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Type 1 collagen and ADP were from Helena Laboratories (Beaumont, TX). The cross-linked collagen-related peptide [CRP; Gly-Lys-Hyp-Gly(Pro-Hyp-Gly)10Lys-Hyp-Gly] was synthesized and cross-linked in the protein core laboratory of Baylor College of Medicine. The thrombin receptor–activating peptide (TRAP) was purchased from Bachem Bioscience (King of Prussia, PA). Human interleukin-6 (IL-6) and soluble IL-6 receptor (sIL-6R) were purchased from the R&D Systems (Minneapolis, MN). Recombinant (r) Syk and rSTAT3 were purchased from Promega (Madison, WI) and Abcam (Cambridge, MA), respectively. The Syk inhibitors I (3,4-methylenedioxy-b-nitrostyrene) and II [3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide] were purchased from EMD Chemicals (Darmstadt, Germany). Actinomycin D was from Sigma (St. Louis, MO).

STAT3 Inhibitors

The STAT3 inhibitor STA21 [(S)-ochromycinone deoxytetrangomycin] was purchased from Enzo Life Sciences (Plymouth Meeting, PA). A stock solution was made by dissolving STA21 in 30% dimethyl sulfoxide (DMSO), and the working solution was made by diluting the stock solution 300-fold in PBS to a final DMSO concentration of 0.1% immediately before use. The vehicle control solution was PBS containing 0.1% DMSO.

The STAT3 inhibitor T40214 is a guanine-rich oligonucleotide (GCGGCGGCAGCGCGGCG). It is in a linear structure in low extracellular [K+] (≤5 mmol/L) but forms a symmetrical G-quartet structure in high intracellular [K+] (≥140 mmol/L) when it is delivered into cells with polyethyleneamine nanobeads (molecular weight, ≥200 kDa). The Sh2 domain of STAT3 to inhibit the tyrosine phosphorylation and dimerization of STAT3 and a scrambled control oligonucleotide (CGGGCGGCAGCGCGGCG) were commercially synthesized (Midland Certified Reagent Co, Midland, TX) and purified by anion-exchange high-pressure liquid chromatography on Q Sepharose followed by pressure filtration in H2O. Immediately before use, T40214 and the control oligonucleotide (1 mg/mL) were coupled to polyethyleneamine nanobeads in an oligo/polyethyleneamine bead ratio of 1 to 2 (wt/wt) with a coupling efficiency of 60% to 70%. The coupled beads were extensively washed with PBS before use.

Agonist-Induced Platelet Aggregation

Washed platelets were incubated with STA21 or vehicle control (0.1% DMSO) for 10 minutes at 37°C and then mixed with purified human fibrinogen (0.2 U/mL). Platelets were recalcificated immediately before being stimulated with type I fibrillar collagen, cross-linked CRP, TRAP, or ADP and monitored for aggregation for 10 minutes at 37°C on an optical aggregometer (Bio/Data, Horsham, PA) with constant stirring at 1200 rpm.

For mouse experiments, C57BL/6 mice (18–22 weeks old) were infused with STA21 or vehicle control daily for 3 days. They were then anesthetized with 3% isoflurane by inhalation to draw blood from the inferior vena cava (0.38% sodium citrate, final concentration). Blood samples were diluted with an equal volume of Ca2+- and Mg2+-free Tyrode buffer and centrifuged at 70g for 20 minutes to obtain platelet-rich plasma. Platelet counts were normalized to 2.5×10⁹/mL with homogeneous plasma before samples were tested for agonist-induced platelet aggregation. The G-quartet STAT3 inhibitor T40214 was also tested in the same fashion.

Expression of the platelet adhesion receptors integrin αIIbβ3, and GP Ibα-IX-V complex was measured by flow cytometry. The surface expression of the activation marker CD62P was measured before and after platelets were stimulated with collagen for 20 minutes at room temperature. The samples were analyzed on an Epic XL-MCL flow cytometer (Beckman Coulter, Miami, FL). The expression of GP 130 and binding of IL-6-sIL-6R complex to platelets were also detected by flow cytometry using a FITC-conjugated mouse anti-human GP 130 (Abcam, Cambridge, MA) and an FITC-conjugated mouse anti-human IL-6 antibody (Abcam).
To quantify potential leukocyte contamination, washed platelets and platelet-rich plasma were incubated with V450 anti-human CD45 (BD Biosciences) for 20 minutes at room temperature. Samples were first analyzed for CD45 positivity and then for particle size in the leukocyte gate on the forward scatter. We did not detect leukocytes in platelet-rich plasma and washed platelet preparations (Figure IA in the online-only Data Supplement).

**Immunoblotting and Immunoprecipitation**

Washed platelets were incubated with either STA21 or 1 of 2 Syk inhibitors for 10 minutes at 37°C before being stimulated with collagen, CRP, or TRAP for 5 to 10 minutes at 37°C. They were then solubilized with a hypotonic lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L Na$_2$VO$_4$, 10 mmol/L Na$_3$VO$_4$, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, and 1% Igepal CA-630, pH 7.5) in the presence of a protease inhibitor cocktail (Materials and Methods in the online-only Data Supplement). Platelet lysates were centrifuged at 13 000g for 15 minutes at 4°C to remove cellular debris. The supernatant was separated on 10% SDS-PAGE and probed for phosphorylated and total STAT isoforms, Syk, and PLC$_{\gamma 2}$ with specific antibodies. The total STAT3 and phosphorylated STAT3 were also probed in lysates from washed platelets pretreated with IL-6 (10 ng/mL) or a complex of IL-6 (10 ng/mL) and an equal molar concentration of si-L6R in the presence or absence of collagen for 5 to 10 minutes at 37°C. For immunoprecipitation, washed platelets were incubated with collagen for 5 minutes at 37°C. Platelet lysates were incubated with antibodies against total and phosphorylated PLC$_{\gamma 2}$ overnight at 4°C, followed by incubation with protein A Sepharose beads (30 µg/mL) for 1 hour at 4°C. Samples were centrifuged at 8000g for 5 minutes to collect protein A beads, which were washed and boiled in an SDS sample buffer. Bead eluates were separated by 10% SDS-PAGE and immunoblotted for STAT3, Syk, and PLC$_{\gamma 2}$. For negative control, nonimmune isotype IgGs were used for immunoblots. In a subset of experiments, platelets were treated with 5 µg/mL actinomycin D, a global transcription inhibitor, for 2 hours at 37°C before stimulation and immunoprecipitation to determine a role of residual transcriptional activity in the interaction of STAT3 with Syk and PLC$_{\gamma 2}$.

**Transfection and Test of HEK293 Cells**

To further evaluate a role of STAT3 in mediating Syk and PLC$_{\gamma 2}$ interaction, human embryonic kidney (HEK) 293 cells were co-transfected with human Syk and PLC$_{\gamma 2}$ cDNAs. These transiently transfected cells were treated with IL-6 for 1 hour in the presence or absence of 20 µmol/L STA21 and then solubilized by a lysis buffer (Cell Signaling Technology, Danvers, MA) 48 hours after transfection. Cell lysates were either separated by SDS-PAGE and probed for STAT3 phosphorylation or used for immunoprecipitation. For the latter, cell lysates were incubated with a STAT3 antibody overnight at 4°C, followed by incubation with protein A Sepharose beads for 1 hour at 4°C. Bead eluates were then separated by SDS-PAGE and probed for Syk, PLC$_{\gamma 2}$, and STAT3.

**Parallel-Plate Flow Chamber Assay**

Citrated blood was perfused over a glass coverslip coated with type I fibrillar collagen (10 µg/mL coating concentration, overnight at 4°C) at a flow rate of 1 mL/min in a parallel-plate flow chamber. Platelet adhesion and aggregation were monitored in real time with time-lapse image acquisition, and the thrombus-covered areas (from a total area of 3×10$^5$ cm$^2$) were quantified offline with the Element Program (Nikon, Melville, NY).

**Generation and Test of Platelet-Specific STAT3-Null Mice**

Megakaryocytes/platelet-specific STAT3-deleted mice (pSTAT3$^{ΔΔ}$) were generated from C57BL/6-Tg(PF4-Cre) mice generated in our laboratory (Materials in the online-only Data Supplement) and crossed with mice carrying STAT3 alleles flanked by loxP sites (STAT3$^{ΔΔ}$, provided by Dr Shizuo Akira, Department of Host Defense, Osaka University). Colonies were expanded by crossing PF4-Cre-STAT3$^{ΔΔ}$ mice with STAT3$^{ΔΔ}$ mice. These mice were genotyped via polymerase chain reaction by amplifying a fragment of the STAT3 gene flanked by loxP sites (primers: 5’ GCTGAAGACAACTCTCCTGTCCTC 3’ and 5’CACACAGAGCACTCAACCTCTCCTC 3’) and a fragment that included the PF4 promoter and the cDNA for Cre recombinase. For data verification, a second strain of pSTAT3$^{ΔΔ}$ mice was also generated using a commercial strain of PF4-Cre mice (Stock No. 008535; Jackson Laboratories, Bar Harbor, ME).

**Collagen-Induced Calcium Influx**

Platelet-rich plasma from STAT3$^{ΔΔ}$ and pSTAT3$^{ΔΔ}$ mice was incubated with 2 mmol/L fura-2/AM for 1 hour at room temperature and then washed. Labeled platelets were suspended in PBS to a final concentration of 1.5×10$^6$/mL. Calcium (final concentration, 2 mmol/L) was added to the platelet suspension immediately before the addition of 0.75 µg/mL collagen. Calcium influx ([Ca$^{2+}$]$_i$) was monitored at 340 nm (excitation) and 505 nm (emission) on a PTI QuantaMaster fluorimeter (Photon Technology International, Birmingham, NJ) for 3 minutes at 37°C with constant stirring.

**Statistical Analysis**

Quantitative data are presented as mean±SEM. Repeated measures ANOVA was used to analyze data from experiments in which comparisons were made between multiple observations from the sample. Standard ANOVA models were fit to data when all observations included in an analysis were independent. Normal probability plots were used to assess the assumption of normally distributed errors. In repeated measures analyses, Huynh-Feldt adjustments were made for cases in which the sphericity assumption came into question. An experiment-wise type I error rate of 0.05 was maintained in each experiment using the Sidak method to adjust P values for testing multiple hypotheses.

**Results**

**STAT3 Inhibitors on Platelet Aggregation**

The STAT3 inhibitor STA21 dose-dependently inhibited ≥50% of platelet aggregation induced by low but not maximal doses of collagen and CRP (Figure 1A and 1B). It also partially blocked the collagen-induced expression of the platelet activation marker CD62P (Figure 1C) but not the release of ATP and serotonin (Figure 1D and 1E). In contrast, STA21 had no effect on ADP- and TRAP-induced platelet aggregation and did not alter expression levels of the adhesion receptors integrin αIIbβ3 and GP Ibα (Figure 1B and IC in the online-only Data Supplement). We then tested the effect of STA21 on platelet thrombus formation by perfusing whole blood over a type I collagen matrix for 1 minute at a flow rate of 1 mL/min, which generated a wall shear stress of 62.5 dynes/cm$^2$ (with a measured viscosity of 6 cP). STA21 at 20 µmol/L, which maximally inhibited collagen-induced platelet aggregation, significantly reduced the number of adherent platelets, whereas the DMSO vehicle control did not (614±24 for STA21 versus 1542±125 for DMSO in 6 random-view fields of ×400 magnification; n=4). Consistent with reduced platelet adhesion and aggregation, thrombus formation on a collagen matrix under flowing conditions was also reduced by 80% by 20 µmol/L STA21 (Figure 1G and 1H).

Because human and mouse STAT3 share >99% of sequence identity, we tested the function of platelets from C57BL/6J
mice that had been infused daily with STA21 or its vehicle control for 3 days. Platelets from STA21 infused mice had reduced collagen-induced aggregation (Figure 1I) without detectable reduction in platelet counts (Figure ID in the online-only Data Supplement).

To validate data generated with STA21, we also tested the oligonucleotide G-quartet T40214, a different class of STAT3 inhibitor that specifically blocked phosphorylation-dependent STAT3 dimerization. Compared with those treated with a scrambled control oligonucleotide, T40214 significantly reduced the aggregation of human platelets induced by 5 µg/mL but not 10 µg/mL collagen (Figure 2A and 2B). Platelet aggregation induced by 50 µmol/L TRAP was not affected (Figure 2C). Platelets pretreated with T40214 also formed significantly smaller thrombi on collagen matrix under a wall shear stress of 62.5 dynes/cm² compared with those treated with a scrambled control oligonucleotide (Figure 2D). Platelets from C57BL/6J mice infused with T40214 had reduced collagen-induced aggregation (Figure 2E). The ex vivo thrombus formation in the cremaster arterioles after photochemical-induced vascular injury was also significantly delayed in T40214-infused mice (Figure 2F).

Collagen-Induced Platelet Aggregation in pSTAT3Δ/Δ Mice

Because STAT3 is ubiquitously expressed, a STAT3 inhibitor infused systemically could interfere with platelet aggregation through its effects on endothelial cells and leukocytes. We therefore generated megakaryocyte/platelet-specific STAT3-deleted mice (pSTAT3Δ/Δ) by crossing the C57BL/6-Tg(CXCL4-cre) (PF4-Cre) mice with STAT3 F/F mice. The selectivity and robustness of Cre expression in the PF4-Cre line were verified by crossing with reporter lines that express β-galactosidase or enhanced yellow fluorescent protein only in cells that experienced Cre recombinase activity (Figure II in the online-only Data Supplement). The platelet-specific deletion of STAT3 was verified by immunoblotting platelet lysates with a STAT3 antibody (Figure IIIA in the online-only Data Supplement). pSTAT3Δ/Δ mice were fertile without detectable physical abnormalities, and their hematologic parameters were comparable to those of STAT3F/F littermates (the Table). Platelets from these pSTAT3Δ/Δ mice had reduced aggregation, CD62P expression, and calcium influx induced by 0.5 and 0.75 µg/mL but not 5 µg/mL collagen (Figure 3A–3F). The rate of thrombus formation was also reduced when blood from pSTAT3Δ/Δ mice was perfused over a

Figure 1. Effects of STA21 on platelet aggregation. Platelet-rich plasma was incubated with STA21 or dimethyl sulfoxide (DMSO) vehicle control (0.1%) for 10 minutes at 37°C and then induced to aggregate with various concentrations of collagen (A; univariate repeated measures for each collagen dose vs baseline; n=9; *P<0.001) or collagen-related peptide (CRP; B; vs baseline; n=3; *P<0.001). Secretion from α-granule was measured by detecting surface expression of CD62P by flow cytometry after platelets were stimulated with 2 µg/mL collagen for 10 minutes (C; univariate repeated measures for each CRP dose vs baseline; n=6; *P<0.001). ATP (D; n=6) and serotonin (E; n=3) released from dense granules were measured by whole-blood aggregometry and ELISA, respectively, in the supernatant of platelets stimulated with 2 µg/mL collagen. The data for A through C are also presented as box plots in Figure V in the online-only Data Supplement. Thrombus formation in vitro was induced by perfusing STA21-treated (20 µM for 10 minutes at 37°C) blood over immobilized collagen for 1 minute at a flow rate of 1 mL/min. Representative images show platelet thrombi in the presence (F) and absence (G) of STA21 (bar=200 µm). The areas covered by thrombi were quantified in 6 random images from each experiment (H; n=3 separate sets of experiments; *P<0.001). For mouse assay, C57BL/6J mice were injected daily with 4 or 8 mg/kg body weight of STA21 or vehicle control through tail veins for 3 days, and collagen-induced platelet aggregation was measured on the third day with an optical aggregometer (I; n=8; *P<0.01).
collagen matrix under arterial shear stress (Figure 3G and 3H). In contrast, TRAP- and ADP-induced platelet aggregation was minimally affected (Figure IIIB and IIIC in the online-only Data Supplement).

Collagen Induced STAT3 Phosphorylation in Human Platelets

Figures 1 through 3 show that blocking STAT3 moderately but specifically inhibits collagen-induced platelet aggregation and thrombus formation. We next assessed whether platelet STAT3 was activated (phosphorylated) after collagen stimulation. Tyr705 and Ser727 of STAT3 are known to be differentially phosphorylated in response to cytokine stimulation in nucleated cells.23,24 We found that collagen and CRP stimulation dose-dependently increased STAT3 phosphorylation at Tyr705 but not Ser727 (Figure 4A and 4B). This collagen-induced STAT3 tyrosine phosphorylation was detectable 0.5 minutes after collagen exposure but reached the maximal level after 5 to 10 minutes (Figure 4C). This collagen-induced STAT3 phosphorylation was dose-dependently blocked by STA21 (Figure 4D). In contrast, TRAP had no effect on the level of STAT3 tyrosine phosphorylation (Figure 4E). Collagen stimulation also did not change the levels of tyrosine phosphorylation of STAT1 and STAT5, the other 2 major STAT proteins in platelets (Figure 4F).

Syk Inhibitors on Tyrosine Phosphorylation of STAT3

We have shown that collagen specifically induced tyrosine phosphorylation of STAT3 but not STAT1 and STAT5 in human platelets. Previous studies have identified prominent roles for Syk and PLCγ2 in collagen-induced calcium mobilization.25 To decipher the relationship between STAT3 and these 2 signal molecules, we evaluated STAT3 phosphorylation in the presence of Syk inhibitors.26,27 Two Syk inhibitors dose-dependently reduced collagen-induced STAT3 phosphorylation (Figure 4G). As expected, this Syk inhibitor was also effective in blocking PLCγ2 phosphorylation induced by collagen (Figure 4H). In contrast, STA21 did not inhibit Syk phosphorylation (Figure 4I) but dose-dependently blocked PLCγ2 phosphorylation in platelets treated by 2 and 5 µg/mL

Table. Hematologic Measurements of pSTAT3Δ/Δ and STAT3F/F Mice*

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<td>Platelet counts, 10⁹/mm³</td>
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*Data are expressed as mean±SEM and were analyzed with 2-way ANOVA (n=27 mice per group).
but not 10 µg/mL collagen (Figure 4J). Together, these data suggest that STAT3 acts downstream of Syk but upstream of PLCγ2.

**Interaction of STAT3 With Syk and PLCγ2**

This spatial relationship among STAT3, Syk, and PLCγ2 was further examined by coimmunoprecipitation experiments. A STAT3 antibody immunoprecipitated Syk and PLCγ2 (Figure 5A) and a PLCγ2 antibody immunoprecipitated Syk and STAT3 (Figure 5B) in lysates of collagen-stimulated platelets. Both antibodies did not immunoprecipitate actin, suggesting that the association of STAT3 with Syk and PLCγ2 was not mediated through actin cytoskeleton. The pretreatment of platelets with the transcription inhibitor actinomycin D did not alter this association (Figure IVC in the online-only Data Supplement). Furthermore, coimmunoprecipitation was also achieved by antibodies specifically against phosphorylated PLCγ2 and STAT3 (Figure 5C). The STAT3 inhibitor STA21 reduced the amount of phosphorylated STAT3 immunoprecipitated by a PLCγ2 antibody (Figure 5D). Consistent with data from human platelets, the collagen-induced phosphorylation of PLCγ2 was significantly reduced in platelets from pSTAT3Δ/Δ mice (Figure 5E).

**IL-6 on Collagen-Induced STAT3 Phosphorylation**

Together, these data suggest that STAT3 interacted with Syk and PLCγ2 to enhance collagen-induced platelet activation and aggregation. A critical implication of this trimolecule interaction could be to facilitate a crosstalk between collagen-induced and inflammatory cytokine–induced signal pathways. This potential crosstalk was examined in human platelets and HEK293 cells. We found that human platelets expressed the common signal transducer of the IL-6 cytokine family gp130 (Figure IV A in the online-only Data Supplement) and that a complex of IL-6 and sIL-6R could be detected on the surface of platelets after 10 minutes of incubation with this complex (Figure IVB in the online-only Data Supplement). The IL-6 and sIL-6R complex but not IL-6 alone induced STAT3 phosphorylation (Figure 6A), which was dose-dependently enhanced by collagen in human platelets (Figure 6B). In contrast to platelets, IL-6 induced STAT3 phosphorylation in HEK293 cells transiently transfected with human Syk and PLCγ2 cDNAs (Figure 6C). STA21 at 20 µmol/L blocked this IL-6–induced STAT3 phosphorylation in HEK293 cells (Figure 6D). Consistent with results from platelet experiments, a STAT3 antibody immunoprecipitated Syk and PLCγ2 from lysates of IL-6–stimulated HEK293 cells (Figure 6E).
Discussion

We have presented several lines of experimental evidence that STAT3 regulates collagen-induced platelet activation and aggregation. First, 2 STAT3-specific inhibitors of different classes, STA21 (a small molecule) and T40214 (a protein binding oligonucleotide G quartet), dose-dependently blocked platelet aggregation induced by low doses of collagen and CRP but not TRAP or ADP. They also inhibited thrombus formation on a collagen substrate under arterial flow and reduced CD62P expression (Figures 1 and 2). Second, platelets from mice deficient in platelet STAT3 (pSTAT3\(^{Δ/Δ}\)) aggregated poorly and had a low level of CD62P expression (Figures 1, 2, and 3), but they reacted normally to ADP and TRAP (Figure III in the online-only Data Supplement). These pSTAT3\(^{Δ/Δ}\) platelets also formed smaller thrombi on a collagen matrix under arterial flow (Figure 3). There are 2 known receptors for collagen on platelets: the integrin \(\alpha_\text{IIb}\beta_1\) and GP VI/Fc receptor \(\gamma\) chain. Our data suggest that STAT3 is involved primarily in GP VI–mediated signaling because STA21 blocked platelet activation induced by not only collagen but also CRP, which specifically targets GP VI. STAT3 phosphorylation was also blocked by inhibiting Syk (Figure 4), a critical tyrosine kinase in the GP VI–mediated signaling pathway. The finding is supported by a report that the Syk inhibitor piceatannol blocked STAT3 phosphorylation in lymphocytic cells. Through these experiments, we have also made 3 novel observations.

First, STAT3 enhanced a collagen-induced intracellular signal that resulted in platelet activation, calcium mobilization, and aggregation. This activity is independent of transcriptional activity because (1) the effects of STAT3 on platelet activation and aggregation occurred as early as 0.5 minutes after collagen stimulation and reached the plateau 5 to 10 minutes after
collagen stimulation, a time period insufficient for significant transcriptional activities, especially in anucleated platelets; (2) STAT3 interacted directly with Syk and PLCγ2, likely forming a trimolecule complex as suggested by coimmunoprecipitation experiments (Figure 5); and (3) the transcription inhibitor actinomycin D did not affect the interaction of STAT3 with Syk and PLCγ2 in collagen-stimulated platelets (Figure IV in the online-only Data Supplement).

Second, STAT3 may act as a protein scaffold in regulating collagen signals. As previously reported, cross-linking GP VI induces the Src kinase–dependent phosphorylation of the immunoreceptor tyrosine-based activation motif, which then recruits and activates Syk.25 Syk activation leads to the phosphorylation of the linker for activation of T cells (LAT) and the adapter Src SH2 domain–containing leukocyte protein of 76 kDa (SLP-76), both of which are required for tyrosine phosphorylation of PLCγ2.32,33 However, there are likely additional proteins linking Syk to PLCγ2 activation because tyrosine phosphorylation of PLCγ2 was reduced but not eliminated in CRP-stimulated LAT−/− platelets32 and because SLP-76 is tyrosine phosphorylated in collagen signaling downstream of Syk but an association between Syk and SLP-76 could not be detected.34 We found that blocking Syk reduced STAT3 phosphorylation but not vice versa (Figure 4) and that STA21...
reduced the amount of STAT3 immunoprecipitated by a PLCγ2 antibody (Figure 5D). Collagen-induced PLCγ2 phosphorylation was reduced in platelets from pSTAT3Δ/Δ mice (Figure 5E). Similarly, an STAT3 antibody immunoprecipitated Syk and PLCγ2 in HEK293 cells that transiently expressed the 2 signal molecules and were stimulated with IL-6 (Figure 6). These data suggest that an activated (dimerized) STAT3 may enhance or accelerate the catalytic interaction between Syk and PLCγ2 by bringing the substrate to the proximity of its kinase (a dimerized STAT3 has 2 SH2 binding sites). In this regard, STAT3 functions as a signal enhancer, not a facilitator. This notion is consistent with the finding that the effects of STAT3 were detected only at low concentrations of collagen or CRP. The notion that STAT3 functions as a protein scaffold is also supported by a report that STAT3 couples phosphatidylinositol 3-kinase with the type 1 interferon receptor in nucleated cells.35

Third, the IL-6–sIL-6R complex but not IL-6 activated STAT3 and enhanced collagen-induced STAT3 phosphorylation in platelets (Figure 6A and 6B), whereas IL-6 alone was sufficient only to activate STAT3 in HEK293 cells (Figure 6C). These data suggest that, unlike HEK293 cells, platelets express the common signal transducer of the IL-6 cytokine family gp130 (Figure IVA in the online-only Data Supplement) but not IL-6 receptor. As a result, IL-6 acts on platelets only when it forms a complex with sIL-6R, which can be released through inflammation-mediated shedding. An IL-6–sIL-6R complex then primes platelets for collagen-induced activation by facilitating or accelerating the catalytic interaction between Syk and PLCγ2. In a similar fashion, IL-15 induces Syk and STAT3 phosphorylation in mouse mast cells.36 Figure 7 schematically illustrates a potential mechanism for IL-6 to enhance collagen-mediated signaling in platelets.

**Conclusions**

We have shown that STAT3 can serve as a protein scaffold to facilitate the catalytic process of activating PLCγ2 by Syk. This novel nontranscriptional activity of STAT3 enhances collagen-induced signaling in platelets, potentially making platelets hyperactive in conditions of inflammation by linking proinflammatory cytokine signals to hemostasis/thrombosis signals. The clinical implications of data presented here remain to be explored, but one could speculate that platelet hyperactivity, which has been widely reported in patients with coronary artery diseases, could be caused by this cross-talk. Furthermore, because hyperactive or activated platelets are insensitive to aspirin, targeting STAT3 could potentially improve aspirin efficacy by reducing inflammation-induced platelet hyperactivity with minimal impacts on the physiologically critical process of hemostasis.

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**Disclosures**

None.

**References**

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CLINICAL PERSPECTIVE

In conditions of inflammation, platelets often deviate from their normal path of activation to become hyperactive in response to various agonists that are in solution or affixed to subendothelial matrix. This platelet hyperactivity is a known risk factor for atherosclerosis and thrombosis, but its underlying mechanisms remain poorly understood. Using specific inhibitors and transgenic mice, we have shown that the signal transducer and activator of transcription 3 (STAT3) is activated in collagen-stimulated human and mouse platelets. Activated STAT3 serves as a protein scaffold to facilitate the catalytic interaction between the kinase Syk (spleen tyrosine kinase) and the substrate PLCγ2, leading to enhanced collagen-induced calcium mobilization and platelet activation/aggregation. These data demonstrate a nontranscriptional activity of STAT3 that facilitates a crosstalk between the proinflammatory cytokine interleukin-6 and hemostasis/thrombosis signals in platelets. This crosstalk could provide a mechanism for platelets to become hyperactive in diverse clinical conditions that share a common pathology of inflammation. Blocking STAT3 and its intracellular signal pathway could potentially reduce and prevent arterial thrombosis. Hyperactive platelets are also known to be insensitive to aspirin. Targeting STAT3 could therefore improve aspirin efficacy by reducing inflammation-induced platelet hyperactivity with a minimal impact on the physiologically critical process of hemostasis.
Signal Transducer and Activator of Transcription 3 (STAT3) Regulates Collagen-Induced Platelet Aggregation Independently of Its Transcription Factor Activity


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MATERIALS

Generation and characterization of platelet-specific STAT3 null mice

C57BL/6J and B6.129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup> (ROSA26-lacZ) mice were purchased from the Jackson Laboratories (Bar Harbor, ME); B6.C-Tg(CMV-cre)1Cgn/J (CMV-Cre) and B6.129X1-Gt(ROSA)26Sor<sup>tm1(EYFP)Cos</sup>/J (ROSA26-eYFP) were obtained from colonies of the Genetically Engineered Mouse Facility of MD Anderson Cancer Center where they have been backcrossed multiple times into C57BL/6 background.

To generate the transgenic C57BL/6-Tg(CXCL4-cre) (PF4-Cre) mouse, the DNA transgenic construct consisted of the 387-bp of the promoter region of human PF4 (nucleotides -312 to +75) followed by the cDNA of Cre recombinase cloned at the start codon of the native gene, and the polyA sequence of the human β-globin gene. Transgenic animals were generated by pronuclear injection of fertilized C57BL/6 oocytes. Colonies were founded from 9 transgenic mice and screened for Cre activity. The line with the most robust and selective expression was backcrossed into C57BL/6J 8 times to dilute undesirable genomic modification introduced by the genetic manipulation.

To evaluate the tissue specificity of Cre recombinase expression, PF4-Cre transgenic mice were crossed with ROSA26-lacZ and ROSA26-eYFP reporter
mice. In these reporters, the cDNA for lacZ or eYFP respectively was introduced into the Gt(ROSA)26Sor locus preceded by a STOP sequence flanked by two loxP sites. In cells expressing Cre, recombination at the two loxP sites excises the intervening sequence allowing the expression of β-galactosidase or eYFP. Bone marrow and different tissues from PF4-Cre;ROSA26-lacZ were fixed and processed for X-gal staining, and blood from PF4-Cre;ROSA26-eYFP was collected and processed for flow-cytometry after labeling. We used F4-Cre, ROSA26-lacZ, and ROSA-eYFP mice as negative controls, and CMV-Cre;ROSA26-lacZ and CMV-Cre;ROSA26-eYFP mice as positive controls.

Megakaryocytes/platelet-specific STAT3 deletant mice (pSTAT3Δ/Δ) were generated by crossing PF4-Cre with mice carrying STAT3 alleles flanked by loxP sites (STAT3F/F; provided by Dr. Shizuo Akira, Department of Host Defense, Osaka University (22)). Colonies were expanded by crossing PF4-Cre;STAT3F/F (pSTAT3Δ/Δ) mice with STAT3F/F mice. These mice were genotyped via PCR by amplifying a fragment of the STAT3 gene flanked by loxP sites (primers: 5'-CCT-GAAGACCAAGTTCATCGTTGTA-3' and 5'-CACACAAGCCATCAAACTCTG-GTCTCC-3'). For data verification, a second strain of pSTAT3Δ/Δ mice was also generated using a commercial strain of PF4-Cre mice (Stock #008535, Jackson laboratories, Bar Harbor, ME) and used to repeat experiments conducted using pSTAT3Δ/Δ mice produced by crossing with the home-generated Cre line.
Mouse experiments were performed according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies of Baylor College of Medicine and the University of Texas MD Anderson Cancer Center.

**Histochemistry for β-galactosidase**

After euthanasia of mice with CO₂, the IVC was ligated and sectioned distally. The animal was perfused via the right ventricle first with 10 ml of PBS and then 10 ml of 0.2% glutaraldehyde in Buffer A (EGTA 5 mM, MgCl₂ 2 mM, 0.1M K-Phos buffer pH 7.3). Femora and tibiae were dissected and bone marrow was flushed and smeared onto slides, and air dried. The kidney, intestines, spleen, liver, and heart were dissected and fixed overnight in 0.2% glutaraldehyde solution at 4°C. Fixed tissues were dehydrated in 30% sucrose, migrated into OTC, and then frozen in OTC. Cryosections (10 μm) and bone marrow smears were incubated 5 min at room temperature in 0.2% glutaraldehyde solution, washed in sodium deoxycholate (0.01%) and Nonidet O-40 (0.02%) in Buffer A, and then incubated overnight at 37°C in Buffer A containing 0.5 mg/ml of X-gal, 10 mM of K₃Fe(CN)₆, 10 mM of K₄Fe(CN)₆, 0.01% of sodium deoxycholate, and 0.02% of Nonidet O-40. After washings, slides were counterstained with eosin and nuclear fast red and mounted.

**Mouse model of thrombosis**
Male C57BL/J6 mice, ~25 – 30g in weight and 12 – 16 wks of age, were anesthetized with pentobarbital (50 mg/kg), underwent tracheotomy, received a jugular venous catheter, and were positioned on a custom tray to carefully expose the cremaster muscle under an upright microscope (Olympus BX50, 40X NA 0.8 water-immersion objective) (34, 35). Following a 30-min equilibration period, FITC-dextran (150 kD, 10 ml/kg of a 5% solution) was injected via the venous catheter and allowed to circulate for ~10 min. After vascular diameter (NIH Image 1.6 public domain software) and mean blood velocity (Optical Doppler velocimeter, Cardiovascular Research Institute, Texas A&M University) were recorded, photochemical injury was induced by exposing ~100 μm of vessel length to epi-illumination (0.6 W/cm²). Time to onset of forming a platelet thrombus and time to flow cessation due to thrombotic vessel occlusion were recorded. The investigator performing the animal experiments was blinded with regard to the treatments or mouse strains.
Supplemental Figure 1: The potential contamination of leukocytes in PRP and washed platelet preparations were determined by flow cytometry. Platelets were first incubated with CD45 for 10 min at room temperature and analyzed first for CD45 positivity and then on particle size (A). Window P2 shows CD45 positive cells that are the size of leukocytes on a forward scatter plot. The bottom panels show counts of platelets (PI window) and leukocytes (P2 window). The plots were representatives PRP (left) and washed platelets (right). Platelet aggregation was induced by ADP or TRAP in the presence of STA21 (B, n = 6). The surface expression of $\alpha_{IIb}\beta_3$ and GP Ib$\alpha$ on resting platelets treated with STA21 for 60
min at 37°C was quantitatively measured by flow cytometry (C). Platelet counts of mice infused with 20 μM of STA21 daily for 3 days were measured immediately before aggregation and thrombus formation assays (D, n = 8).
Supplemental Figure 2: Characterization of PF4-Cre mice: PF4-Cre and CMV-Cre mice were crossed with the reporter lines ROSA26-lacZ and ROSA26-eYFP in which β-galactosidase or eYFP was expressed only in cells with Cre activity. (A) Bone marrow smears and tissue sections from ROSA26-lacZ, PF4-Cre (top row, negative control), CMV-Cre;ROSA26-lacZ (middle row, positive control) and PF4-Cre;ROSA26-lacZ (bottom row) were incubated with X-gal. β-galactosidase activity reflecting Cre activity was absent in negative controls, ubiquitous in positive controls, and restricted to megakaryocytes in PF4-Cre;ROSA26-lacZ mice (bar = 20 μm). (B) Whole blood from ROSA26-eYFP (negative control), CMV-Cre;ROSA26-eYFP (positive control) and PF4-
Cre;ROSA26-eYFP mice was incubated with α-mCD41-APC, α-mCD45-PE-Cy7 and α-mTER119-APC-Cy7 and analyzed by flow-cytometry. The left scattergram shows CD41⁺ (platelets, P, green), CD45⁺ (white blood cells, W, blue), and mTER119⁺ (RBC, R, red) cells. The middle scattergram shows most cells expressing eYFP being platelets. The right scattergram shows most CD41⁺ cells being eYFP⁺ (99.3%) and most eYFP⁺ cells being CD41⁺ (98.8%). (C) Flow-cytometry of PRP from PF4-Cre;ROSA26-eYFP mice (green) shows that most cells are eYFP⁺ when compared to PRP from ROSA26-eYFP mice (light green). The insert shows that almost all cells were platelets (CD41⁺, red). The images are representative of 6 mice per line.
Supplemental Figure 3: Platelet functions of pSTAT3Δ/Δ mice:

Platelets from pSTAT3Δ/Δ and STAT3F/F mice were lysed, separated on 10% of SDS-PAGE, and probed for STAT3 with a monoclonal antibody; β-tubulin was used as a loading control (A). The aggregation of platelets from pSTAT3Δ/Δ and STAT3F/F mice was induced by ADP (B, n = 26) or TRAP (C, n = 26).
Supplemental Figure 4: (A) GP 130 was detected on the surface of human platelets by flow cytometry using a mouse anti-human GP 130 antibody (red line). The isotype control antibody (blue line) was a mouse IgG2. (B) Detection of IL-6-sIL-6R complex on human platelets in citrate PRP after incubation with the complex for 10 min at room temperature (Mann-Whitney Rank Sum Test, n = 5, *p<0.001). (C) The effect of Actinomycin D on collagen-induced STAT3 interaction with Syk and PLCγ2 in platelets. Washed platelets were first incubated with 5 μg/ml of actinomycin D for 2 hrs at 37°C and then stimulated with 5 μg/ml of collagen for 10 min. Platelets were lysed and platelet lysates were incubated with a STAT3 antibody followed by protein A beads for immunoprecipitation as described in the method section. Co-immunoprecipitated proteins were immunoblotted for Syk and PLCγ2. Non-immune IgG was used as negative control.
Supplemental Figure 5: Dose responses of STA21 on aggregation of human platelets induced by fibrillar collagen at 2 μg/ml (A), 5 μg/ml (B), and 10 μg/ml (C). STA21 was also tested for platelet aggregation induced by 1 μg/ml (D), 2 μg/ml (E), and 4 μg/ml (F) of CRP.