The protein tyrosine phosphatase PTPN7 is a negative regulator of ERK activation and thromboxane generation in platelets

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Protein tyrosine phosphatase nonreceptor type 7 (PTPN7), also called hematopoietic protein tyrosine phosphatase, controls extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase in T lymphocytes. Because ERK1/2 plays an important role in regulating thromboxane A_2 (TXA_2) generation in platelets, we investigated the function of PTPN7 in these cells. Using immunoblot analysis, we detected PTPN7 in both human and mouse platelets but not in PTPN7-null mice. PTPN7 KO mouse platelets exhibited increased platelet functional responses, including aggregation, dense granule secretion, and TXA_2 generation, compared with platelets from WT littermates, upon stimulation with both G protein–coupled receptor (GPCR) and glycoprotein VI (GPVI) agonists. Using the GPCR agonist AYPGKF in the presence of the COX inhibitor indomethacin, we found that PTPN7 KO mouse platelets aggregated and secreted to the same extent as WT platelets, suggesting that elevated TXA_2 is responsible for the potentiation of platelet functional responses in PTPN7-KO platelets. Phosphorylation of ERK1/2 was also elevated in PTPN7 KO platelets. Stimulation of platelets with the GPVI agonist collagen-related peptide along with the COX inhibitor indomethacin did not result in phosphorylation of ERK1/2, indicating that GPVI-mediated ERK phosphorylation occurs through TXA_2. Although bleeding times did not significantly differ between PTPN7-null and WT mice, time to death was significantly faster in PTPN7-null mice than in WT mice in a pulmonary thromboembolism model. We conclude that PTPN7 regulates platelet functional responses downstream of GPCR agonists, but not GPVI agonists, through inhibition of ERK activation and thromboxane generation.

Platelets are primary mediators of hemostasis and thrombosis, and their activation is tightly regulated under normal physiological conditions. Upon vascular injury, circulating platelets bind to exposed subendothelial collagen, which leads to initial activation of platelets. Activated platelets secrete their granular contents, such as ADP, and generate TXA_2,[2] which is a positive feedback activator of platelets (1).

TXA_2 is a lipid mediator that acts in an autocrine, paracrine fashion to amplify the initial signal and stabilize thrombus formation (2, 3). Patients with TXA_2 deficiency have a mild bleeding disorder (4). On the contrary, high levels of TXA_2 can lead to pathological conditions such as myocardial infarction (5) and stroke (6). Aspirin, an inhibitor of thromboxane synthesis, improves mortality in patients experiencing myocardial infarction (7). The above examples reiterate the importance of TXA_2 generated by platelets. Therefore, it is essential to understand the regulation of TXA_2 generation in platelets.

ERK is a crucial mediator of TXA_2 generation in platelets. Studies have shown that extracellular signal-regulated protein kinase 1/2 (ERK1/2) plays a major role in TXA_2 generation downstream of protease-activated receptors (PARs) (8) and ADP receptors (P2Y1 and P2Y12) (9, 10). ERK phosphorylates cytoplasmic phospholipase A2 (cPLA2) on serine 505, which is required for activation of cPLA2 (11, 12). Activated cPLA2 is involved in the hydrolysis of membrane phospholipids to release arachidonic acid. Prostaglandin G/H synthase and TXA_2 synthase convert the free arachidonic acid to TXA_2. Given the importance of ERK in TXA_2 generation in platelets, it is crucial to understand the mechanisms of ERK regulation in platelets. Very little is known about ERK regulation in platelets via protein tyrosine phosphatases (PTPs). In lymphocytes, ERK activation is regulated by a PTP known as PTPN7. Thus, we wanted to evaluate the role of PTPN7 in the regulation of ERK in platelets.

PTPN7 (alternatively called hematopoietic PTP) is a cytoplasmic protein tyrosine phosphatase originally cloned from human T lymphocytes (13, 14). PTPN7 is a 38-kDa protein consisting of a C-terminal catalytic domain and a short N-terminal extension that contains the kinase interaction motif. PTPN7 is expressed in cells of hematopoietic lineage, such as neutrophils, megakaryocytes, erythrocytes, and lymphocytes (15). T cells from PTPN7 KO mice show hyperphosphorylation

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[2] The abbreviations used are: TXA_2, thromboxane; ERK, extracellular signal-regulated protein kinase; PAR, protease-activated receptor; PTP, protein tyrosine phosphatase; GPVI, glycoprotein VI; GPCR, G protein–coupled receptor; CRP, collagen-related peptide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ANOVA, analysis of variance; TXB_2, thromboxane B_2.

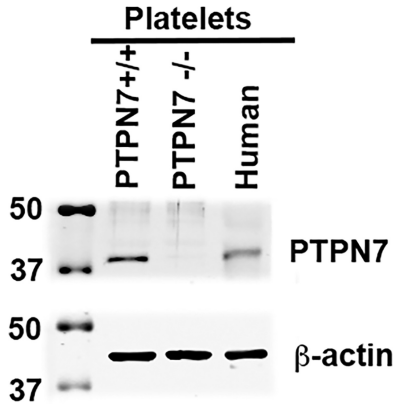

Figure 1. Expression of PTPN7 in human and mouse platelets. Mouse lysates of WT and PTPN7 KO platelets were loaded in the *first* and *second lane*, respectively, and human platelet lysate was loaded in the *third lane*. The blot was probed with PTPN7 antibody, and the same blot was reprobed with β-actin antibody. The data are representative of at least three independent experiments.

of ERK, which indicates that PTPN7 dephosphorylates ERK and thereby negatively regulates T cell activation (15, 16).

Mass spectrometry has demonstrated the presence of PTPN7 in platelets (17, 18); however, the presence of PTPN7 has never been evaluated using biochemical techniques. In this study, we evaluated the presence of PTPN7 in human and mouse platelets. We used PTPN7 knockout mice to investigate the role of PTPN7 in platelet activation. We show that PTPN7 is a negative regulator of ERK and, therefore, of TXA_2 generation and platelet functional responses.

Results

PTPN7 is expressed in mouse and human platelets

PTPN7 is present as a 38-kDa cytoplasmic protein in lymphocytes (13, 14, 19). Its presence in platelets has been shown using proteomics approaches (17, 18) but never using biochemical techniques. To assess the presence of PTPN7 in platelets, we used an antibody that recognizes the N terminus of PTPN7. In Western blot analysis using platelet lysates, we observed that PTPN7 is present as a 38-kDa protein in WT mouse and human platelets but not expressed in PTPN7 KO platelets (Fig. 1). Thus, we conclude that PTPN7 is expressed in platelets.

PTPN7 deficiency results in enhanced platelet functional responses

Because we demonstrated the presence of PTPN7 in platelets, we evaluated the role of PTPN7 in platelet functional responses using PTPN7 KO mice. However, prior to that analysis, we measured blood cell counts in PTPN7 and WT mice and found that PTPN7 deletion did not alter blood cell counts (Table 1). Platelets have two important classes of receptors: ITAM-based receptors, such as GPVI receptors, and GPCRs, such as PARs and ADP receptors (1). Upon stimulation of WT and PTPN7 KO platelets with lower concentrations of the PAR4 agonist AYPGKF, the ADP receptor agonist 2-MesADP, or the GPVI receptor agonist CRP, the extent of aggregation and the amount of ATP secretion were significantly greater in PTPN7 KO mouse platelets compared with platelets from the WT littermates (Fig. 2). PTPN7 KO mouse platelets that were stimulated with higher concentrations of AYPGKF, 2-MesADP, or CRP showed higher dense granule secretion compared with the WT, although the extent of aggregation was similar (Fig. 2). These data suggest that PTPN7 plays an important role in regulating platelet functional responses, such as aggregation and secretion, in platelets.

Table 1
Blood cell counts are unaltered in PTPN7 knockout mice
WBC, white blood cell; LY, lymphocyte; NE, neutrophil; MO, monocyte; RBC, red blood cell; PLT, platelet; MPV, mean platelet volume.

Parameter	$PTPN7^{+/+}$	$PTPN7^{-/-}$
WBC ($10^3/\mu l$)	7.18 ± 0.55	6.19 ± 0.62
LY ($10^3/\mu l$)	6.08 ± 0.51	5.11 ± 0.65
NE ($10^3/\mu l$)	0.69 ± 0.04	0.67 ± 0.04
MO ($10^3/\mu l$)	0.55 ± 0.13	0.40 ± 0.04
RBC ($10^6/\mu l$)	9.37 ± 0.30	10.12 ± 0.07
PLT ($10^3/\mu l$)	721 ± 44	806 ± 24
MPV (fl)	4.10 ± 0.07	4.18 ± 0.02

To confirm whether the enhanced platelet aggregation and secretion in PTPN7 KO mouse platelets were due to enhanced TXA_2 generation, both WT and PTPN7 KO mouse platelets were stimulated with an agonist, and the TXA_2 levels were measured using a Thromboxane B_2 (TXB_2) (a stable analog of TXA_2) assay, a competitive immunoassay. PTPN7 KO mouse platelets showed a significant increase in TXB_2 levels compared with WT samples when stimulated with all agonists (Fig. 3*A*). Therefore, we conclude that TXA_2 generation is potentiated in PTPN7 KO platelets. We also performed flow cytometry to examine p-selectin expression and active GPIIb/IIIa expression (JON/A binding) on WT and PTPN7 KO platelets. Stimulation with 200 μM AYPGKF resulted in enhanced p-selectin expression and JON/A binding (Fig. 3, *B* and *C*). Stimulation with 1 μg/ml CRP caused no significant change in p-selectin expression or JON/A binding, although the trend mirrored that of AYPGKF.

Inhibition of thromboxane generation normalizes PTPN7 knockout platelet responses

Because the aggregation and secretion responses were potentiated in PTPN7 KO mice, we evaluated whether the potentiation was due to enhanced TXA_2 generation. For this purpose, we pretreated WT and PTPN7 KO mouse platelets with 10 μM indomethacin, a cyclooxygenase inhibitor that inhibits TXA_2 generation, followed by stimulation with AYPGKF or CRP. We observed that the extent of aggregation and the amount of dense granule secretion in PTPN7 KO mice were the same as in the WT when the platelets were pretreated with indomethacin (Fig. 4). This result suggests that the potentiation of aggregation and secretion is due to enhanced TXA_2 generation.

ERK is hyperphosphorylated in PTPN7 knockout mouse platelets

Because ERK has been known to be regulated by PTPN7 in lymphocytes (15, 16), we investigated whether PTPN7 also regulates this MAPK in platelets. ERK was found to be hyper-phosphorylated in PTPN7 KO mouse platelets upon AYPGKF stimulation that were pre-treated with or without indomethacin (Fig. 5*A*). PTPN7 KO platelets stimulated with CRP also showed hyper-phosphorylation of ERK. However, in the pres-

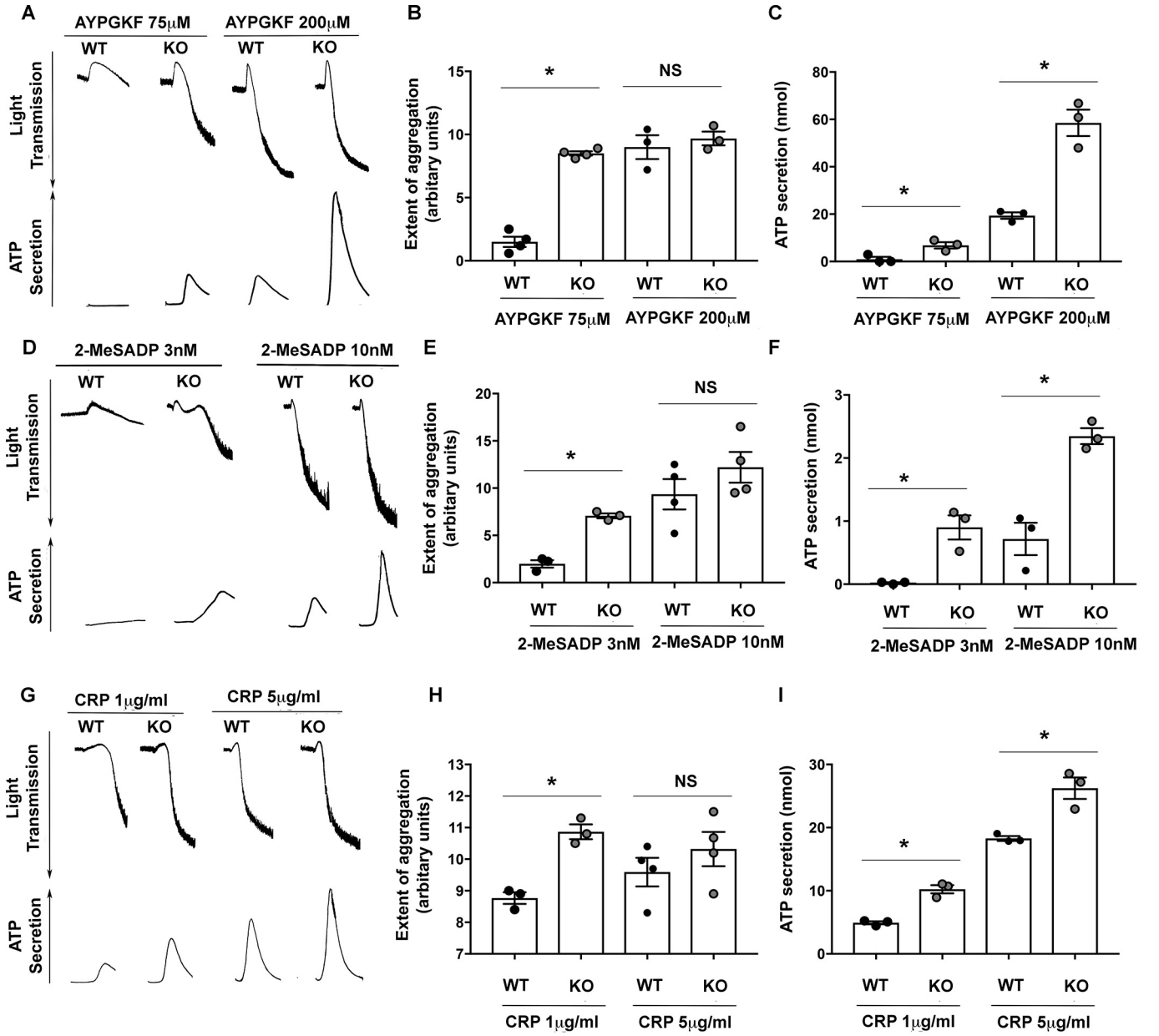

Figure 2. Platelet function is enhanced in PTPN7 knockout mice. Washed mouse platelets were stimulated with low and high concentrations of GPCR and GPVI agonists at 37 °C under stirring conditions. *A*, AYPGKF (75 μM and 200 μM). *B* and *C*, quantitation of the extent of aggregation and ATP secretion in *A*. *, $p <$ 0.05. *NS*, not significant. *D*, 2-MeSADP (3 nM and 10 nM). *E* and *F*, quantitation of the extent of aggregation and ATP secretion in *D*. *, $p < 0.05$. *G*, CRP (1 μg/ml and 5 μg/ml). *H* and *I*, quantitation of the extent of aggregation and ATP secretion in *G*. *, $p < 0.05$ for the indicated comparison. The data are representative of at least three independent experiments ± S.E. Two-way ANOVA revealed a significant interaction between the effects of genotype (WT *versus* PTPN7 KO) and agonist on platelet aggregation ($F = 6.57$, $p = 0.0006$) and secretion ($F = 4.357$, $p = 0.006$).

ence of indomethacin, both WT and PTPN7 KO stimulated with 5 μg/ml of CRP showed no ERK phosphorylation (Fig. 5*B*). These results indicate that ERK phosphorylation occurs downstream of GPVI only by the action of feedback agonists, consistent with the previous observations (20). In addition, based on the aggregation results in the presence of indomethacin, we conclude that GPVI effects are not directly regulated by PTPN7.

PTPN7 is also known to regulate p38 MAPK in B-lymphocytes (19). Because platelets express both ERK and p38 MAPK, we evaluated the role of PTPN7 in p38 regulation. We observed that p38 MAPK is phosphorylated to the same extent in both WT and PTPN7 KO mouse platelets (Fig. 6, *C* and *D*). Because MEK is upstream of ERK in PAR signaling (21, 22), we also evaluated MEK activation by checking MEK phosphorylation. We observed that the activity of MEK was the same in PAR-activated WT and PTPN7 KO platelets (Fig. 6, *A* and *B*). Thus, we conclude that PTPN7 selectively regulates ERK in platelets without regulating its upstream kinase MEK.

PTPN7 KO mice have impaired thrombosis

Because we observed potentiation of platelet functional responses with GPCR and GPVI agonists in PTPN7 KO mouse platelets, we evaluated the implications of PTPN7 deficiency *in*

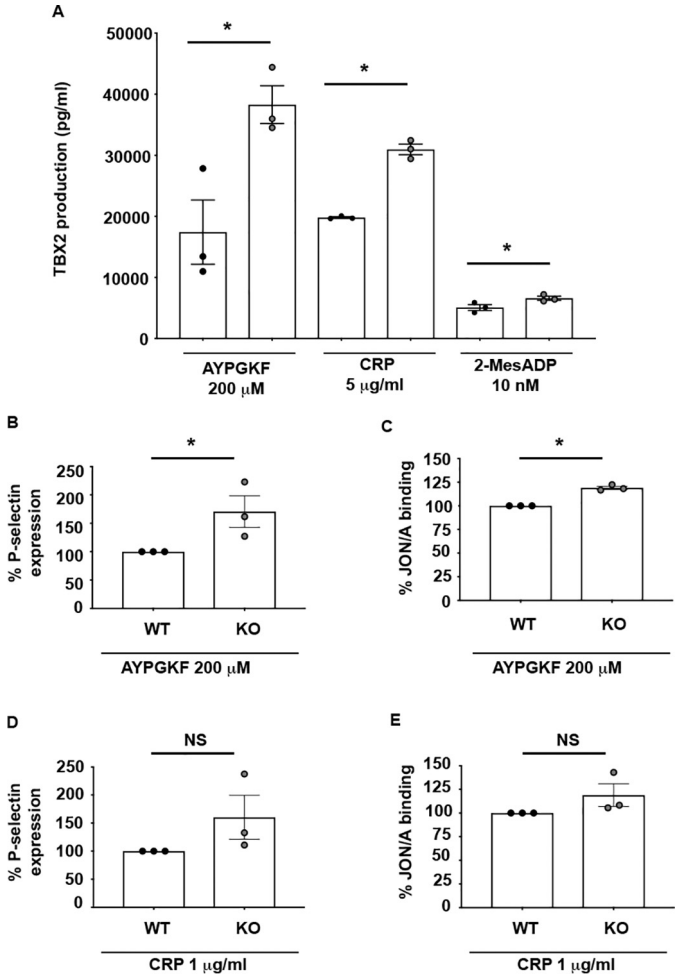

Figure 3. Thromboxane generation is enhanced with PTPN7 deletion. *A*, WT and PTPN7 KO platelets were stimulated with AYPGKF (200 μM), CRP (5 μg/ml), and 2-MesADP (10 nM) for 3.5 min at 37 °C under stirring conditions. Reactions were terminated, and the generated TXB_2 levels were measured as described under "Experimental procedures." Data are represented as percent maximal TXB_2 generated in WT controls. Each *column* is the average of at least three independent experiments ± S.E. *B–E*, flow cytometry analysis of p-selectin exposure or JON/A binding following stimulation of either AYPGKF or CRP. PTPN7 data were normalized to the WT, and statistics were calculated using raw mean fluorescent intensity values. *, $p < 0.05$ for the indicated comparison; *NS*, not significant. No significant interactions were found using two-way ANOVA ($F = 3.77$, $p = 0.54$).

vivo. We evaluated hemostatic function by measuring bleeding times in WT and PTPN7 KO mice. Bleeding times were measured as the time for blood flow to stop after cutting 3 mm of the mouse tail. We observed that there were no significant differences in the average bleeding times between WT and PTPN7 KO mice (Fig. 7*A*). Finally, we evaluated *in vivo* thrombosis using a pulmonary embolism model. In this model, collagen and epinephrine are injected i.v., which causes pulmonary embolism. We observed that time to cessation of respiration was significantly lower in the PTPN7 KO mice compared with the WT (Fig. 7*B*). Thus, we conclude that platelet responses are potentiated *in vivo* in PTPN7-null mice.

Discussion

PTPN7 KO mice have been used previously to characterize the role of PTPN7 in T cell signaling (15). T cells show enhanced ERK activation in PTPN7 KO mice, indicating that PTPN7 is a negative regulator of T-cell receptor-induced T cell activation. Similarly, ERK is hyperphosphorylated in K562 myelogenous leukemia cells undergoing megakaryocytic differentiation when subjected to inhibition by antisense for PTPN7 (23). In B cells, PTPN7 negatively regulates p38 MAPK activation (19). Based on these previous studies, we evaluated the role of PTPN7 as a regulator of MAPK activation in platelets using PTPN7 KO mice.

Platelets express MAPKs such as ERK1/2 (24, 25) and p38 MAPKs (26, 27). ERK inhibition by U0126, a MEK inhibitor, leads to inhibition of cPLA2 activation and TXA_2 generation downstream of PARs (8) and ADP receptors (9), demonstrating that ERK1/2 is required for cPLA2 activation and, consequently, TXA_2 production. ERK is hyperphosphorylated downstream of PAR activation in PTPN7 KO mouse platelets. As a result, there is enhanced TXA_2 generation in PTPN7 KO mouse platelets.

Previous studies showed that ERK phosphorylation downstream of the GPVI receptor is solely dependent on feedback signaling by TXA2 and ADP (20). Roger *et al.* (20) showed that, in the presence of indomethacin, ERK phosphorylation is negligible in collagen-stimulated platelets. Our studies show that ERK is hyperphosphorylated downstream of GPVI receptor activation in PTPN7 KO mouse platelets; however, in the presence of indomethacin, ERK phosphorylation is abolished in both WT and PTPN7 KO platelets stimulated with CRP. Thus, our data are in sync with previously published results. Therefore, potentiation of ERK phosphorylation in PTPN7 KO mouse platelets downstream of the GPVI receptor is due to enhanced TXA_2 generation in PTPN7 KO platelets.

When TXA_2 production is blocked by indomethacin, the extent of PAR-mediated aggregation and secretion in PTPN7 KO mice is similar to the WT. Under these conditions, ERK phosphorylation is greatly inhibited in both WT and PTPN7 KO mice. This shows that TXA_2 generated upon activation of PARs feeds back on platelets and contributes to ERK phosphorylation via secondary signaling. However, even in the presence of indomethacin, ERK remains hyperphosphorylated in PTPN7 KO platelets downstream of PARs. These data suggest that PTPN7 KO mouse platelets show enhanced ERK phosphorylation in PAR primary signaling. Therefore, PTPN7 KO mouse platelets also show enhanced TXA_2 generation. Similarly, the GPVI-mediated aggregation and secretion responses are normalized in indomethacin-treated PTPN7 KO mouse platelets. Therefore, we conclude that the potentiation of aggregation and secretion in PTPN7 KO platelets is due to enhanced secondary stimulation by TXA_2.

Apart from ERK, platelets also express p38 MAPK, which is activated downstream of thrombin (25, 28), and collagen (26, 27). Several studies have suggested that p38 MAPK is essential for cPLA2 activation and TXA_2 generation (25), but other studies using the selective inhibitor VX-702 have shown that p38 MAPK plays no role in agonist-induced platelet activation (29). This study demonstrates that p38 MAPK phosphorylation is comparable in WT and PTPN7 KO platelets upon PAR and GPVI receptor stimulation. These data suggest that PTPN7 selectively regulates ERK activation in platelets.

Last, we evaluated the physiological implications of PTPN7 deficiency in mice. We used a pulmonary embolism model,

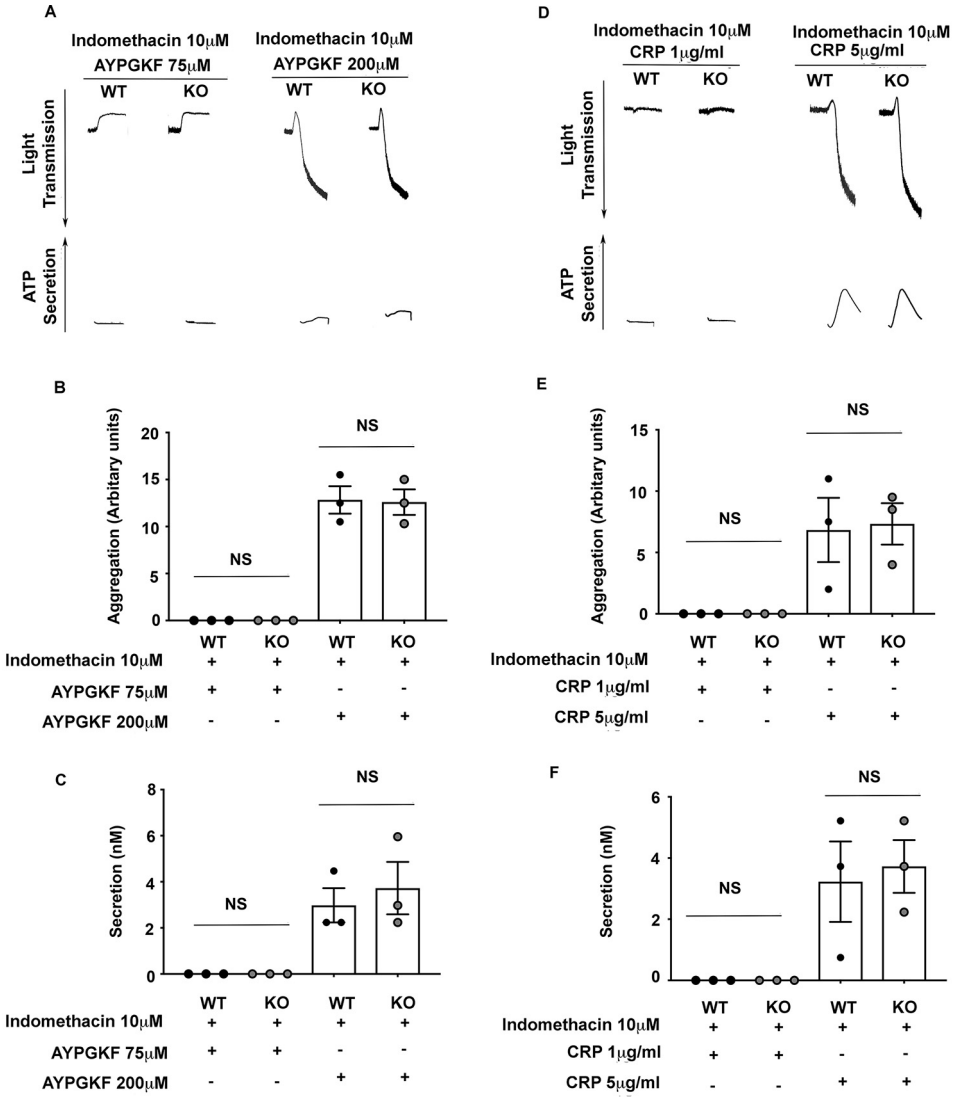

Figure 4. The enhanced platelet responses observed in PTPN7 knockout mice are due to increased thromboxane generation. Washed mouse platelets were pretreated with 10 μM indomethacin and stimulated with low and high concentrations of GPCR and GPVI agonists at 37 °C under stirring conditions. *A*, AYPGKF (75 μM and 200 μM). *B* and *C*, quantitation of the extent of aggregation and ATP secretion in *A*. *D*, CRP (1 μg/ml and 5 μg/ml). *E* and *F*, quantitation of extent of aggregation and ATP secretion in *D*. The data are representative of at least three independent experiments ± S.E. No significant interaction between agonist and genotype were found with respect to aggregation ($F = 0.028$, $p = 0.993$) or secretion ($F = 0.129$, $p = 0.942$) using two-way ANOVA. *NS*, not significant.

which involves direct activation of platelets *in vivo* by injecting collagen and epinephrine into mice, to evaluate *in vivo* thrombosis. Our results indicate that PTPN7 KO mice were more resistant to pulmonary thromboembolism than controls. Intriguingly, PTPN7 KO mice showed normal tail bleeding times. Therefore, based on the pulmonary embolism model, we concluded that PTPN7 plays a significant role in *in vivo* thrombosis; however, PTPN7 is dispensable for primary hemostasis, as indicated by tail bleeding. In conclusion, we have established that PTPN7 negatively regulates ERK activation and, thereby, TXA_2 generation in platelets.

Experimental procedures

Antibodies and reagents

Apyrase (grade VII), indomethacin, and 2-MesADP were obtained from Sigma-Aldrich (St. Louis, MO). The hexapeptide AYPGKF was custom-synthesized at Invitrogen. Collagen-related peptide (CRP) was purchased from Dr. Richard Farndale (University of Cambridge). Antibodies for phospho-tyrosine ERK1/2 (catalog no. M9692) and total ERK (catalog no. M5670) were purchased from Sigma. PTPN7 (hematopoietic PTP) antibody (catalog no. sc-21008) and total PLCγ2 (B-10, catalog no. sc-5283) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pMEK1/2 Ser-217/221 (41G9) antibody (catalog no. 9154T), P-S PKC substrate antibody (catalog no. 2261S), and phospho-p38 MAPK antibody (Thr-180/Tyr-182, D3F9, catalog no. 4511T) were purchased from Cell Signaling Technology (Danvers, MA). Total p38 MAPK antibody (catalog no. TA3263349) was purchased from Origene (Rockville, MD). Luciferin–luciferase reagent was purchased from Chrono-Log (Havertown, PA). Ferric chloride anhydrous (catalog no. 153499) was purchased from MP Biomedicals. All other reagents were of reagent grade, and deionized water was used throughout.

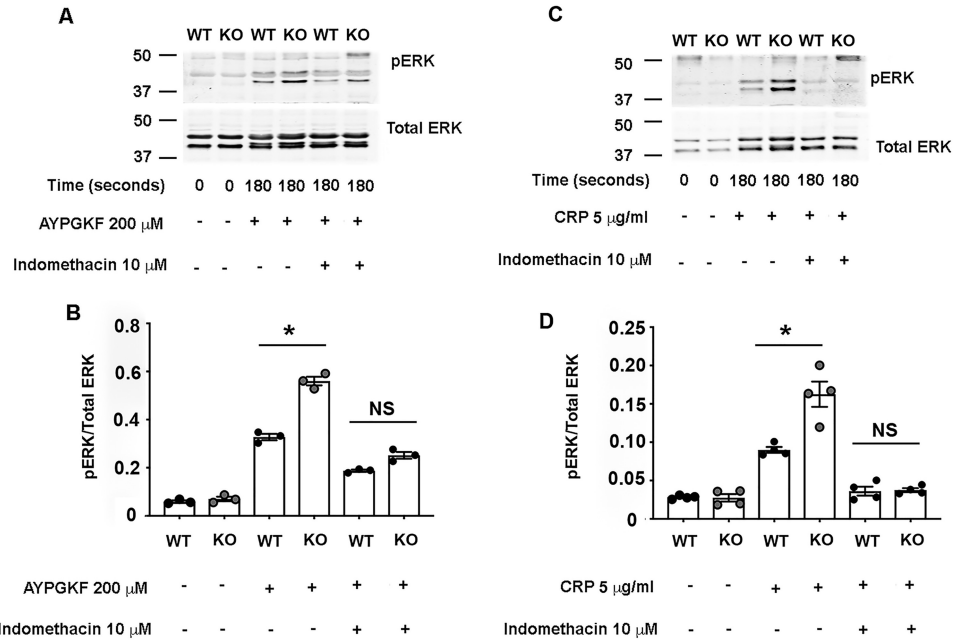

Figure 5. ERK phosphorylation is enhanced in AYPGKF-treated PTPN7 platelets in the absence of thromboxane. *A* and *C*, platelets from WT and PTPN7 KO mice were treated with or without 10 μM indomethacin and stimulated with AYPGKF (*A*, 200 μM) or CRP (*C*, 5 μg/ml). Platelet lysates were collected for Western blot analysis and probed for pERK (Thr-202/Tyr-204). The same blot was probed with total ERK antibody as a protein loading control in each lane. *B* and *D*, quantitation for phosphorylation of ERK. The data are representative of at least three independent experiments ± S.E. *, $p < 0.05$; *NS*, not significant.

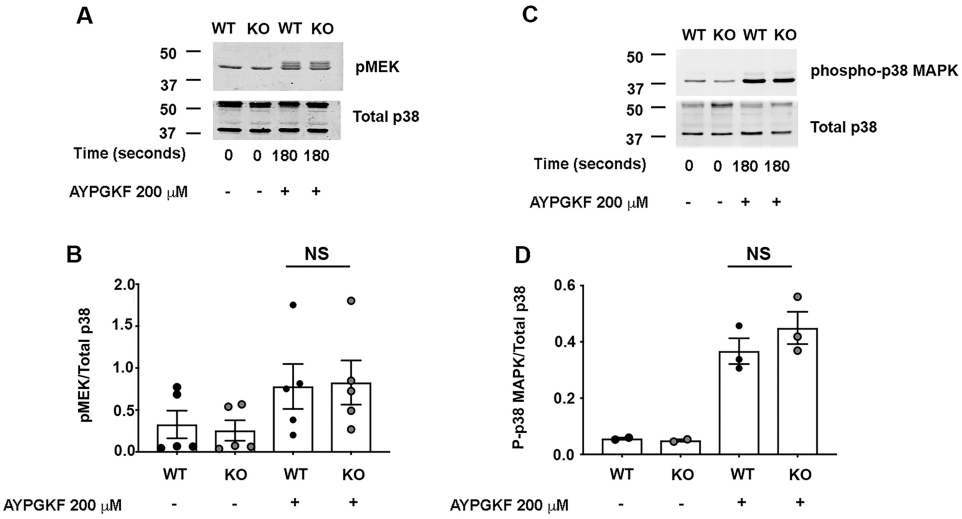

Figure 6. MEK and phospho-p38 phosphorylation remain unchanged in AYPGKF-treated PTPN7 knockout mouse platelets compared with WT platelets. Platelets from WT and PTPN7 KO mice were treated with AYPGKF (200 μM). *A* and *C*, platelet lysates were collected for Western blot analysis and probed for pMEK (*A*) and phospho-p38 (*C*), and the same blots were probed with total p38 antibody as a loading control. *B*, quantitation of phosphorylation of MEK. *D*, quantitation of phosphorylation of p38. The data are representative of at least three independent experiments ± S.E. *NS*, not significant.

Human blood donors

Platelet lysates originating from human donors were used for identification of PTPN7. Experiments involving human donors were approved by the Temple University institutional review board. All studies involving human subjects abided by the Declaration of Helsinki principles.

Animals

PTPN7 knockout mice were obtained from the Riken Research Institute (Japan). They were bred in the central animal facility of Temple University Medical School. All the experiments involving animals were approved by the Temple University Institutional Animal Care and Use Committee (4567).

Isolation of mouse platelets

Mouse blood was collected as described previously (30). Blood was drawn via cardiac puncture into a one-tenth volume of 3.8% sodium citrate. Blood was spun at 100 × *g* for 10 min, and the platelet-rich plasma was separated. The remainder of blood was mixed with 400 μl of 3.8% sodium citrate and spun for another 10 min at 100 × *g*. The resulting platelet-rich plasma was combined, 1 μM prostaglandin E1 was added, and samples were centrifuged for 10 min at 400 × *g*. Platelet-poor plasma was removed, and the platelet pellet was resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES, and 0.2 units/ml

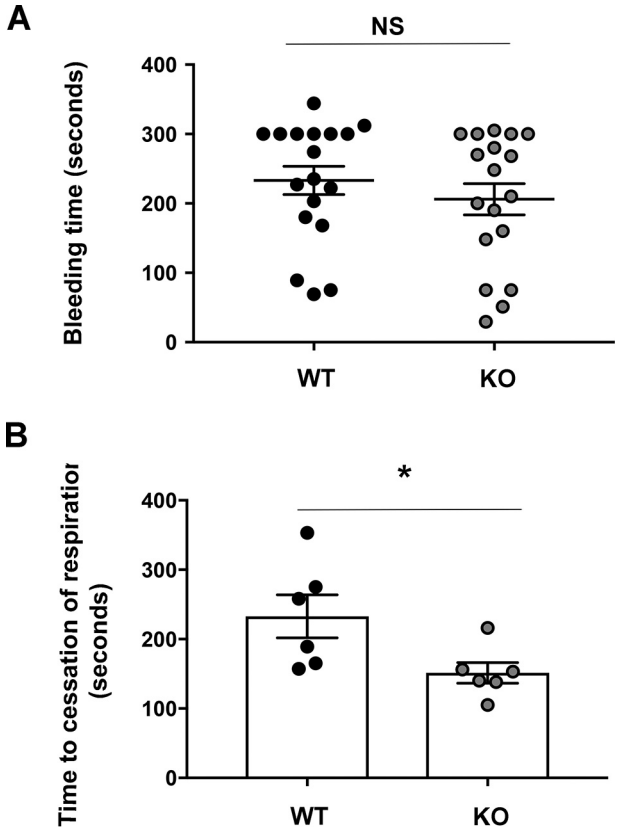

Figure 7. PTPN7 deletion is prothrombotic. *A*, bleeding time was measured in WT and CD45 KO mice following excision of the distal 3 mm of the tail. *B*, WT and CD45 KO mice were anesthetized and injected either with PBS or appropriate amounts of collagen and epinephrine, and time to cessation of respiration was recorded. *, $p < 0.05$ (Student's *t* test). The data are representative of at least three independent experiments ± S.E. *NS*, not significant.

apyrase (pH 7.4)). Platelet counts were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas, TX). Platelet counts were adjusted to 1.5×10^8 platelets/ml.

Aggregometry

Aggregation of 0.25 ml of washed platelets was analyzed using a lumiaggregometer (Chrono-Log Corp. Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37 °C for the indicated time.

Thromboxane generation assay

0.25 ml of washed platelets were stimulated with agonist for 3.5 min. The reaction was stopped by snap-freezing. The samples were thawed and centrifuged at 10,000 × *g* for 10 min to pellet the cells, and the supernatant was diluted 1:500. The diluted samples were used to evaluate thromboxane generation using a TXB_2 EIA kit from Enzo Life Sciences (catalog no. ADI-901-002).

Western blotting

Platelets were stimulated with agonists or vehicle control for the appropriate time under stirring conditions at 37 °C. The reaction was terminated by addition of 1/10 of a volume of 6.6 N $HClO_4$ and placed at 4 °C. The samples were centrifuged at 10,000 × *g* for 5 min, and the protein precipitate was washed with 0.5 ml of deionized water. The samples were again centrifuged as above, and the protein pellets were solubilized in sample buffer containing 0.1 M Tris base, 2% SDS, 1% (v/v) glycerol, 0.1% bromphenol blue, and 100 mM DTT. The samples were incubated at 95 °C for 10 min prior to loading onto the gel. Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman Protran, Pittsburgh, PA). Membranes were blocked with Odyssey blocking buffer for 1 h at room temperature, incubated overnight at 4 °C with the desired primary antibody, and then washed four times with 1× Tris-buffered saline and Tween 20 (TBST). Membranes were then incubated with the appropriate secondary IR dye-labeled antibody (1:10,000) for 60 min at room temperature and washed four times with TBST. Membranes were imaged using a LI-COR Odyssey IR imaging system.

Pulmonary thromboembolism

Mice were weighed, anesthetized, and injected i.v. with 400 μg/kg of collagen and 60 mg/kg epinephrine or PBS (control). The time to cessation of respiration was recorded. Chicago Sky Blue dye was perfused through the right ventricle of the heart. The lungs of the mice were examined to verify that pulmonary embolism occurred, as the dye is excluded from the lungs if the circulation is obstructed because of thrombosis (data not shown).

Bleeding time

Mice (4–5 weeks old) were anesthetized using isoflurane. Four millimeters of the tail were excised and immediately immersed in 0.9% isotonic saline at 37 °C. The bleeding time was defined as the time required for blood flow into the saline to stop.

Statistical analysis

Statistical analysis was performed using either two-way ANOVA or a Student's *t* test depending on the dataset. The method of statistical analysis is indicated in the figure legends. $p < 0.05$ was considered statistically significant. Data are expressed as mean ± S.E.

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