Supplementary Data

Supplementary Materials and Methods

Antibodies and reagents

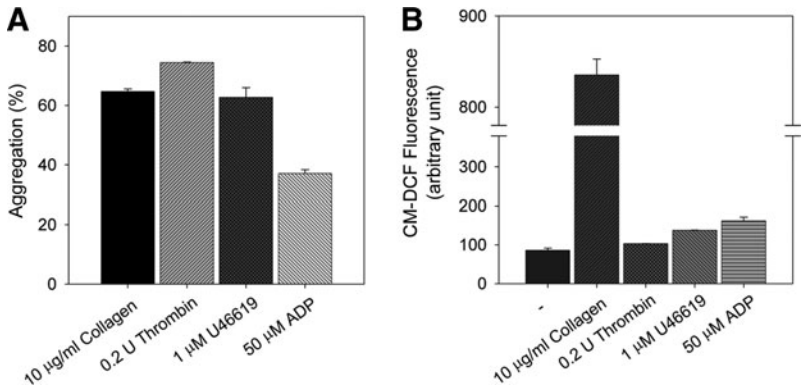
Anti-human CD41-FITC was from Beckman. Anti-mouse CD41-FITC and isotype control antibody (IgG1-FITC) were from BD-Biosciences. Thrombin was from Chrono-Log. U46619 was from Calbiochem. ADP, apocynin, 2-acetylphenothiazine, and VAS2870 were from Sigma-Aldrich. Polyclonal antibody to glycoprotein VI was from Santa Cruz Biotechnology. The synthetic CRP peptide GCP*(GPP*)$_{10}$GCP*G (where P* denotes a hydroxyproline amino acid residue) was cross-linked through N- and C-terminal cysteine residues as previously described (1).

Measurement of platelets purity using the flow cytometry

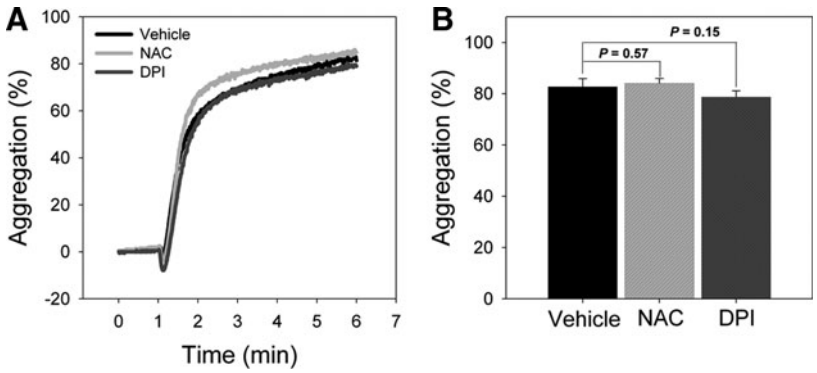
The isolated platelets were incubated with FITC-conjugated anti-human CD41 (CD41-FITC; Beckman), anti-mouse CD41 (CD41-FITC; BD-Biosciences), or isotype control antibody (IgG1-FITC) for 15 min in the dark. The reaction was stopped by adding ice-cold PBS. A FACSCalibur flow cytometer was used for all of the analyses with a minimum of 5×10^4 cells per sample for each measurement. CD41 on the platelets was measured at 530 nm (FL1). Data were analyzed with WinMDI software.

Supplementary References

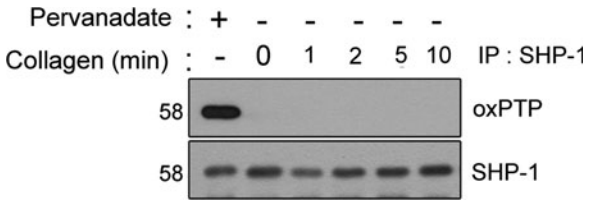
1. Asselin J, Knight CG, Farndale RW, Barnes MJ, and Watson SP. Monomeric (glycine-proline-hydroxyproline)10 repeat sequence is a partial agonist of the platelet collagen receptor glycoprotein VI. *Biochem J* 339 (Pt 2): 413–418, 1999.



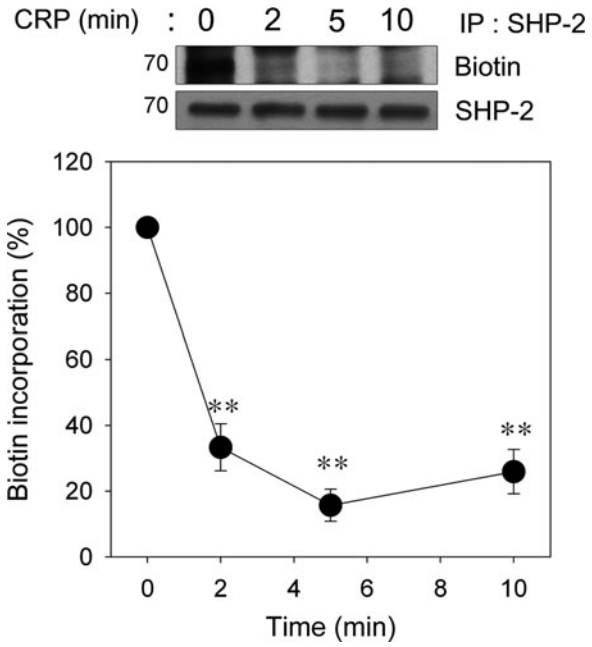
SUPPLEMENTARY FIG. S1. Effects of various agonists on aggregation and cellular reactive oxygen species (ROS) level in human platelets. Washed **(A)** or CM-H_2DCFDA-loaded **(B)** human platelets were stimulated with collagen (10 μg/ml), thrombin (0.2 U/ml), U46619 (1 μM), or ADP (50 μM) for 5 min. Platelet aggregation was demonstrated by the change in light transmission **(A)**. The intracellular ROS level was measured by a spectrofluorophotometer **(B)**. Data are presented as mean ± S.D. of triplicates.



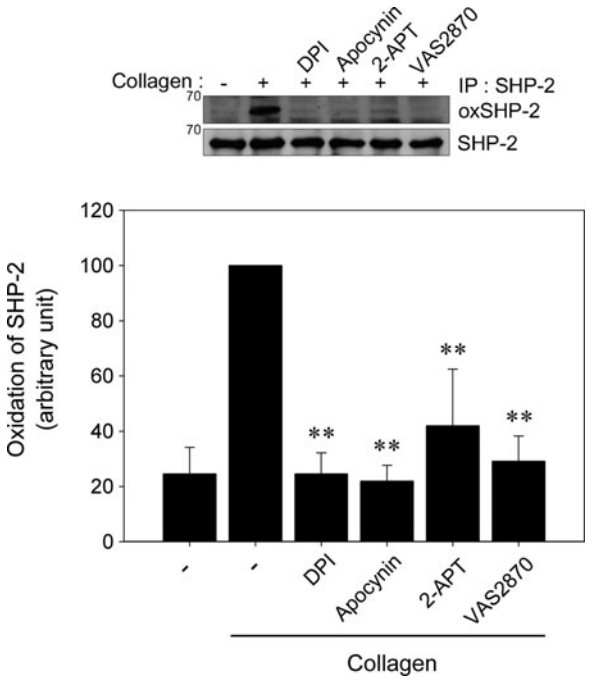
SUPPLEMENTARY FIG. S2. Effects of N-acetylcysteine (NAC) or diphenyl iodonium (DPI) on thrombin-induced aggregation in human platelets. Washed human platelets were preincubated for 5 min in the presence of vehicle, NAC (1 m*M*), or DPI (50 μM) as indicated and then stimulated in an aggregometer with thrombin (0.2 U/ml) under constant stirring for 5 min. Platelet aggregation was assessed and expressed as described in Figure 1A. **(A)** The representative aggregation peaks from three independent experiments are shown. **(B)** The quantitative data are the mean ± S.D.



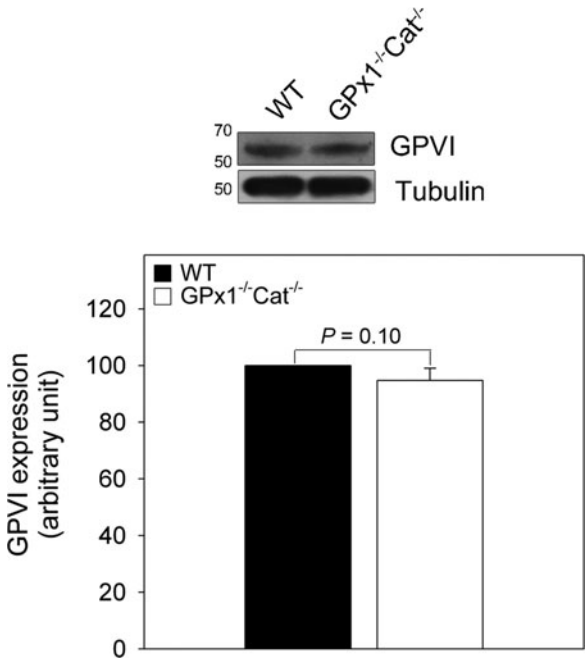
SUPPLEMENTARY FIG. S3. SH2 domain-containing PTP (SHP)-1 is not oxidized in collagen-stimulated human platelets. After pervanadate (100 μM) or collagen (10 μg/ml) stimulation for the indicated times, human platelets were lysed and immunoprecipitated with antibody specific for SHP-1. SHP-1 oxidation was detected as described in Figure 2B. The immunoblots shown are representative of three independent experiments.



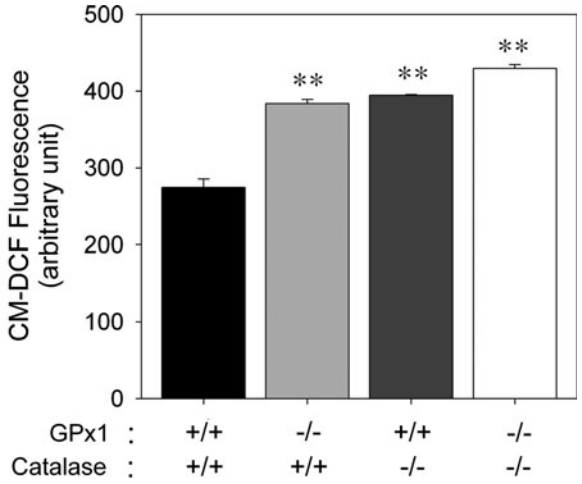
SUPPLEMENTARY FIG. S4. Collagen-related peptide (CRP) leads to SHP-2 oxidation in human platelets. The human platelets were stimulated with CRP (2 μg/ml) for the indicated times. The cell lysates were labeled with PEO-iodoacetyl biotin. Biotin incorporation into SHP-2 was assessed as described in Figure 2C. The immunoblots shown are representative of three independent experiments. The quantitative data are represented by the percent change in biotin incorporation *versus* an unstimulated control, after being normalized to the amount of SHP-2. The data are the mean ± S.D. ($n = 3$; $^{**}p < 0.01$ *versus* unstimulated control).



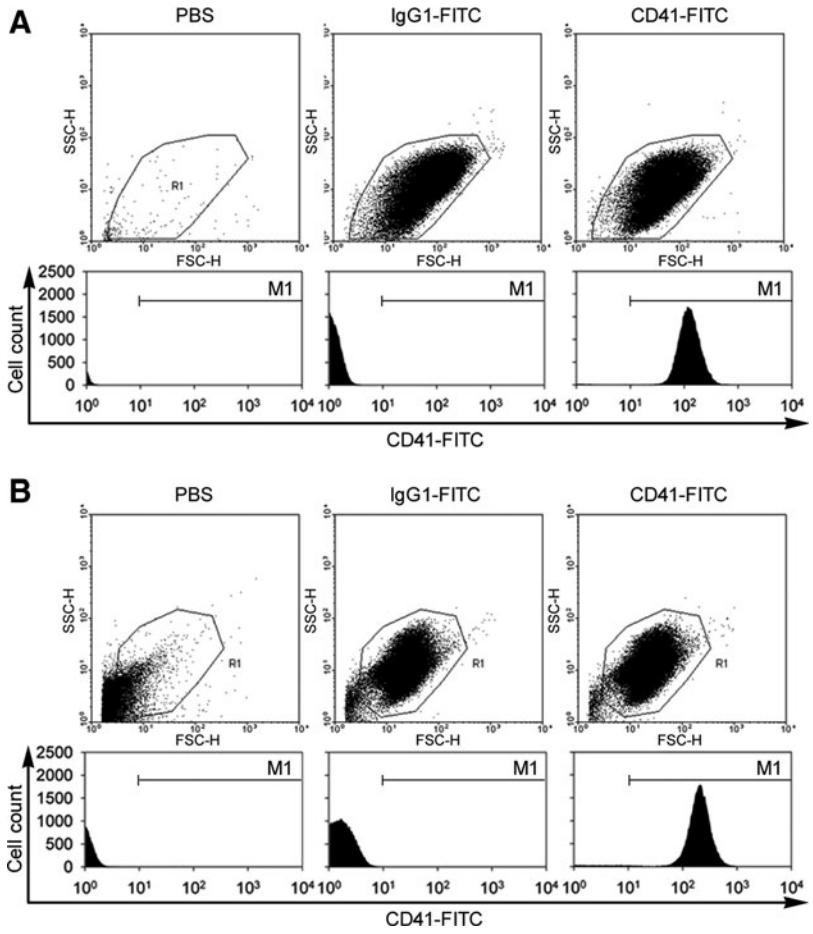
SUPPLEMENTARY FIG. S5. Effects of NADPH oxidase inhibitors on SHP-2 oxidation in collagen-stimulated human platelets. After preincubation with DPI (50 μM), apocynin (100 μM), 2-acetylphenothiazine (2-APT, 0.5 μM), or VAS2870 (100 μM) for 5 min, the human platelets were stimulated with collagen (10 μg/ml) for 2 min. SHP-2 oxidation was assessed as described in Figure 2A. The immunoblots shown are representative of three independent experiments. The quantitative data are the mean ± S.D. $n = 3$; $^{**}p < 0.01$ *versus* stimulated control for an arbitrary amount of oxidized SHP-2 after being normalized to the total amount of SHP-2.



SUPPLEMENTARY FIG. S6. Glutathione peroxidase 1 (GPx1)/catalase double deficiency does not influence the expression of glycoprotein VI (GPVI) in platelets. Washed platelets from wild-type (WT) and GPx1/catalase double-deficient ($GPx1^{-/-}Cat^{-/-}$) mice were lysed and immunoblotted with antibodies specific for GPVI or tubulin. Tubulin expression was used as a control for loading. The immunoblots shown are representative of three independent experiments. The quantitative data are the mean ± S.D. ($n = 3$).



SUPPLEMENTARY FIG. S7. Collagen-induced intracellular ROS generation in GPx1- or/and catalase-deficient platelets. CM-H_2DCFDA-loaded WT, GPx1$^{-/-}$, Cat$^{-/-}$, and GPx1$^{-/-}$Cat$^{-/-}$ platelets were stimulated with collagen (10 μg/ml) under constant stirring for 5 min, and fluorescence was monitored using a spectrofluorophotometer. The quantitative data are the mean ± S.D. $n = 3$; $^{**}p < 0.01$ of the CM-DCF fluorescence.



SUPPLEMENTARY FIG. S8. The purity of isolated platelets is evaluated using flow cytometry. Isolated human **(A)** or mouse **(B)** platelets were stained with isotype control antibody (IgG1-FITC) or CD41-FITC antibody. Purity of isolated platelets was analyzed using flow cytometry. (*Upper*) Forward- and side-scatter scales (FSC and SSC) were set to logarithmic to better distinguish platelets (R1 gate) from debris and other cells (red blood cells or white blood cells). (*Lower*) The histograms represent fluorescence intensity (FL1) of CD41 on platelets within the R1 gate. Representative data from three independent experiments are shown. Morphology and surface marker staining confirmed that platelet purity was >99%.