Platelet protease-activated receptor (PAR)4, but not PAR1, associated with neutral sphingomyelinase responsible for thrombin-stimulated ceramide-NF-κB signaling in human platelets

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Supplementary Appendix

Design and Methods

Reagents and antibodies

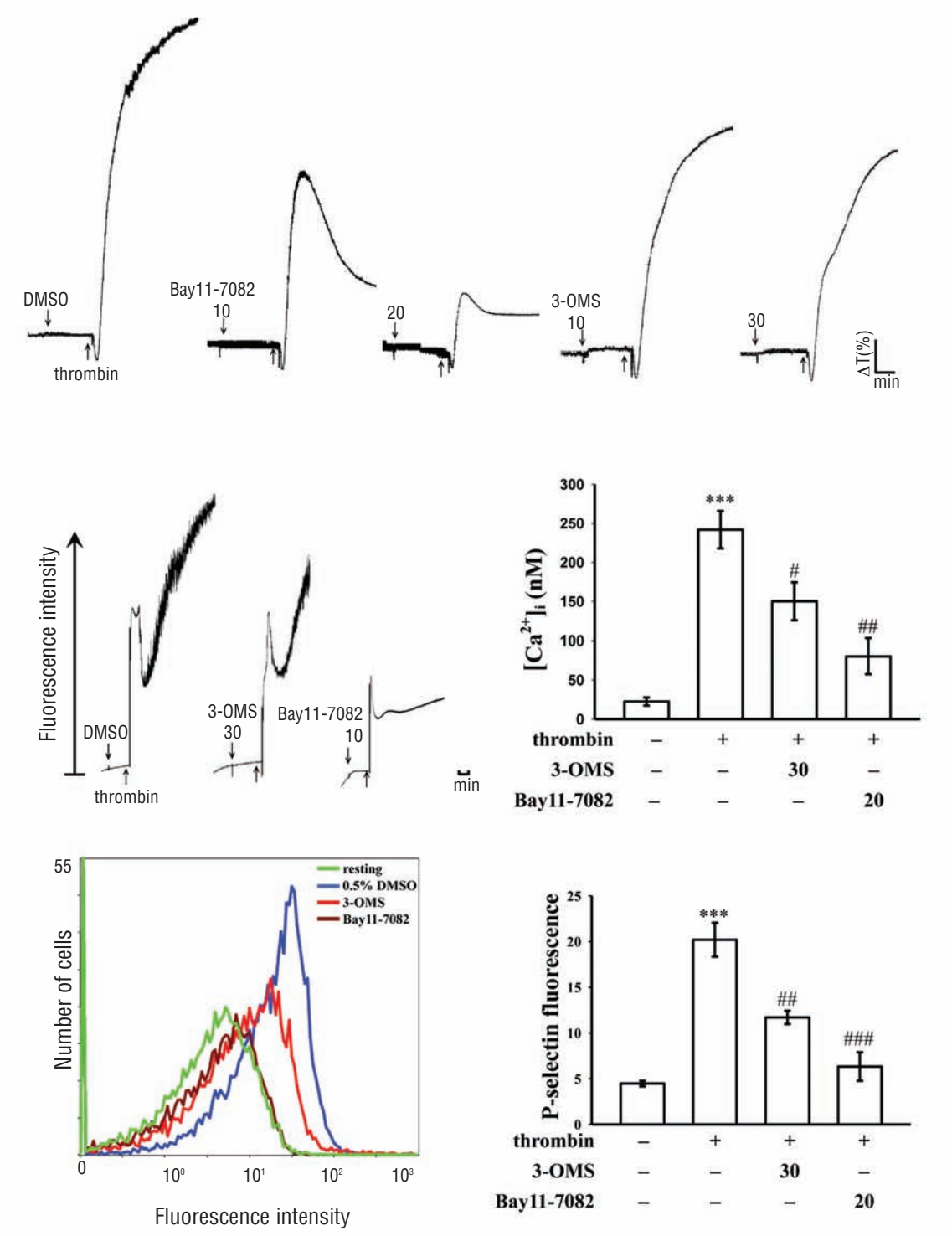
Thrombin, PAR1-AP (SFLLRN-NH_2), PAR4-AP (AYPGFK-NH_2), C2-ceramide, dihydroceramide C2 (DHC), PD98059, SB203580, SP600125, and anti-SMPD3 (nSMase2) monoclonal antibody (mAb) were purchased from Sigma Chemicals (St Louis, MO, USA). SCH79797 and trans-cinnamoyl-YPGKF-NH2 (tcY-NH2) were purchased from Tocris Bioscience (Ellisville, MO, USA). Fura 2-AM was purchased from Molecular Probe (Eugene, OR, USA), and the anti-thrombin R mAb, anti-PAR4 polyclonal antibody (pAb), and the protein A/G plus-agarose were obtained from Santa Cruz (Santa Cruz, CA, USA). The anti-phospho IKK (Ser^{180}/Ser^{181}) pAb, anti-phospho I B , anti-phospho-p38 mitogen-activated protein kinase (MAPK) Ser^{182}, and the anti-p38 MAPK mAbs were purchased from Cell Signaling (Beverly, MA, USA). The anti-human P-selectin (CD62P)-fluorescein mAb was purchased from R&D Systems (Minneapolis, MN, USA), and the anti-tubulin mAb was obtained from NeoMarkers (Fremont, CA, USA). The Hybond-P PVDF membrane, ECL Western blotting detection reagent and analysis system, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, and sheep anti-mouse IgG were obtained from Amersham (Buckinghamshire, UK). The 3-O-methyl-sphingomyeline (3-OMS) was purchased from Biomol (Plymouth Meeting, PA, USA), and the C16:0, C17:0, C18:0, C24:0, and C24:1 types of ceramide were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Immunoblotting study

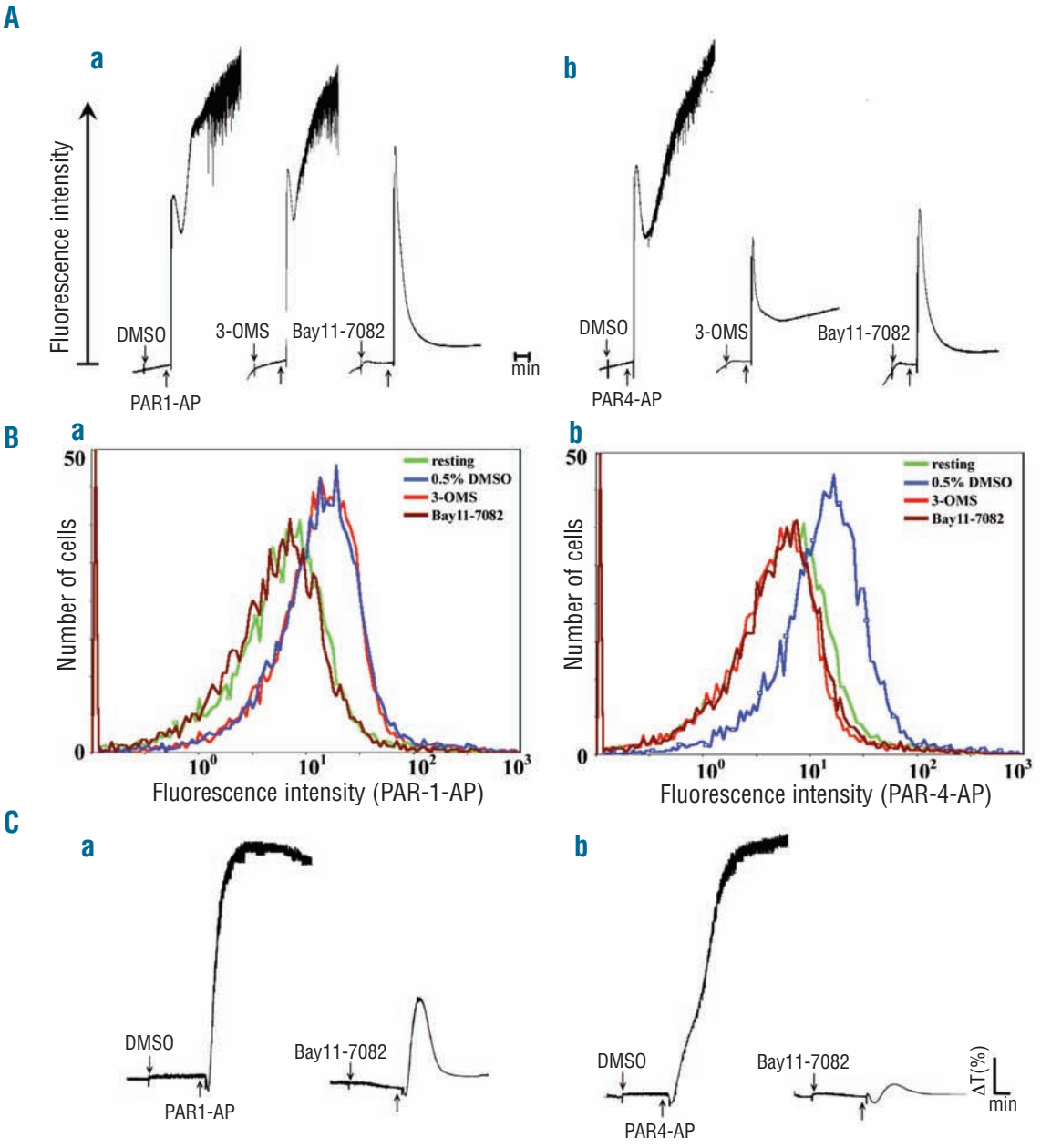
Washed platelets (1×10^9 cells/mL) were pre-incubated with reagents for 3 min followed by the addition of agonists to trigger platelet activation. The reaction was stopped, and platelets were immediately resuspended in 200 L of lysis buffer for 1 h. Lysates were centrifuged at 5000 g for 5 min. Samples containing 80 μg of protein were separated by 12% SDS-PAGE; proteins were electrotransferred by semi-dry transfer (Bio-Rad, Hercules, CA, USA). Blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl, and 0.01% Tween 20) containing 5% BSA for 1 h and were then probed with various primary antibodies. Membranes were incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system. A bar graph showing the ratios of semiquantitative results was obtained by scanning reactive bands and quantifying the optical density, using videodensitometry software (Bio-profil; Biolight Windows Application V2000.01, Vilber Lourmat, France).

Statistical analysis

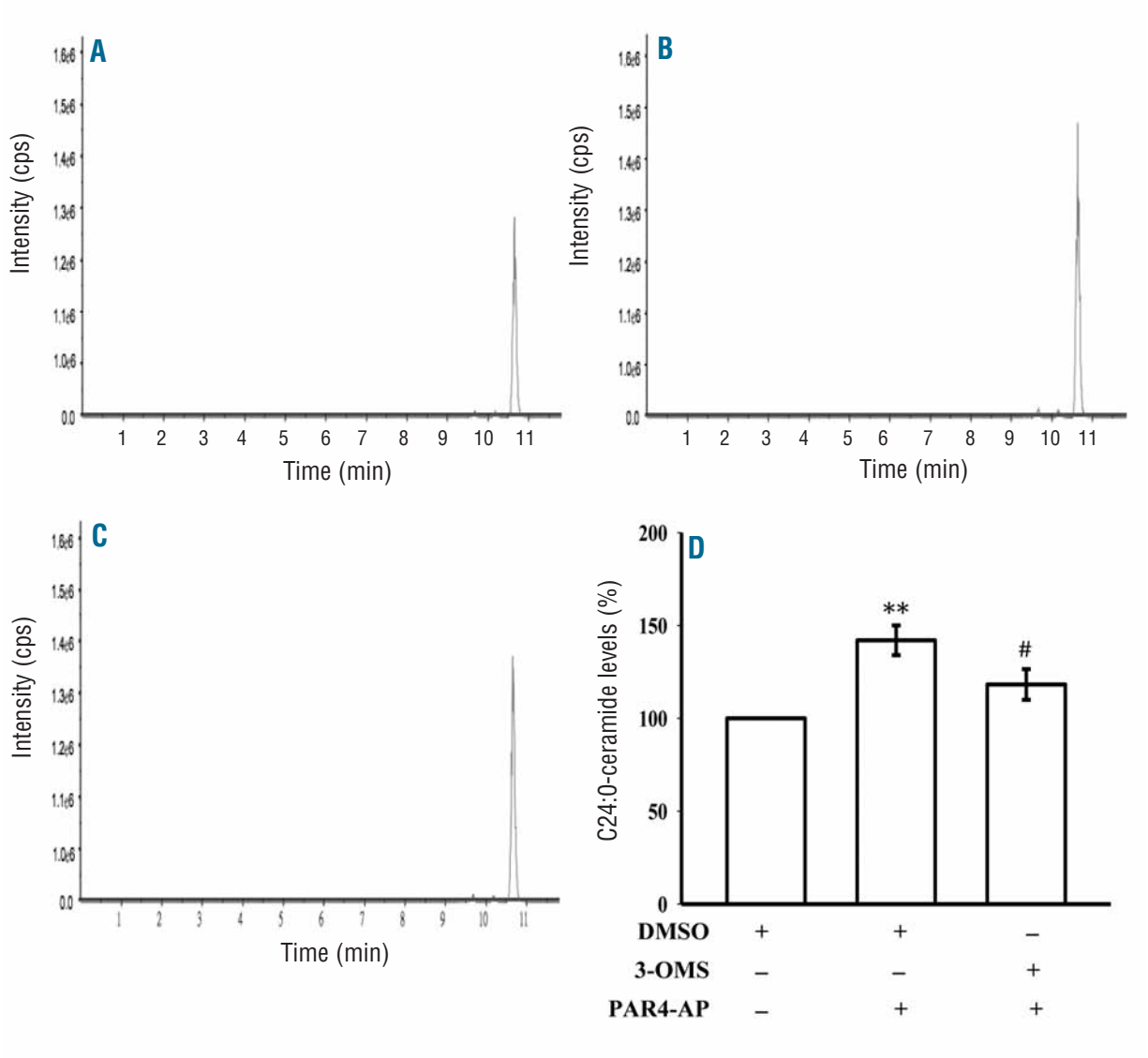
Experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations (n). Values of n refer to the number of experiments, each made with different blood donors. Paired Student's t-test was used to identify significant differences among the *in vivo* groups for platelet plug formation. Other results were assessed by analysis of variance (ANOVA). If ANOVA indicated a significant difference among group means, each group was compared using the Newman-Keuls method. $P<0.05$ was considered statistically significant.



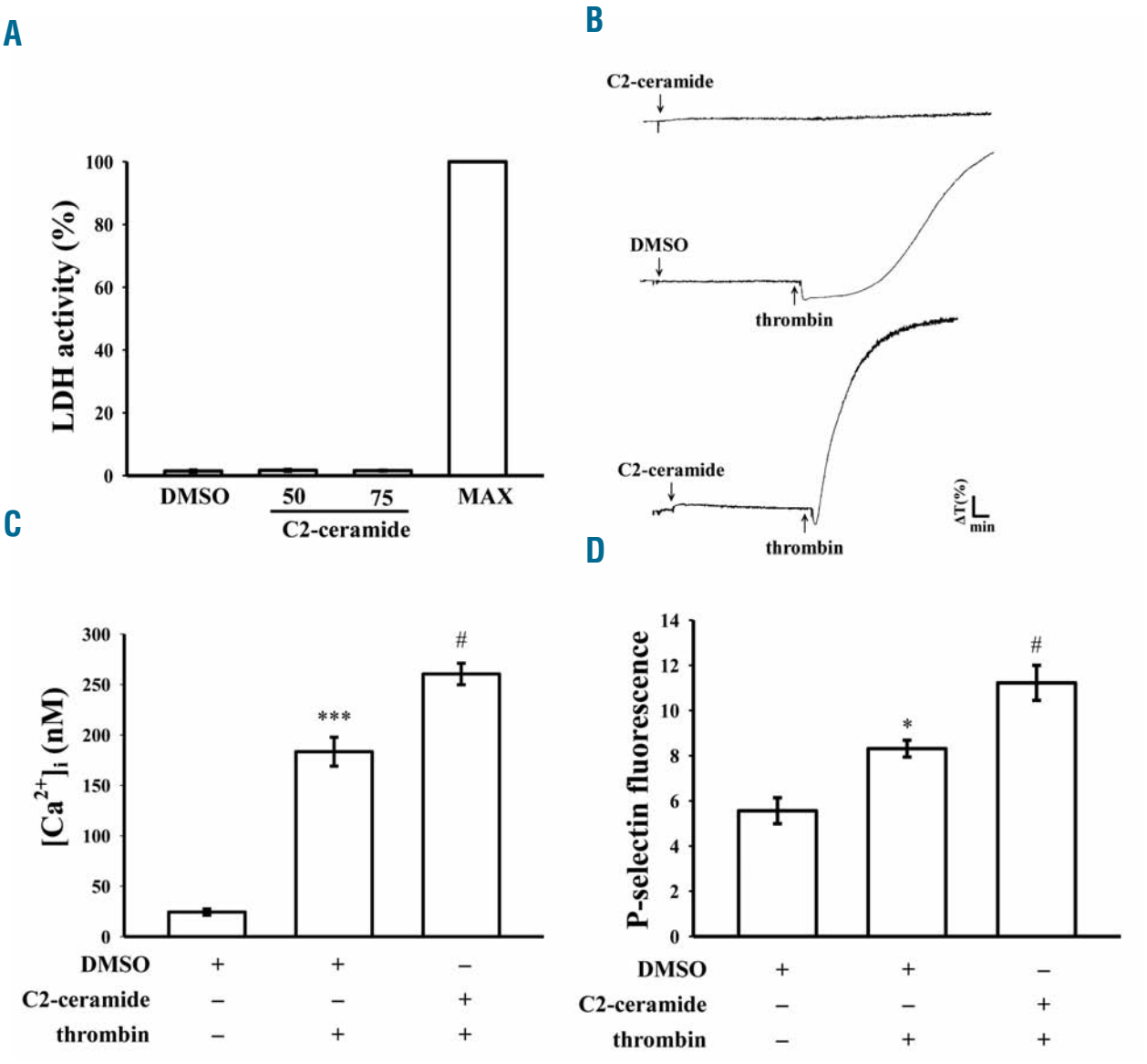
Online Supplementary Figure S1. 3-OMS and Bay11-7082 regulation of platelet aggregation, relative $[Ca^{2+}]_i$ mobilization, and P-selectin expression in thrombin-activated platelets. Washed platelets ($3.6x10^8$/mL) were preincubated with Bay 11-7082 (10 and 20 μM), 3-OMS (10 and 30 μM), or a solvent control (0.5% DMSO). Thereafter, thrombin (0.05 U/mL) was added to trigger (A) platelet aggregation, (B) relative $[Ca^{2+}]_i$ mobilization, and (C) P-selectin expression. The solid lines in C represent the fluorescence profiles of anti-human P-selectin monoclonal antibody (mAb) in the resting platelets (green line), or in platelets pre-incubated with 0.5% DMSO (blue line), 3-OMS (30 μM, red line), or Bay11-7082 (20 μM, brown line) before addition of anti-human P-selectin-fluorescein mAb, followed by thrombin (0.05 U/mL) treatment. Profiles A, B, and C are representative examples of 4 similar experiments. Data in B and C are presented as the means ± S.E.M. (n=4); ***P<0.001, compared with the control (resting) group; #P<0.05, ##P<0.01, and ###P<0.001, compared with the thrombin-treated group.



Online Supplementary Figure S2. OMS and Bay11-7082 regulation of platelet activation stimulated by PAR1-AP or PAR4-AP in washed human platelets. Washed platelets ($3.6x10^8$/mL) were pre-incubated with 3-OMS (30 μM) or Bay11-7082 (20 μM), or a solvent control (0.5% DMSO). Thereafter, PAR1-AP (1 μM) or PAR4-AP (25 μM) was added to trigger (A) relative $[Ca_{2+}]^i$ mobilization, (B) P-selectin expression, and (C) platelet aggregation. The solid lines in B represent the fluorescence profiles of resting platelets (green line), or in platelets pre-incubated with 0.5% DMSO (blue line), 3-OMS (red line), or Bay11-7082 (brown line), followed by (a) PAR1-AP or (b) PAR4-AP treatment. Profiles A, B, and C are representative examples of 4 similar experiments.



Online Supplementary Figure S3. Effect of 3-OMS in C24:0-ceramide formation in PAR4-AP-stimulated platelets by LC-MS/MS analysis. Resting platelets (A; 1x10^6/mL) or platelets were pre-incubated with a solvent control (B; 0.5% DMSO) or 3-OMS (C; 30 μM), followed by the addition of PAR4-AP (25 μM) to trigger C24:0-ceramide formation. The full procedure for LC-MS/MS analysis is described in the main text *Design and Methods* section. (D) indicates the C24:0-ceramide formation shown as the means ± S.E.M. (n=4); $^{**}P<0.01$, compared with the DMSO group; #$P<0.05$, compared with the DMSO+PAR4-AP group.



Online Supplementary Figure S4. Effects of C2-ceramide on lactate dehydrogenase (LDH) release and platelet activation in washed human platelets. (A) Washed platelets ($3.6x10^8$/mL) were pre-incubated with 0.5% DMSO or C2-ceramide (50 and 75 μM) for 30 min, and a 10- L aliquot of supernatant was deposited on a Fuji Dri-Chem slide LDH-PIII for the LDH assay. A maximal value (MAX) of LDH was observed from sonicated platelets. For the other experiments, washed platelets ($3.6x10^8$/mL) were pre-incubated with 0.5% DMSO or C2-ceramide (50 μM), followed by the addition of thrombin (0.03 U/mL) to trigger (B) platelet aggregation, (C) relative $[Ca_{2+}]^i$ mobilization, and (D) P-selectin expression. The profiles (B) are representative examples of 4 similar experiments. (A, C, and D) Means ± S.E.M. (n=4). *P<0.05 and ***P<0.001, compared with the DMSO group; #P<0.05, compared with the DMSO + thrombin group.