Supporting Information

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SI Materials and Methods

Preparation of Washed Platelets. Blood was collected by venipuncture from healthy volunteers who had provided written consent (St. Thomas's Hospital Research Ethics Committee, reference 07/Q0702/24) into trisodium citrate (0.32% final). Platelet-rich plasma was obtained from whole blood by centrifugation ($230 \times g$, 15 min), and platelets were isolated by further centrifugation ($1{,}100 \times g$, 10 min) in the presence of prostaglandin I_2 (PGI_2; 1 μg/mL). The resulting pellets were washed twice in Tyrode's buffer, at pH 7.4, containing Hepes (20 mM; Sigma), resuspended to 3×10^8 platelets/mL, and allowed to rest for at least 30 min for the effects of PGI_2 to dissipate.

Light Transmission Aggregometry. Washed platelet suspensions were incubated (30 min, room temperature) with the $P2Y_{12}$ receptor blocker prasugrel-active metabolite (PAM; 3 μM; a kind gift of AstraZeneca) or vehicle (0.5% DMSO). Aggregation to thrombin (1 U/mL; Sigma), collagen-related peptide [CRP-XL 1 μg/mL (a kind gift of Richard Farndale, University of Cambridge, Cambridge, United Kingdom), in the presence of 1 mg/mL human fibrinogen (Sigma)], Horm collagen (3 μg/mL; Nycomed; in the presence of 1 mg/mL human fibrinogen) or epinephrine (10 μM; Chronolog) was measured in a Bio/Data PAP-8E turbidimetric aggregometer (37 °C, 1,200 rpm stir speed, 4 min) after preincubation (1 min, 37 °C) with the NO donor diethlyammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA/NONOate, Sigma) or vehicle. Although readily soluble in water, DEA/NONOate spontaneously decomposes to NO at physiological pH and was therefore dissolved in 10 mM NaOH. The addition of NaOH (1 mM final) did not alter the pH of the platelet suspension buffer. The half-life of DEA/NONOate (2 min) is such that ~75% of total NO release would occur over the course of a 4-min aggregation experiment.

As required, and in parallel with PAM, platelets were additionally pretreated with milrinone (1 μM; Tocris Bioscience), *erythro*-9-2-hydroxy-3-nonyladenine (10 μM; Tocris Bioscience), sildenafil (10 μM; a kind gift of Adrian Hobbs, The William Harvey Research Institute, London), wortmannin (200 nM; Tocris Bioscience), aspirin (30 μM; Sigma), or N5- [imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride (L-NAME) (10 μM; Sigma). In some experiments, L-arginine (100 μM; Sigma) was added 5 min before thrombin (1 U/mL) to stimulate platelet NOS activity. In other experiments, 8-(4-chlorophenylthio)guanosine-3′, 5′cyclic monophosphorothioate, Rp-isomer (Rp-8-pCPT-cGMPS) (200 μM) or KT5823 (5 μM) was added 10 min before thrombin (1 U/mL) to inhibit cGK activity.

96-Well Plate Aggregometry. In some experiments, platelet aggregation was assessed in 96-well plates, as we have previously reported (1–4). Briefly, washed platelets were treated as described earlier and then plated out as 50-μL aliquots into individual wells of a 96-well microtiter plate (Nunc). DEA/NONOate (0.1 nM–100 μM) or vehicle (0.01 M NaOH) was then added to the wells, and the plate was gently mixed for 1 min. The platelets were then activated by the addition of agonists, thrombin (0.5 or 1 U/mL) or CRP (0.5 or 1 μg/mL in the presence of 1 mg/mL human fibrinogen), and vigorous shaking (1,200 rpm at 37 °C for 5 min) on a microplate shaker (BioShake IQ, QUANTIFOIL Instruments). Absorbance was then measured at 595 nm (Sunrise microplate reader; Tecan), and percentage platelet aggregation was calculated with reference to the absorbance of Tyrodes-Hepes buffer taken as a surrogate for 100% aggregation.

Isobolographic Analysis. Using data from 96-well platelet aggregation experiments, curves of inhibition of thrombin/CRP-induced platelet aggregation by DEA/NONOate in the presence of increased concentrations of PAM or ticagrelor were produced. These data were fitted to a logistic equation using least-squares method (GraphPad Prism 5), and for each curve, the concentration required to inhibit aggregation by 25%, 50%, and 75% was calculated. In some cases, to demonstrate 50% or 75% inhibition, this required interpolation of the curve beyond experimental data. From these data, isobolograms were plotted for each level of inhibition. To do this, for each inhibitor curve, the concentration of PAM/ticagrelor was plotted on the X-axis, and the concentration of DEA/NONOate on the Y-axis. Thus, a scatter plot is produced showing the combinations of DEA/NONOate and PAM/ticagrelor that produce a given level of inhibition. For comparison, a theoretical additive relationship is plotted that represents a straight line connecting the inhibitory concentrations of either PAM or ticagrelor and DEA/NONOate applied alone. The construction of these isobolograms is detailed in Fig. S5.

Whole-Blood Aggregometry. Whole blood, collected into lepirudin (25 μg/mL final; Celgene), was incubated with PAM (3 μM) or vehicle (0.5% DMSO) for 30 min at room temperature. Platelet aggregation to the thrombin receptor activating peptides SFLLRN-amide (10 μM; Bachem) and AYPGKF-amide (30 μM; Bachem) was monitored by impedance aggregometry (Multiplate; Dynabyte) in blood diluted 1:1 with saline, and preincubated (1 min, 37 °C) with DEA/NONOate or vehicle.

ADP+ATP Secretion. Washed platelets, preincubated with PAM or its vehicle, as described earlier, were incubated with DEA/NONOate or vehicle and then activated with thrombin (1 U/mL) and shaking (1,200 rpm, 37 °C; BioShake IQ) in a white 96-well microplate. After 2 min, phosphocreatine (1.5 mM; Sigma), creatine phosphokinase (3,000 U/mL; Sigma), and Chrono-Lume reagent (1:5 vol/vol; Chronolog) were added, and plates were incubated for a further 2 min before the luminescence of each well was read in a Berthold Mithras LB940 multimode plate reader.

P-Selectin Expression and Glycoprotein IIb/IIIa Activation. Washed platelets, preincubated with PAM or its vehicle, as described earlier, were incubated with DEA/NONOate or vehicle and then activated with thrombin (1 U/mL) under nonstirring conditions at 37 °C. After 2 min, the reaction was stopped by dilution with a 10-fold excess of 25 μg/mL lepirudin to halt thrombin activity. Platelets were immediately stained with anti–CD61-allophycocyanin (eBioscience), PAC-1-FITC (BD Bioscience), and anti–P-selectin-PE (eBioscience) for 15 min at 4 °C and then fixed in 2% (vol/vol) formalin (Sigma). PAC-1-FITC and anti–P-selectin-PE immunoreactivity was measured by flow cytometry using a FACSCalibur instrument (Becton Dickenson) by determining the mean fluorescence intensity in the appropriate channel of 15,000 CD61-APC+ events. The specificity of PAC-1 binding was determined using RGDS blocking peptide (Sigma) and by blocking platelet activation with prostacyclin (1 μM). The specificity of anti–P-selectin staining was confirmed by replacement of the antibody with an irrelevant isotype-matched control and by blocking platelet activation with prostacyclin (1 μM). Example control data are shown in Fig. S12.

Akt Phosphorylation. Light transmission aggregometry was performed as described earlier, and the reaction was stopped at 2 min by the addition of an equal volume of 25 mM Tris buffer con-

taining 1% (vol/vol) Nonidet P-40, 5% (vol/vol) glycerol, 150 mM NaCl, 5 mM $MgCl_2$, and 2× protease inhibitor mixture (Roche Applied Bioscience). Samples were sonicated (15 s) and denatured (95 °C, 5 min), and protein concentration was determined by the bicinchoninic acid method. Protein samples (20 μg/lane) were separated by SDS/PAGE, blotted to nitrocellulose membranes, and probed with antiphospho(Ser473)-Akt (New England Biolabs) or anti–pan-Akt primary antibodies (New England Biolabs). Immunoreactivity was visualized using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Dako) and enhanced chemiluminescent detection. Equal protein loading was confirmed by reprobing with anti–β-actin antibodies (Dako). Blots were quantified by densitometry with ImageJ software (National Institutes of Health) and expressed as ratio of intensity of phospho(Ser473)-Akt to total Akt after normalization of each to β-actin band intensity.

Rap1b Activation. Light transmission aggregometry was performed as described earlier, with a washed platelet concentration of 1×10^9/mL, and the reaction was stopped 15 s after the addition of thrombin (1 U/mL) with a 5-fold excess of 25 mM Tris buffer containing 1% Nonidet P-40, 5% glycerol, 150 mM NaCl, 5 mM $MgCl_2$, and 1× protease inhibitor mixture. Samples were snap frozen, and protein concentration was determined by the bicinchoninic acid method. Active Rap1-GTP was purified by RalGDS affinity pull-down, using a commercially available kit (Thermo Scientific). As a control, a sample of raw lysate was treated with an excess of GTPγS before pull-down. Both the eluted Rap1-GTP and raw lysate were separated by SDS/PAGE, blotted to nitrocellulose membranes, and probed with an anti-Rap1 antibody (Thermo Scientific). Immunoreactivity was visualized, using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Dako) and enhanced chemiluminescent detection. Bands corresponding to Rap1-GTP were quantified by densitometry with ImageJ software (National Institutes of Health) and expressed as a fraction of total Rap1 content of raw platelet lysate.

cAMP and cGMP Measurements. Light transmission aggregometry was performed as described earlier, and at 5 min, platelets were lysed with Triton-X-100 (0.625% final) and treated with isobutylmethylxanthine (500 μM final) and potassium fluoride (0.5 M final). cAMP and cGMP concentrations were determined by homogenous time-resolved fluorescence-based competitive immunoassays (Cisbio).

Vasodilator Stimulated Phosphoprotein Phosphorylation. PAM- or vehicle-treated washed platelets were stimulated with thrombin (1 U/mL) in the presence of DEA/NONOate or vehicle. After 4 min, the reaction was stopped with methanol-free formaldehyde (2% final; Fisher Scientific). Platelets were permeabilized (0.2% Triton X-100; Sigma) and incubated with anti–vasodilator stimulated phosphoprotein (VASP)-P(Ser239) primary antibody (Enzo Lifesciences), Alexa647-conjugated secondary antibody (Invitrogen), and FITC-conjugated anti-CD42b (eBiosciences), for 30 min each, in turn, before resuspension in 0.9% saline. VASP-P(Ser239) immunoreactivity was measured by flow cytometry, using a FACSCalibur instrument (Becton Dickenson) by determining the mean VASP-P-Alexa647 fluorescence of 15,000 CD42b-FITC+ events. The specificity of staining was confirmed using a specific blocking peptide (Santa Cruz Biotechnology) and by omission of the primary antibody. Example control data are shown in Fig. S12.

Calcium Mobilization. Platelets were loaded with Fura-2 by incubation with Fura-2 acetoxymethylester (Fura-2 AM) (5 μM, 30 min, 37 °C; Sigma) in platelet rich plasma (PRP) before washing and treatment with PAM or vehicle, as described earlier. Thrombin (1 U/mL)-induced intracellular calcium mobilization, in the presence of DEA/NONOate or vehicle, was measured over the course of 3 min as changes in the ratio of 510 nm emission after excitation at 340 nm and 380 nm in a Perkin-Elmer LS940B fluorometer, as previously described (5). Total Ca^{2+} flux over this time was measured as the area under the trace.

Statistics and Data Analysis. Unless otherwise stated, data were analyzed by two-way ANOVA, using GraphPad Prism 5 for Mac OS X, and differences were considered significant if $P < 0.05$. Summary data (IC_{50}, EC_{50}) were obtained by fitting of data to a logistic equation and tested by Student *t* test (2 groups) or one-way ANOVA (>2 groups). The reader should note that in some cases where only partial responses were obtained at supraphysiological concentrations of DEA/NONOate, inhibitory potencies were interpolated.

1. Armstrong PC, et al. (2009) Utility of 96-well plate aggregometry and measurement of thrombi adhesion to determine aspirin and clopidogrel effectiveness. *Thromb Haemost* 102(4):772–778.
2. Armstrong PC, et al. (2008) Aspirin and the in vitro linear relationship between thromboxane A2-mediated platelet aggregation and platelet production of thromboxane A2. *J Thromb Haemost* 6(11):1933–1943.
3. Chan MV, Armstrong PC, Papalia F, Kirkby NS, Warner TD (2011) Optical multichannel (optimul) platelet aggregometry in 96-well plates as an additional method of platelet reactivity testing. *Platelets* 22(7):485–494.
4. Chan MV, Warner TD (2012) Standardised optical multichannel (optimul) platelet aggregometry using high-speed shaking and fixed time point readings. *Platelets* 23(5): 404–408.
5. Jones S, et al. (2010) The plasma membrane calcium ATPase modulates calcium homeostasis, intracellular signaling events and function in platelets. *J Thromb Haemost* 8(12):2766–2774.

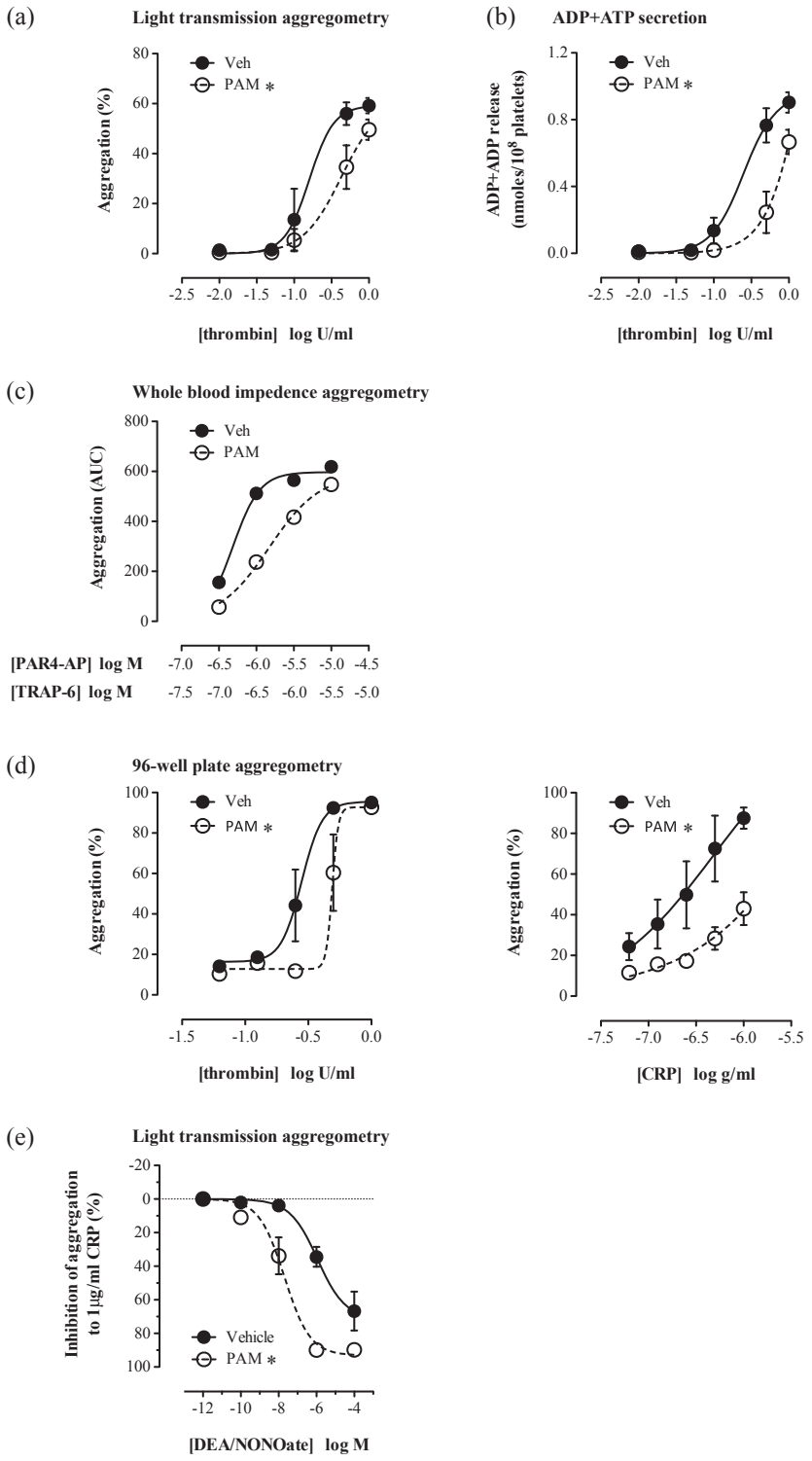

Fig. S1. Effect of $P2Y_{12}$ blockade alone on sensitivity to platelet agonists in different assays. Concentration-response curves to thrombin, protease-activated receptor (PAR)-activating peptides, and CRP in the presence and absence of PAM (3 μM) for washed platelet light transmission aggregometry (*A*), washed platelet luminometric ADP+ATP secretion assays (*B*), whole-blood impedance aggregometry (*C*), or washed platelet 96-well plate aggregometry (*D*). These curves demonstrate the requirement for high, but not supramaximal, concentrations of agonists in experiments with DEA/NONOate to provide a baseline aggregation that is only minimally altered by $P2Y_{12}$ blockade. (*E*) CRP (1 μg/mL)-induced aggregation of washed platelets, determined by changes in light transmission, that was inhibited to a modest degree by the NO donor, DEA/NONOate. This inhibitory effect was greatly potentiated in platelets pretreated with the $P2Y_{12}$ receptor blocker, PAM (3 μM). *$P < 0.05$ by two-way ANOVA for the effect of PAM (n = 3–4).

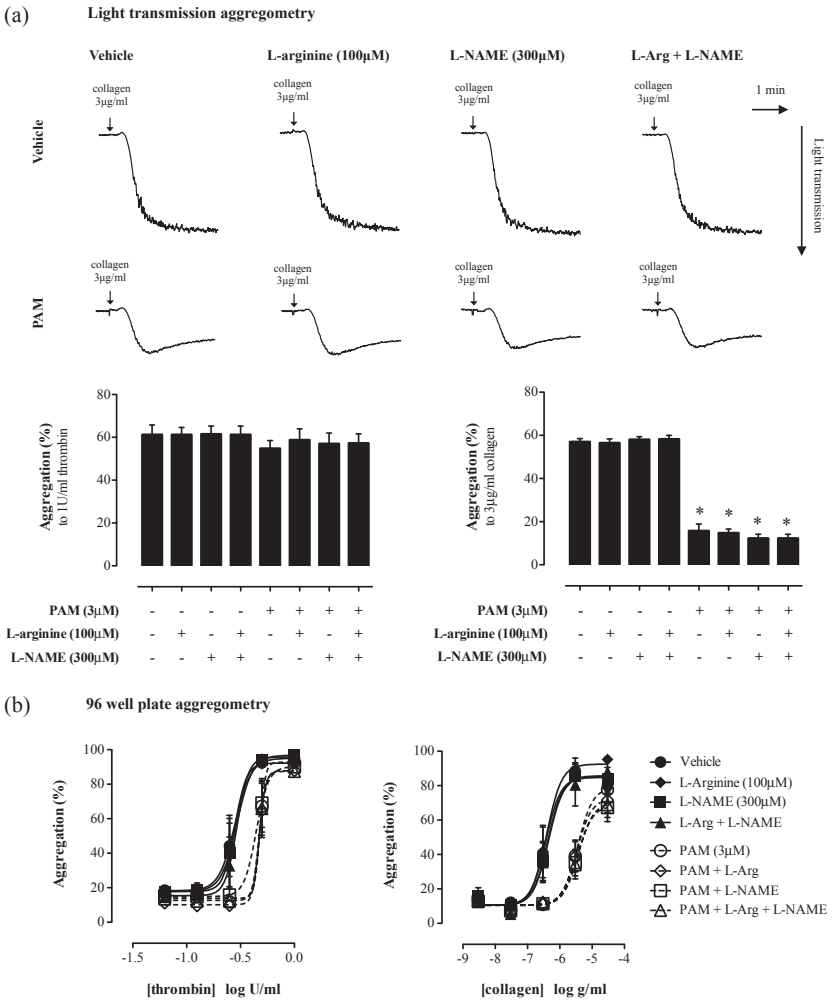

Fig. S2. Endogenous NO does not contribute to platelet aggregation responses in this system in the presence or absence of $P2Y_{12}$ blockade. Thrombin (1 U/mL)- or collagen (3 μg/mL)-induced aggregation of washed platelets, measured by light transmission aggregometry (*A*), was not influenced by addition of L-arginine (100 μM, 5 min incubation) or L-NAME (300 μM, 30 min incubation) to boost or inhibit platelet-derived NO production. Collagen, but not thrombin-induced aggregation, was markedly inhibited by PAM (3 μM). $P2Y_{12}$ receptor blockade did not influence the responses to L-arginine or L-NAME treatment. Parallel experiments using 96-well plate aggregometry (*B*) and a range of thrombin and collagen concentrations similarly found no effect of L-arginine or L-NAME on aggregation responses in the presence or absence of $P2Y_{12}$ receptor blockade. $^*P < 0.05$ by one-way ANOVA vs. vehicle ($n = 4$).

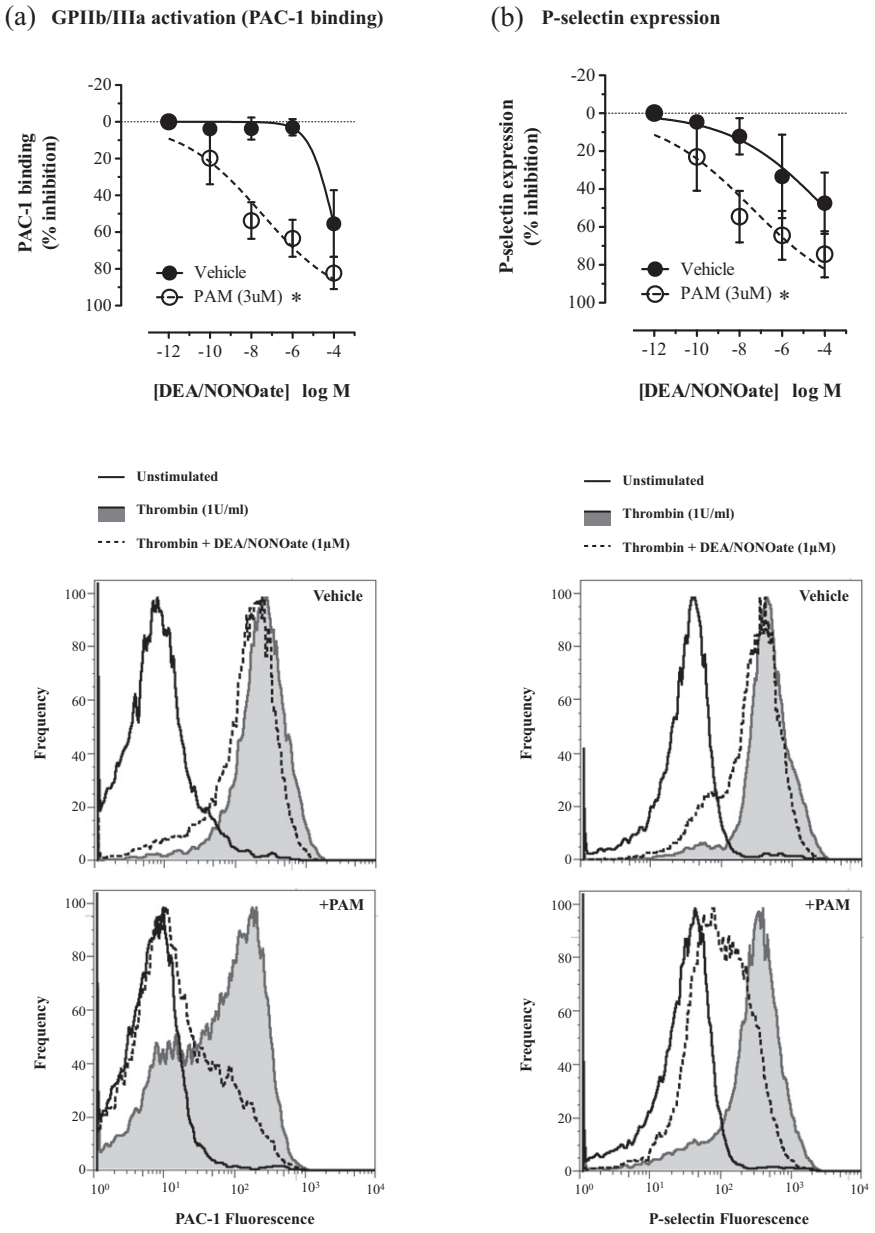

Fig. S3. Blockade of the $P2Y_{12}$ receptor sensitizes platelets to the inhibitory effect of NO against thrombin-induced GPIIb/IIIa activation and alpha granule secretion. Using flow cytometry thrombin (1 U/mL), stimulated GPIIb/IIIa activation was measured by PAC-1 binding (*A*), and alpha granule secretion was measured by P-selectin expression (*B*). DEA/NONOate produced concentration-dependent inhibitions of both of these responses, and these inhibitions were greatly increased by pretreatment of platelets with the $P2Y_{12}$ receptor blocker PAM (3 µM). $*P < 0.05$ by two-way ANOVA for the effect of PAM ($n = 4$).

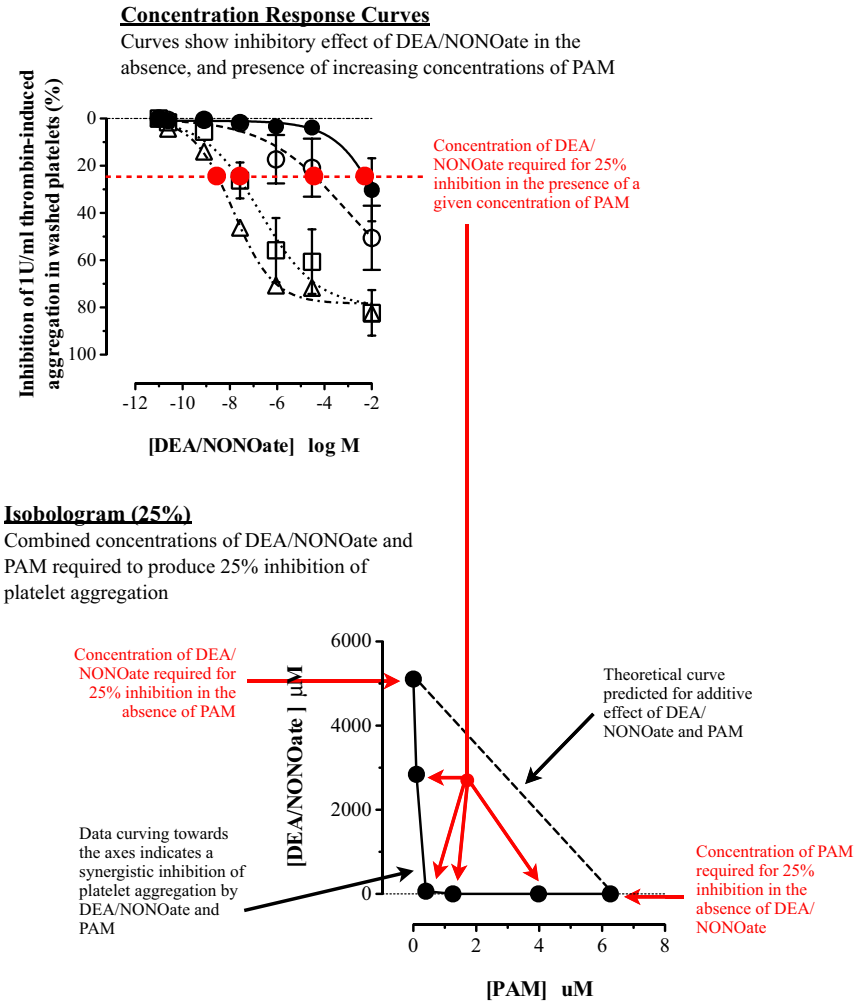

Fig. S4. Construction of isobolograms from matched concentration-response curves. To perform isobolographic analysis, multiple matched 96-well plate aggregation experiments were performed, and inhibition curves were generated showing the concentrations of DEA/NONOate required to inhibit aggregation responses in the presence of specific concentrations of $P2Y_{12}$ receptor blocker. From curves fitted to these data, the concentration of DEA/NONOate required to produce 25%, 50%, and 75% inhibition in the presence of each concentration of $P2Y_{12}$ receptor blocker was calculated. The values were used to construct isobolograms, in which the concentration of $P2Y_{12}$ receptor blocker was plotted on the X-axis and the concentration of DEA/NONOate required to produce a given effect (e.g., 25% inhibition) plotted on the Y-axis. This gives a curve showing the combination concentrations of DEA/NONOate and $P2Y_{12}$ receptor blocker required to produce a given response (e.g., a 25% isobole). This curve is compared with the theoretical linear relationship connecting the concentrations of DEA/NONOate and $P2Y_{12}$ receptor blocker required individually to produce that same effect; that is, the theoretical line corresponding to an additive relationship. If the experimental data are distorted toward the axes away from the theoretical additive line, a synergistic (supraadditive) relationship is demonstrated.

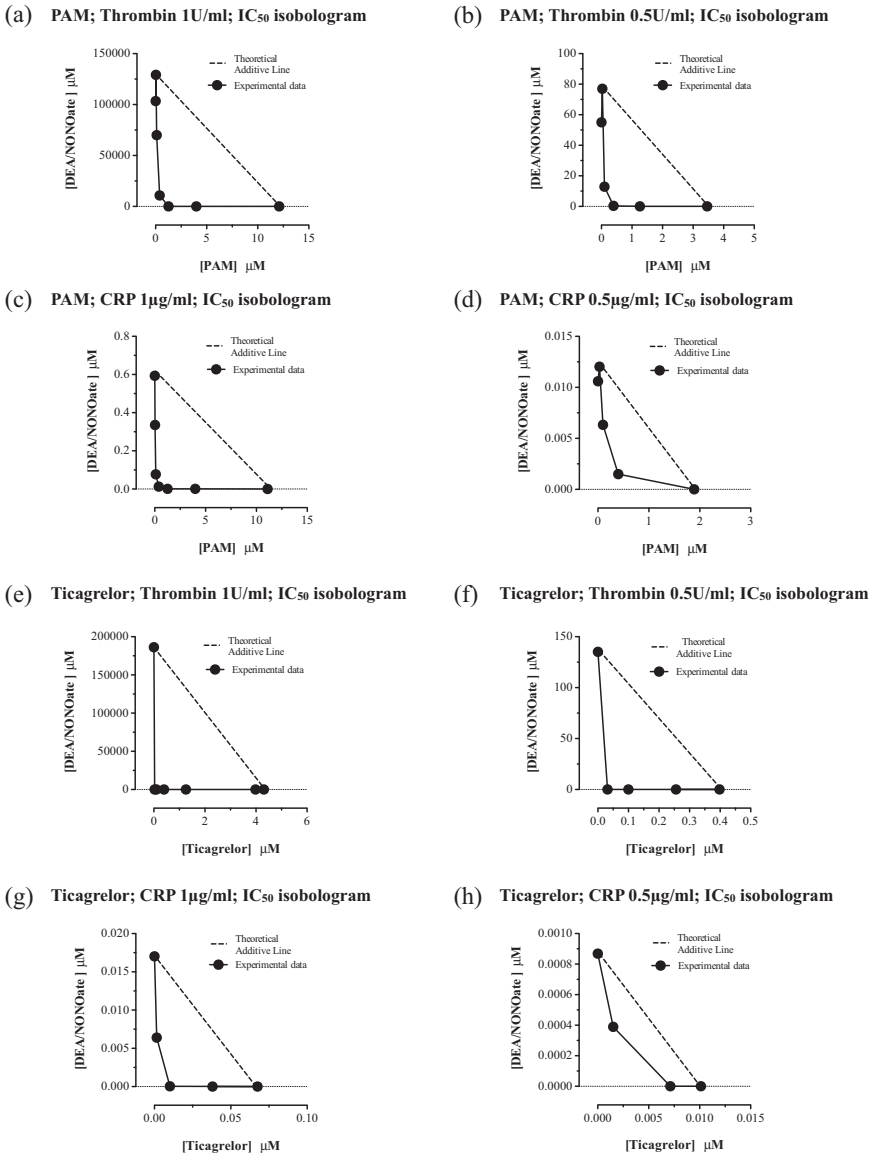

Fig. S5. IC_{50} isobolograms demonstrate that the interaction between $P2Y_{12}$ receptor blockade and NO is synergistic for inhibition of thrombin- and CRP-induced aggregation. Isobolographic analyses of the combinations of the $P2Y_{12}$ receptor blockers PAM (*A–D*) or ticagrelor (*E–H*) and DEA/NONOate required to produce a 50% inhibition of platelet aggregation stimulated by (*A* and *E*) thrombin 1 U/mL, (*B* and *F*) thrombin 0.5 U/mL, (*C* and *G*) CRP 1 µg/mL, or (*D* and *H*) CRP 0.5 µg/mL demonstrate, in all cases, a highly synergistic relationship. This high level of synergism is demonstrated by the markedly greater effect of the drug combinations (isoboles curved toward the axis) than the linear relationship predicted by the arithmetic sum of the effect of either drug alone ($n = 5–6$ for each point).

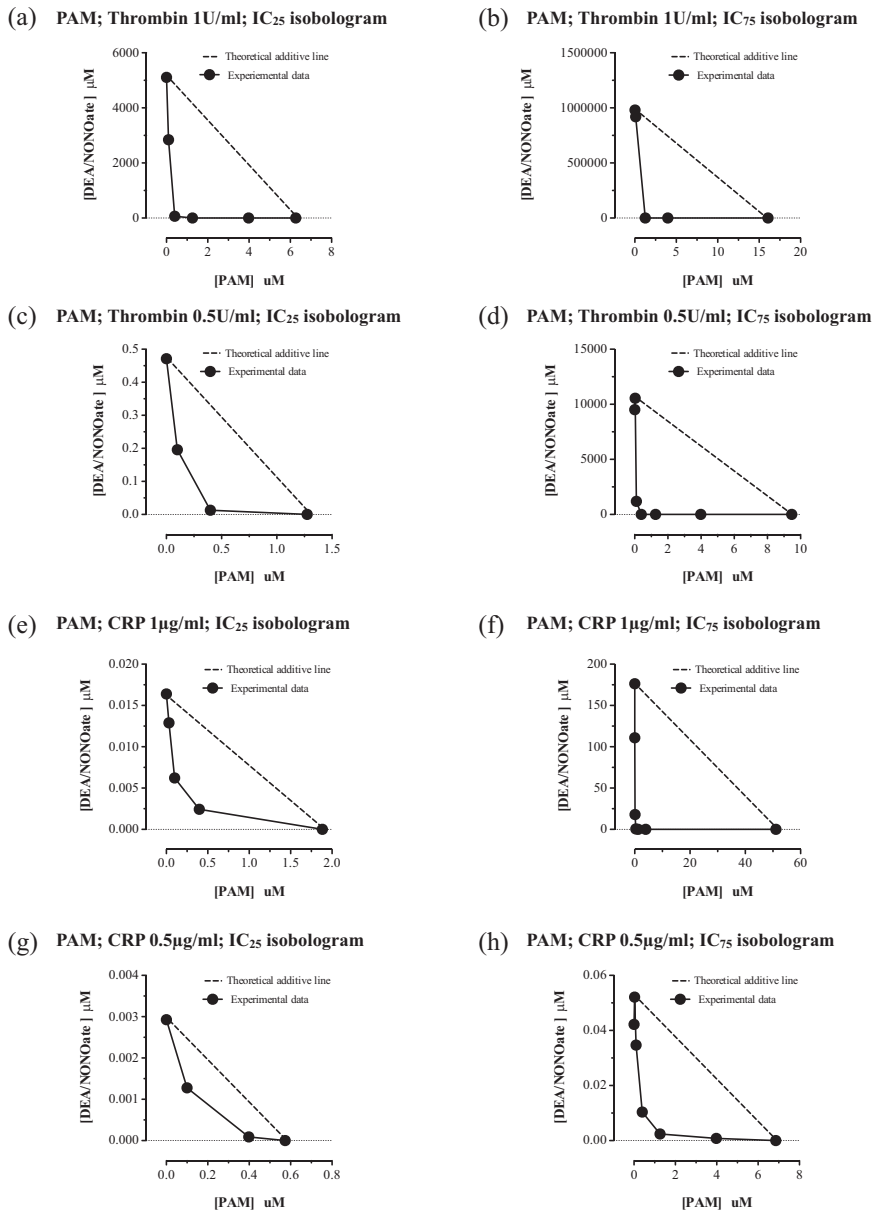

Fig. S6. IC_{25} and IC_{75} isobolograms demonstrate that the interaction between $P2Y_{12}$ receptor blockade and NO is synergistic for inhibition of thrombin- and CRP-induced aggregation. Isobolographic analyses of the combinations of PAM and DEA/NONOate required to produce 25% (*A*, *C*, *E*, and *G*) and 75% (*B*, *D*, *F*, and *H*) inhibition of platelet aggregation stimulated by thrombin 1 U/mL (*A* and *B*), thrombin 0.5 U/mL (*C* and *D*), CRP 1 µg/mL (*E* and *F*), or CRP 0.5 µg/mL (*G* and *H*), display a highly synergistic relationship. This high level of synergism is demonstrated by the markedly greater effect of the drug combinations (isoboles curved toward the axis) than the linear relationship that is predicted by the arithmetic sum of the effect of either drug alone (n = 5–6 for each point).

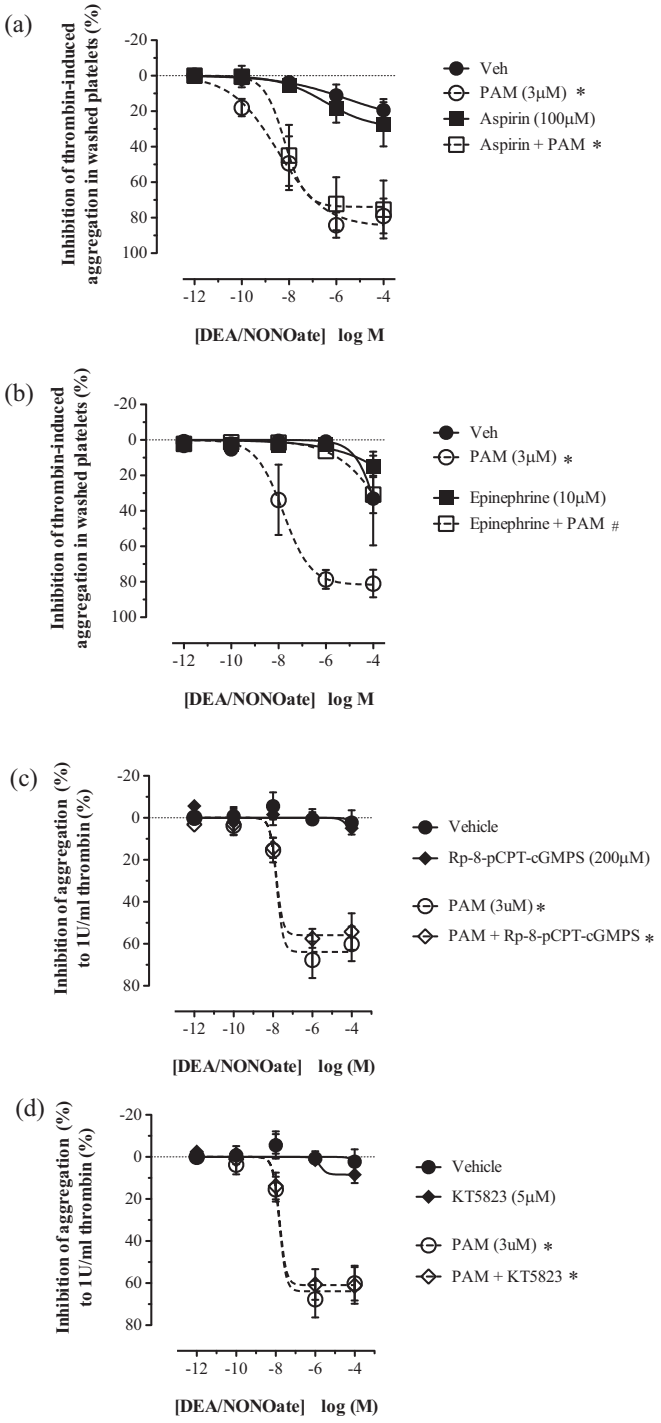

Fig. S7. The synergistic inhibition produced by NO and $P2Y_{12}$ receptor blockade is independent of thromboxane formation but dependent on $G\alpha_{i/z}$ signaling and roles of cGMP-dependent protein kinases. Thrombin (1 U/mL)-induced aggregation of washed platelets was studied by light transmission aggregometry. As previously observed, PAM (3 μM) potentiated the inhibitory effect of DEA/NONOate in this system. Blockade of thromboxane A_2 formation by the cyclooxygenase inhibitor aspirin (100 μM) had no effect on the sensitivity of platelets to DEA/NONOate either in the absence or presence of PAM (*A*). In contrast, addition of epinephrine (10 μM), which restores $G\alpha_{i/z}$ signaling in the presence of $P2Y_{12}$ receptor blockade, completely reversed the ability of PAM to potentiate the antiplatelet effects of DEA/NONOate in this assay (*B*). Two putative cGMP-dependent protein kinase (cGK) inhibitors, Rp-8-pCPT-cGMPS (200 μM) (*C*) and KT5823 (5 μM) (*D*) failed to reverse the inhibition of platelet aggregation produced by the combination of DEA/NONOate and PAM. This may reflect either the involvement of cGK-independent platelet inhibition pathway or the limitations of these experimental tools. $^{*}P < 0.05$ by two-way ANOVA vs. control; $^{\#}P < 0.05$ by two-way ANOVA vs. the same treatment in the absence of epinephrine ($n = 3$–4).

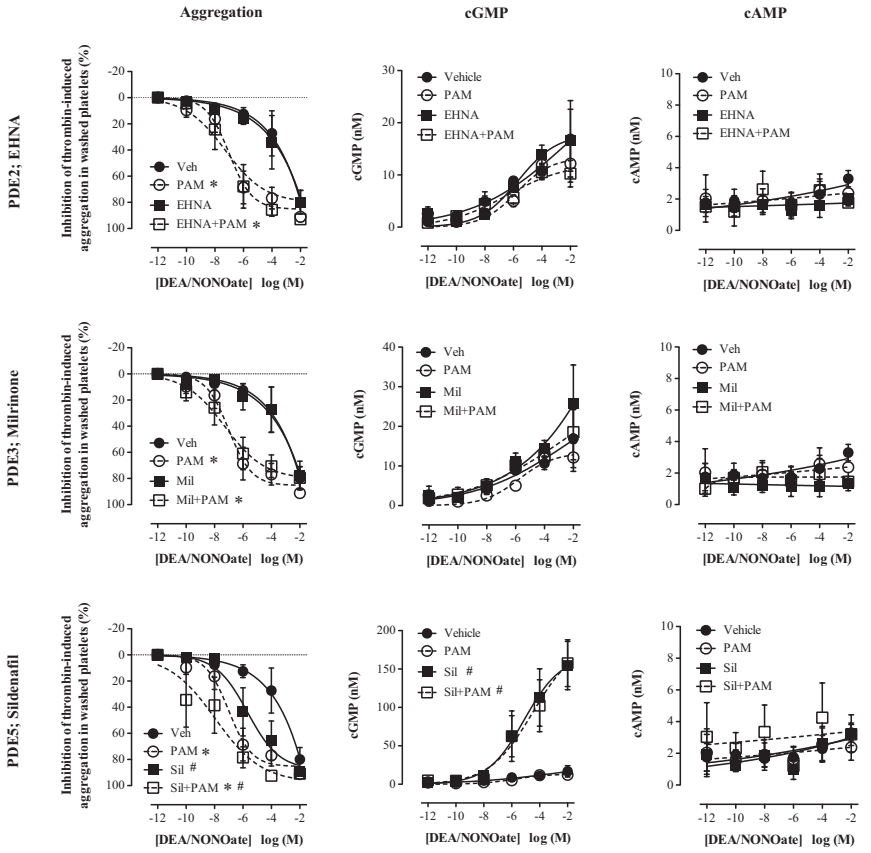

Fig. S8. Inhibition of PDE2, PDE3, or PDE5 does not affect the ability of $P2Y_{12}$ receptor blockade to potentiate the inhibitory effect of NO. Aggregation was measured in thrombin (1 U/mL)-stimulated washed platelets as changes in light transmission, after which, platelets were lysed and cAMP and cGMP content measured by immunoassay. Pretreatment of platelets with the PDE2 inhibitor *erythro*-9-2-hydroxy-3-nonyladenine (10 μM) or the PDE3 inhibitor, milrinone (Mil; 10 μM), had no effect on the ability of DEA/NONOate to inhibit platelet aggregation or stimulate platelet cGMP and cAMP formation. $P2Y_{12}$ receptor blockade by PAM (3 μM) potentiated the antiplatelet effect of DEA/NONOate regardless of concurrent PDE2/3 inhibition. In contrast to *erythro*-9-2-hydroxy-3-nonyladenine and milrinone, the PDE5 inhibitor sildenafil (Sil; 10 μM) enhanced the ability of DEA/NONOate to inhibit aggregation; this was accompanied by enhanced cGMP, but not cAMP, accumulation. Addition of PAM further increased the sensitivity of platelets to DEA/NONOate, but the magnitude of this increase in potency was similar to that seen in the absence of sildenafil, suggesting an additive interaction. $^{*}P < 0.05$ by two-way ANOVA vs. the same treatment in absence of PAM. $^{\#}P < 0.05$ by two-way ANOVA vs. the same treatment in absence of PDE inhibitor ($n = 4$–8).

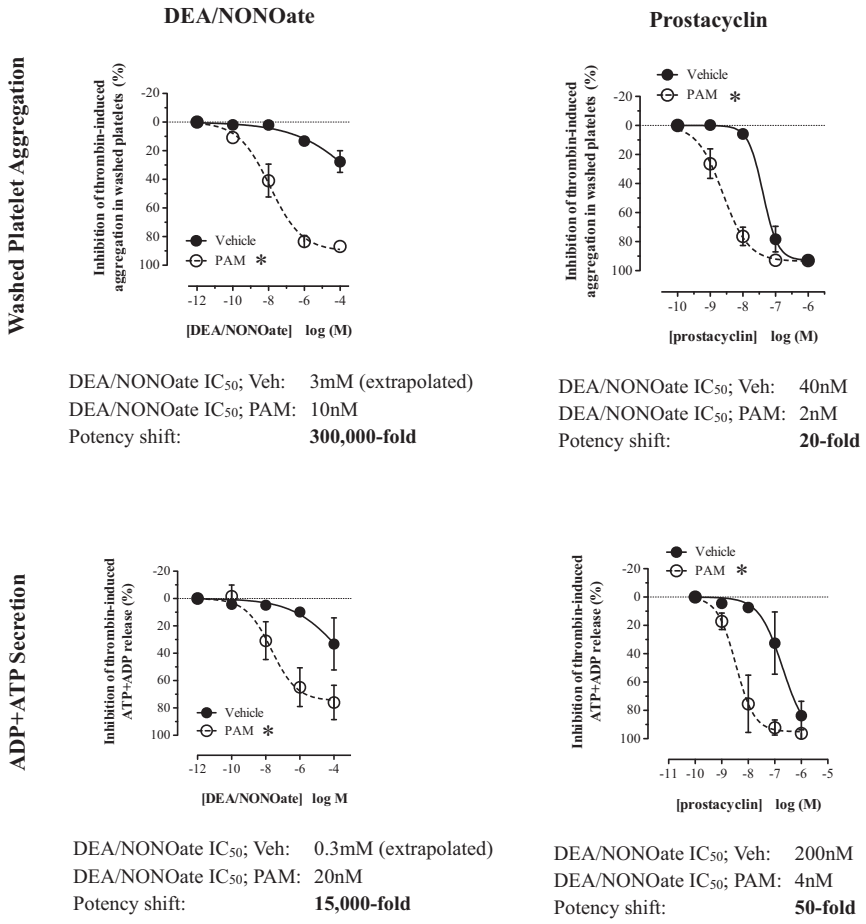

Fig. S9. The interaction between $P2Y_{12}$ receptor blockade and NO is much greater than that between $P2Y_{12}$ receptor blockade and prostacyclin. Thrombin (1 U/mL)-induced aggregation of washed platelets was studied by light transmission aggregometry, and ADP+ATP secretion by luminometry. As previously observed, DEA/NONOate produced only a weak inhibition of these responses, whereas prostacyclin was a more potent and effective inhibitor. Blockade of $P2Y_{12}$ receptors by PAM (3 μM) increased the sensitivity of platelets to both DEA/NONOate and prostacyclin in both assays. The magnitude of this potentiation was, however, far greater for DEA/NONOate (15,000- to 30,000-fold shift) than that observed with prostacyclin (20- to 50-fold shift). $*P < 0.05$ by two-way ANOVA for the effect of PAM ($n = 4–8$).

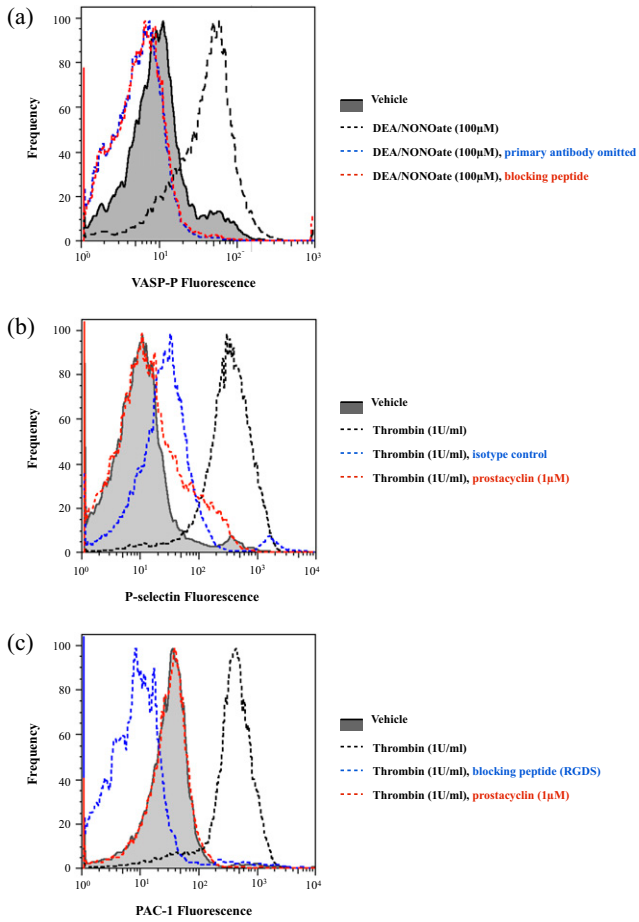

Fig. S10. Representative control data for flow cytometry experiments. VASP phosphorylation (Ser239) was measured in washed platelets by flow cytometry (*A*). An increase in VASP-P(Ser239)-associated fluorescence was observed after treatment of platelets with DEA/NONOate (100 μM), but this increase in fluorescence intensity could be abolished by either omission of the primary antibody or preincubation of the primary antibody with a specific blocking peptide. Alpha granule secretion was measured in washed platelet by flow cytometry for P-selectin expression (*B*). An increase in P-selectin-associated fluorescence was observed after treatment of platelets with thrombin (1 U/mL), but this could be prevented by treatment of platelets with prostacyclin (1 μM) or restored to almost baseline levels by substituting the primary antibody with an irrelevant isotype-matched control antibody. GPIIb/IIIa activation was measured in washed platelets by flow cytometry for PAC-1 binding (*C*). An increase in PAC-1-associated fluorescence was observed after treatment of platelets with thrombin (1 U/mL), but this could be reversed by treatment of platelets with prostacyclin (1 μM) or by preincubating the PAC-1 antibody with RGDS blocking peptide. Histograms are representative of $n = 3$.