The role of neuraminidase 1 and 2 in glycoprotein Ibα-mediated integrin αIIbβ3 activation

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Supplementary Information

The role of neuraminidase 1 and 2 in glycoprotein Ibα-mediated integrin $\alpha_{IIb}\beta_3$ activation

Running head: *The role of neuraminidases in platelet function*

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Number of supplementary Figures: 4

Reagents and sources

We used the following products (with sources): collagen, ristocetin and arachidonic acid (AA, Helena Laboratories, Beaumont, TX, U.S.A.), human α-thrombin, fibrinogen (plasminogen, VWF-, fibronectin-depleted (Enzyme research Laboratories, South Bend, IN, U.S.A.), mono-sialo-dihexosylganglioside (GM3, Avanti Polar Lipids, Alabaster, Alabama, U.S.A.), VWF FXIII-free (Abcam, Melbourne, VIC, Australia), prostaglandin I2 (PGI_2, Cayman Chemical, Ann Arbor, MI, U.S.A.), collagen (NEU-activity assay, Chrono-log corporation, Havertown, PA, U.S.A.), *O*-sialo-glycoprotein Endopeptidase (OSGE, Cedarlane, ON, Canada). ADP, 2′-(4-Methylumbelliferyl)-α-*D*-*N*-acetylneuraminic acid sodium salt hydrate (MUNANA), *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), *N*-acetyl-*D*-glucosamine (GlcNAc), Arg-Gly-Asp-Ser (RGDS) peptide, Triton-X-100, Native human D-Dimer protein and bovine serum albumin (BSA), apyrase, BAPTA-AM-AM, indomethacin, all sourced from Sigma/Merck (Darmstadt, Germany). Peptide-*N*-Glycosidase F (PNGase F, PNGase, New England Biolabs, San Francisco, CA, U.S.A), recombinant neuraminidase (*Clostridium perfringens*, Roche, Sydney, NSW, Australia), laminin (mouse, Life Technologies, Carlsbad, CA, U.S.A.) and fixation buffer (4% paraformaldehyde, BioLegend, San Diego, CA, U.S.A.), IbiTreat microchamber μ-slide VI 0.4 (μ-slide VI0.4, IBIDI, Martinsried, Germany) and ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Antibodies were obtained (with sources): anti-factor V (Haematologic Technologies Inc., Essex Junction, U.S.A.), anti-lysosomal-associated membrane protein-1 (LAMP-1), anti-fibrinogen and anti-VWF (Abcam, Cambridge, MA, U.S.A.), anti-NEU1, anti-NEU2, anti-β-Galactosidase (Santa Cruz Biotechnology, Dallas, TX, U.S.A.), anti-NEU1, anti-mitochondria (Abcam), membrane stain CellBrite 640 (Biotium, Fremont, CA, U.S.A.). Anti-P-selectin antibody (microscopy) and secondary antibodies anti-mouse (Alexa (A)488-conjugated), anti-rabbit (A647) and anti-goat (A647), all sourced from Thermo Fisher, anti-P-selectin-PE (CD62P) and PAC-1-FITC (BD Biosciences, San Diego, CA, U.S.A). Glycan binding lectins *Ricinus communis* agglutinin-1 (RCA-1), wheat germ agglutinin (WGA) and Wheat Germ Agglutinin (WGA), *Erythrina cristagalli* (ECL), Peanut agglutinin (PNA), *Maackia amurensis* lectin-1 (MAL-1), *Sambucus nigra* (SNA) were conjugated to fluorescein and all sourced from Vector Laboratories (Burlingame, CA, U.S.A.).

Methods

Platelet incubations

As large volumes were required, apheresis platelets (Day 1 post-collection and 100% in autologous plasma) were used and diluted to 200x10^9/mL in Tyrode's. Platelets were perfused 5 times (continuous flow) through a microchannel, flow rate of 10,000s^{-1} (59.5mL/min using equation: γ (s^{-1})= 176.1φ x flow rate (γ=flow rate (mL/min) and φ= viscosity (1.047). Prior to perfusion, platelets were stimulated ±risto (0.5mg/mL, 5min 37°C).

NEU activity

NEU activity was measured in plasma, in presence of 75µM sodium acetate (pH 4.5), 0.1% Triton X-100, 0.5mM 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MUNANA) in 96-wells plates after 10min (λ_{ex}=365nm, emission λ_{em}=450nm). NEU activity was measured following Triton-X-100 permeabilisation (0.1%).

Intracellular localisation of NEU1 and NEU2

Platelets were stained with the following antibodies (final concentrations/dilutions) anti-LAMP-1 (1 µg/mL), anti-mitochondria (1.25 µg/mL), anti-factor V (FV, 10 µg/mL), anti-NEU1 (20 µg/mL), anti-P-selectin (1/50), CellBrite 640 (1/50) and anti-NEU2 (1/200, all O/N, 4°C).

Legends Supplementary Tables and Figures.

Suppl. Figure S1: Lectin binding and NEU expression following various treatments.
A) Washed platelets were pre-incubated +/- OSGE (80 µg/mL) or PNGase (10,000 U/mL+1/10 reaction buffer, 60 min, 37°C) and stimulated (VWF (10 µg/mL) + ristocetin (1.2 mg/mL), VWF/risto) and binding to fluorescein-conjugated MAL-1, ECL, PNA, SNA and RCA-1 lectins was measured by flow cytometry (n=3). Data +/- SEM. **B**) Washed platelets were stimulated with VWF/risto with or without recNEU (0.2 U/mL). Fluorescein-conjugated lectins MAL-1, ECL, PNA, SNA and RCA-1 (5 µg/mL) were added and samples were measured. Data was normalised and is shown as % of unstimulated control. *p<0.05 significant against unstim, (n=3, 1-way ANOVA). **C**) Washed platelets were stimulated with ristocetin only (1.2 mg/mL risto) or VWF/risto prior to measurement of binding to fluorescein-conjugated lectins by flow cytometry (n=3). **D**) Washed platelets were stimulated with α-thrombin (0.1 U/mL) and binding to fluorescein-conjugated lectins was measured by FACS (n=4).

Suppl. Figure S2: Granule and lysosome release following VWF-mediated GPIbα activation.
Washed platelets were stimulated with VWF/risto (Table 1), with or without recNEU (0.2 U/mL), calcium (1 mM), fibrinogen (500 µg/mL) and OSGE (80 µg/mL) and **A**) LAMP-1 (n=3), **B**) P-selectin (n=4) and **C**) PAC-1 binding (n=3) was measured. Results are shown as mean fluorescent intensities (MFI) values. Data+/- SEM. *p<0.05 significant against unstim; † = significant against control (1-way ANOVA).

Suppl. Figure S3: DANA inhibits desialylation.
PRP was incubated with/without NEU inhibitor DANA (1 mM, 15 min, 37°C) prior to addition of recNEU (0.2 U/mL, 30 min, 37°C) and fluorescein-conjugated RCA-1 lectin was added. Results are shown as MFI values +/- SEM. *p<0.05 significant against control, (n=3, paired *t*-test).

Suppl. Figure S4: Intracellular location of NEU2.
Unstimulated and stimulated (VWF/risto) non-permeabilised washed platelets were stained for NEU1 (red) and intracellular proteins (green): β-Galactosidase (β-Gal), LAMP-1, factor V (FV), mitochondria (Mt), visualised by fluorescence microscopy. Mitochondrial protein is not retained on the platelet membrane.

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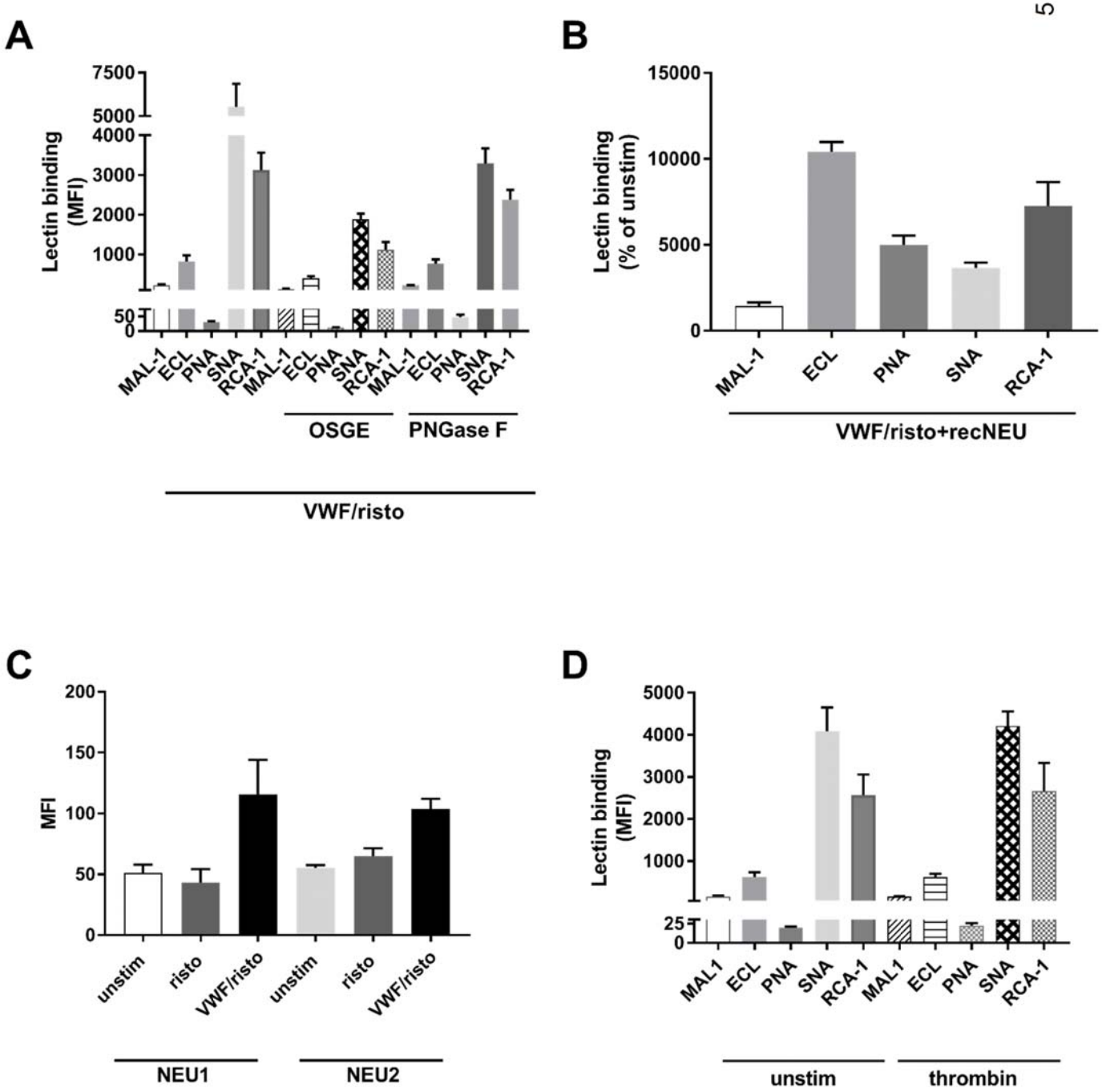

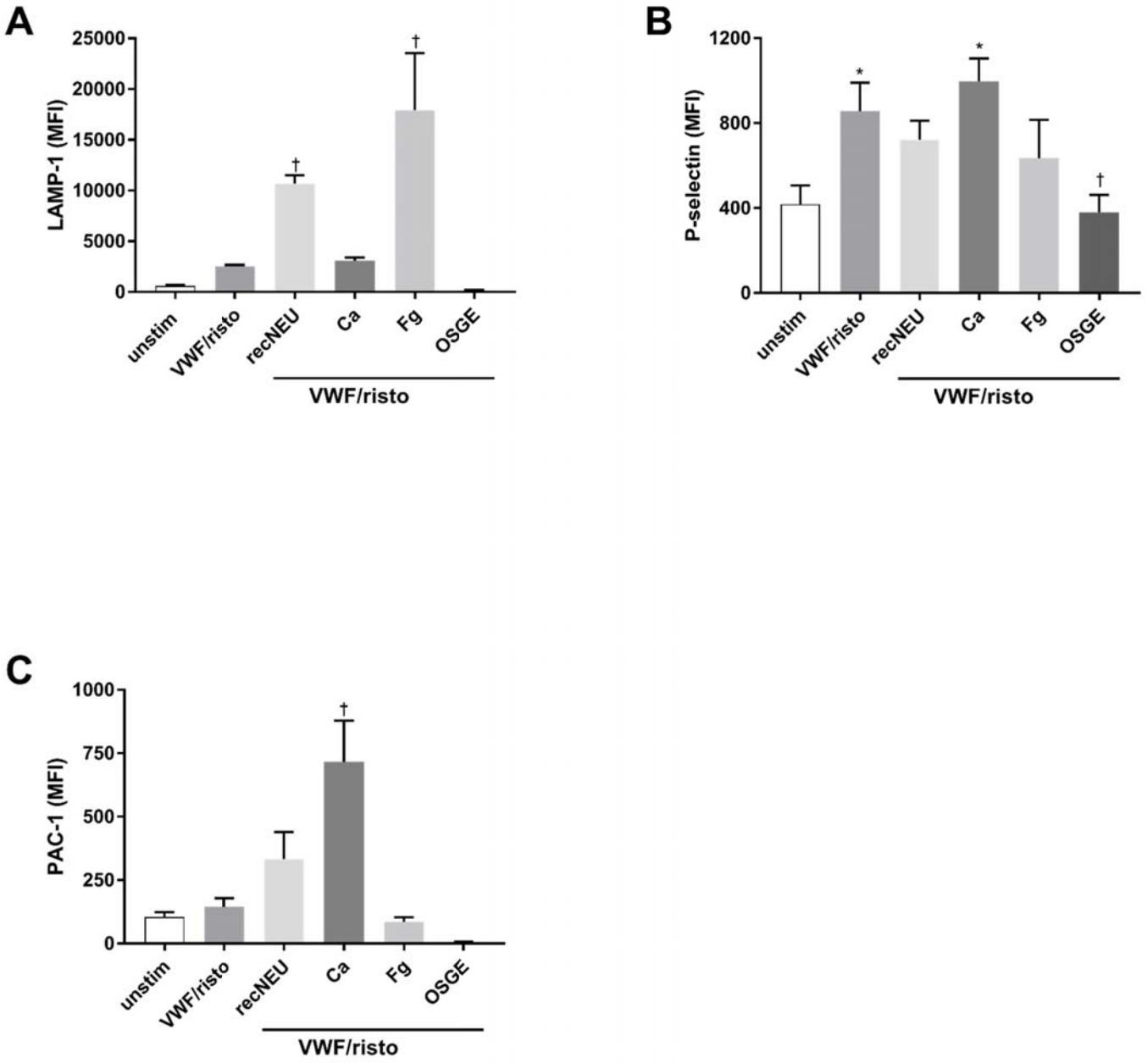
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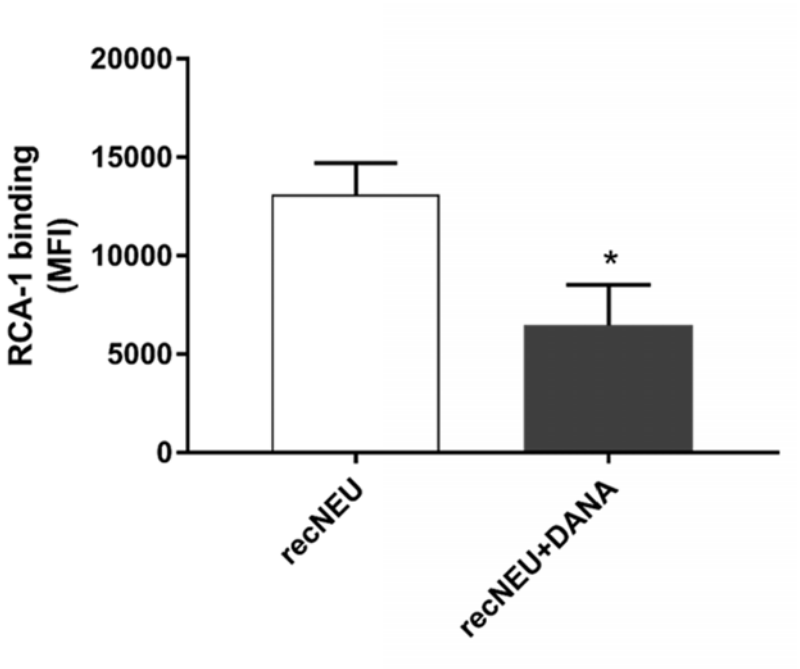

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