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Assessment of Neonatal, Cord, and Adult Platelet Granule Trafficking and Secretion

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Abstract

Despite the transient hyporeactivity of neonatal platelets, full-term neonates do not display a bleeding tendency, suggesting potential compensatory mechanisms which allow for balanced and efficient neonatal hemostasis. This study aimed to utilize small-volume, whole blood platelet functional assays to assess the neonatal platelet response downstream of the hemostatic platelet agonists thrombin and ADP. Thrombin activates platelets via the protease-activated receptors (PARs) 1 and 4, whereas ADP signals via the receptors P2Y₁ and P2Y₁₂ as a positive feedback mediator of platelet activation. We observed that neonatal and cord blood-derived platelets exhibited diminished PAR1-mediated granule secretion and integrin activation relative to adult platelets, correlating to reduced PAR1 expression by neonatal platelets. PAR4-mediated granule secretion was blunted in neonatal platelets, correlating to lower PAR4 expression as compared to adult platelets, while PAR-4 mediated GPIIb/IIIa activation was similar between neonatal and

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AUTHOR CONTRIBUTIONS

A.T.P.N., J.S., A.Z., W.F.B., D.B., O.J.T.M., and K.M.H. conceived and designed the research; A.T.P.N., K.R.J., A-L.I.S., L.E.M., and A.Z. performed experiments; A.T.P.N., J.S., A.D.R., L.E.M., and A.Z. analyzed data; A.T.P.N., J.S., W.F.B., D.B., O.J.T.M. and K.M.H. interpreted results of experiments; A.T.P.N., J.S., A.D.R., A-L.I.S., and L.E.M. prepared figures; A.T.P.N., J.S., O.J.T.M., and K.M.H. drafted the manuscript; A.T.P.N., J.S., W.F.B., D.B., O.J.T.M., and K.M.H. edited and revised manuscript. A.T.P.N., J.S., M.B., A.D.R., A-L.I.S., L.E.M., A.Z., W.F.B., D.B., A.M., O.J.T.M., and K.M.H. approved final version of manuscript.

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adult platelets. Under high shear stress, cord blood-derived platelets yielded similar thrombin generation rates but reduced phosphatidylserine expression as compared to adult platelets. Interestingly, we observed enhanced P2Y₁/P2Y₁₂-mediated dense granule trafficking in neonatal platelets relative to adults, although P2Y₁/P2Y₁₂ expression in neonatal, cord and adult platelets were similar, suggesting that neonatal platelets may employ an ADP-mediated positive feedback loop as a potential compensatory mechanism for neonatal platelet hyporeactivity.

Keywords

platelet; neonatal hematology; hemostasis; protease-activated receptors

INTRODUCTION

Activation of platelets requires agonist stimulation of several G protein-coupled receptors (GPCRs), resulting in rapid calcium mobilization, α -and dense granule release, cytoskeletal reorganization, thromboxane A₂ (TxA₂) release, and conformational changes in the glycoprotein (GP) IIb/IIIa complex to facilitate platelet aggregation [1]. Thrombin is the most potent platelet GPCR activator, signaling through protease-activated receptors (PARs), and structural differences between PAR1 and PAR4 result in distinct functional responses in human platelets [2]. The coordinated action of PAR1 and PAR4 leads to intracellular signaling in adult platelets that is critical for the downstream secretion of adenosine diphosphate (ADP) from platelet dense granules [1,2]. ADP release results in a positive feedback loop via the GPCRs P2Y₁ and P2Y₁₂ [3]. The secreted ADP activates P2Y₁/P2Y₁₂, further potentiating PAR-mediated platelet activation [4].

As platelet P2Y₁/P2Y₁₂ receptors are known to synergize with PARs to amplify platelet activation [3,4], several anti-platelets have been developed targeting these receptors. Clopidogrel was developed as an ADP-receptor antagonist and has been used as a successful antiplatelet therapeutic since the late 1990s; the PAR1 antagonist SCH 530348 (Vorapaxar) was developed as an antiplatelet drug, and while early clinical trials were halted due to increased major bleeding events, this drug received FDA approval in 2014 for use in reducing thrombotic cardiovascular events in patients with a history of myocardial infarction or with peripheral arterial disease [5]. As running comparable clinical trials is not feasible in the neonatal population, the recommendations for use and dosage for anti-platelet agents are often extrapolated from studies in adults despite the fact that in pediatric patients, and in particular neonates, the hemostatic system is still maturing and the platelet response to thrombin and ADP is ill-defined [6].

Currently, neonatal platelets are characterized as being hyporesponsive as compared to adult platelets. Several studies have linked neonatal platelet hyporesponsiveness with deficient synthesis of TxA₂ [7] and impaired signal transduction downstream of TxA₂ receptors [8], impaired mobilization of intracellular calcium [9], and decreased PAR1, PAR4 [10] and GPIIb/IIIa expression [11] levels by neonatal platelets as compared to adults. In our previous work evaluating platelet function in healthy neonates, neonatal platelet α -granule secretion and GPIIb/IIIa activation were markedly reduced to PAR1 stimulation while still responsive

to P2Y₁/P2Y₁₂ receptor stimulation with ADP [12]. These findings suggest that PAR1-mediated responses are impaired in neonatal platelets, yet the amplification of platelet activation via ADP feedback loop is still functional. Questions remain with regard to the potential explanation for the impaired neonatal PAR1 response, whether neonatal PAR4 response is also impaired, and the degree of PAR1 and PAR4 crosstalk with P2Y₁/P2Y₁₂ in mediating neonatal platelet activation.

Understanding the mechanisms underlying neonatal hyporeactivity is requisite in part for pediatric clinical practice to inform decisions on the management of bleeding complications as well as management or prevention of thrombosis and use of antiplatelet agents, especially in the Neonatal Intensive Care Unit. In the present study, we set out to assess the neonatal platelet response downstream of the hemostatic platelet agonists thrombin and ADP utilizing whole-blood functional assays.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma-Aldrich except as noted. For static adhesion assays and super-resolution microscopy, anti-CD63 (MX-49) was from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-MRP4 (D1Z3W) was from Cell Signaling (Danvers, MA, USA). Alexa Fluor secondary antibodies were from Life Technologies (Carlsbad, CA, USA). ADP and TRAP-6 (SFLLRN) were from Tocris Bioscience (Bristol, UK). PAR4 peptide (AYPGKF) was from Abgent (San Diego, CA, USA). For platelet receptor screening, PAR4 antibodies (14H6 and 5F10) [13] were a generous gift from Dr. Marvin Nieman (Case Western Reserve University, Cleveland, OH, USA). P2Y₁ (APR-009) and P2Y₁₂ (APR-012) antibodies were from Alomone Labs (Jerusalem, Israel). Flow cytometry antibodies for P-selectin (CD62P), PAC-1, and phosphatidylserine (Annexin V) staining were obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Study Population

Full-term neonates were at least 38 weeks of gestational age, with APGAR scores ranging from 7–9 at 1 minute, and 9–10 at 5 minutes. Neonates were excluded from the study if presenting with a major congenital anomaly, maternal history of diabetes mellitus, maternal history of immune thrombocytopenia, known drug or alcohol exposure, intraventricular hemorrhage, intravenous antibiotics, pre-eclampsia or chorioamnionitis.

The adult control group consisted of self-reported healthy adults aged 18–50 years. Adults were excluded from the study if presenting with a history of abnormal hemostasis or have taken medication known to affect platelet function in the two weeks prior to participation. Prior to blood sample collection, consent was obtained from the adult donors and/or parents in accordance with Oregon Health & Science University and Stony Brook University Institutional Review Board-approved protocols. All blood samples were de-identified prior to sample processing.

Blood Collection and Preparation

Briefly, blood was drawn by venipuncture from healthy adult donors into sodium citrate (0.38% w/v) or 10% acid citrate dextrose (ACD-A; for shear experiments). Blood was drawn from healthy, full-term neonates at 24 hours of life via heel stick into sodium citrate (0.38% w/v) following a 3-minute application of an infant heel warmer. Samples were collected after obtaining blood for a newborn screen, and were obtained by scooping blood from the puncture site as previously described [12]. Umbilical cord blood samples were obtained from healthy, full-term neonates immediately after clamping post-Caesarean section delivery and mixed with sodium citrate (0.38% w/v) or citrate phosphate dextrose (CPD; for shear experiments) (Supplemental Fig. 1).

After collection of cord blood samples, three suspensions were prepared: whole blood, platelet rich plasma (PRP), and gel-filtered platelets (GFP). For high shear experiments, PRP was prepared by centrifuging whole blood for 4.5 minutes at $650 \times g$. GFP samples were prepared by filtering PRP through a column of Sepharose 2B beads into HEPES-buffered modified Tyrode's solution with 1 mM sodium citrate and 0.1% bovine serum albumin ("platelet buffer"), as previously described [14,15]. GFP and PRP were diluted to a final platelet count of 20,000/ μ l in platelet buffer, whereas whole blood was diluted in platelet buffer in a similar proportion to that of the diluted PRP. All suspensions were mixed with 3 mM CaCl_2 for 10 minutes prior to shear experiments (Supplemental Fig. 1). Platelet suspensions were prepared in polypropylene tubes using buffer containing 0.1% bovine serum albumin and placed on a gentle rocker after recalcification to minimize contact activation effects.

Platelet Activation Assay in Response to Biochemical Agonists

P-selectin and PAC-1 expression on platelet surface was detected using fluorescence-activated cell sorting (FACS) as previously described [12]. Briefly, adult, neonatal, and cord citrated whole blood was diluted with modified HEPES/Tyrode buffer (129 mM NaCl, 12 mM NaHCO_3 , 2.9 mM KCl, 20 mM HEPES, 1 mM MgCl_2 , 0.34 mM Na_2HPO_4 , pH 7.3; supplemented with 5 mM glucose). Diluted whole blood was then incubated with antibodies (Abs) against P-selectin (APC CD62P) and activated integrin GPIIb/IIIa (FITC PAC-1) in the presence of select agonists (10 μ M ADP, 10 μ M TRAP-6, 200 μ M AYPGKF), or vehicle control (HEPES/Tyrode) for 20 minutes at room temperature (RT). BD Cytifix Buffer was added to each sample and incubated at RT for 5 minutes. Samples were finally diluted with PBS, measured by FACS, and analyzed using FlowJo software (Ashland, OR). A platelet-size gate was set based on forward and side scatter to exclude larger white blood cell and red blood cell populations. All samples were subjected to flow cytometry for the same duration, and percentage of P-selectin expression of PAC-1 binding was normalized to the total platelet count (at least 5,000 platelets) detected from each sample. Nonspecific signal was eliminated using APC and FITC anti-IgG as controls.

Platelet Dense Granule Secretion Assay

ATP released from platelet dense granules was used as a surrogate marker for ADP secretion, and measured as light output generated by an ATP-luciferin-luciferase reaction as previously described [4]. Briefly, adult, neonatal, and cord citrated whole blood was diluted

with modified HEPES Tyrode buffer prior to incubation with select agonists (10 μ M ADP, 10 μ M TRAP-6, 200 μ M AYPGKF) or vehicle (HEPES/Tyrode) for 30 seconds at 37°C under orbital shaking in a white, flat bottom 96-well plate (Corning Costar, Tewksbury, MA, USA). Detection reagent Chronolume (Chrono-Log Corporation, Havertown, PA, USA) was added to the wells and sample luminescence was immediately detected using an Infinite M200 spectrophotometer (TECAN, Mannerdorf, Switzerland).

Static Adhesion Assay and Superresolution-Structure Illumination Microscopy

Glass-bottom dishes (MatTek) were coated with poly-L-lysine and washed with PBS. Vehicle (HEPES/Tyrode) or select agonists (10 μ M ADP, 10 μ M TRAP-6, 200 μ M AYPGKF) were incubated with adult or neonate whole blood for 10 minutes prior to fixation with 4% paraformaldehyde (PFA) for 10 minutes and seeded onto immobilized poly-L-lysine for 1 hour at RT. Adherent platelets were permeabilized with blocking solution (1% Fraction V BSA and 0.1% SDS in PBS), then stained with indicated primary Abs (anti-CD63 and anti-MRP4 in blocking solution) overnight at 4°C. Alexa Fluor secondary Abs were added in blocking solution for 2 hours prior to mounting with Fluoromount G (Southern Biotech, AL, USA). Platelets were imaged using super-resolution structured illumination microscopy (SR-SIM) with a Zeiss 100 $\times$ oil immersion 1.46 NA lens on a Zeiss Elyra PS.1 microscope as previously described [16] .

Platelet Receptor Screening

Diluted whole blood was incubated with 10 μ g/mL anti-PAR4 Abs (14H6 and 5F10), anti-PAR1 blocking Ab (ATAP2), P2Y₁ (APR-009) or P2Y₁₂ (APR-121) Abs. Next, samples were stained with a polyclonal FITC anti-IgG Ab (Biocytex Kit, Marseille, France), followed by measurements on a FACS Canto II system (Beckton Dickinson, CA, USA) and analyzed using FlowJo. Number of molecules per platelet was extrapolated by comparing the mean fluorescence intensity (MFI) of the sample to the MFI of a calibrator system using beads coated with increasing and accurately known quantities of immunoglobulins IgG.

Exposure of Platelets to Shear Conditions

GFP, PRP and whole blood preparations were exposed to constant shear stresses for 4 minutes in a computer-controlled hemodynamic shearing device (HSD) [17]. For thrombin generation measurement, only GFP suspensions were exposed to shear stresses of 1, 10, 30, 50, and 70 dyne/cm², and samples were collected every minute using a LabView-controlled syringe pump (PSD/8, Hamilton, Reno, NV) connected to the HSD via a 28-gauge PTFE tube [18]. For flow cytometry measurements, PRP and whole blood suspensions were exposed to high shear stresses of 70 dynes/cm² for 4 minutes, with samples collected prior to and at the end of shear exposure.

Thrombin Generation in Sheared Platelets

Thrombin generation for sheared GFP samples was measured using a modified prothrombinase-based Platelet Activation State (PAS) assay that uses acetylated prothrombin to measure rate of thrombin generation [19]. PAS absorbance readings were normalized against fully activated GFP, generated by sonicating quiescent GFP at 10 W for 10 s with a

Branson Sonifier 150 with a microprobe (Branson, MO, USA) [15] on each day. Normalized PAS values represent the PAS as a fraction of the full activation and allow comparison of data obtained on different days regardless of the full thrombin generation potential for each sample. The change in platelet activation state, PAS, was calculated over the 4 min duration of shear stress exposure.

Platelet P-selectin Expression and Phosphatidylserine Exposure under Shear

Sheared whole blood and PRP suspensions were assessed for platelet activation using anti-CD62P (PE) and Annexin V (FITC). Unsheared suspensions were used as controls. Whole blood or PRP samples were diluted with platelet buffer and FcR blocking reagent (human, Miltenyi Biotec, Auburn, CA) and stained with anti-CD62P for 15 minutes in the dark at 4°C. Separately, whole blood or PRP samples were diluted with Annexin V buffer and stained with Annexin V for 15 minutes in the dark at RT. Samples were supplemented with platelet buffer or Annexin V buffer to a final volume of 500 µl at 4°C, scanned using FACS (BD Accuri C6, BD Biosciences, Franklin Lakes, NJ), and analyzed using Kaluza (Beckman Coulter Life Sciences, Indianapolis, IN). Nonspecific signals were eliminated using PE and APC anti-IgG1κ as controls.

Statistical Analysis

Data were analyzed using GraphPad PRISM 4.0 software (San Diego, CA, USA). To determine statistical significance, one-way ANOVA, or 2-tailed Student's t-test and Tukey post-hoc test were used for comparison between treatments. Data are represented as mean ± standard error of the mean (SEM) of at least 3 independent experiments. For all comparisons, $p < 0.05$ was used to establish significance.

RESULTS

Measurement of Platelet α-Granule Secretion

Platelet activation initiates α-granule secretion resulting in P-selectin expression on the platelet surface [6]. We first investigated the neonatal hemostatic system by assessing platelet activation and α-granule secretion in the presence of agonists to the PAR1/4 or P2Y₁/P2Y₁₂ receptors. Citrated adult, neonate peripheral, and cord whole blood were incubated with an APC-labeled anti-CD62P mAb in the presence of platelet agonists or vehicle prior to fixation and evaluation by flow cytometry. The platelet population was gated from the red blood cell and leukocyte populations based on size (forward scatter-FSC) and granularity (side scatter-SSC) (Fig. 1A). Resting platelets were used to determine baseline P-selectin expression levels, with eFluor-labeled anti-CD31 mAbs used as the platelet marker. The same population gates defined using resting platelets were used to determine the degree of platelet activation in stimulated samples (Fig. 1A). Baseline levels of platelet activation was equivalent for adult, neonatal, and cord blood samples ($n=3$).

Adult platelet P-selectin expression significantly increased in response to the G-protein coupled receptor (GPCR) PAR-1 agonist TRAP-6, PAR-4-agonist AYPGKF, P2Y₁/P2Y₁₂-agonist ADP, or a combination of ADP and TRAP-6 or AYPGKF ($p<0.001$; Fig. 1B). We then investigated the response of neonatal peripheral platelets to these agonists, as measured

by P-selectin expression levels, and observed no significant increase above baseline in P-selectin expression in response to TRAP-6, AYPGKF, or a combination of ADP and TRAP-6 or AYPGKF. In contrast, cord blood-derived platelet P-selectin expression significantly increased above baseline upon stimulation with TRAP-6, AYPGKF, ADP, or a combination of ADP and TRAP-6 or AYPGKF ($p<0.001$). However, the degree of α -granule secretion remained blunted as compared to adult platelets for agonists TRAP-6, ADP, or the combination of ADP and TRAP-6 ($p<0.01$; Fig. 1B), while responses to AYPGKF, or the combination of AYPGKF and ADP were equivalent for cord and adult platelets.

We then subjected adult and cord blood samples to high shear stress using a hemodynamic shearing device (HSD). P-selectin expression was monitored in response to 4 min of shear exposure of 70 dyne/cm². Platelet activity was defined as the percentage of platelets staining positive for PE-labeled CD62P. In platelet-rich plasma (PRP), P-selectin positive events were similar for both sheared adult and cord blood-derived platelets ($n=3$, Fig. 1C). Similar results were observed in whole blood (Fig. 1C), suggesting that cord-derived and adult platelet α -granule secretion in response to shear was similar at the conditions tested.

Measurement of Platelet Integrin Activation

“Inside-out” signaling following platelet activation causes conformational changes that facilitate the conversion of GPIIb/IIIa to a high affinity state for ligand binding, primarily fibrinogen [20]. Conformational changes in GPIIb/IIIa can be quantified by measuring the binding of the antibody PAC-1, which selectively recognizes the active form of GPIIb/IIIa. Citrated adult, neonate, and cord whole blood were incubated with a FITC-labeled PAC-1 mAb in the presence of platelet agonists or vehicle prior to fixation and evaluation by flow cytometry (Fig. 2A). We did not observe any differences in PAC-1 binding among all samples at resting conditions (Fig. 2B). Adult platelets exhibited a significantly increased level of activated GPIIb/IIIa in response to TRAP-6, AYPGKF, ADP, or the combination of ADP and TRAP-6 or AYPGKF ($p<0.0001$). Neonatal platelets also exhibited a significant increase in GPIIb/IIIa activation above baseline in response to AYPGKF, ADP, or the combination of ADP and TRAP-6 or AYPGKF ($p<0.05$). In contrast, neonatal platelet GPIIb/IIIa activation remained blunted in response to TRAP-6 as compared to adult platelets ($p<0.0001$), while AYPGKF elicited a similar response in neonates as compared to adult platelets. Addition of exogenous ADP in combination with TRAP-6 or AYPGKF neonatal platelets induced the same degree of PAC-1 binding as compared to adult platelets under these conditions (Fig. 2B). Cord-derived platelet integrin activation increased significantly in response to AYPGKF, ADP, and ADP in combination with TRAP-6 or AYPGKF ($p<0.05$), but not in response to TRAP-6 stimulation alone (Fig. 2B). Integrin activation in cord blood-derived platelets, while being slightly lower, remained statistically similar to adult platelets, except in response to TRAP-6 ($p<0.05$).

Measurement of Platelet Dense Granule Secretion

ADP is stored at high concentrations in platelet dense granules and is secreted upon platelet activation to further amplify platelet activation downstream of the P2Y₁ and P2Y₁₂ receptors [21]. We next assessed neonatal, cord, and adult blood-derived platelet dense granule secretion by measuring ATP release [4]. Since the constant ratio of ATP:ADP in dense

granules is 2:3, ATP can be used as a surrogate marker for ADP secretion. We observed no differences in baseline ATP level among all samples (n=5, Fig. 3A).

Adult platelet dense granule release increased significantly in response to TRAP-6, AYPGKF, or a combination of ADP and TRAP-6 or AYPGKF ($p<0.0001$), but not to ADP alone (Fig. 3A). A significant increase in dense granule release was observed following stimulation of neonatal platelets with ADP, or the combination of ADP and AYPGKF ($p<0.01$), but not to TRAP-6, AYPKF alone, or the combination of ADP and TRAP-6. Neonatal platelet dense granule secretion was reduced as compared to adult platelets in response to TRAP-6, AYPGKF, or a combination of ADP and TRAP-6 ($p<0.001$). Cord blood-derived platelets secreted ATP in response to ADP, or a combination of ADP and TRAP-6 or AYPGKF ($p<0.0001$), but not to TRAP-6 or AYPGKF alone. Compared to adult platelets, cord blood-derived platelets exhibited a similar degree of dense granule secretion in response to all agonists, except AYPGKF ($p<0.05$; Fig. 3A).

We then utilized an immunofluorescence approach and superresolution-structured illumination microscopy (SR-SIM) to quantify trafficking of platelet dense granules in adult and neonatal platelets, using the dense granule marker CD63 (red) together with a marker for the multidrug resistance protein 4 (MRP4, green), a signaling molecule transporter in platelets. Under resting condition, we observed punctate, granular-like staining structures (red) for both adult and neonatal platelet dense granules (Fig. 3B). TRAP-6 induced translocation of CD63 toward the platelet plasma membrane in adult platelets, and to a lesser extent in neonatal platelets (Fig. 3B). Interestingly, ADP induced a dramatic translocation of CD63 in neonatal platelets, while no differences were observed in adult platelets (Fig. 3B). Stimulation of platelets with the PAR-4 agonist AYPGKF elicited a higher expression of dense granule markers at the platelet periphery in adult platelets, while no differences were observed in neonatal platelets (Fig. 3B). Ortho images of dense granules along the Z-axis indicate that TRAP-6 elicits degranulation of dense granules in adult platelets, while ADP promotes the greatest degree of degranulation in neonatal platelets, resulting in a decrease in the concentration and dispersion of CD63 observed at the platelet membrane (Fig. 3C).

To quantify the translocation of dense granules to the cellular periphery, we next performed an analysis of SR-SIM images using Imaris (Bitplane AG, Zurich, Switzerland). Each dense granule was rendered using a spot detection algorithm, and the platelet center of mass was also determined by the software. In Figure 3D, we show the distance between each dense granule and the platelet center of mass, quantifying the translocation of dense granules within each platelet. In adult platelets, we found that the distance of granules from the center of the platelet significantly increased in response to TRAP-6 (n=3, $p<0.0001$) but not to ADP or AYPGKF in adults. Neonatal platelet granule movement significantly increased in response to ADP and TRAP-6 (n=3, $p<0.0009$), but not to AYPGKF. As compared to adult platelets, neonatal platelets exhibited significantly higher translocation of dense granules in response to ADP, while a reduced extent of granule movement in response to TRAP-6 ($p<0.05$); both adult and neonatal platelet dense granule trafficking was largely unresponsive to stimulation with AYPGKF (Fig. 3D).

Quantification of Platelet Receptors

We next utilized flow cytometry to measure levels of PAR1, PAR4, P2Y₁, and P2Y₁₂ receptors on adult (n=6), neonatal peripheral (n=5), and cord-derived platelets (n=4). Our data confirm that neonatal platelets express significantly lower PAR1 levels than adult platelets, and show that neonatal platelets also express significantly lower levels of PAR4 receptors as compared to adult platelets ($p<0.005$). The levels of P2Y₁ or P2Y₁₂ receptors were similar between neonate and adult platelets (Fig. 4A). Similarly, the levels of P2Y₁, or P2Y₁₂ receptors expressed by cord blood-derived platelets were equivalent to those observed as for adult platelets. Yet, PAR1 and PAR4 receptor expression levels on cord blood-derived platelets was significantly lower as compared to adult platelets ($p<0.05$, Fig. 4A). These data demonstrate that cord and neonate platelets express significantly less PAR1 and PAR4 as compared to adult platelets, providing a potential mechanism in part for the blunted response to PAR agonists are observed for neonate and cord platelets as compared to adult platelets.

Thrombin Generation and Platelet Procoagulant Activity under Shear Stress

Activated platelets facilitate blood coagulation by flipping of plasma membrane leaflets and exposing procoagulant phosphatidylserine (PS). It has been demonstrated that fluid shear stress significantly enhances agonist-induced PS exposure in platelets [22]. Binding of coagulation factors (F) Xa and Va to PS promotes prothrombinase assembly on the platelet surface and enhances proteolytic activity of FXa to catalyze thrombin generation [23,24]. To quantify thrombin generation, we acetylated human prothrombin such that upon activation by prothrombinase this species retains the ability to bind to the platelet surface and cleave a chromogenic peptide substrate yet is incapable of cleaving PAR receptors to activate platelets [19,25]. Gel-filtered platelets from both adult and cord-derived blood were exposed for 4 minutes to five levels of constant shear stress (n=6, Fig. 4B, left). Both adult and cord blood-derived platelets (Fig. 4B, left) yielded similar increases in platelet activation state (PAS), a measure of normalized thrombin generation rate, consistent with prior reports [26]. Adult platelets showed a significant increase in PAS after a 4 min exposure to 50 and 70 dyne/cm² as compared to baseline levels at 1 dyne/cm². The baseline levels for cord blood-derived platelets were lower, and as a result a significant PAS increase was observed at all shear stress levels (Fig. 4B). Still, the total thrombin generation rate, a measure of the full platelet activation potential, for adult platelets was 80.73 ± 4.30 mAbs/min (n=42), but significantly lower at 50.90 ± 2.82 mAbs/min (n=33, $p<0.05$) for cord blood-derived platelets (Fig. 4B, right), suggesting that cord blood-derived platelets may be less procoagulant overall.

Negatively-charged PS was quantified by measuring Annexin V binding via flow cytometry analysis. In PRP, a significant increase in Annexin V binding was observed in response to shear for adult but not cord-blood derived platelets (n=3, $p<0.01$, Fig. 4C). The level of Annexin V binding observed for cord blood-derived platelets following exposure to shear was significantly lower as compared to adult platelets ($p<0.01$, Fig. 4C). In whole blood, sheared cord blood-derived platelets exhibited a small yet significant increase in Annexin V positive events as compared to the unsheared control (n=3, $p<0.01$), while the dramatic increase in shear-induced Annexin V binding to adult platelets observed in PRP was lost in whole blood (Fig. 4C).

DISCUSSION

Several studies have indicated that neonatal platelets have blunted responses to platelet agonists, decreased granule secretion, reduced fibrinogen binding, and decreased platelet aggregation as compared to adult platelets, and that this hyporesponsive phenotype persists for up to several weeks after birth [6,27,28]. Nevertheless, despite our observation that neonatal platelets were less procoagulant in response to shear, bleeding times and platelet function analyzer-100 (PFA-100) closure times under shear in healthy full-term neonates are shorter than in adults, indicating that neonates exhibit more effective primary hemostatic functions [29]. This may be due in part to enhanced neonatal platelet interactions with the vessel wall due to higher hematocrits, higher mean corpuscular volumes, higher vWF concentrations, and the presence of ultra-large vWF multimers in neonatal blood as compared to adults [27,30,31]. Yet, while most healthy newborns do not manifest a bleeding tendency, an increased prevalence of hemostatic disturbances including thrombocytopenia and coagulopathy is observed in premature and sick newborns, which may result in life-threatening bleeding [27]. The potential role of the inherent neonatal platelet hyporeactivity to these hemostatic disturbances has not been fully examined in the clinical management of this population. Understanding the functional phenotype of neonatal platelets may help guide diagnosis and inform treatment options specifically tailored to the neonatal population.

This study focused on the neonatal platelet response downstream of thrombin and ADP as their respective receptors, PAR1/4 and P2Y₁/P2Y₁₂, are major targets of antiplatelet therapies in the adult population. Of relevance, drug dosing of antiplatelet agents in the neonatal population still largely relies on data extrapolated from adult clinical trials. In agreement with previous reports [11,12,32,33], despite similar total P-selectin content and GPIIb/IIIa expression, we observed significantly blunted granule secretion and integrin GPIIb/IIIa activation in response to the PAR-1 agonist TRAP-6 in cord and peripheral neonatal platelets as compared to adult platelets. These functional differences may be attributed in part to the observation that neonatal platelets express lower levels of PAR1 as compared to adult platelets [33]. A similar narrative of hypofunctional response to PAR4 agonists and correspondingly reduced PAR4 levels was observed for neonatal platelets. Yet, neonatal platelets still exhibit robust GPIIb/IIIa activation and dense granule secretion but not α -granule secretion to ADP, which may be due to different regulatory effects that the actin cytoskeleton exert on dense granules as compared to α -granules [34,35]. Perhaps this shift in responsiveness from thrombin to ADP provides a selection advantage during development and the trauma of birth. The functional difference and responsiveness of neonatal platelets as compared to adult platelets should be taken into account when managing bleeding complications through transfusion of adult platelets prophylactically into neonates for fear of potentiating a hypercoagulable, prothrombotic state.

There are several limitations to this study. The sample sizes used in our current study were limited to 3–4 samples per test due to limited availability of neonatal samples, together with the inherently small volume constraint in heel-stick samples. Moreover, the mode of blood collection differed between neonatal, cord-blood derived and adult samples. Subsequently, limited conclusions can be drawn from this study with regards to the normal population distribution of neonatal platelet functional responses and comparison of similarities and

differences between neonatal, cord blood-derived and adult platelets. In light of these limitations we aimed to assess platelet responses to PARs and P2Y₁/P2Y₁₂ receptor agonists using whole blood assays as compared to cord, neonatal peripheral and adult peripheral platelets, demonstrating utility and feasibility of our small-volume assays. Neonatal platelets exhibited a hyporeactive phenotype in response to PAR1/4 agonists, indicative of a loss of sensitivity to the potent platelet agonist thrombin. Neonatal platelets may compensate for this to retain their hemostatic function by enhancing their dense granule trafficking and secretion in response to ADP. Understanding the functional differences between neonates and adults and the mechanisms during development is necessary for the diagnosis and treatment of hemostatic disorders in the neonatal population as well as the development of platelet transfusion guidelines.

Supplementary Material

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ABBREVIATIONS

ATP	adenosine triphosphate
ADP	adenosine diphosphate
BSA	bovine serum albumin
FACS	fluorescence-activated cell sorting
GFP	gel-filtered platelets
GP	glycoprotein
HSD	hemodynamic shearing device
MFI	mean fluorescence intensity
MRP4	multidrug resistance-associated protein 4
PAR	protease-activated receptor
PAS	Platelet Activation State
PBS	phosphate buffered saline
PFA	paraformaldehyde

PRP	platelet-rich plasma
SDS	sodium dodecyl sulfate
SR-SIM	super-resolution structured illumination microscopy
TRAP-6	thrombin receptor activator peptide-6
vWF	von Willebrand Factor

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Figure 1. Effects of G-protein coupled receptor agonists on platelet α -granule secretion. (A) Representative flow cytometry forward (FSC), side scatter (SSC), P-selectin (APC CD62P) and eFluor CD31 fluorescence intensity of resting and TRAP-6-activated adult, neonatal peripheral, and cord blood. Representative fluorescence intensity of resting and activated platelets are in response to HEPES/Tyrod buffer and TRAP-6, respectively. (B) P-selectin expression in adults, neonatal, and cord blood in response to vehicle or ADP, TRAP-6, protease-activated receptor 4 agonist (AYPGKF), or a combination of agonists. (C) P-selectin expression in platelet-rich plasma (PRP) and whole blood in response to high

shear stress. PRP and whole blood suspensions were exposed to high shear stresses of 70 dynes/cm² for 4 minutes. Data are represented as mean $\pm$ SEM of at least 3 independent experiments; * p <0.05 compared to adult vehicle; [†] P <0.05 compared to cord vehicle; $\square$ p <0.05 adults vs. neonatal/cord blood.

Figure 2. Effects of G-protein coupled receptor stimulation on platelet integrin activation. (A) Representative flow cytometry of activated GPIIb/IIIa (FITC PAC-1) and eFluor CD31 fluorescence intensity of resting and TRAP-6-activated adult neonatal peripheral, and cord blood. (B) PAC-1 expression in resting and stimulated adult, neonatal, and cord platelets in response to ADP, TRAP-6, AYPGKF, or a combination of agonists. Data are represented as mean $\pm$ SEM of at least 3 independent experiments; * p <0.05 compared to adult vehicle; # p <0.05 compared to neonatal vehicle; † p <0.05 compared to cord vehicle; $\square$ p <0.05 adults vs. neonatal/cord blood.

Figure 3. Dense granule secretion and distribution of CD63 and MRP-4 in whole blood activated with G-protein coupled receptor agonists.

(A) Dense granule secretion from resting and stimulated platelets treated with ADP, TRAP6, AYPGKF, or select combination of agonists in adults, neonatal, and cord blood. Values are mean $\pm$ SEM of raw luminescence ($\times 10^6$) of at least 3 independent experiments. (B) Dense granule trafficking using CD63 as dense granule marker (red), together with ATP transporter marker MRP4 (green), visualized by SR-SIM. Scale bar = 1 μ m. (C) Ortho view of Z-stack images of platelet dense granule (CD63-red) upon stimulation with TRAP-6 (adult) and ADP (neonate), demonstrating platelet degranulation leading to dispersion of CD63 signal.

Scale bar = 2 μ m. **(D)** Images captured in Z-stacks using ZEISS software were processed and analyzed using Imaris software to render dense granules using a spot detection algorithm. A center of mass was determined for each platelet in the field of view. Each data point on the graph represents the distance between a single granule and the center of mass of such corresponding platelet. * $p < 0.05$ compared to adult vehicle; # $p < 0.05$ compared to neonatal vehicle; † $p < 0.05$ compared to cord vehicle; □ $p < 0.05$ adults vs. neonatal/cord blood.

Figure 4. Quantitative assessment of select platelet G-protein coupled receptors and shear-induced platelet activation.

(A) Protease activated receptors (PARs) 1 and 4, and ADP receptors (P2Y₁, P2Y₁₂) expression levels in adult platelets measured by flow cytometry. Data are represented as mean $\pm$ SEM of at least 3 independent experiments; $\square$ $p < 0.05$ adults vs. neonatal/cord blood. (B) Platelet activation state (PAS) was measured every minute for the 4 min duration in adult and cord platelets (left). Change in PAS, Δ PAS, was calculated over the exposure, normalized to the respective 1 dyne/cm² controls; * $p < 0.05$ vs. adults at 1 dyne/cm²; $\dagger$ $p < 0.05$ vs. cord blood at 1 dyne/cm². PAS values were normalized with thrombin generation values

obtained by sonication of adult and cord-derived platelets (right). $\square p < 0.05$ adult vs. cord blood. (C) Sheared whole blood and PRP suspensions were stained for Annexin V (FITC) and subjected to flow cytometry. $*p < 0.05$ vs. adult vehicle; $^{\dagger}p < 0.05$ vs. cord vehicle; $\square p < 0.05$ adult vs. cord blood.