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Heme induces human and mouse platelet activation through C-type-lectin-like receptor-2

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Intravascular hemolysis is a serious complication developed in many severe infections such as malaria, sepsis (1), typical hemolytic uremic syndrome (2) and congenital diseases such as sickle cell disease (3). Hemolysis is accompanied by thrombocytopenia, platelet activation, platelet-leukocyte aggregates, inflammation, vascular obstruction and organ damage. During hemolysis, red blood cell destruction leads to the release of molecules such as ADP, hemoglobin and heme which exert potent prothrombotic and proinflammatory actions.

Under physiological conditions, free heme is scavenged by the plasma protein hemopexin, and is subsequently catabolized by heme oxygenase-1 into carbon monoxide, biliverdin and ferrous iron (Fe$^{2+}$). However, acute or/and chronic hemolysis exhausts the scavenging system leading to an increase in free heme in the blood. Upon release, reduced heme is rapidly and spontaneously oxidized in the blood into ferric (Fe$^{3+}$) form, hemin, with increased levels observed in hemolytic diseases. It was recently shown that hemin induces human platelet activation and platelet death through ferroptosis, an intracellular iron-mediated cell death (4). However, the mechanisms and receptors involved in platelet activation by hemin are not known.

In this study, we investigated hemin-mediated human and mouse platelet activation in vitro. An increase in labile (i.e. weakly bound and free) heme/hemin is detected in patients with hemolytic diseases with plasma concentrations ranging between 2 and 50 µM (5, 6). Similar concentrations of heme are detected in mice with chronic or acute hemolysis such as sickle cell mice or Escherichia coli-induced hemolysis (1, 7). In this study, we show that hemin (2-12 µM) induces rapid aggregation of human washed platelets, while aggregation is slower and reduced in magnitude at higher (≥ 25 µM) concentrations (Figure 1A, B). Platelet aggregation was associated with increased P-selectin expression and GPIIbIIIa activation as measured by flow cytometry (Figure 1C, D). High (≥ 25 µM) but not lower concentrations of hemin significantly increased phosphatidylserine (PS) exposure on platelets as assessed using Annexin V staining (Figure 1E). Moreover, high concentrations of hemin induced toxicity as measured by increased levels of lactate dehydrogenase (LDH) in the supernatant of activated platelets (Figure 1F). Recent data have shown that a high concentration of hemin (25 µM) triggers lipid peroxidation and human platelet death through ferroptosis but not apoptosis or necroptosis (4). Whether the increase in PS exposure modulates the contribution of platelets to the coagulopathies observed in hemolytic diseases requires further investigation.

Hemin is known to bind to Toll-like receptor (TLR) TLR4 on endothelial cells resulting in endothelial cell activation including secretion of Weibel-Palade bodies (3). However, blocking TLR4 signaling in platelets using TAK-242 did not alter activation by hemin, suggesting a TLR-4-independent pathway of activation (Figure 1H). At low concentrations, human platelet aggregation by hemin was blocked by inhibitors of Syk (PRT-060318), Src family kinases (PP2) and Btk (Ibrutinib), and partially by GPIIbIIIa inhibitor eptifibatide (Integrilin) (Figure 1G, H). These results suggest that
Hemin induces human platelet activation via an immunoreceptor-tyrosine-based activation-motif (ITAM) receptor-based pathway (8). Indeed, low concentrations of hemin provoked phosphorylation of Syk and PLCγ2 (Figure 1I). In order to identify the receptor triggering platelet activation by hemin, we used recombinant dimeric forms of CLEC-2 and GPVI (hFc-CLEC-2 and hFc-GPVI, respectively) as a blocking strategy. Pre-incubation of hemin with hFc-CLEC-2 but not hFc-GPVI abolished platelet aggregation by hemin identifying CLEC-2 as the receptor mediating human platelet activation by hemin (Figure 1G, H). These results also suggest a direct interaction between hemin and CLEC-2. However, while we believe that the inhibition is likely the result of competition between recombinant and platelet CLEC-2, we cannot rule out that recombinant CLEC-2-hemin complex may interfere with CLEC-2 clustering thereby inhibiting platelet activation. Platelet aggregation was not altered by AYP1 (9), an antibody that blocks the interaction between CLEC-2 and podoplanin, providing indirect evidence that hemin binds to a distinct site on CLEC-2 to podoplanin (Figure 1H). Protoporphyrin IX, the precursor of heme, and cobalt hemtoporphyrin were shown to bind to CLEC-2 and inhibit podoplanin-CLEC-2 interaction without inducing platelet activation (10). The type of interactions by which different porphyrins selectively bind to CLEC-2 and induce platelet activation requires further investigation. Platelet aggregation mediated by low concentrations of hemin (6.25 μM) was not altered by cyclooxygenase (COX) (indomethacin) or P2Y12 (cangrelor) inhibitors, showing that, in contrast to podoplanin, secondary mediators are not required for hemin-mediated platelet activation (Figure 1G, H). This may reflect the ability of hemin to induce higher oligomers of CLEC-2 increasing signal transduction. Platelet aggregation does not depend on oxidative stress as hemin-mediated platelet aggregation was not altered by the antioxidant N-acetyl cysteine (NAC) (Figure 1H, S1). At higher concentrations (>25 μM), platelet aggregation was not inhibited by integrilin or inhibitors of Src, Syk and Btk, suggesting that higher concentrations of hemin induce agglutination (Figure S2). Increasing the concentrations of inhibitors did not alter platelet agglutination/aggregation (not shown). The distinct mechanisms of platelet activation by low and high concentrations of hemin might be related to formation of hemin aggregates at high concentrations, which might exert different activities as compared to monomeric or dimeric hemin present at low concentrations.

Hemin induces mouse platelet aggregation, albeit at a slightly higher concentration (Figure 2A). Similar to human platelets, aggregation by low concentrations of hemin was inhibited by inhibitors of Src, Syk and Btk tyrosine kinases, recombinant mouse Fc-mCLEC-2 and GPIIbIIIa blockade (Figure 2B, C). In contrast, inhibitors of TLR4, COX and P2Y12 had no effect on platelet aggregation by hemin (Figure 2C). Platelet aggregation by hemin was significantly reduced in CLEC-2-deficient platelets confirming a key role for CLEC-2 (Figure 2D, E). However, deletion of CLEC-2 did not inhibit platelet shape change suggesting that one or more other receptors support platelet activation by hemin in mice.
The fact that recombinant CLEC-2 inhibits hemin-mediated platelet aggregation suggests a direct interaction between hemin and CLEC-2. Using surface plasmon resonance-based technique, we demonstrated that hemin binds to both murine and human recombinant CLEC-2 with $K_D$ values of $\sim200$ nM in both cases (Figure 3A, C). Hemin binding to mouse CLEC-2 was characterized by kinetic rate association ($k_a$) and dissociation ($k_d$) constants of $k_a = 2.77\pm0.08 \times 10^4$ mol$^{-1}$ s$^{-1}$ and $5.76\pm0.09 \times 10^{-3}$ s$^{-1}$. Similar values of kinetic rate constants were determined for hemin binding to human CLEC-2, $k_a = 2.65\pm0.09 \times 10^4$ mol$^{-1}$ s$^{-1}$ and $k_d = 5.06\pm0.05 \times 10^{-3}$ s$^{-1}$. The interaction of hemin with human and mouse CLEC-2 was further confirmed by UV-vis absorbance spectroscopy (Figure 3B, D). The differential absorbance spectra profiles revealed significant shifts of the spectra of hemin towards higher wavelength by human and mouse CLEC-2 demonstrating a direct binding. The differences in the spectral changes of hemin in the presence of human and mouse CLEC-2 can be explained by distinct residues coordinating heme's iron. The blue shift in the Soret region observed with mCLEC-2 suggests the involvement of sulfur containing amino acid (cysteine) (11) whereas the red shift observed with human CLEC-2 suggests histidine coordination.

Altogether, these results show that hemin is an endogenous agonist for CLEC-2 leading to platelet activation through activation of integrin GPIIbIIIa at low concentrations and agglutination at high concentrations. Recombinant CLEC-2 inhibits platelet activation by both mechanisms suggesting that this may represent a novel form of therapeutic intervention to inhibit platelet activation in hemolytic disease.

**Ethical approval**

Ethical approval for collecting blood from healthy volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175). Mice experiments were performed in accordance with UK laws [Animal (Scientific Procedures) Act 1986] with approval of the local ethical committee and UK Home Office approval (PPL P0e98D513).

**Platelet preparation and aggregation:**

Human and mouse washed platelets were prepared as previously described (12, 13). Platelet aggregation was assessed using light transmission aggregometry for 6 minutes (ChronoLog, Havertown, PA, USA). Fresh synthetic hemin solution was freshly prepared (Frontiers Scientific, USA) for each experiment. PP2 (Tocris/Biotechne Ltd), PRT-060318 (Caltag medsystems), indomethacin (Sigma-Aldrich), cangrelor (The Medicines Company), Ibrutinib (Stratech Scientific), N-Acetyl-L-cysteine (Sigma-Aldrich) and TAK-242 (Sigma-Aldrich) were preincubated for 5 min at 37°C with platelets prior to addition of hemin. Human (h) and mouse (m) CLEC-2 and glycoprotein VI (GPVI) were produced as Fc fusion proteins (hFc-CLEC-2, hFc-GPVI, Fc-mCLEC-2) in human embryonic kidney HEK293 cells and
purified using chromatography affinity. Recombinant human and mouse CLEC-2 were preincubated with hemin for 15 min at 37ºC before addition to platelets. Following aggregation, platelets were centrifuged at 1000g for 10 minutes and LDH was measured in the supernatant using CyQUANT™ LDH Cytotoxicity Assay (ThermoFisher Scientific). CLEC-2 deficient mice (CLEC-1bfl/fl PF4cre) and littermate controls were previously described (14).

Flow cytometry and western blot:

Platelet activation (10^6 platelets) by different concentrations of hemin (20 minutes at 37ºC) was assessed by flow cytometry with antibodies against CD62P (PE/Cy5 anti-human CD62P Antibody, biolegend) and activated GPIIbIIIa (Alexa Fluor® 647 anti-human CD41/CD61 antibody PAC1, Biolegend) using BD Accuri C6 Plus flow cytometer (BD Bioscience). For western blot, Phospho-Syk (Tyr525/526) (C87C1) Rabbit mAb (#2710), Phospho-PLCγ2 (Tyr759) antibody (#3874), Syk antibody (#2712) and PLCγ2 antibody (#3872) were used (Cell signalling). Protein phosphorylation was assessed after 6 minutes of aggregation as previously described (12).

Recombinant CLEC-2 binding to hemin:

The binding of human and mouse dimeric CLEC-2 to hemin was performed at 25 ºC and assessed using surface plasma resonance (SPR) and UV-spectroscopy methods (15).

Contributions:

JHB, MC performed experiments and analyzed data; YD, AS, EM generated reagents; LTR contributed to data interpretation; JDD contributed to research design, performed experiments and interpreted data; SPW contributed to research and data interpretation and provided reagents; JR designed and performed research, collected data, analyzed and interpreted data and wrote the manuscript. All authors reviewed and approved the manuscript.

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Conflict of interest
The authors have no conflict of interest to declare.

References:


Figure 1: Hemin mediates human platelet activation through CLEC-2.

(A, B) Human washed platelets (2x10^8/ml) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2mM). Platelet aggregation was assessed for 6 minutes using light transmission aggregometry (n=5). (C, D) For flow cytometry analysis, 1x10^8 platelets were incubated with different concentrations of hemin for 20 minutes at 37°C. Platelet activation was determined by flow cytometry using (C) anti-P-selectin and (D) GPIIbIIIa PAC-1 antibodies (n=4). Data is shown as fold increase of median of fluorescence (MFI) of treated platelets over control (absence of hemin). (E) Phosphatidylserine exposure was assessed using Annexin V staining. (F) LDH levels were measured in the supernatant following platelet aggregation (6 minutes). Platelet lysate was used to detect the total level of LDH in platelets. (G, H) Platelet aggregation by hemin was assessed using Btk inhibitor Ibrutinib (500nM), Src family kinase inhibitor PP2 (20μM), Syk inhibitor PRT-060318 (20μM), TLR4 inhibitor TAK-242 (10μM), P2Y_{12} inhibitor Cangrelor (10μM) or cyclooxygenase inhibitor indomethacin (10μM). Platelets were preincubated with different inhibitors for 5 minutes at 37°C prior to the addition of hemin. Dimeric CLEC-2 (hFc-CLEC-2, 20μg/ml) or dimeric GPVI (hFc-GPVI, 20μg/ml) were preincubated with hemin for 20 minutes at 37°C prior to addition to platelets (n=5). (H) Histogram data are shown as mean ± SD. (I) Protein levels of the phosphorylation of Syk and PLCγ2, and total Syk and PLCγ2 in platelet lysates were determined by western blot after 6 minutes aggregation. Western blots are representative of 5 independent experiments. The statistical significance was analyzed using a one-way ANOVA with Tukey’s multiple comparisons test using Prism 8 (GraphPad Software Inc, USA). Significance is shown compared to control (absence of hemin), *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001.

Figure 2: Hemin induces mouse platelet aggregation. (A) Mouse washed platelets (2x10^8/ml) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2mM). Platelet aggregation was assessed using light transmission aggregometry. (B, C) Hemin-mediated platelet aggregation (12.5 μM hemin) was assessed in the presence of Ibrutinib (2μM), PP2 (20μM), PRT-060318 (20μM), TAK-242 (10μM), Cangrelor (10μM), recombinant mouse CLEC-2 (Fc-mCLEC-2, 10μg/ml) (n=4). (D, E) Washed platelets from Wild type (WT) or CLEC-2 deficient (CLEC-1b^{+/+} PF4cre, CLEC-2 KO) were incubated with hemin (12.5 μM) in the presence of Ca^{2+} (2mM) (n=5). (E) Histogram data are shown as mean ± SD. The statistical significance was analyzed using a one-way ANOVA with Tukey’s multiple comparisons test using Prism 8. Significance is shown compared to control ****p<0.0001.

Figure 3: Hemin binds to mouse and human recombinant CLEC-2. (A, C) For SPR, real-time interaction profiles were obtained after injection of increasing concentrations of hemin (19.5 – 625 nM) over recombinant mouse (A) and human (C) CLEC-2 immobilized on CM5 sensor chip. The association and dissociation phases were followed for 5 min, each. The black lines show the experimental data, the grey lines depict the fit obtained using Langmuir global analyses model. (B, D)
For UV-vis absorbance spectroscopy, differential spectra were generated after titration of (B) mouse or (D) human dimeric CLEC-2 (2µM) with increasing concentrations of hemin (0.5 – 16µM). The differential spectra were obtained after subtraction of the spectra of hemin at a given concentration from the spectra of the same concentration of hemin in the presence of the protein. The measurements were done at 25 °C in optical cell with 10 mm light path.
Figure S1: Hemin-mediated human platelet activation does not depend on oxidative stress. (A, B) Human washed platelets (2x10^8/ml) were incubated with hemin (6.25 μM) in the presence of Ca^{2+} (2mM). N-acetyl cysteine (NAC, 100 μM) was incubated with platelets for 5 minutes prior to addition of hemin. Platelet aggregation was assessed for 6 minutes using light transmission aggregometry (n=3).

Figure S2: Recombinant human CLEC-2 (hFc-CLEC-2) inhibits platelet agglutination by high concentrations of hemin. Human washed platelets (2x10^8/ml) were incubated with hemin (50μM). Platelet aggregation was assessed using light transmission aggregometry. Ibrutinib (500nM), PP2 (20μM), PRT-060318 (20μM), TAK-242 (10μM), Cangrelor (10μM), Indomethacin (10μM) were preincubated for 5 min with platelets prior hemin addition. Recombinant hFc-CLEC-2 (50μg/ml) was preincubated with hemin for 15 minutes at 37°C prior addition to platelets. Histogram data are shown as mean ± SD. The statistical significance was analyzed using a one-way ANOVA with
Tukey’s multiple comparisons test using Prism 8 (GraphPad Software Inc, USA). Significance is shown compared to control \(*p<0.05\).