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

Intravascular hemolysis is a serious complication developed in sever infections such as malaria, sepsis,\(^5\) typical hemolytic uremic syndrome\(^6\) and congenital diseases such as sickle cell disease.\(^7\) 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 heme induces human platelet activation and platelet death through ferroptosis, an intracellular iron-mediated cell death.\(^8\) However, the mechanisms and receptors involved in platelet activation by heme are not known.

In this study, we investigated heme-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 \(\mu\)M and 50 \(\mu\)M.\(^9\) Similar concentrations of heme are detected in mice with chronic or acute hemolysis as such as sickle cell mice or Escherichia coli-induced hemolysis.\(^7\) In this study, we show that hemin (2-12 \(\mu\)M) induces rapid aggregation of human washed platelets, while aggregation is slower and reduced in magnitude at higher (\(\geq 25\) \(\mu\)M) concentrations (Figure 1A, B). Platelet aggregation was associated with increased P-selectin expression and GPIIbIIa activation as measured by flow cytometry (Figure 1C, D). High (\(\geq 25\) \(\mu\)M) but not lower concentrations of heme significantly increased phosphatidylserine (PS) exposure on platelets as assessed using Annexin V staining (Figure 1E).

Moreover, high concentrations of heme 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 heme (25 \(\mu\)M) 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.\(^7\) However, blocking TLR4 signaling in platelets using TAK-242 did not alter activation by heme, suggesting a TLR-4 independent pathway of activation (Figure 1H). At low concentrations, human platelet aggregation by heme was blocked by inhibitors of Syk (PYK-060318), Src family kinases (PP2) and Btk (Ibrutinib), and partially by GPIIbIIa inhibitor epifibatide (Integrilin) (Figure 1G, H). These results suggest that heme induces human platelet activation via an immunoreceptor-tyrosine-based activation-motif (ITAM) receptor-based pathway.\(^8\) Indeed, low concentrations of heme provoked phosphorylation of Syk and PLC\(\gamma\)2 (Figure 1I). In order to identify the receptor triggering platelet activation by heme, we used recombinant dimeric forms of C-type-lectin-like receptor-2 (CLEC-2) and collagen receptor glycoprotein VI (GPVI) (hFc-CLEC-2 and hFc-GPVI, respectively) as a blocking strategy. Pre-incubation of heme with hFc-CLEC-2 but not hFc-GPVI abolished platelet aggregation by heme identifying CLEC-2 as the receptor mediating human platelet activation by heme (Figure 1G, H). These results also suggest a direct interaction between heme 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 heme binds to a distinct site on CLEC-2 to podoplanin (Figure 1H). Protoporphyrin IX, the protomer of heme and other hematoporphyrins 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 heme (6.25 \(\mu\)M) was not altered by cyclooxygenase (COX) (indomethacin) or P2Y12 (cangrelor) inhibitors, showing that, in contrast to podoplanin, secondary mediators are not required for heme-mediated platelet aggregation (Figure 1G, H). This may reflect the ability of heme to induce higher oligomers of CLEC-2 increasing signal transduction. Platelet aggregation does not depend on oxidative stress as heme-mediated platelet aggregation was not altered by the antioxidant N-acetyl cysteine (NAC) (Figure 1H; Online Supplementary Figure S1). At higher concentrations (>25 \(\mu\)M), platelet aggregation was not inhibited by integrin or inhibitors of Src, Syk and Btk, suggesting that higher concentrations of heme induce agglutination (Online Supplementary 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 heme might be related to formation of heme aggregates at high concentrations, which might exert different activities as compared to monomeric or dimeric heme 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 heme was inhibited by inhibitors of Src, Syk and Btk tyrosine kinases, recombinant mouse Fc-mCLEC-2 and GPIIbIIa blockade (Figure 2B, C). In contrast, inhibitors of TLR4, COX and P2Y12 had no effect on platelet aggregation by heme (Figure 2C). Platelet aggregation by heme 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 heme in mice.

The fact that recombinant CLEC-2 inhibits heme-mediated platelet aggregation suggests a direct interaction between heme and CLEC-2. Using surface plasmon resonance-based technique, we demonstrated that heme binds to both murine and human recombinant CLEC-2 with \(K_d\) values of ~200 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 \(k_a=2.77\pm0.80\times10^{4} \text{ mol}^{-1} \text{s}^{-1}\) and \(5.76\pm0.9\times10^{5} \text{s}^{-1}\).
Similar values of kinetic rate constants were determined for hemin binding to human CLEC-2, $k_a=2.65\pm0.09\times10^4$ mol$^{-1}$ s$^{-1}$ and $k_d=5.06\pm0.05\times10^{-3}$ 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 activa-
Figure 2. Hemin induces mouse platelet aggregation. (A) Mouse washed platelets (2x10^9/mL) were incubated with increasing concentrations of hemin in the presence of Ca^{2+} (2 mM). 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 (2 μM), PP2 (20 μM), PRT-060318 (20 μM), TAK-242 (10 μM), Cangrelor (10 μM), recombinant mouse C-type-lectin-like receptor-2 (Fc-mCLEC-2, 10 μg/mL) (n=4). (D, E) Washed platelets from wild-type (WT) or Clec-2 deficient (Clec-1bfl/fl PF4cre, Clec-2 KO) were incubated with hemin (12.5 μM) in the presence of Ca^{2+} (2 mM) (n=5). (E) Histogram data are shown as mean ± standard deviation. 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 C-type-lectin-like receptor-2. (A, C) For surface plasma resonance, real-time interaction profiles were obtained after injection of increasing concentrations of hemin (18.5–625 nM) over recombinant mouse (A) and human (C) C-type-lectin-like receptor-2 (CLEC-2) immobilized on CM5 sensor chip. The association and dissociation phases were followed for 5 minutes, 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 (2 μM) with increasing concentrations of hemin (0.5–16 μM). 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.
tion through activation of integrin GPIbIIIa 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 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 POe98D513).

Human and mouse washed platelets were prepared as previously described. Platelet aggregation was assessed using light transmission aggregometry for 6 minutes (ChronoLog, Haverton, PA, USA). Fresh synthetic hemolysis solution was freshly prepared (Frontiers Scientific, USA) for each experiment. P2 (Tocris/Biotechnie Ltd, PRT-060318 (Caltag medsystem), indomethacin (Sigma-Aldrich), cangrelor (The Medicines Company), ibrituniab (Stratech Scientific), N-Acetyl-L-cysteine (Sigma-Aldrich) and TAK-242 (Sigma-Aldrich) were preincubated for 5 minutes (min) at 37°C with platelets prior to addition of hemin. Human (h) and mouse (m) CLEC-2 and GPVI were produced as FeC 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 37°C before addition to platelets. Following aggregation, platelets were centrifuged at 1,000g for 10 min 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.

Platelet activation (106 platelets) by different concentrations of hemin (20 min at 37°C) was assessed by flow cytometry with antibodies against CD62P (PE/Cy5 anti-human CD62P antibody, biolegend) and activated GPIbIIbIIIa (Alexa Fluor® 647 anti-human CD41/CD61 antibody PAC1, Biolegend) using BD Accuri C6 Plus flow cytometer (BD Bioscience). For western blotting, Phospho-Syk (Tyr759) antibody (#2712), Syk antibody (#2710), and PLCγ (C87C1) Rabbit mAb (#2710), Phospho-PLCγ (Tyr525/526) (C87C1) Rabbit mAb (#2710), and PLCγ antibody PAC1, Biolegend) using BD Accuri C6 Plus flow cytometry affinity. Recombinant human and mouse CLEC-2 were preincubated with hemin for 15 min at 37°C before addition to platelets. Following aggregation, platelets were centrifuged at 1,000g for 10 min 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.

The binding of human and mouse dimeric CLEC-2 to hemin was performed at 25°C and assessed using surface plasma resonance and UV-spectroscopy methods.

**References**