Excess of heme induces tissue factor-dependent activation of coagulation in mice

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ABSTRACT

An excess of free heme is present in the blood during many types of hemolytic anemia. This has been linked to organ damage caused by heme-mediated oxidative stress and vascular inflammation. We investigated the mechanism of heme-induced coagulation activation *in vivo*. Heme caused coagulation activation in wild-type mice that was attenuated by an anti-tissue factor antibody and in mice expressing low levels of tissue factor. In contrast, neither factor XI deletion nor inhibition of factor XIIa-mediated factor XI activation reduced heme-induced coagulation activation, suggesting that the intrinsic coagulation pathway is not involved. We investigated the source of tissue factor in heme-induced coagulation activation. Heme increased the procoagulant activity of mouse macrophages and human PBMCs. Tissue factor-positive staining was observed on leukocytes isolated from the blood of heme-treated mice but not on endothelial cells in the lungs. Furthermore, heme increased vascular permeability in the mouse lungs, kidney and heart. Deletion of tissue factor from either myeloid cells, hematopoietic or endothelial cells, or inhibition of tissue factor expressed by non-hematopoietic cells did not reduce heme-induced coagulation activation. However, heme-induced activation of coagulation was abolished when both non-hematopoietic and hematopoietic cell tissue factor was inhibited. Finally, we demonstrated that coagulation activation was partially attenuated in sickle cell mice treated with recombinant hemopexin to neutralize free heme. Our results indicate that heme promotes tissue factor-dependent coagulation activation and induces tissue factor expression on leukocytes *in vivo*. We also demonstrated that free heme may contribute to thrombin generation in a mouse model of sickle cell disease.

Introduction

Heme is an iron-protoporphyrin complex that plays a central role in oxygen and electron transfer, and is an important functional group in various proteins, for example myoglobin and hemoglobin. As such, heme is critical to oxygen transport, drug metabolism, respiration and signal transduction.[1] Free heme is liberated from hemoglobin released from lysed red blood cells in several pathological states, including bacterial infections, hemolytic anemias, and hemoglobinopathies.[1] The plasma proteins haptoglobin and hemopexin scavenge hemoglobin and heme, respectively, and clear them from the blood.[2] Unfortunately, these protective mechanisms can become overwhelmed in many pathological states, resulting in complete depletion of hemopexin and very high levels of free heme in the blood.[1,3] For example, in sickle cell disease patients, chronic hemolysis results in steady state blood heme levels around 4 μM, but these levels can reach 20 μM in some patients.[4]

An excess of free heme is highly toxic, because it catalyzes the formation of reactive oxygen species (ROS), leading to oxidative stress and cell injury.[1,5,6] Free heme is highly lipophilic and can intercalate in the lipid membranes of the vascular endothelium. These two processes result in endothelial cell (EC) activation, recruiting leukocytes to the endothelium, and initiating an inflammatory response that damages the endothelium and increases vascular permeability.[5]

Tissue factor (TF) is the primary initiator of the extrinsic coagulation cascade, and the TF:FVIIa complex activates both FX and FIX, resulting in thrombin generation, fibrin deposition and platelet activation.[7] TF is constitutively expressed by perivascular cells and is sequestered from the blood, but upon tissue injury it can initiate clotting.[8] Inducible TF expression is also observed on monocytes in various pathological conditions, including sepsis[9,10] and sickle cell disease.[11,12] Heme has been demonstrated to induce TF expression and activity on endothelial cells *in vitro*.[13] Furthermore, TF expression has been observed on the lung endothelium in mouse models of sickle cell disease.[14,15] We previously demonstrated that, in mouse models of sickle cell disease, TF contributes to both coagulation activation and vascular inflammation.[14]

In this study, we investigated the mechanism by which excess of free heme contributes to the activation of coagulation in a mouse model of heme overload.

Methods

Mice

Male and female C57BL/6J mice (10-12 weeks old) were used for dose response and time course studies. Generation of $TF^{flox/flox}$, Tie-2

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Cre + and $TF^{flox/flox}$, LysM Cre+ mice,[16] low TF mice ($mTF^{-/-}$, hTF^{+}) and heterozygous littermate controls ($mTF^{+/-}$, hTF^{+}),[17] and $FXI^{-/-}$ mice[18] have been previously described. Human TF (HTF) mice, expressing no mouse TF but normal levels of human TF, have also been previously described.[19] To generate human-mouse TF chimeras, C57Bl/6J mice were lethally irradiated with two doses of 7 Gγ (4 h apart) with a 137Cs irradiator (Mark I Irradiator, J. L. Shepherd & Associates) then injected with bone marrow (BM) (4-5 x 10^6 nucleated cells) from HTF mice via the retro-orbital (RO) sinus. These mice, referred to as HTF^{BM}/WT, express human TF on hematopoietic cells and mouse TF on all non-hematopoietic cells. Mice recovered for 12 weeks before heme treatment. All animal studies were approved by the University of North Carolina Animal Care and Use Committees and complied with National Institutes of Health guidelines.

Preparation and administration of heme

Porcine heme (Sigma Aldrich, St. Louis, MO, USA) was prepared at 25 mM in 0.1M NaOH and 0.1M Tris-HCl (final pH 8.0), sterile filtered (0.22 μm) and diluted to 10 mM in saline. Vehicle buffer was prepared by diluting sterile-filtered 0.1M NaOH and 0.1M Tris-HCl (pH=8.0) in saline. Mice were anesthetized with isoflurane, and administered heme via RO injection at doses from 0-35 μmol/kg.[5] These doses are based on the predicted molarity of heme in blood based on the estimated blood volume of the mouse.[20]

Administration of antibodies

C57Bl/6J mice were treated with rat anti-mouse TF (1H1) or control rat IgG antibodies (20 mg/kg) by intraperitoneal injection 30 min before heme administration.[16] 1H1 is a monoclonal rat anti-mouse TF antibody that competes for binding of factor VII.[21] Chimeric HTF^{BM}/WT mice were administered anti-human TF (HTF1, 10 mg/kg), anti-mouse TF (1H1, 20 mg/kg) or control IgG (10 mg/kg) 30 min before heme.[22] To inhibit the intrinsic coagulation pathway, C57Bl/bJ mice were treated with mouse IgG or the murine monoclonal antibody 14E11 (6 mg/kg) via intraperitoneal (IP) injection 30 min before heme administration. This antibody inhibits FXIIa-dependent activation of FXI and had an anticoagulant effect in a mouse model of sepsis.[23,24]

Hemopexin treatment of sickle cell mice

We used the Berkley mouse as a model of sickle cell disease.[25] Human hemopexin (Athens Research Technology, Athens, GA, USA) (500 μg in 100 μL PBS per 25 gram of body weight; 280 μmol/kg) was administered to 5-month old male mice via tail vein injection twice a week for two weeks.

Sample collection and analysis

Mice were anesthetized by isoflurane, and blood was drawn from the inferior vena cava (IVC) into syringes containing 3.8% sodium citrate (final ratio 9:1) and centrifuged at 4000 x g for 15 min at 4°C. Plasma was collected and immediately stored at -80°C. Thrombin-antithrombin (TAT) complexes were analyzed using an ELISA kit (Enzygost TAT micro, Siemens Healthcare Diagnostics, Malvern, PA, USA).

Analysis of procoagulant activity and TF staining

Isolation of human PBMCs and mouse white blood cells, the 1-stage clotting assay and TF staining are standard procedures in our laboratory,[14,26] and are described in detail in the *Online Supplementary Methods*.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All data are presented as mean plus or minus SEM. For 2-group comparisons of parametric data, Student *t*-test was performed. For comparisons of more than two groups, one- or two-way ANOVA with Bonferroni *post hoc* analysis were performed. Non-normally distributed data were analyzed via Kruskal-Wallis test followed by Wilcoxon-Mann Whitney *post hoc* analysis. $P<0.05$ was considered significant for all experiments.

Results

Heme causes a dose- and time-dependent activation of coagulation in wild-type mice

Circulating levels of hemopexin in wild-type mice are very high (0.5-1.2 g/L) and provide protection against free heme.[27] In contrast, in sickle cell mice, plasma levels of hemopexin are significantly reduced.[28] To study the contribution of free heme to the activation of coagulation, we first determined the minimal amount of heme required to deplete hemopexin levels in wild-type mice. We found that neither 3.5 μmol/kg nor 17.5 μmol/kg heme affected plasma levels of hemopexin, whereas injection of 35 μmol/kg of heme resulted in almost complete depletion of hemopexin in the plasma 6 h after injection (Figure 1A and B). This observation is consistent with previously reported effects of high doses of heme on plasma levels of hemopexin.[6] Importantly, heme increased plasma levels of TAT in a dose-dependent manner with the highest administered dose (35 μmol/kg) producing a 2.7-fold increase in TAT levels over control (Figure 1C). All further experiments were performed with the 35 μmol/kg dose of heme. In a time course experiment, 35 μmol/kg heme caused significant coagulation activation as early as 1 h after administration, which was maintained throughout the course of the experiment (Figure 1D).

Heme-mediated coagulation activation is dependent on the extrinsic but not the intrinsic pathway

To determine the role of TF in heme-dependent coagulation activation, we used the rat anti-mouse TF blocking antibody 1H1. Mice were treated with rat IgG or 1H1 (25 mg/kg, IP) 30 min prior to heme injection (35 μmol/kg, RO). Inhibition of TF with 1H1 attenuated coagulation activation in heme-treated mice (Figure 2A). As an independent approach, we used low TF mice that expressed very low levels of human TF (1% of wild-type levels) but not mouse TF ($mTF^{-/-}$, HTF^{+}). Similar to anti-TF antibody-treated mice, plasma TAT levels were significantly reduced in heme injected low TF mice compared to controls ($mTF^{+/-}$, HTF^{+}) (Figure 2B).

Next, we investigated if the intrinsic pathway contributes to heme-mediated activation of coagulation. Thirty minutes prior to heme administration, wild-type mice were treated with either mouse IgG or the murine monoclonal antibody 14E11 (6 mg/kg, IP), which blocks FXIIa-dependent activation of FXI.[24] Coagulation activation was not attenuated in 14E11-treated mice (Figure 2C). Furthermore, FXI-deficient mice were not protected from heme-induced coagulation activation (Figure 2D).

Heme induces TF expression on leukocytes

Tissue factor expression has been observed in leukocytes isolated from sickle cell patients[11] and sickle cell mice.[14] Therefore, we determined if heme can induce TF expression on leukocytes. RAW 264.7 mouse macrophages were treated with various concentrations of

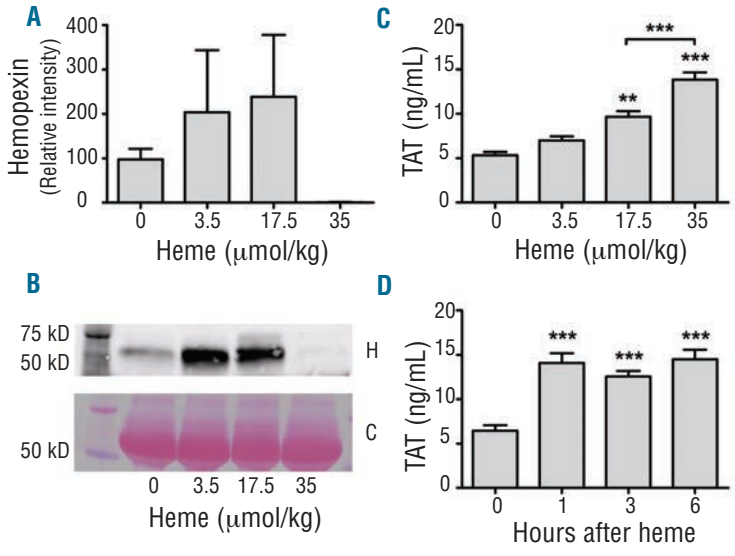

Figure 1. Heme depletes plasma hemopexin levels and activates coagulation. C57BL6/J mice were administered vehicle or heme via retroorbital injection and plasma was collected. (A) Quantification of plasma hemopexin levels 6 h after the injection of the indicated dose of heme (n=4 per group). Data represents fold change *versus* untreated mice. (B) Representative western blot of plasma hemopexin (H). Blots were stained for Coomassie (C) as a loading control. (C) Dose response of coagulation activation as measured by thrombin-antithrombin (TAT) 6 h after various doses of heme (n=4-5/group). (D) Plasma TAT levels measured 1, 3 or 6 h after injection of 35 μmol/kg of heme (n=5-10/group). Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

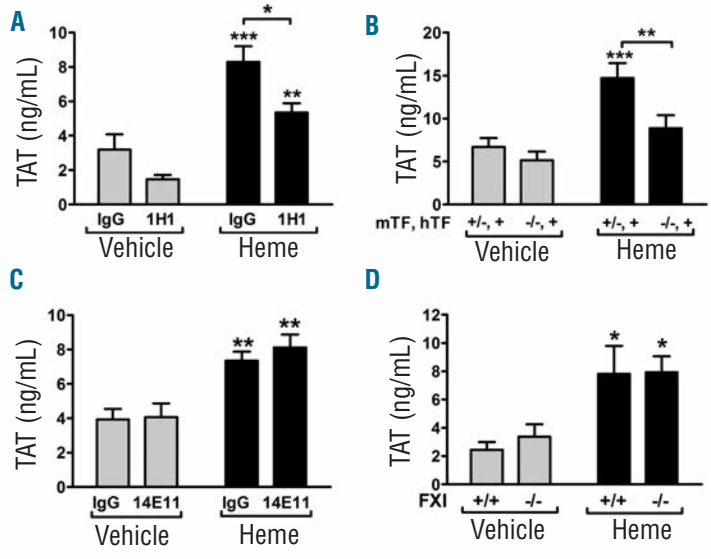

Figure 2. Heme dependent coagulation activation requires tissue factor (TF). (A) Plasma TAT levels in C57BL6/J mice 6 h after vehicle (gray bars) or heme (black bars, 35 μmol/kg) injection (n=5-9). Control rat IgG or 1H1 antibody (25 mg/kg, IP) were injected 30 min prior to heme injection. (B) Plasma TAT levels in control (mTF$^{+/-}$, hTF^{+}) or low TF (mTF$^{-/-}$, hTF^{+}) mice 6 hours after heme injection (n=9-12). (C) Plasma TAT levels in C57BL6/J mice 6 h after vehicle (gray bars) or heme (black bars, 35 μmol/kg) injection (n=5-9). Control mouse IgG or 14E11 antibody (6 mg/kg, IP) were injected 30 min prior to heme injection (n=6-10). (D) Plasma TAT levels 6 h after administration of vehicle or heme in C57BL6/J or FXI$^{+/-}$ mice (n=5). Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. Asterisks over bar indicate significance *versus* respective vehicle-treated control; asterisks over line indicate significance between two groups.

heme (0-50 μM) for 6 h. Heme caused a dose-dependent increase of procoagulant activity (PCA) in RAW 264.7 cells (Figure 3A). PCA was also increased in human PBMCs (Figure 3B). Furthermore, using immunohistochemistry, we analyzed TF protein expression on leukocytes isolated from vehicle or heme-treated mice. We observed TF positive leukocytes isolated from the blood of heme-treated mice but not control mice (Figure 3C). In addition, positive TF staining was observed on human PBMCs treated with heme *in vitro* (Figure 3D). Based on the morphology, TF-positive staining was observed in both monocytes and neutrophils.

Moreover, we analyzed whether heme induces TF expression in lung endothelial cells. Immunohistochemistry revealed TF-positive staining in epithelial and perivascular cells in both control and heme-treated mice, but we did not observe TF staining on endothelial cells in any vessels in the lung using this method (Figure 4). Perivascular TF can be exposed to circulating clotting factors as a result of vascular damage. To determine whether heme damages the vascular endothelium, we measured vascular permeability using the Evans Blue method. In heme-treated mice, we observed an increase in vascular permeability in the heart (1.6±0.2-fold *vs.* control; *P*=0.041), lung (2.4±0.6-fold *vs.* control; *P*=0.026), and kidney (1.3±0.2; *P*=0.05), but not liver, consistent with previous reports.[6]

Contribution of different cellular sources of TF to the heme-induced coagulation activation

To determine the cellular source of TF contributing to heme-induced activation of coagulation, we first analyzed the effect of TF deletion in myeloid cells. TF$^{flox/flox}$, LysM Cre+ and control TF$^{flox/flox}$ mice were injected with heme and plasma TAT levels were analyzed 6 h after injection. We found that deletion of TF from myeloid cells had no effect on heme-dependent activation of coagulation (Figure 5A). Plasma TAT levels were also not attenuated in

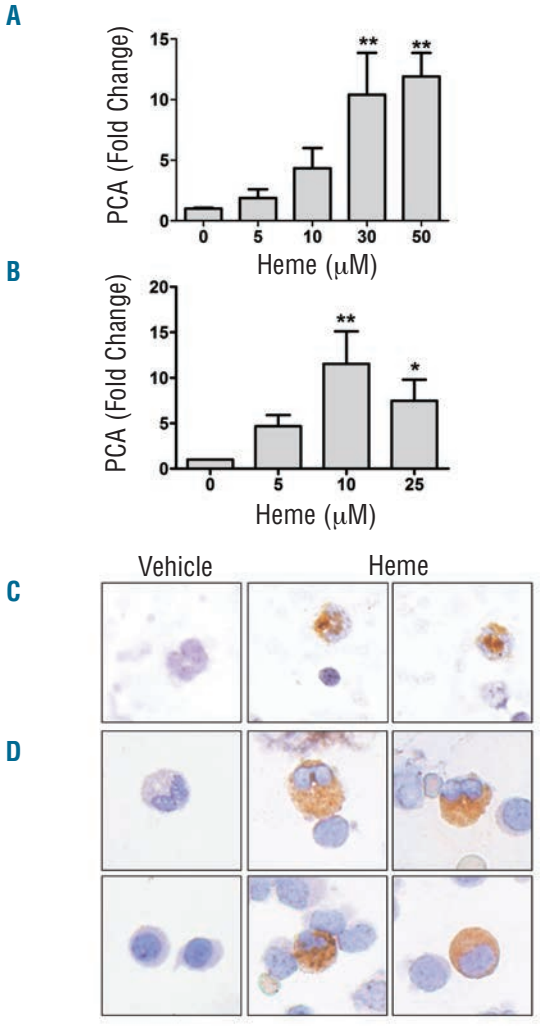

Figure 3. Heme induces tissue factor (TF) expression and activity on leukocytes. (A) Procoagulant activity of RAW 264.7 mouse macrophages treated with the indicated dose of heme for 6 h (n=3). (B) Procoagulant activity of human PBMCs treated with the indicated dose of heme for 24 h (n=4). Data are the mean fold change *versus* vehicle-treated cells, represented as mean±SEM. ***P*<0.01 *versus* 0 μM heme. (C) TF staining (brown) on white blood cells isolated from mice 6 h after heme injection. (D) TF staining (brown) on human PBMCs treated *ex vivo* with 10 μM heme for 24 h.

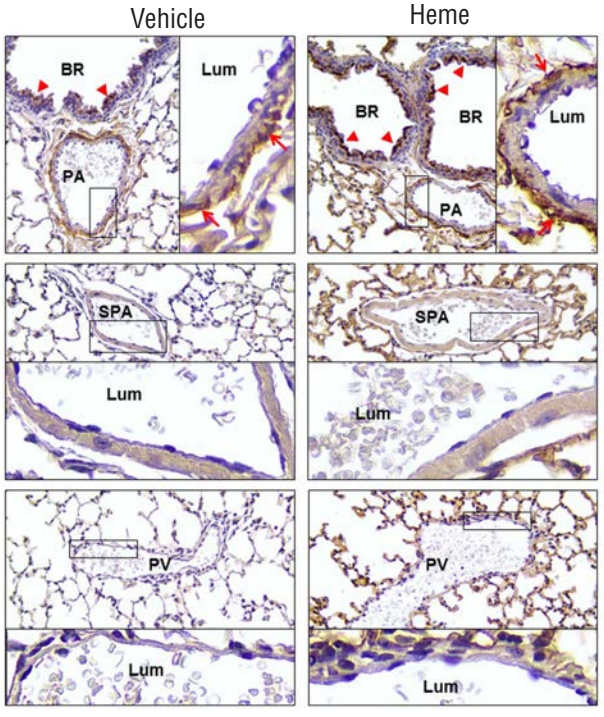

Figure 4. Tissue factor (TF) expression in lung after administration of heme. Representative lung sections demonstrating TF expression in vehicle- and heme-treated mice. TF stains as brown. Low magnification images are 400X, and insets are 400% larger than originals. BR: bronchus; PA: pulmonary artery; SPA: small pulmonary artery; PV: pulmonary vein; Lum: lumen of vessel. Arrowheads indicate epithelial TF and arrows indicate perivascular TF.

heme-injected $TF^{flox/flox}$, Tie2 Cre+ mice in which the TF gene is deleted in both hematopoietic cells and endothelial cells (Figure 5B).

Since heme-induced vascular permeability may result in the exposure of perivascular TF to circulating factor VII/VIIa, we next investigated the contribution of this source of TF to heme-induced activation of coagulation. We generated chimeric mice that express human TF on hematopoietic cells and murine TF on non-hematopoietic cells (WT recipient mice with bone marrow from HTF mice) and used species-specific antibodies to target the different sources of TF. Mice were treated with either IgG, 1H1 or a combination of 1H1 and HTF-1 (mouse anti-human TF antibody) antibodies. Interestingly, inhibition of non-hematopoietic TF with 1H1 had no effect on heme-mediated coagulation activation. However, combining 1H1 and HTF-1, to block both non-hematopoietic and hematopoietic sources of TF, respectively, prevented the activation of coagulation by heme *in vivo* (Figure 5C).

Effect of hemopexin treatment on plasma TAT levels in sickle cell mice

In sickle cell disease and other hemolytic anemias, plasma hemopexin levels are depleted by the excess circulating heme. We hypothesized that excess free heme can contribute to the hypercoagulable state observed in sickle cell disease. Therefore, we investigated whether increasing the hemopexin levels in the circulation could attenuate coagulation in a mouse model of sickle cell disease. Consistent with our previous study,[25] plasma TAT levels were significantly elevated in sickle cell mice compared to non-sickle controls (Figure 6). Administration of hemopexin into sickle mice resulted in a 30% decrease of plasma TAT levels; however, this change was not statistically different from TAT levels in vehicle-treated sickle mice (Figure 6). This partial reduction in plasma TAT levels suggests that free heme might play a minor role in the hypercoagulable state observed in sickle cell mice.

Discussion

We have demonstrated that an excess of circulating free heme can deplete plasma hemopexin levels and trigger systemic thrombin generation in wild-type mice. Furthermore, using both pharmacological and genetic approaches, we found that heme-mediated coagulation

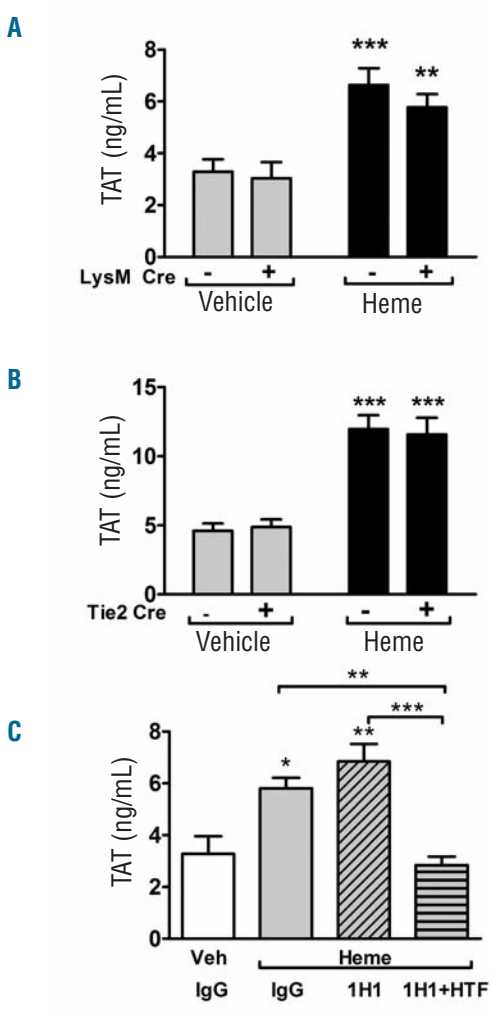

Figure 5. Role of myeloid, hematopoietic and endothelial cell tissue factor (TF) in coagulation activation. (A) Plasma TAT levels in $TF^{flox/flox}$, LysM Cre^{-} or $TF^{flox/flox}$, LysM Cre^{+} mice 6 h after vehicle or heme (35 μmol/kg) injection (n=5-11). (B) Plasma TAT levels 6 h after vehicle or heme injection in $TF^{flox/flox}$, Tie-2 Cre- or $TF^{flox/flox}$, Tie-2 Cre^{+} mice (n=5-8). (C) Plasma TAT levels 6 h after vehicle vehicle or heme injection in HTF^{BM}/WT mice treated with rat IgG (10 mg/kg), 1H1 (20 mg/kg) or HTF-1/1H1 (10 mg/kg and 10 mg/kg, IP) antibodies (n=5-11/group). Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. Asterisks above the bar indicate significance *versus* vehicle/IgG-treated group; asterisks over line indicate significance between two groups.

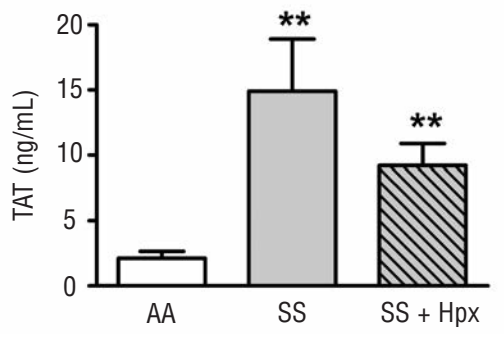

Figure 6. Hemopexin attenuates coagulation activation in sickle cell mice. Five-month old male Berkley sickle cell mice ($m\alpha^{-/-}$, $m\beta^{-/-}$, hβSS) were administered saline or hemopexin (280 μmol/kg intravenous) twice a week for two weeks, and plasma TAT levels were analyzed 16 h after the last injection (n=6-13). Data are presented as mean±SEM. ***P*<0.01 *versus* wild-type (AA) mice by Kruskall-Wallace test.

activation was TF-dependent. It is known that TF expression can be up-regulated on leukocytes during many diseases. Therefore, to identify the cellular sources of TF that contribute to heme-mediated activation of coagulation *in vivo*, we first determined whether heme is capable of inducing TF expression on mouse leukocytes. Indeed, heme treatment caused a dose-dependent increase in procoagulant activity in a mouse macrophage cell line, and TF-positive leukocytes were isolated from heme-treated mice. Despite that, we did not observe an increase in TF-dependent PCA in leukocytes isolated from heme-treated mice (*data not shown*). The reasons for not seeing the increase in TF-dependent PCA are the very small number of TF-positive monocytes in the heme-treated mice, and the possibility that TF-positive leukocytes become activated, migrate outside the vessels, and, therefore, cannot be isolated from blood. In fact, we occasionally observed TF-positive, monocyte-like cells in the lung sections from heme-treated mice (*data not shown*). Furthermore, heme treatment also increased PCA and TF expression on human PBMCs. These data indicate that excess free heme can up-regulate TF expression on leukocytes. Consistent with these findings, we previously showed that in sickle cell disease, monocyte TF expression is positively correlated with markers of hemolysis and coagulation activation.[11] Surprisingly, deletion of TF from all myeloid cells had no effect on heme-induced coagulation activation in mice, indicating that leukocytes are not the dominant source of TF in these experimental conditions.

Since others have demonstrated that heme induces TF expression and activity on endothelial cells *in vitro*,[13] we first tested whether heme can induce endothelial TF expression *in vivo*. We focused on the lung vasculature, because it has been reported to be the only vascular bed demonstrating increased TF staining by endothelial cells in mouse models of sickle cell disease.[15] However, we did not observe any TF-positive endothelial cells in the lung vasculature of mice treated with heme. Consistent with this result, combined deficiency of TF in both hematopoietic and ECs did not attenuate heme-mediated activation of coagulation. These data suggest that the increased expression of endothelial cell TF observed in sickle cell mice is not mediated only by free heme. However, we cannot exclude that induction of TF in lung endothelial cells requires prolonged exposure to free heme. Further studies should determine the mechanism of increased TF expression by lung ECs in sickle cell mice.

The fact that deletion of TF from both endothelial and hematopoietic cells did not attenuate heme-dependent coagulation activation led us to hypothesize that heme may damage the vascular endothelium and expose extravascular sources of TF. Indeed, we found increased vascular permeability in the heart, lung and kidneys of heme-treated mice. However, inhibition of the non-hematopoietic source of TF alone had no effect on coagulation activation induced by heme. It was only when both non-hematopoietic and hematopoietic cell TF were inhib-

ited that coagulation activation was attenuated. These data suggest that both monocyte and perivascular TF contribute to heme-mediated coagulation activation *in vivo*. Importantly, the data presented here differ from observations in a mouse model of endotoxemia, where LPS-induced coagulation activation was partially attenuated by inhibition of either hematopoietic or non-hematopoietic sources of TF.[16] This difference is most likely explained by the fact that the level of coagulation activation in endotoxemic mice was much greater than in heme-treated mice, which allowed us to reveal the partial contribution of TF expressed by both cell types.[29]

It has recently been proposed that in addition to cellular TF, procoagulant microparticles (MPs) may contribute to the propagation of coagulation in various pathological conditions.[30,31] MPs are small membrane vesicles released from activated or apoptotic cells. The procoagulant properties of MPs are due to the presence of phosphatidylserine (PS) with or without TF on their surface.[30] Platelet- and erythrocyte-derived MPs, which are TF negative, contribute to thrombin generation in a FXII/FXI-dependent manner, whereas thrombin generation induced by TF-positive leukocyte-derived MPs requires FVII but not FXII.[32,33] In our dose titration and time course experiment, we did not observe any increase in the total number of PS-positive MPs at 1, 3 or 6 h after heme treatment (*data not shown*), which may explain the lack of FXI contribution to the heme-induced activation of coagulation. Furthermore, it is unlikely that increased levels of free heme can lead to the activation of FXII because treatment of heme-injected mice with 14E11 antibody (an inhibitor of FXIIa-mediated activation of FXI), had no effect on plasma TAT levels. These data strongly support the notion that heme-induced coagulation activation is predominantly driven by TF.

Excess heme has been linked to the pathology of a variety of hemolytic anemias. To examine if the excess of heme can contribute to the activation of coagulation in sickle cell disease, we treated sickle cell mice with recombinant hemopexin. Treatment with hemopexin partially attenuated coagulation activation in sickle cell mice, suggesting that the excess of circulating heme in sickle cell mice might contribute to the hypercoagulable state. Our data contribute to a growing body of evidence suggesting that restoring hemopexin levels in sickle cell mice can be beneficial. Ghosh and colleagues showed that excess heme causes acute chest syndrome in sickle cell mice, and that administration of hemopexin, to reduce plasma levels of free heme, protected sickle mice from acute chest syndrome.[28] Restoring hemopexin also suppressed heme-mediated liver congestion,[6] endothelial activation, oxidative stress and cardiovascular dysfunction in sickle cell mice.[3] Finally, hemopexin attenuated vascular stasis and vaso-occlusion in sickle cell mice.[20] These findings all implicate that administration of hemopexin might have therapeutic potential for sickle cell patients.

Heme has also been suggested to have anticoagulant properties. Recently, it has been shown that heme can bind FVIII *in vitro* and inhibit its procoagulant properties.[34] However, heme could only inhibit the procoagulant functions of FVIII when administered in a molar excess and in the absence of von Willebrand Factor (VWF) which is a natural chaperone of FVIII.[34] This suggests that an excess of heme might have an anticoagulant effect, but this remains to be tested *in vivo*. However, Belcher and colleagues recently demonstrated that heme rapidly triggers the release of VWF from EC both *in vitro* and *in vivo*,[20] which would diminish the anticoagulant properties of heme and support the procoagulant role that we observed.

In summary, our data indicate that an excess of heme promotes systemic thrombin generation in mice that is dependent on the extrinsic coagulation pathway but not the intrinsic pathway. Furthermore, we demonstrate that excess heme can increase TF expression and procoagulant activity on leukocytes both *in vitro* and *in vivo*, and that heme increases vascular permeability and exposes extravascular TF. Using cell-type specific knockouts and chimeric mice, we showed that mice are protected from heme-induced coagulation activation only when all cellular sources of TF are inhibited. Finally, we demonstrated that hemopexin partially attenuated thrombin generation in sickle cell mice, suggesting that excess heme influences coagulation, and might contribute in part to the thrombotic complications of sickle cell disease.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. Toxicol Lett. 2005;157(3):175-188.
2. Schaer DJ, Buehler PW, Alayash AI, et al. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood. 2013;121(8):1276-1284.
3. Vinchi F, De Franceschi L, Ghigo A, et al. Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases. Circulation. 2013; 127(12):1317-1329.
4. Jison ML, Munson PJ, Barb JJ, et al. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. Blood. 2004;104(1):270-280.
5. Wagener FA, Eggert A, Boerman OC, et al. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. Blood. 2001;98(6):1802-11.
6. Vinchi F, Gastaldi S, Silengo L, et al. Hemopexin Prevents Endothelial Damage and Liver Congestion in a Mouse Model of Heme Overload. Am J Pathol. 2008; 173(1):6.
7. Pawlinski R, Pedersen B, Erlich J, et al. Role of tissue factor in haemostasis, thrombosis, angiogenesis and inflammation: lessons from low tissue factor mice. Thromb Haemost. 2004;92(3):444-450.
8. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol. 2007;27(8):1687-1693.
9. Drake TA, Ruf W, Morrissey JH, et al. Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. J Cell Biol. 1989;109(1):389-395.
10. Gregory SA, Morrissey JH, Edgington TS. Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Molecular and cellular biology. 1989;9(6):2752-2755.
11. Setty BN, Key NS, Rao AK, et al. Tissue factor-positive monocytes in children with sickle cell disease: correlation with bio-

markers of haemolysis. Br J Haematol. 2012;157(3):370-80.
12. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood. 2003;102(7): 2678-2683.
13. Setty BN, Betal SG, Zhang J, et al. Heme induces endothelial tissue factor expression: potential role in hemostatic activation in patients with hemolytic anemia. J Thromb Haemost. 2008;6(12):2202-2209.
14. Chantrathammachart P, Mackman N, Sparkenbaugh E, et al. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. Blood. 2012;120(3):636-646.
15. Solovey A, Kollander R, Shet A, et al. Endothelial cell expression of tissue factor in sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin. Blood. 2004;104(3):840-846.
16. Pawlinski R, Wang JG, Owens AP 3rd, et al. Hematopoietic and nonhematopoietic cell tissue factor activates the coagulation cascade in endotoxemic mice. Blood. 2010;116(5):806-814.
17. Parry GC, Erlich JH, Carmeliet P, et al. Low levels of tissue factor are compatible with development and hemostasis in mice. J Clin Invest. 1998;101(3):560-569.
18. Gailani D, Lasky NM, Broze GJ Jr. A murine model of factor XI deficiency. Blood Coagul Fibrinolysis. 1997;8(2):134-144.
19. Pawlinski R, Tencati M, Holscher T, et al. Role of cardiac myocyte tissue factor in heart hemostasis. J Thromb Haemost. 2007;5(8):1693-1700.
20. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood. 2014; 16;123(3):377-390.
21. Kirchhofer D, Moran P, Bullens S, et al. A monoclonal antibody that inhibits mouse tissue factor function. J Thromb Haemost. 2005;3(5):1098-1099.
22. Wang JG, Manly D, Kirchhofer D, et al. Levels of microparticle tissue factor activity correlate with coagulation activation in endotoxemic mice. J Thromb Haemost. 2009;7(7):1092-1098.
23. Tucker EI, Verbout NG, Leung PY, et al. Inhibition of factor XI activation attenuates inflammation and coagulopathy while improving the survival of mouse polymicrobial sepsis. Blood. 2012;119(20):4762-4768.
24. Cheng Q, Tucker EI, Pine MS, et al. A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. Blood. 2010;116(19):3981-3989.
25. Sparkenbaugh EM, Chantrathammachart P, Mickelson J, et al. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. Blood. 2014;123(11):1747-1756.
26. Kasthuri RS, Glover SL, Jonas W, et al. PF4/heparin-antibody complex induces monocyte tissue factor expression and release of tissue factor positive microparticles by activation of FcgammaRI. Blood. 2012;119(22):5285-5293.
27. Tolosano E, Altruda F. Hemopexin: structure, function, and regulation. DNA Cell Biol. 2002;21(4):297-306.
28. Ghosh S, Adisa OA, Chappa P, et al. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. J Clin Invest.2013;123(11):4809-4820.
29. Pawlinski R, Pedersen B, Schabbauer G, et al. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. Blood. 2004;103(4):1342-1347.
30. Owens AP 3rd, Mackman N. Microparticles in hemostasis and thrombosis. Circ Res. 2011;108(10):1284-1297.
31. Key NS, Chantrathammachart P, Moody PW, et al. Membrane microparticles in VTE and cancer. Thromb Res. 2010;125 Suppl 2:S80-83.
32. van Beers EJ, Schaap MC, Berckmans RJ, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica. 2009;94(11):1513-1519.
33. Van Der Meijden PE, Van Schilfgaarde M, Van Oerle R, et al. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost. 2012;10(7):1355-1362.
34. Repesse Y, Dimitrov JD, Peyron I, et al. Heme binds to factor VIII and inhibits its interaction with activated factor IX. J Thromb Haemost. 2012;10(6):1062-1071.