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HEME INDUCES ENDOTHELIAL TISSUE FACTOR EXPRESSION: POTENTIAL ROLE IN HEMOSTATIC ACTIVATION IN PATIENTS WITH HEMOLYTIC ANEMIA

Running Head: Heme Induces Endothelial Tissue Factor Expression

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SUMMARY

Objectives: We explored the possibility that heme, an inflammatory mediator and a product of intravascular hemolysis in patients with hemolytic anemia including sickle cell disease, could modulate hemostasis by an effect on endothelial tissue factor (TF) expression. *Methods:* Levels of TF mRNA, protein, and procoagulant activity were measured in heme-treated endothelial cells. *Results:* Heme induces TF expression on the surface of both macro- and micro-vascular endothelial cells in a concentration-dependent manner with 12- to 50-fold induction noted (ELISA assays) between 1 and 100 μM heme ($P<0.05$). Complementary flow cytometry studies showed that the heme-mediated endothelial TF expression was quantitatively similar to that of TNF-α. Heme also up-regulated the expression of TF mRNA (8- to 26-fold), protein (20- to 39-fold) and procoagulant activity (5- to 13-fold) in endothelial cells in a time-dependent manner. Time-course of heme-mediated TF antigen expression paralleled induction of procoagulant activity with antibody blocking studies demonstrating specificity for TF protein. IL-1α, and TNF-α are not involved in mediating the heme effect, since antibodies against these cytokines, and IL-1-receptor antagonist failed to block heme-induced TF expression. Inhibition of heme-induced TF mRNA expression by sulfasalazine and curcumin suggested that the transcription factor NFκB is involved in mediating heme-induced TF expression in endothelial cells. *Conclusions:* Our results demonstrate that heme induces TF expression by directly activating endothelial cells and that heme-induced endothelial TF expression may provide a pathophysiologic link between the intravascular hemolytic milieu and the hemostatic perturbations previously noted in patients with hemolytic anemia including sickle cell disease.

KEY WORDS: Coagulation, Endothelial cells, Heme, Hemolysis, Sickle Cell Disease, Tissue Factor

INTRODUCTION

Numerous studies have identified that hemostasis is perturbed in patients with hemolytic anemias including sickle cell disease (SCD). Hemostatic abnormalities observed in SCD include evidence for enhanced thrombin generation [1], increased circulatory levels of tissue factor (TF) antigen and procoagulant activity [2,3], and circulating endothelial cells and endothelial- and monocyte-derived microparticles that are positive for TF [4,5]. TF, a cell surface receptor for factor-VII/VIIa, is the physiologic initiator of blood coagulation, forming a complex with circulating factor-VIIa, activating factor-X with subsequent thrombin generation [6]. Although TF is not constitutively expressed on endothelium or monocytes *in vivo*, its expression can be induced by a variety of agonists with pathophysiologic relevance to hemolytic anemias including the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), hypoxia, shear stress, growth factors, endotoxin and reperfusion injury [7-9].

Erythrocytes, that undergo hemolysis intravascularly, release heme and hemoglobin into the circulation with documented studies suggesting that both heme and cell-free hemoglobin may modulate disease severity in patients with SCD by decreasing the bio-availability of the cyto-protective mediator nitric oxide (NO) [10-12]. Other studies have shown that heme can activate endothelial cells *in vitro*, up-regulating the expression of surface adhesion molecules such as VCAM-1, ICAM-1 and E-selectin [13]. Since many cytokine-induced down-stream signaling events in endothelial cells are common to both TF induction and adhesion molecule expression, we hypothesized that heme could affect hemostasis by modulating endothelial TF expression. We report that heme at pathologically relevant concentrations induces TF mRNA and protein in

endothelial cells. We show that TF produced in response to heme is expressed on the cell surface, and that it is functional.

MATERIALS and METHODS

Materials: Three TF-specific antibodies including a rabbit polyclonal and two mouse monoclonal (clones hTF-1 and TF9-10H10) antibodies were used in this study. The rabbit polyclonal antibody and the mouse monoclonal antibody hTF-1 were kindly provided by Dr Ronald Bach (VA Medical Center, Minneapolis, MN), and the clone TF9-10H10 was obtained from Calbiochem (La Jolla, CA). Details of these antibodies and other reagents used are described in the Supplementary Section.

Culture of Endothelial Cells and THP-1 Cells: Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Lonza, Walkersville, MD) and cultured according to the manufacturer's protocol. Cells at passages 2 to 4 were used. All experiments were performed using endothelial growth medium containing 50µg/ml polymyxin-B (EGM-polyB), unless otherwise indicated. Representative experiments also were performed using human lung microvascular endothelial cells (HLMECs, passages 2 to 4) from Clonetics. Endothelial cells were incubated in the absence or presence of the desired agonist (1-100µM heme, 10ng/ml IL-1α, or 10ng/ml TNF-α) for various times (0.5-8 hours), and then analyzed for TF mRNA, TF protein or TF procoagulant activity. As a positive control for inducible TF expression, we used THP-1 cells (a human monocytic leukemia cell line, ATCC, Manassas, VA), activated with LPS in the absence of polymyxin-B.

***Preparation of Heme*:** Fresh working stocks of 10mM heme were prepared in 0.05N NaOH, diluted to desired concentration in EGM-polyB, and pH adjusted to 7.4. Endotoxin-free water was used in the preparation of all reagents. Endotoxin levels in reagents and media were assessed using a limulus assay kit (Lonza, Wakersville, MD).

Analysis of Endothelial TF Protein: Cell surface TF expression was assessed using two complementary methods including an ELISA-based assay (intact cell monolayer) and flow cytometry (cell suspension) as previously described for endothelial adhesion receptors [**14**]. Total TF protein level was assessed using Western blotting of cellular proteins. While the monoclonal anti-TF antibodies, TF9-10H10 and hTF-1, were used in ELISA and/or flow cytometry procedures, the rabbit polyclonal anti-TF antibody was employed in Western analysis (for details refer to supplementary section).

Analysis of Endothelial TF mRNA: TF mRNA levels in control and treated endothelial cultures were measured using a two-step semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay employing the primers 5'-TGT-GAC-CGT-AGA-AGA-TGA-ACG-GAC-3' (forward) and 5'-CCA-CTC-CTG-CCT-TTC-TAC-ACT-TGT-3' (reverse), which yielded a 380-bp PCR product for TF. Identity of PCR fragment from the endothelial TF transcript was confirmed by sequencing the purified PCR fragment, and matching the sequence with published TF mRNA (for details refer to supplementary section).

TF Functional Activity: TF procoagulant activity on intact endothelial monolayers or in cell lysates prepared from control and treated cultures were measured using a TF activity assay kit (American Diagnostica, Stanford, CT). To confirm that the conversion of factor-X to factor-Xa was TF-mediated, in parallel experiments either the cell

monolayers (~50,000 cells) or endothelial lysates (2.5μg protein) were pre-incubated with the monoclonal anti-TF antibody hTF-1 (15μg) for 30-minutes, and assayed for TF activity.

Statistical analysis: Statistical evaluation was performed using Sigmastat (Jandel, San Rafael, CA). All values presented are mean±SD. Multiple group comparison was done using either one-way ANOVA or the Kruskal-Wallis test, as appropriate, and if significant, group-wise comparisons were performed with the Bonferroni or the Dunn's test. Differences at $P<0.05$ were considered statistically significant. Paired-group comparison was performed using either the paired t-test, or the Mann-Whitney rank sum test, as appropriate.

RESULTS

Effects of Heme on Endothelial Surface TF Antigen Expression: Using flow cytometry and ELISA-based assays, we investigated whether heme induced endothelial surface TF expression. As depicted in Figure-1A, heme up-regulated TF expression on intact HUVECs in a concentration-dependent manner with 12-, 20-, and 50-fold induction noted at 1, 10, and 100μM heme, respectively, compared to media controls as assessed by ELISA. The effects noted at all heme concentrations were statistically significant at $P<0.05$ or <0.01. A similar concentration-dependent effect of heme on TF expression also was noted on micro-vascular endothelial cells using HLMECs as a representative cell system (Figure-1B). In parallel experiments, the positive control TNF-α increased TF expression by ~80-fold on both macro- and micro-vascular endothelial cells. Cell surface TF expression was also confirmed using complementary

flow cytometry employing two different monoclonal anti-TF antibodies: TF9-10H10 (Figure-1C) and hTF-1 (Figure-1D). Results demonstrate that significant numbers of cells acquired TF-positivity following activation with heme (22% and 21% cells with TF9-10H10 and hTF-1, respectively). Cells stimulated with the positive control, TNF-α exhibited 27% and 26% TF-positivity, respectively.

Effects of Heme on Endothelial TF mRNA Expression: In preliminary experiments we found that heme induced TF mRNA expression in a concentration-dependent manner with 8-, 15-, and 26-fold up-regulation noted at 1, 10 and 100μM heme, respectively. Since maximal responses with heme on both TF mRNA and TF protein expression (Figure-1) were found at 100μM, our subsequent experiments were performed using 100μM heme. This agonist induced TF mRNA expression in a time-dependent manner with maximal responses between 2 and 3 hours (Figure-2A). TF induction in response to the positive controls IL-1α and TNF-α occurred earlier when compared to heme with TF mRNA already present in endothelial cells within 30-minutes following cytokine exposure. Profiles of TF mRNA expression in endothelial cells and THP-1 cells are shown in Figure-2B. Results demonstrate that total RNA from activated endothelial cells and THP-1 cells contained TF mRNA yielding an RT-PCR fragment of 380-bp. Identity of the endothelial PCR fragment was confirmed by DNA sequence analysis which showed 100% identity to the published TF mRNA sequence. Densitometric analyses demonstrated that incubation of endothelial cells with 100μM heme for 2-hours up-regulated TF mRNA levels by approximately 17-fold (n=4) compared to un-stimulated control cells (Figure-2B), while increases of 29-fold and 48-fold were noted in cells incubated with TNF-α, and IL-1α, respectively (Figure-2B).

Effects of polymyxin-B on LPS-, heme-, and IL-1α-induced endothelial TF expression were evaluated. While polymyxin-B had no inhibitory effect on either heme-, or IL-1α-induced endothelial TF expression, LPS (1μg/ml)-induced TF mRNA expression was inhibited by 86% (supplementary Figure-S1). In addition, heme used in our experiments was free of any detectable endotoxin as assessed by the limulus assay (<0.01EU/ml in test medium containing 100μM heme).

Effects of Heme on Endothelial total TF Protein Levels: Heme induced TF protein expression in a time-dependent manner with 20- to 39-fold stimulation over controls noted between 4 and 7 hours with a peak response at 4-hours similar to that noted with cytokines. The latter agonists, however, induced significant protein expression by 2-hours (Figure-2C). Western blots from representative experiments showing the profiles of TF protein and β-tubulin (the loading control) from endothelial cells, and THP-1 cells are shown in Figure-2D. Results demonstrate that only activated endothelial cells and THP-1 cells expressed TF protein. Densitometric analyses of the blots demonstrated that incubation of endothelial cells with 100μM heme for 4-hours up-regulated TF protein expression by approximately 35-fold (n=4) compared to a 70-fold and 88-fold increase in cells incubated with TNF-α, and IL-1α, respectively (Figure-2D).

Effects of Heme on Endothelial TF Functional Activity: TF expressed in endothelial cells in response to heme (100μM) was functionally active as assessed by the ability of cell lysates to convert factor-X to factor-Xa in the presence of exogenously provided factor-VIIa. Heme-treated endothelial cells expressed TF procoagulant activity in a time-dependent manner with 5- to 13-fold increases over control between 4 and 7 hours with peak activity at 4-hours (Figure-3A). The time-course of TF activity

paralleled the time-course of TF antigen expression in heme-treated cells (Figure-3A and Figure-2C). Similar time-course profiles were noted with TNF-α and IL-1α (Figure-3A). Blocking experiments with anti-TF (Figure-3B) demonstrated that more than 90% of procoagulant activity with all three agonists (heme, TNF-α, and IL-1α)-treated cells was specifically TF-associated. Since TF procoagulant activity in cell lysates measures total cellular activity including cell surface, encrypted and intracellular TF, we further evaluated whether heme induces the expression of functional procoagulant TF on intact endothelium. Results demonstrate that both heme and cytokines induced cell surface TF procoagulant activity with 2.6-, 7.2-, and 11.9-fold increases noted over media control (Figure-3C). Measured surface procoagulant activity was, however <10% of the total activity in both heme- and cytokine-activated endothelial cells (Figures-3B and 3C).

Effects of Curcumin and Sulfasalzine on Heme-Induced TF mRNA Expression: In an attempt to assess whether the transcription factor NFκB was involved in mediating heme-induced TF expression, we evaluated the effects of inhibitors of NFκB activation including sulfasalazine and curcumin [**15,16**]. Following pretreatment of endothelial cells for 30-minutes with sulfasalazine (0.2mM) or curcumin (10μM), monolayers were incubated for 2-hours in the absence or presence of 100μM heme. Results presented in Figure-4 demonstrate that both sulfasalazine and curcumin inhibited heme-induced TF mRNA expression in endothelial cells suggesting that NFκB is involved in mediating heme-induced TF expression.

DISCUSSION

We explored the possibility that heme, a product of intravascular hemolysis in patients with hemolytic anemias, could modulate hemostasis by an effect on endothelial

TF expression. Our results demonstrate that heme, at pathologically relevant concentrations, induces TF expression (both message and protein) in endothelial cells, and that it is biologically active.

TF, a 47-kDa transmembrane glycoprotein, the cellular receptor and cofactor for coagulation factor-VII/VIIa, and the predominant initiator of blood coagulation [6], can be expressed on activated monocytes and endothelial cells [7]. Expression on the endothelium can be induced by a variety of agonists including inflammatory cytokines IL-1 and TNF-α [7]. While multiple nuclear transcription factors are involved in TF induction in endothelial cells, the agonists IL-1α and TNF-α mediate their effects through activation of AP-1 and NFκB [7,17]. Since heme, like IL-1 and TNF-α, appears to activate NFκB, inducing the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelial cells *in vitro* [13,18], we hypothesized that heme could modulate TF expression. We demonstrate that heme induces expression of both TF mRNA and functionally active TF protein with time-courses not too dissimilar to that of IL-1α and TNF-α (Figures-2A, 2C and 3A), although the cytokine response occurred somewhat earlier. Further, the heme effect on endothelial TF expression was not mediated via release of the endogenous cytokines IL-1α and TNF-α from the test endothelial cells (supplementary results). *In vitro* studies have demonstrated that both sulfasalazine and curcumin inhibit NFκB activation in epithelial cells and endothelial cells [15,16], and curcumin, in addition, may affect activation of AP-1, Egr-1 and SP-1 [16]. In preliminary experiments we further assessed whether heme-induced TF expression was modulated by sulfasalazine and curcumin. Both modulators blocked heme-induced TF mRNA expression (Figure-4) suggesting that NFκB is one of the

transcription factors involved in mediating the observed heme-effects. In this context it is interesting to note that a recent study by Hasan and Schafer has demonstrated that induction of Egr-1 expression by heme caused TF expression in vascular smooth muscle cells [19]. Using flow cytometry and ELISA, we also show that heme induces cell surface expression of TF antigen on both macro-, and micro-vascular endothelial cells (Figures-1A and 1B), and that the cell surface expression of TF by heme was comparable to that induced by TNF-α (Figures-1C and 1D). Since plasma heme levels in patients with intravascular hemolytic anemia such as SCD and paroxysmal nocturnal hemoglobinuria (PNH) have been documented to range between 20μM and 600μM [10,20], and since significant changes in TF expression reported here were noted at heme concentrations as low as 1μM, the heme-effects observed in our study appear to be pathologically relevant. In addition, relevance may also be extended to situations associated with acute elevations in levels of plasma hemoglobin such as cardiopulmonary bypass. These conditions carry an increased thrombotic risk [20-22].

Since endotoxin can induce TF expression in endothelial cells even at very low concentrations, use of endotoxin-free reagents and media in our experiments was crucial to delineate the heme-effect. Previous studies have shown that polymyxin-B inhibits endotoxin-induced cell-activation including TF expression in human aortic endothelial cells [23]. We have therefore performed all studies in media containing polymyxin-B. In addition, in preliminary experiments we evaluated the effects of polymyxin-B on agonist-induced TF expression, and confirmed that this agent blocked LPS-mediated, but not heme- or cytokine-induced, TF expression in HUVECs (Figure-S1). Moreover, limulus assay on the media used in our studies was noted to be free of

any detectable endotoxin. We further evaluated whether heme (like hemoglobin [24]) could potentiate LPS-induced TF expression. We found no such enhancement (supplementary results).

Although laboratory and clinical evidence demonstrate enhanced thrombin generation in patients with hemolytic anemia including SCD, the factor(s) that mediate these abnormalities have not been identified. Our results demonstrate that heme induces TF expression at concentrations potentially achievable in the microcirculation. Gladwin and coworkers have documented that intravascular hemolysis produces a state of resistance to endogenously produced, or exogenously delivered NO, due to the stoichiometric oxidation of endogenous NO by cell-free plasma hemoglobin with resultant inhibition of vasodilatation [11]. Decreased NO bioavailability, with consequential impairment in the salutary effects of NO-induced inhibition of platelet aggregation, may be operative in the recently reported enhancement of platelet activation in SCD-related pulmonary hypertension [25]. Our findings serve to further expand the paradigm of hemolysis-induced NO-dependent endothelial dysfunction to additionally include abnormalities in hemostasis based on heme-induced perturbations in fluid-phase hemostasis. In this context it is interesting to note that the study by Solovey *et al* [9] has demonstrated pulmonary endothelial TF expression in sickle transgenic mice following experimental reperfusion injury, a model for SCD-related vaso-occlusion. While no studies to date have reported cause and effect relationship between intravascular hemolysis and thrombogenic risk in patients with hemolytic anemia, a previous preliminary report cited an association between the prothrombotic fragment F1.2 and abnormal transcranial Doppler flow velocities in children with SCD

[26]. In addition, a recent study by deLatour *et al* has addressed the thrombotic history of 460 patients with PNH [21]. These authors reported episodes of thromboses in 116 of 454 accessible patients including the Budd-Chiari syndrome, thrombosis of the central nervous system, and leg thrombosis suggestive of an association between hemolysis and hemostatic perturbations. Other relevant heme effects reported to date include modulation of inflammatory tone by activating neutrophils triggering their oxidative burst, and the production of reactive oxygen species [18,27,28]. Our results taken together with other heme-related published findings may thus provide a critical link between the hemolytic milieu of SCD, coagulation activation and inflammation, and further reiterates that interventions aimed at minimizing intravascular hemolysis may provide therapeutic benefit.

ADDENDUM

BNYS – involved in hypothesis formulation, designed the study, supervised and performed select experiments, performed statistical analyses of the data, and wrote the paper. SGB – performed mRNA-related work and provided assistance in Western analyses. JZ – provided assistance in Western analyses. MJS – involved in hypothesis formulation, reviewed study design and data, and provided critical review of manuscript.

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REFERENCES

1. Stuart MJ, Setty BNY. Hemostatic alterations in sickle cell disease: relationships to disease pathophysiology. Pediatr Pathol Mol Med 2001; **20**:27-46.

2. Key NS, Slungaard A, Dandelet L, Nelson SC, Moertel C, Styles LA, Kuypers FA, Bach RR. Whole blood tissue factor procoagulant activity is elevated in patients with sickle cell disease. Blood 1998; **91**:4216-4223.

3. Mohan JS, Lip GY, Wright J, Bareford D, Blann AD. Plasma levels of tissue factor and soluble E-selectin in sickle cell disease: relationship to genotype and inflammation. Blood Coagulation Fibrinolysis 2005; **16**:209-214.

4. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in sickle cell anemia. J Clin Invest 1998; **101**:1899-1904.

5. Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, Koopmeiners L, Key NS, Hebbel RP. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003; **102**:2678-2683.

6. Nemerson Y. Tissue factor and hemostasis. Blood 1988; **71**:1-8.

7. Camerer E, Kolstø A-B, Prydz H. Cell biology of tissue factor, the principal initiator of blood coagulation. Thrombosis Res 1996; **81**:1-41.

8. Mazzolai L, Silacci P, Bouzourene K, Daniel F, Brunner H, Hayoz D. Tissue factor activity is upregulated in human endothelial cells exposed to oscillatory shear stress. Thromb Haemost 2002; **87**:1062-1068.

9. Solovey A, Kollander R, Shet A, Milbauer LC, Choong S, Panoskaltsis-Mortari A, Blazar BR, Kelm Jr RJ. Hebbel RP. Endothelial cell expression of tissue factor in

sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin. Blood 2004; **104**:840-846.

10. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon III RO, Schechter AN, Gladwin MT. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nature Medicine 2002; **8**:1383-1389.

11. Minneci PC, Deans KJ, Zhi H, Yuen PST, Star RA, Banks SM, Schechter AN, Natanson C, Gladwin MT, Solomon SB. Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin. J Clin Invest 2005; **115**:3409-3417.

12. Kato GJ, McGowan V, Machado RF, Little JA, Taylor VI J, Morris CR, Nichols JS, Wang X, Poljakovic M, Morris Jr SM, Gladwin MT. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension and death in patients with sickle cell disease. Blood 2006; **107**:2279-2285.

13. Wagener FA, Feldman E, deWitte T, Abraham NG. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. Proc Soc Expt Biol Med 1997; **216**:456-463.

14. Setty BNY, Gayen-Betal S. Microvascular endothelial cells express a phosphatidylserine receptor: a functionally active receptor for phosphatidylserine-positive erythrocytes. Blood 2008; **111**:905-914.

15. Wahl C, Liptay S, Guido A, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998; **101**:1163-1174.

16. Pendurthi UR, Williams JT, Rao LVM. Inhibition of tissue factor gene activation in cultured endothelial cells by curcumin. Suppression of activation of transcription factors Egr-1, AP-1 and NFκB. Arterioscler Thromb Vasc Biol 1997; **17**:3406-3413.

17. Mackman N. Regulation of the tissue factor gene. FASEB J 1995; **9**:883-889.

18. Arruda MA, Rossi AG, deFreitas MS, Barja-Fidalgo C, Graca-Souza AV. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide-3-kinase, MAPK, and NF-κB. J Immunol 2004; **173**: 2023-2030.

19. Hasan RN, Schafer AI. Hemin upregulates Egr-1 expression in vascular smooth muscle cells via reactive oxygen species ERK-1/2–Elk-1 and NF-κB. Circulation Research 2008; **18**:42-50.

20. Hartmann RC, Jenkins DE, McKee L. Paroxysmal nocturnal hemoglobinuria: clinical and laboratory studies relating to iron metabolism and therapy. Medicine 1966; **45**:331-363.

21. deLatour RP, Mary J-Y, Slanoubat C, Terriou L, Etienne G, Mohty M, Roth S, deGuibert S, Cahn J-Y, Socie G. Thrombosis in patients with paroxysmal nocturnal hemoglobinuria. Blood 2007; **118**:1072A.

22. Davis CL, Kausz AT, Zager RA, Kharasch ED, Cochran RP. Acute renal failure after cardiopulmonary bypass is related to decreased serum ferritin levels. J Am Soc Nephrol 1999; **10**:2396-2402.

23. Fei H, Berliner JA, Parhami F, Drake TA. Regulation of endothelial cell tissue factor expression by minimally oxidized LDL and lipopolysaccharide. Arterioscler Thromb 1993; **13**:1711-1717.

24. Roth RI. Hemoglobin enhances the production of tissue factor by endothelial cells in response to bacterial endotoxin. Blood 1994; **83**:2860-2865.

25. Villagra J, Shiva S, Hunter LA, Machado RF, Gladwin MT, Kato GJ. Platelet activation in patients with sickle cell disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free hemoglobin. Blood 2007; **110**:2166-2172.

26. Styles L, deJong K, Vichinsky E, Lubin B, Adams R, Kuypers F. Increased RBC phosphatidylserine exposure in sickle cell disease patients at risk for stroke by transcranial Doppler screening. Blood 1997; **90**:604a.

27. Porto BN, Alves LS, Fernandez PL, Dutra TP, Figueiredo RT, Graca-Souza AV, Bozza MT. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem 2007; **282**:20221-20229.

28. Graca-Souza AV, Arruda MAB, deFreitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. Blood 2002; **99**:4160-4165.

FIGURE LEGENDS

Figure-1: Effects of Heme and TNF-α on Surface Expression of TF Protein on Endothelial Cells:

Analysis by ELISA. Following incubation of HUVECs (**panel-A**) or HLMECs (**panel-B**) for 4-hours in the presence or absence of the indicated concentrations of heme or TNF-α, surface TF expression was assessed by ELISA. Results presented are the means±SD from 6 (panel-A) or 3 (panel-B) experiments. All experiments presented were performed with unfixed cells. We have also performed ELISA assays using fixed cells, and found that the TF expression profile in response to activation with heme and cytokines was similar to unfixed cell preparations (i.e. increased TF expression in activated endothelial cells compared to unstimulated medium control). An increased expression of TF on unstimulated control cells was however noted on un-fixed cells (an increase of approximately 20%) compared to fixed cells. *represents $P<0.05$ or <0.01 compared to media control.

Analysis by Flow Cytometry. Following incubation of HUVECs for 4-hours in the absence (**panels-Ci**/**Di**), or presence of 10ng/ml TNF-α (**panels-Cii**/**Dii**) or 100μM heme (**panels-Ciii**/**Diii**), cells were harvested, labeled with anti-TF antibody TF9-10H10 (**panel C**) or hTF-1 (**panel D**), or an equivalent amount of an isotype-matched negative immunoglobulin control, and analyzed. Histogram profiles of FITC-fluorescence from cells labeled with anti-TF antibody (gray histogram) and isotype-matched negative control (solid black line) are shown. The positive histogram-region, M1, is defined using cells labeled with the isotype control. Positivity, shown in each panel, is the difference

in M1 between the cells labeled with anti-TF and the isotype control. Results presented are from a representative experiment repeated thrice with similar results.

Figure-2: Effects of Heme and Cytokines on TF mRNA and Protein Levels in HUVECs:

Panel-A: *Time-dependent changes in TF mRNA levels*: Following incubation of HUVECs with the desired agonist (10ng/ml TNF-α, 10ng/ml IL-1α, or 100μM heme) for times indicated, total RNA was isolated, and analyzed for TF mRNA by RT-PCR. The experiment was repeated twice with similar results.

Panel-B: *Heme and cytokines up-regulate endothelial TF mRNA expression*: HUVECs were incubated in the absence (lane-1), or presence of 10ng/ml TNF-α (lane-2), 10ng/ml IL-1α (lane-3), or 100μM heme (lane-4) for 2-hours, total RNA isolated, and then analyzed for TF mRNA. RT-PCR products of TF mRNA from THP-1 cells (a positive control for TF induction) incubated in the absence (lane-5) or presence of 100ng/ml LPS (lane-6) are shown for comparison. β-Actin mRNA, a constitutively expressing transcript, was co-amplified with TF mRNA as an endogenous control for TF mRNA quantitation using previously described primers [14]. The RT-PCR products of TF mRNA (380-bp), and β-actin mRNA (687-bp) from a representative gel are shown at the top. Band intensities of PCR fragments were determined densitometrically and expressed as a ratio of TF to β-actin mRNAs. Message ratio in the unstimulated medium control was 0.26±0.12. Values (expressed as fold-change compared to media control) are the means±SD from four experiments. *represent $P<0.05$ compared to control.

Panel-C: *Time-dependent changes in TF protein levels*: Following incubation of HUVECs with the desired agonist (10ng/ml TNF-α, 10ng/ml IL-1α, or 100μM heme) for times indicated, total cellular proteins were isolated, and analyzed for TF protein by Western blotting. The experiment was repeated thrice with similar results.

Panel-D: *Heme and cytokines up-regulate endothelial TF protein expression*: HUVECs were incubated in the absence (lane-1), or presence of 10ng/ml TNF-α (lane-2), 10ng/ml IL-1α (lane-3), or 100μM heme (lane-4) for 4-hours and then analyzed for TF protein. Immunoblots from THP-1 cells incubated in the absence (lane-5) or presence of 100ng/ml LPS (lane-6) are shown for comparison. Equal protein loading was checked by re-probing the stripped Immunoblots for β-tubulin protein. TF and β-tubulin (βT) protein bands from representative immunoblots are shown at the top. Intensities of protein bands were determined densitometrically and expressed as a ratio between TF and β-tubulin protein. This ratio in the unstimulated medium control was 0.06±0.06. Values (expressed as fold-change compared to media control) are the means±SD from four experiments. *represent $P<0.05$ compared to control.

Figure-3: Effects of Heme and Cytokines on Endothelial TF Functional Activity:

Panel-A: *Time-Dependent Changes in TF Procoagulant Activity*: Following incubation of HUVECs with the desired agonist (10ng/ml TNF-α, 10ng/ml IL-1α, or 100μM heme) for times indicated, total cellular proteins were extracted and analyzed for TF procoagulant activity. The experiment was repeated thrice with similar results.

Panel-B: *Heme and cytokines up-regulate endothelial TF procoagulant activity in Cell Lysates:* Following incubation of HUVECs for 4-hours with the desired agonist (10ng/ml TNF-α, 10ng/ml IL-1α, or 100μM heme), cell lysates were prepared. Total cellular proteins (2.5μg) were incubated in the presence (black bars) or absence (cross-hatched bars) of a mouse monoclonal anti-TF antibody (hTF-1, 15μg) for 30-minutes, and then assayed for TF functional activity. Results are the means±SD from five experiments. **P<0.05 or <0.01 compared to media control. *P<0.005 by paired t-test compared to respective no antibody treatment controls.

Panel-C: *Heme and cytokines up-regulate endothelial TF procoagulant activity on Intact Cell Monolayers:* Following activation of HUVECs (~50,000 cells in wells of a 96-well plate) for 4-hours with the desired agonist (10ng/ml TNF-α, 10ng/ml IL-1α, or 100μM heme), monolayers were incubated in the presence (black bars) or absence (cross-hatched bars) of a mouse monoclonal anti-TF antibody (hTF-1, 15μg) for 30-minutes, and then assayed for TF functional activity. Results presented are from a representative experiment repeated twice with similar results.

Figure-4: Effects of Sulfasalazine and Curcumin on Heme-Induced Endothelial TF mRNA Expression:

Following pretreatment of HUVECs for 30-minutes with sulfasalazine (0.2mM, lane-3) or curcumin (10μM, lane-4), monolayers were incubated for 2-hours in the absence (lane-1) or presence of 100μM heme (lanes 2-4). Total RNA was extracted and analyzed for TF mRNA. β-Actin mRNA was co-amplified with TF mRNA as an endogenous control for TF mRNA quantitation. The RT-PCR products of TF mRNA

(380-bp), and β-actin mRNA (687-bp) are shown at the top corner. Bars represent densitometric readings of the ratio between TF and β-actin messages. Results presented are from a representative experiment repeated twice with similar results.