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ORIGINAL ARTICLE

Lipopolysaccharide-Induced Hemolysis Is Abolished by Inhibition of Thrombin Generation but Not Inhibition of Platelet Aggregation

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Abstract—In human sepsis, hemolysis is an independent predictor of mortality, but the mechanisms evoking hemolysis have not been fully elucidated. Therefore, we tested the hypotheses that (1) lipopolysaccharide (LPS)-induced hemolysis is dependent on thrombin generation or platelet aggregation and (2) red cell membranes are weakened by LPS. Anesthetized male Wistar rats were subjected to LPS or vehicle for 240 min. The effects of hemostasis inhibition on LPS-induced hemolysis were investigated by use of the thrombin inhibitor argatroban or the platelet function inhibitor eptifibatide. Free hemoglobin concentration, red cell membrane stiffness and red cell morphological changes were determined by spectrophotometry, atomic force microscopy, and light microscopy. Efficacy of argatroban and eptifibatide was assessed by rotational thrombelastometry and impedance aggregometry, respectively. LPS markedly increased free hemoglobin concentration (20.8 μmol/l ± 3.6 *vs.* 3.5 ± 0.3, $n = 6$, $p < 0.0001$) and schistocytes, reduced red cell membrane stiffness, and induced disseminated intravascular coagulation. Inhibition of thrombin formation with argatroban abolished the increase in free hemoglobin concentration, schistocyte formation, and disseminated intravascular coagulation in LPS-treated animals. Eptifibatide had no inhibitory effect. The LPS evoked decrease of red cell stiffness that was not affected by argatroban or eptifibatide. LPS causes hemolysis, schistocyte formation, and red cell membrane weakening in rats. The thrombin inhibitor argatroban but not the platelet inhibitor eptifibatide abolished hemolysis and schistocyte formation. Thus, LPS-induced hemolysis

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Abbreviations: LPS, Lipopolysaccharide

depends on disseminated intravascular coagulation, possibly enhanced by red cell membrane weakening. Clinical studies are necessary to investigate whether thrombin antagonists can decrease hemolysis and mortality in sepsis.

KEY WORDS: lipopolysaccharide; hemolysis; hemostasis; red cell membrane stiffness.

BACKGROUND

We previously demonstrated that increased free hemoglobin plasma concentration is an independent predictor for sepsis mortality [1], and a causal role of free hemoglobin in the pathogenesis of sepsis has been demonstrated in animals [2]. The deleterious effects of free hemoglobin can be explained by the well-known toxicity of cell free hemoglobin and its degradation products [3]. Concerning the pathophysiological mechanisms leading to hemolysis, an involvement of disseminated intravascular coagulation (DIC), which is common in sepsis, has been suggested [4]. Infusion of thrombin was demonstrated to form fibrin strands capable of slicing red blood cells leading to so-called schistocytes in rats [5]. Such fibrin strands have also been demonstrated in humans with disseminated intravascular coagulation [6]. Furthermore, we recently demonstrated as another potential mechanism contributing to LPS-induced hemolysis *in vivo,* that lipopolysaccharide (LPS), the main constituent of the Gram-negative bacterial cell membrane, is incorporated in membranes in a receptor-independent manner and decreases red cell osmotic resistance and membrane stiffness [7, 8]. Interestingly, TLR-4 signaling and an involvement of the complement system as the mechanisms leading to the LPS-induced hemolysis were excluded in this latter study [7].

In the present study, we tested in anesthetized rats the hypotheses that (1) LPS-induced hemolysis depends on thrombin generation or platelet aggregation and (2) red cell membranes are weakened by the endotoxin.

METHODS

Animals

Male Wistar rats (430 to 470 g) were obtained from the central animal care unit of the University Hospital Essen and kept under standardized conditions of temperature (22 °C ± 1), humidity (55% ± 5), and 12-h/12-h light/dark cycles with free access to food (ssniff-Spezialdiäten, Soest, Germany) and water. All animals received humane care according the standards of the Federation of European Laboratory Animal Science Association. The experimental protocol had been approved based on the local animal protection act with the permit number Az.: 84-02.04.2013.A015.

Anesthesia, Analgesia, and Instrumentation

Rats were anesthetized with isoflurane in O_2 (5% isoflurane for induction and 1.5–2% to maintain anesthesia), treated with ketamine (50 mg/kg s.c.) and lidocaine (5 mg/kg s.c.) for analgesia, and subjected to surgical preparation of the right inguinal region for catheter placements into the right femoral artery and vein. Catheters were perfused with Ringer's solution (arterial 3 ml/h, venous 7 ml/kg/h). Mean arterial pressure, heart rate, and respiratory rate of the spontaneous breathing rats were monitored continuously. Body temperature was measured using a rectal probe and was kept above 37 °C. After 240 min, *i.e.*, at the end of the experiments, animals were sacrificed by cardiac incision under deep isoflurane anesthesia.

Experimental Groups and Blood Collections

Experiments were performed using 6 rats per experimental intervention over a total period of 240 min each. LPS, argatroban, and eptifibatide were dissolved in Ringer's solution and administered intravenously using a syringe pump (Perfusor-Secura FT; B. BRAUN, Melsungen, Germany).

The experimental interventions were as follows: infusion of Ringer's solution as a time control, infusion of LPS alone (1 mg/kg/h), infusion of LPS (1 mg/kg/h) and argatroban (50 μg/kg/min) combined, and LPS (1 mg/kg/h) and eptifibatide (180 μg/kg bolus and 2 μg/kg/min) combined.

At different time points (baseline, 60, 120, 180, and 240 min), the blood was collected from the femoral artery into a self-filling arterial sampler (0.7 ml) containing 80 IU electrolyte-balanced heparin (PICO50) Radiometer Medical (Brønshøj, Denmark) for measurements of free hemoglobin concentration, platelet function, red cell stiffness and volume, or into a syringe containing sodium citrate (0.3 ml; 3.13% Eifelfango, Bad Neuenahr-Ahrweiler, Germany) for thromboelastometry examinations. To

compensate for the blood sample-induced blood loss, 1 ml of Ringer's solution was infused after taking each blood sample. Hematocrit decreased from 42.7% ± 2.4 at baseline to 32.7% ± 3.7 at 240 min. No changes in arterial pressure and heart rate were observed with this procedure.

Free Hemoglobin Concentration

For the measurement of free hemoglobin concentration, platelet poor plasma was obtained by centrifugation (3000*g*, 15 min) and stored at − 80 °C. Free hemoglobin concentration was measured spectrophotometrically from the absorption of the hemoglobin *Soret* band (μQuant, Bio-Tek Instruments GmbH, Friedrichshall, Germany) [9].

Assessment of Coagulation and Platelet Function

Sufficient inhibition of coagulation by argatroban was monitored by thromboelastometry of citrated whole blood samples (ROTEM®, TEM International, München, Germany) according to the manufacturer's recommendations. Coagulation was initiated by recalcification with 20 μl 0.2 *M* $CaCl_2$. For evaluation of consumption of hemostatic factors, the maximum clot firmness (MCF) was determined.

Efficacy of platelet aggregation inhibition was measured by impedance aggregometry. Heparin-anticoagulated samples were activated with collagen according to the manufacturer's protocol (Multiplate®, Verum Diagnostica, München, Germany). Platelet aggregation was assessed by determination of the area under the curve and expressed as arbitrary units (A.U.).

Pharmacological Effects of Argatroban and Eptifibatide

As information on argatroban's effects on hemostasis in rats is scarce, the efficacy of argatroban was assessed by measuring the thromboelastometry clotting time (CT). In vehicle-treated animals, clotting time did not change over 240 min (309 s ± 28 *vs.* 319 ± 32; n.s.). LPS in the absence of argatroban caused a shortening of the clotting time (178 s ± 12 after 240 min). In LPS- and argatroban-treated rats, clotting time was prolonged to 623 s ± 71.

To determine the efficacy of eptifibatide (180 μg/kg bolus +2 μg/kg/min) on platelet aggregation inhibition, impedance aggregometry was used. The collagen-induced aggregation averaged 476 A.U. ± 28 and 24 A.U. ± 5 in the absence and presence of eptifibatide (240 min), respectively, demonstrating complete inhibition of platelet aggregation by eptifibatide.

Red Cell Morphology and Schistocyte Formation

Peripheral blood smears were prepared by the wedge slide technique. To assess red cell morphology, light microscopy was performed on an inverted microscope (Zeiss IM35, Carl Zeiss AG, Oberkochen, Germany) using a magnification of × 400. Images were taken using a Canon 1000D single-lens reflex camera (Canon, Krefeld, Germany). Changes in red cell morphology were compared with time-related samples between the different experimental interventions. Definition of schistocytes was according to the International Council for Standardization in Hematology (ICSH). Schistocytes were quantified as the percentage of undamaged red cells per 1000 counted cells at the end of the series in a blinded manner [10, 11].

Red Cell Membrane Stiffness and Red Cell Volume

Red cell membrane stiffness was measured using the atomic force microscopy nanoindentation technique ("Nano Wizard", JPK Instruments, Berlin, Germany) and noncontact/tapping mode high resonance frequency (NCH) cantilevers (NanoWorld, Neuchatel, Switzerland) [7]. An intermittent contact mode at a frequency of 240–270 kHz was used for the imaging. The scan rate was adjusted to 0.75 Hz. The tip was horizontally positioned over the red cells' convex peripheral border and brought in contact with the cell membrane. During the force-tapping mode, the cantilever holder was moved vertically over a distance of 200 nm towards the carrier surface within 5 s. Force mapping was conducted with cantilevers and spring constants of approx. 42 N/m. The cantilever deformation was used to calculate the actual tip position subject to the individual force applied to the red cell membrane. To determine mean red cell volume in LPS and vehicle-treated animals, respectively, and to exclude relevant volume changes potentially altering their membrane stiffness, a Coulter Counter (Sysmex CA 500, Sysmex GmbH, Norderstedt, Germany) was used.

Chemicals and Materials

LPS (*Escherichia coli* serotype 0111:B4) and argatroban (100 mg/ml) were purchased from Sigma-Aldrich (St. Louis, USA), and eptifibatide (2 mg/ml) from Millenium Pharmaceuticals (Cambridge, USA). Isoflurane (Florene) was from Abbott (Wiesbaden, Germany), ketamine 10% from Ceva (Düsseldorf, Germany), lidocaine 1% from AstraZeneca (Wedel, Germany), and Ringer's solution from Fresenius Kabi (Bad Homburg, Germany).

Portex catheters (0.58-mm inner diameter, 0.96-mm outer diameter) were purchased from Smiths Medical International (Hythe, UK). Medical oxygen was obtained from Air Liquide (Düsseldorf, Germany).

Data Analysis and Statistics

Experiments were performed with 6 animals per experimental intervention. Biochemical assays were run in triplicate. Analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6 (GraphPad Prism Software Inc., San Diego, CA). Data are presented as means and standard deviation (SD). Since the D'Agostino-Pearson normality test revealed a normal distribution of variables, statistical analysis was performed using one-way or two-way analysis of variance, as appropriate, with post hoc Bonferroni correction. An *a priori* alpha error p of less than $0.05/n$ was considered statistically significant.

RESULTS

LPS-Induced Increase in Free Hemoglobin Concentration

Free hemoglobin concentration increased in LPS-treated animals from a baseline value of 3.5 μmol/l ± 0.3 to 7.1 μmol/l ± 1.5 after 180 min and to 20.8 μmol/l ± 3.6 after 240 min ($n = 6$, $p < 0.0001$; Fig. 1a). In vehicle-treated rats, no increase in free hemoglobin concentration was detectable. Inhibition of fibrin generation by argatroban abolished the LPS-induced increase in free hemoglobin concentration (Fig. 1b). In contrast, inhibition of platelet aggregation by eptifibatide did not decrease LPS-induced hemolysis (Fig. 1c), although inhibition of platelet aggregation was very profound (see the "METHODS" section).

LPS-Induced Coagulation Disturbance

LPS infusion to rats over 240 min, in contrast to vehicle, decreased clot firmness as evidenced by representative thrombelastograms (Fig. 2) with the decrease in maximum clot firmness becoming apparent after 180 min of LPS infusion. After 240 min, clot firmness had decreased from 71 mm ± 12 to 45 ± 8 ($n = 6$, $p < 0.0001$), suggesting consumption of coagulation factors (Fig. 3). In contrast, no decrease in maximum clot firmness occurred in vehicle-treated rats.

To investigate the effect of an inhibition of fibrin generation on LPS-induced disseminated intravascular coagulation, LPS-treated animals were treated additionally with the direct thrombin antagonist argatroban over the experimental period of 240 min (Fig. 4). Notably, the LPS-induced decrease in maximum clot firmness was abolished by the thrombin antagonist. Maximum clot firmness was 71 mm ± 12 at baseline and 63 mm ± 5 ($n = 6$, $p = 0.67$) after 240 min of combined infusion of LPS and argatroban.

LPS-Induced Alterations in Red Cell Morphology

Morphology of red cells obtained from rats treated with vehicle, argatroban, eptifibatide, LPS, LPS and argatroban combined, and LPS and eptifibatide combined was determined by light microscopy. In comparison with vehicle-treated rats, red cell shape was irregular in LPS-treated animals (compare Fig. 5a, c). Moreover, schistocytes were detectable in LPS-treated rats (6.2% ± 1.2; Fig. 5d). In LPS-treated rats, the schistocyte formation was abolished by argatroban (Fig. 5e), but not by eptifibatide (Fig. 5f). In absence of LPS, neither argatroban nor eptifibatide affected the morphology of red cells in comparison with vehicle (compare Fig. 5a–c). Statistical evaluation of the schistocyte frequency with experimental interventions is shown in Fig. 6.

LPS-Induced Alterations in Red Cell Membrane Stiffness

Red cell membrane stiffness was assessed by the atomic force nanoindentation technique at the beginning and 240 min later at the end of the animal experiments. In LPS-treated rats, red cell stiffness decreased from 230 μN/μm ± 26 to 76 ± 6 ($n = 6$, $p < 0.0001$) after 240 min but remained unchanged with vehicle infusion. Neither argatroban nor eptifibatide affected the membrane weakening in LPS-treated rats (Fig. 7). LPS-induced alterations in membrane stiffness were unexplained by red cell volume changes, as shown by measurements of vehicle- and LPS-treated red cells (54.0 fl ± 2.4 *vs.* 54.6 fl ± 1.8, $n = 6$, n.s.).

Effects of LPS on Arterial Pressure and Heart Rate

Arterial pressure and heart rate were continuously monitored in all experimental groups. As shown in Fig. 8, mean arterial pressure and heart rate remained in the physiological range in all experimental groups

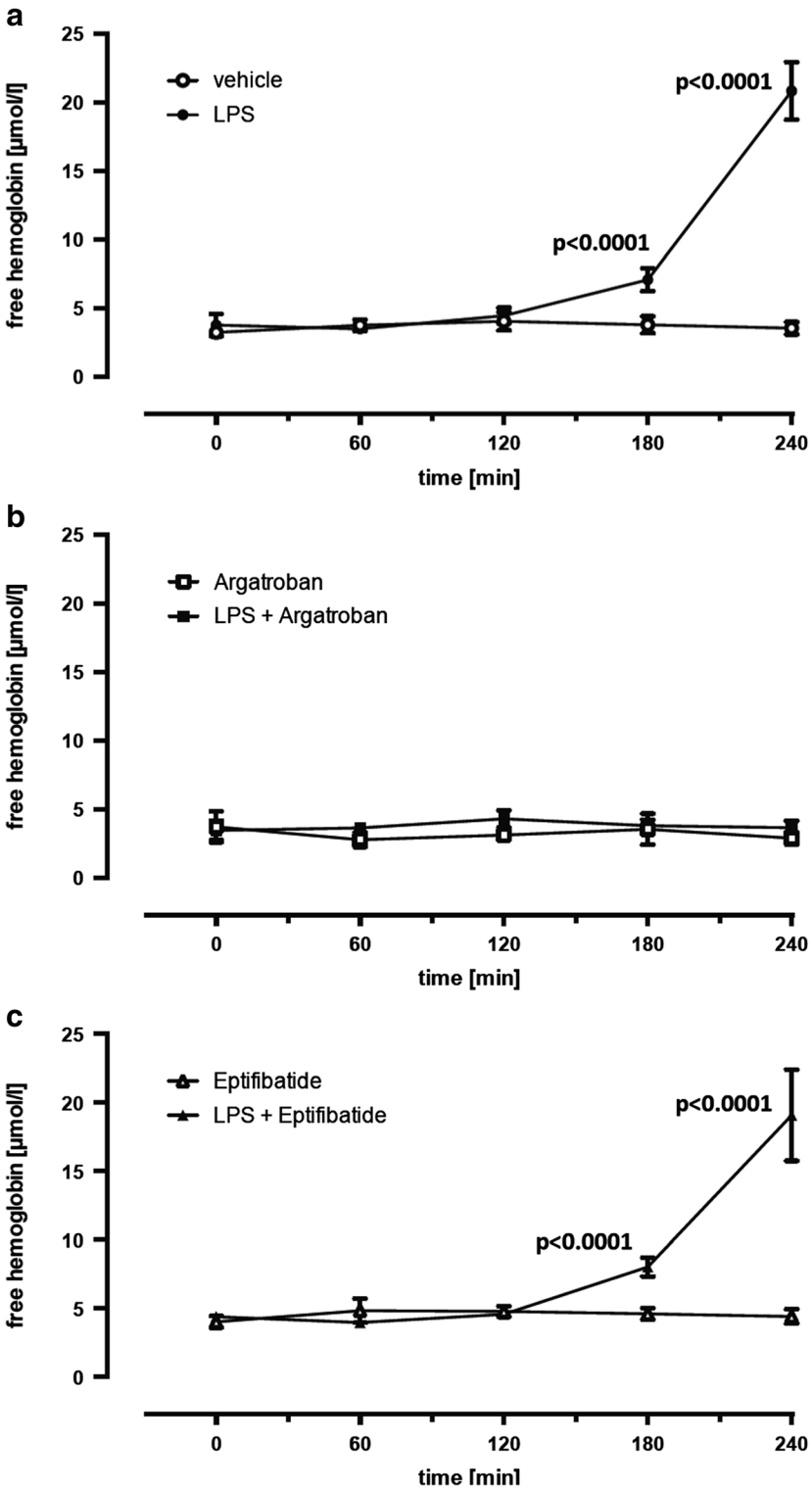

Fig. 1. Effects of LPS on free hemoglobin concentration. LPS (1 mg/kg/h) induced a time-dependent increase of free hemoglobin concentration during endotoxinemia (**a**). Inhibition of thrombin generation with argatroban (50 µg/kg/min) abolished the LPS-induced hemolysis (**b**), while inhibition of platelet aggregation by eptifibatide (180 µg/kg bolus + 2 µg/kg/min) had no effect (**c**). Means ± SD; $n = 6$ per group.

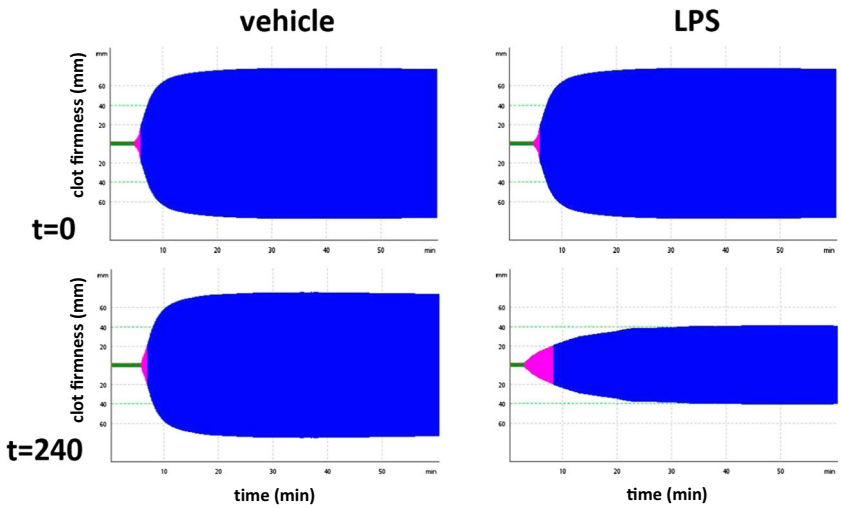

Fig. 2. Effects of LPS and argatroban on whole blood thromboelastometry. Hemostasis was measured by rotational thromboelastometry in whole blood samples obtained from LPS- and vehicle-treated rats, as shown by these representative examples at baseline and after 240 min. The thrombelastograms show the development of clot firmness after initiation of coagulation by recalcification of citrated whole blood samples. Maximum clot firmness was not affected in vehicle-treated rats. In contrast, LPS (1 mg/kg/h) markedly reduced maximum clot firmness, demonstrating consumption of clotting factors by disseminated intravascular coagulation.

within 240 min excluding an endotoxin shock as the reason for the observed increases in free hemoglobin concentration (Fig. 8). In the control group, no change was observed in both variables, while LPS induced a biphasic effect: an increase in arterial pressure after 2 h was followed by a slight decline during the further time course. Moreover, the heart rate slightly increased in LPS-treated animals during the experiment.

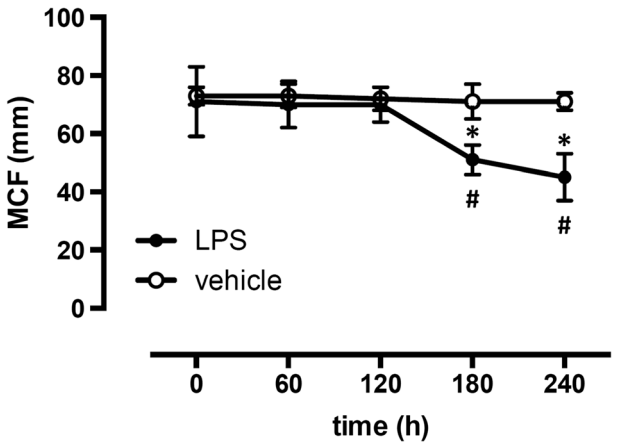

Fig. 3. Effect of LPS on maximum clot firmness. Rats were treated with LPS (1 mg/kg/h) and vehicle, respectively, and citrated whole blood samples were drawn at several time points. Rotational thrombelastometry demonstrated that maximum clot firmness was markedly reduced after 180 and 240 min, thus demonstrating massive consumption of coagulation factors caused by disseminated intravascular coagulation. Means ± SD; $n = 6$. $^{*}p < 0.0001$ *vs.* vehicle; $^{\#}p < 0.0001$ *vs.* $t = 0$.

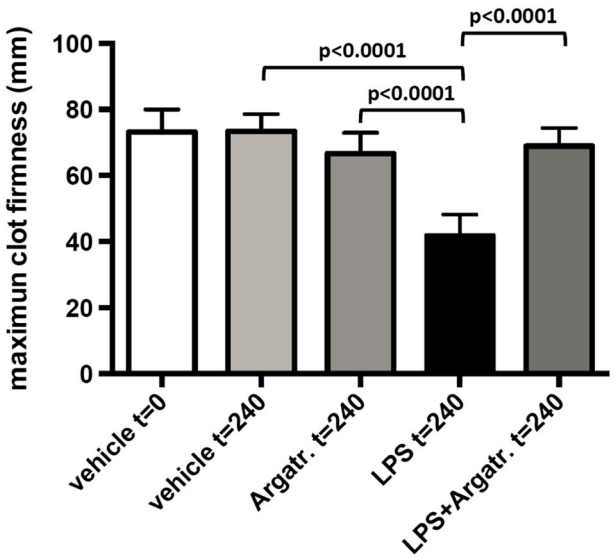

Fig. 4. Effect of argatroban on LPS-induced disseminated intravascular coagulation. Rats were treated with LPS (1 mg/kg/h) or vehicle in the presence or absence of argatroban (50 μg/kg/min), and whole blood samples were drawn after 240 min. Rotational thrombelastometry demonstrated that argatroban abolished the LPS-induced reduction of maximum clot firmness and thus disseminated intravascular coagulation. Means ± SD; $n = 6$ per group.

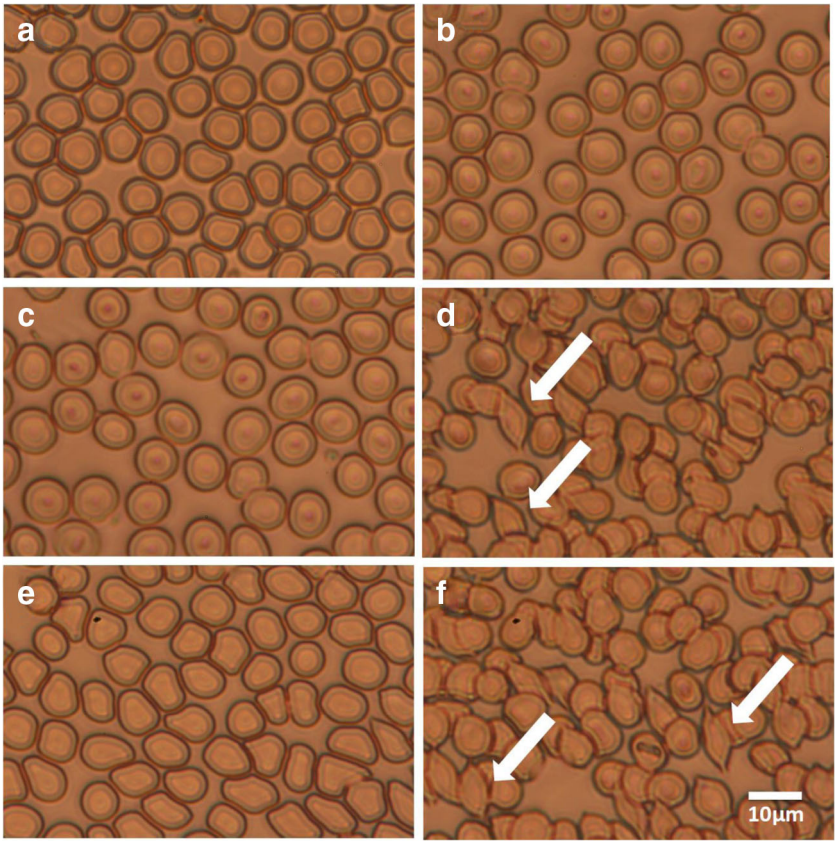

Fig. 5. Effects of LPS on red blood cell morphology. Representative blood smears obtained from rats infused with vehicle (**a**), argatroban (**b**), eptifibatide (**c**), LPS (1 mg/kg/h) (**d**), LPS (1 mg/kg/h) and argatroban (50 µg/kg/min) combined (**e**), and LPS (1 mg/kg/h) and eptifibatide (180 µg/kg bolus + 2 µg/kg/min) combined (**f**) for 240 min. LPS infusion results in irregular morphology of red cells as well as schistocyte formation (see arrows). Argatroban, but not eptifibatide, inhibited schistocyte formation in LPS-treated rats.

DISCUSSION

The present study demonstrates that LPS induces both hemolysis and red cell membrane weakening in rats. Remarkably, inhibition of coagulation by a thrombin antagonist abolished hemolysis, whereas inhibition of platelet aggregation did not. Thus, intravascular coagulation, potentially eased by LPS-evoked red cell membrane weakening, is required for LPS-evoked hemolysis.

Hemolysis is an important cause of morbidity in many diseases. In patients with sepsis, we were the first to demonstrate that free hemoglobin concentration is associated with mortality [1]. Importantly, the increase of free hemoglobin concentration was not only an independent but also the best predictor of nonsurvival with an odds ratio of 6.1. These results were confirmed by another group [12]. Moreover, there is experimental evidence for the importance of hemolysis in endotoxemia and sepsis [2].

Several mechanisms explaining the deleterious effects of hemolysis have been proposed in literature. First, extracellular hemoglobin is a danger molecule and activates innate immune system *via* TLR-4 [13, 14]. Second, free hemoglobin is an effective NO-scavenger, inhibits NO-induced vasodilation, and evokes microvascular perfusion disturbances [15]. Third, release of iron from free hemoglobin induces radical formation with consecutive modifications of lipid, proteins, and DNA as well as inflammation [16]. Fourth, such iron ions can support bacterial growth, as bacteria are dependent on iron [17–20]. Fifth, release of microparticles caused by red cell damage may induce inflammation and disseminated intravascular coagulation [21].

While there is good evidence for the pathophysiological consequences of hemolysis, little information on the causes of hemolysis in sepsis is available. In the present *in vivo* study, we demonstrate that LPS induces hemolysis, schistocyte formation, disseminated intravascular

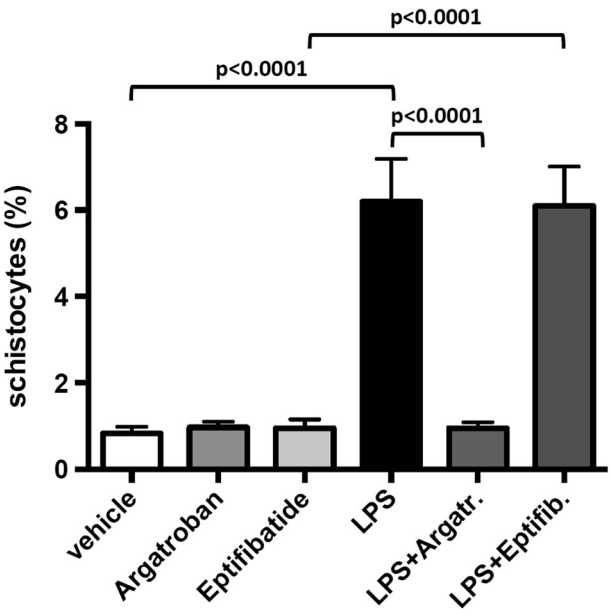

Fig. 6. Effects of argatroban and eptifibatide on the LPS-induced schistocyte formation. LPS (1 mg/kg/h) induced a marked increase in schistocyte count in rats treated with LPS which was abolished by the thrombin antagonist argatroban (50 μg/kg/min). In contrast, inhibition of platelet aggregation with eptifibatide (180 μg/kg bolus + 2 μg/kg/min) did not affect the LPS-induced schistocyte formation.

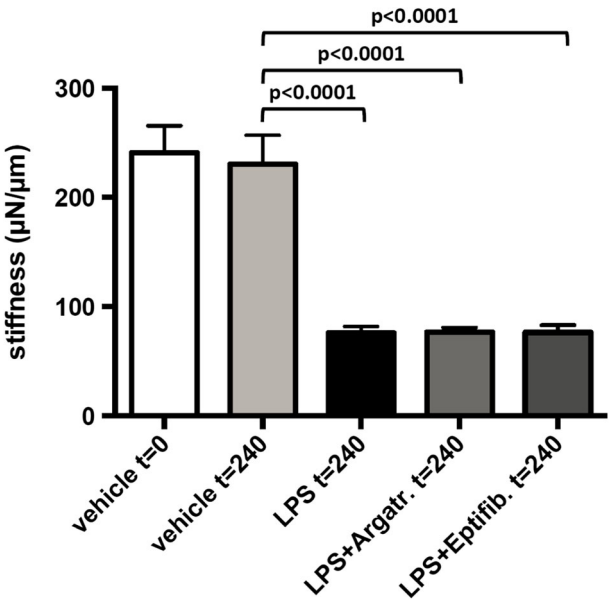

Fig. 7. Effects of LPS, argatroban, and eptifibatide on red cell membrane stiffness. While red cell membrane stiffness was not altered by vehicle infusion into rats for 240 min, LPS (1 mg/kg/h) markedly reduced membrane stiffness. LPS-induced membrane weakening was neither affected by argatroban (50 μg/kg/min) nor by eptifibatide (180 μg/kg bolus + 2 μg/kg/min). Means ± SD; $n = 6$ per group.

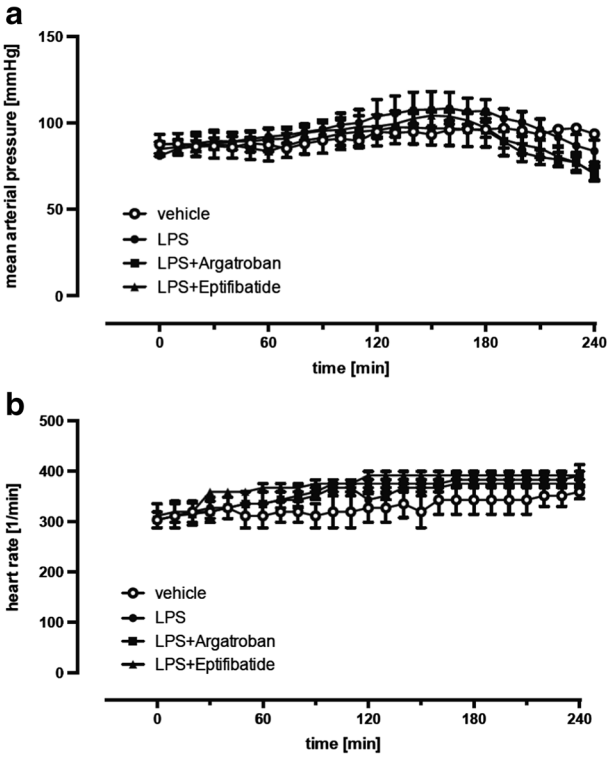

Fig. 8. Effects of LPS on the heart. Mean arterial pressure (**a**) and heart rate (**b**) in rats remained within the physiological range in both vehicle- and LPS-treated animals. Means ± SD.

coagulation, and red cell membrane weakening. Hemolysis, schistocyte formation, and disseminated intravascular coagulation were all abolished by a thrombin antagonist demonstrating a causal link between coagulation and red cell damage. In accordance with our results, early findings had suggested the generation of schistocytes by fibrin strands after thrombin infusion in rats and in a patient with microangiopathy [5, 6]. Interestingly, inhibition of platelet function with eptifibatide did not affect LPS-induced hemolysis thus excluding a pathophysiological importance of primary hemostasis.

An increase in free hemoglobin was detectable after 2 h in the present study. The time lack can most likely be explained by the fact that LPS was infused continuously and that a certain red cell membrane concentration might be necessary to induce hemolysis. Moreover, it is conceivable that the incorporation of LPS micelles into red cell membranes might be a slow process. In accordance with the findings on the time course of hemolysis, the reduction of maximum clot firmness was detectable after a lag period of 2 h. The lag phase might be explained by the accumulation of the continuously infused LPS in the circulation and the complex changes in hemostasis in inflammation.

LPS infusion also induced a marked weakening of red cell membranes, which was not affected by inhibition of coagulation and thus constitutes a second different mechanism likely contributing to hemolysis. These *in vivo* findings are in complete accordance with our recent *in vitro* studies demonstrating that LPS incorporation both into artificial vesicles and red cell membranes leads to membrane weakening and hemolysis [7, 8]. Importantly, a TLR-4-mediated innate immune system signaling and an involvement of the complement system were excluded in the latter study [7].

It is an important question whether the observed inhibition of hemolysis by argatroban might be applied to the clinical setting with a therapeutic intention. For the evaluation of hemostasis, thrombin inhibitor efficacy, and disseminated intravascular coagulation, we used rotational thrombelastometry. The method allows an integrative view on hemostasis and fibrinolysis of small whole blood samples and has been demonstrated to predict diagnosis and prognosis of human sepsis [22, 23]. In the present study, argatroban infusion in LPS-treated animals doubled the clotting time but allowed fibrin to be generated. Furthermore, argatroban increased clot firmness due to the inhibition of disseminated intravascular coagulation in LPS-treated rats. From these thrombelastometry findings, it can be concluded that the effects of argatroban in rats are similar to the effects of the thrombin inhibitor when used in clinical dosages in humans. It is thus conceivable that argatroban therapies might be beneficial to stop both hemolysis and disseminated intravascular coagulation. Our results on the beneficial action of argatroban are supported by recent experimental evidence demonstrating that argatroban improved capillary perfusion in the intestinal microcirculation and reduced leukocyte adhesion [24, 25]. It is important to state that LPS treatment of rats in the present study does only in part reflect the pathophysiology in sepsis for several reasons: (1) the observation period was restricted, (2) endotoxinemia is different from infection, and (3) antiinflammatory mechanisms, relevant for the prognosis of sepsis, are not measured. Therefore, clinical studies are warranted to determine the effects of argatroban on hemolysis and mortality.

CONCLUSION

Thus, LPS causes hemolysis, red cell membrane weakening, and schistocyte formation in rats. Interestingly, inhibition of coagulation, but not inhibition of platelet aggregation, abolished hemolysis and disseminated intravascular coagulation but did not affect red cell membrane weakening. Thus, intravascular coagulation, potentially eased by red blood cell membrane weakening, is required for LPS-induced hemolysis. The data reveal an important crosstalk between inflammation, coagulation, and hemolysis in sepsis. Clinical studies are warranted to investigate whether thrombin inhibitors reduce hemolysis, inflammation, and mortality in human sepsis.

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AUTHORS' CONTRIBUTIONS

SB and KR-F participated in the animal experiments and data analysis. MN and CM performed the atomic force experiments. SB and MH wrote the manuscript. JP corrected the manuscript. All authors read and approved the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests.

Ethical Approval. The experimental protocol had been approved based on the local animal protection act with the permit number Az.: 84-02.04.2013.A015.

REFERENCES

1. Adamzik, M., T. Hamburger, F. Petrat, J. Peters, H. de Groot, and M. Hartmann. 2012. Free hemoglobin concentration in severe sepsis: methods of measurement and prediction of outcome. *Critical Care* 16 (4): R125.
2. Larsen, R., R. Gozzelino, V. Jeney, L. Tokaji, F.A. Bozza, A.M. Japiassú, D. Bonaparte, M.M. Cavalcante, A. Chora, A. Ferreira, I. Marguti, S. Cardoso, N. Sepúlveda, A. Smith, and M.P. Soares. 2010. A central role for free heme in the pathogenesis of severe sepsis. *Science Translational Medicine* 2 (51): 51ra71.
3. Rother, R.P., L. Bell, P. Hillmen, and M.T. Gladwin. 2005. The clinical sequelae of intravascular hemolysis and extracellular plasma

hemoglobin: a novel mechanism of human disease. *JAMA* 293 (13): 1653–1662.
4. Levi, M., M. Schultz, and T. van der Poll. 2010. Disseminated intravascular coagulation in infectious disease. *Seminars in Thrombosis and Hemostasis* 36 (4): 367–377.
5. Bull, B.S., and I.N. Kuhn. 1970. The production of schistocytes by fibrin strands (a scanning electron microscope study). *Blood* 35 (1): 104–111.
6. Heyes, H., W. Köhle, and B. Slijepcevic. 1976. The appearance of schistocytes in the peripheral blood in correlation to the degree of disseminated intravascular coagulation. An experimental study in rats. *Haemostasis* 5 (2): 66–73.
7. Brauckmann, S., K. Effenberger-Neidnicht, H. de Groot, M. Nagel, C. Mayer, J. Peters, and M. Hartmann. 2016. Lipopolysaccharide-induced hemolysis: evidence for direct membrane interactions. *Scientific Reports* 6: 35508.
8. Nagel, M., S. Brauckmann, F. Moegle-Hofacker, K. Effenberger-Neidnicht, M. Hartmann, H. de Groot, and C. Mayer. 2015. Impact of bacterial endotoxin on the structure of DMPC membranes. *Biochimica et Biophysica Acta* 1848 (10 PT A): 2271–2276.
9. Soret, J.-L. 1883. Analyse spectrale: Sur le spectre d'absorption du sang dans la partie violette et ultra-violette. *Comptes Rendus* 97: 1269–1270.
10. Zini, G., G. d'Onofrio, C. Briggs, W. Erber, J.M. Jou, S.H. Lee, S. McFadden, J.L. Vives-Corrons, N. Yutaka, J.F. Lesesve, and International Council for Standardization in Haematology (ICSH). 2012. ICSH recommendations for identification, diagnostic value, and quantitation of schistocytes. *International Journal of Laboratory Hematology* 34 (2): 107–116.
11. Palmer, L., C. Briggs, S. McFadden, G. Zini, J. Burthem, G. Rozenberg, M. Proytcheva, and S.J. Machin. 2015. ICSH recommendations for the standardization of nomenclature and grading of peripheral blood cell morphological features. *International Journal of Laboratory Hematology* 37 (3): 287–303.
12. Janz, D.R., J.A. Bastarache, J.F. Peterson, G. Sills, N. Wickersham, A.K. May, L.J. Roberts 2nd, and L.B. Ware. 2013. Association between cell-free hemoglobin, acetaminophen, and mortality in patients with sepsis: an observational study. *Critical Care Medicine* 41 (3): 784–790.
13. Bloom, O., H. Wang, S. Ivanova, J.M. Vishnubhakat, M. Ombrellino, and K.J. Tracey. 1998. Hypophysectomy, high tumor necrosis factor levels, and hemoglobinemia in lethal endotoxemic shock. *Shock* 10 (6): 395–400.
14. Su, D., R.I. Roth, and J. Levin. 1999. Hemoglobin infusion augments the tumor necrosis factor response to bacterial endotoxin (lipopolysaccharide) in mice. *Critical Care Medicine* 27 (4): 771–778.
15. Weinberg, J.A., S.R. Barnum, and R.P. Patel. 2011. Red blood cell age and potentiation of transfusion-related pathology in trauma patients. *Transfusion* 51 (4): 867–873.
16. Belcher, J.D., J.D. Beckman, G. Balla, J. Balla, and G. Vercellotti. 2010. Heme degradation and vascular injury. *Antioxidants & Redox Signaling* 12 (2): 233–248.
17. Pishchany, G., A.L. McCoy, V.J. Torres, J.C. Krause, J.E. Crowe Jr., M.E. Fabry, and E.P. Skaar. 2010. Specificity for human hemoglobin enhances Staphylococcus aureus infection. *Cell Host & Microbe* 8 (6): 544–550.
18. Tullius, M.V., C.A. Harmston, C.P. Owens, N. Chim, R.P. Morse, L.M. McMath, A. Iniguez, J.M. Kimmey, M.R. Sawaya, J.P. Whitelegge, M.A. Horwitz, and C.W. Goulding. 2011. Discovery and characterization of a unique mycobacterial heme acquisition system. *Proceedings of the National Academy of Sciences of the United States of America* 108 (12): 5051–5056.
19. Bullen, J.J., H.J. Rogers, P.B. Spalding, and C.G. Ward. 2005. Iron and infection: the heart of the matter. *FEMS Immunology and Medical Microbiology* 43 (3): 325–330.
20. Litwin, C.M., and S.B. Calderwood. 1993. Role of iron in regulation of virulence genes. *Clinical Microbiology Reviews* 6 (2): 137–149.
21. Reid, V.L., and N.R. Webster. 2012. Role of microparticles in sepsis. *British Journal of Anaesthesia* 109 (4): 503–513.
22. Adamzik, M., K. Görlinger, J. Peters, and M. Hartmann. 2012. Whole blood impedance aggregometry as a biomarker for the diagnosis and prognosis of severe sepsis. *Critical Care* 16 (5): R204.
23. Adamzik, M., T. Langemeier, U.H. Frey, K. Görlinger, F. Saner, H. Eggebrecht, J. Peters, and M. Hartmann. 2011. Comparison of thrombelastometry with simplified acute physiology score II and sequential organ failure assessment scores for the prediction of 30-day survival: a cohort study. *Shock* 35 (4): 339–342.
24. Schaer, D.J., P.W. Buehler, A.I. Alayash, J.D. Belcher, and G.M. Vercellotti. 2013. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. *Blood* 121 (8): 1276–1284.
25. Tsen, A., L.A. Kirschenbaum, C. LaRow, R. Khan, S. Kurtz, S. Bansal, and M.E. Astiz. 2009. The effect of anticoagulants and the role of thrombin on neutrophil-endothelial cell interactions in septic shock. *Shock* 31 (2): 120–124.