Hemoglobin Enhances the Production of Tissue Factor by Endothelial Cells in Response to Bacterial Endotoxin

By Robert I. Roth

Human endothelial cells respond to bacterial endotoxin (lipopolysaccharide [LPS]) with changes that transform the endothelium into a surface with prominent procoagulant properties. Production of tissue factor (TF) in response to LPS is a major alteration that favors coagulation. Biologic activities of LPS have previously been shown to be enhanced by the presence of hemoglobin. Therefore, the ability of human hemoglobin (Hb) to modulate TF production by cultured human umbilical vein endothelial cells (HUVEC) was investigated. Cell-free Hb (10 mg/mL), either purified native (HbA, or chemically cross-linked (aaHb), was incubated with LPS (0.1 μg/mL), and the mixtures were then added to HUVEC in culture. TF activity was quantified with a clotting assay and TF protein was measured with an enzyme-linked immunosorbant assay. Hb preparations greatly enhanced the production of TF activity (11- to 25-fold greater than TF produced by HUVEC alone) compared with minimal TF activity generated by LPS alone (only twofold greater than HUVEC alone). The enhancement of LPS-induced TF activity was Hb concentration-dependent over a range of 1 to 100 mg/mL. Cross-linked aaHb also greatly enhanced the production of TF protein compared with TF protein generated by LPS alone (12-fold greater v 3.5-fold greater than HUVEC alone, respectively). The enhancement of LPS-induced TF protein was Hb concentration-dependent over a range of 0.1 to 2 mg/mL. Enhancement of TF activity by Hb required new protein synthesis. These results show that human Hb can augment the ability of LPS to induce endothelial cell TF and suggest that hemolysis associated with disseminated intravascular coagulation during sepsis may further stimulate coagulation. In addition, these results suggest a potential mechanism for generalized thrombosis in animals that has been associated with the infusion of cell-free Hb for resuscitation.

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study, the effect of Hb on the production of TF by endothelial cells in response to bacterial endotoxin was examined.

MATERIALS AND METHODS

Reagents. Actinomycin D and cycloheximide were obtained from Sigma (St Louis, MO). Sterile tissue-culture plastic ware was obtained from Becton Dickinson (Mountain View, CA).

Hb. Purified human hemoglobin \( \alpha_2 \) (Hb\( \alpha_2 \)), prepared by ion exchange high pressure liquid chromatography of purified human Hb, as described previously,\(^2\) was provided by collaborators at the Blood Research Division of the Letterman Army Institute of Research (BRD/LAIR; San Francisco, CA). Human cross-linked cell-free hemoglobin (aqHb), covalently cross-linked between the two \( \alpha \)-chains with bis(3,5-dibromosalicyl) fumarate as described previously,\(^2\) also was provided by collaborators at BRD/LAIR. These Hb preparations contained less than 0.4 EU/mL endotoxin (referenced to Escherichia coli LPS B, O55:B5; Difco Laboratories, Detroit, MI), as determined by the Limulus amebocyte lysate test,\(^2\) and did not contain demonstrable erythrocyte stroma, as shown by phosphorus analysis and reverse-phase high pressure liquid chromatography. Hb concentrations were determined spectrophotometrically.

Endotoxin. E coli O26:B6 (W) LPS was purchased from Difco Laboratories.

HUVEC. HUVEC and endothelial cell culture media containing 2% fetal bovine serum, 10 ng/mL epidermal growth factor (EGF) and 1 ng/mL hydrocortisone were purchased from Clonetics (San Diego, CA). HUVEC were plated in 96-well microtiter plates (Nunc; Applied Scientific, South San Francisco, CA) at a seeding density of 3,500 cells/cm\(^2\) (5,000 cells/well) and were grown to confluency at 37°C and 5% CO\(_2\). HUVEC were used at less than 6 passages.

TF procoagulant assay. TF activity was quantified with a plasma recalcification assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 \( \mu \)L E coli LPS alone, Hb\( \alpha_2 \), or aqHb alone, or Hb-LPS mixtures in 90 \( \mu \)L media/well. Standard incubations were conducted using 10 \( \mu \)g/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (\( \times 3 \)) and the HUVEC were freeze-thawed twice and sonicated in 50 \( \mu \)L phosphate-buffered saline for 2 minutes at room temperature. To each well then was added 50 \( \mu \)L normal human citrated plasma and 50 \( \mu \)L calcium (25 mmol/L), and after 8 minutes, turbidity was measured at 340 nm in a 37°C temperature-controlled plate reader (Kinetic-QLC; Whittaker Bioproducts Inc, Walkersville, MD). TF activity was calculated from the turbidity generated in plasma (the mean from 6 to 8 replicate wells) by a standard curve established with dilutions of rabbit brain thromboplastin (Baxter Corporation, Miami, FL). The turbidity generated at 8 minutes by 1:100 diluted thromboplastin was defined as 1 TF arbitrary unit.

TF protein assay. Confluent HUVEC monolayers in 96-well plates were incubated for 4 hours at 37°C with 10 \( \mu \)L E coli LPS alone, Hb\( \alpha_2 \), or aqHb alone, or Hb-LPS mixtures in 90 \( \mu \)L media/well. Standard incubations were conducted using 2 \( \mu \)g/mL Hb. Experiments were performed with 6 to 8 replicate wells. Wells were then washed with media (\( \times 3 \)), and the HUVEC were freeze-thawed twice and sonicated in 50 \( \mu \)L phosphate buffered saline; 10 \( \mu \)L Triton X-100 was added (final concentration, 1%), and incubations were continued overnight at 4°C. The solutions were then removed from the plates and centrifuged for 15 minutes (Microfuge B; Beckman Instruments, Inc, Palo Alto, CA). TF was quantified by enzyme-linked immunosorbant assay (ELISA) using a murine antihuman TF monoclonal antibody (Inutib; generously provided by American Diagnostica, Inc, Greenwich, CT) according to the manufacturer’s directions.
Functional activity and increased antigenic concentrations of TF protein by a sensitive ELISA assay. In an experiment representative of 6 independent studies, TF protein increased in response to LPS (1 pg/mL) for 4 hours in the absence or presence of actinomycin D (ACT) or cycloheximide (CY; each 10 µg/mL), and TF activities then were determined with a plasma recalcification assay. The means and 1 SD of 8 tissue culture-plate wells are presented.

Table 1. Relative Procoagulant Activities of Chemically Different Glycolipids

<table>
<thead>
<tr>
<th>Lipid A*</th>
<th>Deep Rough Mutant</th>
<th>Rough LPS Mutant</th>
<th>Rough LPS S. minnesota Lipid A</th>
<th>Smooth LPS</th>
<th>Smooth LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>8</td>
<td>18</td>
<td>4</td>
<td>6</td>
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Various types of purified LPS were incubated with HUVEC for 4 hours, and TF activities were determined. The LPS that elicited the least TF response by the HUVEC (S. minnesota lipid A) was assigned a relative procoagulant activity of 1, and the bioologic activities of the other LPSs are presented in comparison to lipid A.

* S. minnesota lipid A.
† S. minnesota S65.
‡ Proteus mirabilis R45.
§ P. mirabilis S1959.
¶ E. coli 055:85.

shown). Similar LPS concentration-dependent TF responses were detected in 8 independent experiments; the maximal TF activities from LPS-treated HUVEC (achieved with 10 µg/mL LPS) were from 7 to 28 times greater (mean, 16 times greater) than the TF activity of untreated HUVEC. TF responses varied considerably when different glycolipids were examined (Table 1). A low TF response was observed with S. minnesota lipid A (which consists of a diglucosamine backbone and seven fatty acyl chains) compared with other LPSs which contained additional saccharide moieties, suggesting that O-chain and core carbohydrates are important for the HUVEC response. We also quantified HUVEC TF protein by a sensitive ELISA assay. In an experiment representative of 6 independent studies, TF protein increased in an LPS concentration-dependent manner from 17 pg/well in untreated HUVEC to 116 pg/well in HUVEC after 4 hr incubation with 10 µg/ml LPS (Fig 1B). Total protein per well was unchanged at 41 ± 4 µg/well. In each of the 6 studies, LPS-treated HUVEC showed both increased TF functional activity and increased antigenic concentrations of TF protein.

To further demonstrate that the TF activity induced by LPS resulted from newly formed protein rather than by a process of enhanced catalytic activity by preexisting TF protein, we examined the effect of protein synthesis inhibitors. LPS-stimulated TF production was completely inhibited by actinomycin D or cycloheximide (Fig 2), providing additional evidence that the cellular procoagulant response to LPS required new protein synthesis.

To test the effect of Hb on the production of HUVEC TF in response to LPS, two Hb preparations were investigated. HbA0 was used because this preparation was native Hb and would be potentially available to interact with the endothelium during in vivo hemolysis of erythrocytes. Hb was used because this chemically stabilized preparation is not susceptible to dissociation of the α-chains of the Hb tetramer and is a form of Hb that is presently being developed as a cell-free blood substitute. Hb (10 mg/mL) was first incubated with LPS for 30 minutes at 37°C, and the mixtures then were added to HUVEC. A low concentration of LPS was used (0.1 µg/mL) so that only modest TF production by HUVEC was generated in response to LPS alone. At this concentration, HUVEC incubated with LPS generated only 2.5-fold greater TF activity than unstimulated HUVEC (Fig 3). HbA0 or αHb alone (10 mg/mL each) did not demonstrably increase the very low levels of TF activity produced by HUVEC in the absence of LPS. In contrast, stimulation of TF production from HUVEC was 11-fold increased in the presence of the LPS-HbA0 mixture and was 25-fold increased in the presence of the LPS-αHb mixture. Preincubation of Hb and LPS longer than 30 minutes before their addition to HUVEC did not alter this response.

The enhanced TF production in the presence of various concentrations of Hb was concentration-dependent, as is
HEMOGLOBIN, LPS, AND HUVEC TISSUE FACTOR

To determine whether the enhancement of LPS-elicited TF production in the presence of Hb was the result of new TF protein production, HUVEC were incubated with LPS and Hb in the presence of actinomycin D or cycloheximide. Each of the protein synthesis inhibitors totally prevented the generation of TF activity, indicating that the mechanism for the Hb enhancement process involved new protein synthesis (Fig 5).

DISCUSSION

Despite the extensive existing knowledge of the structure of human Hb and its function within the erythrocyte, relatively little is known about pathophysiologic interactions involving extraerythrocytic Hb and other blood elements and host tissues. Clinical evidence of renal, vascular, and reticuloendothelial system damage during hemolytic episodes has suggested that extraerythrocytic Hb (and, in particular, Hb breakdown products such as hematin) can be toxic. However, the direct effects of Hb have been difficult to determine because the contribution to the observed toxicities from erythrocyte membrane components is also felt to be of major importance. Similarly, in determining the mechanism of organ toxicity associated with LPS-mediated intravascular coagulation with resultant hemolysis, it is difficult to distinguish the contribution of Hb to the observed deleterious effects from the contribution by LPS. The potential for LPS and cell-free Hb to interact in the blood stream adds additional complexity to an understanding of the consequences of extraerythrocytic Hb.

The current efforts to develop cell-free Hb as a transfusion product have allowed detailed investigation of the interactions between Hb and LPS. Previously, we had shown that there is a biologically significant interaction between Hb and LPS.
LPS, with resultant activation of host effector mechanisms. In those studies, mixtures of Hb and LPS were shown to synergistically activate human mononuclear cells and enzymatic coagulation mechanisms. LPS biologic activity in these model systems was clearly shown to be enhanced by Hb. Therefore, modification of LPS by Hb is a process with substantial clinical relevance. In addition, we have recently shown that Hb and LPS form bimolecular complexes and have shown that large LPS aggregates are dissociated on binding to Hb.

Because of the prothrombotic actions of LPS on the vascular endothelium, it was important to investigate the potential ability of Hb to modify this critical interaction during endotoxemia. The present studies showed that HbAα significantly increased TF activity in HUVEC in response to LPS. This effect was observed at concentrations of Hb (1 to 2 mg/mL) that can be encountered in plasma during clinical and experimental endotoxemia. This Hb preparation did not contain detectable erythrocyte stroma that could potentially elicit a HUVEC response. The enhanced TF activity resulted from new TF protein synthesis. The mechanism of this effect is uncertain because the mechanism of LPS signal transduction in HUVEC is not known. However, the demonstration in our laboratory that Hb binds LPS and causes LPS dissociation suggests that disaggregated and/or protein-bound LPS has an increased ability to interact with HUVEC LPS receptors and trigger the procoagulant response. Thus, the Hb enhancement effect may represent the result of presentation to the endothelial cell of a "modified" (eg, disaggregated) LPS. Additionally, a number of other potential mechanisms may be involved. Preliminary studies in our laboratory have shown that LPS induces the denaturation of Hb to methemoglobin and hemichrome, species of Hb that may show altered biologic activity and produce oxygen-free radicals during their formation.

Based on these results using HbAα, it is reasonable to propose that hemolysis caused by LPS-mediated disseminated intravascular coagulation may constitute a positive feedback loop to amplify coagulation. Additionally, enhancement of other LPS biologic activities by HbAα, such as mononuclear cell cytokine production, may contribute to the often fatal consequences of low level endotoxemia (during which LPS concentrations are frequently measured in the 10 to 100 pg/mL range). The present study also showed enhancement of the TF response to LPS by ααHb, a cross-linked preparation of human Hb that is a leading candidate for use as a blood substitute. These findings raise two concerns for the human use of cell-free Hb. Firstly, contamination of Hb preparations by environmental LPS is difficult to avoid. Because large volumes of Hb would be required for resuscitation, the procoagulant consequences of LPS contamination in the Hb preparations could limit the use of ααHb. In patients receiving 1/10th blood volume of ααHb for resuscitation, blood Hb levels ≥10 mg/mL would be achieved. At these concentrations of Hb, substantial enhancement by Hb of the procoagulant activity of any LPS in the circulation would be expected based on our results. Secondly, ααHb would likely be infused into some patients with coexisting endotoxemia. Endotoxemia could arise from gram-negative bacteremia, from reticuloendothelial system dysfunction, and/or from increased gastrointestinal tract translocation of LPS into the circulation secondary to hypotension or trauma. Consequently, ααHb infusion could potentially enhance the systemic pathologic effects of underlying endotoxemia. Because of the demonstration of the in vitro consequences of the interaction between LPS and Hb, which provide a basis for potential thrombotic effects, it is important that future studies investigate the ability of Hb to enhance LPS-induced coagulation in vivo.

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