Redox active hemoglobin enhances lipopolysaccharide-induced injury to cultured bovine endothelial cells

Felice D’Agnillo

Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Submitted 17 February 2004; accepted in final form 16 June 2004

D’Agnillo, Felice. Redox active hemoglobin enhances lipopolysaccharide-induced injury to cultured bovine endothelial cells. Am J Physiol Heart Circ Physiol 287: H1875–H1882, 2004. First published June 17, 2004; 10.1152/ajpheart.00164.2004.—The interaction of cell-free hemoglobin with lipopolysaccharide (LPS) is thought to aggressate the pathophysiology of sepsis and/or septic shock. This study examines the possible modulatory role of cell-free hemoglobin on LPS-induced apoptosis of cultured bovine aortic endothelial cells. Experiments were performed with or without fetal bovine serum, a source of LPS-binding protein and soluble CD14. In the absence of serum, LPS alone or coincubated with purified bovine hemoglobin (BvHb), human hemoglobin (Hb), or α-cross-linked Hb (αHb) did not induce apoptosis. In the presence of serum, LPS induced significant apoptosis. LPS combined with BvHb, Hb, or αHb produced the same extent of apoptosis as LPS alone. To examine whether the H2O2-driven redox activity of hemoglobin alters LPS-induced apoptosis, glucose oxidase was added to the system to generate a subtoxic flux of H2O2. The combined treatment of LPS, glucose oxidase, and BvHb, Hb, or αHb enhanced apoptosis compared with LPS alone. These findings support a possible mechanism whereby the redox cycling of hemoglobin, and not its direct interaction with LPS, contributes to the hemoglobin-mediated enhancement of LPS-related pathophysiology.

apoptosis; ferryl hemoglobin; hydrogen peroxide; glucose oxidase

CELL-FREE HEMOGLOBIN resulting from intravascular hemolysis or the administration of hemoglobin-based oxygen therapeutics is thought to exacerbate the pathophysiology of sepsis or endotoxemia. Previous studies implicate the interaction of hemoglobin with lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and the primary mediator of gram-negative sepsis (3, 26, 39–41). Understanding how hemoglobin enhances the effects of LPS may be critical in the management of sepsis and/or septic shock given the likely occurrence of hemoglobin-LPS interactions during trauma, infection, injury, or surgery.

Several studies have demonstrated that hemoglobin enhances the toxicity or lethality of LPS. Rabbits infused with hemoglobin containing LPS exhibited increased mortality compared with rabbits infused with hemoglobin or LPS (41). Intraperitoneal injection of LPS combined with an intravenous infusion of unmodified or cross-linked hemoglobins induced greater mortality in mice compared with the hemoglobin or LPS alone (39, 40). Intravenous administration of α-cross-linked human hemoglobin (αHb) and LPS caused significantly greater mortality and cardiovascular dysfunction than αHb or LPS alone in a rabbit model of nonlethal endotoxemia (26). Coinjection of purified hemoglobin and LPS enhanced the LPS-induced ocular inflammatory response in rabbits (30). Increased LPS sensitivity in hypophysectomized rats was linked to an increase in circulating plasma hemoglobin levels (3). Another recent study (20) reported increased plasma levels of CD163, an acute phase protein that binds hemoglobin-haptoglobin complexes, after LPS infusion in humans.

Vascular endothelium plays many important roles in homeostasis, inflammation, and infection. By virtue of their direct contact with the bloodstream, endothelial cells are likely targets for cell-free hemoglobin and LPS-mediated injury. Host inflammatory cytokines and reactive oxygen and/or nitrogen species have been implicated in LPS-induced vascular damage in vivo (2, 17, 29, 37). One of the proposed mechanisms to explain the synergism between hemoglobin and LPS implicates the production of TNF-α, an inflammatory cytokine that mediates many of the pathological responses to LPS. Several reports indicate that hemoglobin augments the LPS-induced TNF-α response of cultured monocytes and in vivo (3, 39).

Previous studies have also shown that hemoglobin enhances LPS-induced tissue factor factor production in endothelial cells, increases LPS reactivity in the Limulus amebocyte lysate assay, and promotes LPS binding to endothelial cells in serum-free medium (23, 25, 33, 34). It has been proposed that the binding of LPS to hemoglobin enhances the biological activity of LPS by promoting the disaggregation of LPS complexes (23). A recent study (5) also showed that αHb can activate pentaacylated LPS mutants possessing low intrinsic biological activity. Despite these findings, Yang et al. (44) reported that LPS binding to globin (hemoglobin minus heme) actually antagonizes the actions of LPS on cultured macrophages and in mice. On the basis of these findings, the latter investigators speculated that the heme moiety of hemoglobin must somehow be involved in the deleterious effects of hemoglobin-LPS interactions. Others have shown that some forms of LPS oxidize native and cross-linked hemoglobin and suggest that LPS-induced oxidation of hemoglobin may contribute the synergism of hemoglobin and LPS (8, 24).

Activation of monocytes and myeloid cells by LPS involves the binding of LPS to serum LPS-binding protein (LBP) followed by interaction of these complexes with the CD14 membrane surface receptor. Endothelial cells lack or express very low levels of membrane CD14; thus LPS activation of cultured endothelial cells is largely dependent on the presence of LPS-binding protein and soluble CD14. These findings support a possible mechanism whereby the redox cycling of hemoglobin, and not its direct interaction with LPS, contributes to the hemoglobin-mediated enhancement of LPS-related pathophysiology.

http://www.ajpheart.org

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of LBP and soluble CD14 in serum-containing medium (2). Previous studies have shown that LPS induces serum-dependent apoptosis of cultured bovine and ovine endothelial cells as well as human endothelial cells when coincubated with protein synthesis inhibitors (2, 7, 12, 18, 21). In the present study, cultured bovine aortic endothelial cells (BAECs) were used to investigate the possible synergism between hemoglobin and LPS. First, the modulatory effects of purified bovine hemoglobin (BvHb), human hemoglobin (Hb), or αxHb on LPS-induced apoptosis in the presence or absence of serum was examined. Second, the possible role that hemoglobin redox reactions play in the enhancement of LPS cytotoxicity was explored.

MATERIALS AND METHODS

Materials. Phenol red-free endothelial basal medium (EBM) containing 5.5 mM glucose and fetal bovine serum (FBS) was obtained from Cambrex-BioWhittaker (Walkersville, MD). LPS (Escherichia coli serotype 0127:B8, 3 EU/μg, cell culture tested, catalog no. L4516), polymyxin B sulfate (catalog no. P4932), propidium iodide (PI), and glucose oxidase (Gx; type X-S derived from Aspergillus niger, 100,000 U/g solid) were obtained from Sigma (St. Louis, MO). Hank’s balanced salt solution (HBSS) containing 5.5 mM glucose and PBS was obtained from Life Technologies (Grand Island, NY). Caspase inhibitor, z-VAD-fmk (no. FK-009), was purchased from Enzyme Systems Products (Livermore, CA).

Hemoglobin solutions. Human Hb cross-linked between the lysine 99 residues of the α-chains (αxHb) was a kind gift from the Walter Reed Army Institute of Research (Washington, DC). This hemoglobin solution, prepared in sterile Ringer acetate buffer, was free of superoxide dismutase and catalase and contained <0.4 EU/ml endotoxin by L. amebocyte lysate assay (19). Chromatographically purified human Hb, free of superoxide dismutase and catalase, was a kind gift from Hemosol (Etobicoke, Ontario, Canada). Purified BvHb, containing <0.05 EU/ml, was obtained from Biopure (Boston, MA). These hemoglobin solutions contained >90% ferrous hemoglobin (Fe(II)).

Endothelial cell culture. BAECs (catalog no. BW-6002) were obtained from Cambrex-BioWhittaker (Walkersville, MD). Cells were grown in endothelial growth medium (EGM-MV) that consisted of EBM supplemented with 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 10 μg/ml bovine brain extract, 5% FBS, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B. Cells grown in 100-mm dishes were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. For harvesting, cells were washed with HEPES-buffered saline and incubated with 0.025% trypsin-0.01% EDTA for 5 min at 37°C. After cell detachment, trypsin-neutralizing agent was added, and cells were centrifuged at 220 g for 5 min at 4°C. For experiments, cells were subcultured in 6-well plates (Costar) and reached confluence within 2 days. Experimental treatments were performed using fully supplemented EGM-MV medium with or without FBS. Cell counts were obtained using a Z1 dual-threshold Coulter counter (Beckman-Coulter; Hileah, FL). Experimental data were obtained from BAECs in their second to sixth passage.

Gx treatment. Stock solutions of Gx (1 mg/ml) were prepared in PBS, pH 7.4. Aliquots were frozen at −80°C until use. The amount of Gx used in these experiments was sufficient to generate 1 μM H2O2/min at 25°C in HBSS-containing reaction mixtures using the O-dianisidine-horseradish peroxidase assay (9). This enzymatic activity produced a steady-state H2O2 concentration of −10 μM under cell culture conditions and produced minimal direct cytotoxicity over the course of 16 h (9, 10).

Analysis of hemoglobin oxidation products. Spectral analysis of hemoglobin was performed using a Hewlett-Packard HP-8453 rapid scanning diode array spectrophotometer. Total hemoglobin concentrations were measured using the Winterbourn method and expressed as heme (μM) or protein (mg/ml) (43). Ferryl hemoglobin was determined by measuring the Na2S-induced formation of sulfhemoglobin. Culture samples (1 ml) were reacted with 2 mM Na2S, and the concentration of sulfhemoglobin was calculated using the extinction coefficient of 10.5 mM−1 at 620 nm.

Phase-contrast and fluorescence microscopy. Cellular and nuclear morphology were visualized by phase-contrast and fluorescence microscopy using an inverted IX-71 microscope system equipped with a 10× digital camera system (Olympus America; Melville, NY). Brieﬂy, treatments were aspirated, and cultures containing mostly adherent and some weakly adherent and detached cells were incubated with 2 ml staining buffer (10 mM HEPES-NaOH, 140 mM NaCl, and 2.5 mM CaCl2; pH 7.4) containing 7.5 μg/ml Hoechst 33342, 15 μl Alexa Fluor 488-annexin V conjugate (Molecular Probes; Eugene, OR), and 2.5 μg/ml PI for 20 min at room temperature. Hoechst 33342, a cell-permeant blue fluorescent DNA dye, was used to visualize nuclear morphology. Alexa Fluor 488-annexin V conjugate was used to detect phosphatidylserine (PS) on the outer leaflet of the plasma membrane. PI, a red fluorescent nucleic acid dye, enters and stains cells with decreased plasma membrane integrity. Excitation light from a mercury arc lamp was passed through a triple band ﬁlter set (U-M61000v2; Chroma Technology; Brattleboro, VT) allowing simultaneous visualization of all three fluorochromes. Fixed cells were centrifuged, washed with 2 ml of 70% ethanol and PBS was obtained from Life Technologies (Grand Island, NY). Caspase inhibitor, z-VAD-fmk (no. FK-009), was purchased from Enzyme Systems Products (Livermore, CA).

Annexin V/PI apoptosis assay. Nonadherent and adherent cells, harvested by trypsinization, were pooled, washed twice in calcium- and magnesium-free HBSS, and resuspended in 1 ml assay buffer containing 10 mM HEPES-NaOH, 140 mM NaCl, and 2.5 mM CaCl2; pH 7.4. Annexin V-FITC (5 μl of 2 μg/ml, Pharmingen; San Diego, CA) and PI (10 μl of 5 μg/ml) were added to a 100-μl aliquot (~1 × 104 cells) of this cell suspension. In most cases, the remainder of the cell suspension (0.9 ml) was immediately fixed in ethanol for later analysis of cell cycle. After a 15-min incubation in the dark at room temperature, stained cells were diluted with 350 μl assay buffer and analyzed using a FACScan flow cytometer (Becton Dickinson Biosciences; San Jose, CA). Data were analyzed from a minimum of 10,000 cells/sample using WinMDI software. The stained cell populations were deﬁned as viable or undamaged cells (annexin V negative, PI negative), cells undergoing early apoptosis (annexin V positive, PI negative), and necrotic or late apoptotic cells (annexin V positive, PI positive).

DNA content measurement and cell cycle analysis. Nonadherent and adherent cells were washed with PBS and ﬁxed with 2 ml of 70% ice-cold ethanol for 30 min on ice or stored at −20°C until analysis. Fixed cells were centrifuged, washed with 10 ml PBS, and resuspended in 0.5 ml staining solution (0.1% Triton X-100, 5 μg/ml PI, and 50 μg/ml RNAse). After 25 min in the dark at room temperature, samples were analyzed by ﬂow cytometry. Data were collected from a minimum of 20,000 events/sample, and analysis was performed on gated populations excluding doublets, triplets, and quadruplets. Proportions of cells in the G1, S, and G2/M phase were quantiﬁed using ModFit LT version 3 software (Verity Software House; Topsham, ME). The fraction of sub-G1 content was calculated as a percentage of total gated events.

Caspase activity. Caspase 3 activity was measured using a ﬂuorometric substrate cleavage assay (ApoAlert Caspase 3, Clontech; Palo Alto, CA). Brieﬂy, nonadherent and adherent cells, harvested by trypsinization, were pooled, washed in HBSS, and resuspended in 60 μl lysis buffer for 10 min on ice. Samples were centrifuged at 10,000 g for 3 min, and supernatants were stored at −80°C. Reaction mixtures containing 50 μl sample, 5 μl conjugated AFC substrate, and 50 μl reaction buffer (with dithiothreitol) were incubated for 60 min at 37°C. Fluorescence measurements were performed using a CytoFluor ﬂuorometric plate reader (PerSeptive Biosystems; Framingham, MA) with excitation and emission ﬁlters of 400 and 530 nm, respectively.

Statistical analysis. Data are presented as means ± SE for replicate experiments. Statistical analysis was performed using the unpaired
with BvHb, Hb, or αHb produced a similar degree of apoptosis as LPS alone (Fig. 1B). Polymyxin B, an antibiotic that binds LPS, completely blocked PS externalization, whereas zVAD-fmk, a general caspase inhibitor, significantly suppressed PS externalization. These results suggest that BvHb, Hb, or αHb does not facilitate apoptosis by LPS in the absence of serum nor alter the extent of LPS-induced apoptosis in the presence of serum.

Previous studies have shown that preincubating LPS with Hb or αHb, for 30 min at 37°C before addition to cells, enhanced tissue factor activity of human umbilical vein endothelial cells compared with LPS alone (25, 33, 34). Similar experiments were carried out to examine whether preincubated mixtures of αHb and LPS could enhance endothelial apoptosis. Figure 2 shows that mixtures of αHb (500 μM) and LPS (0.1 μg/ml), preincubated for 30 min at 37°C, induced the same degree of apoptosis as LPS alone or LPS coincubated with αHb in the presence of serum-containing medium. None of the treatments induced significant apoptosis in serum-free medium (Fig. 2). These results further suggest that the physical interaction of hemoglobin and LPS does not mediate or enhance LPS-induced apoptosis in this cell culture system.

Redox active hemoglobin driven by H$_2$O$_2$ enhances LPS-induced apoptosis. To test whether the redox activity of hemoglobin enhances LPS cytotoxicity in serum-containing medium, experiments were conducted with Gx, an enzyme that produces H$_2$O$_2$. Ferrous (Fe$^{2+}$) and ferric (Fe$^{3+}$) forms of hemoglobin react with H$_2$O$_2$ to generate the highly reactive ferryl species (Fe$^{4+}$) (9, 10, 15, 16). Previous work has shown that low and continuous fluxes of enzymatically-generated H$_2$O$_2$ sustain the ferryl-ferric redox cycle and promote ferryl-mediated oxidation reactions in biological systems (9, 10).

RESULTS

LPS-induced endothelial apoptosis: role of serum and hemoglobin. The induction of apoptosis was determined by analyzing the externalization of PS on the surface of cells, an event commonly associated with the early stages of apoptosis. Figure 1 shows the effects of LPS, BvHb, Hb, and αHb on the induction of apoptosis in the presence or absence of serum-containing medium. In the absence of serum, LPS alone or combined with any of the hemoglobins did not significantly increase PS externalization (Fig. 1A). In the presence of serum, LPS induced significant apoptosis (Fig. 1B). LPS combined two-tailed Student’s t-test with SAS JMP (version 3.2) software (SAS Institute; Cary, NC).

Fig. 1. Endothelial cell apoptosis by phosphatidylserine (PS) externalization assay. Cells were treated with 50 μM (0.8 mg/ml) bovine hemoglobin (BvHb), human hemoglobin (Hb), or α-cross-linked Hb (αHb) with or without 1 μg/ml LPS in FBS-free medium (A) or medium containing 5% FBS (B). In B, cells were also treated with LPS and either 40 μM z-VAD-fmk (zVAD) or 5 μg/ml polymyxin B (PB). After 16 h, adherent and nonadherent cells were pooled and analyzed for PS externalization, as described in MATERIALS AND METHODS. For each treatment, the percentages of early apoptotic cells [annexin V positive, propidium iodide (PI) negative] are shown as means ± SE for a minimum of 3 independent experiments (n = 3–6). *P < 0.001 and #P < 0.05 vs. medium alone; †P < 0.05 vs. LPS alone.

Fig. 2. Effect of preincubation of αHb and LPS on endothelial cell apoptosis. Cells were treated with 0.1 μg/ml LPS alone or in combination with 500 μM (8 mg/ml) αHb in FBS-free medium (left) or medium containing 5% FBS (right). Cells were also treated with mixtures of αHb (500 μM) and LPS (0.1 μg/ml) that were preincubated for 30 min at 37°C before addition to cells. PS externalization was measured after 16 h, as described in MATERIALS AND METHODS. The percentages of early apoptotic cells (annexin V positive, PI negative) are shown as means ± SE for a minimum of 3 independent experiments (n = 3–6). *P < 0.001 vs. medium alone.
Figure 3 shows the level of Na$_2$S-detectable ferryl hemoglobin in cultures containing BvHb, Hb, or $\alpha$Hb with or without Gx and/or LPS after a 1-h incubation. In the absence of Gx, the ferryl species was not detected in any of the hemoglobin-containing treatments. As expected, Gx promoted significant ferryl formation in BvHb, Hb, and $\alpha$Hb-containing treatments. LPS had no effect on ferryl formation in any of the hemoglobin mixtures in the presence or absence of Gx after 1 h (Fig. 3) and over the course of 16 h (data not shown).

Figure 4A shows that endothelial cells incubated with Gx alone or in combination with BvHb, Hb, or $\alpha$Hb did not undergo significant apoptosis. In contrast, the combination of LPS, Gx, and either BvHb, Hb, or $\alpha$Hb enhanced PS externalization compared with LPS alone by 2.3-, 1.9-, and 2.1-fold, respectively. Treatment with LPS and Gx produced the same degree of apoptosis as LPS alone, indicating a requirement for hemoglobin in the enhancement mechanism. The inclusion of catalase (100 U/ml), a scavenger of H$_2$O$_2$, in treatments containing $\alpha$Hb, Gx, and LPS inhibited the enhancement effect (27 ± 4% vs. 24 ± 2% for LPS alone), indicating a requirement for H$_2$O$_2$-driven processes. Catalase also inhibited redox active BvHb- and Hb-mediated enhancement of apoptosis, whereas it had no effect on apoptosis induced by LPS alone (data not shown). In separate experiments, it was found that as little as 10 $\mu$M $\alpha$Hb and Gx produced enhancement of LPS-induced apoptosis. Experiments were also performed with LPS concentrations ranging from 0.001 to 1 $\mu$g/ml. Figure 4B shows that a similar degree of apoptotic enhancement was produced by redox active $\alpha$Hb at 0.1 and 1 $\mu$g/ml LPS. Treatment with 0.001 $\mu$g/ml LPS alone did not induce apoptosis; however, a detectable increase in apoptosis was stimulated by redox active $\alpha$Hb at this LPS concentration.

Phase-contrast and immunofluorescence microscopy was used to further identify the nature of cell death (Fig. 5). In control cultures, cells did not stain for externalized PS and displayed normal nuclear morphology by Hoechst staining (Fig. 5B). In experimental cultures, there was an accumulation of rounded and shrunken cells that exhibited intense membrane staining for externalized PS and bright Hoechst staining of condensed and fragmented nuclear material, another key feature of apoptosis (Fig. 5, D and F). These micrographs further confirm the onset of apoptotic cell death in cultures treated with LPS alone and the combination of LPS with hemoglobin and Gx.

**Time course analysis of PS externalization, caspase 3, and cell cycle distribution.** Measurements of PS externalization and caspase 3 activity were performed at different time intervals to further define the involvement of redox active hemoglobin in the enhancement of LPS-induced apoptosis. LPS induced a rapid increase in PS externalization that leveled off after 4 h (Fig. 6A). The combination of LPS, Gx, and $\alpha$Hb showed a similar early increase in PS externalization at 4 h followed by significant enhancement of PS externalization after 8 h. Consistent with these findings, LPS produced the same increase in
caspase 3 activity as the combination of LPS, Gx, and ααHb at 4 h, whereas the latter treatment induced a twofold greater increase in caspase 3 activity compared with LPS alone after 16 h (Fig. 6B).

Cell cycle analysis was also performed to identify and correlate cell cycle alterations with the onset of apoptosis. Figure 7, A and B, shows that LPS produced an increase in the portion of G1 phase cells and a decrease in S phase cells, respectively. By 16 h, the percentages of G1 and S phase cells in LPS-treated cultures were 84 ± 2% and 8 ± 2%, respectively, compared with 62 ± 3% and 25 ± 2% in control cultures. During the initial 4 h of LPS treatment, the increase in sub-G1 content suggests an increase in apoptosis (Fig. 7D). During this time, the fraction of G2/M cells remained constant but elevated compared with control, suggesting that the increase in sub-G1 events arises from apoptosis of S phase cells. The decrease in G2/M cells after 8 h was not accompanied by apoptosis because no additional cell loss was noted in the sub-G1 population (Fig. 7D). Treatment with ααHb and Gx with or without LPS also produced an accumulation of cells in the G1 phase and a significant reduction of S phase cells. The combination of ααHb and Gx produced a rise in G2/M cells observed between 4 and 8 h, and the subsequent decrease in G2/M cells was accompanied by a small detectable increase in sub-G1 content at 16 h. Contrastingly, the combination of LPS, Gx, and ααHb induced a marked increase in G2/M cells by 8 h, and the subsequent drop in G2/M cells after 12 h paralleled a significant accumulation of sub-G1 content. Taken together, these results suggest that the enhancement of LPS-induced apoptosis by redox active hemoglobin arises from cells that become arrested in G2/M and subsequently undergo apoptosis.
DISCUSSION

Cell-free hemoglobin aggravates LPS lethality in vivo via mechanisms that are not completely understood. Although previous studies have implicated the direct interaction of hemoglobin with LPS in the enhancement of certain biological effects of LPS, the present study shows that coincubation of BvHb, Hb, or αHb with LPS does not promote apoptosis in the absence of serum nor alter the extent of LPS-induced apoptosis in the presence of serum. The key observation of the present study is that redox active hemoglobin, driven by a low level of oxidative stress, significantly exacerbates LPS-induced apoptosis. This latter finding provides support for a mechanism that could contribute to hemoglobin-mediated enhancement of LPS lethality, particularly given the oxidative stress conditions associated with sepsis.

Oxidative stress, resulting from the overproduction of reactive oxygen species and/or the depletion antioxidant defenses, is thought to play a key role in the organ injury and/or dysfunction associated with sepsis (29, 37). Xanthine oxidase and phagocyte NADPH oxidase, enzymatic sources for super-
oxide and H\textsubscript{2}O\textsubscript{2}, have been implicated in the overproduction of reactive oxygen species in sepsis and endotoxemia (6, 29, 37, 38). In this context, enzymatic H\textsubscript{2}O\textsubscript{2} delivery by Gx is considered a more physiologically relevant system to simulate oxidative stress than bolus H\textsubscript{2}O\textsubscript{2} addition (1, 9, 32). Increased levels of thiobarbituric acid-reactive substance, an indirect lipid peroxidation marker, have been observed in clinical and experimental sepsis (29, 31, 42). Marked depletion of antioxidants such as ascorbate, vitamin E, and glutathione has also been reported (22, 28, 29). Previous studies have shown that ascorbate and glutathione protect against ferryl-mediated cellular injury; thus it is conceivable that depletion of these antioxidants during sepsis could increase susceptibility to redox active hemoglobin-mediated enhancement of LPS pathophysiology (9, 10, 14).

In addition to oxidants such as H\textsubscript{2}O\textsubscript{2}, the rapid reaction of cell-free hemoglobin with nitric oxide (NO) may also play an important role in sepsis. Increased NO production principally through the increased expression of inducible NO synthase is thought to mediate many of the adverse hemodynamic consequences in severe sepsis (2). Some investigators have sought to exploit the NO-scavenging ability of hemoglobin in the development of cellular hemoglobin-based therapeutics for sepsis (4). However, clinical trials with a relatively nonspecific NO synthase inhibitor suggest that nonspecific NO scavenging in septic shock may have deleterious effects (27). The scavenging of NO by hemoglobin may also have important endothelial implications given the reported beneficial effects of NO on the integrity of the endothelium (7, 11).

Vascular injury characterized by endothelial cell apoptosis is thought to be a factor in sepsis pathophysiology, and studies have proposed direct and indirect roles for LPS in this process (2). LPS-induced apoptosis of murine endothelium in vivo has been reported (13). The LPS-stimulated production of TNF-\(\alpha\) is also an important contributing factor in sepsis pathophysiology. Previous studies have shown that hemoglobin augments the LPS-induced TNF-\(\alpha\) response of cultured monocytes and in vivo, an effect thought to contribute to the synergistic toxicity of hemoglobin and LPS (3, 39). In a murine endotoxemia study (39), the infusion of \(\alpha\)Hb before or concurrent with the injection of LPS increased both plasma TNF-\(\alpha\) levels and lethality compared with LPS alone. However, the latter study also showed that \(\alpha\)Hb infusion 10 h after LPS did not aggravate TNF-\(\alpha\) levels yet still increased lethality compared with LPS alone. This implies that hemoglobin enhancement of LPS-induced TNF-\(\alpha\) production cannot be considered the sole death enhancement mechanism in these animals and that other hemoglobin-mediated processes likely participate in the exacerbation of LPS lethality. Another study (44) showed that globin (hemoglobin minus heme) binds to LPS and reduces both LPS-stimulated TNF-\(\alpha\) release by macrophages and toxicity in mice, which further supports a heme-based enhancement mechanism. Previous studies (8, 24) reported that the physical interaction of hemoglobin with some forms of LPS enhances the rate of hemoglobin oxidation. LPS has also been shown to bind ferric and ferrous iron (35). Roth et al. (36) further demonstrated that LPS oxidizes ferrous iron and postulated that the interaction of LPS with iron in the vicinity of the heme pocket could initiate oxidation and lead to subsequent heme destabilization. In the present study, LPS did not enhance ferryl hemoglobin formation in the presence or absence of Gx.

This suggests the enhancement of LPS-induced apoptosis by redox active hemoglobin cannot be attributed to a stimulatory effect of LPS on ferryl hemoglobin formation. Indirect evidence for the formation of ferryl hemoglobin in an experimental peritonitis model has been reported (45).

The reaction of hemoglobin with Gx-generated H\textsubscript{2}O\textsubscript{2} produces a highly reactive ferryl species that is capable of oxidizing a variety of biomolecules (9, 10, 15, 16). A previous study (9) showed that endothelial cells treated with \(\alpha\)Hb and Gx in serum-free medium triggered G2/M cell cycle arrest followed by apoptosis. In contrast, the present study showed that treatment with \(\alpha\)Hb and Gx in serum-containing medium dramatically inhibited cell cycle progression without inducing significant apoptosis. One possible explanation for these differing outcomes may be that one or more components of serum directly interfere with the H\textsubscript{2}O\textsubscript{2}-driven redox cycling of hemoglobin and/or ferryl-mediated oxidation reactions. Another possibility is that the serum-driven proliferative status of endothelial cells influences the cellular response to redox active hemoglobin. Interestingly, LPS coincubated with \(\alpha\)Hb and Gx in serum-containing medium produced G2/M cell cycle arrest followed by apoptosis. This suggests that LPS may antagonize the antiapoptotic effects of serum and sensitize endothelial cells to injury pathways induced by redox active hemoglobin under serum-free conditions. This observation lends casual support to the idea that LPS exposure may increase host susceptibility to hemoglobin-mediated injury.

The present findings provide support for a mechanism that could be a contributing factor in the synergistic toxicity of hemoglobin and LPS. A better understanding of these processes may ultimately be helpful in managing sepsis-related pathologies under circumstances where hemoglobin-LPS interactions are likely to occur such as trauma, surgery, or with the use of hemoglobin-based oxygen therapeutics. Given the substantial evidence pointing to the development of oxidative stress in sepsis and endotoxemia, it is reasonable to speculate that such an oxidative environment could facilitate the redox cycling of hemoglobin. Further research will be required to clearly define the role of redox active hemoglobin in sepsis or endotoxemia and to test whether certain antioxidant strategies can be effective in minimizing the hemoglobin-mediated enhancement of LPS lethality.

ACKNOWLEDGMENTS

The author thanks A. I. Alayash, Y. Jia, and P. Buehler (Center for Biologics Evaluation and Research, Food and Drug Administration) for helpful discussions and critical reading of the manuscript.

The opinions and assertions contained herein are the scientific views of the author and are not to be construed as policy of the United States Food and Drug Administration.

REFERENCES

1. Antunes F and Cadenas E. Cellular titration of apoptosis with steady state concentrations of H\textsubscript{2}O\textsubscript{2} submicromolar levels of H\textsubscript{2}O\textsubscript{2} induce apoptosis through Fenton chemistry independent of the cellular thiol state. Free Radic Biol Med 30: 1008–1018, 2001.


H1882 HEMOGLOBIN AND LPS-INDUCED ENDOTHELIAL CELL INJURY


