Methemoglobin is a potent activator of endothelial cells by stimulating IL-6 and IL-8 production and E-selectin membrane expression

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Liu, Xueying, and Zoltán Spolarics. Methemoglobin is a potent activator of endothelial cells by stimulating IL-6 and IL-8 production and E-selectin membrane expression. Am J Physiol Cell Physiol 285: C1036–C1046, 2003.—Infection and injury are frequently accompanied by hemolysis. Endothelial cells are direct targets of free Hb or its oxidative derivatives, including methemoglobin (MHB) and hemin. This study tested whether Hb or its derivatives alter cytokotkine (IL-8) and cytokine (IL-6) production and the membrane expression of cell adhesion molecule (E-selectin) in human umbilical vein endothelial cells (passages 2–4, HUVECs). E-selectin membrane content and IL-6 and IL-8 release were quantified by ELISA; cellular mRNA levels were determined by RT-PCR. MHB in vitro resulted in a dose (1–50 μM)- and time (2–16 h)-dependent increase in E-selectin membrane content and IL-6 and IL-8 release in HUVECs. The stimulatory effect of MHB (12 μM) on E-selectin membrane expression and IL-6 and IL-8 release was similar to that produced after treatment with TNF-α (5 ng/ml) and IL-1β (0.25 ng/ml). In contrast, Hb or hemin had no effects. As expected, MHB, Hb, and hemin markedly induced heme oxygenase-1 expression in HUVECs. Haptoglobin, cytochalasin D, and actinomycin inhibited the MHB-induced responses, whereas zinc protoporphyrin IX (a heme oxygenase inhibitor) or desferoxamine (an iron chelator) did not inhibit MHB-induced responses. MHB also increased cellular mRNA levels of E-selectin, IL-6, and IL-8. MHB treatment activated cellular NF-κB and NF-κB inhibitors; N-acetyl cysteine, SN50, and caffeic acid phenylethyl ester inhibited the MHB-induced responses. These data indicate that MHB is a potent activator of endothelial cells through NF-κB-mediated upregulation of cell adhesion molecule expression and heme oxygenase and cytokine production. MHB-induced endothelial cell activation may have clinical significance after infections, hemolysis, or methemoglobinemia.

human umbilical vein endothelial cells; cytokine; chemokine; adhesion molecule; hemolysis; hemoglobin; hemin; nuclear factor-κB

INJURY AND INFECTION may be accompanied by intravascular or intracellular hemolysis (6, 23). Anemia develops relatively early after sepsis, which, at least partially, is the result of an elevated clearance of damaged red blood cells (RBCs) and subsequent processing of hemoglobin by the reticuloendothelial system (7, 10, 27). Acute hemolysis may manifest systemically, leading to a rise in free blood hemoglobin. Free hemoglobin or heme released into the circulation or into the interstitial space in wounds is promptly converted to methemoglobin or hemin by oxidants (22). This process is more pronounced at inflammatory sites, where activated macrophages and neutrophils accumulate and release considerable amounts of reactive oxygen or nitrogen species. Nitric oxide, which is produced in large quantities by a variety of cells during infections, may also directly convert hemoglobin to methemoglobin within the RBCs, and this conversion of hemoglobin to methemoglobin parallels a decrease in RBC deformability (3, 15, 20, 42). Therefore, intravascular or intracellular hemolysis of rigidified RBCs may result in the release of free methemoglobin during infections (45). It is evident that, under these conditions, endothelial cells are among the first potential cellular targets of free hemoglobin, methemoglobin, or their derivatives (17, 39).

It is well known that hemoglobin and hemin sensitize endothelial cells to oxidative damage. It has been shown by several independent studies that the presence of heme or hemin augments H2O2-mediated endothelial cell injury (1, 11, 22, 34). It has also been demonstrated that hemoglobin, methemoglobin, and hemin markedly upregulate heme oxygenase (HO)-1 (2, 18, 48). Heme metabolism through HO produces carbon monoxide and free iron, which have been shown to play an important role in the regulation of vascular tone and oxidant damage, respectively (1, 33). HO-1 has also been shown to function as a chaperone and is considered to be a heat shock protein with cytoprotective effects upon its induction (4, 8, 13). These previous investigations mainly focused on the sensitizing effects of hemoglobin, heme, and hemin on oxidative stress and associated cell injury; however, only a few studies have investigated the potential role of hemoglobin derivatives in the inflammatory response by endothelial cells.

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Studies testing the effects of liposome-encapsulated hemoglobin, a potential blood substitute, have demonstrated that free circulating heme or hemin stimulates IL-6 expression in macrophage-rich tissues (49). Acute hemolysis increased blood cytokines in humans (12). Hemoglobin was also shown to increase IL-8 release by polymorphonuclear phagocytes (29) and to augment IL-1β-induced TNF-α release by macrophages (43a). Hemin in vivo induced neutrophil migration, IL-8 release, and oxidative burst (16). Whereas heme was shown to potentiate TNF-α-induced ICAM-1 and E-selectin expression by endothelial cells (43), no study compared the effects of hemoglobin, methemoglobin, and hemin on a representative set of endothelial cell activation markers. Therefore, it remains unknown whether hemoglobin or methemoglobin results in the activation of pathophysiologically relevant genes representative of endothelial cell activation in addition to their well-known stimulatory effects on HO-1 expression and associated changes in oxidative stress. Because, during infections, methemoglobin appears in RBCs that may be released into the circulation after hemolysis or free hemoglobin may be converted to methemoglobin at injury sites, methemoglobin-induced endothelial cell activation is a potentially important event in the cascade of the inflammatory response (17, 45). Thus we hypothesized that free hemoglobin and methemoglobin result in the activation of endothelial cells to different degrees. To test this hypothesis, we compared the effects of hemoglobin, methemoglobin, and hemin on the production of a representative set of endothelial cell inflammatory markers, including the chemokine IL-8 and the proinflammatory cytokine IL-6. We also compared their effects on the expression and membrane content of the adhesion molecule E-selectin in endothelial cells. We found that whereas hemoglobin, methemoglobin, and hemin are efficient activators of HO-1 expression, only methemoglobin...
stimulates the production of the investigated set of endothelial cell inflammatory markers.

MATERIALS AND METHODS

Reagents. Endotoxin-free, cell culture-grade buffers, media, and reagents were used. Recombinant human TNF-α, mouse anti-human E-selectin antibody, alkaline phosphatase-conjugated rat anti-mouse antibodies, and ELISA kits for human IL-6 and IL-8 were obtained from BD Biosciences (San Diego, CA); recombinant human IL-1β from R&D systems (Minneapolis, MN); anti-NF-κB p65 and p50 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); endothelial cell growth medium and trypsin-EDTA, neutralization solutions, and HEPES-buffered saline solution from Cambrex (Walkersville, MD); N-acetyl cysteine (NAC), caffeic acid phenylethyl ester (CAPE), SN50, and SN50M from Calbiochem (San Diego, CA); RT-PCR kit from Roche (Nutley, NJ); TriReagent for RNA isolation and Formazol from Molecular Research Center (Cincinnati, OH); gel shift assay reagents from Promega (Madison, WI); phenol red-free Hank’s balanced salt solution and Dulbecco’s phosphate-buffered saline (PBS) from Life Technologies (Grand Island, NY); methanol-free formaldehyde (10%) from Polysciences (Warrington, PA); fetal bovine serum from Irvine Scientific (Santa Ana, CA); and human methemoglobin, hemoglobin, hemin, and all other reagents and chemicals of the highest grade available from Sigma-Aldrich (St. Louis, MO).

Cell isolation and culture. Human umbilical vein endothelial cells (HUVECs; Cambrex) and HUVECs isolated from umbilical cords were used in the experiments. HUVECs were subcultured into eight-well glass slides (40,000 cells/well). Cells were fixed with acetone-methanol (1:1, vol/vol). After two washes (PBS), 5% goat serum was added as a blocker (0.5 ml/well), and the cells were incubated for 10 min. After two additional washes (PBS), cells were incubated with anti-human von Willebrand factor for 1 h, washed twice, and then incubated with anti-mouse antibody conjugated with FITC for 30 min in the dark. Anti-human IgG was used in parallel as negative control. Cells were analyzed under a confocal fluorescent microscope. von Willebrand factor-related antigen-positive cell preparations were subcultured and used in the experiments.

Treatment protocols and assays. HUVECs were subcultured into 96-well plates 16–18 h before the experiments. Before the experiment, medium was replaced with fresh medium before the treatment regimens were initiated. Methemoglobin, hemoglobin, and hemin solutions were prepared for every experiment. All agents used in the experiments were dissolved in medium as vehicle, unless otherwise stated. After various treatments, supernatants were collected for analyses. IL-6 and IL-8 in cell medium were determined by ELISA according to the manufacturer’s protocol. Standards were run on each ELISA plate and analyzed in parallel with the test samples.

E-selectin membrane content was determined on confluent cells. After removal of conditioned medium, cells were fixed

![Fig. 2. Methemoglobin results in elevated E-selectin (A), IL-6 (B), and IL-8 (C) mRNA levels in endothelial cells. Cells were incubated in the presence of 12.5 μM methemoglobin, 12.5 μM hemoglobin, or 5 μM hemin. TNF-α (5 ng/ml) served as positive control in parallel incubations. After 3 h of incubation, total cellular RNA was isolated and subjected to RT-PCR. In parallel, GAPDH expression was also determined and run together with the target mRNA, serving as control for amplification and even sample loading. D: in the same mRNA preparations, expression of heme oxygenase-1 (HO-1) was also determined and served as additional positive control. Gels depict 1 typical finding of 5 experiments from 3 independent cell preparations. Veh, vehicle.](image-url)
by addition of 1.5% paraformaldehyde in PBS (pH 7.4) and incubated for 15 min. After two washes (PBS), cells were incubated with 2% bovine serum albumin in PBS for 30 min (blocking). Cells were incubated with mouse anti-human E-selectin antibody (0.2 μg/ml) in the presence of 1% bovine serum albumin in PBS for 60 min. After three washes, 1.500 diluted alkaline phosphatase-conjugated anti-mouse antibody was added, and the cells were incubated for 60 min. Color reaction was developed in the presence of p-nitrophenyl disodium phosphate (0.1 ml/well). After 30 min, the reaction was terminated by addition of 0.05 ml of 3 M NaOH. Optical density was measured in a microplate reader at 405 nm.

RNA determinations. The final cell pellets containing 10^6 endothelial cells were lysed in 2.5 ml of TriReagent. Total cellular RNA was isolated according to the manufacturer’s protocol. The final RNA pellet was dissolved in 0.05–0.1 ml of Formazol (Molecular Research Center). Deoxyligonucleotide primers used for HO-1 (5), GAPDH (5), E-selectin (28), IL-6 (41), and IL-8 (35) determinations were synthesized in the Molecular Biology Core Laboratory of the University of Medicine and Dentistry of New Jersey. GAPDH expression controls were carried out throughout all RT-PCR reactions. The sequences of the employed oligonucleotide primers were as follows: 5'-GCT CAA CAT CCA GCT TGA GG-3' (forward) and 5'-GAC AAA GTT CAT GGC CCT GGG A-3' (reverse) for HO-1, 5'-GTC TTC ACC ACC ATG GAG AA-3' (forward) and 5'-ATC CAC ACT CTT CGT GGT GG-3' (reverse) for GAPDH, 5'-GAT GTG GGC ATG TGG AAT GAT G-3' (forward) and 5'-AGG TAC ACT GAA GGA TCT GG-3' (reverse) for E-selectin, 5'-TGA ACT CCT TCT CCA CAA GCG-3' (forward) and 5'-TCT GAA GAG GTG AGT GGC TGT C-3' (reverse) for IL-6, and 5'-GGA CAA GAG CCA GGA AGA AAC CAC C-3' (forward) and 5'-GCA ACC CTA CAA CAG ACC AC-3' (reverse) for IL-8.

Serial dilutions of RNA samples were subjected to RT-PCR using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Roche, Branchburg, NJ). After RT, Mn2+ was chelated and MgCl2 was added for the amplification steps (2.0 mM) in the presence of dNTP mixtures (2 mM each). Amplification was carried out in the GeneAmp 2400 instrument (Perkin-Elmer) as follows: RT at 70°C for 15 min and PCR at 95°C for 1 min, 95°C for 10 s, and 60°C for 15 s for 35 cycles followed by 60°C for 7 min. The mixture was cooled to 4°C to terminate the reaction and stored in -20°C. Aliquots of the reaction samples were subjected to PAGE.

Electrophoretic mobility shift assays. After the treatments, the cells were washed and then detached by trypsinization. After they were neutralized and washed, 3 × 10^6 cells were suspended in 0.1 ml of whole cell extraction buffer and then centrifuged at 16,000 g for 10 min at 4°C. The supernatant was collected, the protein concentration was determined, and the supernatant was stored in aliquots until NF-κB determinations. The double-stranded NF-κB consensus oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was end-labeled with 10 μCi of [γ-32P]ATP at 222 TBq/mmol (Amersham, Arlington Heights, IL). Binding reactions containing 35 fmol of oligonucleotide and 10 μg of cellular protein were carried out for 20 min at room temperature. For competition reactions, a 100-fold excess of unlabeled oligonucleotide was added 5 min before addition of the labeled probe. For supershift analysis, 1 μg of each antibody was added to the reaction mixture, and the mixture was incubated for 30 min before addition of the radiolabeled probe. After the binding reactions, samples were subjected to nondenaturing 4% PAGE in low ionic strength buffer (80 mM Tris-borate and 2 mM EDTA). Gels were vacuum dried, and signals were quantified using the PhosphorImager SI analyzer and Imagequant version 4.1 program (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Statistical calculations were performed using JMP software (SAS Institute, Cary, NC). Results were analyzed using ANOVA followed by t-test for pairwise comparisons or Tukey-Kramer’s test for multiple comparisons. Statistically significant difference was concluded at P < 0.05.
RESULTS

Methemoglobin activates endothelial cells. Methemoglobin resulted in a concentration-dependent increase in E-selectin membrane content (Fig. 1A) and IL-6 and IL-8 release (Fig. 1, B and C, respectively) in endothelial cells. The effect of methemoglobin reached a plateau at 30–50 μM. For comparison, the effects of TNF-α and IL-1β at approximately their ED₅₀ were also determined in parallel incubations. The IL-6 and IL-8 responses induced by methemoglobin at approximately its ED₅₀ (10 μM) were similar to the TNF-α- and IL-1β-induced responses, whereas the increase in E-selectin membrane expression was more pronounced after cytokine than after methemoglobin treatment. The TNF-α- and methemoglobin-induced responses were time dependent up to 16 h (data not shown). Hemoglobin and hemin had no stimulatory effects on E-selectin membrane expression or IL-6 and IL-8 release compared with methemoglobin (Fig. 1B). Hemoglobin or hemin at up to 50 μM remained ineffective in stimulating a response. The effects of methemoglobin and TNF-α were additive when administered simultaneously at lower concentrations (5 μM methemoglobin + 0.2–1 ng/ml TNF-α); however, combined treatments at maximal doses were not additive (data not shown). Combined methemoglobin and IL-1β treatments resulted in similar observations (data not shown).

Methemoglobin acts through increasing gene transcription. To test whether methemoglobin results in elevated expression of these activation markers, we determined cellular mRNA levels using semiquantitative RT-PCR after these treatments. Under nonstimulated conditions (vehicle), the cellular levels of E-selectin, IL-6, and IL-8 mRNA were below the detectable levels (Fig. 2). Methemoglobin administration resulted in readily detectable, elevated cellular mRNA for E-selectin, IL-6, and IL-8. The effect of 12.5 μM methemoglobin on E-selectin and IL-8 mRNA expression was similar to that observed after TNF-α (5 ng/ml) treatment. Furthermore, IL-6 mRNA levels were more efficiently stimulated by methemoglobin than by TNF-α, in agreement with the more pronounced increase in IL-6 protein release after treatment with methemoglobin than after TNF-α treatment. As expected, and in agreement with previous observations, hemin, hemoglobin, and methemoglobin markedly increased HO-1 mRNA expression, whereas they did not increase E-selectin, IL-6, or IL-8 mRNA levels in endothelial cells (Fig. 2).

Fig. 4. Effect of haptoglobin and Zn protoporphyrin IX (ZnPP IX) on methemoglobin-induced responses in endothelial cells. A: effects of methemoglobin and haptoglobin-neutralized methemoglobin. Cells were incubated for 4 h in the presence of vehicle (PBS), haptoglobin alone (0.71 mg/ml), methemoglobin (5 μM), or methemoglobin neutralized by haptoglobin. [Methemoglobin was incubated with haptoglobin (1:2 wt/wt) for 20 min in medium before administration to cells.] B: effect of the HO-1 inhibitor Zn protoporphyrin IX on methemoglobin-induced responses. Cells were preincubated with 100 μM Zn protoporphyrin IX for 5 min before administration of methemoglobin. Values (means ± SE, n = 6 from 2 independent cell preparations for each study) are expressed as percentage of the methemoglobin-induced response. *Significantly different from methemoglobin.
To further elucidate whether methemoglobin acts through transcriptional stimulation, we tested the effect of the transcriptional inhibitor actinomycin D on the methemoglobin-induced responses. Methemoglobin-induced responses were inhibitable by actinomycin D (Fig. 3). The inhibitory effects of actinomycin D on the TNF-α- and methemoglobin-induced E-selectin expression and IL-6 and IL-8 production were similar (Fig. 3).

Mechanistic aspects of methemoglobin effects. In subsequent experiments, we tested whether methemoglobin uptake and heme metabolism through HO-1 are required for its stimulatory actions on endothelial cells. Haptoglobin, a natural methemoglobin-binding protein, decreased methemoglobin-induced E-selectin membrane content and IL-6 and IL-8 production (Fig. 4A).

The HO inhibitor zinc protoporphyrin IX caused no inhibition of methemoglobin effects (Fig. 4B); rather, it augmented the methemoglobin-induced responses. In contrast, zinc protoporphyrin IX treatment did not augment the TNF-α- or IL-1β-induced E-selectin membrane expression and IL-6 and IL-8 production (data not shown). We also tested the effect of the iron chelator desferroxamine on the methemoglobin-induced responses. Desferroxamine had no effects on the methemoglobin-induced increase in E-selectin membrane content or IL-6 and IL-8 release (data not shown).

To assess whether internalization of methemoglobin is required for the described responses, we tested the effects of cytochalasin D, an endocytosis inhibitor, on the methemoglobin- or TNF-α-induced responses. Cytochalasin D markedly inhibited the methemoglobin-induced increase in E-selectin expression and IL-6 and IL-8 production (Fig. 5). The effect of cytochalasin D was less pronounced on the TNF-α- than on the methemoglobin-induced E-selectin response. Furthermore, cytochalasin D did not inhibit TNF-α-induced IL-8 production (Fig. 5). The inhibitory effect of cytochalasin D on the IL-1β-induced response was similar to that observed after TNF-α treatment (not shown).

Because activation of the transcription factor NF-κB plays an important role in the regulation of E-selectin production and IL-8 and IL-6 expression in endothelial cells, we used gel shift analyses to test whether methemoglobin results in activation of NF-κB. TNF-α treatment in parallel incubations and analyses served as positive control. Methemoglobin treatment increased NF-κB binding to endothelial cells (Fig. 6A). However, the oligonucleotide-protein complex showed a faster migration pattern than samples from TNF-α-treated controls (Fig. 6B, lane 5 vs. lane 3). Preincubation of cell protein extracts with the mixture of the antibodies against the p50 and p65 subunits of the NF-κB complex resulted in a supershift in methemoglobin- and TNF-α-treated cells (Fig. 6B, lane 3 vs. lane 4 and lane 6 vs. lane 5). Use of antibodies against the p50 and p65 subunits individually also resulted in shifts (not shown), providing additional evidence that the proteins interacting with the NF-κB consensus sequence contain the p50-p65 heterocomplex after methemoglobin treatment.
Because activation of NF-κB is partially mediated through redox alterations, we tested the effects of the antioxidant NAC on methemoglobin- and TNF-α-induced endothelial cell responses. NAC had a concentration-dependent inhibitory effect on the methemoglobin- or TNF-α-induced E-selectin membrane content and IL-6 and IL-8 production (Fig. 7). The inhibitory effect of NAC showed a similar pattern of concentration dependence on the methemoglobin- and TNF-α-induced E-selectin and IL-8 responses.

Finally, we tested the effects of SN50, a specific cell-permeable peptide that interacts with the NF-κB translocation sequence (26), as well as CAPE, an inhibitor of NF-κB activation and translocation (30), on the methemoglobin-induced response. CAPE (Fig. 8A) and SN50 (Fig. 8B) inhibited methemoglobin- or TNF-α-induced E-selectin membrane content and IL-6 and IL-8 production in a concentration-dependent manner.

To assess potential toxic effects of the treatment regimens, lactate dehydrogenase content in the collected cell medium was determined. The treatment protocols did not result in cell detachment, elevated lactate dehydrogenase release, or noticeable changes in cell morphology in the incubation times employed in this study (data not shown).

**DISCUSSION**

This study demonstrates for the first time that methemoglobin results in activation of endothelial cells by stimulating membrane expression of the adhesion molecule E-selectin and production and release of the chemokine IL-8 and the proinflammatory cytokine IL-6. The effects of 10–30 μM methemoglobin are similar to the effect of TNF-α and IL-1β at approximately their ED₅₀. A variety of pathological conditions may result in methemoglobinemia, including intoxication by xenobiotics, dietary nitrites, or genetic defects, in-
thelial cell activation and injury, increased sensitivity to oxidative stress, and elevated blood cytokines (47).

The fact that methemoglobin markedly increased cellular mRNA levels of E-selectin, IL-6, and IL-8, together with the observation that actinomycin D inhibited the response, suggests that the elevated mRNA levels are the result of increased gene transcription, rather than increased mRNA stability, after methemoglobin administration. It is noteworthy that the general pattern of methemoglobin effects on E-selectin and IL-8 expression is similar to that observed after treatment with TNF-α or IL-1β. These observations and the fact that NAC or inhibitors of NF-κB resulted in a similar inhibitory pattern on methemoglobin- and TNF-α-induced responses suggest that the signal transduction pathways resulting in endothelial cell activation are shared and/or converge after methemoglobin, TNF-α, or IL-1β treatments. This finding is supported further by the observation that combined treatments with methemoglobin and TNF-α or IL-1β were additive only when they were administered at submaximal concentrations.

This is the first report indicating that methemoglobin activates NF-κB in endothelial cells. Although the rate of migration of the NF-κB complex after methemoglobin treatment was different from that observed after TNF-α, the fact that antibodies against the p50 and p65 subunits resulted in a similar supershift after TNF-α and methemoglobin treatments confirmed that NF-κB becomes activated under these conditions. IκB kinases, as well as the p65 subunit, are subject to redox-dependent regulation by specific intracellular kinases (36). Several independent studies in a variety of cell types demonstrated that NAC treatment prevents TNF-α-induced activation of IκB kinases and also inhibits serine phosphorylation and translocation of p65 (31, 32, 37). The findings that treatment with NAC, as well as SN50 and CAPE (26, 30), caused a similar inhibition pattern on the TNF-α- and methemoglobin-induced increased E-selectin membrane expression and IL-6 and IL-8 production further support the potential involvement of NF-κB activation in the methemoglobin-induced response.

It is also known that elevated gene expression of HO-1 is not dependent on NF-κB, inasmuch as the promoter for HO-1 lacks an NF-κB consensus sequence (13). These facts suggest that the signaling pathway of hemin, hemoglobin, and methemoglobin resulting in elevated HO-1 expression is distinct from the mechanisms causing elevated cytokine, chemokine, or adhesion molecule expression after methemoglobin treatment. It remains to be determined whether the faster migration of the NF-κB complex after methemoglobin treatment is an artifact associated with the interference of trace amounts of methemoglobin in cellular extracts or is a unique characteristic of methemoglobin-mediated NF-κB activation with functional significance.

The fact that the inhibition of HO-1 by zinc protoporphyrin IX did not diminish the methemoglobin-induced responses indicates that carbon monoxide generation, free intracellular iron, or downstream intermediates of hemin catabolism are not required for mediation of the stimulatory effects of methemoglobin on E-selectin membrane expression and IL-6 and IL-8 production. In contrast, the observed stimulatory ef-

![Fig. 7. N-acetyl cysteine (NAC) inhibits increase in E-selectin membrane content and IL-6 and IL-8 release induced by methemoglobin and TNF-α. Endothelial cells were incubated for 4 h with methemoglobin (12.5 µM) or TNF-α (5 ng/ml) in the absence or presence of 3–50 mM N-acetyl cysteine. Values are means ± SE from 2 independent cell preparations determined in triplicate.](http://ajpcell.physiology.org/)
fектs of HO-1 inhibition suggest that the accumulation of metabolites upstream of the HO-1 step may have contributed to the observed effects. Alternatively, under HO-1-inhibited conditions, the absence of carbon monoxide or intracellular iron may have had an augmenting effect on methemoglobin actions, in agreement with previous observations (40).

The exact mechanism of methemoglobin action causing the activation of endothelial cells remains to be further elucidated. However, the observations that hemoglobin or hemin had no stimulatory effects on IL-6, IL-8, or E-selectin expression, chelating iron in the cell medium had no inhibitory effects, and haptoglobin inhibited the methemoglobin-induced responses indicate that the presence of methemoglobin molecules per se is required for the activation of endothelial cells. It is well known that hemin is responsible for HO-1 induction in endothelial cells (1, 4, 8, 22, 48). The observation that hemoglobin, methemoglobin, or hemin caused elevated HO-1 expression indicates that hemin catabolism proceeded after all three of these treatments. The fact that only methemoglobin was efficient in stimulating IL-6, IL-8, and E-selectin expression suggests that the quaternary structure of methemoglobin plays a role in the observed effects. The quaternary structure of methemoglobin is different from that of oxy- or deoxyhemoglobin (21). Furthermore, the tetrameric form of hemoglobin or methemoglobin dissociates into αβ dimers in dilute solutions, with midpoint equilibrium at 6 μM (21). Formation of methemoglobin by oxidants such as ferricyanide or nitrates also results in the appearance of hybrid intermediates (38). The fact that haptoglobin, which binds predominantly the αβ dimmers, but not the tetrameric form, resulted in 50% inhibition at near-midpoint equilibrium concentration of methemoglobin suggests that the dimeric configuration of methemoglobin is effective, but other biologically active forms may also be present. Exact elucidation of this question requires further studies.

The inhibitory effect of cytochalasin D on IL-6 and IL-8 release and E-selectin expression suggests that internalization of methemoglobin is necessary for its actions. However, cytochalasin D, in addition to inhibiting endocytosis, may also affect the processing and transport of de novo synthesized cellular proteins (44). This is presumably reflected in its inhibitory effects on IL-6 release and E-selectin membrane expression after TNF-α or IL-1β treatments. Thus it remains to be elucidated whether endocytosis of methemoglobin by endothelial cells is an obligatory requirement for the observed effects.

Taken together, we conclude that the interaction between methemoglobin and endothelial cells results in increased production and release of IL-6 and IL-8 and elevated membrane expression of E-selectin. The methemoglobin-induced cell activation does not require the metabolism of hemin through the HO-1 pathway but, rather, is mediated through the activation of
signaling mechanisms initiated at the cell membrane level or through intermediates of methemoglobin catabolism upstream of HO-1 action. These observations indicate that the presence of free methemoglobin in blood or interstitial fluid at inflammatory or injury sites may contribute to endothelial cell activation. Elevated chemokine release, together with an increased expression of adhesion molecules, by endothelial cells may promote phagocyte recruitment, whereas increased cytokine production can modulate cellular responses in an autocrine or a paracrine fashion. Methemoglobin-induced endothelial cell activation may be a clinically important event after intoxications accompanied by methemoglobinemia or during infections, especially when acquired or genetic conditions predisposing to hemolysis or diminished antioxidant activity are also present.

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DISCLOSURES

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