Polymerized Hemoglobin Induces Heme Oxygenase-1 Protein Expression and Inhibits Intercellular Adhesion Molecule-1 Protein Expression in Human Lung Microvascular Endothelial Cells

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BACKGROUND: Our clinical trials using a polymerized hemoglobin solution (PolyHb) as a red cell substitute in severely injured patients suggested that this hemoglobin-based oxygen carrier has a systemic antiinflammatory effect. Heme oxygenase-1 (HO-1) has recently been shown to be cytoprotective, and is known to be induced by heme moieties. We investigated the effects of this hemoglobin-based oxygen carrier on HO-1 induction and proinflammatory activation of pulmonary endothelium.

STUDY DESIGN: Human lung microvascular endothelial cells were grown to confluence and preincubated with either cell media (control) or with an equal volume mixture of polymerized hemoglobin/cell media (experimental). The cell cultures were subsequently stimulated with lipopolysaccharide. HO-1 expression was detected by protein immunoblot and further quantified by ELISA; intercellular adhesion molecule-1 protein expression was measured by flow cytometry.

RESULTS: Polymerized hemoglobin induced synthesis of HO-1 protein in human lung microvascular endothelial cells and, concurrently, inhibited lipopolysaccharide-induced intercellular adhesion molecule-1 protein cell surface expression.

CONCLUSIONS: Polymerized hemoglobin attenuates lipopolysaccharide-stimulated expression of intercellular adhesion molecule-1 protein, which is associated with upregulation of the cytoprotective protein HO-1 in human pulmonary endothelial cells. This antiinflammatory effect offers a novel mechanism by which hemoglobin-based oxygen carrier solutions may be exploited therapeutically as resuscitative fluids. (J Am Coll Surg 2005;201:579–584. © 2005 by the American College of Surgeons)

In clinical trials, polymerized hemoglobin (PolyHb) has been shown to be a clinically useful red cell substitute.[1,2] In severely injured patients, its use has been associated with decreased postinjury circulating proinflammatory cytokines compared with those in resuscitation using stored packed red blood cells.[3] Although this finding may be from avoidance of the proinflammatory effects of packed red blood cell transfusion, an alternative hypothesis is that PolyHb has a protective antiinflammatory effect beyond its intended role as an oxygen carrier. Heme oxygenase-1 (HO-1) is the inducible isoform of the heme oxygenase protein and can be synthesized by nearly all cell types. Normally, HO-1 expression is low or undetectable, but in response to a variety of stimuli, including heme moieties, this isoform is rapidly synthesized. HO-1 activity in experimental models has been shown to be cytoprotective by decreasing cellular inflammation.[4,5] The postinjury systemic inflammatory response promotes neutrophil infiltration into tissues, which is central in the pathogenesis of organ dysfunction. Intercellular adhesion molecule-1 (ICAM-1) mediates neutrophil infiltration by facilitating its firm adhesion to endothelium. ICAM-1 is rapidly expressed on

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Abbreviations and Acronyms

HBOC = hemoglobin-based oxygen carrier
HMVEC = human lung microvascular endothelial cell
HO-1 = heme oxygenase-1 protein
ICAM-1 = intercellular adhesion molecule-1 protein
LPS = lipopolysaccharide
MFI = mean fluorescent intensity
PBS = phosphate buffered saline
PolyHb = polymerized hemoglobin

the cell surface during endothelial activation by a number of inflammatory stimuli.[6] Recognizing the unique vulnerability of the lung in the postinjury systemic inflammatory response, we tested our hypothesis that PolyHb has an antiinflammatory effect in human lung microvascular endothelial cells (HMVECs). Given the structural similarities of PolyHb to heme, we investigated its effects on HO-1 induction and on subsequent ICAM-1 expression in HMVECs stimulated by the inflammatory stimulus, lipopolysaccharide (LPS).

METHODS

Endothelial cell culture

Primary human lung microvascular endothelial cells (HMVECs, Cambrex Corp) were grown to 80% to 90% confluence in 12-well cell culture plates using Clonetics EGM-2MV complete cell media (Cambrex Corp). On the day of use, cells were incubated with fresh cell media as controls or with a 50% (v/v) mixture of cell media and PolyHeme (Northfield Laboratories) as the experimental PolyHb group. The ratio of cell media to PolyHb approximated the relative concentration of PolyHb that the pulmonary endothelium of injured patients at risk for acute lung injury would be exposed to during emergent transfusion.[1]

HO-1 protein detection

Cells were incubated with the PolyHb mixture for varying time periods, ranging from 2 hours to 10 hours. At the end of each incubation period, the cells were washed with phosphate buffered saline (PBS) and cell lysates collected. As a positive control for HO-1 induction, hemin (100 μM, Sigma-Aldrich) was incubated with the cells and cell lysates prepared in similar fashion after the varied incubation periods. Protein concentrations were determined by the bicinchoninic acid assay and gel samples were loaded with equal protein concentration and HO-1 protein expression determined by immunoblot detected by chemiluminescence (Pierce). A rabbit polyclonal antibody to human HO-1, the appropriate secondary antibody, and recombinant human HO-1 protein, as an additional positive control for the HO-1 antibody, were purchased from Stressgen. ELISA was used to quantify the amount of HO-1 protein present in cell lysates after the varied incubation periods (Stressgen).

ICAM-1 surface expression

Based on the time course of HO-1 induction, HMVECs were preincubated with the PolyHb mixture or cell media alone (control) for 5 hours, then washed and stimulated for 6 hours with 20 ng/mL or 100 ng/mL LPS (*E coli* serotype 055:B5, Sigma Aldrich) in fresh cell media. After LPS stimulation, the cell monolayers were washed with PBS and detached using Accutase enzyme detachment medium (eBioscience). Cells were centrifuged at 4°C at 1,100 rpm and the supernatant was aspirated. Cells were then resuspended with PBS and incubated in the dark with CD54-FITC (mouse antihuman ICAM-1, clone 84H10) or FITC-isotype for 30 minutes at 4°C (Immunotech). After the antibody incubation, the cells were fixed with 4% paraformaldehyde. ICAM-1 cell surface expression was determined by flow cytometry using a Cytomics FC500 (Beckman Coulter).

Statistical analysis

Data are reported as mean ± SEM and were compared by analysis of variance using the Fisher's protected least significant difference procedure for post hoc comparisons. Statistical significance was established at $p < 0.05$.

RESULTS

HO-1 expression

PolyHb induced the synthesis of HO-1 protein in HMVECs in a time-dependent fashion as shown by protein immunoblot (Fig. 1A). Cell lysates collected at 5-, 8-, and 10-hour incubation periods with PolyHb showed an incremental increase in the intensity of HO-1 protein. As a positive control, hemin incubation upregulated HO-1 protein in a similar time course (10-hour hemin incubation shown in Fig. 1A). Measurements of HO-1 protein (ng/mL) present in cell lysates by ELISA corroborated the results of the protein immunoblot (Fig. 1B). HO-1 protein concentration increased with the 5-hour incubation with PolyHb and continued to rise up to 10 hours (PolyHb