Detection of a ferrylhemoglobin intermediate in an endothelial cell model after hypoxia-reoxygenation

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McLeod, Laurie L., and Abdu I. Alayash. Detection of a ferrylhemoglobin intermediate in an endothelial cell model after hypoxia-reoxygenation. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H92–H99, 1999.—A cell culture model of bovine aortic endothelial cells attached to microcarrier beads was used to study the interaction of diaspirin cross-linked hemoglobin (an oxygen-carrying blood substitute) with hypoxia-reoxygenation. Hemoglobin (200 µM) and hypoxia-volume restriction (3–5 h), together and separately, caused toxicity in this model, as measured by decreased cellular multiplying efficiency. Hemoglobin (60 µM) caused a reduction in hydrogen peroxide concentration and an increase in lipid peroxidation above that induced by hypoxia alone. Incubation of hemoglobin with endothelial cells caused transient oxidation of hemoglobin to its highly reactive and toxic ferryl species after ≈3 h of hypoxia, followed by 1 h of reoxygenation. Lipid peroxidation, which may occur in the presence of ferrylhemoglobin, also occurred after 1 h of reoxygenation. Hemoglobin caused a dose-dependent decrease in intracellular glutathione concentration, suggesting that it caused an oxidative stress to the cells. However, addition of ascorbate, α-tocopherol, or trolox did not decrease hemoglobin oxidation in the presence of normal or hypoxic cells. It is concluded that diaspirin cross-linked hemoglobin forms a ferryl intermediate in the absence of any exogenously added oxidant and contributes to the oxidative burden experienced by endothelial cells after hypoxia-reoxygenation, a condition that is likely to be encountered during trauma and surgery when hemoglobin solutions are used as perfusion agents.

Hemoglobin-based oxygen carriers; endothelium; antioxidants

Interactions of heme proteins with tissue have been suggested to occur in a variety of pathophysiological states, including blast pressure injury (19), ischemia-reperfusion (16), and cerebral hemorrhage (33). In addition, a variety of oxygen-carrying blood substitutes are being developed, based on modified forms of hemoglobin, which also have the potential to interact with vascular tissue (4, 15). Because the toxicity associated with exposure to native hemoglobin has been ascribed to dissociation of hemoglobin into its dimeric subunits, current design strategies for modified hemoglobins include chemical and genetic manipulation to facilitate retention of their tetrameric form (15). These modifications, which include chemical cross-linking of either the α- or β-subunits, polymerization, and genetic amino acid substitution, have been shown to improve the intravascular retention of hemoglobin, to improve its oxygen “off-loading” capabilities, and to decrease its renal toxicity. New and more challenging problems with toxicity and efficacy remain, however, including vasoconstriction, short intravascular half-life, and rapid autoxidation associated with free radical-mediated toxicity, which have been attributed to the redox properties of the heme group (4, 15).

Little is known about the interaction of heme proteins with cellular oxidants and antioxidants in vivo or with oxidative disease states such as ischemia-reperfusion. Oxidation of hemoglobin by endothelial cells has been correlated with oxidative stress and cell injury (27). Reactions with nitric oxide (6) and hydrogen peroxide (17) have been shown to alter the oxidation states of hemoglobin, and reductants such as glutathione and ascorbate, traditionally considered to be antioxidants, can directly oxidize oxyhemoglobin in the presence of oxygen (19). The reaction of hemoglobin with an oxidant, such as hydrogen peroxide, is also expected to cause cooxidation of other cellular macromolecules, such as unsaturated lipids (21). These oxidative reactions of hemoglobins and of chemically stabilized hemoglobins make excellent tools for uncovering the mechanisms of heme-related toxicity in vivo (2, 5).

Hemoglobins exist in several oxidation states. Oxymyoglobin (Hb2+), the oxygen-carrying form, can be autoxidized to methemoglobin (Hb3+), a reaction that can be accelerated by low concentrations of hydrogen peroxide. In the presence of hydrogen peroxide, higher oxidation products of hemoglobin may also be formed, which include ferrihemoglobin (Hb4+), a strong oxidant that can be detected spectrophotometrically, and a transient, globin-associated radical that can only be detected by electron paramagnetic resonance spectroscopy (25, 40). Modification of hemoglobin structure, as is done during the manufacture of blood substitutes, can, in some instances, alter its tendency to form Hb4+ or extend the length of time that it remains in this oxidized state (2).

One widely studied modified hemoglobin is α-DBBF, a stroma-free human hemoglobin, which is stabilized by cross-linking its α-chains with bis(3,5-dibromosalicyl)fumarate (38). It has demonstrated an improved capacity to off-load oxygen at normal tissue oxygen tensions, but it has also exhibited altered redox activity in the presence of hydrogen peroxide in vitro, including a prolonged capacity to remain in the ferryl oxidation state after exposure to hydrogen peroxide (5).

Endothelial cells produce hydrogen peroxide under normal conditions and to a greater extent after anoxia or ischemia followed by reperfusion (24, 34, 49). Endothelial cells have been shown to be a major site of free...
radical production after ischemia-reperfusion (50), as well as a site of oxidative damage (45) and a source of hydrogen peroxide, lipid hydroperoxides, and nitric oxide (26, 36), which are known to react with hemoglobin. Endothelial cells have been used as a model to study potential oxidative mechanisms of toxicity of hemoglobin-based oxygen carriers (18). Modified oxyhemoglobins, including α-DBBF, were shown to be only mildly toxic in normal endothelial cells, whereas ferrylhemoglobin, produced by the reaction of oxyhemoglobin with hydrogen peroxide, induced rapid morphological changes and DNA fragmentation indicative of apoptosis (18).

With the use of a model of endothelial cells grown on microcarrier beads, it is possible to restrict both oxygen and media volume (hypoxia-volume restriction) by letting the cells settle in a test tube followed by reoxygenation-volume replacement (24). Changes in lactate accumulation, pH, calcium flux, hydrogen peroxide production, fatty acid release, and lipid peroxidation have been monitored over time in this model and were found to be dependent on the length of the period of hypoxia-volume restriction (24). This model is now being used to study potential mechanisms of toxicity of hemoglobin-based oxygen carriers during ischemia-reperfusion and to monitor hemoglobin-mediated oxidative reactions. We report, for the first time, the detection of ferrylhemoglobin in a time frame that corresponds closely with peroxide production and lipid peroxidation after hypoxia followed by reoxygenation in an endothelial cell culture.

METHODS

Cell culture model. Bovine aortic endothelial cells (BAEC) were isolated from a freshly slaughtered animal, subcloned, and used before passage 13 (24). The phenotype of these cells was confirmed by the expression of factor VIII antigen, by low-density lipoprotein uptake, and by morphological examination. BAEC were grown to confluence on suspended collagen-coated Cytodex 3 beads (Sigma, St. Louis, MO) in DMEM (GIBCO) containing 10% fetal bovine serum (GIBCO), penicillin-streptomycin-neomycin mix (Sigma), 25.0 mg/l heparin, 2 mM glutamate, and 12.5 mg/l ascorbate. Hydrated Cytodex 3 microcarrier beads (0.08 g) were added to 10⁶ cells in a culture dish, and BAEC were allowed to grow to confluence.

To imitate ischemia, 2 × 10⁶ cells on beads were pipetted into a conical centrifuge tube and allowed to settle, and the excess media were removed to a separate flask (hypoxia-volume restriction). The headspace of the centrifuge tube was flushed with N₂ containing 5% carbon dioxide and incubated at 37°C undisturbed for the specified period of time. To imitate reperfusion, the cells and beads were resuspended in their original oxygenated medium for 1 h. Hydrogen peroxide production was measured in cell medium (1 ml) diluted with modified Hanks’ balanced salt solution (2 ml), using a horse-radish peroxidase assay, which measures the reaction of the enzyme with hydrogen peroxide to oxidize p-hydroxyphenyl acetic acid (p-HPA) (34). The formation of a fluorescent complex with excitation at 323 nm and emission at 400 nm was measured using a Photon Technology International (PTI) Delta Scan (Brunswick, NJ). The assay was standardized with known quantities of hydrogen peroxide. In experiments in which hemoglobin was added to some samples in the normoxic medium, controls minus p-HPA were used to determine that hemoglobin did not interfere with the assay by directly cooxidizing p-HPA.

Measurement of cell survival (plating efficiency). Cell survival was determined after reoxygenation by measurements of 24-h plating efficiency (24). Replating efficiency was determined by pipetting aliquots of cell suspension onto 12-well plates containing fresh complete medium at 37°C followed by a 24-h incubation under standard culture conditions. The number of surviving, attached cells was determined by washing three times with PBS, trypsinizing the cultures, and counting aliquots of the cell suspension with a Coulter counter (Hileah, FL).

Glutathione concentration. Hemoglobin (0–400 μM) was incubated with 5 × 10⁶ cells for 3 h at 37°C. The cells were then washed, trypsinized, and incubated in DMEM with 40 μM monochlorobimane for 10 min (7). Fluorescence was recorded at 461 nm using a PTI Delta Scan, with excitation set at 380 nm, as the percentage present in controls.

Lipid peroxidation. Hypoxic cells were resuspended in their original oxygenated medium for 60 min after the final removal of the overlying medium and immediate extraction (24). The lipid extracts were analyzed at 234 nm for conjugated dienes, an indicator of lipid peroxidation (37).

Hemoglobin solution. α-DBBF hemoglobin was a gift from the Walter Reed Army Institute of Research (Washington, DC). α-DBBF is a human-derived stroma-free hemoglobin stabilized by cross-linking of the α-subunits with bis[3,5-dibromosalicyl]fumarate as previously described (38). The oxygen transport characteristics of α-DBBF are close to those of human blood with a PaO₂ of 28 mmHg. Other functional and oxidation reaction properties of this protein have been published previously (5). Hemoglobin concentrations were based on heme concentration, which was determined spectrally using a Hitachi U-2000 spectrophotometer. Multicomponent analysis was used to calculate the percentage of oxy, met, and ferryl forms of hemoglobin (48).

Oxidation and detection of ferrylhemoglobin in endothelial cells. Hypoxic cells were resuspended in their original oxygenated medium plus hemoglobin. Oxidation of hemoglobin was monitored spectrophotometrically (48), during the volume replacement period, in cells subjected to 0 or 3 h of hypoxia-volume restriction. Multicomponent analysis was used to calculate the percent of oxy, met, and ferryl forms of hemoglobin (48).

Ferrylhemoglobin was also detected by its reaction with sodium sulfide (Na₂S) as it was formed from hemoglobin in cell medium (9). Na₂S (2 mM) was added to the normoxic medium at 60 min and incubated for 30 min before spectrophotometric detection of sulfhemoglobin at 620 nm using a Perkin Elmer Lambda 18 dual-beam spectrophotometer (Norwalk, CT). The concentration of sulfhemoglobin was estimated using the extinction coefficient of sulfymyglobin (ε₆₂₀–640nm = 10.5 M⁻¹ cm⁻¹) (9).

Antioxidants. α-Tocopherol (100 μM) was added to BAEC during growth in 0.5% ethanol, and the excess was washed.
away with fresh medium before the initiation of hypoxia (24). Ascorbate (100 µM) or trolox (100 µM) was added during reoxygenation in the presence of 60 µM hemoglobin.

Statistical analyses. Data are expressed as means ± SE. Analyses were performed using ANOVA and Student’s paired t-test. *P < 0.05 was considered significant. All calculations were performed with JMP 3.2.1 (SAS Institute, Cary, NC).

RESULTS

Initial studies were done to establish dose-response curves for hypoxia and to confirm the presence of anaerobic metabolism. Lactate production was increased in BAEC during hypoxia, and its accumulation in cell medium was dependent on the duration of hypoxia (Fig. 1A). Hydrogen peroxide, a reduction product of superoxide, accumulated in cell medium after variable periods of hypoxia followed by 1 h of reoxygenation, and its production was also dependent on the duration of hypoxia (Fig. 1B).

Toxicity curves were established for α-DBBF hemoglobin administered during reoxygenation using an endothelial cell replating efficiency assay (Fig. 2). Survival was defined as the ability of endothelial cells to remain intact and attached to a culture dish for 24 h following treatment and was shown to be dependent on the duration of hypoxia (24). The addition of 60 or 100 µM hemoglobin during the reoxygenation period did not significantly affect the survival of cells. The addition of α-DBBF hemoglobin (200 µM) during reoxygenation increased the toxicity of both control cells and cells subjected to varying periods of hypoxia-reoxygenation (Fig. 2).

Lipid peroxidation is a measure of oxidative stress and sublethal toxicity. After variable periods of hypoxia, followed by 1 h of reoxygenation, lipid peroxidation was found to be correlated with the duration of treatment (Fig. 3). Addition of 60 µM α-DBBF hemoglobin, a concentration that was not found to significantly affect cell survival when added during the reoxygenation period, caused a significant increase in lipid peroxidation after ∼2 h of hypoxia followed by 1 h of

Fig. 1. A: lactate production by bovine aortic endothelial cells (BAEC) after hypoxia-reoxygenation. Cells were resuspended in 100 µl of PBS after specified periods of hypoxia. Lactate levels were measured immediately using a Sigma diagnostic kit. Content of lactate in the medium was measured as NADH production, catalyzed by lactic dehydrogenase, using lactate and excess NADH as substrates. Lactate concentration is expressed as means ± SE of 3 separate experiments (ANOVA). *Significantly different from control (P < 0.05). B: hydrogen peroxide production by BAEC after hypoxia-reoxygenation. Cells were resuspended in their original oxygenated medium for 1 h after specified periods of hypoxia. Hydrogen peroxide concentration, measured in cell medium using horseradish peroxidase assay, is expressed as means ± SE of 3 separate experiments (ANOVA). *Significantly different from control (P < 0.05).
reoxygenation. Lipid peroxidation after hypoxia was found to be a transient phenomenon with a maximum at 1–2 h of reoxygenation in this system (data not shown). Neither desferrioxamine (200 µM) nor EDTA (100 µM) affected the magnitude of this effect (data not shown).

Hydrogen peroxide accumulation in cell media, as shown in Fig. 1B, was decreased in both normal and hypoxic-reoxygenated cells by 60 µM hemoglobin (Fig. 4). This is consistent with the reported pseudoperoxidase activity of hemoglobin. Like true peroxidases, hemoglobin consumes hydrogen peroxide and organic peroxides as it cycles between oxidation states (31).

Glutathione depletion was not observed during the early stages of reoxygenation in this model (data not shown), possibly because of the relatively low levels of hydrogen peroxide produced at this early stage of reoxygenation. Three hours of incubation with hemoglobin, however, caused depletion in a concentration-dependent manner of intracellular glutathione (Fig. 5), an antioxidant that is involved in cellular defense against both hydrogen peroxide and lipid hydroperoxides (7).

Time-dependent changes in the oxidative state of hemoglobin were monitored in the presence of normal or hypoxic-reoxygenated cells. In these experiments, normal or hypoxic cells were incubated with 60 µM hemoglobin, and spectra were recorded in the visible and Soret regions (Fig. 6A). The oxidation of hemoglobin began immediately, as evidenced by the loss of absorption at the 577- and 541-nm bands typical of oxyhemoglobin, yielding a final spectrum of ferrihemoglobin at 24 h with a characteristic absorption at 630 nm. The disappearance of the ferrous (Fe²⁺) form is accompanied by a burst in the ferrylhemoglobin, as witnessed by the appearance of a characteristic peak at 545 nm and a flattened region between 600 and 700 nm (5), and possibly by other nonferric hemoglobin oxidation products. Figure 7 shows the results of a subse-
sequent multicomponent analysis in which the proportions of ferric (Fe$^{3+}$) and ferryl (Fe$^{4+}$) species only are plotted as a function of time. Only a slight reduction in the total amount of heme in solution, as calculated by the sum of the three hemoglobin oxidation forms, was observed throughout the incubation period.

There was also a steady rise in methemoglobin over a 24-h period of incubation. Methemoglobin concentrations increased twice as rapidly (10.7 vs. 5.6 µM/min) in hypoxic-reoxygenated vs. normal cells and remained higher in those cells until nearly 24 h later, when most hemoglobin was in the methemoglobin state in both normal and reoxygenated cells.

The presence of ferrylhemoglobin was confirmed via its derivitization to sulfhemoglobin using Na$_2$S (9). Na$_2$S was added to the culture medium at 60 min or at 20 h of reoxygenation, and sulfhemoglobin formed via reaction of ferrylhemoglobin with Na$_2$S was measured at 620 nm (Fig. 6B). No changes in endothelial morphology were observed during this 30-min terminal assay. Experiments performed using medium removed from endothelial cells and added to Na$_2$S yielded identical results (data not shown). Sulfhemoglobin formation was greater in hypoxic-reoxygenated cells at 60 min of treatment than in control cells. If Na$_2$S was added at 20 h of reoxygenation, when most of the hemoglobin has returned to its ferric (Hb$^{3+}$) state, no sulfhemoglobin was formed in either control or treated cells. Addition of the antioxidants trolox (100 µM) or ascorbate (100 µM) did not significantly affect sulfhemoglobin production under these conditions (Table 1). However, addition of 100 µM α-tocopherol to the growth medium of BAEC, followed by washing away any excess α-tocopherol before initiation of reoxygenation or normal cell incubation with hemoglobin, significantly increased sulfhemoglobin formation after 60 min of reoxygenation or 60 min of incubation with normal BAEC (Table 1).

**DISCUSSION**

Recently reported animal and human studies have shown that a number of organs can be the target of toxicity when chemically or genetically altered hemoglobins are administered (1, 15, 20, 46). Endothelial cells, because of their primary position in the vasculature, are considered to be a target of damage after hemoglobin administration (8, 27, 29), as well as after ischemia-reperfusion (45, 30). Endothelial cells are also a source of a variety of oxidants, under normal and ischemic conditions (24, 34, 49). Because the level of these oxidants may vary during changes in physiological
pressed as percentage of sham-treated controls; these mechanisms.

endothelial cells (24). were also observed in this model using rabbit aortic period. These results are consistent with data obtained from ischemic tissue in other mod-

The reported toxicities of cross-linked hemoglobin (15), which are also more subtle than death of exposed vascular cells, may involve inflammatory or signaling processes by one of these mechanisms.

One mechanism of lipid peroxidation by hemoglobin has been shown to involve the highly reactive ferryl oxidation state, which has been shown to abstract an allylic hydrogen from unsaturated fatty acids (21). Another mechanism may involve heme loss and subsequent release of iron. Because neither desferrioxamine nor EDTA exhibited an effect on conjugated diene formation in this system, it was assumed that heme iron loss from the cross-linked αDBBF under our mild experimental conditions was negligible. This is not surprising, since cross-linking of hemoglobin with the reagent bis(3,5-dibromosalicyl)fumurate tends to stabilize the hemoglobin molecule and to minimize heme loss (44).

Both ferrylhemoglobin, which can be detected by optical spectroscopy (48), and a globin-associated radical, which can be detected by electron paramagnetic resonance (25), are known to be formed via the reaction of oxy- and methemoglobin with hydrogen peroxide. In vitro, the concentration of hydrogen peroxide that is necessary to produce similar changes is 2–10 times the concentration of hemoglobin (2). Under our experimental conditions, however, no hydrogen peroxide was added to the media except that produced by cells themselves, <10 µM (Fig. 2). This is therefore an example of a biological oxidation of hemoglobin that mimics the more familiar reactions of hemoglobin seen in simpler mixtures (5, 11, 18). Recent studies suggest a mechanism composed of three steps that account for the oxidation of hemoproteins by peroxide: 1) initial oxidation of oxy- to ferrylheme, 2) autoconversion of ferryl intermediate to ferricheme, and 3) reaction of ferricheme with additional peroxide to regenerate the ferryl intermediate, creating a pseudoperoxidase catalytic cycle (3).

It is also possible that lipid peroxidation, which is a free radical chain reaction, provides both the target of hemoglobin oxidation and an alternate source of electrons, lipid hydroperoxides (13), which can cause cysc oxidation and reduction of hemoglobin. Although lipid peroxidation can be terminated normally by reaction with an antioxidant such as α-tocopherol (21) or through destruction of hydroperoxides by glutathione peroxidase (42), the presence of hemoglobin may amplify the level of peroxidation that takes place before termination occurs (21). In these experiments, α-tocopherol, which has been shown to localize to the cell membrane (24), was ineffective in preventing hemoglobin oxidation (Table 1).

Antioxidants administered at various times during ischemia or reperfusion in vivo have variable effectiveness in reducing reperfusion injury, depending on a multitude of conditions, including the duration of ischemia, the age of the animal, and the model studied (for review see Reimer and Jennings (35)). In addition, traditional “antioxidants,” depending on their oxidation potential in relation to other reactants, may behave as oxidants (14), as has been demonstrated for α-tocopherol in low-density lipoprotein (43) and ascorbate or glutathione in the presence of oxyhemoglobin (19). In general, the presence of a reduct active hemoglobin complicates a tissue model, which already includes fluctuating levels of superoxide, hydrogen peroxide, organic peroxides, and nitric oxide. Hemoglobin con-

<table>
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<th>Additions</th>
<th>n</th>
<th>Normal Cells, % of Control (µM)</th>
<th>Hypoxic Cells, % of Control (µM)</th>
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<tr>
<td>None</td>
<td>19</td>
<td>100 (2.9)</td>
<td>107.6 ± 1.9 (3.1 ± 0.06)*</td>
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<tr>
<td>Ascorbate</td>
<td>16</td>
<td>96.0 ± 2.0 (2.8 ± 0.06)</td>
<td>105.7 ± 4.2 (3.1 ± 0.12)</td>
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<tr>
<td>Trolox (100 µM)</td>
<td>16</td>
<td>98.3 ± 1.8 (2.9 ± 0.05)</td>
<td>110.5 ± 4.0 (3.2 ± 0.12)*</td>
</tr>
<tr>
<td>α-Tocopherol (100 µM)</td>
<td>3</td>
<td>114.9 ± 4.3 (3.3 ± 0.12)*</td>
<td>141.1 ± 11.2 (4.1 ± 0.32)†</td>
</tr>
</tbody>
</table>

Values are means ± SE sulfhemoglobin concentration (µM) expressed as percentage of sham-treated controls; n = no. of experiments. α-Tocopherol, ascorbate, or trolox was added as described in METHODS. Hypoxic or control cells were resuspended in their original oxygenated medium, plus 60 µM hemoglobin, with antioxidant or no addition. Na₂S (2 mM) was added at 1 h of perfusion in both normal and hypoxic-reoxygenated cells and allowed to incubate for 30 min before quantitation at 620 nm. *Significantly different from normal cells, no addition; †significantly different from hypoxic cells, no addition (P ≤ 0.05).
Ferrylhemoglobin formation after hypoxia-reoxygenation

Bernard J. Adams

The opinions and assertions contained herein are the scientific policy of the United States Food and Drug Administration.

We thank Francine Wood for technical assistance.

The references are as follows:


