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Forum Review

Blood Substitutes and Redox Responses in the Microcirculation

ANN L. BALDWIN

ABSTRACT

Transfusion of hemoglobin-based blood substitutes, designed for their plasma expansion and oxygen transport capabilities, has resulted in some major problems, such as organ dysfunction, during clinical trials. Experimental evidence demonstrates that these hemoglobins damage tissue by producing highly reactive oxygen species. Although cell-free hemoglobin may present a low risk to people with normal redox status, patients who are sick and have a poor antioxidant status may be at risk. Oxidative damage is particularly dangerous in the microcirculation because excess leakage of plasma components into the interstitium will disturb the fluid balance between blood and tissue and alter the kinetics of delivery of intravascularly injected drugs, and endogenous enzymes and hormones, to various tissues. In this review, the redox chemistry of hemoglobin-based blood substitutes is briefly described, and their effects on cultured endothelial cells, and on the exchange properties of the microvasculature, are discussed. Taking into account the possible mechanisms by which oxidative damage can occur, various methods to reduce the deleterious effects of blood substitutes *in vivo* are evaluated. Finally, several possible cell signaling pathways that are triggered in endothelial cells, in response to modified hemoglobins, are considered in terms of protecting microvascular function. *Antioxid. Redox Signal.* 6, 1019–1030.

INTRODUCTION

CELL-FREE HEMOGLOBIN Hb-based oxygen carriers, such as diaspirin cross-linked Hb (DBBF-Hb), glutaraldehyde-polymerized Hb (PolyHbBv; Oxyglobin™), and O-raffinose-polymerized Hb (O-R-PolyHb; Hemolink™), have been proposed as blood substitutes for transfusions due to their plasma expansion and oxygen transport capabilities. Apart from their use after accidents or major surgery, such substitutes could also be used to alleviate anemia in patients with hematocrits too high to qualify for blood transfusions. In fact, PolyHbBv (Oxyglobin) is currently FDA-approved for veterinary use in the U.S.A., and its human counterpart has recently been approved for clinical use in humans in South Africa. Hb-based blood substitutes have the added advantages that they can be easily purified, stored for relatively long periods of time, and used in patients of all blood types.

However, a number of largely unresolved problems were found during preclinical trials and development of some of these Hb-based substitutes. These include cardiovascular/hemodynamic effects, gastrointestinal changes, immune cell activation, coagulation changes, oxidative stress, and decreased host resistance to overwhelming infection (41, 80). Preclinical studies reported the detection of myocardial lesions in a number of animal models infused with the Baxter Health Care Inc. product, diaspirin cross-linked Hb (DCLHb). These lesions were characterized by a mild to moderate focal-to-multifocal myocardial degeneration and/or necrosis in a highly vascularized portion of the myocardium (18). Baxter has recently terminated its clinical development of this product due to increased fatalities in the test group (72). A dose-response study performed on dogs by Biopure Corp. has shown that Oxyglobin increases arterial oxygen content in the face of normovolemic anemia and produces transient clinical signs (skin discoloration, discolored stools, nausea, vomiting). In addition, histopathology of Oxyglobin administration includes activation of tissue macrophages in multiple organs. Hemolink has recently been withdrawn from Phase III clini-

Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ.

cal trials in cardiac bypass grafting because it produced adverse cardiac events. In addition, numerous animal studies have demonstrated that the administration of extracellular Hb derivatives may lead to a variety of undesirable side effects (16, 30, 69, 88).

There is some experimental evidence to support the idea that modified Hbs, injected *in vivo*, can cause tissue damage and organ dysfunction by producing highly reactive oxygen species (ROS) (5, 41, 76). ROS, such as superoxide anion radical ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2), are by-products of autoxidation. Some Hb-based blood substitutes have been shown to oxidize more readily than Hb in red blood cells in response to chemical modifications aimed at lowering oxygen affinity (95).

REDOX CHEMISTRY OF Hb-BASED BLOOD SUBSTITUTES

Fenton reaction

In the Hb molecule, the hemes are normally bound in pockets from which water is largely excluded. Conformational changes that open up the heme pockets and allow greater access of water and small anions favor the conversion of the heme iron to the ferric or "met" state. The reaction (autoxidation), which also produces $O_2^{\bullet -}$, occurs spontaneously in modified Hbs. A previous study showed that 72 h after infusion of glutaraldehyde-polymerized bovine Hb into an animal, almost 40% of circulating Hb is in the met form (50). In guinea pigs, it was shown that methemoglobin (metHb) formation increased linearly up to a plateau of 30–40% at 12 h following infusion of a Hb conjugated to carboxylate dextran (28). It has been estimated that metHb levels greater than 10% significantly decrease the ability of Hb to deliver oxygen to tissues (51). $O_2^{\bullet -}$ is also produced from the respiratory burst of phagocytes that are activated by Hb; it then undergoes the dismutation reaction, in the presence of superoxide dismutase (SOD), which is contained within the cells, with consequent production of H_2O_2. The H_2O_2 is normally decomposed by its reaction with catalase or with glutathione peroxidase. However, these enzymes are inhibited by $O_2^{\bullet -}$ (15). The metHb then reacts with H_2O_2 to release free iron and free heme (70), which decomposes H_2O_2 to hydroxyl radical ($OH^{\bullet}$) and hydroxyl ion (OH^-) by the Fenton reaction (58):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Intact Hb does not catalyze $OH^{\bullet}$ production (39). It is the loosely bound iron released from heme protein that is able to promote $OH^{\bullet}$ formation. The Fe^{3+} that is formed can react with $O_2^{\bullet -}$ to produce more Fe^{2+}:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

This Fe^{2+} then undergoes the Fenton reaction to produce more $OH^{\bullet}$. Thus, the combination of H_2O_2 and Fe^{2+} represents a highly toxic potential that contributes to peroxidation in Hb-containing systems. One $OH^{\bullet}$ can result in the conversion of many hundred fatty acid side chains into lipid hydroperoxides (38). The $OH^{\bullet}$ can also be formed by another mechanism. When metHb is produced from oxyhemoglobin (oxyHb), $O_2^{\bullet -}$ is released (58, 91), which can then react with H_2O_2 to $OH^{\bullet}$ by the Haber–Weiss reaction. This reaction is catalyzed by the presence of free iron:

$$O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$$

However, it should be emphasized that Fenton chemistry with cell-free Hb, and the resulting Haber–Weiss reaction, are largely speculative *in vivo* because $OH^{\bullet}$ *per se* has not been identified in tissue and correlated with cellular damage. Iron is also capable of catalyzing the production of alkoxy and peroxy radicals from lipid peroxides, and the production of these reactive species could contribute to tissue injury (36).

In normal blood, the reservoir of heme iron is compartmentalized within the erythrocyte, which limits its ability to act as a catalyst. To keep metHb levels in the erythrocytes below 1%, there are enzymatic pathways in place that reduce the ferric iron back to its normal ferrous state or inhibit the oxidation process. The major reducing pathway is the cytochrome-*b*5-methemoglobin reductase system. This enzyme system is NADH-dependent and is responsible for >95% of the erythrocytes' reducing capacity. The other enzymatic pathway that can reduce metHb is NADPH-methemoglobin reductase. This accounts for <5% of the normal erythrocyte reducing capacity. Erythrocytes also contain ascorbate, which consumes oxygen free radicals in the plasma and protects the red cell membrane from lipid peroxidation. In addition, erythrocytes are rich in antioxidant enzymes, such as SOD and catalase, which inactivate the $O_2^{\bullet -}$ and H_2O_2, respectively (91). These enzymes are in close proximity to Hb within the erythrocytes, and although Hb can release catalytic iron when exposed to oxidant stress, injury is limited because the enzymes react with the resultant ROS before they reach the cell membrane. Other authors (73) have stated that free Hb, even in small amounts, accelerates the peroxidation of arachidonic acid and lipids within red cell membranes. Therefore, it is likely that Hb will promote the oxidation and destruction of a variety of biomolecules, quite likely magnifying cell injury and death in areas of active inflammation. It has been postulated that plasma contains concentrations of ascorbic acid and glutathione that may serve as reducing agents when an acellular Hb-based blood substitute is used in plasma under transfusion conditions (27). However, a favorable reduction potential difference must exist between the reducing agent and the blood substitute (*i.e.*, the blood substitute should have a positive potential with respect to the plasma reducing agent). Experiments performed using the naturally polymerized acellular *Lumbricus* Hb showed that this molecule exhibited a positive reduction potential (versus Ag/AgCl), whereas PolyHbBv (FDA-approved for canine anemia) showed a negative potential.

Ferryl Hb

It has been postulated that the excess H_2O_2 reacts with ferrous Hb and metHb, *in vivo*, to initiate further oxidation cycles resulting in the formation of highly reactive ferryl Hb (78). Specifically, when ferrous Hb ($HbFe^{2+}$) reacts with H_2O_2, it donates two electrons to the H_2O_2, to form ferryl Hb ($HbFe^{4+}$) and OH^-. In ferryl Hb, the iron center is at a higher oxidation state. Despite its transient nature, Fe^{4+} heme can

peroxidize lipids, degrade carbohydrates, and modify proteins (34). The interaction of ferryl Hb with H_2O_2 results in the formation of rhombic heme, which is considered to be one of the best measures of the toxicity of a blood substitute (63). The rhombic heme in which the geometry of the iron is distorted, due, in some instances, to the chemical modification of the protein, then initiates a cascade of oxidative side reactions resulting in the formation of free iron.

Reactions involving nitric oxide ($NO^{\bullet}$)

NO, which is constitutively produced by endothelial cells, and is produced by macrophages when they are activated, can have either a protective or a deleterious effect on tissue in the presence of Hb. The deleterious effect can arise in two ways. One way is from its interaction with $O_2^{\bullet -}$ to form peroxynitrite anion ($ONOO^-$):

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^-$$

The amount of $O_2^{\bullet -}$ present determines the amount of $ONOO^-$ that is formed. The concentration of $O_2^{\bullet -}$ in the circulation is kept extremely low by a high concentration of SOD. A balance between $NO^{\bullet}$ and $O_2^{\bullet -}$ is therefore maintained under physiological conditions, and the reaction between the two to form $ONOO^-$ is limited. When the circulation is perfused with a modified Hb, the Hb reacts very quickly with the $NO^{\bullet}$, and the resulting reduction in $NO^{\bullet}$ drives the formation of $ONOO^-$. On protonation, $ONOO^-$ decomposes to the highly reactive $OH^{\bullet}$. The other way that $NO^{\bullet}$ can have a deleterious effect is by its reaction with oxyHb to form metHb:

$$Hb(Fe\text{-}O_2) + NO^{\bullet} \rightarrow Hb(Fe^{3+}) + NO_3^-$$

MetHb is unstable, and when it reacts with H_2O_2, the heme may detach from the molecule. Once released from metHb, the heme may easily penetrate the endothelial membrane and activate heme oxygenase, which causes heme breakdown and release of free iron. Free iron can then catalyze production of $OH^{\bullet}$.

The protective effect of $NO^{\bullet}$ can arise in three ways. Firstly, it can combine with oxoferrylHb, $Hb(Fe^{4+}{=}O)$, which is a powerful oxidant produced by the reaction of Hb with H_2O_2:

$$Fe^{2+}Hb + H_2O_2 \rightarrow Hb(Fe^{4+}{=}O)$$

This oxidant causes tissue injury, and so by scavenging this agent, $NO^{\bullet}$ protects the tissue:

$$Hb(Fe^{4+}{=}O) + NO^{\bullet} \rightarrow Hb(Fe^{3+}) + NO_2^-$$

Secondly, $NO^{\bullet}$ can scavenge the damaging oxidant $ONOO^-$:

$$ONOO^- + NO^{\bullet} \rightarrow NO_2 + NO_2^-$$

It is obvious from these reactions that the role played by $NO^{\bullet}$, whether it acts as a prooxidant or as an antioxidant, depends on the relative amounts of $NO^{\bullet}$ and ROS that are present. The fact that Hb has a high affinity for $NO^{\bullet}$ has the potential for upsetting an existing balance between $NO^{\bullet}$ and ROS. If $NO^{\bullet}$ is scavenged by Hb and ROS, the resulting depletion of $NO^{\bullet}$ can reduce concentrations of intracellular *c* guanosine 3',5'-cyclic monophosphate (cGMP) (60). In cultured endothelial cells, it has been shown that a reduction in cGMP leads to contraction of actomyosin filaments and possible cell contraction to produce a widening of intracellular clefts (66). This effect of lack of $NO^{\bullet}$ on cell junctions is supported by previous experiments showing that microvascular perfusion with nitric oxide synthase (NOS) inhibitors caused venular leakage (7). Thus, the third way in which $NO^{\bullet}$ protects the endothelium is to ensure junctional integrity by maintaining levels of cGMP.

EFFECTS OF Hb-BASED BLOOD SUBSTITUTES ON ENDOTHELIAL CELLS

The endothelial cells lining the vasculature are in direct contact with infused Hb-based blood substitutes. They are particularly sensitive targets that, when damaged, will disrupt vessel permeability and tone. Coculture of Hb with endothelial cells generates an oxidative stress to which the cells respond by synthesizing protective enzymes, or by dying (14). Under conditions of mild oxidative stress, DBBF-Hb can induce growth arrest in cultured endothelial cells, ultimately leading to apoptotic and necrotic cell death (24). On the other hand, normoxic cells exposed to DBBF-Hb expressed augmented heme oxygenase (HO) activity (61, 62). HO is a heme-degrading enzyme that is responsible for catabolizing the heme moiety of senescent Hb, and may constitute a protective response against oxidative stress in endothelial cells. In these experiments, the iron chelator, deferoxamine (DEF), reduced the HO activity, indicating that it was related to heme loss by metHb, or iron-mediated oxygen radical formation (61). In another study, prolonged exposure of endothelial cells to metHb made them resistant to oxidant-mediated injury and to the accumulation of lipid peroxidation products (90). Thus, cultured endothelial cells appear to be able to protect themselves against iron-mediated oxidative stress.

One concern relevant to the use of Hbs as blood substitutes is that *in vivo* production of ferryl Hb may occur under conditions of ischemia and reperfusion in patients with a diminished ability to control oxidative reactions of Hb. The ferryl intermediate (Fe^{4+}) has been detected in the medium of endothelial cells grown on microcarrier beads, subjected to cycles of hypoxia followed by "reperfusion" with DCLHb (26). The presence of the Fe^{4+} correlated well with an increase in lipid peroxidation. Results from these studies also reveal that $NO^{\bullet}$ (produced from exogenous L-arginine) serves as an antioxidant in controlling the levels of ferryl HB, and thus may determine the extent of tissue injury. Another study showed that the formation of DBBF-$HbFe^{4+}$ was correlated to cytotoxicity in an endothelial cell culture model of ischemia–reperfusion and in cells that lack their antioxidative mechanisms such as glutathione (23). Other authors who exposed human endothelial cells to various bovine Hb solutions also came to the conclusion that cell membrane peroxidation was caused by the formation of ferryl Hb (77). By using this system, it was found that release of free iron from Hb molecules following exposure to H_2O_2 was very limited (94). It was concluded that the oxidation of heme iron in $HbFe^{2+}O_2$ probably results in formation of a ferryl Hb species that directly promotes membrane oxidation and peroxidation.

However, although Hb-induced oxidative stress may involve production of both $OH^{\bullet}$ and ferryl Hb species, the actual extent of the oxidative stress depends on the specific way in which the Hb has been chemically modified as demonstrated by a study using human coronary artery endothelial cells (79). In these experiments, the activation of nuclear transcription factor, NF-κB, in the endothelial cells was used as a measure of oxidative stress. Hb that was polymerized with glutaraldehyde produced a rapid translocation of NF-κB to the nucleus, indicating activation, but Hb cross-linked intramolecularly with *o*-adenosine triphosphate and intermolecularly with *O*-adenosine, and combined with reduced glutathione, produced very little activation.

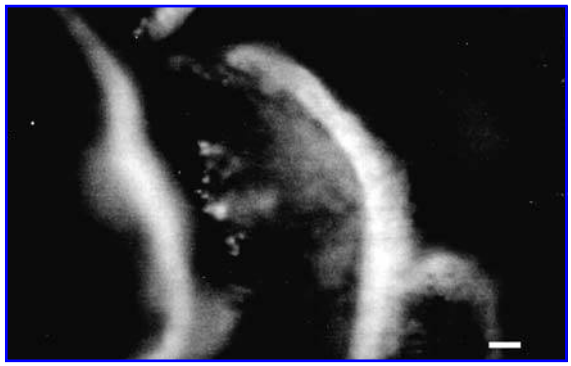

FIG. 1. Photomicrograph of mucosal surface of rat intestine after intravascular injection of 5 ml of DBBF-Hb (10 mg/ml), and suffusion with dihydrorhodamine 123 that fluoresces only in the presence of ROS. Narrow regions of high fluorescence correspond to the epithelium. Scale bar = 25 μm.

RESPONSES OF THE MICROCIRCULATION TO Hb-BASED BLOOD SUBSTITUTES

Increased permeability

It is well known that excess ROS oxidize lipids of the cell membranes. Lipid peroxidation damage of membrane components is thought to play an important role in increasing capillary permeability (33). When Hb-based blood substitutes are injected into the circulation, excess ROS, such as H_2O_2, $O_2^{\bullet -}$, $OH^{\bullet}$, and Fe^{4+}, will form if the Hb increases its oxidation state. As H_2O_2 can easily diffuse across cell membranes (40), and $O_2^{\bullet -}$ can traverse membranes via the chloride anion channel (86), it is likely that these ROS will leave the microvasculature and gain access to other cells in the tissue. This action can have deleterious effects. For example, if the ROS reach mast cells in the interstitium, they will cause the mast cells to degranulate (48) and release a selection of inflammatory mediators, including histamine. Many studies show that histamine increases microvascular permeability (4, 31, 42, 89, 93). In addition, degranulating mast cells release eosinophil and neutrophil activating factors, causing these cells to release more ROS. Surprisingly, only two studies have been performed to determine whether injection of a Hb-based blood substitute into the circulation significantly increases the accumulation of ROS in surrounding tissue (5, 10). In these studies, a bolus injection of DBBF-Hb into the circulation of rats was found to rapidly produce excess ROS, as detected by fluorescence of dihydrorhodamine 123, in the intestinal mucosa (Fig. 1), but results with PolyHbBv were no different from controls that were given a bolus injection of saline. However, it is not even necessary for Hb to be present in a transfusion fluid for excess ROS to form. Endothelial cells produce ROS, such as H_2O_2 and $O_2^{\bullet -}$ (20), partly through autoxidations of mitochondrial electron transport chain constituents (29). In addition, it has been shown that endothelial cells release ROS in response to oxidative stress (96). Usually, these ROS do not produce tissue damage because they are balanced by intracellular antioxidants, such as SOD and catalase, which reside within endothelial cells (44) and red blood cells (91). However, when resuscitation fluids lacking ROS scavengers, such as pasteurized plasma protein solution or 0.9% saline, are injected into the circulation, peroxyl radical trapping capacity is decreased (59). This does not occur when fresh plasma or donor red blood cells are used. It has been suggested that the decreased antioxidant capacity is a result of dilution of the blood by the resuscitation fluids. If free iron is present, as may be the case if a modified Hb is used, then it may react with the ROS by the Fenton reaction to produce $OH^{\bullet}$, as described previously. Ferryl heme iron (Fe^{4+}), the most reactive intermediate of Hb, can also form (20).

Although it is known that formation of the unstable form of Hb, metHb, proceeds rapidly after injection of modified Hbs into the circulation, and that ROS increase microvascular permeability, very few studies have been performed to determine whether injection of Hb-based blood substitutes increase microvascular permeability. Previously, it was shown that bolus injection of DBBF-Hb, in rats, increased venular leakage to bovine serum albumin (BSA) (Fig. 2), produced mast cell degranulation in the rat mesentery (3), and also caused detachment of intestinal epithelial cells from each other and from the basement membrane (2, 6) (Fig. 3). Such changes are characteristic of an inflammatory response (4, 92). This is not surprising because mast cell degranulation, as observed after injection of DBBF-Hb, causes release of histamine and other inflammatory mediators, which then damage cell membranes. It is disad-

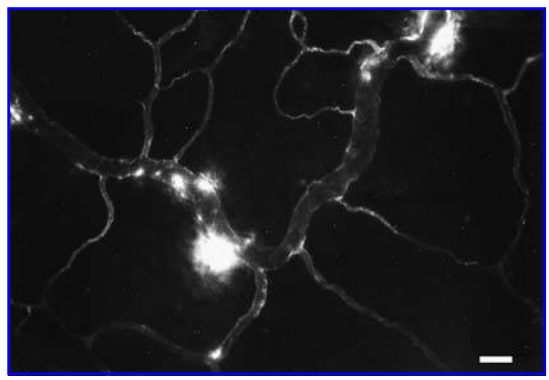

FIG. 2. Photomicrograph of part of a microcirculatory network in the rat mesentery after infusion for 10 min with DBBF-Hb (10 mg/ml) followed by fluorescently labeled BSA for 1 min. The fluorescent tracer can be seen leaking out of regions of venules that are damaged. Scale bar = 25 μm.

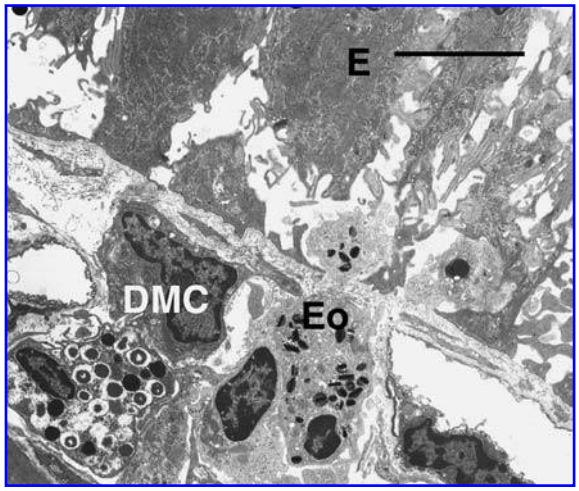

FIG. 3. Electron micrograph of a section through the intestinal mucosa of a rat, previously injected intravascularly with 5 ml of DBBF-Hb. The epithelial cells (E) have partly separated from each other and from the basement membrane. A degranulated mast cell (DMC) is visible, and an eosinophil (Eo) is migrating through the epithelial basement membrane. Scale bar = 5 μm.

vantageous for a potential blood substitute to cause microvascular leakage, because the substitute itself will rapidly leave the circulation, and in addition, alterations in transvascular exchange of plasma proteins will disturb the fluid balance between blood and tissue. Increased microvascular leakage also changes the kinetics of delivery of intravascularly injected drugs, and of endogenous enzymes and hormones, to various tissues. In cases in which microvascular exchange is compromised by the condition itself, such as traumatic injury, it is important that transfusion with a blood substitute does not worsen the existing microvascular leakage.

In another study, it was shown that mesenteric microvascular leakage to BSA, mesenteric mast cell degranulation, and epithelial disruption were significantly lower in animals treated with PolyHbBv compared with DBBF-Hb (10). However, even in animals treated with PolyHbBv, the average number of leaks per unit length of venule was still significantly greater than for controls that were perfused with HEPES-buffered saline with 0.5% BSA. It could be argued that PolyHbBv did not cause as much damage as DBBF-Hb because of its size; PolyHbBv contains a heterogeneous mixture of polymeric (95%) and nonpolymeric (5%) species ranging in size from 32 to 500 kDa (68), whereas DBBF-Hb is not polymerized and has a molecular mass of 64 kDa. Therefore, it might be expected that PolyHbBv would not extravasate as easily as DBBF-Hb. However, it is unlikely that the molecular size affects the damage caused by the modified Hb. Hb bound to polyethylene glycol (PEG-Hb), which has a molecular Stokes–Einstein radius of 250 Å, as opposed to 50 Å for DBBF-Hb, was shown to extravasate from the mesenteric microcirculation within minutes after injection (2). It is more likely that PolyHbBv causes less damage than DBBF-Hb because PolyHbBv is more resistant to irreversible oxidative processes, which include the formation of long-lived ferryl species and subsequent heme degradation and iron loss (63).

There is some evidence to demonstrate that microvessels are sensitive to iron-induced oxidative damage, and it has been hypothesized that iron-catalyzed oxidant production is important in the genesis of microvascular injury after ischemia–reperfusion. A previous experiment (81) showed that the iron chelator, DEF, and the iron-binding protein, apotransferritin, both significantly attenuated the increase in microvascular permeability produced by ischemia and reperfusion of the rat hindquarter. With regard to Hb-based blood substitutes, use of an iron chelator was found to significantly reduce microvascular leakage caused by perfusion with DBBF-Hb (8). These experiments indicate that release of free iron from the Hb, and subsequent formation of ROS, may produce microvascular damage *in vivo*, even though release of free iron in response to H_2O_2 in an endothelial cell culture environment has been found to be limited (94). Additional evidence to support the idea that Hb mediates microvascular damage by an iron-dependent mechanism is provided by a study in which isolated perfused rat lungs were subjected to oxidant injury using *tert*-butyl hydroperoxide (75). In these experiments, it was found that addition of Hb to the perfusate increased the microvascular damage, and that *tert*-butyl hydroperoxide also caused Hb to release large quantities of free iron *in vitro.* The iron binding protein, apotransferrin, prevented the Hb-associated increase in capillary permeability.

In contrast to the experiments previously described demonstrating that perfusion with modified Hb increases microvascular permeability, another study showed that hypervolemic infusion and isovolemic exchange transfusion (50%) of hamsters, with DCLHb, did not enhance microvascular leukocyte–endothelium interaction or endothelial permeability (64). However, in these experiments, the fluorescent tracer molecule that was used to assess microvascular leakage, fluorescein isothiocyanate (FITC)-dextran, had a molecular weight of 150,000, whereas the tracers used in the other studies were smaller (10, 75, 81). Thus, it is possible that FITC-dextran was too large to extravasate through the endothelial gaps produced by Hb. However, a more likely explanation for the discrepancy concerns the method by which microvascular leakage was assessed. In this study, the extravasated FITC-dextran was quantified as the quotient of average fluorescence intensity outside the vessel versus that inside the vessel segment. In other studies, it was demonstrated that macromolecular extravasation from the microcirculation in response to modified Hbs and to inflammatory mediators occurs in very discrete sites along the venules (4, 10, 31, 89, 93). Therefore, it is possible that these small discrete areas of venular leakage were missed in the measurements of the average fluorescence intensity outside each vessel segment.

METHODS TO REDUCE OXIDATIVE DELETERIOUS EFFECTS OF BLOOD SUBSTITUTES *IN VIVO*

The diagram in Fig. 4 depicts the various mechanisms by which modified Hbs can cause tissue damage. From this diagram, it is clear that there are at least three different types of agents that can be administered to reduce endothelial dam-

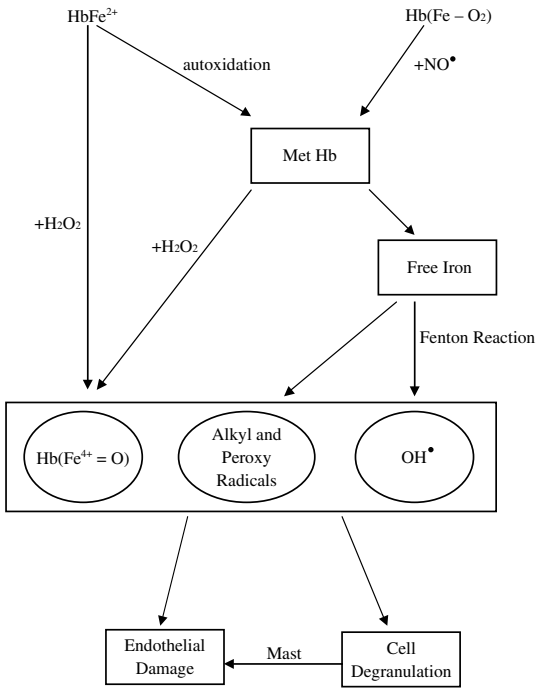

FIG. 4. Possible mechanisms by which modified Hbs can cause microvascular damage.

age: (1) antioxidants, (2) iron chelators, and (3) mast cell stabilizers. It is also possible that NO• donors could help reduce microvascular injury. Although, as shown in Fig. 4, NO• reacts with Hb(Fe-O_2) to produce metHb, it can also stabilize the endothelium by increasing production of cGMP.

Antioxidants

Several methods are currently being developed to effectively scavenge ROS. One approach is to incorporate SOD and catalase into cross-linked Hb (25, 71). This technique ensures that the free radical scavengers are in close contact with the source of the ROS, the Hb, and also effectively addresses the problem that SOD has a short half-life in blood (10–40 min). An alternative way of increasing the half-lives of SOD and catalase is to bind them to PEG. PEG-SOD has a half-life of several days, and PEG linkage increases the half-life of catalase from 2 to 50 h (19). Another antioxidant that has been investigated for use with blood substitutes is nitroxide. Nitroxides are able to catalytically dismutate $O_2^{\bullet -}$, and thus can act as potent antioxidants. They exist in three oxidation states: (1) the nitroxide, (2) the oxoammonium cation, and (3) the reduced nitroxide (hydroxylamine). The nitroxide is oxidized to (2) by $O_2^{\bullet -}$, and a second $O_2^{\bullet -}$ can reduce (2) back to (1) so that the cycle can perpetuate generation of molecular oxygen from $O_2^{\bullet -}$ (46). However, some nitroxides are cleared rapidly from the circulation, and most are cleared within a few hours after intravenous infusion. In addition, nitroxides vary greatly in their ability to function as antioxidants, more specifically as SOD- and catalase-like mimics, in the presence of heme-protein-associated Fe^{3+} and Fe^{4+}. For these reasons, a polynitroxylated Hb-based carrier has been developed in which nitroxide molecules are covalently bound to Hb so that the circulatory half-life of the nitroxide molecules is greatly increased (74). At this time, there is no published record of the ability of Hbs, conjugated with antioxidants, to minimize oxidative tissue damage.

Recently, the protective effect of selenium on Hb-mediated lipid peroxidation has been investigated for use with blood substitutes (78). The rationale for using selenium is that it is a very powerful antioxidant. Selenium is thought to act as an antioxidant in the body because it is a component of the enzyme, glutathione peroxidase, which catalyzes removal of H_2O_2 (84). Hb's oxidative reactions are very complex, and so total protection cannot be achieved using OH• scavengers exclusively. Chen and Lin (21) gave rats selenium oxide daily for 14 days and observed increases in oxyHb/ferrous and decreases in metHb in the blood. As they also saw increased concentrations of glutathione peroxidase activity in the blood of rats fed SeO_2, they concluded that this enzyme must be responsible for the change in the oxy:met ratio of the Hb. Simoni *et al*. (78) demonstrated that treatment with Na_2SeO_3 was very effective in the prevention of oxidative damage induced by Hb. However, they only found slightly increased concentrations of glutathione peroxidase in the livers of the selenium-supplemented rats. In a more recent study, it was shown that Na_2SeO_3 was effective in significantly reducing the intestinal epithelial damage (5) and microvascular leakage (11) associated with bolus injection of DBBF-Hb in rats. *In vitro*, Na_2SeO_3 reduced the oxidation rate of DBBF-Hb while in the presence of oxidants (11). Thus, it appears that Na_2SeO_3 moderates Hb-induced damage at least partly through its interactions with the Hb, and that there is no need for glutathione peroxidase to be involved in the process. Selenium can directly alter the redox state of modified Hbs such that the oxy:met ratio is increased within a very short time period. A later study showed that sodium selenite did not significantly affect leak number or area, in preparations perfused with PolyHbBv, and only reduced leak number in preparations perfused with O-R-PolyHb (13). *In vitro*, Na_2SeO_3 significantly reduced the oxidation rate of DBBF-Hb in the presence of oxidants, had little effect on PolyHbBv, and increased the oxidation rate of O-R-PolyHb. These results suggest that Na_2SeO_3 moderates Hb-induced damage, at least partly, through its redox interactions with the heme sites in the Hb molecules, and that accessibility of the heme site to Na_2SeO_3 governs those interactions. These experiments show that selenium compounds will only be useful adjuncts to Hb-based blood substitutes for which it can be shown, in advance, that the oxidation state is significantly reduced by interaction with selenium.

Iron chelators

One strategy that could be used to minimize the deleterious effects of free Hb in the circulation would be to use an iron chelator. Several iron chelators, such as DEF and deferiprone, are in clinical trials and are fairly safe for *in vivo* use (39, 45). DEF is used in the treatment of ROS-induced diseases, such as myocardial reperfusion injury (17) or rheumatoid arthritis. It is slow to penetrate into cells because

it is hydrophilic, and so millimolar concentrations are required to chelate intracellular iron. However, extracellular iron can be chelated with micromolar concentrations of DEF (35). DEF may also have $OH^{\bullet}$ or $O_2^{\bullet -}$ scavenging properties in addition to its ability to chelate iron (39), and so experiments involving DEF should be interpreted with caution. If low concentrations of DEF are used, Halliwell (37) concludes that the direct scavenging of oxidants will only make a small contribution to the protective effect of DEF against tissue damage. Paller (67) showed that DEF reduced renal dysfunction and accompanying free radical-mediated lipid peroxidation during heme-protein-induced acute renal failure. In an ovine model of exchange transfusion, metHb concentrations of a gluteraldehyde-polymerized bovine Hb increased from 3% to 40% in only 24 h (50). DEF also prevents oxyHb-induced endothelial and smooth muscle cell cytoskeletal injury (22). Deferiprone has a low molecular weight and, unlike DEF, rapidly enters cells. It has been used on patients with myelodysplasia and thalassemia major and is considered an acceptable alternative to DEF. However, there is little published information on the effect of iron chelation on the tissue damage induced by Hb-based blood substitutes. Such information would clarify whether or not the Fenton reaction plays a major role in the oxidative tissue damage caused by Hb-based blood substitutes and would aid in the development of superior products. If the Fenton reaction is responsible for the damage to the intestine produced by modified Hbs, then chelation of the free iron should reduce the damage.

Phosphorothioate oligodeoxynucleotides (PS-ODNs) can also be used as heavy metal chelators. The product C-10 PS-ODN, developed by AVI BioPharma (Corvallis, OR, U.S.A.), has been shown to have a high affinity for iron and facilitate iron excretion (54). PS-ODN has the advantage over the more widely used chelator, DEF, because treatment with DEF is costly, requires parenteral administration, and has side effects associated with its use. Recently, experiments were performed to determine if 2-min pretreatment with C-10 PS-ODN oligonucleotide, followed by coadministration of C-10 PS-ODN and DBBF-Hb, would reduce the microcirculatory leaks produced by DBBF-Hb in the rat mesentery (8). This procedure was very successful, the leak number and leak area per unit length of venule being reduced to 2% and 0.6%, respectively, of the values obtained with DBBF-Hb alone. However, systemic administration of C-10 PS-ODN did not prevent DBBF-Hb-induced damage to the epithelium of the intestinal mucosa, but made it worse (9), indicating that some types of Hb-induced tissue injury may occur by means other than release of free iron. Spectrophotometric measurements of DBBF-Hb oxidation *in vitro* indicate that the presence of PS-ODN may increase the transformation of DBBF-Hb to its ferryl form (Table 1). As DBBF-Hb-induced epithelial damage was worsened in the presence of PS-ODN, this indicates that the epithelium may be particularly vulnerable to ferryl Hb. Several direct strategies are emerging aimed at cycling ferryl back to ferric hemes by stimulating a catalase-like activity of hemoproteins using nitroxide (46) or the addition of Trolox, a vitamin E analogue known for its antiferryl activity (32).

Mast cell stabilizers

Previous studies have shown that intravascular injection of some modified Hbs, such as DBBF-Hb and PEG-Hb (3, 6), but not PolyHbBv or O-R-PolyHb (Hemolink) (10), cause degranulation of mast cells in the mesentery of the rat. As mast cells release inflammatory mediators such as histamine that are known to cause microvascular leaks (93), and as mast cells are strategically placed in the tissue close to venules, it could be hypothesized that mast cell degranulation would exacerbate the leaks caused by injection of a modified Hb. However, use of a mast cell stabilizer only significantly decreased the number, and not the area, of venular leaks (unpublished data; Table 2), suggesting that mast cells may be responsible for creating the smallest leaks. For this reason, it is unlikely that administration of mast cell stabilizers would significantly reduce the microvascular damage produced by modified Hbs. These experiments do, however, indicate that leak formation is a direct effect of the Hb, and is not mediated through mast cells.

Supplemental $NO^{\bullet}$

Opinions of whether or not $NO^{\bullet}$ reduces microvascular permeability are varied. In noninflamed vessels, $NO^{\bullet}$ has been shown to decrease permeability, and inhibition of $NO^{\bullet}$ increased permeability (1, 4, 47, 49). On the other hand, the role of $NO^{\bullet}$ in altering vascular permeability under inflamed conditions seems to contradict these results (43, 55, 85). This dichotomy is not surprising considering the number of different reactions that can occur with $NO^{\bullet}$ in biological systems. Only one study has been published to determine the effects of $NO^{\bullet}$ on Hb-induced increases in microvascular permeability (12). In this study, the mesenteric microcirculation of rats was pretreated with a $NO^{\bullet}$ donor, diethyltriamine nitric oxide adduct (DETA/NO), before perfusing with DBBF-Hb to determine if the microvascular leakage was decreased. Similar to the study using a mast cell stabilizer (Table 2), only the number of leaks was significantly reduced, the leak area per

TABLE 1. PERCENTAGES OF DIFFERENT OXIDATION STATES OF DBBF-HB UNDER DIFFERENT CONDITIONS

Condition	*$Fe^{2+}Hb$*	*$Fe^{3+}Hb$*	*$Fe^{4+}Hb$*
DBBF-Hb	86.7%	9.0%	4.3%
DBBF-Hb + PS-ODN	89.1%	7.2%	3.7%
DBBF-Hb + H_2O_2 (20 min)	23.8%	26.9%	49.2%
DBBF-Hb + H_2O_2 + PS-ODN (20 min)	16.1%	24.9%	59.0%

Table 2. Effects of Mast Cell Stabilizer and NO• Donor on Hb-Induced Microvascular Leakage

Treatment	*Number of leaks* ($\times 10^{-3}$) (μm^{-1})	*Area of leaks* ($\mu m^2\ \mu m^{-1}$)
DBBF-Hb	2.04 ± 0.26	0.13 ± 0.04
DBBF-Hb + cromolyn	0.50 ± 0.14	0.10 ± 0.04
DBBF-Hb	2.19 ± 0.11	0.09 ± 0.02
DBBF-Hb + DETA/NO	0.77 ± 0.05	0.05 ± 0.02

unit length of venule remaining the same. However, only one concentration of DETA/NO was used in this study (10^{-6} *M*), and it is possible that a different concentration of DETA/NO could promote a more protective effect of NO• in this microvascular system.

Summary

To summarize, from Fig. 4, it is obvious that in order to prevent oxidative tissue damage by Hb-based blood substitutes during transfusion, the formation of, and the effects of, different nitrogen- and oxygen-derived radicals must be prevented. To prevent formation of such radicals, it is necessary to reduce the tendency of the Hb to oxidize, either by chemical modification during manufacture or by addition of an appropriate reducing agent at the time of infusion. To prevent the deleterious effects of the radicals, at least with respect to the microvascular exchange, the use of an iron chelator, PS-ODN, appears to be effective. However, as PS-ODN has other functions apart from iron chelation, further research is needed in this area. Alternatively, oxidants could be scavenged downstream of the iron catalysis reaction, by using catalase and SOD. Cross-linking these agents to the Hb molecule is advantageous because it restricts them to the exact location where the radicals are produced. Ideally, a therapeutic agent would scavenge all deleterious radicals, whether or not their production is catalyzed by iron, and would be able to cross biological membranes. One possible candidate that has been suggested is Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable nitroxide that attenuates the effects of $ONOO^-$. Many compounds used to suppress the formation or effects of $ONOO^-$ are not specific because they interfere with the formation of other oxygen-derived radicals (87). Thus, it is impossible to predict whether the beneficial effects of Tempol are due to the prevention of formation of $ONOO^-$, or secondary to prevention of formation of OH•. Unlike recombinant SOD, which is not able to cross biological membranes, Tempol permeates biological membranes and accumulates in the cell cytosol.

PERSPECTIVES

Biological peroxides, such as H_2O_2, $ONOO^-$, and lipid peroxyl radicals, such as are formed after transfusion of Hb-based blood substitutes, have been implicated as regulators of redox-sensitive cell signaling pathways (82). Alterations in these cell signaling pathways can affect endothelial cell function. For example, when endothelial cells are treated with H_2O_2, prior to coculture with smooth muscle cells, generation of cGMP is suppressed, suggesting that NO• synthesis by endothelial cells has been inhibited (53). A likely mechanism by which oxidants can inhibit NOS activity has been suggested (56). Lipid peroxides spontaneously break down to yield 4-hydroxynonenal, which activates phospholipase D in endothelial cells, leading to stimulation of protein kinase C. Protein kinase C can inhibit NOS activity by a variety of mechanisms, including increased endothelial production of $O_2^{\bullet -}$,which binds to NO•. Some of the cellular signaling cascades that occur in response to Hb are protective. For example, exogenous addition of $ONOO^-$ decreased P-selectin expression and protected against ischemia–reperfusion injury (65). Secondly, prolonged exposure of endothelium to heme or metHb renders them resistant to oxidant challenge. The endothelium defends itself by producing the heme degrading enzyme, HO-1, and the iron binding protein, ferritin (90). Finally, use of selenium compounds with modified Hbs, apart from altering the oxidation state of the Hb, may also down-regulate the signaling pathway by which tumor necrosis factor activates the NF-κB transcription factor, a measure of oxidative stress (52). Such protective cell signaling cascades could be utilized in future studies to alleviate the oxidative damage caused by Hb-based blood substitutes.

ABBREVIATIONS

BSA, bovine serum albumin; cGMP, guanosine 3',5'-cyclic monophosphate; DBBF, *bis*(3,5-dibromosalicyl) fumarate; DBBF-Hb or DCLHb, diaspirin cross-linked hemoglobin; DEF, desferrioxamine; DETA/NO, diethylenetriamine nitric oxide adduct; Fe^{2+}, ferrous; Fe^{3+}, ferric; Fe^{4+}, ferryl; Fe^{4+}=O, oxoferryl; FITC, fluorescein isothiocyanate; Hb, hemoglobin; HO, heme oxygenase; H_2O_2, hydrogen peroxide; metHb, methemoglobin; NF-κB, nuclear factor-κB; NO•, nitric oxide; NOS, nitric oxide synthase; $O_2^{\bullet -}$, superoxide anion radical; OH• , hydroxyl radical; OH^-, hydroxyl ion; $ONOO^-$, peroxynitrite; O-R-PolyHb, *O*-raffinose-polymerized Hb; oxyHb, oxyhemoglobin; PEG, polyethylene glycol; PolyHbBv, glutaraldehyde-polymerized Hb; PS-ODN, phosphorothioate oligodeoxynucleotide; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Address reprint requests to:
Ann L. Baldwin, M.D.
Department of Physiology
College of Medicine
University of Arizona
Tucson, AZ 85724-5051

E-mail: abaldwin@u.arizona.edu

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