ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 9, 2011

DOI: 10.1089/ars.2010.3447

REVIEW ARTICLE

Blood Aging, Safety, and Transfusion: Capturing the "Radical" Menace

Paul W. Buehler, Elena Karnaukhova, Monique P. Gelderman, and Abdu I. Alayash

Abstract

Throughout their life span, circulating red blood cells (RBCs) transport oxygen (O_2) primarily from the lungs to tissues and return with carbon dioxide (CO_2) from respiring tissues for final elimination by lungs. This simplistic view of RBCs as O_2 transporter has changed in recent years as other gases, for example, nitric oxide (NO), and small molecules, such as adenosine triphosphate (ATP), have been shown to either be produced and/or carried by RBCs to perform other signaling and O_2 sensing functions. In spite of the numerous biochemical and metabolic changes occurring within RBCs during storage, prior to, and after transfusion, perturbations of RBC membrane are likely to affect blood flow in the microcirculation. Subsequent hemolysis due to storage conditions and/or hemolytic disorders may have some pathophysiological consequences as a result of the release of Hb. In this review, we show that evolution has provided a multitude of protection and intervention strategies against free Hb from "cradle" to "death"; from early biosynthesis to its final degradation and a lot more in between. Furthermore, some of the same naturally occurring protective mechanisms can potentially be employed to oxidatively inactivate this redox active protein and control its damaging side reactions when released outside of the RBC. *Antioxid. Redox Signal.* 14, 1713–1728.

Introduction

When compared to other cells, human red blood cells (RBCs) have modest but important metabolic activities that include maintenance of cationic pumps and 2, 3-diphosphoglycerate (2, 3-DPG) levels which are essential for the allosteric modulation of O_2 binding to hemoglobin (Hb) and the control of Hb oxidation. RBCs house a number of reductive enzymes that keep Hb in the reduced functional form and maintain membrane integrity. However, since RBCs lack the ability to *de novo* synthesize and replenishes these enzymes, an oxidant/antioxidant imbalance may occur as these cells advance in age. RBCs may be stored for up to 42 days under controlled conditions before transfusion (Circular of information for the use of human blood and blood components. AABB, American Red Cross, America's Blood Centers, and the Armed Services Blood Program. Bethesda, MD. AABB.2009. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/default.htm, accessed on December 29, 2010) AABB Circular, 2009). However, numerous changes occur in RBCs during storage, collectively referred to as the ''storage lesion'' that can alter their biological function.

In an attempt to evaluate the efficacy of RBC transfusion, a recent systematic review and meta-analysis was carried out on 45 observational studies that involved 272,596 patients (71). In 42 of the 45 studies, the risk of transfusion outweighed the benefit. Overall, transfusion was associated with 70% increase in death and 80% increase in risk of infection. The question of why any rational physician would ever transfuse a patient was raised in accompanying editorial to this paper (26). However, a counter argument was made in favor of and to the benefits of blood transfusion and was centered on the fact that major progress has been made in improving blood safety during the past 20 years and that the risk of human immunodeficiency virus and hepatitis C has fallen to approximately 1 in 2–3 million (6).

According to the most recent transfusion-related fatalities that were reported to the FDA, there was an increase in transfusion-related acute lung injury (TRALI) fatalities from RBCs. For the same blood products, fatal hemolytic transfusion reactions seem to be declining, whereas reported fatalities attributed to microbial infection remained the same. Albeit the exact incidence of TRALI is not known, it has been estimated that 1 in 5000 transfusions could demonstrate this adverse outcome. TRALI has been the most common cause

Division of Hematology, Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA), Bethesda, Maryland.

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency, determination, or policy.

of transfusion related fatalities reported to the FDA since 2005 (http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ReportAProblem/TransfusionDonationFatalities/ucm204763.htm, accessed on December 29, 2010). Many efforts are currently being made to reduce the incidence of TRALI. It was recently recommended that females should be excluded as plasma donors during an international forum on TRALI (111). In addition to the efforts to reduce TRALI, efforts are also being made to reduce pathogens in RBC transfusion products. Pathogen inactivation in RBCs has been studied and is still being investigated; however, there are no licensed methods yet available (20, 57).

It was recently highlighted in the literature by several retrospective analyses that older RBCs at time of transfusion in certain patient groups may lead to adverse outcomes and ultimately death (12, 58). In addition, it has also been reported that transfusion of older RBCs promoted cancer progression in an animal study (9). These reports on "older" RBCs and their deleterious effects raise the question "What is considered an older RBC?" Logically, one would be inclined to think that these are cells near the end of their 42 day storage period. Surprisingly, RBCs begin to undergo biochemical and physiological changes early into storage, as in case of the reported cancer study in rats, or 15 days and older for the retrospective analyses in critically ill patients (9). Consequently, other retrospective analyses have been performed to evaluate the outcome of infusing older versus fresh RBCs into noncritically ill patients (88, 109). The consensus is that there exists a correlation between transfusing older blood and adverse events. However, it should be taken into consideration that in patients receiving low volumes of blood, the age of the RBCs may be negligible when compared to the severely injured or critically ill patients who are transfused with large volumes. In addition, the mechanism(s) underlying the toxicity of stored RBCs must be understood to achieve safe storage limits.

Among the well-documented pathophysiologic consequences of RBC storage are decreased deformability, impaired blood flow, impaired O_2 delivery to tissue, hemolysis, and imbalance in nitric oxide (NO) homeostasis. Storage-related changes in RBC membrane, specifically deformability in human packed RBCs stored in CPDA-1 at 4°–8°C over a 4-week period have been investigated. It was shown that human RBC deformability decreases significantly by 34% after 4 weeks of storage. However, metabolic rejuvenation restored RBC deformability to control levels (fresh RBCs) (28). Several recent investigations have focused on the effects of storage conditions on O_2 delivery, including the direct measurements of local perfusion and microvascular O_2 distribution when 28-day stored RBCs are introduced into anemic normovolemic hemodiluted animals. Circulation of stored RBCs in these hemodiluted animals resulted in significantly malperfused and under oxygenated microvasculature that was not detectable at the systemic level (107).

Hemolysis, a measure of RBC destruction, is an important risk factor as RBCs age and in the case of hemolytic anemias. Hemolysis-associated pulmonary hypertension, transfusion-associated lung Injury, post-perfusion renal and cerebral dysfunction, morbidity and mortality of stroma-free Hb-based blood substitutes, and aged blood associated mortality in trauma are well-documented consequences of decompartmentalization of Hb (86). Despite efforts to better understand the storage of RBC, these measures remain the most useful predictors of RBC survival and function (86). Multiple biochemical components of RBC storage-induced changes were recently quantified, including hemolysis. It was shown that cell-free Hb in stored blood increases steadily in the medium reaching approximately 0.02 m*M* at the end of the 6 week storage and reaches to approximately 1 m*M* immediately after infusion in circulation, consistent with other previous reports (16). Activated leukocytes could be a source for oxygen free radicals and their potential contribution towards RBC hemolysis, alloimmunization, and febrile reactions, and this has led to discussions about universal leukocyte reduction. Currently, in the United States, a leukocyte reduced blood component is defined as a component containing less than 5×10^6 residual donor leukocytes per final product (13).

A recent investigation on the effects of RBCs after prolonged storage in a mouse transfusion model showed that transfusion of stored RBCs (14 days), or washed stored RBCs, increases plasma non-transferrin bound iron, produces acute tissue iron deposition, and initiates inflammation and endotoxinemia. In contrast, the transfusion of fresh RBCs, or transfusion of stored RBC-derived supernatant, ghosts, or stroma-free lysates, does not produce these effects (48).

The impact of RBC storage on NO homeostasis is less clear but was more visible in recent scientific and popular press. One line of investigation promoting the physiological role of intraerythrocytic s-nitrosolthiol Hb (SNO) at Cysβ93 has been proposed to play a critical role in maintaining RBCs ability to dilate blood vessel, thus maintaining blood flow (90). Accordingly, stored blood and after the first few hours after collection undergoes a gradual loss of its ability to release NO from Cysβ93 and that stored blood must be compensated for the loss in NO before infusion. Another alternative source for NO in blood that involves Hb is nitrite. In this case, it has been suggested, based in large part on *in vitro* observations, that Hb enzymatically reduces nitrite to NO which could also be used to rejuvenate old RBCs (69). The role of NO or its metabolites in restoring NO homeostasis, preventing hemolysis-induced vasculopathy as well as its therapeutic applications remains, however, largely speculative. NO is an autacoid molecule that acts largely in the immediate microenvironment, and its long term and global vascular reach may have been overestimated (112).

Relevant biochemical, structural, and functional changes related to RBC storage is multifactoral and complex in nature. Impact of storage on Hb oxygenation and NO homeostasis is likely to be transient and uncertain of significance. In contrast, perturbations of RBC membrane are likely to adversely affect blood flow in the microcirculation. More importantly, Hb loss due to storage and or induced hemolysis may represent a more serious safety problem that need to be fully understood and controlled.

The focus of this article is to review recent advances in transfusion practices, with a particular focus on the role of free Hb released from aged RBCs and/or during pathological events leading to hemolysis. The role of natural protective pathways operative in the early genesis of Hb formation as well as the role of endogenous protective mechanisms in controlling the redox toxicity associated with free Hb during RBC circulation are reviewed. It is hoped that lessons can be drawn from these naturally occurring and effective antioxidant mechanisms in the design of future interventions to control Hb toxicity.

Current Transfusion Practices Using Stored Red Blood Cells

At present, the blood supply is safer than any time in the history of transfusion in the United States and is considered to be among the safest in the world (29). RBCs can be stored and transfused when stored in an approved anticoagulant and/or additive solution for up to 42 days and stored under refrigeration (1°–6°C) (Circular of information for the use of human blood and blood components. AABB, American Red Cross, America's Blood Centers, and the Armed Services Blood Program. Bethesda, MD. AABB. 2009. http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm and AABB's Technical Manual. 16th edition (93)). When collected in a sterile or "closed" system, the storage requirements and expiration dates of the RBCs vary significantly when compared to being collected or processed in an "open" system. Table 1 shows selected RBC components with their respective storage conditions and shelf lives (93). Due to the limited allowable storage period, it is not surprising that the RBC inventory is subject to recurring highs and lows. Both scenarios are of concern, because a periodic shortage is potentially life threatening. On the other hand, when the inventory is high, blood products may be lost due to outdating. In addition to storing RBCs under refrigeration, they may be stored for 10 years when stored frozen in compliance with Federal regulations and AABB guidelines (47).

Approximately 40 years ago, frozen RBCs were used for transfusion. At the time, it was anticipated that with concurrent advances in technology their use would increase, however this did not materialize (87). At present, active research continues to focus on extending the shelf life of RBCs without compromising their integrity and effectiveness. A few examples of ongoing investigations are the storage of RBCs under anaerobic conditions and the creation of universal donor RBCs using either conventionally collected RBCs or progenitor cell types that can be driven into mature RBCs (39).

As previously mentioned, RBCs undergo biochemical and physiological changes during storage. It has been investigated and well documented that some of these changes are reversible, such as 2,3-DPG levels. The function of 2,3-DPG is to stabilize deoxyHb in order to allow RBCs to deliver O_2 at increased levels of tissue PO_2. A rapid decline of 2,3-DPG occurs after 7 days of storage. However, when RBCs are transfused, the 2,3-DPG levels are restored in patients within a day (up to 48 h) of transfusion (108). This is illustrated in Figure 1. Nevertheless, questions remain regarding changes to the RBC that may occur during storage and are not restored after transfusion, for example, cellular deformability. When do such changes occur? Do they occur during the first 14 days of storage or after 30 days? Moreover, it should be noted that the distribution of the RBC age in the circulation is presumed to be approximately Gaussian, with an average centered at their circulatory half life. Therefore, "fresh" blood has a fraction of RBCs at the end of their cycle, which may contribute to Hb levels in the circulation early on.

A loss of cellular deformability and intravascular hemolysis or inflammation could contribute to clinical adverse events when RBCs reach critical storage durations (43, 48). Thus, biochemical and physiological changes as shown in Figure 1 are an inevitable occurrence during RBC storage. Therefore, at the end of storage, there will be RBCs that are able to carry and deliver O_2 and there will be RBCs that will no longer be able to carry out this function. We have recently shown that during routine cold storage of AS-5 preserved RBCs, the RBCs retained their ability to carry oxygen as they age. Using equilibrium and rapid mixing kinetic measurements, we showed for the first time that cells as old as 42 days of storage largely preserved their *in vitro* interactions with oxygen. Other tested parameters such as 2,3-DPG, ATP etc. decreased, whereas percent hemolysis, extra cellular lactate levels, etc. increased during the 42 days of storage as has been well documented in the literature (40).

Storage Preservation, Hemoglobin Oxidation, and Their Impact on Red Blood Cell Survival

With a few exceptions, the encapsulation of Hb within the RBC in the animal kingdom can be viewed as nature's response to the toxicity of Hb, while ensuring O_2 delivery to critical organs and tissues. The RBC therefore not only protects Hb from the body's proteolytic degradative activities but it also protects the body from Hb redox toxicity and provides an efficient vehicle for O_2 sensing and transport. The impact of Hb loss from RBCs during intravascular hemolysis in several hemolytic conditions and its contribution to human diseases is well established and has recently been reviewed (see (96) for review).

Lesser known human health complications have been reported with the transfusion of old RBCs that can be attributed specifically to the oxidation of Hb and subsequent loss of the

Table 1. Maximal Storage Periods for Selected Red Blood Cell (RBC) Components Under Refrigeration (1–6°C) According to the AABB Technical Manual, 16th Edition

Red Blood Cell Components:[a]	*Storage Period:*		
	21 Days or 28 Days	35 Days	42 Days
RBCs, Leukocytes reduced	21 Days: ACD; CPD;CP2D	CPDA-1	AS-1;AS-3; AS-5
RBCs, irradiated	Original expiration or 28 days from date of irradiation, whichever is sooner		
Apheresis RBCs, Leukocytes reduced		CPDA-1	AS-1;AS-3; AS-5

[a]Frozen RBC components, deglycerolized and rejuvenated RBCs are not included in this table. Consult the AABB Technical Manual, 16th Edition, Table 9-1 for a complete list of all blood components. ACD, acid-citrate-dextrose; AS, additive solution; CPD, citrate-phosphate-dextrose; CP2D, citrate-phosphate-dextrose-dextrose; CPDA-1, citrate-phosphate-dextrose-adenine.

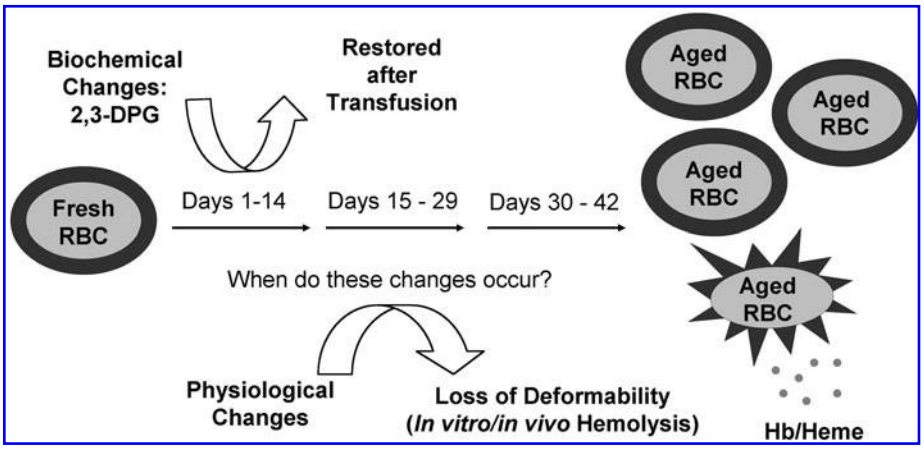

FIG. 1. Biochemical and physiological changes of aging RBCs. RBCs can be stored in an approved additive solution and transfused up to 42 days when stored at 1°–6°C. During the 42-day storage period, biochemical and physiological changes occur (*e.g.*, a rapid degradation of 2,3-DPG). However, 2,3-DPG is restored in patients after transfusion (108). In each RBC unit there are RBCs present of different ages (at different stages of senescence) and therefore, during the 42-day storage period, different rates of changes will occur. Overall, it seems that some of the other observed changes such as a decrease in pH and ATP seem to occur gradually over the 42-day storage period. This suggests that the majority of RBCs in a unit after weeks of storage still have relatively normal biochemical properties and account for a normal *in vivo* survival after transfusion (70).

protein from the RBCs (54). This is due in large part to the presence of several mechanisms within human RBCs that presumably control the spontaneous oxidation of the iron center of Hb. The redox hiatus within RBCs in the face of the continuous reversible binding of O_2 to Hb is brought about in large part by active antioxidative enzymatic machinery within these cells. When ferrous (oxy) Hb is spontaneously oxidized (auto-oxidation) or by chemically induced oxidation, the latter is recycled back to the ferrous functional form so that in the steady state the amount of intracellular metHb is kept below 1%. The metHb is reduced by NADH-cytochromeb5-metHb reductase. In addition, reduction can be done by several dependant metHb reductases and direct reduction by intracellular ascorbate and glutathione. The buildup of $O_2^{\bullet -}$ and H_2O_2 as Hb undergoes auto-oxidation are controlled by the abundant enzymes, superoxide dismutase ($O_2^{\bullet -}$ scavenger) and catalase (H_2O_2 scavenger) within the RBCs. In addition to catalase, glutathione peroxidase1 (GPX1) and peroxiredoxin (Prx) II are also involved in H_2O_2 elimination within the RBC (18).

However, in spite of these natural antioxidative and protective mechanisms operating within RBCs, it has been shown that young RBCs contain more potassium and sodium, have a greater cell volume, and have a lower density than older cells. Immature RBCs appear to be more resistant to hemolysis in hypotonic media than older cells (18). A progressive decrease in activities of several enzymes of the glycolytic pathway and of the hexose monophosphate shunt pathway and in concentrations of organic phosphate esters is associated with aging of mature mammalian RBCs. Although removal mechanisms of senescent RBCs from circulation exist, the survival of RBCs *in vivo* may still be determined by the stability the enzyme proteins that are required for maintenance of the reduced functional state of Hb (18).

Unlike the well-documented Hb-mediated oxidative reactions and subsequent oxidative injuries *in vivo*, little is known about the extent of Hb oxidation during *ex vivo* storage of RBCs. The formation of cross-links between Hb and membrane proteins, a process known to be initiated by Hb oxidation, and oxidative changes that accompany this process were recently demonstrated in RBCs subjected to prolonged hypothermic storage (59).

A number of possible scenarios in which Hb oxidation can be implicated in the storage lesion was recently suggested (54). First, in packed RBC units which contain 42–80 g of Hb, the concentration of Hb that is clinically functional may be reduced over time because of storage lesions, especially in units reaching their maximum shelf life (49). Second, cell senescence and changes in RBC reducing power (*i.e.*, depletion of endogenous antioxidants) can accelerate the rate of Hb auto-oxidation and elevate the concentration of intracellular ROS. Third, the level of molecular O_2 within the RBC unit that is available for redox reactions can also be reduced (113) [For recent discussion of auto-oxidation reactions of Hb and its relationship to oxygen, see (91)].

Finally, the storing vehicle, including the blood bag and the additive solutions, may also alter the Hb oxidative state (113).

Recent studies confirm the role of Hb oxidation in promoting storage lesions (59). RBCs preserved in citrate–phosphate dextrose–adenine storage solution units for up to 6 weeks suffered oxidative injury characterized by the attachment of denatured Hb, presumably hemichromes, to membrane phospholipids and cytoskeleton proteins, such as spectrin. They also reported that traces of denatured Hb were present in microparticles released from the cell membrane throughout storage (60). The incidence of Hb-induced membrane damage increased as a function of storage period, reaching significant levels of Hb-membrane adducts after 35 days.

Extracellular Hb oxidation and quality of RBC relative to preoperative blood salvage has recently been examined. Despite washing, extracellular Hb concentrations remained high (up to 0.7 g/l in a given blood bag) and was associated with a decrease of haptoglobin (Hp) in patients, despite a concomitant inflammatory syndrome. Accordingly, it was recommended that hemolysis must be limited during preoperative blood salvage in order to prevent exposure to oxidized Hb and its metabolites that may trigger cellular injury (43).

Potential Mechanisms and the Origin of Hb Oxidation Within RBCs

Recently, Rifkind and his team (75) reported a set of experiments that shed some light on the role and origin of Hb oxidation within RBCs and how this may potentially be related to the process of aging. They showed that ROS generated in the cytosol are normally neutralized by abundant antioxidant enzymes. However, H_2O_2 generated by the membrane-bound Hb is not accessible to the cytosolic antioxidants and reacts to generate fluorescent heme degradation products *in vitro*. For the first time, these studies established a pathway for oxidative stress associated with Hb auto-oxidation, despite the extensive antioxidant system in RBCs. These byproducts of Hb oxidation and oxidative chemistry that occur at the membrane surface may be released from the RBC to affect nearby tissues and/or react with the RBC membrane, altering RBC function and possibly contributing to the removal of RBCs from circulation (75).

Extensive data exist in the literature which suggest that membrane changes seen in RBCs are mostly likely due to hemoglobin oxidation, rapid heme loss, and then uptake of heme into the membrane (67, 95). However, most recent data point out that the role of the membrane in the formation of H_2O_2 and the resultant heme degradation products are result of noncystolic oxidation of Hb, as depicted in Figure 2, based on the proposal by Rifkind *et al.* (75). The primary event that triggers this reaction cascade begins with the binding of Hb to the cytoplasmic end of band 3 on the RBC membrane. Since deoxyHb has a higher affinity for band 3 (98) than oxyHb, hypoxic conditions found in the microcirculation will favor binding to the membrane. Partial oxygenation of Hb that enhances binding to the membrane also dramatically increases the rate of Hb auto-oxidation (92). Auto-oxidation of Hb bound to the membrane produces a pool of H_2O_2 that is relatively inaccessible to catalase, a cytoplasmic enzyme. This pool of H_2O_2 will increase under hypoxic conditions with the increased binding of Hb to the membrane. Although H_2O_2 in the region of the membrane can be removed by peroxiredoxin and GPX (68), the activity of this enzyme is dependent on the reducing power of NADPH/NADH, which declines with age of cells and for various pathological conditions. Under these conditions, the H_2O_2 reacts with nearby Hb to initiate a redox cycle in which ferric/ferryl Hb and possibly its radical protein, resulting in a self-destructive cycle that leads to the formation heme degradation products before it diffuses into the cytoplasm to react with catalase.

The clinical consequences of excessive amounts of free Hb in the circulation have recently been described (96). Hb acts as a NO scavenger (30, 81) and, once released from RBCs, it will immediately react with NO, depleting plasma levels of this important signaling and vasodilator that has been shown to affect regulation of blood flow, smooth muscle responses, and intravascular thrombosis. This has clearly been documented with free Hb in animals (19) and also when chemically or genetically modified Hbs known as blood substitutes infused in patients experiencing blood loss (101).

Extracellular Hb can rapidly oxidize to metHb due to the process of auto-oxidation, reactions with cellular oxidants, and through its interactions with NO (4). Accumulation of metHb and possibly other oxidative byproducts can be enhanced if endogenous mechanisms to eliminate it, such as Hp-mediated clearance, are exhausted (19). MetHb and its denatured products, such as heme, have been shown to intensify the inflammation response of vessel endothelial cells and to promote atherosclerosis through the oxidation of low-density lipoproteins.

However, the pathological effects of blood storage on endothelial function have been in recent years attributed solely to disruption of NO homeostasis (41, 64). NO is an

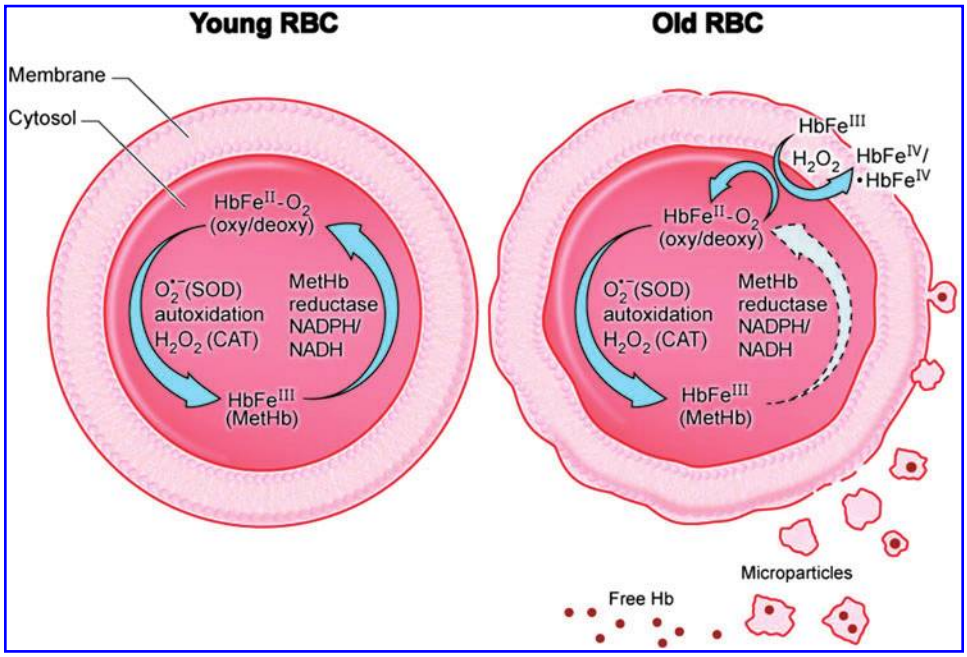

FIG. 2. Mechanisms and origin of Hb oxidation within RBC. Proposed biochemical and oxidative changes that stored RBCs can undergo with time. In young RBCs (*left*), antioxidative enzymes can control reactive oxygen species (ROS) resulting from Hb auto-oxidation and oxidative reactions in the cytosol. As RBC advances in age (*right*), its reductive capacity is reduced with time. Enhanced oxidative reactions occurring at the membrane surface of the RBC that are unhindered by cystolic reductive enzymes will perpetuate oxidative changes sustained by Hb's radical chemistry, leading to the formation of highly reactive and damaging ferryl radical species (75). Hb can be carried to the outside of RBCs by released microparticles that ultimately lead to free Hb into plasma (60). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

important signaling as well as a vasodilator diatomic gas produced by the vascular system (30, 81). The reaction is primarily with the heme group that can be completed within a few seconds with a profound consequence (*i.e.*, blood vessel constriction and elevation in both systematic and pulmonary blood pressures: approximate mean arterial blood pressure changes ranges between 15 and 30 mmHg). However, blood pressure elevations seen after infusion of free Hb "blood substitutes" in both animals and humans appear to follow a predictable path that can return to normal within 2 hours.

It has been argued recently that reduced NO bioavailability, as a result of free Hb in plasma, can augment thrombosis, microcirculatory perturbations, or injury in patients with a compromised vascular system. Accordingly, NO therapies in the form of NO donors or by inhibition of NO synthetic pathways or by modifying Hb Hb/RBCs to become a source of NO have been advocated (41).

Similar approaches in which Sildenafil was given to patients with sickle cell anemia to control pulmonary blood pressure triggered by free Hb has been, however, very disappointing and these trials had to be stopped prematurely because of the increased frequency of pain crises in these patients (25). Bunn and a number of researchers, who are actively involved in sickle cell disease research and management, came up recently against clinical trails that were designed to increase the bioavailability of NO for sickle cell patients with clinical manifestations which were related to plasma Hb. They further argued that NO levels are either not crucial or are only one of many factors that influence the pathophysiology of sickle cell disease (25).

In order to fully appreciate the complexity and the multitude of naturally occurring mechanisms that are designed to protect against Hb toxicity within the human body, it is important that a good understanding of the synthesis, regulation, and protection against the radical chemistry originating from the heme in its free and complexed forms is reviewed within the following sections. It is hoped that a lesson or two can be learned from these naturally occurring antioxidant mechanisms that can be applied therapeutically to safely controlling free Hb in hemolytic disorders and/or when found in storage lesions.

Enzymatic Activities of Hemoglobin: Hemoglobin as a Radical Enzyme

Hb is one of the most studied and characterized hemoproteins. While the function of Hb is primarily to carry O_2 from the lungs to tissues, our understanding of the physiological function of Hb has changed over the past decade. Because of the catalytic nature of some of these newly reported reactions, Hb has been given the title "honorary enzyme" with radical enzymatic and pseudoenzymatic activities and because of its toxicity; in some cases, Hb has been referred to as the "rogue" enzyme. These enzymatic activities include nitric oxide dioxygenase, nitrite reductase, and peroxidase/pseudoenzymatic were recently reviewed by Reeder (89).

Since the discovery in the 1980s that a diatomic gas such as NO is the endothelial-derived relaxing factor (EDRF), there has been an explosion in research carried out and published on the physiological role of NO and the many biological molecules that interact, carry, or destroy it.

NO reacts avidly with RBC's oxyHb (HbO_2) and muscle myoglobin (MbO_2) to form stoichiometric nitrate (NO_3-) and ferric (Met) Hb or Mb (Eq.1) (31).

$$NO + HbFe^{II}\text{-}O_2 \rightarrow HbFe^{III} + NO_3{}^- \qquad \text{(Eq. 1)}$$

This reaction is critical to human physiology affecting NO metabolism, signaling, and toxicity. This reaction also hampered the application of cell-free Hb, developed as blood substitutes and has been reported to be responsible for pulmonary hypertension in patients with sickle cell anemia. Free Hb, unlike RBCs, reaches NO production sites (*i.e.*, the vascular endothelium quite readily and reacts rapidly with NO).

Nitrite ($NO_2{}^-$) can react with deoxyHb ($HbFe^{II}$) to form NO and ferric Hb according to Equation 2.

$$NO_2{}^- + HbFe^{II} + H^+ \rightarrow NO + HbFe^{III} + OH^- \qquad \text{(Eq. 2)}$$

The NO formed in this reaction can then bind to another deoxyHb to form NO heme-bound nitrosylHb. Several oxidative intermediates, including ferrylHb, have been involved in these reactions (83). Accordingly, nitrite, a naturally occurring circulating small molecule, can be readily converted to NO inducing vasodilatation under hypoxic conditions. Hb as a functional nitrite reductase can therefore be of potential therapeutic values in sickle cell disease and other cardiovascular indications (89).

Binding and release of NO has been suggested to be under allosteric control of Hb, because the reactivity of the signal amino acid, Cys β93, and that this reaction is conformation dependant according to this thesis. The allosteric transition in Hb from the tense (T) state (deoxygenated) to R (relaxed) oxygenated state promotes the release of an NO group from Hb's hemes to its thiols (Cysβ93) forming Hb-Cys β93 NO (SNO-Hb).

$$Hb[Fe^{II}NO]\text{-}Cys\beta93 + 2O_2 \rightarrow Hb[Fe^{II}O_2] \text{-} Cys\beta93\text{-}SNO + O_2{}^{\bullet -} + H^+ \qquad \text{(Eq. 3)}$$

Accordingly, O_2 can serve as an electron acceptor resulting in $O_2{}^{\bullet -}$ production. RBC SNO-Hb has been suggested to contribute to RBCs principal function, O_2 delivery via regulation of blood flow, and therefore the RBC can be harnessed for therapeutic purposes, including the reversal of RBC aging (103).

The pseudoperoxidase activities of Hb and myoglobin (Mb) have been under intense investigation for centuries *in vitro*, and recent evidence is accumulating that these reactions do occur *in vivo* with some serious consequences (89). It has been known for some time that Hb can react with H_2O_2 resulting in a complex redox chemical reactions. First, H_2O_2 oxidizes ferrous Hb to generate the higher oxidation state of the protein, (ferryl) ($Hb^{IV}=O_2{}^-$) and when reacting with the ferric protein, a protein-based cation radical ($^{\bullet}Hb^{IV}=O_2{}^-$) is formed:

$$Hb^{II} + H_2O_2 \rightarrow Hb^{IV}=O_2{}^- + H_2O \qquad \text{(Eq. 4)}$$

$$Hb^{II} + Hb^{IV}=O_2{}^- + H^+ \rightarrow 2Hb^{III} + OH^- \qquad \text{(Eq. 5)}$$

$$Hb^{III} + H_2O_2 \rightarrow {}^{\bullet}Hb^{IV}=O_2{}^- + H_2O \qquad \text{(Eq. 6)}$$

Lessons Learned from Cell-Free Hemoglobin Developed as Blood Substitutes

Many laboratories, over many years, have searched for ways to prepare safe and effective cell-free Hb that will perform similar functions outside RBCs. For use as blood substitutes, also commonly known as cell-free Hb-based O_2 carriers (HBOCs), Hb must be capable of cooperative O_2 uptake and delivery with appropriate transition from relaxed R (oxy)- and tense (T) –(deoxy) state affinities, and of maintaining reasonably long functionality in circulation without adverse side effects.

Intracellular O_2 carriers prevail in the vertebrate kingdom, due to the advantages derived from packaging reductants and allosteric effectors together with Hb within RBCs. In the absence of this packaging, HBOCs as decompartmentalized Hbs are more readily lost to the circulation by renal filtration, and lack the mechanisms to maintain the reduced functional heme. More recently it has been shown that packaging Hbs within red blood cells also helps avoid NO scavenging and concomitant increases in blood pressure (66).

Chemically and/or genetically altered HBOCs have so far demonstrated efficacy in proof of concept preclinical studies and reasonable risk in toxicology studies involving normal animals. However, HBOCs have not yet demonstrated a favorable risk to benefit ratio in human clinical trials (for a more recent review of the subject, see Ref. 3). It remains to be seen if the reduced allosteric properties of these HBOCs make them ineffective as O_2 carriers *in vivo*. The physiological consequence of the reduced cooperativity and reduced Bohr and chloride effects of the HBOCs could be significant in affecting tissue acid–base balance, O_2 delivery to tissues, and CO_2 transport to the lungs.

The reduced allosteric responses and enhanced rates of auto-oxidation exhibited by the HBOCs make them problematic for use in an extracellular environment. The auto-oxidation process is a source for nonfunctional metHb, as well as reactive oxygen species ($O_2^{\bullet -}$ and H_2O_2). Recent research shows that auto-oxidation of Hb can also be a source of highly reactive ferryl heme and heme degradation products (76). Nitrite-induced oxidation of air-equilibrated HBOCs can also be a source of metHb and ferryl Hb (83). Cellular toxicity attributed to ferryl heme includes promotion of lipid peroxidation, lactate dehydrogenase release, and DNA fragmentation, phenomena recognized as markers of cell injury and death by apoptosis and necrosis (27). The globin-based ferryl Hb radical was also detected in whole normal blood (105).

In vivo studies from our laboratory and others have indeed shown that unhindered oxidation reactions and ferric Hb accumulation occur in animals from which blood was exchanged transfused with HBOCs (22). These reactions occur at much higher rates in animals such as guinea pigs that lack endogenous reductive mechanisms, such as ascorbic acid, as opposed to animals that are enzymatically capable of producing ascorbate (22). Indirect EPR measurements of ferryl radicals in rabbits infused with HBOCs were reported recently (32). Subtle oxidative changes at the amino acids levels in proteins after infusion of HBOCs or stroma-free Hb were recently identified by more sensitive mass spectrometric methods (53).

Protection Against Hemoglobin from Cradle to the Grave

Figure 3 illustrates the diverse and complex physiological pathways that are deployed in the mammal system to control Hb oxidative reactions and the proteins that have been specifically designed to lessen the toxicities associated with the Hb and the by products of Hb oxidative reactions, for example, heme (discussed here) and iron (discussed elsewhere, see (13) for review). These control mechanisms span from early erythropoiesis to heme degradation in macrophages and many other pathways as outlined in Figure 3.

Alpha hemoglobin stabilizing protein

Toxicity of Hb molecule is driven in large part by its redox active heme prosthetic group. However, even at the very early erythropoietic developmental stages and when heme is incorporated into its respective α or β chains, the α chain in particular is less stable than its β chain counterpart of the protein. In erythroid cells, ROS react with αHb, causing its breakdown and precipitation, and also damage other cellular constituents. Both α and β globin gene loci are located on chromosome 16 and 11 which produce α-and β-globin mRNA and α- and β-globin polypeptides, respectively. These two combine to form a Hb dimer and when combined a full functional Hb tetramer is formed. The intrinsic instability of the α chain has been recognized for some time. However, it was only recently that this process was shown to require a helper or chaperon protein, the alpha Hb stabilizing protein (AHSP). This protein now we know plays a critical role in stabilizing the α subunit of Hb molecule.

In vivo evidence confirming the critical role of AHSP came from studies reported in mice where the ASHP gene was completely ablated, that is, the ability of ASHP protein-coding was removed to ensure complete loss of protein expression in homozygous-null (AHSP−/−) animals. In these animals, mild hemolytic anemia with shortened RBCs survival was observed in which excess Heinz bodies, indicative of denatured Hb, were also found. Moreover, AHSP−/−RBCs produced excessive ROS, and exhibited oxidative damage to endogenous proteins, contrary to heterozygous (AHSP+/−). RBCs were normal in number, appearances, and life span (110). More recent work showed that AHSP also act a molecular chaperone to stabilize nascent α-globin for the final HbA assembly. It promotes native folding of the apo-α-globin and its assembly into αHb in solution and stabilized a pool of free αHb (115).

The three-dimensional structure of AHSP was determined based on studies that utilized resonance spectroscopy and X-ray crystallography (Fig. 4) (36). AHSP forms an elongated three α-helix bundle fold. The binding of αHb was determined to be at the C-terminus of helix 1, the loop connecting helices 1 and 2, and the N-terminal part of helix 2. These studies also revealed that AHSP complexes with αHb in the same region as one of the two β chains of HbA at the α_1 β_1 interface region which is located directly opposite to the αHb heme pocket.

The proposed AHSP-mediated stabilization of αHb have been described by a series of elegant experiments performed by Weiss and his group (for review, see (74)). The α-stabilizing protein minimizes the deleterious effects of the free α-subunit by limiting its prooxidant activity. AHSP binds to oxy-α-Hb, alters the structure of the heme, and induces rapid auto-oxidation, which generates H_2O_2. This is followed by the formation of α Fe-III bis-histidyl complex that prevents further redox cycling and the formation of the ferryl subunit. This hemichrome form of α-Hb is resistant to further oxidation and

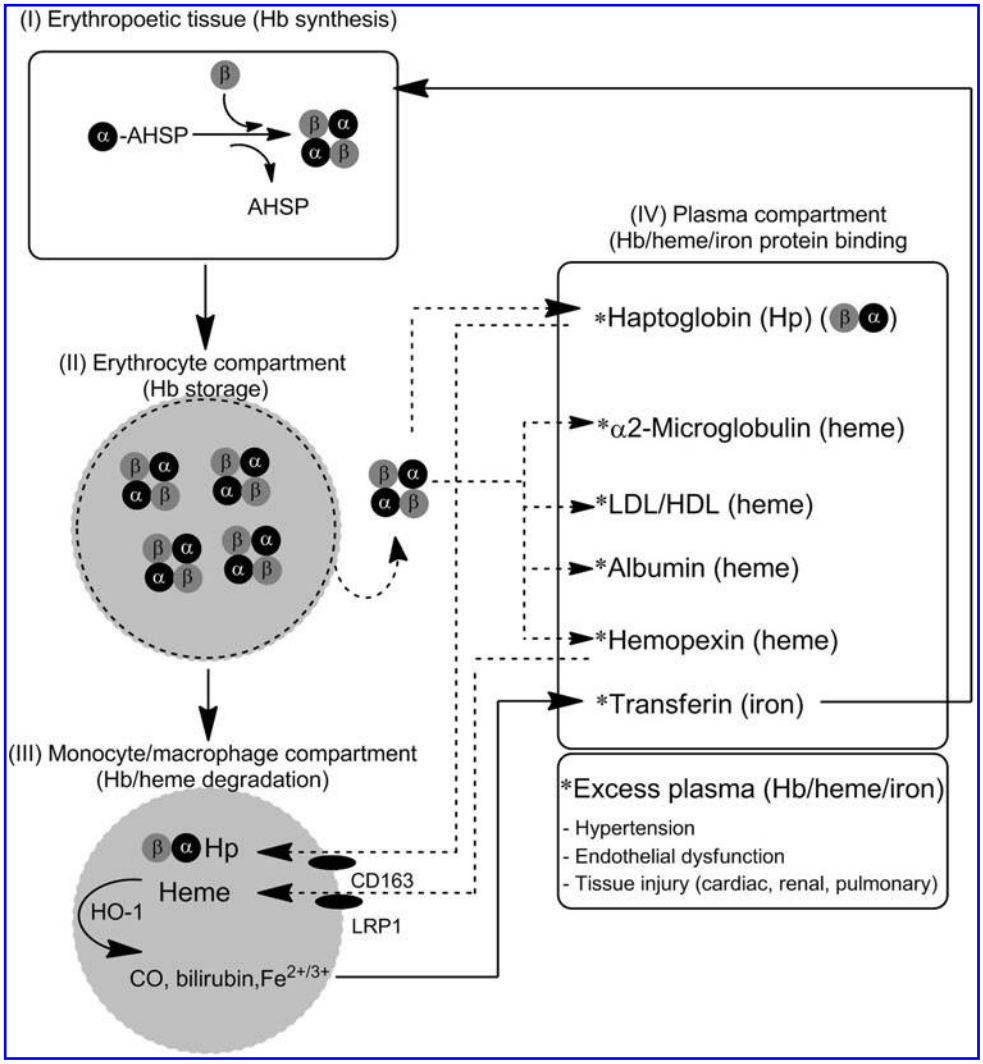

FIG. 3. Protection of hemoglobin from cradle to the grave. Detoxification systems throughout the different compartments in human physiology that provide protection against Hb/heme/iron are outlined. These processes and key proteins that trigger these activities are indicated within each compartment. Alpha hemoglobin stabilizing protein (AHSP) provides protection against oxidative damage to α subunit during early erythropoiesis. Haptoglobin (Hp) and CD163 receptors on macrophages coordinate Hb dimers clearance when Hb is released from aging RBCs or during hemolysis. Once Hb dimers are cleared by macrophages, heme oxygenase (HO) degrades heme released from Hb into iron, bilirubin, and carbon monoxide (CO). When heme is released into plasma, several proteins with different affinities to heme collectively bind and clear heme from circulation. Some of these proteins that are reviewed here include: hemopexin (HPX), high and low density proteins (HDL/LDL), albumin, and α_1- microglobulin (α_1-M). Transport, storage, and metabolism of iron, the by product of heme degradation is briefly outlined here and described fully elsewhere (see (14) for review).

heme loss, because of the sixth coordinate position of the heme iron is occupied and unable to generate ROS (36). It was recently reported that the recovery of this inert form of αHb subunit can be accomplished by further reduction to the ferrous functional form prior to it binding to its subunit counterpart, the β chains of HbA (116).

Similar to AHSP-induced changes within α subunit of Hb detailed above, the reorganization of the heme pocket region into a hexacoordinated configuration via the distal-proximal histidines was also found in naturally occurring neuroglobin (found in neurons) and cytoglobin (expressed in all tissues). It is interesting to note that Hp, similar to AHSP binds to a specific region on the protein close to the heme pocket. However, in the presence of oxidants and unlike ASHP which structurally reorganizes the heme pocket, Hp accomplishes redox stability by short circuiting the emerging and damaging radicals from the heme (Cooper et al., unpublished studies).

AHSP–αHb interactions described thus far may have clinical and biological relevance in β-thalassemia. AHSP has been reported to act as a genetic modifier in this disease. The loss of AHSP may worsen β-thalassemia, most likely by destabilizing excess free αHb, apo-α-globin, or likely both. AHSP also provide a selective advantage for the survival of red cells, especially when there are excess of either α- or β-globin present. Moreover, because of its effects on preventing α-globin denaturation, AHSP may also provide an additional selective advantage to the RBC under conditions of oxidative stress that may lead to hemolysis.

Haptoglobin

The primary mammalian Hb binding proteins circulating in plasma are Hp-α_2–sialoglycoproteins made up of Hp-α and Hp-β globin chains and collectively termed Hp. While all mammalian species possess Hp protein or the gene(s) to express it, only humans are known to express phenotypically differing forms originating from two gene variants (Hp1 and Hp2). Differences occur only in gene variants controlling for Hp-α globin chains and therefore the designation is dependent on the presence of Hp-α^1 or Hp-α^2 globin chains resulting in Hp 1-1 ($\alpha^1\alpha^1$), 2-1 ($\alpha^2\,\alpha^1$), and 2-2 ($\alpha^2\alpha^2$) (62). Hp-α globin chains are primarily involved in disulfide bond formation with Hp-α^1

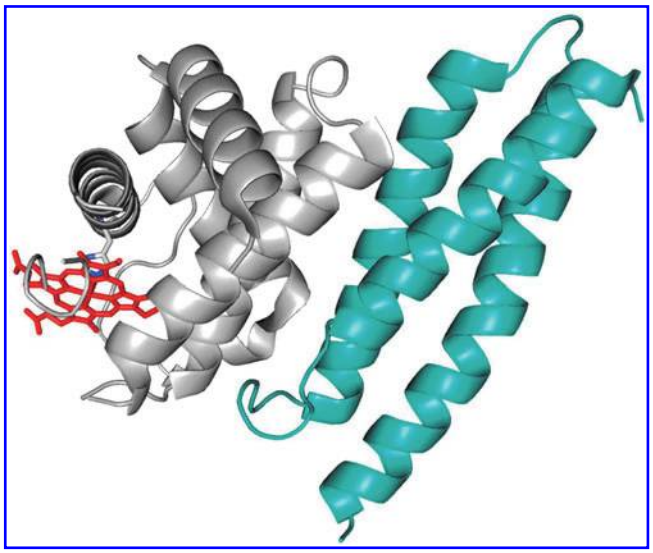

FIG. 4. Crystal structure of alpha-hemoglobin stabilizing protein bound to ferrous hemoglobin alpha-subunit. AHSP on the *right* adopts an elongated three-helix bundle, whereas α-subunit is composed of seven α–helices. AHSP binds αHb on the side of the molecule opposite the heme pocket. In the ferrous–αHb–AHSP complex, The F-helix (*right to the heme*) is distorted and the heme surface is open to interactions with solvent. On oxidation, the iron atom is reformed to generate more oxidatively stable bis-histidyl configuration. The structure was derived from PDB file 1Y01 (35). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

chains forming single disulfide bonds with an adjacent Hp-α chain, and each physically is associated with one Hp-β globin chain. Hp-α^2 chains differ in that they can form two disulfide bonds and are therefore polymeric mixtures with each Hp-α globin associated with one Hp-β globin chain (84). The Hp-β chains are involved in the binding of Hb dimers with contact sites previously identified in both Hb-α and Hb-β globin chains (55, 114). Recent studies with chemically crosslinked and polymerized Hbs have demonstrated the critical role of exposed Hb-α chains in Hp 1-1 and Hp 2-2 binding (23). The binding of Hp 1-1 to human dimeric Hb is reported to occur rapidly ($k = 5.5 \times 10^5\ M^{-1}\ sec^{-1}$) with high affinity and a very small dissociation rate constant, while Hp 2-1 and Hp 2-2 bind Hb dimers more slowly (77). Hp 1-1 is therefore considered to be the most efficient Hb plasma binding protein and can remove Hb in a ratio of 1:1 (Hp 1–1:Hb). Together Hps circulate as mixtures in human plasma in a approximate concentration range from 30 to 200 mg/dl (99). Most mammalian species possess only Hp 1-1 at varying plasma concentrations, only certain nonhuman primates and humans posses additional phenotypes, suggesting a need for evolutionary divergence in non-human primates and humans.

Hp is often termed an "antioxidant protein" based on two critical properties. First, Hp binds free Hb and rapidly directs it toward downstream clearance pathways, preventing tissue distribution. Second, Hp can protect bound Hb's damaging influences in peroxidative environments such that heme emanated radical generation is prevented from causing damage to the Hb protein and the surrounding environment. Previous *in vitro* studies have shown that H_2O_2 induces Hb protein damage is initiated by amino acid oxidation and later heme pocket, α helical structural damage, protein–protein and protein–heme crosslinking with increased H_2O_2 exposure (55). Similar effects are observed *in vivo* within various tissue compartments with exposure to extracellular Hb (21). However, the oxidative effects cause by H_2O_2 are attenuated once the Hb–Hp complex is formed, suggesting a unique antioxidant role of Hp in addition to role as Hb binding protein.

The effective removal of Hb by Hp 1-1 from circulation has been associated with reduced risk long-term vascular-related sequela from numerous pathological conditions having hemolytic components (65). As a result, exogenous administration of Hp used as a Hb scavenging therapeutic could be particularly useful in the event of mild to moderate hemolytic conditions once low levels of endogenous Hp are saturated.

Macrophage scavenger receptor (CD163)

Once extracellular Hb complexes with Hp in circulation, the complex is rapidly cleared by monocyte/macrophage cell surface cysteine rich scavenger receptors identified as CD163 (61). Clearance can take place within the circulation or in the liver; however, the process appears to be saturable and designed to accommodate removal of only complex within a limited but not well-defined concentration range (34). Recent work revealed that when Hb–Hp complex was administered to guinea pigs and dogs at increasing concentrations, the circulating half-life increased by approximately 50-fold, while the nonrenal clearance was decreased by a similar magnitude (19). Therefore, after saturation of CD163, clearance of the complex is dictated by the pharmacokinetics of Hp. Independent of Hp complex formation, Hb can interact directly with CD163 with a low affinity (Kd approximately 400 fold less than Hb–Hp) (80). The process is dependant on direct interaction with N-terminal amino acids of Hb's β globin chain and may be a relevant clearance pathway for extracellular Hb in persons with anhaptoglobinemia (97).

The interaction and uptake of either Hb–Hp or Hb via the CD163 receptor into monocytes/macrophages leads to the breakdown of heme via heme oxygenase (HO). This cascade is the essential next step leading to iron and heme detoxification.

Heme oxygenase

The microsomal heme oxygenase system consists of two primary isoforms including HO-1, HO-2, and a third isoform, HO-3, of little known human relevance. HO-1 is the inducible while HO-2 is the constitutive isoform in mammalian species and both play a critical role in heme metabolism. The stability of heme containing proteins plays a critical role in HO-1 regulation (11) and this process of heme breakdown is initiated by heme in the following sequence:

(1) heme + O_2 + NADPH → Fe^{II} + carbon monoxide (CO) + biliverdin (iron release)
(2) biliverdin → bilirubin (heme catabolism)
(3) Fe^{II} → ferritin → hemosiderin (tissue iron storage)
(4) Fe^{III} → transferrin (plasma iron binding and transport)

In reaction (1), the process is enzymatically controlled by the microsomal heme oxygenase system made up of HO and NADPH-P450 reductase, while reaction (2) is controlled enzymatically by biliverdin reductase. The sequestration and storage of Fe^{II} in process (3) following reaction (1) occurs

initially in ferritin and later as the aggregated ferritin/protein complex, hemosiderin.

The role of HO as a protective enzymatic system is established extensively throughout the literature, and as a result HO has been a target for upregulation in several disease states. The end products of the HO-catalyzed reaction biliverdin and its metabolite bilirubin are believed to possess antioxidant potential by removing reactive oxygen species such as $O_2^{\bullet -}$ and hydroxyl radical (•OH) (104). Additionally, ferritin has demonstrated cellular protective effects via antioxidant activity on the vascular endothelium (10), while CO can function as a vasodilatory gas similar to NO by increasing cyclic guanosine monophosphate (cGMP), leading to decreased vascular resistance (78, 79). However, the majority of scientific effort in the area of HO induction by heme focuses on low and controlled levels which could differ significantly from Hb exposures that can take place during both acquired and genetic hemolytic anemias and when Hb is released from stored RBCs. Figure 5 summarizes the interaction of Hb with Hp, uptake by cell surface CD163 and Hb metabolic breakdown by HO.

Heme Storage and Transport

In circulation, free heme released from Hb upon hemolysis of RBCs is instantly oxidized to its ferric state (often called hemin) which may catalyze the formation of ROS that result in the generation of oxidized forms of low density lipoproteins (52). High and low density lipoproteins (HDL and LDL), hemopexin (HPX), and serum albumin (SA) bind most of the free heme in plasma (Fig. 6). It is known that more than 80% of heme immediately intercalates into LDL and HDL, whereas the remaining ~20% of heme are taken up by albumin and HPX. However, the majority of LDL/HDL-bound heme will be gradually transferred from lipoproteins to HPX and albumin. HPX has a higher binding affinity to heme than albumin does, slowly binds most of heme, and transports it to specific receptors on parenchymal liver cells where it undergoes receptor-mediated endocytosis (51).

The dissociation of heme from Hb, including dissociation from alpha and beta subunits of methemoglobin, and measured rate constants for dissociation and heme transfer to lipoproteins, albumin, and hemopexin have been extensively investigated (see Refs. 24, 45, 38, 46, and 82).

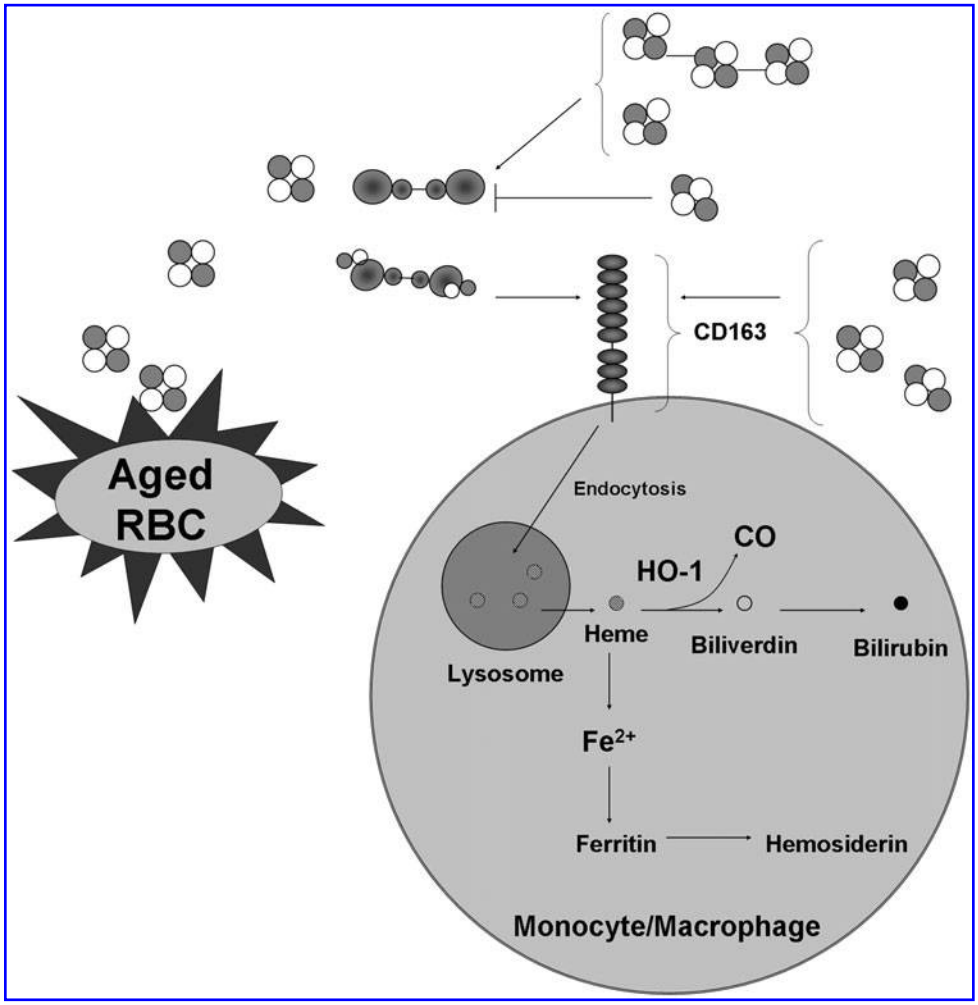

FIG. 5. Clearance of hemoglobin by haptoglobin/CD163. Aged red blood cells represent a potential source of extracellular hemoglobin (Hb) (Tetramer, α-globin, *white circles*; β-globin, *gray circles*). (*Top left*) Hb can then bind to circulating haptoglobin (Hp) (*Center*: *gray-filled oval* in shape of a dumb-bell). Hb is forced to dimerize and bind to Hp, which is then bound to monocyte/macrophage cell surface CD163 (shown as a nine domain cysteine rich scavenger receptor tethered to membrane of monocyte/macrophage) and taken up into the cell. The progression of catabolism of heme (*cross-hatched circles*) and generation of critical by-products (carbon monoxide (CO), biliverdin (*small gray circle*)/bilirubin (*filled circle*)) and sequestration of iron (Fe^{2+}) by binding proteins are shown. *Right top* shows the interaction of chemically modified Hbs with Hp (see (23) for detailed interaction) demonstrating that β crosslinked and polymerized Hbs bind relatively well to Hp, while α crosslinked Hbs do not. The interaction Hb with CD163 in the absence of Hp can proceed at a low affinity with native and crosslinked Hbs but not larger multimeric Hbs (97).

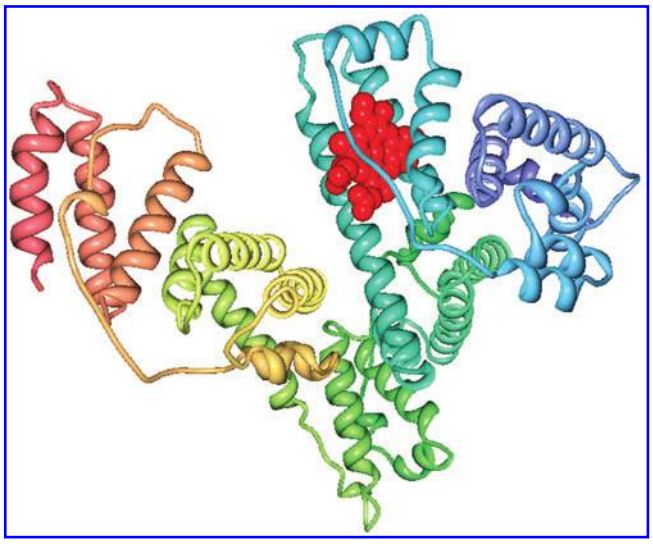

FIG. 6. Albumin and heme complex. The heme albumin complex was derived from PDB crystal structure 109X for the HA–myristate–heme complex (117). Heme (*space-filling representation*) occupies a hydrophobic D-shaped cavity in subdomain 1B, also suitable for binding fatty acids. Heme binding to albumin is stabilized by π–π stacking interaction provided by the two tyrosine residues, Y161 which is in coordination with ferric heme iron, and Y138 whose phenolic hydroxyl is directed to the exterior. Basic amino acid residues H146 and R114 provide additional stabilization via electrostatic interactions and K190 via salt-bridges with heme propionate groups. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Table 2 summarizes the nature of heme linkages in Hb, in the tetrameric and dimeric forms and in those proteins that handle free heme derived from Hb. As can be seen among plasma proteins, HPX has the tightest linkage ($K_a \sim 1.9\times10^{13}$ M^{-1}) with heme via bis-His complex that is stabilized by hydrophobic and electrostatic interactions within the heme pocket. The affinity albumin towards heme is much lower ($K_a \sim 5\times10^{7}$ to 2×10^{9} M^{-1}); however, this has been compensated by a remarkable abundance of albumin.

Hemopexin

HPX serves as the primary specific carrier of plasma heme and participates in its clearance by transporting it to the liver, and thus it functions as a major plasma protector against oxidation because the heme–HPX complex is completely inactive as an oxidant (44). HPX is an acute phase plasma protein, and its plasma levels vary, from 8 μM to 21 μM (102). HPX competes with LDL for heme, and therefore it may also regulate Hb oxidative activity (42). After transporting the heme through a receptor on the parenchymal cell, the intracellular HPX is recycled to its intact free form and released into the blood stream (for review, see Ref. 7).

High and low density lipoproteins

Given the importance of LDL and HDL in cardiovascular diseases, research efforts focused on the investigation of these lipoproteins have provided a wealth of valuable information during the last decade, including crystal structure and biochemical characterization. Earlier studies pointed out that free heme associates with proteins and lipids of the lipoprotein particles at multiple sites (72). However, the transient heme binding to HDL and LDL and the physiological mechanism by which lipoproteins become oxidized remain unclear. Published data suggest that heme released from Hb into the circulation may compromise vascular endothelial cell integrity through oxidative modification of LDL that activates cell protective proteins, HO-1, and ferritin (52). Whereas plasma lipoproteins, HDL and LDL, have a very high affinity to heme (K_d in between 10^{-10} M and 10^{-11} M) and rapidly accept most of it, these plasma compartments are the most sensitive to heme-induced oxidation. It is therefore important that the heme release from HDL and LDL and its transition to HPX proceed faster than the oxidation of lipoproteins triggered by heme (73). Incorporation of heme into the LDL particle results in peroxidation of the hydrophobic core lipids of the lipoprotein, whereas free iron promotes oxidation of the surface lipids only (106). Recently, it was shown that the heme-CO monomer binds to the high affinity site of apolipoprotein B (ApoB) on the LDL particle. Yet the precise mechanism by which heme associates with LDL and HDL remain to be determined [see recent reviews (85,100)].

Serum albumin

Due to its unique structure, albumin possesses remarkable binding capacities for many endogenous and exogenous molecules in the plasma (33). Therefore, it is involved in a variety of metabolic processes, including the maintenance of plasma antioxidant capacity by scavenging ROS and reactive nitrogen species (RNS) and lipid peroxidation byproducts (94)

Table 2. Heme Linkage with Proteins and Lipoprotein

Proteins	*Type of heme linkage (Fe^{II}/Fe^{III} state)*	*K_d constants*
High and low density lipoproteins (HDL/LDL)	Transient binding to the lipoprotein (Fe^{III})	$K_d \sim 10^{-11}$ M to 10^{-10} M[73]
Hemopexin (Hpx)	Hexacoordinate with low spin (Fe^{III})	$K_d: < 10^{-13}$ M[1]
Albumin (SA)	Pentacoordinate with high spin (Fe^{III})	$K_d: \sim 10^{-8}$ M[1]
Heme oxygenase-1 (HO-1)	HO-1 binds and catabolizes heme into biliverdin, free Fe^{II} and CO	N/A[a]
α_1-Microglobulin (α_1M)	Binding at hydrophobic pocket of lipocalin β-barrel (Fe^{II} to 0)	$K_d \sim 10^{-6}$ M[63]

[a]In cooperation with NADPH cytochrome P450 reductase.

and the sequestering of free heme (8). Although the albumin affinity for heme is significantly lower than that of lipoproteins and HPX, due to its abundance, albumin greatly contributes to suppressing of free heme toxicity.

Earlier studies on albumin interactions with heme revealed one primary binding site of high affinity that is specific to heme and several weaker binding sites (15). A crystal structure resolved for albumin–heme–myristate complex (1:1:4) identified a single heme molecule bound to the hydrophobic D-shaped cavity in subdomain 1B, which is known to be a fatty acid binding site, whereas myristate was bound to six other fatty acid binding sites (117). Heme accommodation in this highly hydrophobic binding site is stabilized by π–π stacking interaction provided by the tyrosine residues Y138 and Y161. The position of phenolic hydroxyl group of Y161 implicates coordination with ferric heme iron, whereas the hydroxyl group of Y138 is directed to the exterior solvent. Similar to Hb, but at the lower degree, albumin provides an additional stabilization of heme by a coordination of the propionate groups by basic residues of the binding site: H146 is involved in electrostatic interaction with one of heme carboxylates, the guanidinium group of R114 with the other (2.8 Å), whereas K190 forms salt bridges with both propionate groups (117). Heme binding to albumin is known to be allosterically modulated; it can be inhibited and even prevented by fatty acids binding at multiple sites, including the competitive binding to the heme site.

α_1-Microglobulin

α_1-Microglobulin (α_1-M) is a small glycoprotein (26 kDa) that belongs to the lipocalin superfamily (2, 37). It participates in heme scavenging due to its interactions with heme and Hb (5). This small yellow-brown protein is charge and size heterogeneous. The exact function of α_1-M is not fully understood. α_1-M binds covalently to many plasma proteins (17). In the circulation, about 50% of α_1-M exists in a 1:1 complex with immunoglobulin A (IgA). Whereas free α_1-M undergoes a proteolytic cleavage, the α_1-M/IgA complex serves as a reservoir in which α_1-M is preserved from proteolysis (due to blocked C34 residue) and glomerular filtration (due to its larger size). α_1-M shares a typical sequence motits for lipocalins eight-stranded β-barrel structure that forms a highly hydrophobic pocket suitable for various small ligands (37). The free truncated α_1-M (with C-terminal tetrapeptide cleaved) binds the heme with $K_d \sim 10^{-6}$ *M* (63). The details of the mechanism of heme degradation by α_1-M are unknown. Noteworthy, the truncated α_1-M carries a heterogeneous mixture of yellow-brown chromophores, presumably degradation products of protoporphyrin, that are bound to cysteine and lysine residues at the rim of the binding cavity, that is, C34, K92, K118, and K130 (5, 17).

Whereas the heme scavenging by LDL/HDL, SA, and HPX seems to be sufficient to minimize the levels free heme in plasma, it is not excluded that other plasma proteins, less abundant than SA and less specific to heme than HPX and SA, may also contribute in the maintenance of heme in protein-associated form, at least transiently. For example, heme association with plasma α_1-antitrypsin is of lower affinity than that of SA (unpublished data), yet it illustrates the idea that some other plasma proteins also may interact with free heme.

Conclusion

Hemoglobin enzymatic activities such as NO removal (dioxygenase), NO production (nitrite reductase), and the consumption of H_2O_2 (peroxidase/pseudoperoxidase) can trigger a number of side reactions that can be detrimental to the protein and the surrounding tissues. However, nature has provided an impressive array of proteins that are capable of dealing effectively with released Hb and/or heme as RBC age or during hemolysis due to disease states, therefore detoxifying Hb and its oxidative byproducts. Indeed, we have recently reported that when stroma-free Hb is complexed with Hp, the Hb–Hp complex will lower blood pressure (due to scavenging of NO by Hb) and prevent heme-mediated tissue toxicity (due to oxidative chemistry of Hb) in dogs and in guinea pigs in which their Hp levels were either induced by drugs or co-infused with Hb, respectively (19). Moreover, and surprisingly, the Hb–Hp complex retained its NO and O_2 binding characteristics. It may prove necessary to explore some of theses naturally occurring antioxidative and clearing mechanisms of acellular Hb which have survived selective evolutionary pressures, as a basis of countering the side-effects associated with free Hb in hemolytic anemias, oxygen therapeutics, and possibly in controlling storage lesions rather that relying on short-lived NO-based therapeutics.

References

1. Adams PA and Berman MC. Kinetics and mechanism of the interaction between human serum albumin and monomeric haemin. *Biochem J* 191: 95–102, 1980.
2. Akerstrom B, Logdberg L, Berggard T, Osmark P, and Lindqvist A. alpha(1)-Microglobulin: A yellow-brown lipocalin. *Biochim Biophys Acta* 1482: 172–184, 2000.
3. Alayash AI. Setbacks in blood substitutes research and development: A biochemical perspective. *Clin Lab Med* 30: 381–389, 2010.
4. Alayash AI. Oxygen therapeutics: Can we tame haemoglobin? *Nat Rev Drug Discov* 3: 152–159, 2004.
5. Allhorn M, Berggard T, Nordberg J, Olsson ML, and Akerstrom B. Processing of the lipocalin alpha(1)-microglobulin by hemoglobin induces heme-binding and heme-degradation properties. *Blood* 99: 1894–1901, 2002.
6. Alter HJ and Klein HG. The hazards of blood transfusion in historical perspective. *Blood* 112: 2617–2626, 2008.
7. Ascenzi P and Fasano M. Heme-hemopexin: A 'chronosteric' heme-protein. *IUBMB Life* 59: 700–708, 2007.
8. Ascenzi P and Fasano M. Serum heme-albumin: An allosteric protein. *IUBMB Life* 61: 1118–1122, 2009.
9. Atzil S, Arad M, Glasner A, Abiri N, Avraham R, Greenfeld K, Rosenne E, Beilin B, and Ben–Eliyahu S. Blood transfusion promotes cancer progression: A critical role for aged erythrocytes. *Anesthesiology* 109: 989–997, 2008.
10. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, and Vercellotti GM. Ferritin: A cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 267: 18148–18153, 1992.
11. Balla J, Jacob HS, Balla G, Nath K, Eaton JW, and Vercellotti GM. Endothelial-cell heme uptake from heme proteins: Induction of sensitization and desensitization to oxidant damage. *Proc Natl Acad Sci USA* 90: 9285–9289, 1993.
12. Basran S, Frumento RJ, Cohen A, Lee S, Du Y, Nishanian E, Kaplan HS, Stafford–Smith M, and Bennett–Guerrero E. The association between duration of storage of transfused

red blood cells and morbidity and mortality after reoperative cardiac surgery. *Anesth Analg* 103: 15–20, 2006.
13. Bassumi WY, Blajchman MA, and Al–Moshary MA. Why implement universal leukoreduction? *Hematol Oncol Stem Cell Ther* 1: 106–123, 2008.
14. Beaumont C and Delaby C. Recycling iron in normal and pathological states. *Semin Hematol* 46: 328–338, 2009.
15. Beaven GH, Chen SH, d' Albis A, and Gratzer WB. A spectroscopic study of the haemin–human-serum-albumin system. *Eur J Biochem* 41: 539–546, 1974.
16. Bennett–Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, Mulherin MA, Zhu H, Buck RD, Califf RM, and McMahon TJ. Evolution of adverse changes in stored RBCs. *Proc Natl Acad Sci USA* 104: 17063–17068, 2007.
17. Berggard T, Thelin N, Falkenberg C, Enghild JJ, and Akerstrom B. Prothrombin, albumin and immunoglobulin A form covalent complexes with alpha1-microglobulin in human plasma. *Eur J Biochem* 245: 676–683, 1997.
18. Bishop C and Suregener D. *The Red Blood Cell. A Comprehensive Treatise* New York: Academic Press; 1964.
19. Boretti FS, Buehler PW, D'Agnillo F, Kluge K, Glaus T, Butt OI, Jia Y, Goede J, Pereira CP, Maggiorini M, Schoedon G, Alayash AI, and Schaer DJ. Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs. *J Clin Invest* 119: 2271–2280, 2009.
20. Brooks JP. Quality improvement opportunities in blood banking and transfusion medicine. *Clin Lab Med* 28: 321–337, 2008.
21. Buehler PW, Abraham B, Vallelian F, Linnemayr C, Pereira CP, Cipollo JF, Jia Y, Mikolajczyk M, Boretti FS, Schoedon G, Alayash AI, and Schaer DJ. Haptoglobin preserves the CD163 hemoglobin scavenger pathway by shielding hemoglobin from peroxidative modification. *Blood* 113: 2578–2586, 2009.
22. Buehler PW, D'Agnillo F, Hoffman V, and Alayash AI. Effects of endogenous ascorbate on oxidation, oxygenation and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and guinea pig. *J Pharmacol Exp Ther* 323: 49–60, 2007.
23. Buehler PW, Vallelian F, Mikolajczyk MG, Schoedon G, Schweizer T, Alayash AI, and Schaer DJ. Structural stabilization in tetrameric or polymeric hemoglobin determines its interaction with endogenous antioxidant scavenger pathways. *Antioxid Redox Signal* 10: 1449–1462, 2008.
24. Bunn HF and Jandl JH. Exchange of heme among hemoglobins and between hemoglobin and albumin. *J Biol Chem* 243: 465–475, 1968.
25. Bunn HF, Nathan DG, Dover GJ, Hebbel RP, Platt OS, Rosse WF, and Ware RE. Pulmonary hypertension and nitric oxide depletion in sickle cell disease. *Blood* 116: 687–692, 2010.
26. Carson JL, Reynolds RC, and Klein HG. Bad bad blood? *Crit Care Med* 36: 2707–2708, 2008.
27. D'Agnillo F and Alayash AI. Redox cycling of diaspirin cross-linked hemoglobin induces G2/M arrest and apoptosis in cultured endothelial cells. *Blood* 98: 3315–3323, 2001.
28. d'Almeida MS, Jagger J, Duggan M, White M, Ellis C, and Chin–Yee IH. A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: Implications for animal models of transfusion. *Transfus Med* 10: 291–303, 2000.
29. Dodd R, Kurt Roth W, Ashford P, Dax EM, and Vyas G. Transfusion medicine and safety. *Biologicals* 37: 62–70, 2009.
30. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, and Lemon DD. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nat Biotechnol* 16: 672–676, 1998.
31. Doyle MP and Hoekstra JW. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem* 14: 351–358, 1981.
32. Dunne J, Caron A, Menu P, Alayash AI, Buehler PW, Wilson MT, Silaghi–Dumitrescu R, Faivre B, and Cooper CE. Ascorbate removes key precursors to oxidative damage by cell-free haemoglobin *in vitro* and *in vivo*. *Biochem J* 399: 513–524, 2006.
33. Fasano M, Curry S, Terreno E, Galliano M, Fanali G, Narciso P, Notari S, and Ascenzi P. The extraordinary ligand binding properties of human serum albumin. *IUBMB Life* 57: 787–796, 2005.
34. Faulstick DA, Lowenstein J, and Yiengst MJ. Clearance kinetics of haptoglobin-hemoglobin complex in the human. *Blood* 20: 65–71, 1962.
35. Feng L, Gell DA, Zhou S, Gu L, Kong Y, Li J, Hu M, Yan N, Lee C, Rich AM, Armstrong RS, Lay PA, Gow AJ, Weiss MJ, Mackay JP, and Shi Y. Molecular mechanism of AHSP-mediated stabilization of alpha-hemoglobin. *Cell* 119: 629–640, 2004.
36. Feng L, Zhou S, Gu L, Gell DA, Mackay JP, Weiss MJ, Gow AJ, and Shi Y. Structure of oxidized alpha-haemoglobin bound to AHSP reveals a protective mechanism for haem. *Nature* 435: 697–701, 2005.
37. Flower DR, North AC, and Sansom CE. The lipocalin protein family: Structural and sequence overview. *Biochim Biophys Acta* 1482: 9–24, 2000.
38. Gattoni M, Boffi A, Sarti P, and Chiancone E. Stability of the heme-globin linkage in alphabeta dimers and isolated chains of human hemoglobin. A study of the heme transfer reaction from the immobilized proteins to albumin. *J Biol Chem* 271: 10130–10136, 1996.
39. Gelderman MP and Vostal JG. Current and future cellular transfusion products. *Clin Lab Med* 30: 443–452, 2010
40. Gelderman MP, Yazer MH, Jia Y, Wood F, Alayash AI, and Vostal JG. Serial oxygen equilibrium and kinetic measurements during RBC storage. *Transfus Med* 20: 341–345, 2010
41. Gladwin MT and Kim–Shapiro DB. Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis. *Curr Opin Hematol* 16: 515–523, 2009.
42. Grinshtein N, Bamm VV, Tsemakhovich VA, and Shaklai N. Mechanism of low-density lipoprotein oxidation by hemoglobin-derived iron. *Biochemistry* 42: 6977–6985, 2003.
43. Gueye PM, Bertrand F, Duportail G, and Lessinger JM. Extracellular haemoglobin, oxidative stress and quality of red blood cells relative to perioperative blood salvage. *Clin Chem Lab Med* 48: 677–683, 2010.
44. Gutteridge JM and Smith A. Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. *Biochem J* 256: 861–865, 1988.
45. Hargrove MS, Singleton EW, Quillin ML, Ortiz LA, Phillips GN, Jr., Olson JS, and Mathews AJ. His64(E7)– > Tyr apomyoglobin as a reagent for measuring rates of hemin dissociation. *J Biol Chem* 269: 4207–4214, 1994.
46. Hargrove MS, Whitaker T, Olson JS, Vali RJ, and Mathews AJ. Quaternary structure regulates hemin dissociation from human hemoglobin. *J Biol Chem* 272: 17385–17389, 1997.

47. Hess JR. Red cell freezing and its impact on the supply chain. *Transfus Med* 14: 1–8, 2004.
48. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, Francis KP, Della–Latta P, Whittier S, Sheth S, Hendrickson JE, Zimring JC, Brittenham GM, and Spitalnik SL. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. *Blood* 115: 4284–4292, 2010.
49. Hogman CF and Meryman HT. Red blood cells intended for transfusion: Quality criteria revisited. *Transfusion* 46: 137–142, 2006.
50. Hrkal Z, Vodrazka Z, and Kalousek I. Transfer of heme from ferrihemoglobin and ferrihemoglobin isolated chains to hemopexin. *Eur J Biochem* 43: 73–78, 1974.
51. Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ, and Moestrup SK. Identification of the receptor scavenging hemopexin–heme complexes. *Blood* 106: 2572–2579, 2005.
52. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, and Balla G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100: 879–887, 2002.
53. Jia Y, Buehler PW, Boykins RA, Venable RM, and Alayash AI. Structural basis of peroxide-mediated changes in human hemoglobin: A novel oxidative pathway. *J Biol Chem* 282: 4894–4907, 2007.
54. Kanias T and Acker JP. Biopreservation of red blood cells: The struggle with hemoglobin oxidation. *FEBS J* 277: 343–356, 2010.
55. Kazim AL and Atassi MZ. Haemoglobin binding with haptoglobin. Localization of the haptoglobin-binding site on the alpha-chain of human haemoglobin. *Biochem J* 197: 507–510, 1981.
56. Kihm AJ, Kong Y, Hong W, Russell JE, Rouda S, Adachi K, Simon MC, Blobel GA, and Weiss MJ. An abundant erythroid protein that stabilizes free alpha-haemoglobin. *Nature* 417: 758–763, 2002.
57. Klein HG. Pathogen inactivation technology: Cleansing the blood supply. *J Intern Med* 257: 224–237, 2005.
58. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, and Blackstone EH. Duration of red-cell storage and complications after cardiac surgery. *N Engl J Med* 358: 1229–1239, 2008.
59. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou–Petersen E, Margaritis LH, and Papassideri IS. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. *J Cell Mol Med* 11: 148–155, 2007.
60. Kriebardis AG, Antonelou MH, Stamoulis KE, Economou–Petersen E, Margaritis LH, and Papassideri IS. RBC-derived vesicles during storage: Ultrastructure, protein composition, oxidation, and signaling components. *Transfusion* 48: 1943–1953, 2008.
61. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, and Moestrup SK. Identification of the haemoglobin scavenger receptor. *Nature* 409: 198–201, 2001.
62. Langlois MR and Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem* 42: 1589–1600, 1996.
63. Larsson J, Allhorn M, and Kerstrom B. The lipocalin alpha(1)-microglobulin binds heme in different species. *Arch Biochem Biophys* 432: 196–204, 2004.
64. Lee JS and Gladwin MT. Bad blood: The risks of red cell storage. *Nat Med* 16: 381–2, 2010.
65. Levy AP, Asleh R, Blum S, Levy NS, Miller–Lotan R, Kalet–Litman S, Anbinder Y, Lache O, Nakhoul FM, Asaf R, Farbstein D, Pollak M, Soloveichik YZ, Strauss M, Alshiek J, Livshits A, Schwartz A, Awad H, Jad K, and Goldenstein H. Haptoglobin: Basic and clinical aspects. *Antioxid Redox Signal* 12: 293–304, 2010.
66. Liao JC, Hein TW, Vaughn MW, Huang KT, and Kuo L. Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc Natl Acad Sci USA* 96: 8757–8761, 1999.
67. Light WR, 3rd and Olson JS. The effects of lipid composition on the rate and extent of heme binding to membranes. *J Biol Chem* 265: 15632–15637, 1990.
68. Low FM, Hampton MB, and Winterbourn CC. Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. *Antioxid Redox Signal* 10: 1621–1630, 2008.
69. Lundberg JO, Weitzberg E, and Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 7: 156–167, 2008.
70. Luten M, Roerdinkholder–Stoelwinder B, Bost HJ, and Bosman GJ. Survival of the fittest? Survival of stored red blood cells after transfusion. *Cell Mol Biol (Noisy-le-grand)* 50: 197–203, 2004.
71. Marik PE and Corwin HL. Acute lung injury following blood transfusion: Expanding the definition. *Crit Care Med* 36: 3080–3084, 2008.
72. Miller YI and Shaklai N. Oxidative crosslinking of LDL protein induced by hemin: Involvement of tyrosines. *Biochem Mol Biol Int* 34: 1121–1129, 1994.
73. Miller YI and Shaklai N. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. *Biochim Biophys Acta* 1454: 153–164, 1999.
74. Mollan TL, Yu X, Weiss MJ, and Olson JS. The role of alpha-hemoglobin stabilizing protein in redox chemistry, denaturation, and hemoglobin assembly. *Antioxid Redox Signal* 12: 219–231, 2010.
75. Nagababu E, Mohanty JG, Bhamidipaty S, Ostera GR, and Rifkind JM. Role of the membrane in the formation of heme degradation products in red blood cells. *Life Sci* 86: 133–138, 2010.
76. Nagababu E and Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: Superoxide production and heme degradation. *Biochemistry* 39: 12503–12511, 2000.
77. Nagel RL and Gibson QH. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J Biol Chem* 246: 69–73, 1971.
78. Ndisang JF, Tabien HE, and Wang R. Carbon monoxide and hypertension. *J Hypertens* 22: 1057–1074, 2004.
79. Ndisang JF, Zhao W, and Wang R. Selective regulation of blood pressure by heme oxygenase-1 in hypertension. *Hypertension* 40: 315–321, 2002.
80. Nielsen MJ, Petersen SV, Jacobsen C, Thirup S, Enghild JJ, Graversen JH, and Moestrup SK. A unique loop extension in the serine protease domain of haptoglobin is essential for CD163 recognition of the haptoglobin-hemoglobin complex. *J Biol Chem* 282: 1072–1079, 2007.
81. Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, and Lemon DD. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med* 36: 685–697, 2004.
82. Pasternack RF, Gibbs EJ, Hoeflin E, Kosar WP, Kubera G, Skowronek CA, Wong NM, and Muller–Eberhard U. Hemin binding to serum proteins and the catalysis of interprotein transfer. *Biochemistry* 22: 1753–1758, 1983.
83. Piknova B, Keszler A, Hogg N, and Schechter AN. The reaction of cell-free oxyhemoglobin with nitrite under

physiologically relevant conditions: Implications for nitrite-based therapies. *Nitric Oxide* 20: 88–94, 2009.
84. Polticelli F, Bocedi A, Minervini G, and Ascenzi P. Human haptoglobin structure and function. A molecular modelling study. *FEBS J* 275: 5648–5656, 2008.
85. Prassl R and Laggner P. Molecular structure of low density lipoprotein: Current status and future challenges. *Eur Biophys J* 38: 145–158, 2009.
86. Raat NJ and Ince C. Oxygenating the microcirculation: The perspective from blood transfusion and blood storage. *Vox Sang* 93: 12–18, 2007.
87. Ramsey G. Frozen red blood cells: Cold comfort in a disaster? *Transfusion* 48: 2053–2055, 2008.
88. Rawn J. The silent risks of blood transfusion. *Curr Opin Anaesthesiol* 21: 664–668, 2008.
89. Reeder BJ. The redox activity of hemoglobins: From physiologic functions to pathologic mechanisms. *Antioxid Redox Signal* 13: 1087–1123, 2010.
90. Reynolds JD, Ahearn GS, Angelo M, Zhang J, Cobb F, and Stamler JS. S-nitrosohemoglobin deficiency: A mechanism for loss of physiological activity in banked blood. *Proc Natl Acad Sci USA* 104: 17058–17062, 2007.
91. Rifkind JM, Ramasamy S, Manoharan PT, Nagababu E, and Mohanty JG. Redox reactions of hemoglobin. *Antioxid Redox Signal* 6: 657–666, 2004.
92. Rifkind JM, Zhang L, Levy A, and Manoharan PT. The hypoxic stress on erythrocytes associated with superoxide formation. *Free Radic Res Commun* 12–13 Pt 2: 645–652, 1991.
93. Roback JD, Combs MR, Grossman BJ, and Hillyer CD. American Association of Blood Banks Technical manual. 2008.
94. Roche M, Rondeau P, Singh NR, Tarnus E, and Bourdon E. The antioxidant properties of serum albumin. *FEBS Lett* 582: 1783–7, 2008.
95. Rose MY, Thompson RA, Light WR, and Olson JS. Heme transfer between phospholipid membranes and uptake by apohemoglobin. *J Biol Chem* 260: 6632–6640, 1985.
96. Rother RP, Bell L, Hillmen P, and Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: A novel mechanism of human disease. *JAMA* 293: 1653–1662, 2005.
97. Schaer DJ, Schaer CA, Buehler PW, Boykins RA, Schoedon G, Alayash AI, and Schaffner A. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. *Blood* 107: 373–380, 2006.
98. Shaklai N, Yguerabide J, and Ranney HM. Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. *Biochemistry* 16: 5585–5592, 1977.
99. Shinton NK, Richardson RW, and Williams JD. Diagnostic value of serum haptoglobin. *J Clin Pathol* 18: 114–118, 1965.
100. Silva RA, Huang R, Morris J, Fang J, Gracheva EO, Ren G, Kontush A, Jerome WG, Rye KA, and Davidson WS. Structure of apolipoprotein A-I in spherical high density lipoproteins of different sizes. *Proc Natl Acad Sci USA* 105: 12176–12181, 2008.
101. Silverman TA and Weiskopf RB. Hemoglobin-based oxygen carriers: Current status and future directions. *Anesthesiology* 111: 946–963, 2009.
102. Smith A and Hunt RC. Hemopexin joins transferrin as representative members of a distinct class of receptor-mediated endocytic transport systems. *Eur J Cell Biol* 53: 234–245, 1990.
103. Sonveaux P, Lobysheva, II, Feron O, and McMahon TJ. Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: Role in oxygen delivery. *Physiology (Bethesda)* 22: 97–112, 2007.
104. Stocker R, McDonagh AF, Glazer AN, and Ames BN. Antioxidant activities of bile pigments: Biliverdin and bilirubin. *Methods Enzymol* 186: 301–309, 1990.
105. Svistunenko DA, Patel RP, Voloshchenko SV, and Wilson MT. The globin-based free radical of ferryl hemoglobin is detected in normal human blood. *J Biol Chem* 272: 7114–7121, 1997.
106. Tribble DL, Krauss RM, Lansberg MG, Thiel PM, and van den Berg JJ. Greater oxidative susceptibility of the surface monolayer in small dense LDL may contribute to differences in copper-induced oxidation among LDL density subfractions. *J Lipid Res* 36: 662–671, 1995.
107. Tsai AG, Johnson PC, and Intaglietta M. Oxygen gradients in the microcirculation. *Physiol Rev* 83: 933–963, 2003.
108. Valeri CR and Hirsch NM. Restoration *in vivo* of erythrocyte adenosine triphosphate, 2,3-diphosphoglycerate, potassium ion, and sodium ion concentrations following the transfusion of acid-citrate-dextrose-stored human red blood cells. *J Lab Clin Med* 73: 722–733, 1969.
109. Weinberg JA, McGwin G, Jr., Marques MB, Cherry SA, 3rd, Reiff DA, Kerby JD, and Rue LW, 3rd. Transfusions in the less severely injured: Does age of transfused blood affect outcomes? *J Trauma* 65: 794–798, 2008.
110. Weiss MJ, Zhou S, Feng L, Gell DA, Mackay JP, Shi Y, and Gow AJ. Role of alpha-hemoglobin-stabilizing protein in normal erythropoiesis and beta-thalassemia. *Ann NY Acad Sci* 1054: 103–117, 2005.
111. Wendel S, Biagini S, Trigo F, Fontao–Wendel R, Taaning E, Jorgensen J, Riisom K, Krusius T, Koskinen S, Kretschmer V, Karger R, Lawlor E, Okazaki H, Charlewood R, Brand A, Solheim BG, Flesland O, Letowska M, Zupanska B, Muniz–Diaz E, Nogues N, Senn M, Mansouri–Taleghani B, Chapman CE, Massey E, Navarrete C, Stainsby D, Win N, Williamson LM, Kleinman S, Kopko PM, Silva M, Shulman I, Holness L, and Epstein JS. Measures to prevent TRALI. *Vox Sang* 92: 258–277, 2007.
112. Winslow RM and Intaglietta M. Red cell age and loss of function: Advance or SNO-job? *Transfusion* 48: 411–414, 2008.
113. Yoshida T, AuBuchon JP, Dumont LJ, Gorham JD, Gifford SC, Foster KY, and Bitensky MW. The effects of additive solution pH and metabolic rejuvenation on anaerobic storage of red cells. *Transfusion* 48: 2096–2105, 2008.
114. Yoshioka N and Atassi MZ. Haemoglobin binding with haptoglobin. Localization of the haptoglobin-binding sites on the beta-chain of human haemoglobin by synthetic overlapping peptides encompassing the entire chain. *Biochem J* 234: 453–456, 1986.
115. Yu X, Kong Y, Dore LC, Abdulmalik O, Katein AM, Zhou S, Choi JK, Gell D, Mackay JP, Gow AJ, and Weiss MJ. An erythroid chaperone that facilitates folding of alpha-globin subunits for hemoglobin synthesis. *J Clin Invest* 117: 1856–1865, 2007.
116. Zhou S, Olson JS, Fabian M, Weiss MJ, and Gow AJ. Biochemical fates of alpha hemoglobin bound to alpha hemoglobin-stabilizing protein AHSP. *J Biol Chem* 281: 32611–32618, 2006.
117. Zunszain PA, Ghuman J, Komatsu T, Tsuchida E, and Curry S. Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. *BMC Struct Biol* 3: 6, 2003.

Address correspondence to:
Abdu I. Alayash, Ph.D., D.Sc.
CBER, FDA
NIH Bldg 29, Rm. 112
8800 Rockville Pike
Bethesda, MD 20892

E-mail: abdu.alayash@fda.hhs.gov

Date of first submission to ARS Central, July 2, 2010; date of final revised submission, Oct 7, 2010; date of acceptance, Oct 14, 2010.

Abbreviations Used

2, 3-DPG = 2, 3-diphosphoglycerate
α_1-M = α_1-microglobulin
ACD = acid citrate dextrose
AHSP = alpha hemoglobin stabilizing protein
AS = additive
CD163 = macrophage scavenger receptor
CO = carbon monoxide
CPDA = citrate-phosphate dextrose adenine
EDRF = endothelial derived relaxing factor
EPR = electron paramagnetic resonance
GPX1 = glutathione peroxidase 1
$Hb^{IV} = O_2^-$, ferryl hemoglobin
$^{\bullet}Hb^{IV} = O_2^-$, ferryl hemoglobin radical
$HbFe^{II}$-O_2 = ferrous (oxy)
$HbFe^{III}$ = ferric hemoglobin
HBOCs = hemoglobin based oxygen carriers
HDL = high density lipoproteins
HO = heme oxygenase
H_2O_2 = hydrogen peroxide
Hp = haptoglobin
HPX = hemopexin
IgA = immunoglobulin A
LDL = low density lipoproteins
MetHb = methemoglobin
NO = nitric oxide
NO_2^- = nitrite
NO_3^- = nitrate
Prx = peroxiredoxin
RNS = reactive nitrogen species
ROS = reactive oxygen species
SA = serum albumin
SNO = s-nitrosolthiol,
TRALI = transfusion-related acute lung injury

This article has been cited by:

1. Elena Karnaukhova, Sonia Silinsky Krupnikova, Mohsen Rajabi, Abdu I. Alayash. 2012. Heme binding to human alpha-1 proteinase inhibitor. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1820**:12, 2020-2029. [CrossRef]
2. Sambuddha Banerjee, Yiping Jia, Claire J. Parker Siburt, Bindu Abraham, Francine Wood, Celia Bonaventura, Robert Henkens, Alvin L. Crumbliss, Abdu I. Alayash. 2012. Haptoglobin alters oxygenation and oxidation of hemoglobin and decreases propagation of peroxide-induced oxidative reactions. *Free Radical Biology and Medicine* **53**:6, 1317-1326. [CrossRef]
3. Binglan Yu, Chong Lei, David M. Baron, Andrea U. Steinbicker, Kenneth D. Bloch, Warren M. Zapol. 2012. Diabetes augments and inhaled nitric oxide prevents the adverse hemodynamic effects of transfusing syngeneic stored blood in mice. *Transfusion* **52**:7, 1410-1422. [CrossRef]