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Toxicological Consequences of Extracellular Hemoglobin: Biochemical and Physiological Perspectives

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Abstract

Under normal physiology, human red blood cells (RBCs) demonstrate a circulating lifespan of ~100–120 days with efficient removal of senescent RBCs taking place via the reticuloendothelial system, spleen, and bone marrow phagocytosis. Within this time frame, hemoglobin (Hb) is effectively protected by efficient RBC enzymatic systems designed to allow for interaction between Hb and diffusible ligands while preventing direct contact between Hb and the external environment. Under normal resting conditions, the concentration of extracellular Hb in circulation is therefore minimal and controlled by specific plasma and cellular (monocyte/macrophage) binding proteins (haptoglobin) and receptors (CD163), respectively. However, during pathological conditions leading to hemolysis, extracellular Hb concentrations exceed normal plasma and cellular binding capacities, allowing Hb to become a biologically relevant vasoactive and redox active protein within the circulation and at extravascular sites. Under conditions of genetic, drug-induced, and autoimmune hemolytic anemias, large quantities of Hb are introduced into the circulation and often lead to acute renal failure and vascular dysfunction. Interestingly, the study of chemically modified Hb for use as oxygen therapeutics has allowed for some basic understanding of extracellular Hb toxicity, particularly in the absence of functional clearance mechanisms and in circulatory antioxidant depleted states. *Antioxid. Redox Signal.* 12, 275–291.

Introduction

Hemoglobin (Hb) is one of the most critical proteins in human physiology. However, the balance between normal physiology and toxicity in the absence of hemoglobinopathy is largely dependent on the physical and biochemical separation between Hb and the extra-erythrocytic environment. Red blood cells (RBCs) uniquely function to protect Hb via a selective barrier allowing gaseous and other ligand transport as well as providing enzymatic mechanisms to maintain Hb in a functional nontoxic state (125). The source of Hb and chemically modified Hb therapeutics mediated toxicity *in vitro* and *in vivo* originates at the heme functional groups that can interact with numerous ligands. In principle these interactions can lead to the formation of ferric (Fe^{3+}), ferryl (Fe^{4+}), ferryl heme radical ($\bullet$H-Fe^{4+}), ferryl protein radical ($\bullet$P-Fe^{4+}), hemichromes, protein radical-induced globin chain cross-links, free heme/iron and nonheme radical species (*e.g.*, lipid peroxides) (1). The pathophysiological relevance of Hb-induced oxidative stress is not well understood, however, many products of Hb oxidation are reported in the literature to occur under *in vitro* or *in vivo* conditions and redox reactions are speculated to be contributing factors to oxidative damage at the vascular endothelium and within tissue. In particular, the interaction with nitric oxide (NO) and heme iron is largely attributed to be the cause of extracellular Hb-induced vasoactivity. However, the full scope of potential mechanisms contributing to Hb-induced hypertension is not completely understood and is likely multifactorial. For example, the physical properties of extracellular Hb, adrenergic/serotonin system interactions, Hb-induced free radicals, prostaglandins, and numerous pathways involving vasoactive peptides likely contribute to the overall hypertensive response to extracellular Hb (23). Moreover, it can not be ruled out that the hypertensive effects of extracellular Hb are pathway specific, depending on the type of vascular tissue. Induction of protective enzyme systems such as heme oxygenase (HO) and ferritin also play a fundamental role in the physiological and pathophysiological responses to extracellular Hb (12, 15). The purpose of the present review is to provide a toxicological perspective of endogenously generated extracellular Hb and exogenously administered chemically modified Hb

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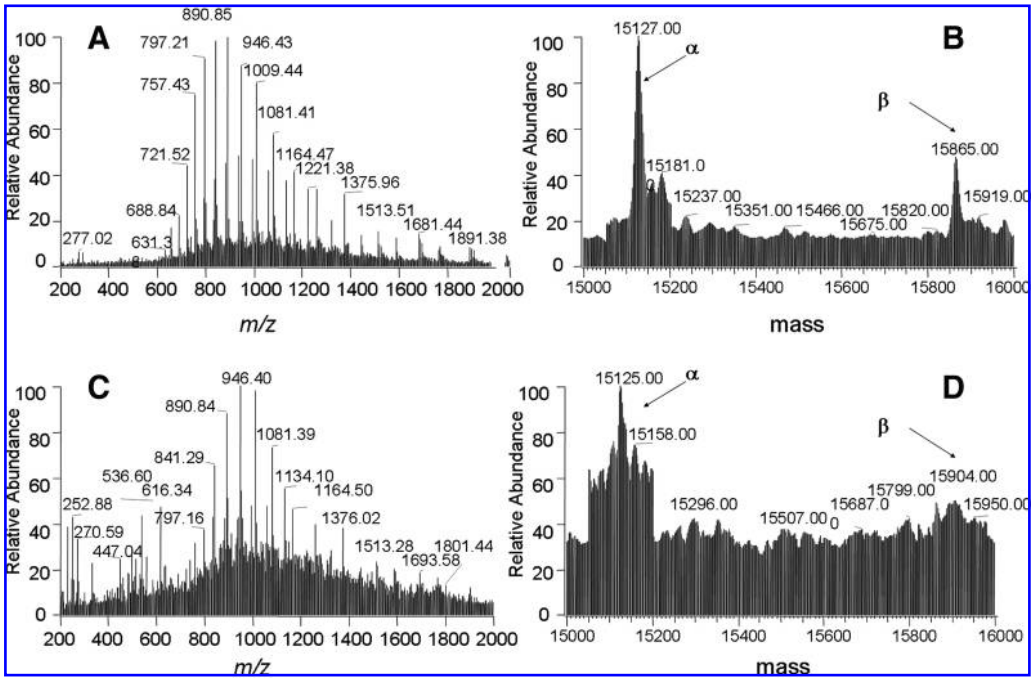

FIG. 1. Mass spectra obtained by LC-electrospray ionization-TOF/MS. (**A**) and (**C**) represent the charge state envelopes of HbA_0 and HbA_0 treated for 1 h at pH 7 with a 2.5-fold bolus of H_2O_2, respectively. The deconvoluted spectra from the acquired data are shown in (**B**) HbA_0 and (**D**) HbA_0:H_2O_2 (1:2.5).

therapeutics. Both sources of extracellular Hb can demonstrate similar *in vitro* and *in vivo* toxicity. Interestingly, chemical modification introduced in Hb often reveals unique characteristics that increase or decrease the toxicity of the native Hb molecule.

Toxicity of Hemoglobin *In Vitro*

Oxidative changes to hemoglobin in the presence of peroxide

The reactions of ferric and ferrous (Fe^{2+}) Hb with prooxidants such hydrogen peroxide (H_2O_2) differ substantially (113, 119). When extracellular Hb reacts with H_2O_2, the formation of a transiently stabilized ferryl heme protein radical (•P-Fe^{4+}) is proposed to occur on the α globin chain's tyrosine 42 (114, 138). The brief stabilization of tyrosyl 42 radicals in adjacent intermolecular α globin chains results in a stable free radical-induced intermolecular α–α globin chain crosslink. This process requires the initial formation of a transient ferryl heme radical (•Heme-Fe^{4+}) generated by the pseudoperoxidative cycle of ferric to ferryl Hb driven by H_2O_2 (28, 115, 116). The process of α globin chain crosslinking preceded by ferryl Hb formation has become a proposed biomarker for the existence of ferryl Hb, a potentially toxic heme iron oxidation state which has been difficult to measure *in vivo* (22, 118, 147). Alternatively, when ferrous Hb is reacted with H_2O_2, a reproducible pattern of β globin chain amino acid oxidations occurs (68). Amino acid oxidations are limited to the β globin chain and include; Cys93(triOx), Cys112(triOx), Met55(diOx), Trp15(diOx and NFK), and several mono- and di-oxidations of histidine residues. Interestingly, no α globin chain amino acid oxidation results from this reaction. The loss of alpha helical structure as determined by circular dichroism and mass spectrometry confirms a loss in β globin conformation. The loss of β globin chain structure is shown following mass spectrometry analysis of purified human Hb (HbA_0) and a 1:2.5 (Hb:H_2O_2) mixture (Figs. 1 and 2). Heme internalization observed as abundant yet nonspecific heme to protein covalent adduct formation and more specific heme-mediated crosslinking occurs between α globin chains at serine 138. Sequencing of two trypsin-generated N-terminal region peptides (α128–α139, $F_{(128)}$LASVSTVLTS*$K_{(139)}$) were found to contain a crosslinker consistent with a modified porphyrin at a mass of 510.95 daltons (68). The involvement of the porphyrin in crosslinking was deduced from mass spectrometry data indicating a complete loss of the heme peak and a shift in the elution time of porphyrin within the total ion chromatogram to co-elute with protein (68). The primary amino acid oxidations of the β globin chain and crosslinking within the α globin chain indicate a reproducible pattern of chemical modification to HbA_0 following bolus exposure to H_2O_2 at physiologic pH (Fig. 3). Haptoglobin (Hp) binding studies revealed that ferric Hb subjected to a range of H_2O_2 concentrations generated end products that were no longer able to bind to Hp or cell surface expressed CD163 (145). This raises a critical question regarding the alternate clearance pathways for extensively damaged Hb and may point toward an investigation of certain Toll-like receptors (TLRs) or pattern recognition receptors such as the receptor for advanced glycation end products (RAGE) (81, 82). Results from the published literature on biochemical toxicity to heme/protein following peroxide reactions with ferric and ferrous Hb are consistent with severe damage to the protein. To this point, it is unclear if these alterations in extracellular Hb are toxic to or protective of the external environment. Interestingly, mammalian and certain plant peroxidases exist with heme cova-

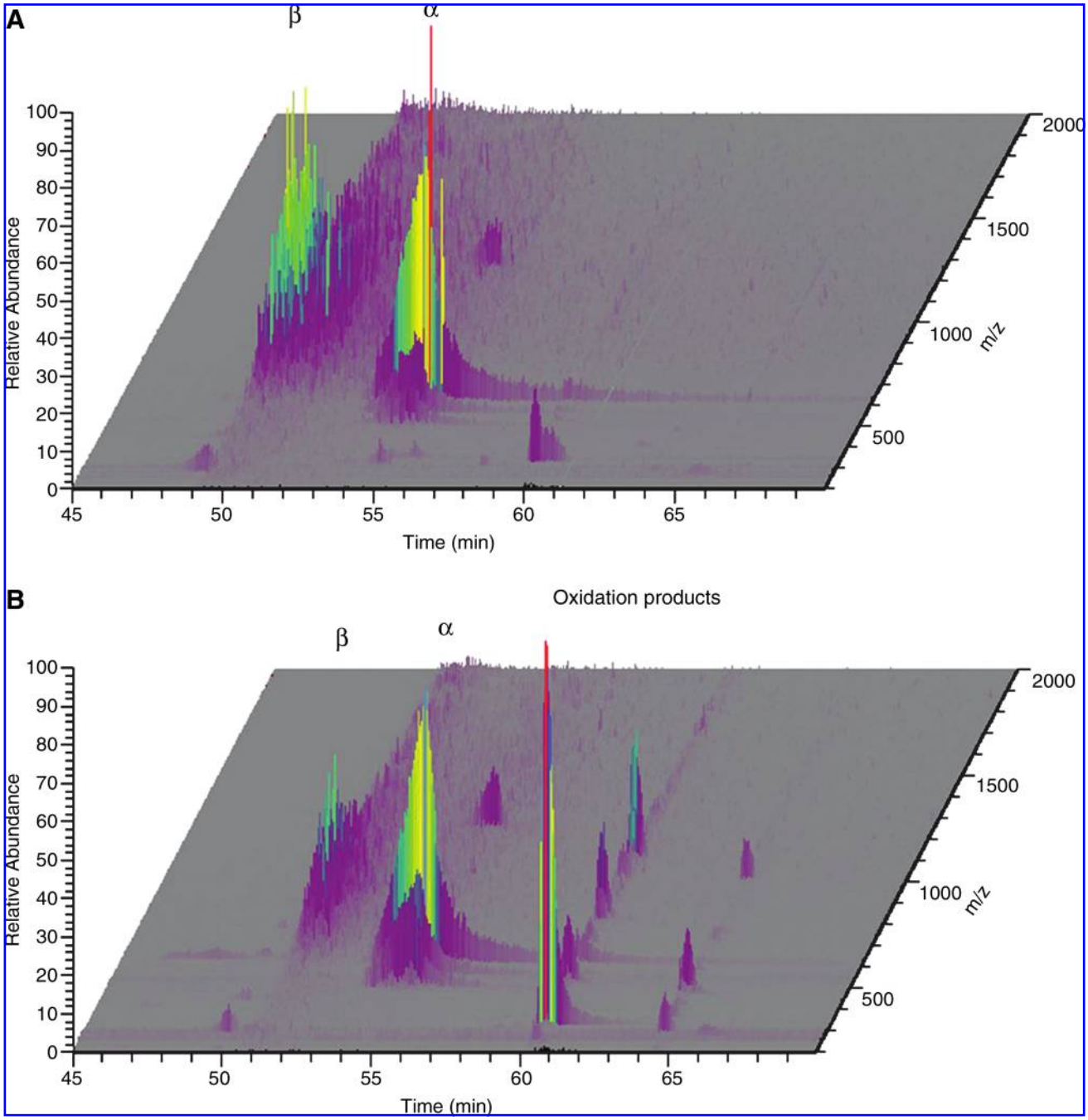

FIG. 2. Three-dimensional LC-electrospray ionization-TOF/MS of (A) HbA_0 and (B) HbA_0 with a 2.5-fold bolus of H_2O_2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

lently bound or adducted within the active site of the enzyme; presumably to prevent damage to the active site from peroxides and reactive byproducts of peroxidase reactions (66, 67, 151). Extracellular Hb *in vivo* exists in both the ferric and ferrous forms, as well as with and without various ligands bound. Therefore, it is possible that many Hb protein modifications identified *in vitro* can be identified within compartments of oxidative stress. Studies using glucose oxidase to generate low continuous fluxes of H_2O_2 have also allowed the redox cycling of ferric and ferryl species to be studied over a longer period of time compared to experiments using bolus H_2O_2 addition, and have also revealed that different chemical modifications can affect the sustainability of these redox reactions (34, 35). Published data suggest that (a) globin chain-induced crosslinking could serve as a protective mechanism to prevent redox cycling of heme iron and prevent the release of toxic radical species, following ferric Hb reaction with H_2O_2 (68); (b) A more complex amino acid oxidation, heme to protein adduct formation and crosslinking appears to occur when ferrous Hb as opposed to ferric Hb is the primary heme

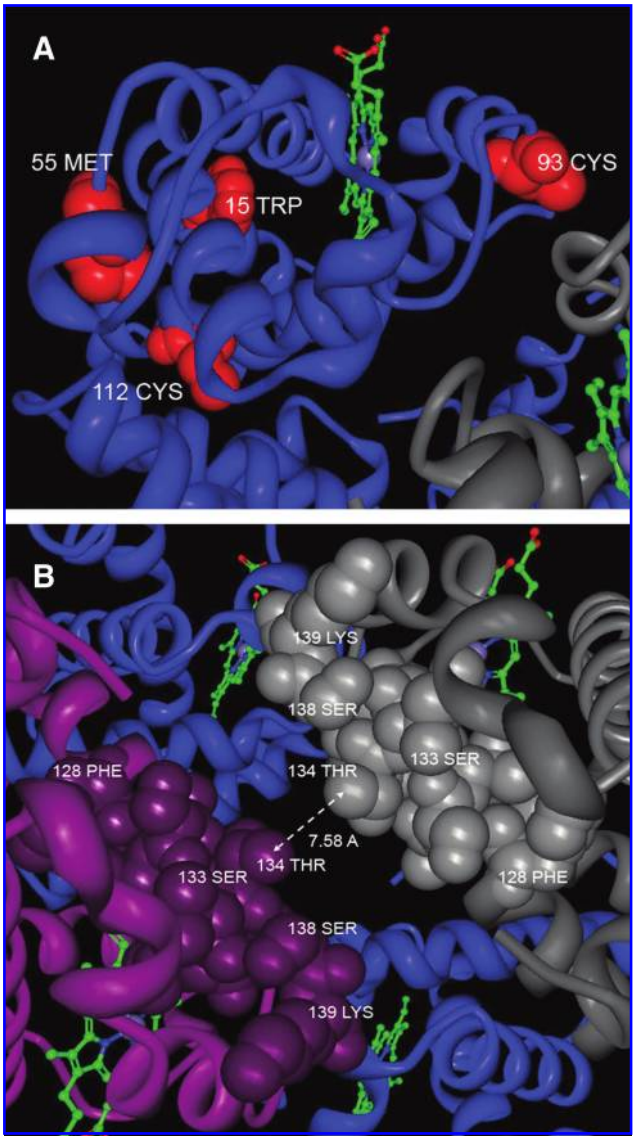

FIG. 3. Ribbon and space filling representations of deoxy HbA_0 (Protein Data Bank code 1GZX). (**A**) The *β* globin chains are shown in *blue* and *α* globin chains are shown in *gray* with oxidized amino acids labeled on the image and shown as *red space filling images* (*β*TRP15), (*β*MET55), (*β*CYS93), and (*β*CYS112). (**B**) The *α* globin chains (*α*1, *purple ribbon* and *α*2, *gray ribbon*) and N-terminal region peptides (*α*128–*α*139, $F_{(128)}LASVSTVLTS^*K_{(139)}$) shown as *purple space filling image* (*α*1) and *gray space filling image* (*α*2). The peptides in the two *α* globin chains of deoxy Hb are 7.58 Angstroms at their closest reactable (*α*1, 134 Threonine (THR) and *α*2, 134 Threonine (THR)). The crosslinker (not shown) is consistent with a modified porphyrin which is reactable at its two carboxyl groups. All hemes are shown as ball and stick images (*green*, carbon), (*blue*, nitrogen) and (*red*, oxygen). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

iron oxidation state exposed to H_2O_2 (68), and (c) detection of amino acid oxidations and crosslink formation may serve as biomarkers for the severity of oxidative insult encountered by extracellular Hb during certain hemolytic states or following the administration of Hb-based therapeutics (22).

Hb-Mediated Endothelial Cytotoxicity and Dysfunction

Acute and repeated long term exposure to Hb aggravates existing atherogenesis and can lead to the onset and progression of endothelial dysfunction, respectively (125, 129). Hb changes the physiology and biochemical behavior of the vascular endothelium and vascular smooth muscle by local oxidative processes and interaction with autacoids that regulate vascular tone. Therefore, endothelial dysfunction is a major concern with chronic hemolytic states, similarly, chemically modified Hb therapeutics with long circulating half lives reach m*M* quantities in plasma and potentially serve as interesting correlates to extracellular Hb exposure and toxicity during extended hemolytic states. Considerable research has focused on the effects of Hb or chemically modified hemoglobin on vascular endothelium.

Endothelial cells are highly susceptible to cytotoxic effects of heme released by Hb (12). Ferric Hb was shown to release heme more readily than ferrous Hb and induced greater endothelial cytotoxicity (9). Activated leucocytes potentiated this effect by oxidizing ferrous Hb to the ferric form (8). Purified Hb or chemically modified hemoglobin in their ferrous forms are not, by themselves, cytotoxic to endothelial cell cultures (33). This contrasts with some studies that have reported that Hb alone activates endothelial cell apoptosis (103). The latter studies, however, employed a commercial Hb with an undetermined purity profile (129), raising the possibility that contaminants contributed to the observed apoptosis. Endothelial cells sustained necrotic injury following treatment with Hb or modified Hb and bolus amounts of H_2O_2 (33). In this setting, ferryl Hb formation correlated directly with the depletion of glutathione (GSH). Endothelial cell culture studies have also employed the glucose oxidase system to enzymatically generate low steady state concentrations of H_2O_2 ranging from 1 to 10 μM that sustained the redox cycling of ferric–ferryl Hb and produced G2/M cell cycle arrest and apoptotic cell death (34) (Fig. 4). This effect was inhibited when the heme iron was blocked with cyanide. The protective effects of catalase, a scavenger of H_2O_2, and ascorbate, a known reductant of the ferryl species, as well as the increased susceptibility of GSH-depleted endothelial cells to ferryl cytotoxicity clearly implicate a role for oxidative stress in this injury process (34, 35). Hb and modified Hbs have also been shown to reduce endothelial barrier function in a manner that correlates with the nature of chemical modification (41). Enhanced autoxidation of chemically modified Hb compared to native Hb correlated with a higher increase in endothelial HO-1 expression (90). Interactions of NO with Hb also modulate endothelial HO-1 induction in a manner that correlates with the redox state of Hb (46).

Toxicity of Hemoglobin *In Vivo*

Animal models

Extracellular Hb is potentially toxic to several organ systems including vascular (8, 9), myocardial (26), renal (99, 110, 111, 128), and CNS (107, 122, 127, 144) tissues. In humans, the extent of toxicity is predominantly dependent on the duration of exposure and the potential for aggravation of co-existing pathophysiology such as atherosclerosis and renal insufficiency. For the purposes of this discussion, the most predom-

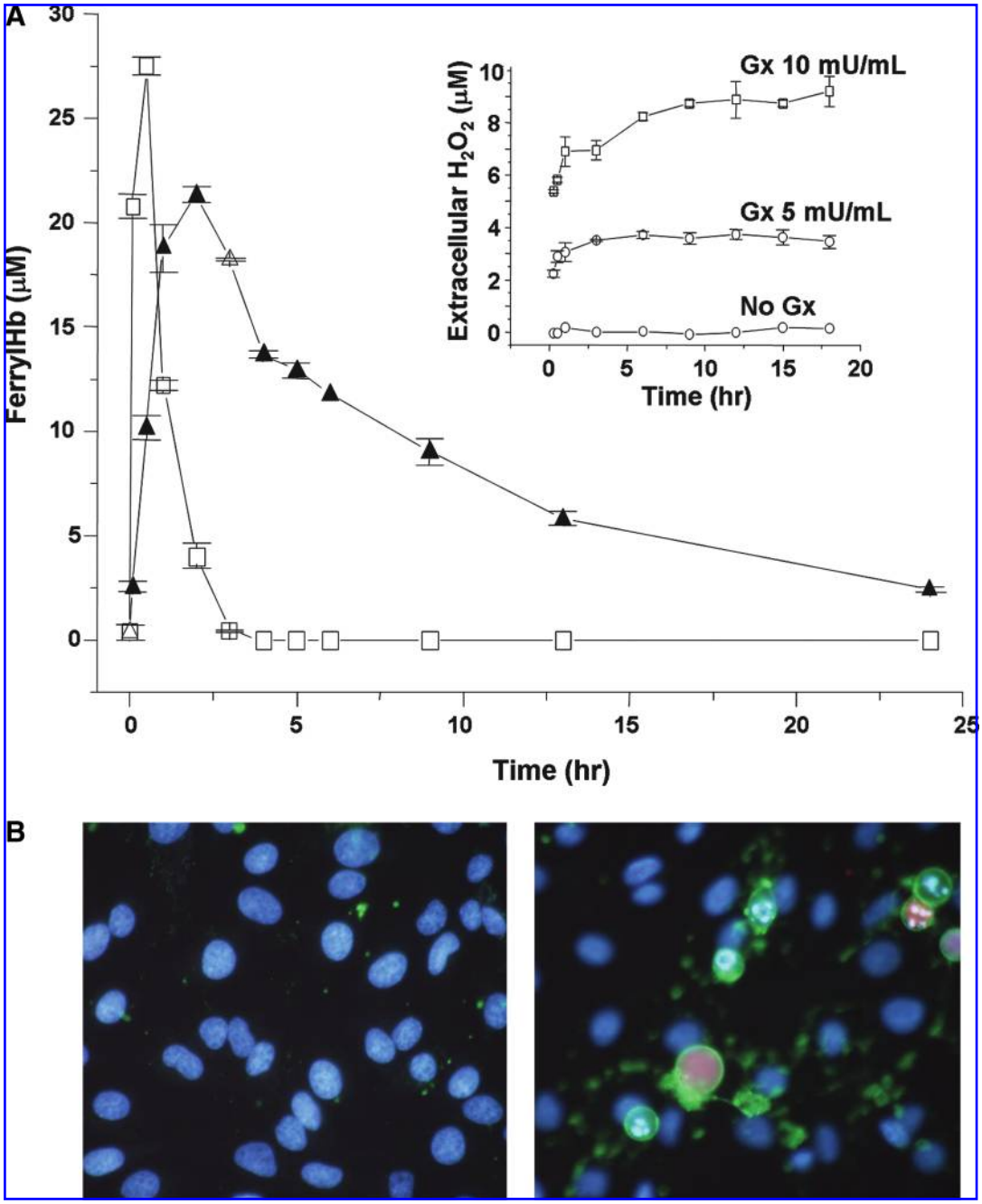

FIG. 4. Generation of hydrogen peroxide, redox cycling of hemoglobin and cellular response. (A) Ferryl Hb formation following the addition of 500 μM H_2O_2 (*open square*) or 10 mU/ml glucose oxidase (*closed triangle*) to medium containing alpha-crosslinked **αα**-DBBF-Hb. Ferryl Hb was measured using the sodium sulfide method. *Inset*: Extracellular accumulation of H_2O_2 over time following addition of 5 or 10 mU/ml glucose oxidase to endothelial cultures. (**B**) Redox cycling of Hb causes endothelial cell death. Control cells or cells treated with αα-DBBF-Hb and glucose oxidase were stained with Hoechst 33342 (*blue*), Alexa Fluor 488 annexin V conjugate (*green*), and propidium iodide (*red*), and visualized by immunofluorescence microscopy. Control cultures showed typical pale blue nuclei staining by Hoechst 33342 and no annexin V or PI staining. Treated cells showed cell rounding, detachment, and condensation of nuclei stained brightly by Hoechst coupled with annexin V plasma membrane staining (apoptosis, *green*) and some PI-stained cells (necrosis, *red*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

inant models of hemolysis in normal and transgenic disease state animals will be discussed.

Sickle cell anemia models have focused on the mechanisms of underlying vascular changes and therapies to reverse sequelae related to sickle cell crisis. The primary goal of creating useful mouse models has been to replace mouse Hb β globin chains with human sickle β globin chains (30, 108, 126). For a detailed comparison of the physiology and pathology associated with various transgenic mouse models see Reference (92). Two examples of predominant and robust models evaluated

in the literature include the S + S Antilles and BERK transgenic sickle cell mouse strains. The S + S Antilles strain are based on C57BL/6 mice but express human α, β^s and $\beta^{s\text{-Antilles}}$ globin transgenes with ~42% of β globin chains expressed as β^s and ~36% as $\beta^{s\text{-Antilles}}$ (15, 16, 43). BERK mice express human α, β^s globin transgenes (15, 108). The BERK mouse represents a severely anemic sickle cell model; however, both transgenic strains experience RBC congestion, inflammation, tissue ischemia, and infarction similar to that observed in sickle cell patients. The advantages of evaluating a less severe model such as the S + S Antilles mouse affords the opportunity to understand oxidative and inflammatory mechanisms underlying the pathology of sickle cell disease. However, most researchers in the field agree that appropriate study designs should rely on two independent transgenic mouse strains as each demonstrates strengths and weakness.

Susceptibility of humans with predominant haptoglobin (Hp) phenotypic prevalence (Hp 1-1 *versus* Hp 2-2 or Hp 2-1 phenotypes) to sequelae of several acute and chronic disease states have been suggested (158). All species carry the Hp 1 gene while humans have evolved to carry an additional Hp 2 gene, allowing for multiple phenotypic combinations such as Hp 1-1, which binds dimeric Hb with high affinity but has a low Hb binding capacity (1:1, Hb:Hp binding). Conversely, Hp 2-2 and 2-1 bind Hb with a lower affinity but a higher capacity (1:1, Hb:Hp binding). Hp 2 phenotypes can more efficiently clear large quantities of Hb to liver parenchymal macrophages compared to the Hp 1 phenotype. The evolutionary need for the emergence of these and more complex phenotypes in humans was presumably driven by disease states such as malaria where high capacity removal of Hb is necessary (6). Interestingly, modern prevalent disease states such as diabetes and atherosclerosis are aggravated by low level hemolysis, and the Hp 1 phenotype is thought to predict better long term prognosis due to more efficient removal of chronic low levels of Hb as previously described by Levy AP *et al*. (76, 77) as well as in the present forum "Haptoglobin: Basic and clinical aspects." As a tool to evaluate Hp phenotype differences and disease progression, the Hp null mice (Hp0), transgenic mice (Hp2), and inbred strains such as the DBA/2 mouse (Hp1) have been used extensively in the evaluation of whole body oxidative stress caused by disease states involving both severe acute and chronic low level hemolysis (3–5, 7, 20, 29, 75, 78–80, 85, 86, 95, 153). Strains of mice vary widely in their expression of Hp. Of the 11 predominant strains of inbred mice, Hp can range from undetectable (AKR and CH3 strains) to 10 mg/mL (DBA/2 strain). Of the 8 remaining strains, plasma Hp levels are ~5 mg/mL (109). Nonetheless, all strains of mice, as well as other mammalian species, carry the gene for Hp 1 with plasma levels varying considerably amongst species. The only species that do not possess a Hp gene are certain birds and reptiles (149); however these species alternatively express a unique Hb binding protein which is potentially similar to soluble CD163. Hp is an acute phase protein which can be induced during inflammatory processes or upon the injection of pro-inflammatory substances. For example, the intramuscular injection of turpentine oil can induce Hp in AKR and C3H mice by 20- to 50-fold. This response can also be observed with injections of India ink, corn oil, paraffin oil, and talcum powder (109, 149). The generation and viability of Hp 0 based on the C57BL/6J background was described by Lim SK *et al*. (79). In the same report an acute drug-induced hemolytic insult created by phenylhydrazine caused death in 55% of Hp (−/−) and only 18% of Hp (+/+) mice, with increased renal tubular necrosis caused by oxidative damage being the primary pathology characterized (79). Interestingly rates of ^{125}I-Hb clearance were similar in both Hp (−/−) and Hp (+/+) mice, suggesting that a mechanism other than Hp was responsible for Hb clearance in Hp (−/−). This observation is likely due to renal clearance mechanisms for Hb that are rapid and high capacity following depletion of Hp (20a). Shortly thereafter, Yang F *et al.* described the expression of the human Hp 2 gene in alveolar macrophages and eosinophils of mice with a CB6F1 background (153). These mice were studied initially to understand the role of Hp in human lung inflammatory diseases and later applied to the study of disease states that lead to endothelial dysfunction, such as diabetes where Hp 1 *versus* Hp 2 phenotypes are believed to have significant impact (3–5, 20, 29, 75, 85, 86). Double knockout mice designed to study heme and Hb toxicity in the absence of hemopexin, the heme binding protein, and Hp are described in the literature and illustrate the importance of the two proteins working in coordination (44, 141, 142, 146).

Animal models designed to evaluate hemodynamic changes as a result of extracellular Hb exposure have employed various species in attempts at elucidating the effects of extracellular Hb on the normal vasculature (39, 87, 88, 155). Additionally, understanding the reversal of Hb-induced hemodynamic aberrations by differing therapeutic agents has been an important purpose for the use of these models. For example, a canine model of acute hemolysis described by Minneci PC *et al.* (87, 88) infused increasing rates of free water at 2, 4, 8, and 16 mL/kg/h. The amounts of free water administered generated plasma concentrations from 20 to 300 μM heme (32–483 mg/dL hemoglobin tetramer). The authors correlated plasma Hb with NO consumption, mean arterial pressure (MAP), and systemic vascular resistance (SVR). The addition of therapies such as nitroprusside and inhaled NO caused a reduction in Hb's vasoactive response, likely based on a complete oxidation of circulating extracellular ferrous Hb to the less NO reactive ferric form. Additional models of intravascular hemolysis have focused on hemodynamic changes by a controlled Hb infusion (20a). By controlling infusions of stroma-free Hb in conscious and anesthetized/instrumented dogs with normal and high haptoglobin levels, consistent concentrations of plasma Hb were achieved. From these studies, an accurate evaluation of hemodynamics could be established and the influence of Hp could be elucidated. Physically or pharmacologically induced hemolysis tends to produce greater variation in extracellular Hb plasma concentrations and generate RBC stoma as well as abnormally shaped RBCs which may contribute to toxicities mistaken for those induced by Hb. In addition, species-specific RBC fragility or deformability may differ from humans; consequently any model of physical or pharmacological induced hemolysis should include a comparison of the animal species RBCs to human RBCs in terms of fragility and deformability. An advantage of these models is that they may be more representative of specific hemolytic disease states. Conversely, models employing direct Hb infusion do not represent specific hemolytic disease states but are favorable for understanding mechanisms of Hb induced toxicity and therapies to attenuate toxicity.

Hemodynamic Changes Induced by Hemoglobin

Infusion of Hb solutions to rodents was first described in the 1920's (13) followed by the administration of a stroma-reduced Hb solution to humans in 1949 (2) and later a stroma-free Hb solution in 1978 (128). In the two later circumstances, infusions of Hb solutions to humans resulted in hypertension, hemoglobinuria, and signs of acute renal failure. One thought prior to exposure of a more refined/purified Hb solution was that residual stroma was leading to adverse hemodynamics and renal toxicity. Nonetheless, this hypothesis was disproven and the Hb molecule outside of the RBC's protective environment was entirely the cause of hemodynamic alterations. After 30 years of research on Hb and Hb therapeutics, there is no absolute mechanism for Hb- induced hypertension. The major hypothesis put forth to explain systemic hemodynamic changes mediated by circulating extracellular Hb focuses on the pharmacologic interaction between Hb and NO (52, 88, 130, 131). However, given the vast range of potential vasoactive substances (*e.g.*, eicosanoids, angiotensin, endothelin, and serotonin), which possess longer pharmacodynamic action when compared to a transient molecule such as NO, it is unlikely that this short-lived diatomic gas is the sole cause of Hb-induced vasoactivity. As a result, other hypotheses describing actions of extracellular Hb on normal physiology must also be considered. Independent of the mechanism(s) driving vasoconstriction induced by extracellular Hb, the results are ultimately limitation of tissue blood flow and tissue oxygenation by RBCs. It is important to note that the development of Hb therapeutics has led to several important hypotheses for mechanisms driving Hb-induced hypertension, including oxygen autoregulatory mechanisms (150), RBC and extracellular Hb physical properties (27), interaction with vasoactive peptides (58, 60, 61, 135) and catecholamine interactions (55–57, 59, 133).

While not considered typical models of hemolysis, the vast number of studies performed to evaluate the hemodynamic toxicity of Hb therapeutics may serve as useful tools in elucidating mechanisms of acute and long term hemolytic events on hemodynamics. Hb therapeutics range in circulating half-life from 4 h to >20 h (45) and therefore may selectively mimic vascular heme/hemoglobin exposure during differing durations of hemolysis. A particularly interesting species-specific response to Hb-based therapeutics is the effects on systolic blood pressure (SBP), diastolic blood pressure (DBP), and MAP. The species-specific effects of a polymerized bovine Hb following a 50% exchange transfusion in conscious male Sprague–Dawley rats and male Hartley guinea pigs are distinct and underline species variation to the pressor effect of Hb (Fig. 5). The baseline blood pressures in each species differ with the rat being greater. However, immediately after the start of transfusion marked increases in the magnitude of change from baseline can be observed between the two species, suggesting that selection of species to mimic human hemodynamic responses to hemoglobin is critical in the evaluation of hemolysis induced hypertension.

Organ Toxicity

The development of Hb therapeutics for the first time has allowed for evaluation of extracellular Hb toxicity under good laboratory practice (GLP). When the results of these studies are contributed to the literature, new light has been shed on potential mechanisms driving the toxicity of Hb. It is important to note that in many toxicology studies evaluating Hb therapeutic safety the common positive control is purified Hb. A unique example related to Hb-induced renal toxicity comes from the work performed Bunn HF *et al*. (24, 25) whose experiments were designed to study renal filtration of Hb; they hypothesized that $\alpha\beta$ dimers of the protein were the principal entities filtered through the glomeruli. In these now classic studies bis(N-maleimidomethyl) ether intramolecularly crosslinked Hb was used as a stabilized tetramer to evaluate the rates of renal excretion of dissociable *versus* nondissociable Hb. As a secondary line of investigation, the authors evaluated this chemically stabilized Hb as a potential volume expanding agent. In this section, we will address Hb-induced organ specific toxicity by referring to the literature and our experimental experiences with both Hb and Hb therapeutics.

Cardiovascular system

Cardiac toxicity (congestive cardiac failure) induced by iron overload is the primary cause of death in thalessemia patients in the absence of adequate chelation therapy (71, 72). In the case of Hb therapeutics and myocardial injury, a disconnect exists in the literature where proof of concept animal models suggest improvement in disease states like myocardial ischemia followed by Hb therapeutic reperfusion (51), while certain animal species exhibit transient myocardial tissue necrosis thought to be related to NO scavenging by Hb (26, 69). Much of the data that exist on Hb myocardial toxicity have been derived from preclinical experience with Diasparin crosslinked Hb ($\alpha\alpha$-DBBF), a bis(3,5-dibromosalicyl) fumarate stabilized Hb tetramer chemically crosslinked between α-globin chains with a four carbon bridge spanning from the α1-Lys 99 to the α2-Lys 99. Following the termination of $\alpha\alpha$-DBBF development, various strategies aimed at understanding myocardial toxicity of $\alpha\alpha$-DBBF from a preclinical perspective were published (26). Characteristics of the myocardial lesions occurring from Hb infusion are primarily focal, but at times there is diffuse degeneration of cardiac myofibers in the left ventricle or the cardiac septum and papillary muscle. The degeneration has been described as coagulative with eosinophilic staining and is believed to resemble anthracycline-type cardiac lesions and catecholamine-induced cardiac lesions. Interestingly, the pathological mechanisms associated with each may be similar in that anthracyclines induce nonresolvable lesions via free radical mechanisms (45), while catecholamine (*e.g.*, epinephrine and norepinephrine) lesions are driven by metabolites such as adrenolutin and adrenochrome which induce reactive oxygen species (14). The lesions caused by $\alpha\alpha$-DBBF are most commonly observed in rhesus monkeys and swine, are reported to affect < 3% of total cardiac tissue, and are resolvable within a 2-week period (26). Nonetheless, the true extent of myocardial involvement can not conceivably be determined by standard pathology evaluation. Lesions were found to occur with a Hb concentration dependence in rhesus monkeys and swine such that administration of 2000 mg/kg or 20 mL of a 10% Hb solution resulted in a mild to moderate pathological grading in 99% of animals dosed (26). While certain species demonstrate variable and inconsistent toxicity (*e.g.*, rabbits and cynomolgus monkeys), other species (mice, rats, and dogs) demonstrate no toxicity (26). The predominant beliefs regarding mechanisms for myocardial

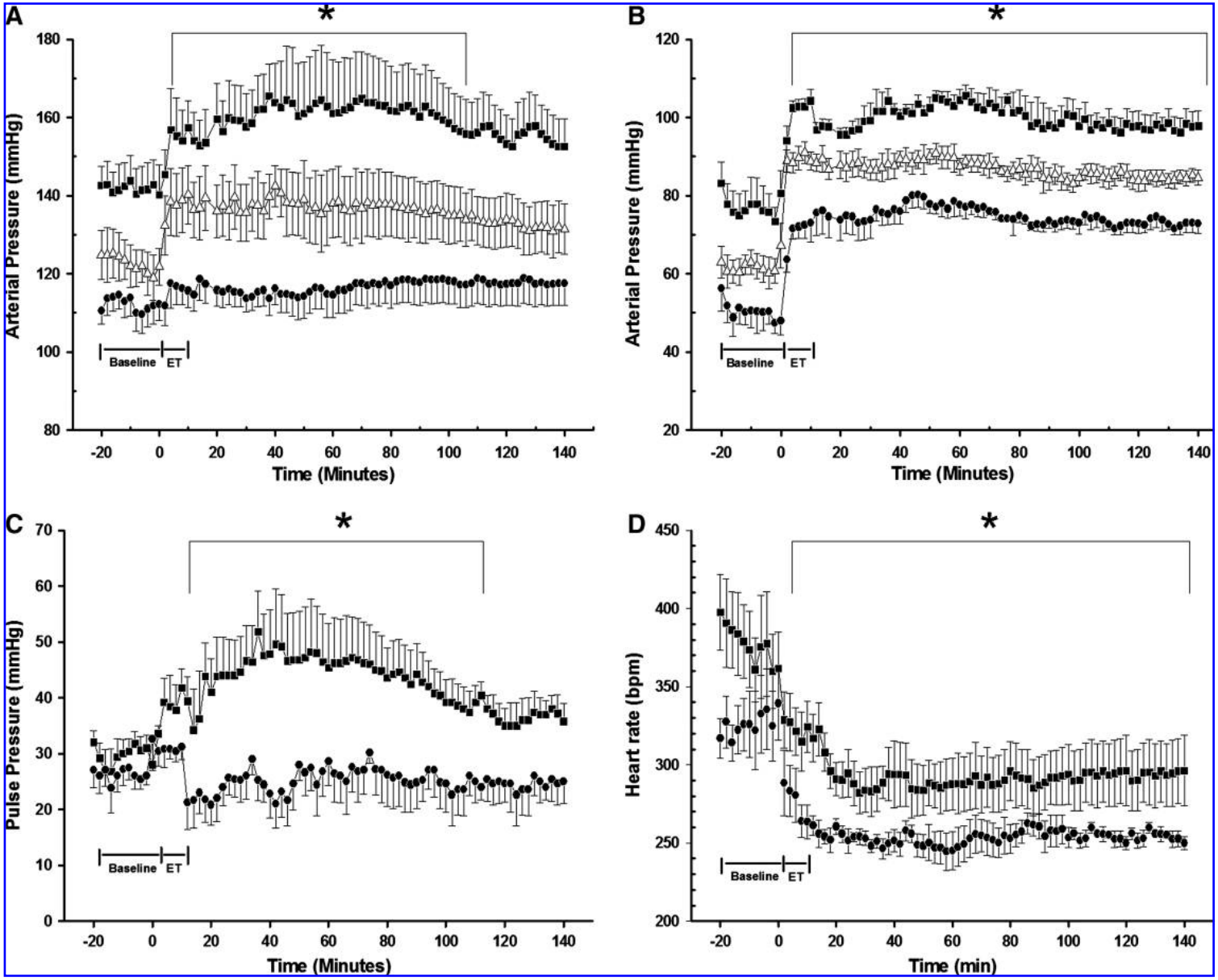

FIG. 5. Blood pressure and heart rate responses in rats and guinea pigs following HBOC transfusion. (**A**) Systolic (*closed square*), diastolic (*closed circle*), and mean arterial blood pressure (*open triangle*) (mmHg) in (A) rats and (B) guinea pigs subjected to a 50% blood for polymerized bovine hemoglobin exchange transfusion. (**C**) Pulse pressure (systolic–diastolic) in rat (■) and guinea pig (●). (**D**) Heart rate (bpm) in rat (■) and guinea pig (●). Data reported as mean ± sem with time frame after exchange transfusion demarcated with an * indicating a significant change from baseline ($P < 0.05$) for mean arterial blood pressure (**A** and **B**), pulse pressure [**C**, guinea pig (●)], and heart rate [**D**, rat (■) and guinea pig (●)].

lesions focused on the interactions of Hb with NO. This appears to be based on the observation that L-NAME caused myocardial toxicity similar to αα-DBBF. This concept was further studied by evaluating the effect of co-administration of several NO donors on lesion formation; however, NO donors were not reported to be effective at attenuating lesions (26). Interestingly, heme was discounted as a source of toxicity based on the inability of cyanomet Hb or co-administration of the iron chelating agent deferoxamine to prevent cardiac lesions. To date, little is known regarding the correlation between Hb-induced myofiber necrosis in animals and reported cardiac events with Hb therapeutics in humans (96). We have evaluated cardiac toxicity in ascorbate and non-ascorbate producing species with a focus on the potential for heme or iron-induced mechanisms of cardiac injury following infusion of Hb (stroma free). Expanding from standard pathology staining methods, our data clearly show cardiac necrosis occurring in the left ventricle of guinea pigs (a non-ascorbate-producing small animal species), with colocalized Perl's iron staining, lipid peroxidation by 4-hydroxynonenal (4-HNE), immunostaining in myofibers as well as the myocardial vasculature (Fig. 6). These observations were consistent

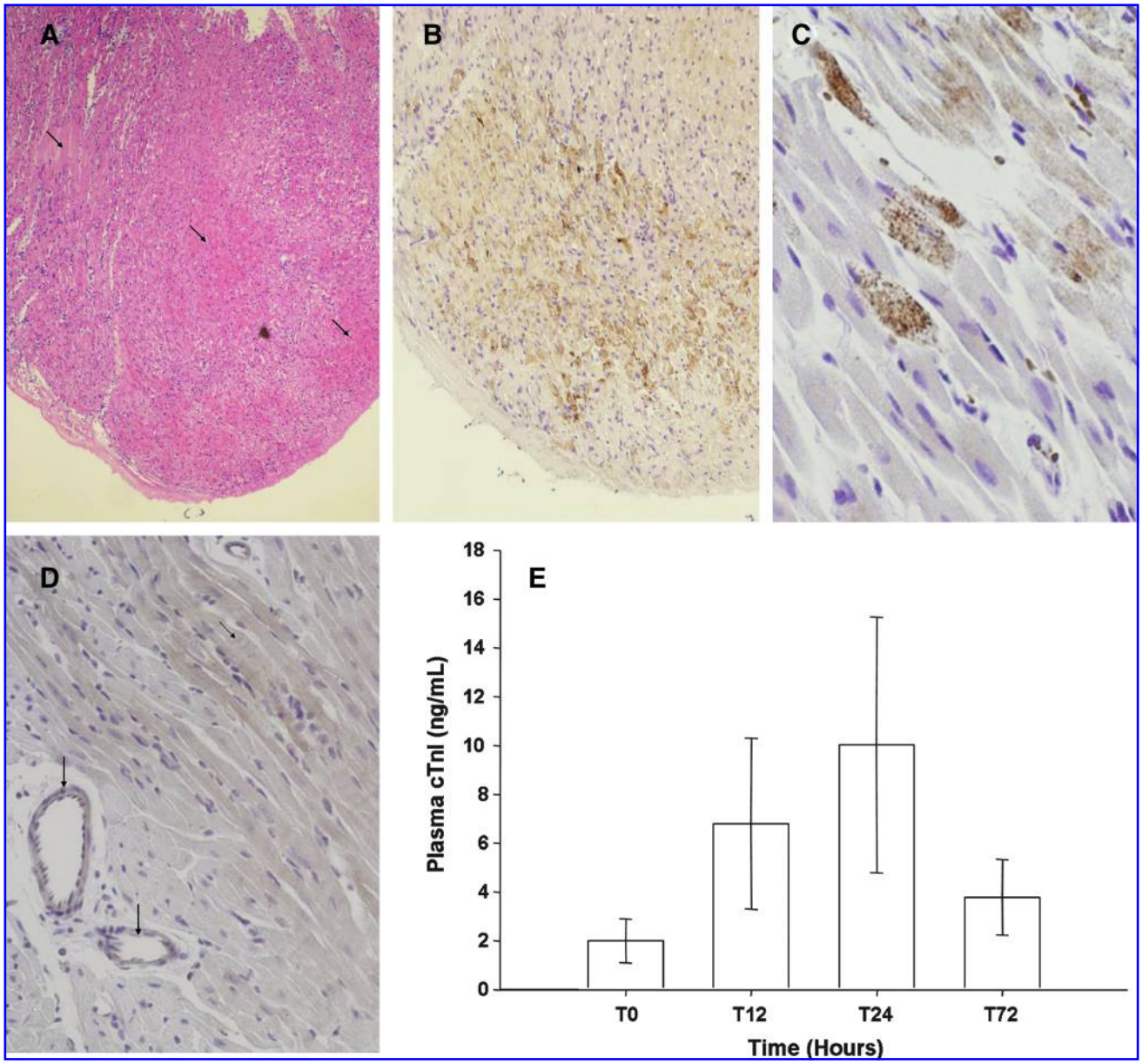

FIG. 6. Cardiac tissue from guinea pigs obtained 24 h following exchange transfusion with a 20% blood for stroma free Hb. (**A**) Hematoxylin and eosin staining showing patches of necrosis (*black arrowhead*) (10×) (**B**, **C**) Sequential tissue slices show colocalization of nonheme iron stained using the Perl's DAB method at (**B**) 10× and (**C**) 40×. (**D**) Immunohistochemical detection of 4-HNE modified protein adducts indicating lipid peroxidation in myocardial tissue and blood vessels (*black arrowheads*). (**E**) Plasma cardiac troponin I (cTnI) determined at baseline, 12, 24, and 72 h post transfusion with polymerized bovine hemoglobin in guinea pigs. Counterstaining was done with hematoxylin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

with the peak cardiac troponin I in the plasma following transfusion with a polymerized bovine Hb (Fig. 6). Vascular changes and endothelial dysfunction are major concerns following the release of Hb into the circulation. The expression of HO-1 in cardiac as well as vascular tissue is consistent with either free heme or Hb entrance into the endothelial or subendothelial space and may be a useful biomarker for acute or continuous exposure to extracellular Hb (Fig. 7).

Central nervous system

Hb-induced neurotoxicity is a major factor in the pathophysiology of intracerebral and subarachnoid hemorrhage. The lysis of extravasated RBCs over a period of hours or days can expose the CNS to significant quantities of Hb or its breakdown products. Concerns over Hb neurotoxicity in the Hb therapeutic field are such that traumatic brain injury (TBI) has remained an exclusionary criterion in clinical trials involving these agents in the United States. Oxidative stress generated from Hb or its breakdown products has long been proposed as an underlying mechanism of Hb-induced CNS damage (127). The link between Hb and vasospasm in the cerebral circulation following subarachnoid hemorrhage has also received significant attention (21, 83, 105). Cultured neurons are susceptible to Hb-induced injury (74, 122). In cocultures of neurons and astrocytes, Hb was toxic to neurons but not astrocytes at relatively low micromolar concentrations (122). Moreover, at subtoxic concentrations in neuronal cultures, Hb increased neuronal susceptibility to excitotoxic injury (123). Significant research has been dedicated to understanding the regulation of HO and other heat-shock genes and their role in modifying neuronal responses to Hb- or heme-induced injury. HO activity, primarily derived from the HO-2 isoform, is exceptionally high in normal brain tissue. HO-1 induction occurs in microglia, astrocytes, and some neurons following subarachnoidal hemorrhage, ischemia, and traumatic brain injury, and in human neurodegenerative diseases. Injection of Hb or blood upregulates HO-1 mainly in microglia throughout the brain, implying that microglia take up extracellular Hb (84). Co-localization of HO-1 and microglia/macrophages in hemorrhagic lesions in human brains were detected as early as 6 h following TBI (18). HO activity has been implicated in the protection of astrocytes and cerebral endothelial cells, but may promote neuronal cytotoxicity (70, 121). Adenoviral transfer of the HO-1 gene provided protection against heme-induced injury to astrocytes and reduced Hb-mediated vasospasm *in vivo* (17, 105, 140). Approaches to selectively transfer of the HO-1 gene to specific cell types like astrocytes may hold interesting promise, given the protective actions afforded by astrocytic HO-1 (17). Paradoxically, some evidence indicates HO-1 may exacerbate early brain injury after intracerebral hemorrhage, and intracerebral injection or systemic administration of HO inhibitors has been shown to reduce Hb- and intracerebral hemorrhage-induced brain edema in rats and pigs (54, 70, 148). HO-2 gene deletion studies showed reduction in hemin-mediated oxidative injury in neurons (120). The latter observations implicate neuronal HO-2 activity as a potential contributor to Hb- or heme-induced toxicity. Iron accumulates in the brain after intracerebral hemorrhage and that systemic deferoxamine reduces brain edema and neurological deficits in this setting (64, 65, 94). Iron chelators such as deferoxamine are also known to protect against oxidative injury via mechanisms independent of iron binding (32, 117). These studies and others suggest that HO-generated iron may be an important causative factor and a possible target for therapeutic intervention in Hb-related CNS disease states (50, 132).

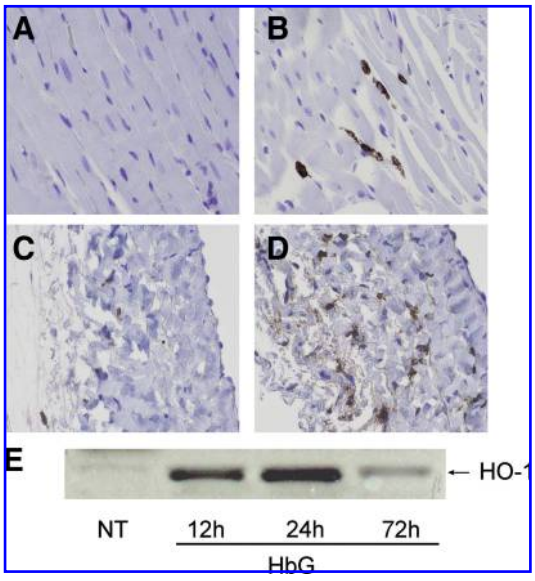

FIG. 7. HO-1 expression following exchange transfusion with polymerized bovine hemoglobin (HbG). Nontreated rat myocardial tissue (**A**) and aorta (**C**) and HbG treated rat myocardial tissue (**B**) and aorta (**D**). HO-1 immunoreactivity is localized primarily in aortic adventitia. (**E**) Time course of HO-1 expression by Western blot analysisin rat heart following HbG transfusion. Counterstaining was done with hematoxylin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Renal system

Renal injury mediated by Hb or its breakdown products has been studied under a number of different settings (143). With respect to Hb infusion models, important distinctions must be made between early studies that employed impure Hb solutions *versus* studies with purified Hb and modified Hb preparations as contaminants such as stromal phospholipids and endotoxin produced significant renal dysfunction (62, 110, 128). Filtered Hb is taken up by proximal tubular cells or excreted once tubular reabsorption capacity is exceeded. Processes that accompany or are thought to contribute to Hb-induced renal injury or dysfunction include (a) tubular obstruction caused heme protein precipitation and tubular cast formation (Fig. 8), (b) ischemic injury resulting from increased renal vascular resistance and vasoconstriction, and (c) heme iron-driven oxidative injury. Renal protection afforded by desferroxamine in some settings have provided some support for the role of iron-mediated renal damage (106). Others have shown that Hb-induced renal injury was exacerbated by aciduria which promotes ferric Hb formation whereas alkaline conditions attenuated injury, suggesting the nature of renal injury may also be dependent on the redox state of Hb (156). Extensive studies have focused on the role of HO-1 in renal protection from Hb- or heme-induced damage (101,

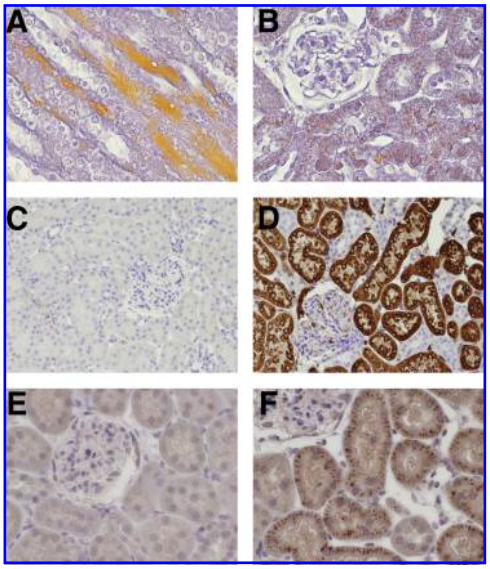

FIG. 8. Renal effects of polymerized bovine hemoglobin in rats. Histochemical detection of HbG by Okajima staining in (**A**) tubular casts and (**B**) proximal tubular cells. Renal HO-1 expression in (**C**) nontreated and (**D**) HbG-treated rats. Immunohistochemical detection of 4-HNE protein adducts in (**E**) nontreated and (**F**) HbG-treated rats. Counterstaining was done with hematoxylin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

143). Filtered Hb induces a remarkable induction of HO-1 in proximal tubular epithelium (Fig. 8). Besides its important role in catabolizing toxic heme, HO generates bile pigments and CO that have antioxidant, anti-apoptotic, vasodilatory, and anti–inflammatory properties. Induction of HO-1 is typically accompanied by ferritin upregulation as a mechanism to sequester liberated iron (11). Evidence for Hb- and/or heme-induced renal oxidative damage to proteins, lipids, and nuclei acids has also been described (100, 143) (Fig. 8).

Pulmonary system

Exposure to extracellular Hb increases pulmonary arterial pressure particular in patients with chronic hemolysis and this has in part been correlated clinically with Hb levels in the plasma (37). Therefore, it is likely the long term and repeated exposure to Hb associated with genetic anemia that induces changes in vascular compliance, elasticity, distensibility, and stiffness (102, 134). Autacoid dysregulation (31, 48, 49) and oxidative stress (63, 124) are likely important in the overall pathology. Short-lived pulmonary hypertensive effects have been observed with administration of certain Hb therapeutics (38) and during acute hemolytic events (157). Interestingly, evaluation of pulmonary parenchyma and stroma following acute administration of Hb-based therapeutics in animals may be associated with acute tissue injury. Similar to other organs such as kidney and heart, intravascular administration of extracellular Hb and Hb therapeutics activate pulmonary HO-1 expression (10, 36, 73). Using a 50% exchange transfusion model with polymerized bovine Hb, we observed intense HO-1 expression and iron accumulation primarily in alveolar macrophages in rat lung within the first 12 h post-transfusion, while 4-HNE protein modifications were observed in pulmonary epithelial cells within the same time frame (Fig. 9). Alterations in lung architecture and extravasation were also notable within the first 12 h but appeared to resolve by 24 h. Others have shown that infusion of ferric Hb produced a more intense pulmonary HO-1 response than ferrous Hb and proposed that this may be related to the greater propensity of ferric Hb to release heme (10).

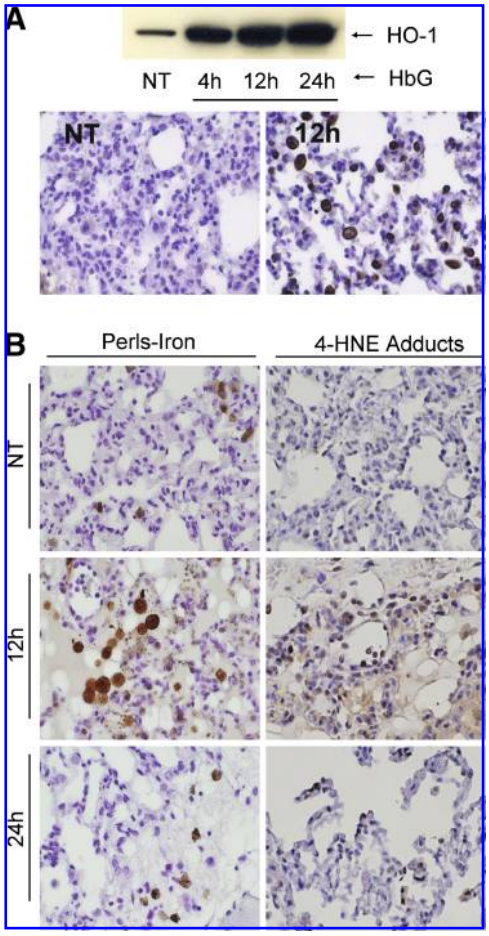

FIG. 9. HO-1 induction, iron deposition, and 4-HNE protein adducts in rat lung following exchange transfusion with polymerized bovine hemoglobin (HbG). (**A**) Pulmonary HO-1 expression by Western blot analysis (*top*) and immunohistochemistry in nontreated (NT) (*bottom, left*) and HbG-treated rats 12 h after transfusion (*bottom, right*). Notable HO-1 immunoreactivity mainly associated with alveolar macrophages following transfusion. (**B**) Perls iron staining with DAB intensification in nontreated (NT) rats and 12 and 24 h following transfusion (*left panel*). Enlarged iron-laden alveolar macrophages and altered pulmonary architecture are notable at 12 h but less evident after 24 h. 4-HNE protein adducts immunoreactivity in nontreated (NT) rats and 12 and 24 h following transfusion (*right panel*). 4-HNE protein modifications in pulmonary epithelial cells are observed at 12 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Potential Methods for Reducing Toxicity

Treatments for acute hemolytic events and chronic genetic hemolytic disorders are limited. Blood transfusions remain

the standard treatment option, particularly in the event of acute hemolysis. In the event of autoimmune hemolytic anemia when transfusion is not effective, Hb therapeutics have been used in some circumstances to successfully restore oxygen delivery and to avoid continuous immune mediated RBC lysis (91). The use of corticosteroids to induce endogenous Hb removal systems (Hp and CD163) has been demonstrated (152). Alternatively, the direct administration of haptoglobin has demonstrated effectiveness in several cases reported from Japanese medical centers with a range of hemolytic etiology (42, 93, 139, 154). Additionally, animal studies have established renal protective effects following Hp infusion during acute hemolysis (104).

Prevention and treatment options for chronic hemolysis in sickle cell anemia and other genetically acquired anemias are also limited. The induction of fetal Hb (Hb F) is the primary focus of small molecules such as 5-azacytidine, butyrates, and hydroxyurea in sickle cell disease (112). Today only hydroxyurea is FDA approved with an indication for the prevention of sickle cell crisis. Interestingly, as many as 40% of responders to hydroxyurea experience benefit absent of increases in Hb F (136). This differential efficacy is thought to be caused by hydroxyurea-induced NO production (53). To this end, other NO based therapies have been studied and used clinically for sequelae of sickle cell anemia. Most notable is inhaled NO gas (INOmax®,100 and 800 ppm) for the treatment of pulmonary hypertension originating from the work of Zapol W *et al.* (19, 47, 137). INOmax is currently FDA approved for term and near term neonates with hypoxic respiratory failure associated with pulmonary hypertension. Additional, NO-based therapies such as L-arginine (40, 89), phosphodiesterase-5-inhibitors (40), and sodium nitrite (87) have been proposed for use in both acute and chronic conditions of hemolysis. Heme-based therapy designed to stimulate HO-1 and potentially trigger CO-mediated vasodilation has also been proposed (9, 12, 15, 97, 98).

Conclusions

The toxicities of extracellular Hb are numerous and the influences of extracellular Hb are observed in a multitude of disease states. Therefore, normal RBC function and intact Hb clearance pathways such as Hp, monocyte/macrophage CD163, HO-1, ferritin, and hemopexin are critical to maintaining protection from the inherently toxic effects of heme and Hb. Heme functional groups are the center of Hb toxicity and can convert to numerous oxidation states and abnormal globin chain structure. Hb and its oxidative byproducts all uniquely contribute to toxic events *in vitro* and *in vivo*. Hb is also notorious for causing vascular changes, the most common and well known being hypertension driven by vascular resistance. Taken together, both oxidative and vasoactive properties of Hb likely encompass the majority of toxicity associated with this important oxygen carrying protein.

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Abbreviations Used

CNS = central nevous system
cTnI = cardiac troponin I
Cys = cysteine
αα-DBBF = diasparin crosslinked hemoglobin
DBP = diastolic blood pressure
Fe = iron
Fe^{2+} = ferrous iron
Fe^{3+} = ferric iron
Fe^{4+} = ferryl iron
GLP = good laboratory practice
GSH = reduced glutathione
Hb = hemoglobin
•H-Fe^{4+} = ferryl heme radical
4-HNE = 4-hydroxynonenal
H_2O_2 = hydrogen peroxide
HO-1 = heme oxygenase
Hp = haptoglobin
LC-electrospray ionization-TOF/MS = liquid chromatography electrospray ionization time of flight mass spectrometry
L-NAME = L-nitro-arginine methyl ester
MAP = mean arterial pressure
Met = methionine
NFK = n-formylkynurenine
NO = nitric oxide
•P-Fe^{4+} = ferryl protein radical
RAGE = receptors for advanced glycation products
RBC = red blood cell
SPB = systolic blood pressure
SVR = systemic vascular resistance
TLRs = toll-like receptors
Trp = tryptophan

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