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Review

Redox Reactions of Hemoglobin and Myoglobin: Biological and Toxicological Implications

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ABSTRACT

Direct cytotoxic effects associated with hemoglobin (Hb) or myoglobin (Mb) have been ascribed to redox reactions (involving either one- or two-electron steps) between the heme group and peroxides. These interactions are the basis of the pseudoperoxidase activity of these hemoproteins and can be cytotoxic when reactive species are formed at relatively high concentrations during inflammation and typically lead to cell death. Peroxides relevant to biological systems include hydrogen peroxide, lipid hydroperoxides, and peroxynitrite. Reactions between Hb/Mb and peroxides form the ferryl oxidation state of the protein, analogous to compounds I and II formed in the catalytic cycle of many peroxidase enzymes. This higher oxidation state of the protein is a potent oxidant capable of promoting oxidative damage to most classes of biological molecules. Free iron, released from Hb, also has the potential to promote oxidative damage via classical "Fenton" chemistry. It has become increasingly evident that Hb/Mb redox reactions or their by-products play a critical role in the pathophysiology of some disease states. This review briefly discusses the reactions of Hb/Mb with biological peroxides, potential cytotoxicity and the impact of these interactions on modulation of cell signaling pathways regulated by these reactive species. Also discussed in this article is the role of heme-protein chemistry in relation to the toxicity of hemoproteins. Antioxid. Redox Signal. 3, 313–327.

INTRODUCTION

HEME-BASED OXYGEN BINDING and delivery proteins are found in a wide variety of organisms, including most vertebrates and a number of invertebrate species. In mammals and many other higher vertebrates, the partnership of hemoglobin (Hb) (the oxygen transport protein) and myoglobin (Mb) (the oxygen storage protein) plays the key role in the distribution of oxygen from the environment to respiring tissues. Both have been extensively studied for decades and have contributed substantially to our knowledge of the relationship between structure and function in proteins (for review, see 5). The heme group, which is the central component providing the oxygen binding function, is identical in both proteins. The substantial differences in functionality between the two proteins are the result of variation in globin structure and subunit organization; providing an excellent example of molecular "engineering." Mb, which consists of a single polypeptide chain with associated heme, binds oxygen reversibly in a simple, noncooperative fashion. The more complex multisubunit structure of Hb (two α chains and two β chains, each with associated heme group) allows for both

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cooperative binding of oxygen and modulation of *in vivo* oxygen affinity by a number of allosteric factors, including pH, organic phosphates, and carbon dioxide (5).

The binding of oxygen by both proteins is dependent on keeping the heme iron in the ferrous state (Fe^{2+}), which, in an oxidizing environment, is not the most stable form. In the absence of cellular reduction systems, the heme iron in both proteins tends to autoxidize to the ferric iron (Fe^{3+}) form, which renders them unable to bind oxygen. This process is roughly equivalent to the "rusting" of metallic iron in an oxidizing environment (83).

In addition to their function in oxygen delivery and storage, both Hb and Mb have been demonstrated to catalyze a variety of oxidative reactions and processes *in vitro* (33). Central to many of these "secondary functions" is a peroxidase-like activity first described by Keilin and Hartree (44). Both ferrous and ferric forms of the proteins are reactive. Hydrogen peroxide (H_2O_2) oxidizes ferric Mb and Hb to an oxoferryl (Fe^{4+}) heme-centered radical and a globin-centered radical, a state similar to peroxidase I. This oxoferryl species is a strong oxidant, capable *in vitro* of oxidizing numerous reducing substrates, including cellular and vascular components.

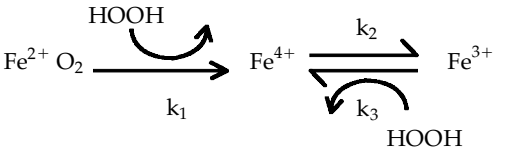
There is increasing evidence that these oxidative processes can, and do, occur in vivo. H_2O_2 is produced in tissues under a variety of conditions, in large part due to leakage from the normal electron transport processes in actively respiring cells (71). Accelerated H_2O_2 production is thought to occur after hypoxia when ischemic tissue is reperfused with oxygenated media (98). Other peroxides of biological significance such as lipid peroxides (LOOH) and peroxynitrite ($ONOO^-$), the product of the reaction of the free radicals nitric oxide (NO) and superoxide ($O_2^{\bullet -}$), can also interact with free Hb and Mb, leading to oxidative processes and potential cellular damage (10).

Vascular endothelium plays a pivotal role in the inflammatory process by coordinating the recruitment of inflammatory cells to sites of injury. Various physiological mediators are utilized in this process (57). Hb and/or Mb are thought to gain access to these sites due to injury-related hemolysis or muscle cell rupture. Interactions of the heme proteins with the vascular system under these conditions of inflammation are now thought to play a key role in the pathophysiology of oxidant-induced vascular injury, including cerebral hemorrhage, blast injury, crush injury, myocardial ischemia/reperfusion, and rhabdomyolysis. Further complications are thought to arise through disruption of key signaling pathways resulting from alteration or destruction of important physiological mediators. In this review, we discuss the potential cytotoxicity associated with the reactions between Hb and Mb with biological peroxides and the impact of these reactions on the modulation of cell signaling pathways.

PEROXIDE REACTIONS WITH Hb AND Mb

H_2O_2

The reactions between the respiratory proteins Hb or Mb and H_2O_2 have been extensively characterized and proceed via a two-electron oxidation process (for review, see 28). Reaction of H_2O_2 with either Fe^{2+} or Fe^{3+} hemoprotein produces the ferryl species, a potent oxidant in which the heme is formally two oxidation equivalents above the Fe^{2+} and is referred to as Fe^{4+} (27). If reactions occur with the Fe^{3+} form, protein-based radicals are also formed (87, 88). The radical represents the second oxidizing equivalent, and both peroxyl and carbon-based radicals are formed simultaneously. A cyclic mechanism including peroxide-dependent oxidation of Fe^{3+} heme and reduction of Fe^{4+} heme and radical has been proposed (4, 73) [Eq. 1]. Hb-based radical species have been detected in whole blood, indicating that H_2O_2 can react with $HbFe^{4+}/HbFe^{3+}$ in red blood cells in the presence of endogenous mechanisms that remove H_2O_2 (88).



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Fe^{4+} is a potent oxidant capable of oxidatively damaging most biological substrates, including

lipids, nucleic acids, and amino acids (12, 27, 31, 45, 61, 76, 78, 97). Formation of Fe^{4+} of both Hb and Mb was correlated to cytotoxicity in an endothelial cell culture model of ischemia/reperfusion and more so in cells that lack their antioxidative mechanisms such as glutathione (21, 51). Recent insights have identified the redox cycle between $MbFe^{3+}$ and $MbFe^{4+}$ as an important modulator of injury in kidneys of rhabdomyolytic rats (58). This added more impetus to research efforts by many groups investigating the effects of oxidative reactions mediated by hemoproteins in other pathological processes. A further example that will be discussed in more detail later includes oxidative damage to low-density lipoprotein (LDL) mediated by Hb, a process that has been correlated with free Hb concentrations in hemodialysis patients (97). Oxidative damage to apoB (the protein component of LDL) is a key event that transforms LDL into a proatherogenic form that in turn promotes atherogenesis.

The globin-based radical of $HbFe^{4+}$ has recently been detected in normal human blood by electron paramagnetic resonance (EPR) (88). Interestingly, the source of H_2O_2 in blood is considered to be the dismutation of $O_2^{\bullet -}$ produced via the autoxidation of Hb. Furthermore, similar to Mb (61), Hb protein radicals may be able to initiate lipid peroxidation reactions directly by abstraction of allylic hydrogens (Patel and Svistunenko, unpublished observations). In addition to intermolecular cross-links, globin radical formation also forms intramolecular cross-links between the heme and amino acids (16, 45). The functional consequences of these modifications are not yet known, but are likely to lead to protein degradation and release of iron. In the case of Mb, the treatment with H_2O_2 leads to covalent alteration of the prosthetic group with concomitant formation of a protein-bound adduct that transforms Mb from an oxygen storage protein to an oxidase, capable of producing additional H_2O_2 (67, 68). Under similar conditions, Hb undergoes similar oxidative modifications, but shows no enzymatic activity (69). Visible absorption, fluorescence, and low-temperature EPR studies recently showed that the reaction of oxyHb with H_2O_2 leads to heme degradation. This process involves the reaction of an additional H_2O_2 molecule with $HbFe^{4+}$ to produce $HbFe^{3+}$ and $O_2^{\bullet -}$ radical anion. $O_2^{\bullet -}$ produced in the heme pocket reacts with the porphyrin entity, resulting in heme degradation and release of iron (60)

LOOH

Oxidation of lipids is implicated in many disease states, including atherosclerosis where peroxidation of fatty acids within LDL is a critical event in transforming this particle into a proatherogenic form (11). Oxidation of membrane lipids can alter membrane fluidity and integrity, eventually leading to cell lysis. Furthermore, specific lipid oxidation products can modulate a variety of signaling processes that affect responses including the control of apoptosis, response to inflammation, and regulation of vascular tone (11, 72, 85). The mechanisms by which LOOH may modulate cell signaling are not known. Possibilities include reactions with peroxidases and/or direct interactions with specific receptors and transcription factors. As discussed above, an example of a disease process relevant to heme protein-dependent oxidative damage to lipids is rhabdomyolysis where Mb-dependent formation of vasoconstrictive F_2-isoprostanes from arachidonic acid peroxidation results in kidney damage and failure (58).

Hb is a potent catalyst of lipid peroxidation. The proposed mechanism (see Fig. 1) involves one-electron redox reactions with LOOH (58, 73). Redox cycling of the iron between the Fe^{3+} and Fe^{4+} oxidation states catalytically decomposes LOOH forming lipid peroxyl and alkoxyl radicals, which then propagate the chain reaction. In addition, $HbFe^{4+}$ (and protein radicals) has the potential to initiate lipid peroxidation directly (61). This has been demonstrated with Mb, and specific binding and oxidation of linoleic acid into the heme crevice have been demonstrated. Whether LOOH can gain access to the heme in native tetrameric Hb is not known, and proposed mechanisms that allow for these interactions include a role for Hb dimers (78) or transfer of heme into the lipid phase (13). Consistent with these concepts, cross-linking strategies that structurally stabi-

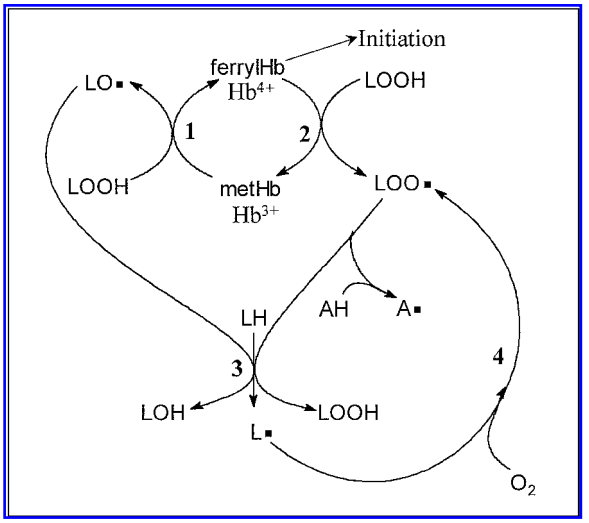

FIG. 1. Proposed scheme for interaction between metHb and membrane LOOH. MetHb reacts with LOOH forming ferrylHb and lipid alkoxyl radicals (LO$^{\bullet}$) (step 1). In turn, ferrylHb reacts with another LOOH reforming metHb and lipid peroxyl radicals (LOO$^{\bullet}$) (step 2). Both LOO$^{\bullet}$ and LO$^{\bullet}$ can propagate the chain of lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid (LH) (step 3). The resultant carbon-centered lipid radical (L$^{\bullet}$) rapidly reacts with oxygen forming more LOO$^{\bullet}$ (step 4). LOH, lipid hydroxide.

lize the Hb tetramer inhibited lipid peroxidation reactions catalyzed by ferric heme (78).

ONOO$^-$

ONOO$^-$, the reaction product of NO and $O_2^{\bullet -}$, has been implicated in numerous pathologies in which an inflammatory component is involved (74). The reaction between $O_2^{\bullet -}$ and NO is diffusion-limited ($k = 6–7 \times 10^9\ M^{-1}\ s^{-1}$) and can compete with Hb-dependent scavenging of NO. Similar to other peroxide-containing molecules, ONOO$^-$ also reacts with Hb. In contrast, however, ONOO$^-$ reacts relatively rapidly ($k = 10^4\ M^{-1}\ s^{-1}$) with $HbFe^{2+}$ (3). This is significantly faster than reactions between $HbFe^{2+}$ and either H_2O_2 or LOOH (73). Reaction with erythrocytes may therefore be a significant route for removal of ONOO$^-$ formed in extracellular compartments in the vasculature. ONOO$^-$ can enter the blood cell either by diffusion through the lipid bilayer or via the anion channel where it then can react with $HbFe^{2+}$ forming $HbFe^{3+}$ (metHb) (22, 49). Concomitantly, tyrosine residues in the protein become nitrated in this reaction (3). Using direct ESR, it was recently shown that in human red blood cells, ONOO$^-$ induced a long-lived singlet signal at $g = 2.004$ arising from Hb (55). Analysis of the visible spectra of Hb/ONOO$^-$ mixture revealed that, in the presence of CO_2, $HbFe^{2+}$ was oxidized to $HbFe^{4+}$, which rapidly decays to a lower iron oxidation state, $HbFe^{3+}$. It was suggested that the $g = 2.004$ radical observed in ESR spectrum was an intermediate formed during $HbFe^{4+}$ decay. Endogenous scavenging of ONOO$^-$ by erythrocytes may thus be viewed as a beneficial process protecting the endothelium from oxidative and/or nitrosative stress incurred under excessive inflammation.

The mechanism of reaction between Hb and ONOO$^-$ was recently studied with Hb in various oxidation and ligation states. The reaction of ONOO$^-$ with $HbFe^{2+}$ led to rapid and complete oxidation of the heme iron to $HbFe^{3+}$ within a 3-s reaction time in a rapid scanning spectrophotometer. When the observation period was extended to 8 s, the spectral footprint of the $HbFe^{4+}$ was indeed detected (3). Bolus addition of a molar excess of ONOO$^-$ to $HbFe^{3+}$, $HbFe^{3+}CN^-$ (cyanomet derivative of Hb), HbCO (carbon monoxy derivative of Hb), and $HbFe^{4+}$ induced no changes in the oxidized iron, but it displaced ligated CO and reduced $HbFe^{4+}$ back to $HbFe^{3+}$. These mechanistic studies also established that ONOO$^-$ oxidizes the heme center of Hb via a direct inner-sphere mechanism (3). The presence of free Hb in close proximity to ONOO$^-$ production sites in the vasculature may therefore contribute to possible *in vivo* toxicity by a two-step mechanism involving (i) direct oxidation of the heme iron and (ii) nitration of the tyrosine residues on the Hb molecule, leading to instability and heme loss (3).

EFFECTS OF Hb ON PEROXIDE-MEDIATED CELL SIGNALING

The biological peroxides discussed herein have been implicated as regulators of redox-sensitive cell signaling pathways (86). For example, reactive species have been implicated in

the regulation of hematopoiesis (80). Several studies have shown that H_2O_2 regulates transcriptional and translational events in many cell types (39, 47, 56). The exact targets that H_2O_2 reacts with, to either stimulate or repress a given pathway, are not known, but downstream targets include the mitogen activated protein kinases and nuclear factor κB (NFκB). These are important components of numerous redox-sensitive signaling pathways that link extracellular stimuli to gene regulation (59). Similarly, lipid peroxides are intermediates in the cascade of reactions forming compounds that mediate an inflammatory response (79). Recently, more specific roles have been reported, including modulation of endothelial cell NO synthase (75).

Relatively little is known of the signaling roles of $ONOO^-$ and, in fact, its production is generally considered a deleterious event. However, recent data indicate that this is not always the case, and at low physiological concentrations (in the nanomolar range) $ONOO^-$ may have cardioprotective functions. For example, exogenous addition of $ONOO^-$ decreased P-selectin expression and protected against ischemia/reperfusion injury (62). Furthermore, endogenous production of this reactive nitrogen species in endothelial cells modulates shear-dependent activation of c-Jun N-terminal kinase (JNK) (95). These effects are not deleterious and, in fact, protect the cell from subsequent oxidative or nitrosative insults.

The effects of reactions between Hb and biologically relevant peroxides in the context of cell signaling have not been explored. The concentrations of cell-free Hb that can be achieved upon administration is high (in the millimolar range in terms of heme) (28) and can potentially compete with endogenous reactions that consume the peroxides mentioned. No systematic studies have been conducted addressing these issues, but reactions between Hb and H_2O_2 generated during reperfusion of ischemic endothelial cells were demonstrated (51). Therefore, similar to NO, the effects of Hb on cell function may be more subtle than oxidative damage mediated by $HbFe^{4+}$ and involve perturbation of redox-sensitive signaling pathways (Fig. 2).

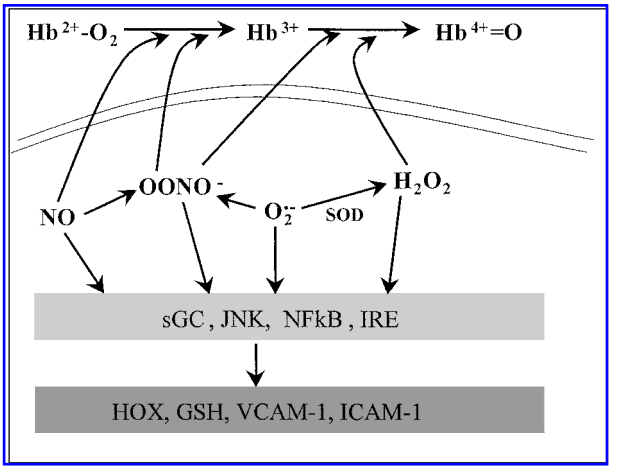

FIG. 2. Potential modulation of cell signaling in endothelial cells exposed to shear stress by cell-free Hb. Shear stress stimulates production of NO and $O_2^{\bullet -}$ in endothelial cells, which react together to form $ONOO^-$. Scavenging of NO by oxyHb is predicted to increase H_2O_2 production from $O_2^{\bullet -}$, a process catalyzed by superoxide dismutase (SOD). OxyHb can be oxidized to metHb by NO, $ONOO^-$, and H_2O_2 (not shown for purposes of clarity). In turn, metHb can modulate $ONOO^-$ and H_2O_2 concentrations by reactions that lead to ferryl Hb ($Hb^{4+} = O$) production. Both reactive oxygen and nitrogen species can regulate transcription and translation processes. The downstream targets of reactive species remain poorly defined. Examples include regulation of soluble guanylate cyclase (sGC), JNK, NFκB, or iron response element (IRE) activity. Examples of specific genes known to be regulated by reactive species include the antioxidants heme oxygenase (HOX) and glutathione (GSH), and the proinflammatory adhesion molecules VCAM-1 and ICAM-1

BIOLOGICAL EXAMPLES OF Hb AND Mb REDOX REACTIONS

Ischemia/reperfusion and myocardial injury

Sustained ischemia results in a complex series of changes, and when ischemia is followed by reperfusion (reoxygenation), this adds further complexity to the pathophysiology of organs. The xanthine oxidase system, a key source for $O_2^{\bullet -}$, is not present in the human myocardium, but present only in endothelial cells in human heart (24). Although activated phagocytes in oxidatively damaged tissue contribute to the oxidant pool, Mb has emerged as an important source of reactive oxygen species in myocardium (39). Like Hb, the autoxidation of Mb represents a potential significant source of $O_2^{\bullet -}$ and H_2O_2 due to the relatively high concentration of Mb within the heart (up to 0.5

m*M*) (89). Mb has been suggested to contribute to ischemia/reperfusion injury through the oxidation of arachidonic acid that can accompany its reaction with H_2O_2 (31).

Recent theoretical calculations of Mb autoxidation in human myocytes containing 150 μM Mb have shown that the rate of $O_2^{\bullet -}$ production would increase by 0.35 μM/min from Mb autoxidation (38). These calculations take into account the degree of tissue oxygenation, normal tissue pH, and a 70% drop in flow rate to mimic ischemic conditions within 15 min of arterial occlusion (38). Thus, it appears that the increased rate of Mb autoxidation caused by conditions relevant to ischemia/reperfusion can be a major source of $O_2^{\bullet -}$ and H_2O_2 in reperfused heart. Redox cycling of H_2O_2 as part of the normal protective mechanisms may not provide total protection during oxidative challenge to muscle. $MbFe^{4+}$ that persists longer in tissue may therefore perpetuate these oxidative reactions (21). Observations that $MbFe^{4+}$ has been detected in isolated ischemic rat hearts (6) and the reported protective role of ascorbate in induced ischemic arrest associated with cardiopulmonary bypass confirm a model for ischemia/reperfusion, whereby $MbFe^{4+}$ would play a key role (32).

Brain injury

Degenerative changes are known to occur in the endothelium of major cerebral arteries in patients who die from cerebral vasospasm after subarachnoid hemorrhage and in animal models of experimental subarachnoid hemorrhage (30). Recent evidence suggests that Hb-induced oxidative stress plays a central role in the pathogenesis of vasospasm after hemorrhage (19, 48). Under normal circumstances, plasma Hb does not cross the blood–brain barrier. Nonetheless, under pathophysiological conditions, such as subarachnoid hemorrhage, head trauma, and stroke, lysed erythrocytes release large amounts of Hb and iron inside the brain. Neurons and glial cells are exposed to Hb and its oxidation products that promote oxidative stress, delayed neurotoxicity, and liquefaction of brain tissue. Two possible mechanisms responsible for Hb side reactions and toxicity in the brain have been suggested. The first mechanism implicates the higher intracellular calcium in brain tissue resulting from Hb exposure, which may affect cell function and may cause cell death (96). Another possible mechanism is Hb-driven free radical damage, specifically DNA damage that has been supported by use of free radical scavengers. *In vitro* exposure of neurons to Hb has been associated with cell death in a dose-dependent manner (18, 63). Hb induced apoptotic changes in endothelial cells, and endothelial cell death might be an important part of vasospasm development in brain.

Although ultrapure Hb, free of any impurities, does not cause apoptotic death in endothelial cell culture (19), induction of subarachnoid hemorrhage in brain of mice by injection of autologous hemolysate over the right parietal cortex was recently shown to induce DNA fragmentation and apoptotic cell death (50). Heme oxygenase (HO-1) immunoreactivity was most abundant in the cortex adjacent to the hemolysate injection site, confirming a heme-mediated origin of this toxicity. Incubation of rat brain homogenates with Hb (0–10 μM), but not hemin, induced lipid peroxidation up to 24 h, and this effect was suppressed by the use of deferoxamine, an iron chelator, and by the use of the NO-releasing compound *S*-nitrosoglutathione (92). The report that Hb-mediated neuronal death was completely blocked by preexposure of cortical cell culture with the anti-ferryl Trolox points to a role for Hb higher oxidation products in brain toxicity (77). This is extremely relevant to the use of cell-free Hb as blood substitutes in patients with a possibly compromised blood–brain barrier, as the central nervous system contains high amount of lipids, which can be a target for lipid peroxidation.

Blast injury

Detonation of explosives or firing of large caliber weapons, as well as accidental occupational explosions, produce high-energy impulse noise (blast) waves characterized by instantaneous sharp increases in atmospheric pressure. Exposure to blast waves can cause injury, predominantly to the gas-filled organs, *i.e.*, lungs. Lung injury is characterized by a me-

chanical damage to alveolar septa and rupture of blood vessels resulting in hemorrhage and edema (26). Biochemically, blast injury results in increased lipid peroxidation, antioxidant depletion, and disruption of calcium transport. Stimulated blast injury in rats exposed to low levels of blast overpressure leads to a contusion-type injury in lungs and appearance of Hb oxidation products in lung tissue, which may act as potential initiators of oxidative stress and lipid peroxidation (26). Another consequence of air blast in lung was reported to be a dramatic drop in both water- and lipid-soluble antioxidants, resulting in a lower total antioxidant capacity (7). A mechanism based on a redox cycling between $HbFe^{4+}$ and $HbFe^{3+}$ has been suggested to account for the observed depletion of ascorbate and reduced glutathione and for the lack of a significant accumulation of the $HbFe^{3+}$ at 60 min after exposure (36).

With the increased use of lightweight body armor and improved helmet design, the incidence of nonpenetrating impact injuries among soldiers on the modern battlefield is expected to increase. The tissue immediately under the affected area can undergo considerable trauma, not dissimilar to a crush injury involving a number of muscle groups and blood vessels. Mb released into surrounding tissue and vasculature can enter the general circulation. Pooling of blood at the injury site can result in large hematomas containing red blood cells that break down and release Hb into the surrounding tissue and vasculature. Both heme-containing proteins (Hb and Mb) can exacerbate tissue damage and have the potential to cause cellular damage and death away from the primary site of injury. The interactions between small quantities of oxidized heme proteins and susceptible target cells were recently investigated in endothelial cell-based model systems (21). Transient oxidation of both Hb and Mb to their respective ferryl forms occurred rapidly and correlated with a drop in the cellular glutathione. $MbFe^{4+}$, but not $HbFe^{4+}$, was observed in samples analyzed at the end of treatment (3 h), which may explain the greater toxicity observed with Mb as compared with Hb. Studies on oxidative mechanisms of different hemoproteins are expected to be related to toxicities caused by impact injuries, which may ultimately help in the development of better strategies against blast injury.

Rhabdomyolysis

Rhabdomyolysis (literally "stripped muscle dissolution") is a biological and clinical condition that takes places as result of Mb disposition in the kidney, causing ~7% of all cases of acute renal failure in the United States. The basis of the renal failure in rhabdomyolysis has been attributed to both intense renal vasoconstriction and renal tubular necrosis (40). Recent animal studies have shown that these events are initiated by a redox cycle between $MbFe^{4+}$ and $MbFe^{3+}$, triggering lipid peroxidation in the kidney without invoking the release of free iron (58). Optical as well as EPR spectra taken in kidney extracts from rats with induced renal damage confirmed the existence of Mb redox forms. The detection of F_2-isoprostane, a potent vasoconstrictor (43, 46), in urine of these animals suggested that lipid peroxidation is a feature of rhabdomyolysis. The reactivity of the $MbFe^{4+}$ was shown to be markedly reduced at alkaline pH, which led the authors to suggest that urine alkalinization, a common clinical treatment, provides a protection against Mb-induced renal injury (58). A follow-up study by the same group of researchers was carried out recently in eight patients with rhabdomyolysis and renal impairment. Urinary excretion of F_2-isoprostanes was strikingly higher in patients than in the normal control group. The $MbFe^{4+}$ formation was assessed by measuring the formation of cross-linked heme in urine collected from these patients (41). Evidence that $MbFe^{4+}$ occurs *in vivo* was obtained by showing that a high percentage of the heme present in the urine of patients with rhabdomyolysis was cross-linked to protein (41).

Earlier studies on the evaluation of renal toxicity of ultrapure bovine Hb-based blood substitute in rats showed sharp increases in creatinine, and higher death at higher doses of Hb. Interestingly, urine alkalinization in these animals ameliorated Hb-mediated renal toxicity by preventing Hb oxidation and precipitation as tubular casts of the oxidized protein (8). It is tempting to speculate that a redox cycle between the $HbFe^{4+}$ and $HbFe^{3+}$states may have

occurred in the kidney of these animals infused with Hb-based blood substitutes similar to that occurring in rhabdomyolytic animals.

Atherosclerosis

Oxidative modification of human LDL is thought to play a major role in the development of atherosclerosis. Free hemin, Hb, Mb, and horseradish peroxidase were reported to promote LDL lipid peroxidation (9, 42, 52, 93). Hb oxidizes lipids by mechanisms that are both peroxide- and heme-dependent. At the high concentration of Hb found in red blood cells, even a minimal vascular hemolysis is believed to be sufficient to yield plasma Hb at the micromolar concentration that can trigger LDL peroxidation (9, 93). These reactions can occur when plasma-heme-binding mechanisms such as haptoglobin, albumin, and hemopexin are overwhelmed. It has been shown that up to 10% of plasma LDL consists of particles of a mildly oxidized LDL, referred to as LDL^-, and that a proportion of this species is linked to the oxidative stress induced during inflammatory events (82). Reactive oxygen species generated during these processes have been proposed agents responsible for LDL oxidation in the artery wall. Free Hb can potentially mediate the LDL^- formation, because Hb-based radicals were found in blood *in vivo* and are well known to initiate lipid peroxidation (53, 70, 78, 94, 97). Recently, Hb released from erythrocytes during hemodialysis has been demonstrated to be covalently bound to LDL (97). A correlation between cell-free Hb and the extent of LDL formation was observed consistent with the ability of cell-free Hb to promote oxidative damage to lipids by interactions with biological molecules.

LOOH and H_2O_2 can initiate a redox cycle between $HbFe^{4+}$ and $HbFe^{3+}$ (53, 54). The oxidation to the $HbFe^{4+}$ has been reported to be accompanied by a protein-based radical, likely involving the amino acid tyrosine. These Hb-based radicals can initiate lipid peroxidation and promote oxidative cross-linking of LDL. In hemodialysis patients, who are at higher risk of developing atherosclerosis, higher LDL^- levels were found during *ex vivo* blood circulation through a model system resembling clinical hemodialysis (97). The increased dityrosine content and the presence of heme in LDL after blood circulation confirmed that Hb-mediated redox reaction, possibly via $HbFe^{4+}/HbFe^{3+}$ transition, may have been responsible for the high LDL^- levels in hemodialysis patients (97).

Blood substitutes

Cell-free Hbs, chemically altered or genetically expressed in microbial host systems, have been developed as oxygen-carrying therapeutics. These site-directed modifications are introduced and serve to stabilize the protein molecules in their tetrameric or polymeric functional form (17). The proposed indications for an oxygen-carrying blood substitute are primarily emergency resuscitation of trauma patients and preoperative hemodilution during surgical procedures. Other likely applications of clinical benefit to patients include the use of these products in treatment of ischemic diseases, tumor sensitization, and the treatment of sickle cell anemia. Animal studies, as well as recent clinical studies, have shown that these proteins probably deliver sufficient oxygen to tissues (17). However, concerns still persist regarding the spontaneous oxidation (autoxidation) of Hb and its redox reactions with tissue oxidants that may potentially impede its clinical usefulness (1). The vascular endothelium is a source of a number of oxidants and has emerged as the primary target of Hb-based toxicity due to its proximity to the circulating protein. Hb, unlike red cells, can diffuse through the endothelial barrier lining the vessel wall, where it can potentially reach NO and disturb the physiological balance between NO, $O_2^{\bullet -}$, $ONOO^-$, and H_2O_2 (1). The presence of cell-free Hb within close proximity to these oxidants may potentially be detrimental to both Hb and the surrounding tissues (Fig. 2).

Diaspirin cross-linked Hb (DBBF-Hb), an intramolecularly cross-linked tetramer with *bis*(3,5-dibromosalicyl)fumarate (DBBF), has been extensively studied *in vitro* and in animal models. In recent years, considerable research and development efforts have been invested in the commercial analogue, DCLHb or

HemAssist, produced by Baxter Healthcare Inc., and its noncommercial analogue, DBBF-Hb, produced by the U.S. Army. The production and development of both Hbs have recently been halted by both the Army and Baxter due to, in the case of DCLHb, reports of excessive fatalities in late clinical trials (81, 84). *In vitro* studies on this Hb revealed that besides modifying ligand interactions, α subunits cross-linking with DBBF can also affect the tendency of this Hb to undergo oxidative modification and the production of $HbFe^{4+}$ in solution (35, 51). The reaction with H_2O_2 produces persistent DBBF-$HbFe^{4+}$ in solution, suggesting that it possesses less effective pseudoperoxidase activity (14). A new radical was recently detected with EPR ($g = 2.006$) in the reaction of metDBBF with H_2O_2, not found in native metHbA (25). The differences in the extent and nature of the radical formed in the cross-linked Hb may have important implications in the amount of oxidative damage it can induce.

In vitro evidence on the detection of DBBF-HbF^{4+} has recently been documented in a number of experimental settings: monolayer of endothelial cells, endothelial cells subjected to ischemia/reperfusion, and cells that lack the antioxidant mechanism, *i.e.*, glutathione (21, 35, 51). DBBF-Hb may have favorable oxygen-binding properties; however, the unique redox chemistry, *i.e.*, suppressed ability to remove H_2O_2 and susceptibility to oxidative damage, may have contributed to its toxicity.

Cell-free Hb may present a low risk to healthy individuals with normal redox status, however, patients with a compromised vasculature and poor antioxidant status, *i.e.*, diabetes, hypertension, myocardial infarction, and acute ischemic stroke, may be at greater risk, as recent clinical trial failures with DCLHb have demonstrated (81, 84). Although the primary event responsible for the microvascular effects of Hb solutions is believed to be the removal of NO by Hb, subsequent oxidative reactions between Hb and oxidants of the vascular system (i.e., H_2O_2 and $ONOO^-$) may potentially lead to a vascular inflammatory cascade of reactions progressing to multiorgan failure (Fig. 2).

PROTECTION STRATEGIES AGAINST HEMOPROTEIN TOXICITY

Inhibition of the reactions between Hb and peroxides

Several antioxidant enzymes and molecules safely detoxify peroxides in both the intra- and extracellular compartments (29). Despite the presence of these systems however, formation of $HbFe^{4+}$ in whole blood has been demonstrated (88). However, $HbFe^{4+}$ does not accumulate and oxidative damage to the red blood cell is likely prevented due to a comproportionation reaction between $HbFe^{4+}$ and $HbFe^{2+}$ (34). The product of this reaction is $HbFe^{3+}$. Coupled with the endogenous metHb reductase system, these reactions represent a protective pathway that can remove peroxide formation without exposing the red blood cell to an oxidative stress (34).

The role of this protective pathway in cell-free Hb solutions is less clear due to the lower concentrations of $HbFe^{2+}$ and the decreased capacity of plasma to reduce $HbFe^{4+}$ back to $HbFe^{3+}$. However, biological reductants present in the plasma may limit oxidative reactions mediated by $HbFe^{4+}$ formed outside the erythrocyte (29). Therapeutically, Hb-based oxygen carriers that do not readily react with peroxides or show decreased $HbFe^{4+}$ reactivity would be good candidates for a blood substitute. Structural modification of Hb can modulate $HbFe^{4+}$ reactivity. For example, cross-linking of the β subunits rather than the α subunits produced proteins that have better pseudoperoxidase activity (14, 69). Surface modification of pyridoxal phosphate cross-linked human Hb with polyoxyethylene produced less $HbFe^{4+}$ than nonconjugated human Hb (90). These results are encouraging from the perspective that $HbFe^{4+}$ reactivity can be modulated. Another very promising strategy is to cross-link Hb with trace amounts of superoxide dismutase and catalase (20). This strategy prevents $HbFe^{4+}$ production and protects against oxidative damage mediated by this Hb derivative. Similar results have been obtained using polynitrosylated Hb that was shown to inhibit reactive oxygen species-dependent activation of leukocyte adherence to the endothelium (64).

Several recently published reports have demonstrated the antioxidant effects of NO, specifically in restoring the hypervalent heme to its less toxic ferric form. Bolus addition of NO protected against *tert*-butyl hydroperoxide-induced oxidative damage in human erythroleukemia K562 cells with different intracellular Hb concentrations (36) and in cardiac myocytes (37). ESR evidence was presented to show that NO exhibits a potent, targetable antioxidant effect against oxidative damage produced by $HbFe^{4+}$ and $MbFe^{4+}$ *in vitro* (36, 37). Incubation of Hb or Mb with bovine aortic endothelial cells subjected to 3 h of hypoxia caused transient oxidation of these hemoproteins to the ferryl form. Formation of the ferryl intermediate was decreased in a concentration-dependent manner by the addition of L-arginine after 3 h of hypoxia. Optimal inhibition of the ferryl heme, possibly due to the antioxidant action of NO, was achieved with 900 μM L-arginine (21).

Lessons learned from studying naturally occurring hemoproteins and recombinant prototypes

Studies on variously modified Hbs developed as blood substitutes provide a window into the unique nature of the heme-protein chemistry. Subtle differences, brought about by changes distant to the heme pocket, can in some cases determine the ability of a given Hb to generate or interact with oxygen and nitrogen free radicals (1, 14). Amino acids in and around the heme pocket of Hb/Mb have been largely conserved thoughout evolution with the exception of some human mutant and animal Hbs. Comparison of the local heme environment in Hbs, which exhibit differences in ligand affinity as a result of species differentiation, chemical alteration, or genetic mutation, has been successfully employed (2, 66). Animal models, such as those facing diverse oxidative environments, may present an interesting model in which the relationship between structure and functional behavior of their oxygen transport/storage proteins and the structural dynamics governing their redox reactions can be determined (15).

The globin-centered radical has a limited lifetime, whereas, in the absence of a suitable reducing substrate, the ferryl radical can persist for minutes or hours, finally autoreducing to the ferric state (or a modified ferric state). The autoreduction process is protein-specific. Thus, although horse and sperm whale Mbs display similar oxygen binding thermodynamics and kinetics, the rate of autoreduction of the Fe^{4+} radical in these proteins differs by a factor of eight (91). This difference has been attributed to the single substitution proximal to the heme of a tyrosine in the sperm whale protein (Tyr^{151}) for the phenylalanine (F) found in the majority of mammalian Mbs. The half-lives of $MbFe^{4+}$ from different species at 25°C were recently determined (33) (*e.g.*, horse, 8 h; dimer of sperm whale Mb, 5 h; sperm whale, 1.5 h). Studies from our labaortoty (Cashon et al., unpublished observations) show that the rates of Fe^{4+} radical autoreduction for the yellowfin tuna and carp Mbs are very much like that seen with the horse Mb, even though the distribution of tyrosine residues differs from that of mammalian proteins. To our knowledge, no studies have been carried out to determine if the Fe^{4+} radical generated in human Mb resembles that from the horse (same configuration of the tyrosine residue) or the sperm whale Mb. This could be of possible significance in studies that extrapolate findings from sperm whale Mb oxidation studies to possible implications in human physiology or pathophysiology.

These observations involving comparisons of Mbs from a wide variety of species seem to suggest an important generalization. There appears to be no absolute correlation between oxygen-binding parameters, stability of the heme iron to autoxidation, and the stability of the Fe^{4+} species formed by the reaction of the protein with H_2O_2, even though the architecture of the oxygen-binding pocket is highly conserved in most species. This reinforces conclusions gained from studies using a wide variety of modified Hbs and mutant Mbs (4, 14). The peroxidative stability of the binding characteristics of the Hb/Mb and the characteristics of the Fe^{4+} species can be modulated separately with the proper choice of perturbation of the heme pocket.

Site-directed mutagenesis is a potentially ef-

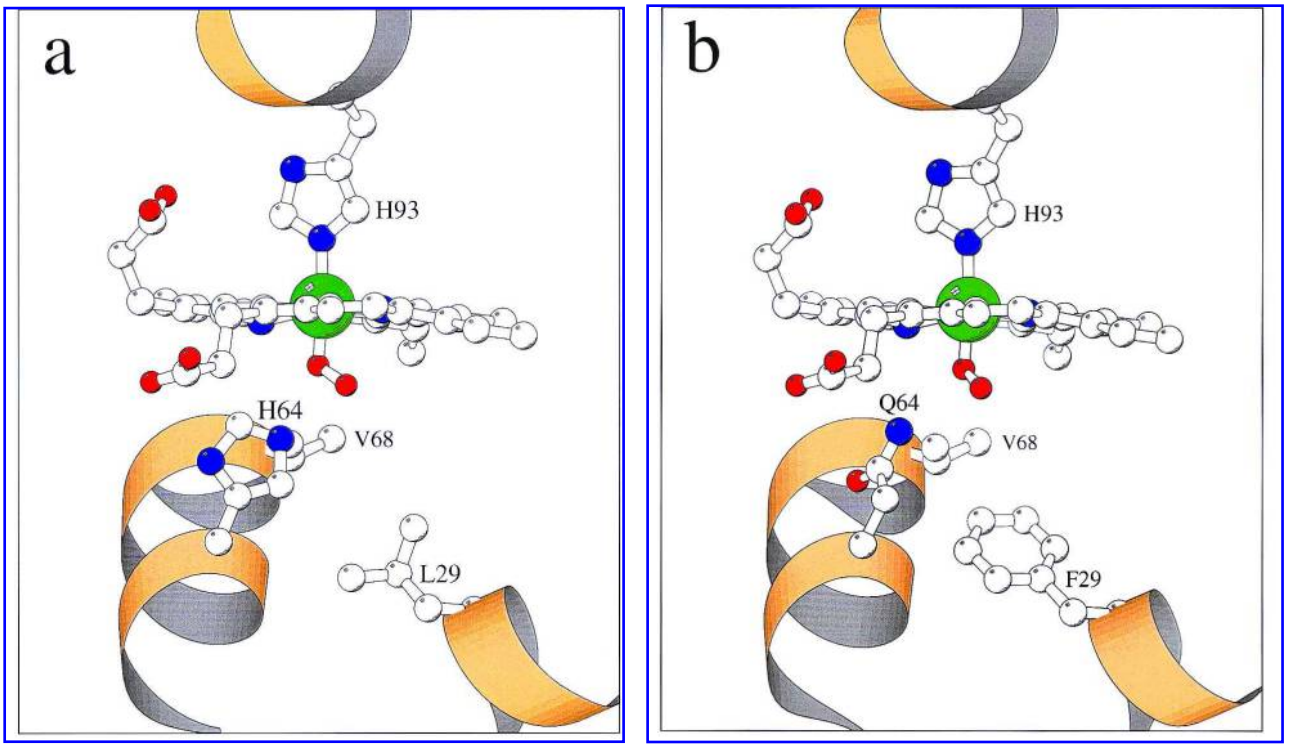

FIG. 3. Structure of heme pocket region of sperm whale Mb and the key amino acid substitutions in the binding pocket. (**a**) The normal binding pocket configuration with histidine (H64) as the distal ligand and leucine (L29) in the back portion of the pocket. (**b**) These key residues are substituted for glutamine (Q) and phenylalanine (F) to produce L29F/H64Q. The spheres represent oxygen (red), iron (green), nitrogen (blue), and carbon (white). Yellow ribbons represent portions of the protein. The coordinates for sperm whale Mb were taken from the Brookhaven protein data bank.

fective tool for engineering Hb for use in transfusion medicine because it allows the fine tuning of protein function and stability. At this point, Mb has provided a simple prototype for these experiments. In native Mb, a distal residue (H64) forms a hydrogen bond with unbound oxygen that is critical to maintaining heme iron in the ferrous state by stabilizing and inhibiting protonation of the bound oxygen (Fig. 3a) (65). Replacement of H64 with a polar

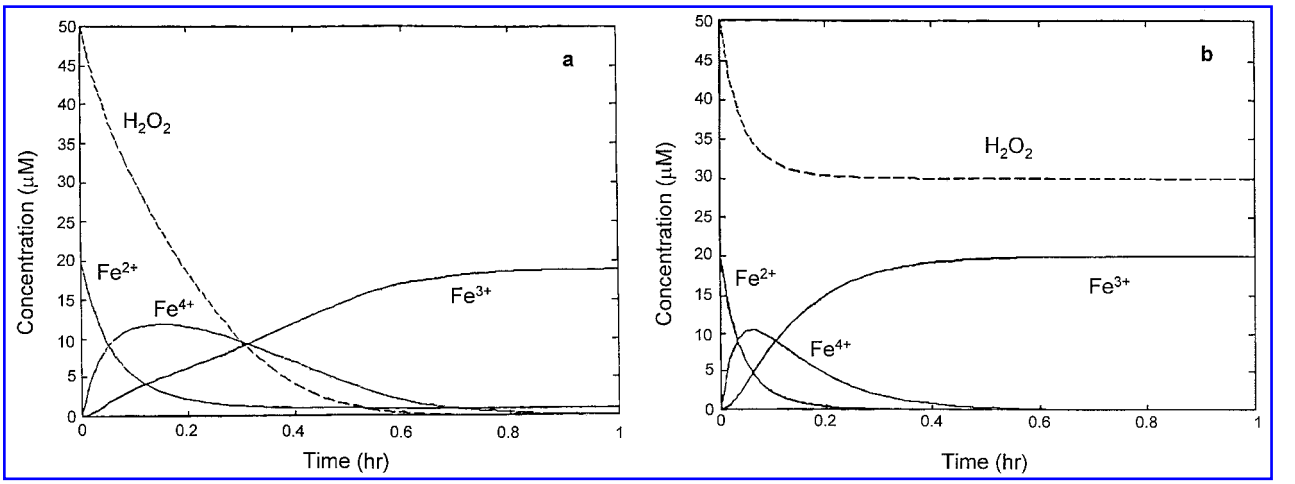

FIG. 4. Fractional changes of oxyMb, metHb, and ferryl Mb during the oxidation of sperm whale Mb (a) and the double mutant L29F/H46Q (b) with H_2O_2. Normalized time courses for the reaction of 20 μM (heme) of the proteins with 50 μM H_2O_2 at 37°C in 50 mM phosphate, pH 7.0.The concentration versus time curves associated with the reaction of the proteins with H_2O_2 are according to a published working model (4).

side chain increases the rate of autoxidation. As the size of the residue at position 29 (leucine) is changed, by replacement with larger phenylalanine (F), so are the rates of autoxidation and oxygen dissociation changed. Double and even triple mutants were also constructed in an effort to minimize or take advantage of the role of specific amino acids in and around the heme pocket (65). Replacing H64 with glutamine (Q) slows the initial oxidation by peroxide and increases the reduction of Fe^{4+} back to Fe^{3+}. Combining the mutation H64Q with L29F results in Mb with enhanced resistance to metMb formation in the absence of antioxidant enzymes (*i.e.*, catalase and superoxide dismutase) due to its own high pseudoperoxidase activity, which rapidly removes any H_2O_2 produced in the initial stages of autoxidation (4) (Figs. 3b and 4). Generally, increasing the polarity of the heme pocket residues at positions B10 and E11 promotes protein stability. These residues act as an "oil-like" coating to inhibit the rusting of the iron by water and oxygen, H_2O_2 and NO. This also suggests that H_2O_2 oxidizes heme iron by an inner-sphere mechanism. It is interesting to note that the double mutation (L29F/H64Q) occurs naturally in the Mb of Asian elephants, and similar multiple replacements have been used to reduce the rate of NO-induced oxidation of both recombinant MbO_2 and blood substitute prototypes without compromising oxygen affinity (23).

ABBREVIATIONS

DBBF, *bis*(3,5-dibromosalicyl)fumarate; DBBF-Hb, diaspirin cross-linked hemoglobin; EPR, electron paramagnetic resonance; Fe^{2+}, ferrous; Fe^{3+}, ferric; Fe^{4+}, ferryl; Hb, hemoglobin; H_2O_2 and HOOH, hydrogen peroxide; JNK, c-Jun N-terminal kinase; LDL, low-density lipoprotein; LOOH, lipid hydroperoxides; Mb, myoglobin; NFκB, nuclear factor κB; NO, nitric oxide; $O_2^{\cdot -}$, superoxide anion radical; $ONOO^-$, peroxynitrite.

REFERENCES

1. Alayash AI. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? *Nat Biotechnol* 17: 545–549, 1999.
2. Alayash AI, Brockner Ryan BA, and Fratantoni JC. Oxidation reactions of human, opossum *Didelphis virginiana* and spot *Leiostomus xanthurus* hemoglobins: a search for a correlation with some structural–functional properties. *Comp Biochem Physiol* 106B: 427–432, 1993.
3. Alayash AI, Brockner Ryan BA, and Cashon RE. Peroxynitrite-mediated heme oxidation and protein modification of native and chemically modified hemoglobins. *Arch Biochem Biophys* 349: 65–73, 1998.
4. Alayash AI, Brockner Ryan BA, Eich RF, Olson JS, and Cashon RE. Reactions of sperm whale myoglobin with hydrogen peroxide. Effects of distal pocket mutations on the formation and stability of the ferryl intermediate. *J Biol Chem* 274: 2029–2037, 1999.
5. Antonini E, and Brunori M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*. Amsterdam, Holland: North-Holland, 1971.
6. Arduini A, Eddy L, and Hochstein P. Detection of ferryl myoglobin in the isolated rat heart. *Free Radic Biol Med* 9: 511–513, 1990.
7. Armstrong KL, Cooper MF, Williams MT, and Elsayed NM. Vitamin E and lipoic acid, but not vitamin C improve blood oxygenation after high-energy impulse noise (Blast) exposure. *Biochem Biophys Res Commun* 253: 114–118, 1998.
8. Atsumi LR, Jacobs EE, Austen WG, and Vlahakes GJ. Ultrapure, stroma-free, polymerized bovine hemoglobin solution: evaluation of renal toxicity. *J Surg Res* 47: 407–411, 1989.
9. Balla G, Jacobs HS, Eaton JW, Belcher JD, and Vercelloti GM. Hemin: a possible physiological mediator of low-density lipoprotein oxidation and endothelial injury. *Arterioscler Thromb* 11: 1700–1711, 1991.
10. Beckman JS, and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271: C1424–C1437, 1996.
11. Berliner JA, and Heinecke JW. The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 20: 707–727, 1996.
12. Cadenas E. Biochemistry of oxygen toxicity. *Annu Rev Biochem* 58: 79–110, 1989.
13. Cannon JB, Kuo FS, Pasternack RF, Wong NM, and Muller-Eberhard U. Kinetics of the interaction of hemin liposomes with heme binding proteins. *Biochemistry* 23: 3715–3721, 1984.
14. Cashon RE, and Alayash AI. Reactions of human hemoglobin A_0 and two cross-linked derivatives with hydrogen peroxide: differential behavior of the ferryl intermediate. *Arch Biochem Biophys* 316: 461–469, 1995.
15. Cashon RE, Vayda ME, and Sidell BD. Kinetic characterization of myoglobins from vertebrates with vastly different body temperatures. *Comp Biochem Physiol* 117B: 613–620, 1997.
16. Catalano CE, Choe YS, and Ortiz de Montellano PR. Reactions of the protein radical in peroxide-treated myoglobin. Formation of a heme-protein cross-link. *J Biol Chem* 264: 10534–10541, 1989.
17. Chang TMS (Ed). *Blood Substitutes: Principles, Methods, Products and Clinical Trials*. Basel, Switzerland: Karger-Landes, 1997.

18. Comair YG, Schipper HM, and Berm S. The prevention of oxyhemoglobin-induced endothelial and smooth muscle cytoskeletal injury by deferoxamine. *Neurosurgery* 32: 58–65, 1993.
19. D'Agnillo F, and Alayash AI. Oxyhemoglobin and apoptosis. *J Neurosurg* 92: 899–902, 2000.
20. D'Agnillo F, and Chang TMS. Polyhemoglobin–superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nat Biotechnol* 16: 667–672, 1998.
21. D'Agnillo F, Wood F, Porras C, Macdonald VW, and Alayash AI. Effects of hypoxia and glutathione depletion on hemoglobin- and myoglobin-mediated oxidative stress toward endothelium. *Biochim Biophys Acta* 1495: 150–159, 2000.
22. Denicola A, Souza JM, and Radi R. Diffusion of peroxynitrite across erythrocyte membranes. *Proc Natl Acad Sci U S A* 95: 3566–3571, 1998.
23. 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.
24. Downey JW, Hearse DJ, and Yellon DM. The role of xanthine oxidase during myocardial ischemia in several species including man. *J Mol Cell Cardiol* 20: 55–63, 1988.
25. Dunne J, Svistunenko DA, Wilson MT, Alayash AI, and Cooper CE. Reactions of cross-linked ferric haemoglobins with hydrogen peroxide. *Biochem Soc Trans* 26: S230, 1998.
26. Elsayed NM. Toxicology of blast overpressure. *Toxicology* 121: 1–15, 1997.
27. Everse J. The structure of heme proteins compounds I and II: some misconceptions. *Free Radic Biol Med* 24: 1338–1346, 1998.
28. Everse J, and Hsia N. The toxicities of native and modified hemoglobins. *Free Radic Biol Med* 22: 1075–1099, 1997.
29. Faivre B, Menu P, Labrude P, and Vigneron C. Hemoglobin autooxidation/oxidation mechanisms and methemoglobin prevention or reduction processes in the bloodstream. Literature review and outline of autooxidation reaction. *Artif Cells Blood Substit Immobil Biotechnol* 26: 17–26, 1998.
30. Findlay JM, Macdonald RL, and Weir BKA. Current concepts of pathophysiology and management of cerebral vasospasm following aneurysmal subarachnoid hemorrhage. *Cerebrovasc Brain Metab Rev* 3: 336–361, 1991.
31. Galaris D, Mira D, Sevanian A, Cadenas E, and Hochstein P. Co-oxidation of salicylate and cholesterol during the oxidation of metmyoglobin by H_2O_2. *Arch Biochem Biophys* 261: 221–231, 1988.
32. Galaris D, Eddy L, Arduini A, Cadenas E, and Hochstein, P. Mechanisms of reoxygenation injury in myocardial infarction: implications of a myoglobin redox cycle. *Biochem Biophys Res Commun* 160: 1162–1168, 1989.
33. Giulivi C, and Cadenas E. Heme protein radicals: formation, fate, and biological consequences. *Free Radic Biol Med* 24: 269–279, 1998.
34. Giulivi C, and Davies KJA. A novel antioxidant role for hemoglobin. The comproportionation of ferrylhemoglobin with oxyhemoglobin. *J Biol Chem* 265: 19453–19460, 1990.
35. Goldman DW, Breyer RJ, Yeh D, Brockner Ryan BA, and Alayash AI. Acellular hemoglobin-mediated oxidative stress toward endothelium: a role for ferryl iron. *Am J Physiol* 275: H1046–H1053, 1998.
36. Gorbunov NV, Elsayed NM, Kisin ER, Kozlov AV, and Kagan VE. Air blast-induced pulmonary oxidative stress: interplay among hemoglobin, antioxidants, and lipid peroxidation. *Am J Physiol* 272: L320–L334, 1997.
37. Gorbunov NV, Tyurina YY, Salama G, Day BW, Claycamp HG, Argros G, Elsayed NM, and Kagan VE. Nitric oxide protects cardiomyocytes against *tert*-butyl hydroperoxide-induced formation of alkoxyl and peroxyl radicals and peroxidation of phosphatidylserine. *Biochem Biophys Res Commun* 744: 647–651, 1998.
38. Gunther MR, Sampath V, and Caughey WS. Potential roles of myoglobin autoxidation in myocardial ischemia–reperfusion injury. *Free Radic Biol Med* 26: 1388–1395, 1999.
39. Guyton KZ, Liu Y, Gorospe M, Xu Q, and Holbrook NJ. Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. *J Biol Chem* 271: 4138–4142, 1996.
40. Holt S, and Moore K. Pathogenesis of renal failure in rhabdomyolysis: the role of myoglobin. *Exp Nephrol* 8: 72–76, 2000.
41. Holt S, Reeder BJ, Wilson MT, Harvey S, Marrow JD, Roberts LJ, and Moore K. Increased lipid peroxidation in patients with rhabdomyolysis. *Lancet* 353: 1241, 1999.
42. Kalyanaraman B, Darley-Usmar V, Struck A, Hogg N, and Parathasarathy S. Role of apoliprotein B-derived radical and alpha-tocopheroxyl radical in peroxidase-dependent oxidation of low-density lipoprotein. *J Lipid Res* 36:1037–1045, 1995.
43. Kang KH, Morrow JD, Roberts LJ, Newman JH, and Banerjee M. Airway and vascular effects of 8–epiprostaglandin F2 alpha in isolated perfused rat lung. *J App Physiol* 74: 460–465, 1993.
44. Keilin D, and Hartree EF. Reaction of methaemoglobin with hydrogen peroxide. *Nature* 166, 513–514, 1950.
45. Kelder PP, Fischer MJ, de Mol NJ, and Janssen LH. Oxidation of chlorpromazine by methemoglobin in the presence of hydrogen peroxide. Formation of chlorpromazine radical cation and its covalent binding to methemoglobin. *Arch Biochem Biophys* 284: 313–319, 1991.
46. Lahaie I, Hardy P, Hou X, Hassessian H, Asselin P, Lachapelle P, Almazan G, Varma DR, Morrow JD, Roberts LJ, and Chemtob S. A novel mechanism for vasoconstrictor action of 8–isoprostaglandin F2 alpha on retinal vessels. *Am J Physiol* 274: R1406–R1416, 1998.
47. Liu RM, Shi MM, Giulivi C, and Forman HJ. Quinones increase gamma-glutamyl transpeptidase expression by multiple mechanisms in rat lung epithelial cells. *Am J Physiol* 274: L330–L336, 1998.

48. Macdonald RL, and Weir BKA. A review of hemoglobin and pathogenesis of cerebral vasospasm. *Stroke* 22: 971–982, 1991.
49. Mallozzi C, Di Stasi AM, and Minetti M. Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. *FASEB J* 11: 1281–1290, 1997.
50. Matz PG, Fujimura M, and Chan PH. Subarachnoid hemolysate produces DNA fragmentation in a pattern similar to apoptosis in mouse brain. *Brain Res* 858: 312–319, 2000.
51. McLeod LL, and Alayash AI. Detection of a ferrylhemoglobin intermediate in an endothelial cell model after hypoxia–reoxygenation. *Am J Physiol* 277: H92–H99, 1999.
52. Miller YI, and Shaklai N. Oxidative crosslinking of LDL protein induced by hemin: involvement of tyrosines. *Biochem Mol Biol Int* 34: 1121–1129, 1994.
53. Miller YI, Felikman Y, and Shaklai N. Hemoglobin-induced apolipoprotein B crosslinking in low-density lipoprotein peroxidation. *Arch Biochem Biophys* 326: 252–260, 1996.
54. Miller YI, Smith A, Morgan WT, and Shaklai N. Role of hemopexin in production of low-density lipoprotein against hemoglobin-induced oxidation. *Biochemistry* 35: 13112–13117, 1996.
55. Minetti M, Scorza G, and Pietraforte D. Peroxynitrite induces long-lived tyrosyl radical(s) in oxyhemoglobin of red blood cells through a reaction involving CO_2 and a ferryl species. *Biochemistry* 7: 2078–2087, 1999.
56. Moinova HR, and Mulcahy RT. An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gamma-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *J Biol Chem* 273: 14683–14689, 1998.
57. Moncada S. Nitric oxide in the vasculature: physiology and pathophysiology. *Ann N Y Acad Sci* 811: 60–69, 1997.
58. Moore KP, Holt S, Patel RP, Svistunenko DA, Zackert W, Goodier D, Reeder BJ, Clozel M, Anand R, Cooper CE, Harvey S, Morrow JD, Wilson MT, Darley-Usmar VM, and Roberts LJ. A causative role for myoglobin induced lipid peroxidation in the renal failure of rhabdomyolysis: effect of alkalinization. *J Biol Chem* 273: 31731–31737, 1998.
59. Muller JM, Rupec RA, and Baeuerle PA. Study of gene regulation by NFκB and AP-1 in response to reactive oxygen intermediates. *Methods* 11: 301–312, 1997.
60. Nagababu E, and Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. *Biochemistry* 39: 12503–12511, 2000.
61. Newman ES, Rice-Evans CA, and Davies MJ. Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochem Biophys Res Commun* 179: 1414–1419, 1991.
62. Nossuli TO, Hayward R, Jensen D, Scalia R, and Lefer AM. Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. *Am J Physiol* 275: H509–H519, 1998.
63. Ogihara K, Zubkov AY, Bernanke DH, Lewis AI, Parent AD, and Zhang JH. Oxyhemoglobin-induced apoptosis in cultured endothelial cells. *J Neurosurg* 91: 459–465, 1999.
64. Okayama N, Park JH, Coe L, Granger DN, Ma LI, Hisa CJC, and Alexander JS. Polynitroxyl $\alpha\alpha$-hemoglobin (PNH) inhibits peroxide and superoxide-mediated neutrophil adherence to human endothelial cells. *Free Radic Res* 31: 53–58, 1999.
65. Olson JS. Genetic engineering of myoglobin as a simple prototype for hemoglobin-based blood substitutes. *Artif Cells Blood Substit Immobil Biotechnol* 22: 429–441, 1994.
66. Ordrias MR, Carson SD, and Wood SC. A resonance Raman study of *Ambystoma trigrinam* hemoglobin: evidence for intra-species hemepocket variations. *Comp Biochem Physiol* 79B: 637–642, 1984.
67. Osawa Y, and Korzekwa K. Oxidative modifications by low levels of HOOH can transform myoglobin to an oxidase. *Proc Natl Acad Sci U S A* 88: 7081–7085, 1991
68. Osawa Y, and Williams MS. Covalent crosslinking of the heme prosthetic group to myoglobin by H_2O_2: toxicological implications. *Free Radic Biol Med* 21: 35–41, 1996.
69. Osawa Y, Darbyshire JF, Meyer CA, and Alayash AI. Differential susceptibilities of the prosthetic heme of hemoglobin-based red cell substitutes: implications in the design of safer agents. *Biochem Pharmacol* 46: 2299–2305, 1993.
70. Paganga G, Rice-Evans C, Rule R, and Leake D. The interaction between ruptured erythrocytes and low-density lipoproteins. *FEBS Lett* 303: 154–158, 1992.
71. Panus PC, Radi PH, Chumley RH, Lillard RH, and Freeman BA. Detection of H_2O_2 release from vascular endothelial cells. *Free Radic Biol Med* 14: 217–223, 1993.
72. Parthasarathy S, Santanam N, Ramachandran S, and Meilhac O. Oxidants and antioxidants in atherogensis. An appraisal. *J Lipid Res* 40: 2143–2157, 1999.
73. Patel RP, Svistunenko DA, Darley-Usmar VM, Symons MCR, and Wilson MT. Redox cycling of human methaemoglobin by H_2O_2 yields persistent ferryl iron and protein based radicals. *Free Radic Res* 25: 117–123, 1996.
74. Patel RP, Mcandrew J, Sellak H, White CR, Jo H, Freeman BA, and Darley-Usmar VM. Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* 1411: 385–400, 1999.
75. Ramasamy S, Parthasarathy S, and Harrison DG. Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J Lipid Res* 39: 268–276, 1998.
76. Rao SI, Wilks A, Hamberg M, and Ortiz de Montellano PR. The lipoxygenase activity of myoglobin. Oxidation of linoleic acid by the ferryl oxygen rather than protein radical. *J Biol Chem* 269: 7210–7216, 1994.
77. Regan RF, and Panter SS. Neurotoxicity of hemoglo-

bin in cortical cell culture. *Neurosci Lett* 153: 219–222, 1993.
78. Rogers MS, Patel RP, Reeder BJ, Sarti P, Wilson MT, and Alayash AI. Pro-oxidant effects of cross-linked haemoglobins explored using liposome and cytochrome *c* oxidase vesicle model membranes. *Biochem J* 310: 827–833, 1994.
79. Salmon JA, and Garland LG. Leukotriene antagonists and inhibitors of leukotriene biosynthesis as potential therapeutic agents. *Prog Drug Res* 37: 9–90, 1991.
80. Sattler M, Winkler T, Verma S, Byrne CH, Shrikhande G, Salgia R, and Griffin JD. Hematopoietic growth factors signal through the formation of reactive oxygen species. *Blood* 93: 2928–2935, 1999.
81. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, Stern KN, and Koudstal PJ. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. *Stroke* 30: 993–996, 1999.
82. Sevanian A, Bittolo-Bon G, Cazzolato G, Hodis H, Hwang J, Zamburlini A, Maiorino M, and Ursini F. LDL^- is a lipid hydroperoxide-enriched circulating lipoprotein. *J Lipid Res* 38: 419–428, 1997.
83. Shikama K. The molecular mechanism of autoxidation for myoglobin and hemoglobin: a venerable puzzle. *Chem Rev* 98: 1357–1373, 1998.
84. Sloan ER, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory M, and Rodman G. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: A randomized controlled efficacy trial. *JAMA* 282: 1857–1864, 1999.
85. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, and Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 3209:915–924, 1989.
86. Suzuki YJ, Forman HJ, and Sevanian A. Oxidants as stimulators of signal transduction. *Free Radic Biol Med* 22: 269–285, 1997.
87. Svistunenko DA, Patel RP, and Wilson MT. An EPR investigation of human methaemoglobin oxidation by hydrogen peroxide: methods to quantify all paramagnetic species observed in the reaction. *Free Radic Res* 24: 269–280, 1996.
88. 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.
89. Sylven C, Tansson E, and Book K. Myoglobin content in human skeletal muscle and myocardium: relation to fiber size and oxidative capacity. *Cardiovasc Res* 18: 443–446, 1984.
90. Talarico T, Swank A, and Privalle C. Autoxidation of pyridoxylated hemoglobin polyoxyethylene conjugate. *Biochem Biophys Res Commun* 250: 354–358, 1998.
91. Uyeda M, and Peisach J. Ultraviolet difference spectroscopy of myoglobin: assignment of pK values of tyrosyl phenolic groups and the stability of ferryl intermediates. *J Am Chem Soc* 20: 2028–2035, 1981.
92. Van Bergen P, Rauhala P, Spooner CM, and Chiueh CC. Hemoglobin and iron-evoked oxidative stress in the brain: protection by bile pigments, manganese and *S*-nitrosoglutathione. *Free Radic Res* 31: 631–640, 1999.
93. Wieland E, Parthasarathy S, and Stienberg D. Peroxidase-dependent metal-independent oxidation of low-density lipoprotein in vitro: a model for in vivo oxidation. *Proc Natl Acad Sci U S A* 90: 5929–5933, 1993.
94. Yoshida Y, Kashiba K, and Niki E. Free radical-mediated oxidation of lipids induced by hemoglobin in aqueous dispersions. *Biochim Biophys Acta* 1201: 165–172, 1994.
95. Young-Mi G, Patel RP, Maland MC, Park H, Beckman JS, Darley-Usmar VM, and Jo H. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of cJun N-terminal kinase in endothelial cells. *Am J Physiol* 277: H1647–H1653, 1999.
96. Zhang H, Weir BKA, and Macdonald RL. Mechanisms of $[Ca^{++}]$ elevation induced by erythrocyte components in endothelial cells. *J Pharmacol Exp Ther* 277: 1501–1509, 1996.
97. Ziouzenkova O, Asatryan L, Akmal M, Tetta C, Wratten ML, Loseto-Wich G, Jurgens G, Heinecke J, and Sevanian A. Oxidative cross-linking of ApoB100 and hemoglobin results in low density lipoprotein modification in blood. Relevance to atherogenesis caused by hemodialysis. *J Biol Chem* 274: 18916–18924, 1999.
98. Zulueta JJ, Kuppusamay P, and Lutty GA. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proc Natl Acad Sci U S A* 85: 4046–4050, 1988.

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1. Boguslaw Lipinski, Etheresia Pretorius, Hester Magdalena Oberholzer, Wendy Jeanette Van Der Spuy. 2012. Iron enhances generation of fibrin fibers in human blood: Implications for pathogenesis of stroke. *Microscopy Research and Technique* **75**:9, 1185-1190. [CrossRef]
2. Chris E. Cooper , Dominik J. Schaer , Paul W. Buehler , Michael T. Wilson , Brandon J. Reeder , Gary Silkstone , Dimitri A. Svistunenko , Leif Bulow , Abdu I. Alayash . Haptoglobin Binding Stabilizes Hemoglobin Ferryl Iron and the Globin Radical on Tyrosine #145. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
3. Gabriella Fanali, Alessandra di Masi, Viviana Trezza, Maria Marino, Mauro Fasano, Paolo Ascenzi. 2012. Human serum albumin: From bench to bedside. *Molecular Aspects of Medicine* **33**:3, 209-290. [CrossRef]
4. Tuulia Tynkkynen, Jaakko Mursu, Tarja Nurmi, Kari Tuppurainen, Reino Laatikainen, Pasi Soininen. 2012. NMR protocol for determination of oxidation susceptibility of serum lipids and application of the protocol to a chocolate study. *Metabolomics* **8**:3, 386-398. [CrossRef]
5. Christelle Kamga, Suhas Krishnamurthy, Sruti Shiva. 2012. Myoglobin and mitochondria: A relationship bound by oxygen and nitric oxide. *Nitric Oxide* **26**:4, 251-258. [CrossRef]
6. Brandon J. Reeder, Dimitri A. Svistunenko, Chris E. Cooper, Michael T. Wilson. 2012. Engineering Tyrosine-Based Electron Flow Pathways in Proteins: The Case of Aplysia Myoglobin. *Journal of the American Chemical Society* 120430071621004. [CrossRef]
7. Tune Wulff, Alfred Jokumsen, Peter Højrup, Flemming Jessen. 2012. Time-dependent changes in protein expression in rainbow trout muscle following hypoxia. *Journal of Proteomics* **75**:8, 2342-2351. [CrossRef]
8. Christine C. WinterbournBiological Chemistry of Reactive Oxygen Species . [CrossRef]
9. Jay F. Storz, Roy E. Weber, Angela Fago. 2012. Oxygenation properties and oxidation rates of mouse hemoglobins that differ in reactive cysteine content. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **161**:2, 265-270. [CrossRef]
10. Naihao Lu, Wei Chen, Jingjie Zhu, Yi-Yuan Peng. 2011. Enhancement of nitrite on heme-induced oxidative reactions: A potential toxicological implication. *Toxicology in Vitro* . [CrossRef]
11. Yusuf Yilmaz, Ebubekir Senates, Talat Ayyildiz, Yasar Colak, Ilyas Tuncer, Ayse Oya Kurdas Ovunc, Enver Dolar, Cem Kalayci. 2011. Characterization of nonalcoholic fatty liver disease unrelated to the metabolic syndrome. *European Journal of Clinical Investigation* no-no. [CrossRef]
12. Ryan Stapley, Dario A. Vitturi, Rakesh P. PatelBiochemistry of Storage of Red Blood Cells 231-241. [CrossRef]
13. Dhiman Maitra, Jaeman Byun, Peter R. Andreana, Ibrahim Abdulhamid, Michael P. Diamond, Ghassan M. Saed, Subramaniam Pennathur, Husam M. Abu-Soud. 2011. Reaction of hemoglobin with HOCl: Mechanism of heme destruction and free iron release. *Free Radical Biology and Medicine* **51**:2, 374-386. [CrossRef]
14. Naihao Lu, Wei Chen, Yi-Yuan Peng. 2011. Effects of glutathione, Trolox and desferrioxamine on hemoglobin-induced protein oxidative damage: Anti-oxidant or pro-oxidant?. *European Journal of Pharmacology* **659**:2-3, 95-101. [CrossRef]
15. Naihao Lu, Puqing Chen, Qin Yang, Yi-Yuan Peng. 2011. Anti- and pro-oxidant effects of (+)-catechin on hemoglobin-induced protein oxidative damage. *Toxicology in Vitro* **25**:4, 833-838. [CrossRef]
16. Paul W. Buehler, Yipin Zhou, Pedro Cabrales, Yiping Jia, Guoyong Sun, David R. Harris, Amy G. Tsai, Marcos Intaglietta, Andre F. Palmer. 2010. Synthesis, biophysical properties and pharmacokinetics of ultrahigh molecular weight tense and relaxed state polymerized bovine hemoglobins. *Biomaterials* **31**:13, 3723-3735. [CrossRef]
17. Ruijuan Du, Bow Ho, Jeak Ling Ding. 2010. Rapid reprogramming of haemoglobin structure-function exposes multiple dual-antimicrobial potencies. *The EMBO Journal* **29**:3, 632-642. [CrossRef]

18. Enika Nagababu, Joy G. Mohanty, Surya Bhamidipaty, Graciela R. Ostera, Joseph M. Rifkind. 2010. Role of the membrane in the formation of heme degradation products in red blood cells#. *Life Sciences* **86**:3-4, 133-138. [CrossRef]
19. N. Botsoglou, E. Botsoglou2 Oxidation and protection of poultry and eggs 50-90. [CrossRef]
20. Paolo Ascenzi, Elisabetta De Marinis, Alessandra di Masi, Chiara Ciaccio, Massimo Coletta. 2009. Peroxynitrite scavenging by ferryl sperm whale myoglobin and human hemoglobin. *Biochemical and Biophysical Research Communications* **390**:1, 27-31. [CrossRef]
21. Marie Grey, Sakda Yainoy, Virapong Prachayasittikul, Leif Bülow. 2009. A superoxide dismutase-human hemoglobin fusion protein showing enhanced antioxidative properties. *FEBS Journal* **276**:21, 6195-6203. [CrossRef]
22. I. V. Gorudko, O. S. Tcherkalina, A. V. Sokolov, M. O. Pulina, E. T. Zakharova, V. B. Vasilyev, S. N. Cherenkevich, O. M. Panasenko. 2009. New approaches to the measurement of the concentration and peroxidase activity of myeloperoxidase in human blood plasma. *Russian Journal of Bioorganic Chemistry* **35**:5, 566-575. [CrossRef]
23. Rung-chi Li, Sofiyan Saleem, Gehua Zhen, Wangsen Cao, Hean Zhuang, Jongseok Lee, Ann Smith, Fiorella Altruda, Emanuela Tolosano, Sylvain Doré. 2009. Heme–hemopexin complex attenuates neuronal cell death and stroke damage. *Journal of Cerebral Blood Flow & Metabolism* **29**:5, 953-964. [CrossRef]
24. Agnieszka Grzelak, Marcin Kruszewski, Ewa Macierzy#ska, #ukasz Piotrowski, #ukasz Pu#aski, B#a#ej Rychlik, Grzegorz Bartosz. 2009. The effects of superoxide dismutase knockout on the oxidative stress parameters and survival of mouse erythrocyt. *Cellular & Molecular Biology Letters* **14**:1, 23-34. [CrossRef]
25. E. V. Bondaryuk, V. V. Senchuk. 2009. Bioaccumulation and biotransformation of flavonols by erythrocytes. *Chemistry of Natural Compounds* **45**:2, 174-178. [CrossRef]
26. Jan Simoni, Grace Simoni, John F. Moeller. 2009. Intrinsic Toxicity of Hemoglobin: How to Counteract It. *Artificial Organs* **33**:2, 100-109. [CrossRef]
27. Hans-Petter Hersleth, Ya-Wen Hsiao, Ulf Ryde, Carl Henrik Görbitz, K. Kristoffer Andersson. 2008. The Influence of X-Rays on the Structural Studies of Peroxide-Derived Myoglobin Intermediates. *Chemistry & Biodiversity* **5**:10, 2067-2089. [CrossRef]
28. M MOZAFFARIEH, M GRIESHABER, S ORGUL, J FLAMMER. 2008. The Potential Value of Natural Antioxidative Treatment in Glaucoma. *Survey of Ophthalmology* **53**:5, 479-505. [CrossRef]
29. József Balla, György Balla, Béla Lakatos, Viktória Jeney, Klára Szentmihályi. 2007. A hemvas az emberi szervezetben. *Orvosi Hetilap* **148**:36, 1699-1706. [CrossRef]
30. Himantha Kushanie Tilakaratne, Baoli Yang, Stephen K. Hunter, Mark E. Andracki, Victor G.J. Rodgers. 2007. Can Myoglobin Expression in Pancreatic Beta Cells Improve Insulin Secretion Under Hypoxia? An Exploratory Study With Transgenic Porcine Islets. *Artificial Organs* **31**:7, 521-531. [CrossRef]
31. Magnus G. Olsson, Maria Allhorn, Tor Olofsson, Bo Åkerström. 2007. Up-regulation of #1-microglobulin by hemoglobin and reactive oxygen species in hepatoma and blood cell lines. *Free Radical Biology and Medicine* **42**:6, 842-851. [CrossRef]
32. Thomas D. Lockwood. 2006. Responsiveness of parasite Cys His proteases to iron redox. *Parasitology Research* **100**:1, 175-181. [CrossRef]
33. Hui-Hong Liu, Yong-Qing Wan, Guo-Lin Zou. 2006. Redox reactions and enzyme-like activities of immobilized myoglobin in aqueous/organic mixtures. *Journal of Electroanalytical Chemistry* **594**:2, 111-117. [CrossRef]
34. Hui-Hong Liu, Yong-Qing Wan, Guo-Lin Zou. 2006. Direct electrochemistry and electrochemical catalysis of immobilized hemoglobin in an ethanol–water mixture. *Analytical and Bioanalytical Chemistry* **385**:8, 1470-1476. [CrossRef]

35. S. M. Dreher-Lesnick, A. Mulenga, J. A. Simser, A. F. Azad. 2006. Differential expression of two glutathione S-transferases identified from the American dog tick, Dermacentor variabilis. *Insect Molecular Biology* **15**:4, 445-453. [CrossRef]

36. Hui-Hong Liu, Guo-Lin Zou. 2006. Electrochemical investigation of immobilized hemoglobin: Redox chemistry and enzymatic catalysis. *Journal of Biochemical and Biophysical Methods* **68**:2, 87-99. [CrossRef]

37. Argirios E. Tsantes , Stefanos Bonovas , Anthi Travlou , Nikolaos M. Sitaras . 2006. Redox Imbalance, Macrocytosis, and RBC Homeostasis. *Antioxidants & Redox Signaling* **8**:7-8, 1205-1216. [Abstract] [Full Text PDF] [Full Text PDF with Links]

38. Raffaella Roncone, Marica Barbieri, Enrico Monzani, Luigi Casella. 2006. Reactive nitrogen species generated by heme proteins: Mechanism of formation and targets. *Coordination Chemistry Reviews* **250**:11-12, 1286-1293. [CrossRef]

39. Hans-Petter Hersleth, Ulf Ryde, Patrik Rydberg, Carl Henrik Görbitz, K. Kristoffer Andersson. 2006. Structures of the high-valent metal-ion haem–oxygen intermediates in peroxidases, oxygenases and catalases. *Journal of Inorganic Biochemistry* **100**:4, 460-476. [CrossRef]

40. Niels B.J. Vollaard, Brandon J. Reeder, Jerry P. Shearman, Patrick Menu, Michael T. Wilson, Chris E. Cooper. 2005. A new sensitive assay reveals that hemoglobin is oxidatively modified in vivo. *Free Radical Biology and Medicine* **39**:9, 1216-1228. [CrossRef]

41. Sara Goldstein, Amram Samuni. 2005. Intra- and intermolecular oxidation of oxymyoglobin and oxyhemoglobin induced by hydroxyl and carbonate radicals. *Free Radical Biology and Medicine* **39**:4, 511-519. [CrossRef]

42. De-Jia Li, Xi-Wen Li, Yu-Xiang Xie, Xiao-Qiang Cai, Guo-Lin Zou. 2005. Identification of intermediate and product from methemoglobin-catalyzed oxidation of o-phenylenediamine in two-phase aqueous?organic system. *Biochemistry (Moscow)* **70**:1, 92-99. [CrossRef]

43. Jack H. Crawford , Balu K. Chacko , Christopher G. Kevil , Rakesh P. Patel . 2004. The Red Blood Cell and Vascular Function in Health and Disease. *Antioxidants & Redox Signaling* **6**:6, 992-999. [Abstract] [Full Text PDF] [Full Text PDF with Links]

44. Li-Hong Yeh , Abdu I. Alayash . 2004. Effects of Cell-Free Hemoglobin on Hypoxia-Inducible Factor (HIF-1#) and Heme Oxygenase (HO-1) Expressions in Endothelial Cells Subjected to Hypoxia. *Antioxidants & Redox Signaling* **6**:6, 944-953. [Abstract] [Full Text PDF] [Full Text PDF with Links]

45. Enika Nagababu , Joseph M. Rifkind . 2004. Heme Degradation by Reactive Oxygen Species. *Antioxidants & Redox Signaling* **6**:6, 967-978. [Abstract] [Full Text PDF] [Full Text PDF with Links]

46. Y JIA. 2004. Oxygen binding and oxidation reactions of human hemoglobin conjugated to carboxylate dextran*1. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1672**:3, 164-173. [CrossRef]

47. Takaaki Hayashi, Koji Uchida, Gen Takebe, Kazuhiko Takahashi. 2004. Rapid formation of 4-hydroxy-2-nonenal, malondialdehyde, and phosphatidylcholine aldehyde from phospholipid hydroperoxide by hemoproteins. *Free Radical Biology and Medicine* **36**:8, 1025-1033. [CrossRef]

48. Heme Proteins 354-361. [CrossRef]

49. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]

50. H Sanders. 2003. Blood meal induces global changes in midgut gene expression in the disease vector, Aedes aegypti. *Insect Biochemistry and Molecular Biology* **33**:11, 1105-1122. [CrossRef]

51. B Cussimanio. 2003. Unusual susceptibility of heme proteins to damage by glucose during non-enzymatic glycation. *Biophysical Chemistry* **105**:2-3, 743-755. [CrossRef]

52. L.-H. Yeh, A. I. Alayash. 2003. Redox side reactions of haemoglobin and cell signalling mechanisms1. *Journal of Internal Medicine* **253**:5, 518-526. [CrossRef]

53. Dario C Ramirez, Yeong-Renn Chen, Ronald P Mason. 2003. Immunochemical detection of hemoglobin-derived radicals formed by reaction with hydrogen peroxide: involvement of a protein-tyrosyl radical. *Free Radical Biology and Medicine* **34**:7, 830-839. [CrossRef]

54. Felice D'Agnillo, Abdu I Alayash. 2002. A role for the myoglobin redox cycle in the induction of endothelial cell apoptosis. *Free Radical Biology and Medicine* **33**:8, 1153-1164. [CrossRef]

55. Shimin Liu, John Connor, Steven Peterson, C. William Shuttleworth, Ke Jian Liu. 2002. Direct Visualization of Trapped Erythrocytes in Rat Brain After Focal Ischemia and Reperfusion. *Journal of Cerebral Blood Flow & Metabolism* 1222-1230. [CrossRef]

56. Kevin P Pavlick, F.Stephen Laroux, John Fuseler, Robert E Wolf, Laura Gray, Jason Hoffman, Matthew B Grisham. 2002. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease1,2 1This article is part of a series of reviews on "Reactive Oxygen and Nitrogen in Inflammation." The full list of papers may be found on the homepage of the journal. 2Guest Editor: Giuseppe Poli. *Free Radical Biology and Medicine* **33**:3, 311-322. [CrossRef]