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Forum Review

Heme Degradation by Reactive Oxygen Species

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ABSTRACT

Heme proteins play a major role in various biological functions, such as oxygen sensing, electron transport, signal transduction, and antioxidant defense enzymes. Most of these reactions are carried out by redox reactions of heme iron. As the heme is not recycled, most cells containing heme proteins have the microsomal mixed function oxygenase, heme oxygenase, which enzymatically degrades heme to biliverdin, carbon monoxide, and iron. However, the red cell with the largest pool of heme protein, hemoglobin, contains no heme oxygenase, and enzymatic degradation of the red cell heme occurs only after the senescent red cells are removed by the reticuloendothelial system. Therefore, only nonenzymatic heme degradation initiated when the heme iron undergoes redox reactions in the presence of oxygen-producing reactive oxygen species takes place in the red cell. Unlike enzymatic degradation, which specifically attacks the α-methene bridge, reactive oxygen species randomly attack all the carbon methene bridges of the tetrapyrrole rings, producing various pyrrole products in addition to releasing iron. This review focuses on the literature related to nonenzymatic heme degradation with special emphasis on hemoglobin, the dominant red cell heme protein. *Antioxid. Redox Signal*. 6, 967–978.

INTRODUCTION

In biological systems, heme proteins perform a number of vital physiological functions that are essential for life. Heme or iron protoporphyrin is the prosthetic group of heme proteins, which are responsible for oxygen transport and storage (hemoglobin, myoglobin), microsomal xenobiotics, drug metabolism, steroid biosynthesis (cytochrome P450), mitochondrial respiration (cytochromes), antioxidant defense enzymes (catalase, peroxidases), and signal transduction processes (guanylate cyclase, CooA, FixL) 18, 25, 30, 36, 41, 52, 71). These diverse functions are performed through oxidation and reduction reactions of heme iron, and the different heme environments resulting from the interactions of the heme with the various proteins (64). Because the redox reactions of heme iron with oxygen generate reactive oxygen species (ROS), heme proteins are a source for the ROS that are thought to contribute to the deleterious effects found in various diseased states and during aging (20, 65, 67, 69). These redox reactions are much faster with free heme than with most heme proteins and, therefore, free heme is more toxic than heme proteins, accelerating membrane peroxidation and damage to cellular macromolecules such as carbohydrates, proteins, and DNA (1, 2, 31, 78).

The most abundant heme protein in nature is hemoglobin. The average human subject has ~750 g of hemoglobin. The limited life span of the red cells (120 days), which have a hemoglobin concentration of 20 m*M*, requires that ~6–8 g of hemoglobin and 300 mg of porphyrin are synthesized every day. As the heme is not recycled, every day ~375 mg of heme is degraded of which 300 mg or 80% comes from hemoglobin. In most cells, heme oxygenase (HO) is responsible for most of the heme degradation (72,75,79). However, the mature red cell and serum contain no HO, and the heme has to be transported to the reticuloendothelial system of the spleen, liver, and kidney in order to be degraded by HO (80). On the other hand, oxyhemoglobin (oxyHb) undergoes redox reactions producing superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which can also mediate heme degradation. Therefore, the possible contribution of heme degradation due to these ROS

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(nonenzymatic) needs to be considered as contributing to heme turnover in red cells. Nonenzymatic heme degradation has already been observed several decades ago although the consequences of these reactions have not received much attention. In this review, we have focused on nonenzymatic degradation of heme with an emphasis on hemoglobin.

HEME BIOSYNTHESIS

Porphyrin contains four pyrrole rings that are linked by methene bridges forming a highly conjugated closed ring system. In protoporphyrin IX, each pyrrole ring has a methyl side chain and either a vinyl or propionic acid side chain (Fig. 1A). Heme is the Fe(II) complex of protoporphyrin IX (Fig.1B). Hematin is the oxidized Fe(III) heme with a water molecule and hydroxide ion in the axial positions. Hemin, the complex formed when chloride ion displaces the water molecule of hematin, is the non–protein-bound form found *in vivo*.

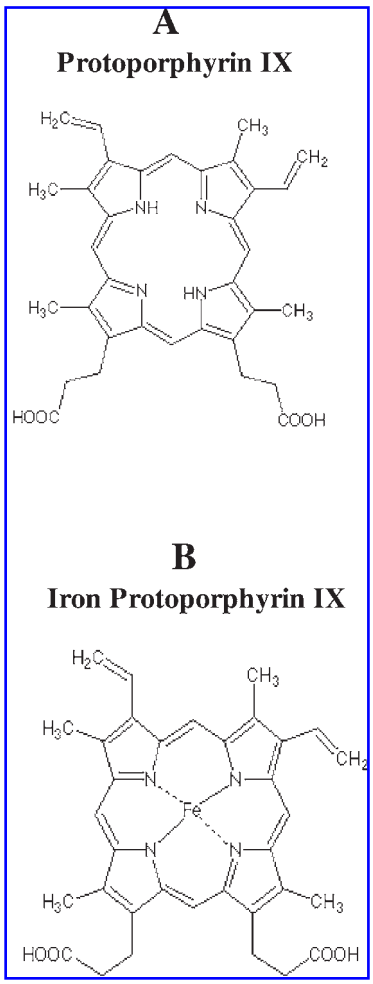

FIG. 1. Molecular structures of protoporphyrin and iron protoporphyrin. (A) Protoporphyrin consists of four pyrrole rings joined together by carbon methene bridges. Each pyrrole has a methyl side chain and either a vinyl or propionic acid side chain. **(B)** Iron binds to the four pyrrole nitrogens, displacing two central hydrogen atoms to form iron protoporphyrin.

Heme biosynthesis involves a series of enzymatic reactions, which occur partly in the mitochondria and partly in the cytosol. Heme synthesis starts with the condensation of glycine and succinyl-CoA by the mitochondrial enzyme δ-aminolevulinic acid synthase to form δ-aminolevulinate (ALA). The cytoplasmic enzyme ALA dehydratase is responsible for condensing two molecules of ALA to form a monopyrrole, porphobilinogen. The combination of four porphobilinogen molecules forms a linear tetrapyrrole. Subsequent enzymatic reactions result in the formation of the cyclic tetrapyrrole uroporphyrinogen. The side chains are subsequently modified until protoporphyrin IX is formed and ferrochelatase catalyzes the binding of iron to the four pyrrole nitrogens with two central hydrogen atoms displaced (Fig. 1B) (22, 72). This heme molecule is incorporated into a protein hydrophobic pocket with the 4-coordinated iron still able to coordinate with two axial ligands forming the active site of heme proteins. In hemoglobin, one axial ligand involves a histidine on the globin and the second axial ligand is available for exogenous ligands. The transport of oxygen involves the reversible binding of oxygen to this axial site in the ferrous form of hemoglobin.

ENZYMATIC HEME DEGRADATION

Most heme degradation studies have emphasized enzymatic degradation. To appreciate the similarities and differences between nonenzymatic heme degradation (the purpose of this review) and enzymatic heme degradation, we have included a short summary of enzymatic heme degradation.

Enzymatic heme degradation (Fig. 2) requires the HO system consisting of three HO isoenzymes and microsomal cytochrome P450 reductase (22, 72, 75, 79, 80). First, HO binds the hemin, which is then reduced to the ferrous heme by NADPH-cytochrome P450 reductase. In the second step, the ferrous heme is complexed with an oxygen molecule that gets reduced to superoxide by another NADPH-cytochrome P450 reductase. In the third step, this superoxide attacks the heme producing ferri-biliverdin IXα by the elimination of the α-methene bridge carbon as carbon monoxide (CO) utilizing another electron from a third NADPH molecule and two oxygen molecules. An additional NADPH-mediated reduction of ferric to ferrous results in the release of iron from biliverdin. The released ferrous iron is efficiently chelated by apoferritin to be stored as ferritin or transported to the bone marrow via transferrin for recycling. Biliverdin reduces to bilirubin by biliverdin reductase. Bilirubin converts to a water-soluble compound by microsomal phase II glucuronidation, and ultimately is eliminated from the body by bile and feces (Fig. 2).

Enzymatic heme degradation converts the prooxidant heme to an antioxidant bilirubin (56).It is, however, important to note that even for enzymatic heme degradation the actual degradation of the heme involves ROS or other free radical species.

It has been shown that heme degradation by HO also produces H_2O_2, which not only degrades the heme nonspecifically without the formation of biliverdin, but also inactivates the HO enzyme (63).

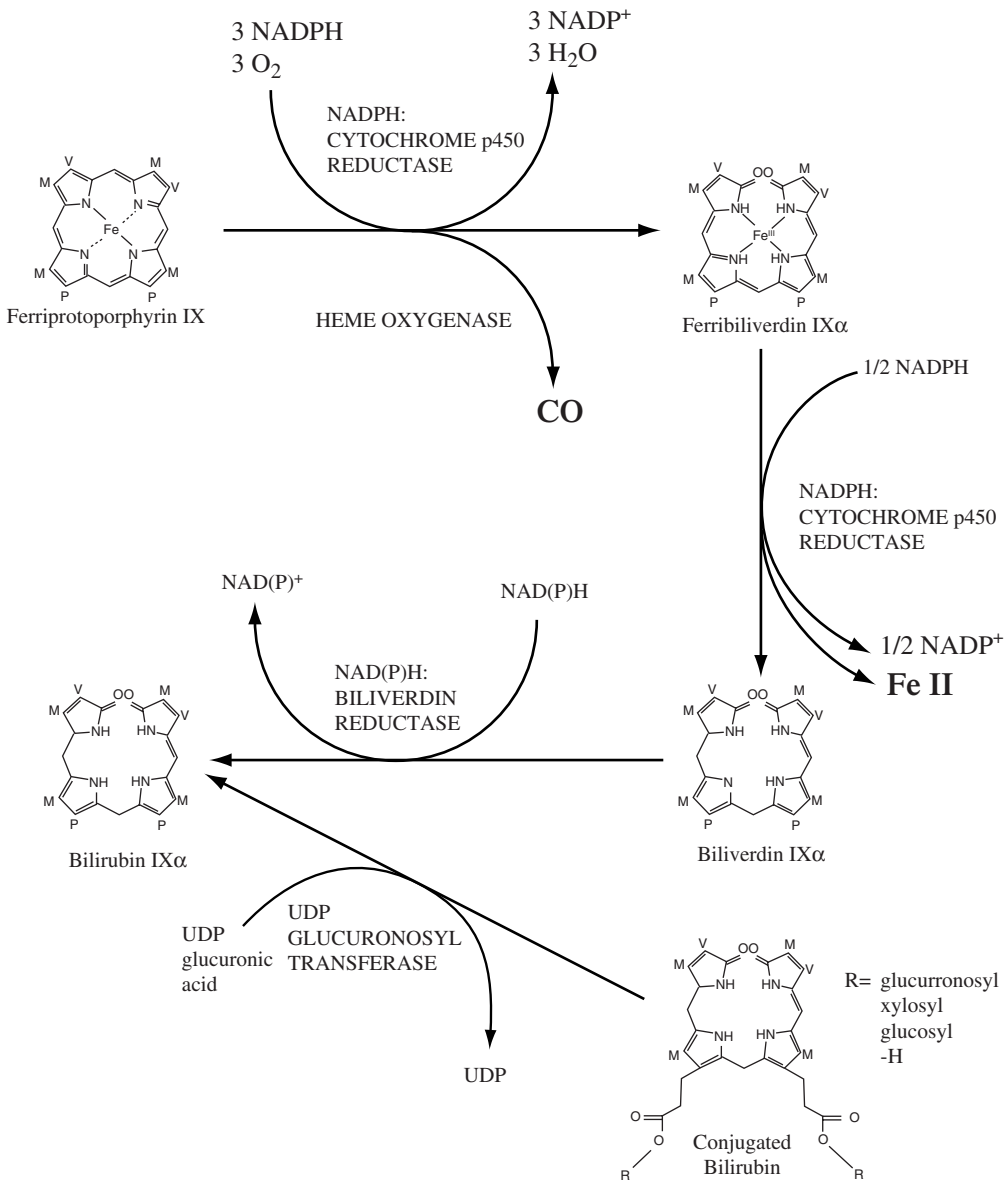

FIG. 2. Microsomal HO-mediated heme degradation. HO along with NADPH cytochrome P450 reductase converts ferriprotoporphyrin IX to biliverdin IX using oxygen and NADPH and releasing water, CO, and iron. Biliverdin is reduced to bilirubin by biliverdin reductase. M = methyl; V = vinyl. [Reproduced from the review (72) with permission from Ryter and Tyrrell].

Heme proteins can also catalyze their own heme degradation. It has been shown that microsomal cytochrome P450 reductase (28) and the cytochrome *c* reductase system (54) degrade their hemes or free heme in the presence of NADPH and oxygen. The NADH-dependent degradation of cytochrome-heme to propentdyopents is reported to take place on the mitochondrial inner membrane, specifically associated with NADH-ubiquinone oxidoreductase (50). Heme degradation mediated by these enzymes is inhibited by catalase, but not by superoxide dismutase (SOD), suggesting that H_2O_2 is involved in the heme degradation. Heme degradation observed in the presence of NADH and lipoamide dehydrogenase at pH 6.5–9.0 was, however, inhibited by both catalase (80%) and SOD (70%) (55).

Several studies (15, 29, 33, 53) have shown that metabolic activation of xenobiotics (*e.g.*, carbon tetrachloride, 2-allyl-2-isopropylacetamide, vinyl chloride) by the liver microsomal cytochrome P450 system produces free radical species, which degrade the heme moiety of microsomal cytochrome P450.

NONENZYMATIC HEME DEGRADATION

Heme degradation by H_2O_2

Degradation of heme to dipyrrolic compounds (propentdyopents) by H_2O_2 was first reported by Fisher and Muller in 1937 (23). Later reports have shown that H_2O_2 degrades the

ferric heme to produce biliverdin IXα and CO in much the same way as HO. It was also shown that subsequent reactions of H_2O_2 with biliverdin yield bilirubin (26, 43). The formation of a peroxidatic [Fe(III)-OOH] intermediate from the reaction of deuteroferriheme with H_2O_2 has been shown to be responsible for the decomposition of H_2O_2 (47).

Brown *et al.* (9) extensively investigated the mechanism of heme degradation by H_2O_2. The oxidation of ferrihemes as a function of pH in the range from 6.5 to 11 indicated an inverse dependence on proton (H^+) levels. Based on this observation, the authors interpreted their results to indicate that the active species, which attacks the heme, is HO_2^-.

$$H_2O_2 \rightleftarrows HO_2^- + H^+ \quad (1)$$

Their model suggests that the ferriheme–peroxide complex results in the intramolecular oxidation of heme to form bile pigments (36).

$$\text{Heme-Fe(III)} + HO_2^- \rightleftarrows \text{Heme-Fe(III)-OOH}^- \rightarrow \text{Bile Pigment} + \text{Fe} + \text{CO} \quad (2)$$

Ascorbic acid also destroys the heme and heme proteins by attacking methene bridges randomly producing mixtures of biliverdin isomers (8, 82). These reports suggest that a mechanism for nonenzymatic heme degradation is similar to that of HO enzymatic heme degradation producing biliverdin and CO. However, subsequent reports did not confirm the formation of CO from H_2O_2-mediated heme degradation. Heme protein enzymes such as cytochrome P450, cytochrome *c* reductase, and mitochondrial NADH-ubiquinone oxidoreductase mediate the heme degradation by the production of superoxide and H_2O_2. As the enzymes do not directly attack the heme, degradation mediated by these enzymes can be considered nonenzymatic degradation. Studies show that heme degradation by these enzymes (11, 17, 28, 50, 54) does not produce CO, further implying that bile pigments are not the primary products.

Schaefer *et al.* (74) studied in detail the degradation products formed during the reaction of hemin with H_2O_2. Six major products characterized by them using mass spectroscopy and NMR were four dipyrrolic propentdyopents, hematic acid, and methylvinylmaleimide. Biliverdin was not found to be an intermediate in the formation of the maleimide and propentdyopent degradation products during this reaction. Groves *et al.* (27) demonstrated the formation of a formal perferryl species [$^+$heme-Fe(IV) = O or heme-Fe(V) = O] from the reaction of heme and H_2O_2. This species has been implicated in the random cleavage of the porphyrin ring to form dipyrroles and monopyrroles (74). Glutathione (GSH) has also been reported to decompose the heme, either in the free form or bound to the protein. This reaction also presumably involves superoxide and H_2O_2, because the reaction is inhibited by catalase and SOD (5).

Even *in vivo,* it has been shown that the amount of CO generated during hepatic heme degradation does not account for all the observed heme degradation (7). Thus, injecting heme and heme proteins into rabbits resulted in the excretion of significant quantities of biliverdin isomers, similar to those obtained after the injection of phenylhydrazine (PH) (39), indicating the random degradation of heme methene bridges. These results indicate the presence of a non-HO mechanism for heme degradation *in vivo*. Whether this involves a non-HO enzymatic mechanism or nonenzymatic heme degradation is not known.

We have shown that the reaction of heme and hemin with H_2O_2 produces two fluorescent products. One product has an excitation wavelength of 321 nm and emission wavelength of 465 nm, and the second product has an excitation wavelength of 460 nm and emission wavelength of 525 nm (57) (Fig. 3). These bands are similar to those found when Fe(II) hemoglobin reacts with H_2O_2 (see below), although considerably higher H_2O_2 concentrations are required with the isolated heme and hemin (57).

Protoporphyrin, without the iron, has a fluorescence excitation wavelength of 400 nm with an emission wavelength of 619 nm, clearly distinct from the fluorescence produced during the reaction of H_2O_2 with heme or hemin. However, even in the presence of a 100-fold excess of H_2O_2, protoporphyrin does not produce those fluorescent bands generated during the reaction with H_2O_2. This observation indicates that the metal center is essential to form the fluorescent products. Potassium superoxide in aprotic solvent systems does, however, produce the same fluorescent products when reacted with either heme or protoporphyrin (59). These results suggest that superoxide is the active species and that the reaction of H_2O_2 with the metal center results in the formation of superoxide. The essential role of superoxide is supported by the reaction of the heme tetrapyrrole rings when superoxide is generated by xanthine oxidase/xanthine (70).

A mechanism for generating superoxide during the reaction of H_2O_2 with heme could involve the formation of a fer-

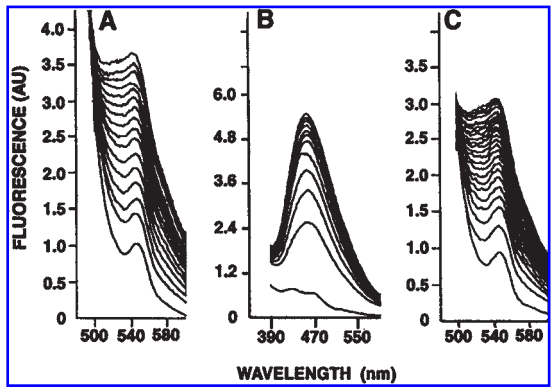

FIG. 3. Fluorescence spectra of the fluorescent products produced during the reaction of heme and hemin with H_2O_2 in 50 m*M* phosphate buffer, pH 7.4, at 21°C. (A) Hemin (50 μ*M*) was reduced to heme by incubating with NADPH (1.0 m*M*) for 30 min in phosphate buffer, pH 7.4 at 21°C. The repetitive emission spectra were scanned at an excitation wavelength of 460 nm following the addition of 1 m*M* H_2O_2 with a time interval of 2 min. The emission of heme at an excitation wavelength of 321 was not measured because of the interference of NADPH fluorescence in that region. The emission spectra of hemin (50 μ*M*) at an excitation wavelength of 321 nm **(B)** and excitation wavelength of 460 nm **(C)** with a time interval of 2 min following the addition of 5 m*M* H_2O_2 are also shown.

rylheme intermediate, which can then react with a second molecule of H_2O_2 to produce Fe(III)-heme and superoxide.

$$\text{Heme-Fe(II)} + H_2O_2 \rightarrow \text{Heme-Fe(IV)} = O + H_2O \quad (3)$$

$$\text{Heme-Fe(IV)} = O + H_2O_2 \rightarrow \text{Heme-Fe(III)---}O_2^{\bullet -} \rightarrow \text{Heme degradation} \quad (4)$$

Support for a reaction scheme involving higher oxidation states of iron comes from the reported consumption of H_2O_2 by ferrihemes (44). The catalase- and peroxidase-like activity of ferrihemes (44,45,48) is explained by the following equation.

$$\text{Heme-Fe(III)} + H_2O_2 \rightarrow {}^{+}\text{Heme-Fe(V)} = O + H_2O \quad (5)$$

The heme-Fe(V) = O represents a Fe(IV) complex with one additional electron removed from the porphyrin ring producing a porphyrin cation, which oxidizes the H_2O_2 to molecular oxygen and water.

$${}^{+}\text{Heme-Fe(V)=O} + H_2O_2 \rightarrow \text{Heme-Fe(III)} + O_2 + H_2O \quad (6)$$

The minor pathway may involve the one-electron oxidation of the H_2O_2 to superoxide anion with the Fe(III)-heme reduced to Fe(II)-heme.

$$\text{Heme-Fe(III)} + H_2O_2 \rightarrow \text{Heme Fe(II)} + O_2^{\bullet -} + 2H^{+} \quad (6A)$$

This superoxide may be responsible for H_2O_2-mediated hemin degradation (Fig. 3). More H_2O_2 is required to degrade the hemin than heme.

Schaefer *et al.* (74) have shown that the formal perferryl can abstract an electron from the methene bridge to form a glycol and regenerate ferric heme. This ferric heme can then react with another molecule of H_2O_2 that oxidatively cleaves the glycol to form amide and α-formyl pyrrolic species. These reactions can be repeated in other methene bridges, resulting in the breakdown of the porphyrins to dipyrroles and monopyrroles.

Heme degradation by hydroperoxides

The reaction of linoleic hydroperoxide with hematin has been studied with a major emphasis on the degradation products of the peroxide (14,16). The major pathway in this reaction involves the one-electron reduction of the lipid hydroperoxide to an alkoxyl radical with the hematin converted to a ferryl heme [Fe(IV)-heme].

$$\text{LOOH} + \text{Fe(III)-heme} \rightarrow \text{LO}^{\bullet} + OH^{-} + \text{Fe(IV)-heme} \quad (7)$$

The minor pathway involves the one-electron oxidation of the hydroperoxide to the hydroperoxyl radical with the hematin reduced to heme.

$$\text{LOOH} + \text{Fe(III)-heme} \rightarrow \text{LOO}^{\bullet} + \text{Fe(II)-heme} \quad (8)$$

These alkoxyl and peroxyl radicals are responsible for hematin mediated lipid peroxidation in membranes, proteins, and DNA (1, 2, 31, 78). At the same time, the alkoxyl and peroxyl radicals are probably responsible for degradation of heme and heme proteins. The reported (40) formation of unknown fluorescence substances when linoleate hydroperoxide reacts with heme can, thus, be attributed to degradation of the heme.

We have also found that organic hydroperoxides (cumene hydroperoxide and tertiary butyl hydroperoxides) degrade heme and hemin to produce the same fluorescent products formed when H_2O_2 reacts with heme or hemin (unpublished observations).

Heme degradation by xenobiotics

PH is known to induce hemolysis by denaturing hemoglobin producing Heinz bodies. It has been shown (38) that during this process PH degrades the porphyrin ring of hemoglobin producing hematinic acid. PH is a more potent reagent for heme degradation and the formation of fluorescent degradation products than H_2O_2 or organic hydroperoxides. Furthermore, the reactive species is not H_2O_2 because catalase and glutathione peroxidase (GSHPX) provide only minimal protection from PH-mediated heme degradation (unpublished observations). Hydroxylamine, divicine, isouramil, acrolein, aniline, and dapsone have also been shown to increase soluble iron when incubated with erythrocytes, indicating appreciable heme degradation (12, 13, 77).

HEMOGLOBIN

The ROS associated with heme degradation (see above) are known to react with hemoglobin. Superoxide in reacting with hemoglobin acts as both an oxidizing and a reducing agent, producing a mixture of methemoglobin (metHb) and oxyHb. OxyHb and metHb also react with H_2O_2 and hydroperoxides to produce ferrylhemoglobin [ferrylHb; HbFe(IV)=O] and oxoferrylhemoglobin [oxoferrylHb; $^{\bullet}$HbFe(IV)=O], respectively (4, 81). FerrylHb and oxoferrylHb are unstable species eventually reduced to metHb.

$$\text{HbFe(II)} + H_2O_2 \rightarrow \text{HbFe(IV)=O} + H_2O + O_2 \quad (9)$$

$$\text{HbFe(III)} + H_2O_2 \rightarrow {}^{\bullet}\text{HbFe(IV)=O} + H_2O \quad (10)$$

A molar excess of H_2O_2 has been reported to degrade the heme moiety of heme proteins such as hemoglobin, metHb, and cytochrome *c*, releasing iron from the heme (24, 32, 34, 35). Although it has clearly been established that the iron can be released from hemoglobin, the evidence for heme degradation during this process is indirect. The uncertainty of heme degradation of intact hemoglobin is based on the insensitivity of the spectral method used to study heme degradation. In evaluating heme degradation, it is also necessary to consider the possible contribution from a reaction of excess H_2O_2 with heme released from hemoglobin (see below). Heme is more readily dissociated from metHb formed during the reaction of H_2O_2 with hemoglobin (10). This dissociation can be further enhanced by damage to the globin, which binds the heme.

Fluorescence formation during the reaction of hemoglobin with H_2O_2

Heme degradation involving the direct reaction of ROS with the intact protein was established when we found that two fluorescent products and free iron are formed during the reaction of H_2O_2 with hemoglobin (57). The excitation and emission wavelengths of these fluorescent products are similar to those observed for the fluorescent products formed when H_2O_2 reacted with heme (Fig. 3) or protoporphyrin IX reacts with superoxide (59). The excitation and emission wavelengths for these bands are distinct from those of the globin fluorescent amino acids tryptophan, tyrosine, or dityrosine, as well as free protoporphyrin. The observation of these fluorescent bands is, therefore, a clear indication that the heme is being degraded. Interestingly, metHb, for which heme dissociation is greater, does not produce fluorescent products during treatment with H_2O_2. Thus, the formation of fluorescent products requires the Fe(II) ferrous state of hemoglobin.

The fluorescent products produced when a 10-fold excess H_2O_2 reacts with hemoglobin corresponds to ~5% of the hemes being degraded. This low level of degradation would be difficult to quantify using changes in the visible spectrum of hemoglobin. Because of the sensitivity of the fluorescent method, it was, however, possible to show that degradation products are produced even when the concentration of H_2O_2 is 50 times lower than that of hemoglobin (58). Although the same products are produced when H_2O_2 reacts with free heme or hemin (57), much higher concentrations of H_2O_2 are needed. This behavior, therefore, clearly indicates that the heme is being degraded while it is still associated with the globin.

The mechanism of heme degradation

Initially, it was thought that hemoglobin could act as a Fenton catalyst (73).

$$\text{Hb(II)} + H_2O_2 \rightarrow \text{Hb(III)} + {}^{\bullet}OH + OH^- \qquad (11)$$

However, subsequent studies have shown that the iron bound to the heme of globin cannot transfer an electron to H_2O_2 to generate hydroxyl ($^{\bullet}OH$) radicals. Any observed $^{\bullet}OH$ radicals presumably result from the reaction of free iron released from hemoglobin as a result of the reaction with H_2O_2 (32, 66). A possible contribution of such a reaction to the observed heme degradation was, however, ruled out. Thus, the addition of excess iron to hemoglobin before adding H_2O_2 does not promote heme degradation, indicating that Fenton chemistry does not contribute to H_2O_2 induced heme degradation.

Instead, the reaction must be associated with the established reaction of H_2O_2 with Fe(II) hemoglobin to produce ferrylHb (Eq. 9). The relationship between ferrylHb and heme degradation was established by showing that compounds that react with ferrylHb, such as sodium sulfide and peroxidase substrates, prevent heme degradation (59). It was also possible to show that heme degradation does not result directly from the formation of ferrylHb. Instead the fluorescent products were shown to form when ferrylHb reacts with an additional molecule of H_2O_2. Thus, when catalase was added after the H_2O_2 had already converted the Fe(II) hemoglobin to ferrylHb, the formation of fluorescent products was inhibited.

Although ferryl species are strong oxidants, the reaction of these species with H_2O_2 has not been considered. To explain our results, we, therefore, proposed that ferrylHb oxidizes H_2O_2 to the superoxide anion with the Fe(IV) being reduced to Fe(III) producing metHb (Fig. 4).

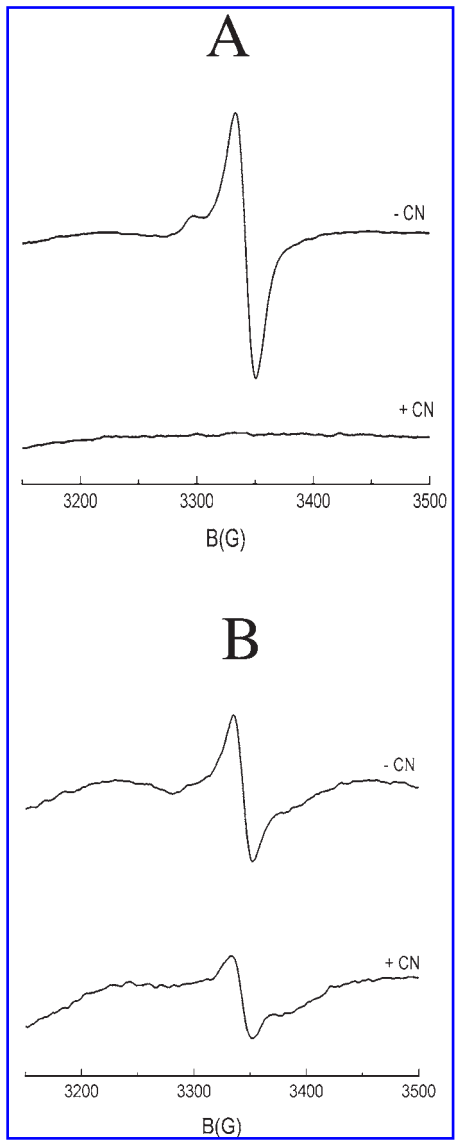

FIG. 4. Free radical production during the reaction of hemoglobin with H_2O_2. (A) MetHb at 450 μ*M* reacted with 4.5 m*M* H_2O_2 in the presence or absence of 5 m*M* sodium cyanide (CN). The formation of oxoferrylHb when metHb reacts with H_2O_2 results in the formation of a protein radical. The formation of this protein radical is completely prevented by the complex of cyanide with metHb. **(B)** Hemoglobin at 450 μ*M* reacted with 4.5 m*M* H_2O_2 in the presence or absence of 5 m*M* cyanide. Even though ferrylHb does not contain a radical, a radical signal is observed when H_2O_2 reacts with hemoglobin. Some of the radical signal is due to the metHb formed, which goes on to react with H_2O_2. Adding cyanide eliminates the contribution of this radical. The residual free radical signal is attributed to superoxide formed by the reaction of ferrylHb with H_2O_2.

$$HbFe(IV)=O + H_2O_2 \rightarrow HbFe(III)\text{---}O_2^{\bullet -} + H_2O \quad (12)$$

Electron paramagnetic resonance has been able to detect this superoxide still retained in the hydrophobic heme pocket (once the superoxide leaves the pocket, it rapidly dismutates and cannot be detected). Superoxide produced in the hydrophobic heme pocket is not only in the right location to react with the tetrapyrrole ring, but because of the aprotic environment of the heme pocket the superoxide has greater reactivity. Enhanced reactivity can also be attributed to the protonation of the superoxide producing the perhydroxyl radical, which is a strong oxidizing agent known to initiate lipid peroxidation. The relatively long lifetime of the superoxide in the heme pocket is explained by the slow rate for the escape of the superoxide from the heme pocket and the inaccessibility of SOD to the heme pocket to scavenge the superoxide. The mechanism of heme degradation is summarized in Fig. 5.

The requirement of Fe(II) hemoglobin for heme degradation

The requirement of Fe(II) hemoglobin for heme degradation can be explained in terms of differences between the reaction of H_2O_2 with ferrylHb produced from Fe(II) hemoglobin (Eq. 12) and the reaction H_2O_2 with oxoferrylHb produced from Fe(III) hemoglobin.

$$^{\bullet}HbFe(IV)=O + H_2O_2 \rightarrow HbFe(III) + O_2 + H_2O \quad (13)$$

OxoferrylHb formed from the reaction of metHb and H_2O_2 oxidizes a second molecule of H_2O_2 by two electrons to produce oxygen, instead of the superoxide formed when ferrylHb reacts, regenerating metHb. This reaction will, therefore continue to use up H_2O_2, and is the basis for the catalase- and/or peroxidase-like activity of Fe(III) heme proteins.

These reactions can be understood in relation to the known peroxidase activity of hemoglobin, which is believed to be analogous to that of horseradish peroxidase. In the classical peroxidase reaction, H_2O_2 withdraws two electrons from the enzyme, producing the intermediate compound 1. This compound withdraws one electron from the substrate, forming compound 2. Then compound 2 withdraws another electron from the substrate to produce the original enzyme and product (21). FerrylHb, the heme iron being one oxidizing equivalent above that of metHb, corresponds to peroxidase compound 2 and can withdraw one electron from the substrate. In the absence of substrates, ferrylHb would oxidize H_2O_2 by abstracting one electron, producing superoxide and metHb (Eq. 9). OxoferrylHb produced during the reaction of metHb with H_2O_2 (Eq. 10) is two oxidizing equivalents above metHb. It is, therefore, equivalent to peroxidase compound 1, except that the second oxidizing equivalent exists on the globin instead of the porphyrin. In the absence of substrates, oxoferrylHb will react with H_2O_2. Although the first step in this reaction may generate superoxide, this superoxide rapidly reacts with the one electron reduced oxoferryl to produce metHb and oxygen. The lifetime of this transient superoxide is much shorter than that produced by the reaction of ferrylHb, and no fluorescent heme degradation is produced. Therefore, metHb in the absence of substrates has catalase-type activity in which one molecule of H_2O_2 serves as a two-electron acceptor and another H_2O_2 molecule serves as a two-electron donor, producing an oxygen molecule and two water molecules and regenerating metHb.

Although H_2O_2 does not produce heme degradation when reacted with metHb, the degradation of Fe(III) hemin has been reported (see above), even though it is less efficient than the reaction with Fe(II) heme (see above). It has been suggested that the reactive species responsible for Fe(III) hemin degradation is the HO_2^- (9), perferryl species (27), or superoxide formed when Fe(III) heme is reduced by H_2O_2 (Eq. 6A). For metHb, which has a greater tendency to form the ferryl species, it is unlikely to form HO_2^- or superoxide. The other difference is the source of the second electron transferred to the H_2O_2 when it reacts with Fe(III) heme. In metHb, this electron comes from the globin and the heme is only in the Fe(IV) state. However, with isolated heme, both

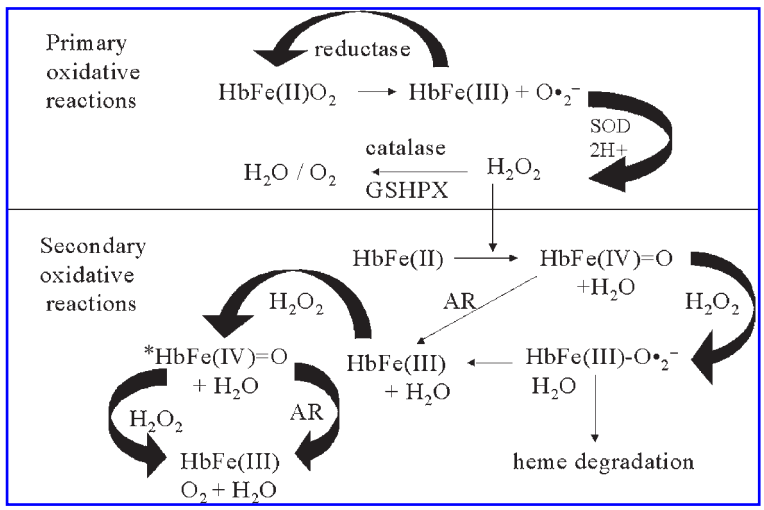

FIG. 5. The proposed mechanism for the degradation of heme during the autoxidation of oxyHb. HbFe(IV)=O, ferrylHb; *HbFe(IV)=O, oxoferrylHb; AR, autoreduction.

electrons come from the heme, resulting in the even more reactive Fe(V) and/or a porphyrin cation (Eq. 6). It is presumably this highly reactive state that is responsible for the reaction with the porphyrin ring. Although this molecule can react with an additional molecule of H_2O_2 to regenerate the Fe(III) heme, it is possible that the reaction irreversibly damaging the heme can compete with the catalase like activity, which regenerates Fe(III) heme.

Heme degradation during autoxidation of oxyHb

OxyHb slowly undergoes autoxidation and produces superoxide and metHb. This superoxide is converted to H_2O_2 by SOD or spontaneous dismutation. Autoxidation, therefore, produces both superoxide and H_2O_2. These reactions explain the steady-state concentration H_2O_2 in red cells, which has been reported to be ~2×10^{-10} *M*. We have reported that there is a direct correlation between the hemoglobin being oxidized and the formation of fluorescent degradation products (58). Catalase and compounds that interfere with ferrylHb formation inhibit most of this heme degradation.

The observation that catalase was more efficient in inhibiting the degradation than SOD indicates that the superoxide formed during autoxidation does not lead to heme degradation. The difference between this superoxide and the superoxide formed when H_2O_2 reacts with ferrylHb (Eq. 12) can be explained by the difference in the exact location of the superoxide in the heme pocket and/or the lifetime of the superoxide in the heme pocket.

These results indicate that the heme degradation that occurs during autoxidation (Fig. 6) and during the reaction of H_2O_2 with hemoglobin proceed by similar mechanisms. As reported earlier (34), a molar excess of H_2O_2 is not required to degrade the heme moiety of hemoglobin. However, these autoxidation studies indicate that when fluorescence is used as a measure of heme degradation, it can be detected even at the very low concentrations of H_2O_2 generated during autoxidation. The use of the sensitive fluorescent technique made it possible to detect low levels of heme degradation missed by other investigators who have studied the reaction of H_2O_2 with hemoglobin (58).

Hemoglobin-based blood substitutes

Chemically modified hemoglobins have been developed as oxygen-carrying therapeutics and are currently being tested in phase II/III clinical trials (3, 68). However, many of these modified hemoglobins have an enhanced rate of autoxidation with an increase in the concomitant heme degradation (Fig. 6) (61). A dichotomy between the rate of autoxidation and heme degradation was, however, found when cross-linked bovine hemoglobin was compared with other blood substitutes. Although the modified bovine hemoglobin has an elevated rate of autoxidation, relatively low levels of heme

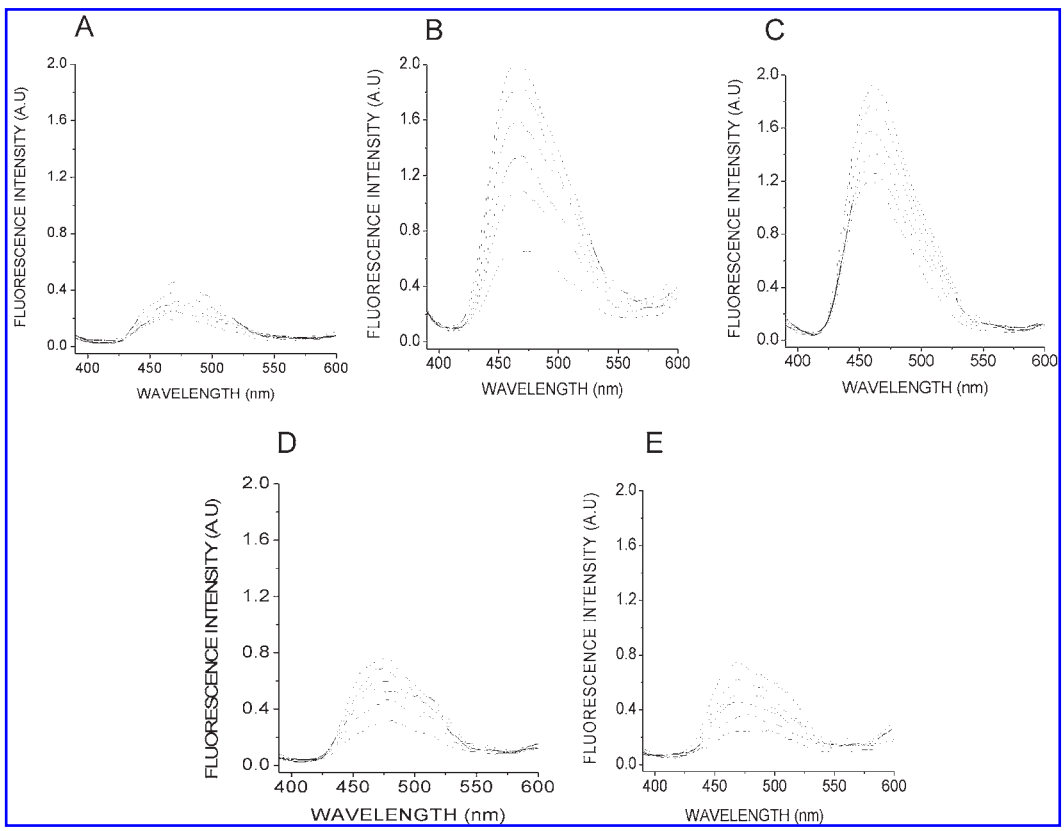

FIG. 6. Fluorescent emission spectra (excitation wavelength 321 nm) of 50 μ*M* of various hemoglobins in EDTA containing 50 m*M* phosphate buffer during incubation at 37 °C. Spectra were taken at 1-h time intervals. **(A)** Hemoglobin A_0; **(B)** human hemoglobin polymerized with *O*-raffinose; **(C)** human hemoglobin cross-linked with *bis*(3,5-dibromosalicyl)fumarate; **(D)** bovine hemoglobin; and **(E)** bovine hemoglobin polymerized with glutaraldehyde.

degradation products were observed. This was explained by differences in the formation of ferrylHb responsible for heme degradation. Some recent data on toxic effects of various blood substitutes suggest that the heme degradation products may be more closely associated with these toxic effects than the levels of autoxidation, making the bovine cross-linked hemoglobin a more useful blood substitute (6).

Oxidized hemoglobin, which is reduced by dithionite, is frequently used as a source of hemoglobin for many experimental purposes. On the basis of fluorescence, we have found heme degradation products in all stored hemoglobin samples (both commercial and prepared in house from flesh blood). For some commercial samples, we have found that as much as 20% of their hemes are degraded. These heme degradation products and free or loosely bound iron present in samples can interfere with many of the assays utilizing hemoglobin.

HEME DEGRADATION IN RED CELLS

The excitation and emission wavelengths of one of the fluorescence bands (excitation 460 nm, emission 525 nm) are close to wavelengths routinely monitored by flow cytometry (excitation 488 nm, emission 530 nm). Therefore, heme degradation in intact red cells can be monitored by flow cytometry. This method was used to follow the formation of fluorescent products during the reaction of H_2O_2 (60), cumene hydroperoxide, and PH (unpublished observations) with red cells. This fluorescence was inhibited by pretreatment of cells with CO or nitrite, but not antioxidants like butylated hydroxytoluene, and α-tocopherol, establishing the involvement of hemoglobin heme degradation, rather than lipid peroxidation, as a source for the observed fluorescence.

The degradation of heme by endogenously generated H_2O_2 in red cells was investigated during incubation of red cells in phosphate-buffered saline at 25°C. Heme degradation begins to be detected only after 40 h of incubation when GSH and catalase levels decreased by 40% and 22%, respectively. However, when GSH was blocked with iodoacetamide, heme degradation was observed without any lag. These results show that although catalase scavenges the major portion of H_2O_2, GSHPX plays a crucial role in protecting the cell from H_2O_2-induced heme degradation (62). It has been suggested that this effect of GSHPX may be related to its ability to neutralize H_2O_2 generated in the region of the membrane by hemoglobin bound to the membrane.

GSH levels are known to reduce by 20–30% during red cell aging (51). The effect of this drop in GSH and the resultant lower GSHPX activity suggests that heme degradation may take place *in vivo* (46).

HEME DEGRADATION *IN VIVO*

Injecting heme and heme proteins into rabbits resulted in urinary excretion of significant quantities of biliverdin isomers, which are similar to those obtained after the injection of PH into rabbits (39). Propendyopents were also found in the urine of infants with prolonged nonobstructive jaundice (42). Oxidation of bilirubin is not the source for these products *in vivo* (49). Therefore, the presence of biliverdin isomers and particularly propendyopents in urine is an indication of nonenzymatic heme degradation. These results suggest that nonenzymatic heme degradation does occur *in vivo*.

The red cells, which do not undergo enzymatic heme degradation, are an ideal system to investigate nonenzymatic heme degradation *in vivo*. It is, however, very difficult to quantify basal levels of fluorescent heme degradation products generated in red cells *in vivo* by flow cytometry. This difficulty arises because of the contribution of instrumental setting to the low levels of fluorescence. It has, however, been possible to bypass this difficulty by measuring steady-state fluorescence of dilute hemolysates (50 μ*M* hemoglobin). Fluorescence with the characteristic excitation and emission wavelengths of both fluorescent heme degradation products has been found. The level of these fluorescent signals depends on how long the cells are in circulation (Fig. 7) as indicated by the twofold higher fluorescence in the most dense (presumably older) fraction than the least dense (presumably younger cells) fraction (unpublished observations). The finding that this basal fluorescence increases when small amounts of H_2O_2 are added to the hemolysate devoid of membranes indicates that the fluorescence is related to heme degradation and not lipid peroxidation.

The presence of fluorescent heme degradation products, possibly dipyrroles, has been found in hemoglobin Koln erythrocytes (19). In sickle erythrocytes (37) and in thalassemia (76), there is evidence of non–heme-bound iron. This iron can originate from heme degradation. Consistent with these reports, we have observed a four- to ten-fold increase in heme degradation products in transgenic mice with sickle hemoglobin and in thalassemic mice (unpublished observations).

Red cells contain an extensive antioxidant system to neutralize the ROS that are generated due to autoxidation of hemoglobin. The detection of fluorescent degradation products in normal fresh blood samples indicates that some of these ROS escape the antioxidant systems and are able to generate heme degradation products. As indicated in terms of the major role of GSHPX (see above), these effects could be attributed to fluctuations in the level of GSH and GSHPX activ-

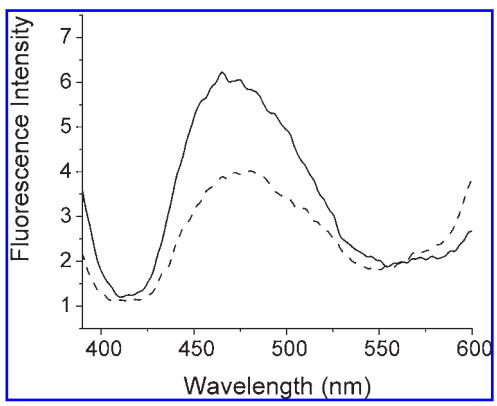

FIG. 7. Fluorescent emission spectra (excitation wavelength 321 nm) of red cells separated by density gradient. The basal fluorescence of least dense cells (---) is compared with the basal fluorescence of densest cells (—).

ity and/or increases in oxidative stress experienced by the organism. The evidence of free iron and higher levels of degradation products with unstable hemoglobins (hemoglobin Koln, sickle hemoglobin, and thalassemia) indicates that the level of degradation products reflects the oxidative stress experienced by the cell. Whereas other aerobic cells have heme-degrading enzymes, as well as enzymes and proteins that dispose of these degradation products, the red cell does not seem to have a system to remove these degradation products. Therefore, these heme degradation products may slowly accumulate on the cell membrane providing an integrated measure of the oxidative stress experienced by the red cell/organism.

In evaluating the significance of red cell heme degradation, it is also necessary to consider possible functional effects on the red cell of these heme degradation products. These products can affect the properties of the membrane influencing red cell deformability and the ability of the red cell to transport oxygen. Our recent studies (unpublished observations) further suggest that these degradation products play a role in the exposure of antigenic sites thought to be responsible for removing cells from circulation.

ABBREVIATIONS

ALA, δ-aminolevulinate; CO, carbon monoxide; ferrylHb, ferrylhemoglobin; GSH, glutathione; GSHPX, glutathione peroxidase; heme, Fe(II)-heme; hemin, Fe(III)-heme; HO, heme oxygenase; H_2O_2, hydrogen peroxide; LOOH, lipid hydroperoxide; MetHb, methemoglobin; $O_2^{\bullet -}$, superoxide radical; $^{\bullet}OH$, hydroxyl radical; oxoferrylHb, oxoferrylhemoglobin; oxyHb, oxyhemoglobin; PH, phenylhydrazine; ROS, reactive oxygen species; SOD, superoxide dismutase.

REFERENCES

1. Aft RL and Mueller GC. Hemin-mediated DNA strand scission. *J Biol Chem* 258: 12069–12072, 1983.
2. Aft RL and Mueller GC. Hemin-mediated oxidative degradation of proteins. *J Biol Chem* 259: 301–305, 1984.
3. Alayash AI. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? *Nat Biotechnol* 17: 545–549, 1999.
4. Alayash AI, Patel RP, and Cashon RE. Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. *Antioxid Redox Signal* 3: 313–327, 2001.
5. Atamna H and Ginsburg H. Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. *J Biol Chem* 270: 24876–24883, 1995.
6. Baldwin AL, Wiley EB, and Alayash AI. Comparison of effects of two hemoglobin-based O_2 carriers on intestinal integrity and microvascular leakage. *Am J Physiol Heart Circ Physiol* 283: H1292–H1301, 2002.
7. Bissell DM and Guzelian PS. Degradation of endogenous hepatic heme by pathways not yielding carbon monoxide. Studies in normal rat liver and in primary hepatocyte culture. *J Clin Invest* 65: 1135–1140, 1980.
8. Bonnett R and McDonagh AF. The meso-reactivity of porphyrins and related compounds. VI. Oxidative cleavage of the haem system. The four isomeric biliverdins of the IX series. *J Chem Soc* [Perkin 1] 9: 881–888, 1973.
9. Brown SB, Hatzikonstantinou H, and Herries DG. The role of peroxide in haem degradation. A study of the oxidation of ferrihaems by hydrogen peroxide. *Biochem J* 174: 901–907, 1978.
10. Bunn HF and Jandl JH. Exchange of heme among hemoglobins and between hemoglobin and albumin. *J Biol Chem* 243: 465–475,1968.
11. Cantoni L, Gibbs AH, and De Matteis F. Loss of haem and haemoproteins during the generation of superoxide anion and hydrogen peroxide: a pathway not involving production of carbon monoxide. *Int J Biochem* 13: 823–830, 1981.
12. Ciccoli L, Ferrali M, Rossi V, Signorini C, Alessandrini C, and Comporti M. Hemolytic drugs aniline and dapsone induce iron release in erythrocytes and increase the free iron pool in spleen and liver. *Toxicol Lett* 110: 57–66, 1999.
13. Comporti M, Signorini C, Buonocore G, and Ciccoli L. Iron release, oxidative stress and erythrocyte ageing. *Free Radic Biol Med* 32: 568–576, 2002.
14. Delcarte J, Jacques P, Fauconnier ML, Hoyaux P, Matsui K, Marlier M, and Thonart P. The homolytic and heterolytic fatty acid hydroperoxide lyase-like activities of hematin. *Biochem Biophys Res Commun* 286: 28–32, 2001.
15. De Matteis F and Unseld A. Increased liver haem degradation caused by foreign chemicals: a comparison of the effects of 2-allyl-2-isopropylacetamide and cobaltous chloride. *Biochem Soc Trans* 4: 205–209, 1976.
16. Dix TA and Marnett LJ. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by hematin. *J Biol Chem* 260: 5351–5357, 1985.
17. Docherty JC, Firneisz GD, and Schacter BA. Methene bridge carbon atom elimination in oxidative heme degradation catalyzed by heme oxygenase and NADPH-cytochrome P-450 reductase. *Arch Biochem Biophys* 235: 657–664, 1984.
18. Dolphin D (Ed). *The Porphyrins*, Vol. 7. New York: Academic Press, 1979.
19. Eisinger J, Flores J, Tyson JA, and Shohet SB. Fluorescent cytoplasm and Heinz bodies of hemoglobin Koln erythrocytes: evidence for intracellular heme catabolism. *Blood* 65: 886–893, 1985.
20. Estabrook RW and Werringloer J. The oxygen sensing characteristics of microsomal enzymes. *Adv Exp Med Biol* 78: 19–35, 1977.
21. Everse J, Johnson MC, and Marini MA. Peroxidative activities of hemoglobin and hemoglobin derivatives. *Methods Enzymol* 231: 547–561, 1994.
22. Ferreira GC. Heme biosynthesis: biochemistry, molecular biology, and relationship to disease. *J Bioenerg Biomembr* 27: 147–150, 1995.
23. Fisher H, and Muller A. *Hoppe-Seyler's Z Physiol Chem* 246: 43–58, 1937.

24. Florence TM. The degradation of cytochrome *c* by hydrogen peroxide. *J Inorg Biochem* 23: 131–141, 1985.
25. Gong W, Hao B, Mansy SS, Gonzalez G, Gilles-Gonzalez MA, and Chan MK. Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. *Proc Natl Acad Sci* U S A 95: 15177–15182, 1998.
26. Gray CH (Ed). *The Bile Pigments.* London: Methuen and Co., 1953, p. 24.
27. Groves JT, Haushalter RC, Nakamura M, Nemo TE, and Evans BJ. High-valent iron-porphyrin complexes related to peroxidase and cytochrome P-450. *J Am Chem Soc* 103: 2884–2886, 1981.
28. Guengerich FP. Destruction of heme and hemoproteins mediated by liver microsomal reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase. *Biochemistry* 17: 3633–3639, 1978.
29. Guengerich FP and Strickland TW. Metabolism of vinyl chloride: destruction of the heme of highly purified liver microsomal cytochrome P-450 by a metabolite. *Mol Pharmacol* 13: 993–1004, 1977.
30. Gunsalus IC, Sligar SG, Nordlund T, and Frauenfelder H. Oxygen sensing heme proteins: monoxygenases, myoglobin and hemoglobin. *Adv Exp Med Biol* 78: 37–50, 1977.
31. Gutteridge JM and Smith A. Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. *Biochem J* 256: 861–865, 1988.
32. Gutteridge JMC. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett* 201: 291–295, 1986.
33. Guzelian PS and Swisher RW. Degradation of cytochrome P-450 haem by carbon tetrachloride and 2-allyl-2-isopropylacetamide in rat liver in vivo and in vitro. Involvement of non-carbon monoxide-forming mechanisms. *Biochem J* 184: 481–489, 1979.
34. Halliwell B and Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 186: 1–85, 1990.
35. Harel S, Salan MA, and Kanner J. Iron release from metmyoglobin, methaemoglobin and cytochrome *c* by a system generating hydrogen peroxide. *Free Radic Res Commun* 5: 11–19, 1988.
36. Hasler JA, Estabrook R, Murray M, Pikuleva I, Waterman M, Capdevila J, Holla V, Helvig C, Falck JR, and Farrell G, *et al.* Human cytochromes P450. *Mol Aspects Med* 20: 1–137, 1999.
37. Hebbel RP. The sickle erythrocyte in double jeopardy: autoxidation and iron decompartmentalization. *Semin Hematol* 27: 51–69, 1990.
38. Hirota K and Sasaki K. Production of hematinic acid by the reaction of hemoglobin with phenylhydrazine: evidence for the oxidative cleavage of heme. *Biol Pharm Bull* 17: 856–858, 1994.
39. Hirota K, Yamamoto S, and Itano HA. Urinary excretion of isomers of biliverdin after destruction in vivo of haemoproteins and haemin. *Biochem J* 229: 477–483, 1985.
40. Iio T and Yoden K. Formation of fluorescent substances from degradation products of methyl linoleate hydroperoxides with amino compound. *Lipids* 23: 1069–1072, 1988.
41. Isaac IS and Dawson JH. Haem iron-containing peroxidases. *Essays Biochem* 34: 51–69, 1999.
42. Itoh S, Ohtaki Y, and Hayashida Y. Propentdyopents in the urine of infants with prolonged non-obstructive jaundice at one-month check-up, well-baby clinic for one-month-old babies. *J Jpn Pediatr Soc* 99: 1112–1116, 1995.
43. Jones P, Prudhoe K, and Robson T. Oxidation of deuteroferrihaem by hydrogen peroxide. *Biochem J* 135: 361–365, 1973.
44. Jones P, Robson T, and Brown SB. The catalase activity of ferrihaems. *Biochem J* 135: 353–359, 1973.
45. Jones P, Mantle D, and Wilson I. Peroxidase-like activities of iron(III)-porphyrins: kinetics of the reduction of a peroxidatically active derivative of deuteroferriheme by phenols. *J Inorg Biochem* 17: 293–304, 1982.
46. Kasapoglu M and Ozben T. Alterations of antioxidant enzymes and oxidative stress markers in aging. *Exp Gerontol* 36: 209–220, 2001.
47. Kelly HC, Davies DM, King MJ, and Jones P. Pre-steady-state kinetics of intermediate formation in the deuteroferriheme–hydrogen peroxide system. *Biochemistry* 16: 3543–3549, 1977.
48. Kremer ML. The reaction of hemin with H_2O_2. *Eur J Biochem* 185: 651–658, 1989.
49. Kunikata T, Itoh S, Ozaki T, Kondo M, Isobe K, and Onishi S. Formation of propentdyopents and biliverdin, oxidized metabolites of bilirubin, in infants receiving oxygen therapy. *Pediatr Int* 42: 331–336, 2000.
50. Kutty RK and Maines MD. Characterization of an NADH-dependent haem-degrading system in ox heart mitochondria. *Biochem J* 246: 467–474, 1987.
51. Lang CA, Naryshkin S, Schneider DL, Mills BJ, and Lindeman RD. Low blood glutathione levels in healthy aging adults. *J Lab Clin Med* 120: 720–725, 1992.
52. Lanzilotta WN, Schuller DJ, Thorsteinsson MV, Kerby RL, Roberts GP, and Poulos TL. Structure of the CO sensing transcription activator CooA. *Nat Struct Biol* 7: 876–880, 2000.
53. Manno M, King LJ, and De Matteis F. The degradation of haem by carbon tetrachloride: metabolic activation requires a free axial coordination site on the haem iron and electron donation. *Xenobiotica* 19: 1023–1035, 1989.
54. Masters BS and Schacter BA. The catalysis of heme degradation by purified NADPH-cytochrome C reductase in the absence of other microsomal proteins. *Ann Clin Res* 17: 18–27, 1976.
55. Matuda S and Nakano K. Heme degradation with participation of the superoxide radical in the presence of NADH and lipoamide dehydrogenase. *Jpn J Med Sci Biol* 37: 171–175, 1984.
56. McGeary RP, Szyczew AJ, and Toth I. Biological properties and therapeutic potential of bilirubin. *Mini Rev Med Chem* 3: 253–256, 2003.
57. Nagababu E and Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. *Biochem Biophys Res Commun* 247: 592–596, 1998.
58. Nagababu E and Rifkind JM. Heme degradation during autoxidation of oxyhemoglobin. *Biochem Biophys Res Commun* 273: 839–845, 2000.

59. Nagababu E and Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. *Biochemistry* 39: 12503–12511, 2000.
60. Nagababu E, Chrest FJ, and Rifkind JM. The origin of red cell fluorescence caused by hydrogen peroxide treatment. *Free Radic Biol Med* 29: 659–663, 2000.
61. Nagababu E, Ramasamy S, Rifkind JM, Jia Y, and Alayash AI. Site-specific cross-linking of human and bovine hemoglobins differentially alters oxygen binding and redox side reactions producing rhombic heme and heme degradation. *Biochemistry* 41: 7407–7415, 2002.
62. Nagababu E, Chrest FJ, and Rifkind JM. Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase. *Biochim Biophys Acta* 1620: 211–217, 2003.
63. Noguchi M, Yoshida T, and Kikuchi G. A stoichiometric study of heme degradation catalyzed by the reconstituted heme oxygenase system with special consideration of the production of hydrogen peroxide during the reaction. *J Biochem* (*Tokyo*) 93: 1027–1036, 1983.
64. Paoli M, Marles-Wright J, and Smith A. Structure-function relationships in heme-proteins. *DNA Cell Biol* 21: 271–280, 2002.
65. Parke DV and Sapota A. Chemical toxicity and reactive oxygen species. *Int J Occup Med Environ Health* 9: 331–340, 1996.
66. Puppo A and Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological Fenton reagent? *Biochem J* 249: 185–190, 1988.
67. Raha S and Robinson BH. Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem Sci* 25: 502–508, 2000.
68. Riess JG. Oxygen carriers ("blood substitutes")—raison d'etre, chemistry, and some physiology. *Chem Rev* 101: 2797–2920, 2001.
69. Rifkind JM, Zhang L, Levy A, and Manoharan PT. The hypoxic stress on erythrocytes associated with superoxide formation. *Free Radic Res Commun* 12–13: 645–652, 1991.
70. Robertson P Jr and Fridovich IA. Reaction of the superoxide radical with tetrapyrroles. *Arch Biochem Biophys* 213: 353–357, 1982.
71. Rodgers KR, Lukat-Rodgers GS, and Tang L. Nitrosyl adducts of FixL as probes of heme environment. *J Biol Inorg Chem* 5: 642–654, 2000.
72. Ryter SW and Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* 28: 289–309, 2000.
73. Sadrzadeh SM, Graf E, Panter SS, Hallaway PE, and Eaton JW. Hemoglobin. A biologic Fenton reagent. *J Biol Chem* 259: 14354–14356, 1984.
74. Schaefer WH, Harris TM, and Guengerich FP. Characterization of the enzymatic and nonenzymatic peroxidative degradation of iron porphyrins and cytochrome P-450 heme. *Biochemistry* 24: 3254–3263, 1985.
75. Shibahara S, Kitamuro T, and Takahashi K. Heme degradation and human disease: diversity is the soul of life. *Antioxid Redox Signal* 4: 593–602, 2002.
76. Shinar E and Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol* 27: 70–82, 1990.
77. Stolze K, Dadak A, Liu Y, and Nohl H. Hydroxylamine and phenol-induced formation of methemoglobin and free radical intermediates in erythrocytes. *Biochem Pharmacol* 52: 1821–1829, 1996.
78. Tappel AL. Unsaturated lipid oxidation catalysed by hematin compounds. *J Biol Chem* 217: 721–733, 1955.
79. Tenhunen R, Marver HS, and Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci U S A* 61: 748–755, 1968.
80. Tenhunen R, Marver HS, and Schmid R. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J Lab Clin Med* 75: 410–421, 1970.
81. Winterbourn CC. Oxidative reactions of hemoglobin. *Methods Enzymol* 186: 265–272, 1990.
82. Yoshida T and Kikuchi G. Sequence by the coupled oxidation of myoglobin with ascorbic acid. *Tohoku J Exp Med* 115: 67–74, 1975.

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