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Antioxidant Mechanisms of Nitric Oxide Against Iron-Catalyzed Oxidative Stress in Cells

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ABSTRACT

Three distinct antioxidant pathways are considered through which iron-catalyzed oxidative stress may be regulated by nitric oxide (NO). The first two pathways involve direct redox interactions of NO with iron catalytic sites and represent a fast response that may be considered an emergency mechanism to protect cells from the consequences of acute and intensive oxidative stress. These are (i) NO-induced nitrosylation at heme and non-heme iron catalytic sites that is capable of directly reducing oxoferryl-associated radicals, (ii) formation of nitrosyl complexes with intracellular "loosely" bound redox-active iron, and (iii) an indirect regulatory pathway that may function as an adaptive mechanism that becomes operational upon long-term exposure of cells to NO. In the latter pathway, NO down-regulates expression of iron-containing proteins to prevent their catalytic prooxidant reactions. Antioxid. Redox Signal. 3, 189–202.

INTRODUCTION

NITRIC OXIDE (NO) has emerged in recent years as a fundamental inter- and intracellular signaling molecule essential for the maintenance of homeostasis. This is due, to a large extent, to its ability to form complexes readily with iron-containing heme and non-heme proteins and its relatively "phlegmatic" behavior toward most biomolecules. It is becoming increasingly clear that redox reactions of NO play a very important role in its regulatory functions in cells and biological fluids. In particular, anti- and prooxidant effects of NO are said to be associated with its numerous protective and toxic functions, respectively.

Antioxidant functions of NO may be realized through a number of direct redox interactions, as well as indirectly via redox-dependent effects on major metabolic regulatory pathways. As a free radical, NO is amazingly reactive toward organic radicals. This feature is responsible for the remarkable antioxidant effectiveness of NO in scavenging lipid peroxyl and alkoxyl radicals and inhibiting peroxidation of different lipids, such as membrane phospholipids and cholesterol (5, 31, 43, 60). Antioxidant effects of NO may be also achieved via its direct

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redox interactions with iron-centers during iron-catalyzed reactions leading to oxidative stress. These include chelation of redox-active metal ions by $NO^{\bullet}$ (31, 40), as well as reduction of oxoferryl species (22, 23). Finally, NO may affect oxidative stress indirectly by down-regulating expression of iron-containing proteins through posttranscriptional mechanisms or up-regulation of antioxidant enzymes, *i.e.*, contributing to antioxidant effects through its action as a signaling molecule. These direct and indirect interactions of NO with three types of iron species that are mainly responsible for the catalysis of oxidative stress reactions in cells and biological fluids—heme proteins, non-heme iron proteins, and "loosely bound" iron readily available for iron chelators—are the focus of our discussion here.

PROOXIDANT ACTIVITY OF HEME PROTEINS

In blood, most iron is bound to hemoglobin (Hb), whereas myoglobin (Mb) represents one of the major reservoirs of iron in some cell types, such as muscle cells (*e.g.*, cardiomyocytes). Mb and Hb react with alkylhydroperoxides (including lipid hydroperoxides) to yield several redox-active species, as well as a number of free radical intermediates, such as alkylhydroperoxyl ($ROO^{\bullet}$), alkyloxyl ($RO^{\bullet}$), and alkyl ($R^{\bullet}$) radicals (10, 32, 67). The oxidation of ferric (met) forms of Mb (Mb-Fe^{3+}) and Hb (Hb-4Fe^{3+}) by hydroperoxides is presumed to proceed via two-electron oxidation to give a long-lived oxoferryl ($Fe^{4+}=O$) species, one oxidation equivalent above the ferric state, plus a transient protein radical (10, 21). There are two (or more) sites for the protein-derived radical: at tyrosine (Tyr^{103}) or tryptophan (Trp^{14}). The Mb Trp^{14} peroxyl radical has been shown to react rapidly with a wide range of proteins to give long-lived secondary radicals on the target protein. Recipient peptides/proteins that contained a tyrosine and/or tryptophan amino acid residue were most susceptible to free radical transfer (11). These protein-to-protein radical transfer reactions may be responsible for protein oxidation (51). Additionally, antioxidants (GSH, ascorbate, Trolox C, vitamin E, and urate) can directly reduce Mb-derived peroxyl radical to produce antioxidant-derived radicals (35). This results in antioxidant depletion. Protein radicals have been shown to react with peribacteroid membrane fractions, with the consequent generation of lipid-derived radicals (35). Apart from short-lived radicals, longer-lived protein radicals associated with oxoferryl hemoproteins may be involved in the initiation of lipid peroxidation (8).

RELEASE OF IRON FROM HEME IN THE MET-Hb/*tert*-BUTYL HYDROPEROXIDE (*tert*-BuOOH) SYSTEM

A plethora of data indicate that two highly reactive iron species are formed during interaction of heme with peroxides, namely oxoferryl (ferryl, perferryl) species and "loosely" bound iron that can be released from the heme. In the absence of oxygen, formation of these species can be described by the following reactions:

$$\text{Met-Hb-Fe}^{3+} + \textit{tert}\text{-BuOOH} \rightarrow \text{Hb-}^{\bullet}\text{heme-Fe}^{4+}=\text{O (Comp I)} + \textit{tert}\text{-BuOH} \quad (1)$$

$$\text{Hb-}^{\bullet}\text{heme-Fe}=\text{O} \rightarrow {}^{\bullet}\text{Hb-heme-Fe}^{4+}=\text{O} \rightarrow \text{fragmentation} \rightarrow \text{Fe}^{2+}\ \text{release} \quad (2)$$

$$\text{Hb-}^{\bullet}\text{heme-Fe}^{4+}=\text{O (Comp I)} + \textit{tert}\text{-BuOOH} \rightarrow \text{Hb-Fe}^{4+}=\text{O (Comp II)} + \textit{tert}\text{-BuOO}^{\bullet} \quad (3)$$

$$\text{Hb-Fe}^{4+}=\text{O (Comp II)} + \textit{tert}\text{-BuOOH} \rightarrow \text{Met-Hb-Fe}^{3+} + \textit{tert}\text{-BuOO}^{\bullet}\ \text{(slow-reaction)} \quad (4)$$

In the presence of oxygen, the reaction of oxygen with protein radical yields the peroxyl radical ($^{\bullet}$O-O-Hb-heme-$Fe^{4+}=O$). In accordance with this scheme, "loosening" of metal is usually associated with oxidation of protein, as has been shown for different classes of superoxide dismutases (39, 73). Self-peroxidation of met-Hb (25) leading to the formation of protein radicals and subsequent protein fragmentation is likely to be the cause of "loosening" and release of iron from the heme. This scheme does not consider the redox state of released iron. It has been shown that free iron produced by heme/perox-

ide interactions is in the ferrous state (9, 33, 52). The mechanisms involved in the formation and release of ferrous (Fe^{2+}) iron from ferryl (Fe^{4+}) species are not fully understood. This, however, is important because ferrous iron is a strong promoter of oxidative stress.

Interestingly, the release of ferrous iron is more characteristic of the interaction of hemoproteins with organic hydroperoxides (cumene hydroperoxide, *tert*-BuOOH) than with hydrogen peroxide (52). This is consistent with the results of Tajima (67), which showed the existence of heme-peroxide complexes. Later, Jinno et al. (38) demonstrated that hemoprotein–butylperoxide complex is rapidly decomposed to non-heme iron. Thus, it seems that investigation of the early stages of interactions of hemoproteins with organic hydroperoxides may give the key to understanding the mechanism(s) of iron release.

It is presumed that the initial product of this reaction is the ferryl heme cation radical (25), although the species has never been directly detected by electron paramagnetic resonance (EPR) spectroscopy. Characterization of this very reactive oxygen intermediate remains a challenging area of research. Recent calculations performed for the heme of cytochromes P450 showed that such a putative reactive oxygen intermediate should contain two parallel unpaired spins on the Fe=O moiety and an antiparallel unpaired spin distributed between the a2u(π) orbital of the porphyrin ring and the π-orbital of ligand atom (46), indicating the existence of an organic radical in the heme iron moiety. Based on kinetic behavior of the peroxyl radical and the protein radical in a met-Hb/hydrogen peroxide model system, Svistunenko et al. (66) suggested that peroxyl radicals with characteristic features at $g = 2.035$ are not formed from protein radicals ($g = 2.0042$). They postulated the existence of a radical intermediate not detectable by EPR, but capable of reacting with oxygen to yield the $g = 2.035$ protein peroxyl radical EPR signal. Notably, the peroxyl radical EPR signal was not saturable by power likely due to its close proximity to the heme iron moiety. In a recent study by Baron et al. (4), a similar EPR signal at $g \approx 2.035$ characteristic of a peroxyl radical was observed (but not assigned) in the reaction system met-Mb/hydrogen peroxide/linoleate immediately after mixing of the reagents. Despite the assignment of the $g = 2.035$ signal to a protein peroxyl radical, an absolutely identical signal was observed during oxidation of *tert*-BuOOH in the absence of protein, clearly indicating that it is derived from the *tert*-$BuOO^{\bullet}$ radical (34).

Thus, it appears that there are two contradicting results on the nature of products formed during interactions of hemoproteins with organic hydroperoxides: (i) EPR-detectable peroxyl radicals do not seem to derive from the protein radical, and (ii) ferrous iron is released from ferryl (Fe^{4+}) heme species, suggesting that the reduction step takes place. At first glance, these two findings seem to be disconnected. Three sets of results, however, permit logical combination of these findings into one scheme: (i) *tert*-BuOOH–heme complex is involved in iron release; (ii) the $g = 2.035$ peroxyl radical and protein radical seem to have a different origin; and (iii) the $g = 2.035$ peroxyl radical EPR signal is detectable very early in the reaction between heme and peroxide, much earlier than the protein radical. Altogether, these facts suggest that a reductive mechanism of iron release during the interaction of heme proteins with peroxides is operational.

To support this hypothesis experimentally, we performed direct EPR studies of met-Hb/*tert*-BuOOH in expectation that this relatively slow reaction will permit detection of the key free radical intermediates, as well as release ("loosening") of ferrous iron. We found no signals in the high-field ($g = 1.95$–2.10) segment of the EPR spectrum at 0 time (Fig. 1, curve 1). After 30 s, an integral peak at $g = 2.035$ and a trough at $g = 2.004$ (species A) was detected in the EPR spectrum (Fig. 1, curve 2). The peak at $g = 2.035$ gradually decayed over time, and a new species (species B) appeared in the EPR spectrum recorded at 5 min (Fig. 1, curve 3). This species had integral peaks at $g = 2.025$ and $g = 2.017$, and a shoulder at $g = 2.010$. The signal intensity of species B was maximal at 15 min (Fig. 1, curves 4 and 5).

Species A is likely a peroxyl radical signal, whose appearance has been well documented for such systems (8, 47, 50, 55, 65, 66) and for a model system containing high concentrations

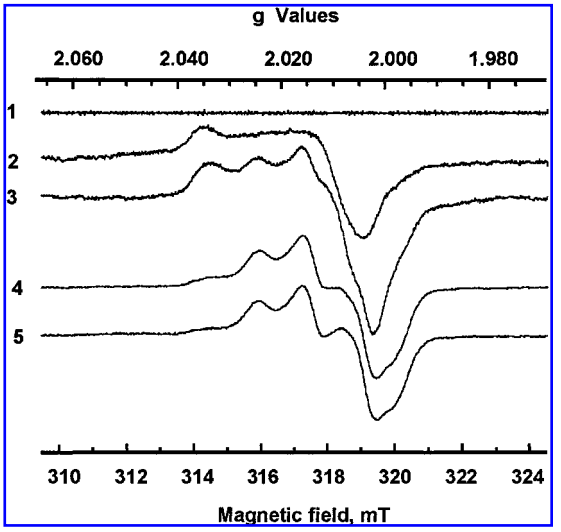


FIG. 1. High-resolution low-temperature EPR spectra of the reaction mixture met-Hb/*tert*-BuOOH acquired at different time intervals after the reagents were mixed. Conditions were as follows: met-Hb (300 μM heme iron), *tert*-BuOOH (1.5 m*M*), disodium phosphate buffer (50 m*M*, pH 7.4 at 25°C). The reaction was started by the addition of *tert*-BuOOH. EPR spectra were recorded at −150°C; gain, 500 and 200; modulation amplitude, 0.5 mT; power, 10 mW; time constant, 0.03 s; scan time, 4 min; scan range, 15 mT. Spectra 1–5 were recorded at 0, 0.5, 5, 15, and 30 min after beginning of the reaction, respectively.

of *tert*-BuOOH and iron ions (34). If this peroxyl radical is the first intermediate of the interaction between *tert*-BuOOH and heme iron, then it must be formed via reaction:

$$\textit{tert}\text{-BuOOH} + Fe^{3+} \rightarrow \textit{tert}\text{-BuOO}^{\cdot} + Fe^{2+} + H^{+} \qquad (5)$$

We were able to obtain similar peroxyl radical signals by one-electron oxidation of *tert*-BuOOH by $K_3Fe(CN)_6$, a strong one-electron oxidant (Fig. 2, inset C). To clarify whether signals observed in the met-Hb/*tert*-BuOOH system are associated with heme iron, we used an EPR power saturation technique to compare relaxation parameters of species A, species B, and peroxyl radical generated in the presence of $K_3Fe(CN)_6$. Species A (detected after 30 s) was not saturable by increasing the power up to 180 mW (Fig. 2, filled squares), whereas species B (Fig. 2, filled diamonds) as well as the EPR signal of peroxyl radical observed in the *tert*-BuOOH/$K_3Fe(CN)_6$ system (Fig. 2, filled circles) were saturated at ~40–100 mW power. This indicates that species A is likely associated with the heme iron moiety of met-Hb in contrast to species B, which is likely a protein-centered radical.

The power saturation technique also permits differentiation between overlapping signals, because different relaxation constants cause a change in the signal features. To clarify whether species A and species B are single signals or a mixture of different signals, we compared the shape of signals obtained at various power values. Each spectrum was divided by a constant corresponding to its intensity, resulting in a series of spectra with constant integral intensity. The series of spectra obtained for species A and species B are presented in Fig. 3. Power saturation did not affect the shape of the signal for species A. This indicates that species A represents a single signal (formed 30 s after beginning of the reaction). In contrast, species B ap-

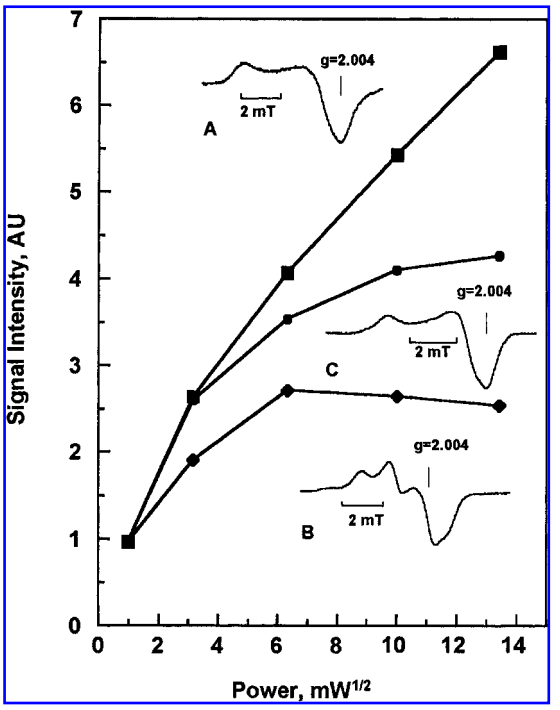


FIG. 2. Power saturation of met-Hb/*tert*-BuOOH EPR signals. Filled squares and spectrum A: EPR signal detected after 30 s of incubation of met-Hb/*tert*-BuOOH at 25°C (species A); filled circles and spectrum C: EPR signals detected in the presence of $K_3Fe(CN)_6$; filled diamonds and spectrum B: EPR signal detected after 30 min of incubation of met-Hb/*tert*-BuOOH at 25°C (species B). EPR spectra were recorded at −150°C; gain, 500 and 200; modulation amplitude, 0.5 mT; power, 10 mW; time constant, 0.03 s; scan time, 4 min; scan range, 15 mT.

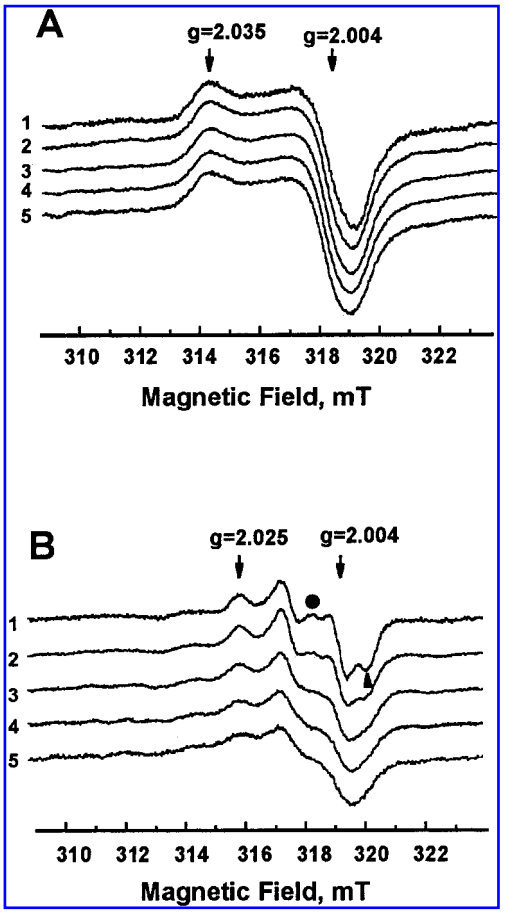


FIG. 3. Serial spectra obtained for species A (A) and B (B) at different values of power. Spectra 1–5 were recorded at 1, 10, 40, 100, and 180 mW, respectively. The spectrum of species B includes features of protein radical, O=Fe^{4+}—protein$^{\bullet}$ (filled circle), and features of protein-centered peroxyl radical, O=Fe^{4+}—protein-O-O$^{\bullet}$ (filled triangle).

pears to be a mixture of at least two signals (see Fig. 3) that can be assigned to protein radical and protein-centered peroxyl radical, as has been well documented (8, 47, 50, 55, 65, 66).

We suggest that species A is the primary radical intermediate formed as a result of iron "loosening" (release) independently of the pathway that yielded species B. This is supported by the following results. We observed neither *tert*-BuO$^{\cdot}$ radicals nor protein radicals that are expected to emerge from one-electron reduction of *tert*-BuOOH by met-Hb at the initial stage of the reaction. Instead, only species A could be seen 30 s after met-Hb and *tert*-BuOOH were mixed. If species A is the primary intermediate of the reaction between heme iron and *tert*-BuOOH, it could be formed only through one-electron oxidation of *tert*-BOOH and reduction of heme iron, respectively. In line with this, generation of species A (but not of species B) was remarkably inhibited when oxy-Hb (Fe^{2+}) was used instead of met-Hb (data not shown). If *tert*-BuOO$^{\bullet}$ is the first reaction intermediate of Fe^{3+} porphyrin with *tert*-BuOOH as has been proposed by Tajima (67) for hydrogen peroxide, then *tert*-BuOO$^{\bullet}$ is expected to be in close proximity to the reduced iron center. In fact, our EPR power saturation experiments demonstrated that species A was indeed associated with heme.

The formation of species A may be related to heme iron reduction and the appearance of Hb(Fe^{2+}). This, however, was not observed in the system (data not shown). Another possibility is "loosening" (release) of ferrous ions from "reductive" interaction between *tert*-BuOOH and Hb. To prove this, we used the Fe^{2+} chelator, *o*-phenanthroline, to detect the ferrous iron. We found that the *o*-phenanthroline-chelatable ferrous iron accumulated over time in the incubation system, and its amount inversely correlated with the concentration of species A (Fig. 4). This implies that species A is likely associated with the "loosening" (release) of ferrous iron.

In summary, our results imply the existence

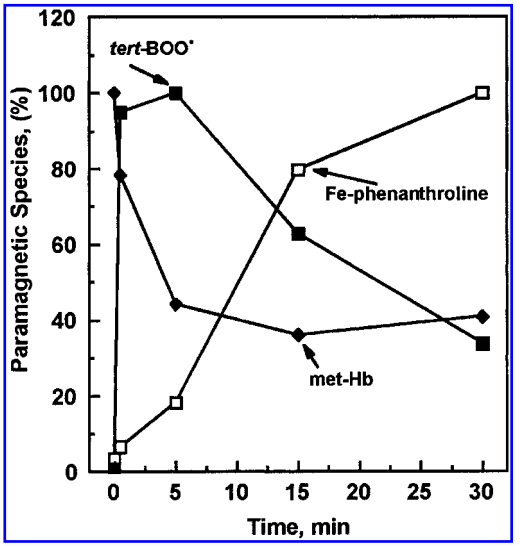
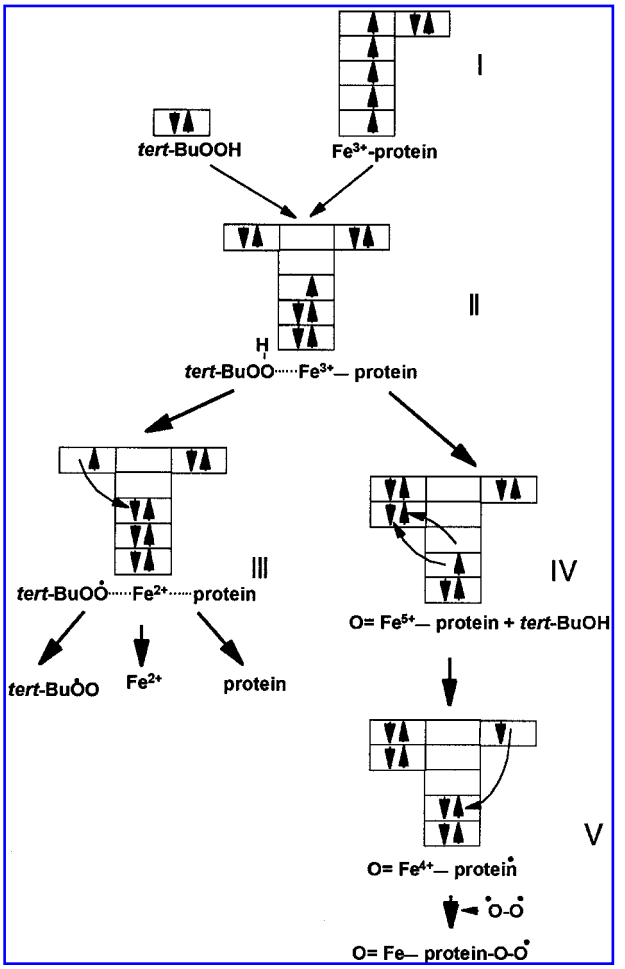


FIG. 4. Time course of met-Hb, species A, and *o*-phenanthroline-Fe^{2+} complex initiated by met-Hb/*tert*-BuOOH. Filled squares: time course of species A; filled diamonds: time course of met-Hb; opened squares: time course of *o*-phenanthroline–Fe^{2+} complex.

of two reaction pathways through which *tert*-BuOOH interacts with met-Hb (Scheme 1). The first one (which is faster) produces *tert*-BuOO$^{\bullet}$ by one-electron oxidation of *tert*-BuOOH (Scheme 1, steps I–III), whereas the second one (which is slower) yields ferryl species by two-electron oxidation of heme iron (Scheme 1, steps I, II, IV, and V). This is in agreement with earlier findings that ferryl-Hb is the only species formed at low *tert*-BuOOH/Hb ratios; with the increase of *tert*-BuOOH/Hb ratio, the release of free iron becomes detectable (8, 48). Simple thermodynamic considerations also indicate that *tert*-BuOOH is more likely to act as an electron donor at high *tert*-BuOOH/Hb ratios, whereas Hb is more likely to be a donor of electrons at high Hb/*tert*-BuOOH ratios. Clearly, achievable concentrations of hydroperoxides (H_2O_2, lipid hydroperoxides) along with the concentrations of heme proteins in cells or biological fluids determine which of these pathways predominate under pathophysiologically relevant conditions.



Scheme 1. Proposed reaction stages during interaction of *tert*-BuOOH with met-Hb. I: The system includes high-spin heme Fe^{3+} ($S = 5/2$) and free *tert*-BuOOH ($S = 0$). II: *tert*-BuOOH and met-Hb form a complex consisting of low-spin heme Fe^{3+} ($S = 1/2$) and *tert*-BuOOH ($S = 0$). III: *tert*-BuOOH reduces heme iron yielding species A, a peroxyl radical ($S = 1/2$), and loosely bound Fe^{2+} ($S = 0$). IV: Two-electron oxidation of heme iron by *tert*-BuOOH yielding perferryl-Hb. The system includes low-spin Fe^{5+} ($S = 1/2$). V: One-electron oxidation of protein by Fe^{5+} yielding Hb-Fe^{4+} ($S = 0$) and species B, representing two radicals, a protein radical ($S = 1/2$) and a protein peroxyl radical ($S = 1/2$).

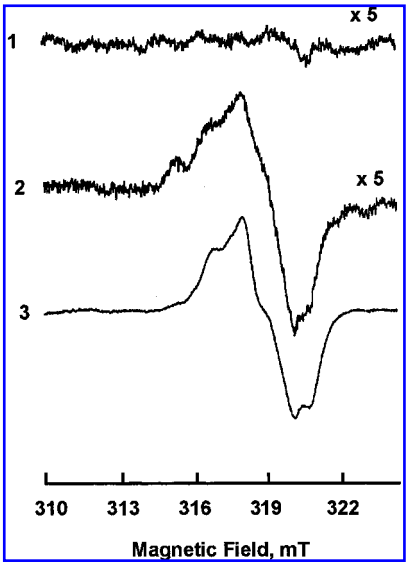


FIG. 5. Low-temperature EPR spectra of K/VP.5 cells treated with *tert*-Bu-OOH. K/VP.5 cells (250×10^6 cells/ml) were incubated in 50 m*M* disodium phosphate buffer, pH 7.4, in the presence of *tert*-BuOOH (80 μmol/10^6 cells) at 25°C. At the indicated time point, cell suspensions were frozen at −150°C and used for EPR measurements. 1, control (no *tert*-Bu-OOH added); 2, cells treated with *tert*-BuOOH (30 s after addition of *tert*-BuOOH); 3, cells treated with *tert*-BuOOH (15 min after addition of tert-BuOOH). EPR spectra were recorded at −150°C; gain, 100 (3) and 500 (1 and 2); modulation amplitude, 0.5 mT; power, 10 mW; time constant, 0.03 s; scan time, 4 min; scan range, 15 mT.

In separate experiments, we performed EPR studies of radical species formed by *tert*-BuOOH in K/VP.5 cells, a subclone of human erythropoetic leukemia K562 cells that contain a relatively high concentration of endogenous Hb [~30–40 pmol/10^6 cells (59)]. We found that after the addition of *tert*-BuOOH to K/VP.5 cells at concentrations close to those used in our model experiments with met-Hb and oxy-Hb, two major species appeared in the EPR spectra (Fig. 5). One signal ($g = 2.035$) was detectable

almost immediately after addition of *tert*-BuOOH (30 s), and the other one ($g = 2.004$) was present in the spectra at both 30 s and 15 min of incubation. Based on the values of g factors and the shape of the signals, they may be tentatively assigned to the *tert*-BuOO$^{\bullet}$ signal and ferryl-protein radical signal, respectively. Thus, essentially very similar mechanisms of Hb/*tert*-BuOOH interactions may operate in cells. Indeed, we were able to detect both *tert*-BuOO$^{\bullet}$ signal and ferryl-protein radical signal as the primary and secondary free radical species formed in the course of the reaction between endogenous (oxy)Hb and *tert*-BuOOH. Although these results strongly suggest that more than one reactive oxidant may be involved in oxidative stress induced by Hb *in vivo*, further studies are necessary to quantitate contributions of the two factors in oxidations of physiologically relevant reductants.

ANTIOXIDANT PATHWAYS OF NO AGAINST IRON-CATALYZED OXIDATIVE STRESS

There are three distinct pathways through which iron-catalyzed oxidative stress may be regulated by NO as presented in our discussion and experimental results below.

NO-induced nitrosylation at heme and non-heme iron catalytic sites protects against oxidative stress

In the first pathway, a direct chemical interaction of NO with hydroperoxides at non-heme and heme iron catalytic sites prevents generation of oxoferryl species and different free radicals, and thus precludes free radical-induced damage of critical biomolecules. The possibility of this pathway has been initially established in simple chemical systems *in vitro* (22, 40).

In the initial experiments, the reactions of met-Mb and met-Hb, oxidized to their respective oxoferryl free radical species (Mb-Fe^{4+}=O and Hb-$4Fe^{4+}$=O) by *tert*-BuOOH, with NO were studied in a nitrogen atmosphere (22). It was shown that NO may reduce both oxoferryl and apoprotein free radical electrophilic centers of Mb-Fe^{4+}=O/Hb-$4Fe^{4+}$=O and eliminate *tert*-butyl(per)oxyl radicals, thus protecting against oxidative damage. Moreover, NO reduced Mb-Fe^{4+}=O/Hb-$4Fe^{4+}$=O to their respective ferric (met) forms and prevented oxidation of lipids [*cis*-parinaric acid (PnA) integrated into liposomes], oxidation of luminol, and formation of the *tert*-butyl(per)oxyl adduct with the spin trap 5,5′-dimethylpyrroline *N*-oxide. NO eliminated the signals of tyrosyl radical detected by EPR and oxoferryl detected by mass spectrometry in the reaction of *tert*-BuOOH with met-Mb (22). Subsequent studies showed that a combination of Hb/*tert*-BuOOH added extracellularly to vascular smooth muscle cells (VSMCs) caused oxidative stress as evidenced by peroxidation of their major phospholipids—phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI). The oxidative stress was accompanied by cytotoxic effects of oxy-Hb/*tert*-BuOOH and met-Hb/*tert*-BuOOH on VSMCs (62). In the presence of an NO donor, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (NOC-15), VSMCs were protected against oxidative stress and cytotoxicity induced by Hb/*tert*-BuOOH. The protective effect of NOC-15 was most likely due to its ability to form NO-heme Hb, hence to prevent the formation of oxoferryl-Hb species (62).

Further experiments demonstrated that this antioxidant function of NO, *i.e.*, its ability to reduce oxoferryl Mb/Hb species, is realized in cells (23, 24). In particular, it was demonstrated that in K/VP.5 cells (a subline of human erythroleukemia K562 cells with elevated intracellular Hb concentrations), *tert*-BuOOH-induced formation of oxoferryl-Hb-derived free radical species was proportional to endogenous Hb concentrations (23). Further, a correlation between the production of oxoferryl-Hb-derived radicals, peroxidation of major membrane phospholipids (PC, PE, PS, PI, and cardiolipin), and cell viability was demonstrated. Most importantly, when the cells were pretreated with an NO donor, NOC-15, that caused nitrosylation of non-heme iron centers and Hb-heme, *tert*-BuOOH was not able to induce either formation of oxoferryl-Hb-derived free radical species or peroxidation of phospholipids in the cells. No cytotoxicity was observed in NOC-15-pretreated cells after incubation with *tert*-BuOOH. These results indicate that NO produces iron-ni-

trosyl complexes whose redox interactions with *tert*-BuOOH prevented generation of oxoferryl-Hb-derived free radical species.

Qualitatively very similar results were obtained with cardiac myocytes (24). Two distinct free radical species—alkoxyl radicals associated with non-heme iron catalytic sites and Mb protein-centered peroxyl radicals, found in low-temperature EPR spectra of cardiac myocytes exposed to *tert*-BuOOH—were completely abolished when the cells were incubated with the combination of NOC-15 and *tert*-BuOOH. NO acted as an effective antioxidant in live cardiomyocytes as it was able to protect cardiomyocytes completely against both *tert*-BuOOH-induced phospholipid peroxidation and cytotoxicity.

Overall, these results on antioxidant effects of NO against oxidative stress induced by combinations of organic hydroperoxides with hemoproteins (Mb or Hb) are summarized in Tables 1 and 2. Notably, formation of NO-heme hemoproteins was essential for antioxidant protection via the mechanism of direct reduction of oxoferryl-derived radicals. The protective effects afforded by nitrosothiols that are known to *S*-nitrosylate hemoproteins were minimal (Table 2).

Intracellular "loosely" bound redox-active iron is available for NO to form nitrosyl complexes

The second pathway is based on the ability of NO to interact with "loosely" bound iron constitutively present in cells or formed as a result of ongoing oxidative stress (see above). Formation of nitrosyl iron complexes inhibits their catalytic prooxidant reactions. This mechanism is similar to the effects of iron chelators, *e.g.*, deferoxamine (DFO).

In plasma, iron is normally bound to proteins mainly due to the concerted action of ceruloplasmin and apotransferrin. The former oxidizes ferrous ions to the ferric form, whereas the latter sequesters ferric ions to form transferrin (19). Transferrin receptors transport iron into cells, and the iron is subsequently stored within ferritin (69). Intracellular iron is mostly tightly bound by iron-storing proteins (e.g., ferritin) and only a small part of total iron, so-called "loosely" bound iron ["free iron" or transit iron pool (36)], may be present as low-molecular-weight iron complexes with a low stability constant. This "loosely" bound iron (which may also include iron "loosely" bound to proteins) is available for chelators, such as DFO and phenanthroline. Normally, iron associated with ferritin does not possess any catalytic activity (1, 37, 57). In contrast, the pool of "loosely" bound iron is capable of catalyzing oxidative stress reactions (2, 27). Therefore, regulation of intracellular iron is deemed to be one of the important mechanisms that determines sensitivity to oxidative stress (6, 29).

One of the effective ways to protect against oxidative stress induced by "loosely" bound iron is to use iron chelators, such as *o*-phenanthroline, EDTA, diethylenetriaminepentaacetic acid, or DFO, that form stable redox-inactive complexes with iron (17, 63). DFO is the most commonly used and pharmacologically efficacious chelator (12, 15, 17, 58, 63). In addition, it is the only iron chelator currently approved for clinical use for treatment of iron overload, including acute iron poisoning and treatment of chronic iron overload in transfusion-dependent anemias, such as β-thalassemia. Based on extensive clinical use of DFO, it was concluded that DFO-available iron can promote oxidative stress (7, 18, 58). Technical difficulties (it is administered by subcutaneous infusion over 8–12 h), as well as problems with compliance, have prompted a search for alternatives to DFO, in particular, iron-chelating molecules active after oral administration (12).

Lately, inhaled NO is broadly used in clinical practice. EPR studies demonstrated that NO and DFO bind the same pools of intracellular iron (45). DFO reacts with intracellular iron to form paramagnetic iron complexes with characteristic EPR signal with $g = 4.3$ (Fig. 6) (44). Treatment of tissue homogenates with sodium nitrite (or gaseous NO) results in appearance of the signal from dinitrosyl iron complexes with $g = 2.03$ (Fig. 6) (70, 71). Administration of DFO prior to sodium nitrite resulted in the $g = 4.3$ EPR signal. Conversely, when DFO was administered after sodium nitrite, only the EPR signal with $g = 2.03$ was detectable (45).

These data demonstrate that the same endogenous iron was available for chelation with DFO or with NO to produce EPR-detectable

Table 1. Effect of NOC-15 on Oxidation of PnA-Labeled Phospholipids Induced by *tert*-BuOOH in Different Cell Lines

Cell line	*tert*-BuOOH (μM)	NOC-15 (μM)	Time (min)	Oxidized PnA (ng/μg of total lipid phosphorus)			
				PI	PE	PS	PC
K/VP.5 erythroleukemia	100	0	60	57.1 ± 7.1	48.2 ± 8.1	58.9 ± 12.8	42.8 ± 2.7
cells (n = 3)	100	40	60	23.2 ± 2.2*	25.4 ± 7.2*	28.6 ± 5.7*	17.8 ± 1.5*
	100	80	60	27.8 ± 2.8	12.5 ± 1.0†	7.1 ± 0.8†	14.3 ± 0.6†
K/VP.5 erythroleukemia cells	100	0	60	62.3 ± 9.3	46.4 ± 6.2	66.1 ± 11.6	44.6 ± 3.4
treated with hemin (25 μM)	100	40	60	7.6 ± 0.3†	16.1 ± 1.4*	32.1 ± 10.1*	11.1 ± 0.7†
(n = 3)	100	80	60	7.1 ± 0.3†	15.0 ± 1.6†	7.1 ± 1.4†	11.1 ± 0.8†
VSMCs treated with oxy-Hb	150	0	60	56.9 ± 2.9	39.7 ± 4.1	56.8 ± 3.1	30.5 ± 3.6
(5 μM) (n = 5)	150	50	60	40.0 ± 3.1*	24.0 ± 1.7*	38.4 ± 1.3*	22.2 ± 3.1
	150	200	60	30.5 ± 3.0*	15.6 ± 1.4*	9.1 ± 6.3†	7.5 ± 4.7*
Cardiomyocytes	150	0	15		9.2 ± 0.6	41.0 ± 5.4	3.4 ± 0.7
(n = 3)	150	150	15		2.4 ± 0.7	0.1 ± 0.1†	1.5 ± 0.6

Cells loaded with PnA (5 μg/10^6 cells) were exposed to *tert*-BuOOH in the presence or absence of NOC-15. NOC-15 was added 15 min prior to induction of oxidative stress. At the end of incubation, cells were harvested and washed, and lipids were extracted and resolved by HPLC. All values are means ± SEM. The amount of intracellular Hb in K/VP.5 cells nontreated and treated with hemin was estimated to be 25 pmol of Hb/10^6 cells and 90 pmol of Hb/10^6 cells, respectively. The amount of intracellular oxy-Mb in cardiomyocytes was estimated to be 50 pmol/10^6 cells.

*$p < 0.05$, †$p < 0.005$ versus *tert*-BuOOH.

TABLE 2. EFFECT OF DIFFERENT NO DONORS ON OXIDATION OF PnA-LABELED PHOSPHOLIPIDS INDUCED BY *tert*-BuOOH IN VSMCs

NO donors	*tert*-BuOOH (μM)	donor (μM)	Time (min)	Oxidized PnA (ng/μg of total lipid phosphorus)			
				PI	PE	PS	PC
None (n = 5)	150	0	60	7.6 ± 0.4	26.3 ± 2.8	6.6 ± 0.4	72.3 ± 8.5
GS-NO (n = 3)	150	200	60	8.0 ± 0.8	25.6 ± 1.5	7.1 ± 1.1	74.4 ± 8.3
NAC-S-NO (n = 3)	150	200	60	7.0 ± 0.7	29.3 ± 1.9	7.6 ± 0.6	78.5 ± 6.4
NOC-15 (n = 5)	150	200	60	4.0 ± 0.4*	10.9 ± 1.0*	2.2 ± 1.5†	37.2 ± 2.3*

Cells loaded with PnA (5 μg/10^6 cells) were exposed to *tert*-BuOOH in the presence or absence of GS-NO, NAC-S-NO, or NOC-15. NO donors were added 15 min prior to induction of oxidative stress. At the end of incubation, cells were harvested and washed, and lipids were extracted and resolved by HPLC. All values are means ± SEM.
*$p < 0.05$, †$p < 0.005$ versus control.

paramagnetic centers. These results also suggest that NO may protect against iron toxicity through binding catalytically redox-active iron that is also available for chelation by DFO. In other words, NO may act as an antioxidant capable of inhibiting oxidative stress induced by "loosely" bound (DFO-chelatable) iron.

NO down-regulates expression of iron-containing proteins to protect against oxidative stress

The third antioxidant pathway for NO is associated with its ability to regulate the intracellular content of iron-containing catalytic sites. Expression of several essential proteins involved in cellular iron homeostasis has been shown to be regulated by iron-regulatory proteins 1 and 2 (IRP-1, IRP-2) binding to specific mRNA sequences—iron responsive elements (IREs) (3, 26, 28, 41, 49, 54, 61, 68). Functional IREs have been identified in the 5′ untranslated regions of ferritin H-chain, ferritin L-chain, erythroid aminolevulinate synthase, mitochondrial aconitase, and succinate dehydrogenase, as well as the 3′ untranslated regions of transferrin receptors (13, 54).

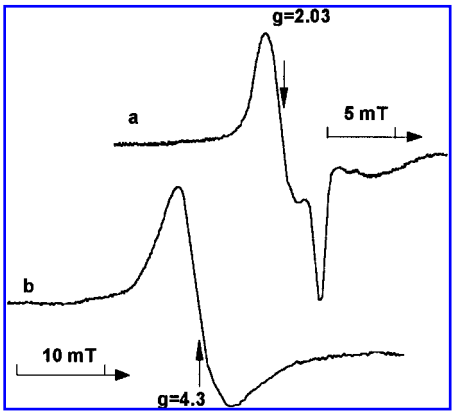


FIG. 6. EPR spectra of dinitrosyl iron complexes (g = 2.03) and DFO–iron complexes (g = 4.3).

NO can directly interact with IRP-1, thus triggering IRE-dependent responses (13, 16). Two different sites on IRP-1 can be targeted by NO. One target site may be the sulfhydryl group of Cys^{437}, which has been shown to regulate the binding of IRP-1 to IREs (16, 30, 64). The second site is the dynamic Fe-S cluster whose removal from the apoprotein is induced by NO binding (53). Hence, NO binding to IRP-1 initiates IRP-1/IRE interactions because only the IRP-1 apoprotein can bind to the IREs (16, 30, 64). Therefore, chronic exposure to NO donors or overproduction of endogenous NO in inducible NO synthase (iNOS)-transduced cells results in decreased cellular content of non-heme and heme-iron by coordinate translational regulatory mechanisms (14, 20, 42, 54, 56). Obviously, cells with decreased levels of endogenous redox-active iron catalytic sites would be less susceptible to oxidative stress.

This has been directly demonstrated in experiments with K/VP.5 cells transduced with a retroviral vector containing the human iNOS gene (K/VP.5-iNOS) (72). K/VP.5-iNOS cells were remarkably less sensitive to the cytotoxic effects of *tert*-BuOOH compared with K/VP.5 cells. It was determined that levels of non-heme and heme (Hb) iron were dramatically decreased in K/VP.5-iNOS cells compared with K/VP.5 cells in line with the sharply decreased intensities of EPR signals of nitrosylated species observed in the presence of endoge-

nously produced NO (by iNOS) or upon addition of exogenous NO (incubation with NOC-15). *tert*-BuOOH-induced oxoferryl-Hb-associated protein-centered free radical species, as well as *tert*-BuO$^{\bullet}$-alkoxyl radicals, were readily detectable in K/VP.5 cells, but were greatly reduced in K/VP.5-iNOS cells. Susceptibility to *tert*-BuOOH-induced cytotoxicity in K/VP.5-iNOS cells was increased by 24-h treatment with an iNOS inhibitor, L-N^{G}-monomethylarginine (L-NMA). NOC-15-mediated protection against *tert*-BuOOH-induced cytotoxicity in L-NMA-treated cells was mediated by direct NO redox interactions with iron catalytic sites, preventing "activation" of *tert*-BuOOH to its cytotoxic reactive species, but was diminished upon addition of NOC-15 to L-NMA-treated cells (72).

In most cells, both direct and indirect regulatory mechanisms of NO are likely operational as effective protective mechanisms against oxidative stress. The pathways that involve direct redox interactions of NO with iron catalytic sites likely represent a fast response that may be considered an emergency mechanism to protect cells from the consequences of both acute and long-term oxidative stress. An indirect regulatory pathway may function as an adaptive mechanism that becomes operational upon long-term exposure of cells to NO (72).

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ABBREVIATIONS

tert-BuOOH, *tert*-butyl hydroperoxide; DFO, deferoxamine; EPR, electron paramagnetic resonance; Hb, hemoglobin; Hb-4Fe^{3+}, methemoglobin; iNOS, inducible nitric oxide synthase; IREs, iron responsive elements; IRP-1 and IRP-2, iron regulatory proteins 1 and 2; K/VP.5-iNOS cells, K/VP.5 cells transduced with a retroviral vector containing the human iNOS gene; Mb, myoglobin; Mb-Fe^{3+}, metmyoglobin; L-NMA, L-N^{G}-monomethylarginine; NO, nitric oxide; NOC-15, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PnA, *cis*-parinaric acid; PS, phosphatidylserine; VSMCs, vascular smooth muscle cells.

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