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Unusual peroxidase activity of polynitroxylated pegylated hemoglobin: Elimination of H_2O_2 coupled with intramolecular oxidation of nitroxides

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ABSTRACT

Polynitroxylated hemoglobin ($Hb(AcTPO)_{12}$) has been developed as a hemoglobin-based oxygen carrier. While $Hb(AcTPO)_{12}$ has been shown to exert beneficial effects in a number of models of oxidative injury, its peroxidase activity has not been characterized thus far. In the blood stream, $Hb(AcTPO)_{12}$ undergoes reduction by ascorbate to its hydroxylamine form $Hb(AcTPOH)_{12}$. Here we report that $Hb(AcTPOH)_{12}$ exhibits peroxidase activity where H_2O_2 is utilized for intramolecular oxidation of its TPOH residues to TPO. This represents an unusual redox-catalytic mechanism whereby reduction of H_2O_2 is achieved at the expense of reducing equivalents of ascorbate converted into those of $Hb(AcTPOH)_{12}$, a new propensity that cannot be directly associated with ascorbate.

1. Introduction

Hemoglobin is essential for aerobic life as an oxygen carrier, yet it can also be harmful – outside of red blood cells (RBCs), acting as a peroxidase. Uninterrupted maintenance of a reduced state of Hb in RBCs is critical to its potential to bind oxygen and prevent cytotoxicity. Numerous attempts have been made to develop Hb-based oxygen carriers (HBOCs) as alternatives to RBCs [1,2]. Structural modifications of Hb by intra- and extra-molecular cross-linking, surface conjugation with polymers, and derivatization with antioxidants has led to the identification of a number of HBOCs with promising pharmacological characteristics [2–6]. One of the recently proposed modifications includes derivatization of Hb with the nitroxide 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) to Hb(2,2,6,6-tetramethylpiperidine 1-oxyl)$_n$ ($Hb(AcTPO)_n$; $n = 10–12$; Ac, covalent linking arm; TPO, 2,2,6,6-tetramethylpiperdine 1-oxyl; TPOH, 1-hydroxy-2,2,6,6-tetramethyl-piperidine). $Hb(AcTPO)_{12}$ is a potent acceptor of electrons that has proven an effective protector against injuries associated with oxidative stress [7–9]. Polynitroxylated, pegylated $Hb–Fe^{III}$ has recently been shown to exhibit both *in vitro* and *in vivo* neuroprotective effects [10].

In plasma, $Hb(AcTPO)_{12}$ has been shown to undergo reduction to its hydroxylamine form $Hb(AcTPOH)_{12}$ [5], whose antioxidant properties have not been well studied. As compared to $Hb(AcTPO)_{12}$, $Hb(AcTPOH)_{12}$ has 12 additional reducing equivalents, which suggests that it can act as a potent antioxidant, particularly against H_2O_2. Here we report that $Hb(AcTPOH)_{12}$ exhibits peroxidase activity whereby H_2O_2 is utilized for intramolecular oxidation of its TPOH residues to TPO.

2. Materials and methods

2.1. Reagents

Bovine $Hb(AcTPO)_{12}$ was obtained from SynZyme Technologies LLC (Irvine, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex 100-treated water or potassium phosphate buffer (pH 7.4). Plasma was isolated from normal blood from a volunteer donor via centrifugation.

Abbreviations: RBCs, red blood cells; Hb, hemoglobin; TPO, 2,2,6,6-tetramethylpiperidine 1-oxyl; TPOH, 1-hydroxy-2,2,6,6-tetramethyl-piperidine; DTPA, diethylenetriaminepentaacetic acid; HBOCs, Hb-based oxygen carriers.

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2.2. Preparation of HB(AcTPOH)$_{12}$

Reduction of Hb(AcTPO)$_{12}$ was carried out at 25 °C for 30 min in 0.1 M phosphate buffer containing 100 μM DTPA and 20 mM ascorbic acid. Hb(AcTPOH)$_{12}$ was separated from low molecular mass compounds via filtration through a protein desalting spin column (Thermo Scientific; Rockford, IL).

EPR measurements were performed on a JEOL JES-FA100 EPR spectrometer (Tokyo, Japan) at 25 °C in gas-permeable Teflon tubing (0.8 mm i.d., 0.013 mm thickness; Alpha Wire Corp.; Elizabeth, NJ). The tubing was filled with 60 μL of sample, double-folded, and placed in an open 3.0 mm (internal diameter) EPR quartz tube. Spectra were recorded under the following EPR conditions: center field, 3350 G; scan range, 75 G; field modulation, 0.79 G; microwave power, 20 mW; time constant, 0.1 s; and scan time, 4 min.

2.3. Assessment of H_2O_2

Quantitative analysis of H_2O_2 was performed with an ISO-HPO-100 mini sensor connected to Apollo 1000 Free Radical Analyzer (World Precision Instruments, Bethesda, MD).

2.4. Fluorescence-monitored oxidation of tyrosine

Formation of dityrosine in PBS buffer (pH 7.4; 25 °C) containing Hb(AcTPO)$_{12}$ or Hb(AcTPOH)$_{12}$ (4 μM), tyrosine (0.15 mM), H_2O_2 (0.048–0.006 mM) and DTPA (0.15 mM) was monitored spectrofluorometrically on a Shimadzu RF-5301PC spectrofluorometer (λ_{ex} = 315 nm; λ_{em} = 420 nm; slit = 5 nm).

2.5. PAGE analysis

Proteins were separated by 10% SDS–PAGE in Tris–glycine buffer. The gels were stained by SilverSNAP stain kit (Termo Fisher Scientific, Inc.; Pittsburgh, PA) according to the manufacturer's protocol. In some experiments, gels were overexposed with developer for better visualization of high molecular weight oligomers.

2.6. Cell culture

SH-SY5Y cells were cultured in DMEM/F-12 supplemented with 10% FBS. Cells were seeded on 12-well plates at a density of 5×10^5 per well and allowed to attach overnight. Cells were first incubated with medium containing Hb(AcTPO)$_{12}$ or Hb(AcTPOH)$_{12}$ (2 μM) and H_2O_2 (100 μM) for 1 h, then washed and further incubated with fresh medium for 23 h. Cells were harvested for caspase 3/7 activity measurements using a Caspase-Glo® 3/7 Assay kit (Promega).

2.7. Statistical analysis

Data are expressed as means (standard deviation of at least triplicate determinations). Changes in variables were analyzed by a one-way ANOVA. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Redox interconversion between Hb(AcTPO)$_{12}$ and Hb(AcTPOH)$_{12}$

Incubation of Hb(AcTPO)$_{12}$ with ascorbic acid in a semi-neutral aqueous solution led to a rapid disappearance of the EPR spectrum of TPO (Fig. 1A1). In this reaction, the reduction of Hb(AcTPO)$_{12}$ to its hydroxylamine form, Hb(AcTPOH)$_{12}$, was confirmed by re-oxidation of the latter with K_3[Fe(CN)$_6$] (Fig. 1A2). Similarly, redox

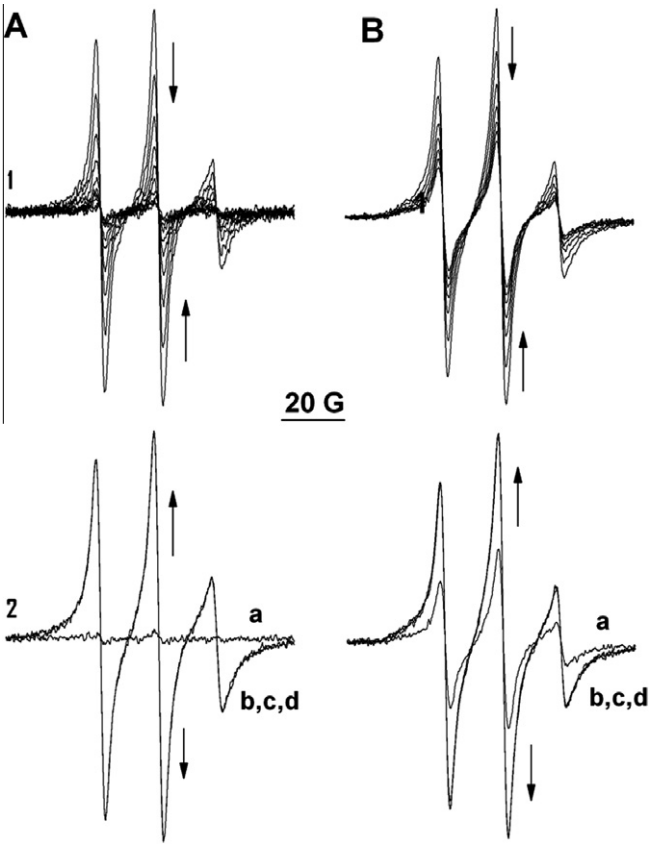


Fig. 1. EPR-monitored reduction of Hb(AcTPO)$_{12}$ by ascorbate and plasma. Reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM DTPA. Hb(AcTPO)$_{12}$, ascorbate and K_3[Fe(CN)$_6$] were used at concentrations of 0.005, 0.2 and 1.5 mM, respectively. Consecutive spectra were recorded with a time interval of 2 min (A) and 10 min (B). Arrows indicate the directions of spectral changes. (A1) Hb(AcTPO)$_{12}$ and ascorbate; (A2a) Hb(AcTPO)$_{12}$ and ascorbate, $t_{incubation}$ = 20 min; consecutive spectra (A2b–d) plus K_3[Fe(CN)$_6$]. (B1) Hb(AcTPO)$_{12}$ and plasma; (B2a) Hb(AcTPO)$_{12}$ and plasma, $t_{incubation}$ = 60 min; consecutive spectra (B2b–d) plus K_3[Fe(CN)$_6$].

interconversion between Hb(AcTPO)$_{12}$ and Hb(AcTPOH)$_{12}$ was observed in human plasma in the absence and presence of K_3[Fe(CN)$_6$] (Fig. 1B1 and 2). These data are in agreement with the studies of Buehler et al., who reported rapid reduction of Hb(AcTPO)$_{12}$ to Hb(AcTPOH)$_{12}$ in the bloodstream of guinea pigs [5]. They further suggest that Hb(AcTPO)$_{12}$ has the potential to decrease the bioavailability of ascorbic acid stoichiometrically to its nitroxide residues.

3.2. Peroxidase activity of Hb(AcTPO)$_{12}$ and Hb(AcTPOH)$_{12}$

Hb–Fe^{III} reacts with H_2O_2 to form ferryl heme and globin radicals where the unpaired electron is localized either on the iron–porphyrin ring or on a tyrosine or cysteine residue ([Hb–Fe^{IV} = O]; reaction 1) [11]. [Hb–Fe^{IV} = O]?has a lifetime ~300 ms and can participate in a number of redox reactions, including with H_2O_2 (reaction 1) [12], while its one electron oxidation leads to the formation of an oxoferryl complex with lifetime ranging from minutes to hours ([Hb–Fe^{IV} = O]; reactions 2) [13,14]. Both [Hb–Fe^{IV} = O] and [Hb–Fe^{IV} = O] are reactive species that can trigger reactions of oxidation with potentially toxic consequences (reactions 2 and 3).

$$Hb{-}Fe^{III} + H_2O_2 \rightarrow [Hb{-}Fe^{IV} = O]^{\cdot} + HO^- + H^+ \quad (1)$$

$$[Hb{-}Fe^{IV} = O]^{\cdot} + H_2O_2 \rightarrow [Hb{-}Fe^{IV} = O] + HOO^{\cdot} \quad (2)$$

$$[Hb{-}Fe^{IV} = O] + [substrate]^- \rightarrow Hb{-}Fe^{III} + HO^- + [substrate]^{\cdot +} \quad (3)$$

The data presented in Fig. 2A indicate that $Hb(AcTPO)_{12}$ and $Hb(AcTPOH)_{12}$ react with H_2O_2 at comparable rates. In the absence of a reducing peroxidase substrate, H_2O_2 triggered covalent protein cross-linking (Fig. 2B). The oligomerization was more pronounced for the nitroxide form than for $Hb(AcTPOH)_{12}$ (molecular mass, 18 kDa); H_2O_2-induced oligomerization is characteristic of hemoproteins and is mediated by recombination of *O*-, *N*- and/or *C*-centered radicals [15,16].

The increased resistance of $Hb(AcTPOH)_{12}$ toward oligomerization suggests that TPOH residues were the main substrates of oxidation reaction driven by [Hb–Fe^{IV} = O] and/or [Hb–Fe^{IV} = O]. In support of this hypothesis, H_2O_2 triggered a quantitative oxidation of the EPR silent hydroxylamine $Hb(AcTPOH)_{12}$ back to its nitroxide form $Hb(AcTPO)_{12}$ (Scheme 1, reactions 1 → 3; Fig. 3A). Recent studies suggest that Hb can catalyze the oxidation of nitroxides by H_2O_2 to oxoammonium cations [17]. However, we did not observe changes in the EPR spectrum of $Hb(AcTPO)_{12}$ (10 μM) upon addition of H_2O_2 (0.2–1 mM; data not shown), which suggests that either reactions 3 → 4 did not occur to any significant extent or TPO^+ was subjected to a rapid oxidation back to TPO.

In blood plasma, both free and protein-bound tyrosine can be oxidized by peroxidases [18,19]. Phagocyte activation triggers a membrane-associated NADPH-oxidase to reduce oxygen to superoxide anion radical, which dismutates to yield H_2O_2. One mechanism of activation of H_2O_2 at sites of inflammation may involve myeloperoxidase secreted by phagocytic white blood cells [20–22]. A similar catalytic function can be envisioned for both $Hb(AcTPO)_{12}$ and $Hb(AcTPOH)_{12}$. Hence, we have assessed the potential of these derivatives of Hb–Fe^{III} to catalyze the oxidation of tyrosine by H_2O_2. The kinetic profile of the process included a reaction lag phase (Fig. 3B), which can be explained by the occurrence of intramolecular oxidation of TPOH residues. At a [TPOH]-to-H_2O_2 ratio of ~2, oxidation of tyrosine did not occur to any significant extent, suggesting that ~6 TPOH residues in $Hb(AcTPOH)_{12}$ were in close proximity to the heme iron and subjected to a preferential oxidation.

3.3. Cell toxicity

SH-SY5Y cells exposed to H_2O_2 responded by significant activation of caspases 3/7 suggesting the onset of apoptosis. Both $Hb(AcTPO)_{12}$ and $Hb(AcTPOH)_{12}$ exerted protection, which, however, was more pronounced for the latter (Fig. 4). Activation of caspases 3 and 7 was not observed in cells incubated with $Hb(AcTPO)_{12}$ or $Hb(AcTPOH)_{12}$ only.

4. Discussion

In RBCs, Hb is maintained in a non-toxic, reduced state (Hb–Fe^{II}) by methemoglobin reductase, while Hb–Fe^{III}-mediated reactions of oxidation are impeded by co-localized superoxide dismutase (SOD), catalase, and glutathione peroxidase [23]. Unconfined Hb–Fe^{II} undergoes oxidation to Hb–Fe^{III}, which sets the stage for a cascade of reactions leading to generation of reactive oxygen species and release of redox active iron following oxidative heme degradation [1,5,23,24]. In addition, Hb–Fe^{III} reacts with H_2O_2 to form ferryl–oxo complexes with peroxidase activity.

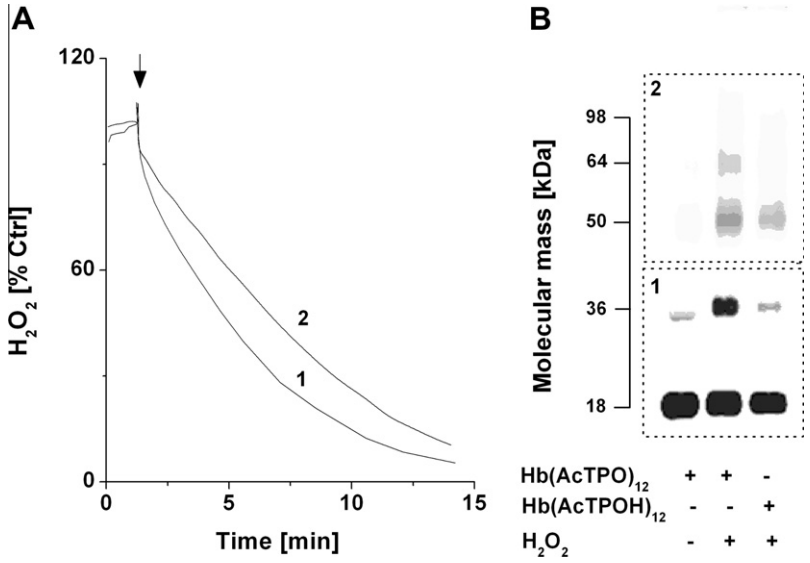
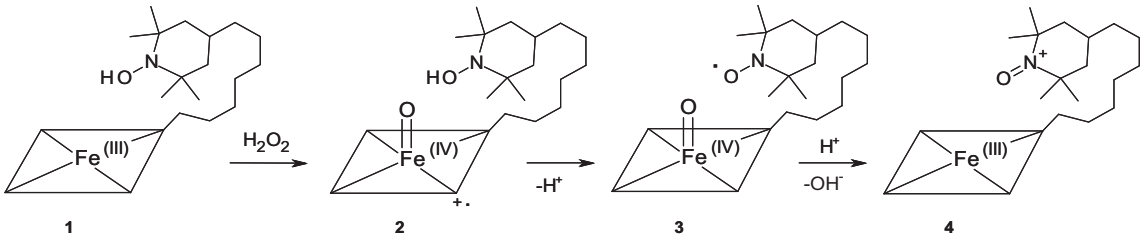


Fig. 2. H_2O_2 mediates oligomerization of $Hb(AcTPO)_{12}$. Reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM DTPA. Quantification of H_2O_2 was performed as described in Section 2. (A) Consumption of H_2O_2 by $Hb(AcTPOH)_{12}$ (trace 1; 0.004 mM) and $Hb(AcTPO)_{12}$ (trace 2; 0.004 mM); arrow indicates the addition of a hemoprotein. (B) SDS–PAGE analysis of the oligomerization of $Hb(AcTPO)_{12}$ (0.004 mM) and $Hb(AcTPOH)_{12}$ (0.004 mM) in the absence and the presence of H_2O_2 (0.05 mM); incubation time, 1 h. In panels 1 and 2, visualization of proteins was performed at different exposure time to developer.



Scheme 1.

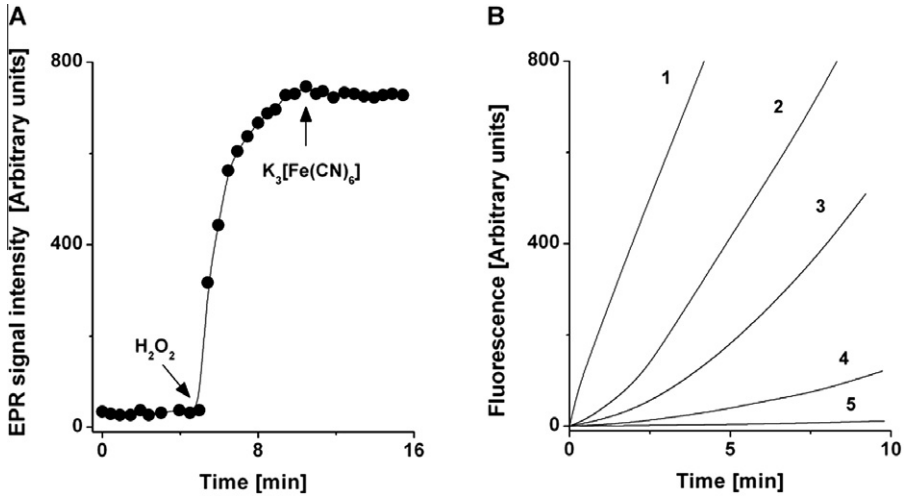


Fig. 3. Peroxidase activity of $Hb(AcTPOH)_{12}$. Reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM DTPA. (A) Time course of the oxidation of $Hb(AcTPOH)_{12}$ (0.01 mM) by H_2O_2 (0.2 mM); $K_3[Fe(CN)_6]$, 5 mM. (B) $Hb(AcTPO)_{12}$ (0.004 mM; trace 1)- and $Hb(AcTPOH)_{12}$ (0.004 mM; traces 2–5)-catalyzed oxidation of tyrosine (0.15 mM) by H_2O_2 (traces (1–5) 0.048, 0.048, 0.024, 0.012 and 0.006 mM, respectively).

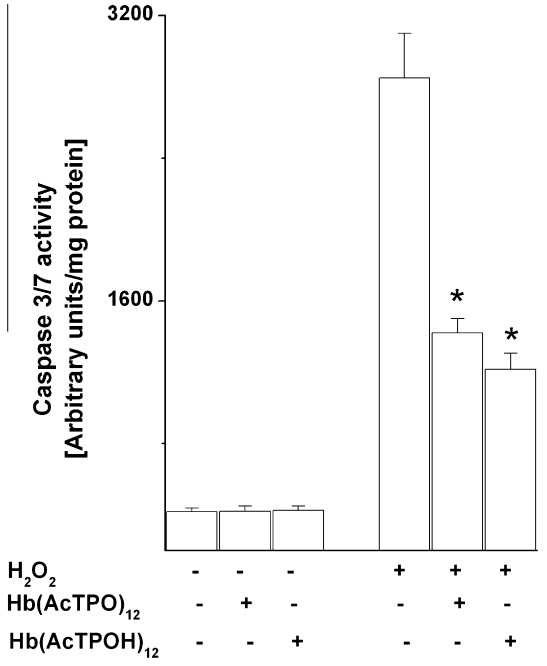


Fig. 4. $Hb(AcTPO)_{12}$ and $Hb(AcTPOH)_{12}$ impede the activation of caspases 3/7 in SH-SY5Y cells exposed to H_2O_2. Cells were incubated with medium containing $Hb(AcTPO)_{12}$ or $Hb(AcTPOH)_{12}$ (0.002 mM) and H_2O_2 (0.1 mM) for 1 h, then washed and further incubated with fresh medium for 23 h. Cells were harvested for caspases 3/7 activity measurements using a Caspase-Glo® 3/7 Assay kit (Promega). The results represent the mean ± SE ($n = 3$; $^*p < 0.01$ vs. the same concentration of H_2O_2).

To reduce the toxicity of HBOCs-mediated redox reactions, derivatives of Hb with catalase, superoxide dismutase, and TEMPO have been synthesized [4–6,8,25]. The synthesis of $Hb(AcTPO)_{12}$ follows studies indicating that the facile redox interconversion between the oxoammonium ($[TEMPO]^+$), nitroxide (TEMPO) and hydroxylamine (TEMPOH) forms of 2,2,6,6-tetramethylpiperidine 1-oxyl mediates the dismutation of superoxide anion radical, facilitates H_2O_2 metabolism by catalase-like actions, and limits formation of $HO^\cdot$ produced by Fenton-like reactions [26]. Although $Hb(AcTPO)_{12}$ has been shown to exert beneficial effects in a number of models of oxidative injury [7–9], its peroxidase activity has not been characterized thus far.

Our data are in agreement with the studies of Buehler et al. [5], who have reported that reduction of $Hb(AcTPO)_{12}$ in the circulation leads to an equilibrium between the nitroxide and hydroxylamine forms of this HBOC. Although this reaction requires ascorbate, an important antioxidant of plasma, $Hb(AcTPOH)_{12}$ may prove effective in elimination of toxic H_2O_2. Ascorbate-derived reducing equivalents converted into those of $Hb(AcTPOH)_{12}$ gain a new propensity of controlling the content of H_2O_2 – a property that cannot be directly associated with ascorbate. Moreover, the presence of extra-reducing equivalents in $Hb(AcTPOH)_{12}$ confers additional resistance against its oxidative degradation/oligomerization by oxidative stress. This is mostly due to the fact that the peroxidase activity of $Hb(AcTPOH)_{12}$ – in contrast to $Hb(AcTPO)_{12}$ – is preferentially directed toward intramolecular oxidation of TPOH residues. This may be particularly important given that the concentration of H_2O_2 can increase in the high micromolar range in patients with severe inflammation. The presence of a significant intramolecular reserve of reducing equivalents – the redox buffering capacity of $Hb(AcTPOH)_{12}$ – may be instrumental in controlling high levels of H_2O_2 which can freely diffuse into different cells and cause toxicity. It could be further speculated that that the nitroxide $Hb(AcTPO)_{12}$ will act as an effective acceptor of electrons during excessive activation of the NADPH-oxidase of inflammatory cells, which leads to a massive production of superoxide radicals. The propensity of $Hb(AcTPO)_{12}$ to act as an electron scavenger may be pharmacologically beneficial in preventing the flow of electrons to dioxygen and suppression of superoxide production under these circumstances. Thus, combinations of $Hb(AcTPO)_{12}$ and $Hb(AcTPOH)_{12}$ whose optimized ratios in plasma may be dictated by levels and sources of oxidative stress may be viewed as a prospectively useful approach for regulation of oxidative stress. In this regard, ascorbate, co-administrated with $Hb(AcTPOH)_{12}$, may turn out to be instrumental in "re-charging" it in the circulation and thus maintaining its protective functions.

Acknowledgments

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