Generation of Hydroxyl Radicals in the Reaction of Ferritin with Hydrogen Peroxide

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Reactive oxygen species (ROS) such as the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) are generated both from endogenous sources such as cellular metabolism or inflammation, and from exogenous sources including molecular oxygen and UV light.[1,2] ROS are involved in normal cell processes such as cell signaling and proliferation. However, when present in excess amounts, ROS damage cellular biomolecules and have been implicated in the etiology of a broad variety of diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic inflammatory disease, and cancer and aging.[3,4]

Iron is one of the most essential transition metals involved in hydroxyl radical generation, as the result of its interaction with H_2O_2 *via* the Fenton-type reaction. The hydroxyl radical is a strongly reactive oxygen, and can damage DNA, and cause lipid peroxidation and protein modification.[5] Iron that is not immediately utilized in the cell is stored in ferritin. However, when iron is regulated improperly, it is potentially toxic, and can lead to cell death. Mammalian ferritin is a large, iron-storage heteropolymer composed of two equivalent subunit types, the light (FTL: ferritin light) and heavy (FTH: ferritin heavy) polypeptides, which are expressed in most cell types.[6-9] It has been recently reported that iron deposition in the brain results from the release of iron from ferritin.[10,11] *In vitro*, the release of iron and protein degradatiobn can be induced by a variety of oxidants.[12]

Iron accumulates progressively with aging in certain regions of the brain; high levels of iron have been previously associated with oxidative stress, which may promote neurodegeneration.[13,14] The results of previous studies have shown that iron released from ferritin and its associated toxicity, is a risk factor for age at the onset of neurodegenerative diseases including Parkinson disease (PD) and Alzheimer disease (AD).[15,16]

In this study, ·OH generation occurring during the reaction of ferritin with H_2O_2 was assessed by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacts with ·OH to form $ABTS^{+\bullet}$. The initial rate of $ABTS^{+\bullet}$ formation was linear with regard to the concentrations of H_2O_2 and ferritin. Free radical scavengers inhibited the formation of $ABTS^{+\bullet}$. $ABTS^{+\bullet}$ formation was also inhibited by low concentrations of the iron chelator, deferoxamine. The results indicated that ·OH generation induced by the reaction of ferritin with H_2O_2 was associated with the release of iron from ferritin.

Figure 1 shows the time-dependent change in optical absorption spectra obtained from a reaction mixture containing 50 μM ABTS, 0.1 mM H_2O_2 and 1 μM ferritin in 10 mM potassium phosphate buffer at pH 7.4. The optical absorption spectra did not change after incubation of 50 μM ABTS with 1 μM ferritin or 0.1 mM H_2O_2 alone (data not shown). Previous pulse radiolysis studies[17,18] have established that ABTS reacts with ·OH radicals at a diffusion-controlled rate to generate a relatively stable $ABTS^{+\bullet}$, which evidences strong electronic absorption abilities. Thus, ferritin can induce the formation of free radicals using H_2O_2. $ABTS^{+\bullet}$ formation increased as a function of time at 415 nm. The initial rate of $ABTS^{+\bullet}$ formation monitored at 415 nm was linear with regard to the concentration of H_2O_2 between 0.1-0.5 mM (Fig. 2, A) and that of ferritin between 0.25-1 μM (Fig. 2, B). The rate of $ABTS^{+\bullet}$ formation is linearly proportional to 2 μM ferritin and 1 mM H_2O_2 respectively (data not shown). The reaction is first order with respect to H_2O_2 and ferritin. These results suggest that ABTS binds to ferritin with a relatively high affinity, an area near the active site where reactive ·OH radicals are produced.

The participation of ·OH in the reaction of ferritin with H_2O_2 was also assessed by examining the protective effects of free radical scavengers such as azide, mannitol, *N*-acetyl-$_L$-

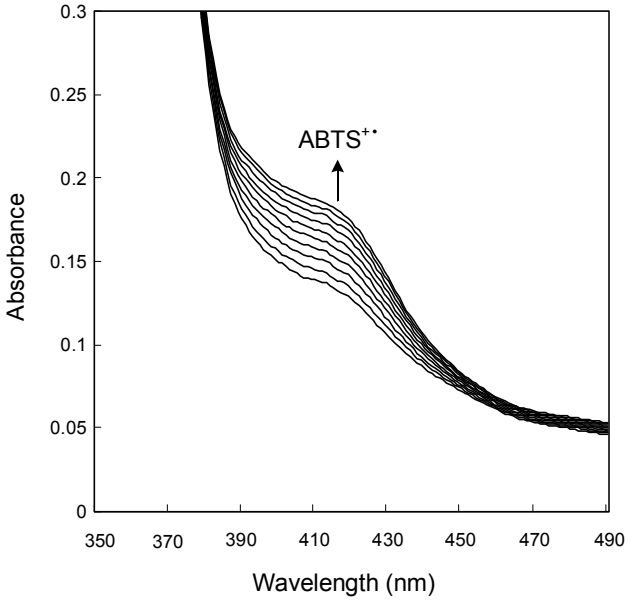

Figure 1. Electronic absorption spectra as a function of time. The spectra were obtained with a solution containing 50 μM ABTS, 0.1 mM H_2O_2 and 1 μM ferritin in 10 mM potassium phosphate buffer at pH 7.4. Each scan took 3 min. The arrows indicate the direction of absorbance change with time.

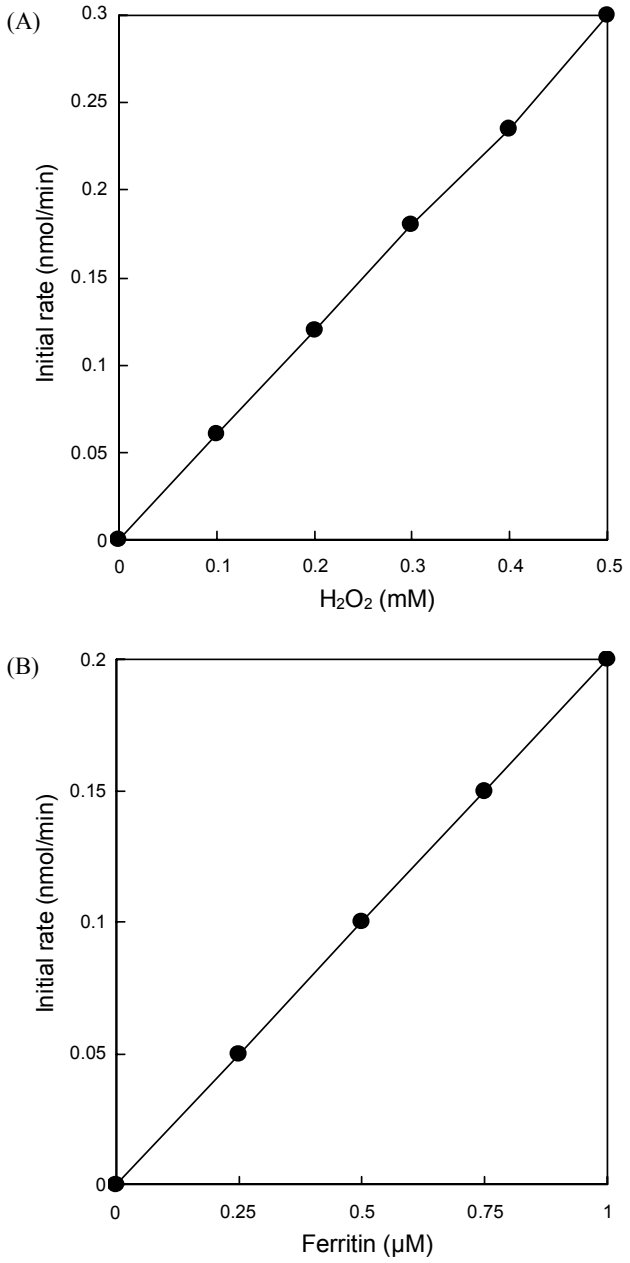

Figure 2. The effects of protein and H_2O_2 concentrations on the rates of ·OH formation determined by using a chromogen, ABTS. (A) The reaction mixture contained 50 μM ABTS, 0.1-0.5 mM H_2O_2 and 1 μM ferritin in 10 mM potassium phosphate buffer at pH 7.4. (B) The reaction mixture contained 50 μM ABTS, 0.25-1 μM ferritin and 0.3 mM H_2O_2 in 10 mM potassium phosphate buffer at pH 7.4.

cysteine and thiourea. The sugar alcohol mannitol, as well as azide and thiol compounds are frequently utilized as hydroxyl radical scavengers (10-100 mM) in laboratory experiments.[3] When ferritin was incubated for 5 min with H_2O_2 in the presence of a variety of free radical scavengers at 37 °C, all scavengers inhibited the generation of ·OH (Table 1). Oxidative stress, occurring as a consequence of increased intracellular levels of ROS, such as H_2O_2, supposedly forms a common pathway resulting in cell death under the aforementioned conditions.[19] *In vivo* the H_2O_2 is probably a direct product of $O_2^{-\bullet}$ dismutation and a variety of oxidase reactions. It has also

Table 1. Effect of radical scavengers on the formation of $ABTS^{+\bullet}$ by ferritin and hydrogen peroxide system.

Hydroxyl radical scavengers	concentration	$ABTS^{+\bullet}$	
		nmol/min	%
None		0.1	100
Azide	10 mM	0.014	14
Mannitol	10 mM	0.072	72
N-acetyl-L-cysteine	10 mM	0.036	36
Thiourea	10 mM	0.011	11

The reaction mixture contained 10 mM potassium phosphate buffer (pH 7.4), 0.5 μM ferritin, 0.3 mM H_2O_2, 50 μM ABTS. The reaction was initiated by addition of H_2O_2 and incubated without or with radical scavengers at 37 °C.

Table 2. Effect of metal chelator on the formation of $ABTS^{+\bullet}$ by ferritin and hydrogen peroxide system.

Metal chelator	concentration	$ABTS^{+\bullet}$	
		nmol/min	%
None		0.1	100
DFX	0.1 mM	0.072	72
	1 mM	0.027	27
	5 mM	0.014	14

The reaction mixture contained 10 mM potassium phosphate buffer (pH 7.4), 0.5 μM ferritin, 0.3 mM H_2O_2, 50 μM ABTS. The reaction was initiated by addition of H_2O_2 and incubated without or with metal chelator at 37 °C.

been noted that the rate of H_2O_2 formation under physiological conditions was 90 μM H_2O_2/min in the liver at 22 °C.[20] Yim *et al.* reported that at least 0.1 mM/min H_2O_2 will be continuously generated under physiological conditions and at a much higher rate under adverse conditions, such as hyperoxia or ischemia and reperfusion.[21] Once formed, H_2O_2 can undergo various reactions, both enzymatic and non-enzymatic. The antioxidant enzymes catalase and glutathione peroxidase act to limit ROS accumulation in cells by breaking down H_2O_2 to H_2O. However, H_2O_2 metabolism can also generate other, more damaging ROS.[22] For example, the endogenous enzyme myeloperoxidase uses H_2O_2 as a substrate to form the highly reactive compound hypochlorous acid. Alternatively, H_2O_2 can undergo Fenton chemistry, reacting with iron ions to form ·OH.[22] Therefore, ·OH gteneration by the reaction of ferritin with, H_2O_2 can exert deleterious effects in cells.

We assessed the effects of metal chelators on ·OH formation *via* the reaction of ferritin with H_2O_2; deferoxamine (DFX) inhibited approximately 28% of the formation of ·OH at 0.1 mM, whereas DFX at 5 mM inhibited 86% of the formation of ·OH (Table 2). The results showed that iron ions might be involved ·OH generation in the reaction of ferritin with H_2O_2. DFX is considered to be a potent free radical scavenger which prevents ·OH generation *via* the Fenton reaction.[23] Iron has been implicated in a variety of diseases; in particular, it has been associated with certain neurological diseases.[24] Hence, iron chelators that can access the brain are high on the wish-list of investigators with an interest in PD,[25]

Alzheimer's disease (AD), and Friedreich's ataxia (FA).[26] Low dose of DFX have been shown previously to improve survival in patients with ischemia-reperfusion injury[27] and erythropoiesis in chronic hemodialysis patients.[28] When DFX was administered as a pretreatment (n = 39; doses of 10 or 30 mg/kg), added to cardioplegic solution (n = 43; doses 0.46 to 1.90 mM), or administered upon reperfusion (n = 52; doses 0.15 to 0.76 mM) and compared with saline controls (n = 25), DFX pretreatment was shown improve survival at each dose from a control value of 44% to 71% and 72% (p less than 0.05), respectively.[27] However, treatment with high doses reduced survival and implied a toxic effect.[27] Therefore, we suggest that the low concentrations of DFX may effectively protect cells against the deleterious conditions associated with ·OH generated in the reaction of ferritin and H_2O_2.

In conclusion, the generation of ·OH in the reaction of ferritin with H_2O_2 was evaluated by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), which was allowed to react with ·OH to form $ABTS^{+\bullet}$. The generation of $ABTS^{+\bullet}$ proved linear with regard to the concentrations of H_2O_2 and ferritin. The protective effects of hydroxyl radical scavengers on the formation of $ABTS^{+\bullet}$ corroborate this mechanism. Because iron chelator inhibited the formation of $ABTS^{+\bullet}$ induced by the reaction of ferritin with H_2O_2, the generation of ·OH was associated with the release of iron from ferritin. Thus, ferritin/H_2O_2-mediated ·OH generation may exert deleterious effects, most notably pathological complications of free radical-mediated diseases.

Experimental Section

Materials. Equine spleen ferritin was purchased from Calbiochem (Darmstadt, German). The commercial ferritin was then further purified *via* gel filtration chromatography using Superose 6 FPLC column (Pharmacia, Sweden). Mannitol, *N*-acetyl-L-cysteine, thiourea and deferoxamine (DFX) were purchased from Sigma (St. Louis, MO). The diammonium salt of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and sodium azide were purchased from Boehringer Mannheim (Mannheim, German). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA). All solutions were treated with Chelex 100 resin to remove any traces of transition metal ions.

Measurement of hydroxyl radical. ·OH was measured by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).[29] ABTS is water-soluble and evidences strong absorption at 340 nm with a molar extinction coefficient ε_{340} of $3.66 \times 10^4\ M^{-1}cm^{-1}$.[30] On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, $ABTS^{+\bullet}$ is conveniently followed at λmax at 415 nm ($\varepsilon_{415} = 3.6 \times 10^4\ M^{-1}cm^{-1}$).[31] The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4) and 50 μM ABTS and 0.1-0.5 mM H_2O_2 and 0.25-1.0 μM protein in a total volume of 1 mL. The reaction was initiated *via* the addition of H_2O_2 and the increase in absorbance at 415 nm was measured by using a UV/Vis spectrophotometer (Shimazu 1601).

Effects of free radical scavenger and metal chelator on the formation of hydroxyl radical. 0.5 μM ferritin was allowed to react with 0.3 mM H_2O_2 in the presence of free radical scavengers (azide, manniotol, *N*-acetyl-L-cysteine and thiourea) or iron chelator (deferoxamine) at 37 °C. ·OH was measured by using a chromogen, 2,2'-azinobis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS).

Replicates. Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

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