

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

Jung Hwan Yoon and Jung Hoon Kang*

Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea. *E-mail: jhkang@cju.ac.kr
Received March 26, 2009, Accepted June 3, 2009

Key Words: Ferritin, Hydroxyl radical, Iron

Reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) 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 degradation 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, $\cdot OH$ generation occurring during the reaction of ferritin with H_2O_2 was assessed by using a chromogen, 2,2'-azino-bis-(2-ethylbenzthiazoline-6-sulfonate) (ABTS), which reacts with $\cdot OH$ to form $ABTS^{\cdot+}$. The initial rate of $ABTS^{\cdot+}$ formation was linear with regard to the concentrations of H_2O_2 and ferritin. Free radical scavengers inhibited the formation of $ABTS^{\cdot+}$. $ABTS^{\cdot+}$ formation was also inhibited by low concentrations of the iron chelator, deferoxamine. The results indicated that $\cdot OH$ 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 μ

M ABTS, 0.1 mM H_2O_2 and 1 μ M ferritin in 10 mM potassium phosphate buffer at pH 7.4. The optical absorption spectra did not change after incubation of 50 μ M ABTS with 1 μ M ferritin or 0.1 mM H_2O_2 alone (data not shown). Previous pulse radiolysis studies^{17,18} have established that ABTS reacts with $\cdot OH$ radicals at a diffusion-controlled rate to generate a relatively stable $ABTS^{\cdot+}$, which evidences strong electronic absorption abilities. Thus, ferritin can induce the formation of free radicals using H_2O_2 . $ABTS^{\cdot+}$ formation increased as a function of time at 415 nm. The initial rate of $ABTS^{\cdot+}$ 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 μ M (Fig. 2, B). The rate of $ABTS^{\cdot+}$ formation is linearly proportional to 2 μ M 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 $\cdot OH$ radicals are produced.

The participation of $\cdot OH$ 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-

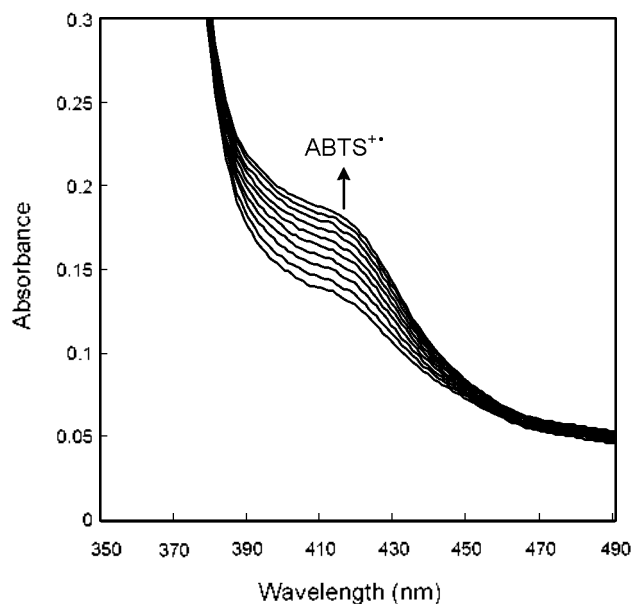


Figure 1. Electronic absorption spectra as a function of time. The spectra were obtained with a solution containing 50 μ M ABTS, 0.1 mM H_2O_2 and 1 μ M ferritin in 10 mM potassium phosphate buffer at pH 7.4. Each scan took 3 min. The arrows indicate the direction of absorbance change with time.

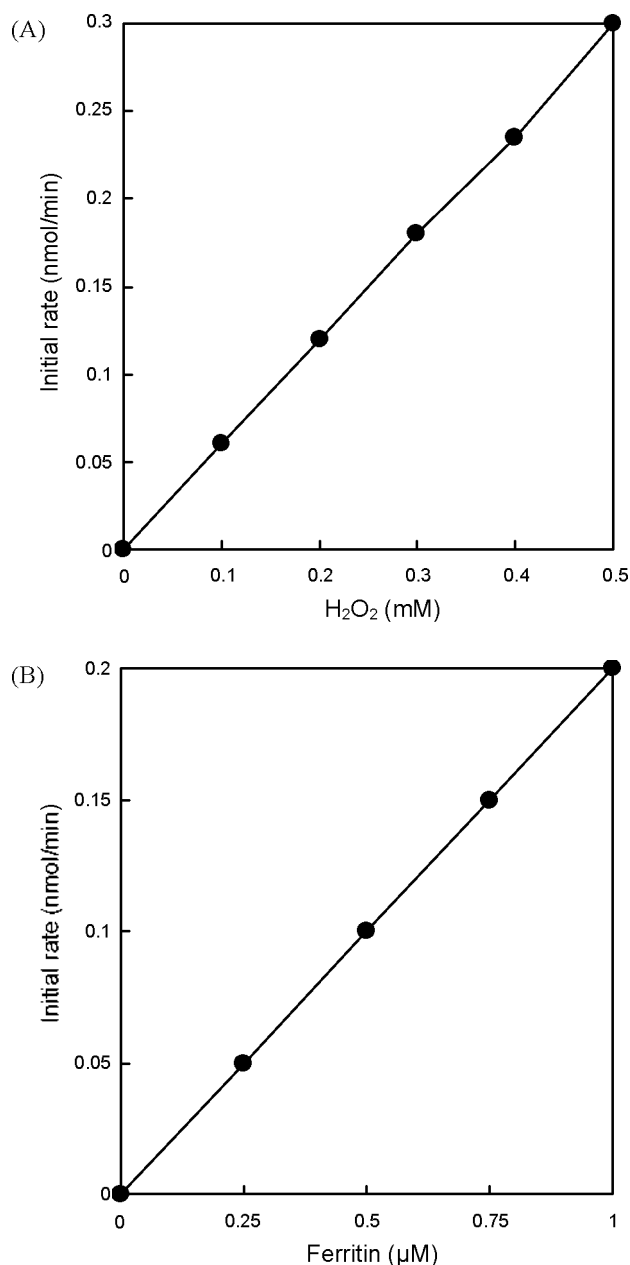


Figure 2. The effects of protein and H₂O₂ concentrations on the rates of $\cdot\text{OH}$ formation determined by using a chromogen, ABTS. (A) The reaction mixture contained 50 μM ABTS, 0.1–0.5 mM H₂O₂ and 1 μM ferritin in 10 mM potassium phosphate buffer at pH 7.4. (B) The reaction mixture contained 50 μM ABTS, 0.25–1 μM ferritin and 0.3 mM H₂O₂ 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 H₂O₂ in the presence of a variety of free radical scavengers at 37 °C, all scavengers inhibited the generation of $\cdot\text{OH}$ (Table 1). Oxidative stress, occurring as a consequence of increased intracellular levels of ROS, such as H₂O₂, supposedly forms a common pathway resulting in cell death under the aforementioned conditions.¹⁹ *In vivo* the H₂O₂ is probably a direct product of O₂⁻ dismutation and a variety of oxidase reactions. It has also

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

Hydroxyl radical scavengers	concentration	ABTS ^{•+}	
		nmol/min	%
None		0.1	100
Azide	10 mM	0.014	14
Mannitol	10 mM	0.072	72
<i>N</i> -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 H₂O₂, 50 μM ABTS. The reaction was initiated by addition of H₂O₂ and incubated without or with radical scavengers at 37 °C.

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

Metal chelator	concentration	ABTS ^{•+}	
		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 H₂O₂, 50 μM ABTS. The reaction was initiated by addition of H₂O₂ and incubated without or with metal chelator at 37 °C.

been noted that the rate of H₂O₂ formation under physiological conditions was 90 μM H₂O₂/min in the liver at 22 °C.²⁰ Yim *et al.* reported that at least 0.1 mM/min H₂O₂ 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, H₂O₂ 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 H₂O₂ to H₂O. However, H₂O₂ metabolism can also generate other, more damaging ROS.²² For example, the endogenous enzyme myeloperoxidase uses H₂O₂ as a substrate to form the highly reactive compound hypochlorous acid. Alternatively, H₂O₂ can undergo Fenton chemistry, reacting with iron ions to form $\cdot\text{OH}$.²² Therefore, $\cdot\text{OH}$ generation by the reaction of ferritin with H₂O₂ can exert deleterious effects in cells.

We assessed the effects of metal chelators on $\cdot\text{OH}$ formation *via* the reaction of ferritin with H₂O₂; deferoxamine (DFX) inhibited approximately 28% of the formation of $\cdot\text{OH}$ at 0.1 mM, whereas DFX at 5 mM inhibited 86% of the formation of $\cdot\text{OH}$ (Table 2). The results showed that iron ions might be involved in the reaction of ferritin with H₂O₂. DFX is considered to be a potent free radical scavenger which prevents $\cdot\text{OH}$ 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 injury²⁷ 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 $\cdot\text{OH}$ generated in the reaction of ferritin and H_2O_2 .

In conclusion, the generation of $\cdot\text{OH}$ 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 $\cdot\text{OH}$ to form $\text{ABTS}^{\cdot-}$. The generation of $\text{ABTS}^{\cdot-}$ proved linear with regard to the concentrations of H_2O_2 and ferritin. The protective effects of hydroxyl radical scavengers on the formation of $\text{ABTS}^{\cdot-}$ corroborate this mechanism. Because iron chelator inhibited the formation of $\text{ABTS}^{\cdot-}$ induced by the reaction of ferritin with H_2O_2 , the generation of $\cdot\text{OH}$ was associated with the release of iron from ferritin. Thus, ferritin/ H_2O_2 -mediated $\cdot\text{OH}$ 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. $\cdot\text{OH}$ 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 $3.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.³⁰ On oxidation, ABTS forms a stable blue-green product presumed to be the cation radical, $\text{ABTS}^{\cdot+}$ is conveniently followed at λ_{max} at 415 nm ($\epsilon_{415} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).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 (Shimadzu 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, mannitol, *N*-acetyl-L-cysteine and thiourea) or iron chelator (deferoxamine) at 37 °C. $\cdot\text{OH}$ 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.

References

1. Yamanashi, B. S.; Hacker, H.; Klintworth, G. K. *Photochem. Photobiol.* **1979**, *30*, 391-395.
2. Shimmura, S.; Suematsu, M.; Shimoyama, M.; Tsubota, K.; Oguchi, Y.; Ishimura, Y. *Exp. Eye. Res.* **1996**, *63*, 519-526.
3. *Free Radicals in Biology and Medicine*; Halliwell, B.; Gutteridge, J. M., Eds.; *Oxford Science*: Oxford, 2007.
4. Evans, M. D.; Dizdaroglu, M.; Cooke, M. S. *Mutat. Res.* **2004**, *527*, 1-61.
5. Welch, K. D.; Davis, T. Z.; Van Eden, M. E.; Aust, S. D. *Free Radic. Biol. Med.* **2002**, *32*, 577-583.
6. Harrison, P. M.; Arosio, P. *Biochim. Biophys. Acta* **1996**, *1275*, 161-203.
7. Theil, E. C.; Matzapetakis, M.; Liu, X. *J. Biol. Inorg. Chem.* **2006**, *11*, 803-810.
8. Theil, E. C. *J. Nutr.* **2003**, *133*, 1549S-1553S.
9. Liu, X.; Theil, E. C. *Acc. Chem. Res.* **2005**, *38*, 167-175.
10. Ong, W. Y.; Ren, M. Q.; Makjanic, J.; Lim, T. M.; Watt, F. J. *Neurochem.* **1999**, *72*, 1574-1579.
11. Nakayama, M.; Takahashi, K.; Komaru, T.; Fukuchi, M.; Shioiri, H.; Sato, Ki.; Kitamuro, T.; Shirato, K.; Yamaguchi, T.; Suematsu, M.; Shibahara, S. *Arterioscler. Thromb. Vasc. Biol.* **2001**, *21*, 1373-1377.
12. Rudeck, M.; Volk, T.; Sitte, N.; Grune, T. *IUBMB Life* **2000**, *49*, 451-456.
13. Thomas, M.; Jankovic, J. *Curr. Opin. Neurol.* **2004**, *17*, 437-442.
14. Zecca, L.; Youdim, M. B.; Riederer, P.; Connor, J. R.; Crichton, R. R. *Nat. Rev. Neurosci.* **2004**, *5*, 863-873.
15. Bartzokis, G.; Tishler, T. A.; Shin, I. S.; Lu, P. H.; Cummings, J. L. *Ann N. Y. Acad. Sci.* **2004**, *1012*, 224-236.
16. Kozirowski, D.; Friedman, A.; Arosio, P.; Santambrogio, P.; Dziewulska, D. *Parkinsonism Relat. Disord.* **2007**, *13*, 214-218.
17. Wolfeden, B. S.; Wilson, R. L. *J. Chem. Soc. Perkin Trans. II* **1982**, 805-812.
18. D'Aquino, M.; Dunster, C.; Wilson, R. L. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 1199-1203.
19. Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7915-7922.
20. Boveris, A.; Oshino, N.; Chance, B. *Biochem. J.* **1972**, *128*, 617-630.
21. Yim, M. B.; Chock, P. B.; Stadtman, E. R. *J. Biol. Chem.* **1993**, *268*, 4099-4105.
22. Wolins, M. S. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 1430-1442.
23. Kamp, D. W.; Graceffa, P.; Pryor, W. A.; Weitzman, S. A. *Free Radic. Biol. Med.* **1992**, *12*, 293-315.
24. Kang, J. H. *Bull. Korean Chem. Soc.* **2008**, *29*, 2395-2398.
25. Kang, J. H. *Bull. Korean Chem. Soc.* **2007**, *28*, 77-80.
26. Whitnall, M.; Richardson, D. R. *Semin. Pediatr. Neurol.* **2006**, *13*, 186-197.
27. DeBore, D. A.; Clark, R. E. *Ann. Thorac. Surg.* **1992**, *53*, 412-418.
28. Lee, C. T.; Liao, S. C.; Hsu, K. T.; Lam, K. K.; Chen, J. B. *Ren. Fail.* **1999**, *21*, 665-673.
29. Radi, R.; Thomson, L.; Rubbo, H.; Prodanov, E. *Arch. Biochem. Biophys.* **1991**, *288*, 112-117.
30. Rush, J. D.; Koppenol, W. H. *J. Am. Chem. Soc.* **1988**, *110*, 4957-4963.
31. Childs, R. E.; Bardsley, W. G. *Biochem. J.* **1975**, *145*, 93-103.