Neurotoxicity Research, Vol. 2, pp. 343-355
Reprints available directly from the publisher
Photocopying permitted by license only

Hydroxyl Radical Production by Ascorbate and Hydrogen Peroxide

ANTHONY J. NAPPI* and EMILY VASS

Department of Biology, Loyola University Chicago, Chicago, IL 60626

(Received November 15, 1999; Revised January 10, 2000; In final form April 11, 2000)

Ascorbate (AH^-) and certain other biological reductants have long been known to produce the cytotoxic hydroxyl radical ($^{\cdot}OH$) when oxidized by hydrogen peroxide (H_2O_2) in the presence of copper or iron catalysts. The present study documents the in vitro production of the $^{\cdot}OH$ solely from the oxidation of AH^- by H_2O_2, independent of mediation by transition metals. Hydroxyl radical generation resulting from the AH^-/H_2O_2 system was quantitatively documented by the specific radical-mediated hydroxylation of salicylic acid, a reaction that was readily assayed with HPLC coupled with electrochemical detection. Two ascorbate-copper complexes (e.g., AH^-/Cu^{2+}-EDTA/H_2O_2 and AH^-/Cu^{2+}-EDTA) and a copper/H_2O_2 system also generated $^{\cdot}OH$, but less effectively than the AH^-/H_2O_2 system. The ability of AH^- and H_2O_2 to generate cytotoxic $^{\cdot}OH$ documents a reaction mechanism that may account for cytotoxic activity in some cellular environments where metal catalysts are lacking.

Keywords: Ascorbate, cytotoxicity, hydrogen peroxide, hydroxyl radical, reactive oxygen intermediates

INTRODUCTION

Reactive oxygen intermediates (ROI) are highly unstable and potentially toxic molecules that are produced under both normal and pathological conditions by several cellular mechanisms. Regulated levels of certain ROI have been shown to be beneficial and even indispensable in cell signaling processes and in defense reactions against microbial infections. However, abnormally high concentrations of ROI, resulting from either exogenous insult or from dysfunctional endogenous controls, can cause oxidative stress that adversely affects cellular metabolism. The ROI most frequently implicated as causative factors in oxidative and peroxidative damage to membrane lipids and other biomolecules include superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and the most aggressive oxygen species, the hydroxyl radical ($^{\cdot}OH$). The latter radical is generated in reactions involving H_2O_2 with either $O_2^{-\cdot}$, NO, or "redox-active" iron or copper (i.e. Fenton reaction) (Eqs. 1–3) (Nappi and Vass, 1998a).

$$H_2O_2 + O_2^{-\cdot} \rightarrow {}^{\cdot}OH + OH^- + O_2 \qquad \text{Eq 1}$$

$$H_2O_2 + NO \rightarrow {}^{\cdot}OH + HNO_2 \qquad \text{Eq 2}$$

$$H_2O_2 + Fe^{2+}(Cu^+) \rightarrow Fe^{3+}(Cu^{2+}) + OH^- + {}^{\cdot}OH \qquad \text{Eq 3}$$

Organisms have acquired in their evolution various mechanisms to protect against toxic oxygen species. Protection against $^{\cdot}OH$-mediated

* Corresponding Author: Dr. A. J. Nappi, Department of Biology, Loyola University Chicago, Chicago, IL 60626. Tel. 773–508–3632, Fax: 773–508–3648, e-mail: anappi@orion.it.luc.edu

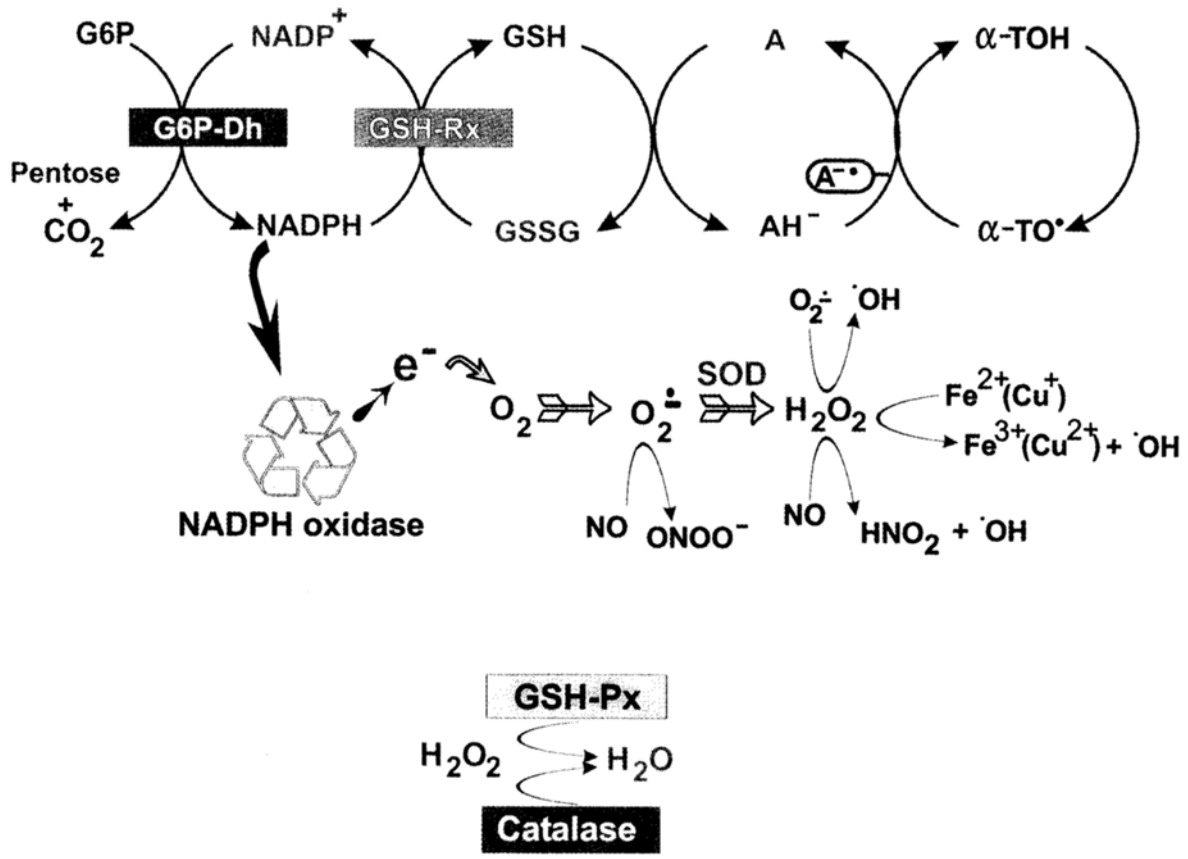

FIGURE 1 In phagocytes and various other cells, an initial monovalent reduction of dioxygen (O_2) catalyzed by NADPH oxidase produces $O_2^{\cdot-}$, which is converted by superoxide dismutase (SOD; EC 1.15.1.1) to H_2O_2. Hydrogen peroxide can react with $O_2^{\cdot-}$, NO, or "redox-active" iron or copper (i.e. Fenton reaction) to produce .OH, which can initiate membrane lipid peroxidation and adversely alter the activity of membrane-bound enzyme systems. The production of ·OH is prevented primarily by the activity of intracellular enzymes, such as catalase (EC 1.11.1.6) and glutathione peroxidase (GSHPx; EC 1.11.1.9) that degrade H_2O_2, and by non-enzymatic antioxidants such as α-tocopherol (vitamin E), reduced glutathione (GSH), and ascorbic acid. GSH is used as a donor of reduction equivalents by GSHPx. Reactions mediated by GSHpx generate oxidized glutathione (GSSG), which is reduced back to GSH by glutathione reductase (GSHRx; EC 1.6.4.2), an enzyme that uses NADPH as an electron donor

damage is attributed to the activity of enzymes such as catalase (EC 1.11.1.6) and glutathione peroxidase (GSHPx; EC 1.11.1.9) that detoxify H_2O_2, and to several non-enzymatic antioxidants including α-tocopherol (α-TO, vitamin E), reduced glutathione (GSH), β-carotene, and ascorbic acid (AH_2; vitamin C) (Fig. 1). α-Tocopherol is an effective radical chain-breaking molecule that blocks membrane lipid peroxidation by quenching peroxyl radicals. The radical scavenging capacity of α-tocopherol is believed to be maintained in large part through the intervention of two reductants, GSH and AH_2 (Niki et al., 1995).

The metabolism of AH_2 and the factors regulating its production are of considerable interest, especially given its purported function as a cellular reductant (Banhegyi et al., 1997). Ascorbic acid has two ionizable -OH groups, and a dissociation constant in water of 7.94×10^{-5} ($pK_a = 4.2$). At physiological pH, the ascorbate anion (AH^-) is the predominant species and

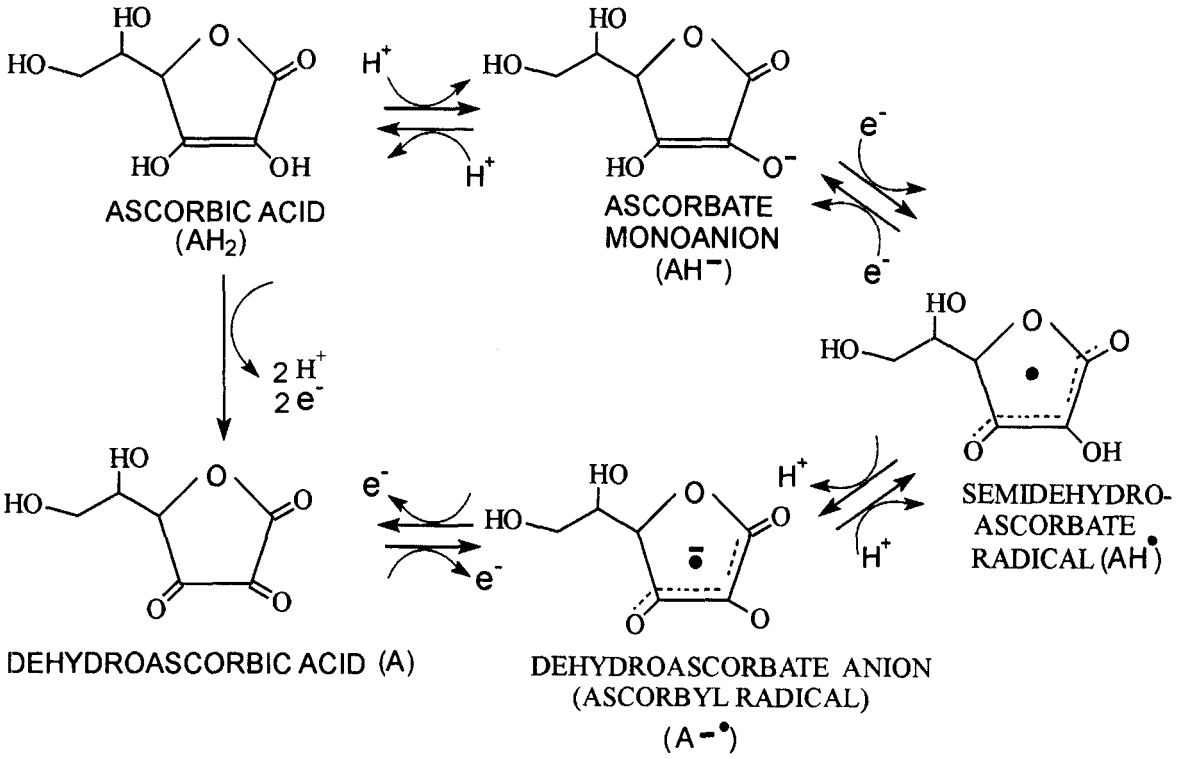

FIGURE 2 Interconversions of AH_2 at physiological pH. Ascorbic acid has two ionizable -OH groups, and a dissociation constant in water of 7.94×10^{-5} ($pK_a = 4.2$). At physiological pH, the ascorbate monoanion (AH^-) is the dominant state. Donation of one electron by AH^- produces semidehydroascorbate radical which is oxidized to form the ascorbyl radical ($A^{-\cdot}$) and dehydroascorbic acid (A). The delocalization of the unpaired electron renders $A^{-\cdot}$ rather unreactive. Dehydroascorbic acid is unstable and breaks down rapidly into diketo-L-gulonic acid, which ultimately produces oxalic and L-threonic acids

undergoes a two-step oxidation to yield dehydroascorbic acid (A) through the formation of an intermediate ascorbyl radical ($A^{-\cdot}$). The interconversions of AH_2 are presented in Fig. 2.

The antioxidant activity of AH^- is due to its ability to react with a variety of free radicals and active oxygen species (Asada, 1996), some of which are illustrated below:

$$AH^- + O_2 \rightarrow A^{-\cdot} + O_2^{-\cdot} + H^+ \quad \text{Eq 4}$$

$$AH^- + O_2^{-\cdot} \rightarrow HO_2^- + A^{-\cdot} \quad \text{Eq 5}$$

$$AH^- + O_2^{-\cdot} + H^+ \rightarrow H_2O_2 + A^{-\cdot} \quad \text{Eq 6}$$

$$AH^- + HO_2\cdot\,(\text{perhydroxyl radical}) \rightarrow H_2O_2 + A^{-\cdot} \quad \text{Eq 7}$$

$$AH^- + \cdot OH \rightarrow H_2O + A^{-\cdot} \quad \text{Eq 8}$$

$$AH^- + O_2^{-\cdot} + H^+ \rightarrow H_2O_2 + A^{-\cdot} \quad \text{Eq 9}$$

$$AH^- + H_2O_2 + H^+ \rightarrow 2H_2O + A \quad \text{Eq 10}$$

$$AH^- + R\cdot\,(\text{alkyl radical}) \rightarrow RH + A^{-\cdot} \quad \text{Eq 11}$$

$$AH^- + \alpha\text{-}TO\cdot\,(\alpha\text{-tocopherol radical}) \rightarrow \alpha\text{-}TOH + A^{-\cdot} \quad \text{Eq 12}$$

In addition to its ability to react directly with ROI, AH^- also provides the reducing equivalents for the enzymatic scavenging of H_2O_2 by ascorbate peroxidase, a universal enzyme in plant cells (Asada, 1996). This reaction, which does not occur in humans and other primates, involves the reduction of H_2O_2 and the concomitant oxi-

dation of AH^- to A. The effectiveness of AH^- as an antioxidant is enhanced by regeneration mechanisms that reduce its oxidation products A and $A^{-\cdot}$. The ascorbate anion can be regenerated by spontaneous disproportionation (Eq 13), or by action of NAD(P)H-dependent and GSH-dependent dehydroascorbate reductases (Fig. 1) (Eqs 14–16).

$$2A^{-\cdot} + H^+ \rightarrow (\text{disproportion})AH^- + A \quad \text{Eq 13}$$

$$2A^{-\cdot} + 2GSH \rightarrow GSSG + 2AH^- \quad \text{Eq 14}$$

$$A + 2GSH \rightarrow GSSG + AH^- + H^+ \quad \text{Eq 15}$$

$$2A^{-\cdot} + GSH + NAD(P)H \rightarrow GSSG + AH^- + NAD(P)^+ \quad \text{Eq 16}$$

Conflicting views exist concerning the stimulatory and inhibitory actions of AH^- on cell growth and development. Several studies have demonstrated cytotoxic activity including DNA-damaging effects when AH^- is present with copper or iron, and H_2O_2 (Andorn et al., 1996; Biaglow et al., 1997; Chakrabarti et al., 1996; Drouin et al., 1996; Elzanowska et al., 1995; Nappi and Vass, 1997; Nowak et al., 1991; Ueda et al., 1996). In reactions involving AH^- and where $^{\cdot}OH$ has been implicated as the cytotoxic molecule, radical production typically has been attributed to the Fenton reaction wherein reduced forms of copper or iron react with H_2O_2 (Andersson and Grankvist, 1995; Biaglow et al., 1997; Dizdaroglu et al., 1991; Drouin et al., 1996; Elzanowska et al., 1995; Grinberg et al., 1995; Hermes-Lima et al., 1994; Kaur et al., 1997), or to the production of reactive derivatives of AH^- metabolism (Juven and Kanner, 1986; Murata et al., 1985). Of considerable interest are reports that document the cytotoxic activity of AH^- in metal ion-free cultures (De Laurenzi et al., 1995; Hisanga et al., 1992; Sestili et al., 1996), and those that also show cytotoxicity to be altered significantly by exogenous catalase and/or superoxide dismutase (Arakawa et al., 1994; Aruoma et al., 1987; Makrigiorgos et al., 1995; Miwa et al., 1988; Murata et al., 1986; Nemoto et al., 1996a; Nemoto et al., 1996b; Schmidt et al., 1993; Sestili et al., 1996; Willekens et al., 1997; Woo and Lee, 1995). These observations suggest that the presence of H_2O_2 may be a critical factor in determining whether AH^- exhibits a stimulatory or an inhibitory effect on the growth of certain cells. This proposal is supported by recent studies showing a positive correlation between increased cellular uptake of AH^- and increased levels of catalase activity. Presumably, augmented catalase activity facilitates the degradation of H_2O_2, levels of which likely are elevated in cells exhibiting an enhanced uptake of AH^- (Nemoto et al., 1996a; Nemoto et al., 1996b). Surprisingly, little attention has been given to identifying the mechanism(s) involved in ascorbate-mediated cytotoxicity when redox-active metals are not present to potentiate Fenton chemistry. This study, which was undertaken to clarify the inhibitory mechanism of ascorbate on cell growth by examining reaction products derived from AH^- and H_2O_2, is the first to document by sensitive and specific electrochemical detection methods the production of $^{\cdot}OH$ solely from the interactions of these two molecules.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions of all components were prepared daily in ultrapure reagent-grade water obtained with a Milli-Q system (Millipore, Bedford, MA) and kept at 4°C for a maximum period of 3 hr.

Generation of $^{\cdot}OH$

Two metal-mediated $^{\cdot}OH$-generating systems were used; an Fe^{3+}-EDTA-H_2O_2 complex (Fenton reaction), and a Cu^{2+}-EDTA/ H_2O_2 system (Nappi and Vass, 1997). The metal-mediated

·OH-generating mixtures were prepared by adding equal volumes of aqueous $FeCl_3$ or $CuCl_2$, and Na_2EDTA to give a final concentration of 0.3 mM in a total volume of 100 µl bicarbonate buffer (100 mM NaCl/ 25 mM $NaHCO_3$; pH 7.2) containing 0.3% H_2O_2 and 5 mM salicylate (2-hydroxybenzoic acid). As shown by others, the redox potential of the metal complex used as a catalyst (i.e., Fe^{2+} or Fe^{3+}) does not affect significantly the yield of ·OH (Florence, 1984). For the ascorbate-mediated production of ·OH, reaction mixtures contained 12µM to 0.5 mM AH^- instead of the metal-EDTA component. The concentration of H_2O_2 used was calculated using 43.6 M^{-1} cm^{-1} as the molar extinction coefficient at 240 nm. Except where noted, incubations with varying concentrations of AH^- or metal catalyst were performed at 22°C. To assess the ability of the AH^- /H_2O_2 complex to generate ·OH independent of metal catalysts, each reaction component was incubated separately for varying periods of time only in metal ion free bicarbonate buffer (pH 7.2). For ·OH inhibition experiments, reaction mixtures contained 1 mM mannitol or methylated catechols (Nappi and Vass, 1998b). All incubations were conducted using disposable polypropylene 1.5 ml vials.

HPLC-ED Analyses of ·OH Production

A sensitive and specific salicylate hydroxylation assay was used in conjunction with high performance liquid chromatography with electrochemical detection (HPLC-ED) to monitor ·OH production (Chiueh et al., 1992; Chiueh et al., 1993; Nappi and Vass, 1996; Nappi and Vass, 1997; Nappi and Vass, 1998a; Nappi and Vass, 1998b; Nappi et al., 1999; Nappi et al., 1995). The products of ·OH-mediated salicylate hydroxylation, which include 2,3- and 2,5-dihydroxybenzoic acid (DHBA) and ortho-catechol, were readily detected and measured by HPLC-ED. Chromatographic standards of these products were compared with the three salicylate hydroxylation products formed by the two ·OH-generating systems. Identifications of co-eluting compounds were confirmed by calculating proportional increases in their peak dimensions following the addition of authentic standards to the mixtures assayed by HPLC-ED. In all tests, 5 µl aliquots of the reaction mixture were analyzed by HPLC-ED. Control experiments were conducted by excluding iron, H_2O_2, NO, or AH_2 as was appropriate for different experiments.

The HPLC system consisted of a Gilson (Madison, WI) 119 UV/Vis spectrophotometer and a Bioanalytical Systems (West Lafayette, IN) LC-4B amperometric detector with a glassy carbon working electrode and a Ag/AgCl reference electrode. The working electrode was maintained at an oxidative potential of +650 mV. Instrument sensitivity was maintained at 50 nA. The solvent system was comprised of 50 mM triethylammonium phosphate and 15% acetonitrile. The pH was adjusted to 2.0 with H_3PO_4 prior to the addition of acetonitrile. All separations were made with an Alltech Spherisorb ODS 5 µm reverse phase column using a flow rate of 1.0 ml/min. Known amounts of different catecholamines occasionally were incorporated into reaction mixtures to serve as internal standards. The correlation coefficient of the calibration curves established for the standards typically was greater than 0.98.

Where appropriate, solutions were pre-treated with Chelex 100 chelating ion exchange resin to remove possible metal contaminants. The chelating resin has a high preference for transition metals such as copper and iron. Also, the specific iron chelator desferrioxamine was incorporated into certain incubation mixtures for comparison with non-treated mixtures to exclude the possibility of ·OH production resulting from iron-mediated Fenton chemistry instead of the interactions of AH^- and H_2O_2. Each experiment was replicated at least 3 times, and the data presented represent the means of the determinations specified. Statistical differences between mean values were evaluated where appropriate

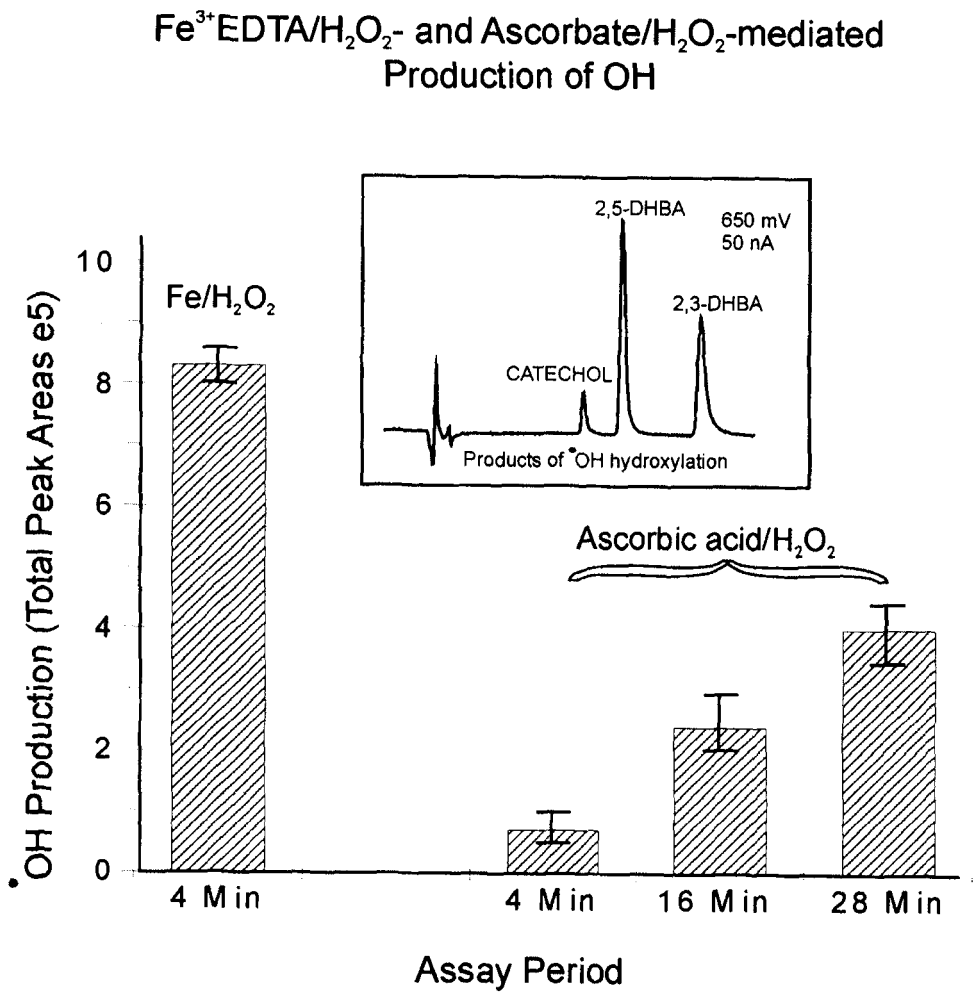

FIGURE 3 Comparison of hydroxyl radical production by the iron-mediated Fenton reaction and by the AH^-/H_2O_2 system. The insert is a representative chromatogram showing the three salicylate hydroxylation products generated by ·OH. Chromatographic conditions were +650 mV, 50 nA full scale, flow rate 1 ml/min

by two-factor analysis of variance (ANOVA), Tukey's test for significant differences, and the Student's *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

In initial investigations, the levels of ·OH produced by the metal-mediated radical generating systems (i.e., Fe^{3+}-EDTA-H_2O_2; Cu^2-EDTA-H_2O_2) were examined and subsequently compared with those formed by the radical generating AH^- mixtures. In each case, quantitative measurements of ·OH produced by these systems were made by integrating the total peak areas detected by HPLC-ED of the three specific products derived from ·OH-mediated hydroxylation of salicylate (Fig. 3). The identity of the salicylate hydroxylation products (catechol, 2,3- and 2,5-DHBA) formed in each reaction was determined by their retention times

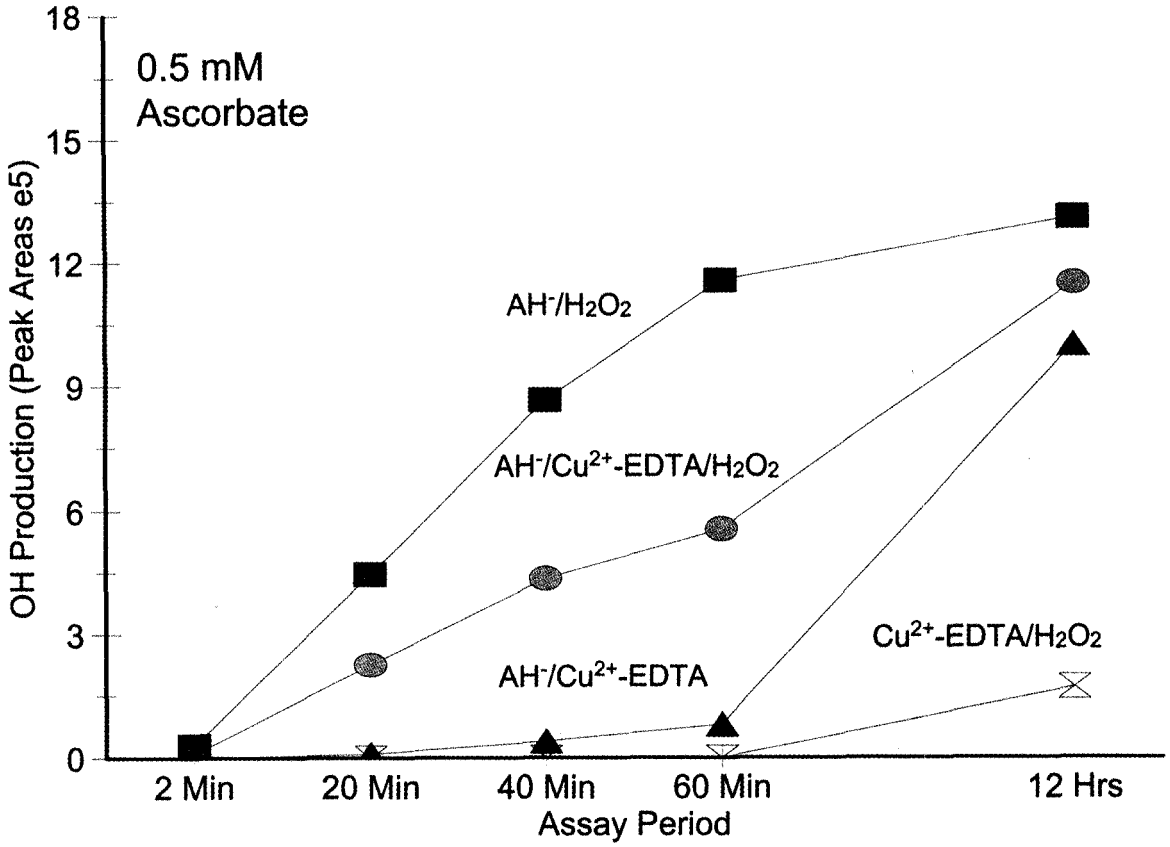

FIGURE 4 Comparison of hydroxyl radical production by various reaction mixtures at different periods of incubation. In the three ascorbate mixtures tested, levels of ·OH were significantly different ($p < 0.01$) at 20, 40, and 60 min post-incubation. Significantly lower levels of ·OH ($p < 0.01$) were produced by the Cu^{2+}-EDTA/H_2O_2 system at all incubation periods tested. Data represent means ($n = 3$)

(9.6, 11.4, and 15.3 min, respectively) and their co-elution with authentic standards in three replicate assays.

Hydroxyl radical production was observed in reaction mixtures containing AH^-/H_2O_2 and AH^-/Cu^{2+}-EDTA/H_2O_2 as early as 2–4 min post-incubation, and considerably later in reaction mixtures containing AH^-/Cu^{2+}-EDTA or Cu^{2+}-EDTA/H_2O_2 (Fig. 4). The amount of ·OH generated by the AH^-/H_2O_2 system was proportional to the duration of incubation (Fig. 3), and increased with increasing concentrations of the reactants, H_2O_2 and AH^- (Figs. 5 and 6). With the concentration of AH^- maintained at 0.5 mM, product formation in 20 min assays was 8.4 times greater with 240 mM H_2O_2 than with 5 mM H_2O_2, and 11.6 times greater at 60 min (Fig. 6). A similar increase in radical production was observed when ascorbate concentrations were increased and the concentration of H_2O_2 maintained at 40 mM (Fig. 6).

Hydroxyl radical production by the AH^-/H_2O_2 system was reduced approximately 40% when mannitol (1 mM) was incorporated into the reaction mixture (not shown). Similar results were obtained with O-methylated cate-

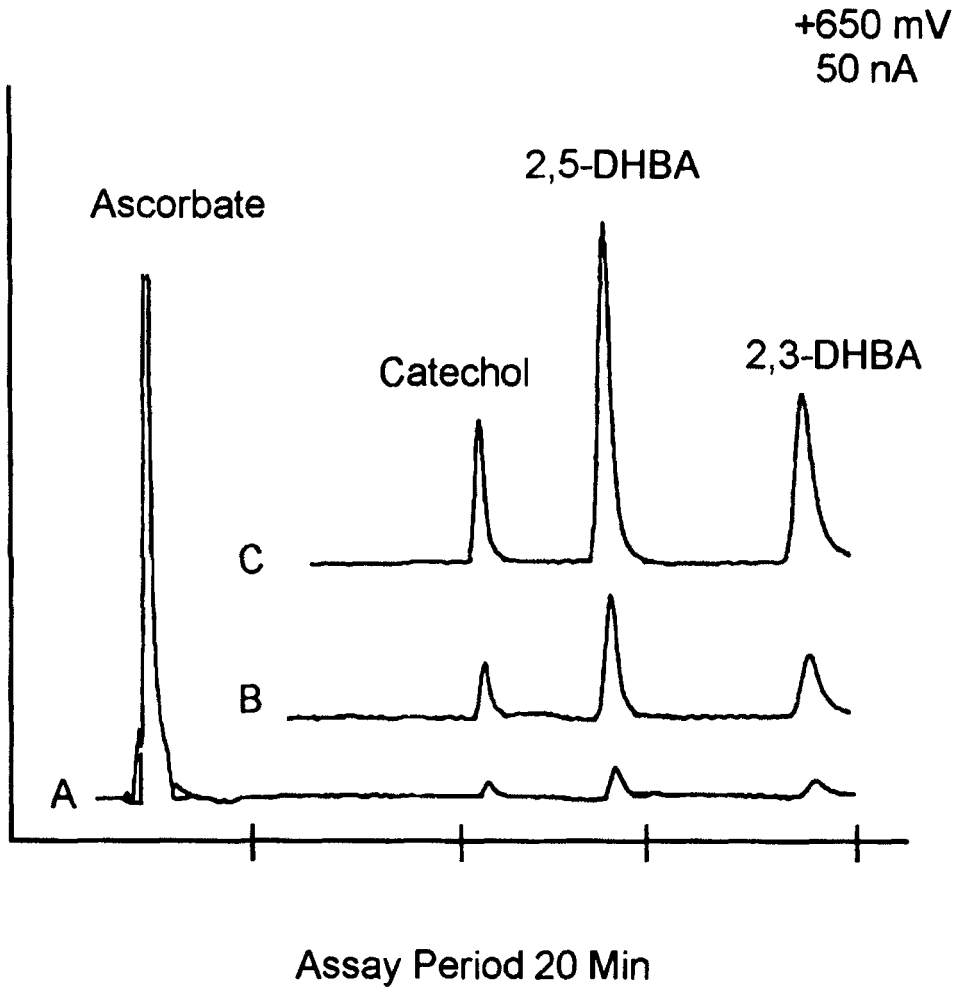

FIGURE 5 Representative chromatograms illustrating the propnt concentrations of the reductant (A = 140 μM; B = 280 μM; C = 430 μM). Chromatographic conditions were +650 mV, 50 nA full scale, flow rate 1 ml/min

chols (Fig. 7), substances previously shown to inhibit iron-mediated ·OH production (Nappi and Vass, 1998b). No ·OH was detected in reaction mixtures containing just bicarbonate buffer and any one of the following components: AH^-, H_2O_2, Cu^{2+}-EDTA, or Fe^{3+}-EDTA, even after 24 hrs incubation. Incubations with desferrioxamine showed no significant differences in ·OH production compared to reaction mixtures lacking the chelate (not presented).

DISCUSSION

Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions (Halliwell and Gutteridge, 1999). Reduced oxygen species, such as H_2O_2, $O_2^{\cdot-}$, and ·OH, inactivate enzymes and damage cellular components causing membrane lipid peroxidation with ensuing production of additional reactive molecules including lipid peroxyl radicals and lipid hydroperoxides. The capacity of the

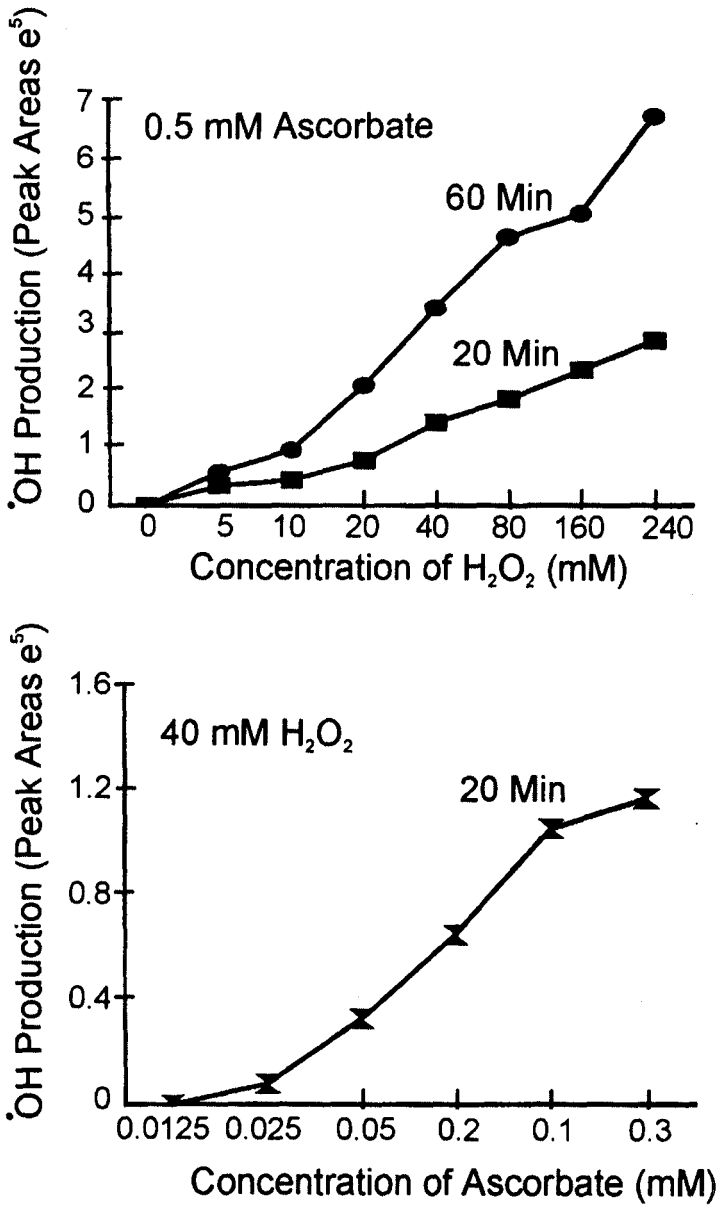

FIGURE 6 Effects of varying the concentrations of H_2O_2 and AH^- on the production of ·OH by the AH^-/H_2O_2 system. Data represent means (n = 3). Chromatographic conditions were +650 mV, 50 nA full scale, flow rate 1 ml/min

cellular antioxidative defense systems to combat the active oxygen species that are produced in excess of what is normally required for signal transduction or metabolism is essential for preventing ROI-mediated oxidative damage.

Biological reductants such as AH^-, GSH, and NADPH have long been known to participate in ·OH production in the presence of H_2O_2 and copper or iron catalysts (Florence, 1984). The present study documents the production of this

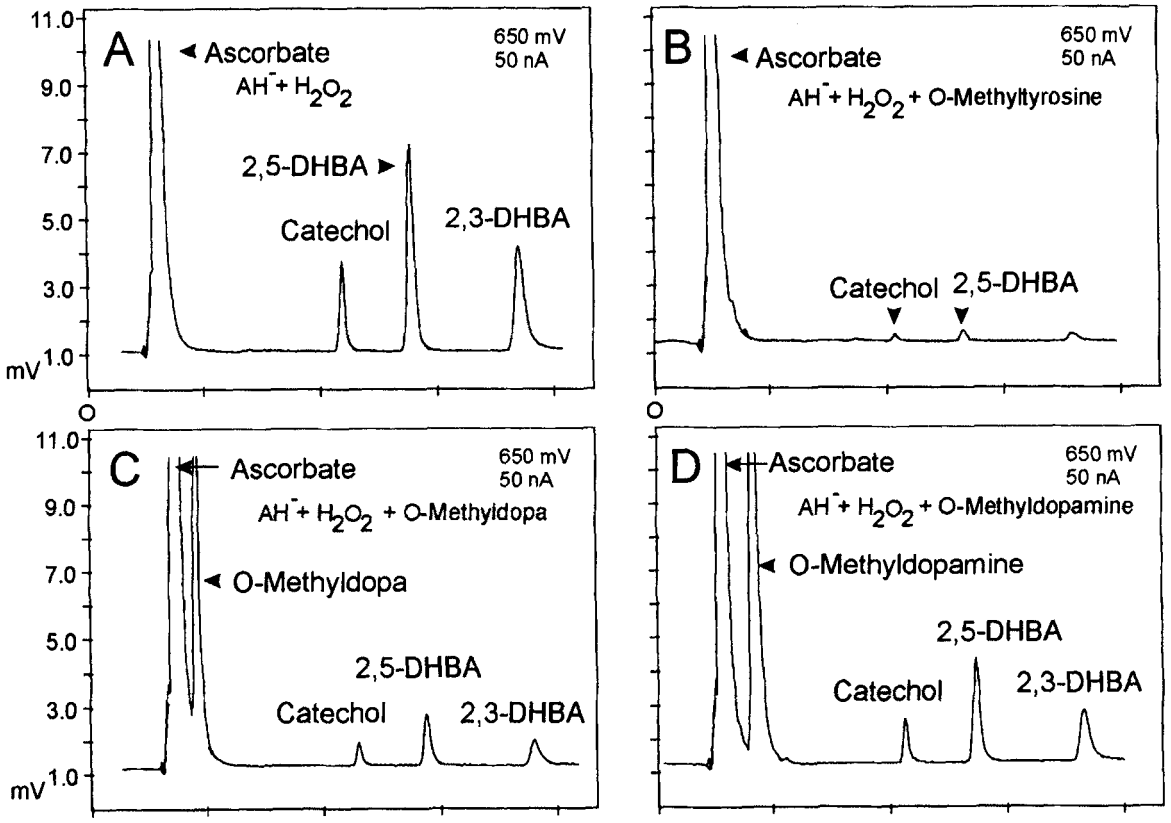

FIGURE 7 Representative chromatograms illustrating the inhibiting effect of O-methylated catechols on ·OH production by the AH^-/H_2O_2 system, a response also observed with Fenton-mediated radical production (Nappi and Vass, 1998b)

cytotoxic radical solely from the oxidation of AH^- by H_2O_2 (Eq 17), independent of mediation by transition metals. Each of the two ascorbate-copper complexes tested (e.g., AH^-/Cu^{2+}-EDTA/H_2O_2 and AH^-/Cu^{2+}-EDTA) also generated ·OH, but they were much less effective in doing so than the AH^-/H_2O_2 system. The mechanism of ascorbate-copper complex catalyzed generation of ·OH involves AH^- reduction of the metal and the formation of $A^{-\cdot}$ (Eq 18), and the ensuing oxidation of the reduced metal by H_2O_2 (Eq 19). Since the concentrations of the reactants used in this investigation were not physiological, the biological relevance of these chemical reactions remains to be determined.

$$AH^- + H_2O_2 \rightarrow A^{-\cdot} + H_2O + {\cdot}OH \quad \text{Eq 17}$$

$$AH^- + Cu^{2+}\text{-EDTA} \rightarrow A^{-\cdot} + Cu^{+}\text{-EDTA} + H^+ \quad \text{Eq 18}$$

$$Cu^{+}\text{-EDTA} + H_2O_2 + H^+ \rightarrow Cu^{2+}\text{-EDTA} + H_2O + {\cdot}OH \quad \text{Eq 19}$$

The lower levels of ·OH seen in metal-mediated reactions (60 min or less in duration) involving AH^- may be due to the fact that the effectiveness of the metal complex Cu^{2+}-EDTA in H_2O_2 oxidations depends, in part, on the competition for the metal ion between the substrate reductant and the catalyst ligand. The reduction of Cu^{2+} by AH^- to Cu^+ not only diminishes the

amount of free AH^- that can be readily oxidized by H_2O_2 to ·OH, but it also leads to the formation of a non-catalytic complex comprised of the reduced metal ion and AH^-. Accordingly, as the H_2O_2-mediated oxidation proceeds, the proportion of metal ions binding to ligand increases, with a concomitant increase in the yield of ·OH. This was considered to be the case in H_2O_2 oxidations catalyzed by a Cu-GSH complex (Florence, 1984).

Since, H_2O_2 can be produced from a variety of sources, including the dismutation of $O_2^{-\cdot}$, leakage from the respiratory chain, phagocytosis, and the metabolism of drugs, H_2O_2 may be considerably more cytotoxic than indicated by its weak in vitro reactivity with most biological molecules (Cohen, 1984). Hydrogen peroxide is an extremely important molecule for cells to manage because it can react readily with $O_2^{-\cdot}$, NO, or transition metals to form ·OH, the most aggressive ROI (Nappi and Vass, 1998a). The capacity of H_2O_2 to generate ·OH solely through redox activity with AH^- inculpates H_2O_2 as a major cytotoxic component, and provides an explanation for frequently observed cytotoxic reactions involving AH^- in cellular environments lacking $O_2^{-\cdot}$ or metal catalysts required for Fenton-type Haber-Weiss reactions. Because of the ubiquitous presence of ascorbate in cellular systems at relatively high concentrations, one-electron reductions by this molecule may represent an important ROI-generating pathway in vivo. In vitro ascorbate has been shown to have various antioxidant properties, protecting various biomolecules against damage by ROI. However, direct evidence that ascorbate functions as an antioxidant in vivo is limited (Halliwell and Gutteridge, 1999). Transition metal-catalyzed oxidations of ascorbate can stimulate lipid peroxidation (Andorn et al., 1996), produce H_2O_2 and ·OH (Aruoma et al., 1991; Biaglow et al., 1997; Chin and Lindsay Robert, 1994; Dizdaroglu et al., 1991; Miura et al., 1994; Tajima et al., 1998), and readily induce additional damage by other free radicals. Similar metal ion-dependant pro-oxidant effects also are manifested by other reducing agents including GSH, NAD(P)H, and α-tocopherol (Halliwell and Gutteridge, 1999). The toxicological significance of the ability of AH^- and other endogenous biological reductants to provide a source of ·OH when oxidized by H_2O_2 is a concern that warrants additional attention.

Finally, it is of interest to note that plants and most animals have the ability to synthesize ascorbate from glucose. Humans, however, lack gulonolactone oxidase, the enzyme that catalyzes the terminal step in ascorbate synthesis. Although the loss of ascorbate synthesizing capacity in humans has been viewed as an inborn error of metabolism (Halliwell and Gutteridge, 1999), there is now a reason to consider this loss a significant evolutionary adaptation, given the fact that the reaction, were it to occur, generates H_2O_2 (Eq 20).

$$\text{L-gulono-}\gamma\text{-lactone} + O_2 \rightarrow \text{L-ascorbate} + H_2O_2 \qquad \text{Eq 20}$$

Acknowledgements

The authors acknowledge support from the National Institutes of Health (GM 59774) and Research Services, Loyola University Chicago.

References

Andersson, M. and Grankvist, K. (1995). Ascorbate-induced free radical toxicity to isolated islet cells. International Journal of Biochemistry & Cell Biology 27, 493–498.

Andorn, A. C., Britton, R. S. and Bacon, B. R. (1996). Ascorbate-stimulated lipid peroxidation in human brain is dependent on iron but not on hydroxyl radical. Journal of Neurochemistry 67, 717–722.

Arakawa, N., Nemoto, S., Suzuki, E. and Otsuka, M. (1994). Role of hydrogen peroxide in the inhibitory effect of ascorbate on cell growth. J Nutr Sci Vitaminol (Tokyo) 40, 219–27.

Aruoma, O.I., Grootveld, M. and Halliwell, B. (1987). The Role of Iron in Ascorbate-Dependent Deoxyribose Degradation Evidence Consistent With a Site-Specific Hydroxyl Radical Generation Caused By Iron Ions Bound to the Deoxyribose Molecule. Journal of Inorganic Biochemistry 29, 289–300.

Aruoma, O. I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. (1991). Copper Ion-Dependent Damage to the Bases in Dna in the Presence of Hydrogen Peroxide. Biochemical Journal 273, 601–604.

Asada, K. (1996). Radical production and scavenging in the chloroplasts. In Photosynthesis and the Environment (ed. N.R. Baker), pp. 123–150. Dordrecht, Kluwer., The Netherlands.

Banhegyi, G., Braun, L., Csala, M., Puskas, F. and Mandl, J. (1997). Ascorbate metabolism and its regulation in animals. Free Radic Biol Med 23, 793–803.

Biaglow, J. E., Manevich, Y., Uckun, F. and Held, K. D. (1997). Quantitation of hydroxyl radicals produced by radiation and copper-linked oxidation of ascorbate by 2-deoxy-D-ribose method. Free Radic Biol Med 22, 1129–38.

Chakrabarti, S., Mahmood, A., Kassis, A. I., Bump, E. A., Jones, A. G. and Makrigiorgos, G. M. (1996). Generation of hydroxyl radicals by nucleohistone-bound metal-adriamycin complexes. Free Radic Res 25, 207–20.

Chin, H.-W. and Lindsay Robert, C. (1994). Ascorbate and transition-metal mediation of methanethiol oxidation to dimethyl disulfide and dimethyl trisulfide. Food Chemistry 49, 387–392.

Chiueh, C. C., Krishna, G., Tulsi, P., Obata, T., Lang, K., Huang, S.-J. and Murphy, D. L. (1992). Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autooxidation in the caudate nucleus: effects of MPP^+. Free Rad. Biol. Med. 13, 581–583.

Chiueh, C. C., Murphy, D. L., Lang, M. K., Tulsi, P. K. and Huang, S.-J. (1993). Hydroxyl free radical (·OH) formation reflected by salicylate hydroxylation and neuromelanin: *In vivo* markers for oxidant injury of nigral neurons. Ann. New York Acad. Sci. 679, 370–375.

Cohen, G. (1984). Oxy-radical toxicity in catecholamine neurons. Neurotoxicology 5, 77–82.

De Laurenzi, V., Melino, G., Savini, I., Annicchiarico-Petruzzelli, M., Finazzi-Agro, A. and Avigliano, L. (1995). Cell death by oxidative stress and ascorbic acid regeneration in human neuroectodermal cell lines. Eur J Cancer 31A, 463–6.

Dizdaroglu, M., Rao, G., Halliwell, B. and Gajewski, E. (1991). Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric Ions. Archives of Biochemistry & Biophysics 285, 317–324.

Drouin, R., Rodriguez, H., Gao, S. W., Gebreyes, Z., TR, O. C., Holmquist, G. P. and Akman, S. A. (1996). Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. Free Radic Biol Med 21, 261–73.

Elzanowska, H., Wolcott, R. G., Hannum, D. M. and Hurst, J. K. (1995). Bactericidal properties of hydrogen peroxide and copper or iron-containing complex ions in relation to leukocyte function. Free Radic Biol Med 18, 437–49.

Florence, T. M. (1984). The Production of Hydroxyl Radical From Hydrogen Peroxide. Journal of Inorganic Biochemistry 22, 221–230.

Grinberg, L. N., Rachmilewitz, E. A., Kitrossky, N. and Chevion, M. (1995). Hydroxyl radical generation in beta-thalassemic red blood cells. Free Radical Biology & Medicine 18, 611–615.

Halliwell, B. and Gutteridge, J. M. C. (1999). Free Radicals in Biology and Medicine, 3 edition. Oxford University Press, Oxford.

Hermes-Lima, M., Wang, E. M., Schulman, H. M., Storey, K. B. and Ponka, P. (1994). Deoxyribose degradation catalyzed by Fe(III)-EDTA: Kinetic aspects and potential usefulness for submicromolar iron measurements. Molecular & Cellular Biochemistry 137, 65–73.

Hisanga, K., Sagar, M.D. and Sharp, F. R. (1992). Ascorbate neurotoxicity in cortical cell cultures. Ann. Neurol. 31, 562–565.

Juven, B. J. and Kanner, J. (1986). Effect of Ascorbic Isoascorbic and Dehydroascorbic Acids On the Growth and Survival of Campylobacter-Jejuni. Journal of Applied Bacteriology 61, 339–346.

Kaur, H., Whiteman, M. and Halliwell, B. (1997). Peroxynitrite-dependent aromatic hydroxylation and nitration of salicylate and phenylalanine. Is hydroxyl radical involved? Free Radic Res 26, 71–82.

Makrigiorgos, G. M., Bump, E., Huang, C., Baranowska-Kortylewicz, J. and Kassis, A. I. (1995). A fluorimetric method for the detection of copper-mediated hydroxyl free radicals in the immediate proximity of DNA. Free Radic Biol Med 18, 669–78.

Miura, T., Muraoka, S. and Ogiso, T. (1994). Effect of ascorbate on adriamycin-Fe-3+-induced lipid peroxidation and DNA damage. Pharmacology & Toxicology 74, 89–94.

Miwa, N., Yamazaki, H., Nagaoka, Y., Kageyama, K., Onoyama, Y., Matsui Yuasa, I., Otani, S. and Morisawa, S. (1988). Altered Production of the Active Oxygen Species Is Involved in Enhanced Cytotoxic Action of Acylated Derivatives of Ascorbate to Tumor Cells. Biochimica et Biophysica Acta 972, 144–151.

Murata, A., Oshima, K., Kubo, T., Yokoo, K., Tanaka, A. and Kato, F. (1985). Mechanism of Synergistic Effect of Ascorbic-Acid and Vitamin K-5 in Inactivation of Phage J-1. Vitamins 59, 203–210.

Murata, A., Suenaga, H., Hideshima, S., Tanaka, Y. and Kato, F. (1986). Hydroxyl Radical As the Reactive Species in the Inactivation of Phages By Ascorbic-Acid. Agricultural & Biological Chemistry 50, 1481–1488.

Nappi, A. J. and Vass, E. (1996). Hydrogen peroxide generation associated with the oxidations of the eumelanin precursors 5,6-dihydroxyindole and 5.6-dihydroxyindole-2-carboxylic acid. Melanoma Research 6, 341–349.

Nappi, A. J. and Vass, E. (1997). Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. Biochim Biophys Acta 1336, 295–302.

Nappi, A. J. and Vass, E. (1998a). Hydroxyl radical formation resulting from the interaction of nitric oxide and hydrogen peroxide. Biochim. Biophys. Acta 1380, 55–63.

Nappi, A. J. and Vass, E. (1998b). Hydroxyl radical formation via iron-mediated Fenton chemistry is inhibited by methylated catechols. Biochim. Biophys. Acta 1425), 159–167.

Nappi, A. J., Vass, E. and Collins, M. A. (1999). Contrasting effects of catecholic and O-methylated tetrahydroisoquinolines on hydroxyl radical production. Biochim. Biophys. Acta. 1434, 64–73.

Nappi, A. J., Vass, E., Prota, G. and Memoli, S. (1995). The effects of hydroxyl radical attack on dopa, dopamine, 6-hydroxydopa, and 6-hydroxydopamine. Pigment Cell Res. 8, 283–293.

Nemoto, S., Otsuka, M. and Arakawa, N. (1996a). Inhibitory effect of ascorbate on cell growth: relation to catalase activity. J Nutr Sci Vitaminol (Tokyo) 42, 77–85.

Nemoto, S., Otsuka, M. and Arakawa, N. (1996b). A positive correlation between catalase activity and ascorbate uptake in the tissues of guinea pigs and cultured cells of mammals. J Nutr Sci Vitaminol (Tokyo) 42, 387–95.

Niki, E., Noguchi, N., Tsuchihashi, H. and Gotoh, N. (1995). Interaction among vitamin C, vitamin E, and beta-carotene. American Journal of Clinical Nutrition 62.

Nowak, D., Piasecka, G., Antczak, A. and Pietras, T. (1991). Effect of Ascorbic Acid On Hydroxyl Radical Generation By Chemical Enzymatic and Cellular Systems Importance For Antioxidant Prevention of Pulmonary Emphysema. Biomedica Biochimica Acta 50, 265–272.

Schmidt, R. J., Chung, L. Y., Andrews, A. M. and Turner, T. D. (1993). Toxicity of L-ascorbic acid to L929 fibroblast cultures: relevance to biocompatibility testing of materials for use in wound management. J Biomed Mater Res 27, 521–30.

Sestili, P., Brandi, G., Brambilla, L., Cattabeni, F. and Cantoni, O. (1996). Hydrogen peroxide mediates the killing of U937 tumor cells elicited by pharmacologically attainable concentrations of ascorbic acid: cell death prevention by extracellular catalase or catalase from cocultured erythrocytes or fibroblasts. J Pharmacol Exp Ther 277, 1719–25.

Tajima, M., Toguchi, M., Kanda, Y., Kunii, S., Hosaka, M., Arakawa, H., Maeda, M., Satoh, K., Asano, K., Kochi, M. and Sakagami, H. (1998). Role of hydrogen peroxide for cell death induction by sodium 5,6-benzylidene-L-ascorbate. Anticancer Research 18, 1697–1702.

Ueda, J.-I., Saito, N., Shimazu, Y. and Ozawa, T. (1996). A comparison of scavenging abilities of antioxidants against hydroxyl radicals. Archives of Biochemistry & Biophysics 333, 377–384.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inze, D. and Van Camp, W. (1997). Catalase is a sink for H2O2 and is indispensable for stress defence in C3 plants. Embo J 16, 4806–16.

Woo, K. I. and Lee, J. (1995). The effects of ascorbic acid on free radical injury in cultured retinal pigment epithelial cells. Korean J Ophthalmol 9, 19–25.