IN VIVO FREE RADICAL FORMATION MONITORED BY THE ASCORBYL RADICAL

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Although oxidative stress is thought to be involved in the pathophysiology of several diseases, little information is available about *in vivo* generation of free radical intermediates. Within this context, we discuss the possibility of monitoring free radical reactions *in vivo* by measuring the levels of endogenous ascorbyl radical, the one-electron oxidation product of vitamin C. Previous studies employing this methodology are briefly summarized. We also present novel results demonstrating that basal levels of ascorbyl radical in human plasma are increased two times upon the addition of 3-morpholinosydnomine N-ethylcarbamide, a sydnonimine capable of generating both nitric oxide and superoxide anion.

Keywords: ascorbyl radical; free radical detection; oxidative stress.

INTRODUCTION

Inherent in oxygen utilization by aerobic organisms is the possibility of damage to biomolecules by free radical species, a phenomenon that has come to be called oxidative stress[1]. Although oxidative stress is thought to be involved in the pathophysiology of several diseases, very little information is available for *in vivo* generation of free radical intermediates. Demonstration of ephemeral free radicals inside a whole organism is a challenging task that has only recently been undertaken and is still being developed.

Since free radicals are usually very reactive species, their half-life in biological systems is extremely short ranging from 10^{-9} s for the hydroxyl radical to 7 s for peroxyl radicals[2]. Consequently, most of the methods used to detect free radicals, even in *in vitro* systems, are indirect, measuring an effect of the radical and not the radical itself (Table 1)[3]. The use of scavengers such as dimethyl sulfoxide and dimethylthiourea for ascertaining hydroxyl radical formation is not unequivocal since dimethyl sulfoxide has several biological effects whereas dimethylthyourea reacts also with hydrogen peroxide, hypochlorous acid, and alkoxyl radicals. Chemiluminescence measurements are also ambiguous since they usually detect excited-end products of free radical-triggered lipid peroxidation. More recent and promising approaches have been the use of scavengers with characterization of the free radical-mediated products by high performance liquid chromatography (Table 1). For instance, the products formed from hydroxyl radical attack on salycilate are known, and their measurement can be used to ascertain hydroxyl radical formation in biological fluids[4]. Particularly important has been the identification and quantitation of products of hydroxyl radical attack upon endogenous scavengers such as DNA bases[5] and proteins[6]. Some of these methods may prove to be useful in clinical situations but more research with experimental models is clearly necessary before standardized methods for measuring oxidative stress in humans are established[3].

Table I. Methods for Free Radical Detection[a]

type	examples
indirect	1. use of exogenous scavengers such as dimethyl sulfoxide, dimethylthiourea,etc
	2. measurement of end-products of lipid peroxidation by chemiluminescence, hydrocarbon exhalation, etc.
indirect but specific	1. use of specific scavengers such as superoxide dismutase, catalase, etc.
	2. use of a scavenger such as salicylate with product characterization by HPLC.
	3. characterization and quantitation of products of free radical reactions with endogenous scavengers.
	4. EPR-spin-trapping
direct	1. EPR

[a]Summarized from references cited in the text.

EPR DETECTION OF THE ASCORBYL RADICAL

Electron paramagnetic resonance (EPR) is the only direct method for detecting free radicals (Table 1) since it is based on the absorption of energy by free radicals in the presence of a magnetic field. Measurement of the absorbed energy in an EPR spectrometer permits the scanning of the characteristic EPR spectrum of the radical (see, for instance, Fig.2). Under physiological conditions, however, most free radicals do not attain steady state concentrations higher than the detection limit of the EPR spectrometer (from 10^{-6} to 10^{-9} M, depending of the radical structure) and consequently their EPR-detection and identification have been performed by spin-trapping[7] or freeze-trapping[8] techniques. In spite of their intrinsic drawbacks, these techniques have been successfully applied in several experimental models, including animals[7], and have contributed to our understanding of several aspects of free radical reactions in biological systems. Although not usually considered as a possibility, the future use of EPR techniques in human medicine cannot be excluded as demonstrated by the pioneering work of Buettner and Chamulitrat in detecting the EPR spectrum of the ascorbyl radical in human synovitis synovial fluid[9].

The ascorbyl radical is the rather stable univalent oxidation product of ascorbic acid (Fig 1), a ubiquitous substance which is required in the diets of humans and several other species that cannot synthesize it from glucose due to the lack of the enzyme L-gulono-γ-lactone oxidase. Many investigators have thought that large doses of ascorbic acid might be beneficial to human health but this proposition is still contro-

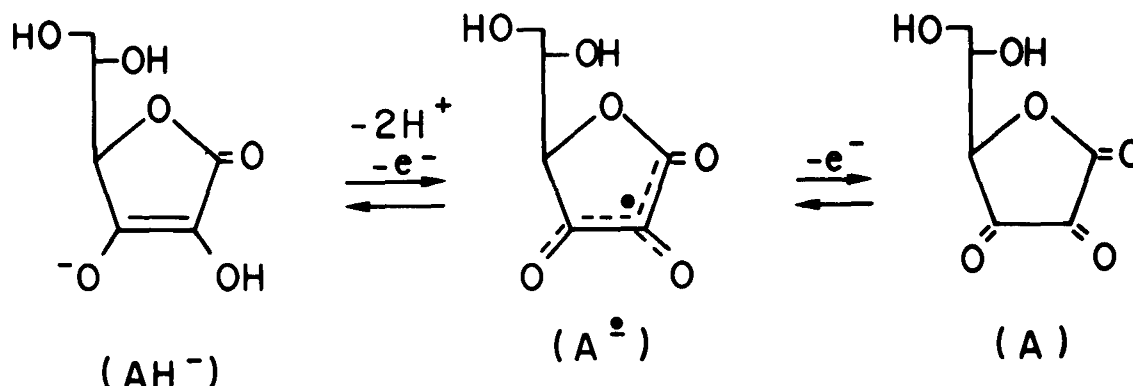

Figure 1. Redox equilibria from ascorbate (AH$^-$) to ascorbyl radical (A$^{\bullet -}$) and dehydroascorbic acid (A).

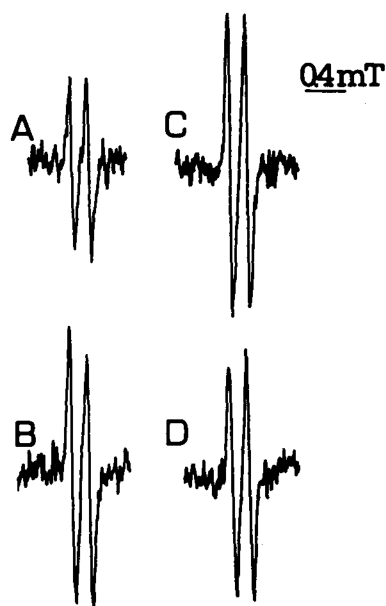

Figure 2. EPR spectra of human plasma endogenous ascorbyl radical obtained before (A) and after (B-D) the addition of 1 mM SIN-1. Blood was carefully collected to avoid hemolysis and all material used was treated as described by Buettner[13] to eliminate transition metal ion contamination. SIN-1 was added to a final concentration of 1mM, the sample was transferred to a flat cell kept at room temperature(about 25°C) and spectra were scanned after 2 min (B), 10 min (C) and 100 min (D). A Bruker ER 200D-SRC was used and the spectra were scanned in the following conditions: microwave power 20 mW; modulation amplitude 0.1 mT, time constant 1 s and scan rate 0.01 mT/s.

versial[10]; there is a general agreement, however, that ascorbic acid acts as an important antioxidant *in vivo*[11].

Ascorbic acid is part of a redox buffer system because of the equilibria that can exist between the reduced species (AH$^-$, ascorbate, pka=4.25), its free radical (A$^{\bullet -}$, ascorbyl) and the fully oxidized species (A, dehydroascorbate) (Fig 1). In reacting with oxidant species such as hydroxyl radical, drug-derived free radicals and transition metal ions, ascorbic acid can form the ascorbyl radical (Fig. 1) which can be easily detected by direct EPR spectroscopy at room temperature (a_H=0.18 mT; see, for instance, Fig. 2). Consequently, it is not surprising that EPR examination of biological fluids such as plasma, urine and bile from experimental animals submitted to several conditions of oxidative stress frequently demonstrates the presence of endogenous ascorbyl radicals[12]. This species was usually considered to be an unavoidable contaminant and only recently did ascorbyl radical levels start to be used to monitor free radical reactions. Such an application was made possible by extensive studies of Buettner on ascorbic acid oxidation[13]. These studies established that the monoanion (AH$^-$), the predominant species at neutral pH, is quite stable in the absence of catalytic transition metal ions, providing the basis of a method for detecting adventitious metal contaminants in buffer solutions[13]. In addition, these studies established the careful procedures for eliminating transition metal ions contamination from the samples and laboratory equipment to be used in ascorbyl radical determinations.

FREE RADICAL REACTIONS MONITORED BY THE ASCORBYL RADICAL

Direct EPR detection of endogenous ascorbyl radicals as a reliable indicator of free radical-mediated injury has been established in different experimental models. For instance, increased levels of endogenous ascorbyl radical have been detected by direct EPR in hairless mouse skin irradiated with 330 nm UV. The signal intensity further increased by topical treatment with chlorpromazine, evidencing that UV radiation induces free radical processes that are exacerbated by the production of free radicals from the drug[13]. The ascorbyl radical has also been detected by direct EPR in the synovial fluid from a patient with synovitis disease; such a detection was considered to be a demonstration of the presence of redox-active iron, which can catalyze oxidative reactions, in synovial inflammation[9]. Increased levels of endogenous ascorbyl radical have also been determined by EPR in human plasma submitted to iron overload[14] and in perfusates of rat hearts submitted to an ischemia/reperfusion sequence[15]. It is also interesting to note that increased production of ascorbyl radical in spruce trees and spinach plants has been associated with oxidative stress leading to forest decline[16].

It would be difficult to determine the levels of ascorbyl radicals in the circulation of experimental animals by direct EPR, but this determination has been achieved by administration of a cytochrome c derivative with a prolonged plasma half life to rats[17]. These studies suggested that a significant amount of ascorbyl radicals might be generated in the circulation of normal animals and that administration of paraquat, a known generator of superoxide radicals, increases the plasma levels of the ascorbyl radical[17]. These results are particularly interesting by suggesting a steady state concentration of ascorbyl radical *in vivo*. Although it has been long known that freshly collected human plasma contains EPR-detectable ascorbyl radical (Fig. 2), it has been difficult to establish if the radical is formed during sample manipulation. Careful

studies, however, are indicating that basal levels of ascorbyl radicals in plasma of rabbits (30 ± 0.7 nM; n=54)[18] or humans (100 ± 30 nM; n= 20)[14] did not vary much. In addition, the ascorbyl radical is stable in plasma for at least 2 h in the absence of transition metal ions and the signal intensity observed in freshly obtained or frozen (at -80^0 °C for at least one month) plasma was the same (unpublished results). By contrast, an increase in the levels of the ascorbyl radical is observed when submitting human plasma to iron overload[14] or to oxidative stress such as that derived from 3-morpholinosydnomine N-ethylcarbamide (SIN-1) decomposition (Fig. 2).

The active metabolite of the vasodilatory drug molsidomine, SIN-1(a gift from Casella AG (Frankfurt, FRG), auto-oxidizes with production of both superoxide anion and nitric oxide at rates of 7.02 uM/min and 3.68 uM/min,respectively, at pH 7.2 and 37°C[19]. Addition of 1mM SIN-1 to human plasma at room temperature led to a fast increase in the concentration of the ascorbyl radical which doubled in about 10 min and slowly decayed (Fig. 2). The maximum increase observed in ascorbyl radical concentration was about two times the basal level (Fig. 2) in plasma from different donors incubated with 1 mM SIN-1, independently of the basal levels of ascorbate and ascorbyl radical; the concentration after 100 min incubation, however, was similar to the basal level of each sample. Although we have not yet studied many human samples, we are obtaining plasma basal levels of ascorbyl radical lower than those previously described for Europeans[14] probably due to different diet habits. The basal levels of ascorbate and ascorbyl measured in the sample employed in the experiment shown in Fig. 2 were 67 μM and 61 nM, respectively, as determined by previously described methods[12c,20]. The important point is that basal ascorbyl radical levels can be determined in the sample from each individual before any manipulation, representing an adequate control for succeeding interventions.

Consequently, ascorbyl radical levels in plasma may be useful for monitoring free radical reactions *in vivo*, particularly in cases where free radical production is discrete, with no extensive damage that could be monitored by less sensitive methodologies[3]. Accordingly, we have been involved in studies demonstrating that increases in blood flow trigger the production of endothelium-derived free radicals in rabbits both *in vivo* and *in vitro*[18]. The spin-trapping technique was used for *in vitro* studies, whereas *in vivo* free radical generation was monitored by plasma levels of the ascorbyl radical[18]. Since ascorbyl radical levels in human plasma drawn from an individual before and after a 1 min forearm ischemia responded to the reactive hyperemia (55% increase), the potential of this methodology for human studies should be explored.

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REFERENCES

1. For recent reviews, see (a) Sies, H. *Angew. Chem. Int. Ed. Engl.*, (1986), **25**, 1058. (b) Floyd, R. A.; *FASEB J.*, (1990), 2587. (c) Halliwell, B., Gutteridge, J. M. C.; *FEBS Lett.*(1992), **307**, 108.
2. Pryor, W. A.; *Ann. Rev. Physiol.*, (1986), **48**, 657.
3. For recent reviews, see (a) Pryor, W. A., Godber, S. S.; *Free Rad. Biol. Med.*, (1991), **10**, 177. (b) Hageman, J. J., Bast, A., Vermeulen, N. P. E.; *Chem.-Biol. Interactions*, (1992), **82**, 243.
4. Grootveld, M.; Halliwell, B.; *Biochem. J.*, (1986), **237**, 499.
5. (a) Dizdaroglu, M.; *Free Rad. Biol. Med.*, (1991), **10**, 225. (b) Shigenaga, M. K.; Ames, B. A.; *Free Rad. Biol. Med.*, (1991), **10**, 211. (c) Park, E-M.; Shigenaga, M. K.; Degan, P.; Korn, T. S.; Kitzeler, J. W.; Weher, C. M.; Kolachana, P.; Ames, B. N.; *Proc. Natl. Acad. Sci.* USA, (1992), **89**, 3375.
6. (a) Stadtman, E. R.; *Free Rad. Biol. Med.*, (1990), **9**, 315. (b) Stadtman, E. R.; *Science*, (1992), **257**, 1220, and references therein.
7. (a) Augusto, O. In *CRC Handbook of Free Radicals and Antioxidants in Biomedicine Vol 3*; Miquel, L., Ed.; CRC Press, Boca Raton (1989); 193. (b) Mottley, C.; Mason, R. P. In *Biological Magnetic Resonance Vol 8;* Berliner, L. J., Reuben, J. Eds; Plenum Press, New York, (1980); 489. (c) Kennedy, C. H.; Maples, K. R.; Mason, R. P.; *Pure & Appl. Chem.*, (1990), **62**, 295.
8. (a) Schaich, K. M.; Borg, D.C. In *Autoxidation in Food and Biological Systems*; Simic, M. G., Karel, M. Eds; Plenum Press, New York (1980); 45. (b) Baker, J. E.; Felix, C. C.; Kalyanaraman, B.; *Proc. Natl. Acad. Sci. USA*, (1988), **85**, 2786.
9. Buettner, G. R.; Chamulitrat, W. U.; *Free Rad. Biol. Med.*, (1990), **8**, 55.
10. Meister, A.; *Biochem. Pharmacol.*, (1992), **44**, 1905.
11. (a) Bendich, A.; Machlin, L. J.; Scandurra, O.; Burton, G. W.; Wainer, D. D. M.; *Adv. Free Rad.Biol. Med.*, (1986), **2**, 419 (b)Halliwell, B. *Free Rad. Res. Comms.*, (1990), **9**,1.
12. For some examples see: (a) Mason, R. P.; Stolze, K.; Morehouse, K. M.; *Br. J. Cancer*, (1987), **55**, Suppl. VII, 163-171. (b) LaCagnin, L. B.; Connor, H. D., Mason, R. P.; Thurman, R. G.; *Mol. Pharmacol.*, (1988), **33**, 351. (c) Knecht, K. T.; Bradford, B. U., Mason, R. P.; Thurman, R. G.; *Mol. Pharmacol.*, (1990), **38**, 26.
13. (a) Buettner, G. R.; *Free Rad. Res. Comms.*, (1986), **1**, 349. (b) Buettner, G. R.; *J. Biochem. Biophys.*, (1988), **16**, 27. (c) Buettner, G. R.; *Free Rad. Res. Comms.*, (1990), **9**, 980.
14. Minetti, M.; Forete, T.; Soriani, M.; Quaresima, V.; Menditto, A.; Ferrari, M.; *Biochem. J.*, (1992), **282**, 459.
15. Pietri, S.; Culcasi, M.; Stella, L.; Cozzone, P. J.; *Eur. J. Biochem.*, (1990), **193**, 845.
16. (a) Stegmann, H. B.; Schuler, P.; Ruff, H. -J. Z. *Naturforsch* , (1989), **45c**, 9. (b) Westphal, S.; Wagner, E.; Knollmuller, M.; Loreth, W.; Schuller, P.; Stegmann, H. B.; *Z. Naturforsch*, (1992), **47c**, 567.
17. Kunitomo, R.; Miyauchi, Y.; Inoue, M.; *J. Biol. Chem.*, (1992), **267**, 8732.
18. Laurindo, F. R. M.; Pedro, M.A.; Barbeiro, H. V.; Pileggi, F.; Carvalho, M. H. C.; Augusto, O.; da Luz, P. L., in preparation.
19. Hogg, N.; Darley-Usmar, V. M.; Wilson, M. T.; Moncada, S.; *Biochem. J.*, (1992), 419.
20. Melhorn, R. J.; *J. Biol. Chem.*, (1991), **266**, 2724.

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