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QUANTITATIVE EFFECTS OF IRON CHELATORS ON HYDROXYL RADICAL PRODUCTION BY THE SUPEROXIDE-DRIVEN FENTON REACTION

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Iron bound to certain chelators is known to promote the conversion of superoxide radicals (O_2^-) to hydroxyl radicals ($HO^\cdot$) by the superoxide-driven Fenton reaction. The production of $HO^\cdot$ by various iron chelates was studied using the reaction of dimethyl sulfoxide and $HO^\cdot$ to produce methane sulphinic acid. Methane sulphinic acid was quantified by use of a simple colorimetric assay and used to determine the amounts of $HO^\cdot$ produced. Superoxide was generated from 200 μM hypoxanthine and 0.05 U/ml xanthine oxidase in the presence of 0–100 μM iron and 100 μM of each chelator. The results of this preliminary investigation illustrate that, at physiological pH, the superoxide-driven Fenton reaction is significantly promoted by iron chelated to EDTA, nitrilotriacetate, and citrate, but is not promoted by the other anions studied.

KEY WORDS: Citrate, deferoxamine, EDTA, nitrilotriacetate, superoxide, xanthine oxidase.

INTRODUCTION

A currently-popular hypothesis explaining the toxicity of superoxide radicals (O_2^-) in biological systems[1–5] is that the O_2^- is converted in the presence of iron to highly toxic hydroxyl radicals ($HO^\cdot$) via the superoxide driven Fenton reaction:

$$O_2^- + Fe^{+++} \rightarrow O_2 + Fe^{++}$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H^+ + Fe^{++} + H_2O_2 \rightarrow HO^\cdot + Fe^{+++} + H_2O.$$

Ferric iron is sparingly soluble at a pH of 7.4 (K_{sp} for $Fe(OH)_3 = 1 \times 10^{-36}$).[6] Therefore, for the above reactions to be biologically significant, the Fe^{+3} must be chelated to remain in solution. Perhaps, when referring to biological systems it is more appropriate to write the above reactions as:

$$O_2^- + A\text{-}Fe^{3-n} \rightarrow O_2 + A\text{-}Fe^{2-n}$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$H^+ + A\text{-}Fe^{2-n} + H_2O_2 \rightarrow HO^\cdot + A\text{-}Fe^{3-n} + H_2O,$$

in which iron is shown complexed to a chelator anion A^{-n}.

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Nomenclature: ADP: adenosine diphosphate; DMSO: dimethyl sulphoxide; EDTA: ethylenediaminetetraacetic acid; NTA: nitrilotriacetate; MSA: methane sulphinic acid.

The recent development of a simple, inexpensive method of measuring $HO^{\cdot}$ in our laboratory[7] has facilitated a re-examination of the effects of various iron chelators on Fenton chemistry. In this method the reaction of $HO^{\cdot}$ radicals with dimethyl sulphoxide (DMSO) to form methane sulphnic acid (MSA),[8-11] is used to determine $HO^{\cdot}$ quantitatively.

$$CH_3SOCH_3 + HO^{\cdot} \rightarrow CH_3SOOH + CH_3^{\cdot}.$$

Dimethyl sulphoxide concentrations of 0.1 to 1.0 M can be used to ensure efficient trapping of nearly 100 percent of $HO^{\cdot}$ radicals generated[12-14] without inhibition of enzymes, such as xanthine oxidase, that generate superoxide. Methane sulphnic acid is detected using a diazonium salt, Fast Blue BB, which forms a yellow complex with the sulphinate anion. This complex can be extracted into an organic solvent and detected spectrophotometrically.

The objective of the present investigation was to study the role of iron in the presence of various chelators on the production of $HO^{\cdot}$ from superoxide.

MATERIALS AND METHODS

Materials were purchased from the following sources: ADP, albumin, hypoxanthine, phytic acid, uric acid, and xanthine oxidase from Sigma Chemical Co. (St. Louis, MO), nitrilotriacetic acid and Fast Blue BB dye from Aldrich Chemicals (Milwaukee, WI), methane sulphinic acid from Fairfield Chemical Company (Blythewood, SC), deferoxamine from Ciba-Geigy, (Summit, NJ), DMSO, EDTA, sodium citrate, Na_2HPO_4 and NaH_2PO_4 from Fisher (Itasca, IL), and $FeCl_3$ from Mallinckrodt (Paris, KY). Absorbances were measured using a Perkin Elmer Lamba 3B spectrophotometer (Norwald, CN).

Superoxide was generated from hypoxanthine and xanthine oxidase in 0.04 M sodium phosphate buffer (pH 7.4), in the presence of 1.0 M DMSO. Stock $FeCl_3$ solutions were prepared in pH 2 HCl; stock solutions of the chelators in the buffer. All solutions were prepared using 5 to 18 MΩ water, pH 7.0, obtained from a Culigan D-45-P reverse osmosis system. Final concentrations in the 2 ml reaction volume were 100 μM chelator (albumin was 0.4 g/ml), 0.2 mM hypoxanthine, 0.05 U/ml xanthine oxidase, and 1.0 M DMSO. Fe^{+3} concentrations varied from 0–100 μM. In addition to the chelator anions being studied, all experimental solutions included 4×10^{-2} M phosphate (present as $H_2PO_4^-$ and HPO_4^{-2}) as well as chloride varying from 1.3×10^{-4} to 7.0×10^{-4}M (since the Fe^{+3} was added as a solution of $FeCl_3$ in HCl). Tubes were prepared by adding, in order, the buffer, DMSO, chelator, $FeCl_3$, and hypoxanthine. The reaction was initiated by adding the xanthine oxidase, mixed for 1 min, and incubated at room temperature for 20 minutes.

The assay for methane sulphinic acid was a modification of a recently published method.[7] The pH of the sample was lowered to 2.5, 100 μl of 30 mM Fast Blue BB dye was added and the solution incubated at room temperature for 10 minutes. The methane sulphinic acid-dye complex was then extracted into 1 ml of a 3:1 mixture of toluene and butanol. The toluene:butanol mixture was washed with 2 ml of butanol saturated water. One hundred microliters of 5% glacial acetic acid in pyridine was added to the organic phase to stabilize the color, and the absorbance of the organic phase at 420 nm determined.

RESULTS

The effects of added iron in the presence of various chelator anions upon $HO^{\cdot}$ generation by the superoxide-driven Fenton reaction are shown in Figure 1. Iron added to solutions of phytate, ADP, urate, and albumin catalyzed only minimal formation of methane sulphinic acid, which was not significntly different from that produced in the presence of phosphate buffer alone. However, methane sulphinic acid generation in the presence of EDTA, NTA, and citrate anions was substantially higher than that in the phosphate buffer alone for all concentrations of iron. Even at zero added iron, the addition of EDTA caused production of methane sulphinic acid, perhaps by chelating trace amounts of Fe^{+3} in the reagents. Methane sulphinic acid production was not detected in the presence of 100 μM deferoxamine. Samples containing all the reagents except xanthine oxidase did not produce detectable amount of methane sulphinic acid (results not shown), verifying that the response required the presence of enzymatically generated O_2^-.

DISCUSSION

In addition to previous reports,[7,14,15] the experiments just described demonstrate the advantages of using DMSO to trap $HO^{\cdot}$ radicals with subsequent colorimetric determination of methane sulphinic acid. Of the previously-available molecular probes for determining $HO^{\cdot}$, the aromatic compounds are the most convenient. When salicylates are used to trap $HO^{\cdot}$, for example, the resulting hydroxylated aromatic derivatives

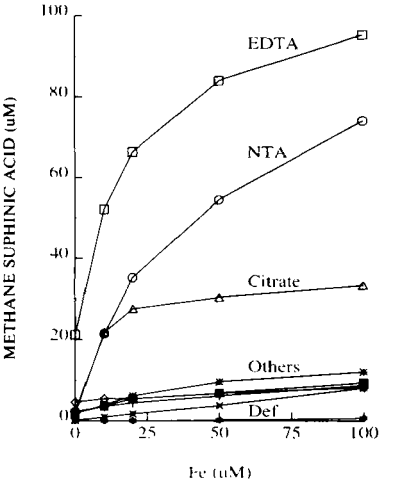

FIGURE 1 Hydroxyl radical production from O_2^- produced from the reaction of xanthine oxidase and hypoxanthine, and varying concentrations of iron, measured as MSA production in 1 M dimethyl sulphoxide, in the presence of 100 μM of various chelators. Data points represent the means of triplicate determinations. Standard deviations for all the experiments were less than 5 μM methane sulphinic acid except when when Fe = 100 μM in the presence of EDTA (SD = 11 μM). Included in "others" are phosphate buffer alone, albumin, ADP, urate, and phytate. NTA = nitrilotriacetate, Def = deferoxamine.

can be detected by colorimetric,[16] gas chromatographic[17,18] or fluorometric[19] methods. DMSO, as a trapping agent for $HO^{\cdot}$, has the advantages of being soluble in both aqueous and organic solvents, of being non-toxic to biological systems,[20–25] and of reacting very rapidly with $HO^{\cdot}$ ($k = 7 \times 10^9$)[26] to yield a single hydroxylated product that has a larger extinction coefficient than that of the hydroxylated aromatic compounds. This method also requires less expensive instrumentation than gas chromatography and fluorescence spectroscopy.

Using this technique in the preliminary studies reported here, we found enhanced production of $HO^{\cdot}$ for iron chelated with EDTA, NTA, and citrate, but low production for iron chelated with ADP-similar to results obtained by Baker and Gebicki.[19] Interestingly, the structures of the three chelators that most efficiently promoted $HO^{\cdot}$ formation (EDTA, NTA, and citrate) all contain oxygen atoms in carboxyl groups that could chelate Fe^{+3}. Chelators that showed little promotion of $HO^{\cdot}$ formation (ADP, phytate, and phosphate buffer) all have phosphate groups available for chelation. Studies by Graf *et al.*[27] have indicated that the best promotors of Fenton chemistry are chelators that have a readily available coordination site, EDTA, NTA, and ADP. No free sites were reported for phytate and deferoxamine. Our studies of the iron chelates of EDTA, NTA, and deferoxamine support their hypothesis; however we observed that ADP was no better than phytate in promoting hydroxyl radical generation. Sibille *et al*[28] have suggested that the reason deferoxamine is so effective in blocking $HO^{\cdot}$ formation is that the strong chelation at all six positions[29] prevents the reduction of Fe^{+3} to Fe^{+2} by O_2^-, and that chelation by tyrosines in lactoferrin and transferrin similary prevents Fe^{+3} reduction, whereas the purple acid phophastases permit the reduction.

In the case of EDTA-iron, the yield of $HO^{\cdot}$ produced by the xanthine oxidase system was substantial and easily measured. However, the promotion of $HO^{\cdot}$ formation by either EDTA or NTA is probably of little biological significance, since neither is found *in vivo*. With the exception of citrate, the $HO^{\cdot}$ generation found with the biologically available chelators tested in this study was approximately one tenth of that with EDTA-iron and was similar to that found with phosphate buffer. This lesser amount of $HO^{\cdot}$ might still be important in a given setting, owing to the extreme reactivity and toxicity of hydroxyl radicals. Normal concentrations of non-protein bound iron in extracellular fluids have been estimated to be less than 5 μM[30] but may be higher in pathological conditions.[31,32] The observation that 20 μM $HO^{\cdot}$ was formed even at 10 μM Fe^{+3} in the presence of citrate is interesting, and suggests that citrate may have physiological significance as a promotor of Fenton chemistry.

Investigation of the iron chelators that may support the superoxide-driven Fenton reaction in complex biological systems deserves much greater attention. Although this reaction is often invoked in theoretical discussions of free radical mediated cellular injury, the importance of the chelator has not been emphasized by many authors.[33–38] If the superoxide driven Fenton reaction is to be confirmed as a pathophysiological mechanism, it is important that a physiologically plausible iron chelator that supports Fenton chemistry be identified.

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References

1. Hamers, M.N. and Roos, D. Oxidative stress in human neutrophilic granulocytes, in *Oxidative Stress*, ed. Helmut Sies, pp. 351–381, Academic Press, London, (1985).
2. Fridovich, I. Superoxide radical: an endogenous toxicant, *Ann. Rev. Pharmacol. Toxicol.*, **23**, 239–257, (1983).
3. Aust, S.D., Morehouse, L.A. and Thomas, C.E., Hypothesis paper – role of metals in oxygen radical reactions, *J. Free Rad. Biol. Med.*, **1**, 3–25, (1985).
4. McCord, J.M. Oxygen-derived free radicals in postischemic tissue injury, *New Engl. J. med.*, **312**, 159–163, (1985).
5. McCord, J.M. and Day, E.D., Superoxide-Dependent Production of Hydroxyl Radical Catalyzed by Iron-EDTA complex, *FEBS Letters*, **86**, 139–142, (1978).
6. Weast, R.C. Ed., *CRC Handbook of Chemistry and Physics, 60th Ed.*, pp. B-220, CRC Press, Inc., Boca Raton, Florida 33431, (1979).
7. Babbs, C.F. and Gale, M.J., Colorimetric asay for methane sulfinic acid in biological samples, *Anal Biochem.*, **163**, 67–73, (1987).
8. Lagercrantz, C and Forshult, S. Trapping of short-lived free radicals as nitroxide radicals detectable by ESR spectroscopy. The radicals formed in the reaction between OH-radicals and some sulphoxides and sulphones, *Acta Chem. Scand.*, **23**, 811–817, (1969).
9. Dixon, W.T., Norman, R.O.C., and Buley, A.L. Electron spin resonance studies of oxidation. Part II. Aliphatic acids and substituted acids, *J. Chem. Soc.*, 3625–3634, (1964).
10. Klein, S.M., Cohen, G. and Cederbaum, A.I., The Interaction of Hydroxyl Radicals with Dimethylsulfoxide Produces Formaldehyde, *FEBS Lettters*, **116**, 220–222, (1980).
11. Klein, S.M., Cohen, G. and Cederbaum, A.I. Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems, *Biochem.*, **20**, 6006–6012, (1981).
12. Repine, R.E., Eaton, J.W., Anders, M.W., Hoidal, J.R., and Fox, R.B. Generations of Hydroxyl Radical by Enzymes, Chemicals, and Human Phagocytes In Vitro, *J. Clin. Invest.*, **64**, 1642–1651, (1979).
13. Yamauchi, N. Kuriyama, H., Watanabe, N., Neda, H., Maeda, M., and Niitsu, Y., Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro, *Cancer Res.*, **49**, 1671–1675, (1989).
14. Babbs, C.F. and Griffin, D. W., Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiological systems, *Free Rad. Biol. Med.*, **6**, 493–503, (1989).
15. Babbs, C.F. and Gale, M.J., Methane sulfinic acid production from DMSO as an indicator of hydroxyl radical production in vivo, in *Free Radicals: Methodology and Concepts*, ed. C Rice-Evans and B Halliwell, pp. 91–121, Richelieu Press, London, (1988).
16. Gutteridge, J.M.C., Richmond, R., and Halliwell, B., Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine, *Biochem. J.*, **184**, 469–472, (1979).
17. Richmond, R. Halliwell, B. Chauhan, J. and Darbre, A. Superoxide dependent formation of hydroxyl radicals: detection of hydroxylation of aromatic compounds, *Anal. Biochem.*, **118**, 328–335, (1981).
18. Halliwell, B. Grootveld, M. and Gutteridge, J.M.C., Methods for the measurement of hydroxyl radicals in biochemical systems: Deoxyribose degradation and aromatic hydroxylation, *Meth. Biochem. Anal.*, **33**, 59–90, (1987).
19. Baker, M.S. and Gebicki, J.M., The effect on pH on yields of hydroxyl radicals produced from superoxide by potential biological chelators, *Arch. Biochem. Biophys.*, **246**, 581–588, (1986).
20. Benville, P.E., Smith, C.E., and Shanks, W.E., Some toxic effects of dimethylsulfoxide in salmon and trout, *Toxicol Appl. Pharmacol.*, **12**, 156–187, (1968).
21. Mason, M.M., Toxicology of DMSO in animals, in *Dimethyl Sulfoxide*, ed. SW Jacob, E.E. Rosen and D.C. Wood, pp. 113–131, Marcel Dekker, Inc., New York, (1971).
22. Maurer, R.R. in *Ciba Foundation Symposium* **52**: *The Freezing of Mammalian Embryos*, p. 116, Elsevier, Amsterdam, (1977).
23. Wilmut, I. and Rowson, L.E.A., Experiments on the low temperature preservation of cow embryos, *Vet. Rec.*, **92**, 686–690, (1973).
24. Willadsen, S.M., Factors affecting the survival of sheep embryos during deep freezing and thawing, in *Ciba Foundation Symposium* **52**: *The Freezing of Mammalian Embryos*, pp. 175–201, Elsevier, Amsterdam, (1977).
25. Ashwood-Smith, M.J., Current concepts concerning radioprotective and cryoprotective properties of dimethyl sulfoxide in cellular systems, *Ann. N.Y. Acad. Sci.*, **243**, 246–256, (1975).

26. Dorfman, L.M. and Adams, G.E., Reactivity of the Hydroxyl Radical in Aqueous Solutions, National Standard Reference Data Series 46 (NSRDS-NBS46), U.S. Government Printing Office, Washington DC, 1973. U.S. National Bureau of Standards
27. Graf, E., Mahoney, J.R., Bryand, R.G., and Eaton, J.W. Iron-catalyzed hydroxyl radical formation, *J. Bio. Chem.*, 3620–3624, (1984).
28. Sibille, J-C, Doi, K., and Aisen, P. Hydroxyl radical formation and iron-binding proteins: stimulation by the purple acid phosphatases, *J. Biol. Chem.*, **262**, 59–62, (1987).
29. Moeschlin S.S. and Schnider, U. Treatment of primary and secondary hemochromatosis and acute iron poisoning with a new, potent iron-eliminating agent (desferrioxamine-B), *N Eng J Med.*, **269**, 57–66, (1963).
30. Halliwell, B. and Gutteridge, J.M.C., Oxygen toxicity, oxygen radicals, transition metals and disease, *Biochem. J.* **219**, 1–14, (1984).
31. Holt, S. Gunderson, M. Joyce, K. Nayini, N.R., Eyster, G.F., Garitano, A.M., Zonia, C., Krause, G.S., Aust, S.D., and White, B.C., Myocardial Tissue Iron Delocalization and Evidence for Lipid Peroxidation After Two Hours of Ischemia, *Ann. Emergency Med.*, **815**, 1155–1159, (1986).
32. Krause, G.S., Joyce, K.M., Nayini, N.R., Zonia, C.L., Garritano, A.M., Hoehner, T.J., Evans, A.T., Indrieri, R.J., Huang, R.R., Aust, S.D., and White, B.C., Cardiac arrest and resuscitation: brain iron delocalization during reperfusion, *Ann Emerg. Med.* **14**, 1037–1043, (1985).
33. Freeman, B.A. and Crapo, J.D. Biology of disease -- free radicals and tissue injury, *Lab. Invest.*, **47**, 412–426, (1982).
34. Bulkley, G.B., The role of oxygen free radicals in human disease processes, *Surgery*, **94**, 407–411, (1983).
35. Southorn, P.A. and Powis, G. Free radicals in medicine I. Chemical nature and biological reactions, *Mayo Clin. Proc.*, **63**, 381–389, (1988).
36. Delmaestro, R.F., An approach to free radicals in medicine and biology, *Acta Physiol. Scand. Suppl.*, **492**, 153–168, (1980).
37. Aust, S.D., Thomas, C.E., Morehouse, L.A., Saito, M. and Bucher, J.R., Active oxygen and toxicity, *Adv. Exp. Med. Biol.*, **197**, 513–526, (1986).
38. Fantone, J.C. and Ward, P.A., Role of oxygen derived free radicals and metabolites in leukocyte dependent inflammatory reactions, *Am. J. path.*, **107**, 397–418, (1982).

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