Free Rad. Res. Comms., Vol. 17, No. 3, pp. 177–185
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A CHELATOR IS REQUIRED FOR MICROSOMAL LIPID PEROXIDATION FOLLOWING REDUCTIVE FERRITIN-IRON MOBILISATION

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(*Received April 1, 1992; in revised form May 19, 1992*)

In the past, antioxidant and chelator studies have implicated a role for iron-dependent oxidative damage in tissues subjected to ischaemia followed by reperfusion. As ferritin is a major source of iron in non-muscular organs and therefore a potential source of the iron required for oxygen radical chemistry, we have determined conditions under which ferritin iron reduction leads to the formation of a pool of iron which is capable of catalysing lipid peroxidation. Under anaerobic conditions and in the presence of rat liver microsomes, flavin mononucleotide (FMN) catalysed the reduction of ferritin iron as shown by both continuous spectrophotometric measurements of tris ferrozine-Fe(II) complex formation and post-reaction Fe(II) determination. The presence of either ferrozine or citrate was not found to alter the time course or extent of ferritin reduction. In contrast, the addition of air to the reactants after a 20 min period of anaerobic reduction resulted in peroxidation of the microsome suspension (as determined with the 2-thiobarbituric acid test) only in the presence of a chelator such as citrate, ADP or nitrilotriacetic acid. These results support the concept that reduced ferritin iron can mediate oxidative damage during reperfusion of previously ischaemic tissues, provided that chelating agents such as citrate or ADP are present.

KEY WORDS: Ferritin iron release, rat liver microsomes, lipid peroxidation, iron chelation, citric acid, flavin mononucleotide.

INTRODUCTION

Measurement of the products of oxidative damage following ischaemia/reperfusion as well as the observed protective effects of antioxidants and iron chelators in some models all indicate that, upon re-oxygenation, there is a burst of oxygen derived free radical (ODFR) production leading to oxidative modification of critical cell components.[1–4] Transition metals catalyse the formation of powerful oxidising agents from oxygen, superoxide and peroxides. Iron is thought to be especially important in biological free radical-mediated oxidations due to its abundance.[5] Work from this laboratory has demonstrated an increase in desferrioxamine-chelatable iron in rabbit kidneys which had been subjected to periods of warm or cold ischaemia.[6,7] This was thought to represent altered intracellular iron homeostasis leading to a subcellular redistribution, the extent and duration of which during subsequent reperfusion was found to correlate inversely with recovery of renal function after autologous transplantation.[7]

The subcellular site(s) of iron mobilisation have not yet been established; however, the iron storage protein ferritin and possibly its degradation product haemosiderin

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are likely candidates in transplantable organs as iron in prosthetic groups would be tightly bound while the endogenous low molecular weight pool of iron is thought to be very small.[8] In cardiac and other muscular tissue, myoglobin, either through H_2O_2-mediated iron release or ferrylmyglobin formation, may provide the iron species which catalyse(s) radical formation.[9,10] Within ferritin, iron is stored as polymeric ferric oxyhydroxide which is released as needed for the synthesis of haem and other proteins requiring iron as a co-factor.[11] The normal physiological mechanisms of ferritin iron release may involve either direct chelation of Fe(III) by cellular agents such as citrate after lysosomal ferritin degradation or reduction to soluble Fe(II) by ascorbate, thiols or other physiological reducing agents [reviewed in ref. 8]. These mechanisms, although kinetically slow, would be operative throughout the range of physiological oxygen tensions and would not be expected to change greatly as a result of the biochemical changes which occur during ischaemia. In contrast, very high rates of reductive iron mobilisation can be achieved in the presence of physiological and xenobiotic semi- and hydroquinones such as those derived from flavin mononucleotide (FMN), flavin adenine dinucleotide, paraquat and the anthracyclines.[12–14] Furthermore, these agents are also generated from their parent quinones in subcellular organelle preparations via NADH and NADPH-dependent processes, but are rapidly oxidised by molecular oxygen thus inhibiting ferritin iron mobilisation under aerobic conditions.[14–16]

In light of the above arguments and the observations that increased levels of chelatable iron occur **during** ischaemia[7] when reductive processes would be active, we have used a previously characterised anaerobic rat liver microsome/NADPH/FMN-driven ferritin iron release system[16] to investigate the potential of reduced ferritin iron as a catalyst of ODFR formation. The use of FMN as a mediator of ferritin reduction is illustrative of the type of iron-release mechanism that would be operative during a period of ischaemia. Levels of suitable redox active compounds *in vivo* have yet to be determined under ischaemic conditions. The generation of ODFR was assessed in this system by measuring thiobarbituric acid reactive products (TBARs) upon aeration of the system. The experiments described here show that although ferritin iron reduction is readily accomplished via an FMN reducing system, lipid peroxidation occurs only in the presence of an iron chelator such as citrate or ADP.

METHODS

Horse spleen ferritin (three times recrystallised, cadmium-free), NADPH, NADP (disodium salt) and glucose-6-phosphate dehydrogenase were obtained from Boehringer Manheim UK (Lewes, East Sussex). Ferritin iron was determined by the method of Drysdale and Munro[17] and found to be 7.2 μmol/mg protein (approx. 3200 iron ions/mol ferritin). A small portion of the ferritin iron (0.02–0.03%) was immediately reducible to ferrozine detectable Fe(II) by ascorbate (5 mM) and likely reflected loosely bound iron which may be an artifact of the commercial ferritin preparation. Subsequent ascorbate-dependent ferritin iron release (0.01–0.02%/min) was linear over a 20 min period, similar to that described by Boyer *et al.*[18] 3-(2-Pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine disodium salt (ferrozine) was purchased from BDH (Poole, Dorset). Glucose-6-phosphate (monosodium, Lot #59F-3901) from Sigma (Poole, Dorset) was used because of

its low redox-active iron content (<20 μmole/mole). Other buffers and reagents were of the highest purity available from BDH Ltd or Sigma.

Rats (200–250 g) were obtained from the Sprague Dawley breeding colony maintained by the MRC Clinical Research Centre, Harrow. Rat liver microsomes were prepared essentially as described previously[19] from a 25% liver homogenate in EDTA/Tris/KCl buffer (1/50/125 mM, pH 7.4). The initial microsomal pellet was washed twice in Tris/KCl buffer (50/125 mM, pH 7.4) and resuspended in Tris/KCl (0.2/150 mM, pH 7.4) at 10–20 mg/ml then kept frozen under liquid nitrogen until use. Protein concentrations of microsomes and ferritin were determined by the method of Lowry[20] using bovine serum albumin as a standard.

Thiobarbituric-reactive substances (TBARs) were determined by the method of Buege and Aust[21] with the addition of 0.02% (w/v) butylated hydroxyanisole to minimize further peroxidation during the heating stage. The absorbance data ($\Delta A_{532}-A_{700}$) were converted to equivalents of malondialdehyde (MDA) using a molar absorptivity of 1.56×10^5. Controls, carried out for each experiment, indicated that none of the reactants, bar NADPH, interfered with the TBA test. The effect of NADPH was limited by maintaining a constant 250 μM concentration by the use of an NADPH-generating system and was, in any event, negligible being approx. 0.3 nmol MDA equivalents/mg microsomal protein under the conditions used. Reactions were carried out at 37°C with rotary mixing in acid-washed glass or new plastic vessels sealed with Suba-Seal septa (Aldrich Chemical Co., Gillingham, Dorset). An anaerobic environment was maintained by constant gassing with nitrogen through a 23 g needle which pierced the septum, a similar needle served as a vent; rapid aeration was accomplished by switching the gas flow to compressed air for 4 min, after which the septum was removed and the mixture exposed to the ambient atmosphere. In addition to other stated components, reactions contained either NADPH (1 mM) or an NADPH-generating system consisting of NADP (250 μM), $MgCl_2$ (10 mM) and glucose-6-phosphate (10 mM) in either Tris/KCl (50/125 mM, pH 7.4) or HEPES/KCl (50/125 mM, pH 7.4). Incubations were initiated by the addition of either NADPH or glucose-6-phosphate dehydrogenase (1.2 units/ml, final concentration).

Spectrophotometric determinations of ferritin iron release in microsomal suspensions were performed using a Uvikon 810P spectrophotometer (Kontron Instruments, Watford, U.K.) either by the continuous monitoring method of Boyer *et al.*[18] or by measuring the amount of ferrozine-chelatable iron after terminating the reactions with either trichloroacetic acid (TCA) or ice cold acetic acid. When the TCA method was used, samples (1 ml) of microsome suspension which had been incubated in the absence of ferrozine were treated anaerobically with 100 mM (15 μl) ferrozine, then mixed 15 s later with 33% TCA (0.5 ml) to quench the reaction. After centrifugation (10 min at 1000 × g) to clarify the solution, $A_{567}-A_{700}$ was determined spectrophotometrically. Reactions which already contained ferrozine received only TCA. In the presence of 22% TCA, the Fe(II)-(ferrozine)$_3$ absorbance maximum shifted to 567 nm with an experimentally determined molar absorptivity of 28500 Au·cm^{-1}. This concentration of TCA caused a gradual loss of absorbance at 567 nm which was less than 5% over 24 h; however, the spectrophotometric readings were all taken within 90 min permitting the use of the above extinction coefficient data to calculate Fe(II) concentrations.

In other experiments, Fe(II) was measured by mixing one volume of sample with four volumes of ice-cold ferrozine/acetic acid/triton X-100 (1.25 mM/7.5 M/0.4%).

The samples were maintained on ice until spectrophotometric measurements were made (within 60 min) and Fe(II) concentrations determined using a molar absorptivity of 27900 $Au \cdot cm^{-1}$ ($A_{562}-A_{700}$ [22]). Under these conditions acetic acid neither prevented the formation of the Fe(II)-(ferrozine)$_3$ complex nor did it alter the spectral properties of the complex in the visible range.

RESULTS AND DISCUSSION

Ferritin Iron Release

Initial studies established that aeration of a rat liver microsome preparation which had been anaerobically incubated with FMN, NADPH and ferritin did not result in increased TBARs formation compared with similarly treated controls which lacked either FMN or the anaerobic step. The results shown in Figure 1, however, demonstrate that this lack of lipid peroxidation was not the result of a failure in ferritin iron reduction. Figure 1A shows the results of a typical ferritin iron release

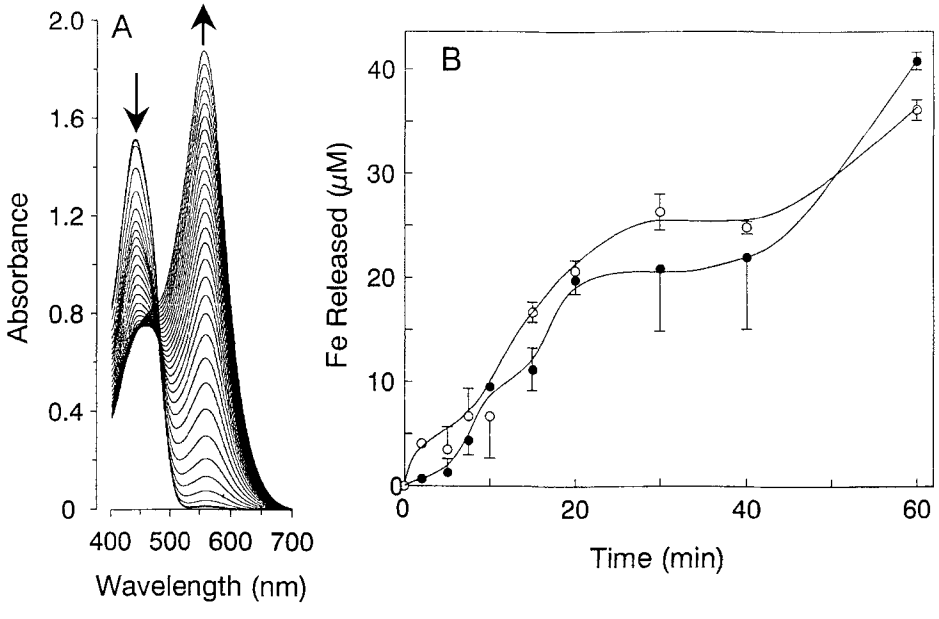

FIGURE 1 **Measurement of ferritin iron reduction.** [A] **Reduction of ferritin and FMN.** Both sample and reference cells contained an anaerobic microsome suspension (0.2 mg/ml in HEPES/KCl buffer) and 200 μM Fe as ferritin, the sample cell additionally contained the NADPH generating system and ferrozine (1 mM). Prior to the addition of FMN (100 μM) and glucose-6-phosphate dehydrogenase (1.7 U/ml) a baseline determination was carried out. Spectra were then recorded over 2 hours at 4 min intervals. [B] **Effect of ferrozine on ferritin iron reduction.** 1 ml anaerobic reaction mixtures contained 0.1 mg microsomal protein, 1 mM NADPH, 100 μM and 200 μM as ferritin in Tris/KCl buffer. Fe(II) concentrations were determined by the TCA method described in the Methods at the indicated time after initiation of the reaction with NADPH. Reactions were carried out in the absence (○) or presence (●) of ferrozine (1.25 mM) in triplicate. Where the SEM was larger than the plotting symbol this is indicated by error bars.

study which employed ferrozine to quantify Fe(II) formation. Note the concomitant decrease in A_{442} and increase in A_{562} as FMN is reduced and the Fe(II)-(ferrozine)$_3$ complex is formed. In order to verify that the presence of ferrozine was not affecting the kinetics of the ferritin iron reduction process, a study was undertaken in which the reaction was carried out in the absence of the chelator and the quantity of Fe(II) formed was then subsequently determined and compared with that generated in the presence of ferrozine. As seen in Figure 1B, similar results were obtained using either procedure indicating that the increase in absorbance due to Fe(II)-(ferrozine)$_3$ complex formation was an accurate measurement of ferritin iron reduction over this timescale. These data show that the lack of pro-oxidant effect in the post-anaerobic incubation period was not the result of a failure in the ferritin reduction process.

Ferritin-Dependent Lipid Peroxidation

The effects of several chelating agents known to permit or potentiate iron-dependent lipid peroxidation were also studied. As shown in Figure 2A, in the presence of NTA, citrate or ADP and following a 20 minute anaerobic incubation, the introduction of air resulted in extensive accumulation of TBA-reactive material. On the other hand if the reaction was performed solely in Tris or phosphate buffer the introduction of air resulted in little or no increase in TBARs, indicating a critical role for a chelator in promoting lipid peroxidation. Figure 2B shows that in addition to chelator (in this case citrate), FMN and NADPH were also absolute requirements for the production of TBARs while exogenous ferritin greatly stimulated the response. The consistent but low level of peroxidation found in the absence of horse spleen ferritin is likely to be due to endogenous ferritin which co-precipitates during the microsome preparation, although a proportion may also be due to a non-haem, non-ferritin mobilisable iron fraction which has been observed by other workers.[23,24]

In order to further clarify the role of chelators in promoting lipid peroxidation following ferritin iron mobilisation, the levels of both Fe(II) and TBARs were followed in the same incubations. The results of this experiment, illustrated in Figure 3, demonstrated that the presence of citrate had no effect on the extent of ferritin iron reduction during the 20 minute anaerobic period. The rate of Fe(II) oxidation upon reaeration was, however, faster in the samples lacking citrate. In contrast, while essentially no accumulation of TBARs occurred in the samples devoid of additional chelator, extensive TBARs generation occurred between 10 and 20 min after aeration of the microsomes containing citrate. This delayed onset of lipid peroxidation is commonly observed in ferrous-dependent systems,[25–27] the length of the delay often being inversely related to the rate of Fe(II) oxidation. The effect of citrate was, therefore, not to potentiate the FMN-dependent reduction of ferritin iron but to render the reduced iron catalytically active.

Citrate may be promoting lipid peroxidation by one of three possible mechanisms: (1) maintaining a pool of redox-cyclable iron, (2) preventing the redeposition of released ferritin iron, or (3) aiding the egress of reduced ferritin iron. Hypothesis (1) is unlikely as Fe(II) in the absence of exogenous chelators promotes microsomal lipid peroxidation,[25] a process which is enhanced by the presence of NADPH or NADPH/FMN.[28] Although citrate may be altering the rate of iron deposition in ferritin, the predominantly L-chain based horse spleen ferritin would not, in any case, be expected to take up Fe(II) at a rate high enough to inhibit lipid peroxidation.[29] On the other hand, ferritin, reduced in the absence of chelators, can contain

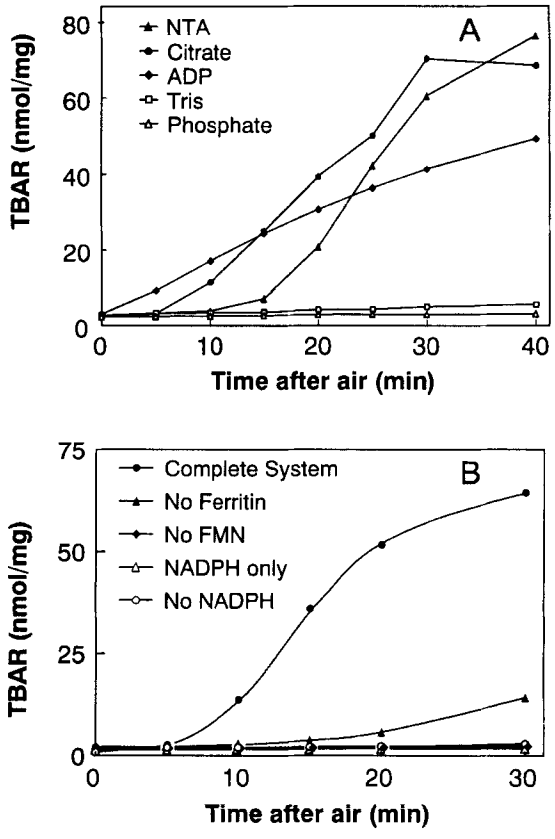

FIGURE 2 **Determinants of ferritin-dependent lipid peroxidation.** [A] **Effect of chelators on lipid peroxidation after ferritin reduction.** Reactions were carried out in tris buffer except when 100 mM potassium phosphate was used (Δ). The incubation mixture contained microsomes (protein concentration of 1 mg/ml), 100 μM FMN, 200 μM Fe as ferritin and, when present, chelator concentrations were 300 μM for citrate and NTA, while ADP was 1 mM. The NADPH generating system was used and reactions were initiated by the addition of 1 U/ml of glucose-6-phosphate dehydrogenase. After 20 min of anaerobic incubation, air was admitted and TBARs formation followed for 40 min as described in the Methods. [B] **Requirements for NADPH, FMN and ferritin.** Reaction conditions were similar to panel [A] with the following exceptions: the buffer was HEPES/KCl, 300 μM citrate was present in all samples and the indicated component was omitted.

considerable amounts of Fe(II).[30] It is therefore plausible to suppose that the primary effect of citrate and the other chelators used in the work presented here is to mobilise Fe(II) from ferritin, forming a catalytically active pool. Aust and co-workers,[31] however, found that a chelator was unnecessary for the ferritin-dependent peroxidation of liposomes when radicals formed by radiolysis were the agents responsible for ferritin reduction. In this case, the membranes were devoid of the integral and surface proteins found in physiological membranes and are perhaps more efficient iron ligands than the microsome preparations used in this study. Monteiro *et al.*[32] have also suggested that chelators are not required for free radical dependent ferritin iron reduction and mobilisation, although they demonstrated this using

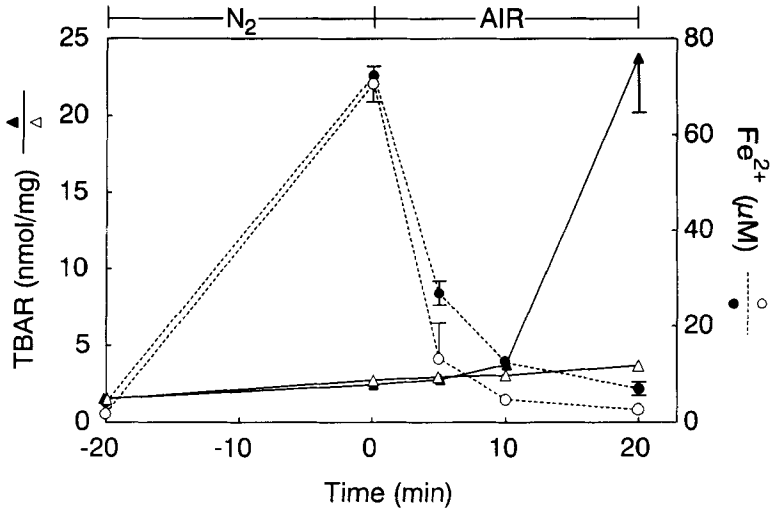

FIGURE 3 **Effect of citrate on ferritin reduction and TBARs formation.** Reaction conditions were as for Figure 2B, the controls (open symbols) lacked citrate. Triplicate reactions were rendered anaerobic until Time = 0 when air was admitted. Error bars indicate the standard deviation when larger than the plotting symbol. Fe(II) was determined by the acetic acid procedure as described in the Methods.

ascorbate to maintain low molecular weight iron in the reduced state, even though ascorbate is itself an iron chelator.

Further studies are in progress to elucidate the role of chelating agents in ferritin-dependent lipid peroxidation and to characterise the nature of FMN-reduced ferritin iron. Although the occurrence of oxygen radical formation as a result of ischaemia/reperfusion and the critical role of iron in catalysing such reactions is not in doubt, the relative importance of this chemistry in the overall pathophysiology of ischaemic damage is not yet known. The source of, and mechanism for the generation of increased levels of (presumably low molecular weight) catalytically active iron are also uncertain. While the intracellular levels of free FMN or other redox-active quinones during ischaemia have yet to be determined, it is known that the rate of ferritin iron reduction is proportional to the FMN concentration down to micromolar levels.[16] Thus, although low levels of endogenous quinones are capable of catalysing ferritin iron reduction under anaerobic conditions, the nature of the physiological chelators and the extent to which their levels are altered under ischaemic conditions will be the factors which determine the degree of ensuing iron-dependent oxidative damage upon reperfusion.

Acknowledgments

The authors wish to thank Dr. G.D. Sweeney (McMaster University) for his valuable discussions. The assistance of Mr. Nick Lane in preparing rat liver microsomes is also gratefully acknowledged. This work was funded by the Medical Research Council (UK).

References

1. J.D. Gower, G. Healing, B.J. Fuller, S. Simpkin and C.J. Green. (1989) Protection against oxidative damage in cold-stored rabbit kidneys by desferrioxamine and indomethacin. *Cryobiology*, **26**, 309–317.
2. A.M. Van der Kraaij, H.G. van Eijk and J.F. Koster. (1989) Prevention of postischaemic cardiac injury by the orally active iron chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and the antioxidant (+)-cyanidanol-3. *Circulation*, **80**, 158–164.
3. S. Marubayashi, J. Dohi, K. Ochi and T. Kawasaki. (1986) Role of free radicals in ischemic rat liver cell injury: Prevention of damage by α-tocopherol administration. *Surgery*, **99**, 184–191.
4. X. Liu, M.R. Prasad, R.M. Engelman, R.M. Jones and D.K. Das. (1990) Role of iron on membrane phospholipid breakdown in ischemic-reperfused rat heart. *American Journal of Physiology*, **259**, H1101–H1107.
5. B. Halliwell and J.M.C. Gutteridge. (1990) Role of free radicals and catalytic metal ions in human disease: An overview. In *Methods in Enzymology* Vol. 186: *Oxygen Radicals in Biological Systems, Part B* (eds. L. Packer and A.N. Glazer), Academic Press, San Diego, pp. 1–85.
6. J. Gower, G. Healing and C. Green. (1989) Measurement by HPLC of desferrioxamine-available iron in rabbit kidneys to assess the effect of ischaemia on the distribution of iron within the total pool. *Free Radical Research Communications*, **5**, 291–299.
7. G. Healing, J.D. Gower, B.J. Fuller and C.J. Green. (1990) Intracellular iron redistribution: An important determinant of reperfusion damage to rabbit kidneys. *Biochemical Pharmacology*, **26**, 1239–1245.
8. R.R. Crichton. (1990) Proteins of iron storage and transport. *Advances in Protein Chemistry*, **40**, 281–363.
9. C. Rice-Evans, G. Okunade and R. Khan. (1989) The suppression of iron release from activated myoglobin by physiological electron donors and by desferrioxamine. *Free Radical Research Communications*, **7**, 45–54.
10. D. Galaris, L. Eddy, A. Arduini, E. Cadenas and P. Hochstein. (1989) Mechanisms of reoxygenation injury in myocardial infarction: Implications of a myoglobin redox cycle. *Biochemical and Biophysical Research Communications*, **160**, 1162–1168.
11. P. Aisen and I. Listowsky. (1980) Iron transport and storage proteins. *Annual Reviews of Biochemistry*, **49**, 357–393.
12. J. Dognin the R.R. Crichton. (1975) Mobilisation of iron from ferritin fractions of defined iron content by biological reductants. *FEBS Letters*, **54**, 234–236.
13. F. Funk, J.-P. Lenders, R.R. Crichton and W. Schneider. (1985) Reductive mobilisation of ferritin iron. *European Journal of Biochemistry*, **152**, 167–172.
14. G.F. Vile and C.C. Winterbourn. (1988) Microsomal reduction of low-molecular-weight Fe^{3+} chelates and ferritin: Enhancement by adriamycin, paraquat, menadione, and anthraquinone 2-sulfonate and inhibition by oxygen. *Archives of Biochemistry and Biophysics*, **267**, 606–613.
15. R. Ulvik and I. Romslo. (1979) Studies on the mobilization of iron from ferritin by isolated rat liver mitochondria. *Biochimica et Biophysica Acta*, **588**, 256–271.
16. B. Rowley and G.D. Sweeney. (1984) Release of ferrous iron from ferritin by liver microsomes: A possible role in the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Canadian Journal of Biochemistry and Cell Biology*, **62**, 1293–1300.
17. J.W. Drysdale and H.N. Munro. (1965) Small-scale isolation of ferritin for the assay of the incorporation of ^{14}C-labelled amino acids. *Biochemical Journal*, **95**, 851–858.
18. R.F. Boyer, T.W. Grabill and R.M. Petrovich. (1988) Reductive release of ferritin iron: A kinetic assay. *Analytical Biochemistry*, **174**, 17–22.
19. J.G. Goddard, D. Basford and G.D. Sweeney. (1986) Lipid peroxidation stimulated by iron nitrilotriacetate. *Biochemical Pharmacology*, **35**, 2381–2387.
20. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
21. J.A. Buege and S.D. Aust. (1978) Microsomal lipid peroxidation. In *Methods in Enzymology* Vol. LII: *Biomembranes* (*Part C: Biological Oxidations*) (eds. S. Fleischer and L. Packer), Academic Press, New York, pp. 302–310.
22. L.L. Stookey. (1970) Ferrozine—A new spectrophotometric reagent for iron. *Analytical Chemistry*, **42**, 779–781.
23. C.E. Thomas and S.D. Aust. (1985) Rat liver microsomal NADPH-dependent release of iron from ferritin and lipid peroxidation. *Journal of Free Radicals in Biology and Medicine*, **1**, 293–300.
24. G. Minotti. (1989) tert-Butyl hydroperoxide-dependent microsomal release of iron and lipid

peroxidation. *Archives of Biochemistry and Biophysics*, **273**, 137–143.
25. J.G. Goddard and G.D. Sweeney. (1987) Delayed ferrous iron-dependent peroxidation of rat liver microsomes. *Archives of Biochemistry and Biophysics*, **259**, 372–381.
26. G. Minotti and S.D. Aust. (1987) An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. *Free Radical Biology and Medicine*, **3**, 379–387.
27. O.I. Aruoma, B. Halliwell, M.J. Laughton, G.J. Quinlan and J.M. Gutteridge. (1989) The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)-iron(III) complex. *Biochemical Journal*, **258**, 617–620.
28. J.G. Goddard. (1989) *In vivo* and *in vitro* studies on the mechanism of iron-dependent lipid peroxidation in liver. Ph.D. Thesis, McMaster University, Hamilton, Ontario, Canada.
29. A. Cozzi, P. Santambrogio, S. Levi and P. Arosio. (1990) Iron detoxifying activity of ferritin. Effects of H and L human apoferritins on lipid peroxidation *in vitro*. *FEBS Letters*, **277**, 119–122.
30. G.D. Watt, R.B. Frankel and G.C. Papaefthymiou. (1985) Reduction of mammalian ferritin. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 3640–3643.
31. D.W. Reif, J. Schubert and S.D. Aust. (1988) Iron release from ferritin and lipid peroxidation by radiolytically generated reducing radicals. *Archives of Biochemistry and Biophysics*, **264**, 238–243.
32. H.P. Monteiro, G.F. Vile and C.C. Winterbourn. (1989) An iron chelator is not required for reductive iron release from ferritin by radical generating systems. *Free Radical Research Communications*, **7**, 33–35.

Accepted by Professor B. Halliwell