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THE CONTROL OF IRON-INDUCED OXIDATIVE DAMAGE IN ISOLATED RAT-LIVER MITOCHONDRIA BY RESPIRATION STATE AND ASCORBATE

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The reaction of iron (II) with H_2O_2 is believed to generate highly reactive species (e.g. $\cdot OH$) capable of initiating biological damage. This study investigates the possibility that the severity of oxidative damage induced by iron in hepatic mitochondria is determined by the level of mitochondrial-H_2O_2 generation, which is believed to be particularly prominent in state-4 respiration.

Iron-induced damage is found to be greater in state-4 than in state-3 respiration. Experiments using uncoupling agents and Ca^{++} to mimic state-3 conditions indicate that this effect reflects differences in the steady-state oxidation-level of the electron carriers of the respiratory chain (and hence the level of H_2O_2-generation), rather than changes in redox potential or transportation of the metal-ion. Evidence is also presented for a mechanism in which Fe(II) and H_2O_2 react inside the mitochondrial matrix.

Ascorbate (vitamin C) is shown to be pro-oxidant in this system, except when present at very high concentration when it becomes antioxidant in nature.

KEY WORDS: Mitochondria, iron toxicity, free radicals, oxidative damage, respiration state, ascorbate.

INTRODUCTION

The toxicity of iron to hepatic mitochondria has been demonstrated in animal models of chronic iron-overload and in isolated mitochondria, and is believed to be mediated by free radicals.[1-3] However, little is known of the precise mechanism by which iron causes such damage to mitochondria, which is believed to include oxidation of lipid, protein and even mtDNA components.[1-4]

The mitochondrion is the subcellular site of the final stages of most biological oxidations, involving the reduction of molecular oxygen to water by four single electron-transfer reactions.[5] During this process, partially-reduced oxygen species remain bound to the cytochrome$_c$ oxidase complex. However, it has been claimed that up to 4% of oxygen consumed by mitochondria escapes from the respiratory chain as a partially-reduced species, the superoxide radical anion ($O_2^{\cdot -}$), to undergo spontaneous or enzymic (via superoxide dismutase) dismutation to hydrogen peroxide [reactions (1) and (2)].[6] Superoxide (and hence hydrogen peroxide) generation is most prominent when electron flux through the transport chain is poor, that is, in state-4 (ADP-limited) respiration.[7] The very presence of the enzymes superoxide dismutase[8] and glutathione peroxidase[9] in the mitochondrial matrix suggests that $O_2^{\cdot -}$ and H_2O_2 generation is an undesirable consequence of using oxygen as the terminal electron acceptor in biological oxidations.

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The $O_2^{\cdot-}$ radical itself is, on the whole, relatively unreactive towards biological molecules; however, by reaction with a redox-active metal-ion (for example, iron or copper) it can lead to generation of the more reactive, and hence damaging, hydroxyl radical ($\cdot OH$) [reactions (3) and (4) (the Fenton reaction), which together constitute the iron-catalysed Haber–Weiss cycle].[10] Therefore, it is likely that $\cdot OH$ is the ultimate damaging agent resulting from errors in mitochondrial oxygen metabolism.

$$O_2 \xrightarrow{+e^-} O_2^{\cdot-} \quad (1)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe(III) + O_2^{\cdot-} \rightarrow Fe(II) + O_2 \quad (3)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^- \quad (4)$$

In mammalian systems the pool of iron which is not incorporated into iron-proteins (for example, haemoglobin) is rendered largely unavailable for participation in free-radical reactions by sequestation in storage or transport proteins (typically ferritin and transferrin).[11] However, to fulfil its biological function iron must be released from such proteins for delivery to the subcellular site required (for example, for incorporation into mitochondrial electron-transport proteins). It is this so-called mobile pool of iron, in which the iron is chelated to low molecular weight ligands (probably ADP, ATP, PP_i, citrate) which is considered responsible for the reduction of H_2O_2 to $\cdot OH$ in biological systems;[12] clearly, there must be a potential hazard in the transportation of iron into mitochondria. In this study we investigate the mechanism of iron toxicity to mitochondria and, in particular, the proposal that in state-4 respiration (in which H_2O_2 production is greatest) mitochondria are more susceptible to damage than in state-3; such conditions may prevail during the re-admission of oxygen to tissues following ischaemia (during which ADP levels would be depleted by general purine catabolism).[13]

Ascorbate (vitamin C) is known to increase profoundly the tissue damage in individuals with iron-overload (for example, thalassaemia patients).[14,15] Ascorbate may act as a pro-oxidant by functioning as the reductant necessary for the mobilisation of iron from ferritin,[16] or by reduction of available iron(III) to iron(II), thus allowing continued reduction of H_2O_2 to $\cdot OH$ [that is, by having the same role as $O_2^{\cdot-}$ in reaction (3)]. On the other hand ascorbate is also believed to function as an antioxidant, one notable role being its ability to repair vitamin E (by hydrogen-atom donation) in biological membranes.[17] The critical role of ascorbate in mitochondrial iron toxicity was also therefore investigated.

MATERIALS AND METHODS

Bovine superoxide dismutase (SOD) and catalase (thymol-free), N-ethylmaleimide (NEM), succinic acid (sodium salt), trizma base, 7-deoxycholic acid, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), bovine serum albumin (BSA) (essentially fatty acid-free) and adenosine 5′-mono-, -di-, and -tri-phosphate were purchased from Sigma Chemical Co. All other chemicals were purchased from Fisons plc.

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Preparation and Incubation of Mitochondria

Male Wistar rats (550–600 g), fed *ad libitum*, were killed by dislocation of the neck and the livers were excised and washed and chopped in ice-cold 0.9% NaCl-2 mM EDTA (diaminoethanetetra-acetic acid, disodium salt), pH 7. Livers were then homogenised using a hand-operated teflon homogeniser in *ca.* 220 ml ice-cold STE buffer (0.3 M sucrose, 5 mM tris-HCl, 2 mM EDTA, pH 7.4). Nuclei and cell debris were removed (20 min. at 600 × g, 2°C) and the mitochondria harvested by centrifugation of the supernatant at 8000 × g for 15 min. Mitochondria were resuspended in *ca.* 25 ml STE buffer and maintained on ice until required. Protein concentration (typically *ca.* 15 mg ml^{-1}) was determined using the Biuret method[18] in the presence of 0.4% 7-deoxycholic acid. Mitochondrial function was checked by determination of the respiratory control ratio;[19] only mitochondria with a respiratory control ratio greater than 4 were used.

Immediately before incubation, mitochondria were pelleted from STE buffer (20,000 × g, 10 min.) and resuspended in KPS buffer [125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4; where indicated (Table 1), the concentration of succinate in this buffer was reduced to 3 mM] to give a concentration of 1 mg protein ml^{-1}.

Enzymes and other reagents were added to the incubation from concentrated stock solutions prepared in KPS buffer with the pH adjusted as necessary. Where indicated, SOD was heat-inactivated by placing in a 90°C water bath for 20 min. (catalase could not be heat-inactivated due to coagulation of the protein upon heating). Reactions with iron were initiated by addition of freshly prepared $FeSO_4 \cdot 7H_2O$ in nitrogen-purged water to the incubation to give a final volume of 5 ml. Incubations were performed in large test-tubes, maintained at 37°C in a shaking water bath.

N–Ethylmaleimide Treatment of Mitochondria

In some experiments mitochondria were pretreated with N-ethylmaleimide to inactivate the $H_2PO_4^-$ translocator. Mitochondria in STE buffer (typically 5 ml, 15 mg protein ml^{-1}), on ice, were preloaded with succinate by addition of 200 μl 200 mM succinate (in KPS buffer, pH 7.4). After 5 minutes excess (*ca.* 30 ml) 10 mM NEM in ST buffer (0.3 M sucrose, 5 mM tris-HCl, pH 7.4) was added. After a further 3 minutes excess (*ca.* 80 ml) 5 mM cysteine (in ST buffer, pH 7.4) was added and the mitochondria centrifuged down (20 000 × g, 10 min.). The mitochondria were then washed once in 5 mM cysteine (ST buffer), once in cysteine-free ST buffer and once in KPS buffer before being resuspended in KPS buffer for incubation.

Determination of TBA-reactive Material

The determination of TBA-reactive material was chosen to assess damage because it is relatively non-specific, and will therefore give a gross indication of damage to lipid, protein, carbohydrate and mtDNA.[20]

Following incubation of mitochondria, a 1 ml sample was removed and added to 1 ml TBA reagent (0.125 MHCl, 1 M trichloroacetic acid, 30 mM TBA). Following heating at 95°C for 15 min. the suspension was centrifuged (8 min, 13000 rpm in the MSE microcentrifuge) and the $A_{535-600nm}$ measured from the supernatant versus a blank (1 ml KPS buffer plus 1 ml TBA reagent, heated at 95°C for 15 min.).

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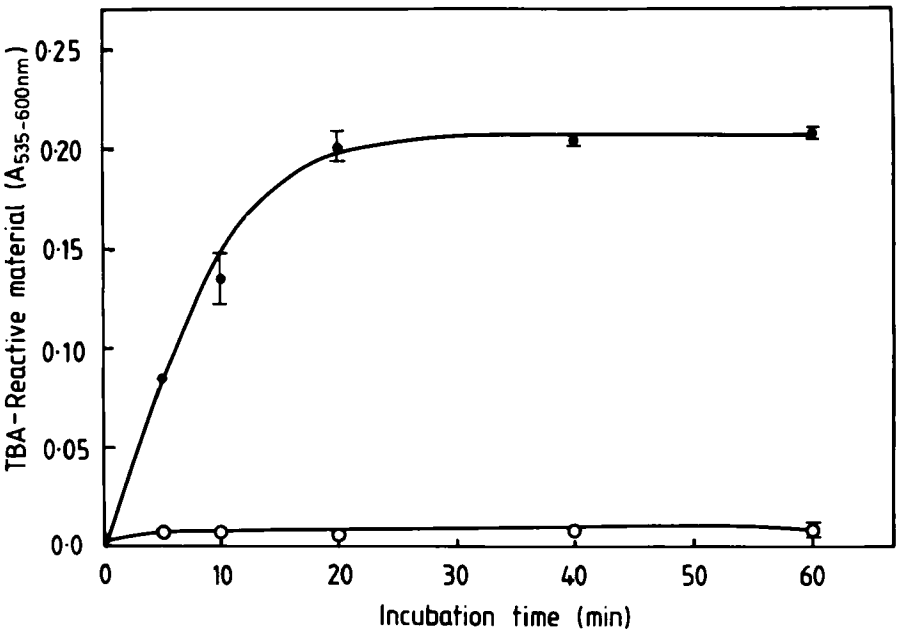

FIGURE 1 Time-dependence of the formation of TBA-reactive material. Mitochondria were incubated (1 mg protein ml^{-1}) in state-4 (125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) in the presence of 25 μM Fe(II) for the times indicated (●). No iron was added to the control incubation (○). Error bars represent ± 1 S.D. (n = 2).

RESULTS

Figure 1 shows a typical time-course of iron-induced formation of TBA-reactive material in state-4 mitochondria; the dose-dependence of this response is demonstrated in Figure 2. Addition of ADP or AMP (which is phosphorylated to ADP by endogenous adenylate kinase), to give state-3 conditions, reduced the level of iron-induced damage compared with the state-4 control, whereas addition of ATP, to inhibit phosphorylation and oxidation, resulted in an increase in iron-induced damage (Table I). The base-level of TBA-reactivity in the absence of added iron is believed to reflect the relatively high levels of iron in phosphate buffers, which may induce the formation of TBA-reactive material or decomposition of endogenous peroxides during the heating stage of the assay (see also Figures 1 and 2).

Although the various adenine nucleotides are expected to change the level of mitochondrial-H_2O_2 generation (via a change in respiration state), and hence the toxicity of iron, they will also chelate the iron, causing relative changes not only in its rate of uptake into the mitochondrial matrix, but also the redox potential (E) for the Fe(III)/Fe(II) couple [for example, the ability of iron(II)-adenine nucleotide complexes to decompose H_2O_2 is in the order ATP > ADP > AMP[21]]. For these reasons, the observed effects of the adenine nucleotides on iron-induced damage cannot necessarily be attributed solely to their effects on H_2O_2-generation.

To increase electron-flux through the respiratory chain (that is, mimic state-3 respiration), *without* a corresponding change in redox potential of the metal-ion, the

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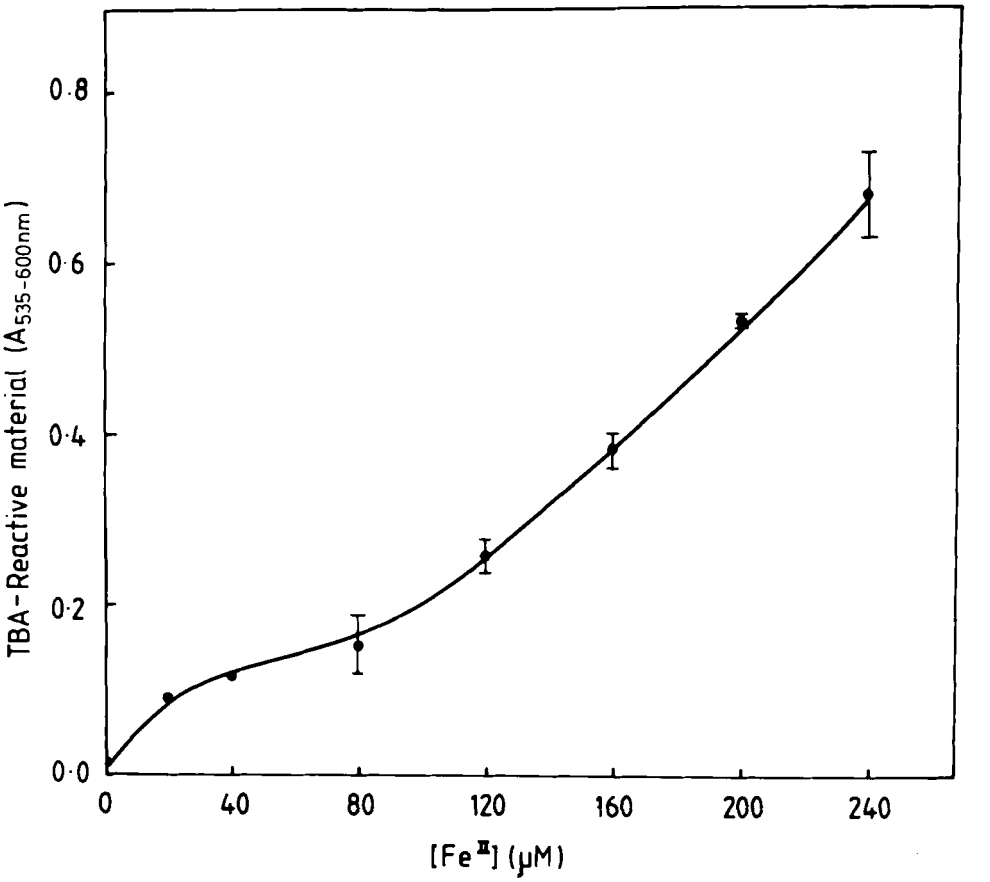

FIGURE 2 The effect of iron concentration on mitochondrial damage. Mitochondria were incubated (1 mg protein ml^{-1}) in state-4 (125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) for 40 min. in the presence of Fe(II) at the concentrations indicated. Error bars represent ± 1 S.D. (n = 3).

uncoupling agents CCCP and 2,4-DNP (2,4-dinitrophenol) were employed. Treatment of mitochondria with these agents leads to a reduction in the damage induced by iron (Table I). CCCP and 2,4-DNP uncouple mitochondria by collapsing ΔpH; since transport of inorganic phosphate into the matrix is driven by ΔpH, these agents may offer protection by inhibiting the associated uptake of iron (as the permeable counter-ion)[22], rather than by reducing H_2O_2-generation via uncoupling. Therefore, in an attempt to establish unambiguously that iron-induced damage to mitochondria is related to the level of H_2O_2-generation (via respiration state), an agent was sought which would induce changes in electron-flux through the respiratory chain without changing the redox potential of iron or its rate of uptake into the matrix.

Mitochondria rapidly sequester free calcium ions by an energy-requiring mechanism driven by the electrical, $\Delta\psi$, (and not ΔpH) component of Δp.[23] Therefore, calcium is not a true uncoupler, but does induce a state-3 condition in the electron-transport proteins, with concomitant oxygen uptake and proton translocation. Fur-

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TABLE I
Effect of adenine nucleotides and uncoupling agents on the iron-induced formation of TBA-reactive material in mitochondria

Addition to[a] incubation	TBA-Reactive Material[b] $A_{535-600nm}$
None (state-4 control)	0.247 ± 0.003
1 mM AMP	0.133 ± 0.003
1 mM ADP	0.194 ± 0.013
1 mM ATP	0.256 ± 0.003
20 μM CCCP	0.119 ± 0.012
20 μM 2,4-DNP	0.079 ± 0.038
Blank (no added iron)	0.006 ± 0.001

[a]Mitochondria were incubated (125 mM KC l, 10 mM $KHPO_4$, 3 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) for 30 min. with 25 μM Fe(II), with the additions indicated. No iron was added to the blank incubation.
[b]Errors represent ± 1 S.D. (n = 3)

thermore, addition of excess Ca^{++} leads to "cycling" of Ca^{++} ions across the inner mitochondrial membrane due to the operation of distinct uptake and release mechanisms, establishing a new steady-state oxidation level in the electron-transport chain components.[24,25] Figure 3 shows that the iron-induced formation of TBA-reactive material in mitochondria is indeed inhibited by prior addition of Ca^{++}, which is attributed to a change in respiration rather than prevention of Fe(II) uptake.

In order to establish that the damage induced by iron is dependent on mitochondrial $O_2^{\cdot-}$ and H_2O_2, protection by SOD and catalase must be demonstrated. However, protection by catalase was poor (28% inhibition), and with SOD a higher level of TBA-reactive material generation was observed (which may be attributed to the protein being a substrate for the formation of TBA-reactive material, as confirmed by the similar behaviour of BSA) [see Figure 4(a)]. We therefore investigated the possibility that the apparent low activity of catalase and inactivity of SOD reflects their failure to gain access into the mitochondrial matrix, which may be the site at which the Haber–Weiss cycle operates. Our approach was to encourage the reactions which we suspect to occur *inside* the matrix of iron-challenged mitochondria (that is, the Haber–Weiss cycle), to take place *outside* the matrix where they should be subject to the effects of exogeneous SOD and catalase. To do this iron must be maintained outside the matrix, ready to react with any $O_2^{\cdot-}$ or H_2O_2 which escapes the endogenous mitochondrial SOD or peroxidases and leaks out to the external medium. Chelating agents (such as EDTA) should not be used to retain the metal-ion outside the matrix (because of changes in redox potential), so we attempted to inhibit the mechanism responsible for transport of iron into the mitochondrial matrix. Since in this system iron is probably transported as the permeable counter-ion accompanying inorganic phosphate uptake,[22] the inorganic phosphate translocator was inhibited with NEM.

Following NEM treatment catalase was found to offer considerable protection against iron, whereas SOD failed to protect [see Figure 4(b)]. BSA (and heat-inactivated SOD) also failed to offer protection, suggesting an enzymic function for catalase, rather than scavenging of $\cdot OH$. Protection of NEM-treated mitochondria by catalase but not SOD suggests that dismutation of $O_2^{\cdot-}$ occurs largely inside the matrix followed by leakage of H_2O_2 into the external medium. This provides direct evidence for the H_2O_2-dependence of damage when iron is not admitted into the

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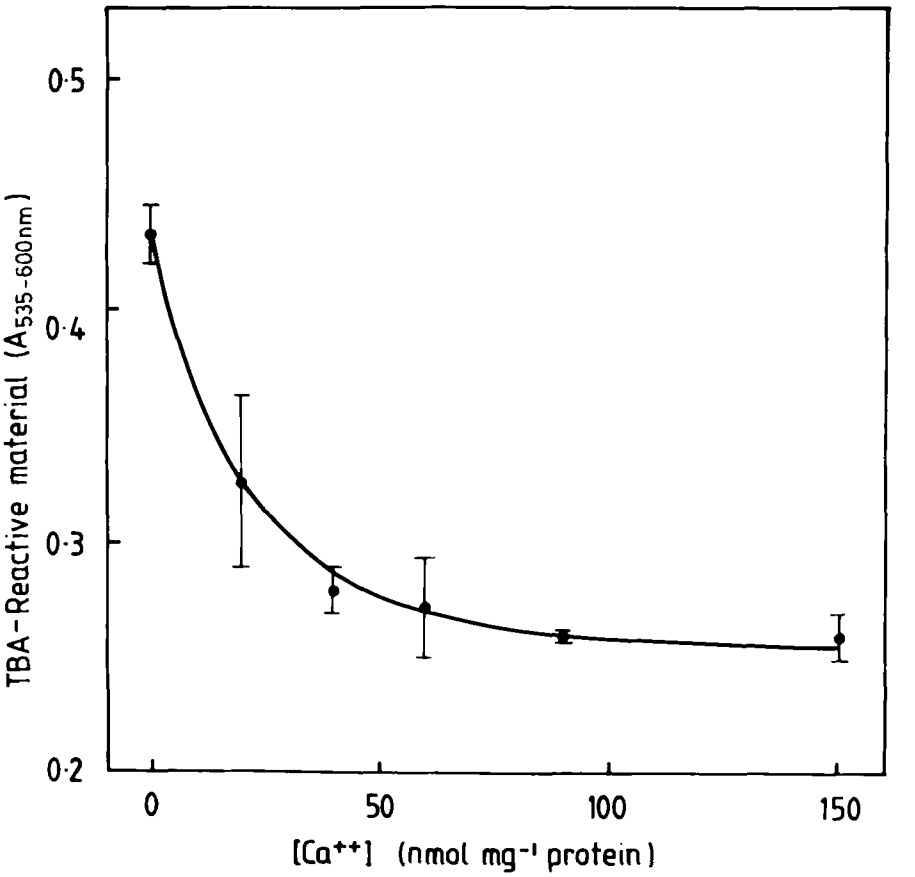

FIGURE 3 The effect of Ca^{++} on iron-induced mitochondrial damage. Mitochondria (1 mg protein ml^{-1}) were incubated (in 125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) for 45 min. with 50 μM Fe(II); Ca^{++} (as $CaCl_2$) was added, before the addition of iron, at the concentrations indicated. Error bars represent $\pm$ 1 S.D. (n = 3).

matrix, and indirect evidence for H_2O_2-dependence when iron is admitted into the matrix of non-NEM treated mitochondria. Also evident from Figure 4 is that the level of damage observed is always lower in NEM-treated mitochondria than in the non-treated controls. This probably reflects the failure of all the H_2O_2 generated in the matrix to escape to the external medium. To confirm that the observed increase in protection by catalase following NEM-treatment of mitochondria is indeed caused by NEM, and not due to some other step in the treatment procedure (for example, washing with cysteine), a control experiment was carried out in which the procedure was followed (see Materials and Methods), but with the omission of NEM.

Following this control-treatment, catalase offered 26% protection from iron, which is comparable to that observed in non-treated mitochondria (28%) (see Table II). These results also show that the control-treatment of mitochondria leads to an overall reduction in damage induced by iron when compared to the non-treated control. NEM-treated and control-treated mitochondria are expected to be internally loaded with cysteine (external cysteine being removed by washing); thiol agents (for example,

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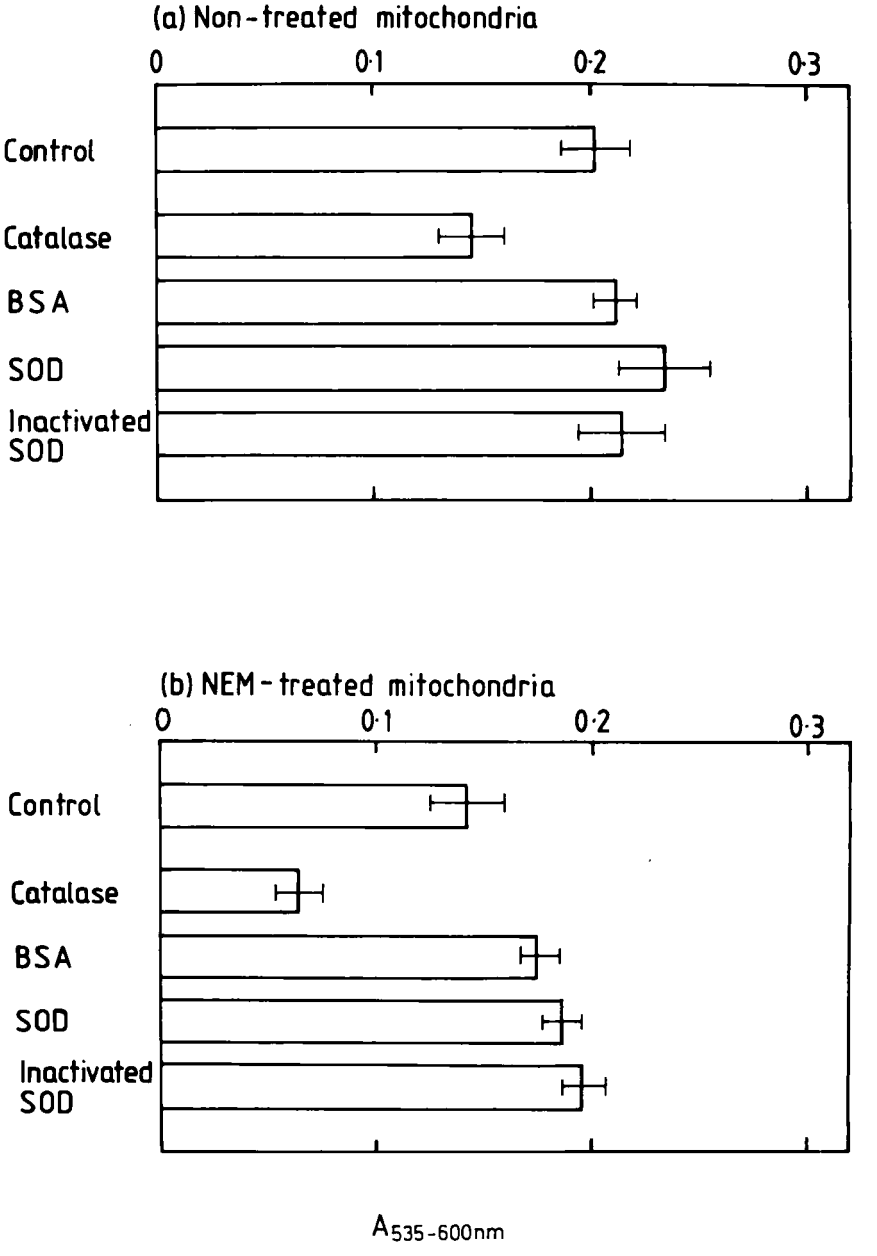

FIGURE 4 The effects of SOD and catalase on iron-induced damage in (a) non-treated and (b) NEM-treated mitochondria (see Materials and Methods). Mitochondria (1 mg protein ml^{-1}) were incubated in state-4 (125 mM KC 1, 10mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) for 40 min. with 25 μM Fe(II). Additions were made as follows: catalase, 2800 units ml^{-1}; BSA, 200 μg ml^{-1}; SOD, 600 units ml^{-1}; heat-inactivated SOD, 200 μg ml^{-1}. No additions were made to the control incubation. Error bars represent $\pm$ 1 S.D. (n = 3).

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TABLE II
Iron-induced damage to non-, control-, and NEM-treated mitochondria: determination of level of damage and protection offered by catalase

Mitochondria[a] used	TBA-Reactive Material[b] $A_{535-600}$		% Inhibition by catalase
	− catalase	+ catalase	
Non-treated	0.202 ± 0.016	0.145 ± 0.015	28
Control-treated	0.091 ± 0.007	0.067 ± 0.013	26
NEM-treated	0.142 ± 0.007	0.064 ± 0.011	55

[a]Mitochondria were incubated (125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 $MgCl_2$, 30 nM rotenone, pH 7.4) for 40 min. with 25 μM Fe(II), in the presence and absence of catalase (2800 units mg^{-1} protein).
[b]Errors represent ± 1 S.D. (n = 3)

cysteine) are known to protect biomolecules from the effects of free radicals,[26] which may account for the low level of damage observed in control-treated mitochondria in which damage is initiated inside the matrix. Cysteine-loading offers less protection to NEM-treated mitochondria because damage is initiated largely *outside* the matrix.

Ascorbate was found to increase the level of iron-induced damage in state-4 mitochondria, particularly at low concentration (Figure 5). Only at relatively high concentration (2 mM) did ascorbate protect significantly.

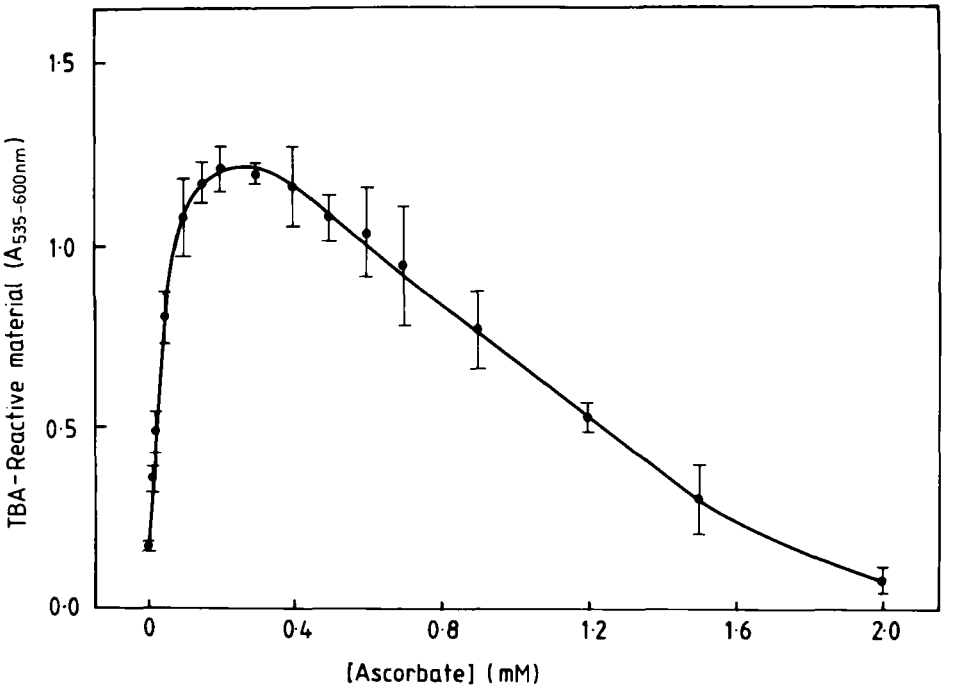

FIGURE 5 The effect of ascorbate on mitochondrial damage. Mitochondria (1 mg protein ml^{-1}) were incubated in state-4 (125 mM KCl, 10 mM $KHPO_4$, 5 mM succinate, 1 mM $MgCl_2$, 30 nM rotenone, pH 7.4) for 60 min. with 25 μM Fe(II). Ascorbate was added as indicated. Error bars represent ± 1 S.D. (n = 3).

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DISCUSSION AND CONCLUSIONS

We have demonstrated that iron induces dose-dependent oxidative damage to isolated rat liver mitochondria. Damage is particularly prominent under conditions of state–4, rather than state–3, respiration. In the living cell, several factors (which may be expected to accompany a shift in respiration state) may account for this apparent difference, for example, a change in the metal-ion redox potential, the metal ion's rate of uptake into the matrix and the steady-state oxidation level of the electron transport system. By isolating the effects of such components, we have shown that the relative levels of iron-induced damage in state–3 and state–4 mitochondria can be explained by a mechanism involving, at least in part, changes in the steady-state oxidation level of the respiratory chain. On the basis of the known dependence of mitochondrial H_2O_2 generation on the oxidation level of the respiratory chain components (see introduction), it is proposed that H_2O_2 is the link between iron-induced oxidative stress and respiration state in isolated rat liver mitochondria. This strongly implies that $\cdot OH$ is the species responsible for the initiation of damage (though the reaction of high-valent iron-oxygen complexes, for example FeO^{2+}, cannot be ruled out). Whatever the identity of the initial oxidant, the biological implication remains the same, namely that iron interacts with mitochondrial-H_2O_2 resulting in biological damage.

The observation that damage is initiated largely inside the mitochondrial matrix (and therefore not subject to the effects of exogeneous SOD and catalase)is possibly a reflection that, at least in the system studied, iron uptake into the matrix is more extensive than H_2O_2 leakage from the matrix. Intra-mitochondrial H_2O_2 is believed to be removed by the selenium-containing glutathione peroxidase of the matrix. However, the demonstration of protection against the effects of iron in NEM-treated mitochondria is evidence for H_2O_2 leakage from mitochondria (and hence escape from the matrix enzyme), and this is consistent with several other studies demonstrating H_2O_2-leakage from intact mitochondria.[7,27,28] Although it has been proposed that 20% of mitochondrial $O_2^{\cdot-}$ escapes from the matrix before dismutation,[29] there is as yet no obvious mechanism for this, and our observation of the failure of exogenous SOD to protect NEM-treated mitochondria from iron fails to support this proposal; this suggests that dismutation occurs inside the matrix followed by H_2O_2-leakage.

The results clearly demonstrate pro-oxidant behaviour of ascorbate, which is probably best explained by a mechanism involving its reduction of Fe(III) to Fe(II) in an ascorbate-driven Fenton reaction [reactions (5) and (6)].

$$AscH^- + Fe(III) \rightarrow Fe(II) + AscH\cdot \quad (5)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^- \quad (6)$$

However, caution must be exercised in the interpretation of this finding in relation to the cell as a whole, in which other processes may modify ascorbate metabolism, rendering it less pro-oxidant and even anti-oxidant (for example, interaction with the pentose phosphate pathway via NADPH and glutathione). Furthermore, the protection offered by ascorbate when present at very high concentration (2 mM) in this system, suggests the occurrence of competing *antioxidant* processes; namely scavenging of $\cdot OH$ and repair of biomolecular radicals (including vitamin E) by ascorbate.

In conclusion, this study provides a chemical basis for the understanding of the role of iron in biological damage. In addition to having immediate relevance to the

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pathophysiology of overt iron-overload diseases (for example, haemochromatosis), an understanding of the pathology of iron may also be important to the understanding of other aetiological processes in which the role of iron is less obvious; for example, the high levels of $O_2^{\cdot-}$ associated with the reperfusion of tissues following ischaemia[30,31] may mobilise iron from intracellular storage proteins to initiate biomolecular damage.

In addition to providing the H_2O_2 necessary for iron toxicity, mitochondria may also play an important role in the pathological processes following initial biomolecular damage. Thus, it is known that oxidative damage to mitochondria (by redox-cycling drugs) leads to their failure to sequester free Ca^{++} ions.[32] Impairment in the control of free Ca^{++} levels in the cell cytosol (due to oxidative damage to mitochondria by, for example, iron) may lead to inappropriate activation of Ca^{++}-dependent enzymes of crucial importance to cellular processes, for example, phospholipase A_2, controlling the arachidonic acid cascade and inflammation,[33,34] and those involved in the control of cell division.[35]

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