

Hydroxyl radical is produced via the Fenton reaction in submitochondrial particles under oxidative stress: implications for diseases associated with iron accumulation

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Mitochondrial dysfunction and reactive oxygen species (ROS) are often implicated in diseases involving oxidative stress and elevated iron. As mitochondria produce ATP by oxidative phosphorylation, ROS by-products are generated from the electron transport chain. Although superoxide and hydrogen peroxide have been thoroughly investigated, little evidence documents hydroxyl radical (HO[•]) production in mitochondria. In order to determine whether HO[•] is generated under oxidative stress conditions by a Fenton-type mechanism, bovine heart submitochondrial particles were examined for HO[•] in the presence and absence of iron ligands, antioxidant enzymes and HO[•] scavengers. HO[•] was measured as 2,3- and 2,5-dihydroxybenzoic acid (DHBA), using HPLC with electrochemical detection. The iron ligand desferrioxamine significantly decreased DHBAs, indicating that HO[•] generation required iron redox-cycling. In addition, results from exogenous SOD and catalase, exogenous hydrogen peroxide, and HO[•]-scavenger studies support a Fenton-type reaction mechanism. The results indicate that increased HO[•] levels occur in mitochondria under oxidative stress and that the HO[•] levels can be modulated with antioxidant enzymes and iron ligands. Our findings together with reports on iron accumulation in degenerative diseases highlight the importance of developing mitochondrial-targeted antioxidants for the therapeutic intervention of diseases associated with mitochondrial dysfunction and oxidative stress.

Keywords: antioxidant enzymes, desferrioxamine, Fenton reaction, hydroxyl radical, iron, oxidative stress, salicylate, submitochondrial particles

Introduction

Energy demands of complex aerobic organisms require the efficient synthesis of ATP by mitochondrial oxidative phosphorylation. In conjunction with ATP synthesis, however, aerobic organisms are exposed to reactive oxygen species (ROS) that are by-

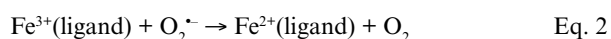
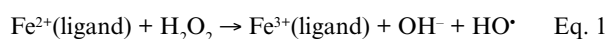
products of the mitochondrial electron transport chain. Under conditions of persistent oxidant production, such as during mitochondrial electron transport chain dysfunction, antioxidant enzymes that remove ROS may be overwhelmed causing oxidative stress. Oxidative stress has been implicated in the pathology of degenerative diseases, including carcinogenesis, hemochromatosis, Parkinson's disease,

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Abbreviations: CAT, catalase; DHBA, dihydroxybenzoic acid; DF, desferrioxamine; HPLC, high-performance liquid chromatography; HO[•], hydroxyl radical; SMPs, submitochondrial particles; SOD, superoxide dismutase

Alzheimer's disease, Friedreich's ataxia and aging itself.¹⁻⁶ Diseases associated with tissue iron accumulation are of particular concern,⁷⁻¹⁰ as iron catalyzes the generation of the highly reactive ROS, hydroxyl radical (HO•).

During oxidative stress, mitochondria may be important sites for sustained HO• production via the Fenton reaction as the precursors and catalysts for Fenton chemistry are co-located within the mitochondrial matrix. Superoxide anion radical is produced in the matrix at complexes I¹¹ and III¹² of the electron transport chain. Hydrogen peroxide is formed from superoxide by Mn-containing superoxide dismutase. Low molecular weight iron is present¹³ and iron ligands that permit iron redox-cycling are abundant.¹⁴ Thus, an HO• flux could be generated in mitochondria by the Fenton reaction (Eq. 1), driven by the reduction of iron by superoxide (Eq. 2) in the presence of an iron catalyst which is chelated to a ligand that facilitates redox cycling. Under mitochondrial oxidative stress conditions which favor Fenton chemistry and iron reduction by superoxide, HO• could cause significant biological damage.



The purpose of this study was to test the hypothesis that HO• is produced via the Fenton reaction in mitochondria under oxidative stress and that mitochondrial HO• production can be modulated by manipulating Fenton reaction conditions.

Materials and methods

Chemicals

All reagents were of the highest grade possible from Sigma-Aldrich Chemical Co. (St Louis, MO, USA), EM Science or JT Baker (VWR, West Chester, PA, USA) and were used as received from the supplier. The HPLC standards, 2,3- and 2,5-dihydroxybenzoic acid, were purchased from Sigma-Aldrich Chemical Co.

HPLC hydroxyl radical assay

Hydroxyl radical was measured by aromatic hydroxylation using salicylate (2-hydroxybenzoate) as a trap according to published methods.¹⁵ The analytes, 2,3- and 2,5-dihydroxybenzoic acid (DHBA), were detected using a high-performance liquid chromatograph (HPLC) equipped with a DHBA-250 column (5 µm particle size; 250 mm × 3 mm i.d. column size) and a Coularray electrochemical detector from ESA, Inc.

(Chelmsford, MA, USA). The elution profile was linear with a mobile phase of 50 mM sodium acetate, 50 mM citric acid, 25% methanol (v/v), and 5% 2-propanol (v/v), adjusted to pH 2.5 with phosphoric acid. The mobile phase was prepared in 18.3 MΩ-cm resistance ultrapure water from a NANOpure system (Barnstead, Dubuque, IA, USA), and was further purified by a C₁₈ cartridge (Waters, Milford, MA, USA) to remove trace organics. The DHBA-250 column temperature was maintained at 27°C and the flow rate at 0.5 ml/min throughout the analysis. DHBA concentrations were calculated by reference to 2,3- and 2,5-DHBA standard curves that were linear with correlation coefficients of $r^2 \geq 0.98$.

Isolation of mitochondria and submitochondrial particles

Bovine heart submitochondrial particles (SMPs) were prepared by differential centrifugation and sonication according to published methods,¹⁶ except that no EDTA was added. The sonication of mitochondrial solutions inverts the inner mitochondrial membrane to form SMPs. This inversion permits the facile analysis of chemical reactions that occur on the matrix face of the inner mitochondrial membrane. The SMPs were uncoupled by several freeze-thaw cycles until additions of ADP in the presence of electron transport chain substrates did not increase oxygen consumption rates. The SMPs were also exhaustively washed to remove matrix components such as mitochondrial DNA and enzymes. All isolation buffers and solutions were treated with Chelex-100 mesh resin (BioRad) to remove trace metals. SMP protein concentrations were determined by the Biuret method with bovine serum albumin as the standard.

Succinate oxidase (EC 1.3.5.1) enzyme assay

Before SMPs were used for experiments, electron transport chain function was assessed with succinate oxidase activity which tests electron flow through complexes II, III, and IV, coenzyme Q, and cytochrome c. Succinate is oxidized by complex II and donates electrons via the electron transport chain to complex IV where oxygen is reduced to water and removed from solution. Thus, succinate oxidase enzyme activity was monitored by measuring rates of oxygen consumption with a Clarke electrode using 1.0 mM succinate as substrate in a 10 mM or 50 mM potassium phosphate buffer pH 7.0 with 1.00 mg SMP protein/ml as previously described.¹⁷ SMP succinate oxidase specific activities ranged between 70–100 nmol O₂/min/mg protein.

SMP metal analysis by ICP-MS

Metal element concentrations were determined using an X-Series ASX-510 Inductively-Coupled Plasma Mass Spectrometer (ICP-MS) from Thermo Scientific Corporation. External metal standards (5–1000 ppb) were measured in triplicate with percentage standard deviations ranging from 3–11% for Fe. Three SMP samples were separated into membrane and supernatant fractions by centrifugation at 105,000 *g* for 55 min. The fractions were digested with nitric acid at 115°C for 20 min. Samples were diluted to a final concentration of 5% nitric acid before analysis in triplicate.

Submitochondrial particle incubation and Fenton chemistry reaction conditions

Submitochondrial particles were used in order to simplify measurement of hydroxyl radical formation on the matrix side of the inner mitochondrial membrane. ADP was used as a physiological iron chelator that occurs in the mitochondrial matrix. When electron transport is inhibited by antimycin A, superoxide levels are known to increase which induces an oxidative stress state.¹⁸ Rotenone was used to stop reverse electron flow from complex II to complex I. The iron and ligand were first added to reaction tubes and the solution was thoroughly mixed by pipette. Following these two additions, buffer was introduced, then salicylate and any other components such as H₂O₂, mannitol and thiourea, SOD, or catalase. The reactant final concentrations were: 1.00 mg/ml, submitochondrial particle protein, 200 μM antimycin A, 2.5 μM rotenone, 10 mM or 75 mM sodium salicylate, 10 mM mannitol or thiourea, 50 U/ml SOD, 100 U/ml catalase and 1.0 mM disodium succinate in a 10 mM or 50 mM potassium phosphate buffer pH 7.0. Ligand effects were tested with 20 μM or 100 μM ADP and 100 μM desferrioxamine (DF) in the presence and absence of 1.0 μM or 2.0 μM FeCl₃. Reactions were incubated at 30.0°C for 0.5 h or 2.00 h, initiated with succinate and stopped with 0.40 M perchloric acid which precipitated mitochondrial protein. Each sample was centrifuged, filtered (0.22 μm, Corning spin-x), and the supernatants were analyzed for dihydroxybenzoic acids by HPLC with electrochemical detection.

Antioxidant enzyme studies: superoxide dismutase and catalase

Active superoxide dismutase (SOD) from bovine erythrocytes (Calbiochem, La Jolla, CA, USA) and catalase from bovine liver were used. Enzymes were inactivated by boiling for 1 h in capped microcentrifuge

tubes. Using perchloric acid to precipitate proteins was found to decrease DHBA solubility and, therefore, was not used in the antioxidant enzyme studies. Instead, proteins were separated from supernatant by filtration. Specifically, after 0.5-h reaction time, 0.6 ml of each sample was filtered by centrifugation in a Spin-x microcentrifuge tube with a 0.22 μm nylon filter and supernatants were analyzed immediately by HPLC.

Statistical analysis

Results for total DHBA values (sum of analytes 2,3-DHBA and 2,5-DHBA) are expressed as mean ± SD of six independent experiments or mean values and propagated errors $\sqrt{SD_1^2 + SD_2^2}$ of triplicate determinations from two independent experiments.

Statistical analyses were performed using Student's *t*-test with the significance set at *P* < 0.05.

Results

In the presence of a physiological iron catalyst Fe(ADP), SMPs generated significantly more HO• as total DHBA under oxidative stress conditions than under normal electron transport chain conditions (Fig. 1). SMPs incubated without electron transport chain inhibitors produced only 4% of the HO• generated in the presence of inhibitors. The relative

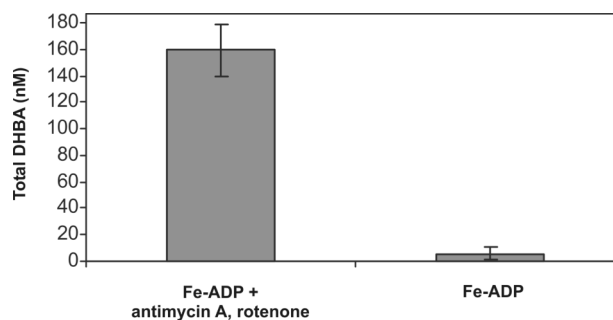


Figure 1 Electron transport chain inhibition increases HO• production in submitochondrial particles. HO• was measured as DHBA in SMPs incubated in the presence and absence of the electron transport chain inhibitors, antimycin A and rotenone. Values are the mean ± SD from six independent experiments (Fe-ADP + AA, rot) and the mean ± range of three independent experiments (Fe-ADP without inhibitors). Mean values are significantly different (*P* ≤ 0.01). Reaction mixtures contained 100 μM ADP, 1.0 μM FeCl₃, 10 mM salicylate, 1.00 mg SMP protein/ml in a 50 mM potassium phosphate buffer pH 7.0. SMP succinate oxidase specific activity was 80 nmol O₂/min/mg protein. AA, antimycin A; DHBA, 2,3- and 2,5-dihydroxybenzoic acid; rot, rotenone. SMPs, submitochondrial particles

Table 1 Critical stability constants for organic ligands and iron

Organic ligand	Equilibrium	Log K Fe ²⁺	Log K Fe ³⁺
Desferrioxamine B ^g	[MHL]/[M] [HL]		30.6
Salicylic acid ^{a,9}	[ML ₂]/[M] [L] ²	11.2 ^a	28.3 ^a
Succinic acid ^e	[ML]/[M] [L]	1.4 ^b	6.9
2,5-DHBA ^e	[M(HL) ₂] [H]/[MHL] [H ₂ L]		2.1 ^a
2,3-DHBA ^f	[ML ₂]/[M] [L] ²		28.7 ^c
ADP ^{d,g}	[ML]/[M] [L]	(for Mg ²⁺ 3.2–3.4)	

Equilibrium log K data are for conditions of low ionic strength, 0.1 M. Equilibrium temperatures are 25°C unless noted:

^a 20°C, ^b 37°C, ^c 27°C.

^d The critical stability constant listed for ADP is for the association with Mg²⁺ as the value for iron was unavailable. Sources: ^e Martell and Smith³³; ^f Martell and Smith³⁴; ^g NIST database.³⁵

DHBA, dihydroxybenzoic acid; L, ligand; M, metal; NIST, National Institute of Standards and Technology, USA.

proportion of SMP-generated HO[•] was iron-ligand dependent, with Fe(desferrioxamine)-catalyzed HO[•] levels significantly decreased as compared to Fe(ADP)-catalyzed levels (Fig. 2). Given its log K (Table 1) and standard reduction potential values,¹⁴ desferrioxamine is known to preferentially bind Fe³⁺ and to inhibit its reduction by superoxide.¹⁹

To test the hypothesis that HO[•] production was dependent on superoxide and hydrogen peroxide, total DHBAs were measured in the presence and absence of exogenous SOD and catalase. The results clearly demonstrate that these antioxidant enzymes, when used individually and together, dramatically decrease DHBA production (Fig. 3). To investigate this hypothesis further, exogenous H₂O₂ was added to SMPs to examine its effects on Fenton reaction products. The results show that total DHBAs were elevated in parallel with increased exogenous H₂O₂ (Table 2). Interestingly, the results also show a trend towards increasing proportions of the 2,5-DHBA isomer and decreasing proportions of the 2,3-DHBA isomer in total DHBA values.

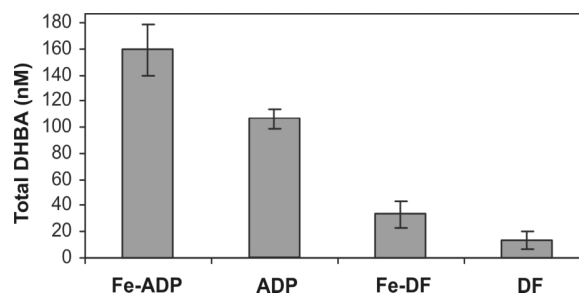


Figure 2 Desferrioxamine diminishes HO[•] production in submitochondrial particles. HO[•] was measured as DHBA in SMPs with ADP or desferrioxamine (DF) in the presence and absence of exogenous Fe³⁺. Values are mean ± SD for Fe-ADP (n = 6) and mean ± propagated errors of triplicate determinations from two independent experiments for ADP, Fe-DF and DF without exogenous iron. All mean values are significantly different from each other (P ≤ 0.01). Reaction mixtures contained 100 μM ADP or desferrioxamine in the presence and absence of 1.0 μM FeCl₃, rotenone, antimycin A, succinate, 10 mM salicylate, and 1.00 mg SMP protein/ml in a 50 mM potassium phosphate buffer pH 7.0. SMP succinate oxidase specific activity was 70 nmol O₂/min/mg protein. DHBA, 2,3- and 2,5-dihydroxybenzoic acid. SMPs, submitochondrial particles

In order to test the hypothesis that a Fenton-type reaction occurred in SMPs, the effects of hydroxyl radical scavengers were investigated. Mannitol and thiourea, with second-order rate constants of $2.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ and $4.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, respectively, were chosen. When equimolar concentrations (10 mM) of salicylate and scavenger were used, the total DHBA mean ± SD (n = 6) or propagated error (n = 2) observed were: salicylate alone, $159 \pm 20 \text{ nM}$ (n = 6); with mannitol, $91 \pm 8 \text{ nM}$ (n = 2); and with thiourea, $55 \pm 3 \text{ nM}$ (n = 2). All mean values were significantly different at P ≤ 0.05. The data demonstrate

Table 2 Exogenous hydrogen peroxide increases HO[•] generation in submitochondrial particles

Additions	Total DHBA (nM)	Increase of total DHBA (%)	Contribution of isomer (%)	
			2,3-DHBA	2,5-DHBA
None	159 ± 20 ^a		39	61
+ 0.50 mM H ₂ O ₂	205 ± 22 ^a	29	36	64
+ 1.00 mM H ₂ O ₂	271 ± 2 ^a	70	30	70

Submitochondrial particle (SMP) reaction mixtures contained rotenone, antimycin A, Fe(ADP), 10 mM salicylate, 1.00 mg SMP protein/ml in a 50 mM potassium phosphate buffer pH 7.0. Electron transport was initiated by the addition of succinate and stopped with 0.4 M perchloric acid. SMP succinate oxidase specific activity was 85 nmol O₂/min/mg protein. Total DHBA values are the mean ± SD for no addition (n = 6) and mean ± propagated errors for experiments with exogenously added H₂O₂ (n = 2). Percentage increase of total DHBA and percentage contribution of DHBA isomers were calculated from nM mean values.

^aAll mean values are significantly different from each other (P ≤ 0.01). DHBA, 2,3- and 2,5-dihydroxybenzoic acid.

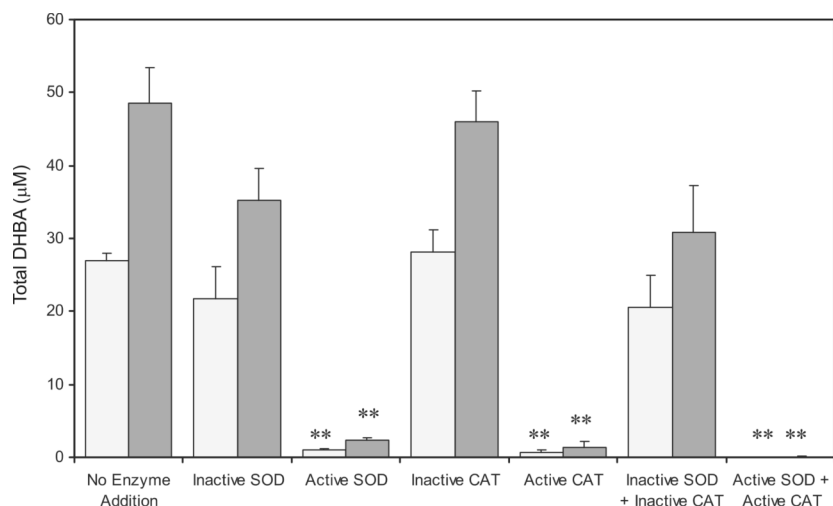


Figure 3 SOD and catalase diminish HO[•] production in submitochondrial particles. HO[•] was measured as DHBA in SMPs in the presence of active enzyme or inactive enzyme (boiled 1 h). Reaction mixtures contained 20 µM ADP, 2.5 µM rotenone, 200 µM antimycin, 1 mM succinate, 1 mg/ml SMP protein, SOD, 50 U/ml, catalase (CAT), 100 U/ml and 75 mM salicylate in the absence (open bars) or presence (gray bars) of 2 µM FeCl₃ in 10 mM potassium phosphate buffer, pH 7.00. Values are mean ± SD of three separate experiments and are normalized to SMP succinate oxidase specific activity of 100 nmol O₂/min/mg protein. Symbols represent values that are significantly different from the corresponding sample with no enzyme addition (P < 0.01). DHBA, 2,3- and 2,5-dihydroxybenzoic acid. SMPs, submitochondrial particles**

that thiourea was the more efficient scavenger, competing with salicylate more effectively than mannitol for reaction with HO[•]. The observed decreases in total DHBA are consistent with the second-order rate constant values, and provide additional support for the hypothesis that HO[•] was generated via a Fenton reaction.

Endogenous iron concentrations appeared to contribute to the total DHBA generated in SMPs as shown in reactions in which ADP was added without FeCl₃ (Figs 2 and 3). Thus, ICP-MS analysis of three SMP samples that were separated into membrane and supernatant fractions was conducted to determine iron content. The endogenous iron found in SMPs in mean nmol Fe/mg protein ± SD were: for membrane fractions, 3.89 ± 0.03 and for supernatant fractions, 0.90 ± 0.06. The mean nmol Fe/mg protein concentration for the supernatant is equivalent to a micromolar concentration under experimental conditions, and indicates that SMPs contained approximately 1 µM endogenous iron in solution. The majority of the endogenous iron (81%) was associated with the membrane fraction, most likely in the form of protein iron–sulfur centers or heme-iron.

Discussion

A growing body of evidence implicates mitochondrial dysfunction in the etiology of degenerative diseases

that are associated with oxidative stress. Mitochondrial dysfunction has been observed in the protein agglomeration that leads to Alzheimer's disease,²⁰ in the dopaminergic neuron loss that occurs in familial and idiopathic Parkinson's disease^{4,21} and in a mouse model of hemochromatosis.⁶ ROS generated from dysfunctional mitochondria may contribute to disease pathology. Under normal physiological conditions, antioxidant enzymes minimize cellular oxidations due to ROS. Our data indicate that exogenously added SOD and catalase significantly decrease HO[•] production in SMPs both in the presence and absence of exogenous iron. When SOD and catalase were added simultaneously, the HO[•] signal was decreased substantially. Our results support the notion that under normal metabolic conditions, little damage is expected from oxidants generated by the mitochondrial electron transport chain and Fenton chemistry. This observation is consistent with measurements of hydrogen peroxide and superoxide in rat tissues under non-oxidative stress conditions.²² Thus, under normal conditions when the precursors to HO[•] are generated at low levels, HO[•] levels will be minimized. However, during the persistent oxidative stress that occurs under pathological conditions, endogenous antioxidant defense systems may not provide sufficient protection. Our data imply that significant increases in HO[•] levels will occur when

electron transport is chemically inhibited or, by analogy, malfunctioning due to disease pathology or conformation abnormalities of electron transport proteins derived from genetic diseases.²³

The mitochondrial matrix sequesters a pool of chelatable iron that could exacerbate oxidative damage during increased ROS production.¹³ Moreover, increased iron storage in mitochondria has been noted in central nervous system diseases as well as in aging animals.⁹

Conditions of increased mitochondrial ROS generation and elevated iron set the stage for HO[•] production via the Fenton reaction. With an estimated half-life of 10⁻⁹ s, HO[•] reacts quickly and indiscriminately with substances that occur within a 93 Å radius.²⁴ The iron chelator, desferrioxamine, has been shown to decrease the cellular damage that is associated with iron-mediated oxidative injury.²⁵ Iron associated with desferrioxamine has a lower standard reduction potential (E° , -0.45 V, Fe³⁺-DF/Fe²⁺-DF) than O₂^{-•} (E° , -0.33 V, O₂/O₂^{-•}), making its reduction by O₂^{-•} thermodynamically unfavorable and, therefore, its participation in Equation 2 unlikely under physiological conditions.¹⁴ By contrast, iron bound to ADP has a reduction potential (E° , +0.10 V, Fe³⁺-ADP/Fe²⁺-ADP) that favors its reduction by superoxide. When desferrioxamine was added to SMPs, a statistically significant decrease in HO[•] production was observed. Consistent with previous work,²⁶ the desferrioxamine-dependent decrease in SMP-derived HO[•] occurred in both the presence and absence of exogenous iron suggesting that SMPs contained an endogenous iron catalyst which was verified by ICP-MS. However, desferrioxamine did not completely remove DHBA production in SMPs. The DHBAs observed in reactions containing desferrioxamine (Fig. 2) can be explained by Fe³⁺ binding to other ligands in the reaction mixture. One possible ligand is salicylate which has a log K value for Fe³⁺ binding that is comparable to that of desferrioxamine (Table 1). In order to trap hydroxyl radical efficiently, salicylate was at high concentrations in the reaction mixtures and was 100–750-fold higher than desferrioxamine. Salicylate is an effective chemical trap for hydroxyl radical,¹⁵ and has been shown to provide protection against oxidative stress induced in paraquat-exposed rats.²⁷ It is plausible that salicylate-bound iron functioned as a Fenton catalyst in our experimental system and as a specific target for HO[•]. Likewise, succinate could have participated in iron binding (Table 1). In addition, under conditions of oxidative stress, superoxide could induce the release of iron from mitochondrial membrane iron–sulfur centers in complexes I or II, as superoxide is known to cause the

release of iron from iron–sulfur centers.²⁸ Subsequent binding of the released iron to organic ligands such as adenine nucleotides or succinate and other Krebs's cycle intermediates would form the Fenton catalyst *in situ*.

Although several reports have been published on the amounts, the location and the membrane-sidedness of superoxide and hydrogen peroxide production in mitochondria,^{12,22,29,30} sparse evidence documents HO[•] formation.^{26,31} Here we report that significantly increased submitochondrial HO[•] production occurs under conditions of oxidative stress caused by electron transport chain inhibition. Iron enhances HO[•] production which is reversed by desferrioxamine, indicating that a redox-cycling iron catalyst is required. The effects of antioxidant enzyme additions, exogenous hydrogen peroxide, and HO[•] scavenger studies strongly support the notion that HO[•] was produced by a Fenton-type reaction. These results underline the importance of developing mitochondrial-targeted antioxidants in the prevention and treatment of diseases associated with mitochondrial dysfunction, oxidative stress and elevated iron.³² In particular, therapeutic strategies that focus on modulating Fenton reaction conditions by removing catalytic iron, superoxide, and hydrogen peroxide could provide beneficial effects by diminishing the formation of HO[•].

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