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IRON STIMULATION OF FREE RADICAL-MEDIATED PORPHYRINOGEN OXIDATION BY HEPATIC AND RENAL MITOCHONDRIA

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Mitochondria from rat liver and kidney catalyze oxidation of uroporphyrinogen in the presence of NADH or succinate and the respiratory chain inhibitor, NaN_3. The rate of porphyrinogen oxidation was substantially accelerated when iron as Fe^{+3}-EDTA was added to reaction mixtures. This effect was partially attenuated by catalase, reduced glutathione (GSH) and other free radical scavengers. These results suggest that iron stimulates free radical-mediated porphyrinogen oxidation by tissue mitochondria under conditions of perturbed mitochondrial respiratory function. These observations suggest a mechanism by which iron could contribute to excess porphyrin excretion in various inherited or chemically-induced porphyrias.

Numerous investigators have demonstrated an essential role of iron in the etiology of chemically-induced hepatic porphyria (1-3), as well as in the clinical expression of porphyria cutanea tarda (PCT) in humans (4,5). Studies on the mechanism(s) of this effect have focused on the action of iron as a specific inhibitor of the porphyrinogen-metabolizing enzyme, uroporphyrinogen decarboxylase (5,6), or as a catalyst in the NADPH-dependent oxidation of reduced porphyrins (porphyrinogens) by tissue microsomes (7-9). Little consideration, however, has been given to the potential role of iron in the exacerbation of free radical-mediated oxidation of porphyrinogens by tissue mitochondria. Such mechanisms seem plausible, however, in light of the central role of mitochondria in cellular oxidative metabolism and the demonstrated capacity of mitochondria from various tissues to generate free radicals such as superoxide anion ($O_2^{\cdot -}$) under conditions of oxidative stress (10-12). A role for iron in the stimulation of free radical-mediated oxidative processes by mitochondria is suggested by the central role played by iron in numerous mitochondrial biochemical processes, and the well-established property of iron to catalyze conversion of $O_2^{\cdot -}$ to more reactive oxygen species such as hydroxyl radical ($OH^{\cdot}$) in vitro (13).

In the present studies we investigated the hypothesis that iron, as Fe^{+3}-EDTA, stimulates the free radical-dependent oxidation of reduced porphyrins by hepatic and

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renal mitochondria in vitro. We report, for the first time, that mitochondria from both liver and kidney, when supplemented with respiratory chain substrates and inhibitors, support substantially increased rates of porphyrinogen oxidation when Fe^{+3} is present in the reaction medium as compared with Fe^{+3}-non-supplemented organelles. These observations suggest that iron may facilitate the oxidation of reduced porphyrins by tissue mitochondria under conditions where mitochondrial respiration is compromised by disease or toxicologic insult.

MATERIALS AND METHODS

Male Sprague-Dawley rats (175-200 gms) were obtained from Tyler Laboratories, Inc., Bellevue, WA. Reduced β-nicotinamine adenine dinucleotide (NADH), sodium azide (NaN_3), rotenone, sodium succinate, ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), hypoxanthine, xanthine oxidase, superoxide dismutase (SOD), catalase (CAT), allopurinol and oxypurinol were obtained from Sigma Chemical Company, St. Louis, MO. Uroporphyrin I was purchased from Porphyrin Products, Logan, UT. All other chemicals and reagents were obtained from standard commercial sources and were of the highest available purity. All solutions were prepared with metal-free deionized water.

Iron-EDTA chelates were prepared by mixing a 100 mM solution of EDTA with an equal volume of 40 mM $FeCl_3$, and appropriate dilutions of this solution were made with deionized water. Uroporphyrinogen was prepared by reduction of the corresponding porphyrin with freshly ground 3% sodium amalgam under N_2.

Mitochondria were prepared from rat liver or kidney cortex essentially as described by Johnson and Lardy (14) using 0.25 M sucrose, 0.05 M Tris buffer, pH 7.5. Pellets were washed twice in the same solution and were suspended 1:1 with respect to original tissue weight in 140 mM KCl. Suspensions were twice frozen at -80° and thawed before use. Protein concentrations were determined by the method of Smith et al (15).

Oxidation of uroporphyrinogen at 37° was followed in 3 cm glass cuvettes using a Perkin Elmer model 552 spectrophotometer by monitoring the increase in absorbance in the Soret maximum (396 nm) against a reference cuvette containing all the components except the porphyrinogen. The $O_2^{\cdot-}$-generating system consisted of 0.2 mM hypoxanthine and 0.1 unit xanthine oxidase in a total volume of 3 ml 0.1 M Tris buffer, pH 7.5. The $OH^{\cdot}$-generating system contained Fe-EDTA (10 uM $FeCl_3$:25 uM EDTA) and 2.5 mM H_2O_2 in 3 ml of the same buffer. Mitochondrial reaction mixtures contained 0.1 M Tris-140 mM KCl buffer, pH 7.5, 150 to 200 ug mitochondrial protein, 0.4 mM NADH or 5 mM succinate and 1 mM NaN_3, in a total volume of 3 ml. Fe-EDTA (1:1 mixture of 40 mM $FeCl_3$ and 100 mM EDTA) and other components were added as indicated in tables and figures. Uroporphyrinogen was added to the sample cuvette after a 5 minute incubation period in a final concentration of 1 uM. Absorbance was monitored for a period of 5 to 15 minutes, depending on the rate of porphyrinogen oxidation under the specified conditions. Rates presented in the figures and tables represent the maximum rates observed under the conditions described. Statistical differences between groups were determined by means of Student's t test.

RESULTS

Initial studies were conducted to demonstrate the oxidation of uroporphyrinogen by $OH^{\cdot}$ or $O_2^{\cdot-}$ in vitro. As indicated in Table 1, uroporphyrinogen (1 uM) was readily oxidized when added to an $OH^{\cdot}$-generating system (Fe-EDTA + H_2O_2). Oxidation of porphyrinogens by $OH^{\cdot}$ has been previously demonstrated (7,16) and proceeds at a rate dependent on the concentrations of either Fe^{+3} or H_2O_2 in the reaction mixture (16).

Table 1. Oxidation of uroporphyrinogen by a hydroxyl radical (OH·) generating system and attenuation by OH· scavengers and GSH

Sample Composition	Porphyrinogen Oxidation Rate (pmoles/min)	Percent Control
Control	695.3 ± 56.1	100
+ Allopurinol (5 mM)	321.0 ± 42.8*	46
+ Oxypurinol (5 mM)	440.3 ± 37.4*	63
+ GSH (1 mM)	632.7 ± 37.7	92
+ GSH (5 mM)	347.5 ± 40.3*	50
+ GSH (10 mM)	36.7 ± 13.4*	5

All cuvettes contained Fe^{+3}(10 uM)-EDTA, H_2O_2 (2.5 mM), 0.1 M TRIS buffer, pH 7.5, and the component indicated in a total volume of 3.0 ml. Oxidation reactions were initiated after a 5 min. incubation period by adding uroporphyrinogen (10 ul) to the sample cuvette to a final concentration of 1 uM and 10 ul of buffer to the reference cuvette. Reactions were conducted at 37°, as described in Materials and Methods.

*$p < 0.05$ with respect to control.

Porphyrinogen oxidation in vitro by OH· is significantly attenuated in the presence of the antioxidant, GSH, or the specific OH· scavengers (17), allopurinol or oxypurinol. Uroporphyrinogen is also readily oxidized in an $O_2^{\cdot-}$-generating system (xanthine oxidase + hypothanthine) (Table 2). This effect is readily prevented by superoxide dismutase (SOD) as well as by GSH. Fe-EDTA, when added to the $O_2^{\cdot-}$ generating system, substantially accelerates oxidation of uroporphyrinogen in vitro. As indicated in Table 2, the rate of porphyrinogen oxidation in the $O_2^{\cdot-}$ generating system with 1 uM iron present was over 17 times that observed in samples with no iron added. This effect was greatly attenuated by GSH or oxypurinol in the reaction mixture.

The capacity of mitochondria from various tissues to generate $O_2^{\cdot-}$ in the presence of specific respiratory chain substrates and inhibitors has been established, although the action of iron on this effect has not been previously described. The effects of iron on NADH-stimulated uroporphyrinogen oxidation by hepatic and renal mitochondria when NaN_3 is used as the respiratory chain inhibitor is illustrated in Figure 1. As shown, the addition of NADH and NaN_3 to liver mitochondria increased the rate of porphyrinogen oxidation to 1.6-times that observed by mitochondria without respiratory chain substrates or inhibitors present. Addition of iron (100 uM final concentration) to hepatic mitochondrial reaction mixtures containing NADH and NaN_3 increased the rate of porphyrinogen oxidation by another 4-fold, to over 6 times that observed in control preparations. Similar observations were made with respect to the effects of iron on renal mitochondria, in which a 7-fold increase in the rate of porphyrinogen oxidation was observed when iron was added in the presence of NADH and NaN_3, as compared with controls.

Table 2. Oxidation of uroporphyrinogen by a superoxide radical ($O_2^{\cdot-}$)-generating system and modulation by iron (Fe^{+3}-EDTA) and antioxidants

Sample Composition	Porphyrinogen Oxidation Rate (pmoles/min)	Percent Control
Control	145.4 ± 68.6	100
+ Superoxide dismutase (24 U/ml)	9.0 ± 9.0*	6
+ GSH (5 mM)	53.5 ± 17.1*	37
+ Fe(10 uM)-EDTA	2516.0 ± 164*	1729
+ Fe(1 uM)-EDTA	1386.7 ± 128*	953
		Percent Fe(1 uM)-EDTA
+ Fe(1 uM)-EDTA + GSH (5 mM)	55.4 ± 32.5**	4
+ Fe(1 uM)-EDTA + Oxypurinol (5 mM)	331.2 ± 19.7**	24

All cuvettes contained xanthine oxidase (0.1 U/ml), hypoxanthine (0.2 mM), 0.1 M Tris buffer, pH 7.5 and the component(s) indicated in a total volume of 3.0 ml. Uroporphyrinogen (1 uM final) was added to both reference and sample cuvettes and the reaction was initiated after a 5 min. incubation period by adding xanthine oxidase to the sample cuvette. Reactions were conducted at 37°, as described in Materials and Methods.

*$p < 0.05$ with respect to control.

**$p < 0.05$ with respect to Fe (1 uM)-EDTA.

Substantial increases in the rates of porphyrinogen oxidation were also mediated by iron when added to mitochondrial preparations in which succinate was employed as the respiratory chain substrate. As shown in Figure 2, iron increased the rate of porphyrinogen oxidation by hepatic mitochondria by 5.5-fold when added to mitochondrial preparations containing succinate and NaN_3, as compared with non-supplemented control preparations. Under the same conditions, iron increased the rate of succinate-stimulated porphyrinogen oxidation by renal mitochondria by 4.5-fold. In contrast, no increased rates of porphyrinogen oxidation were observed when mitochondria from either tissue were incubated with Fe-EDTA in the absence of respiratory substrates and inhibitors, or by any combination of Fe-EDTA, substrate or inhibitor in the absence of mitochondria (data not shown).

Studies were conducted to evaluate the effects of various free radical scavengers and antioxidants on Fe-catalyzed porphyrinogen oxidation by hepatic and renal mitochondria. Results presented in Table 3 demonstrate that SOD did not effectively prevent Fe-stimulated porphyrinogen oxidation in mitochondrial reaction mixtures and, in fact, slightly increased the rate promoted by liver mitochondria. Similarly, the hydroxyl radical scavengers, allopurinol and oxypurinol, were only moderately effective in preventing Fe-stimulated porphyrinogen oxidation. In contrast, GSH attenuated

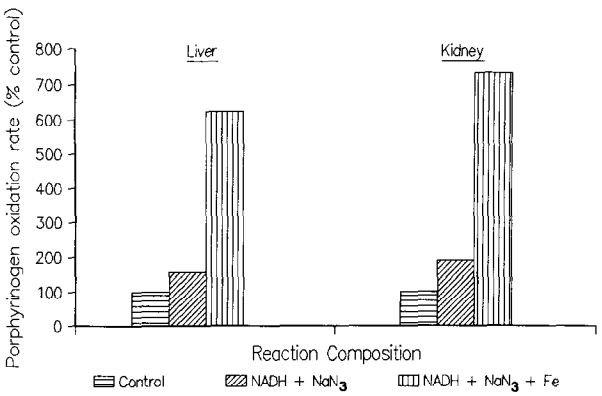

Figure 1. Effects of iron on NADH-stimulated porphyrinogen oxidation by hepatic and renal mitochondria in the presence of NaN_3 as a respiratory chain inhibitor. Reaction mixtures contained 0.1 M Tris-140 mM KCl buffer, pH 7.5, 0.4 mM NADH, 1 mM NaN_3, Fe^{+3}(100 uM)-EDTA(250 uM) and 150-200 ug mitochondrial protein in a total volume of 3 ml. Reactions were initiated after a 5 min. incubation period by addition of uroporphyrinogen to sample cuvettes in a final concentration of 1 uM. Rates of porphyrinogen oxidation by control (no NADH, NaN_3 or Fe added) hepatic and renal mitochondria were 37.4 $\pm$ 3.9 and 48.1 $\pm$ 10.0 pmoles/min/mg protein, respectively.

porphyrinogen oxidation in a dose-related manner when added to reaction mixtures of either liver or kidney mitochondria.

In separate experiments it was determined that catalase (CAT) is substantially inhibited by NaN_3 at the concentration (1 mM) used in mitochondrial studies. Accordingly, CAT was ineffective in preventing porphyrinogen oxidation in mitochondrial reaction mixtures in which NaN_3 was present. However, when rotenone (10 uM) was substituted for NaN_3 as a respiratory chain inhibitor in mitochondrial reaction mixtures, CAT (240 U/ml) effectively reduced the rate of porphyrinogen oxidation to 69 and 74% of rates promoted by hepatic or renal mitochondria, respectively, with NADH (0.4 mM), rotenone and Fe (1 uM) present.

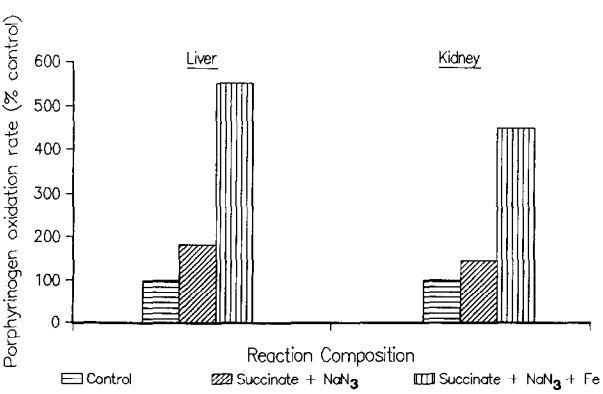

Figure 2. Effects of iron on succinate-stimulated porphyrinogen oxidation by hepatic and renal mitochondria in the presence of NaN_3 as a respiratory chain inhibitor. Reaction mixtures contained 0.1 M Tris-140 mM KCl buffer, pH 7.5, 5 mM succinate, 1 mM NaN_3, Fe^{+3}(100 uM)-EDTA(250 mM) and 150-200 ug mitochondrial protein in a total volume of 3 ml. Reactions were conducted as described in Figure 1 legend.

Table 3. Effects of free radical scavengers and antioxidants on iron-stimulated uroprophyrinogen oxidation by hepatic and renal mitochondria

Sample Composition	Porphyrinogen oxidation rate (pmoles/min/mg protein)			
	Liver	% Control	Kidney	% Control
Control (NADH+NaN_3+FeEDTA)	198 ± 23	100	302 ± 25	100
+ SOD (120 U/ml)	224 ± 29	113	266 ± 43	88
+ Oxypurinol (5 mM)	183 ± 26	93	221 ± 29*	73
+ Allopurinol (5 mM)	176 ± 19	89	242 ± 32	80
+ GSH (1 mM)	178 ± 10	90	130 ± 18*	43
+ GSH (5 mM)	49 ± 16*	25	70 ±17*	23
+ GSH (10 mM)	39 ± 6*	20	43 ± 3*	14

All cuvettes contained NADH (0.4 mM), NaN_3 (1 mM), Fe^{+3}(100 uM)-EDTA (250 uM), 150 to 200 ug protein and 0.1 M Tris-140mM KCl buffer, pH 7.5, in a total volume of 3 ml. Additional components were added at final concentrations indicated prior to addition of Fe-EDTA and NaN_3. Reactions were conducted at 37°, as described in Materials and Methods.

*$p < 0.05$ with respect to control.

DISCUSSION

Previous studies from this laboratory (16) and others (7,18) have demonstrated that porphyrinogens are readily oxidized in vitro in the presence of either OH· or $O_2^{\cdot -}$ - generating systems. In the latter case, this effect is greatly enhanced by addition of iron to the reaction mixture, consistent with the recognized property of iron and other transition metals to catalyze the reduction of $O_2^{\cdot -}$ to more reactive oxidizing species, including the highly reactive OH· (13). The attenuation of this effect by hydroxyl radical scavengers such as allopurinol and oxypurinol confirms the participation of OH· in the accelerated oxidation of porphyrinogen when iron is present in the $O_2^{\cdot -}$ - generating system.

The capacity of mitochondria from various tissues to generate $O_2^{\cdot -}$ and other oxidants has been described by several investigators (10-12). However, this is the first report, to our knowledge, which describes the direct oxidation of porphyrinogens by mitochondrial-generated reactive oxidants and the enhancement of that effect by iron. The precise reactive oxidant species responsible for porphyrinogen oxidation by mitochondria in the presence of iron are not known, but may include a variety of oxidants known to arise from the interaction of iron with biological tissues (13,19,20). Thus, although SOD was ineffective in preventing porphyrinogen oxidation in mitochondrial reaction mixtures, CAT afforded some protection against porphyrinogen oxidation, suggesting the iron-catalyzed formation of H_2O_2 from mitochondria-generated $O_2^{\cdot -}$. Additionally, the rate of porphyrinogen oxidation by hepatic and renal mitochondrial-generated oxidants was moderately reduced by both allopurinol and

oxypurinol (Table 3), suggesting at least the partial participation of OH· in this reaction. Other types of oxidizing species which could arise through the action of OH· on iron complexes have been suggested (19). It is feasible, therefore, that multiple reactive oxidants are involved in the oxidation of porphyrinogens by mitochondria, the precise nature of which remains to be determined. The existence of such species as metal-bound complexes, which bind in such a manner that prevents reaction to scavengers but still allows their interaction with porphyrinogens, has been suggested by Bucher et al (19), and may explain the inability of SOD and CAT to effectively prevent porphyrinogen oxidation in mitochondrial reactions. Alternatively, the inability of these enzymes to permeate partially damaged mitochondrial membranes to sites where oxidant formation arises, could also contribute to their relative ineffectiveness in this respect. The finding that uroporphyrinogen is readily oxidized by mitochondrial preparations in the present test system suggests that reactive oxidants formed by the interaction of iron with mitochondria are of sufficient strength to oxidize cytosolic constituents. That the concentration of GSH, a potent attenuator of porphyrinogen oxidation in vitro (16), is approximately 10-fold less in mitochondria as compared with in the cytosolic fraction (unpublished findings) may contribute to the potency of mitochondrial-generated reactive oxidants formed.

The finding that iron stimulates accelerated porphyrinogen oxidation by oxidatively compromised mitochondria may be relevant to the clinical manifestations of inherited and chemically-induced porphyrias, inasmuch as numerous porphyrinogenic drugs and chemicals are capable of uncoupling or perturbing mitochondrial oxidation reactions in a manner which could elicit reactive oxidant formation in vivo (21). It is tempting to speculate on the basis of the observations reported herein, for example, that compromise of hepatic mitochondrial respiratory capacity by chronic ethanol exposure (22), coupled with hepatic iron overload, might cause accelerated oxidation of porphyrinogens by mitochondrial-generated reactive oxidants, contributing to the accumulation and excretion of highly carboxylated porphyrins observed in PCT (23). That oxidatively compromised kidney mitochondria have a substantial capacity to oxidize reduced porphyrins is also of interest, inasmuch as the kidney is the principal target organ of numerous porphyrinogenic trace metals and other chemicals which have the capacity to disrupt mitochondrial biological oxidation reactions (21). Should such interactions promote the formation of reactive oxidants by kidney mitochondria leading to accelerated porphyrinogen oxidation, such events could account for the high levels of porphyrins observed to accumulate in cortical nephrons during specific chemical exposures. Evidence that the kidney is a principal source of urinary porphyrins excreted in various inherited and chemically-induced porphyrias has been provided by numerous investigators (recently reviewed in reference 24).

In conclusion, the present results demonstrate that mitochondria from liver and kidney promote porphyrinogen oxidation under conditions of impaired respiratory chain electron transport, and that this effect is substantially accentuated by iron. These results suggest a potential mechanism by which iron could contribute to the excess

porphyrin excretion observed in various inherited or chemically-induced porphyrias, independently of proposed effects of iron on heme biosynthetic pathway enzymes or microsomal oxidative processes.

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