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Mild iron overload effect on rat liver nuclei

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Abstract

A single injection of iron-dextran significantly increased iron content in plasma, whole liver, cellular cytosol and liver nuclei. In vitro nuclear rate of Fe^{3+}-EDTA reduction was not affected by the treatment. Membrane-bound enzymatic activities in the nuclei were measured after iron overload. Both NADPH- and NADH-dependent cytochrome *c* reductases were slightly decreased after iron overload, but cytochrome P_{450} was undetectable after 6 h of iron supplementation. The contents of lipid- and water-soluble antioxidants were measured in isolated nuclei from control and iron-overloaded rats. α-Tocopherol and β-carotene co-elutant were decreased by 40% and 83%, respectively after 6 h of treatment. Nuclear glutathione content was not affected. The rate of generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical-like species by isolated rat liver nuclei, were decreased by 50%, 40% and 60%, respectively after 6 h of iron supplementation. An identical qualitative response to iron overload was observed with NADPH and NADH. The inactivation of nuclear cytochrome P_{450}, the significant loss in lipid-soluble antioxidants (α-tocopherol and β-carotene) and the decrease in enzyme-dependent oxygen radical generation, suggest that the increase in catalytic active iron induced by iron overload could affect the cellular nuclei functionality.

Keywords: Iron overload; Liver nuclei; Antioxidants; Oxygen radicals

1. Introduction

Previous enzymological and immunological studies have suggested the similarity among some of the drug-metabolizing enzymes of the nuclear envelope and the endoplasmic reticulum (Thomas et al., 1979), even though the presence of some

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nuclear-metabolizing activity distinct from that found in microsomal systems (Romano et al., 1983) has been established. The mechanism of microsomal reduction of iron has been a subject of controversy. It is now accepted that reduction of iron chelates by the microsomal electron transport system includes the activities of NADPH-cytochrome P_{450} reductase and cytochrome P_{450} (Morehouse and Aust, 1988). Moreover, it has been found that rat liver microsomes or a reconstituted mixed function oxidase system consisting of NADPH-cytochrome P_{450} reductase and cytochrome P_{450} in dilaurylphosphatidylcholine micelles reduced Fe^{3+}-ADP, even under anaerobic conditions (Morehouse and Aust, 1988). Besides forming a competent electron transport chain capable of mixed function oxidase activity, coupling of cytochrome P_{450} with the reductase is proposed to result in increased O_2^- production (Kuthan et al., 1978; Parkinson et al., 1986).

The nucleus contains both NADPH-dependent (Romano et al., 1983) and NADH-dependent (Kano and Nebert, 1980) electron transfer systems and can catalyze in vitro reduction of ferric complexes (Kukielka et al., 1989), produce O_2^- (Yusa et al., 1984; Vartanyan and Gurevich, 1989) and undergo lipid peroxidation in the presence of either NADPH (Baird et al., 1980) or NADH (Kukielka et al., 1989).

Iron overload in the liver has been associated with injury, fibrosis and cirrhosis (Bacon and Britton, 1989). In experimental models of iron overload in rats, the occurrence of lipid peroxidation in whole liver (Galleano and Puntarulo, 1992), isolated cellular fractions (mitochondria, microsomes and lysosomes) (Burkitt and Gilbert, 1989; Rice-Evans et al., 1989; Bacon and Britton, 1990; Sevanian et al., 1990), cells (Poli et al., 1987), and tissue homogenates (Arthur et al., 1988) suggest that oxidative damage is responsible for the toxic effects of iron. Although the findings of many studies have implicated the hydroxyl radical in iron toxicity — Burkitt and Mason (1991) reported in vivo ESR evidence in support of this proposal — it was pointed out that secondary reactions could occur leading to the formation of artifacts during sample collection (Aust et al., 1993). Several mechanisms in which excess hepatic iron causes cellular injury have been proposed, but iron-induced peroxidative injury to phospholipids of organelle membranes is a potential unifying mechanism underlying the major theories of cellular injury in iron overload. It has also been postulated (Bacon and Britton, 1989) that iron-induced oxidative damage to nucleic acids may play a role in some forms of hepatotoxicity due to excess iron, nevertheless the effect of excess iron in vivo on isolated nuclei was not studied.

Cytosolic ferritin is a major storage form of iron, containing ~20% of all iron in the mammal, but a small transit pool of low molecular weight iron chelates is present within the cell (Bacon and Britton, 1989). In isolated nuclei, ferritin is considered the main physiological source of iron and it was suggested that in iron-loaded mouse hepatocytes there is a slow passage of ferritin molecules through the nuclear pores (Smith et al., 1990). Increased release of iron from intranuclear or cytosolic ferritin, could, along with cytosolic iron, provide a source of iron for catalyzing reactions that may affect nucleus integrity after iron overload.

In the current study, experiments were carried out to evaluate the effect of acute mild iron overload in vivo on the activity of membrane-bound enzymes, antioxidant content and enzyme-dependent oxygen radical generation by rat liver nuclei.

2. Materials and methods

Male Wistar rats weighing ≈200 g and fed Purina Chow diet were injected i.p. with iron-dextran (500 mg/kg body weight). Control animals were injected with saline solution. Rat liver nuclei were prepared by the method of Spelberg et al. (1974). Electron microscopic examination was used to assess the purity and ultrastructure of the isolated rat liver nuclei. Essentially intact nuclei with prominent nucleoli and distinct intact outer membranes were present. Mitochondria were not evident and disrupted vesicular membranes were minimal. Total iron content in plasma, whole liver and isolated liver nuclei was determined spectrophotometrically, employing bathophenanthroline (Brumby and Massey, 1967), after treating the samples with HNO_3:$HClO_4$ (1:1) solution (Rothman-Sherman and Tschiember-Tissue, 1981).

α-Tocopherol was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems amperometric detector with a glassy-carbon working electrode at an applied oxidation potential of 0.6 V (Lang et al., 1986). Samples from plasma, liver and isolated liver nuclei were added to 1 ml methanol and extracted with 3 ml hexane. After centrifugation at 1500 × *g* for 10 min, the upper phase was removed and evaporated to dryness under nitrogen. Samples were dissolved in 0.3 ml methanol:ethanol (1:1). β-Carotene content was assessed by HPLC processed in identical experimental conditions as described above (Lang et al., 1986). The peak assigned to β-carotene in the chromatograms was identified by co-elution with β-carotene standards.

In vitro rate of reduction of Fe^{3+}-EDTA was monitored in a reaction system containing 0.1 M phosphate buffer, pH 7.4, ~100 μg nuclear protein, 0.1 mM NADPH or NADH, 50 μM Fe^{3+}-EDTA (1:2) and 5 mM 2,2′-bipyridyl in a final volume of 1 ml. During the reduction under air, the re-oxidation of ferrous iron is inhibited by the 2,2′-bipyridyl and the formation rate of the stable ferrous-(bipyridyl)$_3$ complex was measured at 520 nm (Egyed et al., 1980). An extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ was utilized to calculate the rates of Fe^{3+}-EDTA reduction.

The activities of NADPH- and NADH-dependent cytochrome P_{450} reductases were measured by the method of Phillips and Langdon (1962) and the content of nuclear cytochrome P_{450} was determined by the method of Omura and Sato (1964). Total nuclear glutathione content was determined according to Akerbon and Sies (1981).

Nuclear membrane O_2^- generation was evaluated by measuring the oxidation of epinephrine to adrenochrome (Misra and Fridovich, 1972). The production of $^{\cdot}OH$ or compounds with the oxidizing power of $^{\cdot}OH$ by nuclei was assayed by measuring the generation of formaldehyde from dimethyl sulfoxide (DMSO) (Cederbaum and Cohen, 1984). The basic reaction system for assaying $^{\cdot}OH$ production consisted of 100 mM potassium phosphate, pH 7.4, 33 mM DMSO, 1 mM NaN_3, 0.1 mM NADPH or NADH and 0.5–1 mg nuclear protein in a final volume of 1 ml. Experiments were carried out in the presence of 50 μM Fe^{3+}-EDTA (1:2) for 30 min before termination with trichloroacetic acid. The production of formaldehyde was determined by the Nash reaction. All values were corrected for zero-time controls in which acid was added prior to the addition of nuclei.

The production of H_2O_2 by rat liver nuclei was determined employing cytochrome-*c*-peroxidase, as previously described (Boveris et al., 1972). The buffers and water used to prepare all solutions were passed through columns containing chelex 100 resin to remove metal contaminants. Where indicated, values refer to means ± S.E.M. Significance was determined using the Student's *t*-test.

3. Results

Total iron concentration was measured after acid digestion of the samples obtained from rats after 2–20 h of iron-dextran administration. After 2 h of iron supplementation, increases of 23- and 3-fold over the control values were detected in plasma and liver, respectively (Table 1). Cytosol (supernatant of 105 000 × *g* centrifugation), increased its iron content from 31 ± 1 to 139 ± 5 nmol/mg cytosolic protein after 4 h of the treatment. Isolated rat liver nuclei showed a significant increase in iron content after 20 h of iron-dextran dose.

In spite of iron deposition, α-tocopherol levels in the liver showed a slight decrease (not significant) probably due to the fact that the liver is an important organ of storage of α-tocopherol. However, after 20 h of treatment, the α-tocopherol content in plasma was decreased by 40% as compared to control values (Table 2), suggesting the development of oxidative stress.

Previous data employing this model (Galleano and Puntarulo, 1992), indicated that both in vitro NADPH-dependent chemiluminescence and TBARS generation, from iron-overloaded liver homogenates, showed a significant increase as compared to controls. Since either oxidative stress developed in the cytosol or iron increase in the nuclei could affect functionality, rat liver nuclei were isolated and characterized after exposure to mild iron overload.

When ferric iron is used as the catalyst for the generation of potent oxidizing species, its reduction to ferrous iron must be considered as an early event for the ultimate generation of $^{\cdot}OH$ or the initiating oxidant. In the presence of either NADPH or NADH, the nuclei were capable of reducing Fe^{3+}-EDTA and iron

Table 1
Iron content in acute iron-overloaded rats

	Iron content		
	Plasma (nmol/ml)	Liver (μmol/g wet liver)	Nuclei (μmol/mg protein)
Control	40 ± 4	1.4 ± 0.1	0.17 ± 0.03
Treated			
2 h	830 ± 40	4.7 ± 0.4	0.22 ± 0.02*
4 h	1650 ± 90	7.9 ± 0.6	0.29 ± 0.03
20 h	1130 ± 90	12.8 ± 0.9	0.49 ± 0.04

Treated rats showed statistically significant differences ($P < 0.01$) as compared with the control group. Where indicated by an asterisk ($P > 0.05$), no significant differences are shown.

Table 2
α-Tocopherol level after iron supplementation

	α-Tocopherol content	
	Plasma (μM)	Liver (nmol/g wet liver)
Control	4.3 ± 0.4	31.0 ± 3.0
Treated		
4 h	3.7 ± 0.3*	30.6 ± 3.0*
20 h	2.6 ± 0.2 (−40%)	28.0 ± 2.6*

Treated rats showed statistically significant differences ($P < 0.01$) as compared with the control group. Where indicated by an asterisk ($P > 0.05$), no significant differences are shown.

overload did not affect significantly the rate of Fe^{3+}-EDTA reduction (Table 3). Since the membrane-bound enzymes NADPH- and NADH-dependent cytochrome *c* reductases can reduce EDTA-chelated iron, their activities were evaluated. Neither of those activities was significantly affected by the treatment, which is consistent with lack of effect on Fe^{3+}-EDTA reduction. However, in vivo iron overload drastically decreased the cytochrome P_{450} content of the membrane. Nuclei isolated from control rats showed a cytochrome P_{450} content of 0.06 ± 0.01 nmol/mg nuclear protein but it was undetectable in nuclei isolated from iron-overloaded rats after 6 h of treatment (Table 3). This effect is consistent with the 40% decrease in microsomal cytochrome P_{450} previously reported after 2 h of iron overload (Galleano and Puntarulo, 1992).

The content of lipid and water-soluble antioxidants was measured in isolated nuclei from control and iron-overloaded rats. α-Tocopherol and β-carotene coelutant were decreased by 40% and 83%, respectively after 20 h of the single dose

Table 3
Effect of iron overload on in vitro Fe^{3+}-EDTA reduction rate, cytochrome *c* reductase activity and cytochrome P_{450} content in nuclei

	Fe^{3+}-EDTA reduction (nmol/min per mg protein)		Cytochrome *c* reductase (nmol/min/mg protein)		Cytochrome P_{450} (nmol/mg protein)
	NADH	NADPH	NADH	NADPH	
Control	3.5 ± 0.3	2.1 ± 0.2	28 ± 1	4.3 ± 0.3	0.06 ± 0.01
Treated					
1 h	3.4 ± 0.3	2.2 ± 0.3	26 ± 2	4.2 ± 0.3	—
2 h	3.6 ± 0.2	2.0 ± 0.2	25 ± 1	4.1 ± 0.2	—
6 h	3.5 ± 0.3	2.1 ± 0.2	20 ± 1*	4.1 ± 0.1	n.d.[a]

Treated rats showed no statistically significant differences ($P < 0.01$) as compared with the control group. Where indicated by an asterisk ($P > 0.05$), significant differences are shown.
[a]Under limit of detection.

Table 4
Antioxidants in rat liver nuclei after iron overload

	α-Tocopherol (pmol/mg protein)	β-Carotene (pmol/mg protein)	Glutathione (nmol/mg protein)
Control	14.8 ± 0.8	2.7 ± 0.3	0.23 ± 0.03
Treated			
20 h	8.8 ± 0.9 (−40%)	0.46 ± 0.07 (−83%)	0.25 ± 0.03*

Treated rats showed statistically significant differences ($P < 0.01$) as compared with the control group. Where indicated by an asterisk ($P > 0.05$), no significant differences are shown.

of iron-dextran. No significant changes were detected in glutathione content of the nuclei (Table 4).

Since iron is strongly involved in oxygen radical generation, the effect of the treatment on the ability of isolated rat liver nuclei to catalyze active oxygen species generation was measured. Both, NADPH- and NADH-dependent O_2^- generation, measured as epinephrine oxidation, were significantly decreased after 2 h of iron supplementation (Table 5). To evaluate the production of species with the oxidizing power of $^{\cdot}OH$, the ability of the nuclei to oxidize chemical scavengers to known products was assayed. In previous studies with microsomes (Cederbaum and Cohen, 1984) and isolated nuclei (Kukielka et al., 1989), formaldehyde was found to be the major product resulting from the reaction of DMSO with $^{\cdot}OH$-like species. Nuclei isolated from iron-overloaded rats showed, in the presence of in vitro added Fe^{3+}-EDTA, a 60% decrease in NADPH-dependent generation of formaldehyde from DMSO, and a 37% decrease in the presence of NADH as co-factor, after 6 h of iron exposure in vivo (Table 5). DMSO oxidation was measured in the presence of 50 μM Fe^{3+}-EDTA, in order to evaluate the maximum ability of isolated nuclei to generate $^{\cdot}OH$-like species after being exposed to the increase in iron content in vivo.

Table 5
Oxygen radical generation catalyzed by rat liver nuclei

	Epinephrine oxidation (nmol/min/mg protein)		DMSO oxidation (nmol/min/mg protein)	
	NADPH	NADH	NADPH	NADH
Control	1.1 ± 0.1	0.47 ± 0.02	0.50 ± 0.05	1.40 ± 0.09
Treated				
1 h	1.1 ± 0.2*	0.44 ± 0.04*	0.48 ± 0.05	0.98 ± 0.08
2 h	0.6 ± 0.1	0.34 ± 0.03	0.40 ± 0.05	0.88 ± 0.09
6 h	0.5 ± 0.1	0.32 ± 0.04	0.22 ± 0.03	0.82 ± 0.09

Treated rats showed statistically significant differences ($P < 0.01$) as compared with the control group. Where indicated by an asterisk ($P > 0.05$), no significant differences are shown.

The NADPH-dependent production of H_2O_2 in the presence of Fe^{3+}-EDTA was 0.63 nmol/min per mg nuclear protein in control nuclei and was decreased by 40% after 6 h of iron dose, thus resembling the effect of iron on $^{\cdot}OH$ generation.

4. Discussion

Treatment of rats with iron-dextran resembles haemochromatosis secondary to iron-loading anaemias (anaemias treated with repeated transfusions) and high iron oral intake (Younes et al., 1969; Powell et al., 1980). In addition to the marked increases in storage iron, it has been suggested that in iron overload there is an increase in the intracellular transit pool of iron which could stimulate free radical generation and lipid peroxidation (Britton et al., 1990). These authors have shown, in chronic iron-overloaded rats that ultrafiltrates (<25 000 molecular weight) of hepatic cytosol had a greater pro-oxidant effect than those from controls. In our model, plasmatic iron content was drastically increased after 4 h of iron-dextran dose, leading to significant increases in iron content in whole liver, cellular cytosol and nuclei. These data suggest that in conditions of acute iron overload, the cellular ability to sequester excess iron in the storage protein is overwhelmed. In vitro studies have shown that iron from both ferritin and hemosiderin (Zuyderhoudt, 1978; Puntarulo and Cederbaum, 1993), can stimulate lipid peroxidation. These studies also suggest that the sudden increase in catalytically active iron in the cytosol by acute iron overload, could result either in small amounts of ferrous iron or in excessive amounts of low molecular weight chelated iron that may have an important role in the initiation of free radical reactions.

Smith et al. (1990) reported that no iron-positive inclusions were detected in the nuclei of periportal hepatocytes within 8 weeks of iron treatment. As a consequence, at short time periods, as those studied here, the cytosolic increase in the content of catalytically active iron could be responsible for promoting the generation of toxic agents that lead to lipid peroxidation. However, besides cytosolic antioxidants, nuclei are protected by their own defense system. Antioxidants, both at the cytosolic and nuclear level, are operative in protecting nuclear membranes. It has been reported that liver nuclei matrices contain water-soluble antioxidants (e.g. GSH) (Bellomo et al., 1992) and membranes contain lipid soluble antioxidants (e.g. α-tocopherol) (Chow, 1991). The data reported here indicate that nuclear α-tocopherol and β-carotene, are primarily affected by cellular excess iron due to acute iron overload. Moreover, if the oxidant acts within the nuclear membrane, a decrease of the lipid-soluble antioxidants could occur with no effect upon GSH. The measured consumption of lipid-soluble antioxidants suggest that oxidative stress conditions could have been developed by the treatment.

Nuclear membranes contain inducible, functionally-active mixed-function oxidase activity, (cytochrome P_{450}, NADPH- and NADH-dependent cytochrome *c* reductase activities) (Romano et al., 1983). The lack of effect of iron overload on the rate of reduction of in vitro added Fe^{3+}-EDTA may reflect the no-effect or the slight decrease on nuclear cytochrome *c* reductases activities. Previously, it has been reported that treatment of rats with phenobarbital or 3-methylcholanthrene increas-

ed 2-fold the total cytochrome P_{450} content, as well as a slight increase in the activities of cytochrome *c* reductase (Puntarulo and Cederbaum, 1992). Associated with the increase in components of the nuclear mixed-function oxidase system was an increase in the production of $^{\cdot}OH$-like species and H_2O_2 (Puntarulo and Cederbaum, 1992), suggesting a critical role for cytochrome P_{450} in oxygen radical generation by nuclei. Nuclear cytochrome P_{450} was undetectable after 6 h of iron-dextran dose, probably due either to oxidative stress associated damage or other side reactions developed by excess iron. The measured decrease in enzyme-dependent O_2^- generation by nuclei could be related to the decrease in cytochrome P_{450} content.

The data presented here indicate that the decrease in O_2^- generation after iron overload lead to a lower H_2O_2 production in the presence of NADPH. Moreover, the H_2O_2 generation rate seems to be the limiting factor for $^{\cdot}OH$-like species production since iron reduction was not affected by the treatment.

The iron content after acute mild iron overload increased to a lesser extent in the nuclei than in the cytosol. In spite of the fact than the increase in nuclear iron would only occur upon increase in the cytosolic content, the measured membrane-related effects could not be ascribed exclusively either to cytosolic or to the nuclear excess iron.

Taken as a whole, these results suggest that stress conditions induced by iron overload in vivo, play an important role in the inactivation of cytochrome P_{450}, in the decrease of membrane antioxidants and in the reduced production of enzyme-dependent active oxygen species by nuclei.

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