Iron(II) induces changes in the conformation of mammalian mitochondrial DNA resulting in a reduction of its transcriptional rate

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Abstract Living isolated mitochondria incubated with iron(II) show a major alteration in mitochondrial DNA (mtDNA) conformational forms as assessed by Southern blot analysis of undigested mtDNA. In the presence of iron(II), form I is transformed into form III in a dose-dependent manner. This alteration in mtDNA conformation shows a strong correlation with a decrease in the mtDNA transcription rate ($r = 0.965$, $P < 0.002$), suggesting that iron(II) load results in double-strand breaks and unwinding of mtDNA, which, in turn, is unable to maintain its normal transcriptional rate.

Key words: Mitochondrial transcription; Iron; Mitochondrial DNA conformation

1. Introduction

Iron is potentially dangerous because it participates in hydroxyl ($^{\bullet}$OH) radical formation. Mitochondria are the major source of superoxide, generated in complex III, which can dismutate to yield hydrogen peroxide. This, in turn, can interact with iron(II) to produce $^{\bullet}$OH radicals [1,2]. These radicals can damage mitochondrial DNA (mtDNA) in different ways, either creating single-strand and double-strand breaks, which change mtDNA conformation leading to unwinding of the supercoiled form, linearization and fragmentation [3–5] or producing oxidative damage which, in turn, can result in misreading by the polymerase during mtDNA replication [3–7]. Alterations in the conformation of mtDNA could result in an impairment of its transcription, which may have important functional consequences for the normal function of the enzymes of the electron transport chain.

The molecular basis of the damage produced by iron accumulation within mitochondria, which may have important pathological implications [8,9], is not well understood, so we hypothesize that iron-initiated oxidative damage produces changes of the topology of mtDNA which could result in an impairment of mtDNA transcription. To test this hypothesis we have studied the effect of iron(II) on the mtDNA conformational forms as well as on its transcriptional rate in an in organello system which has been used to study the dynamics of mtDNA transcription in mammalian mitochondria [10–12].

2. Materials and methods

2.1. Mitochondrial isolation

Hearts of adult male Wistar rats were used as a source of mammalian mitochondria. Briefly, hearts were homogenized in 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA), 10 mM Tris–HCl, pH 7.4. The homogenate was centrifuged at $200 \times g$ for 10 min, and the supernatant was centrifuged at $5000 \times g$ for 10 min. The resulting pellet was washed once with homogenization medium, and once with incubation medium containing 100 mM KCl, 75 mM sorbitol, 25 mM sucrose, 5 mM $MgCl_2$, 0.05 mM EDTA, 10 mM phosphate, 10 mM Tris, pH 7.4. All procedures were carried out at 4°C and the resulting mitochondrial fractions were incubated to perform in organello mtDNA transcription and Southern blot analysis in the present of iron(II).

2.2. Iron(II) treatment and in organello mtDNA transcription

In organello transcription was performed as previously described with minor modifications [13]. Briefly, samples containing 1 mg of mitochondrial protein were incubated in 0.5 ml of transcription medium containing 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 0.5 mg BSA, 50 μM UTP, 50 μM CTP, 20 μCi [α-^{32}P]UTP (400–600 Ci/mmol). Iron(II) oxidative stress was induced by adding ferrous chloride ranging from 0 to 3.2 mM [14]. After 1 h of incubation in a rotary shaker, iron-induced oxidative stress was stopped by the addition of 50 mM EDTA, and samples were pelleted at $13\,000 \times g$ during 1 min, and washed twice with 10% glycerol, 10 mM; Tris–HCl, pH 6.8, and 0.15 mM $MgCl_2$. Intramitochondrial nucleic acids were purified as described [15], by SDS–pronase treatment and phenol/chloroform/isoamyl alcohol extraction. Electrophoretic analysis of newly synthesized mitochondrial RNAs was performed by loading equal amounts of total mitochondrial nucleic acids on 1.4% agarose slab gels. Deionized methylmercuric hydroxide (CH_3HgOH, Serva) was added to the gel, because this agent improves the electrophoretic resolution of mitochondrial RNA species [16].

Gels were stained with ethidium bromide, but only after electrophoresis to avoid artifactual changes in the topology of mtDNA due to ethidium bromide. After staining, gels were dried and exposed to autoradiography at −70°C. Autoradiograms were analyzed in a PDI desktop scanner (DNA 35, PD Images) supported by PDI scanning software (Quantity One).

2.3. Analysis of the conformational forms of mtDNA after iron(II) treatment

Conformational analysis of mtDNA was performed on samples of iron(II)-treated mitochondria. After mitochondrial lysis and extraction, nucleic acids were treated with DNase-free RNase, and loaded on a 1.4% agarose–CH_3HgOH gel. After electrophoresis, mtDNA was transferred onto nylon membranes and Southern blot analysis was performed using a ^{32}P-labelled probe containing the sequence of the mitochondrial 16S rRNA gene, as described [17]. Side-by-side *Bam*HI-

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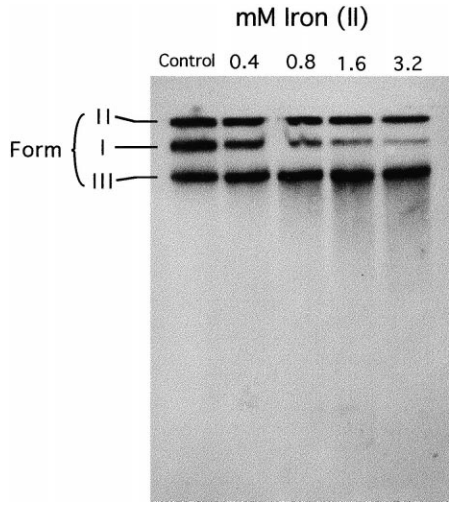

Fig. 1. Southern blot analysis of mtDNA from mitochondria incubated with increasing amounts of iron(II). The pattern of bands corresponds to the three archetypal forms of mtDNA.

digested samples were used as control. Conformational forms I, II and III of mtDNA were quantified on autoradiograms using the same system as for the in organello transcription gels.

3. Results

Incubation of mitochondria with iron(II) results in a dramatic change in the pattern of the conformational forms of mtDNA as assessed in ethidium bromide-stained gels and by Southern blot analysis of undigested mtDNA. Mammalian mitochondria have three archetypal conformations: form I, supercoiled; form II, open relaxed; and form III, linear [18,19]. Fig. 1 shows a dramatic reduction of form I in the Southern blot analysis of undigested mtDNA from iron-treated mitochondria. Increasing amounts of iron(II) (from 0 to 3.2 mM) result in a reduction of form I (which is the most abundant in vivo). This reduction leads to a parallel increase of form III, suggesting an iron(II)-induced modification of supercoiled into linear mtDNA with the generation of mtDNA fragments, as shown by the increase in background below the form III band (see also Fig. 2a).

Fig. 2b shows the effect of iron(II) on the amount of newly synthesized mtRNA species. In this gel it is possible to recognize the set of mitochondrial RNA species previously described in rat liver mitochondria (both processed and unprocessed transcripts) [12,13]. There was a linear decrease of the mtRNA synthesis rate as the iron(II) concentration increased, and processing of mtRNA was not affected by iron(II) treatment. Interestingly, this reduction in mitochondrial transcription activity parallels the modification of mtDNA form I into form III induced by iron(II) treatment (Fig. 2a), and when data were subjected to regression analysis (mtDNA form I vs. total mtRNAs), they showed a strong correlation ($r = 0.965$, $P < 0.002$). To confirm that the reduction in the mtDNA transcription was due to a true decrease of the synthesis rate rather than RNA degradation, a time course experiment (Fig. 3) was performed in the presence of 0.8 mM iron(II). Results indicate that 0.8 mM iron(II)-treated mitochondria are unable to maintain their transcriptional activity after 20 min of exposure, while control mitochondria maintain their transcription activity up to 1 h of incubation.

Iron(II) can be oxidized extremely fast in the presence of a phosphate-containing buffer [20]. Therefore, in order to determine whether the effects observed were due to the oxidation of iron(II) to iron(III), with the subsequent formation of a highly reactive intermediate, or merely due to a direct effect of iron-(III), mitochondria were incubated in the presence of iron-(III). Equimolar amounts of iron(III) did not show any alteration of either the conformational forms or the transcription rate of mtDNA (data not shown).

4. Discussion

Superoxide anion radicals (SAR) are produced in living mitochondria during the electron transfer of complexes I and III of the respiratory chain. SAR can, in turn, reduce iron(III) to iron(II) and maintain the Fenton reaction yielding $^{\bullet}OH$ radical which could nick the DNA strands producing breaks and unwinding of the mtDNA [3,4,18,21–23]. With the present experimental model, we previously demonstrated that mitochondria maintain electron transfer and coupling

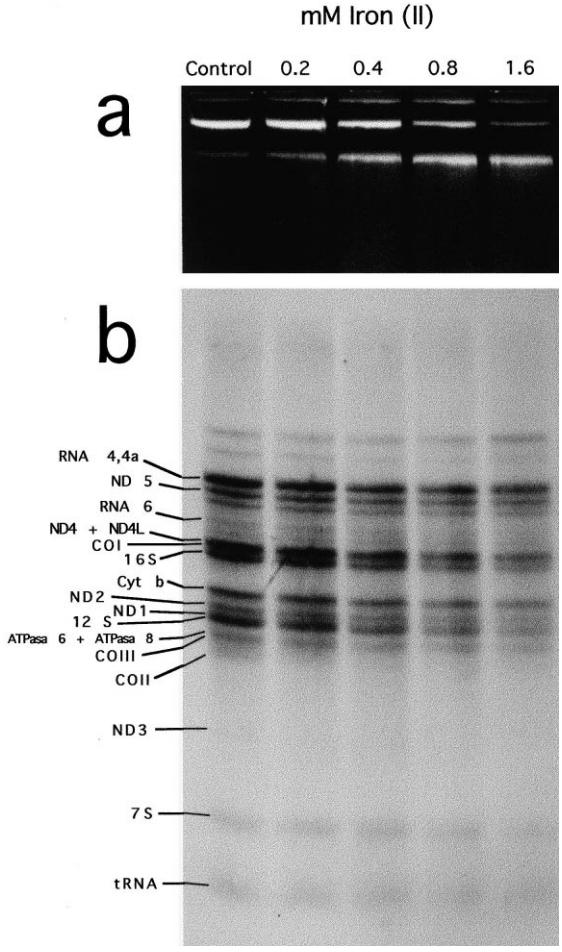

Fig. 2. Electrophoretic patterns in agarose–CH_3HgOH slab gels of heart mitochondrial RNA synthesized in isolated organelles in the presence of increasing concentrations of iron(II). a: Ethidium bromide staining of the upper part of the gel showing the different conformations of the mtDNA. b: Autoradiogram of the same gel, showing the newly synthesized RNA species. Abbreviations indicate the mtRNA species previously identified in mammalian heart mitochondria (see [5]).

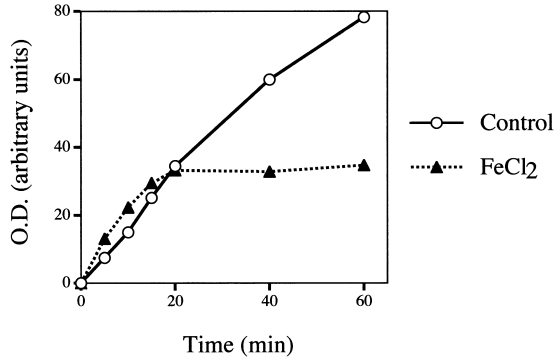

Fig. 3. Time course experiment of total mtDNA transcription rate in the absence or presence of 0.8 mM iron(II).

activity, so hydrogen peroxide is formed and the exogenous addition of iron(II) amplifies the Fenton reaction [10,14].

The dramatic change found in the topology of mtDNA (mainly a conversion of form I into form III) is consistent with the model that proposes that iron(II) binds to the exterior of the double helix, damaging DNA [24]. In relationship to this damage, two DNA binding models for iron have been suggested: either binding to phosphate residues or coordination in a sequence- (base-) specific manner [25]. This iron–mtDNA binding hypotheses has been proved by Yafee et al. [19] who showed, by high-resolution field-emission in-lens scanning electron microscopy, that when respiring mitochondria are incubated with iron they form iron colloids bound to their mtDNA.

The conformational change of form I into form III and the generation of mtDNA fragments occurs in a dose-dependent manner and at 3.2 mM iron(II) form I is almost completely converted into form III, while form II is unaffected by iron treatment. These results suggest that iron-induced mtDNA damage is affecting mainly the supercoiled form. Double-strand breaks should occur at random positions along mtDNA and once per molecule in most cases, in order to generate the linear full-length form. In some molecules, however, more than one double-strand break occurs, giving rise to mtDNA heterogeneous fragments. Both ethidium bromide postelectrophoresis-stained gels and Southern blot analysis show the same pattern consistent with this interpretation.

The alteration in the topology of mtDNA is closely correlated ($r = 0.965$) with a decrease in its transcriptional rate, suggesting that this impairment in the capacity of mtRNA synthesis is mainly due to the conversion of supercoiled into linear DNA. Interestingly, the pattern of newly synthesized mtRNAs seems to be the same as in control mitochondria (including RNA-processed bands), indicating that the decrease in the total transcription rate is due to a true decrease of the total mtRNA synthesis rate. This is consistent with the results of our time course experiment, since in iron(II)-treated mitochondria there is no de novo synthesis of mtRNAs after 20 min of incubation, while the synthetic rate is maintained up to 1 h in control mitochondria, suggesting irreversible damage of the transcription machinery. This decrease in the synthesis of mtRNA species could have important functional consequences, as the mtDNA synthesizes 13 polypeptides, all of them subunits of the oxidative phosphorylation system complexes, leading to a decrease in energy production [26]. This hypothesis is consistent with previous works that showed abnormalities in the activities of the enzymes of the electron transport chain in iron-labeled mitochondria [8,27–29]. Particularly, an increased iron deposition in mitochondria accompanied by impaired respiration and ATP production has been recently reported in patients with Friedreich's ataxia [8,9]. In the same direction, the expression in a yeast model for this human disease of a protein that prevents iron accumulation in mitochondria is able to rescue the lost respiratory function [30].

This is the first study reporting a close correlation between changes in the topology of mtDNA and impairment of its transcription activity, and further expands our knowledge of the effects of iron damage on mtDNA. Furthermore, the iron(II)-induced mtDNA damage with reduction of the transcription activity, described in the present work, should provide a useful model to study the protective mechanisms of mammalian mitochondria from free radical-induced damage on mtDNA.

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