

Disabled early recruitment of antioxidant defenses in Friedreich's ataxia

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Friedreich's ataxia (FRDA) results from a generalized deficiency of mitochondrial iron–sulfur protein activity ascribed to mitochondrial iron overload. However, iron overload appears to be a late event in the disease. Here we show that neither superoxide dismutases nor the import iron machinery was induced by an endogenous oxidative stress in FRDA patients' fibroblasts in contrast to control cells. Superoxide dismutase activity was not induced in the heart of conditional frataxin-KO mice either. This suggests that continuous oxidative damage to iron–sulfur clusters, resulting from hampered superoxide dismutase signaling, is causative of the mitochondrial deficiency and long term mitochondrial iron overload occurring in FRDA.

INTRODUCTION

Friedreich's ataxia (FRDA), the most common autosomal recessive ataxia (1/30 000 live births), also causes a severe hypertrophy of the myocardium. This disease is the consequence of a GAA repeat expansion within the first intron of the frataxin gene (1). The frataxin protein localizes to the mitochondrial inner membrane, and its absence results in a mitochondrial iron overload (2,3) and a generalized deficiency of iron–sulfur cluster-containing proteins (ISPs) (i.e. respiratory chain complexes I, II and III, and aconitases) in endomyocardial biopsies of FRDA patients (4). A similar ISP enzyme deficiency has recently been observed in heart muscle of conditional frataxin knockout mice, but the loss of enzyme activity has been shown to precede any detectable iron accumulation in the mitochondria (5). Consistently, while iron deposit and enzyme deficiency have been reported in post-mortem heart and brain tissues (6,7), we failed to detect any significant enzyme deficiency in cultured skin fibroblasts or skeletal muscle from FRDA patients (4). If one hypothesizes that iron overload is not primitive in FRDA, the question of whether the specific loss of ISP activity is due to an impaired addition or to a progressive release of iron from iron–sulfur clusters then arises. Considering that these clusters are exquisitely sensitive to superoxide radicals (8), the disease

causing mechanism could then consist of a decreased superoxide-scavenging activity in FRDA. We therefore tested the ability of control and FRDA patients' cultured skin fibroblasts to resist the oxidative stress caused by mitochondrial superoxide radical overproduction.

RESULTS

Oligomycin triggers oxidative stress that induces superoxide dismutases (SODs) and loss of mitochondrial membrane iron-containing enzyme activity in control cells

We first devised experimental conditions to endogenously produce superoxides without direct involvement of cell or mitochondrial initial iron content, since this latter might vary between control and FRDA fibroblasts. Superoxides were therefore produced through the monovalent reduction of molecular oxygen by electrons diverted from the respiratory chain by inhibiting ATPase with oligomycin. ATPase inhibition by oligomycin or decreased activity resulting from the NARP mutation in ATPase 6 is known to trigger a high proton motive force across the inner mitochondrial membrane, thus favoring an increased superoxide formation (9,10). The intricate effects of the oligomycin-induced oxidative stress were first studied in control fibroblasts. Increasing superoxide production by oligomycin caused a >4-fold induction of both mitochondrial and cytosolic SOD activities after 16 h in control fibroblasts (Fig. 1). Antimycin A (60 µM), a specific inhibitor of complex III, was also found to induce a 3-fold increase of both mitochondrial and cytosolic SODs in these control cells. We next selected oligomycin for further studies. According to the increase of enzyme activity, a 2–3-fold increase of SOD-1 and SOD-2 mRNA levels by oligomycin was simultaneously observed (Fig. 2). However, the induction of SOD activity failed to protect membrane-bound iron-containing respiratory chain enzymes against the oligomycin-induced stress, while protecting soluble iron-containing aconitase activity (Fig. 3 and Table 1). Indeed, the activity of complex III (but not glycerol-3-phosphate dehydrogenase, which does not contain iron) was markedly reduced in control fibroblasts, resulting in a marked decrease of the quinol-cytochrome *c* reductase (QCCR) to glycerol-3-phosphate dehydrogenase

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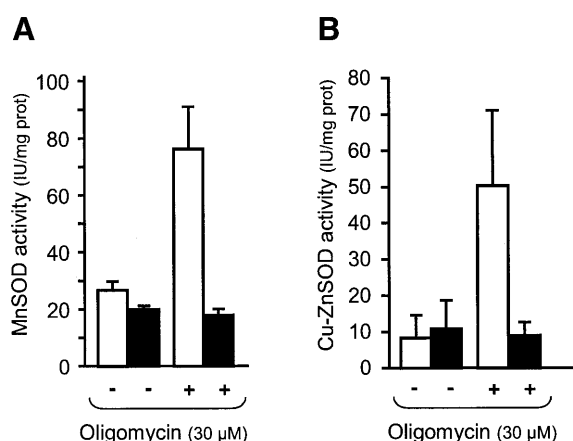


Figure 1. Effect of oligomycin on superoxide dismutase (SOD) activities of control and FRDA fibroblasts. Three independent measurements were performed on three control (open bars) and three FRDA patient (closed bars) fibroblasts. The cells were grown under standard culture conditions (-) or in the presence of 30 μM oligomycin (+) for 16 h. (A) Mitochondrial SOD activity (MnSOD) obtained by inhibiting the cytosolic SOD (Cu-ZnSOD) with 2 mM KCN. (B) Cytosolic SOD activity (Cu-ZnSOD; total SOD minus MnSOD).

activity ratio after 16 h incubation with oligomycin (Fig. 3). Cytochrome *c* oxidase was also found unaffected after 16 h incubation with oligomycin (data not shown). The specific protection of soluble versus membranous enzymes against oxidative stress afforded by SOD was further demonstrated using reduced iron (5 nM FeCl_3) as a pro-oxidant, in an *in vitro* system (Table 2). It was first shown that iron alone brought about a rapid loss of activity of respiratory chain complex II (a membrane-bound superoxide-sensitive iron-sulfur cluster protein) when added to a human heart homogenate. Such a loss of activity could be fully prevented by hydrophobic antioxidants, such as idebenone (60 μM) or ubiquinone (50 μM CoQ_4 , 8 μM CoQ_{10}), as long as succinate was simultaneously added to reduce the quinone. Interestingly enough, adding SOD plus catalase did not afford any protection to this membranous enzyme. When iron was first displaced to the soluble phase by adding a chelator (2 mM EDTA), complex II was fully protected, but soluble aconitase was readily inactivated. Under this condition, adding SOD plus catalase fully protected aconitase. Quinone plus succinate only protected after a 5 min pre-incubation (data not shown). This emphasizes the crucial importance of each antioxidant location *vis-à-vis* the location of the targeted pro-oxidant source and provides a clue to understand why the induction of SOD failed to protect membranous enzyme from the oligomycin-induced stress. A 2 day incubation with oligomycin finally resulted in a 50% lethality in control cells. Most interestingly, adding FeCl_3 (48 μM) plus transferrin (10 μM) to oligomycin in the culture medium fully rescued the cells. This unexpected result demonstrates, but does not explain, the protective role of cell iron against an oligomycin-induced oxidative stress. Bearing in mind the pro-oxidant effect of iron, increasing iron import in case of an oxidative stress appears paradoxical *a priori*. However, recent studies on yeast mutant strains lacking the Cu-ZnSOD have also shown an increased iron import as an adaptation to

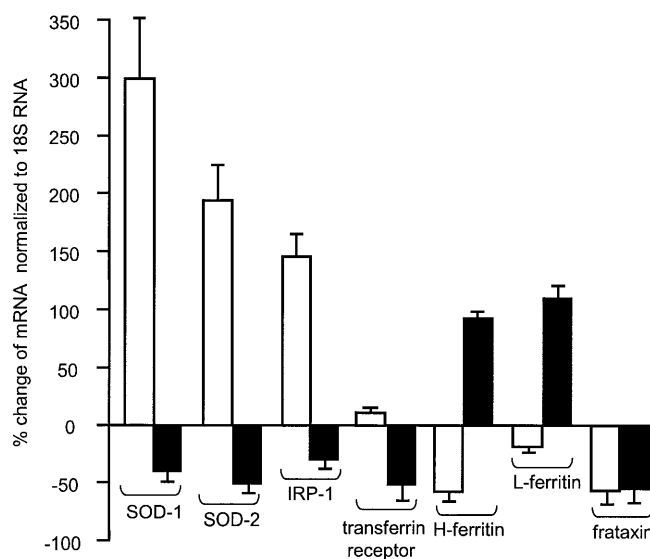


Figure 2. Changes in various gene expressions triggered by oligomycin-induced oxidative stress in control and FRDA fibroblasts. Total RNAs were extracted from three control (open bars) and three FRDA patient (closed bars) fibroblasts grown for 16 h with or without 30 μM oligomycin and estimated by real-time PCR quantification were normalized to the 18S mRNA. Two amplifications were performed with each oligonucleotide pair. Results are expressed as percent of changes of mRNA contents measured in cells grown in the absence of oligomycin.

Table 1. Effect of cell iron and oligomycin-induced oxidative stress on aconitase activity in patient and control fibroblasts

Incubation (16 h)	Aconitase activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	
	Patients ($n = 3$)	Controls ($n = 3$)
-	7.7 ± 0.5	7.6 ± 0.6
Desferoxamine (80 μM)	n.d.	n.d.
Transferrin (10 μM) and FeCl_3 (48 μM)	7.5 ± 0.6	6.6 ± 0.6
Oligomycin (30 μM)	6.7 ± 0.6	9.1 ± 0.9

n.d., not detectable.
Values are means \pm 1 SD.

decreased cellular antioxidant defenses as if the cell machinery was attempting to reconstitute iron-sulfur cluster-containing enzymes that are continuously inactivated by the oxidative stress (11).

Oligomycin-induced oxidative stress affects cellular iron import and storage

Since injuring iron-containing membrane proteins (e.g. iron-sulfur proteins and/or hemoproteins) might increase the demand for cell iron import, we investigated the effect of the oligomycin-induced oxidative stress on iron import and storage in control fibroblasts (Fig. 2). Cell iron homeostasis is under the transcriptional control of several genes while the

Table 2. Effect of pro-oxidant (Fe^{2+}) and anti-oxidant (idebenone, CoQ_4 , SOD) molecules on membrane (respiratory chain complex II) and soluble (aconitase) enzymes in heart homogenates

Condition	Iron-induced loss of enzyme activity (% of initial activity)	
	Complex II	Aconitase
5 nM Fe^{2+}	-72 ± 3	0 ± 0.1
5 nM Fe^{2+} + 6 U SOD + 6 U catalase	-68 ± 3	0 ± 0.3
5 nM Fe^{2+} + 2 mM EDTA	-2 ± 1	-83 ± 3
5 nM Fe^{2+} + 2 mM EDTA + 6 U SOD + 6 U catalase	0 ± 0.1	$+5 \pm 1$
5 nM Fe^{2+} + 60 μM idebenone + 10 mM succinate	-1 ± 0.4	n.m.
5 nM Fe^{2+} + 50 μM CoQ_4 + 10 mM succinate	-1 ± 1	n.m.
5 nM Fe^{2+} + 50 μM CoQ_{10} + 10 mM succinate	-1 ± 0.4	n.m.

CoQ , ubiquinone; SOD, purified MnSOD from *Escherichia coli*; n.m., not measured.

Values are means of three replicates ± 1 SD.

aconitase/iron responsive protein (IRP) ratio is known to exert a post-transcriptional control on several key elements of the iron-import machinery and cellular storage (12). The balance between IRP and its catalytically active form, aconitase, depends upon cytosolic iron content. High iron shifts the equilibrium towards aconitase, whereas iron depletion favors the IRP form. The latter binds the iron responsive elements (IREs) located at the 3' or 5' ends of untranslated regions of several mRNAs for proteins involved in iron homeostasis. IRP/IRE interactions thus stabilize the transferrin receptor mRNA through binding at the 3' end, while binding of IRP to the 5' end of the ferritin reduces its translation (12). IRP-1 mRNA, but not aconitase activity (Table 1), was consistently increased in control cells cultured for 16 h with 30 μM oligomycin (Fig. 2). The IRP:aconitase ratio was therefore shifted towards more iron being imported following oligomycin-induced oxidative stress in control fibroblasts. Accordingly, the amount of transferrin-receptor mRNA known to be stabilized by IRP was slightly increased, while H- and L-ferritin mRNAs were decreased resulting in a reduced iron storage capacity of control cells. Noticeably, a parallel decrease of ferritin and frataxin mRNAs was observed under oligomycin-induced stress conditions, which might denote that these proteins play a similar role of iron storage/detoxification in cytosol and mitochondria, respectively, as proposed previously (13). However, the recent report of the occurrence of a specific ferritin-like protein in human erythroblast mitochondria rather supports the idea that frataxin plays a different role in the mitochondria (14).

Free cytosolic iron does not mediate SOD induction resulting from oligomycin-induced oxidative stress

Because oligomycin-induced oxidative stress affected cell iron import and storage, we finally studied the potential role of free cytosolic iron in both mediating SOD signaling and oxidative

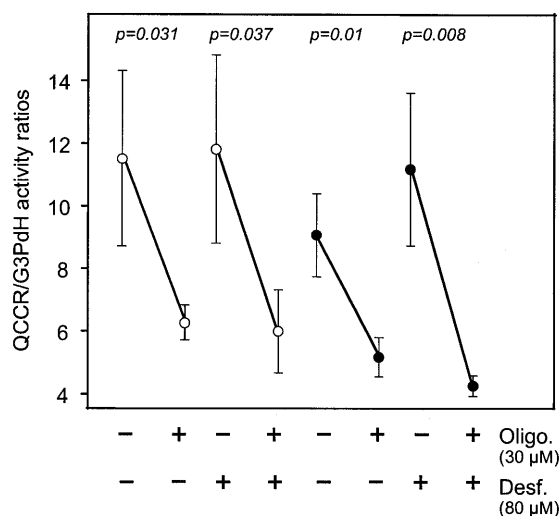


Figure 3. Effect of oligomycin on quinol-cytochrome *c* reductase (QCCR; complex III) to glycerol-3-phosphate dehydrogenase activity ratio in control and FRDA fibroblasts. Enzyme activities were measured as described in Materials and Methods in cells grown in the absence (-) or in the presence (+) of 30 μM oligomycin (Oligo.) for 16 h and/or 80 μM desferoxamine (Desf.) on three control (open circles) and three FRDA patient (closed circles) fibroblasts.

stress by depleting cytosolic iron by desferoxamine (80 μM , 16 h). On one hand, desferoxamine did not significantly decrease either basal SOD activity or SOD induction triggered by oligomycin (not shown), suggesting that cytosolic iron content does not signal SOD induction in human fibroblasts. On the other hand, the loss of iron-containing membrane protein activity was not counteracted by desferoxamine, indicating that free cytosolic iron was not mediating superoxide toxicity under oligomycin-induced oxidative stress conditions (Fig. 3).

Taken together, these data suggest that ATPase inhibition by oligomycin normally results in a superoxide overproduction and a potent SOD induction. Yet, the enhanced SOD activity was unable to protect iron-containing membrane proteins against an oligomycin-induced oxidative stress. The loss of iron-containing membrane protein activity, in turn, increased cell iron demand and iron import and decreased iron storage capacity (Fig. 4).

Oligomycin does not induce SODs or iron import in FRDA fibroblasts

We first carried out a detailed comparison of mitochondrial properties between control and FRDA fibroblasts that failed to reveal any significant difference (data not shown). In particular, similar cell respiration and substrate oxidation rates were measured in both control and FRDA fibroblasts, and an identical sensitivity to oligomycin was measured using succinate as a substrate, associated with high respiratory control values (>6). The activity of the respiratory chain complexes II–IV and the tricarboxylic acid cycle enzymes (aconitase, isocitrate dehydrogenase, citrate synthase and fumarase) were found to be similar in both cell types. Accordingly, cultured skin fibroblasts from controls and patients were found to grow similarly

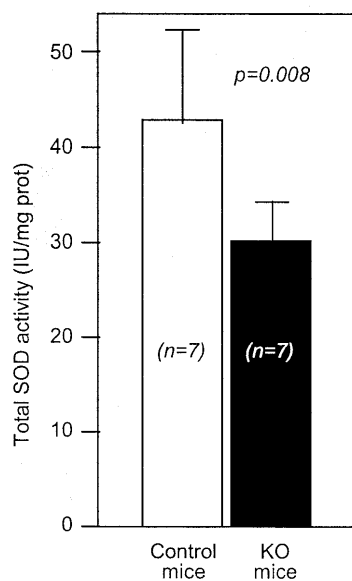


Figure 4. Decreased SOD activity in the heart of 7–10-week-old conditional frataxin knockout mice. Total SOD activity (mitochondrial MnSOD plus cytosolic CuZnSOD) was found to be significantly lower in the heart of 7–10-week-old KO mice (closed bar) compared with age-matched control mice (open bar). Because it predictably results in an increased oxidative stress, the severe deficiency of the respiratory chain previously documented in these KO mice (5) should trigger an even higher SOD induction than in control mice.

in a culture medium selective for respiratory-competent cells, i.e. devoid of glucose, uridine and pyruvate.

We next studied the effect of oligomycin-induced stress in FRDA fibroblasts. In contrast with control fibroblasts, FRDA fibroblasts grown for 16 h with oligomycin failed to display any induction of the mitochondrial or cytosolic SOD activity (Fig. 1). Similarly, antimycin A (60 μ M) failed to induce mitochondrial or cytosolic SODs in patient fibroblasts. Accordingly, SOD-1 and SOD-2 mRNA levels did not increase, and even slightly decreased in FRDA fibroblasts after a 16 h incubation with oligomycin (Fig. 2). As a result, patient's cells were significantly more sensitive to the oligomycin-induced oxidative stress than controls, cell death averaging 80% after 16 h incubation with oligomycin. Adding iron (48 μ M FeCl₃ plus 10 μ M transferrin) fully rescued FRDA fibroblasts as observed in controls, while a 100% cell death was observed when these cells were grown with oligomycin plus desferoxamine (80 μ M; data not shown). It is noteworthy that cell iron deprivation induced by desferoxamine also resulted in a reversible loss of aconitase activity in FRDA fibroblasts (Table 1), denoting that the machinery controlling cell iron import and storage was normally functioning in FRDA cells. Accordingly, a quite similar sensitivity to iron was observed in control and FRDA fibroblasts grown in RPMI medium added with 10 μ M transferrin and/or 48 μ M FeCl₃. After a 72 h incubation, no difference in cell death could be observed either between control and FRDA fibroblasts or between FRDA fibroblasts grown in basal and supplemented media (transferrin and/or iron).

However, at variance with controls, IRP1 and transferrin mRNAs decreased in FRDA fibroblasts exposed to an oligomycin-induced oxidative stress, while both H- and L-ferritin mRNAs increased (Fig. 2). Thus, in contrast with control cells, FRDA fibroblasts reacted to the oxidative stress by decreasing iron-import machinery and lowering iron-storage capacity. In contrast, frataxin mRNA levels were decreased upon oligomycin-induced oxidative stress in both FRDA and control fibroblasts (Fig. 1). Noticeably, frataxin mRNA levels in cultured skin fibroblasts from the three FRDA patients vary from 20 to 25% of controls, a higher value than those measured in skeletal muscle, cerebral and cerebellar cortex, and lymphoblastoid cell lines of most FRDA patients with similar size GAA triplet expansions in the frataxin gene (1).

SODs are not induced in the respiratory chain-deficient, hypertrophic heart of conditional frataxin knockout mice

The results obtained on human cultured skin fibroblasts suggest that, for as yet unknown reasons, FRDA cells submitted to an oligomycin-induced oxidative stress failed to normally increase SOD activity and iron import, and to decrease iron storage capacity, despite parallel loss of membrane-bound iron-containing enzyme activity. In keeping with this, it is worth remembering that no increased SOD transcripts were detected in a mutant yeast strain deleted for the yeast frataxin homolog, YFH1 (15). We therefore next investigated heart homogenates from conditional frataxin knockout mice with progressive hypertrophic cardiomyopathy, which presented a severely deficient activity of the ISPs (aconitases and succinate-ubiquinone oxidoreductase) in the heart muscle (5). Despite severe respiratory chain defect, lower SOD activity was found in hearts of 10-week-old knockout mice than in age-matched control mice (Fig. 4).

Human cell model for high-throughput assay of drugs in FRDA

So far, no FRDA patient cells (cultured fibroblasts or lymphoblastoid cell lines) were found to present sufficient phenotype for testing drug efficiency. We therefore tried to take advantage of the hypersensitivity of FRDA patient cells to oxidative stress to devise conditions allowing a simple and rapid assay of drug efficiency. As established above, patient cells displayed a higher sensitivity to oligomycin-treatment than control cells. Yet, both cell types ultimately died, although with a distinct kinetic. We therefore studied the differential effect of several other pro-oxidant molecules [H₂O₂, *tert*-butyl hydroperoxide (*t*-BH), butyl sulfonide, trifluoroperazine, menadione] on both attached cells and on cell plating efficiency. While we consistently found a higher sensitivity of patient attached cells to these various pro-oxidant compounds compared with control, the differences were not quantitatively reproducible and/or discriminating enough to provide a confident tool for testing protecting molecules. In contrast, the study of plating efficiency revealed a spectacular difference between patient and control cells (Table 3). While control cells were readily able to attach (>95%) in the presence or absence of 3 mM *t*-BH after 24 h, <3% of patient cells were found plated at a similar time point in the presence of *t*-BH. This provides a simple parameter (the number of attached

Table 3. Effect of *tert*-butyl hydroperoxide (3 mM) on plating efficiency (24 h) of FRDA patient and control cultured skin fibroblasts

Condition	Plating efficiency of cultured skin fibroblasts at 24 h	
	FRDA	Control
RPMI 1640 + 10% calf serum	100	100
RPMI 1640 + 10% calf serum + 3 mM <i>tert</i> -butyl hydroperoxide	2 ± 0.3 ^a	97 ± 3 ^a

^aPercent of plating in the absence of *tert*-butyl hydroperoxide. Values are means of three replicates ± 1 SD.

cells) to rapidly screen molecule libraries and possibly identify promising new compounds for future drug therapy.

DISCUSSION

Taken together, the above data support the view that decreased or absent frataxin impairs early antioxidant defenses with no SODs induction resulting in higher cell lethality in response to oxidative stress (Fig. 5B). In keeping with this, lethality by both apoptosis and necrosis, but no detectable iron accumulation, was noted in mouse embryos homozygous for a frataxin exon 4 deletion (16). The mechanism by which absent or decreased frataxin results in disabled early antioxidant defenses, which will require delineation of the poorly known SOD signaling in human cells, remains to be established. Nevertheless, SOD signaling through a release of free mitochondrial iron to the cytosol should however be disregarded, as decreasing cytosolic iron by desferoxamine did not hamper oligomycin-induced SOD induction in control cells. Yet, our observation may account for the previously reported increased sensitivity of FRDA fibroblasts to exogenous pro-oxidants (H₂O₂) (17) that could hardly be ascribed to increased cell or mitochondrial iron content, which did not significantly differ between control and FRDA cells. Because no cell models for FRDA are available so far, we also took advantage of the hypersensitivity of patient cells to oxidative stress to devise conditions which should allow high-throughput assay for potential identification of promising molecules in this disease.

The primary involvement of superoxide injury is consistent with a number of observations in FRDA. First, neurons and cardiomyocytes, the most targeted cells in FRDA (1), have limited antioxidant defenses and have been accordingly shown to be highly sensitive to a decreased SOD activity in several models (18). Secondly, *in vitro* studies on cultured cardiomyocytes have shown that superoxide overproduction is sufficient to cause cell hypertrophy (19), which provides a mechanism for the heart hypertrophy in FRDA and mouse models. Several reports demonstrate an increased oxidative stress in FRDA, including higher levels of plasma malondialdehyde (20) and presence of urinary-excreted oxidized DNA (21). An early implication of superoxides in FRDA could also explain the overlapping neurological presentation of FRDA and inherited vitamin E deficiency, despite a lower incidence of heart disease in the latter (22). Finally, a primary role of superoxide

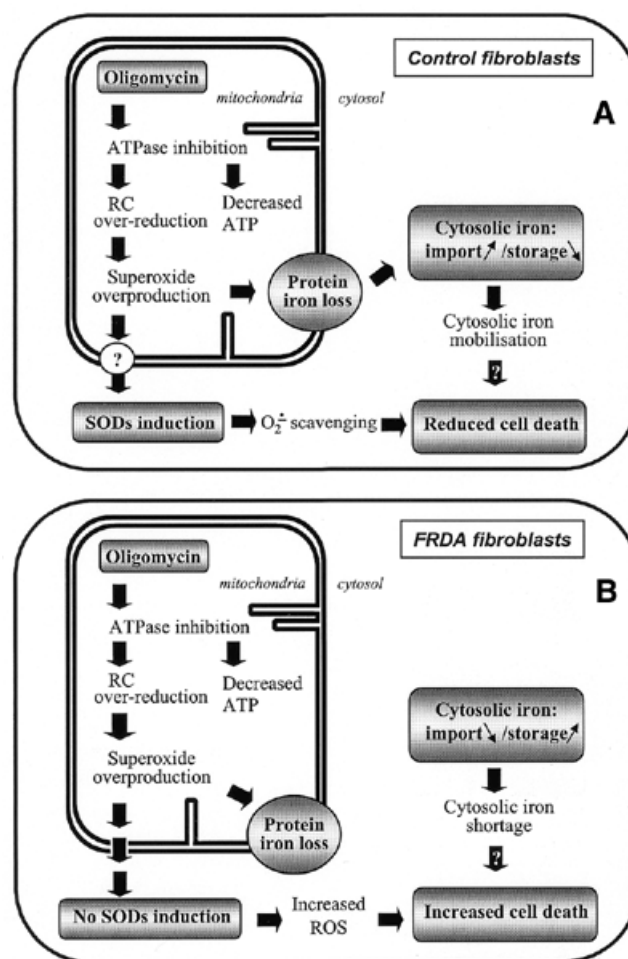


Figure 5. Response of control (A) and FRDA fibroblasts (B) to an oligomycin-induced oxidative stress. (A) Oligomycin-induced ATPase inhibition in control fibroblasts triggers both SOD induction and cytosolic iron mobilization, slowing down the cell death process. (B) Decreased frataxin content in FRDA fibroblasts impaired both SOD induction and cytosolic iron import, ultimately leading to massive cell death. The role of frataxin in SOD induction and the role of cytosolic iron in protecting against cell death remain to be elucidated.

in the pathogenesis of FRDA might account for the efficiency of idebenone, an antioxidant short-chain homolog of ubiquinone, in decreasing heart hypertrophy and urinary-excreted oxidized DNA in FRDA patients (21,23).

MATERIALS AND METHODS

Cell cultures

Fibroblast cultures were established from skin biopsies of three controls (aged 20–24 years) and three FRDA patients (aged 19–24 years). The size of GAA triplet expansion in the first intron of the frataxin gene was established by Southern blot and was roughly similar in the three patients (2.1/2.7, 2.3/3 and 3/3 kb for patients 1, 2 and 3, respectively). Frataxin mRNA levels in cultured skin fibroblasts from the three FRDA patients varied from 20 to 25% of controls. The fraction of

residual frataxin transcript present in the FRDA fibroblasts compared with control cells (normalized to 18S RNA) was estimated using quantitative PCR (see below, Molecular analyses). Cells were grown in RPMI 1640 (Life Technologies SARL, Cergy Pontoise, France) supplemented with glutamax (446 mg/l), 10% fetal calf serum, 100 µg/ml streptomycin, 100 IU/ml penicillin, 200 µM uridine and 2.5 mM sodium pyruvate, at 37°C under standard conditions (24). For the sake of comparison, all studies were carried out on cells harvested after 9–10 population doublings.

Cell iron content, cell death, respiration and enzyme activities

The cell iron content was spectrophotometrically measured after iron extraction, reduction by dithionite and reaction with bathophenanthroline disulfonate in 1 ml of Tris-HCl buffer (0.1 M, pH 7.3) as described previously (25). Cell death was estimated each 24 h by fibroblast permeability to Trypan blue (0.5%). Intact fibroblast respiration and mitochondrial substrate oxidation by digitonin-permeabilized fibroblasts were polarographically studied (26). Heart homogenates were prepared as described previously (23). The activity of the respiratory chain complexes II–IV and the tricarboxylic acid cycle enzymes (aconitase, isocitrate dehydrogenase, citrate synthase and fumarase) were spectrophotometrically measured as described previously (4,26). Total SOD activity (Cu-ZnSOD plus MnSOD activities) was determined by monitoring the autooxidation of pyrogallol at 420 nm (27). Pre-incubation of the sample with 2 mM cyanide for 20 min at room temperature was used to inhibit Cu-ZnSOD, allowing the determination of the specific activity of the MnSOD, and by subtraction of this latter from the total SOD activity, estimation of the Cu-ZnSOD activity. Activities were assayed on supernatant (10 000 g for 5 min) of freeze-thaw extracts treated with Triton X100 (1% final concentration) in 50 mM KH₂PO₄ (pH 7.8). Alternatively, the SOD measurements were carried out on ethanol-chloroform (17%/0.11% final) extracts resulting in similar values. SOD activities were expressed as IU/mg protein.

Molecular analyses

Total RNAs were extracted by treating 10⁶ cells with 1 ml of Trizol reagent according to the manufacturer's recommendations (Total RNA isolation reagent, Life Technologies SARL). Reverse transcription was performed on 5 µg of DNase-treated RNAs using random hexamer primers (GeneAmp-RNA PCR core kit, Roche Molecular System, Branchburg, NJ). SOD1, SOD2, IRP1, transferrin receptor, H- and L- ferritin, frataxin and 18S RNA transcripts were quantified by real-time PCR with the LightCycler system using the FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) on 8 ng of reverse transcribed RNAs. Oligonucleotide sequences were determined with Primer Express software (Perkin Elmer, Warrington, UK): SOD1, 5'-tgggcaaagtggaatga-3' and 5'-caccacaagcacaagcactc-3'; SOD2, 5'-ggacaaacctcagccctaacg-3' and 5'-tttgatgctccagcaactc-3'; IRP1, 5'-tatggctgcacatgacatgcat-3' and 5'-cacaggttcaggtaaaggccc-3'; transferrin receptor, 5'-cacacctggattccctcctt-3' and 5'-tgaccgagatggtgaaactg-3'; H-ferritin, 5'-agcacacctggagacagt-3' and 5'-ggaaattagcccgagccttag-3'; L-ferritin, 5'-ttctatttcgac-

cgcgatgat-3' and 5'-cagttcgcggaagaagtgg-3'; frataxin, 5'-gcccaagcagcctcaattgt-3' and 5'-cccgcgcttctaaaattcta-3'; 18S RNA, 5'-cgccgctagagtgaaattc-3' and 5'-cttccgctctgctcgtctt-3'. PCR amplifications were performed by an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and a last extension at 72°C for 15 s.

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