Frataxin activates mitochondrial energy conversion and oxidative phosphorylation


*Joslin Diabetes Center, Harvard Medical School, Research Division, Boston, MA 02215; and †Beth Israel Deaconess Medical Center, Division of Endocrinology and Metabolism, Department of Medicine, Boston, MA 02215

Contributed by C. Ronald Kahn, August 22, 2000

Friedreich’s ataxia (FA) is an autosomal recessive disease caused by decreased expression of the mitochondrial protein frataxin. The biological function of frataxin is unclear. The homologue of frataxin in yeast, YFH1, is required for cellular respiration and was suggested to regulate mitochondrial iron homeostasis. Patients suffering from FA exhibit decreased ATP production in skeletal muscle. We now demonstrate that overexpression of frataxin in mammalian cells causes a Ca2+-induced up-regulation of tricarboxylic acid cycle flux and respiration, which, in turn, leads to an increased mitochondrial membrane potential (ΔΨm) and results in an elevated cellular ATP content. Thus, frataxin appears to be a key activator of mitochondrial energy conversion and oxidative phosphorylation.

Materials and Methods

Cell Culture. 3T3-L1 preadipocytes were obtained from American Type Culture Collection (lot no. F-154099) and cultured in DMEM with 4.5 g D-glucose/liter (BRL) and 10% heat inactivated bovine calf serum (HyClone). Transfected cells were maintained in media as above containing 2 mg/liter puromycin (Sigma). Differentiation was induced as described previously (27) by using media containing puromycin.

Constructs and Transfection. Human frataxin cDNA was cloned into BamHI and EcoRI sites of pBluescript KS (Stratagene) after PCR amplification with specific primers carrying the appropriate restriction sites at their 5’ ends from reverse transcribed human heart mRNA (CLONTECH). After exclusion of PCR-induced random mutations by sequencing, the full-length cDNA was subcloned into BamHI and EcoRI sites of pBabePuro, a retroviral expression plasmid (28), generating pBabePuro-hFrataxin. This construct was transfected into ecotrophic Phoenix producer cells, and the supernatant was used to infect 3T3-L1 cells, which were transferred into puromycin-containing media 48 h after initiation of infection. Control cells were generated similarly, except for the pBabePuro-hFrataxin was replaced by the original pBabePuro as a vehicle.

Northern blotting was done according to standard procedures (29) by using QuikHyb solution (Stratagene) and hybridization at 68°C with a mouse frataxin probe generated by PCR from the 3’ half of the cDNA (for Fig. 1a) and an α2 probe (a kind gift of B. Spiegelman, Dana Farber Cancer Institute) for Fig. 1e. High stringency washes were performed at 45°C and 60°C, respectively. RNA samples were extracted from confluent tissue culture dishes by using Ultraspec (Biotecx Laboratories, Hous-
RNA, thus the intensity of the transgenic human frataxin mRNA per lane was determined photometrically at 540 nm.

Glycerol, which was converted into glycerol-1-phosphate, which in turn was oxidized by dihydroxyacetone phosphate and hydrogen peroxide. The reduction of the latter was quantified photometrically at 540 nm.

Triglyceride quantification was performed by using the GPO (glucose oxidase-peroxidase) method (31). Glycerol quantification was performed by using the GPO kit (Sigma). Briefly, sonicated cellular extracts containing triglycerides were treated with lipoprotein lipase generating free fatty acids, which were quantified enzymatically (32).

Mitochondrial respiration was quantified by transferring mitochondria (0.5 mg mitochondrial protein) in a 100-ml medium (as above) into a 1-ml Clark-type oxygen electrode chamber at 37°C (36). After recording the basal respiration, respiration was either detuned cells into a 1-ml Clark-type oxygen electrode chamber at 37°C (36). After recording the basal respiration, respiration was then measured in the presence of excess Mn2+(final concentration: 2 nmol mitochondrial protein). One minute after addition of rotenone and succinate, Ca2+-uptake was initiated by addition of 200 mM Ca2+ with 4Ca2+ (2000 dpm/nmol) as tracer. To induce Ca2+-efflux, 5 mM EGTA (Sigma) was added 5 min after addition of Ca2+ (42). At the required times, 50-μl aliquots were withdrawn, vacuum filtered through Millipore filters (0.45 μm pore size), and rinsed twice with 1 ml of cold MSH buffer (as above). The radioactivity remaining on the filters was determined in a liquid scintillation counter.

Mitochondrial membrane potential was measured by rhodamine 123 (Sigma) staining performed in the tissue culture media for 10 min at a final concentration of 10 μg/liter at 37°C. Cells were then washed twice for 2 min each with phenol red-free medium and subjected to digital fluorescence microscopy (Zeiss Axioinvert AXS100TV) at 488 nm excitation wavelength. Quantification of fluorescence was performed by using a software package openlab v2.0, based on three independent slides for each cell line evaluating three areas of each slide.

Protein content was measured photometrically by using the Bradford method (43).

Statistical analyses were performed by using spss for Windows, release 9.0.0, applying independent t tests assuming unequal variances. Differences were considered significant whenever P < 0.05. Error bars are depicted as plus two standard error means (+2 SEM).

Results and Discussion

Overexpression of Frataxin Increases Triglyceride Synthesis.

We have created a 3T3-L1 adipocyte model (44) overexpressing human frataxin by means of retroviral transduction (Fig. 1a). When these cells were differentiated into adipocytes by using a standardized protocol (27), they accumulated triglyceride in a time-dependent manner (Fig. 1b). At the time of full differentiation, 14C-labeled glucose was quantified per time in 340 nm.

Glucose oxidation assay was performed as described (38), except that hydroxylamine was replaced by KOH for capturing radiolabeled 14CO2.

Cellular ATP was measured by using a luciferase-based assay for cellular extracts (Sigma).

Southern blotting (29) was performed by using a probe for the mitochondrially encoded gene cytochrome c oxidase subunit II (a gift of Zhidan Wu, Dana Farber Cancer Institute), and the high stringency wash was done at 55°C. DNA samples were extracted from confluent dishes by proteinase K digestion and subsequent precipitation in 2-propanol. Precipitates were redisolved in 1× restriction buffer appropriate for the endonuclease PstI (Roche Molecular Biochemicals) and subjected to PstI digestion overnight. Loading was 10 μg DNA per lane, which was confirmed by optical density and computerized comparison of ethidium bromide-stained 0.9% agarose gels.

Isolation of mitochondria was performed by dounce homogenization of cells and a subsequent differential centrifugation method (39). Mitochondrial pellets were washed twice in a buffer containing 210 mM D-mannitol, 70 mM sucrose, and 5 mM K+-Hepes, pH 7.4 (MSH buffer). The final pellet was resuspended in MSH buffer and kept on ice for further experiments.

4Ca2+-uptake of isolated mitochondria was monitored by the radioisotope technique (40). Briefly, mitochondria (0.5 mg/ml) were incubated at 25°C in 1 ml of MSH buffer (as above) with continuous stirring. A total of 5 μM rotenone (Sigma) and 2.5 mM potassium succinate (prepared from succinic acid and KOH; both from Sigma) were subsequently added to energize the mitochondria. Where indicated, ruthenium red (Sigma), a specific inhibitor of mitochondrial Ca2+-uptake (41), was added (final concentration: 2 nmol/mg mitochondrial protein). One minute after addition of rotenone and succinate, Ca2+-uptake was initiated by addition of 200 μM Ca2+ with 4Ca2+ (2000 dpm/nmol) as tracer. To induce Ca2+-efflux, 5 mM EGTA (Sigma) was added 5 min after addition of Ca2+ (42). At the required times, 50-μl aliquots were withdrawn, vacuum filtered through Millipore filters (0.45 μm pore size), and rinsed twice with 1 ml of cold MSH buffer (as above). The radioactivity remaining on the filters was determined in a liquid scintillation counter.

Mitochondrial membrane potential was measured by rhodamine 123 (Sigma) staining performed in the tissue culture media for 10 min at a final concentration of 10 μg/liter at 37°C. Cells were then washed twice for 2 min each with phenol red-free medium and subjected to digital fluorescence microscopy (Zeiss Axioinvert AXS100TV) at 488 nm excitation wavelength. Quantification of fluorescence was performed by using a software package openlab v2.0, based on three independent slides for each cell line evaluating three areas of each slide.

Protein content was measured photometrically by using the Bradford method (43).

Statistical analyses were performed by using spss for Windows, release 9.0.0, applying independent t tests assuming unequal variances. Differences were considered significant whenever P < 0.05. Error bars are depicted as plus two standard error means (+2 SEM).

Results and Discussion

Overexpression of Frataxin Increases Triglyceride Synthesis.

We have created a 3T3-L1 adipocyte model (44) overexpressing human frataxin by means of retroviral transduction (Fig. 1a). When these cells were differentiated into adipocytes by using a standardized protocol (27), they accumulated triglyceride in a time-dependent manner (Fig. 1b). At the time of full differentiation, 14C-labeled glucose was quantified per time in 340 nm.

Glucose oxidation assay was performed as described (38), except that hydroxylamine was replaced by KOH for capturing radiolabeled 14CO2.

Cellular ATP was measured by using a luciferase-based assay for cellular extracts (Sigma).

Southern blotting (29) was performed by using a probe for the mitochondrially encoded gene cytochrome c oxidase subunit II (a gift of Zhidan Wu, Dana Farber Cancer Institute), and the high stringency wash was done at 55°C. DNA samples were extracted from confluent dishes by proteinase K digestion and subsequent precipitation in 2-propanol. Precipitates were redisolved in 1× restriction buffer appropriate for the endonuclease PstI (Roche Molecular Biochemicals) and subjected to PstI digestion overnight. Loading was 10 μg DNA per lane, which was confirmed by optical density and computerized comparison of ethidium bromide-stained 0.9% agarose gels.

Isolation of mitochondria was performed by dounce homogenization of cells and a subsequent differential centrifugation method (39). Mitochondrial pellets were washed twice in a buffer containing 210 mM D-mannitol, 70 mM sucrose, and 5 mM K+-Hepes, pH 7.4 (MSH buffer). The final pellet was resuspended in MSH buffer and kept on ice for further experiments.

4Ca2+-uptake of isolated mitochondria was monitored by the radioisotope technique (40). Briefly, mitochondria (0.5 mg/ml) were incubated at 25°C in 1 ml of MSH buffer (as above) with continuous stirring. A total of 5 μM rotenone (Sigma) and 2.5 mM potassium succinate (prepared from succinic acid and KOH; both from Sigma) were subsequently added to energize the mitochondria. Where indicated, ruthenium red (Sigma), a specific inhibitor of mitochondrial Ca2+-uptake (41), was added (final concentration: 2 nmol/mg mitochondrial protein). One minute after addition of rotenone and succinate, Ca2+-uptake was initiated by addition of 200 μM Ca2+ with 4Ca2+ (2000 dpm/nmol) as tracer. To induce Ca2+-efflux, 5 mM EGTA (Sigma) was added 5 min after addition of Ca2+ (42). At the required times, 50-μl aliquots were withdrawn, vacuum filtered through Millipore filters (0.45 μm pore size), and rinsed twice with 1 ml of cold MSH buffer (as above). The radioactivity remaining on the filters was determined in a liquid scintillation counter.

Mitochondrial membrane potential was measured by rhodamine 123 (Sigma) staining performed in the tissue culture media for 10 min at a final concentration of 10 μg/liter at 37°C. Cells were then washed twice for 2 min each with phenol red-free medium and subjected to digital fluorescence microscopy (Zeiss Axioinvert AXS100TV) at 488 nm excitation wavelength. Quantification of fluorescence was performed by using a software package openlab v2.0, based on three independent slides for each cell line evaluating three areas of each slide.

Protein content was measured photometrically by using the Bradford method (43).

Statistical analyses were performed by using spss for Windows, release 9.0.0, applying independent t tests assuming unequal variances. Differences were considered significant whenever P < 0.05. Error bars are depicted as plus two standard error means (+2 SEM).

Results and Discussion

Overexpression of Frataxin Increases Triglyceride Synthesis.

We have created a 3T3-L1 adipocyte model (44) overexpressing human frataxin by means of retroviral transduction (Fig. 1a). When these cells were differentiated into adipocytes by using a standardized protocol (27), they accumulated triglyceride in a time-dependent manner (Fig. 1b). By the time of full differentiation at day 9, cells constitutively overexpressing frataxin accumulated 1.8 times more triglyceride (P = 0.015) (Fig. 1c). To confirm this observation by an independent method, the rate of triglyceride synthesis from 14C-labeled glucose in the media was quantified (Fig. 1d). This assay revealed a parallel increase in 14C-labeled organic soluble material in those cells overexpressing frataxin (P = 0.032). Northern blot analysis for aP2 mRNA, a marker of terminal adipocyte differentiation, demonstrated a 2.2-fold increase when normalized for total RNA (Fig. 1e and f), consistent with the increased triglyceride accumulation as described above. These observations may be explained by an increase in energy conversion as well as by an activation of the cellular differentiation machinery (as indicated by Fig. 1e).

Frataxin Regulates Krebs Cycle Activity.

To investigate whether frataxin leads to an increase in fat storage in adipocytes by causing an increase in uptake of energy or a more efficient conversion of glucose into lipid, we determined the flux of glucose and its tricarboxylic metabolites in this system. Cells overexpressing frataxin demonstrate a 1.5-fold increase of the uptake of nonmetabolizable 2-deoxyglucose, both in the presence and absence of insulin (P = 0.044 and 0.028, respectively) (Fig. 2a). Although the storage of glycogen in these cells was unchanged (data not shown), lactate accumulation, an end-point
of anaerobic glycolysis, was decreased by 75% in cell lysates ($P = 0.002$) (Fig. 2b) and in the tissue culture media (data not shown) of the cells overexpressing frataxin. The decreased lactate levels together with increased glucose uptake and normal glycogen content indicate an elevated pyruvate metabolism. The rate-limiting step in pyruvate conversion is activity of the PDH complex. Previous studies have suggested that PDH activity is reduced in FA patients (48). Conversely, the activity of PDH was increased 1.7-fold in cells overexpressing frataxin ($P = 0.002$) (Fig. 2c), consistent with an increase in acetyl-CoA in these cells.

Acetyl-CoA is normally metabolized in the tricarboxylic acid (TCA) cycle, but is shuttled into the cytosol to be metabolized into triglycerides when synthesized in amounts exceeding the capacity of the TCA cycle (consistent with the results in Fig. 1). Therefore, we hypothesized that TCA cycle activity was maximized in the frataxin overexpressing cells. This hypothesis is supported by the finding that the activity of ICDH was up-regulated 1.8-fold ($P = 0.001$) (Fig. 2d). This is consistent with previous reports (45) suggesting increased activity of another TCA cycle enzyme, 2-ketoglutarate dehydrogenase, in tissues of FA patients. Furthermore, when cells were grown in media containing radioactively labeled $^{14}$C-glucose, frataxin overexpressing cells showed a 1.8-fold increase in $^{14}$CO$_2$ release compared with control cells ($P = 0.027$), also indicating an increased TCA cycle activity (Fig. 2e).

**Frataxin Increases Mitochondrial Ca$^{2+}$ Uptake.** One potential common denominator that might account for activation of PDH and ICDH would be an elevation in mitochondrial Ca$^{2+}$ content.
since both of these enzymes, as well as the mitochondrial dehydrogenases, are activated by Ca$^{2+}$ (46). To test this hypothesis in viable mitochondria, we measured calcium transport into isolated mitochondria as previously described (47). Dynamic $^{45}$Ca$^{2+}$-uptake was increased by 2.2-fold ($P < 0.001$) (Fig. 2f, right pair of bars), an effect which could be blocked by prein- cubation with ruthenium red (Fig. 2f, left pair of bars), a specific blocker of the mitochondrial calcium uniporter. Furthermore, the specific uptake could be reversed by using the Ca$^{2+}$-chelator EGTA (data not shown). Thus, overexpression of frataxin elevates mitochondrial Ca$^{2+}$ content potentially leading to increased TCA cycle activity as described above (Fig. 2 c–e). This elevated Ca$^{2+}$ content might additionally indicate an increase in mitochondrial membrane potential ($\Delta \psi_{\text{mt}}$) because Ca$^{2+}$-transport into mitochondria is a secondary process driven by electrochemical forces (48).

**Frataxin Activates Mitochondrial Respiration in Mammalian Cells.** Previous studies in yeast suggest a decrease in mitochondrial respiration following inactivation of the frataxin homologue YFF1 (9, 18). To confirm these findings in a reciprocal model, we quantified mitochondrial respiration in the 3T3-L1 cells overexpressing frataxin. Indeed, oxygen consumption was increased by more than 2-fold in cells overexpressing frataxin when compared with control cells ($P = 0.018$) (Fig. 3 a and b, left pair of bars). These data are consistent with the above-mentioned observations in yeast. To determine whether this increase in respiration in our mammalian model could be explained by an elevated mitochondrial capacity, mitochondrial respiration was uncoupled in the cells by treatment with FCCP. Interestingly, oxygen uptake was significantly increased in cells overexpressing frataxin compared with control cells following this treatment ($P = 0.022$) (Fig. 3 a and b, right pair of bars), suggesting increased electron transport activity in the cells overexpressing frataxin.

**Frataxin Elevates the Mitochondrial Membrane Potential.** Increased cellular respiration indicates an activated electron transport chain within the inner mitochondrial membrane, suggesting the presence of an elevated mitochondrial membrane potential ($\Delta \psi_{\text{mt}}$) in cells overexpressing frataxin. Although the biochemical function of frataxin is widely unknown, the protein has been demonstrated to be associated with mitochondrial membranes (9). To evaluate whether $\Delta \psi_{\text{mt}}$ is increased in cells overexpressing frataxin, we stained the cells with the fluorescent rhodamine 123, a dye whose accumulation in mitochondria is directly proportional to $\Delta \psi_{\text{mt}}$ (49). Rhodamine-stained cells overexpressing frataxin showed a increased fluorescence of mitochondria when compared with control cells (Fig. 3c). On computerized quantitation, the intensity was increased 2.5-fold in cells overexpressing frataxin, indicating an elevated $\Delta \psi_{\text{mt}}$ ($P = 0.001$) (Fig. 3d).

**Frataxin Activates OXPHOS.** We and others have described decreased ATP production in postexercise skeletal muscle in FA patients (6, 7). The reasons for this observation are unknown. The findings of an elevated $\Delta \psi_{\text{mt}}$ in cells overexpressing frataxin might provide an explanation for the observations in humans. Because $\Delta \psi_{\text{mt}}$ is known to be the driving force of ATP-synthesis by the enzyme F$_{1}$F$_{0}$-ATPase, and $\Delta \psi_{\text{mt}}$ appears to be elevated subsequent to overexpression of frataxin, we hypothe- sized that this might lead to an increase in ATP content. Indeed, spectrophotometric quantitation of cellular ATP content revealed a 1.9-fold increase ($P = 0.015$) (Fig. 3e). To exclude that the elevation in mitochondrial capacity and in OXPHOS of frataxin overexpressing cells could be explained by an increased number of mitochondria, we quantified the relative number of these organelles by using Southern blotting for cytochrome c oxidase subunit II, a gene exclusively encoded in the mitochondrial DNA (Fig. 3f). This approach showed no difference in the amount of mitochondria, an observation consistent with previously published data obtained in muscle extracts from FA patients (45). Furthermore, Western blot analysis of cytochrome c content in the mitochondrial fractions of both cell lines indicated no difference in mitochondrial biogenesis (data not shown).

Taken together, the data suggest that frataxin is an activator of OXPHOS in eukaryotic cells. Because the identification of frataxin as the site of genetic basis for FA, considerable effort has been made to explain the molecular pathogenesis of this disorder. Previous data obtained in a yeast knock-out model for the frataxin homologue YFF1 suggest a respiratory deficit (9, 17, 18). This leads to a growth-deficient or petite-phenotype in the...
yeast (50). The present finding that frataxin is an activator of OXPHOS in mammalian cells suggests that a deficiency of frataxin in tissues of patients with FA would lead to a defective OXPHOS. This view is supported by the recently published findings that the FA-phenotype in humans is associated with reduced ATP-levels in skeletal muscle during exercise (6, 7). Additionally, a coenzyme Q derivative, idebenone, has been shown to ameliorate cardiac dysfunction in FA (51). This clinical observation is consistent with our findings that overexpression of frataxin results in an increased ΔΨm because a similar increase of ΔΨm is found following coenzyme Q10 treatment of hepatocytes, which also protects the cells from oxidative stress (52). Lastly, FA appears to be clinically indistinguishable from inherited α-tocopherol (vitamin E) deficiency (http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?277460), suggesting a close bididachaniiy and a decrease in phenotypic relationship between a diminished antioxidant defense (i.e., vitamin E deficiency) and a decrease in ΔΨm in FA as would be predicted by the present study, and as observed in cultured fibroblasts derived from FA patients (M.R., M.S., M.F.P., and C.R.K., unpublished observations). Conversely, frataxin might regulate mitochondrial iron efflux (20) indirectly by activation of the ATP-dependent iron-transports ATMI in yeast (53) and ABC7 in humans (54), respectively. This would suggest that regulation of OXPHOS by frataxin is the primary effect and leads to secondary alterations in mitochondrial iron homeostasis commonly believed to be the cause of FA.

In summary, our data indicate that frataxin activates OXPHOS and energy conversion and suggest that reduced levels of frataxin in FA patients primarily result in a disease of ATP deficiency rather than an alteration in mitochondrial iron metabolism. This might explain why tissues exclusively dependent on oxidative metabolism, i.e., neuronal and heart tissues (55), are most severely affected in patients with FA (ref. 1; and http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?229300). These findings might additionally explain the metabolic disturbances associated with FA, such as diabetes mellitus and insulin resistance. Clearly, further studies will be needed to investigate implications for both cell biology and disease.

We thank Christoph Richter for helpful comments, Bruce M. Spiegelman and members of his lab for the ap2- and COXII-probes, and Morris F. White for the use of his digital microscope. M.R. was supported by Köln Fortune Grants 88/97 and 88/97V of the University of Cologne. M.F.P. was supported by a Swiss National Science Foundation Fellowship. A.J.Y. was a Howard Hughes Medical Institute Medical Student Research Training Fellow. This work is supported by National Institutes of Health Grant DK 45935 (to C.R.K.).