Mitofusin-2 (Mfn2) is a mitochondrial membrane protein that participates in mitochondrial fusion in mammalian cells and mutations in the Mfn2 gene cause Charcot–Marie–Tooth neuropathy type 2A. Here, we show that Mfn2 loss-of-function inhibits pyruvate, glucose and fatty acid oxidation and reduces mitochondrial membrane potential, whereas Mfn2 gain-of-function increases glucose oxidation and mitochondrial membrane potential. As to the mechanisms involved, we have found that Mfn2 loss-of-function represses nuclear-encoded subunits of OXPHOS complexes I, II, III and V, whereas Mfn2 overexpression induced the subunits of complexes I, IV and V. Obesity-induced Mfn2 deficiency in rat skeletal muscle was also associated with a decrease in the subunits of complexes I, II, III and V. In addition, the effect of Mfn2 overexpression on mitochondrial metabolism was mimicked by a truncated Mfn2 mutant that is inactive as a mitochondrial fusion protein. Our results indicate that Mfn2 triggers mitochondrial energization, at least in part, by regulating OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein.

INTRODUCTION

Mitochondria play a central role in many cellular functions, which include apoptosis, cell proliferation and differentiation, bioenergetics, and metabolism of amino acids and lipids. In connection with energy metabolism, mitochondrial oxidation of fuels results in electrons being transferred by NADH and FADH to the respiratory chain used by complexes I, III and IV to pump protons outside the inner membrane and creating an electrochemical potential gradient. These protons re-enter via ATP synthase with the resulting energy being used to drive conversion of ADP to ATP. Respiration and oxidative phosphorylation are coupled activities and oxygen consumption is strongly dependent on the availability of ADP. This explains why cells match rates of substrate oxidation and ATP production to rates of ATP utilization. In spite of this, several evidences suggest that the existence of additional levels of control of mitochondrial energy metabolism that are superimposed to the chemiosmotic model of operation. Thus, ADP levels change little or do not change at all under conditions when cell respiration is markedly stimulated (1) and ATP concentrations are controlled by factors other than ADP availability in the presence of high extracellular glucose concentrations in β-pancreatic cells (2). In addition, theoretical considerations indicate that ATP production rates are controlled by factors other than ATP utilization such as rates of fuel oxidation (3). The molecular mechanisms involved in those additional levels of control of mitochondrial energy metabolism in cells other than brown adipocytes are largely unknown. Mitochondria frequently form tubular structures or networks (4) which are crucial for the inheritance of mitochondrial DNA. The mitochondrial network is a highly dynamic structure that is regulated through changes in the rates of mitochondrial fission and fusion. Mitochondrial fusion in yeast and Drosophila depends on the integrity of the mitochondrial transmembrane GTPase, Fzo protein (5–7). Mammalian cells express two homologues named mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) (8,9). Mice deficient in either Mfn1 or Mfn2 die in midgestation probably because
of impaired placental function caused by a marked reduction in the number of polyploid giant cells of the placental trophoblast (10). In addition, mouse embryonic fibroblasts derived from Mfn2 or Mfn1 homozygous mutant embryos show fragmented mitochondria (10). Disorganization of the mitochondrial network in muscle cells has also been reported after knock-down of Mfn2 by antisense overexpression (11). In contrast, overexpression of Fzo or mitofusins causes perinuclear clustering of mitochondria (8,11,12) and co-expression of Mfn2 and a dominant negative mutant of Drp1 leads to the generation of mitochondrial tubules (8). Mfn2 has been reported to operate as a cell proliferation suppressor both in vivo and in vitro by cell-cycle arrest in the G0/G1 phases (13). Mfn2 may be also a crucial protein in mitochondrial metabolism as Mfn2 repression reduces glucose oxidation in cells (11). In addition, mutations in Mfn2 cause Charcot–Marie–Tooth type 2A, an autosomal dominant neuropathy (14). This disease is characterized by chronic axonal neuropathy, with nerve biopsy findings of chronic axonal atrophy and regeneration (15,16). Primary axonal involvement is reflected by normal or slightly reduced motor and sensory nerve conduction velocities and decreased amplitudes of evoked motor and sensory nerve responses (17). These data support the notion that Mfn2 loss-of-function causes axonopathy possibly by impairing energy production along the axon.

In this study, we have analyzed the impact of up-regulation and knock-down of Mfn2 protein in energy metabolism in muscle cells and the nature of the mechanisms involved. Mfn2 deficiency reduces fuel oxidation and mitochondrial membrane potential and represses nuclear-encoded subunits of OXPHOS complexes I, II, III and V. An inverse pattern of changes was detected in cells in response to Mfn2 up-regulation. In addition, the effect of Mfn2 overexpression on mitochondrial metabolism was mimicked by a truncated Mfn2 mutant inactive as a mitochondrial fusion protein. In all, we conclude that Mfn2 stimulates the rate of fuel oxidation in cells, at least in part, by regulating OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein.

RESULTS

Effect of Mfn2 loss-of-function on the oxidation of glucose, pyruvate and palmitate and on mitochondrial membrane potential in muscle cells

To study the functional role of Mfn2 in mitochondrial metabolism, we induced a 50% repression of Mfn2 by antisense overexpression in L6E9 myotubes (Fig. 1A). Mfn2-knock-down muscle cells showed a reduced mitochondrial network (11) (data not shown). Mfn2 repression was associated with decreased rates of pyruvate, glucose or palmitate oxidation (Fig. 1B–D). Under these conditions, glucose transport was markedly enhanced in AS cells (Fig. 1E) and, in parallel, lactate production was increased by 53% in AS cells (data not shown); conversely, glucose incorporation into glycogen was significantly depressed (Fig. 1F). The alterations in substrate oxidation were not due to a reduction in mitochondrial mass, as estimated by staining with N-acrydine orange (Fig. 1G) and by the abundance of the mitochondrial proteins porin or COX-IV which remained unaltered in total homogenates from AS cells (data not shown). To evaluate whether mitochondrial membrane potential (ΔΨ) was also altered in Mfn2-knock-down cells, we stained them with JC-1, a cationic dye that accumulates in mitochondria in a potential-dependent manner. In parallel to the alterations in substrate oxidation, ΔΨ in the presence of the distinct substrates was markedly depressed in AS cells (Fig. 1H and I). These data are in keeping with previous observations indicating that the repression of Mfn2 in 10T1/2 cells reduce glucose oxidation and oxygen consumption (11). In all, our data indicate that Mfn2 loss-of-function reduces the activity of Krebs cycle and of the electron transport chain in such a way that the energy metabolism of the cell is compensated by a higher rate of glucose uptake and glycolysis and a lower rate of glycogen synthesis.

Effect of Mfn2 loss-of-function on the expression of the subunits of the OXPHOS system

To determine the nature of the mechanisms involved in the changes in mitochondrial metabolism in response to Mfn2 repression, we focused on the protein composition of mitochondria. The abundance of mitochondrial protein components of distinct respiratory complexes was assayed by western blot in mitochondrial extracts obtained from control and AS muscle cells. More specifically, we assayed subunit p39 from complex I (encoded by nuclear DNA), protein p70 from complex II (encoded by nuclear DNA), p49 (core 2 subunit encoded by nuclear genome) from complex III, COX-I (encoded by mitochondrial DNA) and COX-IV subunits of complex IV (encoded by nuclear DNA), the ω subunit of complex V (encoded by nuclear DNA) and porin (encoded by nuclear DNA). Mfn2 repression caused a marked inhibition of the expression of the subunits of complexes I, II, III and V (Fig. 2A). In contrast, no alterations in the abundance of complex IV subunits or porin were detected in AS cells (Fig. 2A). In keeping with these observations, the enzymatic activity of components I + III and III decreased in AS cells (Fig. 2B). These data indicate that Mfn2 loss-of-function causes a specific alteration in the expression of subunits that participate in complexes I, II, III and V, which leads to reduced activity of several components of the OXPHOS system.

In order to determine whether these alterations in OXPHOS expression were triggered by changes in the expression of the nuclear co-activator PGC-1α, we determined the abundance of this protein in extracts from control and AS cells. Results indicated no change in PGC-1α in response to Mfn2 repression (data not shown).

Next, we studied whether muscle from an Mfn2-deficient animal model also showed altered expression of mitochondrial proteins. Knock-out mice for Mfn2 are not viable (10) and heterozygous knock-out mice do not show alterations in Mfn2 expression in skeletal muscle (data not shown). On the basis of this, we analyzed skeletal muscle from obese Zucker rats in which Mfn2 repression is parallel to a deficient extension of the mitochondrial network (11). Mitochondrial extracts were obtained from lean and obese rats and the expression of several subunits of the OXPHOS system was...
assayed by western blot. The muscle from obese rats showed a reduced expression of subunits of complexes I, II, III and V (Fig. 3). In contrast, no alterations in the abundance of complex IV subunits or porin were detected in Zucker rats (Fig. 3 and data not shown). Thus, the alterations in the content of subunits of the OXPHOS system in muscle from Mfn2-deficient obese rats were very similar to those detected in Mfn2-knock-down muscle cells.

Effect of Mfn2 overexpression on mitochondrial metabolism and mitochondrial composition

To assess whether Mfn2 gain-of-function is sufficient to enhance mitochondrial activity, we overexpressed this protein in cells either by transient transfection or by retroviral infection (Fig. 4). Overexpression of Mfn2 in HeLa cells caused perinuclear aggregation of mitochondria (Fig. 4A), which is consistent with the fusion activity of this protein (8,9,12). Under these conditions, overexpressed Mfn2 was localized in mitochondria (Fig. 4A and B). Mfn2-overexpressing cells showed a marked enhancement of mitochondrial membrane potential (Fig. 4C) and an increase in the rate of glucose oxidation (Fig. 4D). Mfn2 gain-of-function in L6E9 myoblasts was also associated with increased expression of several subunits of complexes I, IV and V (Fig. 4E). In all, Mfn2 gain-of-function activated mitochondrial metabolism and increased the expression of subunits of the OXPHOS system.
Effects of a truncated form of Mfn2 on mitochondrial metabolism

Mfn2 overexpression caused perinuclear aggregation and activation of mitochondrial metabolism. Previous studies have demonstrated that the C-terminus of Mfn is crucial for the oligomerization and, as a consequence, a C-terminal truncated form of Mfn2 has no longer capacity to induce mitochondrial fusion (12,18). To determine whether the metabolic effects of Mfn2 are linked to its effects as a mitochondrial fusion protein, we generated a truncated Mfn2 mutant (hMfn2Δ602–757) that lacks amino acid residues from 602 to 757 and, therefore, has no transmembrane domain or the normal C-terminal end (8,9,12). In agreement with previous observations, overexpression of hMfn2Δ602–757 in HeLa cells did not alter the morphology of mitochondrial filaments, indicating that it had no mitochondrial fusion activity (Fig. 5A). Truncated Mfn2 was localized in mitochondria (80% of total) and in cytosol (20% of total) as assessed by western blot assays by using specific antibodies and by densitometric analysis of four independent experiments. Control for protein loading was also checked by Ponceau staining on Immobilon filters. Values are expressed relative to control levels (mean of control group set to 100). Double and single asterisks indicate a statistically significant difference between control and Zucker rats at \( P < 0.01 \) and \( P < 0.05 \), respectively.

DISCUSSION

Our data strongly indicate that Mfn2 has two biological roles. On the one hand, it is crucial in mitochondrial fusion and the elimination or the knock-down of Mfn2 expression greatly reduces the extent of the mitochondrial network (10,11). However, Mfn2 also affects mitochondrial metabolism and its expression level regulates mitochondrial membrane potential, fuel oxidation and the OXPHOS system (Fig. 6). Given...
that a truncated mutant form of Mfn2 that is inactive as a mitochondrial fusion protein maintains its capacity to activate mitochondrial metabolism, we support the view that the metabolic accelerator and the mitochondrial fusion roles of Mfn2 are separate. This hypothesis may explain the exquisite sensitivity of the neuronal cell to a partial Mfn2 loss-of-function, as recently reported in Charcot–Marie–Tooth type 2A neuropathy, a disease that shows autosomic dominant inheritance (14). On the basis of our data, we propose that Mfn2 loss-of-function participates in triggering the pathological mechanisms that cause disease in highly oxidative tissues such as the nervous system, skeletal muscle or heart (20).

The pattern of metabolic changes associated with up-regulation of Mfn2 indicates that Mfn2 is sufficient to trigger mitochondrial function. Thus, in myoblasts with a limited oxidative capacity, Mfn2 gain-of-function caused an increase in the rate of glucose oxidation and a parallel increase in mitochondrial membrane potential, which indicates augmented pyruvate oxidation in mitochondria and enhanced Krebs cycle and oxidative phosphorylation (Fig. 6). In addition, Mfn2 repression led to a decrease in the oxidation rates of glucose, pyruvate and palmitate and reduced mitochondrial membrane potential in myotubes (Fig. 6). These observations indicate lower pyruvate oxidation, β-oxidation of fatty acids, Krebs cycle and oxidative phosphorylation in cells showing reduced expression of Mfn2 (Fig. 6). Under these conditions, the alteration in mitochondrial metabolism is strong enough to force other metabolic adaptations such as an increase in glucose uptake and in glycolysis and a reduced incorporation of glucose into glycogen (Fig. 6). In all, our data permit to conclude that Mfn2 tightly controls fuel oxidation by mitochondria so up-regulation or down-regulation of Mfn2 modifies accordingly mitochondrial metabolism.

The effects of Mfn2 loss-of-function on mitochondrial metabolism are not a consequence of reduced mitochondrial mass. This conclusion is in part based on the observation that the mitochondrial accumulation of N-acridine orange was not reduced in cells with repressed Mfn2 expression. As it has been reported that N-acridine orange in mitochondria is altered by major alterations in mitochondrial membrane potential (21), and Mfn2 loss-of-function reduces this parameter, we also determined the abundance in total cell lysates of two mitochondrial markers, porin and COX-IV; these two proteins showed normal expression levels in muscle cells with repressed Mfn2.

The mechanisms by which Mfn2 regulates the oxidation of substrates seem to be dependent on the expression of components of the OXPHOS system. Thus, Mfn2 loss-of-function

Figure 4. Human Mfn2 overexpression enhances mitochondrial metabolism and alters mitochondrial composition. (A) HeLa cells were transfected with myc-tagged version at the N-terminus of human Mfn2 and 48 h later the mitochondrial network was observed by incubation with a monoclonal antibody against cytochrome c oxidase subunit I (COX-I) or polyclonal antibody against Mfn2 and further incubation with a Texas red-labelled secondary antibody and an Oregon green-labelled secondary antibody, respectively (scale bar, 10 μm). The signals of mitochondrial marker COX-I and Mfn2 co-localize (merging). T, transfected cells; NT, non-transfected cells. (B) Mitochondrial fractions of HeLa cells transfected with myc-tagged version at the N-terminus of hMfn2 (T) or with an empty vector (C) were obtained by differential centrifugation and were processed by western blot with antibodies against Mfn2, the myc epitope or porin. (C) ΔΨ of HeLa cells transfected with human Mfn2 or with an empty vector (C) was measured with DiIC₅₄(5). Results are mean ± SE of four independent experiments and are expressed as a percentage of control values. Differences between control and hMfn2 cells were statistically significant at P < 0.05. (D) Glucose oxidation was determined in control cells (C) and cells overexpressing hMfn2. During the experiment, cells at logarithmic growth stage were cultured in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 μCi ¹⁴C-(U)-d-glucose (Amersham Pharmacia), and ¹⁴CO₂ was trapped and measured. Results are mean ± SE of four independent observations. Differences between control and hMfn2 cells were statistically significant at P < 0.01. (E) Mitochondrial fractions were obtained from control L6E9 myoblasts (C) and from cells overexpressing hMfn2 and processed by western blot as mentioned in legend from Figure 2A.
Figure 5. A truncated form of Mfn2 enhances mitochondrial metabolism in the absence of effects on mitochondrial network. (A) HeLa cells were transfected with His-tagged version at the N-terminus of human truncated Mfn2 and 48 h later the mitochondrial network was observed by incubation with a monoclonal antibody against cytochrome c oxidase subunit I (COX-I) or polyclonal antibody against Mfn2 and further incubation with a Texas red-labelled secondary antibody and an Oregon green-labelled secondary antibody, respectively (scale bar, 10 μm). The signals of mitochondrial marker COX-I and Mfn2 co-localize (merging). T, transfected cells; NT, non-transfected cells. (B) Mitochondrial (MT) and cytosolic fractions (Cytosol) of HeLa cells transfected with the truncated form of Mfn2 (T) or with an empty vector (C) were obtained by differential centrifugation and were processed by western blot with antibodies against Mfn2, His tag or against porin. (C) HeLa cells were transfected with human truncated Mfn2 or with human Mfn2 and 48 h later mitochondrial fractions were subjected to the purification of outer mitochondrial membranes (OMM) and mitoplasts by osmotic disruption. Fractions were processed by western blot with antibodies against Mfn2, COX-I or porin. (D) ΔΨ of HeLa cells transfected with human truncated Mfn2 (hMfn2Δ602–757) or with an empty vector (C) was measured with DiIC1(5). Results are mean ± SE of four independent experiments and are expressed as a percentage of control values. Differences between control and hMfn2 cells were statistically significant at P < 0.05. (E) Glucose oxidation was determined in control cells (C) and cells overexpressing hMfn2Δ602–757 (truncated form). During the experiment, cells at logarithmic growth were cultured in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 μCi 14C-(U)-D-glucose (Amersham Pharmacia), and 14CO2 was trapped and measured. Results are mean ± SE of four independent experiments. Differences between control and hMfn2 cells were statistically significant at P < 0.05. (F) HeLa cells overexpressing hMfn2Δ602–757 (truncated Mfn2) and cells transfected with the empty vector (C) were visualized by electron microscopy. Control cells showed mitochondria with typical, orthodox configuration, i.e. normal matrix density and regularly spaced and oriented cristae. Cells overexpressing the truncated form of Mfn2 showed condensed state of mitochondria with twisted, rounded and condensed cristae, thereby affecting the internal mitochondrial structure in such a way that the intermembrane space expanded in conjunction with a decrease in the mitochondrial matrix. Asterisks denote mitochondria. Scale bar 1 μm.
represses the expression of nuclear-encoded subunits of complexes I, II, III and V and, in keeping with these observations, reduces the activity of complexes I + III and complex III (Fig. 6). The reduction detected in the 70 kDa subunit from complex II was relatively modest (18%) and was parallel to a slight reduction in complex II activity (12%), which it did not reach statistical significance.

In contrast, Mfn2 gain-of-function up-regulates the subunits of complexes I, IV and V (Fig. 6). Thus, Mfn2 participates in defining the specific composition of the respiratory complexes that operate in mitochondria and, consequently, is a key element in the biology of these organelles. The effects of Mfn2 loss-of-function and gain-of-function on OXPHOS expression are not perfectly symmetrical: while loss-of-function reduced subunits of complex II and III, Mfn2 up-regulation did not cause any alteration in these subunits and alternatively, Mfn2 up-regulation enhanced subunit of complex IV whereas loss-of-function did not modify its expression. Detailed understanding of the mechanisms involved in the effects of Mfn2 are required to provide a molecular explanation for the lack of symmetry. Independently of the mechanisms involved, it may be proposed that the signals derived from Mfn2 are necessary but not sufficient in the regulation of the expression of some OXPHOS subunits.

The effects of Mfn2 on OXPHOS composition are not due to cellular changes in the expression of the nuclear co-activator PGC-1α which is a crucial regulator of genes involved in mitochondrial energy metabolism and mitochondrial biogenesis (22–24).

As to the mechanisms that may explain the effect of Mfn2 on OXPHOS expression, we consider two distinct alternatives: (a) Mfn2 may interact with the components of the OXPHOS system and may participate in the assembly of mitochondrial complexes or in the formation of supercomplexes (25–27) and (b) Mfn2 may induce signals that affect the expression of nuclear genes encoding OXPHOS subunits. The two hypotheses are not mutually exclusive and they are currently under investigation.

In this study, we have found that the transient transfection of the truncated version of Mfn2 (hMfn2Δ602–757) was
localized in cytosol and in the outer mitochondrial membrane as assessed by co-localization with mitochondrial markers in immunofluorescence assays, by subcellular fractionation and by sub mitochondrial fractionation followed by western blot assays. These data do not agree with a previous study in which a truncated form of human Mfn2 (hMfn2Δ604–757) was also transiently transfected in HeLa cells and localized in cytosol in immunofluorescence assays probably because mitochondrial markers were not used (12). As to the localization of Mfn2 that is necessary to regulate OXPHOS, we have shown that wild-type Mfn2 is only localized in the outer mitochondrial membrane, whereas the truncated form hMfn2Δ602–757 is detected both in the outer mitochondrial membrane and in the cytosol. On the basis of these data, we propose that the copies of Mfn2 located in the outer mitochondrial membranes must be crucial in promoting the regulation of OXPHOS expression in cells.

Mutations in Mfn2 gene cause Charcot–Marie–Tooth type 2A neuropathy (14,28) and Mfn2 expression is repressed in skeletal muscle from obese subjects or in obese Zucker rats (11 and this study). On the basis of the observations that Mfn2 loss-of-function causes profound alterations in OXPHOS expression, mitochondrial oxidation or glucose utilization by cells, it is likely that alterations in Mfn2 expression or in Mfn2 function may have pathological implications. In this regard, it is remarkable that Mfn2 repression causes a reduced expression of NDUFA-9 subunit of complex I as well as reduced complex I activity in muscle cells and an impaired complex I activity has been associated to enhanced reactive oxygen species production and to the development of neurodegenerative diseases. Thus, cybrids from patients with sporadic Parkinson disease show a deficit in complex I which can be stably transmitted (29) and maternal descendants in families with maternal patterns of inheritance of Parkinson disease have lower complex I activity (30). In addition, compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine that inhibit complex I activity, replicate most features of Parkinson disease (31). Alzheimer disease has also been associated with deficiencies in complex I (32). In all, we propose that differences in the activity and/or expression of Mfn2 may participate in the susceptibility to develop degenerative disease characterized by deficient mitochondrial function.

MATERIALS AND METHODS

Plasmids and retroviral constructs

To construct human Mfn2 (hMfn2) with the Myc epitope tag, the Mfn2 ORF was cloned into pCMV-Tag3 vector (Stratagene) between BamHI and XhoI sites. The untagged hMfn2 was cloned into the plasmid pAdEasy (Clontech), which encloses two independent expression cassettes expressing Mfn2 and GFP. Each expression cassette was driven by a CMV promoter and contained an SV40 polyadenylation signal. The truncated form of hMfn2, hMfn2Δ602–757, was generated using PCR. cDNA was amplified using the forward primer: 5’-ATACACCTCGAGTTATTACC TGGATGTCAGAGGCCAG-3’. PCR products were pooled, subjected to agarose gel electrophoresis and eluted from the gel. This material was digested with an excess amount of restriction enzymes BamHI and XhoI and ligated into the expression vector pcDNA4/HisMaxA (Invitrogen). These constructs were used in the studies of transient transfection. Transfected cells were identified by GFP fluorescence. Cells transfected with the empty vector were used as controls.

To generate a retroviral expression construct for hMfn2, the Mfn2 ORF was cloned into the retroviral vector pSF91EGFP (Phoenix-MMULV vector, Stanford University Medical Center), whereas the empty vector was used as a control. The retroviral expression vectors were transfected into 293Eco cells. Retroviral stocks were harvested 48 h after transfection and used to infect cells. One millilitre of the retroviral stock was used to infect 6 × 10⁵ cells and 48 h after infection, cells were collected and processed for further assays.

Production of adenovirus

Recombinant adenovirus expressing GFP, βGAL or antisense Mfn2 cDNA fragment were generated by homologous recombination, as previously described (11). The mouse Mfn2 sequence between nucleotides 1 and 370 was cloned in antisense orientation in the shuttle plasmid pdelE1sp1A and co-transfected with pJM17 into HEK 293 cells to achieve homologous recombination. Individual plaques were isolated and checked for mRNA expression after infection of HEK 293 cells. Recombinant adenoviruses were further amplified in HEK 293 cells, purified by cesium chloride gradient centrifugation, dialysed against 1 mM MgCl₂, 10 mM Tris pH 7.4, 10% glycerol and stored at −80°C. DNA was isolated from purified viral particles and the recombinant viral genome was verified by restriction enzyme analysis. Viral stocks were titrated by infecting HEK 293 cells with serial dilutions of the preparation and counting plaque forming units (pfu) on the monolayer 15 days after infection. L6E9 myotubes were infected with adenoviruses at 100 pfu/cell.

Cell culture and transfection

HeLa cells and L6E9 muscle cells were maintained in DMEM with 10% fetal bovine serum, 100 U/ml streptomycin, 100 U/ml penicillin. L6E9 cells were grown in monolayers to the stage of myotubes, as previously described (33), in DMEM containing 2% fetal bovine serum and the same antibiotics, at 37°C in an atmosphere of 5% CO₂–95% air. HeLa cells were transfected with the calcium phosphate technique, as previously reported (33,34).

Immunofluorescence techniques

Cells grown on coverslips were fixed for 20 min with 3% paraformaldehyde in phosphate-buffered saline (PBS), then washed three times in PBS and incubated for 10 min in PBS containing 50 mM NH₄Cl, 10 min in PBS containing 20 mM glycine and 30 min in PBS containing 10% normal goat
serum (GS). Subsequently, coverslips were incubated with primary antibodies at 0.5–1.0 μg/ml (anti-COX-I subunit, Molecular Probes, Leiden, The Netherlands; anti-Mfn2) diluted in PBS containing 10% GS for 1 h at room temperature. After washing in PBS, the coverslips were incubated with fluorochrome-conjugated (Oregon Green or Texas Red) goat secondary antibodies for 45 min. Cells were washed three times in PBS prior to mounting in immunofluore medium (ICN Biomedicals Inc., Aurora, OH, USA). Confocal images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 63× objective at the Scientific and Technical Services, University of Barcelona.

**Measurement of mitochondrial membrane potential and mitochondrial nonyl acridine orange accumulation**

JC-1 is a cationic fluorescent dye (green as monomer; 539 nm) that accumulates in mitochondria in a potential-dependent manner. High concentrations of the dye accumulate in these organelles and red fluorescent aggregates are subsequently formed (597 nm). The concentration of JC-1 green monomers in the mitochondria increases proportionally to the membrane potential, and when the concentration of these monomers reaches a certain level (~240 mV) these J-aggregates are formed. Thus, mitochondrial membrane potential (ΔΨ) can be measured by the determination of red-to-green emission irrespective of the number of mitochondria measured per image (34,35). To determine differences in ΔΨ, the dual emission of the JC-1 was observed and red/green fluorescence ratio measured. Cells were incubated with 1 μM JC-1 (Molecular Probes) diluted in DMEM + 2% FBS for 20 min at 37°C. The cells were washed twice in PBS and the fluorescence emitted was analyzed by flow cytometry. The accuracy of the JC-1 potentiometric method was validated using FCCP (10 μM) and oligomycin (0.5 μg/ml) to assess minimal and maximal ΔΨ values either by confocal microscopy or by flow cytometry (data not shown). For measuring the ΔΨ with the probe 1,1’,3,3’,3’,3’-hexamethyldiodocarbo cyanine iodide [DiIC1(5)] (Molecular Probes), cells were incubated with 250 ng/ml DiIC1(5) diluted in DMEM for 30 min at 37°C, then washed twice in PBS and the fluorescence emitted was then analyzed at 675 nm by flow cytometry. The accuracy of the DiIC1(5) potentiometric method was validated using FCCP (10 μM) and oligomycin (0.5 μg/ml) to assess minimal and maximal ΔΨ (data not shown). To measure mitochondrial mass, cells were incubated with 100 ng/ml nonyl acridine orange (NAO, Molecular Probes) diluted in DMEM for 30 min at 37°C, then washed twice in PBS. The fluorescence emitted was then analyzed at 580 nm by flow cytometry.

**Subcellular fractionation**

Mitochondrial and cytosolic fractions were obtained by differential centrifugation of HeLa or L6E9 cell homogenates. Cells were washed twice with PBS, scraped, pelleted and resuspended in ice-cold buffer containing 250 mM sucrose, 10 mM Heps, pH 7.4, 1 mM EGTA and the protease inhibitors PMSF, leupeptin, pepstatin A and aprotinin. Cells were homogenized using a Dounce homogenizer with a tight fitting Teflon pestle (15 strokes). The homogenates were centrifuged at 700g for 3 min to remove nuclei and unbroken cells, and the post-nuclear supernatants were centrifuged at 1500g for 15 min to obtain a pellet highly enriched in mitochondria. Mitochondrial enrichment was assessed by the abundance of the protein marker porin and enrichment values were near 1.8-fold when compared with total homogenates. The post-mitochondrial supernatant was further centrifuged at 100 000g for 90 min to obtain a cytosolic fraction. All fractions were stored at −80°C.

Mitochondria from the skeletal muscle of Zucker rats were isolated by differential centrifugation. Tissues were rinsed in a buffer containing 100 mM KCl, 5 mM MgCl2, 5 mM EGTA and 5 mM sodium pyrophosphate at pH 6.8, and thereafter homogenized by polytron at 1400 rpm in a buffer containing 250 mM sucrose, 50 mM KCl, 5 mM EDTA, 1 mM sodium pyrophosphate pH 7.4 (buffer A). All steps were performed at 4°C. The suspension was centrifuged at 1300g for 10 min to sediment out nuclei and large cellular debris, and the mitochondria were recovered by centrifugation at 9000g for 15 min. After washing the mitochondrial pellet twice in buffer A to minimize microsomal contamination, the mitochondria were resuspended and stored at −80°C.

In some studies, outer mitochondrial membranes and mitoplasts were purified by the osmotic disruption method. To disrupt outer mitochondrial membranes, 100 μg of mitochondria from HeLa cells or from HeLa cells overexpressing either hMfn2 or hMfn2Δ602–757 were diluted with 9 vol of 20 mM Hepes–KOH buffer, pH 7.4 and incubated at 0°C for 20 min. Mitoplasts were recovered by centrifugation at 15 000 × g for 15 min at 4°C. The outer membrane-rich fraction was recovered after centrifugation of the above supernatant at 144 000g for 20 min at 4°C. The different fractions were analyzed by western blot analysis.

**Western blot assays**

SDS–PAGE electrophoresis and further densitometric analysis were used to estimate the levels of Mfn2, subunits of the OXPHOS system, porin and actin in control and cell extracts or in fractions obtained from skeletal muscle. Thus, samples were run in a 10% acrylamide/bisacrylamide SDS–PAGE and electrophoresed onto nylon filters. These filters were probed with specific antibodies raised against subunits of complex I (anti-NDUFA9, Molecular Probes), complex II (anti-Fp, 70 kDa subunit, Molecular Probes), complex III (anti-core 2, Molecular Probes), complex IV (anti-CO I and anti-CO IV, Molecular Probes), complex V (anti-subunit α from H2–F1–ATP synthase, Molecular Probes), porin (Calbiochem, La Jolla, CA, USA), β-actin (antibody AC-15, Sigma-Aldrich) and Mfn2. Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins were used as secondary antibodies. The specific proteins were detected by the ECL western blotting detection analysis system (Amersham). The signal obtained with each antibody was quantified on autoradiography films using a laser densitometer.

**Electron microscopy**

HeLa cells transfected with the truncated form of Mfn2 and with the empty vector were fixed in 2% paraformaldehyde

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and 0.0128% glutaraldehyde for 30 min. After fixation, cells were scraped off the dish, washed in PBS and post-fixed in 2% osmium tetroxide for 10 min. Cells were dehydrated by rising with concentrations of ethanol, and, after passage through propylene oxide, the blocks were embedded in Epon 812 following the standard technique (36). Semithin, 1 μm cross-sections were stained with toluidine blue. Representative cell samples were chosen, and ultrathin 60–90 nm cross-sections were cut and mounted on bare copper grids. The staining was done with uranyl acetate and lead citrate. The samples were examined under a Hitachi 6600 AB at an accelerating voltage of 80 kV.

Measurement of metabolic and enzymatic activities

Glucose, pyruvate of palmitate oxidation was measured in cells by incubation in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 μCi 14C-(U)-glucose (306 mCi/mmol, Amersham Pharmacia) for glucose oxidation, 2 mM pyruvate and 0.6 μCi 14C-(U)-pyruvate (50 mCi/mmol, Amersham Pharmacia) for pyruvate oxidation, or 0.1 μCi 14C-(U)-palmitic acid (60 mCi/mmol, Amersham Pharmacia) for palmitate oxidation. Cells were incubated for 180 min and 14CO2 was trapped and measured, as described (11).

For the measurement of glucose transport, cells were washed twice in a transport solution (20 mM Hepes, 137 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 0.0128% glutaraldehyde for 30 min. After fixation, cells were scraped off the dish, washed in PBS and post-fixed in 2% osmium tetroxide for 10 min. Cells were dehydrated by rising with concentrations of ethanol, and, after passage through propylene oxide, the blocks were embedded in Epon 812 following the standard technique (36). Semithin, 1 μm cross-sections were stained with toluidine blue. Representative cell samples were chosen, and ultrathin 60–90 nm cross-sections were cut and mounted on bare copper grids. The staining was done with uranyl acetate and lead citrate. The samples were examined under a Hitachi 6600 AB at an accelerating voltage of 80 kV.

Measurement of metabolic and enzymatic activities

Glucose, pyruvate of palmitate oxidation was measured in cells by incubation in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 μCi 14C-(U)-glucose (306 mCi/mmol, Amersham Pharmacia) for glucose oxidation, 2 mM pyruvate and 0.6 μCi 14C-(U)-pyruvate (50 mCi/mmol, Amersham Pharmacia) for pyruvate oxidation, or 0.1 μCi 14C-(U)-palmitic acid (60 mCi/mmol, Amersham Pharmacia) for palmitate oxidation. Cells were incubated for 180 min and 14CO2 was trapped and measured, as described (11).

For the measurement of glucose transport, cells were washed twice in a transport solution (20 mM Hepes, 137 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 2 mM pyruvate, pH 7.4) and then incubated for 10 min with a transport solution that contained 0.1 μmol 2-deoxy-D-glucose and 1 μCi of 2-deoxy-D-[3H]glucose uptake (10 mCi/mmol, Amersham Pharmacia). To determine background labelling, cells were incubated for 10 min with ice-cold 50 mM glucose in PBS containing the same specific activity of 2-deoxy-D-[3H]glucose. Uptake was stopped by the addition of 2 vol of ice-cold 50 mM glucose in PBS. Cells were washed twice in the same solution and disrupted with 0.1 M NaOH, 0.1% SDS. Radioactivity was determined by scintillation counting. Each condition was run in quadruplicate.

Enzymatic activities of NADH:cytochrome c oxidoreductase (complex I, co-enzyme Q and complex III: CI + II), succinate:cytochrome c reductase (complex II, co-enzyme Q and complex III: CII + III), decylubiquinol:cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV), and citrate synthase were determined according to described radiometric or spectrophotometric methods (40–42). In those assays, protein was determined according to Lowry et al. (43).

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Conflict of Interest statement. None declared.

REFERENCES


