Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism: a novel regulatory mechanism altered in obesity.

Daniel Bach*, Sara Pich*, Francesc X. Soriano*, Nathalie Vega†, Bernhard Baumgartner*, Josep Oriola‡, Jens R. Daugaard*, Jorge Lloberas§, Marta Camps*, Juleen R. Zierathφ, Rémi Rabasa-Lhoret†, Harriet Wallberg-Henrikssonφ, Martine Laville†, Manuel Palacín*, Hubert Vidal†, Francisca Rivera†, Martin Brand¶ and Antonio Zorzano*#

*, Parc Científic de Barcelona and Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Spain. Phone: 34-93-4021519; Fax: 34-93-4021559; e.mail: azorzano@bio.ub.es

†, Institut National de la Santé et de la Recherche Médicale Unité-449 and Centre de Recherche en Nutrition Humaine de Lyon, Faculté de Médecine R.T.H. Laennec, F-69372 Lyon, France

‡, Servei Hormonal, Hospital Clínic i Provincial, Barcelona, Spain

§, Departament de Fisiologia (Biología del Macrófago, Immunologia), Facultat de Biologia, Universitat de Barcelona, Spain

φ, Department of Clinical Physiology, Karolinska Hospital, Stockholm, Sweden

¶, MRC Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK

#To whom correspondence should be addressed

Running title: Mitofusin-2 and mitochondrial metabolism
SUMMARY.

In many cells and specially in muscle, mitochondria form elongated filaments or a branched reticulum. We show that mitofusin 2 (Mfn2), a mitochondrial membrane protein that participates in mitochondrial fusion in mammalian cells, is induced during myogenesis and contributes to the maintenance and operation of the mitochondrial network. Repression of Mfn2 caused morphological and functional fragmentation of the mitochondrial network into independent clusters. Concomitantly, repression of Mfn2 reduced glucose oxidation, mitochondrial membrane potential, cell respiration and mitochondrial proton leak. We also show that the Mfn2-dependent mechanism of mitochondrial control is disturbed in obesity by reduced Mfn2 expression.

In all, our data indicate that Mfn2 expression is crucial in mitochondrial metabolism through the maintenance of the mitochondrial network architecture, and reduced Mfn2 expression may explain some of the metabolic alterations associated with obesity.
INTRODUCTION.

In many cell types, especially muscle fibers, mitochondria form tubular structures or networks (1, 2). In fibroblasts and cardiomyocytes, mitochondrial filaments conduct mitochondrial membrane potential (3, 4). The electrical activity of the mitochondrial network in large cells such as muscle fibers may permit the movement of energy from the cell periphery to the cell core.

There is evidence in Saccharomyces, Aspergillus and mammalian cells that mitochondrial filaments are highly dynamic structures (5-7). In yeast this dynamics is regulated by changes in the rates of mitochondrial fission and fusion. Based on the isolation and analysis of yeast mutants that are defective in mitochondrial division and fusion, several laboratories have isolated proteins that mediate mitochondrial fission and fusion (8, 9).

Mitochondrial fusion in yeast and Drosophila depends on the integrity of the fzo gene that encodes a mitochondrial transmembrane GTPase (10, 11). Thus, loss of function of fzo causes fragmentation of mitochondrial tubules in yeast (11) and alteration of spermatogenesis and male sterility in Drosophila (10). Yeast Dnm1p and Mgm1p proteins (12-14) are structurally related to the dynamin GTPase and also contribute to mitochondrial fission (15, 16). Dnm1p operates in conjunction with Mdv1/Fis2/Gag3 and Mdv2/Fis1 to regulate mitochondrial fission (16-18). Since mammals have genes that are homologous to fzo, Dnm and Mgm1p (19-22), such fusion and fission events may also be critical for the architecture of mitochondrial networks in mammalian cells.

Tissues with high and variable rates of aerobic metabolism such as skeletal muscle or heart show a more prominent development of the mitochondrial network (1, 2), although this remains unexplained. In addition, the extent of the mitochondrial network is regulated by hypoxic conditions (23). These data suggest that the mitochondrial network per se or proteins that participate in the generation of the mitochondrial network may be involved in the control of mitochondrial energy metabolism.

In this study, we screened differentially expressed genes in skeletal muscle from obese Zucker rats and we isolated the fzo homologue mitofusin 2 (Mfn2). We demonstrate that
Mfn2 expression is crucial to the maintenance of the morphology and operation of the mitochondrial network and in mitochondrial metabolism. We also show that this regulatory mechanism is disturbed in obesity.
EXPERIMENTAL PROCEDURES.

Subtractive Hybridization.
Message RNA was extracted from *gastrocnemius* muscle of control (fa/+) and obese (fa/fa) rats with oligo(dT)\_20-cellulose columns, as described (24). Complementary DNA was prepared from 2 \( \mu \)g of mRNA using Superscript II (Life Technologies). PCR-Select cDNA Subtraction kit (Clontech) was used to select for genes that are down-regulated in the obese. The cDNA fragments obtained were arrayed on 96-well plates and screened using labelled cDNA from lean or control rats.

Northern Blot.
Northern blot assays from 20 \( \mu \)g total RNA were performed as described (25) using as probes the \(^{32}P\) labelled C31 cDNA fragment (321 bp) obtained from the subtractive hybridization or the \(^{32}P\) labelled stem-cell factor cDNA fragment obtained by PCR. The C31 cDNA fragment is identical to the nucleotide sequence 2,098-2,419 of the rat U41803 (GenBank)

Quantitative PCR.
Retrotranscription and competitive-quantitative PCR was performed from 0.1 \( \mu \)g of total RNA from *vastus lateralis* muscle from lean and obese subjects, as described (26). Briefly, for each sample Mfn2 mRNA and an internal competitor at different known concentrations were co-amplified with the same pair of primers: 5’-atgcatccccacttaagcac-3’ and 5’-ccagagggcagaactttgtc-3’. Mfn2 concentration was extrapolated from the point at which both products are amplified to the same extent.

Subjects and protocols.
Thirteen healthy lean volunteers (51±2 years; BMI: 23±1 kg/m\(^2\); Blood glucose: 4.9±0.1 mmol/l; Plasma insulin: 34±2 pmol/l) and nine obese volunteers (46±2 years; BMI: 34±1 kg/m\(^2\); Blood glucose: 4.9±0.1 mmol/l; Plasma insulin: 62±6 pmol/l) participated in the study. Percutaneous biopsies of the *vastus lateralis* muscle were obtained before a 3-h
euglycemic hyperinsulinemic clamp (insulin infusion rate of 12 pmol/kg/min), as described elsewhere (27). Insulin-stimulated glucose utilisation was determined as reported (26).

Western blot.
A rabbit antibody against the Mfn2 specific peptide LGPKNSRRALMGYNDQVQRP was purchased from Research Genetics. Anti-Porin antiserum was used as a mitochondrial marker. Proteins from total homogenates or fractions enriched in mitochondria were resolved in 10% SDS-PAGE and transferred to Immobilon sheets. Incubation with antibodies and ECL detection was performed as described (25).

Immunofluorescence techniques and mitochondrial staining.
Cells grown on coverslips were fixed for 20 min with 3% paraformaldehyde in 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-COXI subunit, Molecular Probes, Leiden, The Netherlands; anti-myc 9E10, ATCC, Manassas, VA) diluted in PBS containing 10% GS for 1 h at room temperature. After washing in PBS coverslips were incubated with fluorochrom-conjugated (Oregon Green or Texas Red) goat secondary antibodies for 45 min. Cells were washed three times in PBS prior to mounting in immunofluorescence medium (ICN Biomedicals Inc., Aurora, OH). MitoTracker Red CXRos and JC-1 (Molecular Probes, The Netherlands) were used to label mitochondria using the manufacturer’s protocol. Briefly, cells were incubated with 0.5 µg/ml mitochondrial dye diluted in DMEM incomplete medium at 37°C for 30 min. Thereafter cells were washed in PBS warmed to 37°C and fixed with 3% paraformaldehyde in PBS or were processed in vivo. To assess mitochondrial connectivity the laser micro-irradiation technique was applied to cause mitochondrial membrane perforation in living cells previously stained with JC-1. A continuous argon laser (at λ=488 nm) with an output of 11.4 mW was used. The duration of the light laser irradiation was 60 s and the diameter of the focused laser spot was 0.5 µm. Cells were viewed by confocal microscopy before and 1.5 and 5 min after laser irradiation. Confocal images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 63 x objective at the Serveis Científico-Tècnics, University of Barcelona.
Measurement of mitochondrial membrane potential.

JC-1 is a cationic fluorescent dye (green colour as monomer; 539 nm) that accumulates in mitochondria in a potential-dependent manner. Its accumulation in mitochondria leads to high concentrations of the dye and the subsequent formation of red fluorescent aggregates (597 nm). The concentration of JC-1 green monomers in the mitochondria increases proportionally to the membrane potential and they form J-aggregates when green monomers concentration reach certain level. This critical concentration is reached when membrane potential exceed -240 mV. This allows the membrane potential measurement by determination of red-relative to green-emitted light irrespective of the number of mitochondria measured per image (28, 29).

To measure differences in mitochondrial membrane potential between control cells and Mfn-2 knock-out cells the dual emission of the potentiometric dye 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 minutes at 37ºC. The cells were washed once in DMEM+2%FBS and viewed, in vivo, at the confocal microscope. Z-series stacks (always at the same non-saturating conditions) were taken in order to measure red (597 nm) and green (539 nm) fluorescence. JC-1 potentiometric method accuracy was validated using FCCP (10 μM) and oligomycin (0.5 μg/ml) to asses minimal and maximal mitochondrial membrane potential either by confocal microscopy as by flow cytometry (data not shown).

Measurement of mitochondrial volume and surface area in skeletal muscle.

The volume and surface area of skeletal muscle mitochondria were measured by the “vertical sections” method (30). Soleus muscles from four obese Zucker rats and four control lean rats were fixed and sectioned in random orientations, always perpendicular to their longitudinal axis, and 80 photographs per group at 5,000 X were taken at random. Photographs were taken excluding subsarcolemmal mitochondria population and always including Z-disc in the field. For each image, mitochondrial surface area and volume were calculated from the number of randomly distributed points and cycloid arcs in a grid. The number of points inside the mitochondria is taken as the volume, and the number of cycloid arcs crossing the mitochondrial outer membrane is taken as the area. The volume/area ratio
is an intrinsic parameter of mitochondria and does not depend on number of mitochondria studied.

**Transient and stable transfection studies.**

10T/2 fibroblasts were grown in DMEM containing 10% FBS. A plasmid enclosing two independent expression cassettes expressing Mfn2 and GFP was prepared for transient transfection. Each expression cassette was driven by a CMV promoter and contained an SV40 polyadenylation signal. Transfected cells were identified by GFP fluorescence. Cells transfected with the empty vector were used as control. For electron microscopy, transfected cells were sorted using flow cytometry; thereafter, GFP-expressing cells were pelleted and processed for Spurr embedding and ultramicrotomy. For stable transfection, IMAGE clone 3746-e24 was digested with KpnI and XhoI and the antisense fragment corresponding to sequence 1-370 from mouse Mfn2 (GenBank accession number AY028170) was purified and cloned into pcDNA3 vector (Invitrogen) between XhoI-KpnI sites. Geneticin selection at 500 µg/ml allowed isolation of individual clones. All transfections were performed with 20 µg of plasmid by the calcium phosphate method (31).

**Production of adenovirus.**

Recombinant adenovirus expressing GFP, lacZ or antisense Mfn2 cDNA fragment were generated by homologous recombination as previously described (32). The mouse Mfn2 sequence between nucleotides 1-370 was cloned in antisense orientation in the shuttle plasmid pdelE1sp1A and co-transfected with pJM17 into 293 cells to achieve homologous recombination. Individual plaques were isolated and checked for mRNA expression after infection of 293 cells. Recombinant adenovirus were further amplified in 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 293 cells with serial dilutions of the preparation and counting plaque forming units (pfu) on the monolayer 15 days after infection.
Glucose oxidation measurements.

Glucose oxidation was measured in cells expressing or not a Mfn2 antisense sequence. Cells were incubated in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.33 \textmu M $^{14}$C-(U)-D-glucose (323 Ci/mol) (Amersham Pharmacia), and $^{14}$CO$_2$ was trapped and measured as described (33).

Measurement of cell respiration rates

Respiration rates were measured using two 2 ml Clark oxygen electrodes (Rank Brothers, Bottisham, Cambridge, UK) at 37 °C and connected to a Kipp & Zonendual-channel chart recorder, assuming 479 nmol O/ml at air saturation. Cell respiration rates were expressed as a function of cell number. Cells were diluted with Krebs-Ringer buffer supplemented with 5 mM glucose and BSA 2% to 4.10^6 cells/ml in a 2 ml Clark electrode at 37 °C. Inhibitors were used at times and concentrations that caused maximal inhibition (data not shown), and measurements were performed at 70 % air saturation after at least 5 min. Respiration insensitive to myxothiazol (200 \textmu M) was defined as non-mitochondrial and respiration sensitive to myxothiazol was defined as mitochondrial. Similarly, respiration sensitive to oligomycin (80 \textmu g/ml) was defined as coupled respiration, and respiration insensitive to oligomycin but sensitive to myxothiazol was defined as respiration driving proton leak or uncoupled respiration.
RESULTS.

Mitofusin 2 (Mfn2) is highly expressed in muscle and is induced in myogenesis.

In a screening designed to identify genes differentially expressed in obese Zucker rats using PCR-select cDNA subtraction, we isolated a 321 bp clone (C31), which hybridized with a 4.7 kb mRNA species in skeletal muscle. C31 cDNA sequence was identical to the sequence in GenBank between nucleotides 2,098 and 2,419 of rat U41803 and 79% identical to the human cDNA sequence D86987 submitted by Nagase et al. to the GenBank and recently identified as mitofusin Mfn2 (22). Multi-alignment of human Mfn2 with other gene products showed that it is 95% identical to the rat Mfn2 protein (U41308), 94% identical to the mouse Mfn2 counterpart (GenBank accession number AY028170) and 47% identical to *Drosophila melanogaster* Mfn2 (GenBank accession number AF355475). Lower identity was found with the fzo gene product from *D. melanogaster* (31% identity) (10) and that from *Saccharomyces cerevisiae* (8% identity) (11). The tissue distribution of human Mfn2 mRNA was examined by Northern blot analysis at high stringency (Figure 1A). The mRNA species of 4.7 kb hybridized with the 321 bp clone. Transcripts were predominant in skeletal muscle and heart but expression was low in brain, kidney and liver (Figure 1A). A similar distribution profile was detected in rat tissues (data not shown).

Based on the abundant expression of Mfn2 mRNA in skeletal muscle, we examined whether the expression was regulated during myogenesis. L6E9 myoblasts barely expressed Mfn2 but a marked induction of Mfn2 mRNA levels was detected with differentiation into myotubes (Figure 1B).

A polyclonal antibody was raised against a Mfn2 sequence, which detected a 110 kDa band in extracts from heart, skeletal muscle, brown adipose tissue and kidney in rats (Figure 2A); the band was specifically washed by an excess of the immunogenic peptide (Figure 2A). Endogenous Mfn2 was enriched in mitochondrial fractions in heart, brown adipose tissue and kidney (Figure 2B), which is in keeping with observations that overexpressed Mfn2 is found in mitochondria (22, 34). Under these conditions, we found that myogenesis caused the induction of Mfn2 protein in L6E9 cells (Figure 2C).
Mfn2 induction parallels the development of the mitochondrial network and its operation as an intralumenally connected system.

Induction of Mfn2 gene expression in L6E9 myotubes was parallel to the development of a very extensive mitochondrial network (Figure 3A and 3B). Evidence for the operation of the mitochondrial network as an intralumenally connected system in muscle cells was obtained by laser microirradiation (3). In myoblast cells, a short portion of a mitochondrial filament became de-energized when one of the cluster-composing mitochondria was damaged by laser (Figure 3C and 3D). As a consequence of the loss of mitochondrial membrane potential, mitochondria became unstained by JC-1. In contrast, illumination of a single mitochondrial filament in L6E9 myotubes caused a generalized loss of mitochondrial membrane potential throughout the mitochondrial network, and affecting areas situated far from the irradiation spot (Figures 6A and 6B). These data indicate the existence of a very extensive intralumenal connectivity in the mitochondria that form the mitochondrial network in myotubes.

Mfn2 controls the generation and operation of the mitochondrial network.

In order to determine the precise role of Mfn2 on the mitochondrial network, we performed studies in which Mfn2 expression was up- and down-regulated. Over-expression of Mfn2 after transient transfection collapsed the network in L6E9 myoblasts and mitochondria clustered around the nucleus (Figure 4A and 4B). Mitochondria in 10T1/2 fibroblasts are also usually organised in filaments, but rarely seen as isolated organelles (Figure 4C). Over-expression of Mfn2 after transient transfection also caused mitochondrial clustering around the nucleus (Figure 4D). This is in keeping with recent observations (22, 34). Transmission electron microscopy revealed that this clustering was not due to the generation of giant mitochondria. Instead, it corresponds to aggregates of mitochondria with normal ultrastructural morphology (Figure 4E and 4F). Both endogenous and over-expressed Mfn2 protein was localized in mitochondria by biochemical and immunofluorescence assays (data not shown). These data indicate that Mfn2 requires additional proteins in order to complete the full mitochondrial fusion process.
In order to determine the impact of the repression of Mfn2 gene expression, we knocked-down Mfn2 expression in muscle cells. To this end, we generated an adenoviral vector encoding a mouse Mfn2 antisense sequence (AS) and this was used to infect L6E9 myotubes. AS adenoviral infection reduced Mfn2 protein levels in mitochondrial extracts (by about 50%) in the absence of alterations in the abundance of Porin (Figure 5A). This effect was specific and infection with an irrelevant adenovirus, encoding β-galactosidase, had no effect on Mfn2 expression (Figure 5A). Control myotubes or myotubes infected with an irrelevant adenovirus had an extensive mitochondrial network with large filaments running longitudinal to the muscle cell (Figures 5B and 5C). In contrast, AS muscle cells showed discontinuity of the mitochondrial network (Figure 5D). Quantitation indicated that 82% of mitochondrial filaments were longer than 5 µm in control cells whereas only 22% of total mitochondrial filaments showed that size in AS cells.

Illumination of mitochondria in AS myotubes was associated with a loss of mitochondrial membrane potential that affected a very reduced extension of the mitochondrial network (Figures 6A and 6B) compared to control cells (Figures 6C and 6D) and supporting the view for a fragmentation of the mitochondrial network into independent functional clusters.

**Mfn2 controls mitochondrial metabolism.**

Next, we examined whether reduction in Mfn2 expression caused alterations in mitochondrial metabolism. Repression of Mfn2 expression mediated by AS adenoviral expression, in L6E9 myotubes reduced glucose oxidation by 30% (Figure 7A). This effect was specific and infection with an adenoviral vector encoding β-galactosidase did not alter glucose oxidation (Figure 7A). One point of note is that not all L6E9 myoblasts are usually differentiated into myotubes in culture, and near 30% of myoblasts remain undifferentiated (33); provided that undifferentiated myoblasts are not infected with adenoviruses, the reduction we observe in glucose oxidation after AS adenoviral infection represents a substantial underestimate of the actual inhibition. In these conditions, Mfn2 repression reduced mitochondrial membrane potential (Figure 7B).

We also stably transfected 10T1/2 cells with a mouse Mfn2 antisense sequence. Mfn2 mRNA and protein levels were lower in antisense clones than in untransfected or mock-transfected cells (Mfn2 protein levels ranged from 32 to 67% of control values in AS...
clones) (Figure 8A and B). Repression of Mfn2 in 10T1/2 cells also reduced glucose oxidation by 30% (Figure 8C). In addition, AS cells showed a 30% reduction in oxygen consumption (Figure 8D). In these conditions, coupled oxygen consumption was unaltered whereas respiration linked to mitochondrial proton leak was significantly reduced in AS cells (Figure 8D). These data indicate that reduction of Mfn2 expression causes alterations in mitochondrial metabolism characterized by reduced cellular oxygen consumption and depressed glucose oxidation.

**Mfn2 expression is repressed in obesity.**

Skeletal muscle in human obesity is characterized by metabolic alterations which include insulin resistance, accumulation of intracellular triglycerides, reduced oxidation processes and impaired glucose-induced thermogenesis (36, 37). In some animal models of obesity such as in obese Zucker rats or ob/ob mice, skeletal muscle shows a metabolic profile characterized by reduced glucose uptake and glucose oxidation, altered partitioning of fatty acids that are incorporated into triglycerides or oxidized, insulin resistance and reduced oxygen consumption (38-43). In particular, the mechanisms by which obesity leads to a deficient muscle substrate oxidation remain unexplained and are concomitant with unaltered mitochondrial activities of pivotal enzymes such as pyruvate dehydrogenase, citrate synthase, succinate dehydrogenase or β-hydroxyacetyl-CoA dehydrogenase (44-46).

Based on the role of Mfn2 on mitochondrial metabolism, we determined its expression in obesity. Expression of Mfn2 mRNA in Zucker obese rats was 34% lower than in lean (Figure 9A). We therefore measured the gene expression of Mfn2 in skeletal muscle from obese human subjects. Obese subjects were normoglycemic and hyperinsulinemic and showed 50% lower insulin-stimulated glucose disposal as assessed by euglycemic-hyperinsulinemic clamp (4.9±0.8 and 10.1±1.1 mg/kg/min for obese and lean subjects, respectively) (27). Total RNA was purified from *vastus lateralis* muscles from subjects, and RT-competitive-quantitative PCR analysis was performed to measure Mfn2 mRNA (Figure 9B). Expression was 36% lower in the obese subjects (Figure 9B). A negative linear relationship between Mfn2 mRNA levels and body mass index was detected when all data were pooled (data not shown).
Next, we determined Mfn2 protein expression in mitochondrial fractions from skeletal muscle. Expression of Mfn2 in Zucker obese rats was 39% lower than in lean (Figure 10A). In addition, Mfn2 expression in skeletal muscle from obese humans was 43% lower than controls (Figure 10B).

We next examined whether the mitochondrial network was modified in skeletal muscle during obesity. To this end, muscles from obese Zucker rats were obtained and processed for electron microscopy and the extent of the mitochondrial network was assessed as the ratio between mitochondrial volume per unit of mitochondrial surface (30). No differences were found in total mitochondrial volume between control and obese groups indicating that the total mitochondrial mass was unaltered (data not shown). In contrast, the density of mitochondria was higher in the obese group (8.2±0.5 and 11.2±0.6 organelles per 10 \( \mu m^2 \) in control and obese groups, respectively, \( P<0.001 \)) (Figure 11A and 11B). This suggests a smaller mitochondrial size and a fragmented mitochondrial network in skeletal muscle during obesity. In keeping with this view, total mitochondrial surface area was 42% higher in the obese group, and so the mitochondrial volume per unit area was lower (25%) in obesity (Figure 11C).
DISCUSSION.

We show that mitofusin 2 is crucial to the maintenance and operation of the mitochondrial network in muscle and non-muscle cells. This is based on the following observations: a) induction of Mfn2 correlates with the generation of the mitochondrial network, b) repression of Mfn2 in L6E9 myotubes (a 50% reduction) causes the morphological fragmentation of the mitochondrial network, c) repression of Mfn2 in myotubes alters the operation of the mitochondrial network, so instead of acting as an intralumenally connected system, it is divided into independent functional units, and d) repression of Mfn2 expression (a 40% reduction) correlates with a reduction of the mitochondrial network in skeletal muscle from obese Zucker rats.

Mitochondrial networks in myotubes operate as single functional systems and damage to one mitochondrial filament collapses the entire mitochondrial network. This operation is unique to myotubes and previous reports on cardiac myocytes or on fibroblasts suggest there are several independent clusters of electrically connected mitochondria per cell (3, 4). Alteration of the functional mitochondrial network in myotubes with lower Mfn2 expression indicates that this protein permits the operation of the mitochondrial network as an intralumenally connected system.

Our data also demonstrate that Mfn2 controls mitochondrial metabolism. This is based on the observations that: a) repression of Mfn2 in myotubes (50% reduction) reduces mitochondrial membrane potential by nearly 60% and glucose oxidation by at least 30%, and b) cellular repression of Mfn2 in 10T1/2 cells (68% reduction) reduces mitochondrial proton leak and cellular oxygen consumption (30% reduction) and glucose oxidation (70% reduction), which occurs in the absence of changes in coupled respiration. Our data indicate that partial abolition of Mfn2 expression modifies mitochondrial metabolism in cells. In this connection, we postulate that Mfn2 helps to maintain the maximal activity of certain components of the respiratory chain so changes in the expression of Mfn2 may destabilise some of those complexes and/or reduce their activity.
We also show that Mfn2 is reduced by nearly 40% in skeletal muscle from obese subjects and from obese Zucker rats. This reduction is close to that obtained in cultured cells by genetic manipulation. In parallel to a reduction of Mfn2 expression, the mitochondrial network and mitochondrial size in skeletal muscle from obese Zucker rats were smaller. In keeping with these observations, smaller mitochondria have been reported in skeletal muscle from obese subjects (47). The downregulation of Mfn2 detected in skeletal muscle from obese subjects or obese rats together with the observation that Mfn2 promotes mitochondrial metabolism may explain some of the metabolic alterations observed during obesity and characterized by impaired muscle glucose oxidation and fatty acid oxidation (36-39) and reduced cytochrome c oxidase (48).

Zucker rats are obese due to a loss-of-function mutation in the leptin receptor (49) and the obesity of this animal model is only partially explained by its hyperphagia so the underlying metabolic unbalance is not understood (50). This is crucial to our understanding of human obesity, a syndrome characterized by leptin resistance (51). In this regard, the observation that obesity shows reduced Mfn2 expression and a reduced mitochondrial network, which results in reduced mitochondrial metabolism in vitro, could prove to be of central importance. Further studies in rodents after recombinant ablation of Mfn2 are required to determine whether it contributes to obesity.

In summary, we have identified mitofusin 2 (Mfn2) as a mitochondrial protein which plays a role in the control of the morphology and electrochemical operation of the mitochondrial network in cells and in mitochondrial metabolism. Thus, cellular repression of Mfn2 decreases mitochondrial membrane potential and the cellular rate of glucose oxidation and represses mitochondrial proton leak, which occurs in the absence of changes in coupled respiration. These observations suggest that Mfn2 plays a role in the pathophysiology of obesity since repression of Mfn2 gene expression in skeletal muscle from obese subjects may account for impaired energy expenditure and impaired muscle oxidative capacity associated with obesity. In all, our data imply a major role of Mfn2 in mitochondrial energy metabolism and thermogenesis, which when deregulated, may be a key risk factor in the development of obesity.
Acknowledgments.

We thank Robin Rycroft for his editorial support. This work was supported by research grants from the Dirección General de Investigación Científica y Técnica (PM98/0197, SAF2002-02125), Fundació la Marató de TV3 (991110), Fondo de Investigaciones Sanitarias (00/0125), grant 1999SGR 00039 from Generalitat de Catalunya, and COST B17 Action. S.P. is recipient of a predoctoral fellowship from the Ministerio de Ciencia y Tecnología, Spain.
REFERENCES.

1. Bakeeva, L.E., Chentsov, Yu.S. and Skulachev, V.P. (1978) Biochim Biophys Acta 501, 349-369.

2. Kirkwood, S.P., Munn, E.A. and Brooks, G.A. (1986) Am. J. Physiol. 251, C395-402.

3. Amchenkova, A.A., Bakeeva, L.E., Chentsov, Y.S., Skulachev, V.P. and Zorov, D.B. (1988) J Cell Biol 107, 481-495.

4. Skulachev, V.P. (2001) Trends Biochem. Sci. 26, 23-29.

5. Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W., and Walter, P. (1997) Mol. Biol. Cell 8, 1233-1242.

6. Suelmann, R., and Fischer, R. (2000) Cell Motil. Cytoskeleton 45, 42-50.

7. Collins, T.J., Berridge, M.J., Lipp, P., and Bootman, M.D. (2002) EMBO J. 21, 1616-1627.

8. Jensen, R.E., Aiken Hobbs, A.E., Cerveny, K.L. and Sesaki, H. (2000) Micros. Res. Tec. 51, 573-583.

9. Shaw, J.M. and Nunnari, J. (2002) Trends Cell Biol. 12, 178-184.

10. Hales, K.G. and Fuller, M.T. (1997) Cell 90, 121-129.

11. Rapaport, D., Brunner, M., Neupert, W. and Westermann, B. (1998) J. Biol. Chem. 273, 20150-20155.

12. Otsuga, D., Keegan, B.R., Brisch, E., Thatcher, J.W., Hermann, G.J., Bleazard, W., and Shaw, J.M. (1998) J. Cell Biol. 143, 333-349.

13. Guan, K., Farh, L., Marshall, T.K. and Deschenes, R.J. (1993) Curr. Genet. 24, 141-148.

14. Shepard, K. and Yaffe, M. (1999) J. Cell Biol. 144, 711-719.

15. Wong, E.D., Wagner, J.A., Gorsich, S.W., McCaffery, J.M., Shaw, J.M., and Nunnari, J. (2000) J Cell Biol 151, 341-352.

16. Mozdy, A.D., McCaffery, J.M. and Shaw, J.M. (2000) J. Cell Biol.. 151, 367-379.

17. Tieu, Q. and Nunnari, J. (2000) J. Cell Biol.. 151, 353-365.

18. Fekkes, P., Shepard, K.A. and Yaffe, M.P. (2000) J. Cell Biol. 151, 333-340.
19. Smirnova, E., Shurland, D.L., Ryazantsev, S.N., van der Bliek, A.M. (1998) J. Cell Biol. 143, 351-358.

20. Delettre, C., Lenaers, G., Griffoin, J.M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, C., Perret, E., Astarie-Dequeker, C., Lasquellec, L., Arnaud, B., Ducommun, B., Kaplan, J. and Hamel, C.P. (2000) Nat. Genet. 26, 207-210.

21. Alexander, C., Votruba, M., Pesch, U.E., Thiselton, D.L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G., Bhattacharya, S.S. and Wissinger, B. (2000) Nat. Genet. 26, 211-215.

22. Santel, A. and Fuller, M.T. (2001) J. Cell Sci. 114, 867-874.

23. Bereiter-Hahn, J. (1990) Int. Rev. Cytol. 122, 1-63.

24. Mora, S., Monden, I., Zorzano, A. and Keller, K. (1997) Biochem.J. 324, 455-459.

25. Castello, A., Rodriguez-Manzaneque, J.C., Camps, M., Perez-Castillo, A., Testar, X., Palacin, M., Santos, A. and Zorzano, A. (1994) J. Biol. Chem. 269, 5905-5912.

26. Auboeuf, D. and Vidal, H. (1997) Anal. Biochem. 245: 141-148.

27. Andreelli, F., Laville, M., Ducluzeau, P.H., Vega, N., Vallier, P., Khalfallah, Y., Riou, J.P. and Vidal, H. (1999) Diabetologia 42, 358-364.

28. Di Lisa, F., Blank, P.S., Colonna, R., Gambassi, G., Silverman, H.S., Stern, M.D. and Hansford, R.G. (1995) J Physiol. 486, 1-13.

29. Damdimopoulos, A.E., Miranda-Vizuete, A., Pelto-Huikko, M., Gustafsson, J.A. and Spyrou, G.. (2002) J. Biol. Chem. 277, 33249-33257.

30. Baddeley, A.J., Gundersen, H.J.G. and Cruz-Orive, L.M. (1986) J. Microscopy 142, 259-276.

31. Santalucia, T., Boheler, K.R., Brand, N.J., Sahye, U., Fandos, C., Vinals, F., Ferre, J., Testar, X., Palacin, M. and Zorzano, A. (1999) J. Biol. Chem. 274, 17626-17634.

32. Bett, A.J., Haddara, W., Prevec, L. and Graham, F.L. (1994) Proc. Natl. Acad. Sci. 91, 8802.

33. Auestad, N., Korsak, R.A., Morrow, J.W. and Edmond, J. (1991) J. Neurochem. 56, 1376-1386.

34. Rojo M., Legros F., Chateau D. and Lombes A. (2002) J Cell Sci 115, 1663-1674.
35. Canicio, J., Gallardo, E., Illa, I., Testar, X., Palacín, M., Zorzano, A. and Kaliman, P. (1998) Endocrinology 139, 5042-5049.

36. Astrup, A., Andersen, T., Henriksen, O., Christensen, N.J., Bulow, J., Madsen, J. and Quaade, F. (1987) Int. J. Obesity 11, 51-66.

37. Kelley, D.E. and Mandarino, L.J. (2000) Diabetes 49, 677-683.

38. Cuendet, G.S., Loten, E.G., Jeanrenaud, B. and Renold, A.E. (1976) J. Clin. Invest. 58, 1078-1088.

39. Kemmer, F.W., Berger, M., Herberg, L., Gries, F.A., Wirdeier, A. and Becker, K. (1979) Biochem. J. 178, 733-741.

40. Crettaz, M., Prentki, M., Zaninetti, D. and Jeanrenaud, B. (1980) Biochem.J. 186, 525-534.

41. Kaplan, M.L. and Oh, S.S. (1991) Int. J. Obesity 15, 809-812.

42. Turcotte, L.P., Swenberger, J.R., Zavitz Tucker, M. and Yee, A.J. (2001) Diabetes 50, 1389-1396.

43. Zorzano, A., Tomàs, E., Camps, M., Gumà, A and Palacín, M. (2002) In “Muscle metabolism”. Zierath, J.R. and Wallberg-Henriksson, H., eds. (Taylor and Francis. London & New York), pp297-312.

44. Wardlaw, G.M., Kaplan, M.L. and Lanza-Jacoby, S. (1986) J. Nutr. 116, 1841-1852.

45. Torgan, C.E., Brozinick J.T.Jr., Kastello, G.M. and Ivy, J.L. (1989) J. Appl. Physiol. 67, 1807-1813.

46. Amessou, M., Fouque, F., Soussi, N., Desbuquois, B., Hainaut, I., Girard, J. and Benelli, C. (1998) Mol. Cell. Endocrinol. 144, 139-147.

47. Kelley, D.E., He, J., Menshikova, E.V., and Ritov, V.B. (2002) Diabetes 51, 2944-2950.

48. Simoneau, J.A., Kelley, D.E., Neverova, M., and Warden, C.H. (1998) FASEB J. 12, 1739-1745.

49. Phillips, M.S., Liu, Q., Hammond, H.A., Dugan, V., Hey, P.J., Caskey, C.J. and Hess, J.F. (1996) Nat Genet 13, 18-19.

50. Buchberger, P. and Schmidt, I. (1996) Am.J.Physiol. 271, R472-R476.

51. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al (1995) Nat Med 11, 1155-1161.
FIGURE LEGENDS.

Figure 1. Mfn2 is abundantly expressed in muscle and induced during myogenesis.
Panel A. A poly(A) RNA (2 µg per lane) membrane containing 12 different human adult tissues was purchased from CLONTECH. Blots were probed with \(^{32}\)P-labeled rat Mfn2 cDNA fragment and washed at stringent conditions. Mfn2 cDNA hybridises to a transcript of approximately 4.7 kb. Proper quality and control of loading was substantiated by hybridisation with human \(\beta\)-actin cDNA, used as a control probe.
Panel B. Total RNA was purified from L6E9 myoblast (Mb) or myotube (Mt) cells. Mfn2 mRNA was detected after hybridisation with a 1200 base-pair SacI human Mfn2 fragment as a cDNA probe. Cytochrome c oxidase subunit-II (COX-II) mRNA was detected after hybridisation with a 500 base-pair fragment as a cDNA probe. The integrity of RNA in each sample used and the relative amounts of RNA loaded in each lane were checked by 28S ribosomal RNA ethidium bromide staining on the same gel.

Figure 2. Mfn2 protein is enriched in mitochondria and undergoes induction in myogenesis.
Panel A. Extracts enriched in mitochondria were obtained from brown adipose tissue (BAT), kidney, skeletal muscle or heart from rats. Mfn2 was detected in Western blot assays by using a polyclonal antibody raised against the peptide LGPKNSRRALMGYNDQVQRP. Western blot assays were performed in the absence or in the presence of the immunogenic peptide at a concentration of 400 µg/ml.
Panel B. Crude homogenates (H) or fractions enriched in mitochondria (Mito) were obtained from heart, brown adipose tissue and kidney in rats. Mfn2 and Porin were detected in Western blot assays by using specific antibodies.
Panel C. Homogenates (H) or fractions enriched in mitochondria (Mito) were obtained from L6E9 myoblasts (Mb) and myotubes (Mt). Mfn2 and Porin were detected in Western blot assays by using specific antibodies. Control for protein loading was also checked by Ponceau staining of protein on Immobilon filters.
**Figure 3. The mitochondrial network in muscle cells.**

Panels A and B. The mitochondrial network was visualized by incubation with a monoclonal antibody against Porin and further incubation with a Texas red-labeled secondary antibody in L6E9 myoblasts (panel A, scale bar, 10 µm) or in L6E9 myotubes (panel B, scale bar, 10 µm).

Panels C and D. L6E9 myoblasts were irradiated with a laser beam to perforate mitochondria and to induce discharge of the membrane potential in the mitochondrial filaments. Pseudocolor images of the green fluorescence emitted by the monomers of JC-1 were taken before (C) and after 5 min (D) of the laser irradiation. Total discharge of mitochondrial membrane potential is detected as destaining of previously stained mitochondria. Arrows show the place of the laser illumination.

**Figure 4. Human Mfn2 over-expression affects the morphology of mitochondrial filaments in muscle cells.**

Panels A and B. L6E9 muscle cells were transiently co-transfected with human Mfn2 and green fluorescent protein (GFP) and 24 h later, the mitochondrial network was visualized by incubation with a monoclonal antibody against cytochrome c oxidase subunit I and further incubation with a Texas red-labeled secondary antibody (panel B, scale bar, 10 µm); transfected cells (GFP positive) were visualized in panel A.

Panels C-F. 10T1/2 cells were transiently transfected with a GFP expression vector (panels C and E) or with a GFP and human Mfn2 expression vector (panels D and F) and 24 h later, the mitochondrial network was visualized by MitoTracker staining (panels C and D, scale bars, 10 µm) or mitochondria were visualised at the electron microscope (panels E and F, scale bars, 1 µm). Asterisks in panels E and F denote mitochondria.

**Figure 5. Mfn2 repression alters the morphology of the mitochondrial network in muscle cells.**

Panel A. Adenoviruses encoding a mouse DNA sequence (nucleotides 1 to 366 from AY028170) in antisense orientation driven by a CMV promoter were generated. L6E9 myotubes were infected with adenoviruses at 100 pfu/cell either encoding β-galactosidase
(β-GAL) or the mouse Mfn2 antisense sequence (AS); some L6E9 cells were not infected (C). Two days after infection, cells were scraped and fractions enriched in mitochondria were obtained. Mfn2 and Porin were detected in Western blot assays by using specific antibodies. Control for protein loading was also checked by Ponceau staining of protein on Immobilon filters.

Panels B-D. Myotubes infected with the AS adenoviral vector (panel D), with adenoviruses encoding β-galactosidase (panel C) or not infected (panel B) were processed for mitochondria staining with JC-1. Green monomer fluorescence images are shown. Results are representative of four independent experiments. Scale bars, 10 μm.

Figure 6. Mfn2 repression alters the operation of the mitochondrial network in muscle cells.

Panels A-D. Myotubes infected with the AS adenoviral vector (panels C and D) or with adenoviruses encoding β-galactosidase (panels A and B) were irradiated with a laser beam to perforate mitochondria and to induce discharge of the membrane potential in the mitochondrial network. Pseudocolor images of the green fluorescence emitted by the monomers of JC-1 were taken before (panels A and C) and after 5 min (panels B and D) of laser irradiation. Total discharge of mitochondrial membrane potential is detected as destaining of previously stained mitochondria. Arrows show the place of the laser illumination. Arrowheads show mitochondrial clusters unaffected after laser illumination in AS cells. Discontinuous lines delineate the myotubes. Scale bars, 10 μm.

Figure 7. Mfn2 repression impairs glucose oxidation and mitochondrial membrane potential in L6E9 myotubes.

Panel A. Glucose oxidation was determined in L6E9 myotubes previously infected with adenoviruses at 100 pfu/cell either encoding β-galactosidase (β-GAL) or a mouse Mfn2 antisense sequence (AS); glucose oxidation was also measured in myotubes that were not infected (C). During the experiment, cells were in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 μCi 14C-(U)-D-glucose, and 14CO2 was trapped and measured. Results are mean±SE of 4 independent observations. * indicates a statistically significant difference between β-GAL and AS cells, at P<0.05.
Panel B. Myotubes infected with the AS adenoviral vector (AS), with adenoviruses encoding β-galactosidase (β-GAL) or not infected (C) were stained with JC-1 potentiometric dye and mitochondrial membrane potential was measured as the ratio between the red fluorescence (from aggregate formation by the concentrated dye in high mitochondrial polarized regions) versus green fluorescence (from JC-1 monomers). Results are mean±SE of eight independent cells. ** indicates a statistically significant difference between β-GAL and AS cells, at P<0.001.

Figure 8. Mfn2 repression impairs glucose oxidation and mitochondrial metabolism in 10T1/2 cells.
Panel A. Total RNA was obtained from wild type 10T1/2 cells or from cells stably transfected with the empty vector (mock) or with a mouse Mfn2 antisense sequence (DNA fragment between nucleotides 1 and 366 of mouse Mfn2 cDNA, AY028170, in antisense orientation and driven by a CMV promoter) (clones AS1 and AS2). Mfn2 mRNA levels were detected by Northern blot analysis.
Panel B. Crude protein extracts were obtained from wild type 10T1/2 cells transfected with the empty vector (mock) or with different mouse Mfn2 antisense clones (AS1 and AS2). Mfn2 protein abundance was detected by Western blot using a specific antibody.
Panel C. Glucose oxidation was determined in wild type 10T1/2 cells, mock and AS cells. During the experiment, cells attached to 25-cm flasks at logarithmic growth were cultured in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose) containing 0.32 iCi 14C-(U)-D-glucose (Amersham Pharmacia), and 14CO2 was trapped and measured. Results are mean±SE of 4 independent observations. Differences between control and AS cells were statistically significant at P<0.05.
Panel D. Oxygen consumption from 2 x 10^6 mock (open bars) or AS (black bars) cells was determined, in a Clark electrode, at different conditions, to calculate total oxygen consumption, mitochondrial or non-mitochondrial oxygen consumption, coupled oxygen consumption (driving ATP synthesis) (coupl. resp.) or uncoupled respiration (proton leak). Cell respiration rates were expressed as a function of cell number. Cells were incubated in suspension and at constant agitation in Hank’s balanced salt medium (2% FBS, 5 mmol/l glucose). Respiration insensitive to myxothiazol (200 µM) was defined as non-
mitochondrial and respiration sensitive to myxothiazol was defined as mitochondrial. Similarly, respiration sensitive to oligomycin (80 µg/ml) was defined as coupled respiration, and respiration insensitive to oligomycin but sensitive to myxothiazol was defined as respiration driving proton leak or uncoupled respiration. Results are mean±SE of 4 independent experiments. * indicates a statistically significant difference between control and AS cells, at P<0.05.

Figure 9. Obesity represses Mfn2 gene expression in skeletal muscle.
Panel A. Total RNA was purified from skeletal muscle from lean and obese Zucker rats. Mfn2 mRNA was detected after hybridisation with a 321 base-pair fragment (nucleotides 2,098-2,418 of rat Mfn2 cDNA, U41803) as a cDNA probe. Northern blot of stem cell factor (SCF) was performed as a control of the relative amounts of RNA loaded in each lane. The integrity of RNA in each sample used were checked by 18S ribosomal RNA ethidium bromide staining on the same gel. Data are mean±SE of six separate observations. ** indicates a statistically significant difference between lean and obese rats, at P<0.01.
Panels B. Total RNA was purified from skeletal muscle from control or obese subjects. Mfn2 mRNA was detected by competitive-quantitative PCR analysis and data were expressed as amol of Mfn2 mRNA per µg of total RNA. Mfn2 mRNA and an internal competitor at different known concentrations were co-amplified with the same pair of primers: 5'-atgcatccccacttaagcac-3' and 5'-ccagagggcagaactttgc-3'. Mfn2 concentration is extrapolated from the point at which both products are amplified to the same extent. Panel B shows the mean±SE of thirteen and nine independent observations in control and obese subjects. ** indicates a statistically significant difference compared with the control group, at P<0.005.

Figure 10. Mfn2 protein is repressed in skeletal muscle from obese Zucker rats and obese subjects.
Panels A and B. Fractions enriched in mitochondria were obtained from skeletal muscle from lean and obese Zucker rats (Panel A) or from control and obese human subjects (Panel B). Mfn2 and Porin were detected in Western blot assays by using specific antibodies. Control for protein loading was also checked by Ponceau staining of protein on Immobilon
filters. Values are expressed relative to control levels (mean of control group set to 100). * indicates a statistically significant difference between lean and obese rats, at P<0.05 (Panel A). Difference between control and obese subjects (Panel B) was statistically significant, at P<0.005.

**Figure 11. Skeletal muscle from obese Zucker rats shows an altered mitochondrial network.**

Soleus muscles from control rats (panel A) or obese Zucker rats (panel B) were processed to perform stereological analysis of mitochondria. The muscles were fixed and sectioned randomly in different orientations and prepared for electron microscopy. Eighty photographs at 5000 enlargements were taken and mitochondrial surface per unit of mitochondrial volume was determined by using the "vertical sections" method. The extent of the mitochondrial network was estimated as a ratio of the mitochondrial volume/surface (panel C). Results are mean+SE of four independent experiments. Scale bars, 1 µm. ** indicates a statistically significant difference between control obese groups, at P<0.01.
Figure 1
Figure 2

A) Mfn2 Ab and Mfn2 Ab + Peptide

B) Heart, BAT, and Kidney

C) Mb and Mt

H Mito Mb Mt
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism: a novel regulatory mechanism altered in obesity

Daniel Bach, Sara Pich, Francesc X. Soriano, Nathalie Vega, Bernhard Baumgartner, Josep Oriola, Jens R. Daugaard, Jorge Lloberas, Marta Camps, Juleen R. Zierath, Remi Rabasa-Lhoret, Harriet Wallberg-Henriksson, Martine Laville, Manuel Palacín, Hubert Vidal, Francisca Rivera, Martin Brand and Antonio Zorzano

J. Biol. Chem. published online February 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212754200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts