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ARTICLE

G. Mingrone · M. Manco · M. Calvani ·
M. Castagneto · D. Naon · A. Zorzano

Could the low level of expression of the gene encoding skeletal muscle mitofusin-2 account for the metabolic inflexibility of obesity?

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Abstract *Aims/hypothesis:* In obesity the cellular capacity to switch from using lipid to carbohydrate and vice versa as the energy substrate, known as 'metabolic flexibility', is impaired. Mitofusin 2 (MFN2), a mitochondrial membrane protein, seems to contribute to the maintenance and operation of the mitochondrial network, and its expression is reduced in obesity. The aim of this study was to verify whether MFN2 might be implicated in the metabolic inflexibility of obesity. *Materials and methods:* Insulin sensitivity was measured in six morbidly obese women before and 2 years after malabsorptive bariatric surgery (BMI 53.3±10.5 vs 30.3±4.0 kg/m^2). Skeletal muscle *MFN2*, *SLC2A4* (formerly known as *GLUT4*), *COX3* (encoding cytochrome c oxidase subunit III) and *CS* (encoding citrate synthase) mRNA levels were measured by real-time PCR. *Results:* Following bilio–pancreatic surgery, significant increases in *MFN2* mRNA (from 0.4±0.2 to 1.7±1.1 arbitrary units [AU], p=0.019) and *SLC2A4* mRNA (0.38±0.12 to 0.76±0.24 AU, p=0.04) were observed, while increases in *COX3* mRNA (from 14.2±6.4 to 20.2±12.5 AU) and *CS* mRNA (from 0.4±0.1 to 0.7±0.3 AU) failed to reach statistical significance. Insulin-mediated whole-body glucose uptake significantly (p<0.0001) increased from 21.2±4.1 to 52.8±5.9 μmol kg fat-free mass^{-1} min^{-1} and glucose oxidation rose from 11.1±2.1 to 37.7±4.7 μmol kg fat-free mass^{-1} min^{-1} (p<0.0001). Levels of *MFN2* mRNA were strongly correlated with the absolute values for the glucose oxidation rate, both during fasting (glucose oxidation =3.55 *MFN2* mRNA + 3.93; R^2=0.92, p<0.0001) and during the clamp (glucose oxidation=18.8 *MFN2* mRNA+34.7; R^2=0.80, p<0.0001). The percentage changes in *MFN2* mRNA were positively correlated with the percentage change in glucose oxidation during the clamp (glucose oxidation percent (%) change=0.3 *MFN2* mRNA percent (%) change +153.2; R^2=0.61, p<0.001). *Conclusions/interpretation:* We propose that the significant increase in *MFN2* mRNA levels may explain the increase in glucose oxidation observed in morbid obesity following bariatric surgery.

Keywords Glucose oxidation · Insulin sensitivity · Metabolic inflexibility · Mitofusin-2 mRNA expression · Morbid obesity

Abbreviations AU: arbitrary unit · BPD: bilio-pancreatic diversion · COX3: cytochrome c oxidase subunit III · CS: citrate synthase · FFM: fat-free mass · IMTG: intramyocytic triglycerides · M: whole-body glucose uptake value · MFN2: mitofusin 2 · OXPHOS: oxidative phosphorylation · RER: respiratory exchange ratio · TBW: total body water

G. Mingrone (✉) · M. Manco · M. Calvani
Department of Internal Medicine,
Catholic University,
Largo A. Gemelli, 8,
00168 Rome, Italy
e-mail: gmingrone@rm.unicatt.it
Tel.: +39-06-30154395
Fax: +39-06-3054392

M. Castagneto
Department of Surgery,
Faculty of Medicine, Catholic University,
Rome, Italy

D. Naon · A. Zorzano
IRB-PCB,
Barcelona Scientific Park,
Barcelona, Spain

D. Naon · A. Zorzano
Department of Biochemistry and Molecular Biology,
Faculty of Biology,
Barcelona University,
Barcelona, Spain

Introduction

The increased content of triglycerides within muscle fibres found in obesity and type 2 diabetes has been implicated in the causality of insulin resistance [1, 2]. In fact, the depletion of intramyocytic triglyceride (IMTG) depots [3, 4], observed in morbidly obese subjects after weight loss, is accompanied by the reversibility of insulin resistance. The

increased levels of IMTGs depend on the imbalance from fatty acid influx and oxidation, the amount of fatty acids exceeding the oxidative capacity of myocytic mitochondria being accumulated as triglycerides.

The skeletal muscle is able to switch between carbohydrates and fat as fuel substrates depending on its energy requirement, this capability, the so-called 'metabolic flexibility', being lost in insulin resistance. In fact, obese and type 2 diabetic subjects display a higher lipid oxidation in insulin-stimulated conditions [5] instead of turning their metabolism towards glucose oxidation.

It was recently demonstrated [6] in obese Zucker rats, a well-known animal model of obesity, that the mitochondrial surface area was significantly greater while the mitochondrial volume was lower, suggesting the presence of a smaller mitochondrial size and a fragmented mitochondrial network in skeletal muscle during obesity. Mitofusin 2 (MFN2) is a protein that mediates mitochondrial fusion in skeletal muscle from rats and humans [6]. MFN2 protein expression in mitochondrial fractions from skeletal muscle of both Zucker rats and obese subjects was found to be significantly lower than in the lean counterparts [6]. This latter observation, coupled with the knowledge that muscle glucose oxidation during hyperinsulinaemia is impaired in obesity [7], might explain some of the metabolic alterations observed in the obese, insulin-resistant condition and might account for the loss of metabolic flexibility observed in obesity, explaining by what means it may be regained.

To the best of our knowledge no data have been reported in the literature concerning the role of MFN2 in glucose energy metabolism in obese subjects after weight loss. In order to clarify the role of this mitochondrial protein in energy metabolism in obesity, we have measured *MFN2* mRNA expression in skeletal muscle biopsies of six morbidly obese women before and 2 years after malabsorptive bariatric surgery and have correlated these data with whole-body glucose uptake and oxidation. In this regard, *SLC2A4* (formerly known as *GLUT4*) mRNA was assayed in order to verify whether it was increased after bilio–pancreatic diversion (BPD) and whether there was a correlation with glucose uptake and *MFN2* mRNA levels.

Furthermore, the expression of genes coding for the mitochondrial enzymes citrate synthase (CS) and cytochrome c oxidase (specifically subunit 3; COX3) was measured to assess muscle oxidative capacity and mitochondrial function. CS and cytochrome c oxidase were chosen because of their importance as flux-generating reactions in the tricarboxylic acid cycle and the electron transfer chain, respectively [8]. In addition, CS is encoded by nuclear DNA, whereas COX3 is encoded by mitochondrial DNA.

Subjects and methods

Study protocol

Subjects The study groups consisted of six severely obese (BMI >40 kg/m^2) female subjects studied on two separate occasions: before and 2 years after BPD. None had impaired glucose tolerance, diabetes mellitus or any other endocrine or non-endocrine disease. At the time of the baseline study, all subjects were on a free diet, with the following average composition: 60% carbohydrate, 30% fat and 10% protein (at least 1 g/kg body weight). This dietary regimen was maintained for 1 week prior to the study. The subjects were essentially sedentary, i.e. they did not perform any regular physical exercise either before or after BPD.

The nature and purpose of the investigation were explained to all subjects before they agreed to participate in the study, which complied with the guidelines of the Hospital Ethics Committee.

The subjects were studied on two separate days, one to perform the muscle biopsy, and one for the determination of insulin sensitivity with the glucose clamp method.

BPD This essentially malabsorptive surgical procedure [9] consists of an approximately 60% distal gastric resection with stapled closure of the duodenal stump. The residual volume of the stomach is around 300 ml. The small bowel is transected at 2.5 m from the ileo–caecal valve, and its distal end is anastomosed to the remaining stomach. The proximal end of the ileum, comprising the remaining small bowel carrying the bilio–pancreatic juice and excluded from food transit, is anastomosed in an end-to-side fashion to the bowel 50 cm proximal to the ileo–caecal valve. Consequently, the total length of absorbing bowel is brought to 250 cm, the final 50 cm of which, the so-called common channel, represents the site where ingested food and bilio–pancreatic juices mix.

Restrictive bariatric surgery, such as a long gastroplasty, allows 20–30 ml pouch limit capacity.

Body composition

At time 0, body weight was measured to the nearest 0.1 kg with a beam scale, and height to the nearest 0.5 cm using a stadiometer (Holatin, Crosswell, Wales, UK). Total body water (TBW) was determined using 0.19 Bq tritiated water in 5 ml saline solution administered as an i. v. bolus injection [10]. Blood samples were drawn before and 3 h after the injection. Radioactivity was determined in duplicate in 0.5 ml plasma using a beta-scintillation counter (Model 1600TR; Canberra-Packard, Meriden, CT, USA). Corrections were made (5%) for non-aqueous hydrogen exchange [11]; water density at body temperature was assumed to be 0.99371 kg/l. TBW (kg) was computed as ^3H_2O dilution space (litres) ×0.95×0.99371. The within-subject CV for this method is 1.5% [12]. Fat-free mass (FFM) in kg was obtained by dividing the TBW by 0.732 [13].

Euglycaemic–hyperinsulinaemic clamp procedure

Peripheral insulin sensitivity was evaluated by the euglycaemic–hyperinsulinaemic clamp procedure [14]. After inserting a cannula in a dorsal hand vein for sampling

arterialised venous blood, and another in the antecubital fossa of the contralateral arm for infusions, the subjects rested in the supine position for at least 1 h. They were placed with one hand warmed in a heated air box set at 60°C to obtain arterialised blood samples. Whole-body glucose uptake (M value in μmol kg FFM^{-1} min^{-1}) was determined during a primed constant infusion of insulin (at the rate of 6 pmol min^{-1} kg^{-1}). The fasting plasma glucose concentration was maintained throughout the insulin infusion by means of a variable glucose infusion and blood glucose determinations every 5 min. Whole-body peripheral glucose utilisation was calculated during the last 40-min period of the steady-state insulin infusion.

Indirect calorimetry Indirect calorimetry was used to estimate the metabolic rate and route of glucose disposal during the last 40 min of the 2-h hyperinsulinaemic–euglycaemic clamp, i.e. at steady-state. A ventilated hood system was used for continuous collection and mixing of expired air. The analysers (Deltatrac II Metabolic Monitor, Datex-Ohmeda Segrate [MI], Italy) were calibrated before and after each procedure using standard gases. The total volume of expired air was corrected for standard temperature and pressure conditions. The electrical outputs were interfaced with a desktop computer, and integrated measurements of oxygen consumption (ml/min) and carbon dioxide production (ml/min), respiratory exchange ratio (RER) and total resting energy expenditure (expressed as kcal/min) were averaged and recorded every 5 min. All of the studies were conducted at the same time of the day during each period of study. All subjects were at rest for at least 2 h before indirect calorimetry measurements. The variation in measurements of oxygen when repeated within 3 days in the same subject was in the range of 2–3%. The analysers and flow-meter were calibrated on a regular basis by burning absolute ethyl alcohol to estimate the respiratory exchange rate respiratory exchange rate (RER;0.66) and using a Tissot spirometer to calibrate gas flow.

The urinary nitrogen level was determined using a timed urinary sample collected throughout the study to calculate non-protein RER. The quantity of urinary urea nitrogen excreted during the study was used as an index of protein oxidation, assuming that 1 g nitrogen equals 6.25 g protein.

Carbohydrate and lipid oxidation rates were calculated by using de Weir's equations [15] as follows:

$$C = 4.55 \cdot \dot{V}_{CO_2} - 3.21 \cdot \dot{V}_{O_2} - 2.87 \cdot N$$

$$F = 1.67 \cdot \dot{V}_{CO_2} - 1.67 \cdot \dot{V}_{O_2} - 1.92 \cdot N$$

where C and F are the carbohydrate and lipid oxidation rates, respectively, in mg/min;

$\dot{V}_{O_2}$

and

$\dot{V}_{CO_2}$

are the oxygen consumption and carbon dioxide production rate expressed in ml/min; and N is urinary nitrogen excretion in mg/min.

The low and high 5-min measurement of oxygen was not used in the mean estimates of energy expenditure or routes of glucose disposal, i.e. oxidative or non-oxidative glucose disposal. Non-oxidative glucose disposal during the clamp was estimated as the difference between total glucose disposal during the euglycaemic clamp studies and glucose oxidation rate as estimated from indirect calorimetry. RER >1.0 was assumed to represent lipogenesis.

Muscle biopsy

After an overnight fast, percutaneous muscle biopsies were obtained from the vastus lateralis portion of the quadriceps femoris muscle after local anaesthesia of skin and fascia with lidocaine. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C for further gene expression analysis.

Total RNA preparation and quantification of *MFN2*, *CS*, *COX3* and *SLC2A4* mRNA

Total extraction and treatment with DNase I was performed with an RNeasy mini kit (Qiagen). Total RNA from muscle samples was stored at −80°C until further assay. Quantification of the mRNAs was done by real-time PCR. Reverse transcription was performed from 100 ng total RNA in the presence of specific antisense primers, under conditions that allow optimal synthesis of first-strand cDNA, as described elsewhere [6]. Quantification of PCR products was performed by measuring fluorescence from the progressive binding of SYBR green I dye to double-stranded DNA using the ABI Prism 7700 sequence detection system (Applied Biosystems). The relative quantification value of PCR transcripts was calculated by using the manufacturer's protocol (comparative Ct method) with normalisation to *PPIA* (the gene encoding cyclophilin A) as endogenous control. The sets of primers that we used were as follows: *MFN2* (5′-CCCCCTTGTCTTTATGCTGATGTT-3′ and 5′-TTTTGGGAGAGGTGTTGCTTATTTC-3′), *PPIA* (5′-CAAATGCTGGACCCAACACAA-3′ and 5′-CCTCCAC AATATTCATGCCTTCTT-3′), *CS* (5′-CCATCCACAGTG ACCATGAG-3′ and 5′-CTTTGCCAACTTCCTTCTGC-3′), *COX3* (5′-CGCCTGATACTGGCATTTTGT-3′ and 5′-TTTTTTTTTTTTTTTTTTTTTTTTAAGACC-3′) and *SLC2A4* (5′-GCTACCTCTACATCATCCAG-3′ and 5′-TGTCTCGAAGATGCTGGTC-3′).

Statistical analysis

Data are reported as means±SEM, unless otherwise specified. The Wilcoxon test was used to compare data from the two groups. We checked the validity of the model using standard tests. These included assessing the distribution of the residuals, testing for normality, and checking the linearity assumptions in the model by means of standard scatter plots. Data analyses were performed with SPSS statistical software (SPSS, Chicago, IL, USA). A two-sided p value of less than 0.05 was considered significant.

Results

The weight loss attained 2 years after BPD was 42.2±7.5% of the initial body weight; in fact the BMI fell from 53.3±10.5 to 30.3±4.0 kg/m^2 (p<0.0001). This weight loss was mainly dependent on fat mass loss (from 60.1±12.2 to 18.8±1.0 kg, p<0.001). Although FFM in absolute terms was also reduced (73.4±14.9 vs 56.7±6.6 kg, p<0.01), its percentage of total body weight was higher than in the formerly morbidly obese state (74.9±2.5 vs 55.1±1.0%, p<0.0001).

Insulin-mediated whole-body glucose uptake significantly (p<0.0001) increased, from 21.2±4.1 to 52.8±5.9 μmol kg FFM^{-1} min^{-1}, in spite of similar circulating levels of insulin reached during the last 40 min of the clamp (497.0±73.1 vs 493.0±59.4 pmol/l, p=n.s.).

The expression of *MFN2* in the mitochondrial fraction from skeletal muscle was significantly (p=0.019) lower before than after BPD, increasing fourfold from 0.4±0.2 to 1.7±1.1 arbitrary units (AU) (mRNA relative to *Cyclophilin*).

The *COX3* mRNA level increased from 14.2±6.4 to 20.2±12.5 AU, while *CS* almost doubled (from 0.4±0.1 to 0.7±0.3 AU). However, due to a large inter-individual variability, we failed to find a statistical significance.

SLC2A4 expression significantly (p=0.04) increased from 0.38±0.12 to 0.76±0.24 AU. *SLC2A4* mRNA levels positively correlated to *MFN2* mRNA concentrations (*SLC2A4* mRNA=0.50 ln *MFN2* mRNA+0.80; R^2=0.66, p<0.001). Furthermore, *SLC2A4* mRNA levels were linearly correlated with the values of whole-body glucose uptake (M=27.17 *SLC2A4* mRNA+21.59; R^2=0.59, p<0.001).

Due to a non-normal distribution of *MFN2* mRNA levels in the population studied, these values were transformed to natural logarithms. A negative relationship (ln *MFN2* mRNA=−0.038BMI+1.30; R^2=0.36, p<0.001) between BMI and *MFN2* mRNA levels was observed as shown in Fig. 1.

Fasting glucose oxidation significantly (p=0.015) increased after BPD, from 1.0±3.6 to 5.3±1.3 μmol kg FFM^{-1} min^{-1}. The values of fasting glucose oxidation were linearly related with the skeletal muscle levels of *MFN2* (fasting glucose oxidation = 3.55 *MFN2* mRNA levels + 3.93; R^2=0.92, p<0.0001) (Fig. 2). No significant correlation was found between fasting lipid oxidation and *MFN2* mRNA.

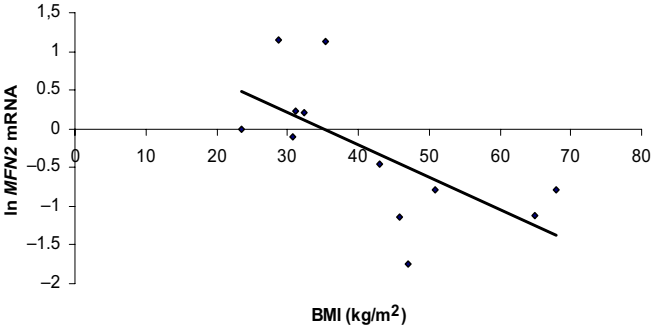

Fig. 1 Negative correlation between BMI and *MFN2* mRNA, expressed in logarithmic form. The equation is as follows: ln *MFN2* mRNA=−0.038×BMI+1.30; R^2=0.36, p<0.001

Whole-body glucose uptake correlated positively with the levels of *MFN2* mRNA (M=14.3ln *MFN2* mRNA+41.0; R^2=0.55, p<0.001).

As a consequence of the large increase in insulin-stimulated glucose uptake, both glucose oxidation and non-oxidative glucose disposal significantly increased (from 11.1±2.1 to 37.7±4.7 μmol kg FFM^{-1} min^{-1}, p<0.0001, and from 10.1±2.1 to 15.1±1.6 μmol kg FFM^{-1} min^{-1}, p=0.001, respectively). The concentrations of *MFN2* were strongly correlated with the absolute values for the rate of glucose oxidation (glucose oxidation = 18.8 *MFN2* mRNA levels + 34.7; R^2=0.80, p<0.0001) as well as the changes in *MFN2* mRNA levels positively correlating with the percentage change in glucose oxidation (glucose oxidation percent (%) change = 0.3 *MFN2* mRNA % change + 153.2; R^2=0.61, p<0.001), as shown in Figs 3 and 4.

Discussion

The present data show for the first time that *MFN2* expression in skeletal muscle from morbidly obese subjects almost quadruples after massive weight loss. The large increase in *MFN2* mRNA levels was associated with the net improvement of insulin-dependent whole-body glucose

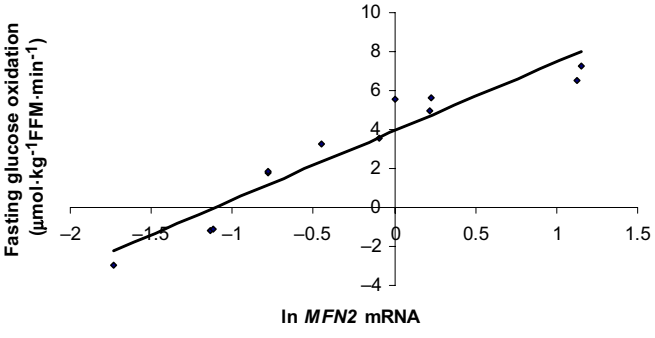

Fig. 2 Linear regression analysis between ln *MFN2* mRNA levels and fasting glucose oxidation. The equation is a follows: fasting glucose oxidation=3.55 *MFN2* mRNA levels+3.93; R^2=0.92, p<0.0001

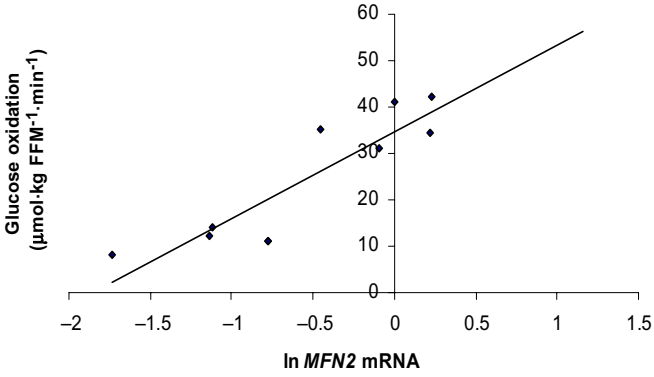

Fig. 3 Linear regression analysis between ln *MFN2* mRNA levels and glucose oxidation during the euglycaemic–hyperinsulinaemic clamp. The equation is as follows: glucose oxidation=18.8 *MFN2* mRNA levels+34.7; R^2=0.80, p<0.0001

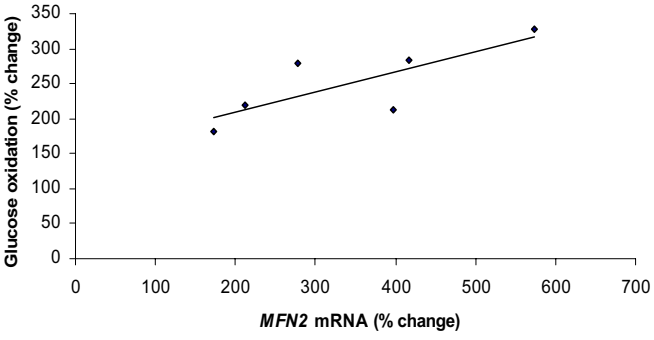

Fig. 4 Linear regression analysis of changes in *MFN2* mRNA as the independent variable and changes in glucose oxidation during the clamp as the dependent variable. The equation is as follows: glucose oxidation percent (%) change=0.3 *MFN2* mRNA % change +153.2; R^2=0.61, p<0.001

uptake and glucose oxidation as well as with the rise in *SLC2A4* expression.

We have previously demonstrated that insulin resistance in morbidly obese patients is positively associated with the IMTG content and with *TNF-α* expression and inversely correlated to *SLC2A4* expression [16]. In agreement with this previous observation [16], whole-body glucose uptake was found to be a linear function of *SLC2A4* expression in skeletal muscle.

MFN2 seems to play a role in the control of the morphology and oxidative capacity of the mitochondrial network [6]. In addition, *MFN2* expression controls the expression of oxidative phosphorylation (OXPHOS) in muscle cells in culture [17]. Therefore, as it was found in morbidly obese subjects before BPD, low *MFN2* expression might explain their low glucose oxidation rate observed during the clamp, while the increase in *MFN2* mRNA levels might account for the increased glucose oxidation found after BPD and weight loss. Factors inducing higher *MFN2* expression might be the same as those determining the increase in *SLC2A4* expression, since these two variables were significantly correlated.

The so-called 'metabolic flexibility' [18, 19] represents a characteristics of mammals to adapt their metabolism to the changes in energy requirements by switching from lipid to carbohydrate oxidation and vice versa. In obese and diabetic individuals a metabolic inflexibility is present, allowing for a failure of skeletal muscle to move appropriately between use of lipid in the fasting state and use of carbohydrate in the insulin-stimulated prandial state. A fundamental component of the metabolic inflexibility is also the reduced capacity for fuel usage in, for example, skeletal muscle, as suggested by reduced mitochondrial size and density found in obesity [20]. However, the reason determining this metabolic inflexibility remains unclear.

Live microscopy studies in yeast (*Saccharomyces cerevisiae*) [21] and plants (onion epidermal cells) [22] has revealed that mitochondrial morphology is continuously remodelled by fission and fusion. In yeast, selective inhibition of either processes significantly modifies mitochondrial size and interconnectivity [23, 24]. Among the best-known proteins involved in mitochondrial dynamics are Fzo/mitofusin, a transmembrane GTPase involved in fusion [25], and Dnm1p/dynamin-related protein 1, a dynamin-related protein involved in fission [26]. Mitochondria fuse with each other by the merging of inner and outer mitochondrial membranes [26]. The complete exchange of matrix proteins by fusion shows that the process of fusion is efficient and that the mitochondrial matrix represents a single cellular compartment.

Two MFN isoforms, MFN1 and MFN2, exist and cooperate in mitochondrial fusion in mammalian cells [27]. While *MFN1* transcripts are highly expressed in heart, *MFN2* mRNA is abundantly expressed in heart and muscle tissue but present at lower levels in many other tissues [27].

MFN2 might represent the link between altered function, as it has been proposed in metabolic inflexibility in obesity, and altered morphology of mitochondria.

The inhibition of *MFN2* expression in myotubes reduces mitochondrial membrane potential, glucose oxidation and expression of OXPHOS subunits and, in parallel, markedly alters the extent of the mitochondrial network [6, 18]. Therefore, the reduced expression of *MFN2* detected in skeletal muscle from obese subjects [6] may allow us to explain the alterations in mitochondrial metabolism that have been reported under this condition. Of great interest is the observation that the normalisation of insulin-mediated glucose disposal after weight loss is accompanied by an increase in *MFN2* expression that translates into the return to normality of glucose oxidation. Our data agree with the findings observed in HeLa cells, where *MFN2* overexpression was accompanied by a marked enhancement of mitochondrial membrane potential and stimulation of glucose oxidation [18].

As far as the expression of *COX3* and *CS* is concerned, a trend towards an increase in their mRNA levels was found, although statistical significance was not detected due to a large intra-individual variability. Due to the four-fold increase in *MFN2* mRNA levels after BPD, we were able to detect a statistical significance, in spite of the dispersion of the data. Mitochondrial ATP production depends on many factors, including the availability of enzyme complexes, ADP and fuel oxidation. Enzymes constitute major

components of the mitochondrial protein complex, and protein synthesis depends on well-coordinated transcriptional regulation of both nuclear and mitochondrial genes [28].

Cytochrome c oxidase [29] is the terminal component of the mitochondrial respiratory chain complex that catalyses the conversion of redox energy to ATP. In eukaryotes, the oligomeric enzyme is bound to the mitochondrial inner membrane with subunits ranging from seven to 13. Thus, its biosynthesis involves a coordinated interplay between nuclear and mitochondrial genomes. The largest subunits, I, II and III, which represent the catalytic core of the enzyme, are encoded by mitochondrial DNA and are synthesised within mitochondria.

CS is involved in oxidative ATP production and is found in direct proportion to muscle mitochondrial content [30, 31]. Kim et al. [32] showed that CS activity was significantly reduced in the vastus lateralis of obese subjects, suggesting that the low mitochondrial content in the skeletal muscle of these subjects contributed to their decrease in muscle oxidative capacity.

The trend towards a rise in *COX3* and *CS* mRNA, although a statistical significance was not reached, might suggest an increase in the skeletal muscle mitochondrial density and oxidative capacity in post-obese subjects, data that are in line with the increased *MFN2* expression found. The observation that *MFN2* mRNA expression is more intensely upregulated by weight loss than the expression of other genes that encode mitochondrial proteins further suggests a major regulatory role of MFN2 in muscle metabolism. This is in keeping with the proposed regulatory role of MFN2 in the morphology and oxidative capacity of the mitochondrial network [6, 18].

In conclusion, although we do not present direct evidence that *MFN2* upregulation translates into actual changes in mitochondrial biology in the muscle of the individuals studied, it is likely that the net improvement in insulin-mediated glucose oxidation observed in post-BPD subjects derives from a skeletal muscle tissue remodelling process with an increase of both the amount and the capacity of mitochondrial oxidation. However, at present it is impossible to discriminate between the roles played by weight loss and lipid malabsorption in this effect. Further studies involving a larger number of subjects are needed to clarify these mechanisms.

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