



Mitochondrial metabolism and insulin resistance



It has been estimated that between 200 and 300 million people worldwide will meet World Health Organisation diagnostic criteria for diabetes mellitus by the end of this decade. This epidemic of predominantly type 2 diabetes has been mediated largely by our shift toward a more sedentary lifestyle, which predisposes us to obesity and insulin resistance. Individuals affected by type 2 diabetes may also exhibit an array of associated undesirable effects, such as hypertension, dyslipidemia and hypercoagulability, which lead to morbidity and mortality from atherosclerotic vascular disease. The co-existence of several of these disorders with insulin resistance constitutes the metabolic syndrome. In Western society, metabolic syndrome diseases are growing at epidemic rates and currently affect approximately 20% of the general population and more than 40% of people over 60 years of age. Recent epidemiological and biological data suggest that the etiology of these diseases shares unexpected and common genetic and biochemical mechanisms.

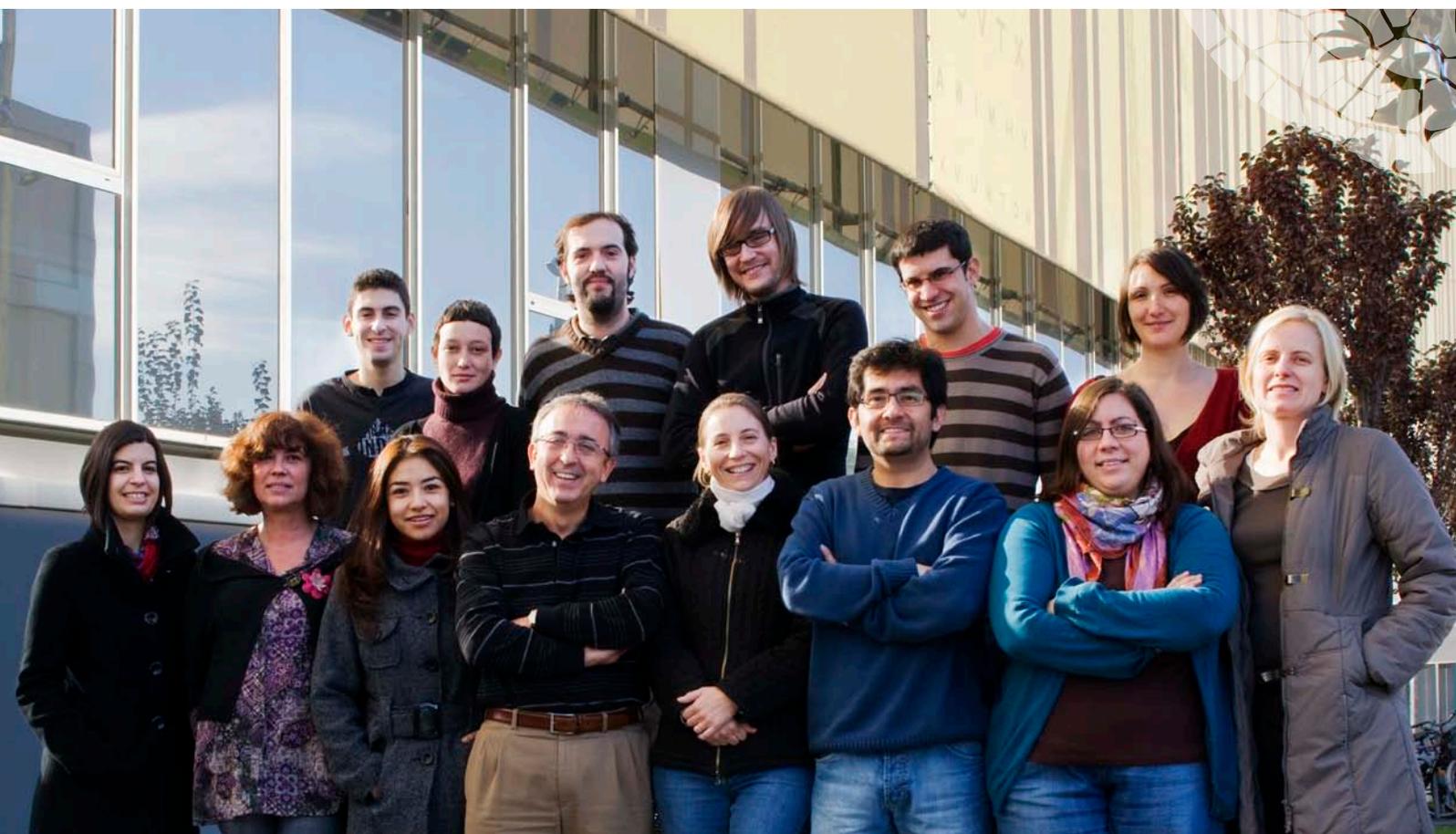
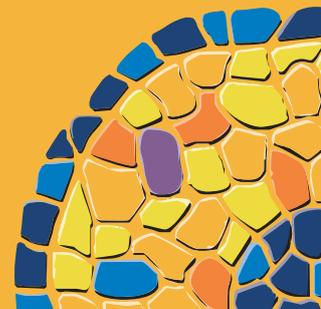
Insulin resistance is strongly associated with obesity, and several mechanisms mediating this interaction have been identified. A number of circulating hormones, adipocytokines (leptin, adiponectin, resistin, plasminogen activator inhibitor-1, interleukin-6, TNF α , and retinol-binding protein 4), and metabolic fuels, such as non-esterified fatty acids (NEFA), originate in the adipocyte and modulate insulin action. An increased mass of stored triglyceride, especially in visceral or deep subcutaneous adipose depots, leads to large adipocytes that are themselves resistant to the capacity of insulin to suppress lipolysis. This resistance results in increased release and circulating levels of NEFA and glycerol, both of which aggravate insulin resistance in skeletal muscle and liver. When chronic, the increased circulating NEFA and other lipids that occur in obesity lead to ectopic fat storage as triglycerides in muscle and liver. Ectopic lipid accumulation has been implicated in insulin resistance, possibly as a result of triglyceride turnover and the production of fatty acid-derived signalling molecules, or of the activation of deleterious intracellular pathways.

There is growing evidence indicating the existence of cross-talk between mitochondrial function and insulin signalling that may be relevant for the pathogenesis of insulin resistance and the disorders characteristic of the metabolic syndrome. Thus, it has been reported that insulin resistance and diabetes are associated with decreased mitochondrial oxidative capacity and at present, there are several lines of evidence supporting the view that alterations in mitochondrial function cause insulin resistance. Experimental evidence linking deficient mitochondrial activity and insulin resistance comes from studies in animals and

in cultured cells. Thus, artificial selection of rats on the basis of low intrinsic exercise capacity for 11 generations has been associated with a high risk of cardiovascular disease or the metabolic syndrome. The decrease in aerobic capacity was associated with a reduction in the amount of oxidative enzymes in skeletal muscle. Studies in knockout mice have also demonstrated that mitochondrial dysfunction caused by long-chain Acyl-CoA dehydrogenase deficiency produces hepatic steatosis and hepatic insulin resistance. Studies in muscle cells in culture have also shown that mitochondrial dysfunction induces aberrant insulin signalling and deficient glucose utilisation. In addition, clinical observations indicate that healthy elderly people have a marked tendency toward insulin resistance, and this resistance is associated with reduced mitochondrial oxidative phosphorylation activity compared with body mass index and activity-matched young individuals. Similarly, insulin-resistant offspring of parents with type 2 diabetes also have impaired mitochondrial function, with mitochondrial adenosine triphosphate (ATP) synthesis being reduced by approximately 30%. These reductions in mitochondrial function were found to be associated with severe muscle insulin resistance and an 80% increase in intramyocellular lipid content. Because these individuals had no abnormalities of systemic or localised rates of lipolysis or plasma concentrations of tumour necrosis factor- α , interleukin-6, resistin, or adiponectin, it is likely that the genetic factor that explains the heritability of type 2 diabetes is connected with the loss of mitochondrial activity in these individuals.

Several mechanisms might contribute to the reduction of mitochondrial activity in insulin-resistant conditions, namely

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changes in mitochondrial density or in mitochondrial function. As to the regulation of mitochondrial density, key nuclear coregulators that are known to control mitochondrial biogenesis include PPAR γ coactivator 1 α (PGC-1 α) and PGC-1 β . Thus, PGC-1 α and PGC-1 β gene expression were recently found to be decreased in muscle of patients with type 2 diabetes and in non-diabetic subjects with a family history of type 2 diabetes. PGC-1 α , like PGC-1 β , induces the expression of genes involved in oxidative phosphorylation in both muscle and liver cells.

Mitochondrial dynamics and mitochondrial metabolism

Mitochondria are dynamic organelles whose morphology is regulated by fusion and fission processes. A growing body of evidence shows the relevance of these shaping processes in the control of mitochondrial activity and cell metabolism. Several

genes encoding mitochondrial fusion and fission proteins have been recently identified. Mammalian proteins involved in mitochondrial fission are Fission 1 homologue protein (Fis1) and Dynamin-related protein 1 (Drp1). Similarly, Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic Atrophy gene 1 (OPA1) are proteins that participate in mitochondrial fusion in mammals. However, there is no evidence to date that demonstrates the capacity of an upstream or transcriptional regulator to shift the balance between mitochondrial fusion and fission events by selective regulation of these proteins.

Several reports provide evidence that Mfn2 protein elicits pleiotropic effects which may be involved in pathology. For instance, Mfn2 is mutated in Charcot Marie Tooth type 2A neuropathy and, interestingly, some of these mutants cause selective defects in mitochondrial fusion, a reduction in mitochondrial axonal transport or defects in mitochondrial coupling, thereby leading to inefficient mitochondria. Defective Mfn2 may also contribute to

impaired mitochondrial function in the context of obesity and type 2 diabetes. This notion is supported by the observation that muscle Mfn2 expression is reduced in patients with these conditions. In addition, we have previously reported that Mfn2 modulates mitochondrial activity through changes in the electron transport chain (ETC) and that this modulation is independent of its role in mitochondrial morphology.

Peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) α and β are important positive regulators of mitochondrial activity and biogenesis in mouse skeletal muscle. Despite these similarities, PGC-1 α and PGC-1 β display low overall sequence identity, with the highest percentages found in two particular domains (activation and RNA recognition domains, with identities of 40% and 50% respectively). Furthermore, key mitochondrial processes, such as organelle biogenesis and uncoupling, are differentially regulated by these homologues. For instance, in C2C12 muscle cells, PGC-1 α , but not PGC-1 β , increases mitochondrial uncoupling, whereas PGC-1 β causes a larger increase in mitochondrial volume than PGC-1 α under the same conditions. In addition, while PGC-1 β expression in distinct tissues is unaffected by physiological processes characterised by increased energy expenditure, such as cold exposure (in brown adipose tissue), fasting (in liver) or exercise (in muscle), PGC-1 α is highly regulated at the transcriptional level under similar physiological challenges. These data suggest that PGC-1 β regulates basal mitochondrial biogenesis, whereas PGC-1 α is involved in regulated mitochondrial activity. In keeping with this view, expression of PGC-1 β is higher than that of PGC-1 α in primary muscle cells under basal conditions.

The functional independency of these homologues in mitochondrial physiology is further illustrated by the phenotypes of PGC-1 α and PGC-1 β knockout (KO) mice. In both animal models, a general defect in the ETC system has been described, thereby demonstrating that PGC-1 α does not fully compensate the effects of PGC-1 β on mitochondria or vice versa. Furthermore, several mitochondrial phenotypes described in the particular case of PGC-1 β -ablated mice cannot be completely explained by impairment of the ETC system. For instance, muscle and liver from PGC-1 β KO mice show a reduction of mitochondrial volume without changes in mitochondria number. This decreased mitochondrial volume together with impaired ETC gene expression may explain the mitochondrial respiration defect found only in muscle strips and not in isolated mitochondria. In keeping with these data, this reduction in mitochondrial size is absent in PGC-1 α KO mice under basal conditions, probably because of normal PGC-1 β expression. Of note, despite all these differences, both genes show a diminished expression in the context of type 2 diabetes, thereby suggesting an impairment of mitochondrial effects selectively regulated by each homologue in this disease.

We previously reported that PGC-1 α induces Mfn2 transcription and that mitochondrial activity regulated by PGC-1 β partly depends on correct Mfn2 expression. However, effects on mitochondrial fusion were not determined. In the light of these results and the PGC-1 β control of basal mitochondrial biogenesis, we have studied whether PGC-1 β regulates Mfn2 transcription and, therefore, whether the mitochondrial dynamics balance can be modulated by transcriptional regulation.

PGC-1 β induces Mfn2 transcription through ERR α coactivation

We have examined whether PGC-1 β regulates the expression of Mfn2 in C2C12 muscle cells. Differentiated C2C12 cells show low levels of PGC-1 β mRNA, as assessed by Northern blot and by real-time PCR. To this end, C2C12 myotubes were transduced either with a mouse PGC-1 β adenovirus or with a control LacZ adenovirus. Mfn2 mRNA levels doubled in PGC-1 β -expressing muscle cells compared to control transduced myotubes. To demonstrate that PGC-1 β directly increases Mfn2 transcription, we transfected 10T1/2 mouse fibroblasts or HeLa cells with a construct containing a 2-kb fragment (-1982/+45) of the Mfn2 promoter fused to a luciferase reporter gene, together with an irrelevant vector (Basal) or mouse PGC-1 β expression vector. PGC-1 β markedly enhanced Mfn2 promoter activity (10.3 \pm 0.9- and 4.2 \pm 0.6-fold over basal Mfn2 promoter activity in 10T1/2 and HeLa respectively). In a previous study, using electrophoretic mobility shift and chromatin immunoprecipitation assays, we showed that ERR α binds to the Mfn2 promoter between nucleotides -459/-396. This DNA region contains three putative boxes with the capacity to bind nuclear receptors, where box 2 is critical for Mfn2 promoter response to PGC-1 α coactivation of ERR α . On the basis of these observations, we determined whether PGC-1 β coactivated ERR α through box 2 in a similar way as PGC-1 α . We transfected 10T1/2 cells with a construct containing a -459/-352 Mfn2 promoter fragment fused to a luciferase reporter gene or with a mutated version of the same fragment that disrupted box 2. We observed

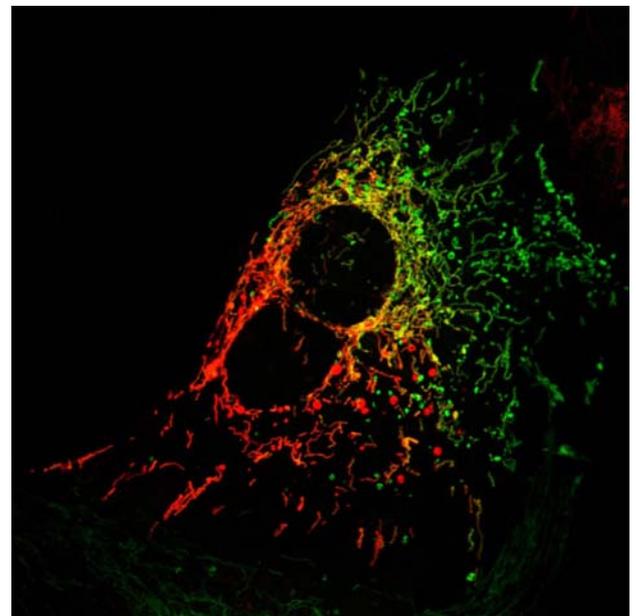


Figure 1. High rate of mitochondrial fusion in myoblasts after overexpression of PGC-1 β . Yellow indicates fusion of green- and red-labelled mitochondria (mitochondrial matrix-targeted green fluorescent protein and red fluorescent protein).

marked coactivation of $ERR\alpha$ by $PGC-1\beta$ in the -459/-352 *Mfn2* promoter fragment. This effect was completely blunted when box 2 was disrupted (11.9 ± 1.09 vs. 2.8 ± 0.6 -fold over basal promoter activity, $p=0.001$, Figure 1C). Furthermore, cancellation of box 2 markedly reduced the activation driven by $PGC-1\beta$ (7.0 ± 0.5 vs. 1.9 ± 0.5 , $p=0.001$) or by $ERR\alpha$ (2.2 ± 0.1 vs. 1.6 ± 0.2 , $p=0.04$), although residual activation was still present. Similar results were obtained when the 2- kb *Mfn2* promoter was cotransfected with $ERR\alpha$ or $PGC-1\beta$.

PGC-1 β causes a large induction of *Mfn2* protein levels

To determine whether $PGC-1\beta$ -mediated *Mfn2* transcription leads to enhanced *Mfn2* protein expression, we transduced C2C12 myotubes with $PGC-1\beta$ or two distinct control adenoviruses at a range of multiplicities of infection (MOI). Total protein extracts and mitochondrial-enriched fractions were obtained and analysed by Western blot. $PGC-1\beta$ induced *Mfn2* in muscle cells and a direct relationship between $PGC-1\beta$ adenoviral dose and *Mfn2* protein induction was detected. $PGC-1\beta$ also increased the cellular content of the constitutive mitochondrial protein Porin, used as a measure of mitochondrial mass. Densitometric quantification of Porin induction at MOI 100 showed a 1.8 ± 0.2 -fold increase in total lysates and a 1.45 ± 0.003 -fold increase in mitochondrial-enriched fractions. Porin induction values in total lysates are consistent with the increase in mitochondrial mass volume reported in $PGC-1\beta$ -overexpressing C2C12 muscle cells.

On the basis of the effects of $PGC-1\beta$ on *Mfn2* and Porin expression, we also analysed whether this coactivator regulates the expression of other proteins involved in mitochondrial dynamics and in the ETC system. $PGC-1\beta$ -transduced myotubes induced the expression of *Mfn1*, *OPA1*, *Drp1* and *Fis1* in mitochondrial-enriched extracts (1.7-, 2.2-, 1.4-, or 2.1-fold over basal values, respectively) to a level similar to that detected for Porin (1.45-fold stimulation). *Mfn2* displayed a significantly higher increase in expression compared to Porin abundance in mitochondrial-enriched extracts (4.3-fold induction). When data were expressed as protein levels relative to Porin expression, we detected significant stimulation of *Mfn2* and *Fis1* only in response to $PGC-1\beta$ overexpression. This superior induction of *Mfn2* protein was also detected in C2C12 myoblasts.

We also analysed several subunits of the ETC system in mitochondrial-enriched fractions from C2C12 myotubes transduced at MOI 100. All the subunits of complexes I, II, III, IV and V studied were induced in response to $PGC-1\beta$, and the extent of induction was similar to that detected in Porin levels (ranging from 1.3- to 2.1-fold increase).

PGC-1 β changes mitochondrial morphology and increases the rate of mitochondrial fusion

As $PGC-1\beta$ induces *Mfn2* expression, we next studied whether this effect was linked to changes in mitochondrial morphology. To this end, we immunofluorescently labelled mitochondria from C2C12 myoblasts transduced with $PGC-1\beta$ or LacZ adenovirus. $PGC-1\beta$ caused an increase in the length of mitochondrial tubules in most myoblasts. This increased mitochondrial size

was also observed by transmission electron microscopy, which also showed normal cristae morphology. To demonstrate that this increase in mitochondrial length was linked to enhanced mitochondrial fusion, we performed a polyethylene glycol-(PEG)-mediated cell fusion assay using two distinct C2C12 lines stably expressing mitochondrial matrix-targeted GFP (mtGFP) or red fluorescent protein (mtRFP). Four hours after PEG addition and in the presence of cycloheximide, a significantly higher percentage (73% vs. 55% in $PGC-1\beta$ and control cells, respectively) of polykaryons from C2C12 cells transduced with $PGC-1\beta$ showed a higher level of mitochondrial matrix content mixing than control LacZ polykaryons (that is to say mtGFP and mtRFP exchange caused by mitochondrial fusion and displayed as yellow mitochondria) (see Figure 1 corresponding to cells after overexpression of $PGC-1\beta$). This higher mtGFP and mtRFP mixing indicated an increase in the rate of mitochondrial fusion induced by $PGC-1\beta$ overexpression. In agreement with previous data, the overexpression of $PGC-1\beta$ in C2C12 myoblasts under these conditions also increased the mitochondrial membrane potential values by ~50%, as measured by using the fluorescent probe JC-1.

***Mfn2* is required for $PGC-1\beta$ -induced changes in mitochondrial morphology**

To study whether the change in mitochondrial morphology induced by $PGC-1\beta$ was mediated mainly through *Mfn2* activity, we used mouse embryonic fibroblasts (MEFs) from wild-type and from *Mfn2* or *Mfn1* KO mice. Overexpression of $PGC-1\beta$ led to an increase in mitochondrial length of wild-type and *Mfn1* KO MEFs, similarly to what was observed in C2C12 myoblasts. Importantly, $PGC-1\beta$ gain-of-function was unable to promote mitochondrial elongation in *Mfn2* KO MEFs, in conditions in which ETC subunits *Cox4* and *Uqcrc2* were increased 1.6- and 1.5-fold respectively. These data demonstrate the requirement of *Mfn2* expression for $PGC-1\beta$ -mediated changes in mitochondrial morphology.

PGC-1 β KO mice show *Mfn2* repression in skeletal muscle and myocardium

We next studied the effects of *in vivo* ablation of $PGC-1\beta$ on *Mfn2* expression in gastrocnemius muscles. *Mfn2* protein levels were reduced by approximately 50% in KO mice. The reduction of *Mfn2* levels was relatively specific as indicated by the absence of major changes in proteins involved in mitochondrial dynamics, *ie*, *Mfn1*, *OPA1*, *Drp1* and *Fis1*. Similar results were obtained using mitochondrial fractions. Reduced *Mfn2* protein expression paralleled lower levels of *Mfn2* mRNA.

Soleus muscle from $PGC-1\beta$ KO mice also showed a decrease in complex IV (subunits *Cox4* and *Cox5b*) mRNA levels. This observation led us to study whether the protein levels of *Cox4* and other subunits of complexes I, II, III and V were also altered in gastrocnemius muscle of this mouse model. These mice displayed a ~40% reduction in complex V (ATP5a1) and III (*Uqcrc2*). Complex IV (*Cox4*) showed a ~25% reduction and complex I (*Ndufa9*) a ~20% decrease while no changes were observed in complex II protein levels (*Sdha*).

The heart is one of the organs with the highest expression of $PGC-1\beta$. Interestingly, heart lysates from KO mice displayed

a clear reduction in Mfn2 protein levels (WT 1.00±0.06, KO 0.57±0.09, p=0.0015; data not shown), thereby suggesting the impairment of this regulatory pathway also in cardiac muscle.

These studies demonstrate that mitochondrial dynamics balance can be shifted towards fusion by transcriptional regulation. More specifically, we show that PGC-1β is a regulator of mitochondrial fusion through its effects of selectively promoting Mfn2 expression upon coactivation of ERRα. This new role of PGC-1β in mitochondrial physiology has been demonstrated using both *in vitro* (muscle cells) and *in vivo* (PGC-1β-ablated mice) approaches. Firstly, we have shown that PGC-1β overexpression in muscle cells regulates mitochondrial dynamics through a mechanism that involves the preferential induction of Mfn2 expression, among other mitochondrial dynamics effectors such as Mfn1, OPA1, Drp1 and Fis1. Furthermore, PGC-1β gain-of-function results in an elongation of mitochondrial tubules, which is linked to increased mitochondrial fusion. Importantly, the ef-

fects of PGC-1β on the promotion of mitochondrial elongation are not observed in Mfn2-ablated cells, thereby demonstrating that Mfn2 activity is essential for PGC-1β-mediated changes in mitochondrial dynamics.

In summary, we provide evidence that the mitochondrial dynamics balance is selectively controlled by a transcriptional regulator, thereby unravelling an upstream mediator of mitochondrial fusion. Furthermore, we also provide evidence of a novel role of PGC-1β in mitochondrial physiology. Given the cross-talk between mitochondrial activity and dynamics, together with reduced Mfn2 and PGC-1β expression in type 2 diabetes, we conclude that the pathway reported here is not only relevant for the thorough explanation of mitochondrial dynamics regulation and the overall mitochondrial effects of PGC-1β but may also provide the basis for the understanding of the alterations of mitochondrial metabolism associated with type 2 diabetes.

SCIENTIFIC OUTPUT

Publications

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Research networks and grants

Adipose tissue: a key target for prevention of the metabolic syndrome

European Science Foundation, COST Action BM0602 (2007-2011)
Principal investigator/Member of the management committee: Antonio Zorzano

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Agency for Administration of University and Research Grants (AGAUR), 2005-SGR-00947 (2005-2008)
Principal investigator: Antonio Zorzano

CIBERDEM-CIBER de Diabetes y Enfermedades Metabólicas Asociadas
Instituto de Salud Carlos III (2007-2011)

Principal investigator/Director of scientific training: Antonio Zorzano

Functional analysis of novel candidate genes of insulin resistance
Spanish Ministry of Science and Innovation, SAF2005-0445 (2005-2008)

Principal investigator: Antonio Zorzano

MITIN-Integration of the system models of mitochondrial function and insulin signalling and its application in the study of complex diseases

European Commission, 7th Framework Programme, FP7-HEALTH-2007-B, 223450 (2008-2011)

Principal investigator: Antonio Zorzano

Collaborations

Early-onset type 2 diabetes and mitochondrial function
John Nolan, St James Hospital, Trinity College Dublin (Dublin, Ireland)

Expression of genes in human adipose tissue
Joan Vendrell, Hospital Joan XXIII (Tarragona, Spain)

Extramitochondrial functions of mitofusin-2
Luca Scorrano, Venetian Institute of Molecular Medicine (Padova, Italy)

Functional analysis of adipose cell proteins
José Manuel Fernández-Real, Trueta Hospital (Girona, Spain)

Mitochondrial dynamics in cardiac cells
Sergio Lavandero, University of Chile (Santiago, Chile)

Mitochondrial fusion in muscle cells
Manuel Rojo, Institut de Biochimie et Génétique Cellulaires-IBGC, (Bordeaux, France)

PGC-1β and mitochondrial dynamics
Antonio Vidal-Puig, Cambridge University (Cambridge, UK)

Type 2 diabetes in morbid obesity and mitochondrial function
Geltrude Mingrone, Catholic University, School of Medicine (Rome, Italy)