Novel mechanisms in the development of insulin resistance and new therapeutic strategies for diabetes

It has been estimated that between 200 million and 300 million people worldwide will meet World Health Organization 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 this disease 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 about 20% of the general population and over 40% of people over 60 years of age. Recent epidemiological and biological data indicate that the etiology of these diseases may share unexpected and common genetic and biochemical mechanisms. A major step towards understanding the metabolic syndrome is the identification of susceptibility genes, which may lead to the acquisition of additional therapeutic targets for future drug design.

In this regard, we study the molecular mechanisms involved in the development of insulin resistance and, in particular, seek to identify novel susceptibility genes for obesity and type 2 diabetes. Our research is structured in three distinct fields: 1) Identification of genes responsible for the development of insulin resistance associated with obesity or type 2 diabetes, with special emphasis on genes implicated in novel mechanisms involving mitochondrial processes (i.e., mitochondrial dynamics), encoding proteins involved in the regulation of nuclear gene expression, or encoding proteins involved in novel signalling events. 2) Analysis of the molecular mechanisms involved in the regulation of glucose transport in muscle and adipose cells and in the development of lipotoxicity. 3) Identification of novel targets and development of new compounds for the treatment of the metabolic syndrome.

Mfn2 regulates mitochondrial metabolism in non-muscle and muscle cells, is down-regulated in obesity and type 2 diabetes and is induced by PGC-1α

In many cell types, especially muscle fibers, mitochondria form tubular structures or networks (Figure 1). There is evidence in Saccharomyces, Aspergillus and mammalian cells that mitochondrial filaments are highly dynamic structures and that they are regulated by changes in the rates of mitochondrial fission and fusion. Studies of gain-of-function and loss-of-function have demonstrated that Mfn proteins regulate mitochondrial fusion in mammalian cells. Thus, overexpression of Mfn2 in cultured cells alters mitochondrial morphology, the effects reported ranging from the generation of reticular structures to extensive perinuclear clustering (Bach et al, 2003). Mouse embryo fibroblasts lacking Mfn2 or Mfn2I display mitochondrial fragmentation (Chen et al, 2003). In addition, Mfn1 and Mfn2 are essential for embryonic development and mice deficient in either gene die in mid-gestation. However, while Mfn2-mutant embryos have a specific and severe disruption of the placental trophoblast giant cell layer, probably caused by a marked reduction in number of placental trophoblastic giant cells, Mfn1-deficient embryos show normal giant cells.

Mfn2 regulates mitochondrial metabolism
Mfn2 is a multifunctional protein and has been reported to inhibit proliferation in smooth muscle cells. Mfn2 also regulates mitochondrial oxidation in
muscle and non-muscle cells. Mfn2 loss-of-function reduces oxygen consumption, mitochondrial membrane potential and oxidation of glucose, pyruvate and fatty acids, without causing changes in mitochondrial mass (Bach et al., 2003; Pich et al., 2005). In addition, Mfn2 gain-of-function activates glucose oxidation and enhances mitochondrial membrane potential. Overexpression of a truncated mutant of Mfn2 that lacks the transmembrane domains and the C-terminal tail also up-regulates glucose oxidation and increases mitochondrial membrane potential, but has no effect on mitochondrial morphology (Pich et al., 2005). These results indicate that Mfn2 participates in mitochondrial oxidation by mechanisms that are independent of its role in mitochondrial fusion.

Regarding the mechanisms responsible for the effects of Mfn2 activity, we have focused on the protein composition of mitochondria. The abundance of mitochondrial protein components of distinct respiratory complexes was assayed in mitochondrial extracts obtained from control and Mfn2 loss-of-function muscle cells. More specifically, we assayed subunit p39 from complex I (encoded by nuclear DNA), protein p70 from complex II (encoded by nuclear DNA), p49 (core 2 subunit encoded by nuclear genome) from complex III, COX-I (encoded by nuclear DNA) and COX-IV subunits of complex IV (encoded by mitochondrial DNA), the β subunit of complex V (encoded by nuclear DNA) and porin (encoded by nuclear DNA). Mfn2 repression caused a marked inhibition of the expression of the subunits of complexes I, II, III and V. In contrast, no alterations in the abundance of complex IV subunits or porin were detected in cells after Mfn2 repression. In keeping with these observations, the enzymatic activity of components I+III and III decreased in Mfn2 loss-of-function cells. These data indicate that Mfn2 loss-of-function causes a specific alteration in the expression of subunits that participate in complexes I, II, III and V, which leads to reduced activity of several components of the OXPHOS system. Mfn2 gain-of-function in L6E9 myoblasts was also associated with increased expression of several subunits of complexes I, IV and V.

In all, evidence indicates that Mfn2 affects mitochondrial metabolism and its expression level regulates mitochondrial membrane potential, fuel oxidation and the OXPHOS system (Figure 2). Given that a truncated mutant form of Mfn2 that is inactive as a mitochondrial fusion protein maintains its capacity to activate mitochondrial metabolism, we support the view that the metabolic accelerator and the mitochondrial fusion roles of Mfn2 are separate. This hypothesis may explain the exquisite sensitivity of the neuronal cell to a partial Mfn2 loss-of-function, as recently reported in Charcot-Marie-Tooth type 2A neuropathy, a disease that shows autosomal dominant inheritance. On the basis of our data, we propose that Mfn2 loss-of-function participates in triggering the pathological mechanisms that cause disease in highly oxidative tissues such as the nervous system, skeletal muscle or heart.

Mfn2 expression is regulated by obesity and type 2 diabetes
Mitochondrial metabolism is altered in skeletal muscle during insulin-resistant states such as type 2 diabetes or obesity. Human type 2 diabetes is associated with reduced capacity to oxidise glucose in the presence of insulin and, more importantly, to oxidise fatty acid in various conditions. A significant decrease in gene expression of subunits participating in complexes I, II, III and IV of the mitochondrial electron transport chain has also been observed in skeletal muscle of type 2 diabetic patients. Reduced glucose oxidation, decreased pyruvate dehydrogenase activity and enhanced pyruvate dehydrogenase kinase have also been reported in skeletal muscle from diabetic rats. Similarly, experimental diabetes in rats reduces gene expression of various subunits of complexes I, III and IV. In keeping with these observations, the respiratory chain is depressed in skeletal muscle from type 2 diabetic patients and oxidative phosphorylation activity is reduced in heart and skeletal muscle in diabetic rats.

Obesity is also characterised by metabolic alterations which involve mitochondrial defects causing reduced oxidation in skeletal muscle. In some animal models

Figure 1. The mitochondrial network in muscle cells. 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 myotubes.
of obesity such as in obese Zucker rats or ob/ob mice, skeletal muscle shows a metabolic profile characterised by reduced glucose uptake and glucose oxidation, altered partitioning of fatty acids that are incorporated into triglycerides, and reduced oxygen consumption.

In keeping with the regulatory role of Mfn2, we have found that skeletal muscle Mfn2 expression is reduced in obesity and this affects men and women to a similar extent (Bach et al., 2005). In addition, we have observed that Mfn2 expression in muscle is inversely proportional to the BMI and directly proportional to insulin sensitivity. We have also found that weight loss induced by biliopancreatic diversion in morbid obese subjects results in a substantial increase in Mfn2 expression in skeletal muscle (Bach et al., 2005). These data indicate that increased adiposity is linked to repression of Mfn2 mRNA in skeletal muscle and this can be reversed by weight loss. Levels of Mfn2 mRNA strongly correlate with glucose oxidation rates during fasting during euglycemic hyperinsulinemic clamp conditions (Mingrone et al., 2005). On the basis of these data, we propose that the increase in Mfn2 mRNA levels explain the increase in glucose oxidation observed in morbid obesity after bariatric surgery.

Our data also indicate that Mfn2 expression is dysregulated in skeletal muscle from type 2 diabetic patients. In fact, a reduction in Mfn2 expression is detected in both obese and nonobese type 2 diabetic patients. Low Mfn2 expression occurs in the presence of reduced expression of the mitochondrial gene COX-III and in the presence of a moderate reduction in citrate synthase mRNA, which suggests the presence of mitochondrial dysfunction.

**PGC-1α, ERR-α and Mfn2 define a mitochondrial regulatory pathway**

Peroxisome proliferator-activated receptor γ (PGC)-1α is a transcriptional co-activator involved in the regulation of genes related to energy metabolism. PGC-1α induces mitochondrial biogenesis and respiration in muscle cells, regulates several aspects of adaptive thermogenesis, gluconeogenesis in liver, and insulin secretion. Overexpression of PGC-1α increases mitochondrial metabolism and this cannot be entirely explained by an increase in mitochondrial mass. In addition, transgenic expression of PGC-1α driven by a muscle specific promoter results in a drastic switch from glycolytic to oxidative fibers. Initially, PGC-1α was described as a tissue-specific co-activator of nuclear receptors but transcription factors of distinct families such as NRF1, MEF2 or FOXO1 are co-activated by this protein. ERRα and GABP are the key transcription factors that regulate the expression of genes of the OXPHOS system mediated by PGC-1α. PGC-1α null mice show, among other defects, reduced mitochondrial function and decreased thermogenic capacity.

We have studied Mfn2 expression under conditions such as exposure to cold or treatment with β3-adrenergic agonists, which stimulate basal energy expenditure and PGC-1α expression. Exposure to cold for 48 h caused stimulation of Mfn2 mRNA levels in skeletal muscle and in brown adipose tissue. Treatment of rats with the β3-adrenergic agonist CL-316243 for a range of times also increased Mfn2 expression in these two tissues.

We have also demonstrated that PGC-1α stimulates Mfn2 gene expression and that this is due to two elements on the promoter that binds ERRα (Soriano et
These data permit us to propose the existence of a regulatory pathway that drives mitochondrial metabolism and is defined by PGC-1α, ERRα and Mfn2 (Figure 3A). The pathway is characterised by a stimulatory action of PGC-1α on the transcription of Mfn2, via co-activation of ERRα. This is supported by the following experimental evidence: a) PGC-1α activates Mfn2 expression in cells; b) the mechanism by which PGC-1α stimulates this expression are dependent on intact ERRα binding in the Mfn2 promoter; c) Mfn2 regulates mitochondrial metabolism; d) PGC-1α action explains the stimulatory effect of cold-exposure or treatment with the β2-adrenergic agonist CL-316243 on Mfn2 expression in muscle and brown adipose tissue.

In addition, we have shown that cold exposure causes an additional increase in PGC-1α in skeletal muscle from mouse knock-outs heterozygous for Mfn2. These results suggest a possible homeostatic Mfn2-induced process that regulates PGC-1α (Figure 3B). This mechanism does not involve a direct effect of Mfn2 as RNAi-induced repression of Mfn2 does not alter PGC-1α expression in C2C12 muscle cells.

Mfn2 is a key target of the nuclear co-activator PGC-1α. Previous studies show that the up-regulation of PGC-1α enhances total mitochondrial membrane potential in cells by increasing mitochondrial number and also by energisation of mitochondria. Mfn2 also enhances mitochondrial membrane potential and several observations support that Mfn2 stimulates mitochondrial proton leak; the effects of Mfn2 are independent of mitochondrial mass. These data support the view that Mfn2 and PGC-1α have common effects on mitochondria. The maintenance of a normal expression of Mfn2 is critical for the stimulatory effect of PGC-1α on mitochondrial membrane potential; in contrast, the effects of PGC-1α on mitochondrial biogenesis are independent of Mfn2. These data suggest that the effects of PGC-1α on mitochondrial energisation require or are mediated by Mfn2. On the basis of the biological roles of Mfn2 reported, we also propose that PGC-1α regulates mitochondrial fusion/fission events and cell proliferation in cells.

Identification of novel substrates of semi-carbazide-sensitive amine oxidase/VAP-1 enzyme

Semiacarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) is a bifunctional protein with copper-containing amine oxidase activity (EC 1.4.3.6) that converts primary amines to aldehydes, with the concomitant production of hydrogen peroxide and ammonia. SSAO/VAP-1 is highly expressed in adipocytes where it is localised mainly in plasma membrane in an insulin-independent manner (Enrique-Tarancon et al, 1998). Substrates of SSAO/VAP-1 exert a variety of insulin-like effects in human, rat and mouse adipose cells. Thus, substrates such as benzylamine or tyramine stimulate glucose transport in isolated human adipocytes. Furthermore, in isolated rat or 3T3-L1 adipocytes, the combination of SSAO substrates at low ineffective vanadate concentrations causes a potent stimulation of glucose transport, GLUT4 recruitment to the cell surface, lipogenesis and inhibition of lipolysis (Zorzano et al, 2003; Marti et al, 2004).

In this regard, in collaboration with Fernando Albericio (IRB Barcelona); Miriam Royo (PCB) and Luc Marti (Genmedica Therapeutics), we have identified potent SSAO/VAP-1 substrates based on arylalkylamines. In a first step, structure activity relationships were studied for SSAO/VAP-1 using a library of arylalkylamine substrates. This analysis has revealed the relevance of the electronic properties of the aryl substituents in the activation or inactivation of the SSAO enzymatic process (Yraola et al, 2006). The experimental data have been contrasted with the findings obtained in previous homology studies in order to elucidate the mechanism and the substrate-binding affinity of SSAO. In addition, the results have been compared with some recent studies about the Amino Oxidase family and reveals new trends in SSAO substrate design. Thus, we have identified a novel compound, 4-phenylbutylamine, as a potent substrate for human SSAO, which can be used in the development of anti-diabetic compounds (Yraola et al, 2006).

A novel signalling pathway responsible for contraction-induced glucose transport in skeletal muscle

Skeletal muscle is the main tissue responsible for insulin-stimulated glucose utilisation in absorptive states. The rate-limiting step for muscle glucose utilisation is glucose transport, which can be rapidly induced by translocation of GLUT4 glucose transporters from intracellular vesicles to the plasma membrane. Muscle contraction and insulin act independently through distinct signalling pathways to induce GLUT4 translocation. Most interestingly, in insulin-resistant states, such as type 2 diabetes, the effects of contraction on glucose uptake are unchanged. Thus, we have focused our research efforts on studying the molecular mechanisms involved in contraction-stimulated glucose uptake.

Muscle contraction induces glucose transport by a mechanism involving local factors within the myocyte. It has been suggested that two main effectors mediate contraction-induced glucose transport, namely activation of AMP-activated protein kinase (AMPK), a metabolic fuel gauge regulated by cellular
energy charge, and transient increases in cytosolic Ca²⁺ within myofibers, as a consequence of plasma membrane and T-tubule depolarisation.

Neuregulin, a growth factor involved in myogenesis, has rapid effects on muscle metabolism. In a manner analogous to insulin and exercise, neuregulins stimulate glucose transport through recruitment of glucose transporters to surface membranes in skeletal muscle (Suárez et al., 2001; Cantó et al., 2004). Like muscle contraction, neuregulins have additive effects with insulin on glucose uptake. In collaboration with Anna Gumà and Carles Cantó (University of Barcelona), we have examined whether neuregulins are involved in the mechanism by which muscle contraction regulates glucose transport.

We have found that muscle contraction stimulates the phosphorylation of ErbB4 and ErbB2 but not ErbB3 receptors (Cantó et al., 2006). This is consistent with the finding that in vitro exposure of skeletal muscle to saturating exogenous neuregulins induces tyrosine phosphorylation of ErbB4 and ErbB2 but induces only weak phosphorylation of ErbB3. These results indicate that ErbB2 and ErbB4 are the main neuregulin receptors in adult skeletal muscle. Caffeine treatment at a concentration that induces Ca²⁺ release to subcontractile levels also mimics contraction effects on ErbB receptor phosphorylation (Cantó et al., 2006).

We have also shown that caffeine-induced increases in cytosolic Ca²⁺-mediate a metalloproteinase-dependent release of neuregulins, which explains the activation of ErbB4 receptors. In addition, activation of ErbB4 is required for Ca²⁺-derived effects on glucose transport and ErbB4 blockade impedes neuregulin- and caffeine-induced glucose transport in incubated soleus muscle (Cantó et al., 2006). In contrast, treatment with specific antibodies that induce ErbB3 blockade do not affect neuregulin- or caffeine-induced glucose transport in soleus muscle.

Blockage of ErbB4 abruptly impairs contraction-induced glucose uptake in slow twitch muscle fibers, and to a lesser extent, in fast twitch muscle fibers. Thus, injection of saturating concentrations of ErbB4 blocking antibody into soleus and EDL muscles before inducing contraction by electrical stimulation of the sciatic nerve inhibited contraction-induced ErbB4 phosphorylation by 86% in soleus and 91% in EDL muscles with a concomitant 71% and 36% impairment in contraction-induced glucose uptake, respectively (Cantó et al., 2006). In conclusion, we provide evidence that contraction-induced activation of neuregulin receptors is required for the stimulation of glucose transport and represents a key component of energy metabolism during muscle contraction.

Figure 3. Proposed model for: a) the regulatory pathway defined by PPARγ co-activator 1α (PGC-1α), ERRα and mitofusin-2, and b) feed-back regulation between Mfn2 activity and PGC-1α expression.

Role of mannan-binding lectin in insulin resistance

Substantial evidence has accumulated over the past decade that the metabolic syndrome is linked to inflammatory pathways. The immune system is constantly exposed to diverse bacterial products. The sensing arm of this immune system is efficient in buffering these bacterial and environmental substances, thereby precluding their interaction with membrane-associated receptors of monocytes/macrophages. Mannan- or mannose-binding lectin (MBL) is a liver-derived serum protein involved in innate immune defence. The ligands for MBL are mannan and N-acetyl glucosamine oligosaccharides, expressed by a wide range of microorganisms. MBL may activate complement by means of the lectin pathway when interacting with MBL-associated serine proteases and can directly opsonise pathogens and enhance the activity of phagocytes by means of novel receptors. Interestingly, there is a correlation between increased concentration of certain complement components (C3) and decreased insulin action. Increased levels of C3 and other inflammatory markers in insulin resistance seem to result from chronic inflammation induced by 'excess' fat tissue but it could also be the result of the relative incapacity to buffer the environmental inducers of inflammation. In this regard, decreased inherited capacity to sense environmental substances would lead to increased
exposure of cell immunity to these products and low-
grade chronic inflammation.

MBL is an excellent model to test this hypothesis. MBL
deficiency is the most frequent immunodeficiency in
humans, and leads to an increased incidence of infec-
tions in subjects with MBL gene mutations. Serum
MBL concentrations vary widely from person to per-
bson because of three variant alleles (B, C, and D,
denoting the substitution of aspartic acid for glycine
at codon 54, the substitution of glutamic acid for
glycine at codon 57, and the substitution of cysteine
for arginine at codon 52, respectively) in the struc-
tural moiety of the functional MBL gene, MBL2, loca-
ted on chromosome 10 in humans. The normal allele
of MBL2 is named A, and the common designation for
the variant alleles is O. Each of the three variant alle-
les influences the stability of the final protein prod-
uct, thereby resulting in reduced serum levels and a
dysfunctional MBL variant with a lower molecular
weight than the normal protein.

In collaboration with José Manuel Fernández-Real and
Joan Vendrell, we have studied the levels of circulat-
ing MBL levels and the frequency of MBL2 mutations
in obese subjects and the metabolic effects of MBL in
muscle. Our results indicate that in non-diabetic
men, age-adjusted serum MBL is significantly lower
among obese subjects than in lean subjects in paral-
lel to increased serum C-reactive protein, interleukin
6 and the soluble fraction of tumour necrosis factor-
α receptor R1 (Fernández-Real et al., 2006). At least,
one MBL2 mutation was present in 48% of obese ver-
sus 39% of non-obese subjects. In addition, the plas-
ma concentration of MBL-A was lower in insulin
resistant obese ob/ob mice than in lean mice of the
same strain in parallel to decreased glucose/insulin
ratio and incubation of rat soleus muscle with human
MBL markedly increased fatty acid oxidation
(Fernández-Real et al., 2006). These findings indi-
cate that MBL, previously thought to function only in
inflammation and the immune system, affects met-
abolic pathways and possibly influences the develop-
ment of insulin resistance and obesity.

**PUBLICATIONS**

<table>
<thead>
<tr>
<th>Authors</th>
<th>Title</th>
<th>Journal</th>
<th>Reference</th>
</tr>
</thead>
</table>

**RESEARCH NETWORKS AND GRANTS**

<table>
<thead>
<tr>
<th>Network/Grant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitofusin-2 gene and risk of obesity or type 2 diabetes</td>
<td>Instituto de Salud Carlos III: 2005-2007</td>
</tr>
<tr>
<td>Molecular pathologies associated to defective activity of membrane transporters</td>
<td>Project Coordinator: Antonio Zorzano</td>
</tr>
</tbody>
</table>
Molecular Medicine Programme

Project Coordinator: Antonio Zorzano

Functional analysis of insulin resistance candidate genes
Plan Nacional de Biomedicina: 2005-2008
Project Coordinator: Antonio Zorzano

Functional and proteomic characterisation of GLUT4 endocytosis in adipocytes
Project Coordinator: Marta Camps

Role of caveolae in the physiology of adipose tissue
Project Coordinator: Marta Camps

OTHER FUNDING SOURCES
Development of compounds with relevant activities in adipose tissue
Provital SA: 2006
Project Coordinator: Antonio Zorzano

COLLABORATIONS
Functional role of SSAO
Christian Carpéné (INSERM U586, Toulouse, France)

Identification of type 2 diabetes susceptibility genes
José Manuel Fernández-Real (Hospital Trueta, Girona, Spain)

Identification of SSAO substrates
Luc Marti (Genmedica Therapeutics, Barcelona, Spain)

Synthesis of novel SSAO substrates
Miriam Royo (Combinatorial Chemistry Unit, Parc Científic de Barcelona, Spain)

Synthesis of novel antidiabetic compounds
Fernando Albericio (IRB Barcelona, Spain)

Hepatic function of Mfn2
Joan Guinovart (IRB Barcelona, Spain)

Project Coordinator: David Chan (California Institute of Technology, Pasadena, USA)
Project Coordinator: Federico Soriguer (Hospital Carlos Haya, Málaga, Spain)
Project Coordinator: Hubert Vidal (INSERM U-449, Lyon, France)
Project Coordinator: Juleen R Zierath (Karolinska Hospital, Stockholm, Sweden)
Project Coordinator: Luc Marti (Genmedica Therapeutics, Barcelona, Spain)
Project Coordinator: Miriam Royo (Combinatorial Chemistry Unit, Parc Científic de Barcelona, Spain)
Project Coordinator: Fernando Albericio (IRB Barcelona, Spain)
Project Coordinator: Joan Guinovart (IRB Barcelona, Spain)

Antonio Zorzano’s group, March 2006.