

Evidence for a Mitochondrial Regulatory Pathway Defined by Peroxisome Proliferator-Activated Receptor- γ Coactivator-1 α , Estrogen-Related Receptor- α , and Mitofusin 2

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Mitofusin 2 (Mfn2) is a mitochondrial membrane protein that participates in mitochondrial fusion and regulates mitochondrial metabolism in mammalian cells. Here, we show that Mfn2 gene expression is induced in skeletal muscle and brown adipose tissue by conditions associated with enhanced energy expenditure, such as cold exposure or β_3 -adrenergic agonist treatment. In keeping with the role of peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α on energy expenditure, we demonstrate a stimulatory effect of PGC-1 α on Mfn2 mRNA and protein expression in muscle cells. PGC-1 α also stimulated the activity of the *Mfn2* promoter, which required the integrity of estrogen-related receptor- α (ERR α)-binding elements located at -413/-398. ERR α also activated the transcriptional activity of the *Mfn2* promoter, and the effects were synergic with those of PGC-1 α . Mfn2 loss of function reduced the stimulatory effect of PGC-1 α on mitochondrial membrane potential. Exposure to cold substantially increased Mfn2 gene expression in skeletal muscle from heterozygous Mfn2 knock-out mice, which occurred in the presence of higher levels of PGC-1 α mRNA compared with control mice. Our results indicate the existence of a regulatory pathway involving PGC-1 α , ERR α , and Mfn2. Alterations in this regulatory pathway may participate in the pathophysiology of insulin-resistant conditions and type 2 diabetes. *Diabetes* 55:1783-1791, 2006

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ERR α , estrogen-related receptor- α ; COX-II, cytochrome c oxidase subunit II; MEF2, myocyte enhancer factor-2; Mfn2, mitofusin 2; PGC, peroxisome proliferator-activated receptor- γ coactivator.

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Peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α is a transcriptional coactivator involved in the regulation of genes related to energy metabolism (1,2). PGC-1 α induces mitochondrial biogenesis and respiration in muscle cells and regulates several aspects of adaptive thermogenesis (3-6), gluconeogenesis in liver (7,8), and insulin secretion (9). Overexpression of PGC-1 α increases mitochondrial metabolism, and this cannot be entirely explained by an increase in the mitochondrial mass (6,10). In addition, transgenic expression of PGC-1 α driven by a muscle-specific promoter results in a drastic switch from glycolytic to oxidative fibers (4). Initially, PGC-1 α was described as a tissue-specific coactivator of nuclear receptors (5), but transcription factors of distinct families such as NRF1 (nuclear respiratory factor-1), myocyte enhancer factor-2 (MEF2), or FOXO1 (forkhead box factor 1) are coactivated by this protein (6,11,12). Estrogen-related receptor- α (ERR α) and GABP α (GA repeat-binding protein- α) are the key transcription factors that regulate the expression of genes of the oxidative phosphorylation system mediated by PGC-1 α (13,14). PGC-1 α -null mice show, among other defects, reduced mitochondrial function and reduced thermogenic capacity (15).

Mitofusin 2 (Mfn2) is a mitochondrial fusion protein (16-19) that is expressed mainly in tissues with high energetic requirements, such as skeletal muscle and heart (16); consequently, it shows an expression pattern similar to that of PGC-1 α . Several lines of evidence indicate that Mfn2 may be involved in the regulation of energy homeostasis. Mfn2-deficient mice die at midgestation, and trophoblast cells, characterized by an active metabolism, show morphologic alterations (17). Mfn2 expression is downregulated in skeletal muscle in animal or human obesity and in type 2 diabetic patients (16,20). Knock-down of Mfn2 in muscle and nonmuscle cells reduces oxygen consumption, glucose oxidation, proton leak, and mitochondrial membrane potential but does not alter coupled respiration (16,21). In addition, regulation of Mfn2 expression alters the expression of oxidative phosphorylation subunits in muscle cells (21).

Here, we report an increase in Mfn2 gene expression under conditions of high energy expenditure mediated by PGC-1 α , and we also demonstrate that PGC-1 α stimulates Mfn2 gene expression and that this is caused by two

elements on the promoter that bind ERR α . In addition, we show that Mfn2 loss of function attenuates the effects of PGC-1 α on mitochondrial membrane potential and that cold exposure causes an additional increase in PGC-1 α in skeletal muscle from mice that are heterozygous knock-out (KO) for Mfn2.

RESEARCH DESIGN AND METHODS

Plasmids. Various fragments of the 5' flanking region of the human Mfn2 gene were amplified and subcloned into the pGL3basic reporter gene vector (Promega) or a pGL3 reporter under the control of a minimal thymidine kinase promoter. Expression plasmids for PGC-1 α (FLAG-tagged PGC-1 α) were a kind gift from Dr. Pere Puigserver (Johns Hopkins Medical School), and plasmid encoding ERR α was a gift from Dr. Diego Haro (University of Barcelona). Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The sequence of the oligonucleotides used is available on request. **Cell culture, Western blot, transfections, and reporter gene assays.** 10T1/2, L6E9, C2C12, and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in subconfluent cultures. Mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum. Myotube differentiation was induced as previously reported (22). Nuclear and mitochondrial fractions were obtained by differential centrifugation of L6E9 cell homogenates (21). Homogenates were also obtained from 10T1/2 and mouse embryonic fibroblast cells. Western blot assays were performed, using specific antibodies against PGC-1 α , Mfn2, subunit α from H⁺-F₁-ATP synthase, porin, and actin. Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins were used as secondary antibodies, and bands were detected by an enhanced chemiluminescence Western blotting detection analysis system (Amersham).

For reporter gene assays, cells were transfected with FuGene 6 transfection reagent (Roche). Luciferase activity was measured 40 h after transfection, as previously reported (22). Expression of reporter genes was normalized to the number of green fluorescent protein-positive cells, which was measured by flow cytometry.

Adenoviral infection. Adenovirus encoding PGC-1 α was a kind gift from Dr. Pere Puigserver (John's Hopkins University). 10T1/2, mouse embryonic fibroblast, and L6E9 (at differentiation day 3) cells were infected with adenoviruses. Cells were harvested 48 h after infection and various measurements performed (16,21).

Animals. Male Wistar rats were subjected to 48 h at 4°C or were injected with the β_3 -adrenergic agonist CL-316243 for 1 or 7 days (1 mg \cdot kg⁻¹ \cdot day⁻¹). Brown adipose tissue and gastrocnemius muscle were removed, rapidly frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Mfn2^{+/-} KO mice (C57BL/6J genetic background) were obtained from the California Institute of Technology (Pasadena, CA). Genotypes were determined by PCR from tail genomic DNA (17). Male mice (9–10 weeks old) were housed individually during exposition to cold (4°C). After 48 h of cold exposure, quadriceps muscle was removed. Animals were treated in accordance with the ethics committee in animal experimentation principles and guidelines of the Barcelona Science Park.

Electrophoretic mobility shift assays. A radiolabeled double-stranded oligonucleotide probe corresponding to the human Mfn2 promoter sequence 5'-TTT CCT CAA AGG CGA CTG AAG GGC AG-3' (-422/-396) was incubated with 3 μ l of in vitro-transcribed and -translated ERR α , and electrophoresis was performed as described (23). Competitor-mutated oligonucleotide 5'-TTT CCT CAT ATG CGA CTG AAG GGC AG-3' differs from wild-type electrophoretic mobility shift assay sequence by the same two-base substitution used in the functional experiments.

Chromatin immunoprecipitation. HeLa cells were transfected by the calcium phosphate method with a FLAG-tagged PGC-1 α expression vector or an irrelevant expression vector. After 48 h, they were cross-linked by formaldehyde, lysed, and subjected to chromatin shearing. Immunoprecipitation was performed with M2 anti-FLAG antibody (Sigma). In other studies, extracts from wild-type HeLa cells were immunoprecipitated with an antibody against human ERR α (PPMX Proteomics). After chromatin immunoprecipitation, DNA was purified by phenol/chloroform extraction. Input (1% of total immunoprecipitated) and immunoprecipitated DNA were subjected to PCR analysis with primers flanking the ERR α site on the Mfn2 promoter (-505/-325 of the human Mfn2 promoter) and the primers for amplification of the cyclophilin gene.

Measurement of specific mRNA levels

Northern blot assays. Total RNA was extracted by using Trizol (Invitrogen). Mfn2 and cytochrome c oxidase subunit-II (COX-II) mRNAs were detected after hybridization with a 1,200-bp *SacI* human Mfn2 fragment and a

500-bp *PstI* fragment as cDNA probes, respectively (16). PGC-1 α mRNA was detected by hybridization with a 500-bp *EcoRI* fragment

Real-time PCR expression analysis. We isolated 2 μ g of total RNA from rat or mice tissues or cell lines with the Trizol reagent (Invitrogen) and used them to synthesize full-length cDNA, in a 20- μ l reaction, using oligo dT and SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed with 0.8 μ l cDNA (diluted 1:10), specific primers (300 nmol/l for most primers, 150 nmol/l for MEF2A, or 150 nmol/l for β -actin), and 2 \times SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 16 μ l. PCR involved 40 cycles of denaturation (95°C, 15 s) and amplification (60°C, 1 min). In all cases, a single PCR product with correct base pair size was detected. Mouse PGC-1 α and β -actin primers were from Kressler et al. (24), mouse MEF2A primers were from Kim et al. (25), and rat PGC-1 α and cytochrome c primers were from Rodgers et al. (26). Sequence of primers is available on request. Quantification of cDNA was performed as reported (27). Results are the means of 5–6 cDNA samples from different observations.

Mitochondrial membrane potential. 10 T1/2 wild-type or mouse embryonic fibroblasts were infected with adenoviral vectors encoding β -galactosidase, green fluorescent protein, or PGC-1 α at different multiplicities of infection, and then, 48 h later, mitochondrial membrane potential was measured with 40 nmol/l DiI₁(5) (Molecular Probes) for 30 min at 37°C. DiI₁(5) fluorescence emission after laser excitation was measured, using a Moflo flow cytometer (Summit Version 3.1 software, DakoCytomation) as previously reported (21).

RESULTS

Mfn2 is induced in skeletal muscle and brown adipose tissue in response to cold exposure or treatment with CL-316243. To study the regulatory profile of Mfn2, we analyzed its expression under conditions such as exposure to cold or treatment with β_3 -adrenergic agonists that stimulate basal energy expenditure and PGC-1 α expression (5,28,29). Exposure to cold for 48 h caused stimulation of Mfn2 mRNA levels in skeletal muscle and brown adipose tissue (1.9- and 2.3-fold stimulation, respectively) (Fig. 1A and B). Under these conditions, the gene expression of the mitochondrially encoded COX-II or cytochrome c were not altered in muscle, whereas β -ATP synthase mRNA was enhanced (Fig. 1B); in brown adipose tissue, COX-II was modestly enhanced in response to cold (35% increase) (Fig. 1B). These results indicate that Mfn2 upregulation was specific. Exposure to cold for 48 h did not increase UCP3 mRNA levels in skeletal muscle (data not shown), which is in keeping with previous reports (30).

Treatment of rats with the β_3 -adrenergic agonist CL-316243 for different times also increased Mfn2 expression. Administration of CL-316243 for 24 h caused a 60% increase in Mfn2 expression in brown adipose tissue, which occurred in the absence of changes in COX-II, β -ATP synthase, or cytochrome c mRNAs (Fig. 1A and B). CL-316243 for 1 week caused a marked stimulation of Mfn2 mRNA levels in muscle (2.2-fold), again in the absence of alterations in COX-II, β -ATP synthase, or cytochrome c mRNA and in the presence of enhanced UCP3 mRNA levels (Fig. 1 and data not shown). Treatment of rats with CL-316243 for 24 h did not cause any alteration in the muscle Mfn2 mRNA (data not shown). Cold exposure and treatment with CL-316243 enhanced PGC-1 α expression in tissues (Fig. 1B).

The effects caused by cold or CL-316243 on Mfn2 expression were not mimicked by incubation of C2C12 muscle cells with analogs of cAMP (8-bromo-cAMP) or with a β -adrenergic agonist (isoproterenol) (data not shown), indicating that the stimulation is independent of cAMP.

PGC-1 α induces Mfn2 expression in muscle cells. We next examined whether PGC-1 α was responsible for the Mfn2 induction under conditions of enhanced energy expenditure. PGC-1 α shows a low expression in cultured

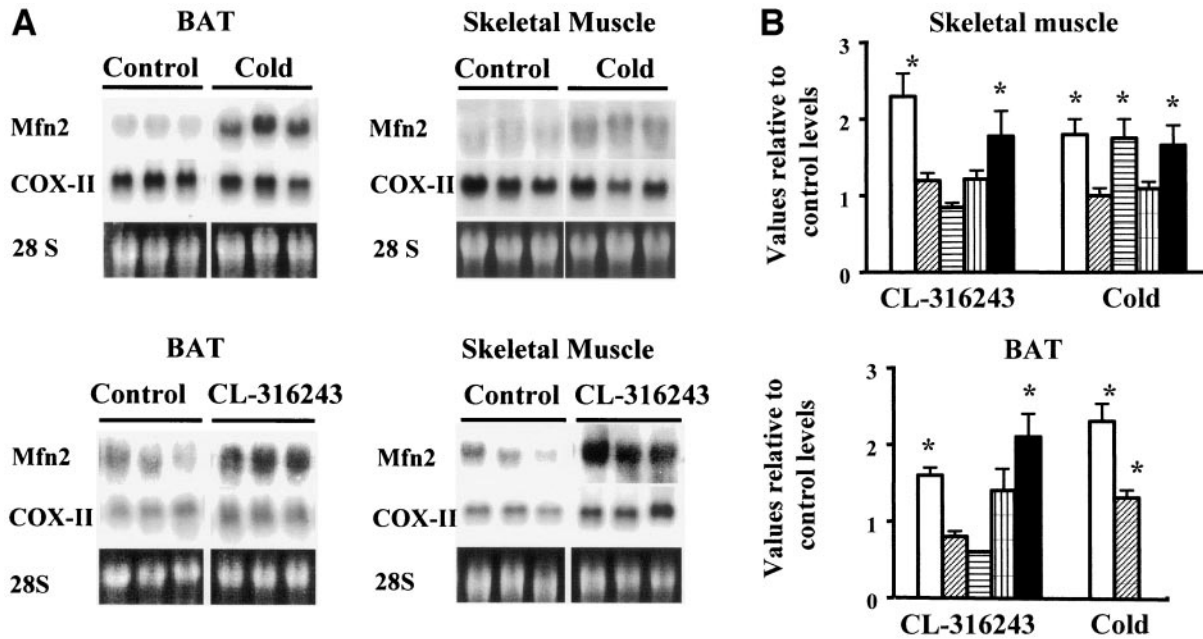


FIG. 1. Expression of Mfn2 is elevated in conditions of increased energy expenditure. Total mRNA was isolated from brown adipose (BAT) tissue and skeletal muscle from control rats or rats exposed to cold for 48 h or after treatment with CL312643 for 1 or 7 days. We applied 18 μg of RNA to gels for the measurement of Mfn2 or COX-II mRNA levels by Northern blot (A). The integrity and the relative amounts of RNA loaded in each lane were checked by 28S ribosomal RNA ethidium bromide staining on the same gel. β-ATP synthase, cytochrome c, and PGC-1α mRNAs were measured by real-time PCR. B: The quantitation of assays and values are relative to the control group for Mfn2 (□), COX-II (▨), β-ATP synthase (▩), cytochrome c (▧), and PGC-1α (■). Data are the means ± SE. *Significant difference compared with the control group, *P* < 0.05.

muscle cells (6); thus, we analyzed the effect of PGC-1α overexpression in differentiated L6E9 myotubes. To this end, myotubes were infected with adenoviruses encoding PGC-1α or β-galactosidase (used as a control), and total

RNA or mitochondrial or nuclear fractions were obtained. Mfn2 mRNA levels were markedly enhanced (near four-fold) in response to PGC-1α expression (Fig. 2A); under these conditions, genes induced by PGC-1α, such as β-ATP

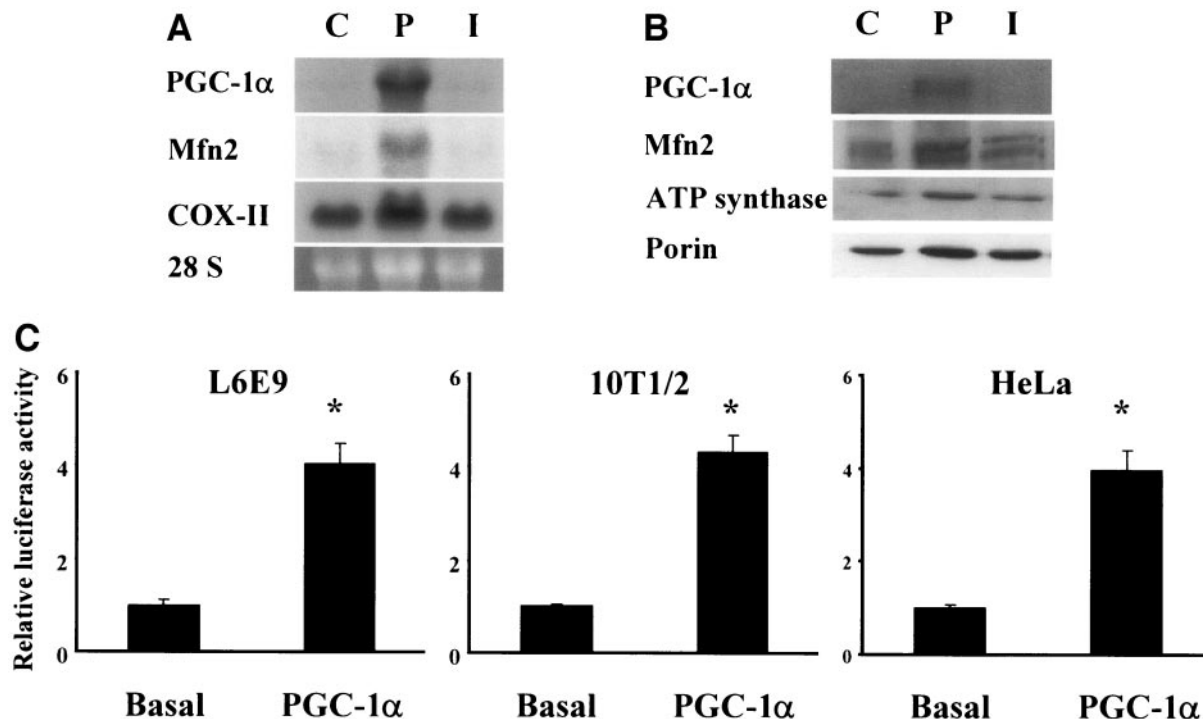


FIG. 2. PGC-1α regulates Mfn2 expression. Total mRNA (A) or mitochondrial extracts (B) were obtained from control L6E9 myotubes, myotubes infected with adenovirus encoding PGC-1α at 200 multiplicity of infection, or with a myotubes infected with an irrelevant adenovirus. We applied 20 μg of total RNA to gels for Northern blot analysis. Specific antibodies were used to detect proteins in Western blot assays. Transcriptional activity of the human Mfn2 promoter (2-kb genomic fragment linked to a luciferase reporter gene) was induced by PGC-1α in L6E9, 10T1/2, and HeLa cells (C). Data are the means ± SE of three to five experiments performed in triplicate. **P* < 0.001. C, control L6E9 myotubes; P, myotubes infected with adenovirus encoding PGC-1α; I, myotubes infected with irrelevant adenovirus.

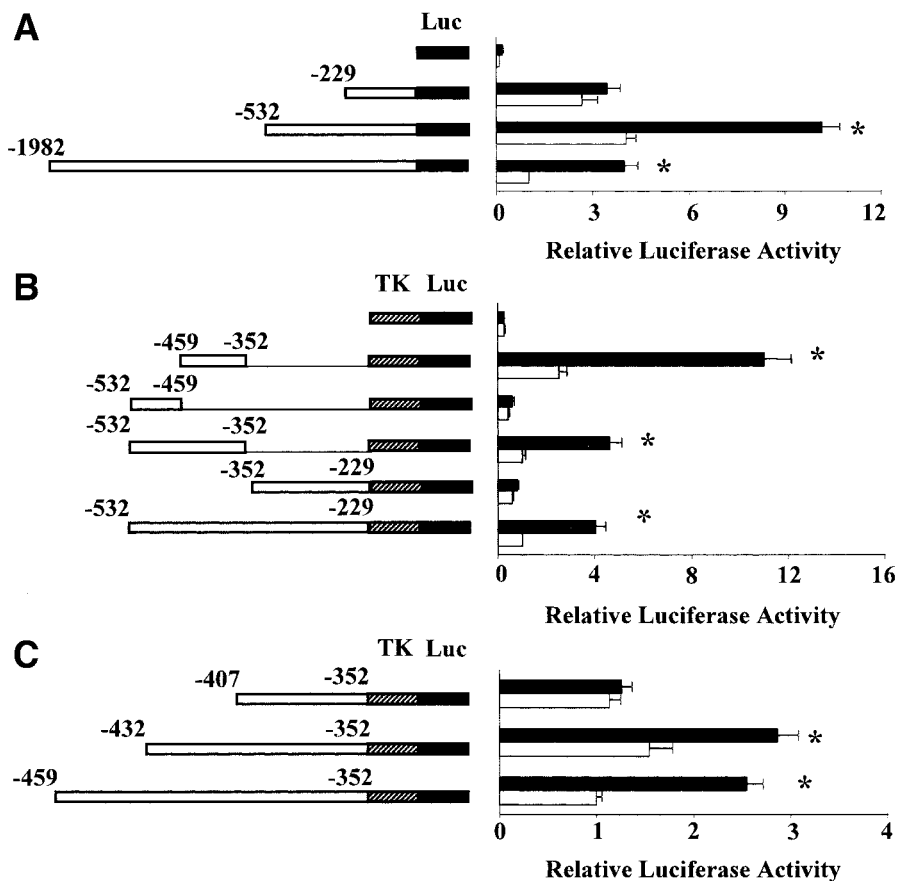


FIG. 3. Mapping the effect of PGC-1 α on the Mfn2 promoter. **A:** $-5'$ deletions of human Mfn2 promoter linked to luciferase reporter gene were performed and constructs transfected into 10T1/2 cells together with an expression vector for PGC-1 α (■) or a control plasmid (□). **B and C:** The fragments of the human Mfn2 promoter indicated were cloned in a luciferase (Luc) reporter plasmid driven by the promoter of thymidine kinase (TK) and transfected into 10T1/2 cells together with an expression vector of PGC-1 α (■) or a control (□) plasmid. Data are the means \pm SE of three to five experiments performed in triplicate. *Significant differences caused by PGC-1 α , $P < 0.001$.

synthase, COX-II, or cytochrome c, were more moderately enhanced (60, 20, and 20% of increase, respectively) (Fig. 2A and data not shown). Mfn2 protein was also markedly increased in response to PGC-1 α expression (3.6-fold increase), and this occurred in the presence of moderate changes in the α subunit of H⁺-F₁-ATP synthase (1.5-fold increase) (Fig. 2B).

To determine whether the effect of PGC-1 α expression was a consequence of transcriptional regulation, L6E9, 10T1/2, or HeLa cells were transiently transfected with a fragment of the human Mfn2 gene promoter ($-1982/+45$) fused to the reporter gene luciferase. Cells transfected with the reporter gene showed low levels of luciferase activity, and cotransfection with PGC-1 α caused a fourfold increase in activity (Fig. 2C). This increase was caused by the presence of specific sequences in the Mfn2 promoter, and cotransfection of PGC-1 α and pGL3-basal promoter containing no promoter sequences to drive the luciferase reporter had no stimulatory effect (data not shown).

The transcriptional effect of PGC-1 α on the Mfn2 promoter requires the integrity of the region $-432/-407$. To determine the *cis*-elements involved in the effects of PGC-1 α on the transcriptional activity of the Mfn2 promoter, 5' deletion constructs of the Mfn2 promoter fused to the luciferase reporter gene were generated and transiently transfected in 10T1/2 cells (Fig. 3A). Deletion from -1982 to -532 of the Mfn2 promoter did not modify the effect of PGC-1 α on luciferase activity (Fig. 3A), and deletion from -532 to -229 cancelled the effect of this coactivator (Fig. 3A). Next, distinct fragments of the promoter were cloned at 5' of the thymidine kinase promoter and fused to the luciferase reporter. The fragment $-532/-229$ was sufficient to permit the response to

PGC-1 α (Fig. 3B), and analysis of deletion fragments revealed that $-532/-352$ and $-459/-352$ retained the capacity to respond to PGC-1 α (Fig. 3B). A smaller fragment $-432/-352$ also responded to this coactivator by enhancing luciferase activity, but the fragment $-407/-352$ did not (Fig. 3C), indicating that the region $-432/-407$ is critical for conferring the capacity to respond to PGC-1 α . **PGC-1 α coactivates ERR α and binds to the Mfn2 promoter.** To identify the specific element that conferred sensitivity to PGC-1 α , we analyzed the sequences corresponding to the $-432/-352$ fragment in human and mouse Mfn2 genes. This fragment was highly conserved, especially the region encompassing $-432/-392$ (Fig. 4A). Visual analysis of elements revealed the putative presence of three binding boxes for nuclear hormone receptors conserved in human and mouse Mfn2 promoters (Fig. 4A). Based on these observations, the three boxes were mutated in the $-459/-352$ -thymidine kinase-luciferase construct, and functional analysis was performed in 10T1/2 cells. Cancellation of the middle box (*box 2*) completely blocked the response to PGC-1 α , whereas mutation in *box 1* did not (Fig. 4B). Mutation of *box 3* or double mutation in *boxes 1* and *3* reduced the stimulatory effect of PGC-1 α compared with the wild-type construct (Fig. 4B). These results indicate that *box 2* is sufficient for activity induced by PGC-1 α and that the maximal activity requires the integrity of a second box, most likely *box 3*. This suggests the operation of a nuclear hormone receptor endogenously expressed in 10T1/2 cells with activity as a monomer. In this regard, transient transfection of cells with thyroid hormone receptor TR α 1 in the presence of T₃ did not enhance the transcriptional activity of the Mfn2 promoter (data not shown).

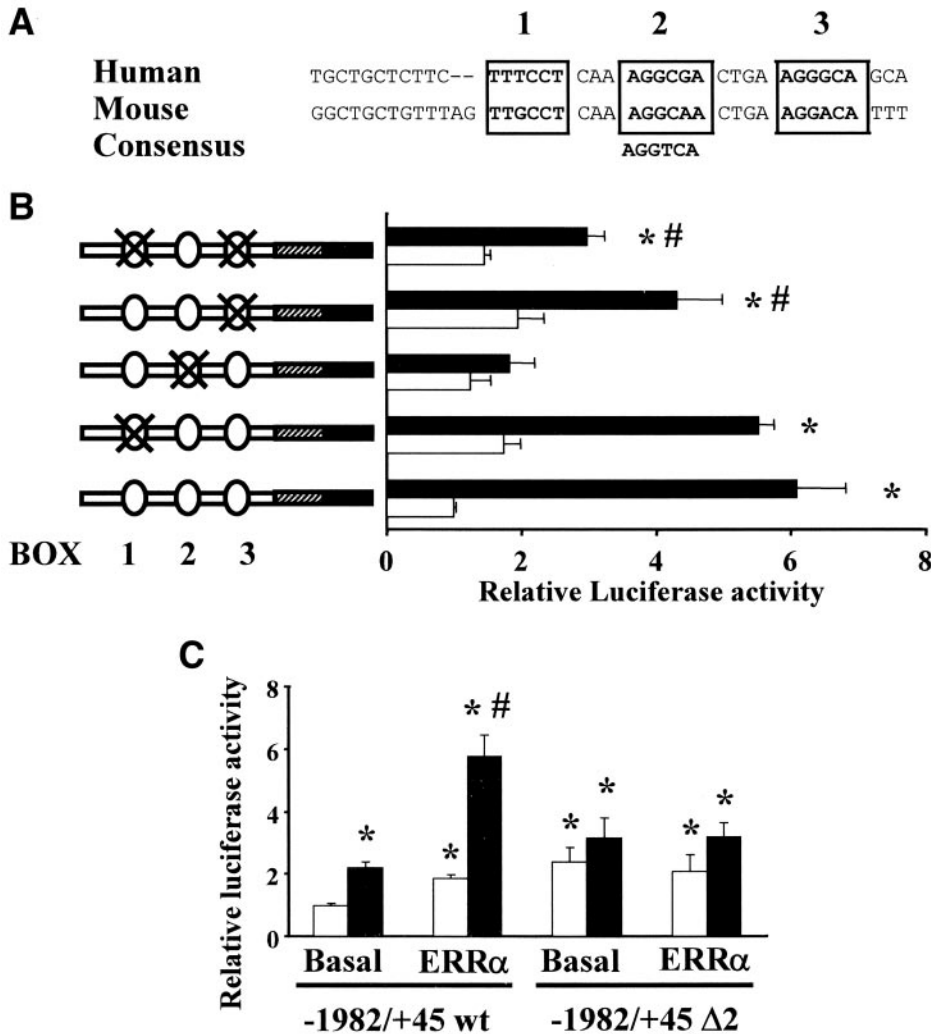


FIG. 4. PGC-1 α coactivates ERR α . **A:** Alignment of the sequences -432/-392 and equivalent regions in human and mouse Mfn2 genes. Boxes indicate putative binding sites for nuclear receptors. **B:** Luciferase activity of the fragment -459/-352 or mutated nuclear receptor box indicated (crossed out) of human Mfn2 promoter cloned in a luciferase reporter plasmid driven by the promoter for thymidine kinase together with an expression vector for PGC-1 α (■) or a control (□) plasmid. **C:** Luciferase activity of 10T1/2 cells transfected with a reporter gene plasmid containing 2 kb of the wild-type (wt) Mfn2 promoter or Mfn2 promoter mutated in box 2 (Δ 2) together with an expression plasmid for PGC-1 α (■) or a control (□) plasmid. *Statistically significant difference compared with the basal group (**B** and **C**), $P < 0.01$. #Statistically significant difference compared with the wild-type constructs (**B**) or with the basal group transfected with PGC-1 α (**C**), $P < 0.01$.

PGC-1 α transactivates oxidative phosphorylation gene transcription through binding to ERR α (13,14). Based on this observation, 10T1/2 cells were transfected with -1982/+45-luciferase plasmids either in the wild-type form or mutated in box 2, in the absence or presence of PGC-1 α and/or ERR α . In the wild-type promoter, ERR α stimulated luciferase activity, and the cotransfection of PGC-1 α and ERR α caused a synergic effect (Fig. 4C). Both actions were suppressed when analyzed in the promoter mutated in box 2 (Fig. 4C). Similar data were obtained when cells were transfected with the -459/-352-thymidine kinase-luciferase plasmid (data not shown).

To determine whether ERR α binds to the Mfn2 promoter, a DNA fragment encompassing the sequence 422/396 was labeled, and electrophoretic mobility shift assays were performed in the presence of ERR α synthesized in vitro (Fig. 5A). A retardation band was specifically detected, and it was competed by a 10- or 50-fold excess of unlabeled oligonucleotide and only partially competed by a mutated form of excess oligonucleotide (Fig. 5A). To confirm that ERR α or PGC-1 α bind to the Mfn2 promoter in vivo, chromatin immunoprecipitation assays were also performed. To this end, extracts from HeLa cells, which endogenously express ERR α (31), were immunoprecipitated with an anti-ERR α antibody, and a fragment of the promoter containing the ERR α -binding element was PCR amplified (-505/-325). Immunoprecipitates specifically

amplified the Mfn2 promoter, thereby indicating the binding of ERR α in vivo (Fig. 5B). In addition, cells were transfected with FLAG-PGC-1 α , extracts were immunoprecipitated with an anti-FLAG antibody, and a fragment of the promoter containing the ERR α -binding element was amplified by PCR. Immunoprecipitates specifically amplified the Mfn2 promoter, indicating the binding of PGC-1 α in vivo (Fig. 5C). In contrast, immunoprecipitates did not amplify a fragment of the cyclophilin gene, used as a negative control (Fig. 5C).

Mfn2 loss of function reduces the effect of PGC-1 α on mitochondrial membrane potential. To determine whether some of the effects of PGC-1 α depend on Mfn2, we studied the impact of Mfn2 loss of function. To this end, we analyzed two different cell models: 10T1/2 cells stably transfected with an antisense form of Mfn2, which show a 50% reduction in Mfn2 (16), and mouse embryonic fibroblasts obtained from Mfn2 KO embryos (17). Infection of wild-type 10T1/2 cells with an adenoviral vector encoding PGC-1 α caused a specific and marked enhancement of mitochondrial membrane potential (Fig. 6A). 10T1/2 cells stably transfected with an antisense form of Mfn2 showed a lower PGC-1 α -stimulated mitochondrial membrane potential (Fig. 6A). Similarly, the stimulatory effect of PGC-1 α on mitochondrial membrane potential was reduced in Mfn2^{-/-} mouse embryonic fibroblast cells (Fig. 6B). The mitochondrial membrane potential that we mea-

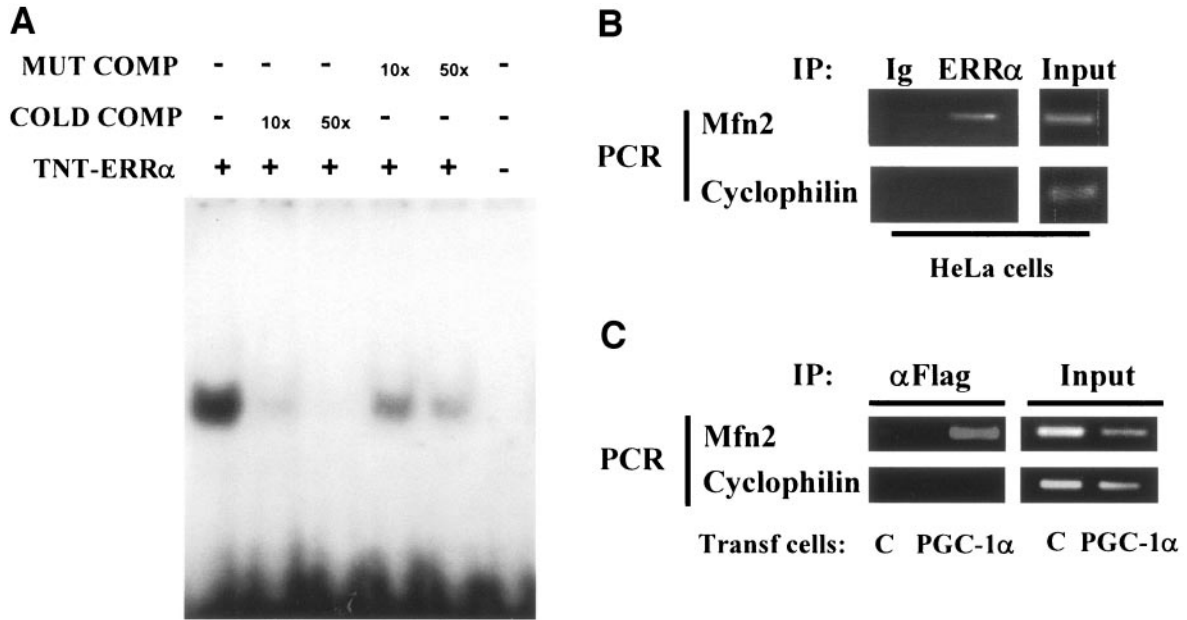


FIG. 5. ERR α and PGC-1 α bind to the Mfn2 promoter. *A*: Electrophoretic mobility-shift assay was performed, using in vitro-transcribed/translated ERR α and radiolabeled probe encoding box 2 from the human Mfn2 promoter. Excess of cold oligonucleotide or mutated (MUT) oligonucleotide was used to compete (COMP) binding. *B* and *C*: Extracts from HeLa cells (*B*) or cells transfected (Transf) with a FLAG-tagged PGC-1 α expression vector or an irrelevant expression vector were chromatin immunoprecipitated (IP), using an antibody directed against ERR α (*B*) or against PGC-1 α (*C*), or an irrelevant antibody (Ig). The input and immunoprecipitated DNA were used as templates for PCR. The image is representative of three to five experiments performed in triplicate. *C*, cells transfected with an irrelevant expression vector.

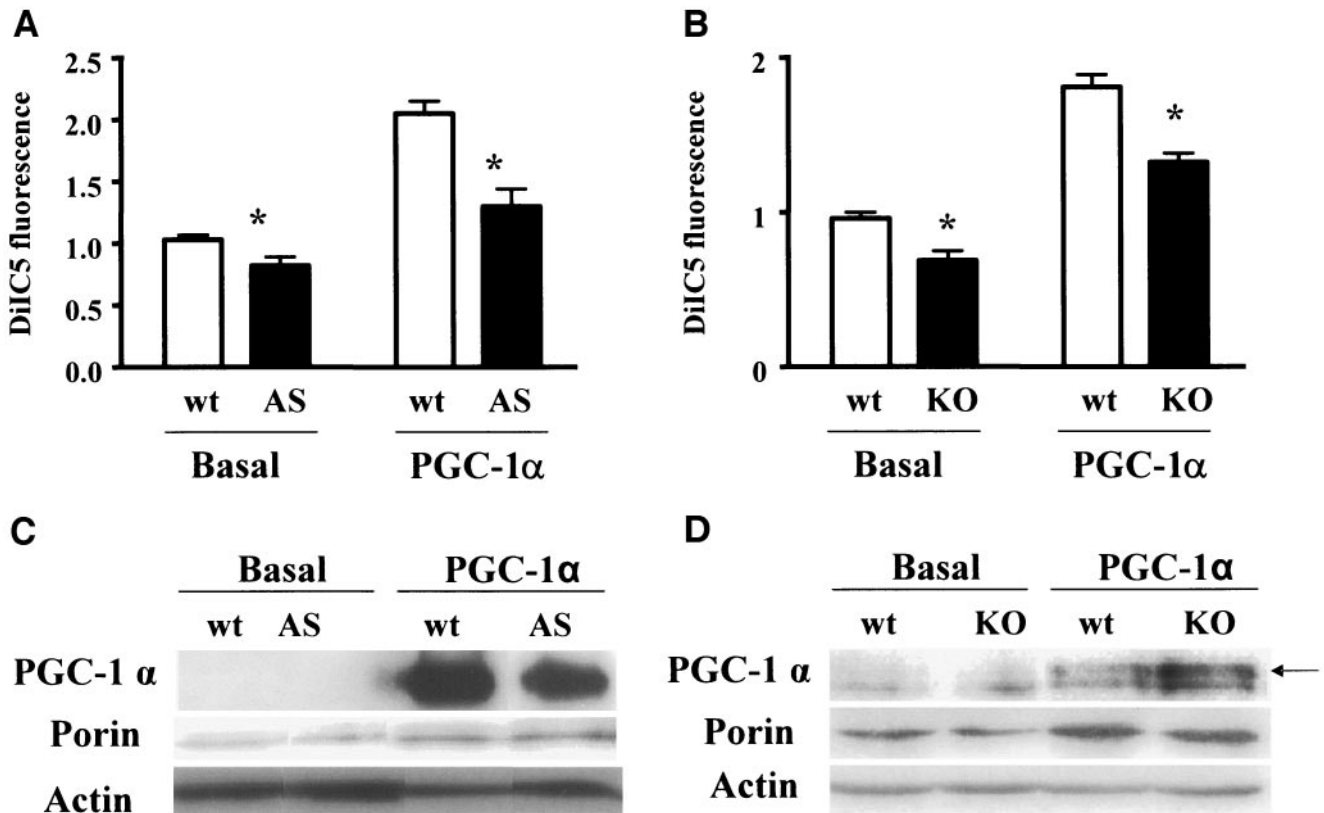


FIG. 6. Mfn2 loss of function reduces the stimulatory effect of PGC-1 α on mitochondrial membrane potential. *A* and *C*: Wild-type 10T1/2 cells (wt) or cells stably transfected with Mfn2 antisense (AS) were infected with adenovirus encoding PGC-1 α (■) or β -galactosidase (□). *B* and *D*: Control mouse embryonic fibroblast cells (wt) or cells obtained from Mfn2^{-/-} KO embryos were infected with adenovirus encoding PGC-1 α (■) or green fluorescent protein alone (□). At 48 h, cells were harvested for measurement of mitochondrial membrane potential and isolation of total homogenates for Western blot assays. Data are the means \pm SE of four to seven experiments performed in triplicate. **P* \leq 0.01 compared with wild-type group.

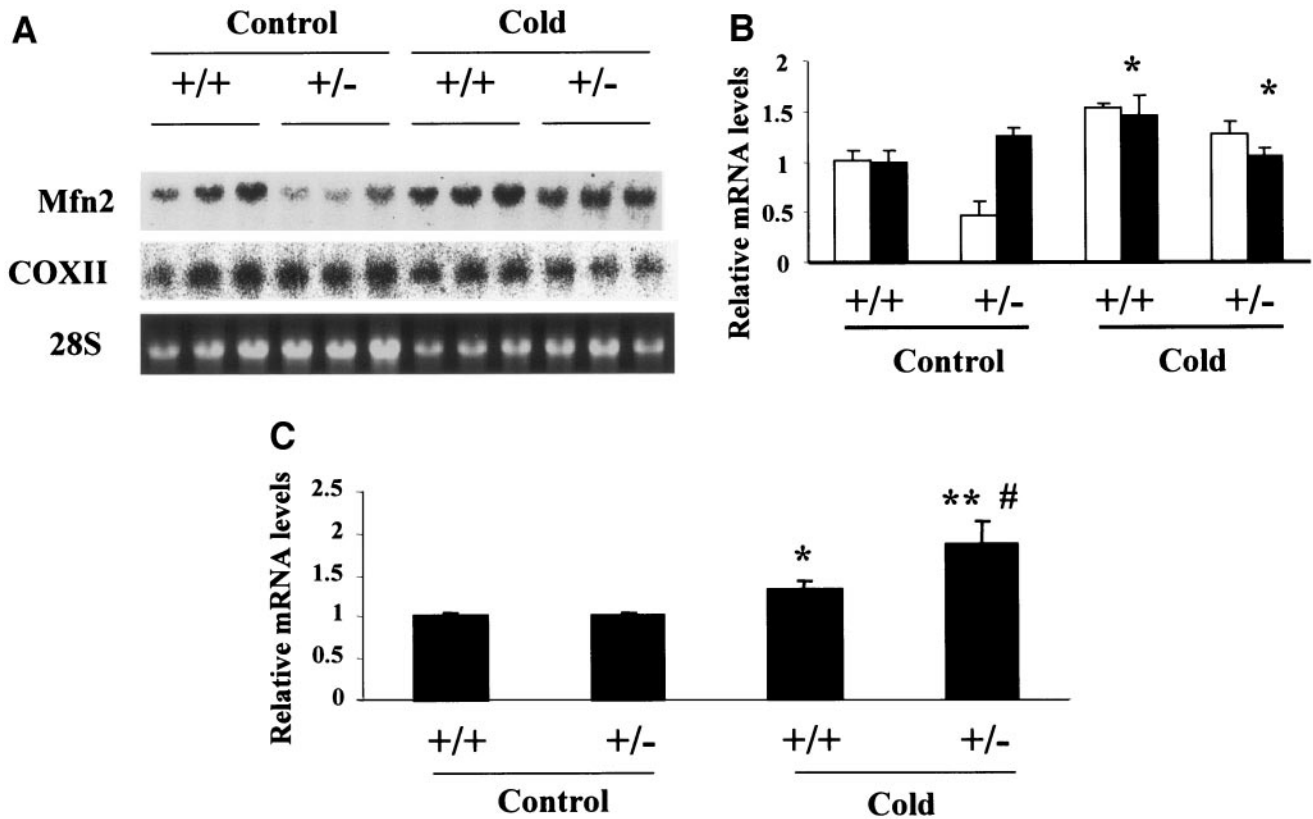


FIG. 7. Cold-exposed *Mfn2*^{+/-} KO mice elevate *Mfn2* gene expression through a higher induction of *PGC-1α* mRNA levels. Total mRNA was isolated from skeletal muscle from control mice (+/+) and heterozygous *Mfn2* KO mice (+/-) under basal conditions or after 48 h of cold exposure. **A and B:** We applied 18 μg of total RNA to gels for the measurement of *Mfn2* (□) and *COX-II* (■) mRNA levels by Northern blot. Data are relative to 28S abundance and represent the means from each value related to control. Data are the means ± SE of five to six observations per group. *Significant difference compared with basal conditions, $P < 0.01$. **C:** Real-time PCR analysis of *PGC-1α* expression. The data of *PGC-1α* are expressed as a ratio to *MEF2A* mRNA. The mRNA values represent the average of five to six mice per group. Data are the means ± SE. *Significant difference compared with basal conditions, $P < 0.05$. **Significant difference compared with basal conditions, $P < 0.001$. #Significant difference between +/+ and +/- groups, $P < 0.05$.

sured in cells is a reflection of the number of mitochondria per cell and their energization state. Because *Mfn2* loss of function did not completely block the effect of *PGC-1α* on mitochondrial membrane potential, we next determined the effect on the abundance of porin, a marker of mitochondrial mass. *PGC-1α* increased the total expression of porin in 10T1/2 and mouse embryonic fibroblast cells (Fig. 6C and D), and *Mfn2* loss of function did not alter this effect (Fig. 6C and D).

***Mfn2* expression is enhanced in skeletal muscle from heterozygous *Mfn2* KO mice in response to cold through a greater induction of *PGC-1α* expression.**

Based on the observation that cold enhances *PGC-1α* in skeletal muscle, we next aimed to determine whether the regulatory pathway defined by *PGC-1α* and *Mfn2* may be altered when only one allele of *Mfn2* was present. To this end, wild-type and heterozygous KO mice for the *Mfn2* gene (+/-) were subjected or not to cold for 48 h and *PGC-1α* and *Mfn2* mRNA levels were measured in skeletal muscle. Under basal conditions, *Mfn2* expression was reduced by near 50% in skeletal muscle from heterozygous mice (Fig. 7A and B), whereas *COX-II* mRNA levels were normal (Fig. 7A and B). Under these conditions, the expression of *Mfn2* protein in skeletal muscle extracts from *Mfn2*^{+/-} KO mice remained unaltered (data not shown). Exposure to cold caused a 60% increase in *Mfn2* mRNA levels in wild-type mice and a greater increase was detected in muscle from *Mfn2*^{+/-} KO mice (threefold

increase) (Fig. 7A and B). Cold did not cause any increase in *COX-II* mRNA levels in muscle (Fig. 7A and B).

Levels of *PGC-1α* mRNA were similar in muscle in both control and *Mfn2*^{+/-} KO mice under basal conditions (Fig. 7C). However, exposure to cold caused a much greater increase in *PGC-1α* expression in muscles from heterozygous *Mfn2* KO mice (70% increase) than in control mice (30% increase) (Fig. 7C).

DISCUSSION

Mfn2 is a protein that shows multiple functions, and some of them are of regulatory nature. *Mfn2* is crucial in mitochondrial fusion, and loss of function greatly reduces the extent of the mitochondrial network in several cell types (16,17). *Mfn2* also inhibits proliferation in smooth muscle cells (32). In addition, *Mfn2* modulates mitochondrial metabolism by regulating mitochondrial membrane potential, fuel oxidation, and the oxidative phosphorylation system (16,21). Thus, in myoblasts with a limited oxidative capacity, *Mfn2* gain of function causes an increased rate of glucose oxidation and a parallel increase in mitochondrial membrane potential, a consequence of augmented pyruvate oxidation, Krebs cycle, and oxidative phosphorylation activities in mitochondria (21). In addition, *Mfn2* repression decreases the oxidation rates of glucose, pyruvate, and palmitate and reduces mitochondrial membrane potential in myotubes (21). As to the

mechanisms involved, the alterations in Mfn2 expression cause a parallel change in the expression of subunits of complexes I, III, and V and do not modify mitochondrial biogenesis (21). In all, available evidence indicates that Mfn2 plays a singular regulatory role in mitochondrial metabolism.

Our data indicate the existence of a regulatory pathway that drives mitochondrial metabolism and is defined by PGC-1 α , ERR α , and Mfn2. The pathway is characterized by a stimulatory action of PGC-1 α on the transcription of Mfn2, via coactivation of ERR α . This is supported by the following experimental evidence: 1) PGC-1 α activates Mfn2 expression in cells, 2) the mechanisms by which PGC-1 α stimulates this expression are dependent on an intact ERR α binding in the Mfn2 promoter, 3) Mfn2 regulates mitochondrial metabolism, and 4) PGC-1 α action explains the stimulatory effect of cold exposure or treatment with the β_3 -adrenergic agonist CL-316243 on *Mfn2* expression in muscle and brown adipose tissue.

In addition, we have found that cold-exposed Mfn2^{+/-} KO mice elevate *Mfn2* expression through a greater induction of PGC-1 α expression, which suggests a possible Mfn2-induced homeostatic process that is aimed at regulating PGC-1 α . The mechanism does not involve a direct effect of Mfn2 because RNA interference-induced repression of Mfn2 did not alter PGC-1 α expression in C2C12 muscle cells (M.L., A.Z., unpublished observations).

Mfn2 is a key target of the nuclear coactivator PGC-1 α . Previous studies show that the upregulation of PGC-1 α enhances total mitochondrial membrane potential in cells by increasing mitochondrial number and also by energization of mitochondria (6,10,33). Mfn2 also enhances mitochondrial membrane potential, and some observations support that Mfn2 stimulates mitochondrial proton leak; the effects of Mfn2 are independent of the mitochondrial mass (16,21). These data support the view that Mfn2 and PGC-1 α share common effects in mitochondria. In this report we also provide evidence that the maintenance of a normal expression of Mfn2 is critical for the stimulatory effect of PGC-1 α on mitochondrial membrane potential; in contrast, the effects of PGC-1 α on mitochondrial biogenesis are independent of Mfn2. These data suggest that the effects of PGC-1 α on mitochondrial energization may require or may be mediated by Mfn2. Based on the reported biological roles of Mfn2 (17,18,21,32,34), we also propose that PGC-1 α may regulate mitochondrial fusion/fission events and cell proliferation in cells.

Regarding the mechanisms by which PGC-1 α stimulates Mfn2 expression, we demonstrate the operation of a transcriptional mechanism that involves the activation of the *Mfn2* promoter, which in turn requires the integrity of ERR α -binding elements located at -413/-398. This conclusion is based on a number of observations, namely 1) ERR α activates the transcriptional activity of the *Mfn2* promoter, and the effects are synergic with those of PGC-1 α ; 2) cancellation of ERR α -binding elements block or diminish the effect of PGC-1 α on promoter activity; 3) ERR α binds in vitro to the ERR α -binding element -410/-405 and in vivo to a Mfn2 promoter fragment centered around the ERR α -binding element; and 4) PGC-1 α binds in vivo the *Mfn2* promoter in a region centered around the ERR α -binding element. Thus, *Mfn2* can be added to the list of genes recently reported to be regulated by the PGC-1 α /ERR α pathway, such as GA repeat-binding protein- α , ERR α , mtTFA, medium-chain acyl-CoA dehydrogenase, ATP synthase, cytochrome c oxidase 5b, isocitrate

dehydrogenase, TIM22 (translocase of inner mitochondrial membrane 22), or carnitine/acylcarnitine translocase (13,14,35).

ERR α is induced by PGC-1 α in cells (36,37) and is also induced in brown adipose tissue and skeletal muscle after 6 h of cold exposure (36). Based on these observations and on the role of ERR α in Mfn2 gene transcription, we propose that ERR α participates in the regulatory pathway that connects PGC-1 α and Mfn2 and regulates mitochondrial function.

Exposure to cold temperature or treatment with the β_3 -adrenergic agonist CL-316243 induces PGC-1 α , which in turn regulates mitochondrial biogenesis and mitochondrial metabolism in both skeletal muscle and brown adipose tissue (5,28,29). Our data indicate that Mfn2 gene expression is also upregulated in skeletal muscle and brown adipose tissue by cold exposure or by treatment with the β_3 -adrenergic agonist CL-316243. The stimulatory effect of cold on Mfn2 expression was similar in skeletal muscle and in brown adipose tissue. However, a distinct profile was detected in brown adipose tissue and skeletal muscle in response to CL-316243 treatment. Although 1 day of treatment was enough to induce Mfn2 in brown adipose tissue, a rapid response was not detected in skeletal muscle, and induction of expression was found after 7 days of treatment. Our observations suggest that the effects of the β_3 -adrenergic agonist CL-316243 in skeletal muscle are indirect and independent of cAMP. The stimulation of Mfn2 gene expression in skeletal muscle and brown adipose tissue under conditions of enhanced energy expenditure may play a relevant role in the adaptive regulation of the mitochondrial metabolism aimed at maintaining energy homeostasis.

The regulatory pathway constituted by PGC-1 α , ERR α , and Mfn2 may be altered in insulin-resistant conditions and particularly in type 2 diabetes. Thus, it has been reported that Mfn2 and PGC-1 α expression is deficient in type 2 diabetic subjects (20,38). In addition, amelioration of insulin sensitivity caused by weight loss in morbidly obese subjects or by acute exercise are associated with increased Mfn2 expression in skeletal muscle (27,39). These data may help to explain the molecular basis for the alterations in mitochondrial function associated with insulin-resistant conditions and type 2 diabetes.

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