Impaired expression of NADH dehydrogenase subunit 1 and PPARγ coactivator-1 in skeletal muscle of ZDF rats: restoration by troglitazone

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Abstract **Type 2 diabetes has been related to a decrease of mitochondrial DNA (mtDNA) content. In this study, we show increased expression of the peroxisome proliferator-activated receptor-α (PPARα) and its target genes involved in fatty acid metabolism in skeletal muscle of Zucker Diabetic Fatty (ZDF) (*fa/fa*) rats. In contrast, the mRNA levels of genes involved in glucose transport and utilization (GLUT4 and phosphofructokinase) were decreased, whereas the expression of pyruvatedehydrogenase kinase 4 (PDK-4), which suppresses glucose oxidation, was increased. The shift from glucose to fatty acids as the source of energy in skeletal muscle of ZDF rats was accompanied by a reduction of subunit 1 of complex I (NADH dehydrogenase subunit 1, ND1) and subunit II of complex IV (cytochrome c oxidase II, COII), two genes of the electronic transport chain encoded by mtDNA. The transcript levels of PPARγ Coactivator 1 (PGC-1) showed a significant reduction. Treatment with troglitazone (30 mg/kg/day) for 15 days reduced insulin values and reversed the increase in PDK-4 mRNA levels, suggesting improved insulin sensitivity. In addition, troglitazone treatment restored ND1 and PGC-1 expression in skeletal muscle. These results suggest that troglitazone may avoid mitochondrial metabolic derangement during the development of diabetes mellitus 2 in skeletal muscle.**—Jové, M., J. Salla, A. Planavila, À. Cabrero, L. Michalik, W. Wahli, J. C. Laguna, and M. Vázquez-Carrera. **Impaired expression of NADH dehydrogenase subunit 1 and PPARγ coactivator-1 in skeletal muscle of ZDF rats: restoration by troglitazone.** ***J. Lipid Res.*** **2004.** 45: **113–123.**

Supplementary key words subunit 1 of complex I • peroxisome proliferator-activated-γ coactivator 1 • subunit II of complex IV • electronic transport chain • Zucker diabetic fatty • peroxisome proliferator-activated receptor • uncoupling protein • dehydrogenase subunit 1 • reduced nicotinamide adenine dinucleotide

Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization and is therefore the major site of insulin resistance in obesity and type 2 diabetes mellitus (1). During the development of insulin resistance in skeletal muscle, a shift in the source of energy from glucose to fatty acids is observed. Transport and metabolism of these substrates is controlled by a class of transcription factors called peroxisome proliferator-activated receptors (PPARs). Three different PPAR subtypes (α, δ/β, and γ) have been identified to date. PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism, such as liver, heart, and skeletal muscle (2). PPARδ/β is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in adipose tissue, whereas other tissues such as skeletal muscle and heart contain limited amounts (2). Naturally occurring fatty acids are activators of all three PPAR subtypes (3, 4). In addition to fatty acids, several synthetic compounds bind and activate specific PPAR subtypes. Thiazolidinediones (troglitazone, ciglitazone, and rosiglitazone), which bind and selectively activate PPARγ (5, 6), are a novel class of insulin-sensitizing agents with antidiabetic activity in vivo.

Recently, it has been demonstrated that fatty acid utilization and expression of several genes involved in fatty acid mitochondrial β-oxidation in skeletal muscle cells are regulated by PPARα (7). In addition, PPARα protein expression is induced during myocyte differentiation, coincident with increased β-oxidation capacity (7). Mitochondrial β-oxidation of these fatty acids renders reducing equivalents in the form of NADH, which are oxidized by the electronic transport chain (ETC), with oxygen serving as the final electron transport acceptor (respiration).

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Abbreviations: ACO, acyl-CoA oxidase; COII, complex IV, cytochrome c oxidase subunit II; CPT-I, carnitine palmitoyltransferase I; CTE, cytosolic acyl-CoA thioesterase; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; ND1, subunit 1 of complex I; NRF-1, nuclear respiratory factor-1; PDK-4, pyruvate dehydrogenase kinase 4; PFK, phosphofructokinase; PGC-1, PPARγ coactivator 1; PPAR, peroxisome proliferator-activated receptor; UCP-2, uncoupling protein 2; ZDF, Zucker diabetic fatty.

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Complexes I, III, and IV of the ETC, but not complex II, couple electron transport to proton pumping and the establishment of a proton and electrochemical gradient. This proton gradient is then used to drive ATP synthesis by complex V in a process known as oxidative phosphorylation. Interestingly, six subunits of complex I [NADH dehydrogenase subunits 1–6 (ND1–6)], one subunit of complex III (cytochrome b, cyt b), three subunits of complex IV [cytochrome c oxidase I–III (CO I–III)], and two subunits of complex V (ATP synthase subunits 6 and 8) are encoded by mitochondrial DNA (mtDNA). Reduced mitochondrial ETC activity causes cellular oxidative stress because electrons accumulate in the early stages of the ETC (complex I and coenzyme Q), where they can be donated directly to molecular oxygen to give superoxide anion (8, 9). Moreover, ETC is linked to the mitochondrial β-oxidation, and inhibition of the former results in inhibition of the latter (10). Therefore, impaired expression or activity of either PPARα or ETC may be mechanisms implicated in lipid accumulation in skeletal muscle, which is the factor that correlates more tightly with insulin resistance (11, 12). In an effort to identify whether abnormal expression of these genes was involved in muscle insulin resistance, we studied their expression in skeletal muscle of Zucker diabetic fatty (ZDF) rats before and after treatment with the insulin-sensitizing drug troglitazone. Increased expression of PPARα and of its target genes was observed in soleus muscle of ZDF rats compared with lean animals. In addition, the expression of two mtDNA-encoded genes involved in ETC [ND1 and complex IV, cytochrome c oxidase subunit II (COII)] was reduced, whereas the expression of the nuclear DNA-encoded gene complex II was not affected. Reduced expression of ND1 and COII in skeletal muscle showed a significant inverse correlation with increased plasma glucose levels, suggesting that reduced utilization of glucose by skeletal muscle was involved in the changes observed. Further, the transcript levels of PPARγ coactivator 1 (PGC-1), which is involved in mitochondrial biogenesis and respiration, were downregulated in skeletal muscle of ZDF rats. Finally, troglitazone treatment restored ND1 and PGC-1 expression in skeletal muscle. These results demonstrate impaired expression of mtDNA-encoded genes of the ETC in skeletal muscle in diabetes mellitus type 2, which can be reversed by thiazolidinedione treatment.

MATERIALS AND METHODS

Animals and treatment

Experiments were carried out in male obese ZDF rats (ZDF/Gmi, *fa/fa*) and lean litter mates ($fa^{+/?}$ or $fa^{+/+}$). They were maintained under standard conditions of illumination (12 h light/dark cycle) and temperature (21 ± 1°C) and fed a diet of Purina 5008 chow. ZDF rats were randomly distributed into two groups. Each group was administered, respectively, either 0.5% carboximethyl cellulose (control group) or 30 mg/kg/day of troglitazone (dissolved in 0.5% carboximethyl cellulose) per os once a day for 15 days. Food and water were given ad libitum. Twenty-four hours after the last administration, rats were killed by decapitation under ketamine (100 mg/kg, ip) anesthesia to collect blood samples and to isolate soleus skeletal muscle and liver. Blood samples were collected in EDTA tubes, and plasma was obtained by centrifugation at 2,200 *g* for 10 min at 4°C. Plasma glucose (Sigma, St. Louis, MO), triglyceride (Sigma), and nonesterified fatty acid (Wako, Germany) levels were determined with A colorimetric test. Leptin (Linco, St. Louis, MO) and insulin (Amersham Biosciences, Barcelona, Spain) were determined by RIA. Tissues were rapidly removed, frozen in liquid nitrogen, and stored at −80°C. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995, 21st July, from the Generalitat de Catalunya.

RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). Relative levels of specific mRNAs were assessed by RT-PCR. cDNA was synthesized from RNA samples by mixing 0.5 μg of total RNA, 125 ng of random hexamers as primers in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus RT (Invitrogen, Barcelona, Spain), 20 U RNAsin (Invitrogen), and 0.5 mM of each dNTP (Sigma) in a total volume of 20 μl. Samples were incubated at 37°C for 60 min. A 5 μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25 μl PCR reaction contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 1.25 μCi [^{32}P]dATP (3,000 Ci/mmol, Amersham Biosciences), 1 unit of Taq polymerase (Invitrogen), 0.5 μg of each primer, and 20 mM Tris-HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60°C). The sequences of the sense and antisense primers used for amplification were: PPARα, 5′-GGCTCGGAGGGCTCTGTCATC-3′ and 5′-ACATGCACTGGCAGCAGTGGA-3′; acyl-CoA oxidase (ACO), 5′-ACTATATTTGGCCAATTTTGTG-3′ and 5′-TGTGGCAGTGGTTTCCAAGCC-3′; muscle-type carnitine palmitoyltransferase I (M-CPT-I), 5′-TTCACTGTGACCCCAGACGGG-3′ and 5′-AATGGACCAGCCCCATGGAGA-3′; cytosolic acyl-CoA thioesterase (CTE), 5′-CAGCCACCCCGAGGTAAAAGG-3′ and 5′-CCTTGAGGCCATCCTTGGTCA-3′; glucose transporter 4 (GLUT4), 5′-GATGCCGTCGGGTTTCCAGCA-3′ and 5′-TGAGGGTGCCTTGTGGGATGG-3′; phosphofructokinase (PFK), 5′-TGACCTCTGGTGGAGATGCCC-3′ and 5′-ATGGACACGCTCTCCCAGGTG-3′; pyruvate dehydrogenase kinase 4 (PDK-4), 5′-GAACACCCCTTCCGTCCAGCT-3′ and 5′-TGTGCCATCGTAGGGACCACA-3′; liver-type CPT-I (L-CPT-I), 5′-TATGTGAGGATGCTGCTT-3′ and 5′-CTCGGAGAGCTAAGCTTG-3′; uncoupling protein 3 (UCP-3), 5′-GGAGCCATGGCAGTGACCTGT-3′ and 5′-TGTGATGTTGGGCCAAGTCCC-3′; UCP-2, 5′-AACAGTTCTACACCAAGGGC-3′ and 5′-AGCATGGTAAGGGCACAGTG-3′; ND1, 5′-CGGCCCATTCGCGTTATTCTT-3′ and 5′-TGATCGTAACGGAAGCGTGGA-3′; COII, 5′-AATCGCCCTTCCCTCCCTACG-3′ and 5′-GGGATGGGTCATGAGTGCAGG-3′; PGC-1, 5′-CCCGTGGATGAAGACGGATTG-3′ and 5′-GTGGGTGTGGTTTGCTGCATG-3′; mitochondrial transcription factor A (mtTFA), 5′-AATTGCAGCCATGTGGAGGGA-3′ and 5′-TTTCTGCCGGGCTTCCTTCTC-3′; nuclear respiratory factor-1 (NRF-1), 5′-TCAACTCCACGGCAGCTGATG-3′ and 5′-AATCGCTTGCTGTCCCACTCG-3′; and adenosyl phosphoribosyl transferase (APRT), 5′-AGCTTCCCGGACTTCCCCATC-3′ and 5′-GACCACTTTCTGCCCCGGTTC-3′. PCR was performed in an MJ Research Thermocycler equipped with a peltier system and temperature probe. After an initial denaturation for 1 min at 94°C, PCR was performed for 20 cycles (PFK, PDK-4), 23 cycles (PPARα in muscle and liver, ACO in muscle and liver, M-CPT-I, L-CPT-I, GLUT4,

UCP-2, PGC-1, NRF-1, mtTFA, COII), 25 cycles (UCP-3), 28 cycles (ND1), and 30 cycles (CTE). Each cycle consisted of denaturation at 92°C for 1 min, primer annealing at 60°C (except 58°C for ACO), and primer extension at 72°C for 1 min and 50 s. A final 5 min extension step at 72°C was performed. Five microliters of each PCR sample was separated on a 1-mm-thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Kodak X-ray films to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPARα: 645 bp; ACO: 195 bp; M-CPT-I: 222 bp; CTE: 224 bp; L-CPT-I: 629 bp; GLUT4: 232; PFK: 163 bp; PDK-4: 168 bp; ND1: 201 bp; COII: 273 bp; UCP-3: 179 bp; UCP-2: 471 bp; PGC-1: 228 bp; NRF-1: 218 bp; mtTFA: 160 bp; and APRT: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study (13). Radioactive bands were quantified by video densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*), which, for most of the target genes analyzed, was included in the same reaction.

Isolation of mitochondria and nuclear extracts

Mitochondrial fraction was obtained as follows. Solei muscles were placed (5% w/v) in ice-cold 0.25 M sucrose buffered with 5 mM *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 7.2) and homogenized with 6–8 strokes using a Teflon glass homogenizer. The homogenate was centrifuged at 8,500 *g* for 10 min. The pellet was resuspended in the sucrose buffer, diluted to the original volume, and centrifuged at 700 *g* for 10 min. The supernatant was decanted and centrifuged at 8,500 *g* for 10 min, and the pellet was washed with 100 mM KCl containing 20 mM K-*N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 7.2) including protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.01 mM leupetin, and 5 μg/ml pepstatin) and resuspended in the same buffer. Protein amounts were determined by the Bradford method (14).

Crude nuclear extracts were isolated using the Dignam method (15) with the modifications described by Helenius et al. (16). Frozen soleus muscles were weighed, transferred to Corning tubes, and ice-cold hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupetin, 5 μg/ml aprotinin, 0.5 mM DTT, 10 mM HEPES, pH 7.9) was added to each sample. The volume was proportional to the weight of the tissue, so as to give 15% homogenates. The tissues were left to thaw in an ice bath and homogenized (2 × 5 s) using a Polytron (Kinematica, Germany). Homogenates were incubated for 10 min on ice and centrifuged (25,000 *g*, 15 min, 4°C). Pellets were washed once with the same volume of hypotonic buffer used in the homogenization step and centrifuged (10,000 *g*, 4°C, 15 min). Supernatants were discarded and pellets were suspended in ice-cold low-salt buffer (25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 20 mM KCl, 20 mM HEPES, pH 7.9) using half of the volume of the hypotonic buffer. Nuclear proteins were released by adding high-salt buffer (25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.2 M KCl, 20 mM HEPES, pH 7.9) drop-by-drop using half of the volume of the low-salt buffer. Samples were incubated on ice for 30 min. During incubation, the tubes were smoothly mixed frequently. Samples were centrifuged (25,000 *g*, 30 min, 4°C), and supernatants were collected in microfuge tubes and stored in aliquots at −80°C. The protein concentration of the nuclear extracts was then measured by the method of Bradford.

Electrophoretic mobility shift assay

Binding activity of mtTFA was assessed by electrophoretic mobility shift assay (EMSA) according to Kanazawa et al. (17). Briefly, a radioactive probe containing the nucleotide sequence of the heavy-strand promoter of mtTFA was prepared by annealing paired oligonucleotides with the sequences 5′-TTTCCTC-CTAACTAAACCCTCTTTAC-3′ and 5′-GTAGGCAAGTAAAGAG-GGTTTAGTTA-3′ and was labeled using [α-^{32}P]dATP (3,000 Ci/mmol, Amersham Biosciences) and DNA Polymerase (Invitrogen). The protein-DNA binding protein reaction was performed at room temperature for 20 min in a volume of 20 μl. The reaction mixture contained 10 μg of mitochondrial extract protein, 100 μg/ml poly dI-dC, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 4% glycerol, and 100,000 cpm labeled nucleotides. Protein-DNA complexes were resolved by electrophoresis on a 8% acrylamide gel and subjected to autoradiography. For competition assays, nonlabeled oligonucleotides were added at 50-fold molar excess to the reaction mixture before the addition of mitochondrial extract protein.

Western blot analysis

Crude nuclear extracts (40 μg for PPARα) and mitochondrial proteins (30 μg for ND1) from soleus muscles were subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA) and incubated overnight at 4°C with 5% nonfat milk solution. Membranes were then incubated with the primary rabbit polyclonal antibody raised against PPARα (dilution 1:1000) (18) or ND1 (dilution 1:200) (Molecular Probes, Eugene, OR). Detection was achieved using the SuperSignal West Dura Trial kit (Pierce Biotechnology, Rockford, IL). Equal loading of proteins was assessed by red phenol staining. Size of detected proteins was estimated using protein molecular mass standards (Life Technologies).

Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) was determined in the mitochondrial fraction by means of the PeroxiDetect kit (Sigma).

Statistical analyses

Results are expressed as means ± SD of five rats. Significant differences were established by Student's *t*-test or ANOVA, according to the number of groups compared. When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Simple correlation analysis was used to evaluate the relationships between ND1 or COII and glucose levels. All statistical analyses were performed using the computer program GraphPad Instat (V2.03, GraphPad Software Inc., San Diego, CA).

RESULTS

Increased PPARα expression in soleus muscle of ZDF rats

Male ZDF and lean rats were sacrificed by 12 weeks of age. By this age, ZDF rats presented established diabetes compared with lean animals, as demonstrated by increased plasma levels of glucose (505 ± 67 vs. 167 ± 37 mg/dl, $P < 0.0001$), triglycerides (649 ± 298 vs. 92 ± 31 mg/dl, $P < 0.01$), nonesterified fatty acids (0.62 ± 0.17 vs. 0.26 ± 0.09 mEq/l, $P < 0.01$), insulin (19.3 ± 7.9 vs. 10.4 ± 2.0 ng/ml, $P < 0.05$), and leptin (11.1 ± 4.8 vs. 1.3 ± 0.2 ng/ml, $P < 0.01$). We next studied the effects of diabetes on PPARα expression in the soleus muscle. PPARα mRNA levels were ~2.5-fold higher ($P < 0.001$) in skeletal mus-

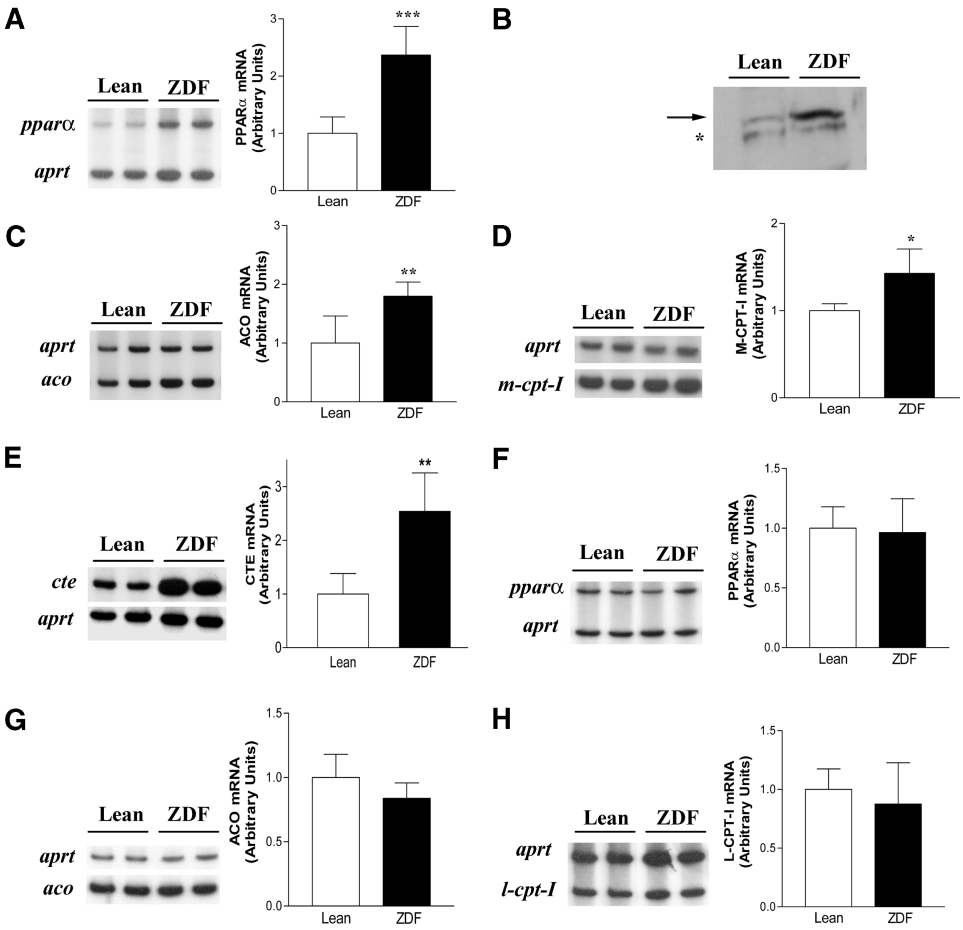

Fig. 1. Increased expression of peroxisome proliferator-activated receptor-α (PPARα) and its target genes in soleus muscle of Zucker diabetic fatty (ZDF) rats. A: Analysis of the mRNA levels of PPARα in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification of the adenosyl phosphoribosyl transferase (APRT)-normalized mRNA levels are shown. Data are expressed as mean ± SD of five rats. B: Immunoblotting of PPARα in soleus muscle of lean and ZDF rats. Nuclear extracts (40 μg of protein) were resolved on a 10% SDS polyacrylamide gel. The blot was analyzed with an affinity-purified anti-PPARα antibody that detects a protein of ~55 kDa corresponding to the PPARα protein (arrow). To show equal loading of protein, a nonspecific protein signal (indicated by an asterisk) from the same blot is included. Analysis of the mRNA levels of acyl-CoA oxidase (ACO) (C), muscle-type carnitine palmitoyltransferase I (CPT-I) (D), and cytosolic acyl-CoA thioesterase (E) in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean ± SD of five rats. Analysis of the mRNA levels of PPARα (F), ACO (G), and liver-type CPT-I (H) in liver of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with lean animals.

cle of ZDF rats than in lean animals (**Fig. 1A**). Significantly increased levels of PPARα protein were also observed in ZDF rats compared with lean animals (Fig. 1B). To evaluate whether the increase in PPARα expression was accompanied by upregulation of its target genes, we determined the transcript levels of ACO, M-CPT-I, and CTE. The mRNA levels of ACO and M-CPT-I, which catalyze, respectively, the rate-limiting step of peroxisomal β-oxidation of fatty acids and the entry of long-chain fatty acids into the mitochondrial matrix, showed a significant induction of ~1.8-fold in ZDF rats (Fig. 1C, D). Likewise, the mRNA levels of CTE, which hydrolyses fatty acyl-CoAs to free fatty acids and CoA, were upregulated (2.5-fold induction, $P < 0.01$) in skeletal muscle of ZDF rats compared with lean animals (Fig. 1E). The upregulation of PPARα and its target genes was specific to skeletal muscle, inasmuch as no changes were observed in liver in the mRNA expression of PPARα, ACO, and L-CPT-I (Fig. 1F–G). Taken together, these data indicate that the expression of PPARα and its target genes involved in fatty acid β-oxidation are upregulated in skeletal muscle of ZDF rats.

Increased expression of UCP-2 in skeletal muscle of ZDF rats

We next evaluated whether the expression of UCPs, mitochondrial membrane proton transporters that uncouple

respiration from oxidative phosphorylation by dissipating the proton gradient across the membrane, was affected in the skeletal muscle of ZDF rats. UCP-2 and UCP-3 are expressed in skeletal muscle under the control of PPAR and, although their physiological roles are not well defined, they have been involved in glucose and fatty acid oxidation. We found a 2.5-fold increase ($P < 0.001$) in UCP-2 mRNA levels in skeletal muscle of ZDF rats, whereas the expression of UCP-3 was unaffected (**Fig. 2A**, B).

Reduced expression of genes involved in glucose uptake and utilization in soleus muscle of ZDF rats

A metabolic feature of fatty acid-induced insulin resistance is reduced uptake and utilization of glucose by skeletal muscle. The mRNA levels of the insulin-responsive GLUT4 and the glycolytic enzyme PFK were reduced by 31% ($P < 0.01$) and by 61% ($P < 0.05$), respectively, in skeletal muscle of ZDF rats compared with lean animals (**Fig. 3**). In contrast, the expression of PDK-4, which suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex (19), increased by 1.7-fold ($P < 0.05$) in skeletal muscle of ZDF rats. These data reflect a shift in the preference and oxidation from glucose to fatty acids in skeletal muscle of ZDF rats.

Impaired expression of mtDNA-encoded genes of the ETC in soleus muscle of ZDF rats

In order to assess whether the reduction in the utilization of glucose affected the expression of genes involved in the ETC, we evaluated the expression of the ND1 and COII, two genes of the ETC encoded by mtDNA. A 68% reduction ($P < 0.05$) was observed in ND1 mRNA levels in soleus muscle of ZDF rats compared with lean animals (**Fig. 4A**). In addition, COII mRNA levels showed reduced expression (41%, $P < 0.02$) in ZDF rats compared with lean animals (Fig. 4B). Protein levels of ND1 were also reduced in skeletal muscle of ZDF rats (Fig. 4C). In contrast, no changes were observed in the transcript levels of the complex II, which is encoded by nuclear DNA (Fig. 4D).

PGC-1 mRNA levels are downregulated in skeletal muscle of ZDF rats

Expression of mtDNA is coordinated by a set of transcription factors and coactivators, including PGC-1, NRF-1, and NRF-2. PGC-1 is involved in the stimulation of the expression of NRF-1 and NRF-2 (20). PGC-1 and NRF-1 also activate the expression of the mtTFA (20), a mtDNA binding protein essential for maintenance, replication, and transcription of mtDNA (21). We evaluated whether altered transcription of mtDNA was the mechanism responsible for the reduced expression of mtDNA-encoded genes involved in ETC. **Figure 5A** shows the mRNA levels of PGC-1 in skeletal muscle. In ZDF rats, a 41% reduction ($P < 0.05$) was observed in PGC-1 transcript levels compared with lean animals. In contrast, no significant changes were observed in the mRNA levels of NRF-1 or mtTFA between ZDF and lean rats (Fig. 5B–D). On the other hand, inasmuch as it has been reported that reduced expression of genes involved in ETC in heart results in increased reactive oxygen species (ROS) generation, which does not alter mtTFA expression but reduces its binding to mtDNA (15), we performed EMSA studies to test this possibility. No changes were observed in the binding of an mtTFA probe to mitochondrial proteins (Fig. 5E). All these findings suggest that with the exception of PGC-1, changes in the expression of transcription factors and coactivators controlling the expression of mtDNA are not involved in the reduced expression of ND1 and COII in ZDF rats.

Reduced expression of ND1 and COII correlates with increased plasma glucose levels

Interestingly, when we studied the relationship between ND1 or COII mRNA levels and plasma glucose concentrations, we found a significant correlation. Thus, ND1 ($r^2 = 0.51$, $P = 0.03$, n = 9), as well as COII ($r^2 = 0.53$, $P = 0.04$, n = 8) mRNA showed an inverse correlation with plasma glucose. This finding suggests that reduced glucose utilization by skeletal muscle contributes, at least in part, to the fall in the expression of mtDNA-encoded genes involved in ETC (**Fig. 6**).

Troglitazone treatment restores ND1 and PGC-1 expression in skeletal muscle of ZDF rats

Finally, we sought to determine whether troglitazone treatment reversed the changes in the expression of genes involved in fatty acid metabolism and ETC. ZDF rats were treated for 15 days with a low dose of troglitazone (30 mg/

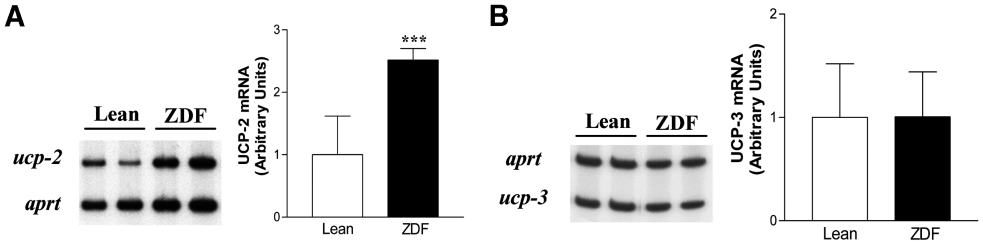

Fig. 2. Increased expression of uncoupling protein 2 (UCP-2) in soleus muscle of ZDF rats. Analysis of the mRNA levels of UCP-2 (A) and UCP-3 (B) in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. *** $P < 0.001$ compared with lean animals.

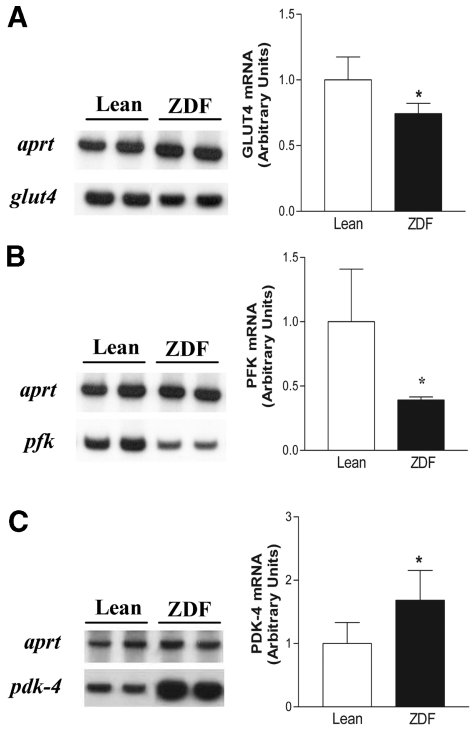

Fig. 3. Reduced expression of genes involved in glucose metabolism in soleus muscle of ZDF rats. Analysis of the mRNA levels of glucose transporter 4 (GLUT4) (A), phosphofructokinase (PFK) (B), and pyruvate deydrogenase kinase 4 (PDK-4) (C) in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. * $P < 0.05$ compared with lean animals.

kg/day). This dose of troglitazone did not significantly reduced plasma glucose levels, although insulin levels fell from 21.7 ± 3.4 in untreated ZDF rats to 13.0 ± 1.8 ng/ml ($P = 0.06$) in troglitazone-treated ZDF rats, suggesting an improvement in insulin sensitivity after drug treatment. Troglitazone did not affect the expression of PPARα or ACO, which showed similar mRNA levels in untreated and troglitazone-treated ZDF rats (data not shown). In contrast, troglitazone treatment reduced PDK-4 mRNA levels compared with untreated ZDF rats, suggesting an increased oxidative glucose disposal in skeletal muscle via the pyruvate dehydrogenase complex (**Fig. 7A**). The mRNA levels of COII showed a slight increase after troglitazone treatment compared with untreated ZDF rats, but differences did not reach statistical significance (data not shown). Further, drug treatment restored the mRNA and protein levels of ND1 to values similar to those present in lean animals (Fig. 7B, C). Interestingly, when we studied the effects of troglitazone treatment on the expression of transcription factors and coactivators controlling the expression of mtDNA, we observed that troglitazone treatment significantly increased PGC-1 and NRF-1 mRNA levels compared with untreated ZDF rats and to lean rats, respectively (**Fig. 8**). Finally, to determine whether the mitochondrial respiratory chain was affected by the changes in ND1 expression, we analyzed the H_2O_2 content in the mitochondrial fraction. H_2O_2 is mainly produced by complex I of the mitochondrial respiratory chain, and its production is abolished by specific inhibitors of complex I, such as rotenone (22). Therefore, we hypothesized that ND1 downregulation would reduce the production of H_2O_2 by mitochondria. We observed that the content of H_2O_2 in mitochondria from ZDF rats (21 ± 3 nmol/mg of protein) was reduced compared with lean rats (61 ± 34 nmol/mg of protein), and troglitazone treatment restored the content of H_2O_2 (71 ± 7 nmol/mg of protein, $P < 0.05$ compared with untreated ZDF rats).

DISCUSSION

It is well known that during the development of fatty acid-induced insulin resistance, a shift in the source of energy is observed from glucose to fatty acids. In agreement with this view, in the present study, we found increased expression of PPARα and its target genes and reduced expression of genes involved in glucose transport and utilization. Increased expression of PPARα and its target genes ACO and M-CPT I has recently been demonstrated in the myocardium of diabetic mice (23). Furthermore, mice with cardiac-restricted overexpression of PPARα showed activation of cardiac PPARα regulatory pathways that resulted in reciprocal repression of glucose uptake and utilization pathways. Thus, overexpression of PPARα in heart led to decreased mRNA levels of GLUT4 and PFK and increased expression of PDK-4. These findings point to the hypothesis that chronic PPARα activation in skeletal muscle or heart may be maladaptive, leading to metabolic disorders in type 2 diabetes mellitus. In support of this hypothesis is the fact that mice lacking PPARα (PPARα$^{-/-}$ apolipoprotein E (apoE)$^{-/-}$) were more resistant to the development of diabetes than were their littermates (PPARα$^{+/+}$apoE$^{-/-}$) (24). However, even in the presence of increased expression of genes involved in fatty acid oxidation regulated by PPARα, lipid accumulation occurs in skeletal muscle in diabetic states, suggesting reduced oxidative capacity (25). In fact, fatty oxidation rates (26) and fatty acid oxidative enzyme activities (25, 26) are up to 50% lower in muscle from obese compared with lean subjects. These reports suggest that impaired fatty acid oxidation precedes muscle lipid accumulation and that in a cellular context of impaired fatty acid oxidation, upregulation of the PPARα pathway may result from increased availability of fatty acids, which are endogenous activators of this transcription factor (3, 4).

The increase in the availability of fatty acids, which leads to a reduced utilization of glucose in the development of insulin resistance, may impair mitochondrial function, favoring fatty acid accumulation in the form of triglycerides within the cells, leading to the generation of ROS or affecting ATP production (8). The mitochondria play a crucial role in cellular physiology, because they generate cellular energy in the form of ATP. This energy is obtained in the ETC by oxidizing reducing equivalents in the form of hydrogen recovered from glucose by the tri-

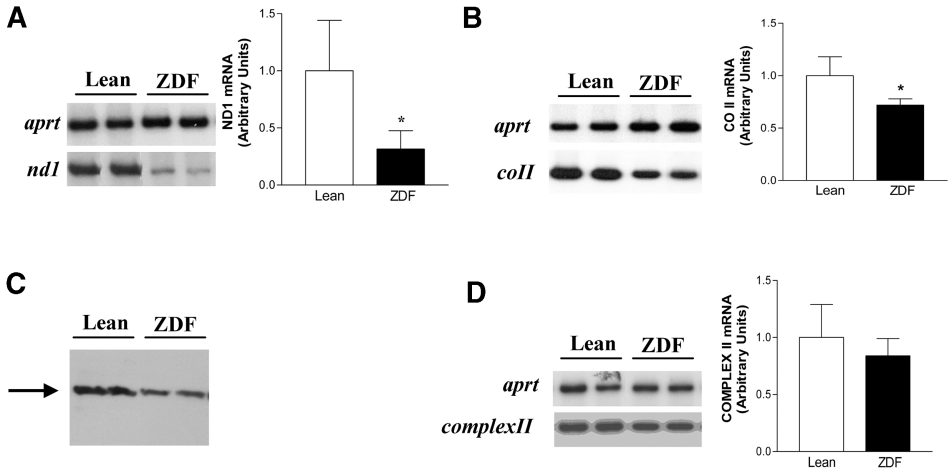

Fig. 4. Reduced expression of mitochondrial DNA genes coding for proteins involved in the electronic transport chain in soleus muscle of ZDF rats. Analysis of the mRNA levels of subunit 1 of complex I (ND1) (A) and of complex IV, cytochrome c oxidase subunit II (COII) (B) in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. C: Immunoblotting of ND1 in soleus muscle of lean and ZDF rats. Mitochondrial extracts (30 μg protein) were resolved on a 10% SDS-polyacrylamide gel. The blot was analyzed with anti-ND1 antibody. Equal protein loading was assessed by phenol red staining of the upper portion of the gel (data not shown). D: Analysis of the mRNA levels of complex II in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. * $P < 0.05$ compared with lean animals.

carboxylic acid cycle and from fatty acids through β-oxidation. Some of the genes of the ETC are encoded by mtDNA, including ND1 and COII. Lee et al. (27) reported that a decrease of mtDNA copy number preceded development of type 2 diabetes, suggesting that this decrease might be related to the pathogenesis of this metabolic disorder. The present work reports reduced expression of the mtDNA-encoded gene ND1 in skeletal muscle of ZDF rats.

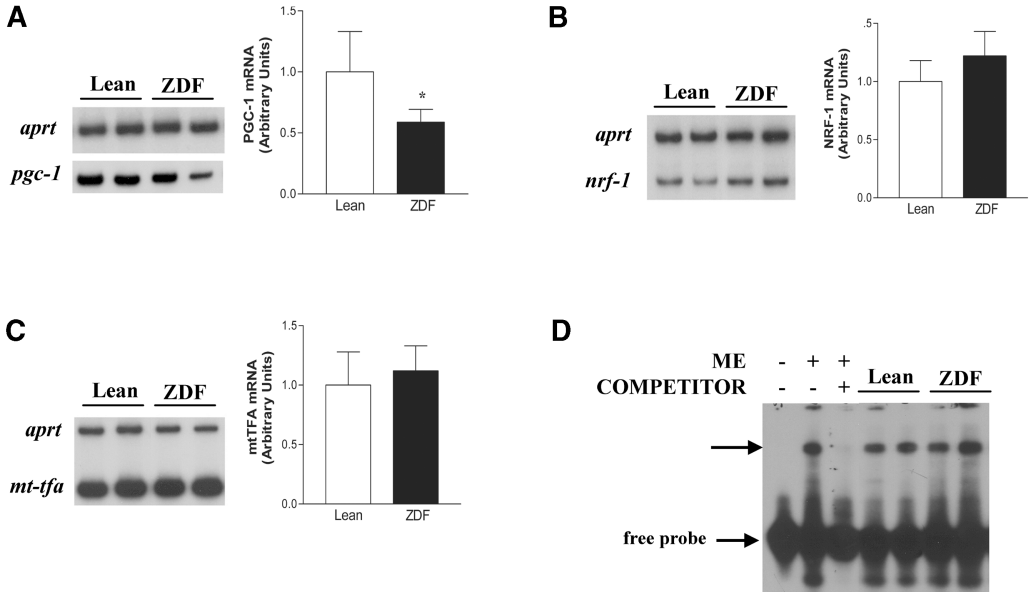

Fig. 5. PPARγ coactivator 1 (PGC-1) mRNA levels are downregulated in soleus muscle of ZDF rats. Analysis of the mRNA levels of PGC-1 (A), nuclear respiratory factor-1 (NRF-1) (B), and mitochondrial transcription factor A (mtTFA) (C) in soleus muscle of lean and ZDF rats. One-half microgram total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. D: Analysis of the binding activity of mitochondrial proteins to an mtTFA probe. Autoradiograph of electrophoretic mobility shift assay performed with a ^{32}P-labeled mtTFA nucleotide and mitochondrial protein extract shows a specific complex, based on competition with a molar excess of an unlabeled probe. * $P < 0.05$ compared with lean animals.

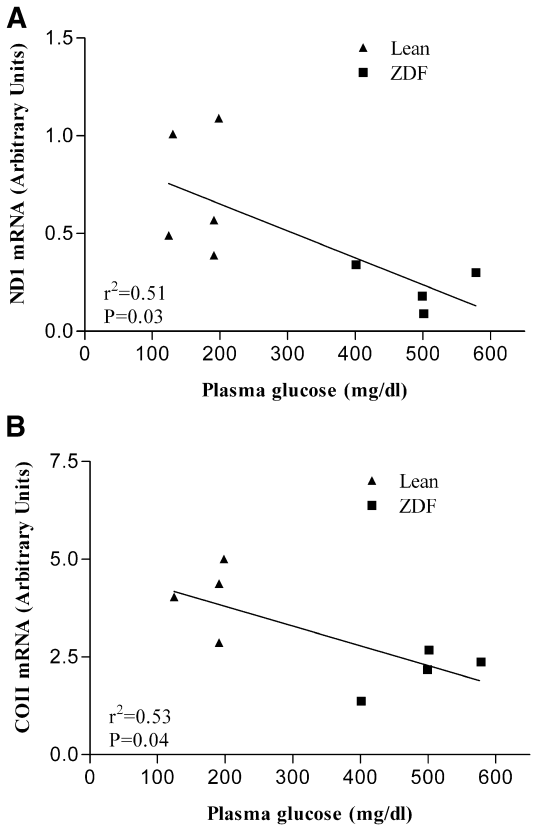

Fig. 6. Correlation between plasma glucose levels and ND1 mRNA levels (A) or COII mRNA levels (B) in lean and ZDF rats. Eight or nine pairs of data, respectively, were analyzed.

The importance of mtDNA in the development of diabetes mellitus is shown by the fact that distinct mutations of mtDNA associate frequently with diabetes. Thus, mutations in ND1 cause diabetes as a prominent phenotype (28). A reduction in the expression of the electron transport system may reduce its activity, leading to varying mitochondrial derangements. For instance, suppression of the electron transport system has been related to deceleration of the tricarboxylic acid cycle in a retrograde fashion (29). Moreover, impairment of the ETC reduces the β-oxidation of fatty acids, because the former exerts control over the latter at different points (10). Therefore, impairment of the expression of ETC may be one of the mechanisms underlying metabolic derangement in skeletal muscle in type 2 diabetes mellitus. Further studies are necessary to clearly establish this point.

Mitochondrial gene expression is regulated by several transcription factors, mainly NRF-1 and mtTFA. The lack of changes in the expression of NRF-1 and mtTFA suggests that these transcription factors are not involved in the reduced expression of ND1 and COII in untreated ZDF rats. In fact, it has been reported in a recent study that the robust induction in mtTFA, NRF-1, and NRF-2 detected in rat hepatoma is accompanied by increased expression of several mtDNA-encoded genes, but not ND1 (30). However, it is important to note that these parameters were measured shortly after the development of hyperglycemia (we used 12-week-old ZDF rats), because in the ZDF rat, a relatively stable level of hyperglycemia is established by 10 to 12 weeks of age. Therefore, it remains to be investigated whether these parameters are altered in ZDF rats with a longer period of established hyperglycemia. In contrast to mtTFA and NRF-1, we have detected a reduced expression of PGC-1 in skeletal muscle. PGC-1 is a coactivator for many factors in the nuclear hormone receptor family, and it has been implicated in mitochondrial biogenesis, respiration, and thermogenesis (31–36). PGC-1 also physically interacts with myocyte enhancer factor 2C to upregulate GLUT4 expression and glucose uptake in L6 cells that overexpress PGC-1 (37). Therefore, PGC-1 downregulation in skeletal muscle of ZDF rats may contribute, at least in part, to the reduction in mtDNA-encoded genes.

In addition to these transcription factors, it has been shown that insulin and glucose flux regulate the expression of mtDNA-encoded genes such as ND1 in skeletal muscle (38). Thus, insulin infusion increased ND1 mRNA levels ~2.5-fold in skeletal muscle, and the ND1 response to insulin correlated with glucose uptake (28–30, 38). Similarly, Kanazawa et al. (17) reported reduced expression of mtDNA-encoded genes (ATP synthase subunit 6 and cytochrome b) in streptozotocin-induced diabetic rat hearts through reduced activity of mtTFA. Insulin treatment completely restored the expression of these genes. In the present study, we found that the reduction in ND1 and COII expression was inversely correlated with glucose levels, indicating that reduced utilization of glucose is responsible, in part, for the reduced expression of these mtDNA-encoded genes. Troglitazone treatment restored ND1 and PGC-1 expression. These effects of troglitazone treatment should be attributed to the improvement in insulin sensitivity afforded by drug treatment. In fact, insulin levels fell from 21.7 ± 3.4 ng/ml in untreated ZDF rats to 13.0 ± 1.8 ng/ml (P = 0.06) in troglitazone-treated ZDF rats, whereas the reduction in glucose levels did not reach statistical significance. In agreement with the improvement in insulin sensitivity in skeletal muscle after troglitazone treatment, we found reduced expression of PDK-4. Upregulation of PDK-4 expression has been associated with an increased fatty acid supply and/or oxidation in skeletal muscle (31). Further, increased abundance of PDK-4 mRNA that is reversed by insulin treatment has been reported in skeletal muscle of streptozotocin-induced diabetic rats (39). Similarly, increased PDK-4 expression and decreased pyruvate dehydrogenase activity have been associated with reduced ATP production (40). Such a defect may impair ATP synthesis, compromising energy-dependent GLUT4 recruitment to the cell surface. Therefore, troglitazone treatment reduces PDK-4 expression, which is associated with increased glucose utilization.

Recently, Patti and coworkers (41) reported data demonstrating decreased expression of PGC-1 and NRF-1 in skeletal muscle from type 2 diabetic patients. Therefore, our results confirm not only that the ZDF rat is a good animal model of type 2 diabetes mellitus for studying changes in gene expression in skeletal muscle, but also, and more im-

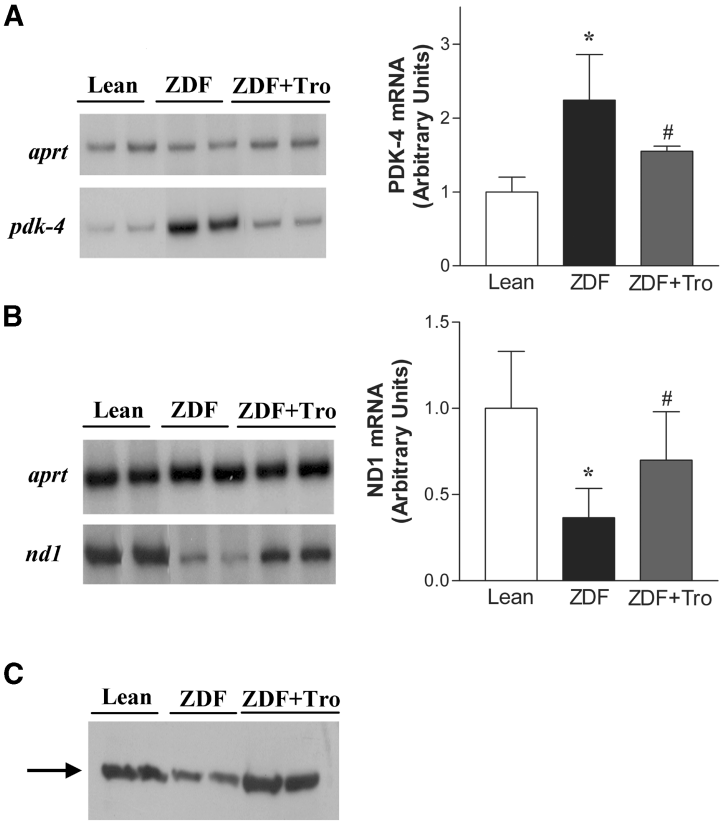

Fig. 7. Troglitazone restores ND1 expression in soleus muscle of ZDF rats. Analysis of the mRNA levels of PDK-4 (A) and ND1 (B) mRNA levels in soleus muscle of lean, untreated ZDF and troglitazone-treated ZDF rats. One-half microgram of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. C: Immunoblotting of ND1 in soleus muscle of lean, untreated ZDF and troglitazone-treated ZDF rats. Equal protein loading was assessed by phenol red staining of the upper portion of the gel (data not shown). * $P < 0.05$ compared with lean animal. # $P < 0.05$ compared with untreated ZDF rats.

portantly, that troglitazone treatment restores PGC-1 expression in muscle. This latter point is particularly interesting inasmuch as detecting and interrupting mitochondrial derangements may contribute to prevention of the onset of overt diabetes.

In summary, in the present study, we have demonstrated that the expression of PGC-1 and the mtDNA-encoded gene ND1 is reduced in skeletal muscle of ZDF rats. Treatment with the thiazolidinedione troglitazone restored the expression of these genes, achieving values similar to those found in lean animals. Because impaired mitochondrial function caused by reduced expression of genes involved in ETC is one of the mechanisms underlying metabolic derangements in skeletal muscle in type 2 diabetes mellitus, the improvement in the expression of ND1 and PGC-1 after troglitazone treatment may prevent these alterations.

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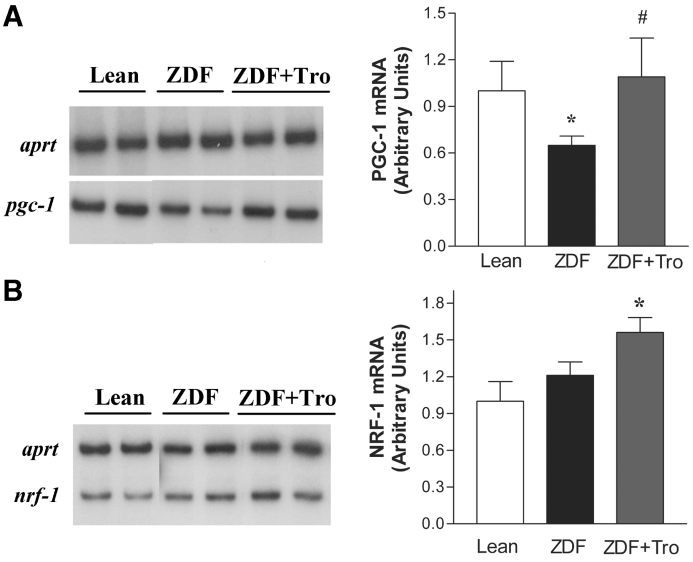

Fig. 8. Troglitazone restores PGC-1 expression in soleus muscle of ZDF rats. Analysis of the mRNA levels of PGC-1 (A) and NRF-1 (B) mRNA levels in soleus muscle of lean, untreated ZDF and troglitazone-treated ZDF rats. One-half microgram of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± SD of five rats. * $P < 0.05$ compared with lean animal. # $P < 0.05$ compared with untreated ZDF rats.

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