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Increased Akt protein expression is associated with decreased ceramide content in skeletal muscle of troglitazone-treated mice

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Abstract

Although it is generally believed that thiazolidinediones ameliorate insulin resistance by lowering circulating free fatty acids, direct effects of these drugs in skeletal muscle may also contribute to their antidiabetic action. We report that troglitazone administration to mice for 1 day increased the protein expression of Akt (two-fold induction, $P < 0.001$) in skeletal muscle without significant changes in the levels of free fatty acids in plasma. Increased Akt protein expression was associated with reduced phospho-AMP-activated protein kinase abundance and with a fall in the phosphorylation of acetyl-CoA carboxylase, which in turn resulted in an increase in the content of muscular malonyl-CoA (2.4-fold, $P < 0.05$) and lactate (1.4-fold, $P < 0.05$). Troglitazone treatment did not affect the mRNA levels of either Akt1 or Akt2, suggesting that a transcriptional mechanism was not involved, but caused a dramatic reduction in the content of muscular ceramides (76%, $P < 0.001$), lipid-derived second messengers known to increase Akt degradation. Our data indicate that troglitazone treatment inhibited de novo ceramide synthesis, since the content of its precursor, palmitoyl-CoA, was reduced (55%, $P = 0.05$). These results were confirmed in C2C12 myotubes, where troglitazone treatment increased Akt protein expression and prevented the reduction of this protein and the increase in ceramide levels caused by palmitate. These findings implicate ceramide as an important intermediate in the regulation of Akt after troglitazone treatment.

Keywords: Akt; PPAR; Troglitazone; AMPK; Malonyl-CoA; Ceramide

1. Introduction

Insulin resistance is a common metabolic abnormality associated with obesity, hypertension and type 2 diabetes mellitus [1]. Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization and is, therefore, the major site of insulin resistance. During the development of insulin resistance in skeletal muscle an impairment of glucose utilization and insulin sensitivity has been related to the presence of increased availability of certain lipid-derived second messengers, such as ceramides, which can attenuate several insulin signaling pathways [2] leading to insulin resistance.

Thiazolidinediones (ciglitazone, pioglitazone, rosiglitazone and troglitazone) are oral antidiabetic agents that improve insulin sensitivity and glucose homeostasis in type 2 diabetic patients as well as in various animal models of diabetes and obesity [3–5]. These drugs act as ligands of the peroxisome proliferator-activated receptor-γ (PPARγ), which belongs to the nuclear hormone receptor superfamily of transcription factors [6,7]. PPARγ is highly expressed in adipose tissue and plays a pivotal role in fat cell differentiation [6,7]. Upon activation by thiazolidinediones PPARγ regulates gene expression by binding as a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) to DNA response elements called peroxisome proliferator-response elements (PPRE), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (direct repeat-1). PPARγ response elements have been identified in the regulatory regions of several genes involved in fatty acid and carbohydrate metabolism [8]. Through these transcriptional changes thiazolidinediones promote accumulation of free

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CTE, cytosolic acyl-CoA thioesterase; M-CPT-I, muscle-type carnitine palmitoyl-transferase; PGC-1, PPARγ coactivator 1; PPAR, peroxisome proliferator-activated receptor

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fatty acids in adipocytes. Thus, this close association of PPARγ-induced lipid lowering with the enhancement of insulin action and the fact that PPARγ is highly expressed in adipose tissues, compared with muscle or liver, have lead to the hypothesis that thiazolidinediones improve muscle insulin action by sequestering lipids in adipocytes, a mechanism that ultimately reduces lipid accumulation in skeletal muscle. Accordingly, the enhanced insulin action in muscle, and perhaps in liver, after thiazolidinedione treatment is generally considered secondary to a systemic lipid-lowering effect via a principal action in adipose tissue [6,9].

However, some evidences also suggest that thiazolidinediones may directly enhance muscle insulin action, independent of the lowering of free fatty acids. Thus, in mice with lipodystrophy in which adipose tissue is essentially absent, troglitazone still improves insulin sensitivity [10]. In agreement with this fact, recent studies in isolated skeletal muscle have shown that thiazolidinediones have direct effects on muscle lipid metabolism independent of PPARγ-mediated gene expression [11]. Therefore, changes in the levels of intracellular lipids and lipid-derived second messengers (such as ceramides) after thiazolidinedione treatment may account for some of the direct antidiabetic actions of these drugs in skeletal muscle. Insulin stimulation of Akt (also referred as protein kinase B) in skeletal muscle is one of the insulin signaling pathways that may be attenuated by ceramides leading to insulin resistance [12]. Akt is a serine/threonine protein kinase that is stimulated by a variety of growth factors including insulin via a multistep pathway involving a phosphatidylinositol 3-kinase-dependent mechanism [13]. Once Akt is phosphorylated and activated, it can promote glucose uptake and subsequent metabolism via translocation of glucose transporter (GLUT) 4 to the plasma membrane [14,15]. In addition, the phosphorylation and inhibition of glycogen synthase kinase 3 by Akt activates glycogen synthase and thereby promotes glycogen synthesis [16]. On the other hand, recent evidences indicate that Akt may be related to fatty acid metabolism through its cross-talk with AMP-activated protein kinase (AMPK), since it has been reported that Akt activation can lead to decreased AMPK activity [17]. AMPK plays an important role in fatty acid metabolism in skeletal muscle since its activation stimulates a rapid phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), leading to a fall in malonyl-CoA concentration, an inhibitor of carnitine palmitoyltransferase I (CPT-I), the enzyme that controls the transfer of long-chain fatty acyl-CoA into mitochondria. Therefore, AMPK activation results in an increase in fatty acid oxidation [18,19]. This cross-talk between Akt and AMPK may be one of the underlying mechanisms responsible for the inhibition of lipid oxidation and the release of lactate (anaerobic glycolysis) in isolated skeletal muscle after troglitazone treatment [11,20].

The aim of the present study was to investigate whether troglitazone treatment affected the expression of Akt and the activity of AMPK in skeletal muscle and their involvement in the effects of this drug on fatty acid and glucose metabolism. The results shown here demonstrate that troglitazone treatment increases Akt protein expression in skeletal muscle, which is associated with a reduction in the activity of AMPK to phosphorylate ACC and an increase in the levels of malonyl-CoA, a known inhibitor of fatty acid oxidation. In addition, we observed that troglitazone treatment reduced the content of ceramides in skeletal muscle, which are involved in the degradation of Akt protein. Further, troglitazone treatment increased Akt protein expression in C2C12 myotubes and prevented the reduction in the expression of this protein and the increase in ceramide levels caused by palmitate. These findings implicate ceramide as an important intermediate in the regulation of Akt afforded by troglitazone treatment.

2. Materials and methods

2.1. Materials

Troglitazone was kindly provided by Glaxo Wellcome.

2.2. Animals and treatment

Twelve male Swiss mice from Harlan (Barcelona) were used. They were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature ($21 \pm 1\ ^{\circ}C$) and fed a standard diet. The mice were randomly distributed into two groups. Each group was administered, respectively, either 0.5% carboximethyl cellulose (control group) or 100 mg/kg/day of troglitazone (dissolved in 0.5% carboximethyl cellulose). Each compound was administered per os once a day for 1 day (1 ml/100 g of body weight). Food and water were given ad libitum. Twenty-four hours after administration, mice were killed under pentobarbitone anesthesia to collect blood samples and to isolate soleus skeletal muscle.

2.3. Plasma determinations

Plasma triglycerides (Sigma), non-esterified fatty acids (Wako) and glucose (Sigma) concentrations were determined by colorimetric tests.

2.4. Cell culture

Mouse C2C12 myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing DMEM and 2% horse serum, which was changed every other day. After 4 additional days, the differentiated C2C12 cells had fused into myotubes. Lipid-containing media were prepared by conjugation of

palmitate with fatty acid-free bovine serum albumin, by a method modified from that described by Chavez et al. [12]. Briefly, palmitate was dissolved in ethanol and diluted 1:100 in DMEM containing 2% (w/v) fatty-acid-free bovine serum albumin. Myotubes were incubated for 16 h in serum free-DMEM containing 2% bovine serum albumin in either the presence or absence of 0.5 mM palmitate and 100 μM troglitazone. After the incubation, RNA was extracted from myotubes as described below.

2.5. RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA 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 reverse transcriptase (Invitrogen), 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 (3000 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′; muscle-type carnitine palmitoyltransferase I (M-CPT-I), 5′-TTCACTGTGACCCCAGACGGG-3′ and 5′-AATGGACCAGCCCCATGGAGA; PPARγc coactivator-1 (PGC-1), 5′-AGAAAGGGCCCGAGCAATCTG-3′ and 5′-AGATGTGCCCCTGCCAGTCAC-3′; Akt1, 5′-GCAAGGGCACCTTTGGGAAAG-3′ and 5′-ACACGCGCTCTCGAGACAGGT-3′; Akt2, 5′-GAGAAGGCCACTGGCCGCTAT-3′ and 5′-CATAGGCGGTCATGGGTCTGG-3′; cytosolic acyl-CoA thioesterase (CTE), 5′-CAGCCACCCCGAGGTAAAAGG-3′ and 5′-CCTTGAGGCCATCCTTGGTCA-3′; and APRT (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-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 23 (PGC-1 and Akt1), 25 (PPARα, M-CPT-I, Akt2) and 27 (CTE) cycles. Each cycle consisted of denaturation at 92 °C for 1 min, primer annealing at 60 °C, and primer extension at 72 °C for 1 min and 50 s. A final 5-min extension step at 72 °C was performed. A volume of 5 μl 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, M-CPT-I: 222 bp, PGC-1: 234 bp, Akt1: 264 bp, Akt2: 167 bp, CTE: 224 bp and APRT: 339 bp). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating 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 [21]. The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

2.6. Immunoblotting

Soleus muscles and C2C12 myotubes were homogenized in cold lysis buffer (5 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5.4 μg/ml aprotinin). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. Protein concentration was measured by the Bradford method. Proteins (50 μg) were separated by SDS-PAGE on 10% separation gels (7% for ACC-P determination) and transferred to Immobilon polyvinylidene diflouride membranes (Millipore). Samples were analyzed for the phophorylation status of Akt on Ser^{473} (Cell Signaling Technology), AMPK on Thr^{172}, ACC on Ser^{79} by Western blot analysis using phosphorylation site-specific antibodies. In addition, total Akt, using an antibody that recognize both the phosphorylated and unphosphorylated proteins (Santa Cruz Biotechnology), heat shock protein (Hsp)27 and Hsp90 (Stressgen Biotechnologies), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling) were measured by Western blot analysis. Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries). Equal loading of proteins was assessed by red phenol staining. Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

2.7. Skeletal muscle malonyl-CoA, palmitoyl-CoA and lactate measurement

Approximately 50 mg of frozen skeletal muscle powder were homogenized in 1 ml of ice-cold 3.6% perchloric acid. The denaturated protein was removed by centrifugation and an aliquot of the supernatant was used directly for analysis of skeletal muscle malonyl-CoA levels by HPLC as previously described [22]. A second aliquot was used for the extraction of lipids, including long-chain acyl-CoAs, with saturated $(NH_4)_2SO_4$ (25 μg/ml of extract) and $CHCl_3$-MeOH (1:2 vol/vol) followed by further $CHCl_3$-MeOH (1:2)

extraction. The resulting supernatants were evaporated and then reconstituted in 1 ml 50 mM KH_2PO_4 (pH 5.3). Extracts were loaded onto a C18 reverse-phase column and long-chain acyl-CoAs were separated following the method described by Ellis et al. [23]. Palmitoyl-CoA detection was performed by comparing sample peak with a standard. Finally, 20 mg of frozen skeletal muscle powder were homogenized in 1 ml of ice-cold 0.5 M perchloric acid and 1 mM EDTA, neutralized to pH 7.0 with 2 M $KHCO_3$ and analyzed for lactate content using a commercial kit (Sigma).

2.8. Hydrogen peroxide determination

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

2.9. Determination of ceramide levels

The content of ceramides in skeletal muscle and C2C12 myotubes was determined by the diacylglycerol kinase method. Briefly, lipids were extracted from approximately 50 mg of skeletal muscle with 600 μl of chloroform, methanol, 1 N HCl (100:100:1). After agitation and centrifugation, the lower phase containing the chloroform-extracted lipids was transferred to a new microfuge tube. Chloroform was evaporated under a N2 stream. Dried lipids were resuspended in 300 μl of 0.1 N KOH in methanol and incubated for 1 h at 37 °C to eliminate diacylglycerol. Then, 300 μl of PBS was added, and lipid extraction was repeated as indicated above. Lipids were resuspended in 100 μl of reaction buffer (150 ng/100 μl cardiolipin, 280 μM diethylenetriaminepentaacetic acid, 51 mM octyl β-D-glucopyranoside, 50 mM NaCl, 51 mM imidazol, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 500 μM ATP, 5 μCi/100 μl [γ-^{32}P]ATP), and 35 ng of diacylglycerol kinase was added to each sample. Reactions were incubated at 30 °C for 30 min and stopped by the addition of 170 μl of stop buffer (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM glucose, 10 mM Hepes, pH 7.2) and 30 μl of 100 mM EDTA. Lipids were extracted again with 1 ml chloroform, methanol, 1 N HCl (100:100:1), resuspended in 40 μl of chloroform, spotted onto silica gel TLC plates (Whatman Inc.), and resolved using chloroform:methanol:acetic acid (65:15:5) as a solvent. Plates were measured in a PhosphoImager (BioRad). Quantification of ceramide mass was obtained by comparison with a standard curve ranging from 0 to 1000 pmol of ceramide-1-phosphate (Sigma), which was processed in parallel to the samples.

2.10. Statistical analyses

Results are expressed as mean ± S.D. of six mice. Significant differences were established by Student's *t*-test or one-way ANOVA, according to the number of groups compared. When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Statistical analyses were performed using the computer program GraphPad Instat (GraphPad Software V2.03) (GraphPad Softwware Inc.).

3. Results

3.1. Troglitazone increases Akt protein expression in soleus muscle of normoglycemic mice

First we determined the effects of troglitazone administration for 1 day (100 mg/kg/day) on several plasma energy substrates. Plasma triglyceride, free fatty acids and glucose levels were not significantly affected by thiazolidinedione treatment for 24 h (data not shown), in agreement with previous studies [24]. We next analyzed the effect of troglitazone on Akt expression in the soleus muscle using both anti-phospho-Akt (Ser-473) and anti-Akt antibodies in the basal state. Thiazolidinedione treatment did not affect the expression of phospho-Akt, which is consistent with the lack of insulin infusion in the animals used in this study (Fig. 1A). In contrast, Akt protein expression was two-fold higher ($P < 0.001$) in skeletal muscle of troglitazone-treated mice than in untreated animals (Fig. 1B).

3.2. Increased Akt protein expression after troglitazone treatment does not reduce the mRNA levels of genes involved in fatty acid oxidation

Since chronic Akt activation has been related to down-regulation of important genes involved in fatty acid oxidation, such as PPARα and PPARγ coactivator-1(PGC-1) [25], we next studied whether the increased protein Akt expression was responsible for the inhibition of fatty acid oxidation reported after troglitazone treatment [11]. The mRNA levels of PPARα, the transcription factor that controls the expression of several genes involved in fatty acid oxidation, were up-regulated (2.7-fold induction, $P < 0.01$) in skeletal muscle of thiazolidinedione-treated mice compared with untreated animals (Fig. 2A). In contrast, no changes were observed in the transcript levels of

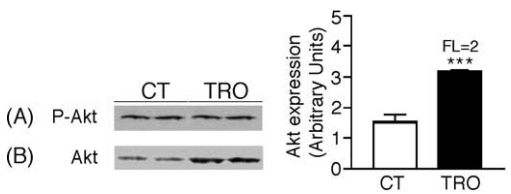

Fig. 1. Increased expression of Akt protein in soleus muscle of troglitazone-treated mice. Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Representative immunoblots using anti-phospho-Akt (ser-473) antibody (A) and anti-Akt antibody (B) are shown. Data are expressed as mean ± S.D. of six mice. $^{***}P < 0.001$ compared with control animals.

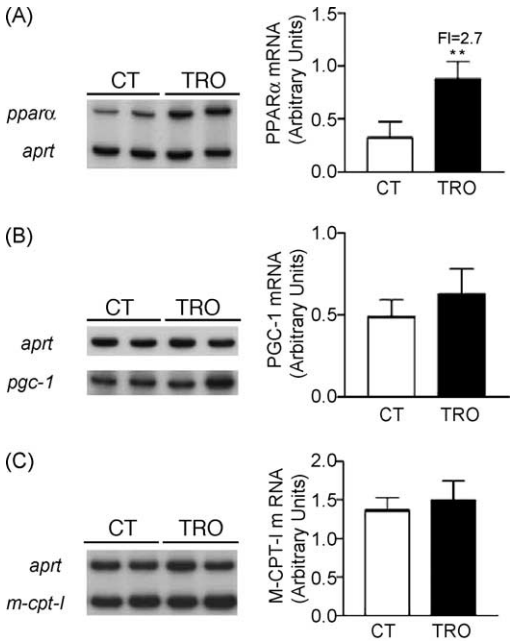

Fig. 2. Troglitazone treatment does not reduce the mRNA levels of genes involved in fatty acid metabolism in soleus muscle. Analysis of the mRNA levels of PPARα (A), PGC-1 (B) and M-CPT-I (C) in soleus muscle of untreated (control) and troglitazone-treated mice. An amount of 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six mice. $^{**}P < 0.01$ compared with control animals.

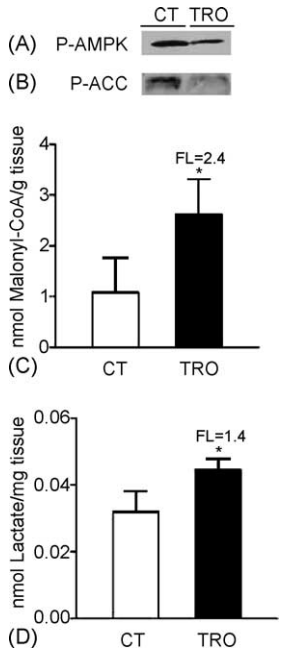

Fig. 3. Effects of troglitazone treatment on AMPK and ACC phopshorylation, malonyl and lactate content in skeletal muscle. Analysis of the phosphorylation of AMPK (A) and ACC (B). Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Equal protein loading was assessed by phenol red staining of the lower portion of the gel (data not shown). Analysis of the intracellular content of malonyl-CoA (C) and lactate (D) in soleus muscle of control and troglitazone-treated mice. Data are expressed as mean ± S.D. of six mice. $^{*}P < 0.05$ compared with control animals.

PGC-1, a coactivator for many factors in the nuclear hormone receptor family that has been implicated in mitochondrial biogenesis, respiration and thermogenesis [26] (Fig. 2B). Similarly, the mRNA expression of muscle-type CPT-I (M-CPT-I), that catalyses the entry of long-chain fatty acids into the mitochondrial matrix for fatty acid oxidation, was not altered by drug treatment (Fig. 2C). Therefore, these data suggest that increased Akt protein expression in skeletal muscle after troglitazone treatment does not result in a down-regulation of genes involved in fatty acid oxidation.

3.3. Increased Akt protein expression is associated with reduced AMPK activity in skeletal muscle of troglitazone-treated mice

Since Akt activation can lead to decreased AMPK activity [17,27] we next evaluated whether the changes in Akt protein expression affected AMPK activity. As shown in Fig. 3A, troglitazone treatment reduced the abundance of phospho-AMPK in skeletal muscle compared with untreated animals. AMPK plays a key role in controlling the switch between carbohydrate and fatty acid utilization in skeletal muscle through phopshorylation of acetyl-CoA carboxylase (ACC), given that this enzyme catalyses the formation of malonyl-CoA, an inhibitor of mitochondrial fatty acid oxidation through feedback inhibition of M-CPT-I. Thus, with reduced phospho-AMPK abundance, one would expect to find a reduction in the phosphorylation of ACC and an increase in the malonyl-CoA levels. Both changes were observed in skeletal muscle of troglitazone-treated animals (Fig. 3B and C). A dramatic fall in the phosphorylation of ACC was observed, whereas the concentration of malonyl-CoA was 2.4-fold ($P < 0.05$) higher in skeletal muscle of troglitazone-treated mice. Furthermore, the decrease in AMPK activity has been related to an increase in lactate production [27] and, according to this fact, a higher content of lactate (1.4-fold, $P < 0.05$) was present in skeletal muscle of troglitazone-treated mice, indicating that drug treatment increased glycolysis (Fig. 3D).

3.4. The increase in Akt protein expression achieved by troglitazone does not involve changes in its mRNA nor Hsp protein levels

Most of the effects caused by activation of the PPARγ transcription factor by troglitazone are mediated through changes in the mRNA expression of its target genes. In order to assess what was the mechanism involved in the observed increase in Akt protein expression after troglitazone treatment, we determined the mRNA levels of Akt. Troglitazone treatment did affect neither the transcript levels of Akt1 nor Akt2 (Fig. 4A and B). These results

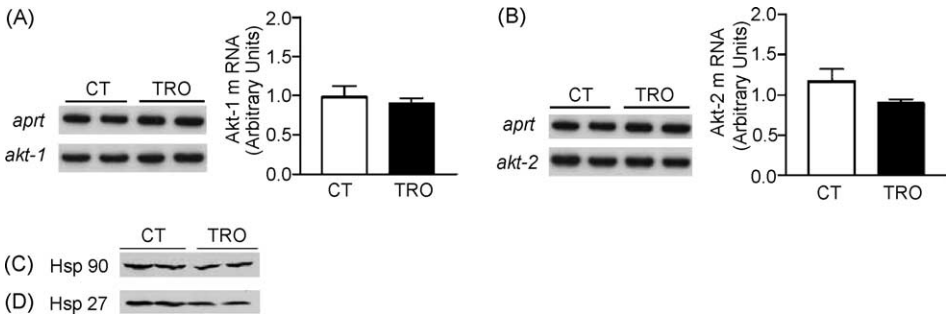

Fig. 4. Troglitazone treatment does not affect the mRNA levels of Akt1 or Akt2 nor Hsp protein expression in skeletal muscle. Analysis of the mRNA levels of Akt1 (A) and Akt2 (B) in soleus muscle of untreated (Control) and troglitazone-treated mice. An amount of 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six mice. Effects of troglitazone treatment on Hsp90 and Hsp27 protein and the generation of H_2O_2 in skeletal muscle. Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Representative immunoblots using anti-Hsp90 (C) and anti-Hsp27 (D) are shown. Data are expressed as mean ± S.D. of six mice.

make unlikely that the changes observed in Akt expression after troglitazone treatment may occur as a result of a transcriptional mechanism and suggest that other mechanisms, such as the control of protein degradation, may be involved. Regarding the degradation of Akt protein it is important to note that Akt binds to heat shock protein 90 (Hsp90), a family of protein chaperones that protect proteins against degradation [28]. Akt forms complexes with Hsp90 and 27, and complex formation with the former facilitates kinase activation by preventing both dephosphorylation and Akt degradation [29]. Therefore, troglitazone may act promoting changes in Hsp expression that would lead to a reduction in Akt degradation. However, when we determined the expression of Hsp90 and Hsp27 no changes were observed in the expression of these proteins (Fig. 4C and D). These data indicate that the effects of troglitazone on Akt expression do not involve a reduction in its degradation as a result of enhanced expression of Hsp proteins.

3.5. The increase in Akt protein expression caused by troglitazone is associated with a reduction in the levels ofpalmitoyl-CoA and ceramides

According to recent studies Akt protein levels are also regulated by ceramide and hydrogen peroxide (H_2O_2) levels, which induce a caspase-3-independent degradation of Akt [30]. Thus, to gain further insight into the mechanism by which troglitazone increases Akt protein expression we determined the levels of H_2O_2 and ceramides in skeletal muscle of troglitazone-treated and untreated mice. Changes in the content of H_2O_2 may activate a wide array of proteases, leading to the degradation of Akt and other proteins, such as PARP [30], a well-established substrate of caspase-3 in vivo [31]. No changes were observed in the content of H_2O_2 in skeletal muscle of troglitazone-treated compared with untreated mice (data not shown), indicating that the cell redox state was unchanged. In agreement with the lack of changes in the levels of H_2O_2 we found that the content of PARP, which shows a similar pattern of degradation that Akt in the presence of increased levels of H_2O_2 [30], was unaffected (data not shown). Ceramides are the other factor governing the degradation of Akt [30]. Palmitoyl-CoA is a precursor of sphingolipid synthesis, since the initial step of de novo ceramide synthesis is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine. When we analyzed the content of ceramides a dramatic reduction (76%, $P < 0.001$) in the skeletal muscle of troglitazone-treated mice compared to untreated mice was observed (Fig. 5A). Moreover, the levels of the precursor of de novo ceramide synthesis, palmitoyl-CoA showed a 55% ($P = 0.05$) reduction in skeletal muscle after troglitazone treatment (Fig. 5B). These results indicate that the increase in the content of Akt protein observed in troglitazone-treated mice may result from the inhibition of de novo ceramide synthesis. The reduction in the content of palmitoyl-CoA may be due to the increase in the mRNA levels of cytosolic acyl-CoA thioesterase (CTE) (3.2-fold induction, $P < 0.05$) (Fig. 5C), which hydrolyses fatty acyl-CoAs to free fatty acids and CoA.

In order to confirm the effects of troglitazone in vivo we performed an additional study in C2C12 myotubes. Treatment with troglitazone for 24 h led to a four-fold increase ($P < 0.01$) in the protein levels of Akt (Fig. 6A), confirming the results obtained in vivo. Further, we studied whether in palmitate-induced insulin resistance troglitazone treatment prevented the reduction in Akt protein expression and the increase in the cellular ceramide content. In myotubes treated with 0.5 mM palmitate for 16 h a 40% reduction ($P < 0.05$) in the protein levels of Akt was observed. In contrast, co-incubation with troglitazone prevented this reduction and even led to a three-fold increase ($P < 0.05$) compared to palmitate-treated cells (Fig. 6B). Finally, the analysis of the ceramide content showed that palmitate caused a 2.5-fold increase ($P < 0.001$) compared to control cells and that in the presence of troglitazone this increase was completely abolished (Fig. 6C).

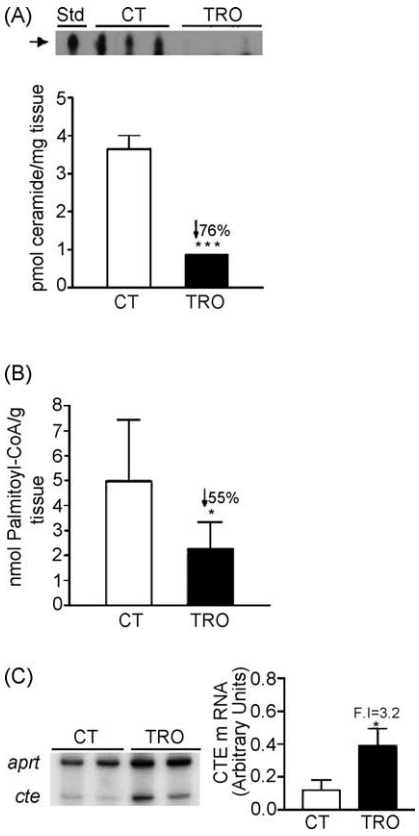

Fig. 5. Troglitazone treatment reduces ceramide levels in skeletal muscle. Measurement of ceramide levels in skeletal muscle of untreated (control) and troglitazone-treated mice. Lipid extracts from skeletal muscle were prepared and assayed for ceramides as detailed in Section 2. (A) Phosphorimage of phosphorylated ceramides from samples and standards, separated by TLC, and its quantification (mean ± S.D.) is shown. (B) Quantification of palmitoyl-CoA levels in skeletal muscle. (C) Analysis of the mRNA levels of CTE in soleus muscle of untreated (control) and troglitazone-treated mice. An amount of 0.5 μg 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 ± S.D. of six mice.

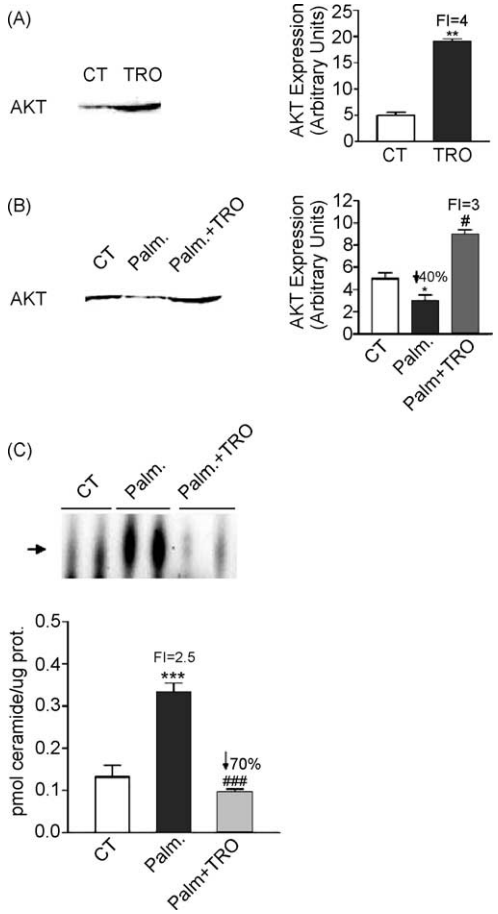

Fig. 6. Troglitazone treatment increases Akt expression and reduces ceramide levels in C2C12 myotubes. (A) Representative immunoblot analysis using anti-Akt antibody of total protein extracts of C2C12 myotubes incubated for 24 h in the presence or in the absence of troglitazone. C2C12 myotubes incubated (16 h) with 0.5 mM palmitate in the presence or in the absence of troglitazone were assayed for (B) immunoblot analysis using anti-Akt antibody or (C) measurement of ceramide levels. Lipid extracts from cells muscle were prepared and assayed for ceramides as detailed in Section 2. Data are expressed as mean ± S.D. of four different experiments. $^{***}P < 0.001$ and $^{*}P < 0.05$ vs. control. ### and # vs. palmitate-treated cells.

4. Discussion

Thiazolidinediones are a recent new class of oral agents for the treatment of type 2 diabetes that improve glycemic control by increasing insulin sensitivity in target tissues, such as skeletal muscle and adipose tissue [9]. Although it is clear that thiazolidinediones work mostly by increasing insulin sensitivity in the periphery, the mechanism(s) by which this occurs remain obscure [32,33]. These drugs are known to ameliorate existing insulin resistance associated with chronic lipid accumulation. It is generally believed that lowering of circulating lipids reduces lipid availability to tissues, resulting in improved insulin sensitivity [6]. In the present study we report that troglitazone treatment increases the expression of Akt protein in skeletal muscle. Akt has been proposed to be an intermediate in the signaling pathway by which insulin, controls glucose uptake [34]. Thus, overexpression of constitutively active forms of Akt induced glucose uptake, GLUT4 translocation, and glycogen synthesis [35,36]. In addition, it has been demonstrated that mice deficient in Akt2 were insulin resistant in muscle and liver [37]. All these data indicate that Akt is a central mediator of the insulin effects. Exposing skeletal muscle cells to free fatty acids, specially saturated free fatty acids, inhibits insulin stimulation of Akt [12]. Interestingly, troglitazone treatment increases the phosphorylation of Akt in skeletal muscle [38], effect that may account for part of the mechanisms leading to improved insulin sensitivity of glucose uptake and glucose storage in skeletal muscle. However, this effect has been related to the reduction in plasma free fatty acids achieved by troglitazone treatment [38]. In contrast, in this study we report that the increase in Akt protein expression occurs without significant changes in the levels of free fatty acids in plasma in the basal state.

Although this study was performed in the soleus, a slow-twitch oxidative muscle, and it may differ in other muscle types, the increase in the expression of Akt in skeletal muscle after troglitazone treatment may have beneficial consequences in the prevention and treatment of type 2 diabetes mellitus. Thus, it has been previously reported that prior treatment with the thiazolidinedione pioglitazone preserved insulin sensitivity in normal rats during acute fatty acid elevation, mainly through protecting liver insulin sensitivity, an effect that was independent of the plasma lipid-lowering of this drug [39]. Our experiments suggest that one additional factor involved in the protection afforded by these drugs against insulin resistance induced by lipid oversupply may be the increase in Akt protein in skeletal muscle. Animals treated with thiazolidinediones would be provided with a high level of Akt in muscle, leading to a preservation of insulin signaling and glucose uptake in lipid-induced insulin resistance. Although the data here presented need to be confirmed in humans, a previous report suggests that a similar effect could be attained by troglitazone in humans. Thus, in agreement with our results, Meyer et al. [38] recently reported that troglitazone treatment showed a tendency to increase Akt protein expression, although differences did not reach statistical significance, in vastus lateralis skeletal muscle of normoglycemic subjects at risk for the development of type 2 diabetes mellitus.

It has been reported that in isolated rat soleus muscle strips troglitazone caused lactate release as well as inhibition of mitochondrial palmitate oxidation by a PPARγ-independent mechanism [11]. Several facts raise the possibility that increased Akt protein expression in skeletal muscle after troglitazone treatment may lead to changes in intracellular lipid metabolism, which are not related to its lipid-lowering action. First, although chronic Akt overexpression has been related with the reduction in the expression of PPARα and PGC-1 [25] we did not find a fall in their expression. These data suggest down-regulation of PPARα and PGC-1 is not involved in the effects of troglitazone on palmitate oxidation and glycolisis. Second, the reported cross-talk between Akt and AMPK [17] may result in changes in intracellular lipid metabolism after troglitazone treatment. In our study we observed that up-regulation of the expression of Akt was accompanied by a reduction in phospho-AMPK content, which is a reflex of a decrease in its activity, leading to a fall in the phosphorylation of ACC and an increase in the malonyl-CoA levels, an inhibitor of mitochondrial fatty acid oxidation through feedback inhibition of M-CPT-I. In agreement with previous studies [27], the decrease in AMPK activity has been associated with an increase in the content of lactate, suggesting that troglitazone may increase the rate of glycolisis, although additional experiments are needed to confirm this point. Therefore, the increase in Akt protein expression after troglitazone treatment may lead to a reduction in AMPK activity and subsequent changes in malonyl-CoA content that may reduce mitochondrial fatty acid oxidation.

Regarding the mechanism of action responsible for the induction in the expression of Akt protein after troglitazone treatment, neither Akt1 nor Akt2 mRNA level was affected by troglitazone, suggesting that transcriptional changes were not involved. Since Akt protein expression is regulated by proteolytic degradation, we next explored whether up-regulation of Akt protein after troglitazone treatment was the result of changes in factors regulating this process. Degradation of Akt is enhanced by generation of H_2O_2 and ceramide [30]. Activation of proteases involved in the H_2O_2-induced degradation of Akt also result in PARP proteolysis. The lack of changes in the intracellular content of H_2O_2 and PARP protein indicates that troglitazone does not affect these factors involved in Akt degradation. In contrast, when we analyzed the content of ceramide, we observed a dramatic fall in the levels of these lipid-derived second messengers after troglitazone treatment. Ceramides are a family of sphingolipids that differ in the fatty acyl moeity and are known to induce insulin resistance and apoptosis in cultured cells [40,41]. Interestingly, a recent study shown that ceramide content is increased in skeletal muscle from obese insulin-resistant humans [42], suggesting that reduction in the content of ceramides attained by troglitazone may contribute to part of its effects. As an intermediate in the sphingomyelin pathway, ceramide can be generated by hydrolysis of sphingomyelin or can be produced by de novo synthesis [43]. Palmitate, once activated to palmitoyl-CoA by acyl-CoA synthase (ACS), is the precursor of de novo ceramide synthesis. The results presented here indicate that troglitazone inhibits de novo ceramide synthesis through reducing the levels of palmitoyl-CoA. Since in our study troglitazone treatment did not lower free fatty acids in plasma it is unlikely that plasma lipid availability may be involved in palmitoyl-CoA changes. In contrast, the data presented here suggest that the reduction in the intracellular content of palmitoyl-CoA may be the result of the increase in the expression of CTE, which hydrolyses fatty acyl-CoAs to free fatty acids and CoA. In addition, we cannot exclude that the well-known inhibitory effect of troglitazone on ACS may also contribute to reduce the content of intracellular palmitoyl-CoA [44].

In summary, in the present study we show that troglitazone treatment increases Akt protein expression in skeletal muscle. The mechanism responsible for the increase in Akt protein expression seems to involve a reduction in de novo ceramide synthesis, since these lipid-derived second messengers accelerate Akt protein degradation. Our results suggest that prior troglitazone treatment may lead to a preservation of insulin signaling and glucose uptake in lipid-induced insulin resistance.

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