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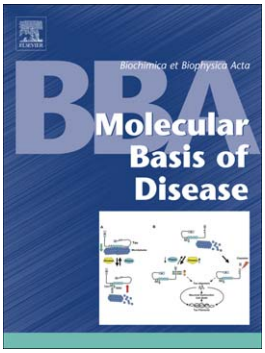

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Abnormal metabolism flexibility in response to high palmitate concentrations in myotubes derived from obese type 2 diabetic patients

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Abstract

Insulin resistance in type 2 diabetes (T2D) is associated with intramuscular lipid (IMCL) accumulation. To determine whether impaired lipid oxidation is involved in IMCL accumulation, we measured expression of genes involved in mitochondrial oxidative metabolism or biogenesis, mitochondrial content and palmitate beta-oxidation before and after palmitate overload (600 µM for 16h), in myotubes derived from healthy subjects and obese T2D patients. Mitochondrial gene expression, content and network were not different between groups. Basal palmitate beta-oxidation was not affected in T2D myotubes, whereas after 16h of palmitate pre-treatment, T2D myotubes in contrast to control myotubes, showed an inability to increase palmitate beta-oxidation ($p<0.05$). Interestingly, acetyl-CoA carboxylase (ACC) phosphorylation was increased with a tendency for statistical significance after palmitate pre-treatment in control myotubes ($p=0.06$) but not in T2D myotubes which can explain their inability to increase palmitate beta-oxidation after palmitate overload. To determine whether the activation of the AMP activated protein kinase (AMPK)-ACC pathway was able to decrease lipid content in T2D myotubes, cells were treated with AICAR and metformin. These AMPK activators had no effect on ACC and AMPK phosphorylation in T2D myotubes as well as on lipid content, whereas AICAR, but not metformin, increased AMPK phosphorylation in control myotubes. Interestingly, metformin treatment and mitochondrial inhibition by antimycin induced increased lipid content in control myotubes. We conclude that T2D myotubes display an impaired capacity to respond to metabolic stimuli.

Key words: lipid overflow, palmitate beta-oxidation, mitochondria, acetyl-CoA carboxylase, AMP activated protein kinase

Abbreviations.

T2D, type 2 diabetes ; IMCL, intramuscular lipid; ACC, acetyl-CoA carboxylase; AMP activated protein kinase, AMPK; AICAR, AMP-mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; FA, fatty-acid; ASM, acid-soluble metabolites; CS,

citrate synthase; HAD, 3-hydroxy-acyl-CoA-dehydrogenase; NRF1, nuclear respiratory factor 1; CPT1, carnitine palmitoyltransferase 1

1. Introduction

During overweight and obesity development, a redistribution of adipose tissue occurs with increased accumulation of visceral fat. This ectopic lipid accumulation has been shown to be related to diverse metabolic diseases including dyslipidemia, cardiovascular disease, insulin resistance and type 2 diabetes (T2D). In healthy people, skeletal muscle accounts ~80% of postprandial glucose disposal. However, during T2D development, skeletal muscle becomes primary significant site of insulin resistance [1, 2]. Several studies have demonstrated a strong negative relationship between ectopic lipids accumulation in skeletal muscle (intramyocellular lipid (IMCL)) and whole body insulin resistance [3-5]. However, the precise mechanisms leading to IMCL accumulation during T2D development are still under investigation. During the last decade, several studies have focused on the implication of mitochondrial dysfunction in insulin resistance development and IMCL accumulation [6-10]. Mitochondrial oxidative phosphorylation was shown to be reduced in the skeletal muscle of type 2 diabetic patients [6-8] and lean insulin-resistant offspring of type 2 diabetic parents [9, 10]. However, more recent studies have shown that mitochondrial function is not altered in T2D skeletal muscle when normalised for mitochondrial content [11] or when diabetic and non-diabetic subjects are matched for body composition [12]. Another hypothesis to explain increased lipid content in skeletal muscle of diabetic patients is an increased fatty-acid (FA) uptake due to increased membrane expression of the major muscle FA transporter FAT/CD36 [13].

Primary human myotubes isolated from type 2 diabetic patients have been shown to express metabolic characteristics that approximate the *in vivo* phenotype of the donor subject [14-19]. This *in vitro* muscle system provides an attractive model in which lipid accumulation can be evaluated apart from the systemic influences of the *in vivo* environment. We have recently shown in this model that an increased membrane expression of FAT/CD36 could explain ~50% of the increase in lipid content in myotubes derived from obese T2D patients [20]. Thus, other mechanisms may be involved in increased IMCL content in myotubes derived from obese T2D patients. Reduced lipid oxidation, particularly oxidation of saturated FA [21, 22], as well as

reduced citrate synthase activity [23], have been shown to be maintained in myotubes established from obese T2D patients. More recently, it has been shown that myotubes derived from obese T2D patients present a decrease in intramyocellular lipid oxidation compared to control myotubes and that this decrease in lipid oxidation was not the result of lower mitochondrial mass [24]. However, the cause of impaired mitochondrial function per se during T2D is not clearly understood and still under investigation.
Among the different treatments for type 2 diabetes, it is important to note that some of them act through the same target. Indeed, physical activity, metformin and thiazolidinediones activate AMP activated protein kinase (AMPK). AMPK is a major metabolic sensor which is activated by an increase in the ratio of AMP/ATP in order to restore energy status of the cell by stimulating ATP-producing pathways (glucose uptake, fatty acid oxidation, and mitochondrial biogenesis) [25-27] and inhibiting ATP-consuming pathways (fatty acid synthesis, glycogen synthesis, and protein synthesis) [28-30]. Interestingly, some studies have shown that the activation of AMPK induces an increase in mitochondrial function and lipid oxidation leading to decreased IMCL content [31-33]. This effect could be the result of an increase in long-chain FA entrance and oxidation in mitochondria due to the reduction in ACC (acetyl-CoA carboxylase) activity, a well known target of AMPK [34, 35]. Taken together these results show that AMPK activation may be an excellent target in order to decrease lipid content in the skeletal muscle of type 2 diabetes patients.
In order to gain further insight into the mechanisms underlying elevated lipid accumulation in T2D primary muscle cells, the aim of our study was to determine whether lipid oxidation is affected in basal condition or after palmitate overload in diabetic myotubes compared to control myotubes and to better understand the mechanisms leading to these defects. We also tested whether AMPK activation could modulate lipid content in these myotubes.

2. Materials and methods

2.1. Primary human satellite cell culture

Skeletal muscle biopsy of the vastus lateralis was performed according to the percutaneous Bergström technique after local anesthesia (xylocaine) [36, 37]. The experimental protocol was approved by the local ethic committee (03/10/GESE, Montpellier, France). Informed and written consent was obtained from all subjects after explanation of the protocol. In the present study, biopsies were taken from four control subjects (age: 49.2 ±2.7 years, body mass index: 24.0 ±1.2 kg/m²) with no familial or personal history of diabetes and from five moderately obese type 2 diabetic patients (age: 53.4 ±2.4 years, body mass index: 31.5 ±0.6 kg/m²). Clinical characteristics of the subjects were previously described [21, 41]. Cell culture of primary human satellite cells was performed as previously described [20]. Cultures were maintained in a growth medium composed of (DMEM, 10% FBS and 1% Ultroser G) and when myoblasts reached confluence, medium was changed (growth medium minus Ultroser G) and the differentiation process occurred until fusion and terminal differentiation into contractile myotubes (8 days). The experiments were performed on passages 2 to 4, after 8 days of differentiation.

2.2. Myotube treatments

Treatments performed on differentiated myotubes are detailed in the figure legends and were done in triplicate for each of the independent cell cultures. The following reagents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France): L-Glutamine, DMEM, antimycin A, AMP-mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), metformin and palmitate. Fetal bovine serum was from Hyclone (Brebières, France). Palmitate was dissolved in chloroform (0.6 mol/l), diluted to 600 µM in DMEM containing 10% of FBS (differentiation medium) and added to the myotubes as previously described [20, 38].

2.3. Lipid accumulation

Lipid accumulation in myotubes was visualized by oil red O (Sigma, Saint-Quentin Fallavier, France) staining as previously described [20, 38, 39]. Oil red O staining was carried for one hour followed by 2 or 3 washes with distilled water. Lipid droplets were then visualized by light microscopy. In order to quantify lipid accumulation in myotubes, oil red O was extracted using isopropanol. The absorbance value was measured using a spectrophotometer set at 490 nm and blanked to untreated cells [20, 38, 39].

2.4. Real-time RT-PCR analysis

Total RNA was isolated with Trizol (Invitrogen, Cergy-Pontoise, France) and single-strand cDNA was synthesized from 2.5 µg of total RNA with random hexamer primers and Superscript II (Invitrogen, Cergy-Pontoise, France). Real-time RT-PCR reactions were carried out with a LightCycler reaction kit (Eurogenetec, Angers, France) in a final volume of 20 µl containing 125 ng of reverse-transcribed total RNA, 500 nM of primers [40], 10 µl of 2x PCR mix and 0.5 µl of SYBR Green. The reactions were carried out in capillaries in a LightCycler instrument (Roche, Rosny-sous-bois, France) with 40 cycles. We determined the relative amounts of the mRNAs studied by means of the second derivative maximum method, with LightCycler analysis software version 3.5 and 18S RNA as the invariant control for all studies.

2.5. Western blots

Cellular extracts were quantified and lysed in Laemmli buffer. 30-50 µg of total proteins were transferred to nitro-cellulose membranes (Schleicher and Schuell, Bioscience, Dassel, Germany). Western blots were performed as described [41]. The following primary antibodies were used: phospho-Ser222-ACC, ACC, phospho-Thr172-AMPK and AMPK (Cell signaling, Paris, France), non-glycosylated protein component of mitochondria (Ab2, Interchim, Montlucon, France); and anti-α-tubulin (Sigma Saint-Quentin Fallavier, France) was used as a loading control. The secondary antibodies were anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase (GE Healthcare, Orsay, France). Proteins were visualized using an enhanced luminescent reagent (Tebu-

Bio), and exposed on film (GE Healthcare, Orsay, France). Expression of proteins was quantified by density analysis using ImageJ Launcher Software.

2.6. Citrate synthase activity

Cellular extracts were homogenized in a solution containing: 10 mM Tris HCl (pH 7.2), 0.1 mM EDTA, 75 mM saccharose and 225 mM mannitol. Citrate synthase activity was measured with 0.5 mM oxaloacetate, 0.3 mM acetyl-CoA, 0.1 mM of 5, 5'-Dithiobis 2-nitro-benzoic acid (DTNB), 100 mM Tris HCL (pH=8.0) and 0.1% Triton 100X. Enzyme activity was monitored by recording the changes in absorbance at 412 nm over 2min at 37°C, and normalized to protein content.

2.7. Immunofluorescence

Differentiated satellite cells were fixed with 4% paraformaldehyde in PBS for 10min, permeabilized with 0.5% triton in PBS for 2min, saturated with 0.5% BSA in PBS for 10min and then incubated with primary antibodies against a non-glycosylated protein component of mitochondria (Ab2, Interchim, Montlucon, France). Secondary antibody was Alexa Fluor 596 anti-mouse (Invitrogen, Molecular Probes, France).

2.8. Palmitate beta-oxidation

Cells were cultured in 96-well plates and differentiated as described above. Differentiated satellite cells were exposed or not to cold palmitate (0.6 mM) for the last 16h of differentiation and then exposed to differentiation medium (DMEM + 10% Foetal Bovin Serum) supplemented with 1% bovine serum albumin, 50 µM palmitate and 9.5 µM (0,09 µCi) [1-^{14}C]palmitate. Identical incubations were conducted on parallel plates that contained no cells. Palmitate beta-oxidation was determined by measuring production of ^{14}C-labeled acid-soluble metabolites (ASM), a measure of tricarboxylic acid cycle intermediates and acetyl esters. After incubation for 30 min at 37°C, reactions were terminated by aspiration of the media and addition of 100 µl of $HClO_4$ at 5% for 15min at room temperature. The ASM was assayed in supernatants of the acid precipitate. Radioactivity of ASM was determined by liquid scintillation counting by use of 4.5 ml of liquid scintillation cocktail (Optiphase 'Hisafe' 3, Perkin Elmer) in scintillation vials. For

protein determination, identical incubations were conducted on parallel plates with the same number of cells.

2.9. Statistical analysis

Statistical analyses were performed using Statview 5.0. Data are means ± SEM. Statistical analyses were performed using Student's t test for unpaired and paired comparison, or ANOVA with Fisher's PLSD post-hoc test for multiple comparisons. $P<0.05$ was considered to be significant.

3. Results

As previously shown by our team, lipid content in the presence of palmitate is increased in myotubes derived from obese T2D patients as compared to myotubes derived from healthy control subjects [20]. Here, we have confirmed this finding (Fig S1A and B, ($p<0.01$)).

3.1. Mitochondrial content and network are normal in T2D myotubes

We examined several mitochondrial parameters between control and T2D myotubes in order to determine whether mitochondrial content is decreased in T2D myotubes.
We first measured the mRNA levels of genes involved in mitochondrial oxidative metabolism (citrate synthase (CS) and 3-hydroxy-acyl-CoA-dehydrogenase (HAD)), of a gene known to mediate the transcriptional control of mitochondrial biogenesis (nuclear respiratory factor 1 (NRF1)) and of a gene involved mitochondrial lipid uptake (carnitine palmitoyltransferase 1 (CPT1)). The expression of all of these genes was not significantly different between control and T2D myotubes (Fig. 1A).

We determined by western blot the expression of a non-glycosylated protein component of mitochondria (Ab2) which has been previously used by others as a marker of mitochondria in subcellular fractions [42] (Fig. 1B). As a second marker of mitochondrial content, we measured the activity of citrate synthase (Fig. 1C). As shown in figure 1B and C, the expression of the non-glycosylated protein component of mitochondria was variable between subjects independently of type 2 diabetes, and citrate synthase activity was not significantly different between control and T2D myotubes. Furthermore, we have performed immunofluorescence experiments to check the specificity of the antibody used in western blot as well as to determine whether the mitochondrial network was different between the two groups (Fig. 1D). As shown in Figure 1D, mitochondria Ab-2 antibody showed a specific mitochondrial "spaghetti"-like staining pattern in the cytoplasm of control as well as in T2D myotubes, without obvious differences in morphology between groups.

Taken together, results presented in figure 1 show that neither mitochondrial content nor the mitochondrial network are affected in differentiated primary muscle cells derived from obese T2D patients.

3.2. Palmitate pre-treatment reveals mitochondrial dysfunction and an abnormal response at the level of ACC phosphorylation in T2D differentiated cells

A reduction in fatty acid oxidation with no difference in mitochondrial content has been observed in T2D myotubes [24]. We measured palmitate beta-oxidation in fully differentiated myotubes without palmitate pre-treatment (-) or with 16h of palmitate pre-treatment (+, cold palmitate, 600 µM). As shown in figure 2A, palmitate beta-oxidation was not significantly different in T2D differentiated cells compared to control cells in the basal state, but was significantly lower in T2D myotubes compared to control myotubes after palmitate overload ($p<0.05$). There was a trend for an increased palmitate oxidation after palmitate pre-treatment compared to basal condition in control myotubes ($p=0.09$), but not in T2D myotubes ($P=0.37$).

We have next monitored ACC phosphorylation (expressed as a ratio of phosphorylated to total protein) (Fig. 2B and C) in 4 cultures of control and 4 cultures of T2D differentiated

muscle cells. In the basal state, ACC phosphorylation was not different between groups (Fig. 2B and C).

We next examined the impact of palmitate pre-treatment (600 µM, 16h) on ACC phosphorylation (Fig. 2D and E). Palmitate pre-treatment was able to increase ACC phosphorylation in control myotubes with a tendency of statistical significance ($p=0.06$) but not in T2D myotubes (Fig. 2D and E).

Figure 2 shows that T2D myotubes are not able to increase palmitate beta-oxidation and ACC phosphorylation to the same degree as control myotubes in response to high concentrations of palmitate pre-treatment. These results suggest that the impairment in palmitate beta-oxidation in response to high level of palmitate pre-treatment in T2D myotubes (Fig. 2A) could be explained by an abnormal response to palmitate at the level of ACC phosphorylation (Fig. 2D and E).

3.3. AICAR or metformin treatments are not able to decrease lipid content in T2D myotubes

To test whether AMPK activation was able to decrease lipid content in T2D myotubes, fully differentiated myotubes were treated with 2 different AMPK activators: AICAR (5-aminoimidazole-4-carboxamide-β-D-ribofuranoside) and metformin (Fig. 3). AICAR treatment for 16h showed a significantly increased AMPK phosphorylation only in control myotubes ($p<0.05$) but not in T2D myotubes, due to a greater variability in the response to AICAR treatment in our T2D cells (Fig. 3 A and B). However, the phosphorylation of ACC was not increased after AICAR treatment in the 2 groups of cells (Fig. 3A and C). Metformin treatment for 16h did not increase either AMPK or ACC phosphorylation in control and T2D myotubes (Fig. 3A, B and C). As a consequence, no decrease in lipid accumulation was observed in T2D myotubes after metformin and AICAR treatments (Fig. 3D, left panel). AICAR treatment also had no effect on lipid content in control myotubes (Fig. 3D, left panel). Surprisingly, long term metformin treatment induced a significant increase in lipid content in control myotubes but not in myotubes from T2D patients (Fig. 3D, right panel).

3.4. Mitochondrial inhibition alters lipid content in control myotubes

AICAR activates AMPK by increasing the intracellular pool of ZMP, an AMPK analogue (for review see [43]). Metformin activates AMPK indirectly by inhibiting complex I of the respiratory chain [44, 45].Thus we sought to examine the effect of antimycin A, a mitochondrial inhibitor (complex III), before challenging cells with a high concentration of palmitate (600 µM for 16h) in order to evaluate if the metformin effect on lipid accumulation could be attributable to mitochondrial inhibition. As shown in figure 4 A and B, after treatment of the cells with antimycin A, palmitate-induced lipid accumulation in control myotubes was significantly increased by 22% ($p<0.05$). Interestingly, no increase in lipid content was found in T2D myotubes after antimycin A treatment which is in accordance with the results shown in Fig. 4 after metformin treatment.

Figure 4 shows that mitochondrial inhibition is sufficient to increase lipid content in control myotubes to the same level as in T2D myotubes.

4. Discussion

Mitochondrial involvement in intramuscular lipid accumulation and T2D development has been widely studied over the last 10 years. Numerous studies have shown a mitochondrial dysfunction in skeletal muscle of T2D patients [6-8] as well as in muscle from persons at risk for developing T2D [9, 10]. However, these results have been refuted in more recent studies showing that mitochondrial function was not altered in T2D skeletal muscle after normalization by mitochondria content [11] or when T2D and control subjects were matched for obesity [12]. Thus, the involvement of mitochondrial dysfunction in T2D is still under investigation. In the present study, we have shown that baseline palmitate oxidation and mitochondrial content were similar between primary human satellite cells derived from healthy control subjects and moderately obese T2D patients. A defect in palmitate beta-oxidation was only revealed when T2D myotubes were challenged with high concentrations of palmitate, and this impaired response to palmitate overload could be the result of a lack of inhibition of ACC activity by palmitate. Thus a mitochondrial dysfunction in T2D myotubes is only revealed upon challenge with saturated fatty acid overload. This result is in accordance with another study showing that myotubes from patients with a history of type 2 diabetes were unable to adapt to a hyperglycaemic–hyperinsulinaemic challenge [46]. Thus, it seems that response to nutrient challenge is impaired in T2D myotubes.

In the present study, we used primary muscle cells in culture to study mitochondrial fatty acid oxidation apart from the systemic influences of the *in vivo* environment. Interestingly, under basal conditions (no palmitate pre-treatment), no evidence for differential expression of genes involved in mitochondrial oxidative metabolism or in genes involved in mitochondrial biogenesis was found between myotubes derived from lean healthy subjects and obese type 2 diabetic patients, confirming results previously described [47]. Furthermore, we found no differences in mitochondrial content (estimated by citrate synthase activity or expression of a mitochondrial protein) or in the mitochondrial network between the two populations of cells. These results differ from previous published findings showing an intrinsic defect in citrate synthase activity [23] but are in accordance with a recent study [24].

Under basal conditions, we found no significant difference in palmitate beta-oxidation in cells derived from obese type 2 diabetic patients compared to those derived from control subjects. This result may be due to a lack of power in consequence of the small n size. However, only one out of 3 T2D myotubes show smaller beta-oxidation values than the control cells. Interestingly, after palmitate pre-treatment for 16h, palmitate beta-oxidation was significantly decreased in T2D myotubes compared to control cells. Palmitate pre-treatment also revealed a greater increase in lipid content in T2D myotubes as compared to control cells, whereas no difference in lipid accumulation was found between the two groups of myotubes in basal conditions (not shown). Thus, control myotubes were able to increase palmitate beta-oxidation after palmitate pre-treatment allowing an only moderate lipid accumulation whereas T2D myotubes showed a smaller response to high palmitate pre-treatment at the level of palmitate beta-oxidation (compared Fig. 2A with Fig. 2B) leading to greater lipid accumulation. The stimulation of fatty acid beta-oxidation by high concentrations of palmitate in control myotubes is in accordance with another study [48]. Moreover, direct inhibition of the electron transport chain by antimycin A and metformin was able to increase palmitate-induced lipid accumulation in control myotubes showing that mitochondrial inhibition can in fact lead to increased lipid content in control myotubes. In contrast, in satellite cells derived from obese T2D patients, neither antimycin A nor metformin treatment lead to an increase in lipid content in T2D myotubes indicating that mitochondrial function was already defective in T2D myotubes or that the lipid accumulation capacity in T2D myotubes was already at its maximum without these treatments.

To obtain mechanistic insights into the modified response to palmitate pre-treatment in T2D myotubes, we monitored changes in P-ACC. Active (dephosphorylated) ACC inhibits fatty acid oxidation by increasing the availability of malonyl-CoA which, in turn, inhibits carnitine palmitoyl transferase 1 (CPT1) and the entrance of long-chain fatty acid in mitochondria. Phosphorylation of ACC inhibits its activity leading to increased fatty acid uptake by mitochondria and, subsequently, increased fatty acid oxidation [49, 50]. In the basal state, no difference in P-ACC or ACC expression was observed between groups. After palmitate pre-treatment, the phosphorylation of ACC tend to be increased in control myotubes which is in accordance with the increase in palmitate beta-oxidation

is those myotubes. Interestingly, in T2D myotubes, no modification of the phosphorylation of ACC was observed after palmitate overload. Thus a lack of inhibition of ACC could explain why palmitate beta-oxidation was not increase in T2D myotubes in response to palmitate overload. Interestingly lack of a significant increase in AMPK phosphorylation was also found in T2D myotubes following AICAR treatment. This result, taken together with the absence of ACC inhibition by palmitate treatment in T2D myotubes suggests that T2D myotubes display a defective response to different stimuli at the level of the AMPK-ACC pathway. These preliminary but really intriguing results need further investigation to better understand the mechanisms leading to this defect in T2D myotubes. Unfortunately, due to the inability of T2D myotubes to activate the AMPK-ACC pathway, we were not able to reduce lipid content in T2D myotubes. Other experiments are necessary to determine whether AMPK is a good target in order to decrease lipid content in T2D myotubes.

Some methodological aspects of the study should be acknowledged.). In the present study, we focused on response to palmitate overload since it is well known that insulin resistance development in response to high FA level is the consequence of high saturated FA level (i.e. palmitate) and not to high unsaturated FA level (i.e. oleate) [51-54]. W acknowledge that the use of 600 µM of palmitate is not physiological since, *in vivo*, circulating free FA are a mix of saturated and unsaturated FA. The best experiment would be to use a cocktail of FA (i.e. oleate + palmitate) since it has been shown that the adverse effect of palmitate on cellular functions can be rescued with the addition of oleate [53, 54]**.** However, our results are in accordance with a recent study using only oleate as FA supply [24].

In summary, this study shows that skeletal muscle cells derived from moderately obese T2D patients exhibited decreased palmitate beta-oxidation and increased lipid accumulation in response to palmitate overload, possibly due to a palmitate-induced inhibition of ACC. In contrast, control myotubes responded to fatty acid overload with an increase in palmitate oxidation, and less lipid accumulation; this response did not reflect an inability of control myotubes to accumulate lipid as demonstrated by the antimycin A and metformin experiments. Studies are needed to evaluate whether the inhibition of ACC phosphorylation in control myotubes is able to reproduce the diabetic phenotype

(i.e. decreased palmitate beta-oxidation and increased myotube lipid content) and to evaluate whether an increase in ACC phosphorylation in T2D myotubes is able to increase palmitate beta-oxidation and decrease lipid accumulation.

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FIGURE LEGEND

Fig. 1. Mitochondrial gene expression, content and network in control and T2D myotubes. A. Expression of genes encoding proteins involved in mitochondrial oxidative metabolism (citrate synthase, CS and 3-hydroxy-acyl-CoA-dehydrogenase, HAD), in transcriptional control of mitochondrial biogenesis (nuclear respiratory factor 1, NRF1) and in lipid metabolism (carnitine palmitoyltransferase 1, CPT1) was evaluated by quantitative real-time PCR in control (n=4) and T2D differentiated satellite cells (n=4). Each value indicates the amount of mRNA expressed relative to 18S RNA. Each experiment was performed in triplicate for each of the 8 independent cell cultures. Data are presented as means ±SEM normalized to mRNA level in control myotubes. B. Representative western blot analysis of a non glycosylated protein component of mitochondria (Ab2) expression in myotubes established from control and T2D myotubes. α-Tubulin was used as a loading control. C. Citrate synthase activity was determined in control (n=3) and T2D differentiated satellite cells (n=3) under basal conditions and expressed relative to protein content. Experiments were performed in duplicate for each of the 6 independent cell cultures. Data are means ± SEM. D. Representative immunofluorescence microscopy of control and T2D myotubes after 8 days of differentiation, incubated with an antibody against a non glycosylated protein component of mitochondria (Ab2) visualized using a monoclonal secondary antibody conjugated to Alexa 546. Scale bar represents 30 μm.

Fig. 2. Effect of palmitate pre-treatment on palmitate beta-oxidation and phosphorylated acetyl-CoA carboxylase expression in control and T2D myotubes. A. Basal palmitate beta-oxidation and palmitate beta-oxidation after 16h of cold palmitate pre-treatment (600 μM) in control (n=3) and T2D differentiated satellite cells (n=3) expressed relative to protein content. Experiments were performed in triplicate for each of the 6 independent cell cultures. Data are means ± SEM. *, $P<0.05$, T2D cells versus control cells. B. Representative western blot analysis of P-ACC in differentiated satellite cells established from 4 control subjects and 4 type 2 diabetic (T2D) patients in the basal state (no palmitate pre-treatment). ACC protein expression was used as a loading control. C. Quantification by density analysis of western blot shown in (A). Data are means ±

SEM. D. Representative western blot analysis of P-ACC in differentiated satellite cells established from 4 control subjects and 4 type 2 diabetic (T2D) patients with (+) or without (-) palmitate pre-treatment (600 µM for 16h). ACC protein expression was used as a loading control. E. Quantification by density analysis of P-ACC and ACC expression in myotubes established from control subjects (n=4) and T2D patients (n=4) with (+) or without (-) palmitate pre-treatment (600 µM for 16h). Data are means ± SEM. P=0.06, palmitate-treated differentiated satellite cells versus untreated cells.

Fig. 3. Effect of AMPK activation on lipid accumulation in control and T2D myotubes. A. Representative western blot analysis of P-ACC and P-AMPK in differentiated satellite cells established from a control subjects and a type 2 diabetic (T2D) patients. AMPK and ACC protein expressions were used as loading controls. B. Quantification by density analysis of P-AMPK (left panel) and P-ACC (right panel) in myotubes with (+) or without (−) AICAR (500 µM) or metformin (2 mM) treatment for 16h, from 4 cultures established from control subjects and 5 cultures established from type 2 diabetic patients (T2D). Data are presented normalized to AMPK and ACC expression, respectively. Data are shown as mean ±SEM. *$p < 0.05$, basal versus AICAR for control cells. C. Quantification of lipid content in control (n=3) and T2D differentiated satellite cells (n=3-4) with (+) or without (-) AICAR (500 µM, left panel) or metformin (2 mM, right panel) treatment (30 min) followed by palmitate treatment (600 µM for 16h). Data are means ±SEM. Each point was assayed in triplicate for each of the 7 independent cell cultures. *, $P<0.05$, T2D cells versus control cells. #, $P<0.05$, metformin and palmitate treatment versus palmitate alone.

Fig. 4. Effect of mitochondrial inhibition on lipid accumulation in control and T2D myotubes. A. Representative phase contrast microscopy of control and T2D myotubes after 16h of palmitate (600 µM) treatment with (+) or without (-) previous treatment with antimycin A (1 µM for 30min). Scale represents 30 µm. B. Quantification of lipid content in control (n=4) and T2D differentiated satellite cells (n=5) with (+) or without (-) antimycin A stimulation (1 µM for 30min) followed by palmitate treatment (600 µM for

16h). Data are means ±SEM. Each point was assayed in triplicate for each of the 9 independent cell cultures. **, p<0.01 palmitate-treated T2D cells versus control cells; #, p<0.05 antimycin + palmitate-treated control versus palmitate-treated control cells.

Fig. S1. Effect of palmitate treatment in control and T2D myotubes. A. Representative phase contrast microscopy of control and T2D myotubes after 16h of palmitate (600 µM) treatment. Scale represents 30 µm. B. Quantification of lipid content in control (n=4) and T2D differentiated satellite cells (n=5) after palmitate treatment (600 µM for 16h). Data are means ±SEM. Each point was assayed in triplicate for each of the 9 independent cell cultures. **, p<0.01 palmitate-treated T2D cells versus control cells.

Figure 1

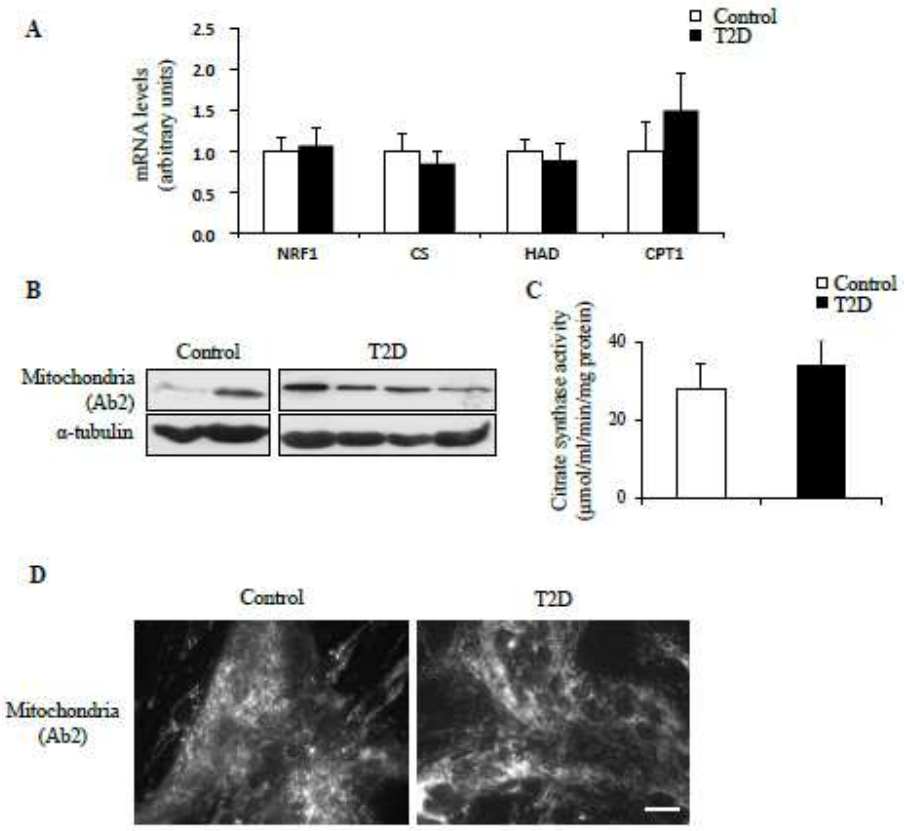


Figure 2

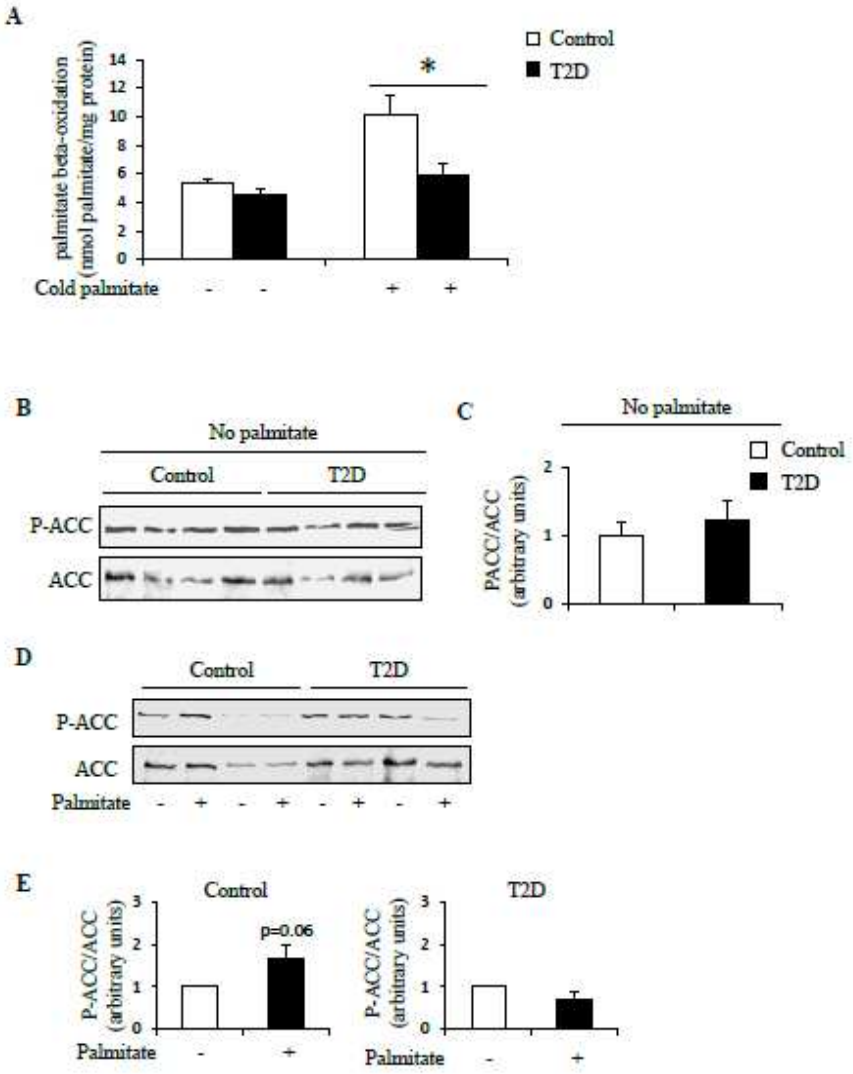


Figure 3

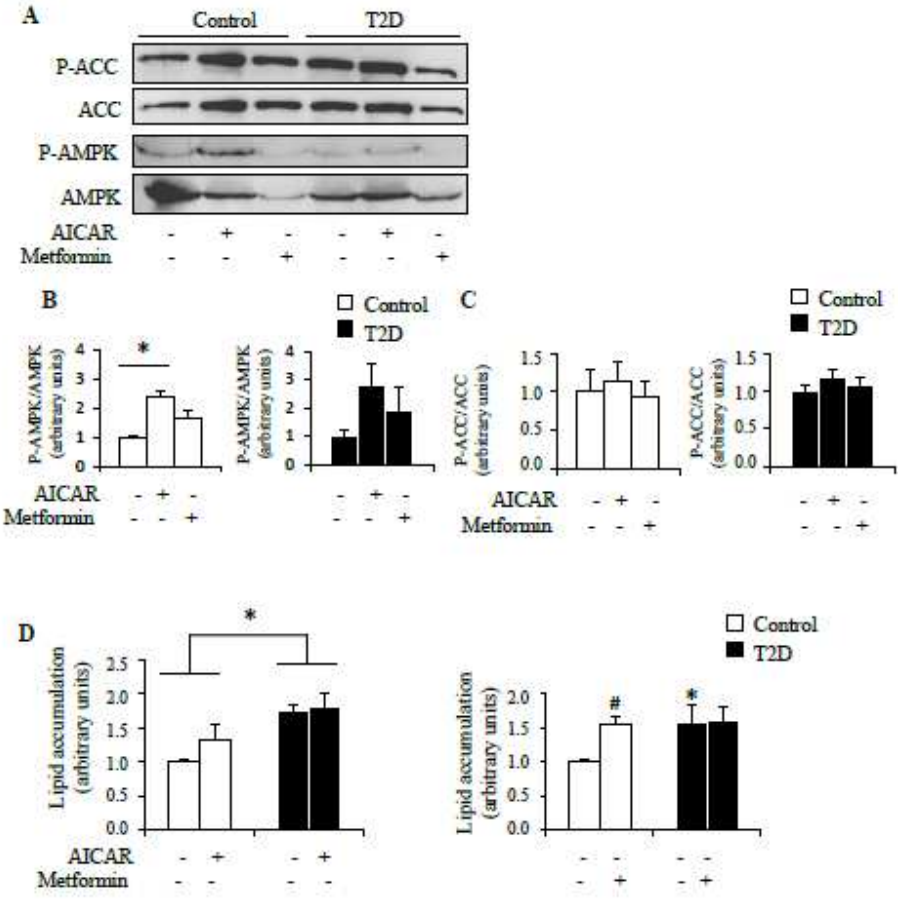
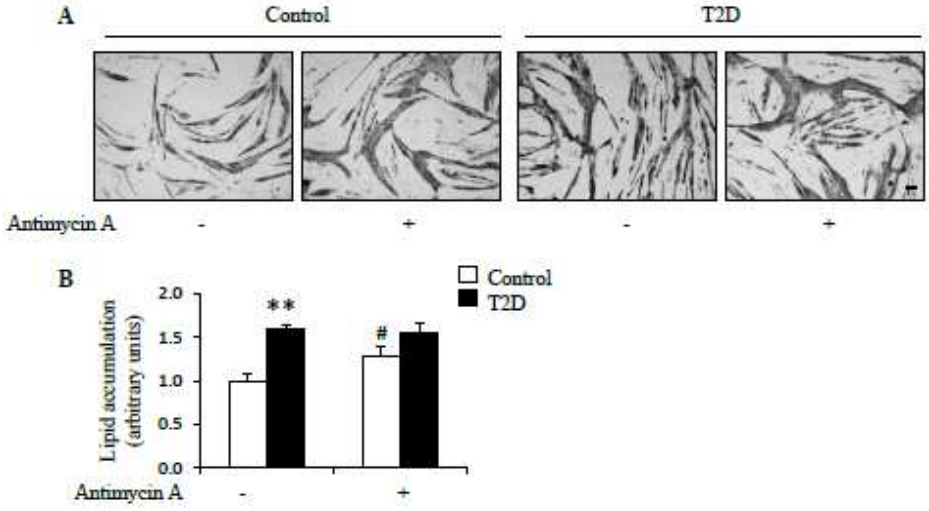


Figure 4



Research highlights

- Mitochondrial content is normal in type 2 diabetic myotubes
- Palmitate oxidation is decreased in type 2 diabetic myotubes compared to control myotubes only in condition of lipid overload
- ACC inactivation by palmitate is impaired in type 2 myotubes
- AICAR and metformin treatments don't activate AMPK or decrease lipid content in type 2 diabetic myotubes
- Mitochondrial inhibition by antimycin or metformin induce an increase in lipid content in control myotubes