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The ceramide transporter CERT is involved in muscle insulin signaling defects under lipotoxic conditions

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Running title: Ceramide transporter and insulin sensitivity

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Abbreviations

Ceramide Transporter :	CERT
Endoplasmic reticulum:	ER
Fatty acid:	FA
Glucosylceramide:	GlcCer
High fat diet:	HFD
Insulin resistance:	IR
Small Interfering-RNA directed against CERT:	siCERT
Plasma membrane:	PM
Membrane contact sites :	MCS
Protein kinase D:	PKD
Sphingomyelin:	SM
SM-synthase-1:	SMS1
Type-2 diabetes:	T2D

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7 figures

Abstract

One main mechanism of insulin resistance (IR), a key feature of type-2 diabetes, is the accumulation of saturated fatty acids (FA) in muscles of obese and type-2 diabetic patients. Understanding the mechanism underlying lipid-induced IR is therefore a crucial challenge. Saturated FA are metabolized into lipid-derivatives called ceramides and their accumulation plays a central role in the development of muscle IR. Ceramides are produced in the endoplasmic reticulum (ER) and transported to the Golgi through a transporter called CERT, where they are converted into different sphingolipid species. We show here that CERT protein expression is reduced in all insulin resistance models studied due to a caspase-dependent cleavage. Inhibiting CERT activity *in vitro* potentiates the deleterious action of lipotoxicity on insulin signaling whereas overexpression of CERT *in vitro* or *in vivo* increases muscle ceramide content and improves insulin signaling. In addition, inhibition of caspase activity prevents ceramide-induced insulin signaling defects in C2C12 muscle cells. Altogether, these results demonstrate the importance of a physiological ER to Golgi ceramide traffic to preserve muscle cell insulin signaling and identifies CERT as a major actor in this process.

Introduction

A worldwide obesity and diabetes epidemic is spreading in humans for four decades now. It is concomitant with alterations of carbohydrate/lipid metabolism, particularly with dyslipidemia, and which have major consequences for cardiovascular diseases and insulin resistance (IR). IR is a metabolic condition in which cells fail to respond to normal levels of insulin and is a key actor of type-2 diabetes (T2D). Numerous studies performed in animals and humans have demonstrated a strong relationship between IR and increased intramyocellular lipid content. Ceramide has been described in many studies as the lipid species involved in muscle insulin resistance (1), although it is worth mentioning that other studies did not find such a relationship and rather privileged diacylglycerols (2).

According to various studies, skeletal muscle accounts for 30 to 70 % of insulin-stimulated glucose disposal in the postprandial state and is thus a primary target for ceramide anti-insulin action (3;4). In the context of visceral obesity, ceramides are primarily produced *de novo* from saturated FA (palmitate) (1). This synthesis takes place in the ER and starts with the condensation of L-serine with palmitoyl-CoA to yield ceramides after several reactions.

Pioneering *in vitro* data showed an involvement of ceramides in the development of IR *via* the direct addition of these lipids on muscle and adipocyte cell lines (5-7). Ceramides inhibit insulin-stimulated glucose uptake and glycogen synthesis by blocking insulin signaling at the level of both IRS1 and Akt (8-11). These results indicate that saturated FAs in cells induce IR *via* ceramide synthesis.

Once ceramides are synthesized *de novo* in the ER, they are transported to the Golgi apparatus and metabolized into other sphingolipids such as sphingomyelin (SM) and glucosylceramides (GlcCer). The intracellular transport of ceramides from the ER to the Golgi involves both ATP-independent and -dependent specific carriers (12). Ceramides intended to be metabolized into GlcCer at the *cis* side of the Golgi apparatus are transported *via* an ATP-

independent vesicular carrier. This carrier is not well characterized, except that its activity is phosphatidylinositol-3-kinase dependent (12). Whereas, to be processed into SM, ceramides are mainly transported from the ER to the Golgi *via* a non-vesicular ATP-dependent transporter called ceramide transporter (CERT) (12). Through CERT, ceramides are extracted from the surface of the ER and transported towards the Golgi where they are metabolized into SM by SM-synthase-1 (SMS1).

Transformation of ceramide into SM may be a critical step in preventing negative actions of ceramides in cells. A metabolomic study demonstrated that reduced levels of plasma C16:1-SM species is predictive of T2D (13). Inhibition of SMS in muscle cells induces a rise in ceramide content and impairs insulin signaling (14). Obese and glucose intolerant individuals show increased muscle ceramide content and lower muscle SM compared to obese and normal glucose tolerant individuals (15).

These data suggest that the biosynthesis of SM from ceramides could be protective for maintaining insulin sensitivity. Since CERT is involved in the transfer of ceramides to the Golgi for the synthesis of SM, we tested the hypothesis that modulation of CERT activity impacts muscle insulin signaling.

Research Design and Methods

Materials

Insulin, palmitate, and BSA were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Gedunin was from Tocris Bioscience (Bristol, UK). Broad caspase inhibitor (Q-VD-OPh) was from Merck Chemicals Ltd (Nottingham, UK). Antibodies against Akt, Akt Ser-473, Akt Thr-308, PKD Ser-916, GSK3 α/β Ser-21/9, ERK Thr-202/Tyr-204, GAPDH, cleaved caspase-3 and -9 were from Cell Signaling (New England Biolabs, Ipswich, USA). The antibody against CERT was from Bethyl Laboratories (Montgomery, TX, USA) and the

one directed against β -actin from Sigma-Aldrich. Secondary horseradish peroxidase antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and the chemiluminescent substrate from ThermoFisher Scientific (Waltham, MA USA). [3 H]-2-deoxy-D-glucose (26.2 Ci/mmol) and D-erythro-[3- 3 H]-sphingosine (Sph) (18.6 Ci/mmol) were from PerkinElmer Life Science (Boston, MA, USA). High performance thin layer chromatography (HPTLC) silica gel plates were from Merck (Darmstadt, Germany). Lipid internal standards (d18:1-12:0 Ceramide, d18:1-12:0 sphingomyelin and d17:1-Sphingosine-1-Phosphate) were obtained from Avanti Polar Lipids (Coger SAS, Paris, France). LCMSMS quality grade solvents were purchased from Fischer Scientific (Illkirch, France).

Culture and transfection of C2C12 muscle cells

C2C12 myoblasts were grown and differentiated as myotubes as described previously (11). Cells were treated with palmitate or oleate conjugated to FA free BSA, as described (16). Both HPA12 and gedunin were reconstituted in DMSO (0.4% final concentration). Control cells were incubated with the same quantity of DMSO. siRNA (25 nM) directed against CERT (Santa Cruz Biotechnology, Dallas, USA) or the same concentration of a non-specific siRNA were transfected for 96 h into C2C12 myotubes using the transfection reagent DharmaFECT (Dharmacon, Cambridge, UK). C2C12 myoblasts were seeded into 12-well plates and were transfected for 48h with a pEGFP N1/hCERT (a gift of Pr. Thierry Levade, Toulouse, France) or pCMV-GFP plasmids (1 μ g per well) using the Transfectin transfection reagent (ATCC, Molsheim, France).

C2C12 glucose transport

Glucose transport was measured by incubating C2C12 myotubes with 10 μ M [3 H] 2-deoxy-D-glucose (1 μ Ci/ml, 26.2 Ci/mmol) for 10 min as previously described (17).

Caspase activity

C2C12 myotubes were treated either with BSA (1.5%) or with palmitate (0.75 mM) complexed with BSA, in the presence or absence of the caspase inhibitor Q-VD-OPh (10 μ M) and the activity of caspase-3/-7 was measured 24 h later using the Apo-ONE kit Homogeneous Caspase-3/-7 Assay (Promega, Madison, USA).

CERT immunoprecipitation

C2C12 cells were lysed and CERT immunoprecipitated from 200 μ g lysates using a CERT antibody. Immunocomplexes were captured by incubation with protein A-agarose beads and solubilized in Laemmli buffer prior to SDS-PAGE and immunoblotted.

[³H]-sphingosine metabolism

C2C12 myotubes were treated with 0.1 or 0.75 mM palmitate in the presence or absence of 10 μ M N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)-dodecanamide (HPA12) (18) for 16h at 37°C, then the cells were pulsed for 2 h with [C3-³H]-sphingosine (0.3 μ Ci/ml) at 10 °C (19). Stock solutions of [³H]Sphingosine in absolute ethanol were prepared and added to conditioned medium. The final concentration of ethanol never exceeded 0.1% (v/v). At the end of the pulse time total lipids were extracted and processed as previously described (19). The methanolized organic phase was analyzed by HPTLC using chloroform/methanol/water (55:20:3 by vol.) as solvent system. Digital autoradiography of HPTLC plates was performed with a Beta-Imager 2000 (Biospace, France) and radioactivity associated with individual lipids was determined using software provided with the instrument. The ³H-labeled sphingolipids were recognized and identified as previously described (19).

Animals

Male C57Bl6 mice (5 weeks old, Charles River Laboratories, Saint Germain Nuelle, France) were adapted to their environment for 1 week prior to the study. Mice were housed with a 12 h light/12 h dark cycle in a temperature-controlled environment and had free access to water and regular diet (energy: 65% carbohydrate, 11% fat, 24% protein) or a high fat diet (HFD) (EF D12492, energy: 21% carbohydrate, 60% fat, 19% protein, gross energy, 24.0 MJ/kg, ssniff Spezialdiäten GmbH, Soest, Germany) for 12 weeks. All procedures were approved by the Regional Ethics Committee for animal experiments no.5 of Ile-de-France (agreement no. 02852.03).

Electrogene transfer in mice

Mice were anesthetized with Aerane (Baxter) and their *tibialis anterior* muscles were injected with 8 U of hyaluronidase 2 h prior to the injection of 15 µg pEGFP N1/hCERT or 15 µg pCMV-GFP plasmids. Six 65 V/cm pulses of 60-ms, with a 100-ms interval, were applied (20). 14 days after gene delivery and before sacrifice, mice were injected or not with 0.75 UI/kg insulin (Actrapid, Novonordisk, La Défense, France) for 15 min. Then, muscles were collected under microscope. All experiments were conducted in accordance with European guidelines for the care and use of laboratory animals and were approved by the institutional animal care and use committee (agreement no. 00315.01).

Lipid extraction

Sphingomyelins and ceramide were extracted according to Bielawski et al (21). Muscles were crushed in an Omni Bead Ruptor 24 apparatus (Omni International, Kennesaw, USA) with 950 µl of saline and circa twenty 1.4 mm OD zirconium oxide beads. An aliquot equivalent to 3 mg muscle (60 µl of lysate) was diluted with 1.94 ml saline and finally spiked with an

internal standard mix containing 30 ng and 125 ng of d18:1/12:0 Ceramide and d18:1/12:0 SM respectively. Lipids were extracted with 2 ml of propanol2/water/ethyl acetate 30/10/60 for 30 min. After centrifugation (1100g, 5 min) the organic phase was kept and the aqueous phase further extracted. After centrifugation both organic phases were combined and evaporated to dryness under vacuum. Samples were solubilized with 200 µl of methanol and transferred to injection vials, again evaporated to dryness under vacuum and finally solubilized with 40 µl of methanol.

Quantification of ceramides and SM by LCMS

Ceramide analysis was carried out on a 1200 6460-QqQ LC-MS/MS system equipped with an ESI source (Agilent technologies, Les Ulis, France) as previously described (22). Samples were injected on a Poroshell C8 2.1x100 mm, 2.7 µm column (Agilent technologies) at a flow rate of 0,3 ml/min, 50°C, and separation was achieved with a linear gradient of (solvent A) formic acid/ammonium formate (0,2 % /1 mM final concentrations) and (solvent B) methanol containing formic acid/ammonium formate 1 mM . Acquisition was performed in positive Single Reaction Monitoring (SRM) mode. Relative quantitation of ceramide related compounds was performed by calculating the response ratio of the considered ceramide to d18:1/12:0 Ceramide, used as internal standard. Two microliter samples were used for quantitation of sphingomyelins.

Human skeletal muscle cells

Biopsies from lean healthy adult volunteers were obtained in the context of agreed preclinical and clinical experiences (23) via the Tissue Bank for Research (Myobank) of the French Association against Myopathies (AFM) in agreement with the French bioethical law (Law No 94-654 of 29 July 1994, amended 22 January 2002). Samples of patients with type-2 diabetes

were obtained from healthy tissue after leg amputation on informed consent. Ethical approval for the use of human muscle tissue was given by the Ethics Committee of Pitié-Salpêtrière hospital (CPP-Ile de France VI-Paris, France). Fresh muscle samples were sliced and dissociated in collagenase. Satellite cells were purified, cultured and differentiated into myotubes, as previously described (16).

Preparation of whole cell lysates

Cells were lysed following experimental manipulation (see figure legends) in an appropriate volume of lysis buffer and frozen at -80°C until required (24).

Real time quantitative RT-PCR

Total RNA was extracted from muscle cells and real-time quantitative RT-PCR analyses were performed as described previously (16). One microgram RNA was retro-transcribed using Superscript II (Invitrogen, Carlsbad, USA). Sequences of sense and antisense primers of the gene to be amplified (CERT) are respectively 5'-TCTGCTTATCTCCTGGTCTCCC-3' and 5'-CGAATCAAGCCAGCCTTGAC-3'.

Immunoblotting

Frozen tissues or cells were homogenized following experimental manipulation in an appropriate volume of lysis buffer, and cell lysates were subjected to SDS/PAGE and immunoblotted as previously described (24).

Statistics

Data were analyzed with GraphPad Prism 6.07 by unpaired or paired two-tailed *t*-test when two groups were compared, and one way Anova followed by Bonferroni's multiple

comparison tests when more than two groups were compared. p value <0.05 was considered significant.

Results

CERT expression is altered in lipotoxic conditions in muscle cells.

Palmitate treatment (0.75 mM) for 16 h induced a 50% decrease in CERT protein expression (fig.1A), concomitantly to a 60% increase in total ceramide content (fig.1B) and a 35% increase in total SM content (fig.1B) in C2C12 myotubes. In *gastrocnemius* muscle lysates from mice fed a HFD (12 weeks) we also observed a 58% decrease in CERT protein content (fig.1C) and a 28% increase in total ceramide content (fig.1D) when compared to controls. However, no difference in total SM content was observed between both groups (fig.1D). We then studied human myotubes differentiated from human satellite cells obtained from either insulin sensitive or T2D donors (10). Figure 1E shows that insulin-stimulated Akt phosphorylation in myotubes derived from muscles from T2D patients was drastically reduced compared to non-diabetic myotubes. Interestingly, a concomitant decrease in CERT expression was observed in T2D patient myotubes compared to control myotubes (fig.1E).

Next, we tested whether the decrease in insulin-induced Akt phosphorylation usually observed after 16 h palmitate exposure (10;11;16) was concomitant to a decreased CERT expression. C2C12 cells were treated with palmitate up to 16 h and insulin-induced Akt phosphorylation and CERT expression were assessed in the same time-frame. Supplemental fig.1A shows that palmitate needed 16 h to induce both defect in insulin signaling (decrease in Akt phosphorylation in response to insulin) and decreased CERT protein content. CERT mRNA levels were not decreased after 16 h palmitate incubation in C2C12 myotubes (supplemental fig.1B) and in muscle of mice fed a HFD compared to control mouse muscles

(supplemental fig.1C), suggesting that the alteration of CERT observed in lipotoxic conditions was post-transcriptional.

Then we tested whether palmitate could act through ceramide production to alter CERT protein expression in muscle cells. C2C12 myotubes were treated with palmitate in the presence of myriocin (inhibitor of the first enzyme of ceramide biosynthesis) for 16h before assessing CERT expression. Decreased CERT protein content observed after palmitate treatment (fig. 1F) was concomitant to an increase in ceramide content in cells (fig.1G). Interestingly, both the decreased-expression of CERT and the increased-ceramide content observed in response to palmitate were completely abrogated in the presence of myriocin (fig.1F-G), suggesting that ceramides produced from palmitate were accountable for the observed CERT alteration in muscle cells.

The type of free FA, saturated or unsaturated, is critical for the development of insulin resistance. While saturated FAs induce insulin resistance (25;26), unsaturated FAs have no deleterious effect and even protect cells from the negative action of saturated FAs (27-29). To determine whether unsaturated FAs exert a protective effect on the expression of CERT in the presence of palmitate, C2C12 myotubes were incubated with palmitate, oleate or linoleate. Supplemental fig.2A shows that while palmitate altered CERT expression, the other two unsaturated FA displayed no significant effects. More interestingly, treatment of C2C12 myotubes with both oleate and palmitate together protected cells against the harmful effect of the latter on CERT expression (supplemental fig.2B).

Overall, these data demonstrate that lipotoxic conditions negatively regulate CERT content and activity in muscle cells.

Influence of the modulation of CERT activity/expression on muscle cell insulin signaling in vitro.

We next determined whether an artificial reduction of CERT function could potentiate palmitate-induced defects in insulin signaling in myotubes. We used a concentration of palmitate (0.1 mM) that had a minimal impact on total ceramide content (supplemental fig.3) and CERT expression (fig.2B). We inhibited the activity of CERT in muscle cells using a CERT inhibitor called N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)-dodecanamide (HPA12) (18). HPA12 inhibited CERT activity through its interaction with the START domain of CERT that usually binds ceramides (30). HPA12 treatment enhanced the ceramide concentration induced by a low concentration of palmitate (supplemental fig.3), suggesting that the inhibition of CERT activity prevented the ceramide produced in the ER to be metabolized into SM in the Golgi. To evaluate the effect of both palmitate and HPA12 on ceramide utilization for the biosynthesis of SM, we studied ceramide metabolism using [³H]-sphingosine as a metabolic precursor, as it is rapidly internalized into cells and N-acylated to ceramide and then metabolized to form SM and GlcCer (31). The experiment was performed at 10 °C, a non-permissive temperature for the ER-to-Golgi vesicle flow, allowing to assess essentially a CERT dependent transport. At that temperature and after a short time pulse, we found comparable levels of radioactivity incorporated in control and palmitate-treated cells (data not shown) and most of the radioactivity remained associated with ceramides (fig.2A). A 0.1 mM or 0.75 mM palmitate treatment inhibited synthesized [³H]-SM levels by 31% and 70% respectively (fig.2A), but did not affect GlcCer biosynthesis (fig.2A). HPA12 treatment mimicked high concentration levels of palmitate and blocked the conversion of ceramide into SM. Addition of 0.75 mM palmitate did not enhance the negative action of HPA12 (fig.2A). Taken together, the data demonstrate that both high palmitate concentration and HPA12 inhibit SM biosynthesis in the Golgi apparatus. We then assessed insulin signaling. At 0.1 mM, palmitate did not affect CERT expression (fig.2B), but only partially blocked SM biosynthesis (fig.2A) and did not inhibit insulin-induced Akt phosphorylation (fig.2B).

However, at the same concentration of palmitate, but in the presence of HPA12, we observed a complete inhibition of ceramide transport from the ER to the Golgi (fig.2A) and an accentuated inhibitory action of the lipid on insulin signaling (fig.2B). Interestingly, at 0.75mM, the negative effect of palmitate on CERT expression and SM biosynthesis was maximal, and HPA12 did not potentiate any further the deleterious action of the lipid on Akt phosphorylation (fig.2B).

Similar results were obtained by using another CERT inhibitor, gedunin which inhibited CERT-mediated extraction of ceramides from the ER membranes (32). Figure 2C shows that, like HPA12, gedunin unmasked the inhibitory action of 0.1 mM palmitate on insulin signaling.

To confirm the importance of the negative effect of a decreased-CERT activity on insulin signaling in muscle cells, we used a small interfering-RNA directed against CERT (siCERT). Figure 3A-B shows that siCERT decreased CERT mRNA and protein content in C2C12 myotubes. Figure 3B demonstrates that 0.1mM palmitate induced only a slight reduction in CERT protein content and had no significant effect on Akt phosphorylation. However, in cells transfected with siCERT, CERT protein was strongly reduced (fig.3B), along with an increased action of palmitate on insulin-induced Akt phosphorylation, confirming that the absence of CERT expression unmasks the action of a low palmitate concentration on insulin signaling (fig.3B).

To demonstrate that a correct ceramide transport from the ER to the Golgi apparatus is essential in preventing the inhibitory effect of ceramides on insulin signaling, we overexpressed CERT in C2C12 myoblasts before treating them with palmitate and insulin. For this experiment, we used myoblasts instead of myotubes because of their higher transfection efficiency. Endogenous CERT expression was identical before and after C2C12 myoblast differentiation into myotubes (data not shown). A 16 h palmitate exposure induced a

7-fold increase in total ceramide content in C2C12 myoblasts (fig.4A) (a much higher increase than in myotubes, e.g. fig.1B). CERT overexpression, however, reduced the total ceramide increase in response to palmitate to 4.1-fold (fig.4A). Overexpression of an exogenous CERT prevented endogenous CERT downregulation in response to a high palmitate concentration (fig.4B). In addition, CERT overexpression also counteracted palmitate's deleterious action on insulin signaling. Indeed, palmitate induced an inhibition of the insulin-induced phosphorylation of Akt, GSK3 and ERK. CERT re-expression, however, induced a major improvement of insulin signaling (fig.4B).

Mechanism of alteration of CERT action in lipotoxic conditions: a PKD and caspase-dependent mechanism.

CERT function is downregulated by phosphorylation on its serine-132 residue by protein kinase D (PKD). PKD is activated by phosphorylation of its serine-960 residue in response to various stresses (33), and CERT phosphorylation on serine-132 by PKD decreases CERT affinity to phosphatidylinositol-4-phosphate in the Golgi, thus reducing ceramide transfer activity (34). Palmitate induced both phosphorylation of PKD on its serine-960 residue (Supplemental fig.4A) and CERT on its serine-132 residue (Supplemental fig.4B). Treatment of cells with a PKD inhibitor (kb-NB-142-70), however, reduced palmitate-induced CERT phosphorylation (Supplemental fig.4B).

A recent study showed that CERT can be cleaved by caspases during pro-apoptotic stress in HeLa cells, resulting in a loss of function of CERT and a decrease in SM *de novo* synthesis in the Golgi (35). Since palmitate activates both caspase-3 and -9 in C2C12 myotubes (36), we wondered whether a similar mechanism could occur in our muscle cell model. To test this hypothesis, we treated C2C12 myotubes with palmitate for 16h in the presence or absence of a broad caspase inhibitor (Q-VD-OPh) (37). Palmitate induced the cleavage of both caspases-

3/-9 (fig.5A) and activity (fig.5B) in C2C12 myotubes, together with a loss of insulin response (fig.5C). Interestingly, Q-VD-OPh, which blocks caspase activity in the presence of palmitate (Fig.5B), prevented the alteration of CERT expression in response to palmitate (Fig.5C), and improved insulin signaling (fig.5C).

We next evaluated whether changes in CERT expression have consequences on glucose metabolism downstream of insulin signaling. Insulin induced a 40% increase in glucose transport in C2C12 myotubes (fig.6). If at 0.1mM, palmitate had no significant effect on insulin-stimulated glucose transport, in the presence of HPA12, palmitate inhibited the insulin-induced stimulation of glucose transport (fig.6). This result confirms the importance of an active ceramide transport to counteract the action of palmitate on insulin signaling. The stimulation of insulin was completely lost when cells were pre-treated with high palmitate concentrations (fig.6). The caspase inhibitor, Q-VD-OPh, however, prevented the inhibition by palmitate of insulin-stimulated glucose transport (fig.6).

Influence of CERT overexpression on muscle insulin sensitivity and ceramide content in vivo.

We fed mice a HFD for 10 weeks and 2 weeks before the end of the diet, we overexpressed a CERT-GFP construct through electrogene-transfer (20) in the *tibialis anterior* muscle of the left leg of the animals (fig.7A). In the right leg, a GFP construct was transferred. Two weeks later we sacrificed the mice and isolated *tibialis anterior* muscles that were overexpressing CERT (GFP-CERT visible under fluorescence microscope, fig.7A). Ceramide species contents (except for C18 and C20) were decreased up to 30% in CERT-overexpressing muscle fibers (fig.7B) demonstrating that CERT overexpression counteracts ceramide accumulation in muscles. We also observed a 10% decrease in total ceramide content (although at $p < 0.0533$). In addition, both caspases-3/-9 were cleaved in HFD muscle mice, and CERT overexpression completely abrogated caspase cleavages (fig.7C), indicating a

decrease in lipotoxicity-induced caspase activation *in vivo*. Next, we assessed insulin signaling in these muscle fibers. CERT overexpression in the *tibialis anterior* muscle prevented endogenous CERT degradation observed in response to lipotoxicity and improved significantly the poor *in vivo* stimulation by insulin of Akt, GSK3 and ERK observed in HFD treated mice (fig.7D).

Discussion

In the present study, we demonstrate that CERT plays a pivotal role for the control of ceramide content and insulin response in muscle cells. In lipotoxic conditions, i.e. in the presence of saturated FA, a decrease in CERT content induces ceramide accumulation in cells through a defective ceramide transport from the ER to the Golgi. This leads to an inhibition of SM synthesis and a concomitant loss in insulin response. Importantly, a direct inhibition of CERT expression/activity has an effect on ceramide content and insulin signaling similar to lipotoxic conditions. Conversely, an increased CERT expression *in vitro* and *in vivo* counteracts the deleterious effects of lipotoxic conditions on muscle insulin signaling. As a reflect of insulin action we show here mainly AKT, GSK3 and ERK phosphorylation. Defects in insulin mediated phosphorylation of these targets are usually concomitant with a loss of tissue insulin sensitivity (38), although a number of studies show a dissociation between Akt phosphorylation and insulin sensitivity (39-41). Thus, it remains to directly demonstrate that CERT activity/expression modulation translates *in vivo* into functional changes of muscle glucose metabolism.

We demonstrate here the direct involvement of CERT in the transport of ceramides from the ER to the Golgi and in their synthesis into SM. However, the fact that the GlcCer content did not change with either palmitate or CERT inhibitor confirms that GlcCers are coming from ceramide transported to the Golgi through the vesicular transport, not through CERT (42). It

is important to stress that we do not observe a decrease in total SM content in cells in lipotoxic condition (fig.1A-B) since the total SM content exceeded the total ceramide content by more than 20-fold in muscle cells (unpublished data).

It appears that a targeted decrease in some ceramide species (C16, C22, C24:1 and C24) without a statistically significant change in total ceramide content was sufficient to modulate the insulin response of the cells in lipotoxic conditions. C16-ceramides have been demonstrated to attenuate the hepatic insulin response (43;44), thus similar ceramide species could also mediate lipotoxicity in muscle cells. However, and in opposite of what was suggested in some studies (40;45), C18-ceramides did not seem to play a role in the inhibition of insulin response in our experimental models. At present we have no explanation for this discrepancy. It is likely that, depending on the relative abundance of specific FA in the HFD, ceramide species are affected differently.

Processing of newly synthesized ceramides to give more complex sphingolipid derivatives such as SM, GlcCer and complex glycosphingolipids (e.g. gangliosides) (46), occurs in the Golgi apparatus. Therefore, an efficient, rapid and regulated transport is required since the $t_{1/2}$ for spontaneous interbilayer movement of ceramide is in the order of days (47). In mammalian cells, transport of ceramides from the ER to the Golgi occurs through two different mechanisms; vesicular and non-vesicular. In yeast, it has been shown that the non-vesicular CERT-dependent transport contributes to 50% of transport ceramide from the ER to the Golgi (48). Our data demonstrate that a default in CERT content in response to lipotoxicity is enough to stop the conversion of ceramide to SM in the Golgi and thus to increase ceramide concentration in cells and to trigger their negative action on insulin signaling. This suggests that the loss in CERT-dependent transport of ceramides from the ER to the Golgi is not compensated by the vesicular transport. However, lack of knowledge about

the mechanism by which this ceramide vesicular transport is regulated precludes any conclusion concerning an effect of lipotoxicity on its function.

A key and original result is that, in lipotoxic conditions, CERT function is modulated through two mechanisms. Firstly, palmitate inhibits CERT activity through phosphorylation on its serine-132 residue by PKD in muscle cells. The importance of this mechanism for the control of CERT activity has already been demonstrated in rat islet β -cells where high dose palmitate treatment increased PKD-induced phosphorylation of CERT and its dysfunction, and deleterious effects on islet β -cells (49). Secondly, CERT protein content is strongly reduced in lipotoxic conditions, and the decreased protein content is secondary to an activation of caspases. The underlying mechanism is not completely resolved but is likely to be mediated by ceramides. Indeed, decreased CERT expression did not occur when the *de novo* ceramide biosynthesis pathway was inhibited (fig.1F). In addition, when CERT was overexpressed in diabetic mouse muscle, ceramide content was decreased (fig.7B) and lipotoxicity-induced caspase cleavage was abrogated (fig.7C). It is likely that the caspase-dependent inhibition of CERT activity prevailed over the PKD-dependent one. Indeed, inhibition of caspase activity restored on its own insulin signaling (fig.5C).

In lipotoxic conditions, GFP-CERT-overexpressing muscle cells displayed higher endogenous CERT concentrations compared to control muscle cells (fig.4B and 7D). This difference in expression could be explained by an enhanced ceramide transport from the ER to the Golgi in CERT-overexpressing cells, resulting in less ceramide accumulated and a decrease in ceramide-induced caspase activation. Thus CERT plays a crucial role in the regulation of sphingolipid metabolism in lipotoxic conditions.

Changes in CERT expression observed in our study are not unprecedented. Indeed, another study has shown that a pro-apoptotic stress (induced by TNF α) can also result in an inactivation of CERT *via* its cleavage by caspases in HeLa cells (35) and results in a

decreased biosynthesis of SM (35). Interestingly, another study demonstrated that the inhibition of the *de novo* ceramide biosynthesis prevents caspase-3 activation in response to palmitate in L6 myotubes (50). As we have observed (fig.5), the authors of the study also showed that inhibition of caspase-3 partially improves insulin-stimulated glucose uptake in palmitate-treated L6 myotubes (50). They did not explain, however, how ceramide-activated caspases could affect insulin signaling but they showed that it is not through proteolysis of insulin signaling proteins (50). Very recently a study conducted in yeast also demonstrated that a non-vesicular ceramide transfer out of the ER prevents the buildup of ceramide content (51). Although *S. cerevisiae* do not express a CERT homologue, under certain conditions, the protein Nvj2p can play a similar role by facilitating lipid exchange between the ER and the Golgi. During ER stress or ceramide overproduction, Nvj2p relocalizes to and increases ER–medial Golgi contacts, facilitating ceramide exit from the ER and preventing ceramide toxic accumulation (51). All of these data strengthen our results in mammalian cells by demonstrating the importance of a functional ceramide transport from the ER to the Golgi to prevent lipotoxicity. Nevertheless, it will be particularly important to completely elucidate CERT regulation in response to an excess of FA.

We previously showed that the mechanisms inhibiting insulin action in the presence of ceramides take place at the plasma membrane (PM) (8;10). A crucial question thus remains: during lipotoxicity, what is the mechanism linking ER-accumulated ceramides to their effects at the PM? One hypothesis would be that ceramides could directly transfer from the ER to the PM at specialized membrane contact sites (MCS). MCS are regions of close apposition between two cellular membranes, and the ER can form MCS with virtually any other organelle within cells. Based on electron microscopy, the ER also has contacts with the PM (46). ER-PM MCS exist in striated muscles between the T-tubule (a specialized plasma membrane network) and the sarcoplasmic reticulum (52). Interestingly, most of the insulin-

regulated glucose transporter GLUT4, gets translocated into T-tubules in response to the hormone (53). As such, ER-T-tubule MCS could form important hubs for lipid metabolism and exchange between membranes. To date, however, no information on sphingolipid transfer from the ER to the PM by such a mechanism is available.

In summary, our study shows that ceramide transport from the ER to the Golgi is altered in lipotoxic conditions and that its artificial recovery prevents ceramide to accumulate and to act negatively on insulin signaling. These results could open avenues to identify new therapeutic targets allowing the amelioration of insulin sensitivity.

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access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest statement: The authors declare that there is no duality of interest associated with this manuscript.

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Figure legends

Figure 1: Effect of lipotoxicity on CERT expression in muscle cells.

C2C12 myotubes were treated with 0.75 mM palmitate (Pal) for 16 h. Cells were collected and (A) CERT protein content and (B) total ceramide and SM levels were assessed. Results are means +/- SEM (n=6). *Significant change (*p<0.05) relative to control. (C) CERT protein content and (D) total ceramide and SM levels were determined from high fat diet (HFD) and chow diet (control) fed mouse *gastrocnemius* muscle. Results are means +/- SEM (n=3). *Significant change (*p<0.05) relative to control muscles. (E) Cultured human control

and diabetic myotubes were treated with 100 nM insulin before being lysed. C2C12 myotubes were treated with 0.75 mM palmitate with or without 10 μ M myriocin for 16 h before being lysed and immunoblotted (F) and total ceramide assessed (G). Results are means \pm SEM (n=3). *Significant change (* $p < 0.05$) relative to untreated cells.

Figure 2: Effect of palmitate and CERT inhibitors on SM synthesis and insulin sensitivity in C2C12 myotubes.

(A) C2C12 myotubes were treated with 0.1 mM or 0.75 mM palmitate (Pal) in the presence of 10 μ M HPA12 for 16 h, and then pulsed with 0.3 μ Ci [$C3\text{-}^3\text{H}$]-sphingosine for 2 h at 10°C. At the end of the pulse, cells were harvested and submitted to lipid extraction and partitioning. The methanolized organic phase and the aqueous phase were analyzed by HPTLC and digital autoradiography of HPTLC. Results are means \pm SEM (n=3). *Significant change (* $p < 0.05$) relative to control. (B) C2C12 myotubes were treated with 0.1 mM or 0.75 mM palmitate (Pal) with or without 10 μ M HPA12 for 16 h. 100 nM insulin was added during the last 10 min of culture and then the cells were lysed. (C) C2C12 myotubes were treated with 0.1 mM palmitate (Pal) with or without 10 μ M or 30 μ M gedunin for 16 h. Insulin (100 nM) was added during the last 10 min of culture and cells were then lysed. Cell lysates were immunoblotted with the indicated antibodies. Histograms show densitometric quantification of both CERT and $^{473}\text{Ser Akt}$. Results are means \pm SEM (n=3). *Significant change (* $p < 0.05$) relative to insulin/palmitate treated cells.

Figure 3: Effect of CERT siRNA on palmitate-induced IR in C2C12 myotubes.

(A) C2C12 myotubes were transfected with a CERT siRNA (25 nM, 72 h) or a scramble siRNA (25 nM, 72 h), then treated with 0.1 mM palmitate (Pal) for 16 h before RNA extraction. CERT mRNA was quantified by qRT-PCR. Results are means \pm SEM (n=3).

*Significant change ($*p<0.05$) relative to control. (B) C2C12 myotubes were transfected with a CERT siRNA (25 nM, 72 h) or a scramble siRNA (25 nM, 72 h), then treated with 0.5 mM palmitate (Pal) for 16 h and 100 nM insulin during the last 10 min of culture before being lysed. Cell lysates were immunoblotted with the indicated antibodies. Histograms show densitometric quantification of both CERT and $^{473}\text{Ser Akt}$. Results are means \pm SEM (n=3). *Significant change ($*p<0.05$) relative to insulin/palmitate treated cells.

Figure 4: Effect of CERT overexpression on palmitate-induced IR in C2C12 myoblasts.

C2C12 myoblasts were transfected with a CERT-GFP plasmid, and then incubated with 0.75 mM palmitate (Pal) for 16 h and 100 nM insulin for the last 10 min. (A) Total ceramide concentration was assessed. Results are means \pm SEM (n=3). *Significant change ($*p<0.05$) relative to palmitate treated cells (B) Cell lysates were immunoblotted with the indicated antibodies. Histograms show densitometric quantification of endogenous CERT, $^{308}\text{Thr Akt}$ and $^{473}\text{Ser Akt}$. Results are means \pm SEM (n=3). *Significant change ($*p<0.05$) relative to insulin/palmitate treated cells.

Figure 5: Involvement of caspases in the regulation of CERT expression and insulin sensitivity in lipotoxic conditions.

(A) C2C12 myotubes were incubated with 0.75 mM palmitate (Pal) for 16h before being lysed. Cell lysates were immunoblotted with the indicated antibodies. (B) C2C12 myotubes were incubated with 0.75 mM palmitate (pal) with or without 20 μM caspase inhibitor (QVDH-OPH) for 16h. Then, caspase activity was assessed. Results are means \pm SEM (n=3). *Significant change ($*p<0.05$) relative to control. (C) C2C12 myotubes were treated with 0.75 mM palmitate (pal) with or without 20 μM QVDH-OPH for 16 h, then with insulin in the last 10 min before being lysed. Cell lysates were immunoblotted with the indicated

antibodies. Histograms show densitometric quantification of both CERT and ⁴⁷³Ser Akt. Results are means +/- SEM (n=4). *Significant change (*p<0.05) relative to insulin/palmitate treated cells.

Figure 6: Effects of modulating CERT activity on insulin-induced glucose uptake in C2C12 myotubes.

C2C12 myotubes were treated with 0.1 mM or 0.75 mM palmitate (Pal) for 16 h with or without HPA12 (10 μM) or QVD-OPh (20 μM). Glucose uptake was assessed after 30 min insulin treatment (100 nM). Results are means +/- SEM (n=5). *Significant change (*p<0.05) relative to insulin.

Figure 7: Consequences of CERT overexpression on insulin signaling and ceramide content in mouse muscle *in vivo*.

(A) The left *tibialis anterior* muscle from 12 weeks high fat diet (HFD) fed mice was transfected with GFP-CERT cDNA, and the right one with a GFP cDNA. After 14 days, mice were injected with insulin for 30 min and sacrificed. *Tibialis anterior* muscles were collected under fluorescence microscope and lysed. (B) Both ceramide species and total ceramides from animal *tibialis anterior* muscles were analyzed. Results are expressed as muscle ceramide fold change when compared to the mean of the control group indicated by a horizontal bar. Results are means +/- SEM (n=7). Significant change (*p<0.05) relative to control *tibialis anterior* muscle. (C) Cell lysates were immunoblotted with the indicated antibodies. Representative blots from three different animals are shown. (D) Cell lysates were immunoblotted with the indicated antibodies. Representative blots and quantifications of ³⁰⁸Thr Akt and ⁴⁷³Ser Akt signals from five animals are shown. Results are means +/- SEM (n=5). *Significant change (*p<0.05) relative to *tibialis anterior* muscle overexpressing CERT.

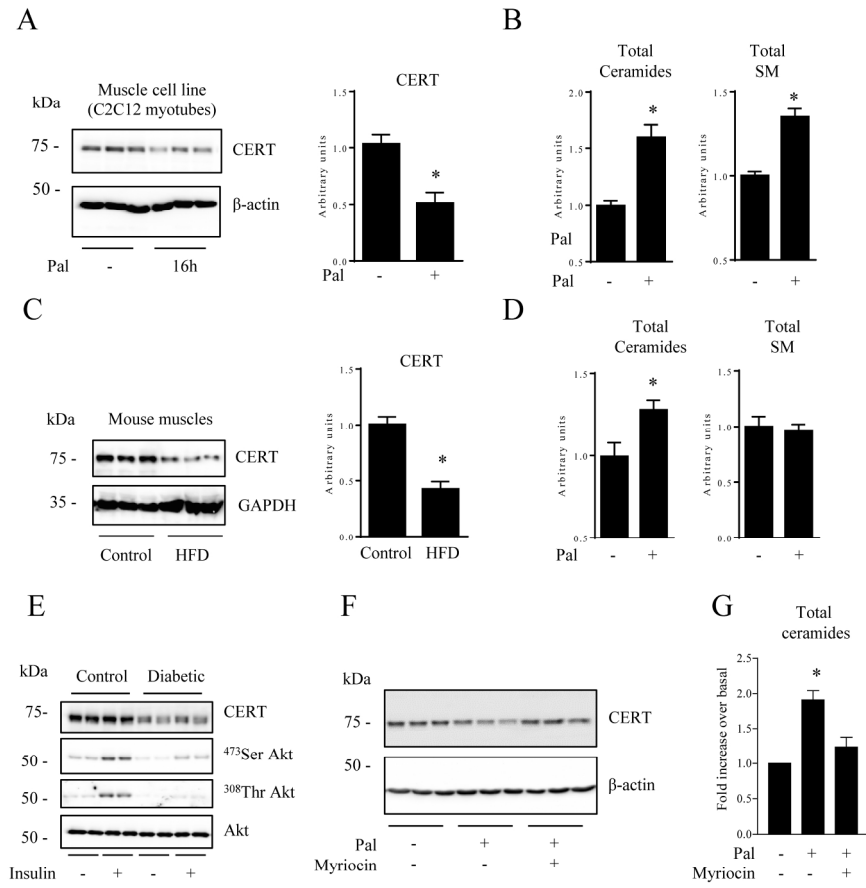


Figure 1

Figure 1

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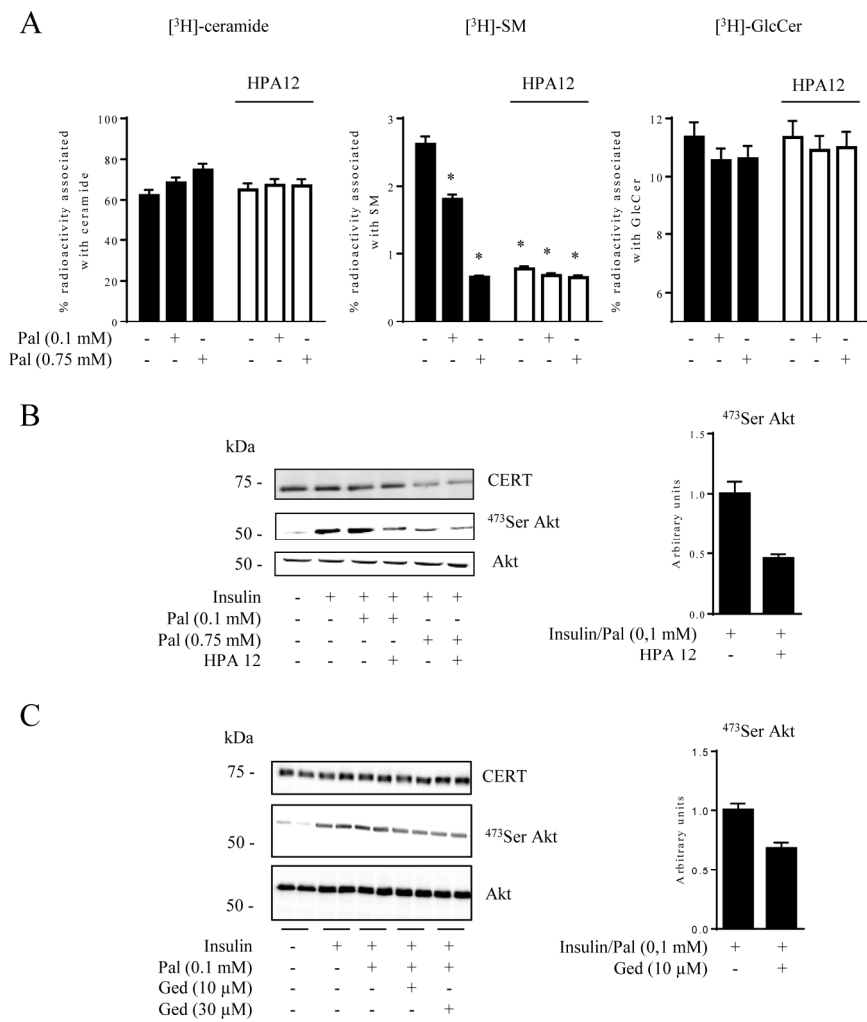


Figure 2

Figure 2

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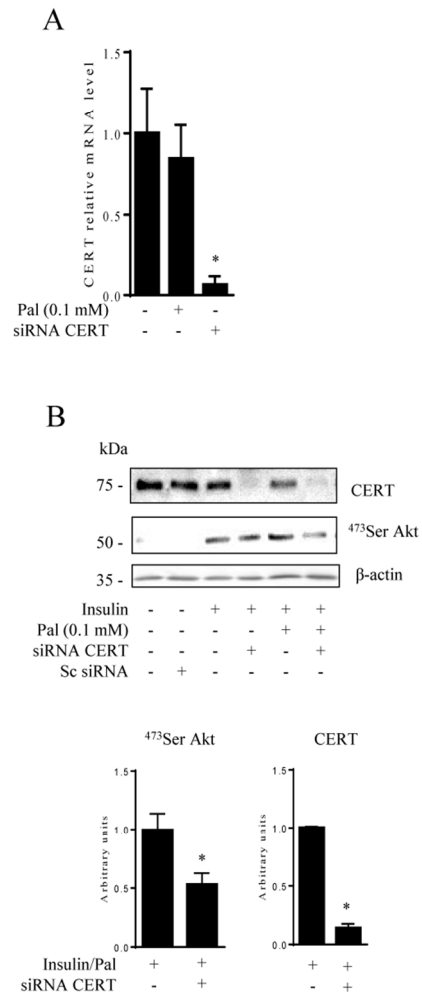


Figure 3

Figure 3

90x123mm (300 x 300 DPI)

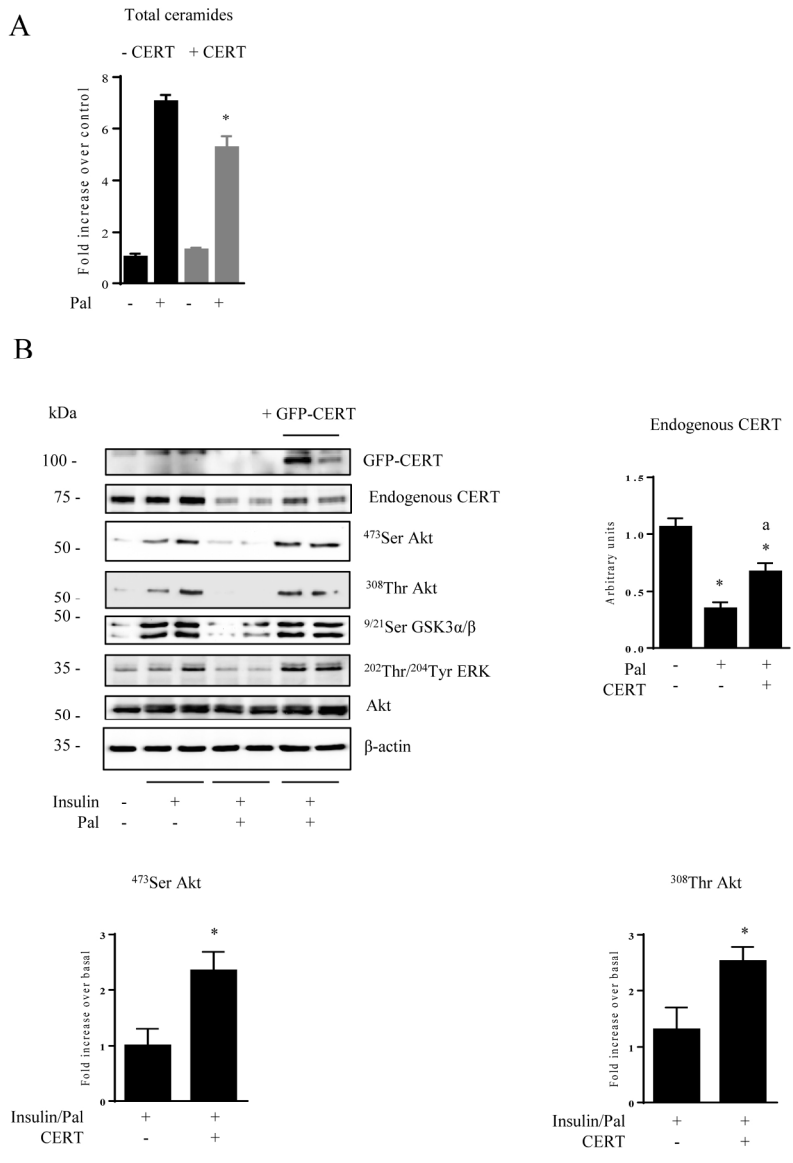


Figure 4

Figure 4

180x241mm (300 x 300 DPI)

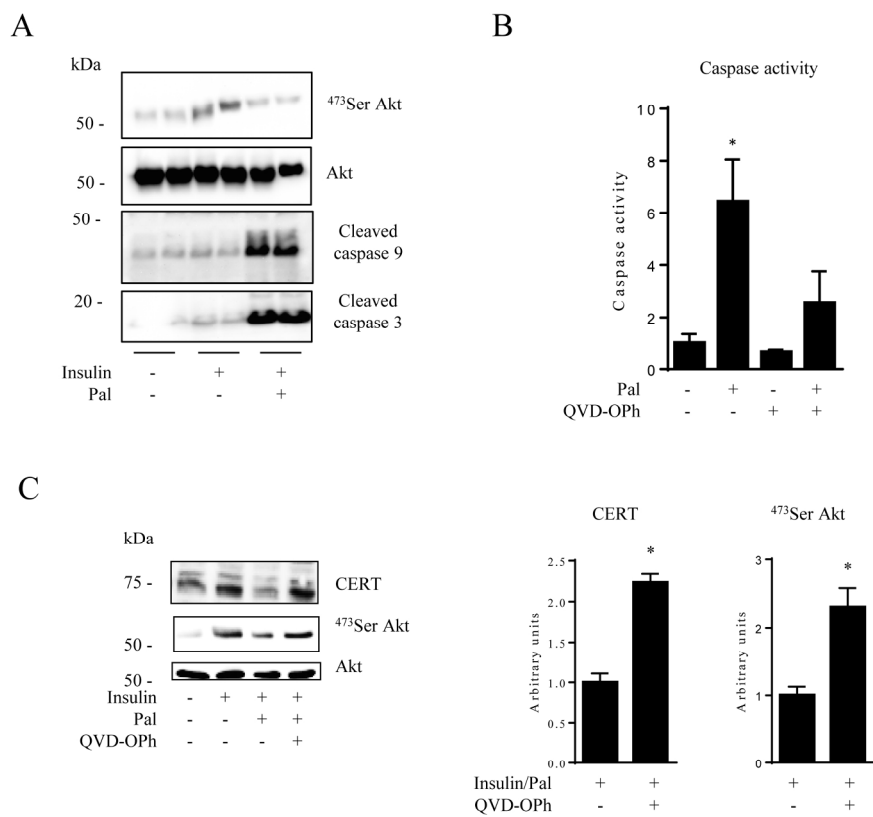


Figure 5

Figure 5

180x224mm (300 x 300 DPI)

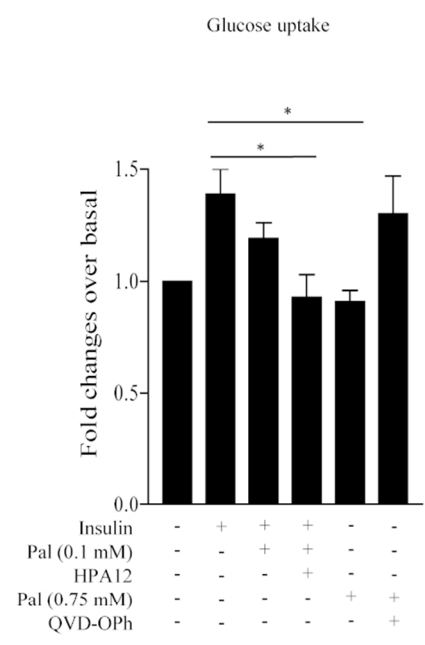


Figure 6

Figure 6

90x107mm (300 x 300 DPI)

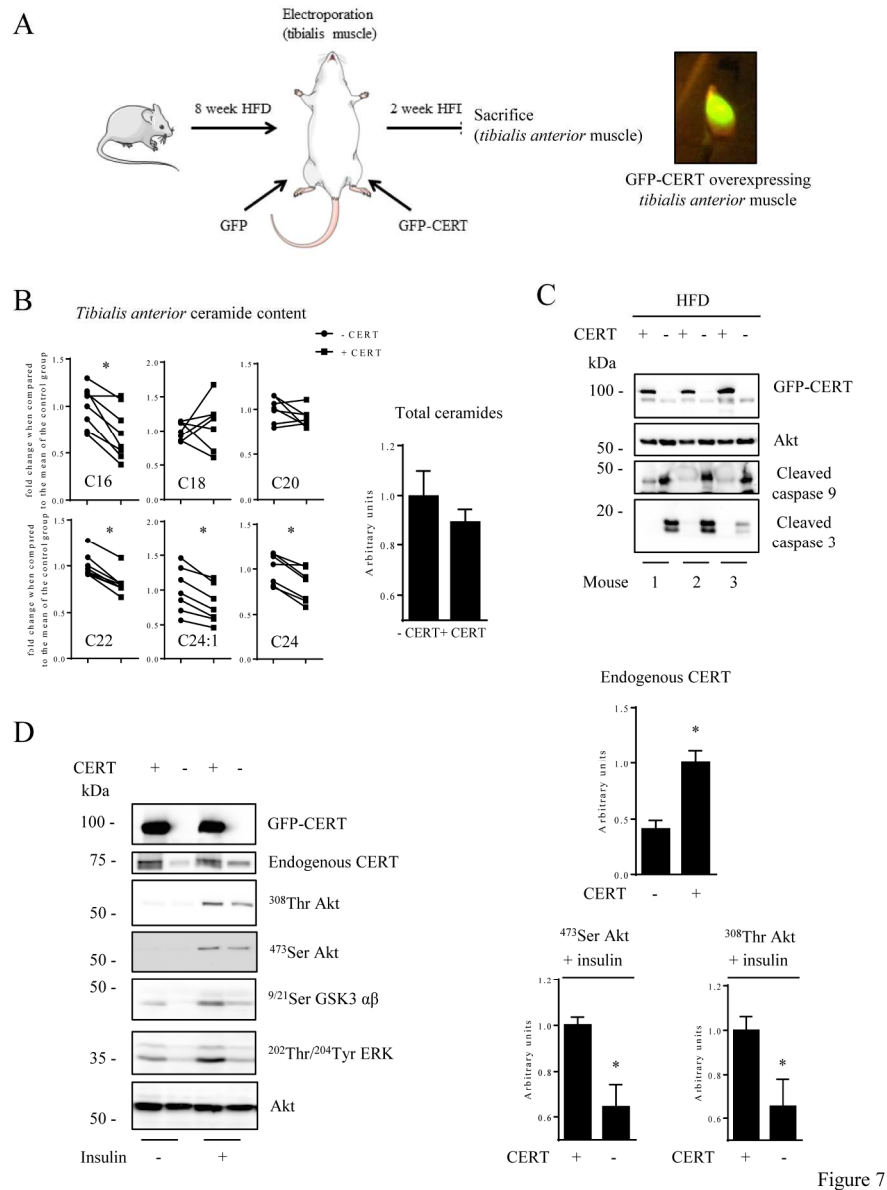
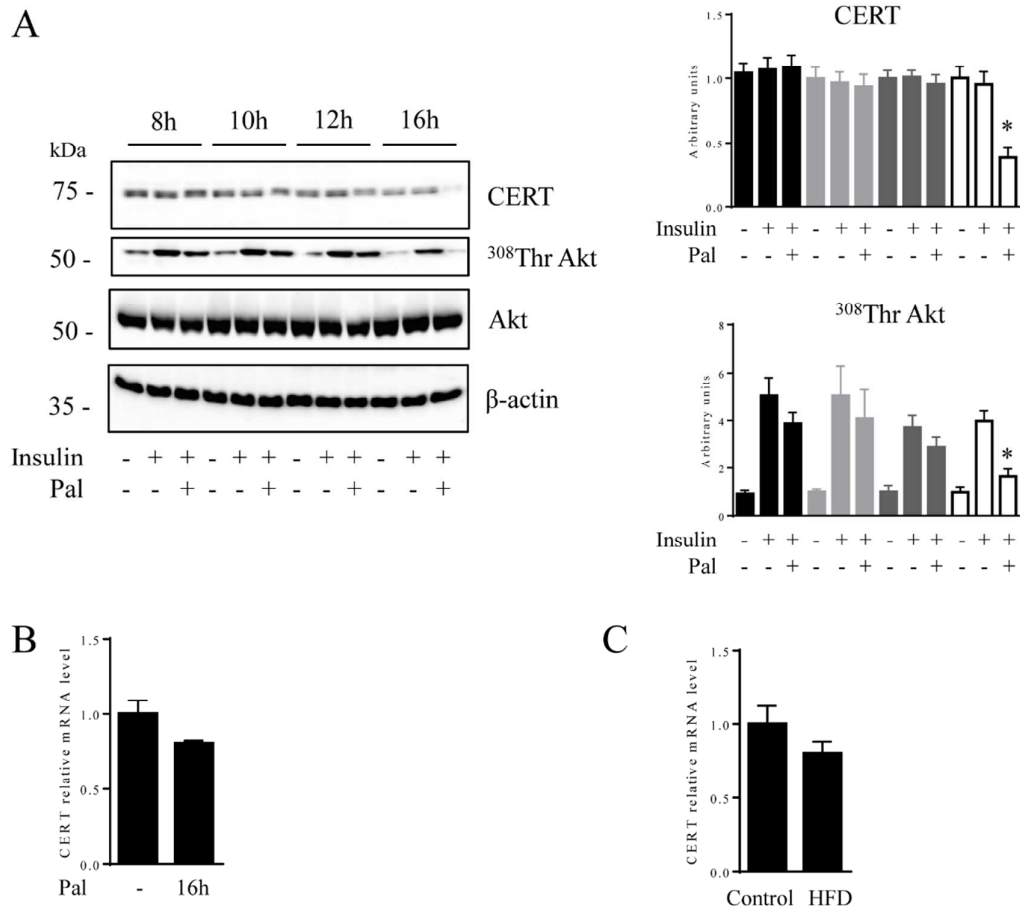


Figure 7

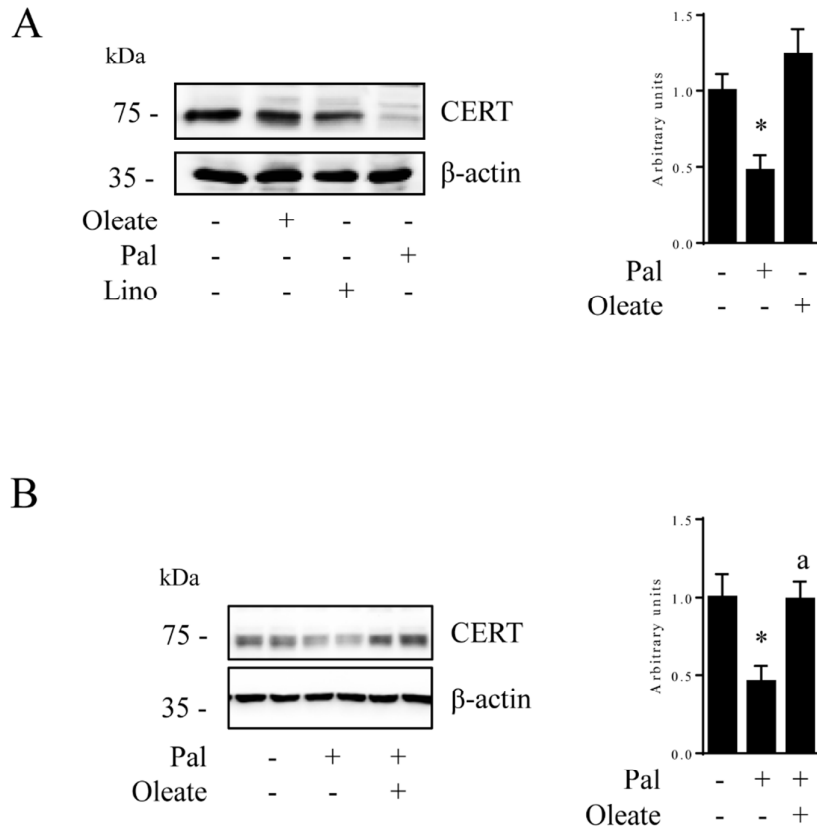
Figure 7

180x236mm (300 x 300 DPI)



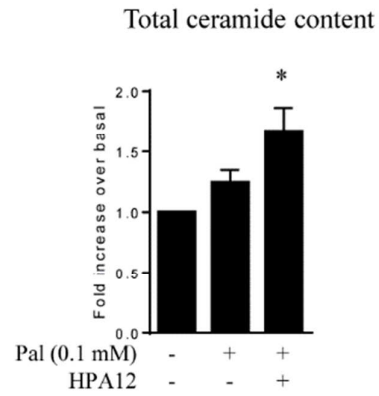
Supplemental figure 1: Effects of lipotoxic conditions on muscle cell CERT content *in vitro* and *in vivo*.

(A) C2C12 myotubes were treated with 0.75 mM palmitate (Pal) from 8h to 16 h, then with insulin during the last 10 min. Lysates were immunoblotted with the indicated antibodies. Blots were quantified and are shown as means \pm SEM (n=6). *Significant change (* p <0.05) relative to insulin alone. Quantification of CERT mRNA levels was performed by qRT-PCR in C2C12 myotubes treated with 0.75 mM palmitate for 16 h (n=6) (B) or in muscles of high fat diet (HFD) and chow diet (control) mice (n=4 to 6) (C). Results are mean \pm SEM.



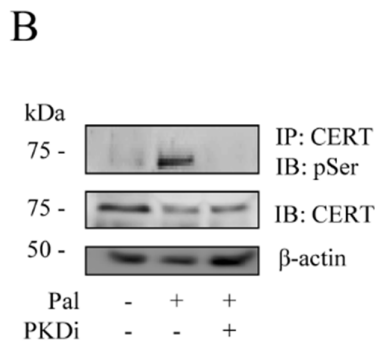
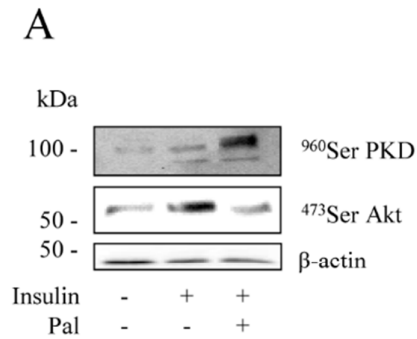
Supplemental figure 2: Effects of saturated and unsaturated fatty acids on CERT protein content in C2C12 myotubes.

(A) C2C12 myotubes were treated for 16 h with 0.3 mM oleate, 0.75 mM palmitate (Pal) or 0.3 mM linoleate (Lino) before being lysed. Histograms show densitometric quantification of CERT. Results are means \pm SEM (n=5). *Significant change (* $p < 0.05$) relative to untreated cells. (B) C2C12 myotubes were treated with 0.3 μ M oleate in the presence or absence of 0.75 mM palmitate (Pal) before being lysed. All cell lysates were immunoblotted with the indicated antibodies. Histograms show densitometric quantification of CERT. Results are means \pm SEM (n=4). *Significant change (* $p < 0.05$) relative to control and ^a (^a < 0.05) relative to palmitate treated myotubes.



Supplemental figure 3: Effects of palmitate and CERT inhibitor HPA12 on total ceramide content in C2C12 myotubes.

C2C12 myotubes were treated with 0.1 mM palmitate (Pal) with or without 10 μ M HPA12 for 16 h. Lipids were then extracted and total ceramides quantified. Results are means \pm SEM (n=4). *Significant change (* $p < 0.05$) relative to untreated myotubes.



Supplemental figure 4: Effects of palmitate on CERT phosphorylation in C2C12 myotubes.

(A) C2C12 myotubes were treated with 0.75 mM palmitate (Pal) for 16 h, then with insulin the last 10 min before being lysed. Lysates were immunoblotted with the indicated antibodies.

(B) C2C12 myotubes were incubated with 0.75 mM palmitate for 16 h, with or without 10 μ M PKD inhibitor (PKDi, kd-NB-142-70). Then CERT was immunoprecipitated and immunoblotted against antibodies directed against phospho-serine, CERT and β -actin.