ELECTRONIC SUPPLEMENTARY MATERIALS

Supplementary Materials and methods

Animals

Human apoC-III transgenic (apoCIII Tg) mice [1] aged 8–12 weeks were used for the isolation of muscle tissue. Mice were housed 3-4 per cage in a temperature-controlled (21 ±1°C) vivarium on a 12-h light-dark cycle. All animals received free access to water and chow diet (LM-485 Mouse/Rat Sterilizable Diet, Harlan Laboratories). All animal care, experimental protocols and procedures were performed in accordance to the Guide for the care and use of laboratory animals, Eighth edition (2011), with approval of the University of Barcelona and Connecticut Internal Animal Care and Use Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA preparation and quantitative RT-PCR

Total RNA was isolated using Ultraspec reagent (Biotecx, Houston, TX, USA). RNA samples were cleaned (NucleoSpin RNA; Macherey-Nagel, Düren, Germany) and checked for integrity by agarose gel electrophoresis. The total RNA isolated by this method was undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR), as previously described. Primer Express Software (Applied Biosystems, Foster City, CA, USA) was used to design the primers (supplemental Table 1).Reverse transcription was performed from 0.5 μg total RNA using Oligo$(dT)_{23}$ and M-MLV Reverse Transcriptase (Life Technologies). The PCR reaction contained 10 ng of reverse-transcribed RNA, 2X IQ™ SYBRGreen Supermix (BioRad, Barcelona, Spain) and 900 nM of each primer. PCR assays were performed on a BioRad MiniOpticon™ Real-Time PCR system. Thermal cycling conditions were as follows: activation of Taq DNA polymerase at 95ºC for 10 minutes, followed by 40 cycles of amplification at 95ºC

for 15 seconds and at 60ºC for 1 minute. Optimal primer amplification efficiency for each primer set was assessed and a dissociation protocol was carried out to ensure a single PCR product.

EMSA

The electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotides for the consensus binding site of PPRE (Santa Cruz Biotechnology). Nuclear extracts (NE) were isolated as previously reported (reference 14 of the manuscript). Oligonucleotides were labeled by incubating the following reaction at 37°C for 2 hours: 2 μl oligonucleotide (1.75 pmol/μl), 2 μl of 5X kinase buffer, 1 μl of T4 polynucleotide kinase (10 U/μl), and 2.5 μl [γ-32P] ATP (3,000 Ci/mmol at 10 mCi/ml). The reaction was stopped by adding 90 μl of TE buffer (10 mmol/L Tris-HCl, pH 7.4, and 1 mmol/l EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (GE Healthcare, Barcelona, Spain) according to the manufacturer's instructions. Five micrograms of crude nuclear protein was incubated for 10 min on ice in binding buffer (10 mmol/l Tris-HCl, pH 8.0, 25 mmol/l KCl, 0.5 mmol/l dithiothreitol, 0.1 mmol/l EDTA, pH 8.0, 5% (v:v) glycerol, 5 mg/ml BSA, and 50 μg/ml poly[dI-dC]) in a final volume of 15 μλ. Then, specific competitor oligonucleotide or antibody for supershift assays were added and incubated for 15 minutes on ice. Subsequently, the labeled probe (100,000 cpm) was added and the reaction was incubated for an additional 15 minutes on ice. Finally, protein-DNA complexes were resolved by electrophoresis at 4°C on 5% (w:v) polyacrylamide gels in 0.5X Tris-borate-EDTA buffer and subjected to autoradiography.

Deoxy-D-glucose,2-[1,2-^{3}H(N)] uptake experiments

C2C12 cells were starved for 24 h and washed twice with HEPES buffer (20 mmol/l HEPES [pH 7.4], 140 mmol/l NaCl, 2.5 mmol/l $MgSO_4$, 5 mmol/l KCl, 1 mmol/l $CaCl_2$). Cells were

preincubated with HEPES buffer for 1 h and incubated for 30 min in the presence or absence of 100 nmol/l insulin, followed by treatment with deoxy-D-glucose,2-[1,2-^{3}H(N)] (37,000 Bq/ml; Amersham Biosciences) for 20 min. The uptake was stopped by adding 10 μmol/l cytochalasin B. After washing with ice-cold 0.9% NaCl three times, cells were lysed with 0.1 mol/l NaOH. Non-specific uptake was measured in the presence of 10μmol/l cytochalasin B and was subtracted from all the values.

Image analysis

The chemiluminescent blots were imaged with the ChemiDoc MP imager (Bio-Rad). Image acquisition and subsequent densitometric analysis of the corresponding blots were performed with ImageLab software version 4.1 (Bio-Rad). The Band Analysis tool of ImageLab software was used to select and determine the background-subtracted density of the bands in all the gels and blots. This software interprets the raw data in three dimensions with the length and width of the band, and the signal emitted from the blot is registered in the third dimension as a peak rising out of the blot surface. The density of a given band was measured as the total volume under the three-dimensional peak.

References:

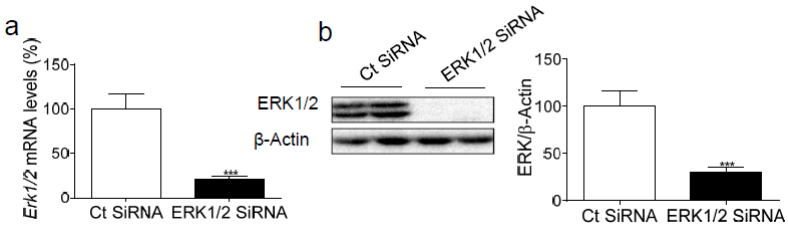
1. Wang F, Kohan AB, Dong HH, Yang Q, Xu M, Huesman S, Lou D, Hui DY, Tso P. Overexpression of apolipoprotein C-III decreases secretion of dietary triglyceride into lymph. Physiol Rep. 2014 Mar 20;2(3):e00247.

Supplementary Table 1. Primer sequences used for real-time RT- PCR

Gene	Primers	
Acox	for	5'-TCTGGAGATCACGGGCACTT-3'
	rev	5'-TTTCCAAGCCTCGAAGATGAG-3'
Aprt	for	5'-CAGCGGCAAGATCGACTACA-3'
	rev	5'-AGCTAGGGAAGGGCCAAACA-3'
Atf3	for	5'-CTGGAGATGTCAGTCACCAAGTCT-3'
	rev	5'-TTTCTCGCCGCCTCCTTT-3'
Bip	for	5`-CAGATCTTCTCCACGGCTTC-3`
	rev	5`-GCAGGAGGAATTCCAGTCAG-3`
Chop	for	5`-CGAAGAGGAAGAATCAAAAACCTT-3`
	rev	5`-GCCCTGGCTCCTCTGTCA-3`
Cpt-1α	for	5`-GCAGAGCACGGCAAAATGA-3`
	rev	5`-GGCTTTCGACCCGAGAAGAC-3`
IκBα	for	5'-CTCACGGAGGACGGAGACTC-3'
	rev	5'-CTCTTCGTGGATGATTGCCA-3'
Il-6	for	5'-ACACATGTTCTCTGGGAAATCGT-3'
	rev	5'-AAGTGCATCATCGTTGTTCATACA-3'
Mcad	for	5`-TGACGGAGCAGCCAATGA-3`
	rev	5`-ATGGCCGCCACATCAGA-3`
Mcp1	for	5'-GCTGGAGAGCTACAAGAGGATCA-3'
	rev	5'-CTCTCTCTTGAGCTTGGTGACAAA-3'
Nq01	for	5'-TATCCTTCCGAGTCATCTCTAGCA-3'
	rev	5'-TCTGCAGCTTCCAGCTTCTTG-3'
Pgc1α	for	5'-AACCACACCCACAGGATCAGA-3'
	rev	5'-TCTTCGCTTTATTGCTCCATGA-3'
Pparα	for	5`-CAAGGCCTCAGGGTACCACTAC-3`
	rev	5`-GCCGAATAGTTCGCCGAAA-3`
Pparβ/δ	for	5`-GCCACAACGCACCCTTTG-3`
	rev	5`-CCACACCAGGCCCTTCTCT-3`
Socs3	for	5'-TTCCCATGCCGCTCACA-3'
	rev	5'-CCCACCCAGCCCCATAC-3'
Tnfα	for	5'-AGCCGATGGGTTGTACCTTGT-3'
	rev	5'-TGAGATAGCAAATCGGCTGAC-3'

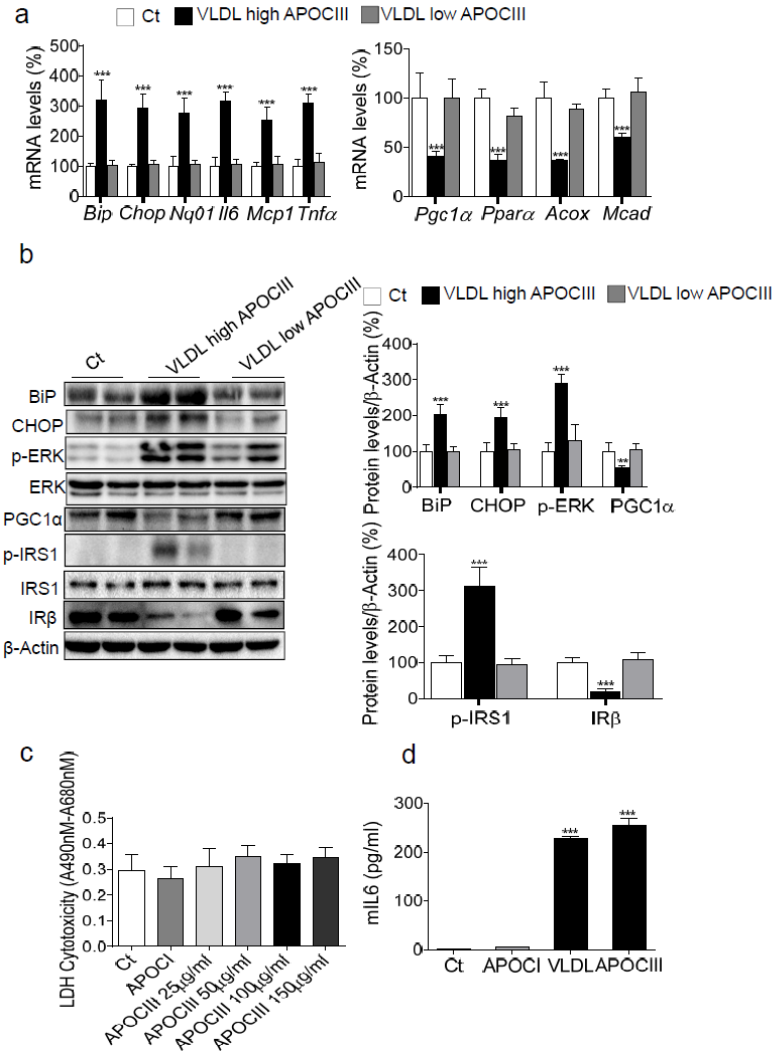
Supplementary Figures

ESM Fig. 1.



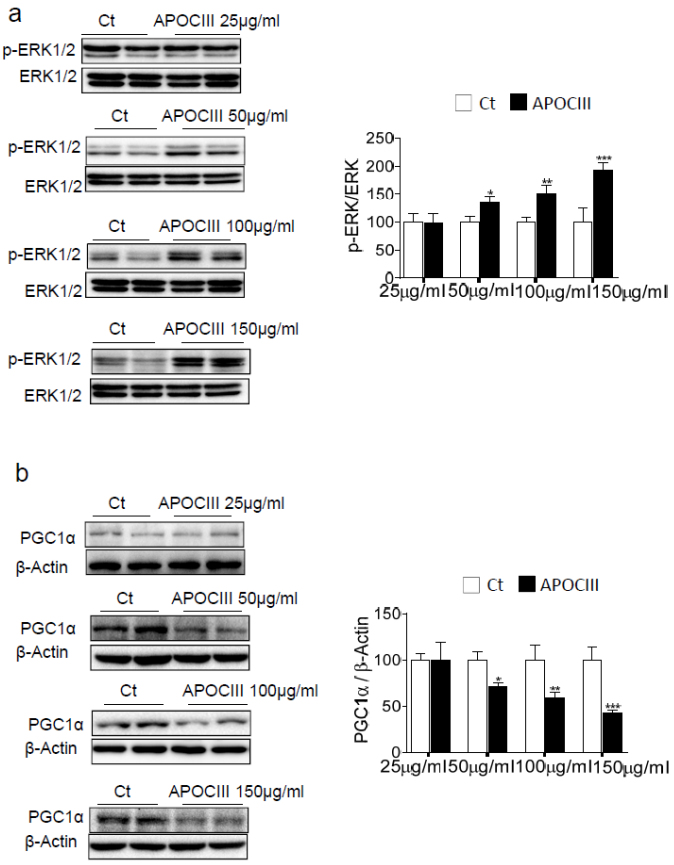
ESM Figure 1. A, mRNA abundance of *Erk1/2* in C2C12 cells transfected with control or ERK1/2 siRNA. B, Phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}) protein levels in C2C12 cells transfected with control or ERK1/2 siRNA. The graphs show quantification expressed as a percentage of control samples ± SD of five independent experiments. Data were compared by Student's t test. ***$p<0.001$, *vs.* control siRNA.

ESM Fig. 2.



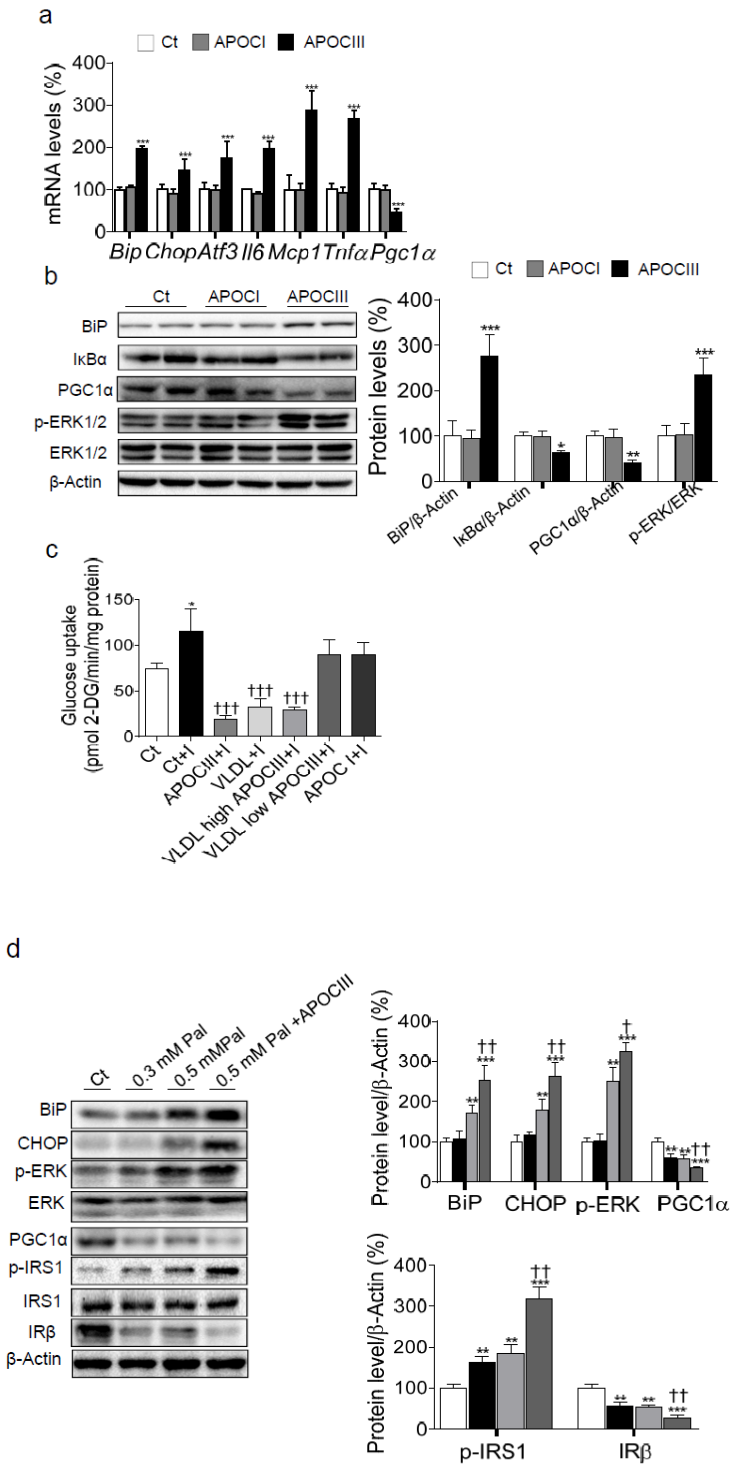
ESM Figure 2. C2C12 myotubes were incubated in the presence or absence (Ct: control) of 300 µg/ml VLDL with high apoCIII (apoCIII/TG=0.0358) isolated from human plasma of hyperlipemic patients or low apoCIII (apoCIII/TG=0.013) isolated from human plasma of normolipemic subjects. A, mRNA abundance of *Bip*, *Chop*, *Nqo1*, *Il-6*, *Mcp-1*, *Tnf-α, Pgc-1α*, *Pparα*, *Acox* and *Mcad*. The graphs show quantification of the *Aprt*-normalized mRNA levels, expressed as a % of control samples ± SD. B, BiP, CHOP, total and phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), PGC-1α, total and phosphorylated IRS1 (Ser^{307}), IRβ and β-actin protein levels. C, ApoCIII does not cause cell toxicity. C2C12 myotubes were incubated in the presence or absence (Ct: control) of 100 µg/ml apoCI and different concentrations of apoCIII for 24 h. Lactate dehydrogenase activity was measured. D, IL-6 secretion to the media. C2C12 myotubes were incubated in the presence or absence (Ct: control) of 100 µg/ml apoCI, 300 µg/ml VLDL or 100 µg/ml apoCIII for 24 h. The graphs show quantification expressed as a % of control samples ± SD of five independent experiments. Data were compared by two-way ANOVA followed by Tukey post-test. ***p<0.001 and **p<0.01 *vs.* control.

ESM Fig. 3.



ESM Figure 3. Concentration-response effects of apoCIII on ERK1/2 and PGC-1α protein levels. C2C12 myotubes were incubated in the presence or absence (Ct: control) of different concentrations of apoCIII for 24 h. Total and phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}) (A) and PGC-1α protein levels (B). The graphs show quantification expressed as a % of control samples ± SD of five independent experiments. Data were compared by Student's t test. ***p<0.001, **p<0.01 and *p<0.05 *vs.* control.

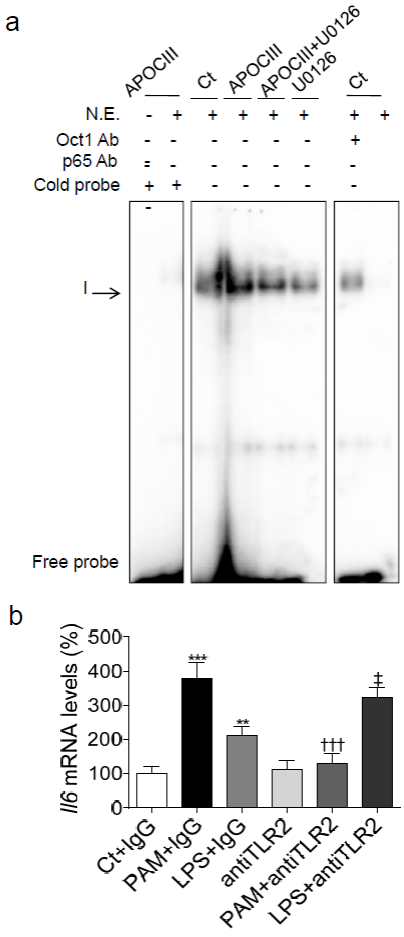
ESM Fig. 4.



ESM Figure 4. C2C12 myotubes were incubated in the presence or absence (Ct: control) of 100 μg/ml apoCI, or 100 μg/ml apoCIII for 24 h. A, mRNA abundance of *Bip*, *Chop*, *Atf3*, *Il-6*, *Mcp-1*, *Tnf-α* and *Pgc-1α*. The graphs show quantification of the *aprt*-normalized mRNA levels, expressed as a % of control samples ± SD of five independent experiments. B, BiP, IκBα, PGC-1α, phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), and β-actin protein levels. C, C2C12 myotubes were incubated in the presence or absence (Ct: control) of 300 μg/ml VLDL with high apoCIII or low apoCIII, 100 μg/ml apoCI, or 100 μg/ml apoCIII for 24 h. 2-DG uptake was assessed

without or with insulin. D, C2C12 myotubes were incubated in the presence or absence (Ct: control) of the saturated fatty acid palmitate conjugated with BSA or 100 μg/ml apoCIII for 24 h. BiP, CHOP, phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}), PGC-1α, total and phosphorylated IRS1 (Ser^{307}), IRβ and β-actin protein levels. The graphs show quantification expressed as a % of control samples ± SD of five independent experiments. Data were compared by two-way ANOVA followed by Tukey post-test. ***p<0.001, **p<0.01 and *p<0.05 *vs.* control. †††p<0.001 ††p<0.01 and †p<0.05 *vs.* cells exposed to 0.5 mM palmitate or cells exposed to insulin.

ESM Fig. 5.



ESM Figure 5. A, C2C12 myotubes were incubated in the presence or absence (Ct: control) of 100 μg/ml apoCIII, 10 μM U0126 or 10 μM U0126 plus 100 μg/ml apoCIII for 24 h. Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and crude nuclear protein extract (NE) from C2C12 myotubes. One main specific complex (I) based on competition with a molar excess of unlabeled probe is shown. The supershift assay performed by incubating NE with an antibody (Ab) directed against the p65 subunit of NF-κB shows a reduction in the band, whereas the band is observed with the unrelated antibody against Oct1. B, The neutralizing antibody against TLR2 is specific for this receptor and it does not block the effect of TLR4. C2C12 myotubes were incubated in the presence of 50 μg/ml of IgG (Ct: control), 20 ng/ml PAM3CSK4 (tripalmitoylated cysteine-, serine-, and lysine-containing peptide) (a TLR2 ligand) or 100 ng/ml *E. coli* lipopolysaccharide (LPS) (a TLR4 ligand) plus 50 μg/ml of IgG, or 20 ng/ml PAM3CSK4 (a TLR2 ligand) or 100 ng/ml *E. coli* LPS (a TLR4 ligand) plus 50 μg/ml of the neutralizing antibody against TLR2 (antiTLR2) for 3 h. mRNA abundance of *Il-6*. The graphs show quantification of the *Aprt*-normalized mRNA levels, expressed as a % of control samples ± SD of five independent experiments. Data were compared by two-way ANOVA followed by Tukey post-test. ***p<0.001 and **p<0.01 *vs.* control. †††p<0.001 vs. PAM3CSK4-exposed cells. ‡ p<0.05 *vs.* LPS-exposed cells.