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Journal of Nutritional Biochemistry 23 (2012) 218–227

Journal of Nutritional Biochemistry

Eicosapentaenoic acid inhibits tumour necrosis factor-α-induced lipolysis in murine cultured adipocytes☆

Silvia Lorente-Cebrián[a], Matilde Bustos[b], Amelia Marti[a], Marta Fernández-Galilea[a], J. Alfredo Martinez[a], Maria J. Moreno-Aliaga[a,*]

[a]Department of Nutrition, Food Science, Physiology and Toxicology, University of Navarra, 31008 Pamplona, Spain
[b]Division of Hepatology and Gene Therapy, Center for Applied Medical Research (CIMA), University of Navarra, 31008 Pamplona, Spain

Received 7 February 2010; received in revised form 29 October 2010; accepted 22 November 2010

Abstract

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid with beneficial effects in obesity and insulin resistance. High levels of proinflammatory cytokine tumour necrosis factor-α (TNF-α) in obesity promote lipolysis in adipocytes, leading to the development of insulin resistance. Thus, the aims of the present study were to analyze the potential antilipolytic properties of EPA on cytokine-induced lipolysis and to investigate the possible mechanisms involved. The EPA effects on basal and TNF-α-induced lipolysis were determined in both primary rat and 3T3-L1 adipocytes. Treatment of primary rat adipocytes with EPA (100 and 200 μM) significantly decreased basal glycerol release ($P<.01$) and prevented cytokine-induced lipolysis in a dose-dependent manner ($P<.001$). Moreover, EPA decreased TNF-α-induced activation of nuclear factor-κB and extracellular-related kinase 1/2 phosphorylation. In addition, the antilipolytic action of EPA was stimulated by the AMP-kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside and blocked by the AMPK-inhibitor compound C. Moreover, we found that EPA stimulated hormone-sensitive lipase (HSL) phosphorylation on serine-565, which further supports the involvement of AMPK in EPA's antilipolytic actions. Eicosapentaenoic acid treatment (24 h), alone and in the presence of TNF-α, also decreased adipose triglyceride lipase (ATGL) protein content in cultured adipocytes. However, oral supplementation with EPA for 35 days was able to partially reverse the down-regulation of HSL and ATGL messenger RNA observed in retroperitoneal adipose tissue of high-fat-diet-fed rats. These findings suggest that EPA inhibits proinflammatory cytokine-induced lipolysis in adipocytes. This effect might contribute to explain the insulin-sensitizing properties of EPA.

Keywords: Lipolysis; Eicosapentaenoic acid; TNF-α; AMPK; HSL

1. Introduction

Obesity and insulin resistance have been associated with high levels of free fatty acids (FFA) from dysregulated triglycerides breakdown (lipolysis) in adipose tissue [1].

Tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine whose production is increased in obesity and is involved in the development of insulin resistance [2]. Increased levels of TNF-α have been associated with high levels of circulating FFA probably due to the fact that this cytokine potently stimulates lipolysis in human, rat and mouse 3T3-L1 adipocytes [3–5] and that contributes to disrupt insulin signalling and to the development of insulin resistance [2].

Tumour necrosis factor-α promotes lipolysis via several mechanisms. Thus, it has been demonstrated that extracellular-related kinase 1 and 2 (ERK 1/2) activation in adipocytes might be involved in the stimulatory effect on lipolysis by proinflammatory cytokines such as TNF-α and interleukin-6 (IL-6) [3,5,6]. Nuclear factor-κB (NF-κB) has also been proposed to mediate TNF-α effects on lipolysis in adipocytes. Indeed, the inhibition of NF-κB abolished TNF-α-induced lipolysis in human adipocytes, suggesting a key role of this transcription factor in the lipolytic effects of TNF-α [7]. Thus, inhibition of

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside; AMPK, AMP-kinase; ATGL, adipose triglyceride lipase; EMSA, electrophoretic mobility shift Assay; EPA, eicosapentaenoic acid; ERK 1/2, extracellular-related kinase 1/2; FFA, free fatty acid; HOMA, homeostasis model assessment; HSL, hormone-sensitive lipase; IL-6, interleukin-6; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PPAR-γ, peroxisome proliferator activated receptor-γ; PUFA, polyunsaturated fatty acids; TNF-α, tumour necrosis factor-α.

☆ Funding: This work has been funded by the Spanish Ministry of Education and Science (AGL2006-04716/ALI), by the University of Navarra (LE/97) and by the Spanish Ministry of Science and Innovation (PCT-A3166019-2007). Silvia Lorente-Cebrián has a predoctoral fellowship FPU from the Spanish Ministry of Education and Science.

* Corresponding author. Department of Nutrition, Food Science, Physiology and Toxicology, University of Navarra, C/. Irunlarrea 1, 31008-Pamplona, Spain. Tel.: +34 948 42 56 00x6558; fax: +34 948 42 56 49.
E-mail address: mjmoreno@unav.es (M.J. Moreno-Aliaga).

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doi:10.1016/j.jnutbio.2010.11.018

NF-κB could be an anti-inflammatory mechanism involved in the regulation of lipolysis in adipocytes that could contribute to improve insulin sensitivity.

The activation of AMP-kinase (AMPK) has also been involved in the regulation of lipolysis in adipocytes. In fact, some studies have demonstrated that AMPK activation decreases lipolysis [8,9]. Moreover, several antidiabetic compounds such as metformin have been shown to inhibit lipolysis [10] and to activate AMPK in adipocytes [11]. This suggests a putative involvement of AMPK activation in the regulation of lipolysis that could contribute to improve insulin sensitivity after administration of insulin-sensitizing compounds.

Hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL)/desnutrin are considered the major lipases involved in triglycerides breakdown in adipocytes [12]. Under a lipolytic stimulus, HSL is phosphorylated in serine residues 563, 659 and 660 which are essential for the activation of HSL and thus for triglycerides breakdown. However, HSL can also be phosphorylated in serine-565, preventing subsequent phosphorylation of other HSL residues and thus decreasing HSL lipase activity. Interestingly, ATGL/desnutrin has a great homology with adiponutrin, an adipocyte-specific protein that was also shown to have lipid hydrolase activity [13]. Adipose triglyceride lipase is up-regulated by fasting, and its expression decreases upon refeeding and with insulin, while adiponutrin shows the opposite pattern [14]. Perilipin is a phosphoprotein that, under basal conditions, is located in the lipid droplet surface of adipocytes. Upon a lipolytic stimulus, perilipin is phosphorylated and binds HSL, which starts the hydrolysis of stored triglycerides, and thus is considered as an essential regulator of lipolysis in adipocytes [15].

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid (PUFA) found in fish and fish oils. Many beneficial effects have been attributed to omega-3 PUFAs in several inflammatory diseases including obesity, insulin resistance and atherosclerosis [16]. Omega-3 PUFAs improve insulin sensitivity and decrease TNF-α production in obesity, although the mechanisms involved have not yet been fully established [17]. In this context, other antidiabetic compounds such as metformin [10] and thiazolidinediones [18], as well as anti-inflammatory agents such as salicylate [19], have been shown to decrease TNF-α-induced lipolysis. This suggests that the improvement of insulin sensitivity by EPA could be related to its ability to inhibit TNF-α-induced proinflammatory effects, including lipolysis.

Therefore, the aim of the present study was to investigate the potential antilipolytic properties of EPA on TNF-α-induced lipolysis and to identify the potential mechanisms that might be involved.

2. Materials and methods

2.1. Materials

Media (Dulbecco's modified Eagle's medium [DMEM]), nonessential amino acids, penicillin/streptomycin, fetal bovine serum (FBS) and nystatin were purchased from Gibco-Invitrogen (Grand Island, NY, USA). Bovine serum albumin (BSA) fraction V and HEPES were from Sigma (St. Louis, MO, USA). Collagen (Purecol) was obtained from Inamed Biomaterials (Fremont, CA, USA). Type I collagenase was supplied by Worthington Biochemical (Lakewood, NJ, USA). Eicosapentaenoic acid and troglitazone were purchased from Cayman Chemical (Ann Arbor, MI, USA), and TNF-α was purchased from Phoenix Peptide (Burlingame, CA, USA). Interleukin-6, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), LY 294002 and PD 98059 were supplied by Sigma (St. Louis, MO, USA). Compound C was purchased from Calbiochem (La Jolla, CA, USA).

2.2. Adipocyte isolation and primary culture

Male Wistar rats (250–280 g in weight) were obtained from the Applied Pharmacobiology Center (CIFA). The animals were housed in cages in temperature-controlled rooms (22°C±2°C) with a light–dark cycle (12 h:12 h). All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use, with the approval of the Ethical Committee for Animal Care and Use at the University of Navarra.

Rats were sacrificed, and epididymal fat depots were removed immediately. Adipocyte isolation and culture were performed as described previously [20]. Briefly, fat was minced with scissors for 90 s in HEPES-phosphate buffer (pH 7.4; containing 5 mM of D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2 \cdot 3H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4 and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer with type I collagenase (1.25 mg/ml per 0.5 g tissue) at 37°C with gentle shaking for 30 min. The resulting cell suspension was diluted in HEPES-phosphate buffer, and the isolated adipocytes were then separated from the undigested tissue by filtration through a 400-μm nylon mesh and washed three times. The isolated adipocytes were then resuspended in DMEM supplemented with 1% FBS and incubated for 30–40 min at 37°C.

Isolated adipocytes (150 μl of 2:1 ratio of packed cells to medium) were then plated on 500 μl of a collagen matrix (Purecol) in six-well culture plates. After incubation for 50 min at 37°C, the culture media with the appropriate treatments were added. Cells were maintained in an incubator at 37°C in 5% of CO_2 for 24 or 96 h. For short-term experiments, media were collected after 24 h. When cells were incubated up to 96 h, aliquots (300 μl) of each media were collected at 24, 48, 72 and 96 h and replaced with fresh medium containing the exact concentration of the treatment. After 96 h, media were collected in order to measure glycerol release.

2.3. Cell culture and differentiation of 3T3-L1 cells

Mouse 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and differentiated as described previously [21]. Briefly, 2 days postconfluence, preadipocytes were cultured for 48 h in DMEM containing 25 mM glucose, 10% FBS, and antibiotics and supplemented with dexamethasone (1 μM), isobutylmethylxanthine (0.5 mM) and insulin (10 μg/ml). After that, cells were cultured with 10% FBS and insulin for 48 h. Cells were then cultured without insulin until day 7–8 postconfluence when cells were differentiated to adipocytes.

2.4. In vitro treatments

Eicosapentaenoic acid was dissolved in ethanol; TNF-α was dissolved in water; AICAR, LY 294002, PD 98059 and compound C were dissolved in dimethyl sulfoxide (DMSO). All compounds were prepared in 1000× stock solutions and then added to the culture media. Control cells were treated with the same amount of the corresponding vehicle (ethanol and/or DMSO). Eicosapentaenoic acid (100 and 200 μM) and troglitazone (10 μM) effects on lipolysis were evaluated in the presence and absence of TNF-α (1–10 ng/ml) and IL-6 (1 ng/ml) at 24 and 96 h alone or in the presence of selective inhibitors. Eicosapentaenoic acid and/or TNF-α and/or AMPK stimulator AICAR was added to the media at the same time. However, when selective inhibitors compound C (20 μM), PD 98059 (50 μM) and LY 294002 (50 μM) were used, adipocytes were preincubated for 1 h prior to the addition of EPA and/or TNF-α [22].

2.4.1. Glycerol measurements

Glycerol released into the media was measured with an Autoanalyzer Cobas-Mira (Roche Diagnostics, Basel, Switzerland) following manufacturer's instructions.

2.5. Preparation of nuclear extracts

For the isolation of nuclear extracts, 5-day postdifferentiated adipocytes were serum-starved overnight (18 h) and then incubated with EPA (100 and 200 μM) and/or TNF-α (1 ng/ml) for 24 h. Nuclei were isolated from 3T3-L1 cells according to the method of Dignam et al. [23] with slight modifications [24]. Briefly, cells were washed and scraped with PBS and centrifuged at 1850*g* for 10 min at 4°C. Cells pellets were resuspended in 5 vol of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol [DTT]) and centrifuged at 1850*g* for 5 min at 4°C. The packed cells were then resuspended in 3 vol of the same hypotonic buffer and allowed swelling for 10 min on ice. The cells were then homogenized with 10 strokes using a glass Dounce homogenizer and centrifuged at 3300*g* for 15 min at 4°C. The pellets obtained were the nuclei. The nuclear extracts were obtained from these nuclei by using the method of Lavery and Schibler [25] with minor differences. The pelleted nuclei were resuspended in a 1.1× extraction buffer (300 mM NaCl, 1 M urea, 1% Igepal CA-630, 1 mM DTT and 25 mM HEPES, pH 7.9), mixed vigorously by vortexing and incubated for 30 min on ice. The extracts were clarified by pelleting the insoluble debris through centrifugation at 15 000*g* for 20 min at 4°C in a microfuge. The supernatants were adjusted to 10% glycerol, rapidly frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined using the autoanalyzer COBAS-Mira (Roche Diagnostics).

2.6. Electrophoretic mobility shift assay

A double-stranded oligonucleotide corresponding to the NF-κB response element (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was end-labeled using [γ-^{32}P]ATP (Perkin Elmer, MA, USA) and T4 polynucleotide kinase (Promega, Madison, WI, USA) according to standard methods. Ten micrograms of nuclear extracts was incubated in a buffer containing 25 mM HEPES, pH 7.9, 10% glycerol and 0.5 mM DTT with 3 μg of poly-deoxy-inosinic-deoxy-cytidylic acid poly [d(I-C)] (Roche) and 5 μg of acetylated BSA (Amersham, United Kingdom) for 30 min at 4°C. A 100-fold excess of specific

competitor was added to some samples. Then the radiolabeled double-strand oligonucleotide (100 000 cpm) was added and incubated for an additional 20 min at room temperature. Oligonucleotide–NFκB binding was determined by electrophoresis in a nondenaturing 4% polyacrylamide gel at 175 V for 3.5 h at room temperature. Gels were dried for 30 min at 80°C and exposed to X-ray film in the presence of an intensifying screen at −80°C.

2.7. Western blot analysis

Extracellular-related kinase 1 and 2 and HSL phosphorylation as well as total ERK 1/2, perilipin, HSL and ATGL protein contents were determined both in mature 3T3-L1 adipocytes (7 days postdifferentiation) and in preconfluent (20%–30%) preadipocytes. Cells were serum-starved overnight (18 h) and then incubated with EPA (100 and 200 μM) and/or TNF-α (1–10 ng/ml) for 30 min. For total ATGL, HSL, and perilipin protein content, 3T3-L1 mature adipocytes were incubated with EPA and/or TNF-α for 24 h. Protein from lysates was heat-denatured in double-strength sodium dodecyl sulphate sample buffer containing DTT before resolution in 7.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Protein immunoblotting was performed as previously described [26]. Briefly, proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) controlled by Ponceau red staining. Membranes were incubated for 1 h in TBS-T (50 mM Tris-HCl [pH 7.6], 200 mM NaCl, and 0.1% Tween 20) with 2% BSA. Proteins were detected by overnight incubation of membrane with specific antibody in TBS-T with 2% BSA and subsequent incubation with horseradish-peroxidase-conjugated protein G (Bio-Rad Laboratories, Inc.) for 1 h. Specific protein bands were visualised using the enhanced chemiluminescence detection system (Amersham) according to manufacturer's instructions. The specific antibodies used in this study — p-ERK 1/2 (Thr-202/Tyr-204), p-HSL (Ser-563 and Ser-565), perilipin, ATGL, HSL and ERK 1/2 — were from Cell Signalling Technologies (Beverly, MA, USA). β-Actin was purchased from Sigma.

2.8. In vivo treatment

Twenty-nine male Wistar rats weighing about 180 g (6 weeks old) were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use and after approval of the Ethical Committee for Animal Care and Use at the University of Navarra (Pamplona, Spain).

Rats were assigned into two dietary groups for 35 days. The control group (n=15) was fed a standard pelleted diet (18% of energy as protein, 76% as carbohydrate and 6% as lipid), and the overweight group (n=14) was fed a high-fat diet ('cafeteria' diet) [17] containing 9% of energy as protein, 29% as carbohydrate and 62% as lipid.

Both dietary groups were divided into two new subgroups. One subgroup from each dietary group received by oral administration EPA ethyl ester (1 g/kg) daily for 35 days (control+EPA: CEPA and overweight+EPA: OEPA groups). The other subgroups of rats (control and overweight) received by oral administration the same volume of water as previously in other studies [17,27].

At the end of the experimental period, rats were euthanized, and white adipose tissue (WAT) depots were collected and frozen (−80°C) for further messenger RNA (mRNA) gene expression analysis.

2.8.1. RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to manufacturer's instructions and incubated with DNA*se* (RNA*se-free*) kit (Ambion, Austin, TX, USA) for 20 min at 37°C. RNA concentrations were measured spectrophotometrically, and its quality was verified by ethidium bromide staining after agarose gel electrophoresis.

2.9. Real-time polymerase chain reaction analysis

For each sample, 1 μg of RNA was reverse-transcribed to complementary DNA (cDNA) by using 200 U of M-MLV Reverse Transcriptase (Invitrogen) and 40 U of RNA*sin* (Promega) according to manufacturer's instructions. The cDNA samples were then frozen at −20°C in several aliquots until gene expression assays were performed.

For quantitative real-time polymerase chain reaction (PCR) analysis, 9 μl of cDNA *per* reaction was used. Reagents for real-time PCR analysis of HSL, perilipin and 18S (TaqMan Gene Expression Assays), ATGL and adiponutrin primer probes (Custom TaqMan Gene Expression Assays) and TaqMan Universal PCR Master mix were purchased from Applied Biosystems (Foster City, CA, USA). The conditions were used according to manufacturer's protocol. Amplification and detection of specific products were performed with the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems). A standard curve was plotted for each primer–probe set with a decimal serial dilution of several cDNA samples to ensure that the end of the reaction for control and different treatment samples was in the middle of the exponential curve of amplification.

Several assays were performed with different housekeeping genes (cyclophilin, β-actin, GAPDH and 18S) in order to determine the best choice for our experimental study. 18S ribosomal primer–probe was used to normalize the expression levels because we observed that 18S showed the lowest variability between samples from different treatments. This was performed for both *in vivo* and *in vitro* studies.

Normalization of the expression levels allowed data to be expressed relative to 18S rRNA, and therefore, any difference in reverse-transcriptase efficiency was compensated. All standards and samples were analyzed as duplicates.

Data were obtained as Ct values (the cycle where the emitted fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number) according to manufacturer's guidelines and used to determine ΔCt values (ΔCt=Ct of the target gene−Ct of the housekeeping gene 18S) of each sample. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method [28].

2.10. Data analysis

Results are given as mean values with their standard errors. Due to the high variability between adipocyte cultures from different rats (*in vitro*), the experimental results from each adipocyte suspension prepared from a single animal were analyzed in relation to a control well from the same suspension. For this reason, the statistical analysis of the data from primary culture adipocytes was performed by repeated-measures analysis of variance followed by a Tukey's *post hoc* test or by a paired Student's *t* test. Linear regression analysis or Pearson's correlation coefficients were calculated to determine relationships between lipases gene expression patterns and several indexes of obesity and insulin resistance. The statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered as statistically significant at P<.05.

3. Results

3.1. Effects of EPA on basal and cytokine-stimulated lipolysis

Eicosapentaenoic acid treatment significantly decreased basal glycerol released to the media in a dose-dependent manner (EPA 100 μM: −18.1%±3.9%, P<.01; EPA 200 μM: −39.9%±5.8%, P<.001) after 96 h of culture (Fig. 1A). This inhibitory effect of EPA on basal lipolysis was observed from the first 24 h of treatment (data not shown). Similarly, adipocytes treated with the peroxisome proliferator activated receptor-γ (PPAR-γ) agonist troglitazone (10 μM) significantly decreased glycerol release after 24 h (−31.2%±6.2%, P<.01) and 96 h of incubation (Fig. 1A).

Treatment with the proinflammatory cytokine TNF-α (1 ng/ml) produced a significant increase on glycerol release at 96 h of culture (TNF-α: +39.7%±4.9%, P<.01; Fig. 1B). Co-treatment with EPA prevented the lipolytic effects of TNF-α (TNF-α+EPA 100 μM: −27.6%±4.7%, P<.001 and TNF-α+EPA 200 μM: −52.6%±4.6%, P<.001 as compared to TNF-α-treated cells), which was also observed from the first 24 h of treatment (data not shown). In the same way, the co-treatment with TNF-α and troglitazone (10 μM) also prevented the lipolytic actions of the cytokine (Fig. 1B).

Similar effects on glycerol release were observed after treatment with another proinflammatory cytokine, IL-6 (Fig. 1C), showing an enhancement on glycerol release after 96 h of treatment (IL-6: +19.9%±6.0%, P<.05). The presence of EPA also inhibited IL-6-stimulated lipolysis (IL-6+EPA 100 μM: −34.7%±3.5%, P<.01 and IL-6+EPA 200 μM: −56.6%±6.9%, P<.001 as compared to IL-6-treated cells). Similarly, troglitazone also decreased IL-6-induced lipolysis.

We also tested the ability of EPA to prevent TNF-α-induced lipolysis in mature 3T3-L1 adipocytes. Treatment with TNF-α for 24 h significantly stimulated (+13.4%±2.9%, P<.05), while EPA did not modify glycerol release in 3T3-L1 adipocytes. However, treatment with EPA prevented TNF-α-induced lipolysis (P<.05) in agreement with the data observed in primary adipocytes (Fig. 1D).

3.2. EPA decreases TNF-α-induced NF-κB binding activity

Nuclear factor-κB has been described to play a critical role in TNF-α-induced lipolysis in adipocytes. To determine the possible mechanisms involved in the antilipolytic action of EPA on basal and cytokine-induced lipolysis, NF-κB binding activity was analyzed by electrophoretic mobility shift assay (EMSA) in 3T3-L1 adipocytes.

As shown in Fig. 2A and B, a strong increase in the abundance of NF-κB–DNA complexes was observed in nuclear extracts from TNF-α-treated adipocytes in comparison with control cells (Fig. 2A, lane 1 vs.

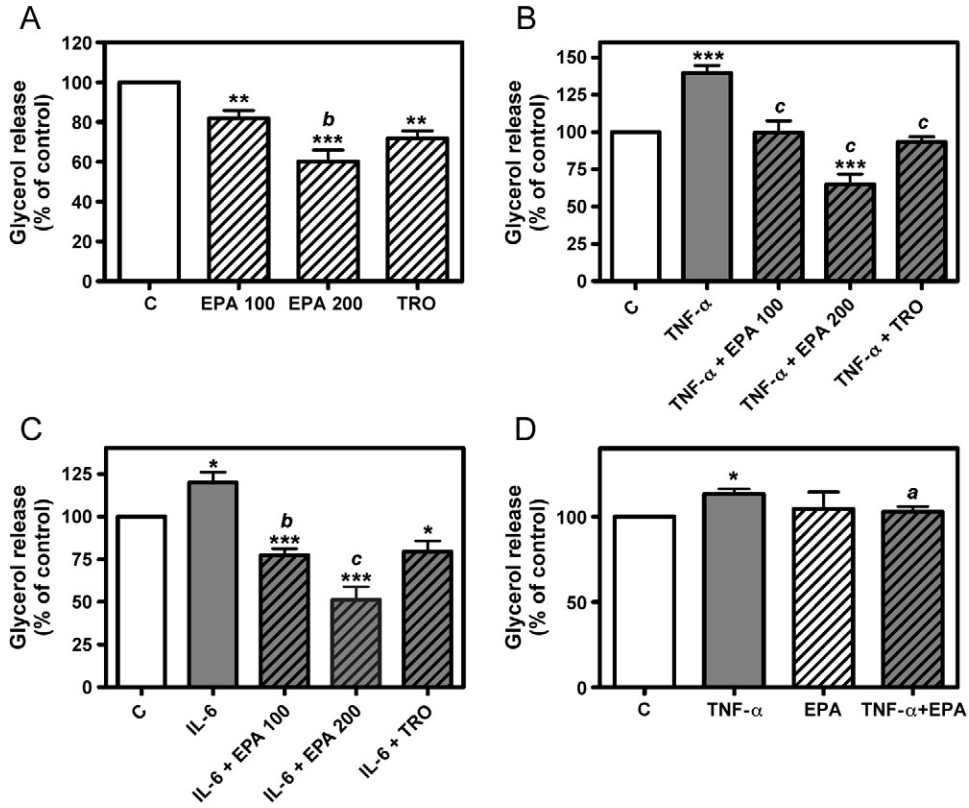

Fig. 1. (A) Effects of EPA (100 and 200 μM) and troglitazone (10 μM) on basal lipolysis determined as glycerol released to the media at 96 h in primary cultured rat adipocytes. (B) Effects of TNF-α at 1 ng/ml and (C) IL-6 at 1 ng/ml alone or in combination with EPA (100 and 200 μM) and troglitazone (10 μM) on basal lipolysis at 96 h in primary cultured rat adipocytes. Results are representative of 8–10 different experiments. (D) Effects of EPA (200 μM) on basal and TNF-α (10 ng/ml)-induced lipolysis in 3T3-L1 adipocytes after treatment for 24 h (five independent experiments). Results are expressed as percentage of glycerol released to the media *P<.05; **P<.01; ***P<.001 vs. control (C) cells; [a]P<.05; [b]P<.01; [c]P<.001 vs. TNF-α- or IL-6-treated cells.

lane 2). Eicosapentaenoic acid 200 μM did not have any significant effect on NF-κB binding activity. However, the presence of EPA 200 μM was able to partially prevent TNF-α-induced NF-κB–DNA binding (lane 2 vs. lane 4), suggesting anti-inflammatory properties for this fatty acid in adipocytes. Similar effects were also observed for EPA 100 μM (data not shown).

3.3. EPA inhibits TNF-α-induced phosphorylation of ERK 1/2

Activation of ERK 1/2 signalling pathway participates in TNF-α-induced lipolysis in adipocytes [3–5]. In order to assess whether EPA interferes in ERK 1/2 phosphorylation, murine 3T3-L1 adipocytes and preadipocytes were treated with EPA (100 and 200 μM) in the presence of TNF-α.

Tumour necrosis factor-α treatment resulted in a positive phosphorylation of ERK 1/2 (Thr-202/Tyr-204) in both adipocytes (Fig. 3A) and preadipocytes (Fig. 3B). Moreover, EPA treatment prevented TNF-α-induced phosphorylation of ERK 1/2 in preadipocytes (completely) and adipocytes (partially).

3.4. AMPK participates in the antilipolytic action of EPA

Previous studies have suggested an antilipolytic role of AMPK activation in adipocytes [9]. Thus, we next tested whether AMPK activation could be involved in the antilipolytic actions of EPA. As shown in Fig. 4A, both EPA and AICAR (an activator of AMPK) significantly reduced basal glycerol release from primary rat adipocytes (EPA 200 μM: −29.3%±6.3%, P<.01 and AICAR: −27.6%±4.9%, P<.05), and this effect was even stronger when both agents were added together (EPA+AICAR: −56.4%±3.0%, P<.01 vs. control cells and P<.05 vs. EPA-treated cells). In addition, co-treatment of EPA with compound C (an inhibitor of AMPK) blocked (P<.05) the antilipolytic effect of EPA on both basal (Fig. 4A) and TNF-α-induced lipolysis (Fig. 4B) in primary rat adipocytes. Moreover, compound C also abrogated the antilipolytic effects of EPA on TNF-α-induced lipolysis in 3T3-L1 adipocytes (Fig. 4C), suggesting an involvement of AMPK activation in the antilipolytic effects of EPA.

However, phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 did not significantly modify the inhibitory effect of EPA on basal (Fig. 4A) and TNF-α-stimulated lipolysis (Fig. 4B). The inhibition of ERK 1/2 with PD 98059 strongly increased the antilipolytic effects of EPA on TNF-α-induced lipolysis in primary rat (Fig. 4B) and 3T3-L1 (Fig. 4C) adipocytes, suggesting that ERK 1/2 activation is essential in the regulation of lipolysis by TNF-α in adipocytes.

3.5. Effects of EPA on HSL, ATGL and perilipin levels

Hormone-sensitive lipase and ATGL, together with perilipin, have been considered the major regulators of lipolysis in adipocytes. In this context, HSL activity is regulated by several mechanisms including reversible phosphorylation in serine residues. Thus, to better elucidate the mechanisms underlying the antilipolytic actions of EPA, we next investigated the effects of EPA on HSL phosphorylation both in Ser-563 and Ser-565 in the presence or absence of TNF-α in mature 3T3-L1 adipocytes (Fig. 5A). Interestingly, EPA treatment (100 and 200 μM) increased HSL phosphorylation in Ser-565, the HSL residue that is phosphorylated by AMPK, both alone and in the presence of TNF-α. However, EPA did not modify the phosphorylation of the

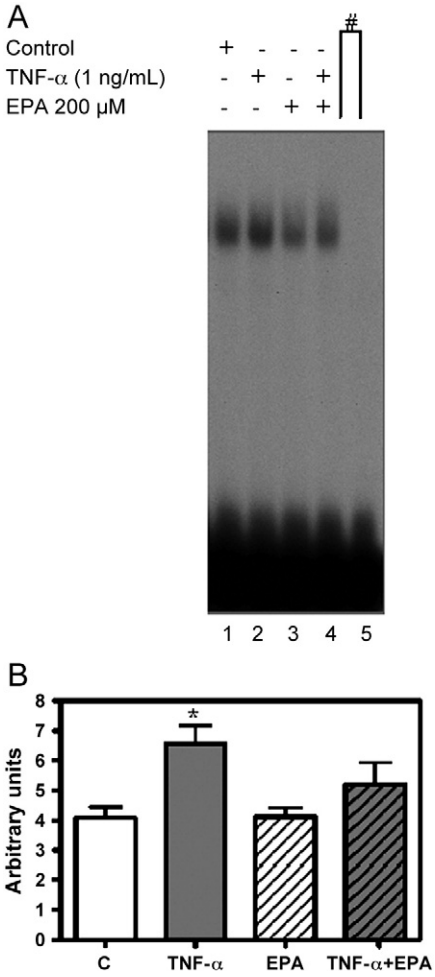

Fig. 2. (A) Effects of treatment with EPA (200 μM) and/or TNF-α (1 ng/ml) on DNA binding ability to NF-κB in 3T3-L1 adipocytes during 24 h. Nuclear extracts and EMSA were prepared as previously described in 'Material and methods'. (B) Arbitrary units (refereed to optical density) showing the intensity of the activation of NF-κB after treatment with EPA and/or TNF-α in mature adipocytes. The # symbol denotes that a competition assay using 100-fold excess of unlabeled oligonucleotide was performed to obtain nonspecific binding activity. Each shift is representative of three independent experiments. *P<.05 vs. control (C).

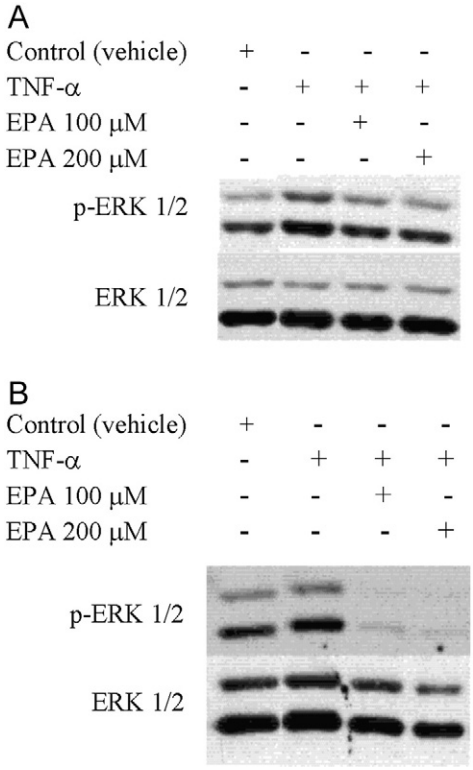

Fig. 3. Analysis of ERK 1/2 activation in 7-day postdifferentiated 3T3-L1 adipocytes (A) and preadipocytes (20%–30% confluence) (B) 30 min after treatment with EPA (100 and 200 μM) in the presence of TNF-α (1 ng/ml). Representative blots of three independent experiments showing phosphorylation on ERK 1/2 in whole cell lysate.

activating residue (Ser-563) of HSL after 30 min of incubation. On the other hand, the proinflammatory cytokine TNF-α did not modify phosphorylation of HSL either in Ser-563 or in Ser-565 at the conditions tested, suggesting that the lipolytic effects of TNF-α are mainly mediated through other mechanisms rather than regulating HSL activity by phosphorylation at these sites.

Contrary to HSL, ATGL lipase activity does not seem to be regulated by phosphorylation [29]. Thus, we further determined whether the inhibitory effect in lipolysis in EPA-treated adipocytes could also be associated with changes in ATGL protein expression. As observed in Fig. 5B, both EPA and TNF-α reduced ATGL protein levels in adipocytes after treatment for 24 h. Similar effects were observed on ATGL mRNA levels in EPA-treated adipocytes (data not shown). In contrast to ATGL, we did not observe any significant effect of EPA on total HSL and perilipin protein content and mRNA levels alone or in the presence of TNF-α after 24 h of treatment (data not shown). However, after a longer incubation period (96 h), a statistically significant down-regulation on HSL, ATGL and perilipin mRNA levels was observed in both EPA- and TNF-α-treated adipocytes (Supplemental Figure 1).

3.6. Effects of EPA-treatment in vivo on HSL, ATGL, adiponutrin and perilipin mRNA in WAT from lean and overweight rats

Next, to better understand the regulation of HSL, ATGL and adiponutrin in obesity and the actions of EPA, we also investigated the gene expression pattern of these main lipases as well as perilipin in WAT of lean and diet-induced overweight rats treated orally with EPA (1 g/kg) for 35 days.

Regarding lipase expression, our data showed that HSL mRNA levels were dramatically decreased (−88.8%±1.4%; P<.001) in the overweight group as compared to control animals (Fig. 6A). The ATGL gene expression was also inhibited, but to a lower extent (−52.6%±4.2% of control group; P<.05) (Fig. 6B), while adiponutrin and perilipin mRNA levels were not significantly modified in these high-fat-fed animals (Fig. 6C and D, respectively).

Eicosapentaenoic acid supplementation was able to prevent the decrease in both HSL and ATGL mRNA expression levels observed in overweight animals (Fig. 6A and B, respectively). In addition, EPA treatment significantly increased adiponutrin mRNA gene expression levels (+84.0±34.0% of control group; P<.05) in this group of animals fed a high-fat diet (Fig. 6C). However, oral supplementation with EPA did not modify HSL, ATGL or adiponutrin mRNA levels in rats fed with a standard diet. Perilipin mRNA levels were not significantly modified by EPA either in overweight or control group (Fig. 6D).

Moreover, a negative correlation was observed between HSL gene expression levels and several markers of obesity and insulin resistance such as body weight (r=−0.620, P=.0003), adiposity (r=−0.496, P=.0061), insulin levels (r=−0.384, P=.047) and homeostasis model assessment (HOMA) index (r=−0.409, P=.03). However, no significant correlations were found between these

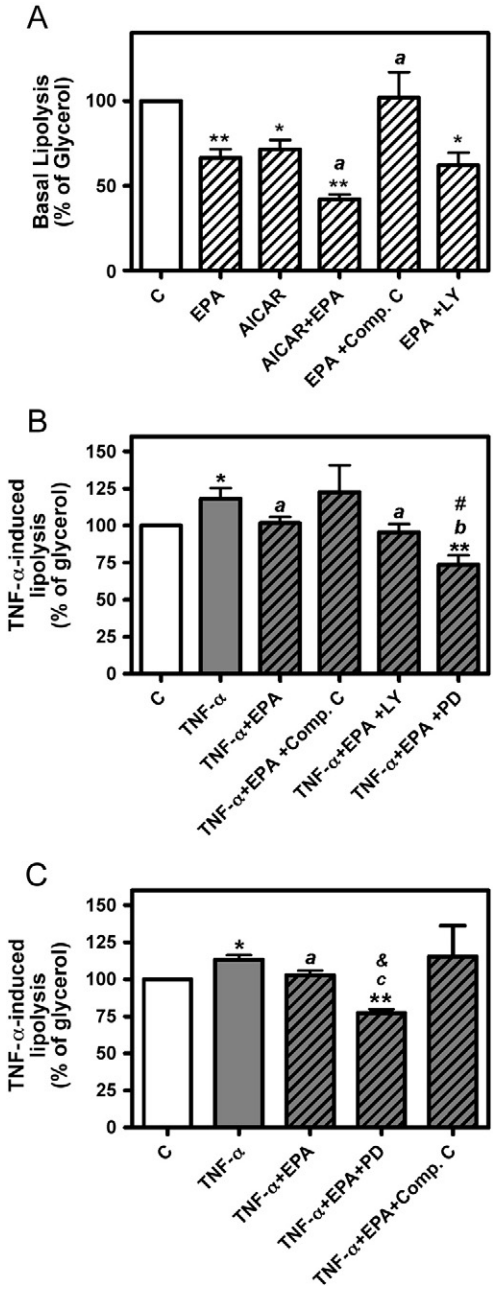

Fig. 4. (A) Effects of an AMPK activator (AICAR; 2 mM), an AMPK inhibitor (compound C; 20 μM) and a PI3K inhibitor (LY 294002; 50 μM) on the antilipolytic effects of EPA after 24 h of treatment in primary cultured rat adipocytes. (B) Effects of several kinase inhibitors: compound C (20 μM), LY 294002 (50 μM) and PD 98059 (50 μM) (an inhibitor of ERK 1/2) in combination with EPA (100 μM) on TNF-α-induced lipolysis after incubation for 24 h in primary cultured adipocytes. (C) Effects of EPA on TNF-α-induced lipolysis alone or in the presence of PD 98059 and compound C after 24 h of treatment in 3T3-L1 adipocytes. Data (mean±S.E.) are expressed as percentage of glycerol released to the media. Results are representative of at least seven different experiments. *$P<.05$; **$P<.01$ vs. control; $^{a}P<.05$; $^{b}P<.01$; $^{c}P<.001$ vs. TNF-α; $^{\#}P<.05$; $^{\&}P<.01$ vs. TNF-α+EPA.

parameters and ATGL and adiponutrin mRNA levels in retroperitoneal fat (Table 1).

4. Discussion

Overproduction of proinflammatory cytokines such as TNF-α and IL-6 is known to induce a strong stimulation of lipolysis in adipocytes, providing an excessive amount of FFA into the blood stream that promotes development of insulin resistance [1,2]. Several studies have suggested that the intake of omega-3 fatty acids, especially EPA, are beneficial on insulin resistance and obesity features in several models of obesity and diabetes in rodents [17,27]. However, the intimate mechanisms sustaining the protective effects of omega-3 fatty acids are still unclear. In the present study, we show the ability of EPA to prevent cytokine-stimulated lipolysis both in primary rat and in 3T3-L1 adipocytes, suggesting that this could be a potential mechanism involved in the insulin-sensitizing properties of EPA. These data are in agreement with previous studies, which have also demonstrated antilipolytic properties for EPA in adipose tissue, being able to attenuate the stimulation of lipolysis by isoprenaline, dexamethasone or a tumour-derived lipid-mobilizing factor [30,31]. However, a recent report has shown that EPA increases basal lipolysis in 3T3-L1 adipocytes [32]. In the present study, we did not observe any significant effect of EPA on basal glycerol release after treatment for 24 h in 3T3-L1 adipocytes. However, EPA exhibited antilipolytic actions on basal lipolysis in primary rat adipocytes. These apparent discrepancies could be due to differences in the type of experimental model (3T3-L1 cells vs. primary adipocytes) and the concentrations of EPA tested (100–200 vs. 300 μM). In this regard, Murata et al. [33] reported that the concentration used in our study (100 μM) is comparable to EPA plasma concentrations after an intake of dietary PUFA.

The present study and others have also shown that some antidiabetic drugs such as troglitazone and metformin decrease basal and TNF-α-stimulated lipolysis in adipocytes [10,18], supporting the fact that inhibition of basal and/or cytokine-stimulated lipolysis is a common mechanism of antidiabetic drugs to improve insulin sensitivity in adipocytes.

Mechanisms regulating lipolysis are multifactorial. Previous data of our group and others have suggested important antiinflammatory properties of EPA in the inflammation induced in adipose tissue by a high-fat diet [17,34,35]. A recent study has also demonstrated that salicylate, a potent anti-inflammatory drug, also decreases TNF-α-induced lipolysis in primary rat adipocytes [19]. In order to evaluate if the antilipolytic actions of EPA are related to its anti-inflammatory properties, we tested the effects of EPA on NF-κB, a pivotal intracellular messenger activated by proinflammatory cytokines, which has been shown to be important for TNF-α-induced lipolysis in adipocytes [7]. We demonstrate for the first time the ability of EPA to inhibit TNF-α-induced activation of NF-κB in adipocytes. Other trials have also demonstrated that EPA prevents NF-κB activation in immune cells such as human monocytic THP-1 cells [36] and murine macrophages [37]. Moreover, other study showed that feeding mice with a diet enriched with n-3 fatty acids prevented NF-κB activation in kidneys [38]. Thus, our data suggest that inhibition of NF-κB activation by EPA could be involved in the ability of this omega-3 fatty acid to attenuate TNF-α-induced lipolysis.

Several studies have demonstrated that ERK 1/2 phosphorylation is important in the regulation of TNF-α-stimulated lipolysis in adipocytes [3-5]. Our data support this finding as we show that the lipolytic effect of TNF-α disappears in the presence of ERK 1/2 inhibitor PD 98059. In addition, we found that EPA prevented TNF-α-induced ERK 1/2 phosphorylation in adipocytes and preadipocytes, in agreement with the decrease observed in glycerol release. In fact, other studies have also shown that EPA decreases ERK 1/2 phosphorylation in hepatoma [33] and smooth muscle [39] cells. However, others have reported that EPA did not decrease ERK 1/2 phosphorylation or even increased ERK phosphorylation in rat cardiomyocytes [40] and human umbilical vein endothelial cells [41]. Nevertheless, disparities in cell physiology might explain the differential regulation of ERK 1/2 phosphorylation and activity by EPA in several cell types. Other investigations have reported that action of antidiabetic and anti-inflammatory drugs, such as metformin and salicylates, involves the inhibition of TNF-α-induced phosphorylation of ERK

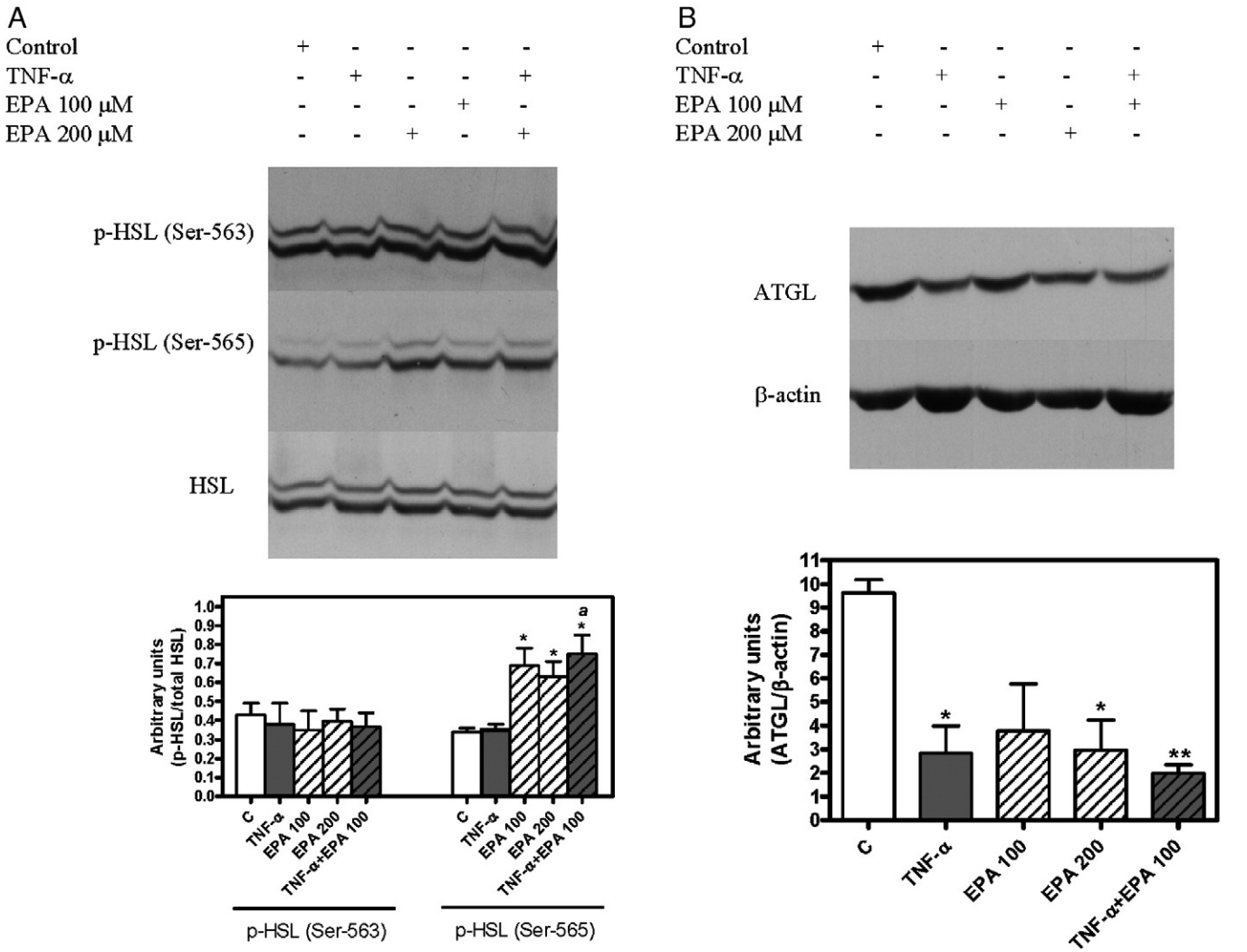

Fig. 5. Effects of EPA (100 and 200 μM) and/or TNF-α (10 ng/ml) on (A) HSL phosphorylation in Ser-563 and Ser-565 (30 min of treatment) and (B) ATGL protein levels (24 h of treatment) in 7-day postdifferentiated 3T3-L1 adipocytes. Representative blots of three independent experiments performed in whole cell lysate. *P<.05 vs. control; [a]P<.05 vs. TNF-α.

1/2 as a mechanism underlying the antilipolytic effect of these compounds in primary rat adipocytes [10,19]. In accordance with these results, our findings suggest that EPA attenuated TNF-α-induced phosphorylation of ERK 1/2 in adipocytes, which may account for EPA's antilipolytic properties.

AMP-kinase is a protein kinase that regulates important metabolic processes of liver, adipose tissue and muscle metabolism [42]. Several studies have suggested that AMPK activation could be involved in the insulin-sensitizing mechanisms of several antidiabetic drugs, such as metformin and thiazolidinediones [11], as well as omega-3 PUFAs [43–45]. In fact, a recent study of our group has demonstrated that EPA strongly stimulates AMPK activation in adipocytes [46]. Most of the studies have shown that AMPK activation inhibits lipolysis in adipocytes [9,47], although others have suggested a potential lipolytic role for AMPK [48]. This apparently contradictory data could be explained because the effects of AMPK activation on lipolysis seem to be time dependent. In fact, a recent study showed that AICAR-induced AMPK activation initially suppressed lipolysis, but a prolonged AICAR treatment led to an increase in lipolysis [49]. This could be surprising regarding our results, which suggested that the inhibitory action of EPA on basal and TNF-α-induced lipolysis after 24 h of treatment could be mediated through AMPK activation. Indeed, incubation with compound C (an inhibitor of AMPK) was able to block the antilipolytic effect of EPA on basal and TNF-α-induced lipolysis. Moreover, a synergistic antilipolytic action was observed with the AMPK-activator AICAR and EPA. However, differences in the type of cell culture, concentration of AICAR used (0.5 vs. 2 mM) and other experimental conditions could explain these differences.

AMP-kinase activation phosphorylates HSL at Ser-565, which inhibits HSL activity and thus prevents lipolysis in adipocytes [8,9]. In this context, we demonstrate that EPA also stimulates HSL phosphorylation on Ser-565, providing further evidence of a direct involvement of AMPK activation on the antilipolytic effects of EPA in adipocytes. Our data indicate that short (30′) and medium-term (24 h) treatment with EPA and/or TNF-α did not modify total HSL and protein contents (both at protein and at mRNA levels), suggesting that early effects on lipolysis relied more on changes in phosphorylation status rather than on a direct regulation of HSL and perilipin transcripts. However, longer treatment (96 h) with EPA as well as with TNF-α induced a significant decrease on both HSL and perilipin gene expression levels. Previous published studies have also shown that TNF-α induced a significant down-regulation on total HSL and perilipin content in human primary adipocytes after longer periods of treatment (48 h) [3,7]. Therefore, these data raise the possibility that HSL and perilipin might be regulated in a time-dependent manner, at least in our murine models of adipocytes.

Nevertheless, EPA could also affect lipolysis by regulating other lipases such as ATGL. We found that treatment with EPA and TNF-α

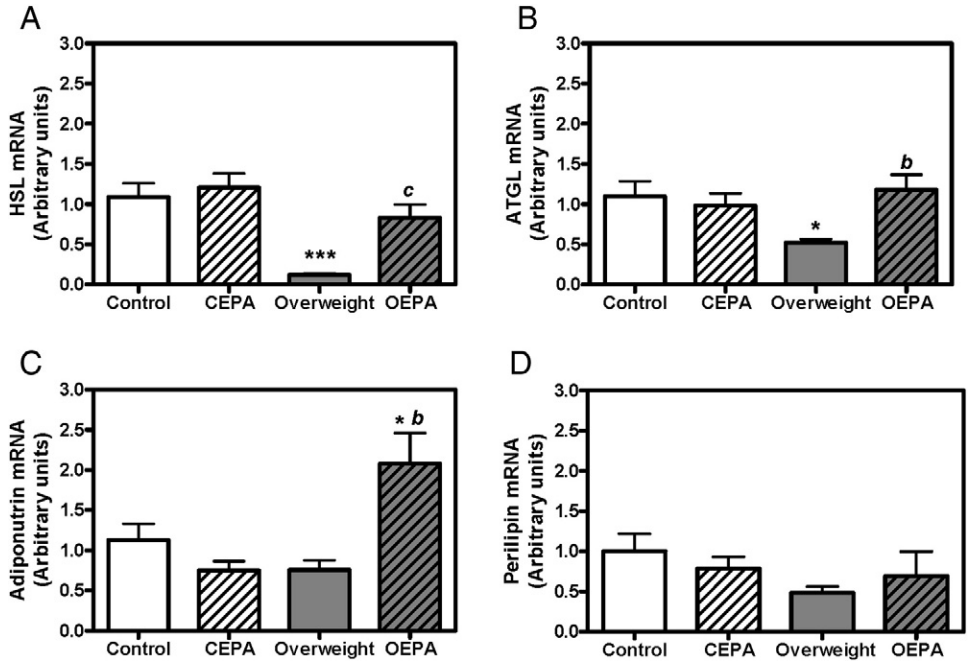

Fig. 6. Effects of oral supplementation with EPA (1 g/kg) on HSL (A), ATGL (B), adiponutrin (C) and perilipin (D) mRNA gene expression in retroperitoneal adipose tissue of rats fed a control diet or a high-fat diet during 5 weeks. *P<.05; ***P<.001 vs. control; ^{b}P<.01; ^{c}P<.001 vs. overweight group. CEPA, control+EPA; OEPA, overweight+EPA.

induced a decrease on ATGL protein levels. This inhibitory effect of TNF-α on ATGL content is in agreement with previously published studies [22,50]. However, these [22,50] and other authors [14] demonstrated that the antilipolytic hormone insulin also down-regulated ATGL in 3T3-L1 adipocytes. These interesting findings suggest that the inhibition of ATGL protein levels and gene expression (data not shown) in adipocytes by EPA could be related to potential insulin-like effects for this fatty acid, as previously described in several studies. Thus, EPA has been shown to up-regulate several insulin-induced activities including insulin receptor substrate-1-associated PI3K and Akt kinase activity in hepatoma cells [33]. Moreover, previous studies of our group have demonstrated that, like insulin, EPA stimulated glucose uptake, glucose oxidation to CO_2 and leptin production [51], as well as Akt phosphorylation [52], in adipocytes. Besides, recent published data in *Caenorhabditis elegans* suggest that ATGL-1 may be a direct inhibitory target of AMPK [53], although further studies are needed to address this possibility in mature adipocytes.

Our study showed that high-fat-diet-induced overweight rats exhibited an inhibition of HSL and ATGL gene expression levels in retroperitoneal adipose tissue, while adiponutrin and perilipin mRNA levels were not significantly modified. This suggests a lipase inhibition, which could promote fat storage and the enlargement of adipose tissue, contributing to increased body weight and adiposity as well as the development of insulin resistance observed in these animals [17]. These results are in agreement with data in human adipose tissue which describe that ATGL and HSL expression is decreased in obese and insulin-resistant subjects [54,55]. These authors described that this decrease in ATGL and HSL mRNA expression is associated with impaired insulin sensitivity. Our data also show that EPA supplementation was able to prevent the decrease in both HSL and ATGL mRNA expression levels observed in overweight animals. Together with the normalization in the gene expression levels of the two lipases, EPA reduced both the insulinaemia and the TNF-α up-regulation observed in adipose tissue of overweight rats as previously reported by our group [17]. In fact, we observed a negative correlation between HSL gene expression levels with basal insulin levels and HOMA, an index of insulin resistance. These data support previous *in vitro* studies suggesting that insulin and TNF-α also play a role in the transcriptional regulation of ATGL and HSL *in vivo*. In fact, EPA did not modify HSL or ATGL mRNA levels in rats fed with a standard diet, which did not exhibit hyperinsulinaemia or TNF-α up-regulation in adipose tissue. In accordance with our findings, Raclot et al. [56] did not find any significant effect of EPA on HSL gene expression levels in retroperitoneal adipose tissue of rats fed a standard control diet. In this context, Festuccia et al. [57] and Kershaw et al. [58] showed that treatment of mice with the PPAR-γ agonist rosiglitazone significantly increased ATGL and HSL mRNA expression, suggesting also that improved insulin sensitivity increases adipose tissue ATGL and HSL expression.

The differential effects of EPA treatment in lipase mRNA levels in lean and overweight rats might be initially surprising. However, in

Table 1
Correlations between HSL, ATGL, and adiponutrin mRNA levels with several indexes of obesity and insulin resistance

	HSL		ATGL		Adiponutrin	
	r	*P*	*r*	*P*	*r*	*P*
Body weight	−0.62	0.0003	−0.257	0.169	0.019	0.917
Adiposity	−0.496	0.0061	−0.115	0.550	0.294	0.121
Basal insulin	−0.384	0.047	−0.263	0.184	−0.115	0.565
HOMA index	−0.409	0.03	−0.265	0.190	−0.105	0.6

Homeostasis Model Assessment Index(HOMA) = [Glucose(mmol / L) × Insulin(mUI / L)] / 22.5.

previous studies with the same experimental groups, we have also reported differential effects of EPA supplementation between control and high-fat-fed rats mainly concerning food intake, leptin production and mRNA levels as well as TNF-α expression and apoptosis in WAT [17]. Therefore, our current data further support the differential effects of EPA supplementation depending on diet composition and physiological/metabolical status of animals, which could be of interest when considering supplementation with omega-3-enriched products.

A question that remains to be addressed is whether the observed changes on lipase expression reflect changes on lipolysis *in vivo*. In a previous study, we determined basal and isoproterenol-stimulated lipolysis in adipocytes isolated from retroperitoneal adipose tissue (*ex vivo*) of the four same experimental groups and found that EPA supplementation did not affect either basal or stimulated lipolysis [17]. These findings indicate that EPA does not modify *ex vivo* lipolysis either in lean or in overweight rats. Moreover, EPA supplementation did not change circulating FFA levels in these animals [17]. However, such data should be considered with caution since plasma FFA levels can be affected by β-oxidation and reesterification processes, which could be somehow regulated also by EPA [59]. In conclusion, with our present and previous data, we cannot totally rule out that there might be some alterations on lipolysis *in vivo* upon EPA supplementation.

Another intriguing aspect is the apparent lack of parallelism between the *in vitro* and *in vivo* data. However, it should be stated that it is not always possible to extrapolate *in vitro* data to *in vivo* situations since there are many limitations, and thus, it might be difficult to correlate findings observed under both approaches. In fact, on the *in vivo* situation, EPA is also affecting adipokine production as well as several metabolic processes not only in adipose tissue [17,34,60] but also in other key metabolic tissues such as liver and muscle [59], which could also contribute in some way to regulate lipase expression in WAT and lipolysis *in vivo*.

Regarding the regulation of adiponutrin, an opposite regulation between ATGL and adiponutrin in adipocytes and adipose tissue has been suggested [14]. In fact, insulin induces a dose-dependent decrease in ATGL expression; while several studies have described that the adiponutrin gene is up-regulated in response to insulin in a glucose-dependent way [61]. The increase in adiponutrin gene expression observed in obese rats that received an oral supplementation with EPA suggests that the regulation of adiponutrin mRNA levels by this fatty acid is not mediated by glucose levels and insulinaemia and might involve other mechanisms.

In conclusion, we have presented new evidence showing that dietary omega-3 EPA directly inhibits TNF-α-stimulated lipolysis in primary rat and 3T3-L1 adipocytes. This antilipolytic effect is mediated through several mechanisms, which involve the inhibition of proinflammatory pathways such as ERK 1/2 and NF-κB, as well as the stimulation of AMPK. We also provide first evidence about the regulatory role of EPA on HSL and ATGL both *in vitro* and *in vivo*. Our data support that the ability of EPA to prevent cytokine-stimulated lipolysis could contribute to explain the improvement in insulin sensitivity observed after EPA treatment.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2010.11.018.

Acknowledgments

This work has been funded by the Spanish Ministry of Science and Innovation (AGL2006-04716/ALI and AGL2009-10873/ALI and PCT-A3166019-2007) and by the University of Navarra (LE/97). Silvia Lorente-Cebrián is supported by a predoctoral grant FPU "Formación de Profesorado Universitario" from the Spanish Ministry of Education and Science. The technical assistance of A. Lorente, V. Ciaurriz and A. Redin is gratefully acknowledged.

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