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A B S T R A C T

Visfatin is an adipokine highly expressed in visceral adipose tissue (AT) of humans and rodents, the production of which seems to be dysregulated in excessive fat accumulation and conditions of insulin resistance. Eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid (PUFA), has been demonstrated to exert beneficial effects within obesity and insulin resistance conditions, which have been further linked to its reported ability to modulate adipokine production by adipocytes. TNF-α is a pro-inflammatory cytokine whose production is increased in obesity and involved in the development of insulin resistance. Control of adipokine production by some insulin-sensitizing compounds has been associated with the stimulation of AMP-activated protein kinase (AMPK). The aim of this study was to examine *in vitro* the effects of EPA on visfatin production and the potential involvement of AMPK both in the absence or presence of TNF-α. Treatment with the pro-inflammatory cytokine TNF-α (1 ng/mL) did not modify visfatin gene expression and protein secretion in primary cultured rat adipocytes. However, treatment of these primary adipocytes with EPA (200 μM) for 24 h significantly increased visfatin secretion ($p<0.001$) and mRNA gene expression ($p<0.05$). Moreover, the stimulatory effect of EPA on visfatin secretion was prevented by treatment with AMPK inhibitor Compound C but not with phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002. Similar results were observed in 3T3-L1 adipocytes. Moreover, EPA strongly stimulated AMPK phosphorylation alone or in

combination with TNF-α in 3T3-L1 adipocytes and pre-adipocytes. These results suggest that the stimulatory action of EPA on visfatin production involves AMPK activation in adipocytes.

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, adenosine 5' monophosphate protein kinase; AT, adipose tissue; DTT, dithiothreitol; EPA, eicosapentaenoic acid; ELISA, enzyme linked immunoassay; FFA, free fatty acids; HOMA, homeostasis model assessment; M-MLV, Moloney-Murine Leukemia Virus; Nampt, nicotinamide phosphoribosyltranferase; PBEF, pre-B cell colony-enhancing factor; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PPAR-γ, peroxisome proliferator-activated receptor-gamma; PUFA, polyunsaturated fatty acid; SDS, sodium dodecyl sulphate.

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INTRODUCTION

Visfatin/Nampt/PBEF is a newly discovered adipokine, which is mainly produced by visceral adipose tissue (AT) in both humans and rodents [1]. Conflicting results have been found regarding the role played by visfatin in obesity and insulin resistance. In fact, some studies have shown that visfatin production is enhanced during obesity and type 2 diabetes development [1, 2], while others have found that plasma visfatin is reduced in obesity [3, 4] and insulin resistance [5], and suggest that visfatin might not be related to insulin sensitivity in humans [6-8].

A further conflicting point concerning visfatins' action is its potential role as an insulin-mimetic which was initially shown by Fukuhara *et al.* [1]. In fact, they observed that visfatin significantly increased glucose uptake and triglycerides storage in 3T3-L1 adipocytes and L6 myocytes as well as decreased glucose production from hepatocytes. Moreover, *in vivo* administration of visfatin to KKAy rodents (obese and insulin-resistant) decreased circulating levels of glucose and insulin and thus, improved insulin sensitivity, which was also observed in streptozotocin-treated mice [1]. However, other investigators have not observed any insulin-mimetic effects of visfatin in adipogenesis, glucose uptake, insulin signalling in adipocytes or glucose lowering effects [9], and the original article [1] has been retracted [10].

Little is known about the regulation of visfatin production by adipocytes. It has been shown that glucose stimulates visfatin release in adipocytes. This stimulatory effect of glucose on visfatin secretion involves the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) pathways [11]. Moreover, visfatin gene expression is up-regulated by glucocorticoids and down-regulated by lipolytic agents (forskolin, isoprenaline and cholera toxin) in 3T3-L1 adipocytes [12, 13]. However, the role played by TNF-α and IL-6 as well as other pro-inflammatory cytokines in visfatin production is still controversial. Indeed, while some studies have observed that TNF-α and IL-6 inhibit visfatin synthesis in 3T3-L1 adipocytes [12, 14] and *in vivo* [15], an assay performed in human adipocytes has shown that treatment with TNF-α induces an up-regulation in visfatin production [16]. In order to better elucidate TNF-α actions on this adipokine, we will analyze its short (24 h) and long-term (96 h) effects on both visfatin secretion and gene expression in a model of primary cultured rat adipocytes anchored to a collagen matrix, which simulates basement membrane attachment, producing a more physiological environment [17].

A regulatory role for dietary fats, such as palmitate, a saturated fatty acid (FFA), and oleate (monounsaturated FFA) on visfatin gene expression has been also described in 3T3-L1 murine adipocytes [18].

Eicosapentaenoic acid (EPA) is a dietary polyunsaturated fatty acid (PUFA) from the omega-3 family (n-3 PUFA). EPA has been reported to exert beneficial effects on health, including improvements of inflammatory conditions, obesity, insulin resistance and type 2 diabetes [19-22]. Some of these actions have been related to the anti-inflammatory properties of n-3 PUFAs [19, 23] and to their ability to modulate adipokine production, such as adiponectin and leptin, both *in vivo* [19, 20] and *in vitro* [17, 24]. In this context, a recent study of our group has suggested that the insulin-sensitizing effects of *in vivo* EPA treatment in cafeteria fed animals could also be related to a stimulatory action on visfatin gene expression in visceral fat [21]. However, the direct effects of EPA on visfatin production have not yet been studied.

Several studies have reported the regulatory role of AMP-activated protein kinase (AMPK) activation on adipokine production, such as adiponectin [25]. In addition, the antidiabetic efficacy of some insulin sensitizers, such as metformin and

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glitazones, involves activation of AMPK [26]. Some studies have suggested that AMPK activation could be involved in EPA-induced improvements in insulin sensitivity [27]. However, controversial results have also been reported suggesting that dietary fish oils do not activate AMP-activated protein kinase in mouse tissues [28]. To our knowledge, there are no studies addressing the ability of EPA to modulate AMPK activation in adipocytes.

In the present study, we examined the direct effects of EPA on visfatin gene expression and protein secretion both in primary rat and in 3T3-L1 adipocytes. Additionally, we also investigated if EPA activates AMPK in 3T3-L1 adipocytes and the potential involvement of this kinase on the EPA's action on visfatin.

MATERIAL AND METHODS

Animals

Male Wistar rats were obtained from the Applied Pharmacobiology Center (CIFA-Pamplona, Spain). The animals were housed in cages in temperature-controlled rooms (22 ± 2ºC) with a light-dark cycle (12 hours: 12 hours). 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.

Adipocyte isolation and primary culture

Adipocytes were isolated from epididymal fat depots of Wistar rats, as previously described [24]. Briefly, AT fragments were digested with type I collagenase at 37ºC with gentle shaking for 30 minutes. The resulting cell suspension was diluted in HEPES buffer and then filtered through a 400 μm nylon mesh. The isolated adipocytes were washed three times and resuspended in low-glucose DMEM (5 mM) supplemented with 1% fetal bovine serum (FBS) and incubated for 30-40 minutes at 37ºC.

The isolated adipocytes were plated on 500 μL of a collagen matrix (Purecol, Inamed Biomaterials, CA) in six well culture plates and after 50 minutes incubation, culture media containing the different treatments were added and cells were cultured for 24 or 96 hours.

Cell culture and differentiation of 3T3-L1 cells

Mouse 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD) and differentiated as described previously [29]. Briefly, two days post-confluence pre-adipocytes were cultured for 48 hours in DMEM containing 25 mM glucose, 10% Fetal Bovine Serum (FBS), antibiotics and supplemented with dexamethasone (1 μM), isobutylmetilxantine (0.5 mM) and insulin (10 μg/mL). After that, cells were cultured with 10% FBS and insulin for 48 hours. Cells were then cultured without insulin until day 6-7 post-confluence when cells were completely differentiated to adipocytes. Prior to the addition of the appropriate treatments, cells were serum starved overnight and then treated.

Treatments

EPA (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol, TNF-α (Phoenix Peptide, Burlingame, CA) was dissolved in water, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, Sigma, St. Louis, MO), LY 294002 (Sigma) and Compound C (Calbiochem, La Jolla, CA) were dissolved in DMSO. All compounds were prepared in 1000x 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). EPA (100 and 200 μM) and/or TNF-α (1 ng/mL) and/or the AMPK stimulator AICAR (2 mM) were added to the media at the same time. However, when the selective

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PI3K inhibitor, LY 294002 (50 μM) and AMPK inhibitor Compound C (20 μM) were used, adipocytes were pre-incubated for 30 minutes with the inhibitors prior to the addition of EPA (200 μM).

Assays

The total amount of visfatin secreted into culture media was determined through the use of an enzyme linked immunosorbent assay (ELISA) kit, for rat/mouse visfatin (Alpco Diagnostics, Salem, NH).

Analyses of mRNA

Total RNA was extracted according to the procedure of Trizol (Invitrogen, Grand Island, NY). Following this, the RNA was incubated with *RNAse-free* DNase kit (Ambion, Austin, TX). 1 μg of RNA was reverse-transcribed to cDNA by using Moloney-Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen). Visfatin mRNA and 18S levels were determined by quantitative real-time polymerase chain reaction (PCR) using the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed as previously described [24]. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

3T3-L1 adipocytes were cultured and induced to differentiate as previously described [29]. AMPK activation was determined both in mature adipocytes (7 days post-differentiation) and in pre-confluent (20-30%) pre-adipocytes. Cells were serum-starved overnight (18 hours) and then incubated with the appropriate treatment.

Cells (0.5×10^6) were washed in PBS, lysed in 200 μL of double strength sodium-dodecyl sulfate sample buffer containing dithiothreitol [60 % glycerol, 150 mM Tris-HCl (pH 6.8), 2 mM DTT, 2 % SDS, Bromophenol blue] and heat-denatured before resolution in 7.5 % sodium-dodecyl sulfate-polyacrylamide gel electrophoresis. Protein immunoblotting was performed as previously described [30]. Briefly, proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) and then stained with Ponceau red solution to verify equal loading of proteins. Membranes were then blocked for 2 hours at room temperature in TBS-Tween-20 [50 mM Tris-HCl (pH 7.6), 200 mM NaCl and 0.1% Tween-20] with 2% BSA (fraction V, Sigma) containing 10% (w/v) non-fat dried milk, washed twice and then incubated overnight with phospho-AMPKα (Thr-172) antibody (Cell Signaling Technology, Beverly, MA) diluted 1:1000. After further washings, membranes were incubated with horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG (Cell Signaling) diluted 1:5000 in TBS-Tween-20 for 1 hour. p-AMPKα (Thr-172) was visualized on Amersham Hyperfilm (GE Healthcare) using the CL+Chemiluminescence kit (GE Healthcare). For reprobing of the Western blots, antibodies were stripped from membranes using the stripping buffer [62.5 mM TrisHCl (pH 6.8) 2% SDS and 100 mM 2-mercaptoethanol] for 30 minutes at 50ºC, washing the membranes with TBS-Tween20, and after making sure that the original signal was removed, we reprobed with AMPKα (Cell Signalling) diluted 1:1000 and incubated overnight. Then, the bands were visualized as mentioned above.

Data Analysis

Results are given as mean values ± standard error. The statistical analysis was performed by repeated measures one-way ANOVA, followed by a Dunnet´s *post* test, or by a two-way ANOVA. Differences were considered as statistically significant at $p<0.05$. The statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

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RESULTS

Effects of EPA on visfatin secretion in primary cultured rat adipocytes

Incubation of primary rat adipocytes with EPA at 200 μM induced an increase on basal visfatin secretion as compared to control cells both at 24 hours (169.7±18.0% of control, $p<0.001$) and 96 hours (122.5±10.8% of control, $p<0.05$) of treatment (Fig. 1A). This stimulatory effect of EPA on visfatin seems to be dose-dependent, since no significant changes on visfatin release were observed when adipocytes were incubated with lower doses of EPA (data not shown).

Taking into account that some studies have described that TNF-α alters visfatin production in adipocytes, we tested the effects of the pro-inflammatory cytokine in our model of primary cultured rat adipocytes and their potential ability to reverse EPA-stimulatory actions on visfatin. However, in our model of cultured adipocytes, the presence of pro-inflammatory cytokine TNF-α (1 ng/mL) did not modify visfatin secretion after short (24 hours) or long-term (96 hours) treatment. Interestingly, the stimulatory effect of EPA 200 μM on visfatin secretion was also observed in the presence of TNF-α in the media.

The expression pattern of the visfatin gene after 24 hours-treatment was similar to those observed for visfatin secretion. Thus, incubation of adipocytes with EPA alone or in combination with TNF-α significantly increased visfatin mRNA gene expression levels (Fig. 1B).

Effects of AICAR, Compound C and LY 294002 on EPA-stimulated visfatin production in primary cultured rat adipocytes and murine 3T3-L1 adipocytes

Using the same model of primary cultured rat adipocytes, we observed that treatment with the AMPK inhibitor Compound C (20 μM) abolished the stimulatory action of EPA on visfatin release. We also determined the effects of EPA (200 μM) in combination with the AMPK stimulator AICAR (2 mM). Interestingly, co-treatment of EPA+AICAR further stimulated visfatin production (163.8±20.1% of control for EPA, $p<0.01$ and 185.1±23.3% of control for EPA+AICAR-treated cells, $p<0.01$), suggesting a certain additive effect of both agents. However, PI3K inhibitor LY 294002 (50 μM) did not significantly modify the stimulatory effect induced by EPA on visfatin secretion (Fig. 2A).

In order to verify the role of AMPK in the stimulatory action of EPA on visfatin, we also tested the ability of Compound C to reverse EPA actions on visfatin in 3T3-L1 adipocytes. In agreement with the data observed in primary adipocytes, incubation of mature 3T3-L1 adipocytes with EPA (200 μM) induced a strong increase on visfatin secretion after treatment during 24 hours (130.2±13.4% of control, $p<0.05$). Interestingly, this effect was significantly prevented ($p<0.01$) by the addition of AMPK-inhibitor Compound C (Fig. 2B) but not with the PI3K inhibitor LY 294002 (data not shown), as previously observed in primary cultured rat adipocytes. The pattern of the effects of EPA on visfatin gene expression was similar to the results obtained for visfatin secretion in 3T3-L1 adipocytes. Thus, visfatin mRNA levels were significantly ($p<0.05$) enhanced after 24 hours of treatment with EPA 200 μM, while the presence of Compound C prevented the stimulatory effect of EPA on visfatin gene expression (Fig. 2C). These findings suggest a potential involvement of AMPK on EPA-stimulated visfatin production in murine adipocytes.

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Effects of EPA on AMPK activation in 3T3-L1 adipocytes

In order to assess whether EPA directly stimulates AMPK activation, 3T3-L1 adipocytes and pre-adipocytes were treated with EPA (100 μM and 200 μM).

Incubation of both 3T3-L1 mature adipocytes (Fig. 3A) and pre-adipocytes (Fig. 3B) with EPA resulted in a positive activation of AMPK in a dose-dependent manner. In contrast, the pro-inflammatory cytokine TNF-α (1 ng/mL) did not phosphorylate AMPK (Thr-172) in 3T3-L1 adipocytes. Interestingly, the presence of EPA (100 and 200 μM) in TNF-α treated adipocytes also stimulated AMPK phosphorylation to similar levels to what was observed in adipocytes treated with EPA alone. As expected, AICAR (2 mM) induced a strong increase on AMPK activation, while the co-treatment with Compound C (20 μM) was able to partially block the AMPK phosphorylation, induced by both EPA and AICAR.

All these results clearly demonstrate the ability of EPA to activate AMPK phosphorylation (Thr-172) in 3T3-L1 cells (both adipocytes and pre-adipocytes).

DISCUSSION

Several studies have reported the ability of n-3 PUFA to improve insulin-sensitivity by controlling adipokine production [22]. In fact, EPA regulates leptin and adiponectin production *in vitro* [17, 24] and *in vivo* [19, 27, 31].

A recent study of our group also demonstrated the ability of EPA ethyl ester administration to prevent the decrease of visfatin gene expression observed in high fat diet-induced obese rats. An inverse relationship with Homeostasis Model Assessment (HOMA), an index of insulin resistance, was also found, suggesting that the insulin-sensitizing effects of EPA could be related to its stimulatory action on visfatin gene expression in visceral fat [21]. Here, the finding that a direct stimulatory effect of EPA on both visfatin gene expression and protein secretion in primary adipocytes is relevant. Thus, it suggests that the up-regulation of visfatin gene expression in visceral AT observed in our previous study after *in vivo* EPA administration was not only due to the reducing effects of EPA treatment on the size of this fat depot, but also by a direct transcriptional up-regulation of visfatin gene by this n-3 PUFA.

Other studies have reported the ability of dietary FA to modulate visfatin gene expression. In contrast to n-3 PUFA EPA, saturated FFA palmitate and monounstaturated FFA oleate, down-regulated visfatin mRNA gene expression in 3T3-L1 adipocytes and pre-adipocytes [18]. Moreover, this down-regulation of visfatin was mentioned as a potential mechanism to directly induce insulin resistance by oleate and palmitate *in vitro* [18]. In this context, it has been also shown that a synthetic mixture including stearic, oleic, linoleic, linolenic and arachidonic acid normalized the increase in visfatin release induced by treatment with the insulin-sensitizing PPAR-γ (peroxisome proliferator-activated receptor-gamma) agonist rosiglitazone, in human isolated adipocytes [32]. These findings suggest a differential regulation of visfatin depending of the type of dietary fat and support our hypothesis that visfatin up-regulation by EPA could be another mechanism by which omega-3 PUFAs may improve insulin sensitivity.

We have tested if incubation with 1 ng/mL of TNF-α, a dose known to induce lipolysis and inflammatory markers in our model of cultured adipocytes (data not shown), was able to reverse the EPA-stimulated visfatin secretion. Our data have revealed that TNF-α did not induce any significant change on visfatin secretion, both in the presence or absence of EPA after 24 and 96 hours of treatment. This fact suggests that in our culture conditions, the stimulatory action of EPA on visfatin seems not to be

related to n-3 PUFAs ability to block the inflammatory pathway [19]. The available data in the literature concerning the regulation of visfatin by pro-inflammatory cytokines are controversial. Indeed, some studies have observed that TNF-α and IL-6 induced a *time*- and *dose*-dependent down-regulation of visfatin gene expression levels in 3T3-L1 adipocytes [12, 14]. However, an assay performed in human adipocytes has shown that visfatin mRNA levels were highly increased after 24-72 h of incubation with TNF-α [16]. The disparity observed may be related to differences in the type (primary *vs* cell lines) and source (rat, mouse or human) of cultured adipocytes, the concentration of TNF-α used and the duration of the treatment.

AMPK is a protein kinase that regulates important metabolic processes, whose activation induces multiple effects on liver, AT and muscle metabolism [33]. AMPK activation has been associated to the regulation of adipokines production, such as adiponectin [25]. Recently, long chain fatty acyl analogs, which are AMPK activators, have been proposed as potential agents for treating type 2 diabetes [34]. Thus, some studies have suggested that AMPK activation could be involved in EPA-induced improvements on insulin sensitivity [27], although there was no data showing evidence of a direct activation of AMPK by EPA in adipocytes. Our present results clearly demonstrated that treatment of adipocytes with EPA strongly stimulated AMPK activation. These data are in accordance with previous published studies which reported that polyunsaturated fatty acids induced activation of this signal transduction protein in liver [35]. Moreover, a recent study also demonstrated an increase in intestinal glucose absorption potentially via acute activation of AMPK after n-3 PUFAs feeding during gestation in pigs [36]. However, other investigators did not find any changes in AMPK phosphorylation in mouse tissues (liver, skeletal muscle and heart) after feeding a diet supplemented with fish oil [28].

An interesting finding of our study was that the stimulatory effect of EPA on visfatin secretion in adipocytes involved AMPK activation pathway, but not PI3K. In support of our study, a recent work performed in myocytes has also demonstrated that cells exposed to AICAR or glucose restriction, which also increase AMPK activity, had increased Nampt/visfatin mRNA expression and protein levels, indicating that AMPK regulates Nampt expression induced by glucose restriction in these insulin-target cells [37].

EPA has been shown to stimulate PI3K pathway in hepatoma cells [38] and adipocytes (data not shown). Moreover, glucose-stimulated visfatin secretion involves the PI3K/Akt pathway [11]. However, the fact that PI3K inhibitor treatment did not modify EPA-stimulated visfatin release either in primary rat and 3T3-L1 adipocytes, suggest that PI3K signalling pathway is not likely to be involved in the stimulatory effect of EPA on visfatin secretion.

In summary, our data show for the first time that EPA increases visfatin expression and secretion in primary adipocytes, and that AMPK activation is intimately involved in the EPA-stimulatory action on visfatin production. These mechanisms could account for the insulin-sensitizer actions of EPA.

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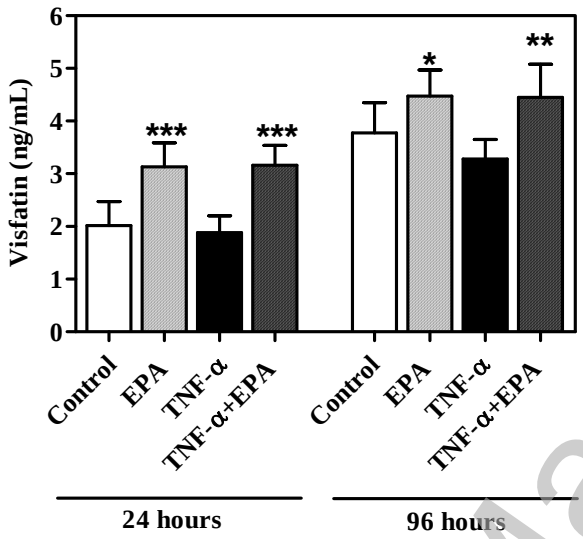
Figure 1:

A)



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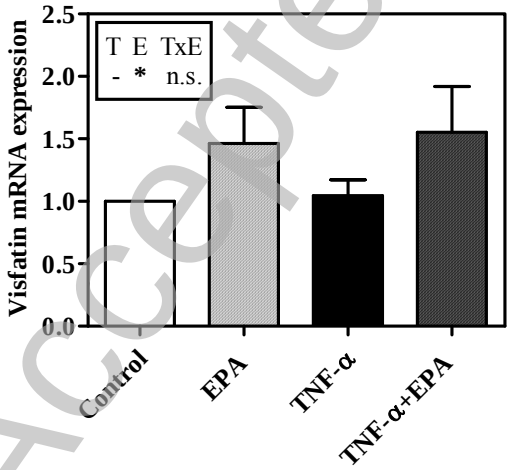
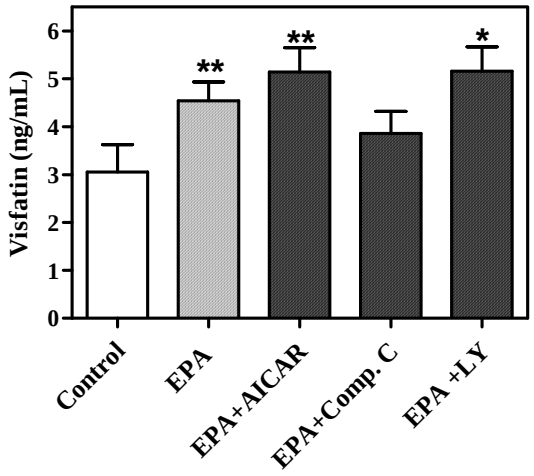


Figure 2:

A)



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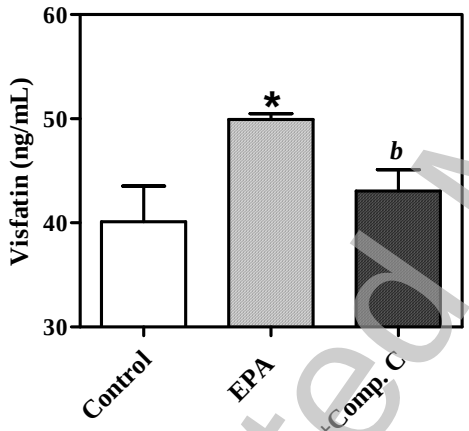

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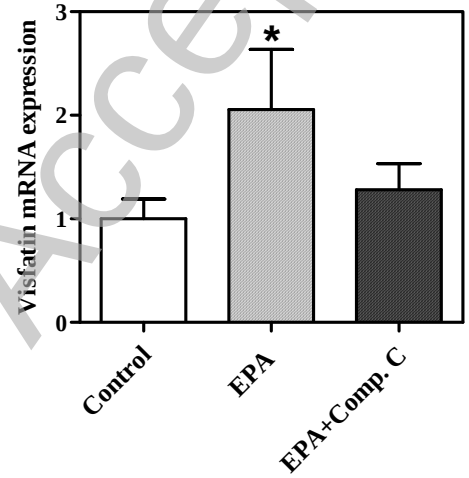

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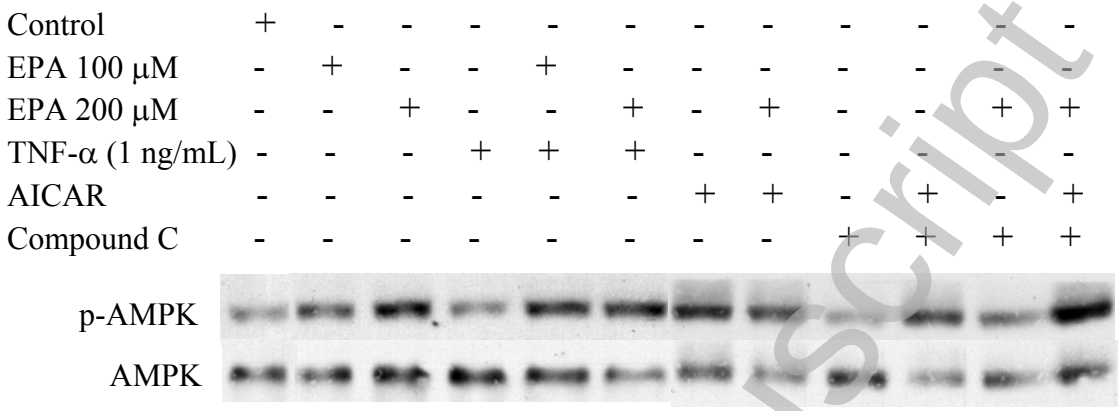
Figure 3:

A) 3T3-L1 adipocytes (7 days post-differentiation).



B) 3T3-L1 pre-adipocytes.

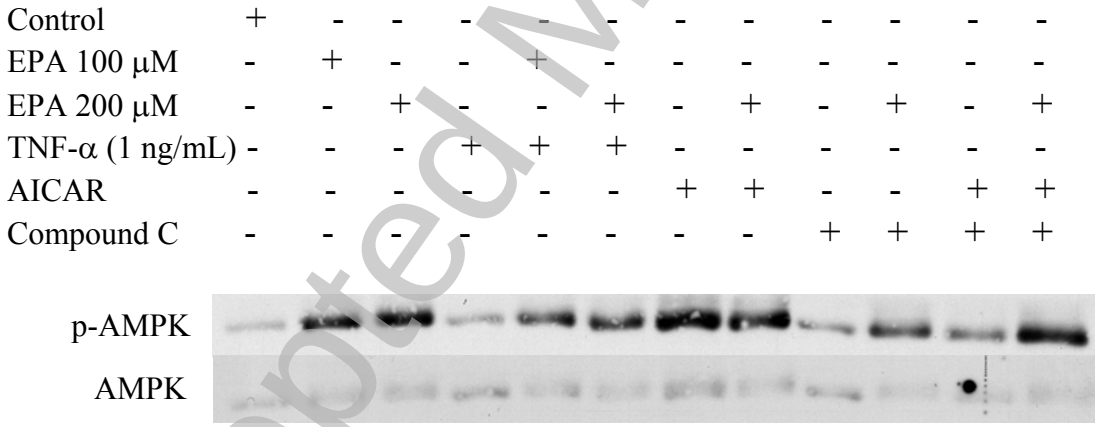


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Figure legends

Figure 1. (A) Effects of EPA (200 μM) and TNF-α (1 ng/mL) on visfatin secretion in isolated primary cultured rat adipocytes over 24 and 96 hours of treatment. *p<0.05; **p<0.01 and ***p<0.001 as compared to control. (B) Visfatin mRNA gene expression levels after 24 hours EPA treatment with and without TNF-α. Gene expression data were analysed by two-way ANOVA (*p<0.05). Results are representative of at least five independent experiments.

Figure 2. (A) Effects of EPA (200 μM) on visfatin secretion alone or in the presence of AICAR (2 mM), Compound C (20 μM), or LY 294002 (50 μM) after 24 hour treatment in primary cultured rat adipocytes. Effects of EPA (200 μM) on visfatin secretion (B) and mRNA gene expression (C) in the absence or presence of Compound C (20 μM) in 3T3-L1 adipocytes. *p<0.05 and **p<0.01 as compared to control cells ^{b}p<0.01 as compared to EPA-treated cells. Results are representative of 4-6 independent experiments.

Figure 3. Analysis of AMPK activation in 3T3-L1 adipocytes (A) and pre-adipocytes (B) 30 minutes after treatment with EPA (100 and 200 μM) in presence and absence of TNF-α (1 ng/mL), AICAR (2 mM) and Compound C (20 μM). Whole cell lysates were subjected to gel electrophoresis and immunoblotted using specific antibodies for p-AMPKα (Thr-172) and AMPKα as described in material and methods. Representative blots of three independent experiments showing the effects of different treatments on AMPK phosphorylation.