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RESEARCH ARTICLE

Eicosapentaenoic acid up-regulates apelin secretion and gene expression in 3T3-L1 adipocytes

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Recent studies have shown the ability of apelin to restore glucose tolerance in obese and insulin-resistant mice. Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid (PUFA) from the omega-3 family that has many beneficial effects in obesity-linked disorders. The aim of this study was to examine *in vitro* the effects of EPA on apelin secretion and gene expression in mature 3T3-L1 adipocytes. Treatment with EPA (100 and 200 μM) significantly increased basal ($p<0.01$) and insulin-stimulated ($p<0.001$) apelin secretion and gene expression in adipocytes. EPA also stimulated Akt phosphorylation, a down-stream target of phosphatidylinositol 3-kinase (PI3K), in 3T3-L1 adipocytes. Moreover, treatment with the PI3K inhibitor LY294002 completely blocked EPA-stimulatory action on apelin mRNA gene expression ($p<0.001$), but not modified the stimulatory effect of EPA on basal apelin secretion. Furthermore, the stimulatory effect of EPA on basal apelin release was also observed in the presence of Actinomycin D and Cycloheximide, suggesting that EPA might also regulate apelin secretion by *via* post-transcriptional mechanisms. These findings suggest that the mechanisms mediating EPA-induced apelin synthesis and/or secretion are complex, involving steps that are PI3K dependent and steps that are PI3K independent.

Keywords:
Adipocytes / Akt / Apelin / Eicosapentaenoic acid / Insulin resistance

1 Introduction

Apelin is a newly identified adipokine produced by adipocytes as well as other cell types such as cardiomyocytes, and whose production is dysregulated in obesity and insulin resistance [1, 2]. Many studies have reported an up-regulation of apelin production in obesity associated to hyperinsulinemia both in humans and in rodents [1, 3, 4] while no changes were observed on apelin production when glucose and insulin levels remained in normal values [1]. Over-production of apelin in obesity has been suggested to be one of the last protections before the development of obesity-linked disorders such as type 2 diabetes and cardiovascular diseases [5, 6]. In fact, apelin restored glucose tolerance and increased glucose utilization in obese and insulin-resistant mice suggesting that apelin represents a promising target in the management of insulin resistance [7]. This supports the increasing interest on the study of the regulation of apelin production by adipocytes.

A strong relationship between adipocyte-secreted apelin and insulin has been suggested. Thus, insulin up-regulated apelin secretion and expression both *in vivo* [1] and *in vitro* [1, 8, 9]. In addition, other studies have demonstrated that the pro-inflammatory cytokine Tumour Necrosis Factor-alpha (TNF-α), which has been described to induce insulin

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Abbreviations: ACE, angiotensin-converting enzyme; **EPA,** eicosapentaenoic acid; **HOMA,** homeostasis model assessment; **MAPK,** mitogen-activated protein kinase; ***n*-3 PUFA,** omega-3 polyunsaturated fatty acid; **PI3K,** phosphatidylinositol 3-kinase; **TNF-α,** tumour necrosis factor-α

resistance in obesity [10], also increased apelin secretion and gene expression in human and mice adipose tissue [2]. Moreover, apelin gene expression is up-regulated by Growth Hormone [8] and down-regulated by dexamethasone in 3T3-L1 adipocytes [9].

Regarding the signaling pathways involved in apelin production in adipocytes, a direct contribution of phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways on apelin production has been suggested [1, 2, 8]. In fact, inhibition of PI3K/Akt and MAPK pathways blocked the stimulatory action of insulin [1], TNF-α [2] and Growth Hormone [8] on apelin secretion and gene expression in adipocytes.

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid (*n*-3 PUFA) that is found in fish and fish oils. Many interesting and important benefits on health have been attributed to EPA such as improvements of inflammatory conditions, obesity, insulin resistance and type 2 diabetes [11–13]. Many of these actions have been related to the ability of *n*-3 PUFAs to regulate adipokines production such as leptin and adiponectin *in vitro* [14, 15] and *in vivo* [16, 17].

In this context, a recent study of our group has demonstrated an up-regulation of apelin mRNA in visceral adipose tissue after EPA supplementation in high-fat fed rats, which was negatively correlated with the homeostasis model assessment (HOMA) index, a marker of insulin resistance. This fact suggested that the insulin-sensitizing properties of EPA *in vivo* might be related to this stimulatory action on apelin gene expression in white adipose tissue [18]. However, it still remains to be established if EPA stimulates apelinergic system by direct or indirect mechanisms.

The aims of this study were to examine the EPA direct effects on basal and insulin-stimulated apelin secretion and gene expression in adipocytes. Besides, we also investigated the potential mechanisms and signaling pathways involved in the regulation of basal apelin production by EPA in adipocytes.

2 Materials and methods

2.1 Cell culture and differentiation of 3T3-L1 cells

Mouse 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD) and were differentiated as described previously [19, 20]. Briefly, 2 days post-confluence pre-adipocytes were cultured for 48 h in DMEM (Invitrogen, Grand Island, NY) containing 25 mM glucose, 10% fetal bovine serum (Invitrogen), antibiotics and supplemented with dexamethasone (1 μM; Sigma, St. Louis, MO), isobutylmethylxantine (0.5 mM; Sigma) and insulin (10 μg/mL; Sigma). After that, cells were cultured with 10% fetal bovine serum and insulin for 48 h and then, adipocytes were cultured without insulin until day 6–8 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 with or without EPA (100 and 200 μM; Cayman Chemical, Ann Arbor, MI) and/or insulin (1.6 nM) for 24 h. When the selective PI3K inhibitor, LY 294002 (Sigma) was used, adipocytes were pre-incubated for 30 min with LY 294002 (50 μM; Sigma) prior to the addition of EPA and/or insulin. Actinomycin D (5 μg/mL; Sigma) and Cycloheximide (10 μg/mL; Sigma) were added at the same time as EPA. Control cells were treated with the same amount of vehicle (DMSO and/or Ethanol).

2.2 Assays

The total amount of apelin released to the media was determined by ELISA for mouse/rat apelin from Phoenix Peptide (Burlingame, CA) [21].

2.3 Analyses of apelin mRNA levels

Total RNA was extracted according to the procedure of Trizol (Invitrogen) and then, 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 Reverse Transcriptase (Invitrogen). Apelin mRNA and Cyclofilin levels were determined by quantitative real-time PCR using the ABI PRISM 7000HT Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed as described previously [15]. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4 Western blot analysis

3T3-L1 cells were cultured and induced to differentiate as described previously [19, 20]. Akt activation was performed in 7 days post-differentiation cells. Cells were serum-starved overnight (18 h) and then incubated with the appropriate treatment.

Western blot was performed [22] using antibodies specific for p-Akt (Ser-473) and Akt from Cell Signalling Technologies (Beverly, MA). Equal loading of samples was confirmed by Ponceau S staining.

2.5 Measurement of intracellular apelin

Intracellular apelin levels were determined in control and EPA-treated adipocytes after 24 h of treatment. Medium was discarded and cells were lysed with 200 μL of lysis buffer (2mM Tris, pH 8, 0.137 mM NaCl, glycerol 10%, 1 mM sodium ortovanadate, 2 mM EDTA, 1 mM PMSF and 1% protease inhibitor) and then incubated for 30 min at 4°C. After the incubation period, cell lysates were centrifuged at 200 *g* for 10 min at 4°C and supernatant was collected in

order to determine intracellular apelin levels. The measurement of apelin concentration in the whole cell lysate was quantified by using apelin mouse/rat ELISA kit from Phoenix Peptide (Burlingame) according to the manufacturer's protocol.

2.6 Data analysis

Results are given as mean values and SDs. The statistical analysis was performed by repeated measures one-way ANOVA followed by Tukey *post hoc* test or by a paired Student's *t*-test in order to determine differences between groups. Differences were considered as statistically significant at $p<0.05$. The statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

3 Results

3.1 EPA stimulates apelin secretion and mRNA expression in 3T3-L1 adipocytes

To determine whether EPA could be directly involved in the regulation of apelin production, we investigated apelin secretion and mRNA expression in 3T3-L1 mature adipocytes.

Treatment of 3T3-L1 adipocytes with EPA (100 and 200 μM) induced a strong increase on basal apelin secretion after 24 h (EPA 100 μM: +227.6±83.5% of control, $p=0.0343$ and EPA 200 μM: +236.8±75.4% of control, $p=0.0044$) (Fig. 1A).

Apelin mRNA levels were also significantly increased by EPA treatment reaching significant differences for EPA 200 μM (+178.0±73.2% of control, $p=0.0333$). These data showed that the stimulatory effect of EPA on apelin gene expression was less potent than the increase observed in apelin secretion, suggesting that EPA's-stimulatory action on apelin secretion is likely to be secondary, only in part, to the increase observed in apelin gene expression (Fig. 1B).

3.2 EPA potentiates insulin-stimulated apelin secretion

Treatment of adipocytes with insulin (1.6 nM) for 24 h induced a significant increase on apelin secretion (+184.7±68.1% of control, $p=0.018$). Interestingly, co-treatment of insulin and EPA (200 μM) further enhanced apelin secretion (+447.1±133.3% of control, $p=0.0003$), suggesting an additive effect of both agents (Fig. 2A). Regarding to the effects on apelin gene expression, treatment with insulin induced an increase in apelin mRNA levels (+136.0±37.5% of control, $p=0.0049$) similarly as the effects observed for apelin secretion. Besides, this

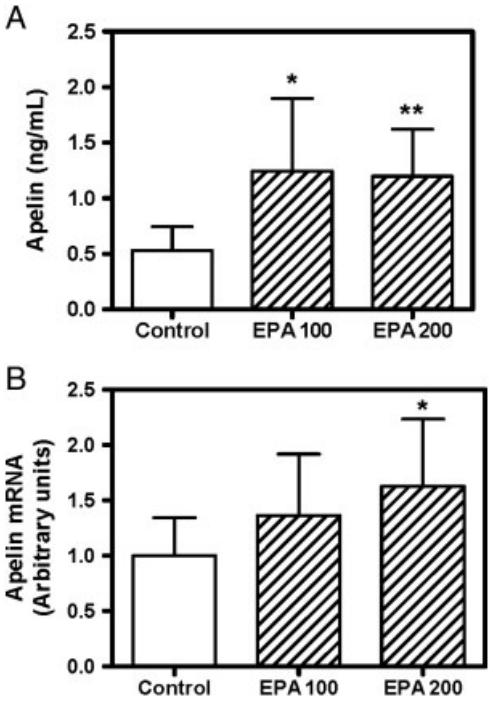

Figure 1. Effects of EPA (100 and 200 μM) on apelin secretion (A) and mRNA levels (B) in mature adipocytes after 24 h of treatment. $^{*}p<0.05$; $^{**}p<0.01$ as compared with control. Results are representative of at least five to six independent experiments. Data are expressed as mean±SD.

stimulatory effect of insulin was also enhanced by the presence of EPA (+205.5±54.2% of control, $p=0.0159$), which is consistent with the increase in apelin production as described previously (Fig. 2B).

3.3 EPA induces Akt phosphorylation and PI3K inhibitor LY 294002 abrogates EPA-stimulated apelin gene expression

Insulin-stimulated apelin production is known to be regulated by several signaling pathways including PI3K/Akt and MAPK. To further investigate the mechanisms involved in EPA's stimulatory effect on the regulation of apelin production, we tested whether PI3K/Akt and/or MAPK could be a potential intracellular target involved in EPA-induced up-regulation of apelin production.

Treatment of mature 3T3-L1 adipocytes with EPA did not modify phosphorylation of ERK 1/2, the main protein involved in MAPK signaling pathway. However, our data showed that incubation of 3T3-L1 mature adipocytes with EPA resulted in a dose-dependent activation of Akt (Ser-473) (Fig. 3A), demonstrating the ability of EPA to activate PI3K/Akt pathway in adipocytes.

We also tested the effects of treatment with the PI3K inhibitor LY 294002 (50 μM) on apelin gene expression and protein secretion. Figure 3B shows that the presence of LY

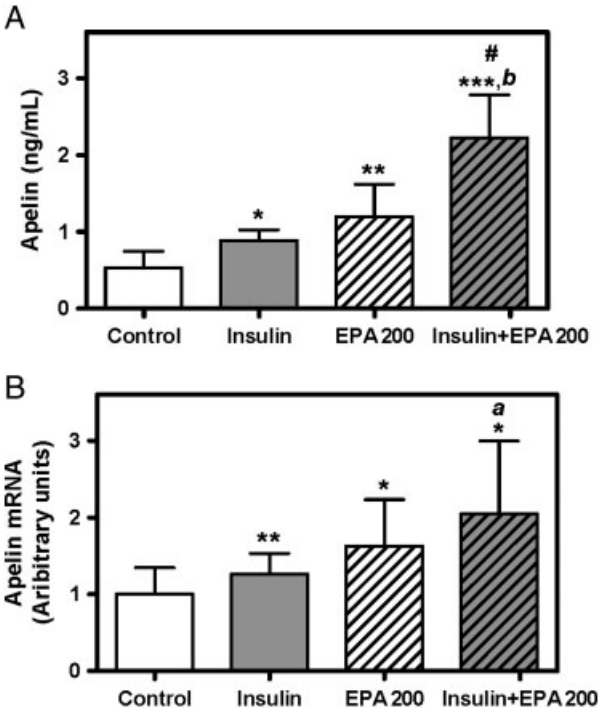

Figure 2. Effects of EPA (200 μM) and insulin (1.6 nM) on apelin secretion (A) and apelin gene expression (B) in mature 3T3-L1 adipocytes after 24 h of treatment. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ as compared with control cells. $^{a}p<0.05$; $^{b}p<0.01$ as compared with insulin-treated cells. $^{\sharp}p<0.05$ as compared with EPA-treated cells (200 μM). Results are representative of at least five to six independent experiments. Data are expressed as mean ± SD.

294002 significantly decreased basal (LY: −92.3 ± 1.0 of control, $p = 0.001$) and EPA-stimulated apelin mRNA levels (EPA 100 μM: −87.6 ± 6.3%, $p = 0.0008$ and EPA 200 μM: −84.4 ± 12.2%, $p = 0.0009$, as compared with control). This fact demonstrates that PI3K/Akt pathway is essential for the intracellular up-regulation of apelin gene expression by EPA in adipocytes.

However, treatment of adipocytes with PI3K inhibitor LY 294002 (50 μM) did not modify the stimulatory effect of EPA on apelin secretion. Surprisingly, LY *per se* also stimulated apelin secretion (Fig. 3C).

3.4 Effects of Actinomycin D and Cycloheximide on EPA-stimulated apelin secretion and gene expression

To further characterize the regulation of EPA-induced apelin production, we next examined the effects of Actinomycin D, a transcriptional inhibitor, and Cycloheximide, an inhibitor of protein synthesis, on EPA-stimulated apelin secretion and gene expression in 3T3-L1 adipocytes.

As expected, the transcriptional inhibition with Actinomycin D completely blocked apelin gene expression

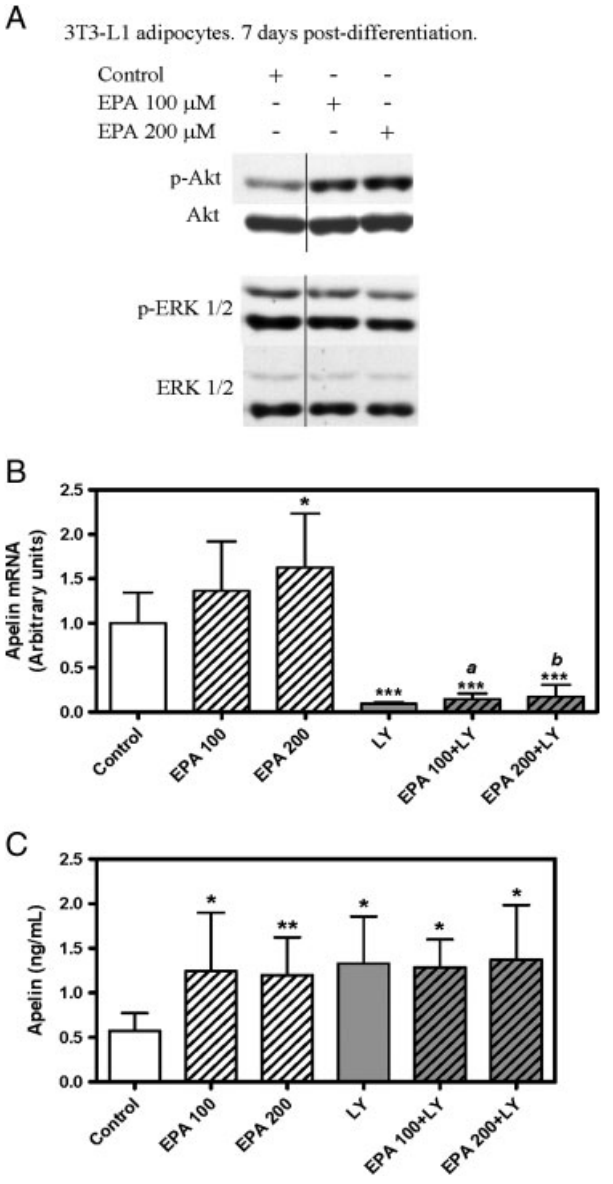

Figure 3. (A) Analysis of Akt (Ser-473) and ERK 1/2 (Thr-202/Tyr-204) activation in mature 3T3-L1 adipocytes after treatment for 30 min with EPA (100 and 200 μM). (B and C) Effects of EPA on apelin mRNA and apelin secretion in 3T3-L1 adipocytes after 24 h of EPA treatment (100 and 200 μM) alone or in the presence of PI3K inhibitor LY 294002 (50 μM). Differentiated 3T3-L1 adipocytes were pre-incubated for 30 min with LY 294002 prior to the addition of EPA. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ as compared with control. $^{a}p<0.05$ and $^{b}p<0.01$ as compared with EPA-treated cells (100 and 200 μM). Results are representative of at least six independent experiments. Data are expressed as mean ± SD.

(Fig. 4A), however, did not modify the stimulatory action of EPA on apelin secretion and even moderately enhanced the total amount of apelin released to the media (Fig. 4B). Interestingly, we also found that apelin secretion (basal and EPA-stimulated) was not modified by treatment

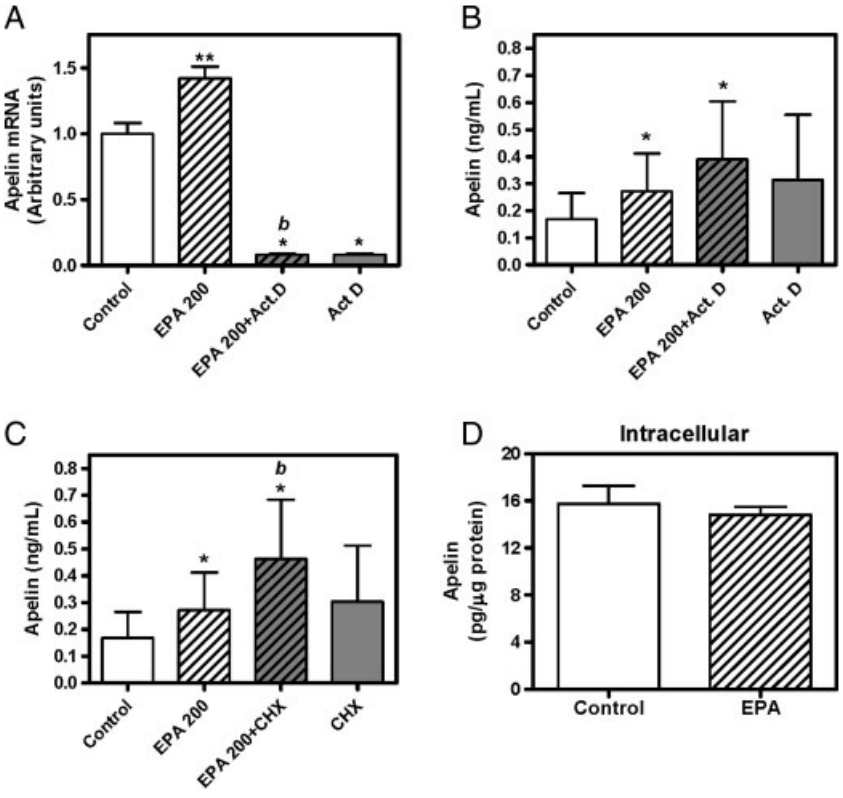

Figure 4. Effects of Actinomycin D (5 μg/mL) on EPA-stimulated apelin mRNA expression (A) and secretion (B) and effects of Cycloheximide (10 μg/mL) on apelin secretion (C) in mature 3T3-L1 adipocytes. (D) Intracellular apelin content in control and EPA-treated adipocytes during 24 h. $^{*}p<0.05$; $^{**}p<0.01$ as compared with control. $^{b}p<0.01$ as compared with EPA-treated adipocytes. Results are representative of three to four independent experiments. Data are expressed as mean ± SD.

with the protein synthesis inhibitor Cycloheximide (Fig. 4C).

In order to test if EPA stimulated the release of a stored cytosolic pool of apelin, we analyzed the intracellular apelin levels. The data showed that EPA treatment during 24 h did not modify intracellular levels of this adipokine (Fig. 4D). No significant changes were either observed after short periods of EPA treatment (15–240 min, data not shown).

4 Discussion

Many studies have reported an up-regulation of apelin production in obesity associated to hyperinsulinemia both in humans and in rodents [1, 3, 4]. However, it has been suggested that the over-production of apelin in obesity could be one of the last protections before the emergence of the obesity-related disorders such as type 2 diabetes [5]. In this context, a recent study of our group has demonstrated that an oral supplementation with EPA ethyl ester induced a significant increase in apelin gene expression in adipose tissue of rats fed a high fat diet. Moreover, HOMA, an index of insulin resistance, was negatively correlated with apelin mRNA suggesting that to some extent, the insulin-sensitizing effects of EPA could be also related to its stimulatory action on apelin gene expression in visceral fat [18], although the potential contribution of apelin coming from other tissues such as heart can not be ruled out.

In this study, we report a stimulatory action of EPA (100–200 μM) on both apelin secretion and gene expression in mature 3T3-L1 adipocytes. These concentrations of EPA were used based on the previously published data concerning tissue distribution of orally administered esterified EPA in animals, suggesting that these doses are comparable to its plasma concentrations after intake of dietary EPA [23]. Moreover, several studies supported the effectiveness of similar concentrations of EPA in adipocyte biology and function, including the regulation of adipogenesis [24], the secretion of different adipokines such as leptin [14, 25] adiponectin [15], and visfatin [20], as well as the suppression of the pro-inflammatory effects of TNF-α in adipocytes co-cultured with macrophages [26].

Our present data in cultured adipocytes suggest that the stimulation of apelin gene expression observed after EPA supplementation *in vivo* is likely to be secondary to the ability of EPA to directly modulate the mechanisms involved in apelin synthesis and secretion by the adipocytes.

Our data agree with previous studies showing that insulin up-regulated apelin production in adipocytes [1, 9]. However, the fold of change in apelin in response to insulin was not as strong as described in other studies [1, 9, 27], because we used a lower concentration of insulin (1.6 nM *versus* 50–100 nM), which is closer to the physiological range of insulin *in vivo*. We also found that the stimulatory action of insulin on apelin secretion and expression was significantly increased in the presence of EPA, suggesting that

EPA and insulin have a certain additive effect on apelin production. Previous studies have demonstrated that the direct stimulation of apelin by insulin is clearly associated with the activation of PI3K/Akt and MAPK pathways [1]. Our data suggest that EPA stimulates apelin by a MAPK-independent manner, since we demonstrate that EPA did not modify phosphorylation of ERK 1/2, the main protein involved in MAPK signalling pathway. However, EPA stimulated the phosphorylation of Akt, a down-stream target of insulin receptor substrate-1 (IRS-1) and a key regulator of insulin signalling pathway [28]. In this way, previously published data in hepatoma cells have also demonstrated the ability of EPA to stimulate both basal and insulin-induced tyrosine phosphorylation of insulin receptor substrate-1, insulin receptor substrate-1-associated PI3K, and its downstream target Akt kinase activity [29]. Moreover, our data demonstrated that the presence of PI3K inhibitor LY 294002 completely abrogated the up-regulation of basal apelin gene expression by EPA-treatment, suggesting that the transcriptional up-regulation on apelin expression induced by EPA is mediated *via* PI3K/Akt pathway. Although, it still remains to be determined, taken together, our present and previous [29] data, it is likely to hypothesize that the stimulatory action of EPA on insulin-induced apelin gene, might be also mediated, at least in part, by activation of PI3K/Akt pathway.

PI3K is a key player in vesicle trafficking in adipocytes and other cell types. It transduces a myriad of growth and metabolic effects of insulin, including, but not limited to, stimulation of glucose transport, gene transcription, and insulin-stimulated protein synthesis [30]. However, the absence of effects of LY 294002 on the amount of EPA-induced apelin secreted to the media, suggest that EPA seems to regulate apelin production also by mechanisms independent of the PI3K/Akt pathway. We also observed that treatment with the PI3K inhibitor increased apelin secretion as much as EPA or co-administration did. These findings suggest that the mechanisms mediating basal- or EPA-induced apelin synthesis and/or secretion are complex, involving steps that are PI3K dependent and steps that are PI3K independent.

The fact that EPA caused a higher stimulation on apelin protein secretion than on gene expression, raised the possibility that EPA might regulate apelin production *via* post-transcriptional mechanisms. In fact, the presence of Actinomycin D did not modify the enhancement induced by EPA on apelin secretion, supporting the involvement of post-transcriptional mechanisms in the regulation of apelin production. Moreover, the lack of effect of Cycloheximide on EPA-stimulated apelin secretion suggests that apelin secretion does not depend on protein synthesis. This fact raises the possibility that apelin might be secreted to the media from a pre-existing pool present in mature adipocytes or that the post-transcriptional stimulation of apelin by EPA might be dependent on apelin degradation rather than altered protein secretion.

Several studies have reported that some adipokines, as leptin, are likely to be secreted in part from a pre-existing intracellular pool in adipocytes [30, 31]. However, our present data argue against the possibility that EPA might enhance the release of a pre-existing cytosolic pool of apelin. A previous study in cardiomyocytes also support that there is a storage form of apelin, although these cells do not have large stores of apelin, and then secretion relies mostly on the expression and synthesis of *de novo* apelin [32]. These apparently conflicting results might be due to differences on the cell type used (cardiomyocytes *versus* adipocytes) and thus, to the regulation mechanism of apelin secretion.

Taken together, all our present data suggest that EPA-induced apelin secretion does not depend on protein synthesis but rather on protein degradation. A similar post-transcriptional regulatory mechanism has been proposed for glucagon-induced aquaporin-8 expression [33]. Vickers *et al.* reported that angiotensin-converting enzyme (ACE2) was able to hydrolyze bioactive apelin-13 and apelin-36 [34]. It has been suggested that *n*-3 PUFA and their metabolites could function as ACE enzyme inhibitors [35, 36]. Moreover, a recent study has demonstrated that ACE2 is expressed in mouse adipocytes and regulated by a high-fat diet [37]. These facts suggest the possibility that EPA could up-regulate apelin secretion in adipocytes by inhibiting ACE2, its hydrolyzing enzyme, although this speculative issue requires further investigation. Based on this hypothesis, inhibition of protein synthesis with Cycloheximide could cause a decrease in the protein involved in apelin degradation, and therefore increasing the amount of apelin available to be secreted. This would explain the increased secretion of apelin in EPA+Cycloheximide *versus* EPA-treated adipocytes.

Our study demonstrates for the first time that apelin production by adipocytes can be regulated by fatty acids. However, it still remains to be determined if the ability of EPA to stimulate apelin is unique for this fatty acid or is also shared by other C:20 fatty acids or other PUFAs. In this context, previous studies of our group and others have observed differential effects on the regulation of other adipokines, such as leptin and visfatin, depending on the type of fatty acid. Thus, we demonstrated that EPA [14] but not DHA (unpublished data) stimulated leptin gene expression and secretion by primary rat adipocytes. In contrast, arachidonic [38], linoleic [39], and conjugated linoleic [40] acids inhibited leptin production. Moreover, in contrast with the *n*-3 PUFA EPA [20], the saturated palmitate and mono-unsaturated oleate down-regulated visfatin mRNA gene expression in 3T3-L1 adipocytes [41].

In summary, the results presented here show that EPA stimulates both basal and insulin-stimulated apelin secretion in adipocytes. EPA-stimulated apelin secretion seems to be mediated by both transcriptional and post-transcriptional mechanisms. Further studies are necessary to more precisely define the molecular components involved in these processes.

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The authors have declared no conflict of interest.

5 References

[1] Boucher, J., Masri, B., Daviaud, D., Gesta, S. *et al.*, Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005, *146*, 1764–1771.

[2] Daviaud, D., Boucher, J., Gesta, S., Dray, C. *et al.*, TNFalpha up-regulates apelin expression in human and mouse adipose tissue. *FASEB J.* 2006, *20*, 1528–1530.

[3] Li, L., Yang, G., Li, Q., Tang, Y. *et al.*, Changes and relations of circulating visfatin, apelin, and resistin levels in normal, impaired glucose tolerance, and type 2 diabetic subjects. *Exp. Clin. Endocrinol. Diabetes* 2006, *114*, 544–548.

[4] Castan-Laurell, I., Vitkova, M., Daviaud, D., Dray, C. *et al.*, Effect of hypocaloric diet-induced weight loss in obese women on plasma apelin and adipose tissue expression of apelin and APJ. *Eur. J. Endocrinol.* 2008, *158*, 905–910.

[5] Castan-Laurell, I., Boucher, J., Dray, C., Daviaud, D. *et al.*, Apelin, a novel adipokine over-produced in obesity: friend or foe? *Mol. Cell. Endocrinol.* 2005, *245*, 7–9.

[6] Carpéné, C., Dray, C., Attané, C., Valet, P. *et al.*, Expanding role for the apelin/APJ system in physiopathology. *J. Physiol. Biochem.* 2007, *63*, 359–373.

[7] Dray, C., Knauf, C., Daviaud, D., Waget, A. *et al.*, Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab.* 2008, *8*, 437–445.

[8] Kralisch, S., Lossner, U., Bluher, M., Paschke, R. *et al.*, Growth hormone induces apelin mRNA expression and secretion in mouse 3T3-L1 adipocytes. *Regul. Pept.* 2007, *139*, 84–89.

[9] Wei, L., Hou, X., Tatemoto, K., Regulation of apelin mRNA expression by insulin and glucocorticoids in mouse 3T3-L1 adipocytes. *Regul. Pept.* 2005, *132*, 27–32.

[10] Hotamisligil, G. S., Shargill, N. S., Spiegelman, B. M., Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993, *259*, 87–91.

[11] Mori, T. A., Bao, D. Q., Burke, V., Puddey, I. B. *et al.*, Dietary fish as a major component of a weight-loss diet: effect on serum lipids, glucose, and insulin metabolism in overweight hypertensive subjects. *Am. J. Clin. Nutr.* 1999, *70*, 817–825.

[12] Mori, T. A., Beilin, L. J., Omega-3 fatty acids and inflammation. *Curr. Atheroscler. Rep.* 2004, *6*, 461–467.

[13] Flachs, P., Rossmeisl, M., Bryhn, M., Kopecky, J., Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin. Sci. (Lond.)* 2009, *116*, 1–16.

[14] Pérez-Matute, P., Marti, A., Martínez, J. A., Fernández-Otero, M. P. *et al.*, Eicosapentaenoic fatty acid increases leptin secretion from primary cultured rat adipocytes: role of glucose metabolism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005, *288*, R1682–R1688.

[15] Lorente-Cebrian, S., Perez-Matute, P., Martinez, J. A., Marti, A., Moreno-Aliaga, M. J., Effects of eicosapentaenoic acid (EPA) on adiponectin gene expression and secretion in primary cultured rat adipocytes. *J. Physiol. Biochem.* 2006, *62*, 61–69.

[16] Flachs, P., Mohamed-Ali, V., Horakova, O., Rossmeisl, M. *et al.*, Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia* 2006, *49*, 394–397.

[17] Pérez-Matute, P., Pérez-Echarri, N., Martínez, J. A., Marti, A., Moreno-Aliaga, M. J., Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor-alpha. *Br. J. Nutr.* 2007, *97*, 389–398.

[18] Moreno-Aliaga, M. J., Pérez-Echarri, M. N., Pérez-Matute, P., Marcos, B. *et al.*, Effects of EPA ethyl ester on visfatin and apelin in control and diet-induced obese (DIO) rats. *Diabetes* 2008, *59*, A2574–PO.

[19] Moreno-Aliaga, M. J., Matsumura, F., Effects of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (p,p′-DDT) on 3T3-L1 and 3T3-F442A adipocyte differentiation. *Biochem. Pharmacol.* 2002, *63*, 997–1007.

[20] Lorente-Cebrian, S., Bustos, M., Marti, A., Martinez, J. A., Moreno-Aliaga, M. J., Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. *Clin. Sci. (Lond.)* 2009, *117*, 243–249.

[21] Porstmann, T., Kiessig, S. T., Enzyme immunoassay techniques. An overview. *J. Immunol. Methods* 1992, *150*, 5–21.

[22] Bustos, M., Beraza, N., Lasarte, J. J., Baixeras, E. *et al.*, Protection against liver damage by cardiotrophin-1: a hepatocyte survival factor up-regulated in the regenerating liver in rats. *Gastroenterology* 2003, *125*, 192–201.

[23] Ishiguro, J., Tada, T., Ogihara, T., Mizota, M. *et al.*, Metabolism of ethyl eicosapentaenoate (EPA-E) in rats and effect of its metabolites on ellagic acid-induced thrombus formation in the stenosed femoral artery of rabbits. *Chem. Pharm. Bull. (Tokyo)* 1988, *36*, 2158–2167.

[24] Tanabe, Y., Matsunaga, Y., Saito, M., Nakayama, K., Involvement of cyclooxygenase-2 in synergistic effect of cyclic stretching and eicosapentaenoic acid on adipocyte differentiation. *J. Pharmacol. Sci.* 2008, *106*, 478–484.

[25] Murata, M., Kaji, H., Takahashi, Y., Iida, K. *et al.*, Stimulation by eicosapentaenoic acids of leptin mRNA expression and its secretion in mouse 3T3-L1 adipocytes *in vitro*. *Biochem. Biophys. Res. Commun.* 2000, *270*, 343–348.

[26] Itoh, M., Suganami, T., Satoh, N., Tanimoto-Koyama, K. *et al.*, Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler. Thromb. Vasc. Biol.* 2007, *27*, 1918–1925.

[27] Glassford, A. J., Yue, P., Sheikh, A. Y., Chun, H. J. *et al.*, HIF-1 regulates hypoxia- and insulin-induced expression of apelin in adipocytes. *Am. J. Physiol. Endocrinol. Metab.* 2007, *293*, E1590–E1596.

[28] Gonzalez, E., McGraw, T. E., Insulin signaling diverges into Akt-dependent and -independent signals to regulate the recruitment/docking and the fusion of GLUT4 vesicles to the plasma membrane. *Mol. Biol. Cell* 2006, *17*, 4484–4493.

[29] Murata, M., Kaji, H., Iida, K., Okimura, Y., Chihara, K., Dual action of eicosapentaenoic acid in hepatoma cells: up-regulation of metabolic action of insulin and inhibition of cell proliferation. *J. Biol. Chem.* 2001, *276*, 31422–31428.

[30] Bradley, R. L., Cheatham, B., Regulation of ob gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes* 1999, *48*, 272–278.

[31] Roh, C., Thoidis, G., Farmer, S. R., Kandror, K. V., Identification and characterization of leptin-containing intracellular compartment in rat adipose cells. *Am. J. Physiol. Endocrinol. Metab.* 2000, *279*, E893–E899.

[32] Ronkainen, V. P., Ronkainen, J. J., Hanninen, S. L., Leskinen, H. *et al.*, Hypoxia inducible factor regulates the cardiac expression and secretion of apelin. *FASEB J.* 2007, *21*, 1821–1830.

[33] Soria, L. R., Gradilone, S. A., Larocca, M. C., Marinelli, R. A., Glucagon induces the gene expression of aquaporin-8 but not that of aquaporin-9 water channels in the rat hepatocyte. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2009, *296*, R1274–R1281.

[34] Vickers, C., Hales, P., Kaushik, V., Dick, L. *et al.*, Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.* 2002, *277*, 14838–14843.

[35] Kumar, K. V., Das, U. N., Effect of cis-unsaturated fatty acids, prostaglandins, and free radicals on angiotensin-converting enzyme activity in vitro. *Proc. Soc. Exp. Biol. Med.* 1997, *214*, 374–379.

[36] Das, U. N., Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective molecules. *Lipids Health Dis.* 2008, *7*, 37.

[37] Gupte, M., Boustany-Kari, C. M., Bharadwaj, K., Police, S. *et al.*, ACE2 is expressed in mouse adipocytes and regulated by a high-fat diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2008, *295*, R781–R788.

[38] Perez-Matute, P., Marti, A., Martinez, J. A., Moreno-Aliaga, M. J., Effects of arachidonic acid on leptin secretion and expression in primary cultured rat adipocytes. *J. Physiol. Biochem.* 2003, *59*, 201–208.

[39] Perez-Matute, P., Martinez, J. A., Marti, A., Moreno-Aliaga, M. J., Linoleic acid decreases leptin and adiponectin secretion from primary rat adipocytes in the presence of insulin. *Lipids* 2007, *42*, 913–920.

[40] Perez-Matute, P., Marti, A., Martinez, J. A., Fernandez-Otero, M. P. *et al.*, Conjugated linoleic acid inhibits glucose metabolism, leptin and adiponectin secretion in primary cultured rat adipocytes. *Mol. Cell. Endocrinol.* 2007, *268*, 50–58.

[41] Wen, Y., Wang, H. W., Wu, J., Lu, H. L. *et al.*, Effects of fatty acid regulation on visfatin gene expression in adipocytes. *Chin. Med. J. (Engl.)* 2006, *119*, 1701–1708.

