Journal of Physiology and Biochemistry (2019) 75:341–349
https://doi.org/10.1007/s13105-019-00685-5

ORIGINAL ARTICLE

The regulation of inflammation-related genes after palmitic acid and DHA treatments is not mediated by DNA methylation

Mirian Samblas[1] · Julia C. Carraro[2] · J. Alfredo Martínez[1,3,4,5] · Fermín I. Milagro[1,3,4]

Received: 12 November 2018 / Accepted: 2 May 2019 / Published online: 19 August 2019

Abstract

Fatty acids (FAs) are known to participate in body inflammatory responses. In particular, saturated FAs such as palmitic acid (PA) induce inflammatory signals in macrophages, whereas polyunsaturated FAs, including docosahexaenoic acid (DHA), have been related to anti-inflammatory effects. Several studies have suggested a role of fatty acids on DNA methylation, epigenetically regulating gene expression in inflammation processes. Therefore, this study investigated the effect of PA and DHA on the inflammation-related genes on human macrophages. In addition, a second aim was to study the epigenetic mechanism underlying the effect of FAs on the inflammatory response. For these purposes, human acute monocytic leukaemia cells (THP-1) were differentiated into macrophages with 12-O-tetradecanoylphorbol-13-acetate (TPA), followed by an incubation with PA or DHA. At the end of the experiment, mRNA expression, protein secretion, and CpG methylation of the following inflammatory genes were analysed: interleukin 1 beta (*IL1B*), tumour necrosis factor (*TNF*), plasminogen activator inhibitor-1 (*SERPINE1*) and interleukin 18 (*IL18*). The results showed that the treatment with PA increased IL-18 and TNF-α production. Contrariwise, the supplementation with DHA reduced IL-18, TNF-α and PAI-1 secretion by macrophages. However, the incubation with these fatty acids did not apparently modify the DNA methylation status of the investigated genes in the screened CpG sites. This research reveals that PA induces important pro-inflammatory markers in human macrophages, whereas DHA decreases the inflammatory response. Apparently, DNA methylation is not directly involved in the fatty acid-mediated regulation of the expression of these inflammation-related genes.

Keywords Epigenetics · Cytokines · Macrophages · PUFA · Saturated fatty acids

Introduction

Fatty acids (FAs) are involved in a variety of functions such as energy metabolism, components of cell membranes and regulators of gene transcription [17]. Circulating and tissue abnormal FA levels have long been recognised to participate in human diseases including inflammation [43]. The excessive amounts of some FAs can be detected by innate recognition receptors, resulting in the activation of inflammatory signalling pathways, and defined as metabolic inflammation [19]. For instance, saturated fatty acids (SFAs), such as lauric and palmitic acid (PA), are able to stimulate inflammation by the activation of toll-like receptor (TLR) signalling pathway [43]. Conversely, diverse monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) may have beneficial effects on inflammation by generating eicosanoids and molecules involved in the resolution of inflammation and healing [26, 27, 43, 48].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13105-019-00685-5) contains supplementary material, which is available to authorized users.

✉ Fermín I. Milagro
fmilagro@unav.es

[1] Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, University of Navarra, Irunlarrea 1, 31008 Pamplona, Navarra, Spain

[2] Department of Clinical and Social Nutrition, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil

[3] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain

[4] Navarra Institute for Health Research (IdiSNA), Pamplona, Spain

[5] IMDEA Food, Research Institute of Food & Health Sciences, Madrid, Spain

Inflammatory process requires a sophisticated regulatory mechanism to carry out functions at signal- and gene-specific levels, where monocytes and macrophages are the main cells involved in the inflammatory response [46]. In general, circulating monocytes migrate to inflamed region and differentiate into monocyte-derived macrophages [28]. Cells must be able to phenotypically adapt continuously their response by the expression, production and secretion of different inflammatory mediators, to an environmental change, such as diet [47]. In this sense, important steps of inflammation as macrophage migration and cytokine secretion are regulated by epigenetic mechanisms. For example, genes coding for interleukins or interferons have been described to be differently methylated in inflammatory disorders [12].

DNA methylation is an extensively studied epigenetic mark in the mammalian genome in relation to aberrant gene expression in disease pathogenesis [41]. Thus, investigations have evidenced that DNA methylation profiles might also be modified by environmental factors including the restriction or supplementation with different nutrients [32, 35]. In this sense, it is understandable that FAs are between those factors that modify cells' methylome, being DNA methylation one of the mechanisms that link FAs and the modification of inflammatory response [30].

To our knowledge, there are scarce studies about the impact of different SFAs and PUFAs on the inflammatory response of human macrophages, neither the role of DNA methylation. Therefore, the present work aimed to investigate the effect of PA and docosahexaenoic acid (DHA) on the expression of inflammatory markers and the possible epigenetic mechanisms underlying the fatty acids' effect on pro-inflammatory genes.

Material and methods

Cell culture, differentiation, and treatments

Human acute monocyte cells (THP-1) were purchased from American Type Cell Culture (ATCC® TIB-202™, VA, USA) and maintained at 37 °C and 5% CO_2 in RPMI-1460 medium (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate, 4500 mg/l glucose, 10% fetal bovine serum (Gibco) and 100 U/ml penicillin, 100 μg/ml streptomycin.

Cells were grown at a density of 6×10^5 cells/ml in a 24-well plate. For THP-1 monocyte differentiation into macrophages, 25 ng/ml of phorbol 12-myristate 13-acetate (PMA) was added to the medium, and the monocytes were incubated for 48 h at 37 °C in a 5% CO_2 incubator. After the differentiation, THP-1 macrophages were treated with palmitic and docosahexaenoic (DHA) acids at a concentration of 80 μM during 30 h at 37 °C based on previous studies [2, 3]. Palmitic acid and DHA were dissolved in ethanol and both of them were then mixed with bovine serum albumin (BSA) in a 1:2 ratio (BSA/fatty acid) such was previously described [15]. Finally, RNA, DNA and culture supernatants were collected for further analysis.

Gene expression

RNA from THP-1 cells was extracted using TRIzol reagent (Life Technologies, CA, USA), following the manufacturer's instructions. RNA concentration was quantified through Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). cDNA was obtained from approximately 1 μg of total RNA using MultiScribe™ Reverse Transcriptase Kit according to manufacturer's protocol (Thermo Fisher Scientific, MA, USA). In quantitative real-time PCR analyses, the relative expression of genes was quantified using Taqman primers, Taqman Universal Master Mix (Applied Biosystems, CA, USA) and ABI Prism 7900HT Sequence Detection System. The amplification was conducted in duplicate in 10 μl with the following steps: 50 °C 2 min, 95 °C 10 min, and 40 cycles of 95 °C 15 s and 60 °C 1 min. The references of the specific predesigned Taqman primers and probes were the following: *IL1B* (Hs01555410_m1), *TNF* (Hs00174128_m1), *IL18* (Hs01038788_m1) and *SERPINE1* (Hs01126606_m1). Gene expression was calculated by using the comparative $2^{-\Delta\Delta Ct}$ method and with *GAPDH* (Hs02758991_g1) as a housekeeping gene. According to this method, the relative gene expression is set to 1 for reference gene expression because ΔΔCt is equal to 0 as 2^0 is 1 [40]. The intra-assay CV was 1.28% for *IL1B*, 0.61% for *TNF*, 0.38% for *IL18* and 0.47% for *SERPINE1*.

Cytokine detection analysis

Cytokine secretion in cell medium was determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits for human TNF-α (DTA00C, R&D Systems, MN, USA), PAI1 (DTSE100, R&D Systems, MN, USA) and IL-18 (#7620, MBL International Corporation, MA, USA). The samples were run in duplicate, and the intra-/inter-assay CV were 4.9/7.6% for TNF-α, 5.7/8.4% for PAI1 and 7.2/7.5% for IL-18. Absorbance was quantified at 450 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland).

DNA methylation assay

DNA isolation from THP-1 macrophages was performed with MasterPure™ DNA Purification Kit (Epicentre, WI, USA) following the manufacturer's guidelines. Genomic DNA was sodium bisulfite-converted using the EpiTect Bisulfite Kit (Qiagen, CA, USA). The following DNA methylation quantification was performed by MassARRAY EpiTYPER

technology (Sequenom Inc., CA, USA), that is a tool for the quantitative analysis of DNA methylation, consisting on a combination of matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry and base-specific cleavage [10]. Four amplicons corresponding to *IL18*, *IL1B*, *SERPINE1* and *TNF* genes covering 26 CpG sites were selected. These sequences were selected based on (i) the sequences had been previously studied by our group or by other groups; (ii) the sequences presented a high number of CpG in the promoter region; (iii) we were able to design primers for amplifying the sequence. The designed primers and the complete amplicon sequences are reported previously [44]. The primers were designed by EpiDesigner software (Sequenom Inc.; http://www.epidesigner.com/start3.html) for the amplicons of interest: *IL18* (chr11: 112,163,853-112,164,105), *IL1B* (chr2: 112,837,566-112,837,895), *SERPINE1* (chr7: 101,127,068-101,127,400), and *TNF* (chr6: 31,575,209-31,575,481). The mean percentage of DNA methylation across each amplicon was measured for each duplicate of CpG sites for each sample.

Transcription factor-binding site analysis

In order to identify the putative transcription factor binding sites in the studied regions of the *IL18* and *TNF* genes, a bioinformatic analysis was performed through LASAGNA-Search 2.0 using TRANSFAC matrices and aligned models, as described elsewhere [24], TFBIND (tfbind.hgc.jp), ALGGEN (alggen.lsi.upc.es) and TRANSFAC® databases.

Statistical analysis

Differences between groups were calculated using Student's *t* test for the direct comparisons between two groups and one-way ANOVA test followed by Dunnett's test for multiple comparisons. Data are presented as mean ± SEM. *p* values less than 0.05 were accepted as statistically significant. Statistical analyses and graphics were performed using GraphPad Prism 5.0 (GraphPad Software, CA, USA).

Results

Treatment with fatty acids altered inflammatory-related gene expression

In the present study, we have tested whether the treatment with FA had any influence on inflammation-related genes in THP-1 macrophages. Specifically, we studied the effect of one saturated fatty acid, PA and one polyunsaturated fatty acid, DHA, on the expression of genes associated with the inflammatory response, such as *IL18*, *TNF*, *IL1B* and *SERPINE1*. Macrophages treated with 80 μM PA increased *TNF* ($p < 0.01$) mRNA levels in comparison with the control (Fig. 1). Moreover, when the macrophages were incubated with 80 μM DHA, the expression of *IL18* ($p < 0.05$) and *TNF* ($p < 0.05$) was lower than the control condition, while the expression of *SERPINE1* ($p < 0.001$) was higher. No statistical changes were observed in the mRNA levels of *IL1B* after the incubation with palmitic acid and DHA.

Effects of fatty acids on cytokine secretion

In order to evaluate the effect of these fatty acids on the inflammatory response, the levels of pro-inflammatory cytokines IL-18, TNF-α and PAI-1 in the medium of THP-1 macrophages were determined. The results evidenced that PA stimulation significantly increased the levels of IL-18 ($p < 0.01$) and TNF-α ($p < 0.05$) in the supernatants of macrophages, when compared with the control group (Fig. 2). Of note, incubation with DHA significantly decreased IL-18 ($p < 0.01$), TNF-α ($p < 0.01$) and PAI-1 ($p < 0.01$) secretion.

Effects of fatty acids on the DNA methylation status of inflammation-related genes

Regarding gene promoter methylation analysis after the incubation with PA, as shown in Fig. 3, only three of the 26 CpG sites analysed, CpG_6 ($p < 0.001$) of *IL18*, CpG_1 ($p < 0.001$) and CpG_7 ($p < 0.001$) of *SERPINE1* genes, showed statistical differences in DNA methylation levels between treated and control groups. When the cells were incubated with DHA, the methylation levels of CpG_6 ($p < 0.01$) of *IL18*, CpG_1 ($p < 0.001$) and CpG_8 ($p < 0.05$) of *SERPINE1* and CpG_4.5.6 ($p < 0.01$) of *TNF* were statistically modified when compared with the methylation percentage of the non-treated macrophages. No changes in methylation were noted in the analysed region of *IL1B* after the treatments.

Discussion

This study was designed to elucidate the hypothesis that the regulation of the inflammatory process of macrophages by FAs is mediated by epigenetic mechanisms (particularly, DNA methylation). The origin of this rationale derives from previous experimental observations [29]. First, some FAs, generally SFAs and *trans* FAs, are associated with a pro-inflammatory phenotype in several diseases [16, 49], while other FAs, commonly MUFAs and PUFAs, may present beneficial effects on the inflammatory response [48]. Nevertheless, a critical question is the mode that FAs conduct the inflammation-related cells to this particular phenotype contributing to the regulation of inflammation. Second, there are increasing inflammation-related pathogenic situations, such as obesity or metabolic syndrome, in which an abnormal

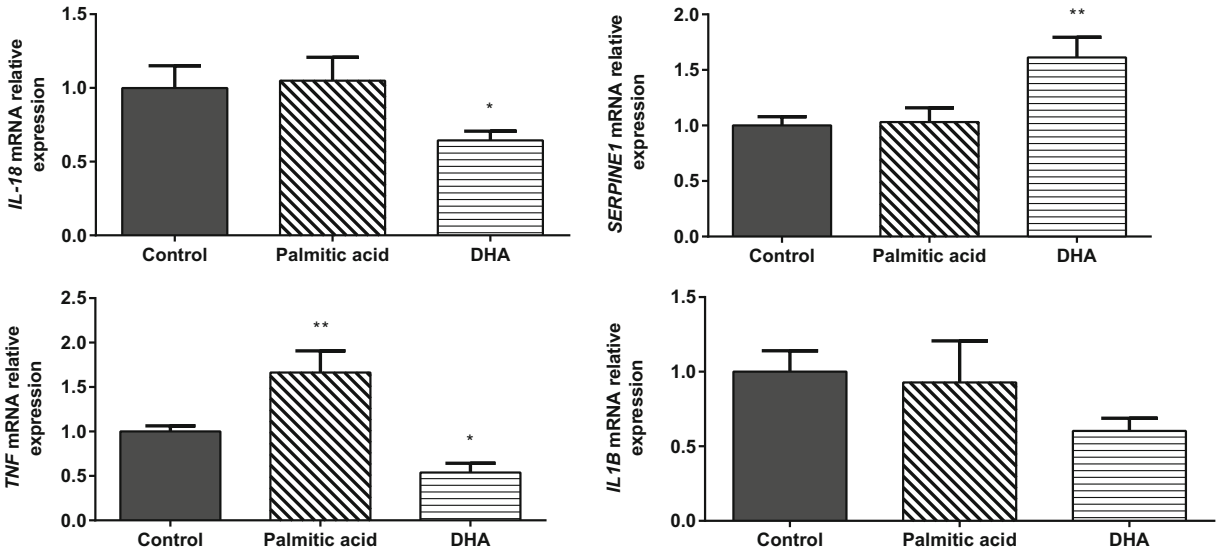

Fig. 1 Effects of palmitic acid and docosahexaenoic acid on the expression of inflammatory genes in THP-1 cells treated with TPA. Results are expressed as means ± SEM ($n = 8$). Differences between groups were analysed by one-way ANOVA followed by Dunnett test. *p value < 0.05, **p value < 0.01 vs control

epigenetic pattern has been observed [18, 29]. DNA methylation may modulate mediators of inflammation including immune cells and inflammatory molecules [13, 39] affecting the balance toward a pro- or anti-inflammatory milieu. Third, recent investigations evidence that PA and DHA supplementation in animals, or other FAs treatment in vitro, is able to modify the methylation profile of cells [4, 38]. However, whether in vitro incubation with PA and DHA leads to regulate the inflammatory response through DNA methylation mechanisms is yet unknown.

The results of the present study indicate that the transcriptional profile induced by PA in THP-1 macrophages is markedly pro-inflammatory compared to DHA, in accordance with published data obtained with these FAs in animal and human models [21, 34]. PA is the most abundant dietary and plasma FA [34], whereas DHA together with eicosapentaenoic acid (EPA) are the main omega-3 FAs [51], but PA and DHA contribute differently to inflammation. The incubation of adipocytes with PA stimulates specific inflammatory and metabolic pathways. PA

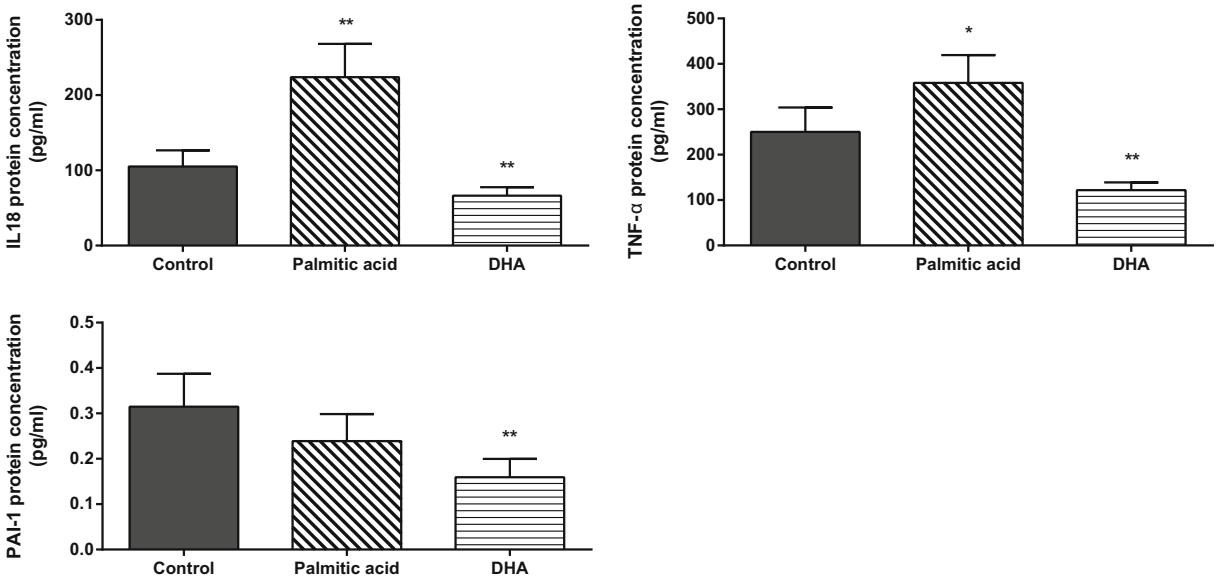

Fig. 2 Effects of palmitic acid and docosahexaenoic acid on the secretion of inflammatory cytokines in THP-1 cells treated with TPA. Results are expressed as means ± SEM ($n = 7$–8). Differences between groups were analysed by one-way ANOVA followed by Dunnett test. *p value < 0.05; **p value < 0.01

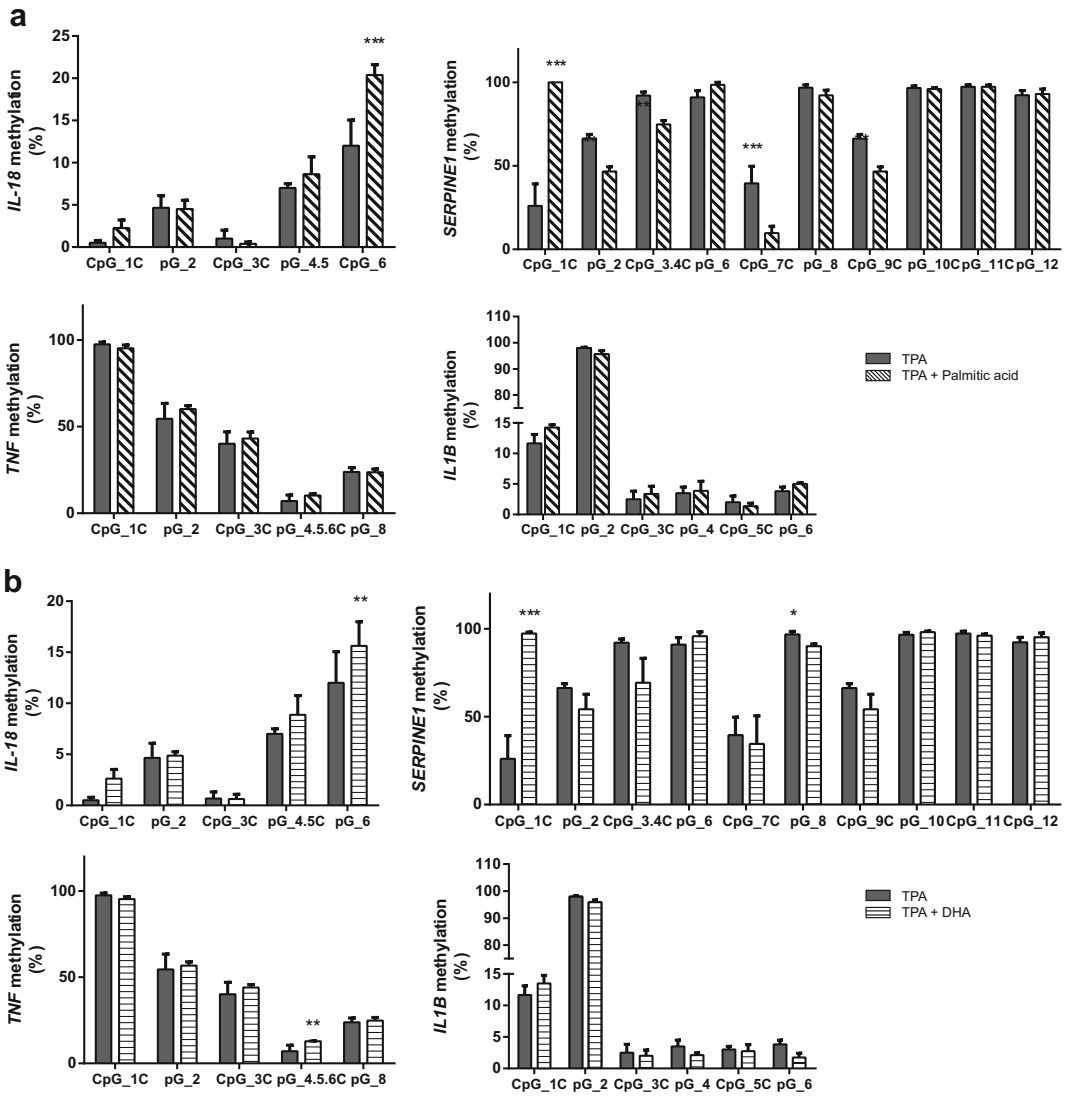

Fig. 3 CpG methylation levels (as percentage) in *IL18*, *SERPINE1*, *IL1B* and *TNF* genes after the incubation of THP-1 macrophages with **a** palmitic acid and **b** docosahexaenoic acid, measured by MassARRAY®. Results are expressed as means ± SEM ($n = 4$). Unpaired Student's *t* test was used to compare each CpG with the control group. **p* value < 0.05; ***p* value < 0.01; ****p* value < 0.001

binds TLR family, which activates gene transcription of inflammatory mediators, resulting in a production of pro-inflammatory molecules such as TNF-α and IL-1β [23]. This role for PA on inflammation can also partly explain the increased risk for metabolic syndrome commonly associated with Western diet consumption [42], which is rich in this SFA [20]. On the other hand, DHA is considered an anti-inflammatory nutrient due to its ability to reduce nuclear factor-κB (NF-κB) activation and the expression of TNF-α in macrophages [33], and also to augment the concentration of the anti-inflammatory cytokine IL-10 [6]. Previous investigations have demonstrated that DHA reduces the production of the classic pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [7]. Regarding these beneficial effects, nutritional strategies usually include supplementation with this omega-3 FA for the prevention and treatment of cardiovascular diseases, obesity or metabolic syndrome [25], which are diseases with an inflammatory component. However, the effects of this FA on inflammatory markers are unclear, perhaps due to differences between study designs, the inflammatory mediators studied or the doses and the duration of the experiments [25], chiefly in healthy human beings [36]. Our results demonstrated that the in vitro supplementation with 80 μM of PA or DHA during 30 h affected the production of the cytokines TNF-α and IL-1β in human macrophages, intensifying or attenuating the inflammatory state of macrophages, respectively.

Consistent with the present results, previous studies observed changes in the expression of inflammation-related genes at similar concentrations and duration of the current experiment, and in the same THP-1 cell model [2, 3, 50]. It is important to underline the fact that the present research investigated the effect of PA and DHA on the inflammatory molecules, in cells under baseline inflammatory conditions, and without induction of inflammation with different strategies (lipopolysaccharide, TNF…). In the majority of in vitro experimental designs, inflammation in cells was stimulated to evaluate the effects of FA [31]. In that condition, the changes in gene expression are thus more important [1]. This feature might be a reason explaining that the effects of FAs are not so clear in the present study as in other studies with stimulated inflammation models [1]. The effects of PA and DHA on the inflammatory response are in part controlled by a combination between the activation of signalling pathways and molecule production in cells. However, the mechanisms of gene expression regulation by FAs remain to be elucidated. To acquire a comprehensive overview of the epigenetic processes modulated by FAs, previous association studies have been carried out between whole genome DNA methylome in human blood cells and SFA and PUFA intake [5, 11]. These investigations evidenced a modulation of DNA methylation levels by FAs. Therefore, one of the aims of this research was to investigate the impact of PA and DHA supplementation on the DNA methylation levels of inflammation-related genes in THP-1 macrophages. Unexpectedly, our results suggested that the addition of 80 μM of PA and DHA did barely induce differences on the DNA methylation levels of the selected genes. We also found that both PA and DHA modified methylation of the CpG sites in the same sense. FA-treated THP-1 cells exhibited significantly higher methylated cytosine levels in *IL18* and *TNF* genes and lower methylated levels in *SERPINE1* gene, as compared to the control ones. Recent studies investigating the DNA methylation modifications by FAs in THP-1 cells indicated that FA supplementation induced distinct DNA methylation modifications uncovering pro- and anti-inflammatory profiles [11, 49]. However, there not apparently exists in the literature any work studying the specific DNA methylation modification of these genes by FAs in THP-1 cells. However, a study of the effect of DHA in DNA methylation of apoptotic genes in a human colon cancer cell line used a dose of 50 μM. This study demonstrated DNA methylation changes after the treatment of DHA at 50 μM and during 12 h [8]. In addition, the treatment of PUFAs at 100 μM also demonstrated changes in DNA methylation patterns in colorectal cancer cell lines but after 6 days of incubation [45]. Regarding time and doses similar to those used in the present study, in 3T3-L1 cells, palmitic acid treatment at 100 μM and 24 h regulated *TNF* expression levels through changes in *TNF* promoter DNA methylation levels [14]. The present trial demonstrates for the first time that PA and DHA hardly induce modifications in the DNA methylation status of the classic inflammatory genes. Concerning previous studies, it is possible that the weak changes in DNA methylation levels of gene promoter may be caused by (i) the short-term treatment at this dose, (ii) we have not studied all the CpG sites of the promoter and (iii) palmitic acid and DHA have weak effect on the DNA methylation of genes studied in the present work. In addition, the higher mRNA expression and lower protein secretion of PAI-1 after DHA treatment may suggest a different post-transcriptional epigenetic regulation of the molecules by this FA. For example, there exists post-transcriptional regulation mediated by miRNAs in which an incomplete complementarity between miRNA and mRNA results in the inhibition of gene translation after DHA treatment [37, 53, 54]. Moreover, in the offspring of rats fed a high-fat diet, the acetylation and dimethylation of H3K9 histone were associated with lower adiponectin and with higher leptin expression [5]. Treatment of M17 neuroblastoma cells with DHA induced increased global acetylation of H3K9 and decreased histone deacetylases 1, 2 and 3. In addition, DHA also decreased global levels of dimethyl H3K4, dimethyl H3K9, dimethyl H3K27, dimethyl H3K36 and dimethyl H3K79. Overall, DHA treatment induces histone changes and modifies gene expression [5]. PA can also affect the covalent modification of histones in pro-inflammatory mechanisms. For example, in murine macrophages, PA modifies histone deacetylase inhibitor resulting in the increase of adipocyte fatty acid binding protein [9].

The present work has shown that CpG_6 of *IL18* and CpG_4.5.6 of *TNF* gene have higher methylation levels comparing with the control one. In order to understand the importance of these CpG sites in the regulation of transcription, four different online databases (TFBIND, ALGGEN, LASAGNA 2.0 and TRANSFAC®) have been used to study putative transcription factor (TF) binding sites in these regions. The bioinformatics analysis of CpG_4.5.6 of *TNF*, and after the combination of the results from the four databases, identified E2F, EGR1, GATA1, MZF1 and Sp1 TFs. In addition, the analysis of CpG_6 of *IL18* determined E2F and HNF3 family (Supplementary Fig. 1). The *TNF* promoter region located between − 170 and − 155 from TSS has been identified as a target for the transcription factors EGR1 and Sp1 and included a major TPA-responsive domain [22]. In vitro studies performed in human and murine cell lines showed that both Egr family and Sp1 bound to the GC-rich motif of the *TNF* promoter [22, 52]. E2F, GATA family and MZF1 TFs are able to interact with methylated CpG sites

and regulate gene expression [55], but no experimental evidence has been found for the binding to *TNF*. E2F and HFN3 family are also putative TFs for *IL18* promoter due to its ability to bind CpG sites, but there is no in vitro or in vivo evidence for this union.

In conclusion, results of the present study strengthen the understanding of the mechanisms by which PA and DHA cause their effect on inflammation of macrophages. The exposure of THP-1 macrophages to 80 μM of either PA or DHA induces changes in gene expression and cytokine secretion that may affect the inflammatory environment of the macrophages, increasing or decreasing inflammatory response, respectively. However, apparently DNA methylation is not directly involved in the fatty acid-mediated regulation of these genes. Further studies in the field, involving epigenetic study of molecules that participate in inflammatory signalling pathways, are required to better understand the mechanisms whereby these FAs influence inflammation.

Acknowledgments We thank the technical assistance of Enrique Buso (UCIM, University of Valencia) for the MassARRAY® measurements.

Funding details This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness under grant AGL2013-45554-R; the Spanish Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (CIBERobn); and the Brazilian Ministry of Education, Culture and Sport under grant BES-2014-068409.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent This article does not contain any individual participants.

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