Docosapentaenoic acid (22:5n-3) down-regulates the expression of genes involved in fat synthesis in liver cells

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Abstract

Previous studies have shown that Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) exhibit triacylglycerol (TAG) lowering effect *in vitro* and *in vivo* by down-regulating the Sterol Regulating Element Binding Protein (SREBP-1c) and reducing the expression levels of lipogenic genes. However, there is no evidence on the effect of Docosapentaenoic Acid (DPA) on SREBP-1c expression levels. DPA is a long chain n-3 fatty acid present in our diet through fish, red meat and milk of ruminant animals. Therefore, this study aimed to elucidate the effect of DPA on liver fatty acid synthesis in an *in vitro* model using rat liver cells. Our results suggested that DPA incubation (50 μM) for 48 hours (like EPA and DHA) caused a significant decrease in the mRNA expression levels of SREBP-1c, 3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A reductase (HMG-CoA reductase), Acetyl Coenzyme A Carboxylase (ACC-1) and Fatty Acid Synthase (FASn) compared with Oleic Acid (OA) and also a decrease in the protein levels of SREBP-1 and ACC-1. A time-course fatty acid analysis showed that DPA and EPA are interconvertable in the cells, however, after 8 hours of incubation with DPA, the cell phospholipids contained mainly DPA. The gene expression profiling of the lipogenic genes repeated at 8 hours confirmed that the inhibitory effect of DPA on mRNA expression levels of the lipogenic genes was most likely due to DPA itself and not due to its conversion into EPA.

INTRODUCTION

Docosapentaenoic Acid (DPA) is a long chain n-3 fatty acid present in our diet through fish, red meat and milk of ruminant animals, although the concentration of fatty acid can vary between species. The concentration of DPA in fish (such as the Australian salmon), red meat (lamb fillet) and bovine milk is 388, 23 and 2.3 mg per 100 g, respectively (1-3). There is limited investigation of the biological effects of n-3 DPA, probably because it has only recently become available commercially in pure form. There has been extensive research on the biological effects of other n-3 LCPs namely Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) which has shown that these LCPs possess numerous beneficial health properties, such as TAG lowering effects and amelioration of hepatic steatosis (4). Previous studies in animals have shown that supplementation with EPA ameliorates hepatic steatosis by down-regulation of the mature form of SREBP-1 protein, thereby reducing the expression of lipogenic genes (4-7). SREBP-1c is a hepatic gene transcription factor with a critical role in controlling the transcription of genes involved in fatty acid synthesis, especially in the liver *in vitro* and *in vivo* (8-10). It has been reported that EPA and DHA inhibit SREBP-1c gene transcription, enhance SREBP-1c mRNA turnover and interfere with the proteolysis processing of SREBP-1c protein (11, 12). Furthermore, in another key tissue concerned with whole body lipid metabolism, it has been shown using the *in vitro* cell model 3T3-L1 adipocytes that EPA suppresses lipid droplet formation by suppression of the expression levels of genes related with lipid droplet function such as Cidea and Stearoyl-CoA Desaturases (13). To date, there is no data available on the effect of DPA on gene or protein expression levels of the main upstream controller of lipogenic genes, SREBP-1c. Recently it was reported in an *in vivo* study that supplementation of *db/db* mice (an animal model of type 2 diabetes) with synthetic tri-DPA for 4 weeks, reduced the mRNA expression levels of

hepatic Acetyl Coenzyme A Carboxylase (ACC-2) and enzyme activities of Fatty Acid Synthase (FASn) and Malic Enzyme (ME) (14). Tri-DPA is not naturally present in diet and that study did not investigate the effect of DPA on SREBP-1c. In the study presented here, the aim was to elucidate the effect of pure DPA on lipogenic gene and protein expression levels in liver cells.

The liver plays a central role in whole body lipid metabolism and adapts rapidly to changes in dietary fat composition. This adaption involves changes in the expression of genes involved in glycolysis, *de novo* lipogenesis and fatty acid elongation, desaturation and oxidation. The liver provides an excellent and relevant model to study the molecular effects of DPA. The specific aims of the current study was to investigate the molecular actions of DPA compared with EPA and DHA on the key players involved in lipogenesis (SREBP-1c, HMG-CoA reductase, ACC-1, Carbohydrate Responsive Element Binding Protein (ChREBP) and FASn) using the rat hepatoma FAO cell line.

MATERIAL AND METHODS

Cell culture

FAO hepatoma cell line is a differentiated cell line derived from the rat hepatoma H4IIEC3 line (15). FAO cells were seeded in 24-well plates at 1 x 10^5 cells per well and grown in RPMI media containing 10% (w/v) FBS and 1% (v/v) penicillin / streptomycin (Invitrogen, CA). After 48 hours, FAO cells were at 70% confluence and at that time treated with the fatty acids as detailed below.

Preparation of n-3 LCP media

The stock solutions of fatty acids were prepared in 100% (v/v) ethanol at a concentration of 20 mM and the working solution was prepared by adding the stock to the RPMI media to achieve a final concentration of 50 µM. Previous studies that have investigated the effects of n-3 LCP in cell culture have successfully used a dose of 50 µM for 48 hours in various cell lines (16-19) including liver cells (17, 18). The working solution was prepared fresh before treating the cells.

Fatty acid treatment of cells

FAO cells were treated with 50 µM of EPA, DPA or DHA (purchased from Nu-Chek Prep Inc., USA) for 48 hours. 50µM OA (Nu-Chek Prep Inc., USA) was used as an unsaturated fatty acid control while ethanol (EC) (0.5%) was used as a vehicle control. Treatments were replenished after 24 hours. The data reported in Fig 3 indicates that the supplemented free fatty acids were successfully incorporated into the FAO cells.

Gene expression analysis of lipogenesis

After treatment, RNA was extracted from the cells and first-strand cDNA was generated from 1 µg total RNA. The levels of mRNA were measured with specific primers designed using Primer Express software package version 3.0 (Applied Biosystems, CA). Real time-PCR was performed in duplicate with reaction volumes of 20 µl, containing SYBR Green 1 (Applied Biosystems, CA). Data was obtained using a comparative critical threshold (Ct) method where the amount of target gene was normalised to the amount of cyclophilin. Cyclophilin was used as a housekeeping gene since its expression levels did not change with the treatments (data not shown).

Protein preparation

After respective fatty acid treatment, the FAO cells were washed twice with ice-cold 1 x Dulbecco's PBS, pH 7.4 (Invitrogen, CA) and were collected in ice-cold modified RIPA lysis buffer (64 mM Tris-HCl, pH 7.4 that contains 150 mM NaCl, 1% (w/v) NP-40, 1 mM EGTA and 0.25% (w/v) sodium deoxycholate). The protease and phosphatase inhibitors (100 mM sodium fluoride, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 mM phenylmethanesulfonylfluoride, 1 µg/ml leupeptin and 1 µg/ml pepstatin) were added to the lysis buffer just prior to addition to cells for solubilisation for 15 min at 4°C. The solubilised and resuspended cells were centrifuged at 9,000 x g for 15 min in a 4°C pre-cooled microcentrifuge. The supernatant (whole cell lysate) was collected into a fresh eppendorf tube, protein content was determined using the BradFord assay (Bio-Rad, PA) (20) and prepared for Western Blotting analysis of proteins of interest as detailed below.

Western Blotting

Equal amounts of protein (30 µg) were reduced in loading buffer containing β-mercaptoethanol and the samples were denatured at 100°C for 2 min. Proteins were resolved by 8% (for ACC-1) or 10% (for SREBP-1c) (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After SDS, fractionated proteins were electrophoretically transferred to nitrocellulose membranes using 1 x Towbin's Transfer buffer. Membranes were blocked overnight at 4°C using 5% (w/v) skim milk in tris-buffered saline and Tween-20 (TBST) for ACC-1 and 5% (w/v) BSA in TBST for SREBP-1c. Membranes were then probed with the relevant primary antibodies (Cell Signalling, Danvers, MA) for 2 hours at room temperature: ACC-1 rabbit monoclonal antibody; Phospho-ACC-1 (Ser79) rabbit monoclonal antibody; rabbit polyclonal anti-SREBP-1 antibody and mouse monoclonal β-actin antibody at a concentration of 1 in 1000 in TBST. After incubation with the primary antibody, membranes were then washed in 6 x TBST for 10 min each at room temperature and the membrane was probed with the relevant secondary antibodies for 2 hours at room temperature (anti-rabbit or –mouse IgG horseradish peroxide) at a concentration of 1:5000 in TBST. The bound antibodies were visualised by enhanced chemiluminescence (ECL) (GE Healthcare, UK) as per manufacturer's instructions. The density of protein bands was quantified using Image J software (NIH, Maryland, USA).

Fatty acid analysis

Lipid extraction - FAO cells in 6-well plate format were treated with the fatty acids as specified above. After 48 hours, the media was aspirated and cells were washed with ice-cold 1 x PBS, pH 7.4 twice. 50µl of trypsin EDTA was added to each well and incubated at 37°C for 5 min. Cells from 3 wells were pooled together and 1.5ml of PBS was added to

trypsinised cells. The cells were then transferred to 10ml glass screw-capped (teflon-lined) centrifuge tubes and tissue lipids were extracted by 2:1 chloroform : methanol, as described by Portolesi et al, 2007 (21). In brief, after addition of chloroform/methanol, the samples were vortexed for 10 min and centrifuged at 1500 x g for 10 min to separate the aqueous and organic phase at room temperature. The organic phase containing the lipid was removed and transferred to a new glass tube and evaporated under a stream of nitrogen. The lipid extract was reconstituted in 150 µl of chloroform and the lipids were then separated by thin layer chromatography (TLC).

Thin layer chromatography (TLC) – The lipid extracts were spotted onto silica gel plates (silica gel 60 G, Merck, Germany) and developed in 85:15:2 (v/v) hexane : diethylether : acetic acid in paper-lined tanks. The lipids were visualised with 0.1% (w/v) 2′,7′ – dichlorofluroescein indicator in ethanol (Scharlau, Spain). The phospholipid band from the samples was scraped off into glass screw-capped tubes and were reacted with 2% H_2SO_4 in 100% methanol for 3 hours at 80°C to form the fatty acid methyl esters (FAME). After the reaction, the FAME were isolated in 100% petroleum ether and stored in glass vials at -20°C prior to gas chromatography.

Gas chromatography - The purified FAMEs were separated by capillary gas liquid chromatography (GLC) using a 50 m x 0.32 mm (I.D.) fused silica bonded phase column (BPX70, SGE, Melbourne, Australia) (22). Fatty acids were identified by comparison with standard mixtures of FAME and results were calculated using response factors derived from FAME standards of known composition.

Statistical analysis

Data calculations and statistical analysis were performed using Statistical Package for the Social Sciences software (SPSS version 17.0; Fullerton, USA). Significant differences between treatment groups were tested using a one-way ANOVA for each type of fatty acid. Normality of distribution was tested using the homogeneity of variance test. If the test was non-significant, the post-hoc comparisons were made using the least significant difference (LSD) test with a significance level of <0.05. If the homogeneity of variance test was significant, the data was analysed using Welch test and post-hoc comparisons were made using Dunnetts T3 test with a level of <0.05 considered as significant.

RESULTS

Effect of n-3 LCP incubation on the expression of lipogenic genes

The effect of the n-3 LCP treatment, for 48 hours, on the expression levels of key genes involved in lipid synthesis in FAO liver cells is shown in Fig 1. This demonstrates that EPA, DPA and DHA treatment resulted in a significant decrease in the gene expression levels of SREBP-1c by 73, 96 and 84 per cent, respectively, compared with OA ($p<0.05$, $n=2$). OA was used as a control fatty acid to show that the effect of n-3 LCP on lipogenic genes was not common to all unsaturated fatty acids. EPA, DPA and DHA treatment also caused a significant decrease in the gene expression levels of HMG-CoA reductase by 53, 93 and 76 per cent, respectively, compared with OA ($p<0.05$, $n=2$). DPA and DHA treatment significantly reduced the mRNA expression levels of ChREBP by 83 and 54 per cent, respectively, relative to OA ($p<0.05$, $n=2$). The mRNA expression levels of ACC-1 and FASn were also decreased significantly ($p<0.05$) by treatment of cells with all three n-3 LCP and the maximum decrease of 86% was observed in FASn expression in the cells supplemented with 50 μM of DPA. The results obtained from the mRNA expression analysis of the two upstream genes in lipogenesis, SREBP-1c and ACC-1, were confirmed by measuring their protein levels (Fig 2). DPA significantly reduced the protein levels of ACC-1 by 72% compared with the vehicle ($p<0.05$, $n=2$). Although EPA and DHA also reduced ACC-1 protein levels by 55 and 48%, respectively, compared with the vehicle, the effect was not significant ($p<0.05$, $n=2$). In the case of SREBP-1c, DPA and DHA significantly reduced the pre-mature SREBP-1c protein levels by 71 and 85% respectively, compared with the vehicle. EPA did not alter the expression of SREBP-1c protein levels in the FAO cells.

Effect of n-3 LCP incubation on the fatty acid composition of FAO liver cells

The fatty acid composition of the total phospholipids (PL) of FAO cells supplemented with n-3 LCP for 48 hours was analysed in order to ensure that the fatty acids supplemented to the cells were being incorporated into the cell PL. The proportion of n-3 LCP in the OA treated cells was <2% of total fatty acids. The results also showed that, the cells supplemented with EPA resulted in significant increase in EPA levels (by 16 fold) and DPA levels (by 9 fold), relative to the OA control ($p<0.05$, n=2) but there was no increase in DHA levels. Similarly, DPA incubation resulted in a significant accumulation of DPA (by 14 fold) as well as EPA (by 9 fold), relative to the OA control in the cell PL ($p<0.05$, n=2). DHA incubation significantly elevated DHA levels (by 12 fold) and EPA levels (by 6 fold) compared with OA control. There was a small but significant increase in DPA in cells treated with DHA (by 1.2 fold). The cells treated with ethanol and OA did not cause any changes in n-3 LCP proportions of the cells.

Effect of short-term n-3 LCP incubation on fatty acid composition of FAO liver cells

The results from the fatty acid analysis of FAO cells conducted at 48 hours suggested that the inter-conversion of DPA to EPA and vice-versa occurs in these cells with either fatty acid provided in the culture medium. Hence, it became important to identify the time-point at which the cells supplemented with DPA contained mostly DPA and little EPA. A time-course of fatty acid analysis was performed at 0, 1, 2, 4, 8 and 24 hours and the cells were treated in exactly similar manner as for the 48 hour analysis. The results showed that the proportion of DPA reached a peak at 8 hours (from 0 to 17%) and then remained constant, however the proportion of EPA formed (from DPA) in the DPA-treated cells at 8 hours was low (3%) as shown in Fig 3.

Effect of short-term n-3 LCP incubation on the expression of lipogenic genes

The results from 48 hour fatty acid analysis of FAO cells showed that at 48 hours the PL of cells treated with DPA contained both DPA and EPA. Therefore it was not clear if the results obtained in gene expression and protein analysis were due to DPA or due to its conversion into EPA. The results obtained from the time-course fatty acid analysis identified that at 8 hours, there was a maximum amount of DPA present in the DPA-treated cells, with a minimal amount of EPA formed from the DPA. Therefore, the mRNA expression levels of the lipogenic genes were re-investigated after 8 hours of DPA incubation. The gene expression results at 8 hours of treatment showed that DPA (like EPA and DHA) significantly down-regulated the expression levels of SREBP-1c, HMG-CoA reductase, FASn and ACC-1 by 54 to 99% ($p<0.05$, $n=2$) (Fig 4). The decrease in expression of ChREBP was not significant.

Discussion and conclusions

DPA is a dietary n-3 LCP present in fish, fish oil, meat and milk of ruminant species. Therefore, it is important to elucidate its molecular effects on lipid metabolism and its metabolism in liver cells. Previous studies have shown that oral supplementation with n-3 LCP ameliorates hepatic steatosis, via down-regulation of mature form of SREBP-1 protein and thereby reducing the expression of lipogenic genes (4-7). However, there is no data available on effect of pure DPA on lipogenic gene and protein expression. The aim of this *in vitro* study was to investigate the effect of DPA on liver fatty acid synthesis compared with EPA and DHA. The second aim was to investigate the incorporation and inter-conversion of these n-3 fatty acids. The results showed that 48 hour incubation of DPA (like EPA and DHA) down-regulated the mRNA expression levels of SREBP-1c and ACC-1. DPA also reduced the protein levels of SREBP-1c and ACC-1 significantly, compared with the vehicle. SREBP-1c is

the main upstream controller of fat synthesis *in vitro* and *in vivo* (8, 23). Therefore, suppression of SREBP-1c levels in the liver cells is expected to reduce lipogenesis.

The SREBPs are synthesised in the endoplasmic reticulum (ER) in the form of a precursor protein. To become transcriptionally active, the SREBP precursor undergoes proteolytic cleavage in the Golgi apparatus to liberate its N-terminal domain, which constitutes the mature transcription factor (24). In the present study we looked at the protein expression level of pre-mature SREBP-1 (120 kDa) and future studies should also investigate the effect of DPA incubation on mature SREBP-1 (66 KDa) to confirm this effect. In the present study, EPA, DPA and DHA also reduced the mRNA expression levels of HMG-CoA reductase, which catalyses a critical step of converting HMG-CoA to mevalonic acid in the cholesterol synthesis pathway (25). Our findings also demonstrated that the gene expression levels of FASn were down-regulated in all n-3-LCP treated cells compared with OA-treated cells. In our study, DPA and DHA treatment significantly decreased the mRNA expression levels of ChREBP in the FAO cells. ChREBP controls the expression of L-pyruvate kinase and the decreased gene expression of ChREBP can thereby limit L-pyruvate kinase, which in turn can restrict the substrate (glucose) available for TAG synthesis. These results are consistent with previously published studies, which have shown that EPA and DHA suppress ChREBP activity by increasing its mRNA decay and by altering ChREBP protein translocation from the cytosol to the nucleus both in primary hepatocyte cultures and in liver *in vivo* (12, 26). However our study is the first investigation to report the inhibitory effect of pure DPA on expression of lipogenic genes in liver cells. These results suggest that DPA may have a role in reducing fat synthesis and thereby TAG synthesis. An *in vivo* study in *db/db* mice (an animal model of type 2 diabetes) recently reported that when 1% tri-linoleate in the control diet was replaced with synthetic tri-DPA for 4 weeks, the hepatic TAG level and total amount was

decreased compared to the control (14). In this study, the efficacy of DPA was greater than that of EPA, but less than that of DHA in the liver. In contrast, EPA had the greatest serum TAG reducing effect. However this study used a form of DPA (tri-DPA) which is not naturally present in our diet. It was also reported that the mRNA expression levels of hepatic ACC-2 were reduced (14). However the protein expression was not investigated. Supplementation of *db/db* mice with tri-DPA (in TAG form) also significantly reduced the hepatic enzyme activities of FASn and malic enzyme (14).

The results from our study also showed that DPA and EPA are interconvertible in the liver cells; however, there was little evidence of conversion of EPA and DPA into DHA. These results are consistent with the previously published studies conducted using endothelial cells (27-29). One study in the literature reported that the media from DPA-incubated endothelial cells contained small amounts of DHA suggesting that DPA was converted to DHA and then released into the media (29). In our study the fatty acid composition of the cell media was not analysed. However, it can also be speculated that the lack of appearance of DHA in the cell PL from EPA and DPA incubation could be due to the fact that FAO hepatoma cells have a reported impaired capacity to beta-oxidise long chain fatty acids (30). It was also observed in our study that DHA incubation led to increased EPA levels, suggesting retro-conversion of DHA to EPA. There is also evidence in the literature on retro-conversion of DHA to EPA from human feeding trials. One study compared the metabolism of EPA and DHA by supplementing volunteers with 6 g/d for 6 days. It was reported that EPA increased the DPA levels in plasma and platelet PL, however DHA levels remained unchanged. DHA feeding increased EPA concentrations in plasma PL implying the occurrence of retro-conversion (31, 32).

It was also of interest to identify the rate of incorporation of DPA into cell PL over time and to identify the rate at which the conversion of DPA to EPA occurred. The time points tested were 0, 1, 2, 4, 8 and 24 hours. It was observed that after 8 hours of treatment, the cells supplemented with DPA contained maximum amount of DPA while minimum amount of EPA. The gene expression results obtained after 8 hours of treatment showed that DPA significantly down-regulated the expression of SREBP-1c, HMG-CoA reductase, FASn and ACC-1 by 54-99% ($p<0.05$) (Fig 4). Since at this time point there was mainly only DPA present in the cell PL and minimal EPA is present, it can be concluded that effect of DPA on these genes was most likely due to DPA itself and not due to its conversion into EPA.

In order to provide biological relevance to the findings with gene and protein expression analysis, functional end-point assays were conducted to measure the cellular TAG levels. However, the results from the current study failed to show an effect of DPA on TAG levels in the FAO cells (data not shown). It could be that the concentrations of the fatty acids used in this study were sufficient to cause a change in gene expression levels and protein levels in the cells but not to induce a change in the TAG amount and/or synthesis in the liver cells.

In conclusion, the data shows that supplementation of liver cells *in vitro* with DPA (like EPA and DHA) down-regulated the expression levels of key genes and proteins involved in fatty acid synthesis. This suggests that DPA should be considered as a biologically active n-3 LCP. DPA is often ignored in the literature in reports from studies because it has been under-studied, yet in all studies involving n-3 LCP measurements in tissues and foods DPA is generally present and in some instances in greater levels than DHA (eg. in ruminant meat and milk). Future studies should investigate the effects of pure DPA on plasma/liver TAG levels in an *in vivo* model such as diet induced hypertriglyceridemia in rats or an animal model of NASH.

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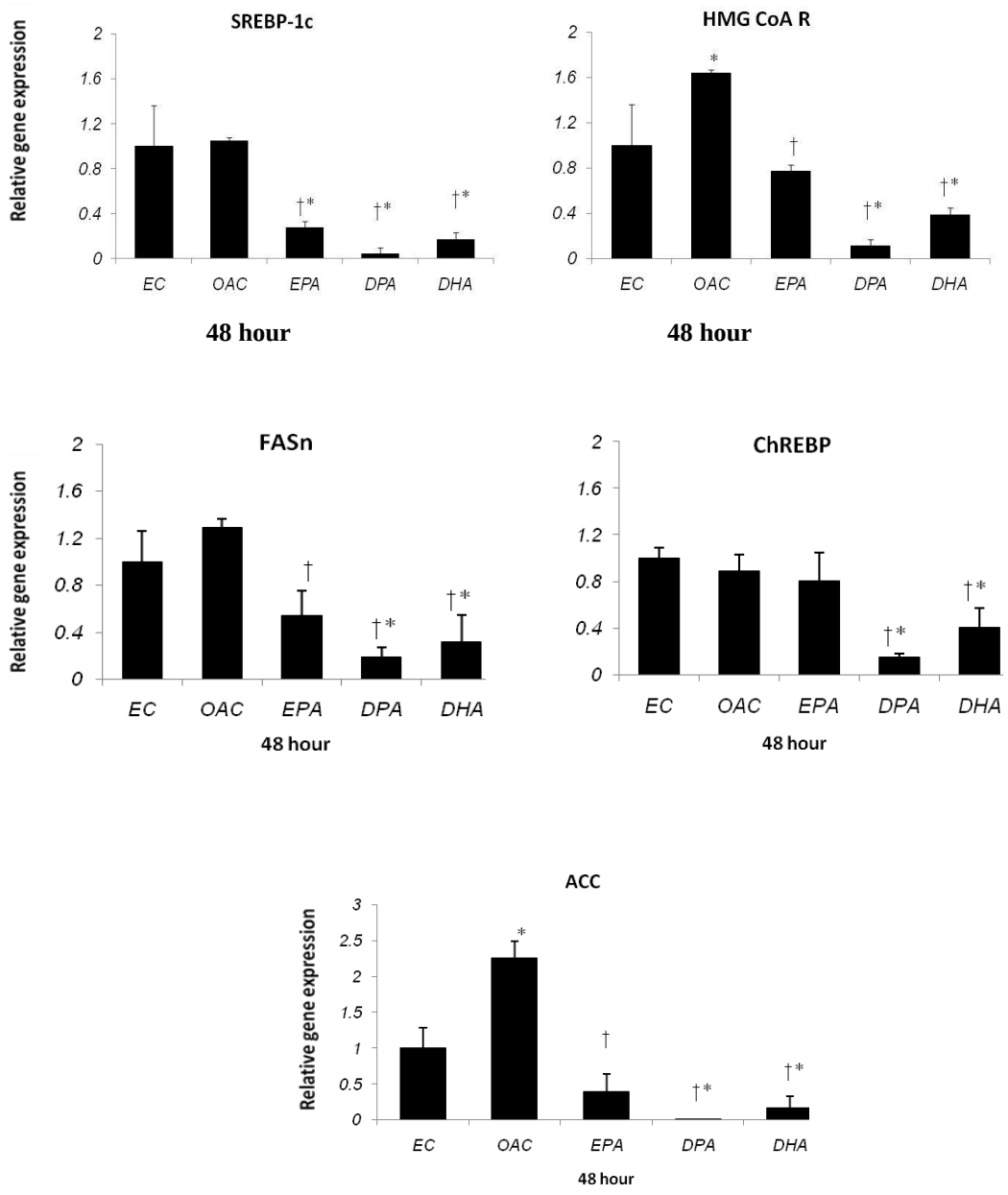

Figure 1: mRNA expression levels of SREBP-1c, HMG CoA reductase, ChREBP, FASn and ACC-1 after treatment of FAO cells with 50µM of EPA, DPA, DHA or OA for 48 hours.
The results are expressed as relative gene expression (mean ± SEM). The data is obtained from 2 independent experiments with n=3 in each experiment. Data was analysed using one way ANOVA and post hoc comparisons were made using LSD. Values significantly different from the vehicle (EC) are denoted with * and those different from OA are denoted as † ($p<0.05$). The vehicle has been assigned a value of 1. EC, ethanol control; OA, oleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

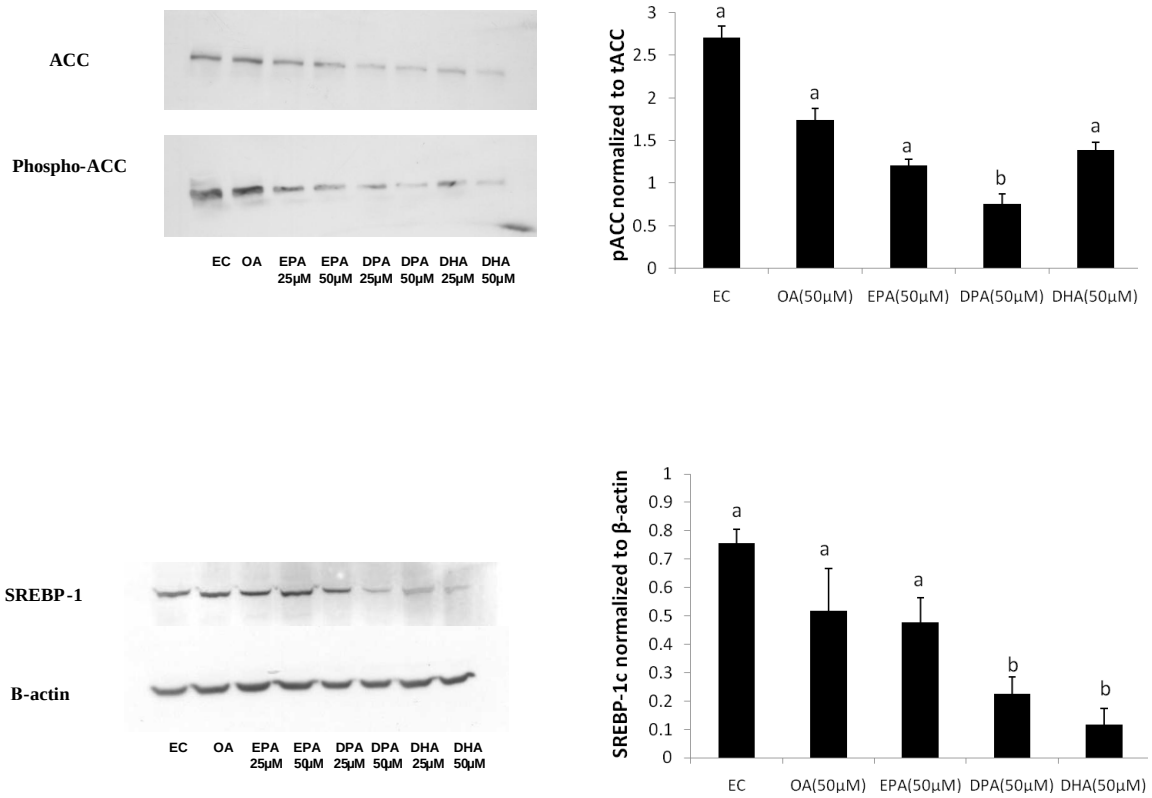

Figure 2: Protein levels of ACC-1 and SREBP-1 after 48 hours of treatment of FAO cells with 50µM of EPA, DPA, DHA or OA or vehicle (EC). 20µg protein was loaded per lane. The graph shows arbitrary units of pACC normalised to tACC and of SREBP-1c normalised to the control β-actin, representing the mean (± SEM) of two independent experiments. Data was analysed using one way ANOVA and post hoc comparisons were made using LSD. Values with different superscript are significantly ($p<0.05$) different to each other. EC, ethanol control; OA, oleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

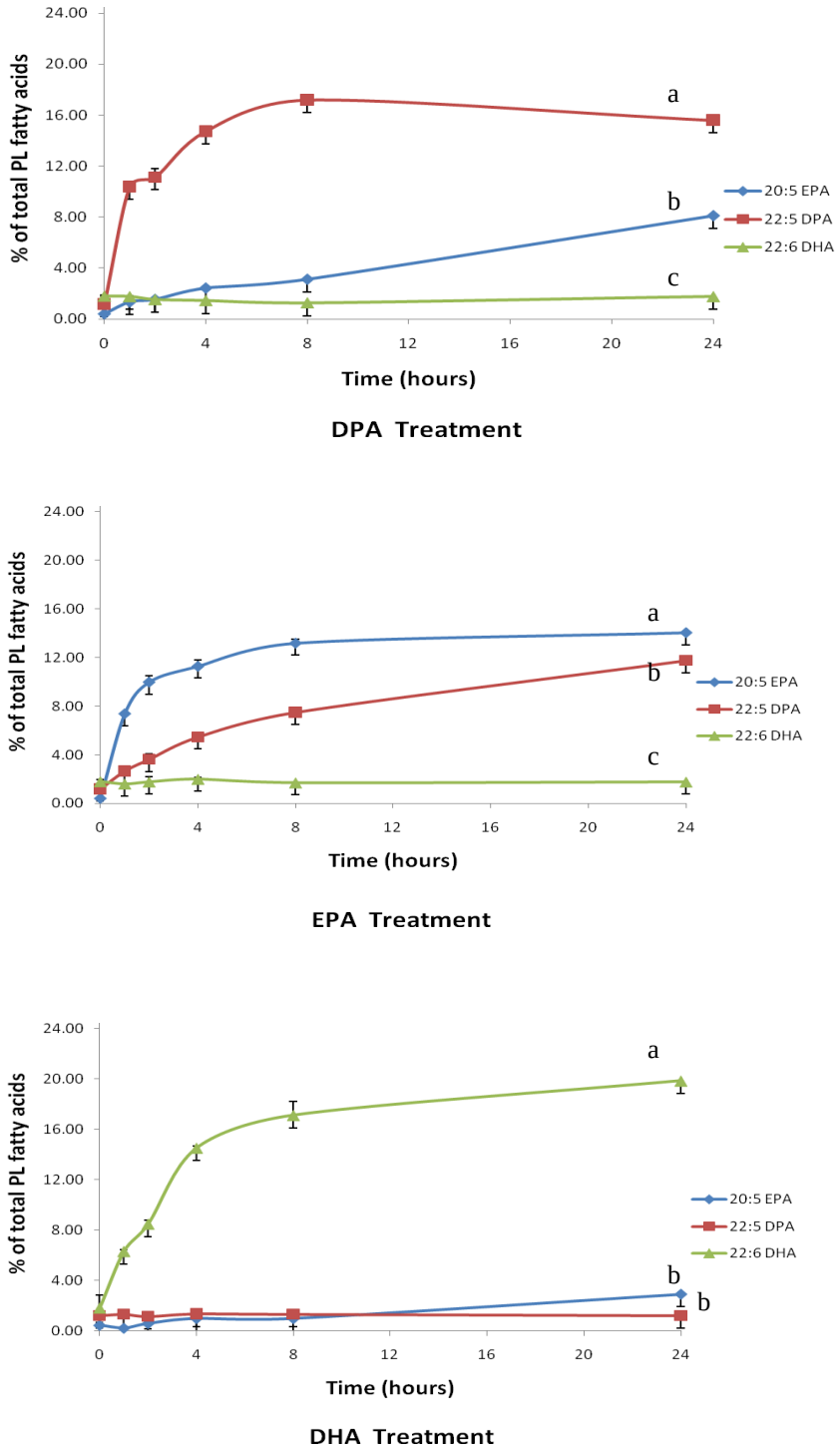

Figure 3: Time course incorporation of fatty acids into phospholipids of FAO cells supplemented with n-3 LCP from 0 to 24 hours.

Fatty acid composition of liver cells supplemented with 50 µM of EPA, DPA or DHA for 0-24 hours. Results are expressed as percentage of total PL fatty acids (mean ± SEM). The results were obtained from two independent experiments with n=6 in each experiment. Data was analysed using one way ANOVA and post hoc comparisons were made using LSD. The values with significant superscripts are significantly different ($p<0.05$).

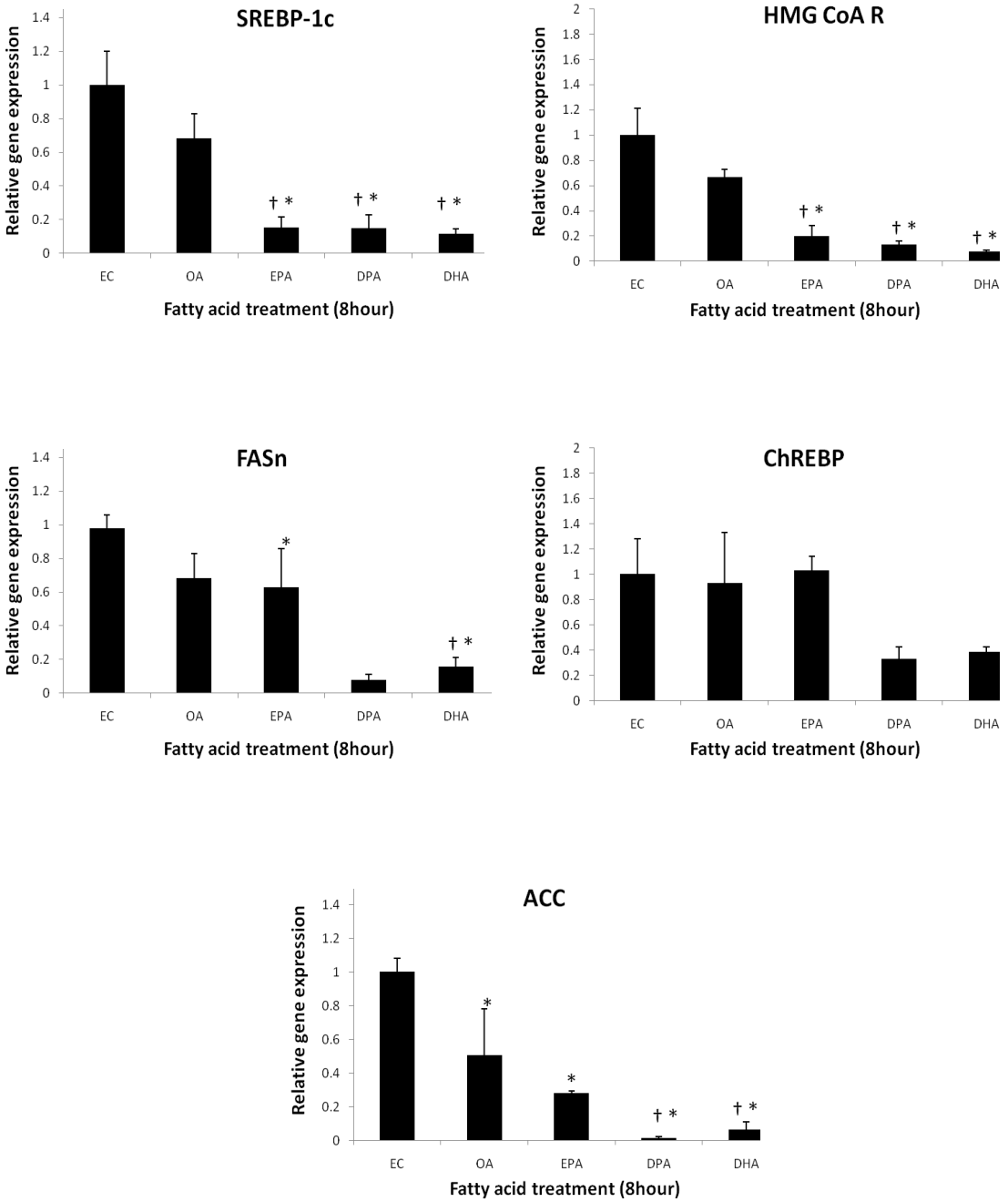

Figure 4: mRNA expression levels of SREBP-1c, HMG-CoA reductase, ChREBP, FASn and ACC-1 after treatment of FAO cells with 50µM of EPA, DPA, DHA or OA or vehicle (EC) for 8 hours. The results are expressed as relative gene expression (mean ± SEM). The data is obtained from 2 independent experiments with n=3 in each experiment. Data was analysed using one way ANOVA and post hoc comparisons were made using LSD. Values significantly different from the vehicle (EC) are denoted with * and those different from OA are denoted as † ($p<0.05$). The vehicle has been assigned a value of 1. EC,ethanol control; OA, oleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

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