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ORIGINAL ARTICLE

n-3 PUFA as Regulators of Cardiac Gene Transcription: A New Link between PPAR Activation and Fatty Acid Composition

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Abstract The fatty acids regulate gene expression directly binding to nuclear receptors or affecting the protein content of transcription factors. In this work, supplementing primary cultures of neonatal rat cardiomyocytes with 60 μM EPA or DHA, we demonstrated by an ELISA assay an increased PPAR β/δ binding to DNA. n-3 PUFA supplementation deeply changed the acyl composition of both cytosolic and nuclear fractions. The high content of total fatty acids, particularly EPA and DHA, and its increase following supplementation suggested a selective accumulation of n-3 PUFAs in the nucleus, supporting the direct interaction of n-3 PUFA with PPAR. The activity of acyl-CoA thioesterase (ACOT), catalyzing the reaction leading to NEFA from acyl-CoA, increased in n-3 PUFA supplemented cells. The NEFA/acyl-CoA ratio is an important regulator of the fatty acid transport to the nucleus and consequent modulation of gene transcription, and although ACOT activity is not the only parameter of this ratio, it is important for the control of the NEFA pool composition. Our data further clarify what happens in cardiomyocytes following n-3 PUFA supplementation, linking the modification of acyl composition to ACOT activity and PPAR activation.

Keywords EPA · DHA · PPAR · Acyl-CoA thioesterase · Neonatal rat cardiomyocytes

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Abbreviations

ACOT	Acyl-CoA thioesterase
A-FABP	Adipocyte fatty acid binding protein
B23	Nucleophosmin
BSA	Bovine serum albumin
CF	Cytoplasmic fraction
ChREBP	Carbohydrate response element-binding protein
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
EPA	Eicosapentaenoic acid
FA	Fatty acid
FABP	Fatty acid binding protein
FCS	Fetal calf serum
HNF-4α	Hepatocyte nuclear factor 4α
HRP	Horseradish peroxidase
HS	Horse serum
K-FABP	Keratinocyte FABP
LXR	Liver X receptors
MEK1	Mitogen-activated protein kinase/extracellular signal-regulated kinase 1
NEFA	Nonesterified fatty acid
NF	Nuclear fraction
NF kappa B	Nuclear factor kappa B
PPAR	Peroxisome proliferator activated receptor
PPRE	Peroxisome proliferator responsive element
PUFA	Polyunsaturated fatty acid
RXR	Retinoid X receptor
SREBP	Sterol regulatory element-binding protein
TNB	2-Nitro-5-thiobenzoic acid
WCL	Whole cell lysate

Introduction

The role of nutrition in the management of diseases has often centered on correcting apparent nutrient deficiencies or meeting estimated nutritional requirements of patients. Recently, further understanding of the underlying mechanisms of various disease processes and how certain nutrients possess pharmacological properties have fueled an interest in exploring how nutritional therapies themselves could modify the behavior of various conditions. Nutrients such as n-3 PUFA have been demonstrated to have at least the potential to modulate diseases. Eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), the n-3 PUFA present in marine foods, have an important role in the prevention of many diseases, particularly cardiovascular diseases (CVD) [1, 2] and the American Heart Association and the European Society of Cardiology recommend a higher fish consumption and/or the daily intake of 1 g n-3 PUFA for primary and especially for secondary prevention [3].

n-3 PUFA preventive activity is related to their hypocholesterolemic, hypotriglyceridemic and anti-inflammatory effect, to the reduction of platelet aggregation and blood viscosity, to the antithrombotic and fibrinolytic activities [4, 5] and to the protection from ischemia/reperfusion-induced cellular damage and arrhythmias [6]. Among the different mechanisms considered at the basis of PUFA effects, the modulation of gene expression [7] is of particular interest. Actually, in a recent work [8] we demonstrated that EPA and DHA supplementation to cultured neonatal rat cardiomyocytes is able to modulate the expression of more than 100 genes, many of them related to cardiac hypertrophy.

The fatty acids regulate gene expression binding directly to nuclear receptors or affecting SREBP, ChREBP and NF kappa B protein content. PPAR are considered important effectors of fatty acid regulation of gene transcription, and recent studies have identified that PPAR have some new effects in CVD [9]. PPAR belong to a ligand-activated nuclear receptor super-family that includes three members (α, β/δ, and γ) encoded by distinct genes, which bind to sequence-specific target elements as an hetero-dimer with the retinoid X receptor (RXR) in the promoter region of target genes [10].

The first aim of the present study was to elucidate if EPA and DHA act simply by increasing PPAR/RXR DNA binding or if they can also increase the biosynthesis of PPAR themselves, so regulating the nuclear abundance of these nuclear factors.

The second aim of this work was to establish a new link between the modification of fatty acid composition induced by n-3 PUFA and their molecular effects.

Experimental Procedure

Material

Fatty acids, Ham F10 medium, fetal calf serum (FCS), horse serum (HS), gentamicin, amphotericin B, BSA, pentadecanoic acid, CelLytic^{TM} M, protease inhibitor cocktail, DTNB, palmitoyl coenzyme A lithium salt, anti-rat β-actin IgG were from Sigma (Milan, Italy). Anti-rat α, β/δ, γ PPAR, nucleophosmin and HRP-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology (Milan, Italy). All other chemicals and solvents were of the highest analytical grade.

Methods

Cell Cultures

Primary cultures of neonatal cardiomyocytes were obtained from the ventricles of 2–4 day old Wistar rats according to Yagev et al. [11]. The research was conducted in conformity with the Public Health Service Policy on Human Care and Use of Laboratory Animals, and the study protocol was approved by The Animal Care Committee of the University of Bologna (Italy). Cells were seeded at a density of 1.5×10^6 cells/ml in 100-mm i.d. Petri dishes in Ham F10 nutrient mixture supplemented with 10% v/v FCS, 10% v/v HS, gentamicin (1%), amphotericin B (1%), and grown at 37°C, 5% CO_2 and 95% humidity. Forty-eight hours from seeding, cardiomyocytes were randomly divided into control and fatty acid supplemented groups. EPA and DHA were dissolved in ethanol and supplemented at 60 μM concentration. Control medium was added with the same volume of ethanol (≤0.1% v/v), to avoid interference due to the vehicle. Media were changed every 48 h and on day 8 from seeding, after a 6-day exposure to fatty acids and at complete confluence, cardiomyocytes were washed three times with 0.9% NaCl and the cells scraped off. Whole cell lysate (WCL) was obtained using CelLytic^{TM} M plus protease inhibitor cocktail (1 μM AEBSF, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A and 14 μM E-64) while the nuclear (NF) and cytoplasmic fraction (CF) were obtained by differential centrifugations as previously reported by Wright et al. [12].

PPAR DNA Binding

The degree of DNA binding of the three PPAR isoforms was determined by an immunosorbent assay (ELISA) utilizing PPAR α, β/δ and γ transcription factor assay kits (Cayman Chemicals, USA) following the manufacturer's instructions. Briefly, a specific double stranded DNA sequence containing the peroxisome proliferator responsive

element (PPRE) was immobilized onto the bottom of the wells of 96-well plates. Aliquots of NF diluted to obtain 50 μg protein in each assay were added to each well, so that PPAR contained in the extract bind specifically to the PPRE. Different PPAR isoforms were detected by the addition of specific primary antibodies directed against PPAR α, β/δ, or γ. A secondary antibody conjugated to HRP was added to provide a sensitive colorimetric readout at 450 nm. DNA binding of all the three isoforms was evaluated in each NF.

Western Blot Analysis

Western blotting was performed on WCL, CF and NF. Aliquots corresponding to 30 μg of proteins were analyzed by SDS–PAGE (12% gel). Proteins were transferred onto a nitrocellulose membrane and probed at room temperature for 60 min with the specific primary antibody anti-α, anti-β/δ, anti-γ PPAR, anti-nucleophosmin (B23) (1:1,000) or anti-β-actin (1:20,000). After further washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG for 30 min. Final detection was performed with an enhanced chemiluminescence (ECL Advance™) Western blotting detection kit (Amersham Biosciences, UK). β-Actin was used in WCL and CF [13, 14], and B23 in NF [15] as loading control.

Fatty Acid Composition

Total lipids were extracted from CF and NF according to Folch et al. [16] and methyl esterified according to Stoffel et al. [17]. Prior to methyl esterification, pentadecanoic acid (C15:0) was added as the internal standard. Fatty acid composition (as methyl esters) was determined by gas chromatography (GC 8000, Fisons, Milan, Italy) using a capillary column (SP 2340, 0.2 μm film thickness) at a programmed temperature gradient (160–210 °C, 8 °C/min) as previously reported [18]. The gas chromatographic peaks were identified on the basis of their retention time ratio relative to methyl stearate and predetermined on authentic samples. Gas chromatographic traces and quantitative evaluations were obtained using a Chrom Card Software (Thermo Electron Scientific, Milan, Italy) computing integrator.

Acyl-CoA Thioesterase Activity

Acyl-CoA thioesterase (EC 3.1.2.2, ACOT) activity was determined spectrophotometrically according to Ofman et al. [19] by measuring the cleavage of the free thiol group of palmitoyl coenzyme A, used as the substrate. The reaction mixture contained 50 mM HEPES, 50 mM KCl, 1 mg/ml BSA and 100 μM palmitoyl coenzyme A lithium salt. Reactions started with the addition of 100 μl of WCL, and after 15 min at 37 °C were stopped by the addition of DTNB at a final concentration of 0.5 mM. DTBN reacted with CoA-SH, and the TNB formed was measured at 412 nm using a spectrophotometer (Beckman Coulter DU730, Milan, Italy). Substrate blanks were run in order to correct for aspecific TNB formation. ACOT activity was calculated from the TNB molar extinction coefficient ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$) and normalized for protein content in the same sample.

Protein Content

The protein content in the samples was determined according to Bradford [20] using BSA as standard.

Statistical Analysis

Data are reported as means ± SD of results obtained in at least three independent cell cultures. Differences were tested for statistical significance by one way ANOVA using Tukey's test.

Results

The evaluation of DNA binding of the three PPAR isoforms revealed a significant increase in EPA and DHA supplemented cardiomyocytes for the β/δ isoform only (Fig. 1).

As evidenced by western blotting analysis, all three PPAR isoforms appeared expressed in WCL (Fig. 2a), and PUFA supplementation did not apparently modify protein

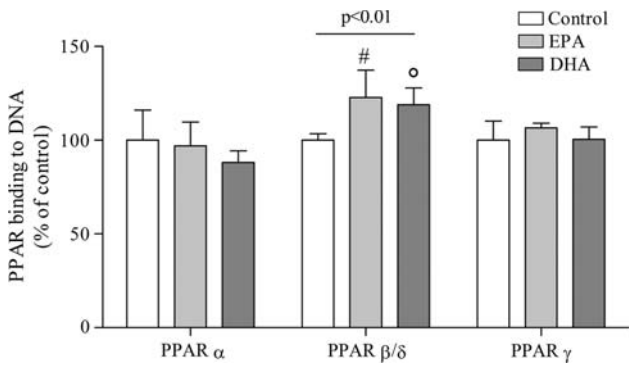

Fig. 1 PPAR binding to DNA in control and EPA or DHA supplemented cardiomyocytes. PPAR binding was measured using aliquots of NF as described in "Methods", and is expressed as percentage of control cells, assigned as 100%. Data are mean ± SD of five samples obtained in independent cell cultures. Statistical analysis was by one way ANOVA (PPAR β/δ $P < 0.01$) with Tukey as post-test (#$P < 0.05$ and °$P < 0.01$ vs. control)

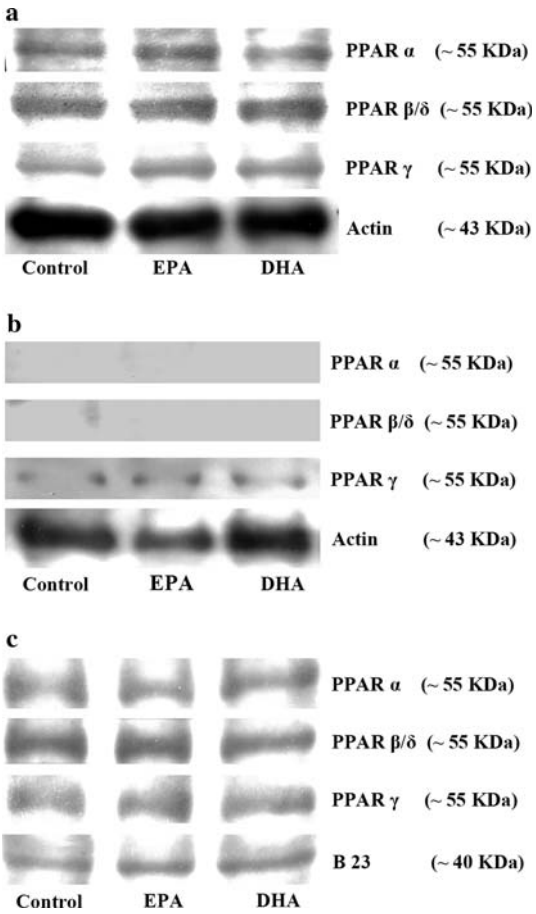

Fig. 2 PPAR α, *β/δ* and *γ* protein expression and subcellular localization in control, EPA and DHA supplemented cardiomyocytes. PPAR protein expression was analyzed in whole cell lysate (**a**), cytosolic fraction (**b**), and nuclear fraction (**c**) using SDS–PAGE as described in "Methods". Pictures are representative of three different western blotting experiments

expression, regardless of the isoform considered. Western blotting analysis in CF (Fig. 2b) and NF (Fig. 2c) clearly showed that all PPAR isoforms were present almost exclusively in the nucleus, while at the cytoplasmic level, a very mild appearance was noted only for the *γ* subtype. Differences due to PUFA supplementation were not apparent either in WCL nor in NF.

The fatty acid composition of CF is reported in Fig. 3a. EPA supplementation led to an enhanced EPA and n-3 docosapentaenoic (C22:5n-3, DPA) relative molar content, without any increase in DHA content. DHA relative molar content increased in CF of DHA supplemented cardiomyocytes. In both cases, incorporation of n-3 PUFA was paralleled by a reduction in C18:1, C18:2n-6, and C20:4n-6 relative molar content. Total concentration of fatty acids in CF was unaffected by PUFA supplementation (Fig. 3b).

Even in NF, supplementation with EPA led to an enhanced EPA and n-3 DPA relative molar content, without any increase in DHA content, while supplemented DHA was significantly incorporated without any appreciable retro conversion to n-3 DPA and EPA (Fig. 4a). Supplementation with both PUFA caused a reduction in C16:0, C18:0, C18:1, and C20:4n-6 relative molar content, more evident in EPA supplemented cells. Furthermore, both n-3 PUFA supplementations increased fatty acid concentration in NF (Fig. 4b).

As shown in Fig. 5 n-3 PUFA supplementation, particularly DHA one, significantly increased ACOT activity.

Discussion

The evaluation of PPAR α, *β/δ* and *γ* protein mass confirmed that all PPAR isoforms are expressed in neonatal rat cardiomyocytes, according to Takano et al. [21] and Planavila et al. [22], and that they are almost exclusively localized at the nuclear level, as reported by Takano et al. [21] and Cheng et al. [23] in neonatal and adult rat cardiomyocytes, respectively. The mild expression of *γ* isoform in CF is in agreement with Burgermeister and Seger [24], who reported the export to and retention in cytosol of this isoform due to its interaction with mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK1) [25]. The enhanced PPAR *β/δ* DNA binding observed in n-3 PUFA supplemented cells could be due to both a PPAR enhanced activity state or to a higher PPAR concentration. Although immunoblotting did not reveal any modification in PPAR protein mass it is difficult to exclude it, since ELISA assays are most sensitive.

EPA and DHA supplementation deeply changed the acyl composition of both CF and NF. In the NF the increase in n-3 PUFA relative content occurred at the expense of saturated C16:0 and C18:0, indicating that supplementation had a stronger effect on the PUFA/SFA ratio in the nucleus than in the cytosol. The high content of total FA, and in particular of EPA and DHA, and its increase following supplementation suggests a selective accumulation of n-3 PUFAs in the nucleus. This is in agreement with Brochot et al. [26], who observed a particularly high incorporation of n-3 PUFAs in the nuclear membrane of cardiac cells after feeding rats an α-linolenic supplemented diet, and supports a direct interaction of EPA and DHA with PPAR, since, in order to act as a bona fide ligand, a compound has to be present within the nucleus in sufficient amounts.

Obviously, for acting within the nucleus, EPA and DHA have to get into the nucleus. In this study, EPA and DHA were delivered as non-esterified fatty acids (NEFA) to the cell from extracellular sources, and CF composition clearly show that they were taken up from media. Once within the cell, free EPA and DHA may continue to diffuse into the nucleus, but more likely they are modified by enzymes for acylation into membrane phospholipids, or they are bound

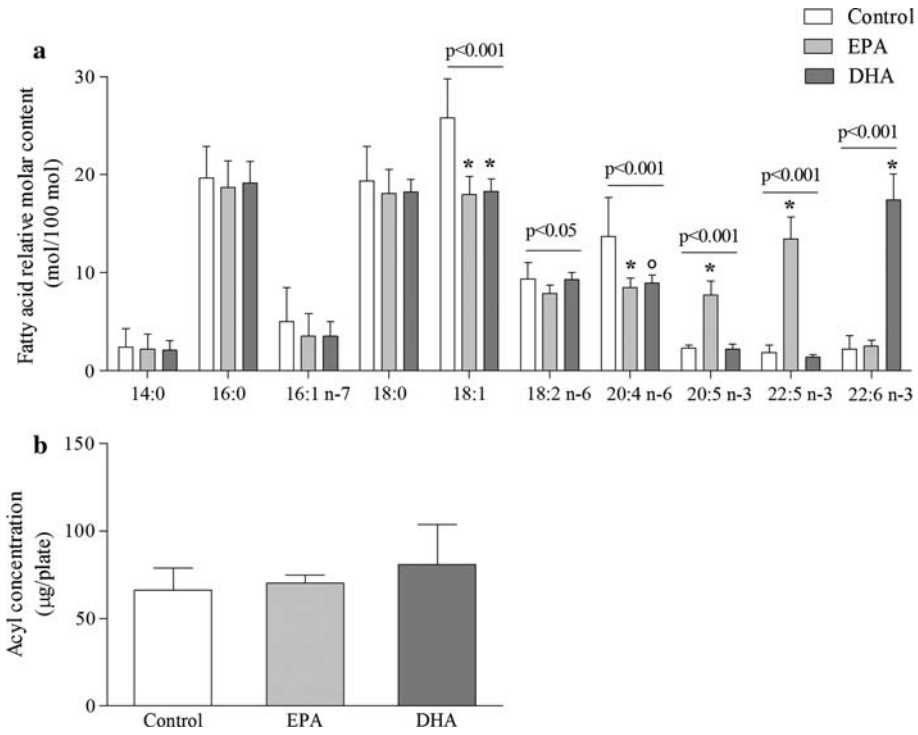

Fig. 3 Fatty acid percent composition and concentration of cytoplasmic fraction in control, EPA and DHA supplemented cardiomyocytes. Cytosolic fraction was separated by centrifugation, lipids were extracted, methylesterified and analyzed by gas chromatography as described in "Methods". Data are expressed as mol/100 mol (**a**) and µg/plate (**b**), and are mean ± SD of at least six samples obtained in three independent cell cultures. Statistical analysis was by one way ANOVA (18:1 $P < 0.001$; 18:2n-6 $P < 0.05$; 20:4n-6 $P < 0.001$; 20:5n-3 $P < 0.001$; 22:5n-3 $P < 0.001$; 22:6n-3 $P < 0.001$) with Tukey as post-test (°$P < 0.01$ and *$P < 0.001$ vs. control)

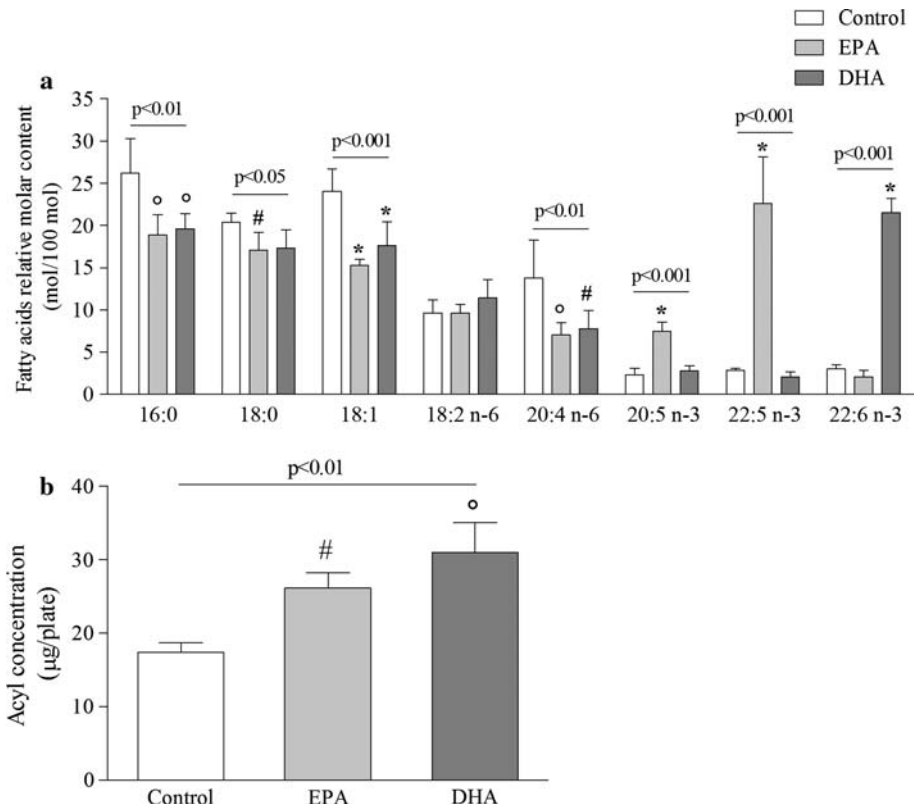

Fig. 4 Fatty acid percent composition and concentration of nuclear fraction in control, EPA and DHA supplemented cardiomyocytes. Nuclear fraction was separated by centrifugation, lipids were extracted, methylesterified and analyzed by gas chromatography as described in Methods. Data are expressed as mol/100 mol (**a**) and µg/plate (**b**), and are mean ± SD of at least six samples obtained in three independent cell cultures. Statistical analysis was by one way ANOVA (16:0 $P < 0.01$; 18:0 $P < 0.05$; 18:1 $P < 0.001$; 20:4n-6 $P < 0.01$; 20:5n-3 $P < 0.001$; 22:5n-3 $P < 0.001$; 22:6n-3 $P < 0.001$) with Tukey as post-test (#$P < 0.05$; °$P < 0.01$ and *$P < 0.001$ vs. control)

by fatty acid binding proteins (FABP). FABP have been shown to strongly bind medium- and long-chain FA [27, 28]. Binding of FA to FABP within the cytoplasm results in the rapid movement of the lipid–protein complex through the nuclear pores into the nucleus [29, 30]. Since FABP bind NEFA with higher affinity than acyl-CoAs [31, 32], and long-chain acyl-CoA esters act as antagonists while long-chain NEFA as agonists for the PPAR [32, 33],

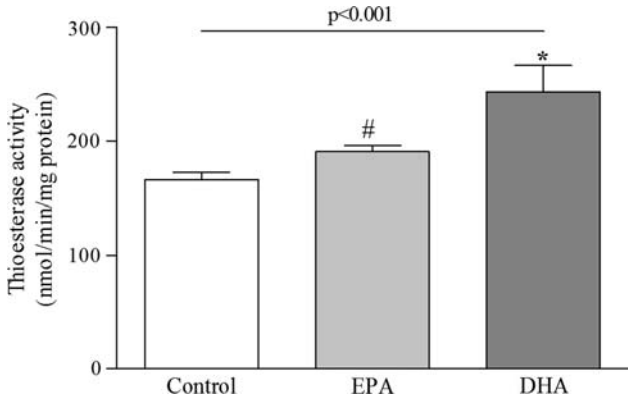

Fig. 5 Acyl-CoA thioesterase (ACOT) activity in control, EPA and DHA supplemented cardiomyocytes. ACOT activity was measured in whole cell extract as described in "Methods". Data are expressed as nmol/min/mg protein, and are mean ± SD of at least six samples obtained in three independent cell cultures. Statistical analysis was by one way ANOVA ($P < 0.001$) with Tukey as post-test (#$P < 0.05$; *$P < 0.001$ vs. control)

the NEFA/acyl-CoA ratio is an important determinant in fatty acid transport to the nucleus and in the regulation of gene transcription. The activity of ACOT, catalyzing the reaction leading to NEFA from acyl-CoA, increased in n-3 PUFA supplemented cells, in agreement with Ramos and Colquhon [34]. Although ACOT activity is not the only parameter for NEFA/acyl-CoA ratio, it is conceivable that it is important for the control of the NEFA pool composition, as also suggested by Pawar and Jump [35] and Mandard et al. [36]. Recently, Dongol et al. [37] reported the presence of a functional PPRE in the promoter region of ACOT gene. Since EPA and DHA are PPAR ligands, they could upregulate ACOT gene transcription, creating a self-maintaining loop that guarantees a high cytosolic NEFA concentration. NEFA are then transferred to the nucleus by FABP, where they can exert their activity as PPAR ligands.

The selective activation of PPAR β/δ is difficult to explain. Tentatively, we can suggest the intervention of FABP. Within the nucleus, FABP may interact with PPAR [28] and apparently donate the fatty acid to activate the PPAR [38]. Tan et al. [38] provided evidence that in adipocytes some FABP act in concert with PPAR and that this activity is highly selective for particular FABP-PPAR pairs: A-FABP specifically enhances the activity of PPAR γ, while K-FABP activates PPAR β/δ. Although further studies are needed to verify if similar pairs are also present in cardiomyocytes, and eventually if they have preferential ligands, this hypothesis could explain the preferential PPAR β/δ activation.

Although the original concept that ligand availability is the sole and primary factor controlling the activity of PPAR has been replaced in recent years, due to the recognition of different control mechanisms of PPAR activity (competition for the dimerization partner RXR and for PPRE sites, recruitment of co-activators and repressors, degree of phosphorylation), it remains the sine qua non of the event. Our data further clarify what happens in cardiomyocytes following n-3 PUFA supplementation, linking the modification of acyl composition to ACOT activity and PPAR activation. This may contribute to the understanding of the protective mechanisms of n-3 PUFA in CVD.

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