FEBS Letters 581 (2007) 923–929

N – 3 PUFAs modulate global gene expression profile in cultured rat cardiomyocytes. Implications in cardiac hypertrophy and heart failure

Alessandra Bordoni[a,*], Annalisa Astolfi[b], Luca Morandi[c], Andrea Pession[b], Francesca Danesi[a], Mattia Di Nunzio[a], Monica Franzoni[b], PierLuigi Biagi[a], Annalisa Pession[c]

[a] *Nutrition Research Center, Department of Biochemistry "G. Moruzzi", University of Bologna, Via Irnerio, 48 – 40126 Bologna, Italy*
[b] *Oncologia ed Ematologia Pediatrica "Lalla Seragnoli" – Policlinico S. Orsola-Malpighi, University of Bologna, Italy*
[c] *Section of Pathology, Department of Oncology – Ospedale Bellaria, University of Bologna, Italy*

Received 11 December 2006; revised 24 January 2007; accepted 26 January 2007

Available online 6 February 2007

Edited by Laszlo Nagy

Abstract In cardiac cells the effects of *n* – 3 PUFAs on the whole genome are still unknown despite their recognized cardioprotective effects and ability to modulate gene expression. We have evaluated the effects of *n* – 3 PUFAs supplementation on the global gene expression profile in cultured neonatal rat cardiomyocytes, detecting many genes related to lipid transport and metabolism among the upregulated ones. Many of the downregulated genes appeared related to inflammation, cell growth, extracellular and cardiac matrix remodelling, calcium movements and ROS generation. Our data allow to speculate that the cardioprotective effect of *n* – 3 PUFAs is related to a direct modulation of genes in cardiac cells.

Keywords: *n* – 3 PUFA; Cultured cardiomyocytes; Gene expression; Eicosapentaenoic acid; Docosahexaenoic acid

1. Introduction

N – 3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are believed to have a protective action against cardiovascular diseases (CVDs), particularly coronary heart diseases, and fish oil feeding has been associated to reduced mortality in several studies [1].

N – 3 PUFAs play a key role in the prevention or progression of human diseases by different mechanisms, i.e. by modulating membrane lipid composition and by affecting metabolic and signal-transduction pathways [2]; nowadays, a direct control of gene expression by PUFAs has been demonstrated [3]. Many studies have been addressed to the understanding of the molecular mechanisms underlying the effects of PUFAs on gene expression in the liver [3], but little is known about the heart, and the effects of EPA and DHA on the whole genome have never been investigated in cardiomyocytes. Nowadays, novel techniques, such as DNA microarrays, enable the study of cardiomyocyte gene expression changes in response to PUFA in a global way. Using this novel approach, we have evaluated the effects of EPA and DHA supplementation on the global gene expression profile in cultured neonatal rat cardiomyocytes.

Although mammals are able to synthesise EPA and DHA from α linolenic acid (ALA), the lack of effectiveness of the precursor fatty acid in CVD has been recently reviewed by Wang et al. [4], so we focused our attention on the two *n* – 3 PUFAs which appear to prevent cardiac diseases.

*Corresponding author. Fax: +39 051 2091235.
E-mail address: alessandra.bordoni@unibo.it (A. Bordoni).

Abbreviations: ALA, α linolenic acid; ADRP, adipose differentiation-related protein; CH, cardiac hypertrophy; CVD, cardiovascular diseases; DECR1, 2,4-dyenoylCoA reductase 1; DHA, docosahexaenoic acid; DPT, dermatopontin; ECH1, enoylCoA hydratase 1; ECM, extracellular and cardiac matrix; EPA, eicosapentaenoic acid; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HF, heart failure; HMGCS2, 3-hydroxy-3-metylglutarylCoA synthase 2; Il-6, interleukin-6; MBEI, model based expression index; MI, myocardial infarction; MMP12, matrix metalloproteinase 12; NEFA, non-esterified fatty acid; NFkB, nuclear factor-kappa B; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SAM, significance analysis of microarrays; STAR, steroidogenic acute regulatory protein; STAT3, signal transducer and activator of transcription 3; TIMP, tissue inhibitors of matrix metalloproteinase; TNFα, tumor necrosis factor-alpha

2. Materials and methods

2.1. Materials

Ham F10 media, fetal calf serum (FCS), horse serum (HS), propidium iodide, EPA and DHA were from Sigma (St. Louis, MO, USA); rat oligo array G4130A and Low RNA input fluorescent linear amplification kit were from Agilent Technologies (Palo Alto, CA, USA); RNAeasy protect miny kit was from Qiagen (Milan, Italy), Cy3-CTP and Cy5-CTP were from Perkin Elmer (Milan, Italy); primers were custom synthesized by Proligo (Sigma-Aldrich, Milan, Italy) and TIB MolBiol (Roche Diagnostics, Milan, Italy); Superscript II RT was from Invitrogen (Milano, Italy).

2.2. Methods

2.2.1. Cell cultures. Primary cultures of cardiomyocytes were obtained from the ventricles of newborn Wistar rats according to Yagev et al. [5]. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NHI Publication No. 85–23, 5 revised 1996). To obtain pure cultures avoiding the presence of fibroblasts, cells were pre-plated twice before the final seeding, and the absence of cells other than cardiomyocytes verified microscopically. After 48 h in control medium (Ham F10 plus 10% FCS and 10% HS), some cells were shifted to a 60 μM EPA or a 60 μM DHA supplemented medium. Fatty acids were dissolved in ethanol, and control medium was added with the same volume of ethanol (0.01% v/v). Media were changed every 48 h; on day 8 from seeding, after 6 day exposure to *n* – 3 PUFAs and at complete confluence, cardiomyocytes were washed three times with 0.9% NaCl and scraped off.

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.070

To verify the incorporation of the supplemented fatty acids, cell lipids were extracted [6] and separated by thin layer chromatography. Spots corresponding to phospholipids were scraped off; fatty acids were methyl-esterified and gas-chromatographed [7].

2.2.2. Cell cycle and apoptosis. Cells were seeded in 24-well plates at a concentration of 200000/well in complete medium. Some cells were shifted to EPA- or DHA-containing medium 48 h after seeding. Two–four replicates were performed for each time point. Viable cells were counted by trypan blue dye exclusion each day for the following 6 days. Apoptosis and cell cycle analysis were performed by cytofluorimetric detection with a FACS Calibur Cytometer (Becton Dickinson, Mansfield, MA) [8] after staining with propidium iodide. Cell cycle distribution was calculated using ModFit software (Verify Software House, Inc., Mansfield, MA).

2.2.3. Microarray analysis. Cells were scraped off in ice cold PBS, treated with RNAlater and homogenized. Total RNA was extracted (RNAeasy Protect mini kit) and analysed on both a spectrophotometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only samples with 28S/18S ratio > 2.0 and no evidence of ribosomal degradation were included. The cRNA was generated by in vitro transcription (Low RNA input fluorescent linear amplification kit), labelled with Cy3-CTP or Cy5-CTP, and hybridized to 22 K-gene arrays (Agilent Rat oligo array G4130A) containing sequences representing over 20000 well-characterized rat transcripts. Direct comparisons were performed between $n-3$ PUFAs supplemented cells versus unsupplemented ones (controls), and each analysis was dye-swapped. Data analysis was performed using R and Bioconductor packages (www.bioconductor.org). Red and green signals were background-subtracted and normalized (Lowess smoother). The whole dataset was filtered to exclude genes poorly expressed in all the samples or not varying (coefficient of variation of at least 2), thus leading to a list of 7110 genes.

To compare control and $n-3$ PUFA supplemented cardiomyocytes, SAM (significance analysis of microarrays) algorithm was used, minimizing potential false positive genes (<3%). Hierarchical clustering was performed using Manhattan distance and complete linkage.

2.2.4. Real time PCR. Validation of selected genes was performed by quantitative real-time PCR with a 5700 Applied Biosystems (Applied Biosystems, Foster City, CA) apparatus. One μg of total RNA was retrotranscribed using random hexamers and Superscript II RT. Specific primer pairs (Table 1) were chosen by Primer Express 2.0 (Applied Biosystems, Foster City, CA). Gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

2.2.5. PPAR activation. The activation of PPARα and β/δ by $n-3$ PUFAs was determined by an immunosorbent assay (ELISA) utilizing PPARα and β/δ transcription factor assay kits (Cayman Chemicals, USA). Nuclear extracts were prepared from cardiomyocytes by differential centrifugation according to Wright et al. [9] and the assay performed following the manufacturer's instruction.

3. Results

In cardiomyocytes, supplemented $n-3$ PUFAs were incorporated into phospholipids, and their amount increased about 10-fold in comparison with control cells (data not shown).

In mammals EPA can be converted into DHA and DHA can be retro converted into EPA [10], both pathways taking place mainly in liver peroxisomes. Consequently, when according to clinical practice fish oil or supplements are administered, it is difficult to assess which effects are due to EPA and which to DHA, since they are inter convertible. Cultured cardiomyocytes are able to synthesise DHA from EPA [11], but no data are reported in the literature about the retro conversion of DHA in cardiac cells. So the rate of the inter conversion between EPA and DHA could be different in cultured cardiomyocytes and in the whole animal, this being an interfering factor in the interpretation of the results. To avoid it, we supplemented cells with 60 μM EPA or 60 μM DHA and performed separated microarray analysis. Then, we compared results obtained with the two different supplementations and considered as modulated only genes upregulated or downregulated in a significant way by both the fatty acids. In this way, we obtained results independent by the rate of inter conversion between the two fatty acids. With the same aim, in RT-PCR, cell growth, apoptosis and PPAR activation experiments we considered data from EPA and DHA supplemented cardiomyocytes all together.

The expression of 122 cDNA was significantly altered in cardiomyocytes grown in the $n-3$ PUFAs enriched medium

Table 1
Sequences of specific primer pairs used for real-time PCR

Gene name	Direct	Reverse
HMGCS2 (3-hydroxy-3-metylglutaryl CoA synthase 2)	CGCATGTCCCCTGAGGAATT	CCAAGTGCCTGGGAAGAGGT
ECH1 (enoylCoA hydratase 1)	AATTCACGGAGGCTGCATTG	GCAGCGTTCCTACATCAGCA
STAR (steroidogenic acute regulatory protein)	GGCCTTGGGCATACTCAACA	CAGCACCTCCAGTCGGAACA
DECR1 (2,4-dyenoylCoA reductase 1)	GGCGTGGAAGCCATGAATAA	TCAAATTTTCCAGTCGGGTCC
MMP12 (matrix metalloproteinase 12)	CATTCTCTGGGCTTCCCTGC	TGAGTTCCTGCCTCACATCGT
DPT (dermatopontin)	AGGCCACTACGGCGAAGAC	CCGGCACATTATGAACTTCCA
GAPDH (glyceraldehyde-3-phosphate-dehydrogenase)	CTTGTGCAGTGCCAGCCTC	CAAGAGAAGGCAGCCCTGGTA

Table 2
Upregulated genes in $n-3$ PUFA supplemented cardiomyocytes with respect to controls

GenBank	UniGene	Gene name	Fold change	LogR
NM_173094	Rn.29594	3-Hydroxy-3-metylglutaryl CoA synthase 2	2.19	1.13
NM_013200	Rn.6028	Carnitine palmitoyltransferase 1b	2.03	1.02
NM_022594	Rn.6148	EnoylCoA hydratase 1, peroxisomal	1.78	0.83
AA899721	Rn.37524	Mitochondrial acylCoA thioesterase 1	1.59	0.67
BI285616	Rn.101967	Adipose differentiation-related protein	1.53	0.61
NM_031558	Rn.11399	Steroidogenic acute regulatory protein	1.51	0.59
NM_057197	Rn.2854	2,4-dyenoylCoA reductase 1	1.38	0.46
NM_145090	Rn.11219	ADP-ribosylation factor GTPase activating protein 1	1.24	0.31

Upregulated genes are identified by Genbank and Unigene code. Differential expression is shown as fold change and log ratio (LogR; base-two logarithm of the expression ratio) between PUFA-treated and untreated cells.

(Supplementary Figure 1); 17 genes were upregulated (8 identified genes, 2 EST, 7 unknown sequences) and 105 were downregulated (39 identified genes, 30 EST, 36 unknown sequences) relative to controls (false discovery rate <3%). Data on gene expression are available on the GEO website (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE4327. Identified upregulated genes are reported in Table 2; identified downregulated genes, listed according to molecular function, are reported in Table 3. To confirm results we performed real-time PCR analysis of some interesting upregulated (HMGCS2, STAR, ECH1, DECR1) and downregulated genes (DPT, MMP12), showing that microarray results are reliable and that PUFA supplementation is able to induce substantial changes in the gene expression profile of cardiac cells (Fig. 1).

Upregulation of genes by PUFAs is considered mainly due to the activation of PPARs. As shown in Fig. 2A, the expression of the three PPAR genes, reported as the mean normalized fluorescence level in the four microarray experiments performed, was not influenced by PUFA supplementation, and the fluorescence level suggested a low expression in all samples. Regarding PPAR activation, no differences were detected in PPARα between control and supplemented cardiomyocytes (data not shown), while PPARβ/δ appeared significantly activated in $n-3$ PUFAs supplemented cells (Fig. 2B).

Table 3
Downregulated genes in $n-3$ PUFA supplemented cardiomyocytes with respect to controls

GenBank	UniGene	Gene Name	Fold change	LogR
Related to lipid metabolism				
NM_031841	Rn.83595	Scd2; stearoyl-Coenzyme A desaturase 2	−0.77	−0.37
Related to inflammation				
NM_053843	Rn.107323	Fcgr3; Fc receptor, IgG, low affinity III	−0.83	−0.27
NM_023025	Rn.44992	Cyp2j4; CYP2J4	−0.82	−0.29
NM_012589	Rn.9873	Il6; interleukin 6	−0.82	−0.29
X73371	Rn.33323	Fcgr2b; Fc receptor, IgG, low affinity IIb	−0.81	−0.30
NM_138900	Rn.4037	C1s; complement component 1, s subcomponent	−0.80	−0.33
NM_012870	Rn.9792	Tnfrsf11b; tumor necrosis factor receptor superfamily, member 11b	−0.78	−0.35
AF159103	Rn.92334	Tnfip6; tumor necrosis factor induced protein 6	−0.76	−0.39
BI289859	Rn.8807	Ignt; I-branching beta-1,6-acetylglucosaminyltransferase	−0.76	−0.39
AA800318	Rn.100285	Serping1; serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	−0.71	−0.49
NM_080698	Rn.8778	Fmod; fibromodulin	−0.71	−0.50
AA858732	Rn.2283	Lyz; lysozyme	−0.64	−0.64
Related to cell survival and ECM remodelling				
BI275292	Rn.9048	Agpt2; angiopoietin-2	−0.86	−0.22
NM_031050	Rn.3087	Lum; lumican	−0.84	−0.26
BM389281	Rn.30828	Scgf; stem cell growth factor	−0.80	−0.33
NM_017066	Rn.1653	Ptn; pleiotrophin	−0.75	−0.41
NM_022177	Rn.54439	Cxcl12; chemokine (C-X-C motif) ligand 12	−0.72	−0.48
CB544481	Rn.2379	Mgp; matrix Gla protein	−0.65	−0.62
NM_053963	Rn.33193	Mmp12; matrix metalloproteinase 12	−0.59	−0.76
BM386671	Rn.8396	Dpt; dermatopontin	−0.51	−0.96
Related to cell proliferation, cell cycle and regulation of transcription				
NM_031335	Rn.28212	Polr2f; polymerase II	−0.85	−0.23
NM_017029	Rn.10971	Nef3; neurofilament 3, medium	−0.83	−0.27
AI103327	Rn.14867	Tcf19; transcription factor 19	−0.82	−0.28
U17565	Rn.33226	Mcmd6; mini chromosome maintenance deficient 6	−0.81	−0.31
AA858882	Rn.82737	Zfp36; zinc finger protein 36	−0.78	−0.35
NM_057144	Rn.11345	Csrp3; cysteine-rich protein 3	−0.77	−0.38
AI012106	Rn.19481	Bteb1; basic transcription element binding protein 1	−0.76	−0.39
NM_138856	Rn.53644	Gs3; putative regulation protein GS3	−0.71	−0.50
NM_019282	Rn.42929	Cktsf1b1; cysteine knot superfamily 1, BMP antagonist 1	−0.66	−0.59
AA964489	Rn.92160	Ceacam10; CEA-related cell adhesion molecule 10	−0.60	−0.73
Related to Ca^{2+} movements and cardiac contractility				
NM_131914	Rn.81070	Cav2; caveolin 2	−0.82	−0.29
NM_022005	Rn.839	Fxyd6; FXYD domain-containing ion transport regulator 6	−0.76	−0.39
Related to ROS production				
AI137330	Rn.98491	Cybb; endothelial type gp91-phox gene	−0.61	−0.71
Other functions				
BM385476	Rn.32168	Damp1; DAMP-1 protein	−0.81	−0.30
AI230347	Rn.7233	Ns5atp9; Ns5atp9 protein	−0.80	−0.32
NM_133594	Rn.103348	Sumo2; SMT3 suppressor of mif two 3 homolog 2	−0.75	−0.42
NM_145093	Rn.38451	Aard; A5D3 protein	−0.73	−0.45
NM_053591	Rn.6051	Dpep1; dipeptidase 1 (renal)	−0.71	−0.50

Downregulated genes are grouped according to their function and identified by Genbank and Unigene code.
Differential expression is shown as fold change and log ratio (LogR; base-two logarithm of the expression ratio) between PUFA-treated and untreated cells.

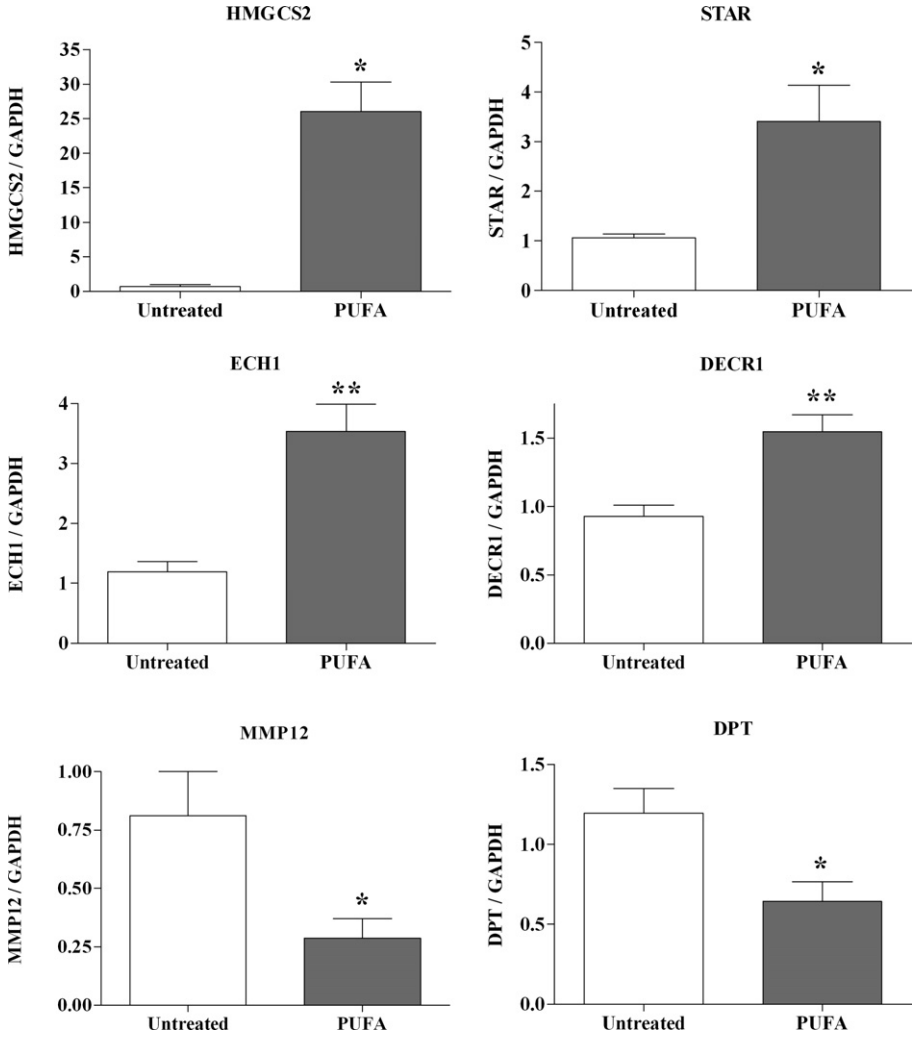

Fig. 1. Differential expression of selected genes in control and $n-3$ PUFA supplemented cardiomyocytes by real-time PCR. Data are means ± S.D. of the results obtained in both EPA and DHA supplemented cardiomyocytes (2–4 biological replicates and 2–4 technical replicates in each condition). The expression of every gene was normalized to GAPDH expression. Significance: Students' t test ($^{*}P < 0.05$; $^{**}P < 0.01$).

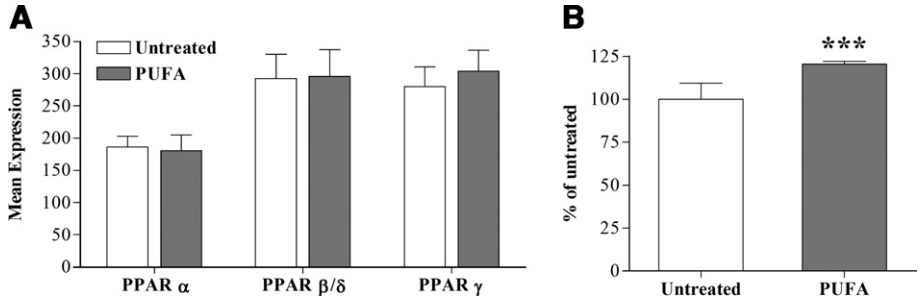

Fig. 2. Expression and activation of PPARs in control and supplemented cardiomyocytes. Data are means ± S.D. of results obtained in both EPA and DHA supplemented cardiomyocytes. (A) Level of expression of PPARα, β/δ, and γ in control and $n-3$ PUFA supplemented cardiomyocytes. The expression of the three PPAR genes is shown as the mean normalized fluorescence level in the four microarray experiments performed. (B) Activation of PPAR β/δ in control and $n-3$ PUFA supplemented cardiomyocytes. Significance: Students' t test ($^{***}P < 0.001$).

Since microarray data suggested an involvement of $n-3$ PUFAs in cardiomyocyte survival and proliferation, we studied the effects of the supplementation on these biological processes. PUFA-supplemented cardiomyocytes showed a modest increase in cell growth at late time points ($P < 0.05$ after 6 days in $n-3$ PUFA supplemented media, Fig. 3A), and no significant alteration in cell cycle distribution (data not shown). PUFA supplementation significantly protected cardiomyocytes from apoptosis (30% apoptosis inhibition already at early time points; Fig. 3B); pictures taken after 1 and 4 days of supplementation confirmed the increase in cell survival due to $n-3$ PUFAs (Fig. 3C and D).

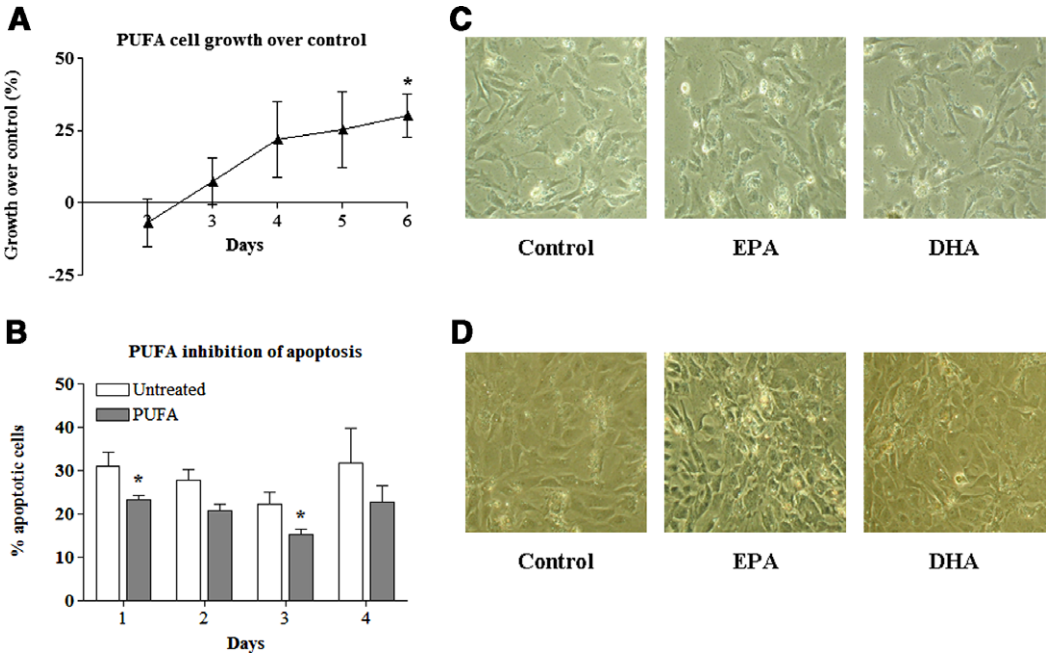

Fig. 3. Effects of PUFA supplementation on cell morphology, growth and apoptosis. Data are means ± S.D. of results obtained in both EPA and DHA supplemented cardiomyocytes. (A) Growth percentage over control of PUFA-treated cells. Each point is the mean ± S.E.M. of 4–8 replicates. Significance: Students' t test ($^*P < 0.05$). (B) Percentage of apoptosis inhibition by PUFA supplementation: each bar is the mean ± S.E.M. of two replicates. Significance: Students' t test ($^*P < 0.05$). (C) and (D) Microscopic observation of cell growth; pictures taken after 1 day (C) and 4 days (D) of supplementation with EPA or DHA.

3.1. Discussion

In cardiac cells $n-3$ PUFA supplementation caused the induction of several genes encoding proteins involved in lipid transport and metabolism, leading to a significant enrichment of transcripts belonging to Gene Ontology categories "lipid metabolism", "fatty acid metabolism" and "lipid transport" (P-value $\ll 0.01$). Particularly, the upregulation of adipose differentiation-related protein (ADRP), CPT1b and ECH1 genes possibly explains the pleiotropic effect reported for $n-3$ PUFAs in the heart, which preferably uses fatty acids as an energy source. ADRP, CPT1b and ECH1 genes, as many genes involved in cardiac energy metabolism, are PPAR-regulated; the observed upregulation of the gene encoding for acylCoA thioesterase, responsible for the cleavage of acylCoAs to the corresponding non-esterified fatty acids (NEFAs) and CoASH [12], may facilitate the $n-3$ PUFA mediated activation of PPARs, since NEFAs are preferential ligands of these nuclear receptors.

PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as heart, while PPARβ/δ is ubiquitously expressed [13]. Recently, Gilde et al. [14], using neonatal rat cardiomyocytes as well as the embryonic rat heart-derived H9c2 cells, clearly demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism, suggesting that PPAR β/δ may play an important role in cardiac disease. In our study PPARα was not activated by EPA or DHA, while PPAR β/δ was significantly activated. This is in agreement with Gilde et al. [14], who demonstrated that PPARβ/δ was fatty acid inducible and activated the expression of PPARα target genes involved in fatty acid utilization in cardiac myocytes, suggesting that PPARα and PPARβ/δ shared similar functions in cardiac cells regarding cardiac fatty acid metabolism. More recently Planavila et al. [15] demonstrated that PPARβ/δ activation inhibits phenylephrine-induced cardiomyocyte hypertrophy in neonatal rat ventricular cardiomyocytes. PPARβ/δ activation also inhibits LPS-induced NF-κB activation through a mechanism that may involve enhanced protein–protein interaction between this PPAR subtype and the p65 subunit of NF-κB. These data indicate that inhibition of the NF-κB signaling pathway may be the underlying mechanism responsible for the inhibition of cardiomyocyte growth. Regarding PPARγ, it has been initially reported that it has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as heart contain limited amounts. Very recently Ding et al. [16] indicates not only that PPARδ is expressed in cardiomyocytes, but also that in these cells it is a negative regulator of TNFα production via suppressing the NF-κB pathway. Our data about PPARγ expression are in agreement with Ding, and although further studies about the activity of $n-3$ PUFAs as PPARγ ligands are needed, it is possible to speculate about the role of this transcription factor in EPA and DHA anti-inflammatory activity in the heart.

Differently from liver, in cardiomyocytes, $n-3$ PUFAs did not downregulate genes involved in glucose metabolism and fatty acid synthesis, apart from stearoylCoA desaturase 2. Unexpectedly, many genes downregulated by $n-3$ PUFAs were related to cellular pathways controlling inflammation response, cardiac remodelling and angiogenesis, and apoptosis.

Chronic induction of inflammatory cytokines, such as TNFα or interleukin-6 (Il-6), and chronic inflammation are associated with the onset and progression of cardiac hypertrophy (CH) [17] and myocardial infarction (MI) [18]. $N-3$ PUFAs are considered potent anti-inflammatory agents [19], and their anti-inflammatory effects may be due to the inhibition of

NFκB and STAT3 activation via PPAR activation [15,20,21]. Although further studies are needed in cardiomyocytes during cytokine-induced inflammation before drawing final conclusion, in the light of the downregulation of inflammatory genes our data confirm the potential anti inflammatory role of $n-3$ PUFAs in cardiac cells.

Both CH and MI are characterised by cardiac and extracellular matrix (ECM) remodelling. Increased collagen synthesis causes fibrosis; metalloproteinases break down collagen, with their actions restricted by TIMPs (tissue inhibitors of matrix metalloproteinases) [22]. Once the metalloproteinase-TIMP balance is upset in favour of increased metalloproteinase activity, collagen crosslinks degenerate so that the hypertrophied left ventricle begins to dilate [23]. The observed downregulation of matrix metalloproteinase 12 in $n-3$ PUFA cells could be supposed to contribute to the counteraction of fibrosis.

Since microarray data indicated a modulation of genes related to cell proliferation, cell cycle and regulation of transcription, some experiments were performed in order to verify the effects of PUFA supplementation on these biological processes. After an initial inhibiting effect, PUFA supplemented cardiomyocytes showed a modest increase in cell growth at late time points, without any significantly alteration in cell cycle distribution. The effect on cell growth could be explained by a significant protection from apoptosis by PUFA treatment. Apoptosis of cardiomyocytes plays a pivotal role in the progression of CH and MI toward heart failure (HF) [24,25], so the decrease in programmed cell death could be one of the protective mechanisms of $n-3$ PUFAs. According to Shimojo et al. [26], we did not find any modification in gene expression of three important molecules related to apoptosis (caspase-3, Bax and Bcl-2); notwithstanding, since the execution of the apoptotic program entails complex interactions between and execution of multiple molecular subprograms, and in the light of our data, a counteraction of programmed cell death by $n-3$ PUFAs has to be carefully considered.

Consonant with overall knowledge, high rates of ROS production [27] and changes in calcium homeostasis are strictly related to CH and MI, too. $N-3$ PUFAs downregulated the gene encoding for GP91phox, the subunit responsible for the catalytic activity of NADPH-oxidase, which is reported to be the main source of ROS production during CH and MI [28], and the gene encoding for caveolin-2, therefore interfering with the biogenesis of caveolae. Recently Morris et al. [29] demonstrated that α1-adrenergic receptor signalling, which activation can initiate arrhythmogenesis, is localized to caveolae in neonatal rat cardiomyocytes.

All together, our microarray data suggest that $n-3$ PUFAs regulate many genes involved in CH and infarction. As a confirmation, analysing published microarray datasets obtained in a rat model of CH [30] and in a murine model of MI [31] and comparing them to our data it appears clear that genes upregulated during MI or CH are downregulated by $n-3$ PUFA and vice versa.

Evidence from epidemiological and randomized controlled trials shows beneficial effects of $n-3$ PUFAs from fish sources on CVD, especially in patients with preexisting CVD. These data have led the American Heart Association Dietary Guidelines committee to recommend to the general population the consumption of at least two servings of fish per week. A recent systematic review of the literature on the effects of $n-3$ PUFAs on cardiovascular disease outcomes and adverse events indicate that, in secondary prevention, most trials reported that fish oil significantly reduced all-cause mortality, MI, cardiac and sudden death, or stroke [4]. In primary prevention, most cohort studies reported that fish consumption was associated with lower rates of all-cause mortality and adverse cardiac outcomes, while the effects on stroke were inconsistent [4].

In this scenario, and apart from clinical data, the debate on $n-3$ PUFA mechanism of action is still open. In this work, utilizing high throughput technologies, we analysed the molecular events due to $n-3$ PUFA supplementation in a global way, reporting for the first time data about the modulation of gene expression profile by these fatty acids in cardiomyocytes. Our data confirm the possibility that the mechanism of action of $n-3$ PUFAs is not only related to the prevention of the metabolic syndrome or to the anti arrhythmic effect due to stabilization of cell membrane, but also to direct effects on cardiac cell genome, and provide a new putative link between $n-3$ PUFA supplementation and prevention of myocardial dysfunctions.

Further studies are needed to elucidate the molecular effects of $n-3$ PUFAs, as well as to verify which nuclear receptors among the many having fatty acids as putative ligands [32], are involved in cardiac cells, but the global vision given by our results may be important for addressing researches toward specific pathways.

Acknowledgment: The authors thank Prof. Lanfranco Masotti (Dept. Biochemistry, University of Bologna) for many useful suggestions and critical discussions and for his encouragement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.01.070.

References

[1] Engler, M.M. and Engler, M.B. (2006) Omega-3 fatty acids: role in cardiovascular health and disease. J. Cardiovasc. Nurs. 21, 17–24.
[2] Hulbert, A.J., Turner, N., Storlien, L.H. and Else, P.L. (2005) Dietary fats and membrane function: implications for metabolism and disease. Biol. Rev. Camb. Philos. Soc. 80, 155–169.
[3] Nakamura, M.T., Cheon, Y., Li, Y. and Nara, T.Y. (2004) Mechanisms of regulation of gene expression by fatty acids. Lipids 39, 1077–1083.
[4] Wang, C., Harris, W.S., Chung, M., Lichtenstein, A.H., Balk, E.M., Kupelnick, B., Jordan, H.S. and Lau, J. (2006) $n-3$ Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. Am. J. Clin. Nutr. 84, 5–17.
[5] Yagev, S., Heller, M. and Pinson, A. (1984) Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro 20, 893–898.
[6] Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.
[7] Stoffel, W., Chu, F. and Ahrens, E.H. (1959) Analysis of long-chain fatty acids by gas-liquid chromatography. Anal. Chem. 31, 307–308.
[8] Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring

thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods 139, 271–279.
[9] Wright, G., Singh, I.S., Hasday, J.D., Farrance, I.K., Hall, G., Cross, A.S. and Rogers, T.B. (2002) Endotoxin stress-response in cardiomyocytes: NF-kappaB activation and tumor necrosis factor-alpha expression. Am. J. Physiol. Heart Circ. Physiol. 282, H872–H879.
[10] Brossard, N., Croset, M., Pachiaudi, C., Riou, J.P., Tayot, J.L. and Lagarde, M. (1996) Retroconversion and metabolism of [13C]22:6$n-3$ in humans and rats after intake of a single dose of [13C]22:6$n-3$-triacylglycerols. Am. J. Clin. Nutr. 64, 577–586.
[11] Lopez Jimenez, J.A., Bordoni, A., Hrelia, S., Rossi, C.A., Turchetto, E., Zamora Navarro, S. and Biagi, P.L. (1993) Evidence for a detectable delta-6-desaturase activity in rat heart microsomes: aging influence on enzyme activity. Biochem. Biophys. Res. Commun. 192, 1037–1041.
[12] Ramos, K.L. and Colquhoun, A. (2001) Evidence for the involvement of polyunsaturated fatty acids in the regulation of long-chain acyl CoA thioesterases and peroxisome proliferation in rat carcinosarcoma. Cell Biochem. Funct. 19, 1–9.
[13] Braissant, O., Foufelle, F., Scotto, C., Dauca, M. and Wahli, W. (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. Endocrinology 137, 354–666.
[14] Gilde, A.J., van der Lee, K.A., Willemsen, P.H., Chinetti, G., van der Leij, F.R., van der Vusse, G.J., Staels, B. and van Bilsen, M. (2003) Peroxisome proliferator-activated receptor (PPAR) alpha and PPARbeta/delta, but not PPARgamma, modulate the expression of genes involved in cardiac lipid metabolism. Circ. Res. 92, 518–524.
[15] Planavila, A., Rodriguez-Calvo, R., Jove, M., Michalik, L., Wahli, W., Laguna, J.C. and Vazquez-Carrera, M. (2005) Peroxisome proliferator-activated receptor beta/delta activation inhibits hypertrophy in neonatal rat cardiomyocytes. Cardiovasc. Res. 65, 832–841.
[16] Ding, G., Cheng, L., Qin, Q., Frontin, S. and Yang, Q. (2006) PPARdelta modulates lipopolysaccharide-induced TNFalpha inflammation signaling in cultured cardiomyocytes. J. Mol. Cell. Cardiol. 40, 821–828.
[17] Mielniczuk, L.M. and Baughman, K.L. (2006) Immune modulation therapy in heart failure. Congest. Heart Fail. 12, 91–96.
[18] Frangogiannis, N.G. (2006) Targeting the inflammatory response in healing myocardial infarcts. Curr. Med. Chem. 13, 1877–1893.
[19] Calder, P.C. (2006) $n-3$ polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am. J. Clin. Nutr. 83, 1505S–1519S.
[20] Zhao, G., Etherton, T.D., Martin, K.R., Vanden Heuvel, J.P., Gillies, P.J., West, S.G. and Kris-Etherton, P.M. (2005) Anti-inflammatory effects of polyunsaturated fatty acids in THP-1 cells. Biochem. Biophys. Res. Commun. 336, 909–917.
[21] Wang, L.H., Yang, X.Y., Zhang, X., Huang, J., Hou, J., Li, J., Xiong, H., Mihalic, K., Zhu, H., Xiao, W. and Farrar, W.L. (2004) Transcriptional inactivation of STAT3 by PPARgamma suppresses IL-6-responsive multiple myeloma cells. Immunity 20, 205–218.
[22] Opie, L.H. (2004) Heart Physiology: From Cell to Circulation, Lippincott Williams and Wilkins, Philadelphia.
[23] Bradham, W.S., Bozkurt, B., Gunasinghe, H., Mann, D. and Spinale, F.G. (2002) Tumor necrosis factor-alpha and myocardial remodeling in progression of heart failure: a current perspective. Cardiovasc. Res. 53, 822–830.
[24] Aukrust, P., Gullestad, L., Ueland, T., Damas, J.K. and Yndestad, A. (2005) Inflammatory and anti-inflammatory cytokines in chronic heart failure: potential therapeutic implications. Ann. Med. 37, 74–85.
[25] Takemura, G. and Fujiwara, H. (2006) Morphological aspects of apoptosis in heart diseases. J. Cell Mol. Med. 10, 56–75.
[26] Shimojo, N., Jesmin, S., Zaedi, S., Soma, M., Maeda, S., Yamaguchi, I., Goto, K. and Miyauchi, T. (2006) Changes in important apoptosis-related molecules in the endothelin-1-induced hypertrophied cardiomyocytes: effect of the pretreatment with eicosapentaenoic acid. Exp. Biol. Med. (Maywood) (2), 932–936.
[27] Sawyer, D.B., Siwik, D.A., Xiao, L., Pimentel, D.R., Singh, K. and Colucci, W.S. (2002) Role of oxidative stress in myocardial hypertrophy and failure. J. Mol. Cell Cardiol. 34, 379–388.
[28] Murdoch, C.E., Grieve, D.J., Cave, A.C., Looi, Y.H. and Shah, A.M. (2006) NADPH oxidase and heart failure. Curr. Opin. Pharmacol. 6, 148–153.
[29] Morris, J.B., Huynh, H., Vasilevski, O. and Woodcock, E.A. (2006) Alpha(1)-Adrenergic receptor signaling is localized to caveolae in neonatal rat cardiomyocytes. J. Mol. Cell Cardiol. 41, 17–25.
[30] Strom, C.C., Kruhoffer, M., Knudsen, S., Stensgaard-Hansen, F., Jonassen, T.E.N., Orntoft, T.F., Haunso, S. and Sheikh, S.P. (2004) Identification of a core set of genes that signifies pathways underlying cardiac hypertrophy. Comp. Funct. Genom. 5, 459–470.
[31] Genomics of Cardiovascular Development, Adaptation, and Remodeling. NHLBI Program for Genomic Applications, Harvard Medical School. URL: http://www.cardiogenomics.org.
[32] Bordoni, A., Di Nunzio, M., Danesi, F. and Biagi, P.L. (2006) Polyunsaturated fatty acids: from diet to binding to PPARs and other nuclear receptors. Genes Nutr. 1, 95–106.