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

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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.

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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.

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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.