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ω6/ ω3 POLYUNSATURATED FATTY ACID SUPPLEMENTATIONS IN RENAL CELL MODEL LEAD TO A PARTICULAR REGULATION THROUGH LIPIDOME FOR PRESERVED ω6/ ω3 RATIOS

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Abstract

Polyunsaturated fatty acids (PUFA) supplementations modify cell lipid composition leading to a change in cell function. However, the effect of PUFA supplementations in renal model cell on the kidney epithelial cells membrane fatty acid profile is not known. Therefore, the purpose of this study was to determine the effects of PUFAs with different ω6/ω3 ratios supplementations in the kidney epithelial cells and the type of supplementation that can be used as cellular protection during kidney transplantation. For that, we used as model the LLCPK-1 cell and determined their membrane fatty acid (FA) composition after supplementation with three different commercial food supplements. These supplements consist of S1: docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) with ω6/ω3 ratio = 0.1, S2: DHA, EPA, linoleic acid (LA) and γ-linoleic acid (GLA) with ω6/ω3 ratio = 2.5, or S3: α-linolenic acid (ALA) and LA with ω6/ω3 ratio near 1. Cells were incubated for 24 hr with 30 µM of ω3 fatty acids from each supplement. Fatty acid composition of control and experimental groups was analysed by gas chromatography after extraction of lipids and fatty acids methylation. The efficiency of cell PUFA supplementation was achieved by showing 2 to 4 fold increases in cell PUFA incorporation. Whatever the supplementation used, the cell saturated fatty acids (SFA) were decreased by 50% following the three supplementations used ($p<0.001$) as compared to control group. These decreases in SFA were compensated in part by increasing monounsaturated fatty acid levels. All these changes were observed with constant of cell ω6/ω3 ratio whatever the supplementations used. These data suggest that the supplements, with long chain polyunsaturated fatty acids or their precursors, lead to important regulation in the lipidome (desaturases and elongases) associated to preserved ω6/ω3 ratios. The fatty acids remodeling may represent an interesting new mechanism by which renal FA homoestasis could occurred.

Key words: LLCPK-1, PUFA, MUFA, SFA, ω6, ω3, lipid, desaturase, elongase, nutrition.

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INTRODUCTION

The polyunsaturated fatty acids (PUFAs) were identified as potential food additives or as drugs for their biological activities. There are many evidences suggesting beneficial actions of PUFA on human health. In fact, previous studies demonstrate the protective effect of PUFAs supplementations against atherosclerotic heart diseases, myocardial infarction (8, 16, 6), cancer (24, 7), neurologic and psychiatric disorders (27, 21, 13) and renal diseases (11). The incorporation of PUFAs in cell membrane induced a change in the membrane fluidity (23) and in the regulation of receptor functions and enzyme activity (15, 12) leading to their physiological protective effects.

The modern western diet is deficient in ω3 fatty acids with ω6/ω3 ratio near 15 to 30 whereas in ancestral and traditional diet this ratio was about 1 (25). In addition, food agencies, scientific societies, national and international organizations recommend diet with ratio of ω6 to ω3 between 1 and 5 (12, 10). This balance of ω6 and ω3 series of PUFAs represents a very important prevention factor in many chronic diseases (cardiovascular diseases, autoimmune diseases, asthma, diabetes and rheumatoid arthritis) associated with the increase of inflammatory mediators. These factors are increased by high ω6/ω3 ratio (increase of ω6 fatty acids intake) thus the objective of decreasing ω6/ω3 ratio (by an increase of ω3 fatty acids intake or supplementation) can be protective (26, 25). More recently the regulation of the desaturation index (C16:1/C16:0) by interacting with lipid desaturases (the delta 9 type) was found to be another way by which PUFAs may be protective (3, 2).

Pig kidney epithelial cells (LLC PK 1) is a cell line model used for studying molecular mechanisms of renal injuries induced by inflammatory process such as Ischemia-Reperfusion during renal transplantation (9). This renal cell model is also used to study plasma membrane transporters, receptors and the signaling pathways related to these membrane compounds (29). Furthermore, the effects of PUFAs supplementation in the cellular lipidome fatty acid profile and regulation are not known in LLCPK-1 model.

The aim of this study was to determine the fatty acid profile and the effects of supplementations of different PUFAs with different ω6/ω3 ratios near dietary recommendation on total fatty acid composition of kidney epithelial cells (LLC PK 1) and which type of supplementation can be used in the future as cellular protection on kidney cells during transplantation.

MATERIALS AND METHODS

Cell culture

Kidney epithelial cell line (LLC PK 1) was obtained from the American Type Culture Collection (Rockville,

MD). They were grown in T-75 flask (Nunc, Merck-Eurolab, France) using Dulbeco's Modified Eafle's Medium (DMEM) (Gibco, Inveitrogen Life Technologie, France) supplemented with 10% fetal calf serum (Gibco) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Sigma-Aldrich, France) at 37°C in a 5% CO_2/95% air humidified atmosphere. When cell cultures reached about 80% confluence, cells were removed using 0.25% trypsin in EDTA (Gibco) and subcultured into 6 well plates. At confluence, cells were serum-starved for 48 hr before experiments.

Cell supplementations

Three commercial supplements (Laboratoires Nutergia, Capdenac, France) with different formulation were used for experiments; S1: docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) with ω6/ω3 ratio = 0.1, S2: DHA, EPA, linoleic acid (LA) and γ-linoleic acid (GLA) with ω6/ω3 ratio = 2.5, or S3: α-linolenic acid (ALA) and LA with ω6/ω3 ratio near 1.Their fatty acids composition are shown in Table 1.

Cells were incubated for 24 hours with 30 µM of PUFA from each supplement complexes with fatty acids free BSA (Sigma-Aldrich, France) or just complete culture medium as control.

Table 1. Fatty acid composition of the three commercial food health supplements with different formulations used for experiments; S1: docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) with ω6/ω3 ratio = 0.1, S2: DHA, EPA, linoleic acid (LA) and γ-linoleic acid (GLA) with ω6/ω3 ratio = 2.5, or S3: α-linolenic acid (ALA) and LA with ω-6/ω-3 ratio near 1.

Fatty acid	S1	S2	S3
C14:0	7.9	4.8	0.03
C16:0	17.6	14.4	5.9
C16:1	9.1	5.6	0.07
C18:0	3.4	4.1	3.9
C18:1 ω9	11	6.6	14.6
C18:2 ω6	2	16	34
C18:3 ω3	1	0.6	38
C18:3 ω6	0.3	7.9	1.7
C20:4 ω6	1.2	0.7	0
C20:5 ω3	19	12.3	0
C22:5 ω3	2.4	1.4	0
C22:6 ω3	12.6	7.5	0
Σ SFA	31	25	10.1
Σ MUFA	24.8	25.7	15.6
Σ PUFA	44	49	74
Σ ω6	4	57	36
Σ ω3	37	22.3	38
ω6 /ω3	0.1	2.5	0.94

Total fatty acid composition

Cellular lipids were extracted with methanol and chloroform according to Bligh and Dyer's methods (5), in presence of buthylated hydroxytoluene (50 µmol/L final concentration, Sigma, St Louis, MO). Fatty acid composition was determinated after methylation with BF3-methanol (Sigma, St Louis, MO) according to Otha *et al*. (22). The fatty acid methyl esters were analyzed by GC on a Perkin Elmer Autosystem XL (Perkin Elmer, Courtaboeuf, France) using an Omegawax 250 capillary column (30m x 0.25 mm i.d., 0.25µm film thickness) (Sigma-Supelco) equipped with a flame ionization detector and the Turbochrom software. Hydrogen was used as carrier gas (8 psig constant pressure). The oven temperature program ranged from 60°C to 215°C with a temperature rise of 45°C/min. Fatty acids were identified by their retention times on the column with respect to appropriate standards (PUFA 2, Sigma-Supelco).

Statistical analysis

Results are expressed as means ± SEM of six experiments. Statistical analysis was done using analysis of variance (ANOVA) followed with the Tukey's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant.

RESULTS

The changes of total fatty acids composition in kidney epithelial cells (LLC PK1) after polyunsaturated fatty acids supplementation using different ω6/ω3 ratio are shown in Table 2. Results are expressed as a percentage of the total identified fatty acids. The two major saturated FA (C16:0 and C18:0) were decreased in a similar manner by 50% in the 3 cell supplementations. The effect of supplementation on relative proportion of cell saturated fatty acids (SFA), are illustrated in figure 1. Experimental groups shown increased level of oleic acid (C18:1) by four fold ($p<0.05$ *vs. control*). Linoleic acid (C18:2 ω6, LA) representing only 0.20% of total cells fatty acids was increased by six fold ($p<0.05$ *vs. control*). S1 and S2 supplementations increased the initial proportion (0.51%) of arachidonic acid (C20:4 ω6, AA) by about 460% and S3 increased it by about 330% ($p<0.05$ *vs. control*). All treatments increased significantly eicosapentaenoic acid (C20:5 ω3, EPA) level by 200% compared to control group. Levels of docosapentaenoic acid (C22:5 ω3, DPA) and docosahexaenoic (C22:6 ω3, DHA) strongly increased by 700% after supplementation with S1 and S2 ($p<0.05$ *vs. control*), this increase was lower (200%) in the S3-incubated group.

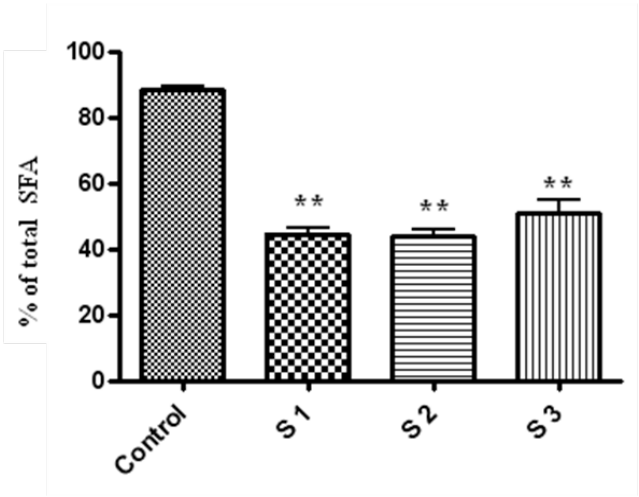

Figure 1. Total amount of saturated fatty acids (SFA) in LLC PK 1 cells incubated 24 hr with different supplementations (S1: DHA and EPA, S2: DHA, EPA, LA and GLA with ω-6/ω-3 ratio = 2.5, or S3: ALA and LA with ω-6/ω-3 ratio = 1). The data represent means ± SEM of six experiments. ** $p<0.001$ *vs.* control.

Table 2. Effect of different supplementations with different ω-6/ω-3 ratio on total fatty acid composition of kidney epithelial cells (LLC PK 1).

	Control	S1	S2	S3
C16:0	31.67 ± 0.31[a]	21.24 ± 0,58[b]	20.77 ± 0,46[b]	24.9 ± 1.4[b]
C18:0	56.47 ± 1.12[a]	23.29 ± 1,02[b]	22.90 ± 1,90[b]	25.7 ± 2,8[b]
C18:1	10.08 ± 1.16[a]	46.20 ± 1,38[b]	48.35 ± 1,60[b]	43.1 ± 3,4[c]
C18:2 ω6	0.20 ± 0.01[a]	1.29 ± 0,05[a]	1.27 ± 0,08[a]	1.19 ± 0.11[a]
C20:4 ω6	0.51 ± 0.08[a]	2.39 ± 0,13[a]	2.25 ± 0,15[a]	1.71 ± 0.27[a]
C20:5 ω3	0.40 ± 0.03[a]	0.96 ± 0,02[a]	0.80 ± 0,03[a]	0.93 ± 0.11[a]
C22:5 ω3	0.23 ± 0.07[a]	1.83 ± 0,29[a]	1.49 ± 0,26[a]	0.95 ± 0.11[a]
C22:6 ω3	0.45 ± 0.13[a]	2.81 ± 0,50[a]	2.12 ± 0,18[a]	1.55 ± 0.28[a]
ΣSFA	88.14 ± .,39[a]	44.53 ± 1,56[b]	43.67 ± 1,92[b]	50.6 ± 4.0[c]
Σ MUFA	10.08 ± 1.16a	46.20 ± 1,38[b]	48.35 ± 1,60[b]	43.1 ± 3.4[c]
ΣPUFA	1.78 ± 0.26[a]	9.28 ± 0,78[b]	7.93 ± 0,48[b]	6.33 ± 0.61[b]
Σ ω3	1.08 ± 0.20[a]	5.60 ± 0,71[b]	4.42 ± 0,38[b]	3.43 ± 0.32[b]
Σ ω6	0.70 ± 0.09[a]	3,68 ± 0,16[b]	3.52 ± 0,22[b]	2.89 ± 0.38[b]
ω6/ω3	0.74 ± 0.16[a]	0,71 ± 0,09[a]	0.82 ± 0,07[a]	0.85 ± 0.11[a]

Values represent the relative amounts, expressed as a percentage, of the total identified fatty acids. The data represent means ± SEM of six experiments. Values in the same line not bearing the same superscript letters ([a, b, c]) were significantly different at $p<0.05$.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

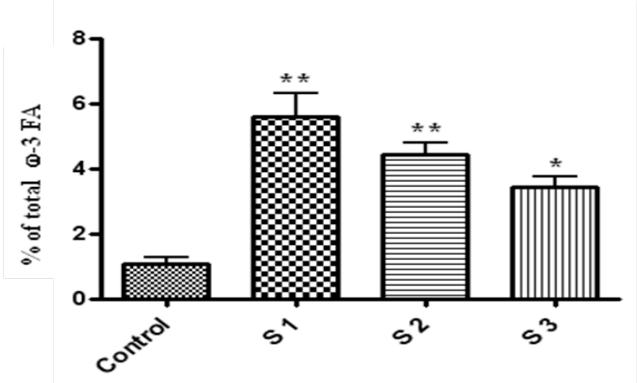

Figure 2. Total amount of polyunsaturated fatty acids ω-3 in LLC PK 1 cells incubated 24 hr with different supplementations (S1: DHA and EPA, S2: DHA, EPA, LA and GLA with ω-6/ω-3 ratio = 2.5, or S3: ALA and LA with ω-6/ω-3 ratio = 1). The data represent means ± SEM of six experiments. * $p<0.01$ *vs.* control, ** $p<0.001$ *vs.* control.

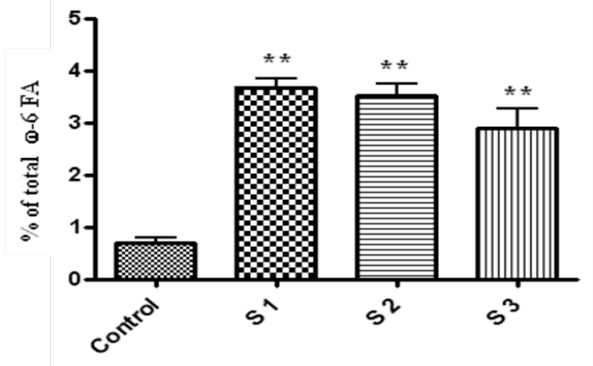

Figure 3. Total amount of polyunsaturated fatty acids ω-6 in LLC PK 1 cells incubated 24 hr with different supplementations (S1: DHA and EPA, S2: DHA, EPA, LA and GLA with ω-6/ω-3 ratio = 2.5, or S3: ALA and LA with ω-6/ω-3 ratio = 1). The data represent means ± SEM of six experiments. ** $p<0.001$ *vs.* control.

Figures 2 and 3 represent relative proportion of cell ω3 and ω6 fatty acids, respectively. These figures showed significative increases by two to four fold of levels of cell ω3 and ω6 fatty acids ($p<0.001$ *vs. control*) following the three supplementations used.

All the changes described above were obtained with a conserved ω-6/ω-3 ratio whatever the supplementation used. No significant difference between values of control and experimental groups was found for this ratio. Furthermore the ω6/ω3 ratio was already below 1 in this renal cell line and this ratio was kept constant when submitted to different PUFA supplementations by modifying all SFA, MUFA and PUFA levels.

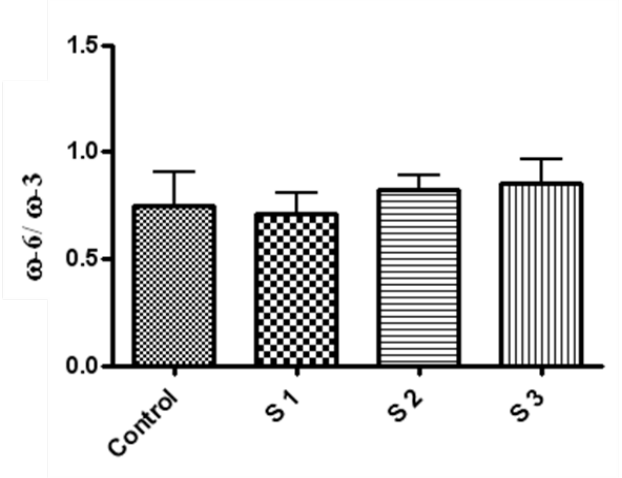

Figure 4. ω-6/ω-3 ratio in LLC PK 1 cells incubated 24 hr with different supplementations (S1: DHA and EPA, S2: DHA, EPA, LA and GLA with ω-6/ω-3 ratio = 2.5, or S3: ALA and LA with ω-6/ω-3 ratio = 1). The data represent means ± SEM of six experiments.

DISCUSSION

We evaluated the effect of different cellular supplementation ω-6/ω-3 ratio on the changes of total fatty acids composition in kidney epithelial cells (LLC PK1). The efficiency of cell supplementation was achieved by showing 2 to 4 fold increases in cell PUFA incorporation. The cell saturated fatty acids (SFA) was decreased by 50% following the three supplementations used ($p<0.001$) as compared to control group. The ω6/ω3 ratio of control LLC PK 1 was 0.65. This value was not significantly changed whatever the PUFA supplementations used.

The western diets are too rich in vegetable oils (rich in LA) that modify the ω6/ω3 ratio to a 30-fold too high and shifted heavily toward ω6 FA (25). These supplementations were achieved with three different commercial human health food supplements, consisting of fish oil ω-3 fatty acids (DHA and EPA) (S1), ω3 and ω6 fatty acids (DHA, EPA, LA and GLA) (S2), or ω3 and ω6 fatty acids precursor (ALA and LA) with ω6/ω3 ratio near 1 (S3). Mixtures of vegetable oils were used to achieve LA, GLA and ALA concentrations and ω6/ω3 ratios. Because ω6 and ω3 FA are not interconvertible in the human body, the effect of direct incubation of our two S1 and S2 supplementations modified both ω6 and ω3 pathways especially at the level of long chain ω6 and ω3 PUFA. We also confirm that the ratio of LA/ALA in our diet is less efficient than fish oil supplementation to increase cellular DHA (20, 17, 1). However the ratio of ω6/ω3 LC PUFAs whatever the supplementation used was kept constant. Furthermore this ω6/ω3 ratio in the LLCPK1 is less than 1. So our hypothesis to mimick in our cell model the PUFA regulation on human nutrition from western diets, could not be apply here. This required an experimental model with altered ω6/ω3 ratio as it is found in western diets.

In the present study ALA was not detected and LA was 0.2 %, AA was almost abundant as EPA in the control culture conditions. In human RBC, the most cell characterized for analysis of PUFA supplementations, LA was almost 100 times as abundant as ALA and AA was almost 30 times as abundant as EPA (19). The model we used is particular in this respect. When fed with PUFA whatever the length of chain of PUFA, we show in our cell model that the 3 supplementations are able to produce DHA and ARA at conversion rate of more 50 %. When fed with ALA or GLA, there is no detectable ALA and GLA changes produced from the fed respectively. When fed with DHA (S1 and S2), there is detectable DHA in cell produced from the fed (2.8% and 2.1% respectively) that corresponds to direct accretion of DHA by the cells since this DHA levels was higher than that measured after ALA/LA supplementation S3 (1.55%). This last supplementation modify the ω3 pathway with more DHA incorporated in LLCPK1 by acting on the metabolic process involving delta 5 and delta 6 and elongases (Elo VL2, Elo VL5) (3, 4).

In this study, we observed a profound and similar change in SFA and MUFA following both PUFAs supplementations. To explore the metabolic significance of these findings, the deep modification of the desaturation index (C18:1/C18:0) possibly through a positive regulation of delta9 desaturase (FAT 5, FAT 6 or FAT 7) could be involved. The ability of PUFAs to regulate the desaturase expression has been presented in human studies (3). In another work (14) the DHA levels were markedly decreased. This is not the case in our study. This could be due to our direct supplementation and cell incorporation alleviating this DHA defect observed in human studies. The C18:1 is a non-essential MUFA that can be synthetized through de novo pathway in which delta9 desaturase appear the rate-limiting enzyme. This regulation through delta9 desaturase could contribute to maintain a homeostasis in the unsaturation level of the cellular membranes (31). Elevated delta9 desaturase in human appears to be associated with metabolic syndrome and represent a target for possible treatments of metabolic syndrome (30). Further studies are needed to understand this regulation of transcription with the different mRNA encoding for desaturase isoforms of the rate-limiting enzyme in MUFA biosynthesis. Following the well-known regulation of delta5 and 6 desaturases as key enzymes in the metabolism of PUFA (18), this study adds a possible regulation of all desaturases in this cell line by PUFA supplementations.

These data suggest that supplementations with long chain polyunsaturated fatty acids or their precursors by having the same effect on the fatty acids remodeling and lipidome in LLC PK 1 cells may represent an interesting new mechanism by which renal FA homeostasis and protection could occurred.

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The author's responsibilities were as follow: JMM for conception of the study design, MF, CF and JMM write the manuscript; MF and CF conduct the cell culture and supplementation; MA conduct the lipid profile analysis and the review of the manuscript. All authors participated in the study and take responsibility for the content of this report. None of the authors had a personal or financial conflict of interest.

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