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ESSENTIAL FATTY ACID METABOLISM IN CULTURED RAT CARDIOMYOCYTES IN RESPONSE TO EITHER N-6 OR N-3 FATTY ACID SUPPLEMENTATION

S. Hrelia[1], J.A. Lopez Jimenez*, A. Bordoni, S. Zamora Navarro*, D.F. Horrobin§, C.A. Rossi, and P.L. Biagi

Department of Biochemistry "G. Moruzzi", University of Bologna (Italy)
*Department of Physiology and Pharmacology, University of Murcia (Spain)
§Efamol Research Institute - Kentville (Canada)

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In this study we demonstrate that cultured rat cardiomyocytes possess the capacity to desaturate/elongate essential fatty acids (EFAs). Alpha-linolenic acid (ALA) conversion to higher metabolites was greater than linoleic acid (LA) conversion, according to the higher affinity of the delta-6-desaturase enzyme for the n-3 than for the n-6 EFAs. Gamma-linolenic acid (GLA) supplementation to the culture medium had no influence on LA conversion; but the addition of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids significantly decreased the formation of interconversion products from LA. The conversion of ALA to higher metabolites was greatly affected by GLA; EPA had no effect on ALA conversion, while DHA significantly inhibited it. Both GLA (converted mostly to dihomo-gamma-linolenic acid) and EPA can be removed from phospholipids and addressed to prostanoid biosynthesis, so avoiding their potential accumulation and the inhibition of their own production. Our data clearly indicate that supplementation of the culture medium with either n-6 or n-3 fatty acids can cause reduced levels of the other series of fatty acids. This effect may be undesirable, since both n-6 and n-3 fatty acids are important in the prevention of coronary diseases.

Changes in the composition of phospholipid fatty acid chains can affect a number of cellular functions, including ion transport and transmembrane signal transduction (1,2). Relative availability of saturated and unsaturated fatty acids for phospholipid synthesis is determined by diet, fatty acid incorporation and activity of elongase and desaturase enzymes. Thus, the capacity of a particular tissue to elongate/desaturate available fatty acid substrates determines in situ control of membrane fatty acid composition. Although the major part of polyunsaturated fatty acid (PUFA) metabolism in the body is thought to occur in the liver, desaturase activity has also been found in brain, adrenal and testis (3,4) and in several types of

[1]Address for correspondence: Dipartimento di Biochimica "G. Moruzzi", Via Irnerio,48 - 40126 Bologna (Italy). Fax: +39 51 351224; E-mail: hrelia@biocfarm.unibo.it.

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primary cell cultures and cell lines (5,6). We previously demonstrated the ability of heart microsomes to desaturate linoleic acid (LA, 18:2 n-6) and to synthesize arachidonic acid (AA) to satisfy partially tissue needs for eicosanoids production and for fatty acids for membrane structure (7).

The metabolites of LA and of α-linolenic acid (ALA, 18:3 n-3) are responsible for most of the biologic effects of the dietary essential fatty acids (EFAs), and these metabolites are involved in a number of factors which may influence coronary heart disease (8). Levels of LA and ALA metabolites may be reduced in coronary patients and LA administration may not be able to raise them (9). Both n-6 and n-3 EFAs are likely to be important in the prevention of atherosclerosis since the common risk factors are associated with reduced desaturation of the parent dietary EFAs (8). PUFAs are probably involved in the development of obstructive coronary heart disease by affecting thrombogenesis and possibly by affecting the level of blood lipids (10). In order to prevent re-occlusion following angioplasty, fish oil has been administered in different clinical trials (11, 12), but without significant effects in comparison with placebo administration. Recovery of cardiac function in rats after coronary occlusion was comparable to that in the control animals receiving lard instead of fish oil (13). Moreover, hearts obtained from rats fed a diet containing 10% w/w fish oil for 12 weeks and perfused, did not perform differently from control hearts (14). The lack of significant results in these studies could lie in the administration of massive amounts of n-3 EFAs only, which could correct the deficit in n-3 series at the cost of introducing a depletion of n-6 series. However, it has been suggested that combined administration of n-6 and n-3 PUFAs has a beneficial effect in preventing restenosis in comparison with placebo (8).

The objective of the present study is to evaluate the cellular metabolism of n-6 and n-3 PUFAs in neonatal rat cultured cardiomyocytes, characterizing the desaturation of both LA and ALA and determining the effect of fatty acid supplementation to the culture medium on cardiomyocyte fatty acid composition.

MATERIALS AND METHODS

Materials. Radioactive materials, [1-^{14}C] LA (53 mCi/mmol), [1-^{14}C] ALA (52 mCi/mmol) of 99% radiochemical purity were purchased from NEN (Boston, MA, USA) and used without further purification. Unlabelled fatty acids, eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA), sera, Ham F10 culture medium and other biochemicals were obtained from Sigma Chem. (St. Louis, MO. USA). Gamma linolenic acid (18:3 n-6, GLA) was a kind gift of Callanish Ltd (Breasclete, Scotland). All chemicals and solvents were of the highest analytical grade
Methods. Primary heart cell cultures were obtained by isolation of cardiomyocytes

from the ventricles of 2-4 days old Wistar rats, as previously reported (15). Before the final seeding in Petri dishes, cells were divided into different groups:

a. control cells, grown in nutrient mixture Ham F10 supplemented with 10% v/v fetal calf serum and 10% v/v horse serum;
b. cells grown in the same medium, but supplemented with 60μM GLA or 60μM EPA or 60μM DHA, in ethanol vehicle.

In control cells, the same ethanol concentration (0.04% v/v) was added to the culture medium. Cells were incubated at 37°C, 95% humidity, 5% CO_2.

Cardiomyocytes were grown in the above mentioned conditions until they appeared completely confluent in a monolayer. No differences in cell size and morphology were detected among the different groups of cardiomyocytes by phase contrast light microscopy. Some dishes of each group were radiolabelled with [1-^{14}C] LA or [1-^{14}C] ALA (1 μCi/dish) for 24 hrs. Then cells were washed three times with control medium supplemented with 10% horse serum and 10% fetal calf serum, and two times with phosphate buffered saline. Preliminary experiments demonstrated that the radioactivity in the medium is completely removed by these washes, and medium is not carried over into the excised cells.

Both labelled and unlabelled cells were scraped off in ice-cold methanol, and total lipids were extracted according to Folch et al. (16). Fatty acid methyl esters were prepared from all samples according to Stoffel (17).

In the radiolabelled cardiomyocytes the desaturating/elongating activities for both LA and ALA were evaluated by separating fatty acid methyl esters on thin layer chromatography plates coated with silica gel G, impregnated with 10% (w/v) $AgNO_3$, and identifying them by comparison with authentic standards. Plates were developed in hexane/diethyl ether (8:2 v/v), and spots made visible under ultraviolet light by spraying with 2',7'-dichlorofluorescein (0.2%, w/v in ethanol). The spots were scraped off into scintillation vials and immediately counted in 10 ml of liquid scintillation mixture (Instagel, Packard) using a 1900 TR Packard liquid scintillation spectrometer.

The fatty acid composition of cardiomyocyte total lipids was determined by gas chromatography (Carlo Erba mod. 4160) using a capillary column (SP 2340, 0.10-0.15 μm i.d.) at a programmed temperature (160-210°C, with an 8°C/min gradient), as previously reported (18).

Data are means ± S.D. of at least 4 different cultures. Statistical differences were evaluated using the Student's t-test.

RESULTS

In order to examine the ability of neonatal rat cardiomyocytes to desaturate/elongate LA and ALA, and to evaluate the influence of GLA, EPA or DHA supplementation on these activities, cells were incubated with radioactive substrates and the percent distribution of radioactivity in the different fatty acid classes was measured.

In figure 1 the desaturating/elongating activities for LA of cardiomyocytes grown in the different media are reported. These activities were evaluated as the conversion of LA to longer and more unsaturated fatty acids, and are expressed as percent radioactivity in the higher polyunsaturated fatty acid (HPUFA) fractions (trienoic plus tetraenoic plus higher polyenoic fractions) divided by the total

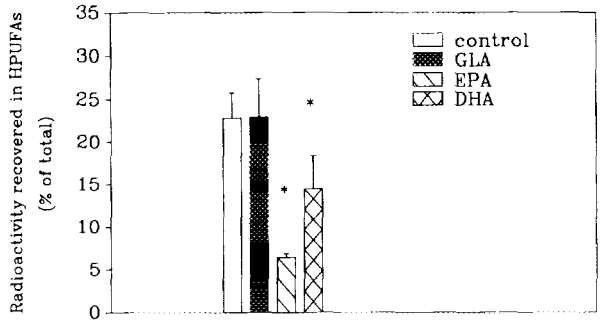

Figure 1. Conversion of LA to longer and more unsaturated fatty acids in cardiomyocytes grown in media supplemented with different fatty acids.
Cells were radiolabelled with 1 μCi [1-^{14}C] LA/dish for 24 hours. After lipid extraction and methyl esterification, fatty acids were separated with argentation TLC. Results are expressed as percent radioactivity recovered in the trienoic plus tetraenoic plus higher polyenoic fractions (HPUFAs)/total radioactivity recovered. Data are means ± S.D. of at least 4 different cell cultures.
Statistical analysis was performed by the Student's t test: *$p<0.001$.

radioactivity recovered. Cardiomyocytes are able to desaturate/elongate LA and about 23% of the radioactivity was recovered in the more unsaturated fractions. The addition of different polyunsaturated fatty acids (GLA, EPA, DHA) influenced to a different degree the relative amount of LA that was utilized for conversion into longer and more unsaturated products. GLA had no significant influence on LA metabolism, while EPA and DHA decreased the conversion of LA from 23% to 6% and 14% respectively.

The desaturating/elongating activities for ALA of cardiomyocytes grown in the different media are reported in figure 2. These activities were evaluated as the conversion of ALA to longer and more unsaturated fatty acids, and are expressed as percent radioactivity in the tetraenoic plus higher polyenoic fractions divided by the total radioactivity recovered. Cardiomyocytes are able to desaturate/elongate even ALA and about 93% of the radioactivity was recovered in the more unsaturated fraction. The addition of GLA, EPA and DHA influenced differently the relative amount of ALA that was utilized for conversion into longer and more unsaturated products. GLA decreased the conversion of ALA from 93% to 61%, DHA decreased the conversion of ALA to 82% while EPA had no effect.

In Table 1 the fatty acid composition of total lipids derived from cardiomyocytes grown in different media is reported. In all groups the supplementation induced marked modifications in the fatty acid pattern in comparison to control cells. Particularly, in GLA supplemented cells, we observed

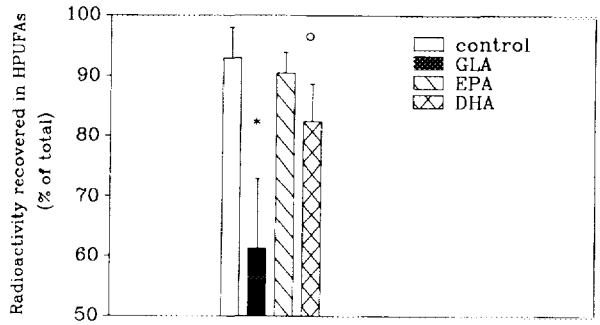

Figure 2. Conversion of ALA to longer and more unsaturated fatty acids in cardiomyocytes grown in media supplemented with different fatty acids.
Cells were radiolabelled with 1 μCi [1-^{14}C] ALA/dish for 24 hours. After lipid extraction and methyl esterification, fatty acids were separated with argentation TLC. Results are expressed as percent radioactivity recovered in the tetraenoic plus higher polyenoic fractions (HPUFAs)/total radioactivity recovered. Data are means ± S.D. of at least 4 different cell cultures.
Statistical analysis was performed by the Student's t test: °$p<0.02$; *$p<0.001$.

very high levels not only of GLA itself, but also of its direct metabolite 20:3 n-6, dihomo-gammalinolenic acid (DGLA). Interestingly, the relative molar content of AA and of other n-6 PUFAs was decreased. In EPA supplemented cells, the direct incorporation of this fatty acid was detected, together with the appearance of a notable amount of the direct metabolite 22:5 n-3. The following step to 22:6 n-3 seems to be blocked, because DHA relative molar content was lower than in control cells. In DHA supplemented cells, the high relative molar content of this fatty acid clearly indicates its incorporation into cellular lipids; no retroconversion to 22:5 n-3 was observed. The unsaturation index increased in all groups of supplemented cells; in fact the relative molar content of saturated and monounsaturated fatty acids decreased in all groups with a concomitant increase of the relative molar content of polyunsaturated fatty acids. This effect was more evident in GLA supplemented cells. The n-6/n-3 ratio significantly increased in GLA supplemented and significantly decreased in n-3 supplemented cells.

DISCUSSION

The factors regulating the unsaturated fatty acid composition of heart lipids are poorly understood. Brenner reported that heart microsomes converted linoleate to GLA (3), and we also demonstrated the ability of heart microsomes to desaturate LA and to synthesize AA to satisfy partially tissue needs for eicosanoid production (7). In this paper we demonstrated that cultured heart myocytes have the capacity

Table 1. Fatty acid composition (mol/100 mol) of total lipids derived from cardiomyocytes grown in media supplemented with different fatty acids

Fatty acid	Control (n=6)	GLA (n=4)	EPA (n=5)	DHA (n=4)
14:0	1.53±0.43	0.87±0.52	1.67±0.12	1.57±0.30
16:0	22.87±2.00	12.05±1.19*	20.90±0.75	21.33±0.79
16:1	2.02±0.69	0.70±0.27°	1.56±0.27	1.18±0.40
17:0	2.54±0.79	0.52±0.32°	1.56±0.26§	1.64±0.54
18:0	17.71±0.94	8.87±0.85*	15.04±0.40*	15.74±1.02§
18:1	16.13±0.38	6.25±0.14*	12.43±0.24*	12.76±0.20*
18:2n-6	9.77±0.55	4.63±0.35*	8.53±0.57°	10.01±0.87
18:3n-6	0.26±0.30	18.19±3.58*	0.27±0.34	tr
18:3n-3	0.58±0.37	0.32±0.22	0.86±0.36	1.03±0.14
20:3n-6	tr	32.92±4.21	tr	tr
20:3n-3	1.86±0.55	tr	1.01±0.11°	1.11±0.02§
20:4n-6	14.21±0.75	9.97±0.95*	6.84±0.66*	7.11±0.55*
20:5n-3	0.53±0.20	0.89±0.55	6.93±0.74*	1.14±0.05*
22:4n-6	3.96±0.40	1.84±0.25*	1.74±0.27*	1.31±0.11*
22:5n-6	0.96±0.23	0.18±0.14*	0.40±0.25°	0.53±0.25§
22:5n-3	2.75±0.37	0.75±0.18*	18.42±1.63*	1.63±0.17*
22:6n-3	3.23±0.33	1.22±0.18*	2.06±0.24*	22.07±1.52*
U.I.	154.3±1.8	233.6±9.1*	212.5±9.4*	223.0±8.4*
n-6/n-3	3.30±0.16	22.56±4.62*	0.61±0.10*	0.70±0.06*
SFAs	45.24±0.61	22.27±1.47*	38.79±1.14*	40.28±1.21*
MUFAs	17.80±0.62	6.95±0.23*	13.98±0.42*	13.93±0.22*
PUFAs	36.81±0.37	70.78±2.12*	46.96±1.40*	45.98±1.23*

Gas chromatographic analysis of fatty acids (as methyl esters) was performed in the conditions reported in Methods. The number of samples examined is reported in brackets. Data are means ± S.D. Statistical analysis was performed by the Student's t test comparing control cardiomyocytes vs. cardiomyocytes grown in supplemented medium: §$p<0.05$; °$p<0.01$; *$p<0.001$.
U.I.= unsaturation index; SFAs = saturated fatty acids; MUFAs = monounsaturated fatty acids; PUFAs = polyunsaturated fatty acids.

to desaturate/elongate essential fatty acids; since our cultures were pure cultures of cardiomyocytes, we established that these desaturating/elongating activities were localized in myocytes and not in other cells such as heart fibroblasts. ALA conversion to higher and more unsaturated metabolites was greater than LA conversion, according to the higher affinity of the rate limiting enzyme, delta-6-desaturase (D6D) for the n-3 than for the n-6 EFAs (19).

GLA supplementation had no influence on LA conversion to more unsaturated fatty acids. Although it has been reported that, in liver microsomes, GLA inhibits D6D activity for LA (20), other authors did not observe any modifications in D6D activity for LA in response to GLA supplementation to animals (21, 22). On the other hand, the addition of both n-3 fatty acids, EPA and DHA, significantly decreased the formation of interconversion products from LA. The conversion of ALA to more unsaturated metabolites was greatly affected by GLA supplementation. When n-3 fatty acids were supplemented to cardiomyocytes, ALA conversion was influenced in different ways depending on the fatty acid added to the culture medium. In fact, EPA supplementation had no effect on ALA conversion, while DHA supplementation significantly inhibited it.

In order to tentatively explain the lack of inhibition of GLA and EPA of their own production, it is important to note that both GLA (which is mostly converted to DGLA) and EPA are substrates for enzymes involved in eicosanoid biosynthesis. These fatty acids can be removed from phospholipids and applied to prostanoid biosynthesis, so avoiding their potential accumulation. DHA is not a substrate for eicosanoid production, and it may be for this reason that it is effective in inhibiting ALA conversion. Furthermore, for many years it has been assumed that DHA was formed by a series of desaturations and elongations involving delta-6, delta-5, and delta-4-desaturases (23). Voss et al. have recently demonstrated that the formation of DHA from ALA involves a new three step mechanism, i.e. an elongation (22:5 n-3 $\rightarrow$ 24:5 n-3), a delta-6-desaturation (24:5n-3 $\rightarrow$ 24:6n-3), and a ß-oxidation (24:6n-3 $\rightarrow$ 22:6n-3) (24). Apparently, it is the same enzyme that catalyses the conversion of ALA and of 24:5 n-3. Therefore, delta-6-desaturation is required only once for EPA production, but twice for DHA biosynthesis. In DHA formation from ALA, delta-6-desaturation is not only the first and rate limiting step, but also the final desaturating step, and this could be one more explanation for the effectiveness of DHA supplementation in reducing ALA conversion.

The fatty acid composition of total lipids derived from cardiomyocytes grown in different media indicates that each supplemented fatty acid was incorporated to a significant extent (table 1). In GLA supplemented cells, GLA was converted to DGLA, but the relative molar contents of AA and other n-6 fatty acids were lower than in control cells, suggesting a partial inhibition of delta-5-desaturase activity. In EPA supplemented cells, EPA was converted to 22:5 n-3, while DHA relative molar content was lower than in control cells, indicating an inhibition of the metabolic pathway leading to DHA. Interestingly, in DHA supplemented cells, no

retroconversion of DHA itself to 22:5n-3 was observed, in contrast to that reported by Mohammed et al. (25) in cardiac myocytes isolated from rats fed fish oil. Anyway, the n-6/n-3 ratio was similar in EPA and DHA supplemented cells, and lower than in control cells.

In conclusion, our data clearly indicate that primary cultured neonatal cardiomyocytes posses an active EFA metabolism and that supplementation of the culture medium with either n-6 or n-3 fatty acids can cause reduced levels of the other series of fatty acids. This effect may be undesirable, since both n-6 and n-3 fatty acids are likely to be important in the prevention of atherosclerosis and that the common risk factors are associated with reduced 6-desaturation of the parent EFAs to their metabolites (8). Although it is possible that the metabolism on n-6 and n-3 fatty acids in cardiomyocytes is not solely regulated by the activity of the desaturation and elongation enzymes, but also by their incorporation into phospholipid and triglyceride fractions, in the light of the reported data a very interesting intervention in the clinical treatment of heart diseases would be to treat with 6-desaturated EFAs of both series, instead of supplementation with n-6 or n-3 fatty acids alone. Therefore the clinical approach of administering a combination of GLA and EPA to patients in order to prevent re-occlusion following angioplasty might effectively be successful.

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