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Contrasting effects of arachidonic acid and docosahexaenoic acid membrane incorporation into cardiomyocytes on free cholesterol turnover

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ABSTRACT

The preservation of a constant pool of free cholesterol (FC) is critical to ensure several functions of cardiomyocytes. We investigated the impact of the membrane incorporation of arachidonic acid (C20:4 ω6, AA) or docosahexaenoic acid (C22:6 ω3, DHA) as ω6 or ω3 polyunsaturated fatty acids (PUFAs) on cholesterol homeostasis in primary cultures of neonatal rat cardiac myocytes. We measured significant alterations to the phospholipid FA profiles, which had markedly different ω6/ω3 ratios between the AA and DHA cells (13 *vs.* 1). The AA cells showed a 2.7-fold lower cholesterol biosynthesis than the DHA cells. Overall, the AA cells showed 2-fold lower FC masses and 2-fold higher cholesteryl ester masses than the DHA cells. The AA cells had a lower FC to phospholipid ratio and higher triglyceride levels than the DHA cells. Moreover, the AA cells showed a 40% decrease in ATP binding cassette transporter A1 (ABCA1)-mediated and a 19% decrease in ABCG1-mediated cholesterol efflux than the DHA cells. The differences in cholesterol efflux pathways induced by AA or DHA incorporation were not caused by variations in ABCs transporter expression and were reduced when ABC transporters were overexpressed by exposure to LXR/RXR agonists. These results show that AA incorporation into cardiomyocyte membranes decreased the FC turnover by markedly decreasing the endogenous cholesterol synthesis and by decreasing the ABCA1- and ABCG1-cholesterol efflux pathways, whereas DHA had the opposite effects. We propose that these observations may partially contribute to the beneficial effects on the heart of a diet containing a high ω3/ω6 PUFA ratio.

1. Introduction

Dietary changes weakly affect the saturated and monounsaturated fatty acid content and composition in the membranes of cardiac ventricular myocytes. However, the omega-3/omega-6 dietary balance strongly influences the docosahexaenoic acid (DHA) *vs.* arachidonic acid (AA) content and composition of membrane phospholipids. An increase in DHA results in an equivalent decrease in AA. These dietary alterations of polyunsaturated fatty acids (PUFAs) in cell membranes influence cardiomyocyte functions by modulating membrane physicochemical properties such as the fluidity, organization and function of membrane proteins. Moreover, PUFAs modulate cardiac eicosanoids formed from arachidonic acid (C20:4 ω6) and eicosapentaenoic acid (C20:5 ω3) [1]. PUFA balance is known to influence several signal transduction pathways in various cell types [2]. Equally important is the ability of PUFAs to modulate cholesterol metabolism, particularly cellular cholesterol homeostasis, which results from a balance between endogenous synthesis, receptor-mediated endocytosis and cholesterol efflux. However, controversial results have been reported in various cell types such as macrophage-derived foam cells, which play a key role in atherosclerosis, as well as fibroblasts and adipocytes [3].

Abbreviations: AA, arachidonic acid; ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; apo, apolipoprotein; CE, cholesteryl esters; DHA, docosahexaenoic acid; FA, fatty acids; FC, free cholesterol; LXR/RXR, liver X receptor/retinoid X receptor; MUFA, monounsaturated fatty acid; PL, phospholipids; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SREBP, sterol regulatory element binding proteins; TG, triglycerides

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Several epidemiological studies have shown the beneficial effect of a diet containing a high ω3/ω6 PUFA ratio in the prevention of cardiovascular disease [4–7]. Many experimental studies have provided evidence that ω3 PUFAs have diverse actions on various cell types such as platelets, endothelial cells, vascular smooth muscle cells and macrophages. Some studies also demonstrated that PUFAs exert several cardiac effects such as the regulation of phospholipase A2 activity [8], the alteration of receptor-mediated phospholipase C activity [9], the modulation of a β-adrenergic transduction mechanism through cAMP [10–12], or an influence on the mitochondrial apoptosis pathway [13]. Nevertheless, to our knowledge, the impact of membrane PUFA composition on cardiomyocyte cholesterol homeostasis has not been investigated. Recently, we reported data on the regulation of cholesterol metabolism in cardiomyocytes. For this, we developed experimental procedures to assess cholesterol synthesis, cholesterol masses, LDL-cholesterol uptake and cholesterol efflux from primary cultures of neonatal rat cardiac myocytes [14]. We demonstrated the key importance of the ABCA1-(ATP binding cassette transporter A1) and ABCG1-mediated cholesterol efflux pathways in maintaining a constant free cholesterol (FC) pool size, which is critical for ensuring several major functions such as membrane domain rigidity, ion exchangers, β-adrenergic signaling [15–17], Ca^{2+} current regulation [18] or MG53 protein-mediated cardiac membrane repair [19].

The purpose of the present study was to evaluate the impact of the membrane incorporation of either AA or DHA as ω6 and ω3 PUFAs on cholesterol homeostasis in cardiomyocytes.

Our results show that cholesterol biosynthesis, ABCA1- and ABCG1-cholesterol efflux pathways, and overall cellular FC pool size are markedly decreased in AA-enriched cardiomyocytes compared with DHA-enriched cardiomyocytes. These findings suggest that the potentially deleterious *in vitro* effects of ω6 PUFA membrane incorporation may partially contribute to the beneficial effects on the heart of a diet containing a high ω3/ω6 PUFA ratio.

2. Material and methods

2.1. Cardiomyocyte primary culture

Cardiac myocytes were prepared from 2- to 4-day-old Wistar rats as previously described [13,14,20]. Briefly, the ventricles were minced and the cardiac cells were dissociated during 7 proteolytic treatments in trypsin at 30 °C. The cells from the last 6 steps were resuspended in culture medium, and the preparation was enriched in myocytes by 2 successive preplating periods (30 and 120 min). The final suspension was seeded on 60 mm Petri dishes (Falcon Primaria; Becton Dickinson, Pont de claix, France; 2×10^6 cells/dish) or on 24-well plates (Falcon Primaria; 12×10^4 cells/well). The cells were grown in standard complete culture medium (Ham's F10 medium supplemented with 10% fetal calf serum, 10% human serum and antibiotics with a free calcium concentration standardized at 1.2 mM) renewed after 24 h and after 48 h thereafter. We previously reported that cardiomyocytes cultured in these conditions contain less than 5% of non-muscle cells [21]. After 96 h in the standard medium, the cells were further grown either in standard medium or in one of the experimental media for 80 h and then subjected to experimental treatments. All experiments were conducted 7–8 days after plating.

2.2. Membrane fatty acid alterations

To investigate the effect of membrane PUFA enrichment, the cardiomyocytes were incubated 80 h with the experimental media containing either AA or DHA (Cayman Chemical Company, Ann Harbor, MI) prepared by adding FA bound to serum albumin to the standard culture medium as previously described [8]. Some cells were kept untreated in standard medium (standard cells). The final molar ratio of FA/albumin was approximately 1.5 and the final concentration of added FA was 90 μM (final albumin concentration: 4.1 g/L). The concentration of 90 μM of FA was selected because it is within the concentration range that our group and others currently use to induce marked effects on several functions of cardiomyocytes without inducing cellular toxicity (60–100 μM) [10–12]. After a 32-h incubation, the cells were further grown for 48 h in either basal medium to produce the standard cardiomyocytes (Std cells) or the PUFA-supplemented media to produce AA- or DHA-enriched cardiomyocytes (AA cells and DHA cells, respectively).

2.3. General experimental design to study the impact of fatty acids

The results of PUFA incorporation on endogenous cholesterol synthesis were evaluated by incubating the cardiomyocytes for 18 h in serum-free medium containing [^{3}H]-acetate. In addition, cardiomyocytes were radiolabeled with [^{3}H]-cholesterol, equilibrated overnight in BSA-serum free medium and then subjected to cholesterol efflux measurements. Moreover, the impact on FA profiles, cholesterol masses, phospholipid and triglyceride contents, and mRNA or protein expression for ABC transporters was determined after overnight equilibration in 0.5% BSA-serum free medium.

2.4. Fatty acid analysis

The cells were harvested in distilled water and lipids were extracted in a 2:1 chloroform-methanol mixture [22]. Phospholipids (PL) were separated from nonphosphorous lipids on silica acid cartridges [23] and the FA were trans-methylated with 7%. BF3-methanol The methyl esters were analyzed as described elsewhere by gas chromatography (GC 3900, Varian, Les Ulis, France) on econocap EC-WAX capillary columns (30 m × 0.32 m, Alltech Associates, Deerfield, IL, USA) coupled to a flame ionization detector [13,24]. A mixture was used to identify FA methyl esters in samples, and the results were expressed as the relative abundance of total FA using heptadecanoic acid (C17:0) as an internal standard.

2.5. Assessment of endogenous cholesterol synthesis

As previously described [14], *de novo* cholesterol synthesis was assayed by thin layer chromatography after an 18-h incubation of the cells with [^{3}H] acetate as a radiolabeled precursor of cholesterol in serum-free medium supplemented with 0.5% BSA. Free cholesterol bands well separated from bands of diglycerides, monoglycerides or phospholipids were quantified by liquid scintillation counting, and the results were expressed as cpm FC/μg protein/18 h.

2.6. Determination of free and esterified cholesterol masses

After cell lysis with 0.2 M NaOH, a sample was removed for protein determination using the BCA method, while the remaining cell lysate was used to quantify FC and cholesteryl ester (CE) contents using HPLC as previously described [14,25]. This method allows the determination of free cholesterol and 7 cholesteryl esters: linoleate, oleate, arachidonate, docosahexaenoate, palmitate, myristate and stearate. In these experimental conditions, cholesteryl eicosapentaenoate and cholesteryl docosapentaenoate, if present, comigrate with cholesteryl docosahexaenoate as a unique cholesteryl-ω3 peak. The results were expressed as nmol cholesterol/mg cell protein.

2.7. Measurement of cellular phospholipid and triglyceride contents

The cells were scraped into ice-cold lysis buffer (10 mM Tris–HCl, 1% Triton X-100, 0.5% Nonidet-P40 with protease inhibitors), and lipids were extracted from cardiomyocytes by the Folch method [22]. Briefly, 4 mL of a 2:1 chloroform–methanol mixture was added into 200–400 μL of cell lysate and vigorously mixed by vortex, followed by the addition of 1 mL of 0.73% NaCl solution to cause phase separation. The