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Human apolipoprotein E allele and docosahexaenoic acid intake modulate peripheral cholesterol homeostasis in mice

Anthony Pinçon[a,b], Jean-Denis Coulombe[a,b], Raphaël Chouinard-Watkins[a,b], Mélanie Plourde[a,b,c,*]

[a]Research Center on Aging, Department of Physiology, Université de Sherbrooke, 1036 Belvédère Sud, Sherbrooke, Québec, Canada, J1H 4C4
[b]Institute of Nutrition and Functional Foods, 2440 Boulevard Hochelaga, Québec City, Québec, Canada, G1V 0A6
[c]Department of Medicine, Université de Sherbrooke, 2500 Boul. de l'Université, Sherbrooke, Québec, Canada, J1K 2R1

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Abstract

Carrying at least one apolipoprotein E ε4 allele (*E4*+) is the main genetic risk factor for Alzheimer's disease (AD). Epidemiological studies support that consuming fatty fish rich in docosahexaenoic acid (DHA; 22:6ω3) is protective against development of AD. However, this protective effect seems not to hold in *E4*+. The involvement of *APOE* genotype on the relationship between DHA intake and cognitive decline could be mediated through cholesterol. Many studies show a link between cholesterol metabolism and AD progression. In this study, we investigated whether cholesterol metabolism is improved in *E3*+ and *E4*+ mice consuming a diet rich in DHA. Plasma cholesterol was 36% lower in *E4*+ mice compared to *E3*+ mice fed the control diet ($P=.02$), and in the liver, there was a significant genotype effect where cholesterol levels were 18% lower in *E4*+ mice than *E3*+ mice. The low-density lipoprotein receptor was overexpressed in the liver of *E4*+ mice. Plasma cholesterol levels were 33% lower after the DHA diet ($P=.02$) in *E3*+ mice only, and there was a significant diet effect where cholesterol level was 67% lower in the liver of mice fed DHA. Mice fed the DHA diet also had 62% less lipolysis stimulated lipoprotein receptor expression in the liver compared to mice fed the control diet ($P<.0001$), but there was no genotype effect. These findings suggest that plasma and liver cholesterol homeostasis and the receptors regulating uptake of cholesterol in the liver are modulated differently and independently by *APOE* allele and DHA intake.

Keywords: Apolipoprotein E; Docosahexaenoic acid; Diet; Cholesterol; Metabolism; Mice

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease modulated by several environmental, physiological and genetic risk factors. The main genetic risk of AD is carrying an ε4 allele of apolipoprotein E (*E4*+). Production of the apolipoprotein E (apoE) protein is controlled by the *APOE* gene for which three different alleles are recognized: ε2, ε3 and ε4 [1]. ApoE production occurs primarily in the liver and in the brain and, to a lesser extent, in macrophages [2–3]. ApoE plays a pivotal role in lipid homeostasis: it regulates cholesterol, triglyceride and phospholipid transport and metabolism *via* interactions with receptors of the LDL family [4]. The low-density lipoprotein receptor (LDLR) is the receptor responsible for the uptake of cholesterol-rich LDL particles [5]. However, LDLR is not the only apoE receptor involved in lipoprotein metabolism. The lipolysis-stimulated lipoprotein receptor (LSR) is a multimeric receptor in the liver that recognizes both apoB and apoE and plays a role in the clearance of both triglyceride-rich particles and LDL [6].

Cholesterol is a key structural molecule of cellular membranes and it is important for brain function because it is involved in synaptic plasticity, learning, memory and neuronal integrity during aging [7]. Molecular evidence points toward a link between peripheral cholesterol metabolism and AD since high levels of plasma cholesterol in mid-life have been associated with a higher risk of developing AD [8]. There is currently no drug to cure or delay cognitive deficits associated with late-onset AD supporting that prevention strategies are urgently needed. A diet containing docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid (PUFA) concentrated in fatty fish, has shown promising results in animals to prevent onset of cognitive decline, but in humans, results are less consistent [9]. The mechanisms explaining why fortification of the diet with DHA might help to prevent cognitive decline might stand on its role in neuronal differentiation [10], neurogenesis [11] and protection against synaptic loss [12]. However, it seems that *E4*+ are not protected against cognitive decline when eating DHA [13–14]. Human and animal studies suggest that higher plasma cholesterol levels are associated with higher risk of cognitive decline [8,15]. Since apoE protein plays a key role in plasma cholesterol homeostasis and since apoE genotype modulates plasma DHA response to a DHA diet [16], we hypothesize that apoE genotype modifies plasma cholesterol levels under a DHA diet. In order to elucidate the multiorgan mechanisms linking *APOE* genotype with cholesterol metabolism, animal models are required.

* Corresponding author. Research Center on Aging, 1036 Belvédère Sud, Sherbrooke, Québec, Canada J1H 4C4. Tel.: +1-819-780-2220 poste 45664; fax: +1-819-829-7141.
E-mail address: Melanie.plourde2@usherbrooke.ca (M. Plourde).

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To date, mice knocked in for human *APOE* isoforms provide a unique and useful tool to characterize dysfunction in lipid metabolism according to *APOE* genotype [17]. Therefore, in this study, we sought to investigate in *E3+* and *E4+* mice whether there is an interaction between a diet rich in DHA and E4 allele on peripheral cholesterol level and on proteins involved in cholesterol metabolism.

2. Materials and methods

2.1. Animals

APOE-targeted replacement mice expressing human *APOE* allele were purchased at Taconic (Hudson, NY, USA). From weaning to 4 months of age, mice were fed a regular chow diet containing 68% (w/w) carbohydrate, 11% (w/w) fat, 21% (w/w) proteins (Teklad 2018; Harlan Laboratories, Indiana, USA). At 4 months, half of the mice were fed a diet containing 0.7% (w/w) DHA (DHA diet; Research Diets Inc., New Brunswick, NJ, USA) while the other half remained on regular chow diet ($n=10$–14/genotype). At 12 months of age, mice were anesthetized with ketamine/xylazine and 100 μl of blood was collected by cardiac puncture in a lithium heparin tube (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 4°C for 5 min at 2000*g*, and plasma was collected and frozen at −80°C. Mice were immediately perfused in the heart with 50 ml of 0.1 M PBS buffer. Liver was fast frozen on dry ice. All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the Institutional Committee of the Centre Hospitalier de l'Université de Laval.

2.2. Cholesterol analysis

Liver was pulverized in powder with a biopulverizer (Biospec products, Bartlesville, OK, USA). Total lipids were extracted using the Folch *et al.* method from a 50-mg sample of liver powder [18]. The liver total lipid extract was then saponified using 1 M KOH/methanol and heated at 90°C for 1 h. To quantify cholesterol, 250 μg of 5α-cholestane (10 mg/ml) was added to the samples before lipid extraction and area under the curve was used to quantify total cholesterol in the samples. Cholesterol was analyzed by gas chromatography. Plasma cholesterol was measured by a commercially available kit (DIM chol-cholesterol flex; Siemens) on a clinical analyzer.

2.3. Western immunoblotting

Total proteins were extracted from a sample of 50 mg of liver powder using 1 ml of extraction buffer containing 50 mM Tris–HCl (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, 0.5% (w/v) and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Tissue was sonicated and centrifuged for 20 min at 100,000*g* at 4°C. Twenty micrograms of proteins was loaded on a 10% Mini-PROTEAN TGX Stain-Free polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked with 5% (w/v) milk in 0.05% (v/v) TBS-tween for 60 min at room temperature and thereafter incubated overnight at 4°C with the following primary antibodies: LDLR (1:1000; Novus, Vancouver, Canada), LSR (1:500; Sigma, Oakville, Canada), LRP1 (1:2000; Abcam, Cambridge, UK) and ApoE (1:500; Novus, Vancouver, Canada). Bands were revealed by chemiluminescence with Luminata Crescendo HRP substrate (EMD Millipore, Billerica, MA, USA) using a peroxidase-conjugated secondary antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA). Densitometry was assed using ChemiDoc MP System (Bio-Rad Laboratories). Total proteins were quantified with the Stain-Free technology (Bio-Rad Laboratories) and used as loading control. This technology is a more robust quantification technique compared to β-actin for western immunoblotting [19–20]. Protein levels of *E3+* mice fed the control diet were standardized at 100%.

2.4. Plasma apoE quantification

ApoE levels were measured in plasma from mice expressing one of the two human *APOE* alleles using a sandwich ELISA (Abcam). Briefly, plasma sample was diluted 1:200 into 1× Diluent N that was provided with the kit. Fifty microliters of sample or standard was loaded into a 96-well plate that had been coated with an anti-apoE antibody. Levels of apoE were performed in duplicate and quantification was performed using the standard curve. Absorbance was measured at 450 nm using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA).

2.5. Liver protein gene expression

RNA in the liver powder was extracted using the RNeasy Minikit (Qiagen, Venlo, Netherlands). RNA purity and integrity were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed at the RNomics Platform, Laboratoire de Génomique Fonctionnelle, University of Sherbrooke, QC, Canada. cDNA synthesis was performed using 1.3 μg of RNA with Transcriptor reverse transcriptase, random hexamers and dNTPs (Roche Diagnostics, Basel, Switzerland). Quantitative PCR was conducted with 10 ng cDNA and 200 nM primer pair solution on a CFX-384 termocycler (Bio-Rad Laboratories). Relative expression calculations of the candidate genes were performed using the housekeeping genes Pum1, Sdha and Txnl4b for mouse cDNA.

2.6. Statistical analysis

Data are expressed as mean ± SEM. Two-way ANOVA with genotype and diet as fixed factors were performed. When there was a significant genotype × diet interaction, subgroup analysis with *t* test was performed to compare differences between genotypes in each dietary group separately and to compare differences between diets in each genotype group separately. Statistical significance was set at $P<.05$.

3. Results

3.1. E3+ mice fed the DHA diet have lower plasma cholesterol levels compared to E3+ mice fed the control diet

For plasma cholesterol, there was a trend toward a diet × genotype interaction ($P=.054$). Plasma cholesterol was 36% lower in *E4+* mice compared to *E3+* mice fed the control diet ($P=.02$; Fig. 1). *E3+* mice fed the DHA diet had 33% lower plasma cholesterol compared to *E3+* mice fed the control diet ($P=.025$; Fig. 1). There was no such significant diet effect in *E4+* mice (Fig. 1). These results suggest that *E4+* mice did not respond to the DHA diet in terms of plasma cholesterol lowering.

3.2. E3+ and E4+ fed the DHA diet have lower hepatic lipoprotein receptor protein and mRNA levels compared E3+ and E4+ mice fed the control diet

There was no diet × genotype interaction on the protein levels and mRNA expression of the LDLR, LSR and low-density lipoprotein receptor-related protein 1 (LRP1). There was a genotype effect on hepatic LDLR protein levels and its mRNA expression levels ($P=.004$ and $P=.026$; Fig. 2A and B). LDLR protein levels were 60–66% higher in *E4+* mice than *E3+* mice whereas mRNA expression levels were 23–33% higher in *E4+* mice than *E3+* mice, and this effect was independent of the diet (Fig. 2A and B). There was no diet effect on the protein levels of LDLR but there was a 35–40% lower expression of LDLR mRNA in mice fed the DHA diet compared to the control diet ($P<.0001$; Fig. 2A and B). There was a diet effect for LSR protein level and its mRNA expression level ($P<.0001$ and $P=.002$; Fig. 2C and D). LSR protein level was 62% lower in mice fed the DHA diet than mice fed the control diet whereas mRNA expression levels were 23–33% lower in mice fed DHA than mice fed the control diet. The diet effects were independent from genotype (Fig. 2C and D). There was no diet or genotype effect for LRP1 protein levels (Fig. 2E).

3.3. E4+ mice have lower plasma apoE and higher liver apoE levels compared to E3+ mice

There was no diet × genotype interaction on the plasma and liver levels of apoE. However, there was an independent genotype effect on apoE levels in the plasma and the liver ($P<.0001$ and $P=.019$; Fig. 3A and B). Plasma apoE levels were ~35% lower in *E4+* mice than *E3+* mice (Fig. 3A), whereas in the liver, they were ~25% higher in *E4+* mice than *E3+* mice (Fig. 3B).

3.4. E3+ and E4+ fed the DHA diet have lower liver cholesterol levels compared to E3+ and E4+ mice fed the control diet

There was no diet × genotype interaction on the levels of cholesterol in the liver. There was a diet effect and a genotype effect for cholesterol level in the liver ($P<.0001$ and $P=.015$; Fig. 4). Cholesterol levels were ~67% lower in mice fed the DHA diet than mice fed the control diet (Fig. 4). Moreover, cholesterol level in the livers were ~18% lower in *E4+* mice than *E3+* mice (Fig. 4).