Eicosapentaenoic Acid Decreases Post-prandial β-Hydroxybutyrate and Free Fatty Acid Responses in Healthy Young and Elderly

RUNNING HEAD: EPA lowers plasma β-hydroxybutyrate response

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Abstract

Objectives: We investigated whether a dietary supplement rich in eicosapentaenoic acid (EPA) increases plasma fasting ketones or postprandial ketone responses in healthy young and elderly subjects.

Research Methods & Procedures: 10 young (22 ± 2 y old) and 10 elderly subjects (75 ± 4 y old) were recruited and participated in two identical study days, one before and one 6 weeks after providing an EPA-enriched supplement (1.4 g/d of EPA and 0.2 g/d of docosahexaenoic acid). On the study days, blood samples were collected at fasting and every hour for 6 h after giving a breakfast. Fasting and post-prandial plasma β-hydroxybutyrate (β-OHB), free fatty acids (FFA), triglycerides, glucose and insulin responses were measured. Fatty acid profiles were assessed in fasting plasma samples before and after the EPA supplement.

Results: After the EPA supplement, postprandial plasma β-OHB responses dropped by 44% in the young and by 24% in the elderly, along with 20% and 34% lower FFA responses in the young and elderly adults, respectively. β-OHB and FFA were positively and significantly correlated in young but not in elderly subjects both before and after the EPA supplement. In both groups, postprandial plasma triglycerides, glucose and insulin were not significantly different after the intake of the EPA supplement. Before and after the EPA supplement, fasting plasma EPA was 50% higher in the elderly but increased by about 5 times in both groups following the intake of EPA supplement.

Conclusions: Contrary to our expectations, EPA supplementation lowered postprandial β-OHB response and, in the elderly subjects, the concentration of postprandial β-OHB was not lowered after the intake of EPA supplement.

Keywords: eicosapentaenoic acid; ketones; β-hydroxybutyrate; free fatty acids; elderly; aging

68 **List of abbreviations**

69 β-OHB – beta-hydroxybutyrate

70 DHA – docosahexaenoic acid

71 EPA – eicosapentaenoic acid

72 FFA – free fatty acids

73 PPAR-α – peroxisome proliferator-activated receptor-alpha

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Introduction

Glucose normally provides about 97% of cerebral energy requirements but when glucose availability is limited, i.e. during fasting or starvation, ketones become important brain energy substrates supplying up to 70% of brain energy requirements [1]. Brain uptake of ketones is directly proportional to their circulating level [2-4]. During healthy aging, brain glucose uptake decreases significantly in specific cortical regions [5], an effect that is more pronounced in elderly with deteriorating cognitive function such as Alzheimer's disease [6]. Therefore, the idea of safely inducing chronic, mild ketonemia has been proposed as a strategy to counteract declining brain glucose uptake and hence hopefully reduce the risk of deteriorating cognition in the elderly [7, 8].

Ketones refer to three molecules: acetoacetate, β-hydroxybutyrate (β-OHB) and acetone. They are produced principally in liver mitochondria from successive condensation of acetyl-CoAs derived from β-oxidation of free fatty acids (FFA). Despite the possible need for a fuel to replace glucose in the aging brain, neither of the common ways of increasing ketone production, i.e. fasting for several days [1] or a very high fat ketogenic diet [9], seems realistic for the elderly. An alternative approach to safely inducing mild ketonemia may be to increase FFA β-oxidation and up-regulate transcription of enzymes involved in ketogenesis, particularly 3-hydroxy-3-methylglutaryl coenzyme A synthase. Both β-oxidation and transcription of 3-hydroxy-3-methylglutaryl coenzyme A synthase are regulated by the nuclear receptor - peroxisome proliferator-activated receptor-alpha (PPAR-α), which is a ligand-activated transcription factor [10].

In vitro studies show that the omega-3 fatty acid – eicosapentaenoic acid (EPA; 20:5ω3) – is a strong natural fatty acid ligand for PPAR-α [11]. Hence, our hypothesis

was that if PPAR-α is involved in activating the β-oxidation of FFA and up-regulating enzymes of ketogenesis and since EPA is a good ligand of PPAR-α [11], ketogenesis should increase after consuming an EPA supplement. Until now, the possible link between activation of PPAR-α by fatty acids such as EPA and increased ketogenesis had not been assessed in humans. The aims of this study were to determine, first, whether supplementation with an EPA-enriched fish oil would increase ketone concentration and, second, whether ketone concentration would differ in the elderly compared to young adults after EPA supplementation.

Given the potentially important clinical application of ketones as alternative cerebral energy substrates in the elderly, but the relative scarcity of information about fasting and postprandial ketone production during healthy aging [12], we compared these parameters in healthy elderly to young adults after giving an EPA supplement. Our approach was to measure changes in plasma FFA and ketone concentration during two identical metabolic study days, one before and one 6 wk after EPA supplementation since a direct measure of ketogenesis enzymes and PPAR-α activation was not possible in human liver.

Subjects and Methods

Subjects: Subjects were recruited in two age groups: 18-25 y old (young) and 70-85 y old (elderly). All subjects were non-smokers and selected for relatively good health. As part of the screening, blood chemistry was assessed after a 12 h overnight fast. Fasting glucose and hemoglobin A_{1c} were used to rule out the presence of glucose intolerance or overt diabetes. A complete blood cell count was used for blood disorders, electrolyte profile, aspartate transaminase and alanine transaminase for liver function/nutritional status, high and low density lipoprotein cholesterol, triglycerides and albumin for nutritional status, C-reactive protein as a marker of inflammatory processes, and thyroid stimulating hormone for thyroid function. Subjects with plasma omega-3 fatty acids higher than 4% of total fatty acids were excluded. Except for glucose and cholesterol, other parameters did not differ significantly between the two groups (**Table 1**).

Approval for the study was obtained from the Research Ethics Committee of the Health and Social Services Center – Sherbrooke University Geriatrics Institute, which oversees all human research done at the Research Center on Aging. All subjects gave informed, written consent before participating. Thirty subjects underwent screening. Complete metabolic data were obtained for 10 young and 10 elderly subjects (5 women and 5 men in each group) who met our inclusion criteria. A group size of ten was sufficient to meet the statistical power ($\beta = 0.80$) needed to achieve a significant difference in doubling fasting plasma ketones after EPA supplementation [13].

Protocol and sample collection: During the study, each subject consumed 4 capsules / day providing a total of 1480 mg/d of EPA and 250 mg/d of docosahexaenoic acid (DHA;

22:6ω3). The capsules used were a commercially-available omega-3 fatty acid supplement (OM3, Isodis Natura, Brussels, Belgium), hereafter designated as the EPA supplement. Subjects participated in two identical metabolic study days, one before and one 6 wk after EPA supplementation. On each metabolic study day, subjects arrived at 7:30 a.m. after a 12 h fast. A forearm venous catheter was installed and was kept patent by flushing hourly with non-heparinized saline after fasting blood draw (defined as time 0) and hourly blood draw for 6 h using a 5 ml latex-free syringe (Becton Dickinson, Franklin Lakes, NJ). The subjects received a breakfast (between time 0 and time 1) composed of eggs, bacon, cheese, one slice of tomato and toast with an average fat content of 23 g or 43% of the breakfast calories and around 38 g of carbohydrates accounting for around 34% of the breakfast calories. Blood samples were transferred immediately to a 5 mL K_2-EDTA coated tube (Becton Dickinson) and kept on ice until the end of the study day when they were all centrifuged at 2300 g for 18 min at 4˚C. Plasma was stored at -20˚C until further analysis. During the study day, water was available *ad libitum* and subjects were asked to remain in a resting position, with short walks allowed.

Plasma fatty acid profile: Plasma total lipids were extracted into 2:1 chloroform/methanol solution, using heptadecanoate as an internal standard. The total lipids were then saponified with 1 mol/L methanolic potassium hydroxide followed by transmethylation of the FFA to fatty acid methyl esters using 14% methanolic boron trifluoride. Fatty acid methyl esters were analyzed using a gas chromatograph (Agilent model 6890, Palo Alto, CA) equipped with a 50 m BPX-70 fused capillary column (SGE, Melbourne, Australia, 0.25 mm i.d., 0.25 μm film thickness). Splitless mode injection and flame ionization

detection were performed at 250°C. The oven temperature program was 50°C for 2 min, increased to 170°C at a rate of 20°C/min and held there for 15 min, increased to 210°C at a rate of 5°C/min and held there for 7 minutes. The inlet pressure of the carrier gas (He) was 233 kPa at 50°C. The identity of individual fatty acids was determined by comparing retention times with standard mixtures of fatty acids (NuChek 68A, NuChek 411, and NuChek 455; NuChek Prep, Inc., Elysian, MN) and a custom mixture of saturated fatty acid standards.

Other analyses: Commercially available reagent kits were used for the analysis of β-OHB (RX Daytona kit; Randox Laboratories Ltd., Antrim, UK), and FFA (Wako Diagnostics, Richmond, VA) and triglycerides and glucose (Dade Behring Inc., Newark, DE) using an automated clinical chemistry analyzer (Dimension XPand Plus, Dade Behring Inc., Newark, DE). Insulin was analyzed by ELISA (Mercodia, Upssala, Sweden) using a microplate reader (model 3550, BioRad, Hercules, CA).

Statistical analysis: Results are given as mean ± SEM in Figures and Tables. Postprandial responses over the 6 h of the study day were defined by areas under the curve calculated for plasma β-OHB, FFA, triglycerides, glucose and insulin (Prism software version 4.0, GraphPad Prism, San Diego, CA) allowing comparison between curves. Since data were not normally distributed and the sample size was small, we used non-parametric tests to compare data. Hence, data for the two age groups were compared by a Mann-Whitney test using SPSS software (version 12.0, SPSS Inc, Chicago, IL). To determine statistical significance following the EPA supplementation,

we used Wilcoxon's signed rank test. To determine age-by-diet interactions, we compared calculated variables (after – before EPA supplement) for fasting β-OHB, FFA, triglycerides, glucose and insulin and for postprandial responses between age groups using a Mann-Whitney test. Correlation between FFA and β-OHB was determined using the Spearman correlation coefficient. Significance was set at $p < 0.05$.

Results

Plasma β-OHB and FFA (Figure 1): After the EPA supplement, fasting plasma β-OHB and FFA were respectively 51% lower ($p = 0.007$) and 35% lower ($p = 0.022$) in young subjects but unchanged in the elderly. Postprandial β-OHB response was significantly lower in both groups after the EPA supplement, with a greater drop (44%) in the young than in the elderly (24%). Similarly, after EPA, the postprandial FFA response was 20% lower in both groups, but did not reach statistical significance in the young subjects ($p = 0.059$). Neither before nor after EPA was β-OHB response statistically different between elderly and young subjects. In contrast, the postprandial FFA response was about 40% higher in the elderly both before ($p = 0.014$) and after ($p = 0.013$) the EPA supplement. Fasting plasma FFA and β-OHB were positively correlated in young ($p < 0.05$) both before and after the intake of EPA supplement but not in elderly (Figure 2).

Plasma triglycerides, glucose and insulin (Figure 1): Before EPA supplementation, fasting plasma triglycerides were similar in the young and the elderly. After EPA supplementation, the elderly had 63% higher fasting triglycerides compared to the young adults ($p = 0.041$) and postprandial plasma triglycerides levels were significantly higher compared to young ($p = 0.041$). In both groups, fasting and postprandial plasma triglycerides were not significantly lower after the EPA supplement. In the elderly, fasting glucose was around 24% higher both before and after EPA supplementation compared to young. In both groups, fasting glucose concentrations and postprandial glucose responses were similar after the intake of EPA supplement. Fasting and postprandial

insulin of elderly was similar to young before and after the EPA supplementation. After EPA supplementation, fasting plasma insulin was 42% and 39% higher in the elderly ($p = 0.017$) and the young ($p = 0.114$), respectively whereas the postprandial insulin response was not significantly different in both groups.

Plasma fatty acid profile (Figure 4): Plasma fatty acid profiles were assessed to evaluate the effectiveness of the EPA supplement in increasing fasting plasma EPA and DHA levels. Before EPA supplementation and compared to the young adults, the elderly had 85% higher plasma EPA but DHA concentration was similar in both groups. After the intake of EPA supplement, fasting plasma EPA concentration was 5.6 and 5.1 times higher in the young and elderly, respectively, whereas fasting plasma DHA was 24% higher only in the young subjects ($p = 0.037$). After the EPA supplement, fasting plasma EPA (mg/L) remained 67% higher in elderly compared to young adults.

Discussion

This study aimed to evaluate the impact of EPA supplementation on fasting and postprandial ketone concentration in both young and elderly adults. Our results suggest that EPA supplementation *reduced* the postprandial β-OHB and FFA responses in both groups. The concentration of ketones in plasma reflects the balance between appearance in and removal from the plasma. Although β-oxidation of β-OHB is similar in young and elderly adults [12], we have no indication whether after an EPA supplementation the β-oxidation of β-OHB would be higher or its removal from plasma would be altered. Therefore, the reason why EPA supplementation lowered rather than raised β-OHB and FFA responses in this study is unclear.

The production of ketones requires increased mobilization of FFA from adipose tissue to the liver by increasing lipolysis in adipose tissue and/or triglyceride-rich lipoproteins [14, 15], coupled with enhancement of the liver's capacity to convert these substrates into β-OHB and other ketones [16]. Since we observed a linear correlation between fasting plasma FFA and β-OHB concentrations in young subjects (Figure 2) [14] and because ketones are produced from FFA β-oxidation, the lower FFA response may therefore have contributed significantly to lowered β-OHB response (Figure 1).

Indeed, we hypothesized that giving an EPA supplement would up-regulate ketogenesis enzymes in the liver leading to increase ketone concentration. However, EPA interacts with at least four families of transcription factors - PPAR-α, liver X receptors, hepatic nuclear factor-4α and sterol regulatory-element-binding protein - and

generates a large range of eicosanoids able to modulate transcription factor activity [17]. It is therefore possible that the EPA supplement may have simultaneously decreased lipolysis through one of the other activated transcription factors since it is controlled by gene transcription in the liver [18]. Two studies in humans [14, 19], reported that an EPA supplement resulted in decreased peripheral lipolysis from adipose tissue thereby reducing the availability of FFA. Moreover, EPA is suggested to lower liver TG synthesis and increase adipose TG clearance thereby reducing the release of FFA by lipoprotein lipase [17]. In this study, EPA supplementation did not significantly lower fasting or postprandial plasma TG (Figure 3). This result may not be so unusual in normolipidic humans since it occurs in about half of placebo-controlled trials [20] and is due to low baseline TG concentration and EPA dose [17]. Hence, increasing plasma β-OHB concentrations in humans appears complex and may require combined strategies for increasing FFA lipolysis (substrate for ketonegenesis) from either adipose tissue and/or triglyceride-rich lipoproteins while simultaneously increasing the liver's capacity to produce ketones.

Since cognitive decline affects the elderly and because ketones are the major alternative brain fuel to glucose, we investigated whether fasting and postprandial ketone concentrations would be *increased* similarly in young and elderly after the EPA supplementation. In our elderly group, neither fasting nor postprandial plasma β-OHB was statistically different from that seen in our young adults but the elderly had a higher postprandial FFA response compared to our young subjects. Moreover, our data do not support a significant correlation of fasting plasma FFA with β-OHB in the elderly either before or after EPA supplementation (Figure 2). Therefore, lipid metabolism, specifically

271 regarding FFA release and/or β-oxidation, is possibly altered during aging [22].
272 However, the ratio of fasting plasma β-OHB/FFA, which is suggested to be a marker for
273 fatty acid β-oxidation capacity and/or ketogenesis in the liver [14], was not statistically
274 different between young and elderly (data not shown).

275 Thus, the higher postprandial response of FFA in the elderly (Figure 1) [22] may
276 be a result of lower β-oxidation in muscle possibly resulting from lower muscle mass in
277 elderly or lower muscle capacity for β-oxidation [22]. Despite possibly altered lipid
278 metabolism in the elderly, our results support a similar concentration of both fasting and
279 postprandial β-OHB response in elderly compared to young adults.

280 EPA incorporation into plasma lipids following an EPA supplementation may differ
281 during aging [23, 24]. In this study, fasting plasma EPA concentration before EPA
282 supplementation was 85% higher in our elderly compared to young adults, which agrees
283 with previous work [23, 24]. The reasons why plasma EPA differs in the young and
284 elderly is unknown [23] but may result from lower β-oxidation of dietary EPA, thus
285 leaving a greater proportion for incorporation into plasma lipids in the elderly [24]. The
286 impact of higher incorporation of EPA into plasma lipids during aging has not yet been
287 fully investigated but should be considered as a possible confounder in results of studies
288 using fish oil supplementation with elderly. Our EPA supplement also provided 200 mg
289 of DHA/d, but this did not significantly raise fasting plasma DHA in our elderly group.
290 This indirectly suggests that 200 mg of DHA/d for 6 wk may not be sufficient to raise
291 fasting plasma DHA in the elderly.

292 We conclude that short term EPA supplementation lowers β-OHB and FFA
293 responses, an effect apparently not influenced by healthy aging.
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Figure Legends

Figure 1: Plasma β-hydroxybutyrate (β-OHB, upper, mean ± SEM) and free fatty acid (FFA, lower) postprandial responses of young and elderly before and 6 wk after the intake of eicosapentaenoic acid (EPA) supplement. Subjects had breakfast between time 0 and time 1 (↑).

*Statistically different for fasting plasma measures, $p < 0.05$.

† Area under the curves significantly decreased after EPA supplement, $p < 0.05$

Figure 2: Correlation between fasting plasma β-hydroxybutyrate (β-OHB) and free fatty acids (FFA) in young and elderly before and 6 weeks after the intake of eicosapentaenoic acid (EPA) supplement. Both correlations in young were statistically significant ($p < 0.05$) while both correlations in elderly were not statistically significant.

Figure 3: Concentration (mg/L) of fasting plasma eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in young and elderly before and 6 weeks after the intake of EPA supplement.

*Statistically significant between young and elderly on same EPA treatment, $p < 0.05$

† Significantly increased after EPA supplement, $p < 0.05$

Figure 1: Plasma triglycerides (upper, mean ± SEM), glucose (middle) and insulin (lower) postprandial responses of young and elderly before and 6 wk after the intake of

382 eicosapentaenoic acid (EPA) supplement. Subjects had breakfast between time 0 and

383 time 1 (↑).

384 *Statistically different for fasting plasma measures, $p < 0.05$.

385