Accumulation of DHA in HepG2 cell membranes is elevated following supplementation with fatty acids found in dairy fat

Introduction

The health benefits of n-3 LCPUFA through all stages of life have emphasised the need to enrich the current Western diet with n-3 LCPUFA. N-3 LCPUFA, particularly DHA, have been implicated in the neurodevelopment of infants (Maclrides et al. 1995; Carlson et al. 1996; Willatts et al. 1998). Epidemiology and human intervention trials also support the relevance of n-3 fatty acids in cardiovascular disease prevention (Hu et al. 1999; Albert et al. 2002; de Lorgeril et al. 1994; Bryan et al. 1999). As a result, the latest health recommendations call for an increase in fish consumption to 2-3 meals/week. The diet can also be enriched with n-3 LCPUFA by consuming modified foods enriched with n-3 LCPUFA or with fish oil supplements. These practices rely heavily on an already declining global fish supply (Dormergue et al. 2005).

Exploitation of this natural resource has reduced catch size, both the number of fish and their maturity (Dormergue et al. 2005). Clearly, an alternative source of n-3 LCPUFA is required to meet the current dietary recommendations for human health.

Vegetable oils, such as linseed and canola, are rich in the n-3 essential fatty acid ALA and may provide an alternative source of these bioactive n-3 LCPUFAs. ALA is a substrate for the fatty acid synthetic pathway, where it is converted to longer chain n-3 metabolites in the body. ALA goes through a series of desaturations and elongations to yield EPA, DPA and DHA. The proposed pathway suggests that increasing the intake of ALA could increase the synthesis and accumulation of DHA in tissues and plasma. Several studies have attempted to increase the endogenous synthesis of EPA and DHA by supplementing the diet with ALA. However, elevating the dietary intake of ALA in animals and humans results in an increase in the level of EPA, but little change in the level of DHA (Sanderson and Roshanai 1983; Mantzioris et al. 1995; Mantzioris et al. 1994; Blank et al. 2002).

Fatty acids downstream of ALA in the fatty acid synthetic pathway are also precursors to EPA and DHA. These include stearidonic acid (18:3n-3), EPA and DPA. Dairy fat contains several fatty acids downstream of ALA, in addition to ALA, which have the potential to be converted to EPA and DHA endogenously. Butter contains 0.8-1.2% ALA, 0.09-0.27% EPA and 0.1-0.22% DPA of the total fatty acids. It is a more sustainable resource than fish oil and can be incorporated into the habitual diet of the general population. We have used the human hepatoma HepG2 cell line to examine the accumulation of EPA and DHA in cells supplemented with ALA, EPA and DPA. The HepG2 cell line is of interest because the liver is the

Abstract

Health authorities have recognised the importance of n-3 long chain polyunsaturated fatty acids (LCPUFA) in maintaining health and it is generally accepted that we need to increase our dietary intake of these fatty acids, particularly eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). Common practice has been to increase our intake of fish, consume fish oil supplements or increase our intake of n-3 enriched foods. These practices rely heavily on a declining global fish supply. Alternative ways to increase our n-3 LCPUFA status are, therefore, imperative. The fatty acids found in dairy fat may provide an alternate source to increase our n-3 LCPUFA status.

We examined the accumulation of EPA and DHA in HepG2 cells supplemented with α-linolenic acid (18:3n-3, ALA), EPA and docosapentaenoic acid (22:5n-3, DPA). There was a dose-dependent increase in the level of ALA, EPA and DPA in HepG2 cell phospholipids following supplementation with these respective fatty acids. The accumulation of DHA, the long chain n-3 metabolite of fatty acid synthesis, was significantly higher in cells supplemented with EPA and DPA compared to those supplemented with ALA. Dairy fats uniquely contain greater amounts of EPA and DPA than ALA and no DHA. This study suggests that the fatty acids found in dairy fats have the capacity to elevate the level of DHA in cell membranes.

Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), foetal bovine serum (FBS), penicillin streptomycin and trypsin were purchased from JRH Biosciences (Victoria). Free fatty acids (FFA) and authentic lipid standards were obtained from Nu-Check-Prep Inc (Elysian, MN). Bovine serum albumin (BSA) was from Sigma (St Louis, MO). All other chemicals and reagents were of an analytical grade.
Cell culture
HepG2 cells (human hepatoma cell line) were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/mL penicillin and 37.5 μg/mL streptomycin. Cells were grown in 75 cm² tissue culture flasks and incubated at 37°C in a 5% CO₂ humidified incubator. At 80% confluence, HepG2 cells were harvested and seeded into 6-well plates at a density of 4 x 10⁵ cells/mL in 2 mL of growth medium. After 72 h, the medium was removed and replaced with serum-free DMEM, supplemented with FFA bound to essentially fatty acid-free BSA. The molar ratio of fatty acid to albumin was 4:1. Stock solutions of free fatty acids were prepared in ethanol at a concentration of 10 mg/mL and diluted in serum-free medium to achieve final concentrations ranging from 0.5-10 μg/mL ALA, EPA or DPA. The media was replaced after 24h. After 48h incubation with albumin-bound fatty acids, the cells were harvested for fatty acid analysis. Cells were used between passage 15 and 20. Cell viability was assessed by trypan blue exclusion.

Fatty acid analysis
Lipids were extracted from cells with chloroform-methanol (2:1, by vol) according to the method of Folch et al. (1957). Fatty acid fractions were separated by thin layer chromatography (TLC) (Silica Gel 60H Merck Darmstadt) using a mobile-phase of petroleum spirits, diethyl ether and glacial acetic acid (180:30:2, by vol). The phospholipid fractions were isolated and methylated with 1% sulphuric acid in methanol. The resulting fatty acid methyl esters were extracted into n-heptane and transferred to vials containing anhydrous Na₂SO₄. Fatty acid methyl esters were measured by gas chromatography on a BPX-70 50 m capillary column coated with 70% cyanopropyl polysilphenylene-siloxane (0.25 μm film thickness and 0.33 mm internal diameter SGE, Victoria) on a Hewlett-Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) fitted with a flame ionisation detector. Helium was the carrier gas and the split-ratio was 20:1. The injection port temperature was 250°C and the detector temperature was 300°C. The initial column temperature was 140°C and increased to 220°C at a rate of 5°C/min. The identity of each fatty acid peak was ascertained by comparison of peak retention time to authentic lipid standards. The relative amount of each fatty acid (% of total fatty acid) was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids.

Results
Following ALA supplementation, the level of ALA in cell phospholipids increased significantly from 0.12±0.11% to 7.05±0.53% total fatty acids (Figure 1). The accumulation of DHA peaked following supplementation with 5 μg/mL ALA and did not increase with higher concentrations. At its peak, the level of DHA in cell phospholipids had increased 1.7-fold, from 2.62±0.06% to 4.44±0.33% total fatty acids (Figure 2).

Following EPA supplementation, the level of EPA in HepG2 cell phospholipids increased linearly with dose, from 0.1±0.01% in control cells to 9.50±1.02% total fatty acids in cell supplemented with the highest dose of EPA (10 μg/mL) (Figure 1). There was a gradual increase in the level of DHA following supplementation with EPA (Figure 2). At 10 μg/mL EPA, the level of DHA in cell phospholipids increased 2.4-fold to 6.28±0.91% total fatty acids. Compared to cells supplemented with ALA, the level of DHA in

![Figure 1: The accumulation of α-linolenic acid (●), eicosapentaenoic acid (□) and docosapentaenoic acid (●), in HepG2 cell phospholipids of cells supplemented with α-linolenic acid, eicosapentaenoic acid and docosapentaenoic acid, respectively. HepG2 cells were seeded and grown as described in materials and methods. After 72 h the medium was replaced with serum free medium containing increasing amounts of each fatty acid bound to BSA. The cells were harvested for lipid analysis following 48 h supplementation with the respective fatty acid. Values are means ± SE of at least three replicates.](image1)

![Figure 2: The accumulation of docosahexaenoic acid (DHA) in HepG2 cell phospholipids following supplementation with α-linolenic acid (●), eicosapentaenoic acid (□) and docosapentaenoic acid (●). HepG2 cells were seeded and grown as described in materials and methods. After 72 h the medium was replaced with serum free medium containing increasing amounts of each fatty acid bound to BSA. The cells were harvested for lipid analysis following 48 h supplementation with the respective fatty acid. Values are means ± SE of at least three replicates. Points with different letters are significantly different between groups at that concentration (p<0.05).](image2)
cell phospholipids, at the equivalent dose of EPA, was significantly greater ($p<0.05$).

The level of DPA in cells supplemented with DPA also increased linearly with dose. The level of DPA in cell phospholipids increased 17-fold from 0.3±0.02% to 5.19±0.27% total fatty acids (Figure 1). Similar to those cells supplemented with ALA and EPA, there was a gradual increase in the level of DHA following supplement with DPA (Figure 2). At 10 μg/mL DPA, the level of DHA in cell phospholipids increased 2.8-fold to 6.97±0.49% total fatty acids. At 10 μg/mL DPA, the level of DHA in cell phospholipids was significantly greater ($p<0.05$) than in those cells supplemented with ALA.

Discussion

This study has demonstrated that the fatty acids found in dairy fats have the capacity to elevate the level of DHA in HEPG2 cell phospholipids above that observed in cells supplemented with ALA. Previous animal and human dietary intervention studies suggest that the fatty acids in dairy fat elevate the accumulation of DHA in tissues. Animals fed a butterfat-based diet have higher levels of EPA and DHA in plasma and tissues compared to those fed a vegetable oil-based diet (Naughton et al. 1988). A study by Courage et al. (1998) supports the finding that dairy fats increase the level of DHA in human tissues. This study evaluated the fatty acid composition of red blood cells of infants fed breast milk, formula or evaporated milk formula at 3 and 6 months of age. In both age groups, the level of EPA and DHA in those infants receiving evaporated milk formula was significantly higher ($p<0.05$) than formula-fed infants. The unique fatty acid composition of dairy fats may promote the conversion of n-3 fatty acid precursors to their longer chain metabolites. Dairy fat has a low linoleic acid (18:2n-6):ALA ratio. ALA is the predominant fatty acid in the Western diet and is also a substrate for the enzymes required to convert precursor n-3 fatty acids to their longer chain derivatives. The high LA:ALA ratio of the Western diet is thought to contribute to the poor DHA status of the population. Moreover, dairy fat contains EPA and DPA, which can be converted endogenously to DHA. As we have shown here, the accumulation of DHA from EPA and DPA into cell phospholipids is higher than from ALA. Other fatty acids characteristic of dairy fat, including conjugated linoleic acid (CLA), may act to enhance the capacity of the fatty acid synthetic pathway in the body, providing a sustainable resource for enriching the n-3 LCPUFA status of the community.

Conclusion

The inclusion of n-3 LCPUFAs in the diet has, in the past, relied heavily on the intake of fish oils. We have provided evidence to suggest that alternative foods rich in n-3 precursors, particularly EPA and DPA, have the capacity to enrich the diet with n-3 LCPUFAs. Dairy fat contains precursor n-3 fatty acids that can be converted endogenously to n-3 LCPUFAs, leading to elevated tissue levels of these fatty acids, conferring the associated health benefits. Dairy fats may, therefore, provide a means for enriching the diet with n-3 LCPUFAs.

References


