Competition between 24:5n-3 and ALA for Δ6 desaturase may limit the accumulation of DHA in HepG2 cell membranes

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Abstract The use of Δ6 desaturase (D6D) twice in the conversion of α-linolenic acid (ALA; 18:3n-3) to docosahexaenoic acid (DHA; 22:6n-3) suggests that this enzyme may play a key regulatory role in the synthesis and accumulation of DHA from ALA. We examined this using an in vitro model of fatty acid metabolism to measure the accumulation of the long-chain metabolites of ALA in HepG2 cell phospholipids. The accumulation of ALA, eicosapentaenoic acid (20:5n-3), docosapentaenoic acid (22:5n-3), and 24:5n-3 in cell phospholipids was linearly related to the concentration of supplemented ALA over the range tested (1.8–72 μM). The accumulation of the post-D6D products of 22:5n-3, 24:6n-3 and DHA, in cell phospholipids was saturated at concentrations of >18 μM ALA. Supplementation of HepG2 cells with preformed DHA revealed that, although the accumulation of DHA in cell phospholipids approached saturation, the level of DHA in cell phospholipids was significantly greater compared with the accumulation of DHA from ALA, indicating that the accumulation of DHA from ALA was not limited by incorporation. The parallel pattern of accumulation of 24:6n-3 and DHA in response to increasing concentrations of ALA suggests that the competition between 24:5n-3 and ALA for D6D may contribute to the limited accumulation of DHA in cell membranes.—Portolesi, R., B. C. Powell, and R. A. Gibson. Competition between 24:5n-3 and ALA for Δ6 desaturase may limit the accumulation of DHA in HepG2 cell membranes. J. Lipid Res. 2007. 48: 1592–1598.

Supplementary key words fatty acid synthesis • essential fatty acids • α-linolenic acid • docosahexaenoic acid

The synthesis of long-chain polyunsaturated fatty acids (LCPUFAs) from α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6) is well documented, yet regulation of the pathway, particularly the regulation of the conversion of ALA to docosahexaenoic acid (DHA; 22:6n-3), remains a focus of investigation. This is attributable to the observation that increased dietary intakes of ALA in animals and humans result in an increased level of eicosapentaenoic acid (EPA; 20:5n-3) but little or no change in the level of DHA in tissues or plasma (1–3). Direct measurement of fatty acid synthesis in humans using labeled ALA has demonstrated the conversion of ALA to EPA and docosapentaenoic acid (DPA; 22:5n-3) with limited conversion to DHA (1, 4–6). There are several plausible explanations for the disparity between the intake of ALA and its conversion to DHA in vivo. Ingested ALA has several metabolic fates, including β-oxidation, carbon recycling, conversion to LCPUFA, and direct incorporation into structural lipids (7). The balance between these metabolic fates influences the accumulation of DHA from ALA in tissues. Additionally, both n-3 and n-6 fatty acids use common enzymes in the synthesis of fatty acids; therefore, the competition between n-3 and n-6 fatty acid substrates for these enzymes is an important determinant of DHA synthesis. ALA and LA are both substrates for Δ6 desaturase (D6D). When both of these substrates are present, there is competition for active sites on D6D, with ALA being the preferred substrate (8). The affinity of D6D for ALA is approximately two to three times that of LA (9). The competition between substrates for D6D also extends to the 24 carbon fatty acids (24:5n-3 and 24:4n-6) at the second use of the enzyme in the synthesis of DHA and 22:5n-6, respectively. Therefore, competition between n-3 and n-6 substrates and the preferential entry of fatty acids into one or more metabolic pathways influences the fatty

Abbreviations: ALA, α-linolenic acid; D6D, Δ6 desaturase; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid.

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acid composition of cell membranes and the accumulation of DHA from ALA.

The study described here used the HepG2 cell line as an in vitro model of fatty acid metabolism to characterize the accumulation of n-3 fatty acids in HepG2 cell phospholipids after fatty acid supplementation. The human hepatoma HepG2 cell line is of particular interest because the liver is the major site of fatty acid synthesis, with a comparatively high level of Δ6D expression (10). By examining the accumulation of LCPUFAs in HepG2 cells after supplementation with fatty acids individually and exclusive of other fatty acids, the competition between fatty acid classes (n-3 vs. n-6) is eliminated. We aimed to examine the accumulation of ALA and its long-chain metabolites in HepG2 cell membranes after supplementation with increasing concentrations of ALA. The dynamics of fatty acid uptake and accumulation were also examined by measuring the accumulation of ALA and its metabolites in HepG2 cell phospholipids over 48 h. We also examined the accumulation of DHA in HepG2 cell membranes when supplied preformed in the cell culture medium.

MATERIALS AND METHODS

Materials

DMEM, FBS, penicillin, streptomycin, and trypsin were purchased from SAFC Biosciences (Victoria, Australia). FFAs and authentic lipid standards were obtained from NuChek Prep, Inc. (Elysian, MN). BSA was from Sigma (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in DMEM supplemented with 10% FBS, 2 mM t-glutamine, 50 U/ml penicillin, and 37.5 U/ml streptomycin (growth medium). 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 onto six-well plates at a density of 4 × 10⁵ cells/ml in 2 ml of growth medium. After 72 h, the medium was replaced and removed with serum-free DMEM supplemented with ALA or DHA, as FFA, bound to fatty acid-free BSA. The molar ratio of fatty acid to albumin was 4:1. Stock solutions of FFA were prepared in ethanol at a concentration of 10 mg/ml and diluted in serum-free DMEM to achieve final concentrations ranging from 1.8 to 72 μM ALA or 3.0 to 30 μM DHA. Concentrations of >72 μM were toxic to HepG2 cells. Moreover, concentrations of >60 μM were considered beyond physiological concentrations (9). Medium was replaced after 24 h. After 48 h of incubation with medium supplemented with fatty acids, the cells were harvested for lipid extraction and fatty acid analysis.

To measure the accumulation of fatty acids over time, HepG2 cells were cultured in DMEM with 10% FBS for 72 h. The medium was then replaced with serum-free DMEM supplemented with 72 μM ALA bound to BSA. Cells were harvested before supplementation, then at 1, 2, 6, 12, 24, and 48 h after incubation with ALA. The fatty acid composition of HepG2 cells maintained in serum-free DMEM, supplemented with the equivalent concentration of BSA, was measured at 1, 2, 6, 24, and 48 h after incubation. Cells were used between passages 15 and 20. Cell viability was assessed by trypan blue exclusion.

Fatty acid analysis

Lipids were extracted from cells with chloroform-methanol (2:1, v/v) according to the method of Folch, Lees, and Sloane-Stanley (11). Fatty acid fractions were separated by TLC (Silica Gel 60F; Merck, Darmstadt, Germany) using a mobile phase of petroleum spirits, diethyl ether, and glacial acetic acid (180:30:2, v/v). Phospholipid, triglyceride, and cholesteryl ester fractions were isolated and methylated with 1% sulfuric acid in methanol. The resulting fatty acid methyl esters (FAMEs) were extracted into n-heptane and transferred to vials containing anhydrous Na₂SO₄. FAMEs 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 0.32 mm internal diameter; SGE, Victoria, Australia) on a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA) fitted with a flame ionization 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 with authentic lipid standards (NuChek Prep). The relative amount of each fatty acid (percentage 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. The majority of fatty acids accumulated in the phospholipid fraction of HepG2 cell lipid extracts, accounting for ~66% of the total fat content. Minimal quantities accumulated in the cholesteryl ester fraction, and ~33% accumulated in triglycerides.

Identification of 24 carbon fatty acids

In HepG2 cells supplemented with ALA, two peaks beyond the retention time of DHA were apparent in the gas chromatography analysis of FAMEs from cell phospholipids. The unknown peaks were suspected to be the chain elongation product of DPA, 24:5n-3, and the Δ6 desaturation product of 24:5n-3, 24:6n-3. Commercial standards for 24:5n-3 and 24:6n-3 are not available; however, these fatty acids occur in the jellyfish Aurelia (12). FAMEs from the phospholipid fraction of HepG2 cells supplemented with ALA were sent to laboratories at the Commonwealth Scientific and Industrial Research Organization Marine and Atmospheric Research (Hobart, Tasmania, Australia) for analysis by gas chromatography, where the retention times of the unknown peaks were compared with FAMEs derived from Aurelia species to reveal that the two unknown peaks had comparable retention times to 24:5n-3 and 24:6n-3 in Aurelia. The mass spectra of the unknown peaks were also examined. FAMEs were analyzed by GC-MS on a Hewlett-Packard 6890 gas chromatograph equipped with a BPX-70 50 m capillary column (0.25 μm film thickness and 0.22 mm internal diameter; SGE) and a 5972 mass selective detector under the conditions described above. A 70 eV ionization potential was applied, and the mass spectrometry acquisition of each sample was set to scan mode. The fragment pattern for the first unknown peak was (major ions at m/z 79, 91, 108, 119, 133, 147, 161, and 186, corresponding to 24:5n-3 as described in Aurelia (12), and also contained the characteristic ion at m/z 108, typical of fatty acids with an n-5 terminal group (13). The mass spectra of the second unknown peak contained 79, 91, 105, 119, 128, 137, 147, 157, and 195, corresponding to 24:6n-3 as described in Aurelia (12). The mass spectra and retention times of these peaks confirmed that they were 24:5n-3 and 24:6n-3, respectively.

Statistical analysis

Fatty acid analyses were performed on means of at least three separate replicates. ANOVA and Bonferroni post hoc statistical
tests (SPSS, Inc., Chicago, IL) were used to compare means after fatty acid supplementation. Statistical significance was defined as $P < 0.05$. Curve fitting was performed using the SigmaPlot curve-fitting program (version 7.101; SPSS, Inc.). Data are expressed as means ± SEM.

RESULTS

Accumulation of n-3 fatty acids

To investigate the accumulation of ALA and its long-chain metabolites, HepG2 cells were supplemented with increasing concentrations of ALA. The accumulation of ALA into cell phospholipids was concentration-dependent, increasing from 0.12 ± 0.11% total fatty acids to 7.05 ± 0.53% total fatty acids (Fig. 2) over the concentration range tested (1.8–72 μM ALA). The accumulation of ALA metabolites, EPA and DPA, was linearly dependent on the concentration of ALA in the medium (Fig. 2). The level of EPA in HepG2 cell phospholipids increased by 47-fold, from 0.15 ± 0.02% total fatty acids in unsupplemented cells to 7.07 ± 0.74% total fatty acids in cells supplemented with 72 μM ALA. The level of DPA increased by 7-fold from 0.25 ± 0.05% total fatty acids in unsupplemented cells to 1.91 ± 0.31% total fatty acids in cells supplemented with 20 μg/ml ALA. In contrast, the level of DHA was maximal after supplementation with 18 μM ALA and did not increase with increasing concentrations of ALA. At its maximum, the level of DHA in cell phospholipids had increased by 1.7-fold from 2.62 ± 0.06% total fatty acids to 4.44 ± 0.33% total fatty acids. The level of DHA in the triglyceride fraction was minimal, representing only 8% of the level of DHA present in cell phospholipids. Fatty acid analysis of the medium after supplementation of HepG2 cells with ALA showed that there was no accumulation of DHA into the medium.
The accumulation of 24:5n-3 and 24:6n-3 in cell phospholipids was also measured (Fig. 3). There was a dose-dependent relationship between the accumulation of the D6D substrate, 24:5n-3, in cell phospholipids and the concentration of ALA in the medium (Fig. 3). The accumulation of the product of D6D, 24:6n-3, however, was curvilinear and paralleled the accumulation of DHA. There was an increase in the level of 24:6n-3, up to 18 μM ALA (Fig. 2). The level of saturated fatty acids increased concomitant with a decrease in the total level of monounsaturated fatty acids in HepG2 cell phospholipids after supplementation with increasing concentrations of ALA, suggesting an exchange in the distribution of fatty acids with ALA supplementation. The total level of n-9 fatty acids decreased with increasing concentrations of ALA. Under conditions of ALA supplementation, the level of n-6 fatty acids did not change. These results are summarized in Table 1.

Accumulation of preformed DHA

To examine whether the limited accumulation of DHA from ALA was attributable to a limitation in the ability of DHA to be incorporated into cell phospholipids, the accumulation of DHA when supplied in the medium was examined. The level of DHA in HepG2 cell phospholipids accumulated readily, increasing from 3.14 ± 0.27% total fatty acids in control cells to 16.26 ± 0.61% total fatty acids in cells supplemented with 30 μM DHA (Fig. 4). The level of DHA that accumulated in cell phospholipids when supplemented directly in the medium was significantly (P < 0.05) greater than when cells were supplemented with ALA, reflecting a limitation in the ability of ALA to be incorporated into cell phospholipids. Values followed by different letters are significantly different from each other (P < 0.05) by Bonferroni post hoc test.

Fig. 3. Accumulation of 24:5n-3 (closed circles) and 24:6n-3 (open circles) in HepG2 cell phospholipids after supplementation with increasing concentrations of ALA. 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 ALA bound to BSA. The cells were harvested for lipid analysis after 48 h of supplementation with ALA. Values are means ± SEM of three replicates. Values marked with different letters are significantly different within each fatty acid group (P < 0.05). Regression curves have been fitted to the data.
increasing concentrations of the 18 carbon precursor, ALA. However, the accumulation of DHA in cell phospholipids was curvilinear, regardless of substrate, and appeared to approach saturation.

Accumulation of n-3 fatty acids over a 48 h time course

The change in fatty acid composition of HepG2 cell membranes over the 48 h supplementation period was investigated. The maximum accumulation of ALA in HepG2 cell phospholipids occurred at 6 h, increasing from 0.29 ± 0.18% total fatty acids before supplementation with ALA to 8.97 ± 0.59% total fatty acids at 6 h (Fig. 5). After 48 h, the level of ALA had equilibrated to 6.26 ± 0.52% total fatty acids. The level of EPA increased steadily over the 48 h supplementation period. The level of DHA remained relatively unchanged (Fig. 5). However, Fig. 6 shows that the level of DHA in HepG2 cells incubated in serum-free DMEM decreased gradually over 48 h. The level of DHA in HepG2 cell phospholipids immediately before supplementation with ALA was 4.26 ± 0.58% total fatty acids. This can be attributed to the content of DHA in DMEM supplemented with FBS, which contains 6.70 ± 0.31% DHA total fatty acids. Incubation of HepG2 cells with 72 µM ALA maintained the initial level of DHA so that there was a net increase in the level of DHA over the 48 h supplementation period compared with control cells, indicating synthesis.

DISCUSSION

The pattern of accumulation of n-3 LCPUFAs after supplementation with ALA in HepG2 cells supports the proposed fatty acid synthetic pathway (14–16) and indicates that the HepG2 cell system is a suitable model for examining the synthesis and accumulation of fatty acids. In this study, we measured the change in phospholipid composition after supplementation, a net effect of synthesis and incorporation, to evaluate the efficacy of various n-3 substrates in altering membrane fatty acid composition. Previous studies have demonstrated the synthesis of...
EPA, DPA, and DHA from radiolabeled ALA in HepG2 cells (17, 18). In this study, the level of ALA in HepG2 cell phospholipids peaked at 6 h and equilibrated by 48 h, yet the accumulation of its metabolites, EPA and DPA, increased steadily. Moreover, the rate of appearance of EPA was much greater than that of DPA and represented the major n-3 fatty acid in cell phospholipids. In contrast, the level of DHA appeared static over the 48 h incubation period. Analysis of HepG2 cells maintained in serum-free medium showed a depletion of DHA from cell phospholipids under these conditions. The supplementation of HepG2 cells with 72 μM ALA maintained the initial level of DHA, indicative of synthesis.

Other cell lines, including human mammary carcinoma cells, ZR-75-1 (19), and human colon carcinoma cells, CaCo-2 (20), have been used to demonstrate the capacity of in vitro cell systems to mimic in vivo fatty acid synthesis. The accumulation of n-3 fatty acids in HepG2 cells described here showed marked similarities with that described in vivo. In a very early study by two pioneers in the field, Mohrhauer and Holman (21) examined the total fatty acid composition of the liver of rats maintained on a fat-free diet supplemented with increasing amounts of pure ALA as the only source of dietary fat. The authors reported a dose-dependent increase in the level of ALA in the livers of supplemented rats, with an accompanying increase in the level of EPA and DPA (21). The level of DHA in liver lipids increased dramatically when ALA provided up to 1% of calories. Further increases in the percentage of calories of ALA, in the absence of competing n-6 substrates, did not significantly increase the accumulation of DHA in liver lipids (21). The patterns of n-3 fatty acid accumulation in rats and HepG2 cells are remarkably similar.

Other in vivo studies have demonstrated the limited accumulation of DHA in cell membranes or plasma lipids after ALA supplementation. One study examined the fatty acid composition of plasma lipids in male volunteers consuming a high-ALA diet (mean intake of 13.0 ± 6.3 g/day, equivalent to 6.5 times the recommended intake in the United States (22) and Australia (National Health and Medical Research Council, 1992; National Heart Foundation, 1999)) and found that the dietary intake of ALA correlated positively with the concentration of ALA, as well as EPA, in plasma lipids but not DHA (2). In hamsters fed increasing amounts of ALA while maintaining the intake of LA, the level of ALA in plasma phospholipids increased linearly with a proportional enrichment of EPA and DPA in plasma phospholipids, but not DHA (23). Counter-intuitively, the percentage of DHA in plasma phospholipids decreased in hamsters fed the highest level of dietary ALA (23). It was also shown in piglets that, although the level of EPA in plasma was directly related to the dietary intake of ALA, greater dietary intakes of ALA did not translate to increased levels of DHA (3). The direct relationship between ALA supplementation and the level of EPA in cell membranes and the poor accumulation of DHA after supplementation with ALA was also evident in HepG2 cells. Even in the absence of competing n-6 substrates, a limitation in the accumulation of DHA after supplementation with increasing amounts of ALA was observed in HepG2 cell phospholipids. Human, animal, and in vitro studies indicate a limitation in the synthesis of DHA from ALA, but the mechanism is unclear. The regulation of DHA synthesis by the 18 carbon fatty acid precursor ALA is surprising and indicates that dietary interventions using ALA-rich dietary fats may not be useful in increasing the level of DHA in tissues.

The synthesis and accumulation of DHA from ALA is a multi-enzyme process; hence, the limited accumulation of DHA from its 18 carbon precursor may arise from regulation at several loci. Research has shown that the enzymatic activity and mRNA expression of the key enzyme in fatty acid synthesis, D6D, varies with hormonal and nutritional manipulation (24–27). Studies showed the greatest activity of D6D when ALA was the substrate, followed by LA and oleic acid (28, 29). Somewhat higher concentrations of LA and ALA were required to obtain a saturable rate of desaturation (30) compared with the conversion of 24 carbon fatty acids, indicating the greater capacity for D6D to desaturate 18 carbon fatty acids over 24 carbon fatty acids. The linear accumulation of EPA in cells supplemented with increasing concentrations of ALA indicates that Δ5 desaturase was not saturated under these conditions and suggests that the regulation is at a point beyond the synthesis of EPA. The dose-dependent accumulation of EPA and 24:5n-3 in cells supplemented with ALA also suggests that the enzymes involved in the elongation of fatty acids were not saturated. The curvilinear accumulation of post-Δ6 desaturation products, 24:6n-3 and DHA, implicates D6D and events after the Δ6 desaturation of substrates in regulating the accumulation of DHA. The data in Fig. 2 suggest that the limited accumulation of DHA in cell phospholipids after supplementation with ALA could be attributable to competition between ALA and 24:5n-3 for active sites on D6D. D6D is used twice in the synthesis of DHA from ALA; therefore, with increasing concentrations of ALA, the Δ6 desaturation of 24:5n-3 to 24:6n-3 will be competitively inhibited. Because D6D has a higher affinity for ALA than 24:5n-3 (14), it is reasonable to suggest that the Δ6 desaturation of 24:5n-3 is inhibited with increasing concentrations of ALA, preventing the synthesis of DHA.

Other processes may also contribute to the level of DHA in cell membranes. The increased incorporation of DHA when supplied in the medium suggests that HepG2 cells have the capacity to incorporate up to 16% of total fatty acids as DHA. Clearly, the level of DHA in cell phospholipids when supplied directly was increased significantly, compared with cells supplemented with ALA, as the fatty acid synthetic pathway was bypassed. The accumulation of DHA in cells supplemented with DHA is an effect of incorporation compared with the net effect of DHA synthesis and incorporation in cells supplemented with ALA. The accumulation of DHA, however, when supplied directly was curvilinear and indicates that the accumulation of DHA, even when it was supplied preformed, approached saturation. The trafficking of fatty acid between organelles and the acylation of DHA into cell membranes
may also be important regulatory steps in the accumulation of DHA.

In conclusion, the limited accumulation of DHA from ALA reported here is consistent with the concept that the synthesis of DHA from ALA is tightly regulated. The linear accumulation of EPA in HepG2 cells after supplementation with increasing concentrations of ALA indicates that the conversion of ALA is not limited at the first Δ6 desaturation step. The accumulation of the immediate Δ6Δ6 substrate, 24:5n-3, was also linear and suggests that the elongation enzymes involved in the conversion of EPA to DPA and of DPA to 24:5n-3 were not saturated. In contrast, the accumulation of the post-Δ6Δ6 product, 24:6n-3, in cell phospholipids was curvilinear and paralleled the accumulation of DHA after supplementation with increasing concentrations of ALA, which tends to suggest the saturation of Δ6Δ6 by ALA. The use of Δ6Δ6 by both 24:5n-3 and ALA suggests that competition between 24:5n-3 and ALA for active sites on Δ6Δ6 directly influences the capacity for cell membranes to accumulate DHA in significant amounts. The pattern of accumulation of n-3 LCPUFAs in HepG2 cells supplemented with ALA implicates the competition between ALA and 24:5n-3 as a major point of regulation in the synthesis of DHA and may explain why ALA intervention trials in animals and humans have been ineffective at increasing tissue levels of DHA.

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