Available at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Arachidonic acid and docosahexaenoic acid supplemented to an essential fatty acid–deficient diet alters the response to endotoxin in rats

Pei-Ra Ling[a], *Alpin Malkan*[b], *Hau D. Le*[b], *Mark Puder*[b], *Bruce R. Bistrian*[a,*]

[a] *From the Laboratory of Nutrition/Infection, Beth Israel Deaconess Medical Center, Boston, MA 02215, USA*
[b] *The Department of Surgery and The Vascular Biology Program, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA*

ARTICLE INFO

Article history:
Received 6 April 2011
Accepted 31 July 2011

ABSTRACT

This study examined fatty acid profiles, triene-tetraene ratios (20:3n9/20:4n6), and nutritional and inflammatory markers in rats fed an essential fatty acid–deficient (EFAD) diet provided as 2% hydrogenated coconut oil (HCO) alone for 2 weeks or with 1.3 mg of arachidonic acid (AA) and 3.3 mg of docosahexaenoic acid (DHA) (AA + DHA) added to achieve 2% fat. Healthy controls were fed an AIN 93M diet (AIN) with 2% soybean oil. The HCO and AA + DHA diets led to significant reductions of linoleic acid, α-linolenic acid, and AA (20:4n6) and increases in Mead acid (20:3n9) in plasma and liver compared with the AIN diet; but the triene-tetraene levels remained well within normal. However, levels of 20:3n9 and 20:4n6 were lower in liver phospholipids in the AA + DHA than in HCO group, suggesting reduced elongation and desaturation in ω-9 and -6 pathways. The AA + DHA group also had significantly lower levels of 18:1n9 and 16:1n7 as well as 18:1n9/18:0 and 16:1n7/16:0 than the HCO group, suggesting inhibition of stearyl-Co A desaturase–1 activity. In response to lipopolysaccharide, the levels of tumor necrosis factor and interleukin-6 were significantly lower with HCO, reflecting reduced inflammation. The AA + DHA group had higher levels of IL-6 and C-reactive protein than the HCO group but significantly lower than the AIN group. However, in response to endotoxin, interleukin-6 was higher with AA + DHA than with AIN. Feeding an EFAD diet reduces baseline inflammation and inflammatory response to endotoxin long before the development of EFAD, and added AA + DHA modifies this response.

1. Introduction

Linoleic acid (LA, 18:2n6) and α-linolenic acid (ALA, 18:3n3) are the 2 essential fatty acids that cannot be synthesized endogenously and thus must be obtained from the diet. Essential fatty acid deficiency (EFAD) typically occurs in the human adult when LA provides less than 1% to 2% of total energy intake [1,2]. Because human diets contain abundant amounts of LA and ALA, the development of EFAD is uncommon. However, EFAD has been observed in premature infants, in patients with chronic malnutrition or malabsorption, or in those receiving prolonged intravenous feeding

Author contributions: PRL and BRB designed and conducted the study, measured all the markers, analyzed data, and had primary responsibility for writing the paper. AM and HDL helped with glucose measurements and tail vein injections. MP provided arachidonic acid and docosahexaenoic acid and, with AM and HDL, participated in study design. All authors reviewed and approved the manuscript for final content.

* *Corresponding author*. Tel.: +1 617 632 8545; fax: +1 617 632 0204.
E-mail address: bbistria@bidmc.harvard.edu (B.R. Bistrian).

0026-0495/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2011.07.017

without essential fatty acids [3-6]. The dietary absence of LA leads to reduced levels of 18:2n6 and its metabolic product, arachidonic acid (20:4n6, AA), in membrane lipids and raises the ratio of 20:3n9/20:4n6 in plasma and tissue lipids [7]. A triene-tetraene ratio (20:3n9/20:4n6) in plasma phospholipids greater than 0.2 is considered pathognomonic for the diagnosis of EFAD, although clinical evidence for EFAD including growth retardation, dry skin, hair loss, increased susceptibility to infection, and severe impairment in the systemic inflammatory response is not generally seen until the ratio is greater than 0.4 [8,9].

Plant oils generally are excellent sources of LA; and many are good sources of ALA but do not contain their distal elongation and desaturation products, such as AA, eicosapentaenoic acid (EPA, 20:5n3), and docosahexaenoic acid (DHA, 22:6n3). However, a modest intake of plant oil provides adequate amounts of AA, EPA, and DHA through metabolic conversion and prevents the development of EFAD. In man, ALA is converted to EPA and particularly DHA at a low rate [10,11]. Therefore, directly providing EPA and DHA is more potent and efficient than ALA for increasing EPA and DHA levels in plasma and tissues [12]. On the other hand, the presence of high amounts of EPA and DHA, which are found in substantial amounts in oily, cold-water fish, significantly reduces AA levels in membrane lipids through preferential incorporation of EPA over AA. However, this situation does not impair essential fatty acid status because Mead acid (20:3n9) levels are similarly lowered, maintaining or even further reducing triene-tetraene ratios. Thus, the relationships among these fatty acids are complex; and the extent to which markers of EFAD are attributable specifically to the deficiency of dietary LA is apparently unclear.

Recent study using 1%, 5%, and 10% of fish oil as the sole fat source in mice for 9 weeks showed that all animals except those in the 1% group gained weight over the study period [13]. In addition, the ratios of 20:3n9/20:4n6 were maintained in the 10% fish oil group similar to the 10% soybean oil group. Although this ratio was still within the reference range in the 5% fish oil group, it significantly increased at 9 weeks as compared with the 10% soybean oil group. These findings suggest that despite inadequate dietary LA intakes (at 0.2% and 0.4% energy), the amounts of AA and EPA + DHA present in fish oil were able to alleviate (5%) and prevent (10%) the development of EFAD [13]. Although the lowering of AA by either EFAD [14] or fish oil [15] can improve the clinical response to endotoxin, EFAD markedly increases the susceptibility to infection and increases mortality [16], whereas fish oil can both reduce infection and improve clinical outcome [17]. This led us to design this study to evaluate the effect of AA and DHA when added to a short-term EFAD diet on the changes in fatty acid profiles, 20:3n9/20:4n6 ratios, and inflammation. The hydrogenated coconut oil (HCO) diet was chosen as the EFAD diet. The amount of added AA + DHA was similar to that found in a 2% fish oil diet, which would not be sufficient to prevent the ultimate development of EFAD based on this prior study [13]. The comparison was made with the soybean oil diet containing abundant amounts of LA and ALA with essentially no AA, EPA, and DHA. The intent was to determine if an EFAD diet would produce changes in fatty acid metabolism and the systemic inflammatory response long before the development of EFAD, and the impact on these parameters following added AA + DHA.

2. Material and methods

2.1. *Animals, diets, and experimental designs*

Weaning male Sprague-Dawley rats were obtained from Taconic Farm (Germantown, NY) and placed in individual cages on a 12:12-hour light-dark photoperiod at 24°C to 26°C for 4 days before the experiments. Tap water and laboratory rat chow (Purina 5008; PharmServe, Framingham, MA) were provided ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

At the end of 4 days of accommodation in the animal facility, a total of 39 animals, weight 110 to 130 g, were randomly assigned to 3 groups: ad libitum feeding for 2 weeks with a modified AIN 93M diet with 2% of soybean oil by weight (AIN group), this same diet with 2% HCO by weight in place of soybean oil (HCO group), or this diet with 1.13% of HCO, 0.039% of AA, and 0.78% of DHA by weight in place of soybean oil (AA + DHA group). The LA content of the HCO and HCO plus AA + DHA diets were thus in trace quantities (0.05% and 0.03% by weight, respectively). The feeding only lasted for 2 weeks, although it is well documented that long-term feeding (8-14 weeks) of EFAD diets is required in rats to achieve EFAD with clinical manifestations [18]. The amounts of AA and DHA were similar to those found in a diet with 2% fish oil by weight. All the diets were of identical caloric density and consisted of 14% casein, 3.5% mineral mixture, 1.0% vitamin mixture, and 0.25% choline bitartrate (Dyets, Bethlehem, PA) (Table 1).

Table 1 – Dietary compositions (grams per kilogram)

	AIN	AA + DHA	HCO
Casein	140	140	140
L-Cystine	1.8	1.8	1.8
Sucrose	120	120	120
Cornstarch	465.7	465.7	465.7
Dextrose	155	155	155
Cellulose	50	50	50
Soybean Oil	20	0	0
HCO	0	11.81	20
AA	0	0.39	0
DHA	0	7.8	0
t-Butylhydroquinone	0.004	0.004	0.004
Mineral mix #210025	35	35	35
Vitamin mix #310025	10	10	10
Choline bitartrate	2.5	2.5	2.5

AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO.

During feeding, body weights and food intakes were recorded every other day. After 2 weeks of feeding, animals were fasted overnight. On the next day, 6 rats from each dietary group received saline tail vein injection; and 7 rats from each dietary group received lipopolysaccharide (LPS) tail vein injection at 1 mg/kg (0111:B4; Sigma, St Louis, MO). At 4 hours after injection with saline or LPS, animals were killed. Blood and pieces of liver were collected for fatty acid analysis. In addition, nutritional markers, including plasma glucose, triglycerides, nonesterified fatty acid (NEFA), and insulin, were measured. In addition, plasma C-reactive protein (CRP), tumor necrosis factor (TNF), and interleukin-6 (IL-6) were also determined as markers of the systemic inflammatory response.

2.2. Analysis of lipids and other markers

The lipids from plasma and the liver were extracted with 6 vol of chloroform-methanol (2:1) by the method of Folch et al [19]. Before the extraction, 30 μL of a 1-mg/mL solution of diheptadecanoyl phosphatidylcholine and 30 μL of a 1-mg/mL solution of triheptadecanoyl glycerol (Nu-Check Prep, Elysian, NY) in chloroform-methanol (1:1, vol/vol) were added as an internal standard to all samples. Triglyceride and phospholipid fractions were isolated by aminopropyl column (Sigma) using chloroform/isopropanol (2:1, vol/vol) and methanol, respectively. The methyl esters were prepared using sodium methoxide and methanol base-boron trifluoride and washed with a saturated NaCl solution. Fatty acid composition was determined by gas chromatography with a Hewlett Packard 5890 II (Hewlett Packard, Palo Alto, CA) using a SUPELCOWAX 10 0.25–mm ID column at a temperature of 150°C to 260°C. Fatty acid methyl ester peaks were identified by comparison of retention times of a standard mixture and quantified using the internal standard. Fatty acid profiles of triglycerides and phospholipids in plasma and liver were determined. Plasma and liver triglycerides were thought to best reflect potential changes in stearyl-Co A desaturase–1 activity as an indicator of de novo lipogenesis. Plasma and liver phospholipids were more likely to reflect changes in essential fatty acid metabolism.

Blood glucose from tail vein was determined by a blood glucose meter (Bayer, Elkhart, IN). Plasma levels of triglyceride and free fatty acid were, respectively, determined by a triglyceride determination kit (Sigma) and a kit for the quantitative determination of NEFA from Wako Chemicals (Richmond, VA). Plasma insulin was determined by a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA). Plasma CRP was measured by rat CRP ELISA kit (Helica Biosystem, Fullerton, CA). Plasma TNF and IL-6 were measured by commercial ELISA kits from Biosource International (Camarillo, CA) and Thermo Scientific (Rockford, IL), respectively.

2.3. Statistics

Results are presented as mean ± SD for fatty acid profiles and as mean ± SEM for nutritional and inflammatory markers. To assess the statistical significance of differences in mean values among the different diets (AIN, HCO, and AA + DHA) and the different treatments (saline and LPS), 2-way analysis of variance with Fisher least significant test was used (SigmaStat 3.0, 2003; SPSS, Chicago, IL). Significance for all analysis was defined as $P \leq .05$.

3. Results

3.1. Food intakes and weight gains

Average daily food intake was about 21 to 22 g/d in all animals. Over 2 weeks of feeding, there were no significant differences in food intakes among the AIN, HCO, and AA + DHA groups. In addition, all of the animals gained weight; and no differences in weight gain were found among the 3 groups.

Based on the average daily food intake, it was calculated that about 2.57% and 0.35% of total caloric intakes per day were, respectively, provided by dietary LA and ALA in the AIN group. In this group, AA intake was estimated at 0.3 mg/d; and DHA was estimated at 0.04 mg/d. Animals fed on the HCO diet received 0.13% of total calories from dietary LA and no calories from ALA, AA, and DHA per day. Animals fed on the AA + DHA diet received 0.08% of total caloric intake from LA and zero intakes from ALA per day. However, in this group, the animals received 1.3 mg of AA (0.3% of total caloric intakes) and 3.3 mg of DHA daily.

3.2. Triene-tetraene ratio in plasma phospholipids

Table 2 lists this ratio in different dietary groups with different treatments. Although all ratios were less than 0.2, the ratio was 0.009 in the AIN group, significantly increased to 0.039 in the HCO group, and 0.034 in the AA + DHA group, respectively. There was no significant difference in 20:3n9/20:4n6 between the HCO and AA + DHA groups. Lipopolysaccharide treatment did not change this ratio in any dietary group compared with saline treatment.

3.3. The changes of palmitic acid (16:0), palmitoleic acid (16:1n7), stearic acid (18:0), and oleic acid (18:1n9) in triglycerides

In plasma triglycerides (Fig. 1A), the AA + DHA group had the highest levels of 16:0 as compared with the AIN and the

Table 2 – Triene-tetraene ratios in plasma phospholipids

	Saline (n = 6)	LPS (n = 7)
AIN	0.009 ± 0.003 *	0.008 ± 0.003 *
AA + DHA	0.034 ± 0.019	0.042 ± 0.014
HCO	0.039 ± 0.003	0.037 ± 0.011

Mean ± SD. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline: a group received saline for 4 hours after tail vein injection; LPS: a group received LPS (1 mg/kg) for 4 hours after tail vein injection.

* $P < .005$, AIN vs all of others.

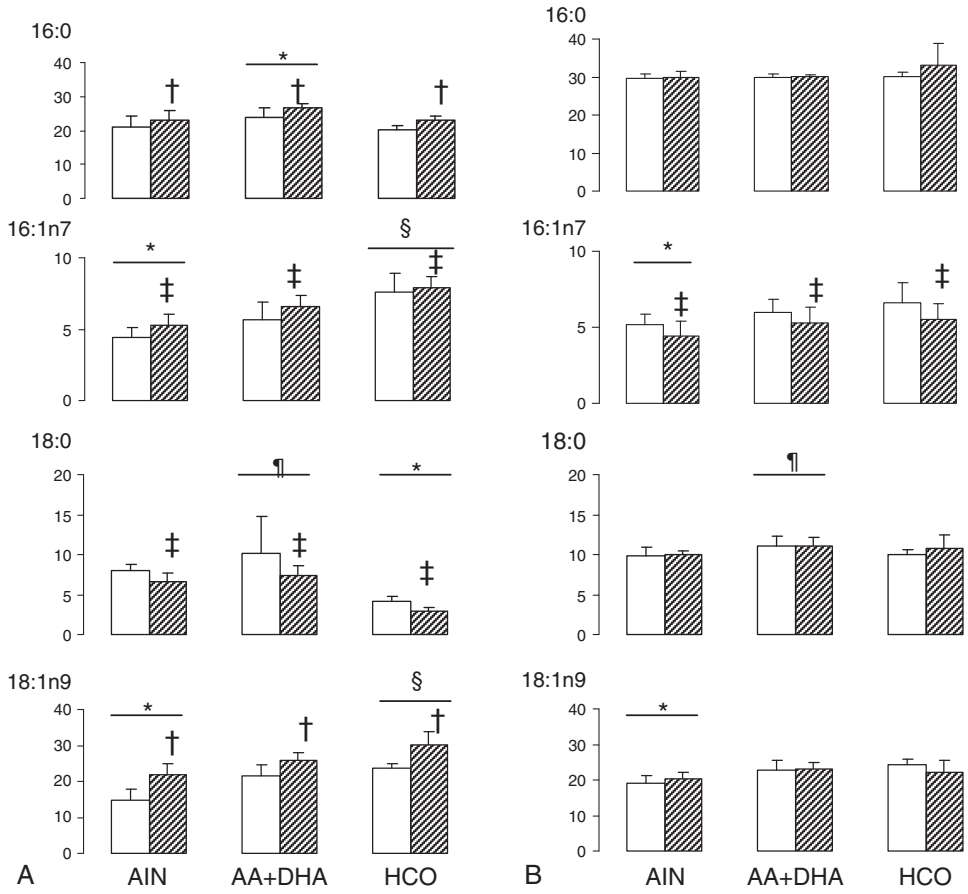

Fig. 1 – The levels of 16:0, 16:1n7, 18:0, and 18:1n9 in plasma (A) and liver (B) triglycerides. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, vs all of other dietary groups; §P < .001, HCO vs AA + DHA; ¶P < .05, AA + DHA vs AIN; †P < .001, LPS vs saline; ‡P < .05, LPS vs saline.

HCO groups ($P < .001$). There were no differences in this fatty acid between the AIN and the HCO groups. However, the lowest level of 18:0 was found in the HCO group ($P < .001$) among the 3 groups; and the level of 18:0 was higher in the AA + DHA group than in the AIN group ($P < .05$). Feeding with both the HCO and the AA + DHA diets significantly increased the levels of 16:1n7 and 18:1n9 in plasma triglycerides as compared with the AIN diet, with significantly greater increases in the HCO group. Lipopolysaccharide treatment significantly increased the levels of 16:0, 16:1n7, and 18:1n9 as compared with saline treatment in all dietary groups. In contrast, LPS significantly decreased the levels of 18:0 in all dietary groups.

In the liver triglycerides (Fig. 1B), no differences were found in 16:0 among groups. The levels of 18:0 were significantly lower in the AIN group than those in the AA + DHA group, but not different from those in the HCO group. Similar to the changes seen in plasma triglycerides, feeding with the HCO and the AA + DHA diets significantly increased the levels of 16:1n7 and 18:1n9 in the liver triglycerides. Unlike the changes in plasma triglycerides, the levels of 16:1n7 and 18:1n9 were not different between the HCO and the AA + DHA groups. Lipopolysaccharide did not change the levels of 16:0, 18:0, and 18:1n9, but did lead to the significant reduction of 16:1n7 in all dietary groups.

Fig. 2 shows the ratio of 16:1n7/16:0 and 18:1n9/18:0 in plasma and liver triglyceride as an index of stearyl-CoA desaturase–1 activity. For 16:1n7/16:0 (Fig. 2A), the highest value in plasma was found in the HCO group ($P < .001$); and no differences were found between the AIN and the AA + DHA groups. In liver triglycerides, no differences in this ratio were found among the 3 groups. Lipopolysaccharide did not change this ratio in plasma triglycerides but significantly decreased this ratio in liver triglycerides in all dietary groups. For 18:1n9/18:0 (Fig. 2B), the highest values in plasma were also found in the HCO group ($P < .001$); and no differences were found between the AIN and the AA + DHA groups. In liver triglycerides, there were no differences in this ratio among the 3 groups. Lipopolysaccharide significantly increased the ratio of 18:1n9/18:0 in plasma in all dietary groups, but significantly more so in the HCO

group, with no effects on this ratio in liver triglycerides found in any dietary group.

3.4. *The changes of palmitic acid (16:0), palmitoleic acid (16:1n7), stearic acid (18:0), and oleic acid (18:1n9) in phospholipids*

In plasma phospholipids (Fig. 3A), the levels of 16:0 were significantly lower in the HCO group as compared with the other dietary groups. No differences were found between the AIN and the AA + DHA groups. There were no differences in 18:0 among the 3 dietary groups. Feeding with the HCO and the AA + DHA diets significantly increased 16:1n7 and 18:1n9 in plasma phospholipids compared with the AIN diet. No differences were found in these 2 fatty acids between the HCO and the AA + DHA diets. Lipopolysaccharide significantly increased the levels of 16:1n7 and 18:1n9 in all dietary groups but significantly decreased the levels of 18:0 as compared with saline in all dietary groups, and significantly more so in the HCO group. Lipopolysaccharide did not change the levels of 16:0 in plasma phospholipids.

In the liver phospholipids (Fig. 3B), similar to the changes seen in plasma phospholipids, the levels of 16:0 were significantly lower in the HCO group as compared with other dietary groups; and no differences were found between the AIN and the AA + DHA groups. Both the HCO and AA + DHA diets significantly increased 18:0 and 18:1n9 compared with the AIN diet. However, only the AA + DHA diet significantly increased the levels of 16:n7. No differences in 16:n7 were found between the AIN and the HCO diets. Lipopolysaccharide significantly increased the levels of 16:n7 and 18:1n9 but significantly decreased the levels of 18:0 in the liver phospholipids. No effects were observed in 16:0.

3.5. *The changes of LA (18:2n6), ALA (18:3n3), AA (20:4n6), EPA (20:5n3), and DHA (22:6n3) in triglycerides*

In plasma triglycerides (Fig. 4A), the HCO and AA + DHA diets, as expected, led to significantly reduced levels of LA, ALA, and AA in plasma triglycerides as compared with the AIN diet. However, the levels of LA and AA ($P = .06$) were not different between the HCO and AA + DHA diets; but the levels of ALA were significantly higher in the HCO diet as compared with the AA + DHA diet. The levels of EPA and DHA were not different among the 3 dietary groups. Lipopolysaccharide significantly increased the levels of ALA but significantly decreased the levels of AA in all dietary groups. Lipopolysaccharide had no impact on the levels of LA, EPA, and DHA in any group.

In the liver triglycerides (Fig. 4B), similar to that seen in plasma, the HCO and AA + DHA diets led to significantly reduced levels of LA and ALA in triglycerides. The levels of EPA were significantly lower in the HCO diet compared with the AA + DHA and AIN diets. The highest levels of DHA were found in the AA + DHA group compared with the other dietary groups ($P < .01$) but were not different between the HCO and AIN diets. Lipopolysaccharide did not have an impact on LA, ALA, AA, EPA, and DHA in liver triglycerides in the different dietary groups.

3.6. *The changes of LA (18:2n6), ALA (18:3n3), AA (20:4n6), EPA (20:5n3), and DHA (22:6n3) in phospholipids*

In plasma (Fig. 5A), the HCO and AA + DHA diets led to significantly reduced levels of LA and ALA in phospholipids. However, unlike the changes seen in plasma triglycerides, the levels of AA and EPA were not different among the 3 dietary groups. The highest levels of DHA were found in the

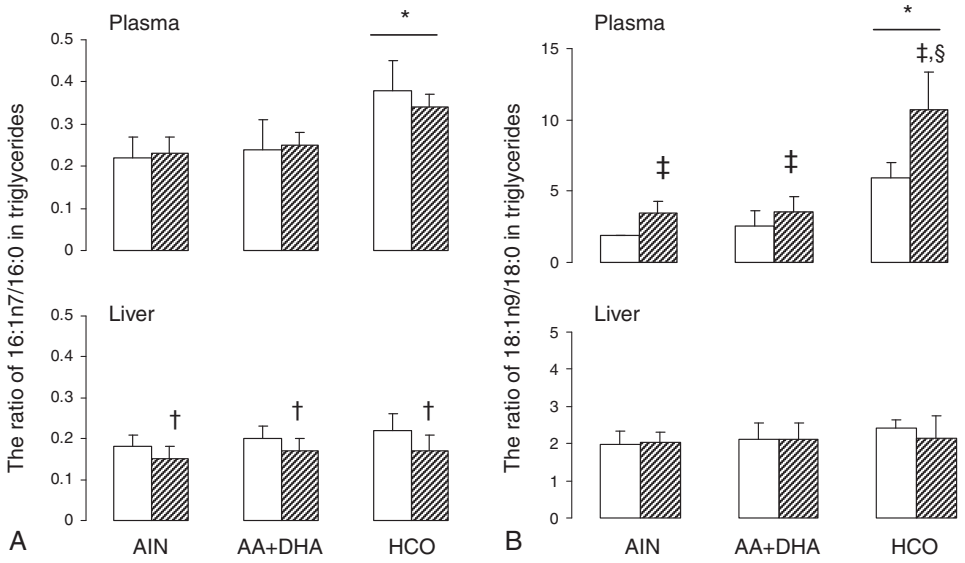

Fig. 2 – The ratios of 16:1n7/16:0 (A) and 18:1n9/18:0 (B) in triglycerides in plasma and liver. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *$P < .001$, AIN vs all of other dietary groups; †$P < .001$, LPS vs saline; ‡$P < .05$, LPS vs saline; §$P < .01$ vs all.

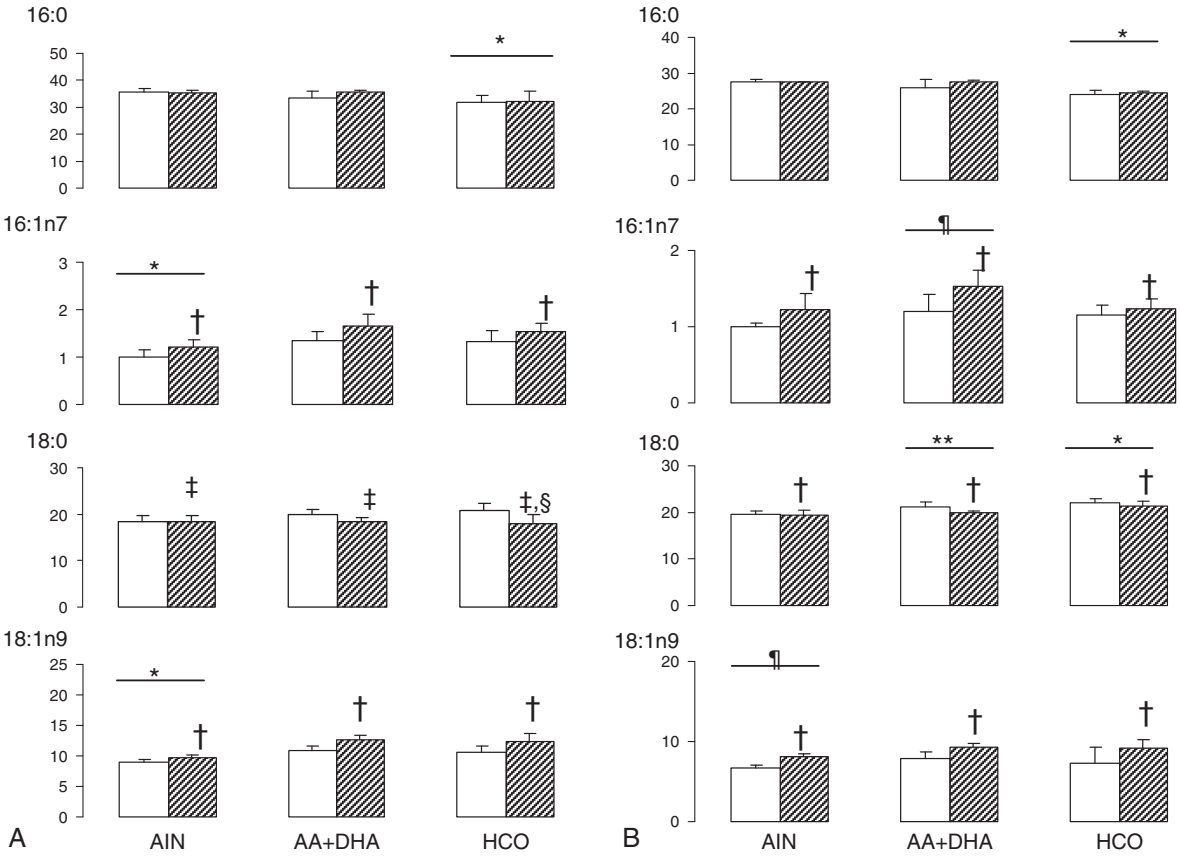

Fig. 3 – The levels of 16:0, 16:1n7, 18:0, and 18:1n9 in plasma (A) and liver (B) phospholipids. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, vs all of other dietary groups; ¶P < .05, vs all of other dietary groups; **P < .05, AA + DHA vs AIN. †P < .001, LPS vs saline; ‡P < .05, LPS vs saline; §P < .05, AIN, LPS vs AA + DHA, LPS.

AA + DHA group compared with other groups ($P < .001$). However, there was significantly more DHA in the HCO group as compared with the AIN group. Lipopolysaccharide significantly reduced AA and had no impact on other fatty acids in the 3 dietary groups.

In liver phospholipids (Fig. 5B), the HCO and AA + DHA diets led to significantly reduced levels of LA and AA compared with the AIN diet. The levels of AA were significantly lower in the AA + DHA group compared with the HCO group. The levels of EPA and DHA were significantly higher in both the HCO and AA + DHA groups as compared with the AIN group. The levels of EPA were significantly higher in the AA + DHA group as compared with the AIN and HCO groups.

3.7. *The changes of Mead acid (20:3n9) in triglycerides and phospholipids*

The levels of 20:3n9 in plasma triglycerides (Fig. 6A) and phospholipids (Fig. 6B) were significantly lower in the AIN group as compared with the HCO and the AA + DHA groups. However, only in the liver phospholipids were significantly lower levels of 20:3n9 found in the AA + DHA group compared with the HCO group ($P < .005$). No differences in this fatty acid were found in liver triglycerides.

3.8. *The changes in selective markers of nutrition and inflammation*

Plasma insulin, glucose, triglycerides, and NEFA were not significantly different among the 3 dietary groups (Fig. 7). Lipopolysaccharide significantly increased the levels of insulin (Fig. 7A) and triglycerides (Fig. 7C), but significantly decreased the levels of glucose in plasma (Fig. 7B). Lipopolysaccharide did not change NEFA levels in plasma in any group.

Fig. 8 presents the changes in plasma CRP (A), TNF (B), and IL-6 (C) in all groups. The highest CRP was found in the AIN group ($P < .001$), and the lowest CRP was found in the HCO group ($P < .005$). Lipopolysaccharide treatment did not change CRP in any dietary group as compared with saline treatment. Without LPS, plasma TNF was detected only in the HCO group. In response to LPS stimulation, plasma TNF was significantly increased in all groups. No differences in plasma TNF were found between the AIN and the AA + DHA groups, but the levels of plasma TNF were significantly lower in the HCO group compared with the AIN group. Trace amounts of IL-6

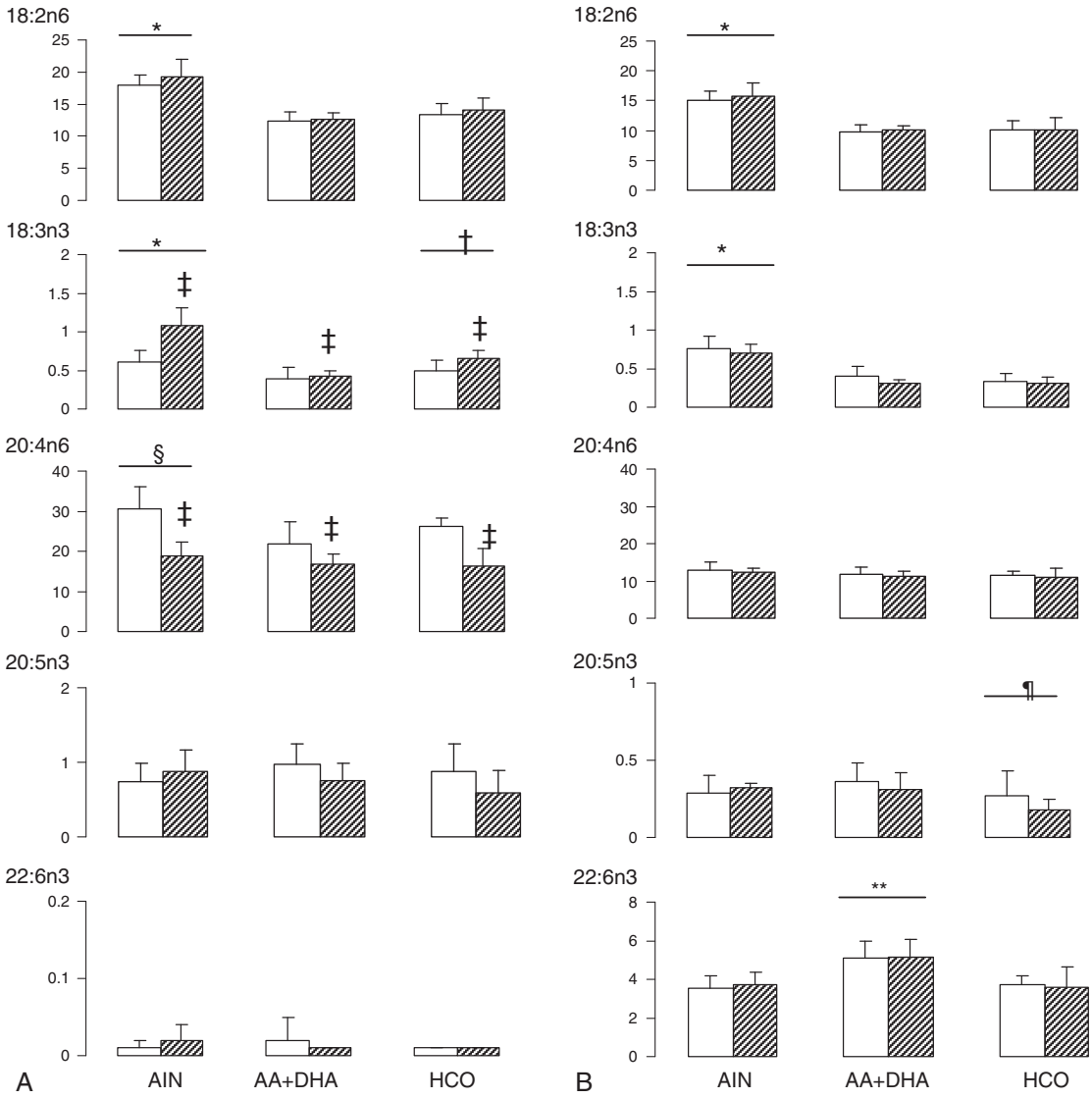

Fig. 4 – The levels of 18:2n6, 18:3n3, 20:4n6, 20:5n3, and 22:6n3 in plasma (A) and liver (B) triglycerides. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, vs all of other dietary groups; †P < .01, HCO vs AA + DHA; §P < .05, vs all of other dietary groups; ¶P < .01, HCO vs AA + DHA and P < .05, HCO vs AIN; **P < .01, AA + DHA vs HCO and AIN. ‡P < .01, LPS vs saline.

were detected in plasma in all groups with saline treatment. Similar to TNF, LPS treatment significantly increased the levels of IL-6 in plasma in all dietary groups. However, the IL-6 levels were significantly lower in the HCO group as compared with the AIN and the AA + DHA groups. The levels of IL-6 were also significantly higher in the AA + DHA group than in the AIN group.

4. Discussion

As expected, feeding with an EFAD diet, either the HCO or the AA + DHA diet, resulted in significant reductions of 18:2n6 and 18:3n3 in both triglycerides and phospholipids in plasma and liver tissue after only 2 weeks of feeding an essential fatty acid depletion diet. In addition, significant amounts of 20:3n9, a metabolite of oleic acid (18:0) indicating the marked reduction in dietary LA, were increasingly produced in plasma and in the liver. In the HCO group, the ratio of 20:3n9/20:4n6 in plasma phospholipids was significantly increased to 0.042, a 4.3-fold increase compared with the ratio found in the AIN group. Although this ratio was still substantially less than 0.2, the state of EFAD was considered to be developing in this group. The rats fed the AA + DHA diet consumed similar amounts of LA (0.08% of total energy intake) compared with those fed the HCO diet (0.12% of total energy intake), which are substantially less than the requirement levels of at least 1%, and with no ALA in either. As a result, similar reductions in 18:2n6 and

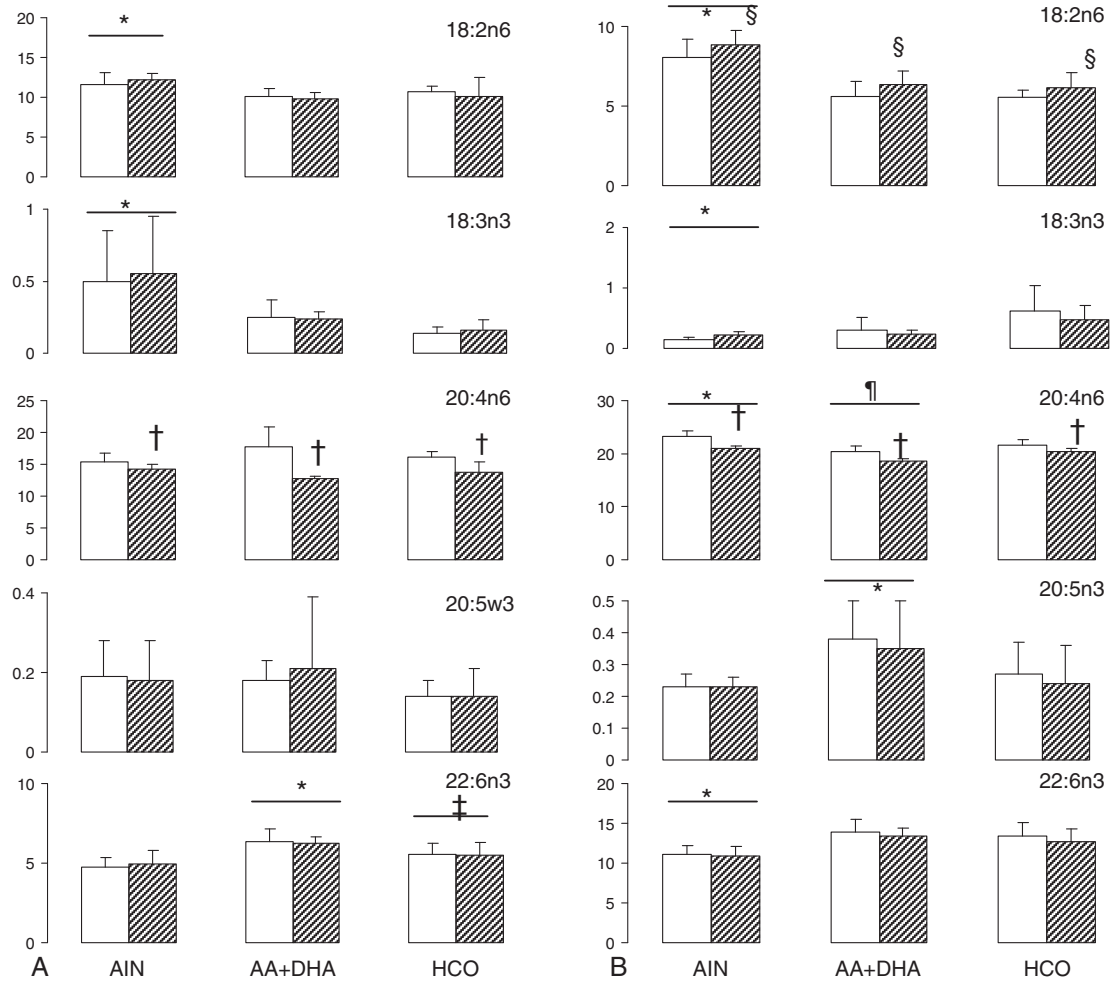

Fig. 5 – The levels of 18:2n6, 18:3n3, 20:4n6, 20:5n3, and 22:6n3 in plasma (A) and liver (B) phospholipids. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *$P < .005$, vs all of others dietary groups; ‡$P < .05$, HCO vs AIN; ¶$P < .001$, AA + DHA vs HCO; †$P < .005$, LPS vs saline; §$P < .05$, LPS vs saline.

18:3n3 in plasma and liver triglycerides and phospholipids were observed in the AA + DHA and the HCO groups compared with the AIN group. As seen in the HCO group, lower levels of AA were also observed in plasma triglyceride and in liver phospholipids in the AA + DHA group compared with the AIN group. The amount of 20:3n9 was also significantly increased in the AA + DHA group compared with that in the AIN group. In the AA + DHA group, the ratio of 20:3n9/20:4n6 was 0.034, a 3.8-fold increase compared with that in the AIN group. Based on the changes in the ratio of 20:3n9/20:4n6 and the reductions in 18:2n6 and 18:3n3, it appeared that the degree of developing EFAD was at a comparable level between the HCO and the AA + DHA groups.

It should be noted, however, that some differences in fatty acid profiles were observed between the HCO and the AA + DHA groups. For instance, a greater reduction of AA was observed in liver phospholipids in the AA + DHA group than in the HCO group, although the animals in the AA + DHA group consumed 1.3 mg/d of AA, whereas animals in the HCO group consumed no AA. The lower AA in liver phospholipids in the AA + DHA group was presumably a consequence of the added DHA in the diet, which by retroconversion increased EPA in liver phospholipids (Fig. 5B), which would displace AA. The levels of 20:3n9 were also significantly lower in liver phospholipids in the AA + DHA group compared with those in the HCO group. Because the intakes of LA and ALA and the amounts of oleic acid in the diet were not substantially different between the HCO and the AA + DHA groups in this study, the only difference was the addition of 0.039% of AA and 0.78% of DHA (0.098% and 1.95% of total caloric intake) by weight in the AA + DHA diet. The reduced amounts of 20:3n9 in liver phospholipids in the AA + DHA group compared with the HCO group indicate that the added AA + DHA either by itself and/or by the production of EPA decreases elongation and desaturation in the ω-9 pathway. Although the ratio of 20:3n9/20:4n6 greater than 0.2 is considered as the principal biochemical marker of EFAD, the current findings suggest that the course

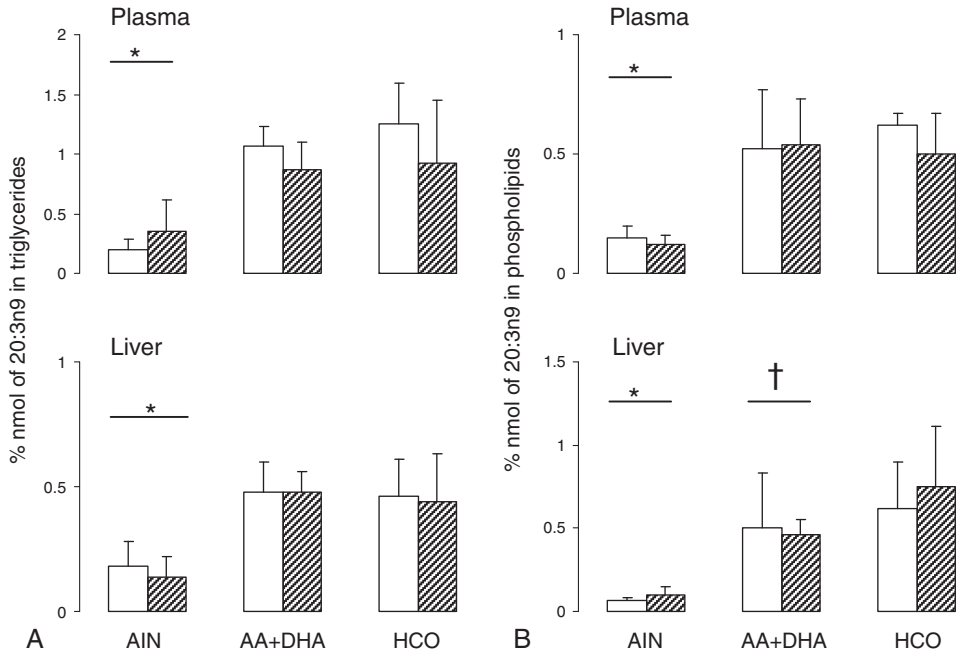

Fig. 6 – The levels of 20:3n9 triglyceride and phospholipid in plasma and liver. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, AIN vs all of other dietary groups; †P < .05, AA + DHA vs HCO.

of biochemical development of EFAD can be slowed down by adding AA and DHA to the EFAD diet.

The potential inhibitory effects of the added AA + DHA to the EFAD diet on stearyl-CoA desaturase–1 were also observed in the differences of 16:0, 16:1n7, 18:0, and 18:1n9 between the HCO and the AA + DHA groups. Consistent with previous studies [20-22], the EFAD diet presumably stimulated the process of de novo lipogenesis in both the HCO and AA + DHA groups, reflected by the increases in 16:0, 16:1n7, 18:0, and 18:1n9 in plasma and liver in these 2 groups compared with the AIN group, although one cannot be certain based on the much higher levels of the saturated fatty acids and the monounsaturated fatty acids in these diets compared with soybean oil. 16:0 is the normal end product of the fatty acid synthetase system, and 18:0 is the elongation product of 16:0. Interestingly, in the AA + DHA group, palmitic acid (16:0) and

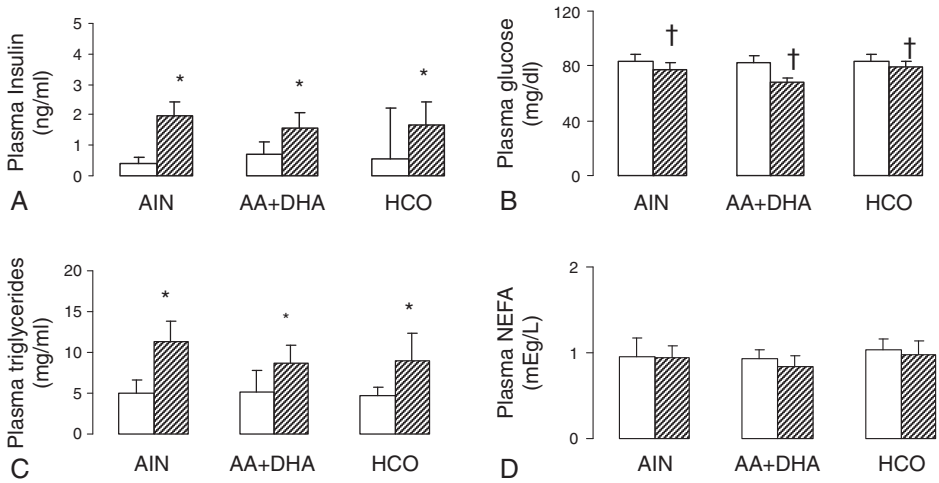

Fig. 7 – Plasma levels of insulin (A), glucose (B), triglycerides (C), and NEFA (D) in different groups. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, LPS vs saline; †P < .05, LPS vs saline.

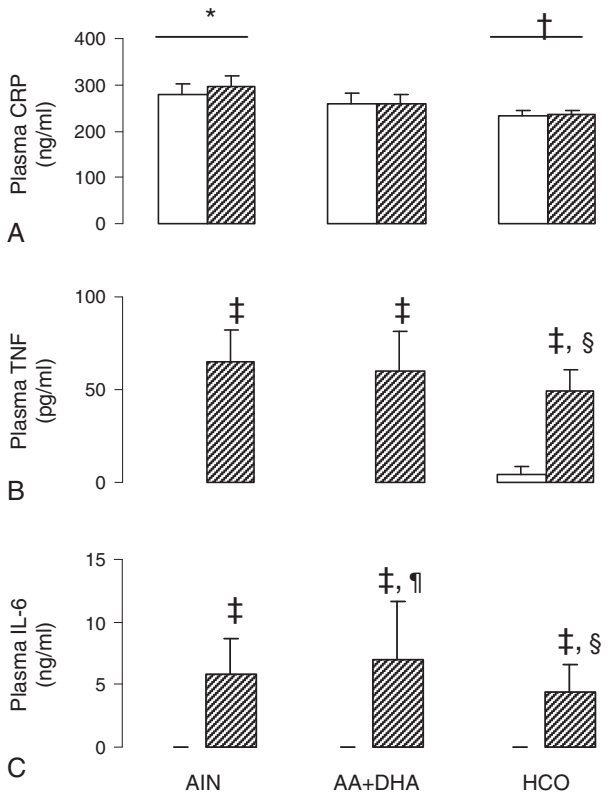

Fig. 8 – Plasma levels of CRP (A), TNF (B), and IL-6 (C) in the liver (D) in different groups. AIN: a modified AIN 93M purified rodent diet with 2% of soybean oil by weight; AA + DHA: a modified AIN 93M purified rodent diet with 1.13% HCO, 0.039% AA, and 0.78% of DHA; HCO: a modified AIN 93M purified rodent diet with 2% HCO. Saline (empty bar, n = 6): a group received saline for 4 hours after tail vein injection; LPS (shady bar, n = 7): a group received LPS (1 mg/kg) for 4 hours after tail vein injection. *P < .001, AIN vs all other dietary groups; †P < .005, HCO vs AA + DHA; ‡P < .001, LPS vs saline; §P < .05, HCO, LPS vs AIN, LPS; ¶P < .05, AA + DHA, LPS vs AIN, LPS and HCO, LPS.

stearic acid (18:0) were significantly higher in plasma triglycerides and phospholipids and also in liver triglycerides compared with those in the HCO group (Figs. 1 and 3). In contrast, the levels of 16:1n7 and 18:1n9 were significantly lower in plasma triglycerides in the AA + DHA group compared with those in the HCO. Because the amounts of 16:0, 16:1n7, 18:0, and 18:1n9 were similar but slightly lower in the AA + DHA compared with the HCO diet, the significantly lower levels of 16:1n7 and 18:1n9 in plasma triglycerides found in the AA + DHA group suggest a relative block in stearyl-Co A desaturase–1 activity from 16:0 and 18:0 as a consequence of the added AA and DHA. As a result, less 16:1n7 and 18:n9 would be formed endogenously in the AA + DHA group compared with the HCO group. Because de novo lipogenesis is one of the important consequences of defined EFAD, the present results further suggest that adding AA + DHA to an EFAD diet can relieve this aspect of beginning development of EFAD as well.

The hypothesis that the added AA + DHA alters the response to an EFAD diet is also supported by the differences found in plasma concentrations of CRP, TNF, and IL-6 between the HCO and AA + DHA groups both at basal and LPS-stimulated states. Feeding with the HCO and the AA + DHA diets significantly reduced CRP concentrations in plasma compared with AIN diet, reflecting reduction in baseline inflammation. This finding is consistent with the previous reports that dietary intakes of LA are positively associated with inflammatory mediators [23,24]. However, at basal and LPS-stimulated conditions, the levels of plasma CRP were significantly higher in the AA + DHA compared with the HCO group (Fig. 8), indicating greater inflammation in the AA + DHA group compared with the HCO group. The failure of CRP to increase in response to endotoxin in any of the groups presumably reflects the timing of blood samples, taken just 4 hours after LPS injection, which would be insufficient to generate an acute phase protein response. In response to LPS challenge, significantly lower levels of TNF and IL-6 were found in the HCO group but not in the AA + DHA group as compared with the AIN group. In fact, plasma TNF concentrations were not different between the AA + DHA and the AIN groups. Plasma IL-6 levels in the AA + DHA group were the highest among the 3 groups. Therefore, it seems that the AA + DHA added to an EFAD diet does restore and in some respects even enhance the capacity to release cytokines in response to LPS challenge in rats fed on EFAD diet. Thus, whereas the AA + DHA group exhibited an anti-inflammatory profile similar to the HCO group before endotoxin, a proinflammatory response in terms of cytokine relative to the HCO group was seen after stimulation. This may be a beneficial reaction for the host in terms of improving resistance to infectious challenge.

In the present study, LPS (1 mg/kg) significantly increased 16:1n7 and 18:1n9 but also significantly decreased 18:0 in plasma triglycerides and phospholipids in all dietary groups. In the liver, LPS significantly decreased 16:1n7 in triglycerides and 18:0 in phospholipids but significantly increased 16:1n7 and 18:1n9 in phospholipids in all dietary groups. These findings are consistent with previous reports that the host response to infection and inflammation is usually accompanied by enhanced hepatic fatty acid synthesis and reesterification of fatty acid in the liver [25,26]. Other findings were that LPS significantly reduced the levels of 20:4n6 in plasma triglycerides and in plasma and liver phospholipids in all dietary groups. Lipopolysaccharide is known to induce the production of phospholipase A2 that releases AA from plasma and tissue lipids and cyclooxygenase-2 enzymes [27] that convert AA to prostaglandins and other mediators for the production and resolution of inflammation [28-30]. Although eicosanoids including prostaglandins and leukotrienes influence cytokine production [31,32], it is not likely that the relative degree of attenuation of the inflammatory response in the EFAD diets was attributable only to the level of AA because hepatic phospholipid AA levels were higher in the HCO diet than the AA + DHA diet with lesser inflammation. Although speculative, the only factors that tracked with the degree of inflammation was the total ω-6 fatty acids in the diet and

the level of phospholipid Mead acid; and both reflect the essential fatty acid status. These noted differences would presumably be even more pronounced with the development of a full biochemical and clinical EFAD state. What is apparent however is that changes in inflammation occur when feeding an EFAD diet compared with one just above the requirement level long before the definitive biochemical evidence of EFAD, and these changes can be further modified by feeding small amounts of the distal products of the essential fatty acids.

In conclusion, our study shows that short-term feeding with a diet markedly deficient in LA without ALA, AA, and DHA changes plasma and liver fatty acid composition in a manner consistent with the beginning development of classic EFAD but long before its clinical manifestations as reflected in reduction in weight gain and other nutrition markers. The animals fed an EFAD diet had a reduced baseline inflammation and dampened inflammatory response to LPS challenge. Adding small amounts of AA and DHA to an EFAD diet modulated these changes, including reduced Mead acid levels and probably stearyl-Co A desaturase–1 activity, and restored certain elements of the systemic inflammatory response consequent to such a diet. Further investigations in rats with fully established EFAD are needed, especially whether providing larger amounts of AA + DHA, or perhaps AA + EPA + DHA, at the level that would be present in a 10% fish oil diet that totally prevents EFAD, for longer periods of time could completely normalize essential fatty acid status while maintaining lower levels of AA as compared with a plant oil–based diet [13]. In that way, perhaps a normal essential fatty acid status, as manifest by a triene-tetraene ratio indistinguishable from that found when provided with a soybean oil diet, with its implications for homeostatic metabolic status, might also lead to further modulation of the systemic inflammatory response with improved clinical outcomes related in part to the reduction of AA levels. Finally, these results demonstrate that important biochemical and physiologic changes occur early in response to the consumption of an EFAD diet, which may have relevance under many clinical circumstances.

Conflict of Interest

There were no interest conflicts.

REFERENCES

[1] Institute of Medicine. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. (2002/2005) Washington DC: National Academy Press.

[2] Das UN. Essential fatty acids—a review. Curr Pharm Biotechnol 2006;7:457-66.

[3] Press M, Kikuchi H, Thompson GR. Essential fatty acid deficiency secondary to intestinal malabsorption. Gut 1972;13:837.

[4] Paulsrud JR, Pensler L, Whitten CF, et al. Essential fatty acid deficiency in infants induced by fat-free intravenous feeding. Am J Clin Nutr 1972;25:897-904.

[5] Riella MC, Broviac JW, Wells M, et al. Essential fatty acid deficiency in human adult during total parenteral nutrition. Ann Intern Med 1974;83:786-9.

[6] Martins FM, Wennberg A, Meurling S, et al. Serum lipids and fatty acids composition of tissues in rats on total parenteral nutrition (TPN). Lipid 1984;19:728-37.

[7] Holman RT. Essential fatty acid deficiency. In: Holman RT, editor. Progress in the chemistry of fats and other lipids, IX. New York: Pergamon Press; 1971. p. 275-348.

[8] Holman RT. Biological activity and requirement for polyunsaturated acids. In: Holman RT, editor. Progress in the chemistry of fats and other lipids. Oxford: Pergamon Press.; 1971. p. 611-82.

[9] Holman RT. Essential fatty acid deficiency in humans. In: Rechcigl Jr M, editor. CRC handbook series of nutrition and food. Section E, 3. West Palm Beach: CRC Press Inc.; 1978. p. 335-68.

[10] Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, decosapentaenoic and docosahexaenoic acids in young women. Br J Nutri 2002;88:411-20.

[11] Plourde M, Cunnane SC. Extremely limited synthesis of long chain polyunsaturates in adults: implication for their dietary essentiality and use as supplements. Appl. Physiol. Nutr. Metab 2007;32:619-34.

[12] Kris-Etherton PM, Grieger JA, Etherton TD. Dietary reference intakes of DHA and EPA. Prostaglandins Leukot Essential Fatty Acids 2009;81:99-104.

[13] Strijbosch RA, Lee S, Arsenault DA, et al. Fish oil prevents essential fatty acid deficiency and enhances growth: clinical and biochemical implications. Metabolism 2008;57:698-707.

[14] Cook JA, Wise WC, Halushka PV. Elevated thromboxane levels in the rat during endotoxic shock: protective effects of imidazole, 13-azaprostanoic acid, or essential fatty deficiency. J Clin Invest 1980;65:227-30.

[15] Mascioli EA, Iwasa Y, Trimbo S, et al. Endotoxin challenge after menhaden oil diet: effects on survival of guinea pigs. Am J Clin Nutr 1989;49:277-82.

[16] Hansen A, Wiese H, Boelsche A, et al. Role of linoleic acid in infant nutrition. Pediatrics 1963;31(suppl):171-92.

[17] Marik PE, Zaloga GP. Immunonutrition in critically ill patients: a systematic review and analysis of the literature. Intensive Care Med 2008;34:1980-90.

[18] Lefknowith JB, Flippo V, Sprecher H, Needleman P, et al. Paradoxical conservation of cardiac and renal arachidonate content in essential fatty acid deficiency. J Biol Chem 1985;260:15736-44.

[19] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.

[20] Williams MA, Tamai KT, Hincenbergs I, et al. Hydrogenated coconut oil and tissue fatty acids in EFA-depleted and EFA-supplemented rats. J Nutr 1972;102:847-55.

[21] Huang MT, Williams MA. Essential fatty acid deficiency and plasma triglyceride turnover in rats. Am J Physiol 1980;238: E499-505.

[22] Ling PR, Leon CED, Le H, et al. Early development of essential fatty acid deficiency in rats: fat-free vs. hydrogenated coconut oil diet. Prostaglandins Leukot Essent Fat Acids 2010;83: 229-37.

[23] Das UN. Can essential fatty acid reduce the burden of disease(s)? Lipids Health Dis 2008;8:7-11.

[24] Poudel-Tandukar K, Nanri A, Matsushita Y, et al. Dietary intakes of alpha-linolenic and linoleic acids are inversely associated with serum C-reactive protein levels among Japanese men. Nutr Res 2009;29:363-70.

[25] Lanza-Jacoby S, Tabares A. Triglyceride kinetics, tissue lipoprotein lipase, and liver lipogenesis in septic rats. Am J Physiol 1990;258:E678-85.

[26] Grunfeld C, Feingold KR. Tumor necrosis factor, cytokines and the hyperlipidemia of infection. Trend Endocrinol Metab 1991;2:213-9.
[27] Akundi RS, Candelario-Jalil E, Hess S, et al. Signal transduction pathways regulating cyclooxygenase-2 in lipopolysaccharide-activated primary rat microglia. Glia 2005;51:199-208.
[28] Lee FP, Jen CX, Chang CC, et al. Mechanisms of adiponectin-mediated COX-2 induction and protection against iron injury in mouse hepatocytes. J Cell Physiol 2010;224:837-47.
[29] Rosenberger TA, Villacreses NE, Hovda JT, et al. Rat brain arachidonic acid metabolism is increased by a 6-day intracerebral ventricular infusion of bacterial lipopolysaccharide. J Neurochem 2004;88:1168-78.
[30] Palomba L, Amadori A, Cantoni O. Early release of arachidonic acid prevents an otherwise immediate formation of toxic levels of peroxynitrite in astrocytes stimulated with lipopolysaccharide/interferon-gamma. J Neurochem 2007;103:904-13.
[31] Kumar GS, Das UN. Effect of prostaglandins and their precursors on the proliferation of lymphocytes and their secretion of tumor necrosis factor and various interleukins. Prostaglandins Leukot Essent Fat Acids 1994;50:331-4.
[32] Calder PC. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fat Acids 2006;75:197-202.