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Dietary Docosahexaenoic Acid Decreases Plasma Triglycerides with Mixed Effects on Indices of β-oxidation

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ABSTRACT

One known effect of long chain n-3 polyunsaturated fatty acids is their ability to decrease plasma triglycerides. However, identification of the specific n-3 fatty acids and the underlying mechanisms responsible for this change remains uncertain. This present study was designed to evalute the effects of moderate levels of dietary docosahexaenoic acid (22 : 6(n-3)) on modulating plasma triglyderides. Male CD-1 mice were maintained for 15 days on identical diets containing either docosahexahexaenoic acid ethyl ester (1.5%, w/w) or linoleic acid (18 : 2(n-6)) ethyl ester (1.5%, w/w). Plasma triglycerides were 40% lower in the docosahexaenoic acid group than in the linoleic acid group. Hepatic carnitine palmitoyltransferase activity (a key regulatory enzyme for mitocondria β-oxidation) was not significantly different between the dietary groups. However, plasma acid soluble acylcarnitine levels(which increase with increasing β-oxidation) were significantly higher in the docosahexaenoic acid group. This data suggests that plasma triglyceride levels are lower in mice fed diets containing moderate levels of docosahexaenoic acid compared to linoleic acid, but this effect on plasma triglycerides is not modulated through an augmentation of mitochondrial β-oxidation. (*Korean J Nutrition* 30(9) : 1067~1072, 1997)

KEY WORDS : docosahexaenoic acid · plasma triglycerides · mice · CPT · plasma acylcarnitine.

Introduction

Recent evidence suggests that elevated concentrations of plasma triglycerides may increase the risk of cardiovascular disease[1]. Fish oils rich in (n-3) polyunsaturated fatty acids (PUFA), in particular eicosapentaenoic acid (EPA, 20 : 5(n-3)) and docosahexaenoic acid (DHA, 22 : 6(n-3)), are very effective in lowering plasma triglyceride concentrations and preventing the formation of atherogenic lesions[2-5]. A number of possible mechanisms have been proposed explaining the triglyceride-lowering effect of (n-3) PUFA[2-5], including increases in hepatic mitochondrial and peroxisomal β-oxidation. However, the roles which EPA and DHA play in modulating plasma triglycerides have yet to be clarified. Dietary EPA, more consistently has been associated with the triglyceride-lowering effect observed with dietary (n-3) PUFA, while the effect of DHA is more controversial[6-10].

Based on limited research and inconclusive evidence, this study was designed to evaluate the effect of dietary DHA on plasma triglyceride concentrations and certain indices of β-oxidation.

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Materials & Methods

1. Animals

Twelve CD-1 male mice (HSD, Indianapolis, IN) (21 – 24g) were upon arrival, randomly divided into

two dietary groups of six animals per group. All mice were maintained on chow diet for 3 weeks, then placed on experimental diets for 15 days .

2. Diets

All diets contained 10% (w/w) fat. The linoleic acid (LA, 18 : 2 (n-6)) content of the diets, as supplied primarily by safflower oil (75% LA) (Sigma, St. Louis, MO) was maintained at approximately 1.3% (w/w) (Table 1 and 2). The filler oils (7%, w/w) were olive oil (3.5%, w/w) (Sigma, St. Louis, MO) and tripalmitin (3.5%, w/w) (Sigma, St. Louis, MO). Each diet contained an additional 1.5% (w/w) of fatty acid ethyl esters (Nu Chek Prep, Elysian, MN). The linoleic aicd diet (LA) contained linoleic acid (LA, 18 : 2 (n-6)) ethyl ester while the experimental diet (DHA) contained an equivalent amount of the ethyl ester of docosahexaenoic acid (DHA, 22 : 6 (n-3)). Diets were prepared in bulk, prepackaged in separate Whirl-Pak bags (NASCO, Atkinson, WI) and stored under nitrogen at −80℃. Alpha-tocopherol (RRR) (2mg/kg diet) (Sigma, St. Louis, MO) was supplemented to the diet as an antioxidant to prevent oxidation of PUFA during storage.

Water and food were provided ad libitum for 15 days. Fresh diet was provided daily and uneaten food was discarded to minimize oxidation prior to consumption. The fatty acid analysis of the diets is presented in Table 2.

Table 1. Experimental design[1)]

	LA group[2)]	DHA group[3)]
AIN-76™ Purified diet(Fat Free)	900	900
Safflower oil	15	15
Olive oil	35	35
Tripalmitin	35	35
18 : 2n-6 ethyl ester	15	0
22 : 6n-3 ethyl ester	0	15
α-Tocopherol(RRR) (mg/g PUFA)	2mg	2mg

1) All components are in units of g/kg diet except for α-tocopherol
2) LA group : Linoleic acid group
3) DHA group : Docosahexaenoic acid group

Table 2. Fatty acid composition in the diet[1)]

	LA group	DHA group
16 : 0	42.0	40.8
18 : 0	1.8	1.8
18 : 1n-9	30.1	29.9
18 : 2n-6	26.2	13.1
22 : 6n-3	ND	14.3

1) All components are in units of g/kg diet
ND : Not detectable

3. Analysis of liver fatty acid composition

Analysis of hepatic phospholipid fatty acid composition was carried out as described by whelan,, et al (1991). Briefly, livers (100mg) were homogenized in 0.8ml cold saline (0.9%). Liver homogenates were resuspended in 3ml methanol/chloroform (2 : 1, v/v), extracted once with chloroform/saline (1 : 1, v/v), and twice with chloroform. Different lipid fractions were separated by thin layer chromatography with chloroform/methanol (8 : 1, v/v) as the developing solvent. The phospholipid fractions were scraped from the thin layer chromatography plates, saponified with 0.5 N methanolic KOH at 86℃ for 8 min, and acidified with 0.7 N methanolic HCL. Following the extraction with hexane, fatty acids were methylated with ethereal diazomethane, and the final samples were resuspended in hexane for gas chromatographic analysis.

4. Plasma triglyceride analysis

Animals fasted overnight and were anethesized by methoxyflurane (Pitman-Moore, Mendelian, IL) inhalation. Blood (1ml) was drawn by cardiac puncture with a syringe containing 3mg EDTA. Whole blood was centrifuged at 2,200×g, at 4℃ for 15min. Plasma was collected and stored at −80℃ for triglyceride analysis the next day. Plasma triglycerides were quantified spectrophotometrically using the triglyceride analysis kit from Sigma (St. Louis, MO).

5. Hepatic carnitine palmitoyl transferase(CPT) analysis

Animals were sacrificed by cervical dislocation. Livers were perfused with cold saline (0.9%) and removed immediately. CPT analyses were carried out using the procedure of Surette, et al.(1992). Briefly, hepatic mitochandria were prepared according to the method of Mcgarry, et al.[12)] and mitochondrial CPT activity was assayed as described by Bieber, et al.[13)]. The reaction mixture contained 0.1 mM palmitoyl CoA, and 8.0mM L-carnitine, 0.25mM 5,5'-dithiobis- (2-nitrobenzoic acid) in a final reaction

volume of 1.0ml. The reaction was initiated with the mitochon-drial preparation (approximately 80μg protein) and rates were determined by monitoring the change in absorbance at 412nm (reaction rates were determin-ed to be stable for 4min).

6. Protein determination

Protein was determined by a modified Lowry assay[14].

7. Analysis of plasma carnitine

Plasma carnitine levels were determined according to the radioisotopic method of Cederblad and Lindstedt[15] as modified by Sachan, et al.[16].

8. Statistical analysis

All of the results were reported as mean plus one standard error (±SEM).

Differences between means were assessed by Student's t-test (Graphpad software, San Diego CA, USA). A p value <0.05 was considered to be significant.

Table 3. Fatty acid composition in liver phospholipid[1]

	LA group	DHA group	p-value
12 : 0	0.50 ± 0.06	0.65 ± 0.06	0.1075
14 : 0	0.35 ± 0.05	0.36 ± 0.05	0.8903
16 : 0	27.97 ± 0.19[a]	29.65 ± 0.18[b]	<0.0001**
16 : 1	1.01 ± 0.07[a]	0.75 ± 0.07[b]	0.0253*
18 : 0	12.15 ± 0.11	12.36 ± 0.15	0.285
18 : 1n-9	8.92 ± 0.18	8.56 ± 0.13	0.136
18 : 2n-6	16.06 ± 0.20	16.05 ± 0.15	0.969
20 : 3n-6	2.00 ± 0.09[a]	1.35 ± 0.09[b]	0.0005**
20 : 4n-6	19.81 ± 0.19[a]	6.02 ± 0.15[b]	<0.0001**
20 : 5n-3	ND[2]	2.02 ± 0.14	
22 : 4n-6	0.35 ± 0.05	ND	
22 : 5n-6	0.70 ± 0.29	ND	
22 : 5n-3	0.34 ± 0.05[a]	0.58 ± 0.06[b]	0.0118*
22 : 6n-3	10.03 ± 0.20[a]	21.71 ± 0.16[b]	<0.0001**

1) All data are mole % and expressed as mean±SEM for six experimental values

2) ND : Not detectable

Values with different letters in the same row are significantly different at *$p<0.05$ and **$p<0.01$

Results

Food intake and weight gain were not significantly different between the two dietary groups[5]. Dietary DHA effectively enriched tissue phospholipids with DHA at the expense of arachidonic acid(AA, 20 : 4(n-6))(Table 3). When DHA was included in the diet, DHA content of hepatic phospholipid increased from 10mol% in the control group to 21.7mol% in the DHA group. Concomitantly, tisssue AA content was 70% lower in the DHA group. It was also observed that inclusion of DHA in the diet resulted in the appearance of EPA in hepatic phospholipids. EPA content of hepatic phospholipids was increased to 2 mol% in the DHA group versus the LA group where EPA was not detectable.

Plasma triglyceride concentration was 40% lower in the DHA group compared to animals supplemented with equivalent levels of LA. Plasma triglycerides decreased significantly from 117.4mg/dl in the LA group to 70.1mg/dl in the DHA group ($p<0.05$) (Table 4).

The type of supplemented PUFA had little effect on CPT activity. When DHA was included in the diet, CPT activity was not significantly different from the LA group (Table 4). However, plasma acid soluble acylcarnitine levels were significantly higher (43% higher) in the DHA group than in the LA group.

Discussion

One unequivocal effect of dietary (n-3) PUFA, in the form of fish oils, is their ability to lower plasma triglycerides. This effect has been clearly eatablished in clinical trials involving humans and in experiments involving animal models[2]. However, some controversy remains as to which of the (n-3)PUFA have the

Table 4. Effect of dietary DHA on plasma TG and indices of fatty acid oxidation[1]

	LA group	DHA group	p-value
Plasma TG(mg/dl)	117.4 ± 7.55[a]	70.1 ± 7.14[b]	0.0011
CPT Activity(nmole/min/mg protein)	67.5 ± 2.37	72.8 ± 1.02	0.0669
Plasma ASAC[2](nmole/ml)	23.4 ± 1.1[a]	33.5 ± 2.5[b]	0.0041

1) All data are expressed as mean±SEM for six experimental values

2) ASAC : acid soluble acylcarnitine

Values with different letters in the same row are significantly different at $p<0.05$

ability to lower circulating triglyceride levels. Dietary EPA has been shown to decrease plasma triglycerides [6)8)10)], while the effects of dietary DHA are more uncertain[6-10]. The objective of this study was to evaluate the effects of DHA on plasma triglyceride concentration. The diets were formulated to carefully control for all components, including proteins, carbohydrates, and lipids. The lipid composition of the diets was identically matched for monounsaturated, saturated, and PUFA content. The important difference in the diets was that moderate levels of DHA replaced LA as the only change. LA was used as the variable in the diets instead of oleic acid to control for the effects of PUFA on triglyceride synthesis. It has been shown that PUFA, and not monounsaturated fats, comparatively, lower the activities of enzymes important in fatty acid synthesis ; however, linoleic acid is a weak hypotriglyceridemic agent compared to (n-3) PUFA[17)18)].

There was 40% lower plasma triglyceride concentration when DHA was substituted for LA in the diet. We believe this reduction would have been more significant if a greater number of animals were used in each dietary group. This observation is supported by reports that hepatic secretion rates for triglycerides are reduced by DHA[5)7)], where DHA was reported to be as effective as EPA[5)]. Several studies also report that dietary DHA suppresses fatty acid synthesis and decreases the activities of lipogenic enzymes[5)7)19)]. However, several studies also reported that dietary DHA does not lower plasma triglyceride concentrations. Triglyceride levels of rats maintained on diets containing DHA ethyl ester(3%, w/w) were not significantly different from matched animals consuming olive oil or LA[6)]. More recently, a study utilizing rats incubated with several levels of DHA ethyl ester reported that plasma triglyceride levels were unaffected by DHA feeding. However, the data in this study is hard to interpret as the body weight changes among the different dietary groups were dramatically different[10)]. In addition, a human study conducted in conjunction with the Tr ϕ mso study assessed the effects of dietary fish oil on plasma triglyceride levels[9)]. This double-blind 10-week intervention trial resulted in significant reduction in plasma triglycerides as a result of fish oil (containing both EPA and DHA) supplementation. Following multivariate analysis of the data, the authors concluded that dietary EPA, and not DHA, was responsible for the changes in triglyceride levels. However, the direct effect of dietary DHA on triglycerides was never determined. The results of the present study suggest that plasma triglyceride levels are lower when diets are supplemented with DHA instead of LA. It has been suggested that the triglyceride -lowering effect of (n-3) PUFA may be modulated, in part, by an increase in fatty acid oxidation ; however, this explanation is also controversial[19)20)21)]. It has been demonstrated that dietary EPA can augment both mitochondrial and peroxisomal β-oxidation[8)]. Similarly, Surette, et al.[5)] reported that plasma triglycerides were negatively, but significantly correlated ($r = -0.97$) with CPT activity following fish oil feeding. However, no changes were observed in peroxisomal β-oxidation[4)]. Of importance is whether EPA and/or DHA are responsible for these observed effects on β-oxidation. This present study suggests that the triglyceride-lowering effect of DHA may not be modulated through an increase in mitochondrial oxidation of fatty acids as determined by CPT activity. Willumsen, et al.[10)] also reported that dietary DHA does not effect hepatic CPT activity, but contrary to our observations, they also observed no changes in plasma triglyceride levels. However, they did report increased peroxisomal β-oxidation following feeding with DHA at dietary levels comparable to our study. Induction of peroxisomal β-oxidation by n-3 PUFA is well documented[8)10)21)]. While we observed no significant change in CPT activity, the plasma acid soluble acylcarnitine levels were significantly increased in the DHA group. Plasma levels of these acylcarnitines are sharply elevated under physiological conditions of accelerated fatty acid oxidation[22)23)]. Peroxisomes can be a source of acylcarni-tines[24)] as liver peroxisomes contain acylcarnitine transferases[25)26)]. Retroconversion of DHA to EPA is believed to be the result of peroxisomal β-oxidation[27)], as peroxisomes contain acyl CoA synthetase specific for long chain fatty acids[28)]. In the present study, the relative abundance of EPA in hepatic phospholipids increased from non-detectable levels in the LA group to 2. 0mol% in the DHA group suggesting significant re-

troconversion of dietary DHA to EPA. Therefore, it is possible that some of the effects observed in this study may be influenced by the endogenous formation of EPA via retroconversion from DHA.

In summary, animals consuming DHA had significantly lower plasma triglyceride levels than those in the LA-supplemented group. Our results suggest that this effect is not modulated by mitochondrial β-oxidation as measured by CPT activity. However, it was observed that plasma acid acyl carnitine levels were higher in the DHA group, suggesting an increase in fatty acid oxidation that may be related to retroconversion of DHA to EPA. These results further suggest that the independent effect of DHA on the modulation of plasma triglycerides has yet to be fully clarified.

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