Original Article *J. Clin. Biochem. Nutr.*, **41**, 184–190, November 2007

Effect of Docosahexaenoic Acid Ingestion on Temporal Change in Urinary Excretion of Mercapturic Acid in ODS Rats

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Received 4 December, 2006; Accepted 1 April, 2007

Summary **We hypothesized a suppressive mechanism for docosahexaenoic acid (22:6n-3; DHA)-induced tissue lipid peroxidation in which the degradation products, especially aldehydic compounds, are conjugated with glutathione through catalysis by glutathione S-transferases, and then excreted into urine as mercapturic acids. In the present study, ascorbic acid-requiring ODS rats were fed a diet containing DHA (3.6% of total energy) for 31 days. Lipid peroxides including degradation products and their scavengers in the liver and kidney were determined, and the temporal change in the urinary excretion of mercapturic acids was also measured. The activity of aldehyde dehydrogenase, which catalyzes the oxidation and detoxification of aldehydes, tended to be higher in the liver of DHA-fed rats. The levels of lipid peroxides as measured by thiobarbituric acid-reactive substances and aldehydic compounds were higher and that of α-tocopherol was lower in the liver, and the pattern of temporal changes in the urinary excretion of mercapturic acids was also different between the *n*-6 linoleic acid and DHA-fed rats. Accordingly, we presume from these results that after dietary DHA-induced lipid peroxidation, a proportion of the lipid peroxidation-derived aldehydic degradation products is excreted into urine as mercapturic acids.**

***Key Words*: Docosahexaenoic Acid, lipid peroxide, mercapturic acid, ODS rats**

Introduction

Docosahexaenoic acid (22:6n-3; DHA) together with eicosapentaenoic acid (20:5n-3; EPA) is the predominant n-3 polyunsaturated fatty acid (PUFA) in fish oils. Consumption of fish oils is particularly associated with a low incidence of atherosclerosis and cardiovascular diseases, and this prophylactic effect is attributed to n-3 PUFAs, such as EPA and DHA [*1–7*]. In recent years, de Urquiza *et al.* [*8*] have reported that DHA may influence neural function through activation of a retinoid X receptor signaling pathway in brain tissue.

However, DHA is very prone to lipid peroxidation because of its unstable chemical structure with six double bonds. It was reported that the relative reaction rate constant of peroxidation was 1, 2, 3, 4 and 5 against unsaturated fatty

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acids in which the number of methylene group among double bonds was 1, 2, 3, 4 and 5, respectively [*9*]. In previous studies, we and other groups showed that DHA ingestion [*10–16*], similar to fish oil ingestion [*17–21*], enhanced the susceptibility of rat liver and kidney to lipid peroxidation and increased the requirement for vitamin E (Vit. E). The enhancement was a function of dietary DHA levels [*22–24*] and was thought to be attributable to the substitution of membrane fatty acids with highly unsaturated DHA, as similarly suggested by Vaagenes *et al.* [*11*].

However, we found that DHA ingestion did not increase the end products of lipid peroxidation, such as lipofuscin, in the liver of rats fed a physiological requirement level of Vit. E [*13*, *15*, *16*]. We hypothesize that there are primarily two suppressive mechanisms for it. One is an antioxidative mechanism that suppresses the generation of lipid peroxides, and the other is detoxification and/or excretion mechanisms that suppress the accumulation of lipid peroxides and their degradation products, particularly reactive aldehydes. The former mechanism is thought to be exerted through increases in ascorbic acid (AsA) and glutathione (GSH) induced by DHA intake [*13*, *14*, *23*], which potentiates reductive recycling of Vit. E, thus maintaining antioxidative potency. However, the DHA-induced generation of tissue lipid peroxides was not suppressed further even after higher doses of Vit. E [*12*, *13*], or after higher doses of AsA and methionine [*15*, *16*]. Methionine is necessary for the synthesis of GSH via cysteine. Accordingly, the antioxidative potency exerted through Vit. E, AsA and GSH is not enough to suppress the generation of lipid peroxides. Additionally, ingestion of DHA did not increase liver glutathione reductase and glutathione peroxidase activities, even though the tissue lipid peroxide levels in DHA-fed rats were higher [*13*, *14*]. These observations suggest that some mechanisms other than antioxidants and antioxidant enzymes are induced after DHA ingestion in order to suppress the accumulation of lipid peroxides and their degradation products leading to end products of lipid peroxidation.

When 4-hydroxy-2-hexenal (4-HHE) [*25*] and 4-hydroxy-2-nonenal (4-HNE) [*26*] as secondary *n*-3- and *n*-6-PUFA derived degradation products of lipid peroxidation, respectively, were injected directly into the blood stream of rats, the mercapturic acids (acetylcysteine conjugates) derived metabolically from conjugates of the aldehydes with GSH were detected in the urine. Therefore, we presume that detoxification and/or excretion of reactive aldehydes derived from dietary DHA-induced lipid peroxidation is a second mechanism for suppressing the gradual accumulation of lipid peroxides and their degradation products, in which glutathione S-transferases (GSTs) firstly catalyze the conjugation of lipid peroxidation-derived aldehydes with GSH. In addition, we hypothesize that another mechanism involving dietary DHA-induced biotransformation mediated by aldehyde dehydrogenase (ALDH) detoxifies reactive aldehydes, resulting in formation of their carboxylic acids [*26*].

In this study, therefore, we focused in particular on detoxification and/or excretion mechanisms for suppressing the accumulation of lipid peroxides and their degradation products induced after DHA intake. Our previous study showed that DHA ingestion elevated the urinary excretion of mercapturic acids on day 28 in Sprague-Dawley rats [*27*]. Hence, we measured here the temporal change in the urinary excretion of mercapturic acids in AsA-requiring Osteogenic Disorder Shionogi/Shi-od/od (ODS) rats after DHA intake. We used this specific rat strain to restrict the effects of an antioxidant AsA as least as possible because our previous studies showed that AsA was increased in tissues by DHA ingestion [*13*, *14*, *23*]. Clarification of the mechanisms that suppress dietary DHA-induced lipid peroxidation is important not only for reasons of safety, but also to formulate a means of efficiently enhancing the physiological effectiveness of n-3 PUFAs.

Materials and Methods

Animals and diets

The experimental procedures used in this study met the guidelines of the animal handling committee in Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Male AsA-requiring ODS rats (CLEA Japan, Tokyo, Japan), lacking l-gulono-γ-lactone oxidase in the AsA biosynthetic pathway [*28*], 6 weeks of age and weighing 120–140 g, were housed individually in stainless-steel wire-bottomed cages kept at a constant temperature of 22 ± 1°C and humidity of 50–60% with a 12 h light-dark cycle. The composition of the experimental diets, based on the AIN-76 purified diet for rats [*29*, *30*], is shown in Table 1. For experimental groups, six rats each were assigned to two groups: LA and DHA groups. The diets were fed for 31 days. The dietary lipid level was 70 g/kg diet. In the LA diet, the level of linoleic acid (18:2*n*-6; LA) was 6.3% of total energy. In the DHA-containing diet, the level of LA and DHA were 2.0 and 3.6% of total energy, respectively. For energy calculation, Atwater energy factors [*31*] were used. The proportion of total PUFA was at almost the same level in the LA- and DHA-containing diets. The Vit. E content as *RRR*-α-tocopherol equivalent, which is calculated by the biopotency ratio of tocopherols [*32*], was 100 mg/kg diet. The dietary AsA level was set at 300 mg/kg diet according to our previous results [*15*]. During the experimental period, each diet was made available to the rats in the evening and was removed the next morning. After being deprived of food overnight, the rats were sacrificed by cardiac puncture. The liver and kidney were promptly excised, and the liver was then perfused with ice-cold isotonic saline via the portal

Table 1. Composition of experimental diets (g/kg diet) and fatty acid composition (g/100 g) of dietary lipids given to ODS rats[1]

Group	LA	DHA
	% total energy	
Linoleic acid	6.3	2.0
DHA	—	3.6
	g/kg diet	
Basic components[2]	930	930
Test lipids[3]	70	70
Safflower oil	35	7
Olive oil	35	42
DHA concentrate[4]	—	21
Fatty acids	g/100 g	
16:0	8.3	7.1
18:0	2.3	2.1
18:1 (*n*-9)	46.8	51.8
18:2 (*n*-6) LA	40.6	13.0
18:3 (*n*-3)	0.3	0.4
20:4 (*n*-6) AA	ND	0.5
20:5 (*n*-3) EPA	ND	0.4
22:5 (*n*-6)	ND	0.2
22:5 (*n*-3)	ND	0.5
22:6 (*n*-3) DHA	ND	22.8
Others	1.3	1.0
PUFA	40.9	37.8

[1] The vitamin E content as *RRR*-α-tocopherol equivalent of the experimental diets was 100 mg/kg diet. ND, not detectable.
[2] The basic components of the diet given to the groups were as follows: casein, 200.0 g; DL-methionine, 3.0 g; cornstarch, 150.0 g; sucrose, 239.7 g; glucose, 240.0 g; cellulose powder, 50.0 g; AIN-76 vitamin mixture [*29*, *30*], 10.0 g; AIN-76 mineral mixture [*29*], 35.0; choline bitartrate, 2 g; ascorbic acid 0.3 g.
[3] Fat energy percentage is 15.7% of total energy.
[4] The purity of the DHA ethyl esters was 93%.

vein. The liver and kidney samples were stored at −80°C until used for the analysis.

Lipid peroxides, α-tocopherol and antioxidant enzymes

The tissue thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Ohkawa *et al.* [*33*] with a minor modification, in which butylated hydroxytoluene was added to the reaction mixture at a final concentration of 0.45 mM. TBARS are expressed in terms of the malondialdehyde (MDA) equivalent. The MDA + 4-hydroxy-2-alkenals (4-HAE) in their free form in tissues were determined using commercial BIOXYTECH®LPO-586™ (OXIS International Inc., Foster City, CA) assay kit. The LPO-586 method is designed to assay MDA in conjunction with 4-HAE in methanesulfonic acid.

α-Tocopherol concentrations in the test lipids and tissues were analyzed by HPLC [*34*].

Activities of GST and ALDH were determined by the method of Jennson *et al.* [*35*] and Pietruszko and Yonetani [*36*], respectively. The protein content was measured by the method of Lowry *et al.* [*37*].

Urinary mercapturic acid excretion

To determine a temporal change in the urinary excretion of mercapturic acids, whole urine was collected on days 0, 7, 14, 21, and 28, respectively. The urinary mercapturic acid was analyzed by HPLC after the extraction by the method of Kress and Pentz [*38*].

Fatty acid composition

Total lipids in the liver and kidney were extracted according to the method of Bligh and Dyer [*39*]. Fatty acid methyl esters of dietary lipids and total tissue lipids were prepared and analyzed by GLC according to a previous study [*15*].

Statistical analysis

Significant difference between the mean values of the LA and DHA groups was evaluated by Student's *t* test. Significant difference among the mean values of urinary mercapturic acids was evaluated by repeated-measures ANOVA. The limit of significance was set at $p<0.05$.

Results

In the ODS rats, no signs of scurvy, such as hemorrhages around the eyes and nose, appeared during the 31 days of the experimental period. The rats consumed 17.3–18.4 g food/d and gained 3.5–3.8 g/d over the 31 days of the experimental period (data not shown). There were no significant differences in food intake, body-weight gain, and liver and kidney weights between the two groups (data not shown).

Various and complex mixtures of compounds derived from in vivo lipid peroxidation, including TBA-reactive precursors which degrade under the acidity and heating conditions used in the assay, react with TBA. These degradable precursors include lipid hydroperoxides, hydroperoxyendoperoxides and so forth. The aldehydic degradation products from *in vivo* lipid peroxidation, such as malondialdehyde, alkenals, alkadienals and so on in their free form, also react with TBA but these free aldehydes are produced after *in vivo* lipid peroxidation has been extensively promoted.

The TBARS and free MDA + 4-HAE levels in the liver were significantly higher with DHA intake as indicated in Table 2. The α-tocopherol level was significantly lower with DHA intake. The GST and ALDH activities did not differ between the two groups although the latter activity tended to be higher with DHA intake.

Table 2. Lipid peroxides and their scavengers in the liver and kidney of ODS rats[1]

Group	LA	DHA
Liver		
TBARS (nmol/g)	62.5 ± 6.2	103.2 ± 18.5*
MDA + 4-HAE (nmol/g)	10.5 ± 2.3	14.2 ± 2.7*
α-Tocopherol (nmol/g)	91.8 ± 16.2	68.3 ± 11.2*
GST (unit/mg protein)	1.8 ± 0.3	1.8 ± 0.3
ALDH (unit/mg protein)	13.1 ± 1.6	15.1 ± 1.6
Kidney		
TBARS (nmol/g)	158.3 ± 7.0	166.6 ± 12.4*
MDA + 4-HAE (nmol/g)	14.1 ± 1.5	14.6 ± 2.7
α-Tocopherol (nmol/g)	37.9 ± 4.0	32.8 ± 11.6*

Abbreviations: ALDH, aldehyde dehydrogenase; GST, glutathione S-transferase; MDA + 4-HAE, malondialdehyde + 4-hydroxy-2-alkenal; TBARS, thiobarbituric acid-reactive substances.

[1] Values are mean ± SD, $n = 6$.

* Significant difference between the LA and DHA groups using Student's t test ($p<0.05$).

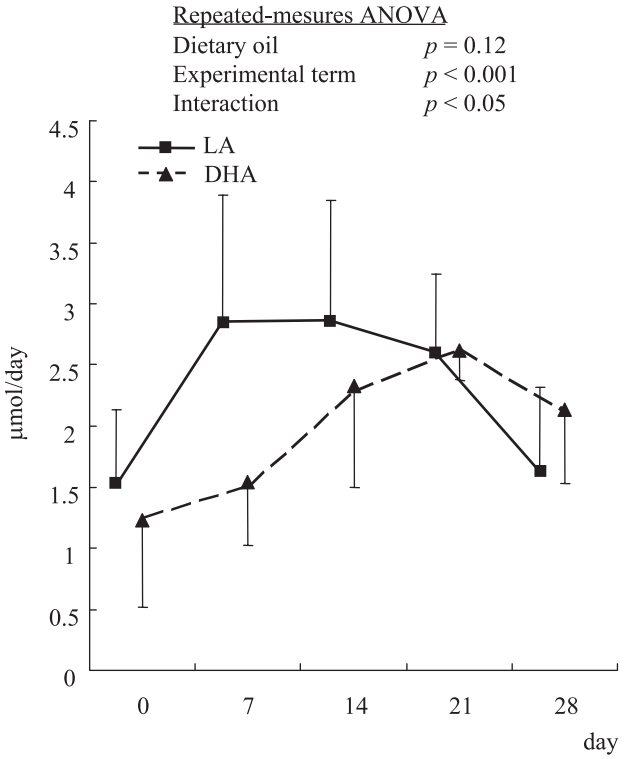

Fig. 1. Time course of urinary excretion of mercapturic acids in ODS rats. Values are mean ± SD, $n = 6$.
* Significant difference between the LA and DHA groups using Student's t test ($p<0.05$). Repeated-measures ANOVA showed a significant difference with the experimental term and a significant interaction between dietary oil and experimental term.

In the kidney, the TBARS level was significantly higher with DHA intake but the free MDA + 4-HAE level did not differ between the two groups (Table 2). The α-tocopherol level was significantly lower with DHA intake.

The urinary excretion of mercapturic acids did not differ significantly between the two groups on days 0, 14 and 21 (Fig. 1), but it was significantly lower in the DHA-fed group than in the LA group on day 7. On day 28, the difference between the two groups was not significant but the excretion tended to be higher with DHA intake. The temporal excretion in the LA group fed the diet rich in *n*-6 LA increased rapidly after the diet intake, was kept in a plateau from day 7 to 21, and then decreased on day 28. While that in the DHA-fed group gradually increased until day 21 and then decreased a little on day 28. Repeated-measures ANOVA showed a significant difference with the experimental term and a significant interaction between dietary oil and experimental term. Therefore, this result indicated that the pattern of changes in the urinary excretion of mercapturic acids was different between the LA- and DHA-fed groups.

The major PUFA compositions of total lipids in the liver and kidney are shown in Table 3. In the liver, the proportions of LA and arachidonic acid (20:4*n*-6; AA) were significantly lower in the DHA group than in the LA group, while those of EPA, 22:5*n*-3, and DHA were significantly higher in the DHA group. In the kidney, the proportion of LA was significantly higher in the DHA group than in the LA group and that of AA was significantly lower in the DHA group. Those of EPA, 22:5*n*-3, and DHA were significantly higher in the DHA group than those of the LA group.

Discussion

In this study, we focused in particular on detoxification and/or excretion mechanisms of lipid peroxidation-derived reactive aldehydes as described in the introduction. To prove these mechanisms, we fed rats a high level of DHA (3.6% of total energy), which is actually hard to ingest everyday in our daily life. But such a model system is in particular necessary to highlight the *in vivo* response and to detect it.

The TBARS and free MDA + 4-HAE levels in the liver were significantly higher and the Vit. E level was lower with DHA intake (Table 2). These results mean that DHA administration stimulated lipid peroxidation to increase TBARS and free MDA + 4-HAE levels in this study. These free aldehydes are produced after *in vivo* lipid peroxidation has been extensively promoted and not combined with macromolecules like

protein. Hence, the MDA + 4-HAE level and their increase following DHA intake were very low compared with those of TBARS (Table 2).

As shown in Fig. 1, repeated-measures ANOVA showed an interaction between the LA and DHA groups. This result indicates that the pattern of urinary excretion of mercapturic acids was different between the two groups in the experimental term and the excretion of mercapturic acids might be promoted gradually after DHA was ingested. The gradual increase of mercapturic acid excretion into urine in the DHA-fed rats reflected that of lipid peroxidation attended by the increases in the DHA levels in tissues. In addition, the excretion on day 28 tended to be higher with DHA intake. This result on day 28 was similar to our previous report [*27*]. We have reported the gene expression of dietary DHA-induced multidrug resistance-associated protein 3 in the liver [*40*], which could mediate the transport of reactive aldehydes conjugated with GSH into blood stream from the liver. Therefore, the conjugates were metabolized to mercapturic acids in the kidney, and then excreted into urine.

However, the total activities of GSTs in the liver on day 31 did not differ significantly with DHA intake in this experiment (Table 2). Ålin *et al.* [*41*] and Hiratsuka *et al.* [*42*] reported that GSTA4-4 (previously called GST 8-8), which is present as a very minor GST protein in rat liver (approximately 1/75 of total activity of GSTs [*41*]), exhibited extremely high catalytic activity towards 4-HNE. Accordingly, even if the total activity of GSTs does not change in rats fed DHA, GSTs might be associated with the excretion of lipid peroxidation-derived reactive aldehydes including 4-HNE derived from oxidation of *n*-6 PUFAs and even 4-HHE derived from oxidation of *n*-3 PUFAs.

Danielson *et al.* [*43*] reported that the specificity constants (k_{cat}/Km) of GST 8-8 (GST A4-4) with 4-HNE was higher than that with 4-HHE. Hubatsch *et al.* [*44*] also obtained similar results. Hence, the excretion of mercapturic acids into urine might have been higher in the LA diet group from the early stage of the experimental period (Fig. 1). Therefore, the temporal change in the urinary excretion of mercapturic acids seems to reflect the difference in substrate affinity between 4-HNE and 4-HHE with GSH for GST. The decrease of the excretion in the LA group on day 28 after the plateau remains to be solved but some other detoxication mechanisms might be induced during the plateau stage. Additional studies are now under way to thoroughly characterize the precise mechanisms.

Alary *et al*. [*26*] and Mitchell and Peterson [*45*] reported an oxidation pathway for the formation of 4-hydroxy-2-nonenoic acid from 4-HNE through catalysis by ALDH, and subsequent conjugation with GSH to mercapturic acid [*26*] and also to mitochondrial β-oxidation. Similarly, a reduction pathway catalyzed by alcohol dehydrogenase was reported for the formation of 1,4-dihydroxy-2-nonene [*26*, *46*] from 4-HNE and subsequent conjugation with GSH to mercapturic acids [*26*]. However, Hartley *et al.* [*47*] and Reichard *et al.* [*48*] indicated that 4-HNE metabolism and detoxication in hepatocytes from normal rat liver were primarily mediated by GST, particularly GSTA4-4, and minor or insignificant by both ALDH and alcohol dehydrogenase, depending on the rat liver cell types used. In this study, the concentrations of free MDA + 4-HAE in the liver were low compared with those of TBARS, and also the difference of the free MDA + 4-HAE concentrations between the two groups was small (Table 2). Therefore, the statistical significance might not be detected in the ALDH activity.

In any case, each mechanism described above may play a role to some degree in the detoxication and/or excretion of lipid peroxidation-derived reactive aldehydes in DHA-fed rats. The details of the metabolic fate remain to be solved.

In the kidney, the TBARS level was significantly higher with DHA intake but the free MDA + 4-HAE level did not differ significantly between the two groups (Table 2). Considering the PUFA composition of the kidney (Table 3), the proportions of *n*-6 PUFAs with lower degree of unsaturation were generally higher, and that of highly unsaturated DHA was lower, than in the liver. In addition, high retroconversion from DHA to EPA was shown in the kidney (Table 3), as has already been reported [*15*, *16*, *49*]. Furthermore, although the Vit. E level in the kidney becomes lower with DHA intake, the extent of the decrease is small compared with that in the liver (Table 2), as similarly observed already [*12–14*,

Table 3. Major polyunsaturated fatty acid compositions of total lipids in the liver and kidney of ODS rats[1]

Group	LA	DHA
	g/100 g	
Liver		
18:2 (*n*-6) LA	11.9 ± 1.3	8.7 ± 0.8*
20:4 (*n*-6) AA	17.1 ± 1.2	5.4 ± 0.3*
20:5 (*n*-3) EPA	ND	2.9 ± 0.5*
22:5 (*n*-3)	0.1 ± 0.1	1.3 ± 0.1*
22:6 (*n*-3) DHA	2.1 ± 0.2	18.5 ± 0.6*
Kidney		
18:2 (*n*-6) LA	8.9 ± 0.1	11.4 ± 0.4*
20:4 (*n*-6) AA	28.4 ± 1.0	14.1 ± 0.4*
20:5 (*n*-3) EPA	ND	6.7 ± 0.4*
22:5 (*n*-3)	ND	0.5 ± 0.1*
22:6 (*n*-3) DHA	1.1 ± 0.1	6.8 ± 0.1*

Abbreviations: AA; arachidonic acid: DHA, docosahexaenoic acid; EPA; eicosapentaenoic acid; LA, linoleic acid; ND, not detectable.
[1] Values are means ± SD, $n = 6$
* Significant difference between the LA and DHA groups using Student's *t* test ($p<0.05$).

23]. Hence, the requirement for Vit. E is low in the kidney. Because of these characteristic profiles and the plausible difference in antioxidant potential, the difference of the lipid peroxide levels in the kidney might have been small between the two groups.

Accordingly, we presume from the results observed herein that a proportion of the lipid peroxidation-derived aldehydic degradation products following intake of DHA might be excreted into urine as mercapturic acids via conjugation with GSH by GST, particularly in the liver. Additionally, this study shows that the pattern of temporal change in the urinary excretion of mercapturic acids was also different between the *n*-3 and *n*-6 PUFA ingestion. Such detoxification and/or excretion mechanisms might play an important role in suppressing tissue damages to occur.

Acknowledgments

We gratefully acknowledge the technical assistance of Miss Kinuyo Iwata. We thank Maruha Corporation, Japan, for the generous gift of the DHA ethyl ester concentrate.

Abbreviations

ALDH, aldehyde dehydrogenase; AA, arachidonic acid; AsA, ascorbic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GSH, glutathione; GST, glutathione S-transferase; 4-HAE, 4-hydroxy-2-alkenals; 4-HHE, 4-hydroxy-2-hexenal; 4-HNE, 4-hydroxy-2-nonenal; LA, linoleic acid; MDA, malondialdehyde; ODS rat, Osteogenic Disorder Shionogi/Shi-od/od rat; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances; Vit. E, vitamin E.

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