The Effect of Docosahexaenoic Acid on Plasma Catecholamine Concentrations and Glucose Tolerance during Long-Lasting Psychological Stress: A Double-Blind Placebo-Controlled Study

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Summary We previously found that docosahexaenoic acid (DHA) intake prevented aggression from increasing at times of mental stress. In the present study, we investigated whether DHA intake modified the plasma catecholamines and cortisol of medical students during a 9-wk period of final exams. We also investigated the effects of DHA intake on a 75g oral glucose tolerance test (oGTT). Fourteen medical students participated in the present study. They were randomly allocated to either control or DHA group in a double-blind manner. Subjects in the control group (4 males and 3 females) took 10 control capsules/d, each capsule containing 280mg of mixed plant oil, and those in the DHA group (4 males and 3 females) took 10 DHA capsules/d containing 1.5g DHA for 9wk, during which subjects underwent more than 20 stressful final exams. At the start and end of the study, plasma catecholamines (epinephrine, norepinephrine (NE) and dopamine) and cortisol were measured; a 75g oGTT was also performed. There were no intra- or intergroup differences in plasma glucose concentrations. However, NE concentrations were significantly reduced after DHA administration (-31%, p<0.03). The other catecholamines and cortisol did not change significantly. The plasma ratio of epinephrine to NE increased in every DHA subject (+78%, p<0.02), and intergroup differences were significant (p<0.03). We conclude that these effects of DHA may be applied to people under long-lasting psychological stress to prevent stress-related diseases.

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Recently, several lines of evidence have indicated that the behavior of both animals and humans is affected by dietary fatty acids. Mice fed an n-3 fatty acid-deficient diet and an n-3-sufficient diet for two generations had different general behaviors as well as sensitivities to behavior-affecting drugs (1). Rats on an n-3-deficient diet showed more aggressive behavior at a brightness-discrimination test than those on an n-3-sufficient diet (2). Behavioral changes have been observed also in rhesus monkeys. N-3-deficient monkeys initiate more bouts of stereo-typed behavior in their home cages than those fed an n-3-sufficient (soybean oil as the only source of fat) control diet (3). Moreover, there is an association between the blood n-3 fatty acid status and behavior problems in children. Investigating 6–12-y-old boys, Stevens et al (4) showed that a greater number of behavior problems, temper tantrums and sleep problems were reported in subjects with lower total n-3 fatty acid concentrations in the plasma phospholipid fraction.

In this context, we performed a randomized placebo-controlled double-blind study using young Japanese students (5) whose n-3 fatty acid intake was much lower than average Japanese people (6). We administered 1.5–1.8 g docosahexaenoic acid (DHA)/d to students of the DHA group for 3 mo. We measured the aggressiveness of the students at the start and end of the study. Because of the presence of stressor at the end of the study (final exams that were of vital importance to students), the averaged aggressiveness was significantly increased in the control group, but it stayed the same as before in the DHA group. From these findings, we concluded that DHA intake prevented aggressiveness from increasing at times of mental stress (5).

Today, a considerable body of evidence links increased aggressive behaviors with decreased central nervous system (CNS) serotonin function (7, 8). Low cerebrospinal 5-hydroxyindoleacetic acid concentration, an indicator of reduced serotonin turnover rate in the frontal cortex (9), has actually been associated with hostility (10) and aggressive behavior (11). Interestingly, there is an association between plasma long-chain polyunsaturated fatty acids including DHA and 5-hydroxyindoleacetic acid in cerebrospinal fluid in healthy volunteers (12). Also, reduced CNS serotonin function increases catecholamine (CA) outflow (7). Therefore, if the aggression-controlling effects by DHA intake (5) are mediated by increased CNS serotonin function, we could expect that DHA administration might modify peripheral CA levels.

Here, we report another intervention study using medical students to test the effect of DHA administration on CA levels during a 9-wk final exam period. In the present study we also performed a 75 g oral glucose tolerance test (oGTT) considering that glucose tolerance might be enhanced during DHA treatment if CA
levels are lowered.

MATERIALS AND METHODS

Subjects. The purpose and protocol of the present study was explained to the students of the four-year class of Toyama Medical and Pharmaceutical University School of Medicine, who had to pass the final exams that lasted for 9 wk (the last week of December through the first week of March) to begin bedside courses in the next school year. Eighteen nonsmoking students (21–25 y of age, 10 males and 8 females) volunteered to enter the present study. They were all medical students and already well accustomed to blood sampling. None of the subjects had participated in any fish oil-intervention studies. They had been judged healthy through physical examinations and interviews by physicians 5 mo before entry to the study. Subjects were not taking any medicines regularly, and their glycated hemoglobin (Hb A₁c) levels were not more than 5.3%, the average being 4.7%. They were allocated to either a control (5 males and 4 females) or DHA (5 males and 4 females) group in a double-blind manner; randomization was stratified according to sexes. Written informed consent was obtained from each subject, and the study was approved by the Pharmacy and Therapeutics Committee (the ethics committee) of Toyama Medical and Pharmaceutical University.

Study design. The present study started a few days before the final exam period and ended a few days before it ended. In the final exam period, there were more than 20 important exams including four exams of pathology that were of vital importance for students because the failure rate was usually higher than other exams in the whole schooling period. Two of the four pathology exams were scheduled at the start of the final exam period, and the other two at the end. Consequently, the subjects were under heavy stress at the starting point of the present study as well as at its end. This was confirmed by interviews with them. Since all the subjects belonged to the same class, they took the same final exams, mostly on the same days. They were asked to maintain their body weights and physical activity levels and to consume their habitual diets during the study. They were instructed to take 10 capsules/day containing either DHA-rich fish oil for the DHA group (1.5 g DHA/d) or control oil for the control group for the study period of 9 wk. Three or four capsules were taken after each meal. Each capsule contained 280 mg of oil with 0.3% α-tocopherol. The fish oil used for the DHA group contained 52.1 wt% DHA, 5.9% eicosapentaenoic acid (EPA), 2.9% arachidonic acid, 7.3% oleic acid, 2.2% stearic acid, 2.5% palmitoleic acid and 7.4% palmitic acid, and was essentially odorless. The control oil was a mixture of 47% olive oil, 25% rapeseed oil, 25% soybean oil and 3% DHA-rich fish oil. The fatty acid composition of the control oil was as follows: 1.7% DHA, 4.6% α-linolenic acid, 21.7% linoleic acid, 56.7% oleic acid, 3.1% stearic acid and 8.8% palmitic acid.

At the start and end of the study, subjects were asked to come to our laboratory in the early morning without having breakfast and to refrain from drinking alcoholic
beverages during the previous day. Blood samples for CA were collected after 30 min of sitting quietly in a chair with an indwelling needle in a forearm vein so that subjects did not feel any puncture pain when samples were collected. Blood sample 1 (30 min after sitting and immediately before glucose intake) consisted of plasma from EDTA-anticoagulated blood for CA (norepinephrine (NE), epinephrine (EP) and dopamine) measurement, serum for insulin and cortisol measurement, and another plasma from blood with heparin, EDTA and NaF for glucose measurement. Plasma and serum were frozen immediately after separation. Those samples were sent to the Special Reference Laboratories (Toyama) for analysis. Packed red blood cells (RBC) were obtained from the EDTA-anticoagulated blood and washed twice with saline containing 0.002% butylated hydroxytoluene. The washed RBC were sent to the Sagami Chemical Research Center for fatty acid analysis of the total phospholipid fraction of RBC. Blood sample 2 (30 min after glucose intake) was used for glucose and insulin analysis, and blood samples 3 (60 min after glucose intake) and 4 (120 min after glucose intake) were for glucose levels alone.

**Fatty acid analysis.** The fatty acid composition of the total phospholipid fraction of RBC was determined as described previously (13) with slight modification. Briefly, total lipids were extracted by the method of Folch et al (14). The total phospholipid fraction was separated by thin-layer chromatography, and its fatty acid composition was analyzed by gas chromatography after trans-methylation.

**Food analysis.** Food intake was calculated with our own software using two sets of a food frequency questionnaire completed at the start and end of the study during the last part of 75 g oGTT. The software was a program of Kishokun (a food questionnaire program, Kan’ei-shuppan, Okayama) modified for local use. The two sets of data were averaged.

**Statistical analysis.** Data are expressed as means ± SD. Statview (Japanese version 4.5) was used for statistical analysis. With regard to the plasma ratio of EP to NE, log values were taken for normalization. Paired t-test was used for intragroup comparisons of the results at start and end of the study; intergroup differences were analyzed by two-way ANOVA. Baseline values of the two groups were compared by unpaired t-test. Differences of $p < 0.05$ were considered significant.

**RESULTS**

**Exclusion of improper subjects**

One male control subject dropped out of the study before the second blood sampling at the end of the study for personal reasons. One female control subject was excluded from the study because she suffered from mild primary shock during the first blood sampling. Two DHA subjects (one male and one female) finished the whole course of the study, but the second blood sampling at the end of the study was performed 2 d after they took their last exams. We, therefore, excluded those subjects before the double-blind code was open. The questionnaire
Table 1. Changes in the fatty acid composition (mol\%) in the total phospholipid fraction in RBC.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control ((n=7))</th>
<th>DHA ((n=7))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>16:0</td>
<td>24.9 ± 3.7</td>
<td>24.3 ± 4.6</td>
</tr>
<tr>
<td>18:0</td>
<td>13.0 ± 0.8</td>
<td>13.5 ± 1.7</td>
</tr>
<tr>
<td>18:1((n-9))</td>
<td>13.4 ± 1.8</td>
<td>13.5 ± 1.9</td>
</tr>
<tr>
<td>18:2((n-6))</td>
<td>9.5 ± 1.2</td>
<td>9.2 ± 2.1</td>
</tr>
<tr>
<td>20:4((n-6))</td>
<td>9.7 ± 2.0</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>20:5((n-3))</td>
<td>1.1 ± 0.5</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>22:5((n-3))</td>
<td>1.7 ± 0.4</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>22:6((n-3))</td>
<td>5.4 ± 1.6</td>
<td>6.1 ± 2.1</td>
</tr>
</tbody>
</table>

Subjects ingested 3 g of either control oil (97% mixed plant oil +3% fish oil) or DHA-rich oil (52% DHA) for 9 wk. There were no significant differences in baseline values between the two groups in any fatty acids. *\(p<0.01\) by paired \(t\)-test, \(p<0.01\) by ANOVA; †\(p<0.003\) by paired \(t\)-test, \(p=0.05\) by ANOVA. The DHA concentration increased in every subject in the DHA group.

performed at the end of the study showed that no subjects of either group complained of any serious adverse effects from taking capsules, and that subjects did not infer the kind of their own capsules more correctly than by chance.

*Combination of data of both sexes*

We could not find any marked differences between the two sexes in any measured items within any group. Consequently, we combined the data of both sexes.

*RBC fatty acid composition and lipid intake*

Changes in the major fatty acids in the RBC total phospholipid fraction are shown in Table 1. In the control group, there were no significant changes in any fatty acids, whereas EPA and DHA increased significantly in the DHA group. The DHA concentration increased in every DHA subject. If capsules were not taken into account, averaged DHA intake from food was 27 and 19 mg/d for the control and DHA groups, respectively (not significantly different). There were no significant differences in the average intake of total lipids and total energy between the two groups.

*Plasma glucose and serum insulin levels*

As shown in Table 2, there were no significant intra- or intergroup differences in the plasma glucose or serum insulin levels.
Table 2. Changes in glucose (mmol/L) and insulin levels (shown in parentheses, U/mL) in an oral glucose tolerance test between the start and end of 9 wk of capsule administration.

<table>
<thead>
<tr>
<th>Sampling time (min)</th>
<th>Control (n=7)</th>
<th>DHA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>0</td>
<td>5.3 ± 0.3</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(7.8 ± 4.2)</td>
<td>(7.7 ± 2.1)</td>
</tr>
<tr>
<td>30</td>
<td>8.1 ± 1.0</td>
<td>8.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(89.0 ± 58.2)</td>
<td>(69.6 ± 16.8)</td>
</tr>
<tr>
<td>60</td>
<td>7.6 ± 2.1</td>
<td>7.7 ± 2.4</td>
</tr>
<tr>
<td>120</td>
<td>6.4 ± 1.5</td>
<td>7.5 ± 2.0</td>
</tr>
</tbody>
</table>

At the start and end of the study, 75 g oGTT was performed. There were no significant differences in baseline values between the two groups at any sampling point. Glycated hemoglobin A1c levels were rather low when the subjects were not under stress; 4.8 ± 0.4% for the control group and 4.6 ± 0.3% for the DHA group.

Table 3. Changes in plasma epinephrine (pmol/L), norepinephrine (nmol/L), dopamine (pmol/L) and cortisol (nmol/L) levels between the start and end of the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=7)</th>
<th>DHA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>187 ± 121</td>
<td>166 ± 117</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>2.01 ± 0.67</td>
<td>1.84 ± 0.70</td>
</tr>
<tr>
<td>Dopamine†</td>
<td>75.8 ± 47.1</td>
<td>37.3 ± 12.4</td>
</tr>
<tr>
<td>Cortisol</td>
<td>384 ± 80</td>
<td>287 ± 113**</td>
</tr>
</tbody>
</table>

Blood samples for hormone measurement were collected after 30 min of sitting quietly with an indwelling needle in a forearm vein of the subjects at the start and end of the study. There were no significant differences in baseline values between the two groups. *Significantly different from baseline (p < 0.03 by paired t-test); there was a tendency that the decrease in the DHA group (−31%) was greater than that of the control group (p < 0.10 by ANOVA). † The detection limits of dopamine were 33 pmol/L; the value of 33 was tentatively assigned for values under the detection limits. **p < 0.10 by paired t-test.
Fig. 1. Changes in plasma EP/NE ratios before and after capsule administration. Subjects took 10 capsules of either placebo or DHA-rich fish oil for 9 wk. Data were log-transformed for normalization. The averaged increase in the DHA group was +78% (10%–188%, mean – SD–mean + SD). * $p < 0.02$ by paired t-test, † $p < 0.03$ by ANOVA.

**CA and cortisol levels**

As shown in Table 3, there were no significant differences in dopamine, EP and cortisol levels between the two groups. NE levels decreased significantly in the DHA group ($-31\%, \ p < 0.03$ by paired t-test, $p < 0.10$ by ANOVA), whereas the levels were stable in the control group. The ratio of EP to NE was increased in every DHA subject ($+78\%, \ p < 0.02$ by paired t-test); the increases were significantly different from the controls ($p < 0.03$ by ANOVA, Fig. 1).

**DISCUSSION**

The duration of the present study was 9 wk. We could not find a longer period of stress, essentially identical to all subjects, in any homogeneous population accustomed to blood sampling and that might volunteer to enter the present study. That was why we chose this 9-wk period of continuous stress. Since the subjects had known the coming 9-wk period of considerable stress long before the start of the present study, they had already been under considerable psychological stress when we started administering capsules. Indeed, their baseline values of plasma NE
were already rather high (Table 3), which means DHA supplementation was able to modulate CA metabolism even after the appearance of stress. This point is noteworthy when applying the results of the present study to daily life where a countermeasure to stress is usually taken only after it starts.

The most important finding in the present study is that plasma EP/NE ratios were significantly increased in the DHA group as compared to the control group. Recently, Christensen and Schultz-Larsen (15) measured basal plasma CA levels of 804 (412 males) people of 70y of age and followed them for 7y. During that period 115 males died. An analysis of plasma CA levels revealed that 10% of the male subjects with high plasma EP and low NE levels had died 7y later, whereas 50% of the male subjects with low plasma EP and high NE had died (15). Plasma EP levels increase during acute mental stress, but no close correlation has been established between plasma EP levels and chronic stress (16). Indeed, psychological stress in patients with duodenal ulcer did not increase plasma EP levels but increased NE levels (17). Basal plasma EP and cortisol levels may represent the adrenal capacity to cope with stress; therefore, an inadequate response of EP to chronic psychological stress may be harmful (16). The plasma CA balance in the DHA group became similar to that of the longevity group of 70-y-old males (15) and did not suggest relative EP insufficiency even after long-lasting psychological stress.

As can be seen from Table 3, the change in plasma NE levels in the DHA group was the major contributor to the increase in EP/NE ratios. Christensen and Schultz-Larsen (15) reported that the mean basal NE values of males who died of cardiovascular diseases (2.43 nmol/L) were significantly higher than those of male survivors (1.95 nmol/L). Consequently, a decrease in plasma NE as shown in the DHA group is likely beneficial to the cardiovascular system. There are several fish oil intervention trials measuring plasma NE. Singer et al (18) randomly allocated 47 hypertensives to the following three groups and treated them for more than 36 wk: Group P was treated with propranolol alone; Group F with fish oil alone; and Group P+F, which was first treated with propranolol, then with propranolol plus fish oil, and finally with propranolol plus fish oil placebo. They found that, after fish oil supplementation, plasma NE levels were reduced to 80% of the baseline value in Group F and 56% of that in Group P+F. The reduction levels of plasma NE were similar to our study (18). However, their findings were exceptional because no other controlled studies have found any significant decreases in plasma NE during fish oil treatment with normal subjects (19, 20) or mild hypertensives (19, 21–23). Although experimental protocols differed from study to study and no direct comparison of these studies seemed possible, the plasma NE levels before fish oil administration were rather high in the study of Singer et al (18) that found the NE-reducing activity of fish oil. Our subjects had also rather high baseline plasma NE levels. Consequently, high pre-values of NE might be one of the factors that determined whether fish oil reduces NE or not. In the present study, we carefully took blood samples for CA, and subjects were medical students.
accustomed to blood sampling. High plasma NE values at the start of the present study were due to the nature of a protocol that had one distinctive feature: the presence of long-lasting stressor from the beginning. This is the first report that has shown that DHA intake modifies plasma CA levels even in normal subjects.

At present the detailed mechanism of how plasma NE levels were reduced by DHA administration is unclear. Available evidence indicates the involvement of the CNS serotonin function as described in the introduction. We therefore suggest that DHA administration restored the depressed CNS serotonin function caused by the stress of the final exams to its normal state, which in turn reduced NE output.

Semafuko et al (24) fed rats diets containing 16 weight % of different oils since pregnancy, and measured NE contents and release from perfused rat hearts. They found that sunflower oil (71% linoleic acid and essentially no α-linolenic acid) caused a significant increase in cardiac NE content and release as compared to coconut oil. Although they did not measure the fatty acid composition of the heart, it is likely that sunflower oil-fed rats had less n-3 fatty acids in the heart than coconut oil (mostly saturated fatty acids)-fed rats because of the competition against n-3 fatty acids by a large amount of linoleic acid in the sunflower oil. Consequently, DHA intake might affect plasma NE levels, at least partly, by modulating the DHA concentrations of peripheral tissues. Young and Walgren (25) has also shown similar effects of another high-linoleic oil, safflower oil, with rats. They fed rats either safflower oil, coconut oil or medium-chain triglycerides; after 14 d on the diet, in vivo synthesis of NE in the heart was likewise higher in safflower oil-fed rats than in coconut oil- or medium-chain triglycerides-fed rats.

Fish oil has not been recommended for diabetic patients without some concern because some but not all of the early studies (mostly non-controlled) of fish oil intervention in diabetic patients showed adverse effects on glucose tolerance (26). However, controlled studies have supported the notion that habitual fish or fish oil intake may be protective against the development of impaired glucose tolerance in a non-diabetic population. Additionally, those controlled studies performed more recently in NIDDM patients (26) and hypertriglyceridemic patients with or without impaired glucose tolerance or diabetes (27) have denied adverse effects on glucose tolerance. When we planned the present study, we anticipated fish oil administration might reduce one or some of the counter-hormones. If so, we could expect preventive effects of DHA against the deterioration of glucose tolerance by a long-lasting psychological stressor. As shown in Table 2, there were no significant changes within any group or between the two groups. Plasma glucose levels before capsule administration were rather high considering that the HbA1c of subjects was no more than 5.3%. This was most likely due to the final exams. Although we could not find any ameliorating effect of DHA-rich fish oil on glucose tolerance, our findings further support the notion that fish oil does not impair glucose tolerance.

In conclusion, DHA-rich fish oil increases plasma EP/NE ratios by decreasing plasma NE levels, for the most part, at times of long-lasting psychological stress, and this effect may be applied to stress-related diseases.
The authors are very grateful to the volunteers of the present study. They volunteered to participate in time- and painstaking blood sampling sessions when they badly needed time to memorize as much as possible for preparing for final exams and their survival at a medical school was at stake. The present study was supported in part by grants from the Japan-United States Cooperative Medical Science Program, and the Special Coordination Funds for Promoting Science and Technology of the Science and Technology Agency of Japan.

REFERENCES


