Fish oil supplementation reduces excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and the 11-dehydrothromboxane B_2/2,3-dinor-6-oxo-$PGF_{1\alpha}$ excretion ratio in adult men

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Dietary supplementation with a fish oil concentrate (FOC) reduced the endogenous synthesis of prostacyclin (PGI_2), as measured by the excretion of its major urinary catabolite, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (PGI_2-M). Thirty-four healthy men (24–57 years old) were given controlled diets and supplements that provided 40% of the energy from fat and a minimum of 22 mg/d of α-tocopherol for two consecutive experimental periods of 10 weeks each. During the experimental periods, the men received capsules containing 15 g/d of a placebo oil (PO) (period 1) or 15 g/d of the FOC (period 2). In addition to the PO or FOC, capsules contained 1 mg of α-tocopherol per g of fat as an antioxidant. The average daily excretion of PGI_2-M during the last week of FOC supplementation (period 2) was 22% less (P = 0.0001) than at the end of the first period. These results are at variance with those reported in comparable human studies conducted by other investigators during the middle and late 1980s. A 20% reduction (P = 0.003) in the 11-dehydrothromboxane B_2 to 2,3-dinor-6-oxo-$PGF_{1\alpha}$ excretion ratio at the end of period 2 in this study demonstrates that a shift of the n-6 to n-3 polyunsaturated fatty acid ratio from 12.5 to 2.3 brings about a substantial modulation of the eicosanoid system. (J. Nutr. Biochem. 4:695–698, 1993.)

Keywords: n-3 fatty acids; prostacyclin; thromboxane-to-prostacyclin ratio; in vivo synthesis; urinary excretion

Introduction

Eicosanoids have been implicated in inflammatory processes, immunological-allergic reactions, blood pressure regulation, oncogenesis, and atherosclerosis and its complications.[1–3] Dietary modifications, if of sufficient magnitude, can significantly modulate the eicosanoid profile and thus influence those processes. Prostacyclin (PGI_2) is a major endogenous vasodilator, an inhibitor of platelet aggregation, and an important regulator of vascular cholesterol content.[4–6] Dietary alteration of the thromboxane/prostacyclin synthetic balance may be one of the mechanisms responsible for the lower incidence of atherothrombotic events observed in populations consuming relatively large quantities of marine foods.[7,8] Accordingly, urinary thromboxane and prostacyclin metabolites are considered indexes of the impact of dietary interventions on the thrombogenic potential.

Conflicting reports have appeared in the literature on the effect of fish oil on the endogenous synthesis of prostacyclin in humans as measured by the excretion of its major urinary metabolite, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (PGI_2-M).[8–14] Divergence of results is possibly rooted in unresolved problems in analytical methodology. In the present study, we determined the effect of an anchovy oil supplement on the excretion of PGI_2-M and the 11-dehydrothromboxane B_2 to PGI_2-M excretion ratio in healthy men.

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Methods and materials

Subjects and diets

This trial is part of a larger study designed to determine the effects of long-chain n-3 fatty acids on a broad spectrum of hematological, immunological, and metabolic variables, including eicosanoid metabolism. The design of the study was published previously,[15,16] and the experimental details described below are those relevant to the results presented in this paper.

Male volunteers, 24–57 years of age, were recruited from the Beltsville, Maryland area of the United States. They were screened to exclude smokers, those with health problems, regular users of prescription drugs and alcohol, and those with unusual dietary habits. This preliminary screening was followed by a complete medical evaluation. Aspirin, aspirin-containing medications, and other anti-inflammatory drugs were not permitted during the study. Acetaminophen was the only analgesic approved for occasional use. Forty subjects were ultimately selected for the larger study, but data for this segment of the study are available from 34 subjects only. All protocols were approved by the Institutional Review boards of Georgetown University School of Medicine and the National Cancer Institute, U.S. Department of Health and Human Services.

A basal diet was developed from common foods to be fed together with 15 g/d of either a placebo oil (PO) or a fish oil concentrate (FOC). The PO was a blend of 48% stripped lard, 40% beef tallow, and 12% corn oil. The FOC (ROPUFA-50%) was a concentrate of refined anchovy oil. Both oils were supplied by Hoffman-La Roche, Inc. (Nutley, NJ USA) and administered in 15 soft gelatin capsules per day. The aggregate of basal diet and oil supplement provided 40% of the energy from fat. The fatty acid composition of the two supplements and the daily nutrients and fatty acid intakes during the placebo and the fish oil periods have been published.[15,16] During the study, subjects were switched from PO to FOC. Replacing PO with FOC shifted the n-6 to n-3 PUFA ratio from 12.5 to 2.3. Total α-tocopherol intake from the basal diet and the two supplements was at least 22 mg/d. Body weight was maintained by adjusting caloric intake. The meals were prepared in the Human Study Facility of the Beltsville Human Nutrition Research Center.

All subjects received the controlled basal diet for a total of 20 weeks, which were divided into two periods of equal length. During period 1, they consumed 15 g/d of PO, 7 g at breakfast and 8 g at dinner. During period 2, the subjects consumed 15 g/d of FOC, administered in two doses as was the PO.

Urine collection

Three consecutive 24-hr urine samples were collected during the last week of each period. Samples were collected in silanized glass bottles and kept on ice during the collection period. After the 24-hr collections were completed, 2% portions of each 24-hr collection were pooled (72-hr urine pools) and stored at −22° C until analyzed.

Measurement of 2,3-dinor-6-oxo-$PGF_{1\alpha}$

Analyses of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ (PGI_2-M) were carried out in duplicate on aliquots of the 72-hr urine pools. This enabled us to assess the mean daily excretion of PGI_2-M during the 72-hr periods.

Urine (20 mL) was diluted with 10 mL of water, spiked with 10 ng of 2,3-dinor-6-oxo-[19,19,20,20-$^2H_4$]$PGF_{1\alpha}$ and acidified to pH 3 with dilute HCl. After standing for 1 hr at room temperature, equal volumes of acidified urine were loaded onto two Chem Elut CE1020 cartridges (Varian Associates, Harbor City, CA USA). After 3–5 min, 40 mL of CH_2Cl_2/AcOEt (4:1, vol/vol) were passed through each column, eluates were collected, and solvents were evaporated. Each residue obtained from solvent evaporation was treated with 150 μL of MeOH, 4 mL of alkalized (NaOH) water (pH 11–12), and the pH was adjusted to 10 if necessary. After standing for 15 min, the solutions were shaken with 5 mL of AcOEt for 60 sec. The upper phases obtained after centrifuging were discarded, and the pH of the lower phases was adjusted to 3 with 0.1 N HCl. After 15 min the acidified solutions were shaken with 5 mL of CH_2Cl_2 for 60 sec, then centrifuged. The upper phases were discarded; the lower (organic) phases were evaporated, and the residues were treated with 3 drops of triethylamine/pyridine/water (1:10:10, vol/vol/vol). After stirring, the solutions were heated at 55° C for 30 min, then treated with 3 drops of EtOH, and evaporated to dryness under dry N_2. The residues were treated with 20 μL of diisopropylethylamine and 20 μL of 35% pentafluorobenzyl bromide (Pierce Chemical Co., Rockford, IL USA) in acetonitrile and heated at 40° C for 30 min. The residues from solvent evaporation were dissolved in 25 μL of MeOH and placed on two silica gel G TLC plates that were 5 cm × 20 cm and 500 μm in thickness (Analtech, Inc., Newark, DE USA). The plates were developed with AcOEt/2,2,4-trimethylpentane (4:1, vol/vol) saturated with 0.1% aqueous acetic acid. Two-cm bands were scraped off where indicated by a reference plate (R_f ca. 0.3). Silica was extracted with 3 mL of 10% MeOH in AcOEt, the solutions were evaporated to dryness, and the residues were treated with 3 drops of a saturated solution of methoxylamine hydrochloride (Aldrich Chemical Co., Milwaukee, WI USA) in pyridine. After heating at 40° C for 60 min, the crystalline residues obtained after thorough evaporation of pyridine were extracted three times with diethyl ether. The ether extracts were evaporated. The residues were treated with 20 μL of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Pierce) in pyridine (1:1, vol/vol) and heated at 40° C for 15 min. The dry residues obtained after evaporation were dissolved in 20 μL of 5% BSTFA/pyridine (1:1, vol/vol) in 2,2,4-trimethylpentane and were thus ready for injection into the GC-MS system. Typically, 2-μL portions were injected.

Gas chromatography-tandem mass spectrometry

Gas chromatography was done with a Varian 3400 instrument operated in the splitless mode with a 30 m × 0.25 mm DB-1 (J. & W. Scientific, Inc., Rancho Cordova, CA USA) capillary column, phase thickness 0.25 μm. Injector temperature was 250° C. The oven was kept at 100° C for 0.5 min after injection, then it was heated to 300° C at the rate of 27° C/min and held at 300° C for 10 min. The chromatograph was coupled to a Finnigan-MAT TSQ-70B mass spectrometer operated in the negative ion detection mode with methane used as ionization gas. The interface temperature was 300° C, and the ion source temperature was 150° C. Methane was supplied at a pressure of 7 Torr, argon collision cell pressure 1 mTorr, collision energy 35 eV, electron energy 70 eV, emission current 0.2 mA. For tandom mass spectrometric analysis we used the pair of daughter fragments at m/z 240 and m/z 244 (P − (90 × 3 + 32 + 44)) of the parent ions at m/z 586 and m/z 590 ([M-PFB]⁻). The interassay coefficient of variation for the PGI_2-M assay is 4.0%.

Statistical analysis

We analyzed the data as paired observations, i.e., the difference between PGI_2-M excretion rate at the end of period 1

(placebo) compared with the rate at the end of period 2 (fish oil). The mean difference was evaluated with the Student's *t* test. The mean difference between the 11-dehydrothromboxane B_2/PGI_2-M excretion ratios was evaluated with the nonparametric signed rank test. 11-dehydrothromboxane B_2 (11-$DTXB_2$) excretion data from each subject have been reported previously.[17]

Results

Intake of fish oil in this study was associated with a reduction of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ excretion. The mean PGI_2-M excretion (ng/24 hr) during the last week of periods 1 and 2 was 156.2 ± 65.2 (n = 34) and 122.3 ± 56.1 (n = 34), respectively. The mean difference (33.9 ng/24 hr) was evaluated with the Student's *t* test, which indicated that the reduction in PGI_2-M excretion was highly significant (P = 0.0001). Only five of the 34 participants showed an increase in PGI_2-M excretion at the end of period 2 (fish oil). The mean 11-dehydrothromboxane B_2 to PGI_2-M excretion ratio during the last week of periods 1 and 2 was 6.0 ± 4.7 and 4.8 ± 2.0, respectively. Evaluation of the mean difference (1.24) with the signed rank test indicated that the difference was significant (P = 0.003). The 11-dehydrothromboxane B_2/PGI_2-M ratio decreased in 25 participants and increased in only nine at the end of period 2. PGI_2-M excretion rates and 11-dehydrothromboxane B_2 to PGI_2-M ratios of each subject for the two feeding periods are shown in *Table 1*.

Table 1 2,3-Dinor-6-oxo-$PGF_{1\alpha}$ (PGI_2-M) excretion rates (ng/24 hr) and 11-dehydrothromboxane B_2 to PGI_2-M excretion ratios during the last week of the indicated diet[a,b]

Subject	PGI_2-M		11-$DTXB_2$ to PGI_2-M	
	Placebo	Fish oil	Placebo	Fish oil
1	242	135	3.3	4.0
2	140	109	6.9	5.6
3	233	196	4.0	3.2
4	192	228	6.1	3.9
5	129	137	4.8	3.3
6	150	135	4.7	5.0
7	115	74	3.4	9.9
8	190	101	3.9	3.4
9	154	99	6.9	7.2
10	155	100	6.0	6.6
11	75	43	0.9	2.6
12	108	77	9.2	8.4
13	83	87	4.7	3.6
14	179	146	7.9	3.8
15	123	81	6.3	4.8
16	71	59	9.1	6.5
17	99	75	12.2	8.4
18	134	81	4.4	3.8
19	92	72	8.9	8.8
20	132	95	8.4	6.4
21	87	76	28.8	6.2
22	203	117	5.2	4.3
23	164	152	4.6	3.0
24	202	144	4.4	3.8
25	164	108	5.5	4.3
26	138	133	5.8	4.1
27	247	193	2.5	2.4
28	161	103	3.0	5.7
29	246	250	1.6	1.3
30	381	273	2.8	2.0
31	184	175	3.6	2.7
32	97	100	6.8	4.9
33	179	162	3.6	3.8
34	62	41	5.4	5.6

[a]Mean daily excretion during a 72-hr period. For details, see Methods and materials.
[b]11-Dehydrothromboxane B_2 (11-$DTXB_2$) excretion data are from A. Ferretti et al.[17]

Discussion

The effect of dietary long-chain n-3 polyunsaturated fatty acids (PUFA) on the synthesis of prostacyclin has been studied extensively in humans and animal models during the last 12 years, but the results have not be consistent. In 1981, Hornstra et al.[18] reported that feeding cod liver oil to rats leads to a considerable reduction in ex vivo formation of vascular prostacyclin. Morita et al.[19] found that aortas from eicosapentaenoic acid-fed mice produce significantly less PGI_2 than controls. Similar studies with animal models conducted during the same period confirmed these observations.[20,21] The results of these early ex vivo investigations are in sharp contrast to those obtained with humans during most of the 1980s and early 1990s. Fisher et al.[9,10] reported that feeding volunteers 40 mL/d of cod liver oil for 24 days (n=6) or 750 g/d of mackerel for three days (n=3) resulted in a significant increase of in vivo production of PGI_2 as measured by excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$. The authors also provided the first direct evidence for in vivo formation of PGI_3 in humans. In contrast to the results of Fischer's study, von Schacky et al.,[11] in a study with six subjects, found that ingestion of cod liver oil at the rate of 10–40 mL/d for 20 weeks left the excretion level of PGI_2-M virtually unaltered. Knapp et al.[12] found that excretion of PGI_2-M in six male atherosclerotic patients fell within the first week of administration of 50 mL/d of Max-EPA (containing 10 g of eicosapentaenoate). The decline continued throughout the study (4 weeks); however, PGI_2-M excretion was not depressed in seven healthy control subjects. Three years later, Knapp and FitzGerald[13] determined that Max-EPA in daily doses of up to 50 mL over a period of 4 weeks did not significantly influence PGI_2-M excretion in eight subjects with essential hypertension. In a multicenter human study, Hornstra et al.[14] found that consumption of 135 g/d of mackerel paste for 6 weeks was not associated with a decrease in the production of PGI_2 as reflected by the excretion of PGI_2-M. One series of measurements, however, indicated a striking increase during the experimental period. Concomitantly, formation of a considerable amount of PGI_3 was inferred from the appearance of 2,3-dinor-6-oxo-17,18-dehydro-$PGF_{1\alpha}$ in urine during mackerel consumption. Finally, Fischer and Weber[8] observed that synthesis of PGI_2 in 20 Eskimos, who normally ingest large amounts of n-3 PUFA, was significantly higher than in 20 age- and sex-matched Danes consuming a Western diet. It is possible that this divergence of results from compara-

ble human studies is due, at least to some degree, to pitfalls in the analytical methods used for the measurement of 2,3-dinor-6-oxo-$PGF_{1\alpha}$. Indeed, we found that the analysis of PGI_2-M presents peculiar problems of reproducibility, possibly stemming from the fact that the molecule can exist in at least three forms in dynamic equilibrium.[22,23] In the present study we conducted all the analyses in a paired fashion, i.e., urine samples collected during periods 1 and 2 from the same subject were processed simultaneously and analyzed by GC-tandem mass spectrometry the same day. Thus, we avoided any adverse consequences of day-to-day changes in instrument performance.

We believe that our study is unique because of the large number of participants involved and because of the length of the dietary treatment. The results demonstrate that decreasing the n-6 to n-3 fatty acid ratio from 12.5 to 2.3 reduces the excretion of PGI_2-M and, presumably, reduces endothelial synthesis of PGI_2. Only five of the 34 subjects that completed the study showed a minimal increase in PGI_2-M excretion at the end of period 2. The decrease of the 11-dehydrothromboxane B_2 to PGI_2-M excretion ratio indicates that, on a percentage basis, 11-dehydrothromboxane B_2 reduction was greater than that of PGI_2-M. Furthermore, fish oil ingestion induces the synthesis of the vasoactive PGI_3,[9,10] thus offsetting the effect of PGI_2 reduction. Conversely, formation of the biologically inactive thromboxane A_3[10] is of no consequence. These three circumstances all operate in the direction of enhancing the antithrombotic effect of fish oil. Hornstra et al.[14] suggested that measurements of urinary metabolites of PGI_2 may not reflect an accurate estimate of vascular PGI_2 synthesis because prostacyclin synthesized in other (rat) tissues, notably stomach and intestines, may contribute to urinary PGI_2-M, and fish oil may influence the relative catabolic rates of PGI_2 and PGI_3. If these circumstances indeed occur in humans, the physiological relevance of diet-induced changes of PGI_2-M (as well as 2,3-dinor-6-oxo-17,18-dehydro-$PGF_{1\alpha}$) excretion would be more difficult to assess.

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