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Fish Oil and Oxidative Stress by Inflammatory Leukocytes

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We investigated the effects of diets with different fatty acid composition upon the oxidative stress of inflammatory leukocytes of rats. After weaning, two groups of rats were fed isoenergetic semipurified diets for five weeks containing 5% of corn oil or menhaden oil. Polymorphonuclear leukocytes from rats fed menhaden oil diet incorporated n-3 polyunsaturated fatty acids into phospholipid membranes at the expense of arachidonic acid. These cells showed diminished superoxide production and, as a consequence, the total antioxidant status in the inflammatory exudate was increased. However, nitric oxide production was not affected by diet. Free malondialdeyde concentration increased in the exudate because of lower mitochondrial activity. These results add new aspects that help clarifying the anti-inflammatory mechanisms of n-3 polyunsaturated fatty acids.

Keywords: Corn oil, inflammation, lipid peroxidation, nitric oxide, n-3 PUFA, superoxide

Abbreviations: AA, arachidonic acid; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HbO_2, oxyhaemoglobin; MDA, malondialdehyde; MO, menhaden oil; L-NIO, N-imino-ethyl-L-ornithine; $^{\cdot}NO$, nitric oxide; $O_2^{\cdot-}$, superoxide; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PMA, phorbol 12-myristate 13-acetate; PMNLs, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; TAS, total antioxidant status

INTRODUCTION

The effect of dietary manipulation on inflammatory mediators is a tool of considerable interest. Fish oil rich in n-3 polyunsaturated fatty acids (PUFA) acts by diminishing synthesis of arachidonate metabolites and by competitive inhibition of cyclooxygenase[1] and lipoxygenase pathways,[2] though other mechanisms may also be involved. Polymorphonuclear leukocytes (PMNLs) accumulate in the area of the acute inflammation, giving a clue to the pathophysiology of this disorder. When n-3 PUFA are incorporated into the incubation medium, human stimulated PMNLs produce less superoxide ($O_2^{\cdot-}$).[3] It has also been demonstrated that dietary fish oil supplementation for six weeks in human volunteers results in a decrease in $O_2^{\cdot-}$ production by isolated PMNLs,[4] but the effect of n-3 PUFA upon the production of nitric oxide ($^{\cdot}NO$) by PMNLs is unknown. Cells from an inflammatory exudate generate $O_2^{\cdot-}$ and $^{\cdot}NO$[5] and both radicals can react to give rise to peroxy-

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nitrite. However, there are few studies upon the production of $O_2^{\cdot-}$ and $^{\cdot}NO$ in incubation conditions that minimise the reaction between them.[5] This study was designed to examine the effects of feeding rats with diets rich in n-6 PUFA and n-3 PUFA upon parameters of the inflammatory response such as the arachidonate-derived prostaglandin E_2 (PGE_2) and the production of $O_2^{\cdot-}$ and $^{\cdot}NO$ by cells from an inflammatory exudate of rats. Other oxidant and antioxidant parameters of the inflamed area were also studied to gain an insight into the reaction pathways.

MATERIALS AND METHODS

Animals and Diets

After weaning, twelve male Sprague-Dawley rats were divided into two randomised groups and fed for five weeks with two isoenergetic semi-purified diets (Table I) containing 50 g of fatty acids/Kg diet, as corn oil (CO) or menhaden oil (MO) (Sigma Chemical Co. St. Louis, MO). Room temperature was maintained at 21–23°C, with 40–60% humidity. The room was lit on a 12-h light:dark cycle. Rats were weighed weekly. Diets provided 75 IU of α-tocopherol/Kg. No other antioxidants were present in the oils or diets. Diets were manufactured weekly and were stored frozen under vacuum. Food was provided to the rats daily and food remains were removed daily. Experimental protocols were reviewed and approved by the Committee of the Faculty of Biology in accordance with the EC guidelines.

TABLE I Composition of semipurified diets

Component	Amount g/Kg diet
Casein[1]	220
DL-Methionine	1
Mineral mix[2]	35
Vitamin mix[3]	10
Cellulose	20
Cornstarch	436
Sucrose	228
Oil	50

[1] Vitamin free delipidated
[2] AIN-93M (ICN Pharmaceuticals, Costa Mesa, CA)
[3] AIN-93VX (ICN Pharmaceuticals, Costa Mesa, CA)

Laboratory Techniques

A granuloma was induced by subcutaneous administration of 6 ml of air, followed 24 h later by 4 ml carrageenin 2% (w/v) in sterile saline into the dorsum of rats as previously described.[6] One day after injection, the exudate present in the inflammatory pouch was harvested with a heparinized syringe and was centrifuged at 800*g* for 10 min. The supernatant was used for measurement of PGE_2, malondialdehyde (MDA), total antioxidant status (TAS), α-tocopherol and retinol concentrations. Cells were used for measurement of $O_2^{\cdot-}$ and $^{\cdot}NO$ production, mitochondrial activity, and membrane fatty acid analysis. Cells were counted after hypo-osmotic lysis of contaminating erythrocytes, resuspended in phosphate-buffered saline (PBS) pH 7.4 without Ca^{2+} or Mg^{2+}, and washed twice in the same buffer. Differential cell counts were performed microscopically after non-specific esterase staining, giving 84% PMNLs, 11% monocytes, and 4.5% lymphocytes and mast cells.

For PGE_2 measurement, the supernatant from the exudate was processed through C18 solid phase Sep-Pack cartridges (Waters, Milford, MA), previously activated with methanol and acidified water (pH 4.0). Eluates from 5 ml of methanol were evaporated under nitrogen and the resulting dried residues were resuspended for the PGE_2 immunoassay analysis (Cayman Chemical Co, Ann Arbor, MI). Free MDA was measured following the HPLC technique of Kawai *et al.*[7] using a 5 μm Lichrocart Lichrosphere RP-18 column (125 mm × 4 mm i.d.) (Merck, Darmstadt, Germany) with a mobile phase consisting of 0.01 M sodium dihydrogephosphate/acetonitrile/isopropanol (50:20:30; v/v/v), and detection at 315 nm. Samples of the supernatant from the exudate reacted with *p*-nitrophenylhydrazine hydrochloride at pH

3.7 at room temperature and 20 µl of the reaction mixture were injected into the chromatograph (Merck-Hitachi). TAS was analysed by using a commercial kit (Randox Laboratories, Crumlin, North Ireland) following the suppliers' directions. The concentration of α-tocopherol was measured by the Burton *et al.* technique[8] with slight modifications that also allowed us to measure retinol. HPLC separation was performed on a 5 µm Lichrocart Lichrosphere RP-18 column (250 mm × 4.6 mm i.d.) (Merck), using retinyl acetate (Sigma) as an internal standard; samples were eluted with methanol at a flow rate of 2 ml/min and their ultraviolet absorption at 295 nm was recorded.

The viability of inflammatory cells before incubation was above 90% as assessed by the Trypan blue exclusion test in both CO and MO fed rats. Inflammatory cells (1×10^6 cells/tube) from the exudate were incubated for 1 h in PBS, pH 7.4 with 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma). Generation of $O_2^{\cdot-}$ was assayed by measuring superoxide dismutase (SOD)-inhibitable reduction of 0.15 mM cytochrome *c* (horse heart type VI. Sigma) in the presence of 0.6 mM N-imino-ethyl-L-ornithine (L-NIO) (Cookson Chemicals LTD, Southampton, United Kingdom). This inhibitor of NO synthase was included in order to avoid underestimation of $O_2^{\cdot-}$ production.[5] Production of $^{\cdot}NO$ was determined by the oxyhaemoglobin (HbO_2) method[9] as modified by Murphy *et al.*[10] in the presence of 15 µM HbO_2, 60 U/ml SOD, 100 U/ml catalase, and 0.6 mM L-arginine (all from Sigma) vs a blank preincubated with 0.6 mM of L-NIO for 30 min. Reactions were stopped by immersing the tubes in ice and cold centrifugation. The supernatants were used to measure the formation of $O_2^{\cdot-}$ at 550 nm (E_{550} = 21.1 mM/cm) and $^{\cdot}NO$ as the change in the absorbance at 578 nm vs 592 nm ($E_{578-592}$ = 11.2 mM/cm). The generation of $O_2^{\cdot-}$ and $^{\cdot}NO$ are expressed as nmol/h/10^6 cells. The production of these radicals was almost entirely due to PMNLs of the inflammatory cell preparation. We also evaluated the mitochondrial activity of inflammatory cells after incubation with WST-1 reagent and PBS for 1 h in tubes designed for this purpose by using a commercial kit from Boehringer Mannheim (Mannheim, Germany). The test is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases.

Membranes from 0.5 ml of the pellet were obtained by haemolysis with distilled water in the presence of 250 µl of EDTA (0.375 M) and 250 µl of ascorbic acid (0.075 M) followed by 5 min centrifugation in eppendorf tubes. Pellets were washed three times in the same solution. Phospholipid fatty acid composition of inflammatory cell membranes was studied by gas chromatography. Total lipids were extracted with chloroform/methanol 2:1 (v/v).[11] Samples were subjected to thin layer chromatography on silica gel plates (Merck) using hexane/diisopropyl ether/acetic acid (80:20:1; v/v/v). After elution, total phospholipids were extracted with methanol/benzene (4:1; v/v)[12] methylated with acetylchloride and heated to 100°C for 60 min. Fatty acid methyl esters dissolved in hexane were analysed using a Perkin Elmer Autosystem gas chromatograph (Perkin-Elmer, Norwalk, CO) equipped with a SP-2330 silica capillary column (30 mm × 0.25 mm i.d.) (Supelco, Bellefonte, PI). Peaks were quantified by comparison of equivalent chain lengths with those of standard fatty acid methyl esters (Sigma). Results are expressed as percentages of total fatty acid peaks retrieved by gas chromatography.

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM) of six rats. Statistical analysis was done using the Student's *t*-test comparing values of rats fed MO vs values of rats fed CO (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

RESULTS

The weight increase was the same in both groups of rats. There were no statistically significant dif-

ferences in the weight of granuloma (7.9 ± 1.5 g and 6.7 ± 1.4 g for CO and MO fed rats respectively) or in the volume of exudate (3.8 ml approximately) or number of cells (225×10^6 cells per exudate approximately).

The data in Table II indicate the values of the parameters measured in the supernatant of the exudate. PGE_2 concentration was reduced (62.5% decrease) in MO fed rats. MDA concentration was significantly higher (363 % increase) in MO than in CO fed rats. TAS was increased in MO fed rats, and concentrations of α-tocopherol and retinol were approximately the same in both groups of rats (1.0 μmol/L and 0.35 μmol/L respectively).

Generation of $O_2^{\cdot-}$ (Fig. 1) by inflammatory cells was significantly suppressed after five weeks of MO diet when compared to CO and represented a reduction of 31% ($P < 0.05$). The $^{\cdot}NO$ released (Fig. 1) was the same in both diets. Mitochondrial activity assessed by the WST-1 test after incubation decreased by 22% in rats fed MO ($P < 0.05$) (data not shown).

The differences in the phospholipid fatty acid composition of inflammatory cell membranes (Table III) reflected the different fatty acid composition of the lipids of both diets. The percentage of oleic acid (18:1n-9) in rats fed MO was higher (16.26%) than in rats fed CO (11.82%) ($P < 0.01$). Conversely, the percentage of linoleic acid (18:2n-6) and arachidonic acid (AA) (20:4n-6) was significantly lower in rats fed MO ($P < 0.001$). Eicosapentaenoic acid (EPA) (20:5n-3), and docosahexaenoic acid (DHA) (22:6n-3), the major n-3 fatty acids derived from MO, represented 6.62% and 2.32% of the total fatty acids in MO fed rats.

TABLE II PGE_2, Malondialdehyde (MDA), Total Antioxidant Status (TAS), α-tocopherol, and Retinol from the Exudate of Rats Fed Corn Oil (CO) or Menhaden Oil (MO) for Five Weeks

	CO	MO
PGE_2 (pmol/L)	187,8 ± 30,3	70,3 ± 11,3*
MDA (μmol/L)	23,8 ± 6,8	110,2 ± 10,7***
TAS (mmol/L)	1,4 ± 0.1	2,2 ± 0.05***
α-tocopherol (μmol/L)	1,4 ± 0,4	1.0 ± 0,1
Retinol (μmol/L)	0,5 ± 0,2	0,3 ± 0,1

Values are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (see text)

DISCUSSION

Dietary administration of fish oil to rats for five weeks leads to incorporation of EPA and DHA into phospholipids of inflammatory cell membranes followed by a reduction in AA. These changes can affect cell functioning in a variety of ways and explain the reduction in PGE_2 concentration as has previously been reported.[1] Lee *et al.*[2] also described the inhibition of the product generation by the 5-lipoxygenase pathway of neutrophils and monocytes, and an attenuation of the LTB_4-mediated chemotaxis and endothelial-cell adherence of neutrophils. However, we observed no reduction in the number of inflammatory cells in the exudate of rats fed MO, nor a reduction in viability as assessed by Trypan blue exclusion.

Although the volume of the exudate and the number of inflammatory cells were the same in both groups of rats, free MDA increased in the exudate of rats fed MO. Besides being a secondary product of non-enzymic peroxidation of membrane PUFA, MDA is also produced in some enzymic processes. It represents the steady state level between its formation and its metabolisation by mitochondria and endoplasmic reticulum. The oxidizability of PUFA by a non-enzymic pathway depends linearly on the number of *bis*-allylic methylenes it contains and on the presence of low-molecular-mass iron chelates. We have previously demonstrated that this form of iron is present in the exudate.[13] Membranes incorporate n-3 PUFA and although they decrease the synthesis of series 2 and 4 of eicosanoids, they increase the synthesis of series 3 and 5. These endoperoxides can give rise to MDA. The reduction of mitochondrial aldehyde NAD^+ dehydrogenases assessed by the WST-1 test can be related to impaired metabolisation of MDA by mitochon-

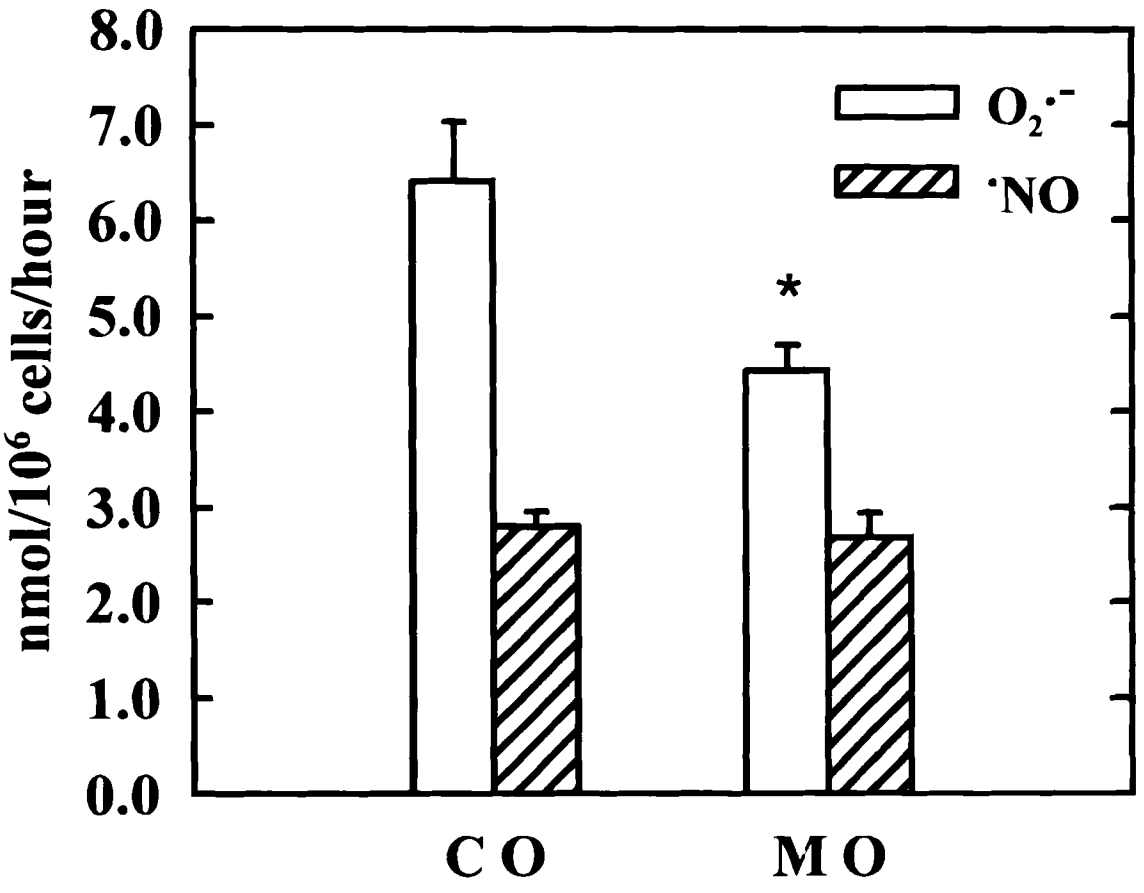

FIGURE 1 Superoxide ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$) generation by polymorphonuclear leukocytes from an inflammatory exudate of rats fed corn oil (CO) or menhaden oil (MO) for five weeks. Values are mean ± SEM. *$P < 0.05$ vs CO group. For details see text.

TABLE III Fatty Acid Composition of PMNL Membranes from Rats Fed Corn Oil (CO) or Menhaden Oil (MO) for Five Weeks

	CO	MO
	%	%
16:0	22,7 ± 0,3	22,9 ± 0,7
16:1n-7	2,6 ± 0,5	4,8 ± 0,6
18:0	20,9 ± 1,4	21,0 ± 1.0
18:1n-9	11,8 ± 0.7	16,3 ± 0.7**
18:1n-7	2,3 ± 0,1	3,4 ± 0.2***
18:2n-6	8,3 ± 0.7	2,7 ± 0.3***
20:4n-6	16,8 ± 1.7	6,9 ± 0.9***
20:5n-3	0,3 ± 0.1	6,6 ± 1.0***
22:5n-3	2,1 ± 0.3	1,6 ± 0,1
22:6n-3	0,4 ± 0,1	2,3 ± 0.3**
24:1n-9	4,5 ± 0.3	4,3 ± 0.5
n-9	16,3 ± 0.8	20,6 ± 1.0*
n-6	28,6 ± 2.3	13,7 ± 1.1***
n-3	2,8 ± 0.3	10,5 ± 1.3**
n-3/n-6	0,1 ± 0,0	0,8 ± 0.1***

dria.[14] All these facts explain the rise of MDA in the exudate.

Leukocytes play an important role in inflammatory processes, and there is growing evidence that ingestion of n-3 PUFA reduces oxidative burst.[4,15,16] There are several reports on the effect of n-3 PUFA upon $O_2^{\bullet-}$ production by PMNLs.[3,4] We found that PMNLs from the inflammatory exudate of rats fed MO generated significantly lower levels of $O_2^{\bullet-}$ than PMNLs from rats fed CO. The reduction of $O_2^{\bullet-}$ generation was usually associated with a reduction in AA availability for cyclooxygenase.[3] We have observed a decrease in PGE_2 accumulated in the exudate of rats fed MO. This, together with the lowered cytokine production[17] observed after consumption of

low-fat, high-fish diet, and the increased levels of MDA that can damage NADPH oxidase, may be responsible for the diminished $O_2^{\cdot-}$ production. Studies upon ·NO production by n-3 PUFA have been carried out using macrophages[18–20] or cultured vascular smooth muscle cells,[21] but not in PMNLs. Attempts were made to relate the increase in ·NO generation in rats fed fish oil to a reduction in AA availability for lipoxygenase.[20] However all studies on ·NO were performed in such conditions that $O_2^{\cdot-}$ was also produced. Since ·NO reacts with $O_2^{\cdot-}$, a reduction in the synthesis of $O_2^{\cdot-}$ may account for the increased activity of ·NO. In the present study, the addition of SOD to the incubating medium, avoided the reaction between both radicals[5] and we found no changes in ·NO generation. As ·NO synthase is a cytosolic enzyme, it is less likely to be attacked by MDA accumulated in the exudate. Although Yaqoob and Calder[18] postulated that inducible ·NO synthase in macrophages could be regulated by fatty acids or by products derived from them, it seems that this situation did not take place in PMNLs. Concentration of α-tocopherol in the exudate, as occurs in plasma, reflects the content of α-tocopherol in the diet, which was the same in both groups. Moreover, the absence of changes in α-tocopherol concentration and the high TAS concentration in the exudate can be related to the decreased $O_2^{\cdot-}$ production.

Dietary fish oil has been shown to diminish clinical manifestations of inflammatory diseases, but may be detrimental with regard to host defence against invading pathogens.[17] Our data demonstrate that the beneficial effects of dietary fish oil in acute inflammatory processes are related to lowered AA metabolites and $O_2^{\cdot-}$ production by inflammatory cells, and consequently to increased antioxidant capacity of the exudate. Furthermore, the increased concentration of MDA in the exudate, probably due to low mitochondrial activity, affects membrane enzymes such as NADPH oxidase but not cytosolic enzymes such as NO synthase.

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