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LABORATORY INVESTIGATION

Fish oil supplementation and essential fatty acid deficiency reduce nitric oxide synthesis by rat macrophages

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Fish oil supplementation and essential fatty acid deficiency reduce nitric oxide synthesis by rat macrophages. Both fish oil-derived ω-3 polyunsaturated fatty acid (ω3 PUFA) supplementation and essential fatty acid (EFA) deficiency have been shown to exert anti-inflammatory effects and, hence, to ameliorate immune-mediated glomerulonephritis. The mechanisms underlying these effects include alterations in the production of eicosanoids, cytokines (that is, tumor necrosis factor, TNF-α) and reactive oxygen species by blood borne cells. Because, in addition to these mediators nitric oxide (NO) is also implicated in glomerular injury, we have examined if both diets affected macrophage NO production as well. Rats were fed a standard chow, an ω3 PUFA-supplemented diet, or an EFA-deficient diet for six weeks before resident peritoneal macrophages were isolated. These cells were exposed to lipopolysaccharide (LPS) and the NO metabolite, nitrite (NO_2^-), was measured in the medium using the Griess reagent. Release of NO_2^- was enhanced by LPS in a dose-dependent manner. With 10 ng/ml LPS challenge, NO_2^- release was reduced by 37% and 57% by ω3 PUFA supplementation and EFA deficiency, respectively. NO_2^- returned to control levels two weeks after the end of diet. Macrophage production of TNF-α responded in a similar manner. Diet-induced reduction of NO_2^- release was neither attributable to a reduction of inducible NO synthase mRNA levels as shown by Northern blot analysis, nor to an increased competition of NO synthase and arginase for the substrate (L-arginine). Indeed, arginase activity of macrophages was even slightly reduced by both ω3 PUFA-supplemented diet and EFA-deficient diet. Diet-induced reduction of NO synthase activity was specific for the inducible form since constitutive NO synthase activity in vascular wall was unaffected by EFA deficiency, and even significantly increased by ω3 PUFA supplementation. These data demonstrate that NO synthesis by rat macrophages is down-regulated by both ω3-PUFA supplemented diet and EFA-deficient diet. This may contribute to the explanation of the anti-inflammatory effects of such dietary treatments.

Modifications of dietary fatty acids have been shown to exert anti-inflammatory effects and, hence, to ameliorate several animal models of renal disease [1, 2]. Such modifications include supplementation with fish oils that are rich in ω3 polyunsaturated fatty acid (ω3 PUFA), particularly eicosapentaenoic acid/docosahexaenoic acid and restriction of the essential fatty acids (EFA). The effects of fish oil in experimental models of lupus nephritis [3, 4], accelerated nephrotoxic serum nephritis [5], and membranous nephropathy [6] are to delay the onset of renal disease, to attenuate the decline in glomerular filtration rate, and to prevent the rise in proteinuria, respectively. Similarly, the beneficial effects of EFA deficiency during the acute and chronic phases of nephrotoxic serum nephritis [7, 8] and in the experimental model of human minimal change disease [9] include limitation of glomerular infiltration by macrophages, improvement of glomerular hemodynamics, and reduction of proteinuria. The mechanism by which dietary fatty acid manipulations exert their effects in inflammation is not clear at this time. One hypothesis is that they reduce the synthesis of arachidonate metabolites potentially involved in glomerular alterations. For example, eicosapentaenoic acid from fish oil is converted by cyclooxygenase and lipoxygenase pathways to thromboxane (TX) A_3 and leukotriene (LT) B_5, respectively. These inflammatory and vasoconstrictive lipids are much less active than the arachidonic acid metabolites TXA_2 and LTB_4 [1]. Beside eicosanoid synthesis, fish oil supplementation affects several other functions of cells involved in inflammation. Replacement of arachidonic acid by eicosapentaenoic acid in the cell membranes of neutrophils, monocytes/macrophages, or resident glomerular and interstitial cells may reduce the formation of platelet activating factor (PAF) [1], reactive oxygen species [10], and cytokines, essentially interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) [11]. Additionally, it can suppress LTB_4- and PAF-stimulated chemotaxis by reducing signal transduction at the level of phospholipase C [12].

Macrophages infiltrating glomeruli in nephrotoxic nephritis [13] and membranous nephropathy [14, 15] also produce L-arginine-derived nitric oxide (NO). This metabolite is implicated in glomerular injury since it may alter the intrinsic glomerular cells and have hemodynamic effects [15]. We therefore investigated the hypothesis that dietary fatty acid manipulations have anti-inflammatory properties by affecting the NO synthase pathway in macrophages as well. To this aim, we compared the effect of ω3 PUFA supplementation and EFA deficiency on the production of nitrite (NO_2^-) and nitrate (NO_3^-), the major stable end products of NO, in rat macrophages exposed *in vitro* to lipopolysaccharide (LPS).

Methods

Animals and diets

Male Sprague-Dawley rats were used in all experiments. From the time of weaning, age-matched animals were fed either a standard rat chow, a standard rat chow supplemented with 15% menhaden oil, a rich source of ω3 PUFA, or an EFA-deficient diet. All the diets were prepared by Ets. L. Pietrement (Provins, France). The fatty acid compositions of the lipids were analyzed

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Table 1. Fatty acid composition of dietary lipids

Fatty acid	Control diet	ω3 PUFA-supplemented diet	EFA-deficient diet
Lauric (12:0)	0.09	—	41.72
Myristic (14:0)	2.12	5.48	17.62
Palmitic (16:0)	21.21	15.77	11.61
Palmitoleic (16.1)	2.82	13.34	0.32
Stearic (18:0)	6.79	3.48	12.39
Oleic (18:1)	24.53	25.00	2.72
Linoleic (18:2)	33.25	6.79	1.02
Linolenic (18:3)	3.60	1.08	0.31
Arachidonic acid (20:4)	—	—	—
EPA (20:5)	—	9.40	—
DCHA (22:6)	—	0.23	—

Numbers represent percent of the total fatty acid content. Abbreviation is DCHA, docosahexaenoic acid.

by Laboratoires des Agriculteurs de France (Paris, France) and are listed in Table 1. The animals were maintained on these diets for two to six weeks before the experiments were performed.

Isolation of peritoneal macrophages

Resident macrophages were obtained by peritoneal lavage with Hank's solution (HBSS, Flow Laboratories, Irvine, UK) containing 20 IU/ml heparin. Cells were washed by centrifugation, then resuspended in MEM (Boehringer, Mannheim, Germany) containing 5% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin/100 μg/ml streptomycin. They were plated at 2×10^6 cells/ml in 0.5 ml medium in 24-well culture plates (Nunc, Roskilde, Denmark) and were allowed to adhere for two hours at 37°C in a 5% CO_2-95% air atmosphere. The nonadherent cells were removed by two washings and the macrophage monolayers were then overlaid with 0.3 ml MEM containing 1% FBS and 0 to 100 ng/ml LPS (*Escherichia coli* 026:B6; Sigma Chemical Co, St. Louis, Missouri, USA). All adherent cells were stained in immunofluorescence with the rat ED1 monocloned antibody (Serotec, Oxford, UK). The concentration of L-arginine in the medium was 0.7 mM. At indicated times, cell-free culture supernatants were harvested before they were assayed for NO_2^-/NO_3^- and TNF-α concentrations.

Determination of inducible NO synthase activity

Inducible NO synthase activity was assayed indirectly by measuring NO_2^-/NO_3^- production. NO_2^- was measured by a colorimetric assay based on the Griess reaction, as previously described [16], with minor modifications. Briefly, 75 μl aliquots of macrophage-conditioned medium were incubated with 150 μl aliquots of Griess reagent (1% sulfanilamide/0.1% naphtylethylene diamine dihydrochloride/2.5% H_3PO_4). The absorbance was read at 540 nm after 15 minutes. NO_2^- concentration was determined with reference to a standard curve by using concentrations from 1.5 to 50 μM sodium nitrite in culture media. In some selected cases, NO_3^- concentration was also measured after reduction of NO_3^- to NO_2^- with nitrate reductase [17].

TNF-α assay

TNF-α biological activity was determined by the assay for cytotoxicity using L-929 cells as previously described [18]. Specificity for TNF-α was demonstrated by neutralization of TNF-α-induced cytotoxicity with anti-mouse TNF-α (10^6 neutralizing U/ml, Genzyme, Cambridge, Massachusetts, USA).

LAF assay

IL-1 biological activity was determined by the standard thymocyte proliferation assay as previously described [19].

Isolation of RNA and Northern blot analysis

Macrophages (20×10^6) were seeded into 100 mm tissue culture dishes and allowed to adhere for two hours at 37°C in a 5% CO_2-95% air atmosphere. After washing, macrophage monolayers were stimulated with 10 ng/ml LPS for four hours. Then, cells were exposed to a lysing buffer (10 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA, 2% sodium dodecylsulfate (SDS), and 250 μg/ml proteinase K (Sigma Chemical) for 45 minutes at 37°C. Total RNA was then extracted by the phenol-chloroform method [20], precipitated with isopropanol and, after dissolution in 10 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA, precipitated again with 3 M LiCl. After two supplementary precipitations with ethanol, RNA concentration and purity were determined by obtaining the A_{260} and A_{280} readings. Agarose gel (0.9%) electrophoresis of total RNA (10 μg/lane) was followed by Northern blot transfer on Gene Screen Plus membrane (New England Nuclear, Boston, Massachusetts, USA).

The membranes were prehybridized for two hours at 42°C with a solution containing 1 × Denhardt's reagent, 0.1% salmon sperm DNA, 50% formamide, 0.5% SDS, 0.6 M NaCl, 0.005 M EDTA, 0.05 M Tris, pH 7.4. Blots were hybridized with a probe for mouse macrophage nitric oxide synthase cDNA (mac-NOS; provided by Dr. Charles J. Lowenstein, Johns Hopkins University, Baltimore, Maryland, USA) [21] that was radiolabeled with dCTP [^{32}P] (3000 Ci/mmol Amersham International, Amersham, UK) by random primer labeling (Megaprime DNA labeling system, Amersham). After hybridization, blots were subjected to autoradiography at −70°C in cassettes with intensifying screens and then reprobed with mouse β actin to ensure that approximately equal amounts of RNA were loaded in each lane. Steady-state mRNA transcript levels were quantified using scanning densitometry.

Arginase assay

Arginase activity of macrophages was measured as described by Russel and Ruegg [22] with minor modifications [23]. Briefly, 0.25 μM L-[guanido-^{14}C]-arginine (53.4 mCi/mmol; NEN, Cambridge, Massachusetts, USA) was added to the culture medium of macrophages together with 300 μM N^G-monomethyl-L-arginine (L-NMMA, Sigma) and 0 to 100 ng/ml LPS. After 48 hours, 0.15 ml of supernatant was removed and added to 0.8 ml of 250 mM acetic acid solution, pH 4.5, containing 100 mM urea and 10 mM L-arginine. After the addition of 0.3 ml of 1 g/ml Dowex resin (HCR-W2, Sigma), the tubes were shaken for one minute and centrifuged at 120 × g for five minutes. Five hundred microliters of supernatant containing ^{14}C urea were removed and added to 3 ml of Aquasafe 300 (Zinsser Analytic, Maidenhead, UK) in counting vials. Percentage conversion of ^{14}C-arginine to ^{14}C-urea was calculated as follows:

$$\frac{(\text{cpm in 0.5 ml supernatant}) - (\text{cpm in medium without cells})}{0.36 \times (\text{cpm in 0.15 ml unseparated medium})} \times 100$$

Determination of constitutive NO synthase activity

Constitutive NO synthase activity was assayed indirectly by measuring the vascular wall content of cyclic guanosine monophosphate (cGMP), as previously described [24]. Rats were killed by cervical dislocation, and thoracic aortae were rapidly excised, rinsed in cold phosphate-buffered saline, and homogenized at 4°C in 10 vol of 0.1 N HCl with an all-glass homogenizer. The homogenates were centrifuged at 15,000 × g for 15 minutes, and supernatants were stored at −20°C until assayed. Radioimmunoassay of cGMP was performed after acetylation of the supernatants, as previously described [25].

Statistical analysis

Results are given as the mean ± standard error of the mean (SEM). The statistical significance of differences between groups was analyzed by the Student's *t*-test of unpaired samples. A P value ≤ 0.05 was considered significant.

Results

The capacity of resident rat peritoneal macrophages to release NO_2^- and NO_3^- was enhanced by exposure to LPS in a dose-dependent manner (Fig. 1). As previously shown, NO_2^- represented in all cases about 60% of the total NO_2^-/NO_3^- present at the time of analysis [26].

On the basis of results obtained in comparable studies [6] ω3 PUFA-supplemented diet and EFA-deficient diet were maintained six weeks before the capacity of macrophages to release NO_2^- and NO_3^- was determined. EFA deficiency and to a lesser extent ω3 PUFA supplementation were associated with a reduction of NO_2^- and NO_2^-/NO_3^- release. Decrease in NO_2^- concentration in the culture medium of macrophages stimulated by 10 ng/ml LPS reached 57% and 37%, respectively. The time course of diet-induced changes in macrophage NO synthase activity was then determined under similar conditions of stimulation. Reduction in NO_2^- release reached significance after six weeks of ω3 PUFA-supplemented diet and five weeks of EFA-deficient diet (Fig. 2). Two weeks after the end of both diets, NO_2^- levels returned to control levels.

Because LPS induces the synthesis of TNF-α and IL-1 which in turn may induce the release of NO_2^-, we considered the possibility that the reduction of LPS-induced NO_2^- release by modifications of dietary fatty acids might reflect a decrease of TNF-α and/or IL-1 generation. First, TNF-α release into the culture medium of macrophages was studied by bioassay. Figure 3 shows that TNF-α bioactivity was detectable, even in the absence of LPS challenge. At the lowest concentrations of LPS, ω3 PUFA supplementation and to a lesser extent EFA deficiency were associated with a reduction of TNF-α levels. To examine the relationship of TNF-α to NO_2^- release, macrophages were stimulated with LPS for 24 hours in the presence of neutralizing antibody to TNF-α. This inhibited TNF-α bioactivity in the culture medium of macrophages by >99%. As shown in Table 2, its addition did not reduce significantly NO_2^- release. IL-1 bioactivity was not detectable in the culture medium of macrophages exposed to LPS concentrations up to 10 ng/ml, ruling out the hypothesis of IL-1 involvement in NO_2^- release under these conditions. At a LPS concentration of 100 ng/ml, IL-1 bioactivity reached 38.6 ± 8.8 U/ml (N = 5). Because of the unavailability of an antibody specific for rat IL-1, we were not able to assess IL-1 involvement in NO_2^- release under that condition.

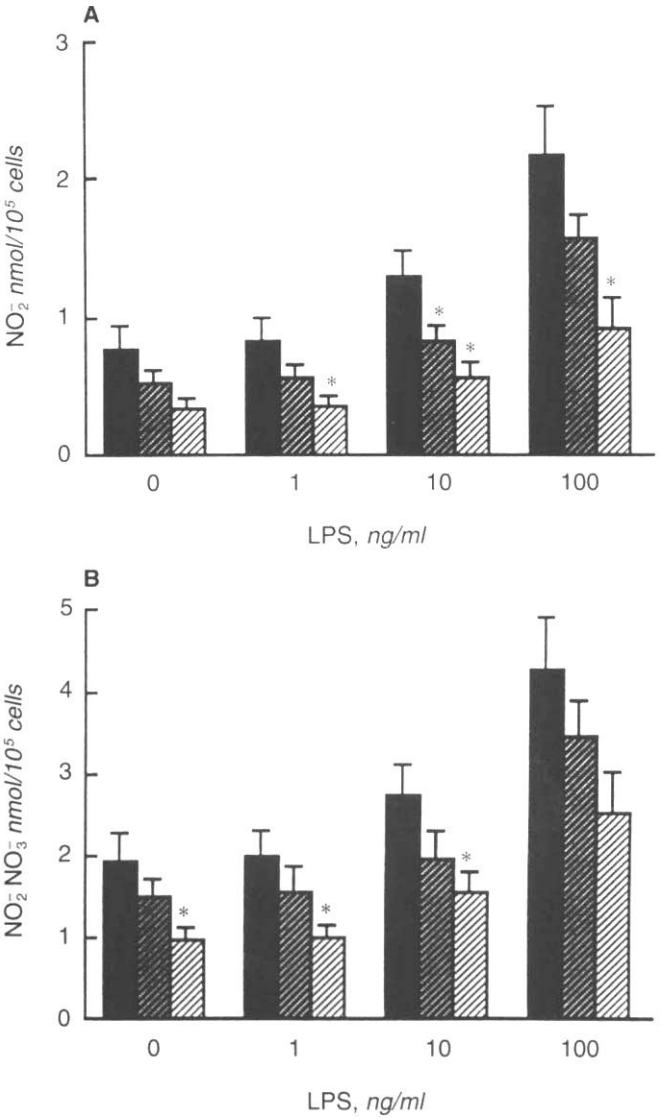

Fig. 1. *Production of NO_2^- (A) and NO_2^-/NO_3^- (B) by resident macrophages isolated from peritoneum after 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨).* Cells were incubated for 24 hours with the indicated concentrations of LPS. Means and SEM of values obtained in 11 to 19 experiments are given. *$P < 0.05$ vs. control diet.

We next determined whether diet-induced reduction of NO_2^- generation was attributable to reduction of inducible NO synthase mRNA levels or to other mechanisms. Macrophages stimulated with LPS expressed detectable amounts of the inducible NO synthase message four hours after stimulation (Fig. 4). This level of expression was not affected by modifications of dietary fatty acids. Another possible explanation for diet-induced reduction of NO_2^- generation was that the arginase pathway also present in macrophages was increased and hence competed for substrate with inducible NO synthase [23]. Thus we studied L-arginine metabolism via the arginase pathway in macrophages under basal conditions and upon exposure to LPS (Fig. 5). Basal arginase

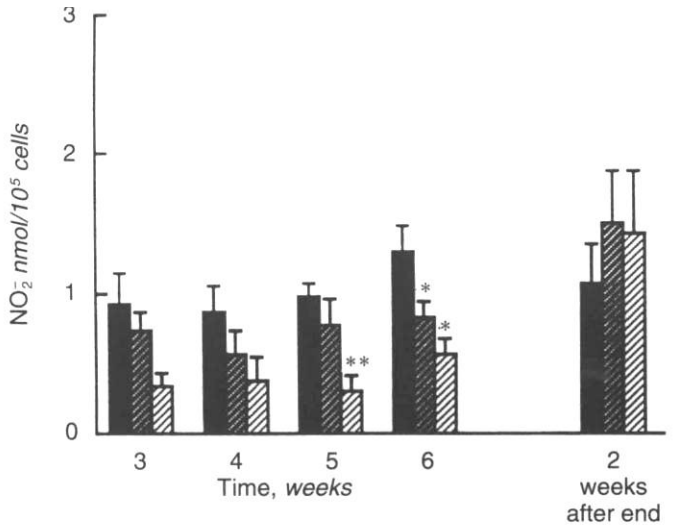

Fig. 2. *Production of NO_2^- by resident macrophages isolated from peritoneum after 2 to 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨), and at 2 weeks after the end of dietary manipulation.* Cells were stimulated with 10 ng/ml LPS for 24 hours. Means and SEM of values obtained in 4 to 19 experiments are given. *$P < 0.05$, **$P < 0.005$ vs. control diet.

Table 2. TNF-α dependence for effects of LPS on NO_2^- release from resident macrophages

	LPS *ng/ml*			
	0	1	10	100
(A) NO_2^- release *nmol/10^5 cells*				
* without anti-TNF-α antibody	0.37 ± 0.10	0.39 ± 0.11	0.52 ± 0.07	0.94 ±0.09
* with anti-TNF-α antibody	0.36 ± 0.10	0.34 ± 0.10	0.49 ± 0.08	0.91 ± 0.09
(B) TNF-α release *mU/10^5 cells*				
* without anti-TNF-α antibody	26.7 ± 12.3	17.7 ± 8.3	34.5 ± 17.9	142.4 ± 42.8
* with anti-TNF-α antibody	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.9 ± 0.9

Cells were incubated for 24 hours under the indicated conditions. Means and SEM of values obtained in 6 experiments are given.

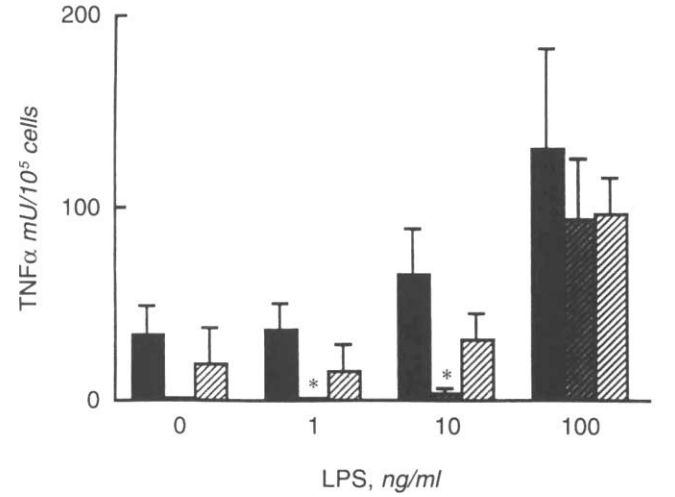

Fig. 3. *Production of bioactive TNF-α by resident macrophages isolated from peritoneum after 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨).* Cells were incubated for 24 hours with the indicated concentrations of LPS. Means and SEM of values obtained in 6 to 13 experiments are given. *$P < 0.05$ vs. control diet.

activity was present (11.8 ± 1.0% conversion of ^{14}C L-arginine to ^{14}C-urea; $N = 7$) and was not affected by LPS. It was not increased but, on the contrary, slightly reduced by ω3 PUFA supplementation and EFA deficiency.

Finally, to dermine whether modifications of dietary fatty acids affected both forms of NO synthase, their effect on constitutive NO synthase activity was determined. This enzyme, located in the endothelium, generates NO that, in turn, activates the soluble guanylate cyclase of vascular smooth muscle, resulting in the generation of cGMP. Thus we measured aortic cGMP levels with a radioimmunoassay kit. Figure 6 shows that ω3 PUFA supplementation increased significantly cGMP levels, whereas EFA deficiency was inactive.

Discussion

The present study was designed to examine whether ω3 PUFA supplementation and EFA deficiency have significant effect on macrophage NO generation, a parameter of immune and inflammatory responses. Because NO is so labile, direct measurements of NO are difficult. Thus we measured the accumulation of NO_2^-/NO_3^-, the major stable end products of NO. Stimulation of resident peritoneal macrophages with LPS resulted in a time-dependent accumulation of NO_2^- in the culture medium, which reached about 2.5 nmol/10^5 cells at 24 hours. This value is almost identical to that reported for the production of NO_2^- by the macrophage cell line J774 [27]. Examination of the effects of ω3 PUFA supplementation and EFA deficiency revealed that both dietary manipulations led to reduced release of NO_2^-/NO_3^- at all the concentrations of LPS tested (Figs. 1 and 2). Because this effect was obvious after a short-time diet (3 weeks) and was absent two weeks after cessation of the diet, it could reflect the involvement of a fast-turnover compartment of fatty acids.

The mechanisms for the diet-induced reduction of NO_2^- release from LPS-activated macrophages are not explained by this study. The main property both diets are known to share is the ability to reduce arachidonic acid availability. In ω3 PUFA-supplemented diet, eicosapentaenoic acid and docosahexaenoic acid compete with arachidonic acid as substrates for cyclooxygenase and lipoxygenase and for the 2-position in membrane phospholipids. This in turn reduces generation of prostaglandin $(PG)I_2$, TXA_2 and LTB_4 [1]. In an EFA-deficient diet, dietary restriction of linoleic acid, the arachidonic acid precursor, results in reduced macrophage generation of PGE_2, TXA_2 and LTB_4 [28]. Because PG may contribute to favor NO synthesis in

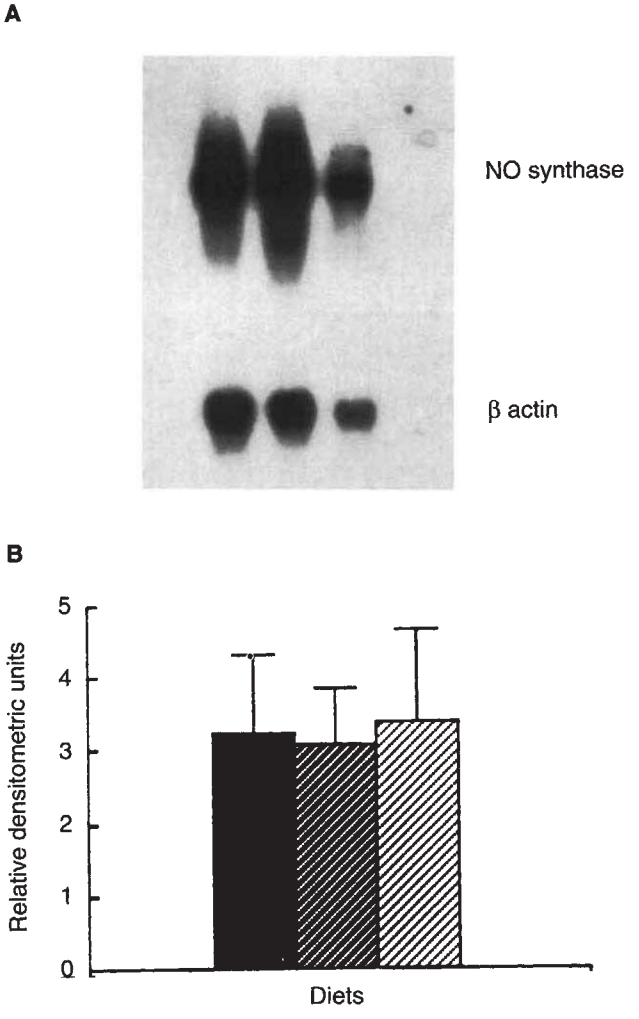

Fig. 4. A. *Inducible NO synthase mRNA levels in resident macrophages isolated from peritoneum after 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨).* Cells were incubated for 4 hours with 10 ng/ml LPS. Total RNA was isolated, and the levels of inducible NO synthase and β actin mRNAs were assessed by Northern analysis. **B.** Amounts of inducible NO synthase mRNA determined in five different blots and adjusted for amount of β actin mRNA in each lane.

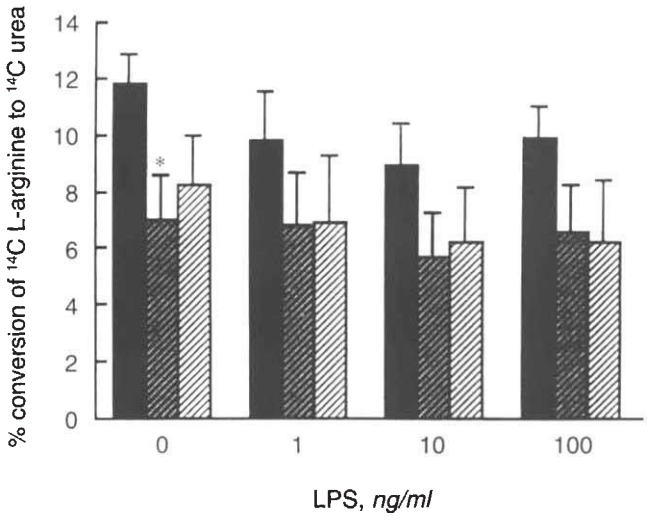

Fig. 5. *Arginase activity of resident macrophages isolated from peritoneum after 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨).* Cells were incubated for 48 hours with the indicated concentrations of LPS. Means and SEM of values obtained in 7 experiments are given. $^*P < 0.05$ vs. control diet.

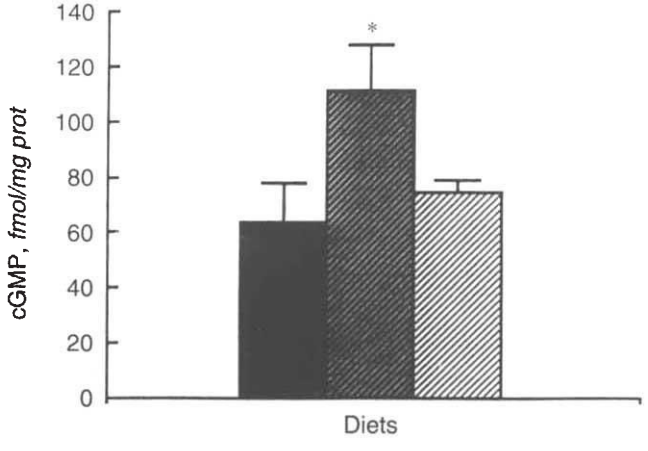

Fig. 6. *Aortic cGMP content after 6 weeks of control diet (■), ω3 PUFA-supplemented diet (▨), and EFA-deficient diet (▨).* Means and SEM of values obtained in 6 experiments are given. $^*P \leq 0.05$ vs. control diet.

response to LPS [29], their reduction may eventually account for the decreased NO inducibility.

The mechanisms by which fatty acid dietary manipulations affect NO_2^- release could also involve reduction in cytokine production, as suggested by previous works. Endres et al [11] reported that dietary supplementation with ω3 PUFA reduced IL-1 and TNF-α synthesis by stimulated human blood mononuclear cells. Similarly, production of IL-1 and TNF-α by mononuclear cells was found to be significantly reduced after the consumption of the low-fat, high-fish diet [30]. Data from the current study show that decreased NO_2^- release is associated with reduction in TNF-α production, as assessed by measuring its soluble and bioactive fraction (Fig. 3). It is clear from the study with anti-TNF-α antiserum that reduction in TNF-α release and decrease in NO_2^- production were probably not linked. These findings are in agreement with those reported by Zhang and Morrison [29]. These authors demonstrated that LPS stimulated TNF-α and NO production in mouse peritoneal macrophages through different biochemical pathways, and that the signal transduction for both pathways was regulated by a pertussis toxin-sensitive factor. Since TNF-α is not involved, diet-induced reduction of NO_2^- production could be attributed to the increased production of cytokines that are inhibitory for inducible NO synthase. They include transforming growth factor β, IL-4 and IL-10 [31]. We are presently carrying out studies examining this hypothesis.

Nitric oxide synthesis in macrophages is complex and involves two successive steps that could be affected by ω3 PUFA supplementation and EFA deficiency. The first step is the expression of inducible NO synthase gene. Its promoter includes various sequences for the binding of transcription factors such as interferon-γ response element and nuclear factor κB [32]. The diets studied failed to modify inducible NO synthase mRNA levels (Fig.

4). The second step includes translation of the gene and expression of the enzyme in the cytosol. Fatty acid dietary manipulations possibly reduce the level and/or the activity of this enzyme. Because of the unavailability of an antibody specific for the protein, we were not able to assess directly its level. But enzyme activity has been determined indirectly. Its diet-induced reduction could be explained by a decrease in the availability of substrate (L-arginine) and cofactors [33]. Regarding macrophage L-arginine metabolism, two pathways have been described, via NO synthase and arginase, respectively [34]. Our results indicate that competition of both pathways for L-arginine is not a molecular explanation for the effect of the diets (Fig. 5). Thus the mechanism by which fatty acid dietary manipulations influence macrophage NO generation still has to be elucidated.

The fact that no reduction in cGMP content of aortic wall was observed after either dietary manipulation (Fig. 6) suggests that diets affected differently the constitutive and the inducible forms of NO synthase. Actually, ω3 PUFA supplementation even increased significantly constitutive NO synthase activity. This effect is consistent with recent observation by McVeigh et al [35], who demonstrated that fish oil supplementation significantly improved the forearm blood flow responses to acetylcholine without altering the responses to glycerol trinitrate in patients with type 2 diabetes mellitus. These improved vasodilator responses have been attributed to the increased release of endothelium-derived NO.

The physiological relevance of diet-induced reduction in inducible NO synthase activity is unknown. Nitric oxide has been proposed to participate in a wide variety of functions in inflammatory reaction [31]. Anti-inflammatory effects are inhibition of platelet aggregation and of leukocyte adhesion to endothelium. Pro-inflammatory effects include vasodilation and cytotoxicity. In fact, NO produced in nmol quantities mediates vascular relaxation, whereas NO produced in mmol quantities mediates cytotoxicity. Because following reduction of NO synthase activity by dietary treatments, NO levels still remained in mmol range, these diets would limit NO-induced cytotoxicity within the glomerulus without modifying hemodynamic responses.

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