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Dietary Fish Oil Enhances Macrophage Production of Nitric Oxide

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Nitric oxide (NO) is recognized as an important mediator of hemodynamic regulation and multisystem organ failure (MOF). Although polyunsaturated fatty acids (PUFA) are known to modify the elaboration of some humoral mediators in MOF, their effect upon NO production has not been evaluated. This study was designed to examine the effect of omega-3 (ω3) and omega-6 (ω6) PUFA on macrophage production of NO, TNF, PGE_2, and PGI_2. Rats were fed diets of 18% by calorie safflower oil (ω6) or fish oil (ω3) for 12 days. Bronchoalveolar macrophages (BAM) were divided into group A (medium only), group B (0.5 μg/ml PGE_2 or PGI_2 + medium) or group C (10 μM indomethacin + medium). Cells were stimulated with 100 U/ml interferon-γ and 10 μg/ml *Escherichia coli*–LPS. In group A, BAM from animals fed ω3 produced significantly more NO (3.64 vs 1.92 μM, $P < 0.05$) and TNF (8.52 vs 1.75 μg/ml, $P < 0.05$) than BAM from ω6-fed animals. The addition of exogenous PGE_2 or PGI_2 (group B) ablated the difference in NO and TNF observed in group A. Indomethacin also (group C) ablated the difference in NO and TNF production seen in ω3- and ω6-fed animals noted in group A. These data demonstrate that PUFA influence BAM production of NO and TNF. Changes in the ω6-derived prostanoids may account for the differences in TNF production, but these data suggest that PGE_2 and PGI_2 are not responsible for the observed differences in NO production. Through its effect upon macrophage NO production, manipulation of exogenous ω3/ω6 PUFA may be of value in the management of patients at risk for development of MOF.

Nitric oxide (NO) is a highly reactive molecule produced in a number of mammalian cells including macrophages, neutrophils, platelets, hepatocytes, endothelial cells, adrenal cells, and neurons [1–8]. Microbial and tumor cell destruction and macrophage induction of lymphocyte proliferation have been linked to the production of NO [2, 9]. Nitric oxide's effects upon vasomotor tone [10, 11], local organ blood flow, and systemic blood pressure make NO suspect as an intermediate in the hemodynamic alterations of multisystem organ failure (MOF). In the lung several cell populations have demonstrated the ability to produce NO under various circumstances. Nitric oxide production by respiratory endothelium can be induced by hypoxia and appears to be involved in the regulation of ventilation–perfusion matching [7]. In our laboratory we have demonstrated that another cell population in the lung, the bronchoalveolar macrophages (BAM), is capable of producing large amounts of NO when exposed to immunomodulators. The role of NO produced by BAM in MOF and ARDS has yet to be investigated.

The interaction between nutrition and the immune response has been of increasing clinical and laboratory interest during the last two decades. Specifically, the observation that altering lipid substrates effects immunosuppressive eicosanoid production in the macrophage has generated many questions regarding the importance of specific polyunsaturated fatty acids (PUFA) upon the host immune response [13, 14]. In our laboratory, we have demonstrated that decreases in peritoneal macrophage PGE_2 production from animals fed diets with omega-3 (ω3) rather than omega-6 (ω6) PUFA, such as fish oils, result in diminished cellular TNF production (unpublished data). Interestingly both exogenous TNF and PGE_2 can stimulate cellular production of NO [15]. Based upon the observations that PGE2 inhibits TNF production, it would seem unlikely that both of these compounds would bring about the same increase in cellular NO production. In order to evaluate the possible role dietary PUFA upon NO production and further evaluate the relationship between endogenous TNF, eicosanoid, and NO, the following set of experiments was undertaken. Given the above stated interactions between PGE_2/TNF and TNF/NO, we hypothesized that macrophage NO production would be decreased in those animals fed predominantly ω6 versus ω3 fatty acids.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Charles River Laboratories; Wilmington, MA) weighing 150–175 g (n = 60) were housed individually in a climate/light cycle controlled facility. All were fed a pelleted fat-free diet for

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3 days. During the next 12 days each animal had free access to one of two nutritionally equivalent diets (Purina Test Diets; Richmond, IN). Group 1 received chow containing 18% by calorie fish oil (Menhaden, Zapata Haynie Corp., 2% ω6/24.6% ω3 PUFA). Group 2 received chow containing 18% by calorie safflower oil (77.1% ω6/0.2% ω3 PUFA). Food and water intake were unrestricted and both food consumption and weight gain were monitored daily.

Macrophage harvest and culture. Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (40 mg/kg). The heart–lung complex was removed through a median sternotomy. The trachea was cannulated and the lungs were lavaged with a total of 50 cc endotoxin-free PBS. This was accomplished by instilling five 10-cc volumes into the trachea and withdrawing the fluid under a constant pressure after each instillation. Returns of less than 35 cc were discarded. The lavage fluid was centrifuged and the cells were washed and counted. The cell pellet was reconstituted in phenol red-free Dulbecco's modified Eagle's medium with 10% Nuserum (Collaborative Biomedical Products, Bedford, MA) and 5 μM L-arginine. Cells were placed in 24-well plates at 500,000 or 1 million cells per well. After 4 hr of standing in 5% CO_2 at 37°C, cell wells were washed, leaving a purified population of BAM attached to the plates. All experiments were approved by and completed under the guidelines set by the Institutional Animal Care and Use Committee of the Children's Hospital Research Foundation.

Stimulation. All wells were stimulated with 100 u/ml IFN-γ and 10 μg/ml LPS (*Escherichia coli* 0127:B8) placed simultaneously into the medium after the 4-hr plating/wash period. Group A cell received no other treatment. In addition group B cells received exogenous and PGI_2 or 0.5 ng/ml PGE_2 (Cayman Chem., Ann Arbor, MI). Group C received 10 μM indomethacin (Sigma Chem. Co., St. Louis, MO) in addition to the IFN-γ and LPS. The additional agents were added at the time of stimulation with the IFN-γ and LPS. All plates were incubated in 5% CO_2 at 37°C throughout the experimental period. The dosages of prostaglandins were approximately 10^6 times that of measured PGE_2 production in BAM in our laboratory.

Assays. Supernatants were collected at four time intervals between 0 and 60 hr. TNF content was determined by the WEHI cytotoxic assay. Both PGE_2 and 6-keto-$PGF_{1\alpha}$ (metabolite of PGI_2) levels were analyzed with ELISA assays (Cayman Chem.). NO formation was recorded by analyzing nitrite in the supernatant. This measurement was accomplished by using the Griess reaction and measuring the developed chromophore at 546 nm against standards of $NaNO_2$ [2, 5]. Supernatants containing indomethacin were measured at dual wavelengths, 546/650 nm (Molecular Devices Kinetic Microplate Reader).

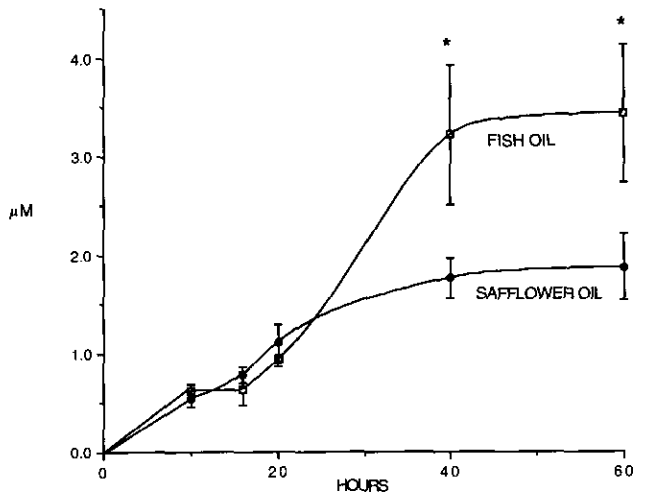

FIG. 1. Nitric oxide production in fish oil-fed (open squares) versus safflower oil-fed (solid circle) animals. Measured as nitrite levels in supernatant (μM) collected at 0–60 hr. Statistically significant differences noted by *, $P < 0.05$.

Statistical analysis. Comparisons between means of the maximum values were made with Student's t test and ANOVA. The data are listed as means ± standard deviations between the diet groups.

RESULTS

Effects of dietary ω3/ω6 PUFA. Group A stimulated BAM from animals fed chow containing fish oil produced significantly more nitric oxide than those fed safflower oil chow (NO: 3.64 μM vs 1.92 μM, $P < 0.05$; Fig. 1). Levels of TNF in the ω3-fed animals were significantly higher than the levels in those fed ω6 PUFA (TNF: 8.52×10^6 pg/ml vs 1.74×10^6 pg/ml, $P < 0.005$; Fig. 2). Supernatant levels of PGE_2 were not significantly different between the two diet groups (PGE_2: ω3, 517 ng/ml vs ω6, 582 ng/ml). Similarly levels of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) did not differ between the PUFA diets (PGI_2: ω3, 82.5 ng/ml vs ω6, 78.9 ng/ml).

Effects of exogenous prostaglandin. In group B, the addition PGE_2 (0.5 ng/ml) to the cell cultures, ablated the differences in NO between the ω3/ω6-fed animals seen in group A (NO: 62.2 μM vs 55.0 μM; Fig. 3). The differences in TNF levels were also abolished with the addition of PGE_2 (TNF: 2.92×10^5 pg/ml vs 2.42×10^5 pg/ml). Exogenous PGI_2 (0.5 ng/ml) gave results similar to those seen with the addition of PGE_2. NO production was not significantly different between the ω3/ω6-fed animals (NO: 54.3 μM vs 52.9 μM). Of note was the significant difference between NO levels in group A (NO: 3.64 μM vs 1.92 μM) as compared to group B (NO/PGE_2: 62.2 μM vs 55.0 μM and NO/PGI_2: 54.3 μM vs 52.9 μM).

Effects of prostaglandin blockade. In group C BAM cultures, 10 μM indomethacin was added to each cell

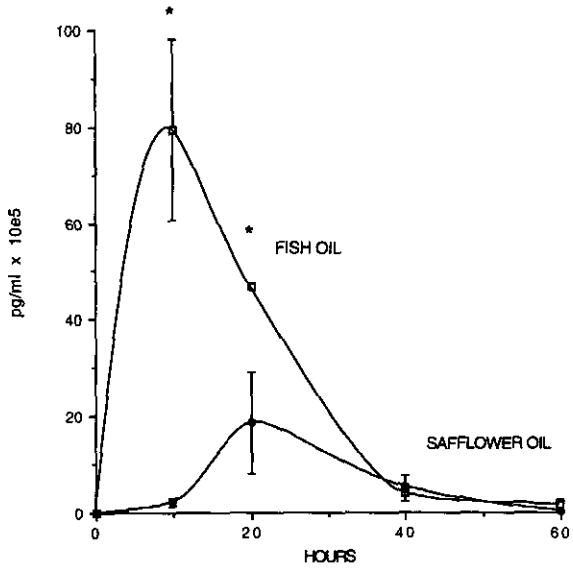

FIG. 2. TNF in fish oil-fed (open squares) versus safflower oil-fed (solid circle) animals. Measured from supernatant (pg/ml × e5) collected at 0–60 hours. Statistically significant differences noted by *, $P < 0.05$.

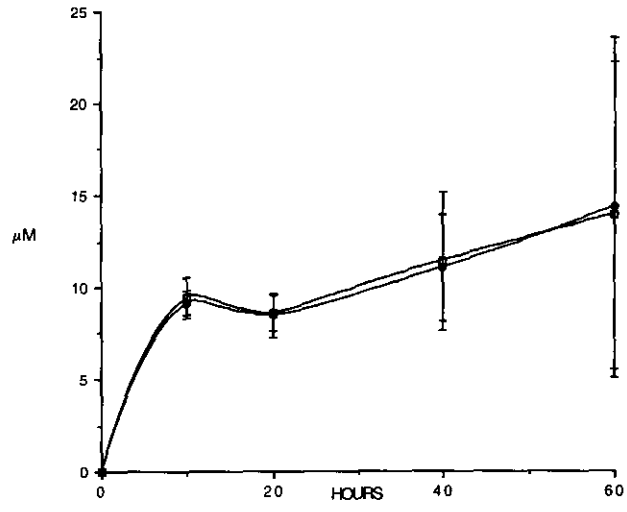

FIG. 4. Nitric oxide production in fish oil-fed (open squares) versus safflower oil-fed (solid circle) animals after indomethacin was added to media. Measured as nitrite level in supernatant (μM) collected at 0–60 hours.

well. No significant differences in NO or TNF production between ω3/ω6-fed animals were present (NO: 15.6 μM vs 17.0 μM, Fig. 4; and TNF: 1.02×10^4 pg/ml vs 5.1 $\times 10^3$ pg/ml, Fig. 5) in this group. Levels of 6-keto-$PGF_{1\alpha}$ were undetectable in the group C cultures. PGE_2 levels were present at decreased levels in comparison to group A (PGE_2: ω3, 388.2 ng/ml vs 517 ng/ml and ω6, 445.9 ng/ml vs 582 ng/ml).

DISCUSSION

Numerous nutritional substances have been evaluated as for their possible roles as immunomodulators. Fish oils rich in eicosapentaenoic acid can have a beneficial effect on various indicators of immune response and the host response to infection as demonstrated in several experimental models [16, 17]. How ω3 PUFA mediate their protective effects is unclear. Series 2 prostaglandins are immunosuppressive but their production can be decreased through dietary substitution of ω3 for ω6 PUFA. In so doing, the inhibitory effects of these prostaglandins on macrophage production of TNF is reduced.

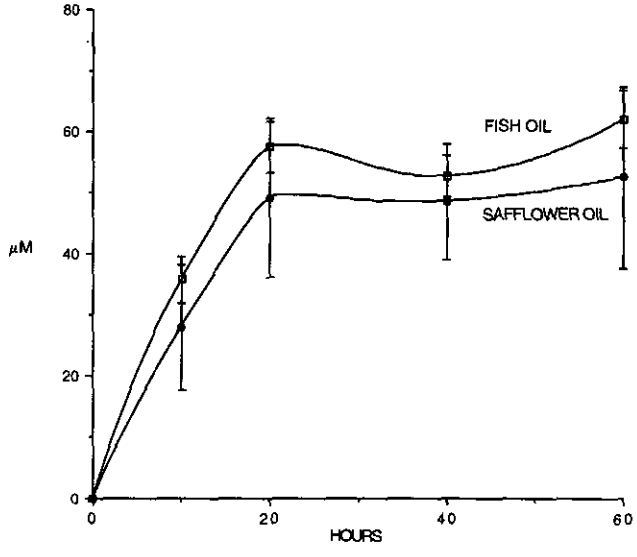

FIG. 3. Nitric oxide production in fish oil-fed (open squares) versus safflower oil-fed (solid circle) animals after exogenous PGE_2 was added to media. Measured as nitrite level in supernatant (μM) collected at 0–60 hours.

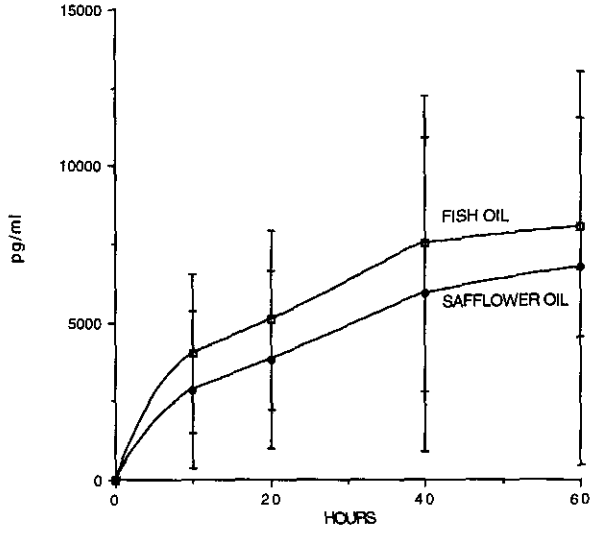

FIG. 5. TNF in fish oil-fed (open squares) versus safflower oil-fed (solid circle) animals after indomethacin was added to media. Measured in supernatant (pg/ml) collected at 0–60 hours.

The data in these experiments demonstrate that PUFA manipulation has a similar effect on BAM NO production. The animals fed diets containing ω3 PUFA possessed BAM that produced larger amounts of NO when stimulated compared to the animals which received ω6 PUFA. Given that TNF production can be altered through dietary PUFA manipulation through the immunosuppressive effects of PGE_2 and that exogenous TNF induces the production of NO, one might assume that the prostaglandins are also directly or indirectly involved in the regulation of NO production. If this were the case, the addition of PGE_2 or PGI_2 should significantly decrease the amount of NO elaborated by the BAM regardless of the animal's dietary PUFA intake. In our experiments the addition of these prostaglandins did not only fail to suppress BAM NO production but in fact resulted in a marked increase in NO elaboration. Furthermore, the addition of indomethacin resulted in levels of NO production greater than controls but less than those seen in the cells treated with PGE_2 or PGI_2.

These results were contrary to those expected and suggest that PGE_2 or PGI_2 are not the primary regulators of NO synthesis from the BAM. Our observations may be partially explained by the superphysiologic doses of exogenous prostanoids that were utilized. It is possible that the prostaglandins and the indomethacin caused a nonspecific cellular response resulting in the induction of NO synthesis. The mechanism of inducible nitric oxide synthase activation has been incompletely investigated. In fact, there has been little success in the activation of NO production of human monocytes *in vitro*. It is entirely likely that a variety of cytokines, prostanoids, pharmacologic agents, and metabolic products are capable of inducing NO production in the rodent macrophage.

The important question to be addressed is the biological significance of the above observations. Many studies have sought to demonstrate the beneficial effects of ω3 PUFA over the ω6 variety in the critically ill patient. We observed that the ω3 PUFA group BAM produced significantly more NO than the ω6 PUFA group. Nitric oxide produced in low concentrations by vascular endothelium is involved in hemodynamic regulation and cell mediated antimicrobial activity. At higher concentrations NO represents a cytotoxic radical molecule involved in cellular immunity and possibly the pathophysiology of septic shock and multisystem organ failure. There is no clear definition as to what amount of NO production is protective versus detrimental to the organism. This investigation supplied data which demonstrate a method of manipulating macrophage NO production by supplying specific dietary PUFA. Further investigations are needed to determine the mechanism by which specific PUFA regulate the macrophage NO production. Similarly examination of the effects of lower doses of exogenous prostaglandins are warranted before further speculations on the relationship between prostaglandins and NO production can be made.

Manipulation of dietary fatty acids in patients at risk for infectious complications may be of value through their effects upon cellular NO production.

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