Dietary n-3 Polyunsaturated Fatty Acids Prevent the Development of Atherosclerotic Lesions in Mice

Modulation of Macrophage Secretory Activities

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We examined the effects of dietary n-3 polyunsaturated and saturated fatty acids on the development of the atherogenic process in mice and on the macrophage ability to secrete several effector molecules that may be involved in the atherogenic process. The secretion of inflammatory proteins such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and the production of lipoprotein lipase (LPL), nitrogen oxide (NO), and prostaglandin E2 (PGE2) were evaluated in peritoneal macrophages isolated from atherosclerosis-susceptible C57BL/6J mice. The mice were assigned at random to three experimental groups: the first group was fed a semi-defined control diet (control diet); the second group was maintained on the control diet supplemented with 10% menhaden oil (menhaden diet); and the third group received the control diet supplemented with 10% palm oil plus 2% cholesterol (saturated fat diet). Macrophages derived from mice fed the menhaden diet showed a suppression of their basal TNF-α mRNA expression and production. They also presented a dramatically decreased ability to express TNF-α and IL-1β mRNAs in response to exposure to lipopolysaccharide (LPS) compared with the macrophages from the control group. LPL mRNA and protein expression were downregulated after 6 and 15 weeks of menhaden-diet feeding. Significantly higher NO production in response to interferon gamma was found, both after 6 and 15 weeks of diet feeding, in the menhaden group compared with the control group. In addition, prostaglandin production and macrophage tumoricidal activity in response to LPS were decreased in this group compared with the control group. Macrophages derived from the saturated fat group did not show any significant alterations in TNF-α, LPL, NO, or PGE2 secretion compared with controls. Interestingly, we observed a progressive increase of the LPS-induced IL-1β gene expression and secretion among macrophages harvested from mice receiving the dietary supplement of saturated fatty acids. At 6 and 15 weeks histologic examination of the atherosclerotic lesions did not reveal any important lesions in the control and menhaden groups, whereas a gradual development of fatty streaks was observed in the saturated fat group. After 15 weeks of diet, the addition of saturated fatty acids to the control and menhaden experimental diets for 10 additional weeks resulted in a major development of lesions in the control group, whereas only slight lesions were observed in the menhaden group. These data indicated a beneficial effect of dietary n-3 polyunsaturated fatty acids on the development of atherosclerosis and underlined possible involvement of cytokines in atherogenesis. (Arterioscler Thromb. 1993;13:1515-1524.)

KEY WORDS • macrophage • dietary fatty acids • atherosclerosis

The role of environmental factors in the incidence of cardiovascular diseases is well-known. Dietary fat is one of the variables that has been associated experimentally and epidemiologically with the development of atherosclerosis. Diets high in cholesterol and saturated fatty acids have been shown to promote the development of atherosclerosis in several species, including the mouse. In contrast, dietary supplementation with n-3 fatty acids has been suggested as preventing or reducing the development of coronary atherosclerosis both in humans and animals.

Several observations support the possibility that macrophages may play an important role in the development of diet-induced atherosclerosis. Diet-induced hypercholesterolemia, which is believed to injure vascular endothelium, is accompanied by the adhesion of circulating monocytes to arterial endothelial cells followed by emigration of the monocytes into the subendothelium, where they differentiate into macrophages. In addition, while macrophage products have been shown to be present in atherosclerotic plaque, cytokines produced by the activated macrophages affect growth and gene expression in the vessel wall.

Previous studies demonstrate that n-3 fatty acids have substantial effects on several cellular constituents of the atherosclerotic plaque, which may account for their...
beneficial effects on atherosclerosis.\textsuperscript{16} In monocytes/macrophages, n-3 fatty acids decrease the production of several factors that may contribute to the development of atherosclerosis.\textsuperscript{17,20} However, while conflicting results have been published concerning the effect of dietary n-3 fatty acids on the synthesis of interleukin-1\(\beta\) (IL-1\(\beta\)) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), many of the effects of n-3 fatty acids on macrophage function remain unknown. Moreover, the cellular mechanisms by which a high-saturated fatty acid diet may favor the development of atherosclerosis are still unclear.

To investigate the possible relation between the dietary modulation of cytokine expression by fatty acids and the development of atherosclerosis, we evaluated the in vivo long-term effects of n-3 and saturated fatty acids on the expression and secretion of several macrophage effector molecules that may affect the atherogenic process. Because mice develop early and advanced atherosclerotic lesions after 6- and 15-week feeding periods with an atherogenic diet,\textsuperscript{21} we monitored the production of TNF-\(\alpha\), IL-1\(\beta\), lipoprotein lipase (LPL), nitrogen oxide (NO\textsubscript{2}), and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) in peritoneal macrophages harvested from mice fed the experimental diets for 6 and 15 weeks.

\section*{Methods}

\subsection*{Animals}
Pathogen-free C57BL/6J mice, 6 weeks of age, were obtained from The Jackson Laboratory, Bar Harbor, Me. Animals were housed in autoclaved cages and given sterilized water and food.

\subsection*{Diets}
On day 0 of the experiments, 10 to 15 mice were randomly assigned to one of three dietary groups (control, menhaden, or saturated fat) and fed the autoclaveable, defined, and semi-purified diets for 6 and 15 weeks. Since the use of commercial stock rations as a control diet is a poor nutritional practice, we designed a semi-defined control diet. The control diet composition percentages, based on weight, were as follows: casein, 24\A of the total metabolizable energy; dl-methionine, 0.3\%; AIN mineral mix, 3.5\%; choline chloride, 0.2\%; dl-\(\alpha\)-tocopherol acetate, 0.12\%; and, to avoid the appearance of an essential fatty acid--deficient effect, corn oil, 5\%, and menhaden oil, 5\%. Menhaden oil (10\%) was added to the diet high in n-3 fatty acids (menhaden), and palm oil (10\%) and cholesterol (2\%) were added to the diet high in cholesterol and saturated fatty acids (saturated fat). Protein, minerals, vitamins, fibers, and calories were given in the same amounts in the diets, which differed only in their fat content. Fresh diet was provided daily to each group to minimize autoxidation. All diets were custom designed and purchased from ICN Nutritional Biochemicals, Cleveland, Ohio.

\subsection*{Chemicals}
Brewer's thioglycollate was purchased from DIFCO, Detroit, Mich. Dulbecco's minimal essential medium (DMEM) was obtained from ICN Biochemicals Inc, Costa Mesa, Calif, supplemented with fatty acid- and endotoxin-free 0.5\% bovine serum albumin (BSA; Boehringer Mannheim, Montreal, Canada), 2 mmol/L L-glutamine (ICN Biochemicals), and penicillin-streptomycin (Flow, McLean, Va). Lipopolysaccharide (LPS) was purchased from Sigma, St Louis, Mo. Interferon gamma (IFN-\(\gamma\)) was purchased from Amgen, Thousand Oaks, Calif.

\subsection*{Macrophages}
Following 6- and 15-week maintenance periods on the specified diets, animals were injected intraperitoneally with 1 mL thioglycollate medium (1\%). Peritoneal exudate cells were harvested 4 days after the injection. The cells were washed by centrifugation and resuspended in DMEM supplemented with 0.5\% BSA (DMEM-BSA). A suspension of 2\times10^9 cells/mL was prepared.

\subsection*{RNA extraction}
Macrophages (1\times10^7) were plated in 35-mm plastic Petri dishes (Falcon, Lincoln Park, NJ). After 2 hours of adherence, cells were washed and cultured for the different time points with the appropriate agents. Macrophages were lysed with guanidine isothiocyanate. Total RNA was purified by centrifugation through a cesium chloride gradient as described by Chirgwin et al.\textsuperscript{22}

\subsection*{Northern blot analysis}
Total RNA (15 \(\mu\g) was separated on 1.2\% agarose gel containing 2.2 mol/L formaldehyde as previously described.\textsuperscript{23} The blots were prehybridized for 18 hours in a prehybridization buffer (Hybri-disol, Oncor, Gaithersburg, Md). The mRNA expression was analyzed by hybridization with \([\alpha^{32}P]dCTP\) (specific activity, ~3000 Ci/mmol; Amer-sham Corp, Arlington Heights, Ill) labeled by nick translation DNA probes. The human LPL probe was prepared in our laboratory. cDNA was obtained from total RNA by using a reverse transcriptase reaction. Two synthetic primers were used to amplify enzymatically a 872-bp region of the LPL probe. The LPL probe was subsequently purified on a low-melting agarose gel. Murine tumor necrosis factor (TNF) and interleukin-1\(\beta\) (IL-1\(\beta\)) were kindly provided by Dr A. Cerami (Rockefeller University, New York, NY) and Dr H. Young (NCI, Frederick, Md), respectively. The actin probe was obtained from Dr D.W. Cleveland (Johns Hopkins University, Baltimore, Md). Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). mRNA levels were quantified by densitometry with a laser densitometers and normalized to actin mRNA signals.

\subsection*{Assays}
\subsection*{Determination of TNF-\(\alpha\) activity}
A double-sandwich enzyme-linked immunosorbent assay (ELISA) as described by Sheehan et al\textsuperscript{24} was used to determine the quantity of TNF-\(\alpha\) in the supernatants of macrophages. Hamster monoclonal antibody to murine TNF-\(\alpha\) was purchased from Genzyme, Boston, Mass, and rabbit polyclonal anti-murine TNF-\(\alpha\) was prepared and purified by standard procedures. Briefly, 2 \(\mu\g per well of a monoclonal antibody against TNF-\(\alpha\) was absorbed in 96-well plates and incubated in the presence of different dilutions of the tested sample or with the TNF-\(\alpha\) standards. After washing, the polyclonal antibody to TNF-\(\alpha\) was added. Anti-rabbit immunoglobulin (Ig) G--peroxidase was added to the wells and incubated for 1 hour. The peroxidase reaction was developed by
adding peroxidase substrate and was read in an automated plate reader (Dynatech, Chantilly, Va).

Determination of IL-1β activity. IL-1β levels were determined by the thymocyte comitogenic assay. Briefly, IL-1β–like activity was determined by the ability of supernatants to stimulate the uptake of tritiated thymidine (³H-TdR) by the D10.1 thymocyte clone in the presence of a suboptimal concentration of concanavalin A (1 μg/mL; Sigma). One unit of IL-1β is defined as the amount of IL-1β required to stimulate 50% maximal proliferation of the D10.1 cells.

Determination of the LPL immunoreactive mass. LPL immunoreactive mass was measured by ELISA using affinity-purified antibodies specific for LPL according to techniques previously described with some modifications. In brief, 1 μg per well of a purified antibody against bovine LPL was absorbed in titer plates (Immunon II, Dynatech or Costar) and incubated overnight with different dilutions of the sample (in phosphate-buffered saline [PBS] or 0.154 mol/L NaCl) or with murine LPL standards. The wells were then washed with PBS and 0.05% Tween 20, and affinity-purified antibody to LPL was added. Anti-rabbit IgG-peroxidase was added to the wells and incubated for 3 hours. The peroxidase reaction was developed by adding peroxidase substrate (0.3 mg/mL o-phenylenediamine and 0.0125% hydrogen peroxide in 0.1 mol/L citrate, pH 4.5). The reaction was stopped with 25 μL of 4 mol/L sulfuric acid, and the results were read in an ELISA plate reader (Dynatech).

Nitrate assays. The estimation of NO₂ produced by macrophages after 18 hours of stimulation by IFN-γ was performed according to the method of Green et al. Results were expressed as nitrite production per total protein content of macrophages.

PGE₂ assays. After an 18-hour treatment of the macrophages with LPS, supernatants were collected and assayed for PGE₂ concentrations by using radioimmunoassay kits (NEN, Mississauga, Ontario, Canada).

Protein assays. Total protein content was estimated according to the Bradford method with some modifications. The Bradford method is a colorimetric assay (Biorad, Mississauga, Ontario, Canada). Results were expressed as micrograms protein per milliliter.

Tumoricidal assays. The tumoricidal activity of macrophages was estimated from their ability to kill ⁵¹I-labeled tumor cells in culture. L929 tumor cells, growing as monolayers in DMEM/10% fetal bovine serum (FBS) were pulsed with ¹³¹I-labeled tumor cells (specific activity, 0.5 μCi/mL) for 18 hours. After extensive washing to remove unincorporated radioactivity, the cells were trypsinized and 5×10⁶ viable cells were added to the macrophage monolayers. Percoll–mononuclear (10⁶ cells in 200 μL DMEM/10% FBS) were allowed to adhere to the surface of 96-well plates. Nonadherent cells were removed by washing with medium. The macrophages were then incubated for 24 hours at 37°C with LPS (final concentration, 5 to 5000 ng/mL). After extensive washing to remove the macrophage activator, 5×10⁶ labeled L929 cells were added and cocultured with the macrophages for 72 hours at 37°C. Each well was then washed, and the cells were lysed by the addition of 200 μL 0.5 mol/L NaOH. The lysate and two additional washes of NaOH (total volume, 600 μL) were combined, and the radioactivity was measured in a gamma counter. Specific cytotoxicity was calculated using the formula

\[
\frac{(\text{Mean cpm L929 Cells}) - (\text{Mean cpm L929 Cells} + \text{Macrophages})}{(\text{Mean cpm L929 Cells})}
\]

Histology

Histological scoring of arterial lesions was performed as described by Stewart-Phillips et al. The aortas of three mice from each experimental group were cut just distal to the sinus valves. Cryostat frozen sections, 10-μm thick, were cut at three different levels, mounted on slides, stained with oil red O, and counterstained with hematoxylin. The sections were examined microscopically, and a similar section of each aorta, which showed valve cusps, was scored from 0 to 10 according to the number of lesions, the average size of the lesions, the number of foam cells, the involvement of the media, and the presence of raised endothelium in the lesions.

Statistical Analysis

Statistical analysis of the results was performed by using a one-way analysis of variance followed by a Bonferonni t test.

Results

Food Intake and Weight Gain

The mice consumed similar amounts of food (~5 g/mouse per day). The weight gains of animals maintained for 6 weeks on the different diets were similar: control, 20.1±0.7 g; menhaden, 20.1±0.1 g; saturated fat, 20.7±0.6 g. Similarly, the weights of mice did not differ significantly after 15 weeks of maintenance on the various diets: control, 23.2±0.5 g; menhaden, 23.4±0.8 g; saturated fat, 23.7±0.1 g.

TNF-α Expression and Secretion

Basal and LPS-stimulated TNF-α expression and secretion in macrophages from mice maintained on control, menhaden, and saturated fat diets for 6 and 15 weeks are presented in Figs 1 and 2, respectively. After 6 weeks of diet, basal TNF-α expression and secretion were similar (P>.05) in macrophages harvested from the three groups of mice (Fig 1). LPS-stimulated TNF-α mRNA expression and secretion did not differ significantly (P>.05) in control and saturated fat group macrophages. In contrast, they were reduced in macrophages harvested from the menhaden group compared with those from the control group (P=.067 and P=.064, respectively) (Fig 1). After 15 weeks of maintenance on the various diets, basal TNF-α mRNA expression and secretion were significantly lower in the menhaden-fed group than in the other groups (P<.05; Fig 2). LPS-induced TNF-α mRNA expression and secretion were about eightfold and threefold less augmented, respectively, in macrophages from menhaden-fed mice than from the other two groups of mice (P<.001 and P<.01, respectively; Fig 2).

IL-1β Expression and Secretion

Basal and LPS-induced IL-1β expression and secretion in macrophages from the control, menhaden, and saturated fat groups after 6 and 15 weeks of diet are presented...
Bar graphs showing basal and lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) mRNA expression in peritoneal macrophages from mice on control (CTL), menhaden oil (MENH), and palm oil (SAT) diets for 6 weeks. Macrophages were stimulated with LPS (5 μg/mL) for 12 hours. Data represent mean ± SEM of three independent determinations. Expression of TNF-α mRNA was quantified by densitometric analysis of autoradiograms obtained after Northern blot analysis. Top, Integrated optical density for TNF-α mRNA expression was normalized to the levels of actin mRNA expression in the same experimental sample. Bottom, Basal and LPS-stimulated TNF-α secretion in macrophages from mice fed the different diets for 6 weeks. Macrophages were stimulated with LPS (5 μg/mL) for 18 hours. Data represent mean ± SEM of three assays from one macrophage preparation. Med indicates medium.

Fig 1.

in Figs 3 and 4, respectively. After 6 weeks of diet, basal and LPS-induced IL-1β expression and secretion did not differ significantly among the various groups, although macrophages from mice fed a saturated fat diet responded to LPS stimulation with an augmentation of IL-1β expression compared with the other two groups (Fig 3). After 15 weeks of diet, the LPS-increased IL-1β expression was similar in the menhaden and control groups (Fig 4). However, we observed a decrease of the LPS-induced...
IL-1β secretion in the menhaden group compared with the control group (P<.01; Fig 4). In contrast, the level of IL-1β mRNA expression and secretion in response to LPS stimulation was higher (P<.001) in macrophages from the saturated fat group than in macrophages isolated from the control group (Fig 4).
LPL Expression and Secretion

We measured basal LPL expression and production in macrophages from mice maintained on the different diets. After 6 weeks of diet feeding, we observed a decrease of LPL mRNA expression and secretion in the menhaden group (50% less LPL compared with animals on the control diet, \( P=.15 \) and \( P<.001 \), respectively; Fig 5). Basal mRNA LPL expression and secretion were not significantly different in macrophages from the saturated fat group compared with the control group. After 15 weeks of maintenance on the diets, the diminution of LPL gene expression and production was still observed in macrophages isolated from the menhaden group compared with the control group (\( P>.05 \) and \( P<.01 \), respectively; Fig 6).

NO\textsubscript{2} Production

We measured the production of NO\textsubscript{2} by IFN-\( \gamma \)-stimulated macrophages isolated from mice fed the control, menhaden, and saturated fat diets. Macrophages obtained from mice fed a diet high in n-3 fatty acids for 6 or 15 weeks exhibited a significantly (\( P<.01 \)) higher NO\textsubscript{2} production in response to IFN-\( \gamma \) than the cells harvested from the control and saturated fat diet mice (Fig 7).

PGE\textsubscript{2} Production

When challenged with LPS, the macrophages from mice fed menhaden oil for 15 weeks secreted about 50% (\( P<.001 \)) less PGE\textsubscript{2} than those harvested from animals maintained on the other diets (menhaden, 70±5 pg/mL; control, 170±5 pg/mL; and saturated fat, 185±10 pg/mL).

Macrophage Tumoricidal Activity

Macrophage monolayers were exposed to increasing concentrations of LPS, and cytolyis of L929 targets was subsequently assessed. When macrophages isolated from mice fed one of the three diets for 6 weeks were exposed to LPS, no differences were found with respect to their tumoricidal function (Fig 8). After 15 weeks, however, the macrophages isolated from the menhaden group were significantly (\( P<.001 \)) less cytolytic (60% decrease) than control and saturated fat macrophages when exposed to high concentrations of LPS (Fig 8).

Histology

After 6 weeks of diet, no mouse fed the control or the menhaden diet developed atheromatous lesions. Very small lesions were observed in these dietary groups after 15 weeks of diets (Table). In contrast, mice maintained on the saturated fat diet had discernible lesions, with an increase in size and number of foam cells with time (6 weeks' score, 4.0±1.6; 15 weeks' score, 7.2±1.0). Supplementation of the control and menhaden diets with saturated fatty acids for 10 additional weeks led to the development of important lesions in the control group but not in the menhaden group (Table).

Discussion

This study demonstrated that long-term feeding with n-3 and saturated fatty acids alters the expression and production of several factors that may represent some important contributors to the cellular cascade of ath-
**P<.07 vs control.

erosclerosis. It represents, to our knowledge, the first report showing a relation between the modulation of macrophage cytokine expression by dietary fatty acids and the development of atherosclerosis. Although peripheral blood monocytes are the cells of choice to predict arterial wall events, their isolation is not feasible in mice. We therefore selected macrophages isolated from the peritoneal cavity. These cells offer the advantage of having a long life span (months), which makes them suitable for a long-term feeding study.

It has been suggested that TNF-α could be involved in the evolution of atheroma. Indeed, this cytokine, which is produced principally by activated macrophages, stimulates new vessel formation and induces hemorrhagic necrosis, features characteristic of evolving atheroma. TNF-α has also been shown to induce procoagulant activity among endothelial cells and to enhance leukocyte adhesion to the vessel endothelium. Moreover, immunoreactive TNF-α has been detected and localized in atherosclerotic lesions.

We demonstrated that LPS-stimulated macrophages isolated from mice fed a diet high in n-3 fatty acids for 6 weeks produce less TNF-α than do the cells harvested from mice fed the control or saturated fat diets. These results accord with those of Endres et al., who reports that n-3 supplementation for the same period of time reduces the amount of inducible production of TNF-α by human peripheral blood monocytes. In addition, we observed a further decrease in the macrophages' ability to produce TNF-α after a longer administration of the diet, suggesting that the maximal effect of n-3 fatty acids on macrophage TNF-α production may be reached slowly during supplementation.

We also investigated the mechanisms underlying the suppression of TNF-α production induced by dietary n-3 fatty acids. The correlation we found between the...
decreased TNF-α gene expression and the diminished production of this cytokine after dietary supplementation with n-3 fatty acids indicated that this diet may alter macrophage TNF-α production by decreasing its mRNA level. Since n-3 fatty acids induce changes in lipooxygenase products, a reduction of macrophage leukotriene production might account for the decrease of the TNF-α secretion. In contrast, since prostaglandins have been shown to inhibit TNF-α production by macrophages, the lower PGE2 production in LPS-challenged macrophages from mice fed a high-n-3 fatty acid diet suggests that alteration of this cyclooxygenase product was not involved in the decreased TNF-α production observed in this group.

In contrast to the dietary effects of n-3 fatty acids, TNF-α expression and secretion in peritoneal macrophages were unaffected by diets high in cholesterol and saturated fatty acids. Since we observed the development of fatty streak-like lesions after 15 weeks of consumption of the atherogenic diet, we postulate that alteration in TNF-α production is not a major factor in the development of early murine atherosclerotic lesions. However, this observation does not preclude a role for a decreased TNF-α production in the protective effect of the n-3 fatty acid diet on atherogenesis. Indeed, the fact that mice fed a 15-week menhaden diet exhibited low basal and stimulated TNF-α responses and did not develop atherosclerotic lesions when later exposed to a saturated fat diet suggests that the reduced production of TNF-α could contribute to the protective effect of n-3 fatty acids on atherogenesis.

It has been previously suggested that IL-1 may participate in the growth regulation in the vessel wall. Indeed, IL-1 induces a variety of responses in endothelial cells, resulting in the stimulation of granulocyte adhesion, an increase in procoagulant activity, and the proliferation of endothelial cells. IL-1 also stimulates the proliferation and secretion of platelet-derived growth factor by smooth muscle cells. It has been suggested, therefore, that IL-1 may play a role in the development of the atherogenic process.

Our results demonstrated that LPS-induced IL-1 production was suppressed after 15 weeks of supplementation with menhaden oil. In accordance with our data, a reduction in total IL-1 synthesis resulting from the dietary intake of n-3 fatty acids has previously been shown to occur in human monocytes. Our results also demonstrated that a high-cholesterol and high-saturated fatty acid diet increases the IL-1 mRNA expression and production by macrophages in a time-dependent manner. The parallel development of atherosclerotic lesions in mice maintained on the saturated fat diet supports the hypothesis that an increase in IL-1 production by macrophages may be involved in the development of the atherogenic process in mice.

The recognition of a role of acute bacterial infections in arterial endothelium injury argues for a biologic explanation for the differences observed in LPS-induced cytokine expression for in vivo development of atherosclerosis. Moreover, one may speculate that if the state of macrophage responsiveness to LPS is indicative of its responsiveness to other, as yet unknown, stimulators in the artery wall, then the role of fatty acids, in terms of resistance to and protection against atherosclerosis, could be at least partially mediated by the macrophage production of cytokines.

LPL has also been suggested as promoting the development of atherosclerosis by producing remnant lipoproteins...
on the endothelial surface.\textsuperscript{42} Recently, macrophages have been shown to express LPL in atherosclerotic lesions.\textsuperscript{14} Our results demonstrated that macrophages isolated from mice fed a diet high in menhaden oil express and secrete significantly less LPL than macrophages isolated from mice fed a control or saturated fat diet. This observation raises the possibility that a relative reduction in macrophage LPL production may represent one of the mechanisms by which n-3 fatty acids favorably affect the atherogenic process. The mechanisms underlying this effect are still unclear. Lipid loading of cells is known to affect LPL mRNA expression in cultured macrophages. In particular, hypertriglyceridemic very-low-density lipoproteins have been shown to enhance the secretion of LPL from macrophages.\textsuperscript{43} Since fish oil decreases the size and neutral lipid content of the particles, and in some cases even the number of very-low-density lipoprotein particles secreted,\textsuperscript{44} the decreased LPL secretion observed after n-3 fatty acid supplementation could be due to the effects of fish oil on lipoprotein metabolism.

In addition to cytokines, macrophages have been shown to produce potent vasodilator products, the nitrogen oxides.\textsuperscript{45} Since atherosclerosis impairs vascular relaxation,\textsuperscript{46} we tested the hypothesis that diets known to affect the development of atherosclerosis may modify NO\textsubscript{3} production by macrophages. We found that supplementation with n-3 fatty acids increased the IFN-\gamma-stimulated production of nitrites by macrophages compared with that observed in the control and saturated fat groups. It has been previously demonstrated that diet-induced atherosclerosis may alter the synthesis of NO\textsubscript{3} in the aorta.\textsuperscript{47} Our results showed that dietary n-3 fatty acids stimulate the production of nitrites by macrophages. This might favorably affect vascular reactivity with respect to atherosclerosis.

The results obtained from the histological examination of aorta supported our hypothesis that the modulatory effect of n-3 fatty acids on macrophage function could at least partially explain their beneficial effect in retarding the development of atherosclerosis. Indeed, they demonstrated that mice maintained on the menhaden diet developed fewer atherosclerotic lesions than the control mice when they were later exposed to an atherogenic diet. Moreover, they supported the possibility that the increase in IL-1 production by macrophages after prolonged feeding with a diet high in cholesterol and saturated fatty acids may represent one mechanism underlying the deleterious effect of this diet on the atherogenic process.

We did not attempt to investigate the mechanisms by which fatty acids may modulate cytokine production. There are multiple mechanisms by which diet fat has the potentiality of affecting biological processes. The first and most obvious mechanism involves the synthesis of structural lipids of altered fatty acid composition that result in alterations in membrane function.\textsuperscript{48} Cellular lipids are also enzymatic substrates for a variety of intracellular metabolic events such as cicosanoid synthesis.\textsuperscript{49} Alterations of cicosanoid synthesis by polyunsaturated fatty acids may also be responsible for some of the dietary fatty acid effect on macrophage gene expression. Another level of biological function potentially altered by a change in dietary fat composition involves a change in hormone binding or responsiveness. Finally, variation in dietary fat intake may also alter expression of nuclear function by changing receptor-mediated stimulation of gene products or by changing the transport of gene products out of the nucleus.\textsuperscript{50} Understanding and using the effects of n-3 fatty acids on the cellular constituents of atherosclerosis may offer new prophylactic strategies against the development of the atherogenic process.

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