Fish Oil Enhances Macrophage Tumor Necrosis Factor-Alpha mRNA Expression at the Transcriptional Level

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Dietary supplementation with fish oil has previously been shown to enhance in vivo and in vitro (macrophage) synthesis of tumor necrosis factor-alpha (TNF-α) in response to bacterial lipopolysaccharide (LPS) stimulation. The studies reported here were conducted to gain insight into the molecular mechanisms of this nutrient-immune interaction by comparing the concentration, rate of synthesis, and rate of decay of TNF-α mRNA upon LPS stimulation of macrophages obtained from mice fed high-fat diets, rich in either fish oil, corn oil, or coconut oil, or a low-fat diet for a period of 4 weeks. The results indicate that compared with the other diet groups, LPS stimulation of macrophages from mice fed fish oil resulted in (1) enhanced levels of mRNA and protein for TNF-α, and (2) increased transcription of TNF-α mRNA as assessed by nuclear run-on assays. Posttranscriptional studies showed that the rate of decay of TNF-α mRNA did not vary significantly for macrophages from mice fed with fish oil as compared with corn oil. Further studies using actinomycin D and cycloheximide suggested that RNA synthesis, but not protein synthesis, was necessary for TNF-α mRNA accumulation. Taken together, the present studies suggest that fish oil enhances macrophage TNF-α mRNA expression at the transcriptional level. Although such TNF-α upregulation may provide a mechanism for the beneficial effects of fish oil in certain inflammatory and immune disorders, it can also underlie its potential deleterious effects if the degree of upregulation leads to exaggerated TNF-α production that exceeds the limits of benefit to reach toxic levels.

DIETARY SUPPLEMENTATION with fish oil has been shown to be beneficial in a variety of immunologic and inflammatory diseases.[1-3] It has been proposed that such beneficial effects may be related to their selective inhibitory effects on the cyclooxygenase and lipoxygenase pathways.[4,5] Since macrophages play a major role in immunoregulation, host defense against infection, and the inflammatory response, recent interest has focused on the effects of such dietary manipulation on the ability of macrophages to secrete mediators of the immune and inflammatory responses, ie, cytokines such as tumor necrosis factor-alpha (TNF-α). These studies have shown that macrophages from mice fed diets rich in fish oils produced more TNF-α and less PGE_2 in response to challenge with bacterial lipopolysaccharide (LPS) than macrophages from mice fed other high-fat diets.[6-8] These results may have important implications for understanding the mechanisms by which fish oil (and n-3 polyunsaturated fatty acids) supplementations may be effective in the alleviation of certain diseases such as arthritis.

The present studies were undertaken to explore at the molecular level the mechanism by which fish oil feeding enhances TNF-α production in response to LPS. Since the expression of mRNA is the crucial first step of protein synthesis, we measured TNF-α protein and mRNA levels upon LPS stimulation of macrophages from mice fed high-fat diets rich in either fish oil (rich in n-3 polyunsaturated fatty acids), corn oil (n-6 polyunsaturated fatty acids), or coconut oil (saturated fatty acids), or fed a low-fat diet. In addition, we assessed whether TNF-α mRNA expression was associated with altered transcriptional or posttranscriptional rates.

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MATERIALS AND METHODS

Animals and Diets

Specific pathogen–free female outbred Swiss Webster mice (BRL, Füllinsdorf, Switzerland) were obtained from the supplier at 3 weeks of age and housed in a temperature-controlled room (23°C) with a 12-hour dark-light cycle. The mice were caged in polycarbonate cages and had access to tap water and a standard laboratory chow (low-fat) diet composed of (wt/wt). 20% protein, 60% carbohydrate, and 4% fat for 1 week before the start of the experiments. Pelleted high-fat diets used in these studies were rich (20% wt/wt) in corn oil, coconut oil, or menhaden fish oil and were purchased from ICN Biomedicals (Costa Mesa, CA). The diets were supplemented with 1% (by weight) vitamin E (DL-α-tocopherol powder, 250 IU/g). With the exception of the high–corn oil diet, all other diets were supplemented with corn oil (1% by weight) to ensure adequate intake of n-6 essential fatty acids. The diets were stored in sealed containers kept at 4°C. Fresh diets were given to mice on a day-to-day basis for 4 weeks.

Peritoneal Macrophages

Mice fed the diets rich in corn, coconut, and fish oils, as well as the low-fat diet, were killed after 4 weeks and their peritoneal macrophages were removed as previously described.[8] Briefly, peritoneal cavities of mice were aseptically washed with ice-cold phosphate-buffered saline (PBS), pH 7.2 (Boehringer, Mannheim, Germany), and peritoneal cells were washed by centrifugation, suspended in medium 199 (Boehringer) supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal calf serum, counted, and transferred onto 24-well dishes (Costar, Cambridge, MA) or 250-mL (75-cm^2) culture flasks (Costar). The cells were incubated for 1 hour at 37°C in a humidified chamber with a 95% air–5% CO_2 atmosphere, and nonadherent cells were removed by repeated gentle washings with prewarmed PBS. The adherent cells (10^6, 2×10^7, or 10^8 cells per flask, depending on the experiments) were then used for further studies.

In Vitro Production of TNF-α

Macrophage monolayers (10^6 per well) were incubated for 24 hours in 1 mL medium 199 containing 2% fetal calf serum without or with LPS (2 μg/mL). At the end of the incubation, the culture medium was removed and stored at −20°C until used for measuring TNF-α levels. TNF-α levels on samples were determined colorimetrically by the TNF-α toxicity for L929 cells using an in vitro assay as previously described.[9] Murine recombinant TNF-α was used as a positive control. One cytotoxic unit was defined as the highest dilution of the test material that caused ≥50% destruction of the monolayer of L929 cells, and the titer was expressed as units per milliliter medium. The detection limit of the assay was 20 U TNF-α/mL. Identity of TNF-α in the test materials was confirmed by neutralization of its cytotoxicity against L929 cells using a rabbit polyclonal antiserum against murine TNF-α.

Measurement of TNF-α mRNA Expression by Dot Blot Hybridization

Macrophages (2×10^7 per flask) were incubated with culture medium containing 2 μg/mL *Salmonella minnesota* LPS (Sigma, St Louis, MO). At intervals of 0, 30, 60, and 120 minutes, cell monolayers were washed with warm PBS and then lysed with 6.5 mL guanidium isothiocyanate solution (4 mol/L, Fluka, Buchs, Switzerland), and total RNA was isolated using a guanidinium isothiocyanate–CsCl ultracentrifugation method.[10] Total RNA was dotted using a dot blot manifold (Bio-Rad, Fullerton, CA), at a concentration of 0.5 μg per dot, onto nylon membranes (Boehringer). Oligonucleotide probes were used to detect the presence of mRNA for the cytokines murine β-actin (5′-GGC TGG GGT GTT GAA GGT CTC AAA CAT GAT CTG GGT CAT CTT-3′)[11] and TNF-α′′ (5′-GGT CAC CCT TCT CCA GCT GGA AGA C-3′).[12] Probes were labeled with [^{32}P]γ-adenosine triphosphate (Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase. The membranes were prehybridized for 3 hours at 55°C in (final concentration of each component buffer) 5× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 10× Denhardt solution, 7% sodium dodecyl sulfate (SDS), 20 mmol/L sodium phosphate, and 100 μg/mL herring sperm DNA. Hybridization was performed at 55°C overnight in the same buffer used for prehybridization. The membranes were stringently washed for 20 minutes at 51°C (twice in 1× SSC, 0.7 mol/L sodium phosphate, 10× Denhardt solution, and 5% SDS, final concentration) followed by two 20-minute washes at 51°C in 1× SSC and 1% SDS. Membranes were dried and exposed to film (Kodak X-OMAT AR, Eastman Kodak, Rochester, NY) up to 7 days at −80°C, after which the film was developed. Intensity of autoradiographed RNA was measured by densitometry.

Nuclear Run-on Assay

Macrophages (10^8 per flask) from mice fed corn oil and fish oils were exposed to 2 μg/mL LPS for 30 minutes, after which the cell monolayers were gently washed with ice-cold PBS and lysed with ice-cold lysis buffer (10 mmol/L Tris hydrochloride, pH 7.4, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, and 0.5% Nonidet P-40). After being kept on ice for 5 minutes, the suspension was centrifuged at 500 × *g* for 5 minutes at 4°C and the supernatant was then discarded.[13] The lysis step was repeated once, and nuclei were collected (10^8 nuclei/mL) and stored under liquid nitrogen using the following buffer: 25% glycerol, 5 mmol/L MgAc, 0.1 mmol/L EDTA, 5 mmol/L dithiothreitol, and 50 mmol/L Tris hydrochloride, pH 8. The following solution was prepared before starting the reactions: 50 μL solution A (2 mmol/L adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate), 0.1 mmol/L *S*-adenosylmethionine, and 200 μCi ^{32}P-uridine triphosphate (>750 Ci/mmol, Amersham) were added to 40 μL solution B (0.6 mmol/L KCl and 12.5 mmol/L MgAc) and 10 μL deionized distilled water. To this solution, 100 μL nuclei suspension was added. The mixture was incubated for 30 minutes, when the reactions were stopped by adding 6.5 mL guanidinium isothiocyanate solution (4 mol/L), and the RNA was isolated using a guanidinium isothiocyanate–CsCl ultracentrifugation method.[10] Residual unincorporated labeled nucleotides were removed by chromatography on a Sephadex G-50 column. Labeled RNA from transcription run-on reactions was hybridized to membranes containing a plasmid-derived 1.4-kb *Pst* I-*Bam*HI insert for murine cachectin/TNF-α[12] kind gift of Bruce Beutler, University of Texas, Dallas, TX) and a plasmid-derived insert containing the murine β-actin cDNA[11] kind gift of Shigeru Sakiyama, Chiba Cancer Center Research Institute, Chiba, Japan). Specific mRNA was quantified by densitometry.

Posttranscriptional Studies

Macrophage monolayers (10^8 per flask) were exposed to 2 μg LPS/mL for 1 hour, after which the monolayers received either actinomycin D alone (5 μg/mL, Sigma), cycloheximide (10 μg/mL, Sigma), or a combination of both reagents. At the intervals 1, 2, and 3 hours after addition of inhibitors, the reactions were terminated and RNA was isolated, dotted onto nylon membranes, and hybridized with oligonucleotide probes for TNF-α and β-actin, and the amount of specific mRNA was determined by film densitometry.

RESULTS

Body Weight

The mean body weight of all groups at the start of the experiment varied between 22.2 and 22.4 g, with a SEM of ± 0.2 g. After 4 weeks' ingestion of test diets, body weights for mice fed fish oil and corn oil diets (47.2 ± 0.7 and 46.8 ± 0.6 g, respectively) were significantly higher

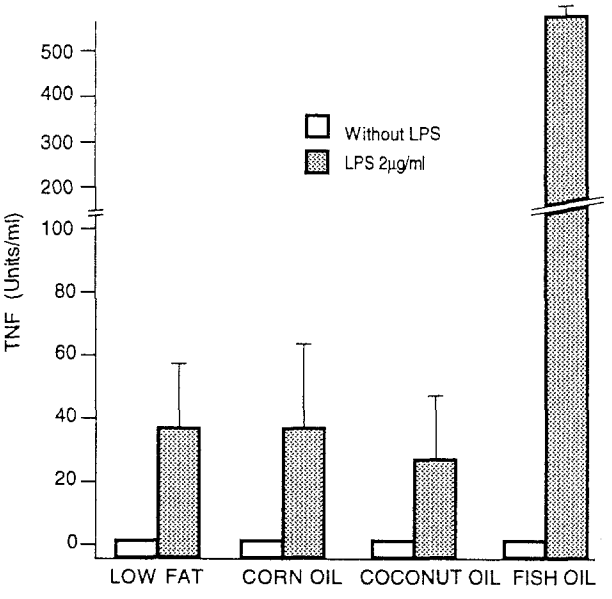

Fig 1. Production of TNF-α in response to LPS by peritoneal macrophages from mice fed different diets. Mean ± SEM for 15 wells. ANOVA indicates a highly significant difference across diets ($P < .001$). Post-hoc pairwise comparisons (using the Neuman-Keuls multiple comparison test) indicate significant differences only between the fish oil group and the 3 other diet groups.

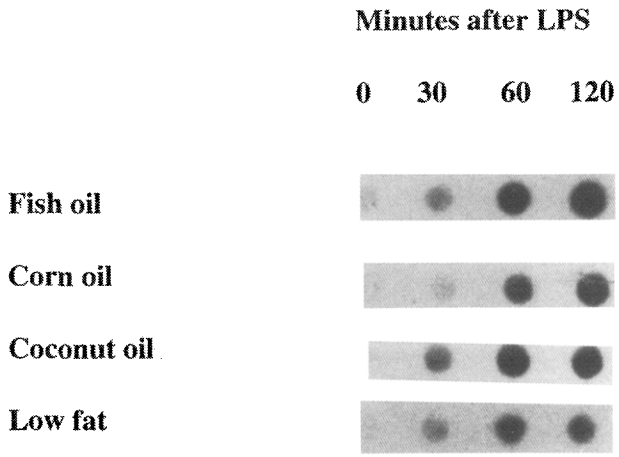

Fig 2. Dot blot analysis of RNA from murine peritoneal macrophages (mice fed different diets) after LPS stimulation (2 μg/mL). RNA from 3 macrophage monolayers were pooled, and 0.5 μg total RNA was analyzed per dot using a ^{32}P-labeled murine TNF-α oligonucleotide probe.

($P < .01$) than for those fed the coconut oil diet and the low-fat diet (43.1 ± 0.7 and 42.6 ± 0.6, respectively).

In Vitro Levels of TNF-α and TNF-α mRNA Expression

Macrophages from mice fed the diet rich in fish oil produced higher levels of TNF-α in response to LPS than macrophages from mice fed the other diets (Fig 1). Similarly, higher levels of mRNA TNF-α, which were particularly evident after 2 hours of LPS exposure, were observed in macrophages from mice fed fish oil than from those fed corn oil, coconut oil, or the low-fat diet (Figs 2 and 3). Compared with the low-fat diet (taken as 1), TNF-α mRNA was increased by 2.3-fold with fish oil and to a lesser extent (by ~1.2- and 1.5-fold) with corn oil and coconut oil, respectively. It is to be noted that in a preliminary study comparing the fish oil and corn oil groups (data not provided), it was found that after peak mRNA levels are reached 2 hours after LPS, there is a decline in mRNA during the subsequent 2 hours—such that the difference between fish oil and corn oil is maintained.

Nuclear Run-on Assay

β-Actin mRNA level did not vary significantly among samples from the test diets (Fig 4). However, TNF-α mRNA levels were dependent on diet type: densitometry readings indicate that the TNF-α mRNA ratio between fish oil and corn oil was 1.7, thereby indicating an increased TNF-α mRNA transcription in macrophages from mice fed the fish oil diet.

Posttranscriptional Studies

To investigate whether the LPS-induced increase of TNF-α mRNA could occur in the absence of transcription, macrophages were treated with actinomycin D, an inhibitor of eukaryotic RNA polymerase I. The results showed no increase in transcription when macrophages were treated with actinomycin D, indicating that RNA synthesis is necessary for TNF-α mRNA accumulation to occur (Fig 5). In addition, actinomycin D was not able to prevent TNF-α mRNA degradation, thereby suggesting that its accumulation is not due to an enhancement of mRNA stability.

To investigate whether active protein synthesis is required for induction of TNF-α mRNA accumulation induced by LPS, macrophages were incubated with cycloheximide, an inhibitor of protein synthesis. The results showed that cycloheximide alone inhibited TNF-α mRNA degradation (Fig 5). Also, this suggests that protein synthesis is not required for TNF-α mRNA accumulation. The combined treatment with actinomycin D and cycloheximide did not inhibit TNF-α mRNA degradation, as found for treatment with actinomycin alone. Calculation of TNF-α mRNA half-life from the actinomycin D–treated macrophage indicated that there are no significant half-life differences between the two diet groups: 26.5 minutes for macrophages of mice fed fish oil and 23.9 minutes for those of mice fed corn oil.

DISCUSSION

The aim of the present studies was to gain insight into the molecular mechanisms by which diets rich in fish oil enhance TNF-α production. Specifically, we determined whether there was an increase in TNF-α mRNA upon LPS stimulation of peritoneal macrophages and, if so, whether this was due to enhancement of transcription or to changes in mRNA degradation. Our results indicate that TNF-α mRNA expression and TNF-α protein production are enhanced upon LPS stimulation of macrophages from mice fed a diet rich in fish oil. This is in line with previous studies

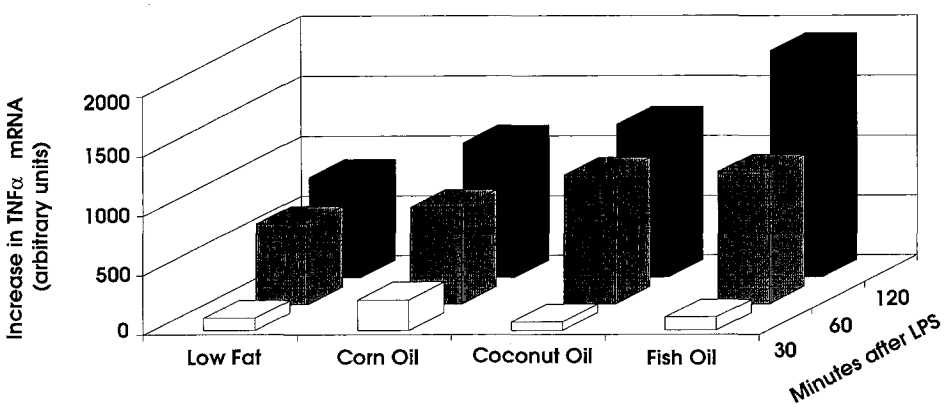

Fig 3. Densitometric readings for dot blots shown in Fig 2, indicate the kinetics of the increase in TNF-α mRNA at various times after LPS stimulation.

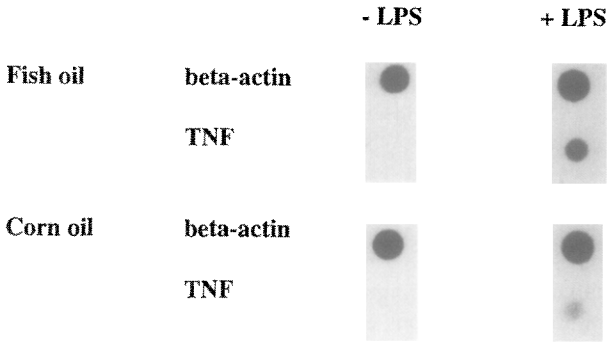

Fig 4. Analysis of TNF-α gene transcription in macrophages from mice fed fish oil or corn oil diets. (−LPS) Resting macrophages; (+LPS) macrophages activated with 2 μg/mL LPS. After 1 hour of exposure to LPS, macrophage nuclei were isolated and run-on assays were performed.

in which an enhancement in the production of TNF-α after LPS stimulation was observed in vitro and in vivo.[6-8]

It should be noted that in response to the LPS challenge, the increase in TNF-α protein in the fish oil group was more than 10-fold the levels in the other three groups, whereas mRNA was increased by only 2.3-fold. In macrophages of mice fed the other high-fat diets, on the other hand, TNF-α protein levels in response to LPS were similar to those obtained with the low-fat diet despite the tendency for TNF-α mRNA to be higher, albeit increases that are less well marked than found with the fish oil group. This apparent discrepancy between alterations in mRNA and in the protein for TNF-α may be explained by the possibility that in addition to an increase in the transcription of the TNF-α gene, there may also be alterations in posttranscriptional events. In the case of coconut oil or corn oil groups, increases in mRNA degradation may have compensated for the relatively small increases in TNF-α mRNA. In macrophages from mice fed fish oil, on the other hand, our data show no changes in mRNA degradation, but alterations at the translational level may also have occurred and contributed to the increase in TNF-α protein. Further studies are warranted to address these issues of posttranscriptional events. It is nonetheless clear from the study presented here that in macrophages from mice fed fish oil, the increase of TNF-α biosynthesis in response to LPS results in part through a specific enhancement of transcription of the TNF-α gene.

One of the potential mechanisms by which TNF-α production and TNF-α mRNA expression are enhanced upon fish oil feeding is through changes in the production of eicosanoids. Fish oil is rich in the long-chain n-3 polyunsaturated fatty acid (C22:6, 3) and docosahexaenoic acid (C20:5, 3). These fatty acids are preferentially incorporated into membrane phospholipids and inhibit the activity of the cyclooxygenase pathway, which metabolizes arachidonic acid to prostaglandins and thromboxane A_2.[14,15] In addition, eicosapentaenoic acid competes with arachidonic acid for metabolism by the lipoxygenase pathway to the leukotrienes.[16] Since depletion of arachidonic acid is accompanied by eicosapentaenoic acid substitution into the membrane phospholipid pool, it follows that upon stimulation, eicosapentaenoic acid rather than arachidonic acid will provide the substrate for prostaglandin and leukotriene production. The end result therefore is a shift in favor of the production of prostaglandins of the 3 and 5 series instead of those of the 2 and 4 series, which have been suggested to be more biologically active. Given evidence that PGE_2 can downregulate the production of TNF-α and

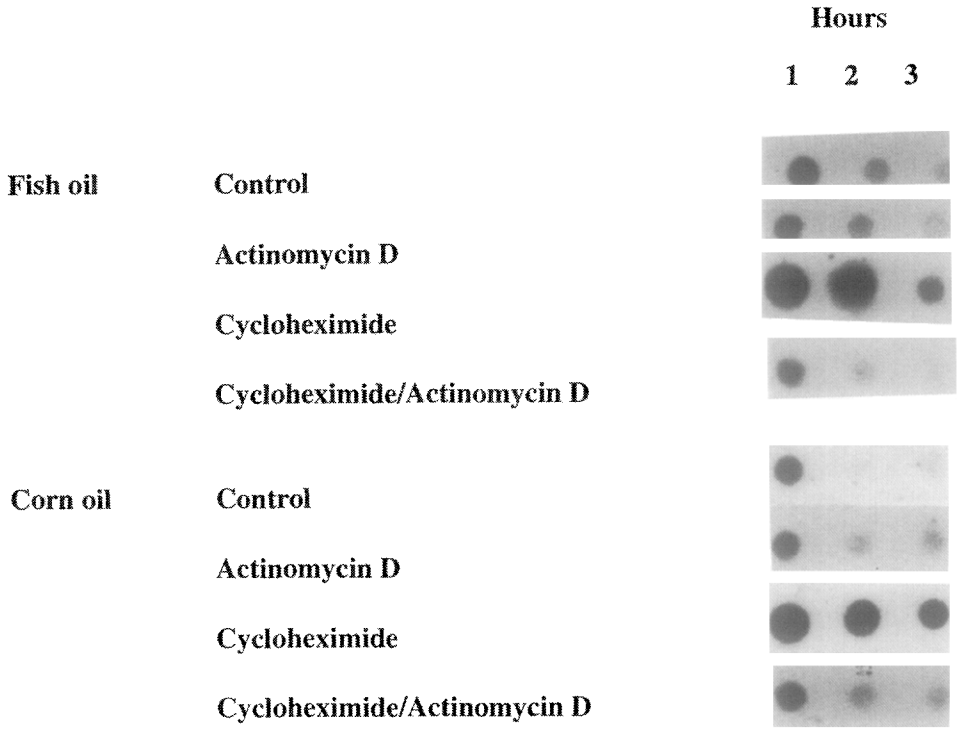

Fig 5. Influence of actinomycin D (5 μg/mL), cycloheximide (10 μg/mL), or a combination of cycloheximide (10 μg/mL) and actinomycin D (5 μg/mL) on TNF-α mRNA expression in macrophages from mice fed corn oil–or fish oil–enriched diets. Experiments were terminated at 1, 2, and 3 hours after addition of the inhibitors.

TNF-α mRNA expression,[17-19] the possibility arises therefore that the enhanced production of TNF-α found in response to fish oil feeding results from a suppressive effect of fish oil on the synthesis of the 2-series of prostaglandins.

Another potentially interesting mechanism is based on the notion that n-3 polyunsaturated fatty acids could have a "priming" effect on TNF-α production.[20,21] Although such a hypothesis requires further assessment, such priming has been shown to occur in phagocytes when they are treated with low concentrations of protein kinase C (PKC) activators.[19] Indeed, PKC has been implicated in the transcriptional regulation of TNF-α gene expression, where PKC activators upregulate TNF-α gene expression and inhibitors downregulate the gene expression.[22] Different fatty acids could thus conceivably modulate PKC activity or other protein kinases either directly[19,23,24] or indirectly by stimulating polyphosphoinositide turnover.[25] It is therefore also plausible that in the presence of LPS, protein kinase(s), through activation of transcription factors such as NF-KB, may be involved in transcriptional control of TNF-α gene expression.

In conclusion, the present studies indicate that in mice fed a diet rich in fish oil (ie, rich in n-3 fatty acids), the enhanced TNF-α production after LPS stimulation of peritoneal macrophages is associated with an increased transcription of the TNF-α gene, but is not related to changes in the rate of mRNA degradation. The macrophages are seemingly in an activated state for TNF-α production, and this increased TNF-α is not due to the existence of mRNA before LPS stimulation. Our results thus indicate that fish oil activates the transcriptional process for TNF-α production in murine peritoneal macrophages. Although such TNF-α upregulation may provide a mechanism for the beneficial effects of fish oil in certain inflammatory and immune disorders, it can also underlie its potential deleterious effects. For example, we have previously shown that in response to challenge with LPS, mice fed a diet rich in fish oil show TNF-α serum levels 10 times greater than in mice fed other diets,[8] and administration of the same diet to mice was associated with increased mortality after infection with *Salmonella typhimurium.*[26] The possibility arises therefore that high fish oil consumption may result in a degree of upregulation leading to exaggerated TNF-α production whereby the limits of benefit are exceeded to reach toxic levels.

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