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Todd E. Novak, Tricia A. Babcock, David H. Jho, W. Scott Helton and N. Joseph Espat

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NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α transcription

TODD E. NOVAK, TRICIA A. BABCOCK, DAVID H. JHO,
W. SCOTT HELTON, AND N. JOSEPH ESPAT
Laboratories of Surgical Metabolism, University of Illinois at Chicago, Chicago, Illinois 60612

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Novak, Todd E., Tricia A. Babcock, David H. Jho, W. Scott Helton, and N. Joseph Espat. NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α transcription. *Am J Physiol Lung Cell Mol Physiol* 284: L84–L89, 2003. First published August 30, 2002; 10.1152/ajplung.00077.2002.—ω-3 Fatty acid (FA) emulsions reduce LPS-stimulated murine macrophage TNF-α production, but the exact mechanism has yet to be defined. The purpose of this study was to determine the mechanism for ω-3 FA inhibition of macrophage TNF-α production following LPS stimulation. RAW 264.7 cells were pretreated with isocaloric emulsions of ω-3 FA (Omegaven), ω-6 FA (Lipovenos), or DMEM and subsequently exposed to LPS. IκB-α and phospho-IκB-α were determined by Western blotting. NF-κB binding was assessed using the electromobility shift assay, and activity was measured using a luciferase reporter vector. RT-PCR and ELISA quantified TNF-α mRNA and protein levels, respectively. Pretreatment with ω-3 FA inhibited IκB phosphorylation and significantly decreased NF-κB activity. Moreover, ω-3-treated cells demonstrated significant decreases in both TNF-α mRNA and protein expression by 47 and 46%, respectively. These experiments demonstrate that a mechanism for proinflammatory cytokine inhibition in murine macrophages by ω-3 FA is mediated, in part, through inactivation of the NF-κB signal transduction pathway secondary to inhibition of IκB phosphorylation.

eicosapentaenoic acid; nuclear factor-κB; signal transduction; tumor necrosis factor-α

FISH OIL EMULSIONS rich in ω-3 fatty acids (FA) have consistently demonstrated anti-inflammatory properties primarily through their effects on the macrophage (MØ) component of the inflammatory response (7, 10). However, studies examining the effects of ω-3 FA on the elaboration of proinflammatory cytokines (PIC) in MØ have been reported with variable results, a likely occurrence from the lack of a pure and consistent source of experimental ω-3 FA substrate (7, 14, 16, 21). As pharmaceutical grade ω-3 FA have not been available, their true biological effects are obfuscated from EPA oxidation, endotoxin contamination, and the inherent suppression of the macrophage inflammatory response by albumin alone (25). A commercially available pharmaceutical grade ω-3 FA emulsion Omegaven recently became available that enables the experimental evaluation of specific mechanisms of ω-3 FA action without the confounding variables of impurity. This fish oil emulsion is currently used as an anti-inflammatory agent for critically ill patients (19, 20). However, the mechanisms of action are not well defined. Moreover, a pure and isoenergetic ω-6 FA emulsion (Lipovenos) is also available for use as a true experimental control, allowing exclusion of the nonspecific effects of lipids on MØ in these experiments.

Previous studies in our laboratory have demonstrated that ω-3 FA emulsions inhibit LPS-mediated TNF-α expression in MØ, although the mechanisms of action are still unknown (1). It is hypothesized that ω-3 FA may modulate NF-κB transcriptional activator proteins, a principal pathway for MØ PIC elaboration (4, 5).

NF-κBs are dimers usually located in the cytoplasm associated with an inhibitor protein (IκB) (2). Under basal conditions, IκB maintains NF-κB in the cytoplasm by preventing display of the nuclear localization sequence (3, 6). When MØs are activated by a large variety of inducers, including endotoxin via the Tlr-4 receptor (18), IκB kinase-α phosphorylates IκB at two serine residues (Ser 32 and 36) allowing dissociation from NF-κB (8, 23, 24). Phosphorylated IκB is subsequently targeted for polyubiquitination and degradation through the 26S proteosome pathway. NF-κB is then free to localize to the nucleus initiating transcription of various PIC genes, most notably TNF-α (14).

The activation of NF-κB plays a vital role in the elaboration of TNF-α; therefore, we hypothesized that ω-3 FA may exert inhibitory effects at a specific point along the NF-κB pathway. The purpose of these experiments was to define a mechanism for ω-3 FA inhibition of MØ TNF-α production following LPS stimulation.

MATERIALS AND METHODS

Materials. Escherichia coli 0111:B4 LPS was purchased from Sigma (St. Louis, MO). The murine MØ cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD). Omegaven (ω-3 FA emulsion) and Lipovenos (ω-6 FA emulsion) were purchased from Fresenius-Kabi (Bad-Homburg, Germany).

Address for reprint requests and other correspondence: N. Joseph Espat, Dept. of Surgery M/C 958, Univ. of Illinois at Chicago, 840 South Wood St., Rm. 435E, Chicago, IL 60612 (E-mail: jespat@uic.edu).

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Cell culture. RAW 264.7 cells were suspended in complete medium, DMEM (Mediatech, Herndon, VA) (supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin). In all experiments, cells were plated in 24-well plates (except transfections, 35-mm plates; nuclear extraction, 100-mm plates) at a density of 1×10^6 cells/well. All experiments were performed in a humidified atmosphere under 5% CO_2 at 37°C.

Experimental design. All experiments adhered to the following protocol unless otherwise noted. Cells were randomly separated into three separate treatment groups: media alone (DMEM), ω-3 FA emulsion, or ω-6 FA emulsion. Cells were allowed to adhere for 2 h and then incubated in the presence of Omegaven (12 mg%, ω-3 FA emulsion), Lipovenos (10 mg%, isoenergetic, isocaloric ω-6 FA emulsion), or DMEM for 4 h. Optimal concentrations for MØ TNF-α inhibition were determined in previous studies (1). The cells were washed twice with DMEM and then stimulated with LPS (1 μg/ml) for 3 h. After LPS incubation, the cells were washed twice with DMEM and then examined as described below.

Measurement of IκB phosphorylation. To investigate the effects of ω-3 FA on IκB phosphorylation, total cellular protein from ω-3-treated RAW cells was analyzed via Western blotting. Cells were lysed with ×1 lysis buffer, 75 μg IκB, and 150 μg (phosphor-IκB) of total protein were separately loaded on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA), along with 0.35 μg of biotinylated protein standard (New England Biolabs, Beverly, MA), and run at 100 V for 70 min. The gel was transblotted to a nitrocellulose membrane (Bio-rad) at 300 mA for 70 min. The membrane was blocked with 5% nonfat dry milk in TBS containing 1% Tween 20 (TBST) for 90 min and then incubated in the presence of the primary antibody specific for IκB or phospho-IκB (New England Biolabs) (1:1,000 dilution, TBST with 5% BSA) overnight at 4°C. The membrane was then washed three times for 10 min each in TBST and subsequently exposed to an anti-biotin antibody (1:1,000 dilution) and anti-rabbit-HRP antibody (1:2,000 dilution) (New England Biolabs) for 1 h. The membrane was then washed again three times with TBST. The protein was detected by incubating the membrane with TMB Stabilized Substrate for HRP (Promega, Madison, WI) for 5 min.

Nuclear protein extraction and EMSA. Cells were grown to confluence (1×10^7 cells) in 100-mm plates and treated as described in *Experimental design*. After endotoxin exposure, cells were washed twice with ice-cold PBS, and total nuclear extract was prepared using the reagents and protocol described by Active Motif LLC (Carlsbad, CA). For the EMSA, T4 polynucleotide kinase, poly(dI-dC), [^{32}P]ATP, and the Sephadex G-50M column were purchased from Amersham Biosciences (Piscataway, NJ). All other reagents for this experiment were obtained from Sigma Chemical unless otherwise specified. The probe was a 24-bp double-stranded construct of the NF-κB consensus sequence (5′-AGGGACTTTCCGCTG GGACTTTCC-3′), which was end-labeled using T4 polynucleotide kinase and [^{32}P]ATP. The labeled probe was purified on a Sephadex G-50M column. For each sample, 5 μg of total nuclear protein were incubated with the labeled double-stranded probe (~50,000 cpm) and 5 μg of poly(dI-dC) in binding buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM dithiothreitol) for 20 min at 25°C. Specific competition was done by adding 100 ng of unlabeled NF-κB double-stranded binding probe to the reaction. The mixtures were run on 5% polyacrylamide gel electrophoresis in ×1 Tris-glycine-EDTA buffer. The gels were then vacuum-dried and exposed to radiographic film (Eastman Kodak, New Haven, CT).

Transfection of luciferase reporter vector. When measuring NF-κB activity, RAW cells were first transfected, before FA exposure, with a luciferase reporter vector (Clontech, Palo Alto, CA) containing four tandem copies of the NF-κB consensus sequence fused to a TATA-like region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter. All transfection media (exclusive of FBS) were supplemented with 1.0 μg/ml protamine sulfate. Protamine has been shown to increase lipid-mediated transfection of MØ by condensing DNA structure into a compact structure (9). In addition, the nuclear localization amino acid signal of protamine increases the nuclear translocation of DNA, thus enhancing luciferase transcription (11). One microgram of NF-κB luciferase vector was transfected with 8 μl of lipid in 200 μl of DMEM via the LipofectAMINE method (Invitrogen, Carlsbad, CA) for 5 h into RAW 264.7 cells. Zero point one microgram *Renilla* luciferase-positive control vector (pRL-SV40: Promega) was cotransfected with the NF-κB luciferase vector to normalize the transfection efficiency. pTAL-Luc (Clontech), substituting for the NF-κB luciferase vector, was used as a negative control. After the 5-h incubation, 1 ml DMEM with ×2 FBS was overlaid onto the cells. Transfection medium was replaced with complete medium at 18 h from start of the transfection.

Measurement of NF-κB activity. Transfected RAW cells were treated as described in *Experimental design* and then lysed with 150 μl ×1 passive lysis buffer (Promega) for 15 min at room temperature. Firefly and *Renilla* luciferase activities were obtained by analyzing 10 μl of protein lysate following the protocol provided by the Dual Luciferase Reporter Assay System (Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) (2-s delay, 10-s count). To adjust for any variation in transfection efficiency, the results were reported as firefly luciferase activity divided by *Renilla* luciferase activity.

RT-PCR analysis of TNF-α message. Total RNA from 4×10^6 treated RAW cells was extracted with 1 ml TRI reagent (Molecular Research Center, Cincinnati, OH). The samples were spun at 16,000 *g* for 10 min at 4°C, and then 700 μl of supernatant were removed and added to 70 μl 1-bromo-3-chloro-propane (Sigma). Samples were vortexed, incubated at room temperature for 10 min, and then spun for 5 min at 4°C. Three-hundred-fifty microliters of the resulting supernatant were added to an equal volume of Phenol/Isoamyl Alcohol/Chloroform (Fischer Scientific, Pittsburgh, PA), vortexed, and spun for 10 min at 4°C. Three-hundred microliters of the resulting supernatant were added to an equal volume of ice-cold isopropanol (Fischer) and precipitated at −20°C overnight. Samples were then spun at 16,000 *g* for 15 min at 4°C, washed with 75% ethanol, and the final RNA precipitant was suspended in 10 μl DEPC-treated water. RNA concentrations were determined spectrophotometrically at 260 nm, while the A_{260}/A_{280} ratio measured the purity of the samples. The first strand cDNA synthesis containing 0.8 μg total RNA was primed with oligo(dT) and 1 cycle was run: 15 min at 42°C, 5 min at 99°C, and 10 min at 5°C. Specific primers (Invitrogen) were used for TNF-α (sense, TCTCATCAGTTCTATGGCCC; antisense, GGGAGTAGACAAGGTACAAC) and GAPDH (sense, AGCCTTCTCCATGGTGGTGAAGAC; antisense, CGGAGTCAACGGATTTGGTCGTAT). The PCR cycling conditions for all reactions were as follows: 94°C for 1 min, 60°C for 35 s, and a final extension period at 72°C for 7 min. Optimal amplification was achieved at 26 cycles for TNF-α and GAPDH. GAPDH served as an internal control. The PCR products were run alongside 0.25 μg of 100-bp molecular standard ladder (Bio-rad) on a 1.5% agarose gel containing ethidium bromide. After separation, the bands

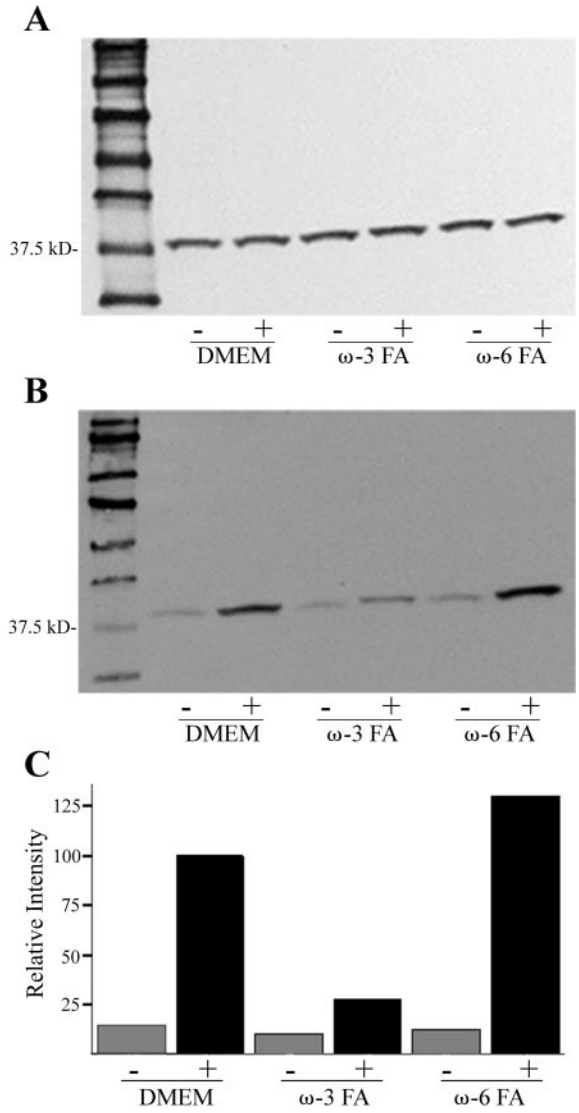

Fig. 1. Representative Western blot analysis of IκB-α (*A*) and phospho-IκB-α (*B*) protein. RAW cells were pretreated with DMEM, ω-3 fatty acids (FA), or ω-6 FA for 4 h, washed, and then exposed to DMEM (−) or endotoxin (+) for 3 h. The cells were subsequently washed and then lysed to obtain cellular protein. Seventy-five grams and 150 g total protein were separately loaded in each lane for IκB (*A*) and phospho-IκB (*B*), respectively. Blots represent 1 of 3 experiments with similar results. *C*: semiquantification of the phospho-IκB blot. The band intensities were quantified using Kodak 1D. DMEM + LPS was set at 100, and background was set at 0.

were visualized under UV light (Fischer Scientific) and analyzed with 1D software (Eastman Kodak). The results were expressed as the relative intensity vs. the control (GADPH).

ELISA for TNF-α. Supernatants from treated RAW 264.7 cells were collected and immediately frozen at −70°C. The samples were subsequently analyzed for TNF-α protein following the protocol provided by R&D Systems (Minneapolis, MN).

Statistical analysis. TNF-α and NF-κB data are presented as means ± SE. The data were analyzed by one-way ANOVA, as well as additional ANOVA post hoc analysis (Tukey and Scheffé's). Statistical significance was defined at the $P < 0.05$ level.

RESULTS

Effects of ω-3 FA on IκB phosphorylation. The amount of total IκB, phosphorylated and unphosphorylated, did not deviate from baseline levels (DMEM + LPS) after 3-h LPS exposure (Fig. 1*A*). The amount of phospho-IκB, however, did change significantly depending on the treatment (Fig. 1, *B* and *C*). A small amount of IκB phosphorylation is evident in nonstimulated MØ for all three treatments. DMEM and ω-6 FA pretreatment, followed by LPS stimulation, exhibited approximately the same moderate increase in phospho-IκB. Conversely, ω-3 FA treatment significantly decreased the amount of IκB phosphorylation; the intensity is similar to the nonstimulated cells.

Effects of ω-3 FA on NF-κB binding and activity. A low basal level of NF-κB binding to the TNF-α-specific consensus sequence was observed in non-LPS-stimulated MØ, whereas LPS exposure generated strong binding of NF-κB in both DMEM- and ω-6-pretreated cells but not after ω-3 FA (Fig. 2). Moreover, the effects of DMEM, ω-3, and ω-6 FAs on NF-κB binding parallel

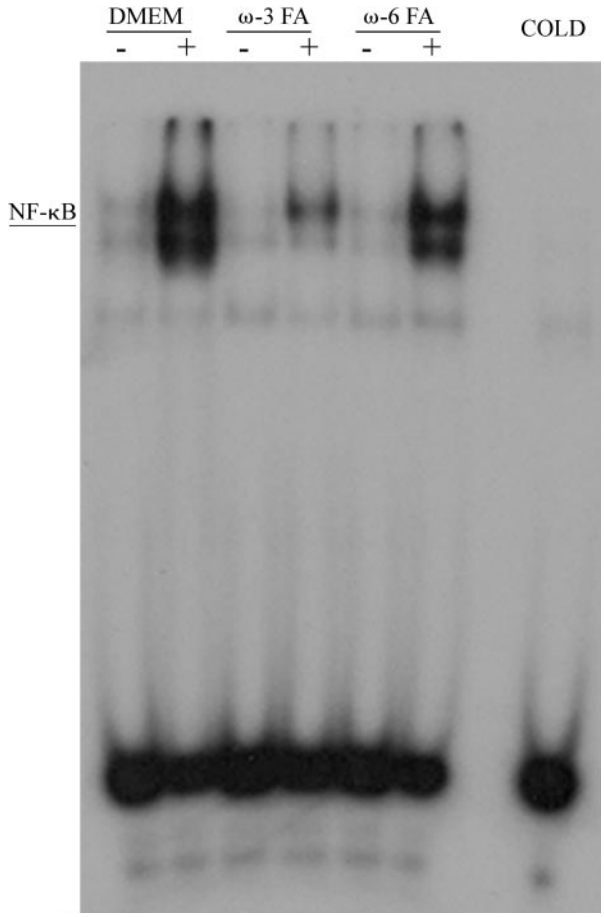

Fig. 2. NF-κB binding activity via EMSA. Confluent cells were pretreated with DMEM, ω-3 FA emulsion, or ω-6 FA emulsion for 4 h, washed, and then exposed to DMEM (−) or LPS (+) for 3 h. Total nuclear protein was subsequently isolated and analyzed by EMSA for NF-κB DNA binding activity using a ^{32}P-labeled double-stranded oligonucleotide of the NF-κB consensus sequence 5′-AGGGACTTTC-CGCTGGGACTTTCC-3′. An additional nonlabeled probe was added in the competition assay (cold).

the results seen in the activity studies (Fig. 3). A low level of NF-κB activity was observed in non-LPS-stimulated MØ. Strong activation of NF-κB was induced after LPS exposure, as shown by the large increase in luciferase activity in both the DMEM- and ω-6-pretreated cells. Conversely, ω-3 pretreatment significantly decreased NF-κB activation by 63% under control levels following endotoxin exposure ($P < 0.01$). It should be noted that transfecting with protamine sulfate in the media did not alter basal luciferase activity and that the pTAL vector (control) produced nominal luciferase activity (data not shown).

ω-3 FA effects on TNF-α message transcription. MØ incubated in the presence of DMEM, ω-3 FA, and ω-6 FA demonstrated similar low levels of constitutive expression of TNF-α mRNA (Fig. 4). After LPS stimulation, cells pretreated with DMEM and ω-6 FA averaged greater than a 100% increase in transcription over basal levels. Conversely, LPS-stimulated cells first exposed to ω-3 FA demonstrated a significant decrease (47%) in TNF-α mRNA levels under the control; this value is only a 15% increase over the control media (DMEM) without LPS. This difference is statistically significant with a P value of <0.01.

ω-3 FA effects on TNF-α protein production. Cells pretreated with either ω-3 FA or ω-6 FA without LPS stimulation did not demonstrate a significant change in TNF-α production from baseline (DMEM). After LPS stimulation, TNF-α production from DMEM- and ω-6 FA-treated cells increased similarly. However, ω-3-treated cells displayed a significant decrease in TNF-α production after LPS stimulation, with a 46% decrease from baseline ($P < 0.01$) (Fig. 5). In addition, it should

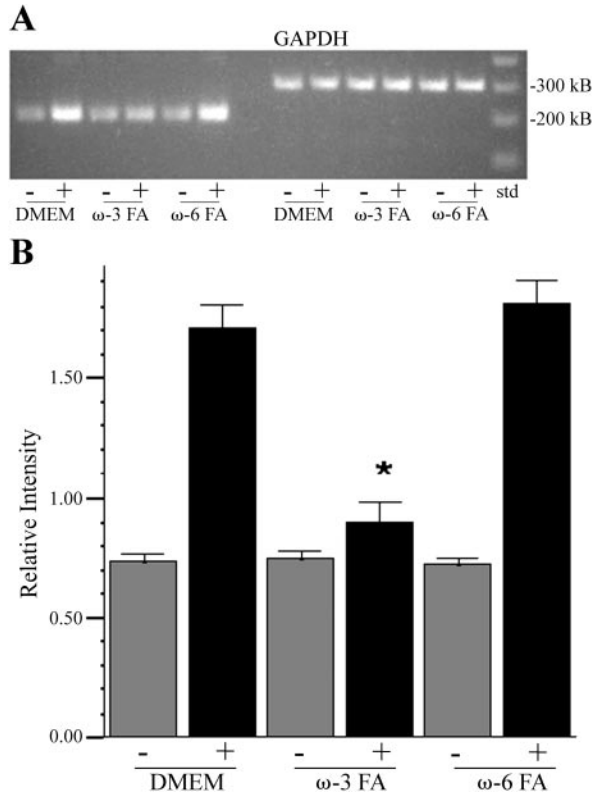

Fig. 4. Effects of ω-FA on RAW cell TNF-α mRNA expression. RAW cells were pretreated with DMEM, ω-3 FA emulsion, or ω-6 FA emulsion for 4 h, washed, and then exposed to DMEM (−) or endotoxin (+) for 3 h. Total RNA was extracted and TNF-α message determined via RT-PCR (*A*). The band intensities were quantified using Kodak 1D software and are illustrated in the bar graph (*B*). The results are expressed as the relative intensity of TNF-α vs. control (GAPDH) ($n = 3$). Data represent means ± SE. *Mean difference is significant at the 0.05 level.

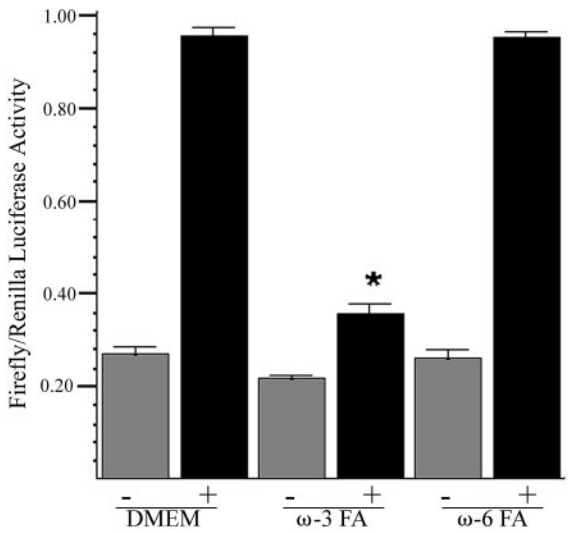

Fig. 3. Luciferase activity of NF-κB reporter vector in RAW cells. One gram of firefly reporter plasmid was transfected, along with 0.01 g of SV40 *Renilla* luciferase reporter as a transfection efficiency control, into 1×10^6 RAW cells for 5 h. The cells were subsequently treated with DMEM, ω-3 FA, or ω-6 FA for 4 h, washed, and then incubated in the presence of DMEM (−) or LPS (+) (1 g/ml) for 3 h. The cells were then lysed and firefly and *Renilla* luciferase activities were determined. Intensity values are reported as firefly/*Renilla* ($n = 3$). Data represent means ± SE. *Mean difference is significant at the 0.05 level.

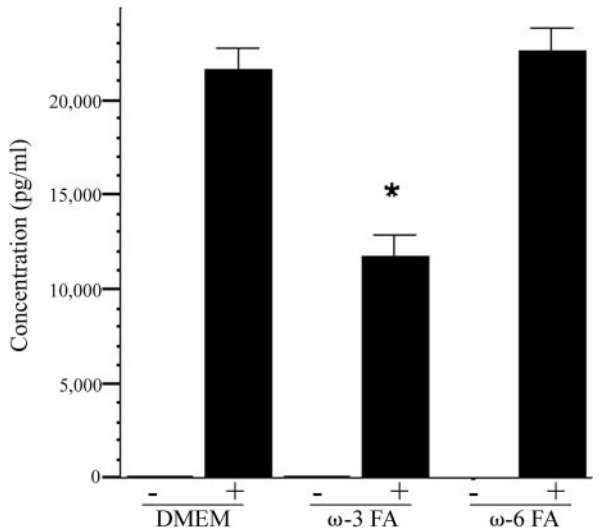

Fig. 5. Effects of ω-FA on RAW cell TNF-α production. Cells were pretreated with DMEM, ω-3 FA emulsion, or ω-6 FA emulsion for 4 h, washed, and then incubated in the presence of DMEM (−) or LPS (+) (1 g/ml) for 3 h. Supernatants were collected and analyzed by ELISA ($n = 3$). Data represent means ± SE. *Mean difference is significant at the 0.05 level.

be noted that the decrease in TNF-α protein is congruent with the decrease in TNF-α message after ω-3 FA treatment, 46 and 47%, respectively.

DISCUSSION

The early inflammatory response is regulated predominately by the MØ component of the immune system and is characterized by an increased production of PIC, mediated, in part, through NF-κB activation (4). ω-3 FA have long been considered as an anti-inflammatory agent. However, past studies have reported variable PIC elaboration. Previously available ω-3 FA sources for experimental evaluation were potentially contaminated or consisted of compound substances likely contributing to the observed experimental discrepancies. For instance, Sato et al. (21) reported that ω-3 FA treatment of human monocytes increased TNF-α production after LPS stimulation, yet Billiar et al. (7) demonstrated a decrease in TNF-α. The recent availability of a pharmaceutical grade ω-3 FA source will now allow for assessment of the cellular mechanisms that define the anti-inflammatory properties of ω-3 FA. This study investigated the effects of ω-3 FA on the elaboration of TNF-α in the context of a LPS-stimulated in vitro murine MØ model.

This report examined the effects of ω-3 FA on the phosphorylation of IκB at serine 32 as a requirement for both NF-κB translocation to the nucleus and activation. The results demonstrate that following LPS stimulation, ω-3 FA pretreatment significantly decreases phosphorylation at serine 32, thus providing a basis for the observed decrease in NF-κB binding and activity following ω-3 incubation. Without the requisite IκB phosphorylation, NF-κB remains inactive in the cytoplasm bound to IκB. Our data indicate that total levels of IκB (phosphorylated and unphosphorylated) do not deviate after a 3-h LPS treatment. These results are consistent with previous reports on the time course for IκB degradation through the ubiquitin-proteosome pathway. Shanley et al. (22) reported that IκB is degraded rapidly after 5 min of LPS exposure but returns to baseline levels within 30 min.

Subsequent experiments examining the effects of ω-3 FA on the NF-κB signal transduction cascade were initiated based on two observations: *1*) ω-3 FA decrease transcription of TNF-α suggesting modulation of an intercellular signal transduction pathway and *2*) NF-κB is an essential transcriptional regulator of inflammatory gene activation, including TNF-α. The experimental data support that ω-3 FA significantly decrease both NF-κB binding to the TNF-α-specific consensus sequence and subsequent activity in response to LPS stimulation compared with both DMEM and ω-6 FA. Moreover, MØ incubated in ω-3 FA-rich media before LPS stimulation produces significantly less TNF-α message elaboration. These data implicate that a major anti-inflammatory mechanism for ω-3 FA is reduction of TNF-α gene transcription, mediated, in part, through inhibition of NF-κB regulatory proteins. As TNF-α protein decreases a proportional amount, the anti-inflammatory effects of ω-3 FA on TNF-α occur primarily at the level of gene transcription. These results are consistent with previous studies examining the effects of ω-3 FA on TNF-α production in an in vitro murine MØ model (14).

The mechanisms modulating cellular events proximal to IκB phosphorylation in the elaboration of TNF-α are still yet to be elucidated. Some studies have suggested that the incorporation of ω-3 FA into the plasma membrane alters the composition of the phospholipid pool, thus modifying the production of inflammatory lipid mediators (e.g., PGE_2) (15, 17). It is thought that ω-3 FA displace arachidonic acid from plasma membranes decreasing its availability as a precursor of inflammation-associated prostanoids. Moreover, Kunkel et al. (13) reported a clear autoregulatory relationship between TNF-α and proinflammatory prostanglandins, particularly PGE_2. Recent studies also suggest that ω-3 FA may act at the level of membrane-bound receptors. Jordan and Stein (12) propose that ω-3 FA may alter the physical and chemical properties of the plasma membrane so that receptor ligand binding is altered. On the basis of these observations, it is possible that ω-3 FA change the sensitivity of the Tlr-4 receptor for LPS, consequently inhibiting transduction of proinflammatory signals into the interior of the MØ. Future experiments are warranted in the investigation of the proximal regulatory mechanisms of ω-3 FA on IκB kinase-α inhibition, specifically the interaction between ω-3 FA, inflammatory prostaglandin elaboration, and Tlr-4 receptor function.

In summary, the data demonstrate that treatment of murine MØ with ω-3 FA significantly decreases IκB phosphorylation at serine 32 and consequently reduces the ability of NF-κB to bind to the TNF-α-specific consensus sequence. As a result, the NF-κB signal transduction cascade is inhibited, and this decreased NF-κB activity is translated into a concomitant decrease in TNF-α mRNA transcription. TNF-α protein elaboration is reduced accordingly. Moreover, the ω-6 FA-treated MØ exhibits similar effects as media alone (DMEM) in all experiments, validating that the observed anti-inflammatory effects on the MØ are exclusive to ω-3 FA and not a result of a general lipid effect. These experiments demonstrate that a mechanism for proinflammatory cytokine transcription inhibition in murine MØ by ω-3 FA is mediated, in part, through inactivation of the NF-κB signal transduction pathway secondary to inhibition of IκB phosphorylation at serine 32.

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