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Role of Nrf2 in Suppressing LPS-Induced Inflammation in Mouse Peritoneal Macrophages by Polyunsaturated Fatty Acids Docosahexaenoic Acid and Eicosapentaenoic Acid

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Abstract: This study is to investigate the role of Nrf2 in suppressing LPS-mediated inflammation in *ex vivo* macrophages by polyunsaturated fatty acids (PUFA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Primary peritoneal macrophages from Nrf2 wild-type (+/+; WT) and Nrf2 knockout (−/−; KO) mice were treated with lipopolysaccharides (LPS) in the presence or absence of DHA or EPA. Quantitative real-time PCR (qPCR) analyses showed that LPS potently induced cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in the macrophages collected from Nrf2 (+/+) wild-type mice. DHA and EPA inhibited LPS-induced COX-2, iNOS, IL-1β, IL-6, or TNF-α, but increased hemeoxygenase (HO-1) expression. DHA was found to be more potent than EPA in inhibiting COX-2, iNOS, IL-1β, IL-6, and TNF-α mRNA expression. DHA and EPA were also found to induce HO-1 and Nrf2 mRNA with a different dose−response. LPS induced COX-2, iNOS, IL-1β, IL-6, and TNF-α in the macrophages collected from Nrf2 (−/−) mice as well, however, DHA and EPA suppression of COX-2, iNOS, IL-1β, IL-6, and TNF-α was attenuated as compared to that in Nrf2 (+/+) macrophages. Taken together, using Western blotting, ELISA and qPCR approaches coupled with Nrf2 (−/−) mice, our study clearly shows for the first time that DHA/EPA would induce Nrf2 signaling pathway and that Nrf2 plays a role in DHA/EPA suppression of LPS-induced inflammation.

Keywords: Docosahexaenoic acid; eicosapentaenoic acid; nuclear factor-erythroid 2-related factor 2; Nrf2; inflammation; antioxidative stress

1. Introduction

Polyunsaturated fatty acids (PUFA) contain two or more *cis* double bonds that are separated by a single methylene group. Omega-3 fatty acids are a family of PUFA that have a final carbon−carbon double bond at the third bond from the methyl end of the fatty acid. Decosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two omega-3 fatty acids and are important nutritional essentials. But, these two omega-3 fatty acids cannot be synthesized by the human body and must be obtained from food.

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Polyunsaturated fatty acids, such as *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) and *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA), have been found to possess beneficial effects in cardioprotection, anti-inflammation, vascular disease prevention, metastatic breast cancer incidence reduction, possible type 2 diabetes therapy, and multiple sclerosis.[1−4] These and other health benefits of DHA and EPA ignited extensive studies in recent years. In November 2004, a prescription form of omega-3 fatty acids (P-O3FA, Omacor capsules, Reliant Pharmaceuticals, Inc., Liberty Corner, NJ) was approved by the US FDA for reducing very high triglycerides in adults (≥500 mg/dL) adjunctive to diet.[5] DHA, the most abundant n-3 PUFA in erythrocyte membranes, was associated with a reduced risk of breast cancer.[6] Supplement of EPA was found to help cancer patients retain muscle mass in a 2009 clinical trial.[7] The anti-inflammatory effects of DHA/EPA have also been well studied in preclinical research[8−12] as well as in clinical research.[13−16] These previous studies explored the effectiveness of DHA/EPA in their anti-inflammation activities, and in addition, our previous study[8] also suggested a possible link to the nuclear factor E2-related factor 2 (Nrf2) signaling pathway.

Nrf2 is a cap "n" collar basic leucine zipper transcription factor. It is crucial to defend against many chemical and biological insults[17] and has been shown to regulate the expression of many genes, including those involved in phase II detoxification and antioxidative stress.[18] Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein (Keap1) under basal conditions. When the cell is challenged by oxidative stress, Nrf2 is released from Keap1 inhibition, translocates to the nucleus, dimerizes with Maf, and activates transcription of genes containing the antioxidant response element (ARE) in the promoter regions of genes. Nrf2 has been reported in playing an important role in lung injury reversal,[19,20] human endothelial cell survival,[21] neuroinflam-

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mation,[22] hyperoxia,[23] lung damage from cigarette smoking,[24] and impaired function of macrophages.[25] Other studies suggest that Nrf2 suppresses inflammation by inhibiting NF-κB activation through regulation of redox balance.[26] In our previous study, we have shown that Nrf2 protects intestinal integrity through the regulation of proinflammatory cytokines and induction of phase II detoxifying enzyme,[27] and that sulforaphane suppressed lipopolysaccharide (LPS)-induced inflammation in the mouse peritoneal macrophages through Nrf2 pathway.[28] In this study, we further evaluated whether Nrf2 would play an important role in DHA/EPA's anti-inflammation mechanism of action in mouse primary macrophages derived from Nrf2 (−/−) and Nrf2 (+/+) mice.

2. Materials and Methods

2.1. Animals, Cell Culture and Reagents. Nrf2 (−/−) mice were backcrossed with C57BL/6J wile-type mice purchased from The Jackson Laboratory (Bar Harbor, ME), as described previously.[28] The genotype of each animal was confirmed by extracting DNA from the tail, and RT-PCR was performed with the primers: 3′-primer, 5′-GGA ATG GAA AAT AGC TCC TGC C-3′; 5′-primer, 5′-GCC TGA GAG CTG TAG GCC C-3′; and lacZ primer, 5′-GGG TTT TCC CAG TCA CGA C-3′. Nrf2 (−/−) mice exhibited bands at 200 bp, while Nrf2 (+/+) mice exhibited bands at 300 bp. The second generations (F2) of 9−12 week old male Nrf2 (−/−) were used in this study. Sex and age matched wild type mice (Nrf2 (+/+)) from The Jackson Laboratory, together with the Nrf2 (−/−) knockout mice, were housed at Rutgers Animal Facility and maintained under 12 h light/dark cycles.

Thioglycolate broth-elicited peritoneal macrophages were described previously.[28] Approximately 0.8 mL of thioglycolate broth was injected intraperitoneally to each mouse, and four days later, the peritoneal macrophages were harvested from the mouse peritoneal cavity with a cold pH 6.8 phosphate buffer solution containing 0.02% EDTA. The collected macrophage cells were centrifuged at 1,000 rpm for 10 min, and the cell pellet was resuspended with 0.083% ammonium chloride solution to remove any red blood cells. The cell numbers were counted, and equal amounts of cells from Nrf2 (+/+) and Nrf2 (−/−) mice were cultured for four to six hours in DMEM medium containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2. For Western blotting protein samples, macrophages were then treated with medium containing DHA/EPA for 6 h. After 6 h of DHA/EPA treatment, the medium containing DHA/EPA was replaced with medium containing 1 μg/mL of lipopolysaccharides (LPS) for an additional 18 h of LPS only treatment. For the experiments for qPCR, ELISA, and NO measurement, after 6 h of DHA/EPA treatment, the medium containing DHA/EPA was replaced with medium containing 1 μg/mL of LPS only for 8 h of LPS treatment. mRNAs from the cells were extracted thereafter (an approximate total of 14 h of treatment as identified as optimum, based on the results shown in Figure 5). Cell culture media were used for ELISA and NO measurements. Negative controls (DMEM with 10% FBS, labeled as No Treatment Control in the figures) and positive controls (DMEM with 10% FBS and 1 μg/mL of LPS, labeled as LPS in the figures) were used in all cell treatment groups. All chemicals including DHA and EPA were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Thioglycolate broth was obtained from Edge Biologicals (Memphis, TN). LPS was derived from *Escherichia coli* 055:B5.

2.2. Protein Extraction and Western Blotting. Following DHA/EPA and LPS treatments for 18 h, peritoneal macrophages were washed with ice-cold PBS and lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO). The cell lysates were centrifuged at 12000*g* for 10 min at 4 °C. The protein concentrations of the whole cell lysate were measured by using Pierce BCA protein assay reagent (Thermo Scientific, Waltham, MA). 20 μg of protein was loaded onto NuPAGE 4−12% electrophoresis gel (Invitrogen). After electrophoresis, the proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membrane at 130 mV. The PVDF membranes were incubated with the selected primary antibodies, the membrane proteins were detected by HRP-conjugated secondary antibodies, and the signals were enhanced with ECL reagents (GE Healthcare). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Measurement of Nitrite (NO) Concentration and the Cytokines. A sensitive fluorimetric assay method described by Misko et al. was used for the NO concentration

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Table 1. Oligonucleotide Primers Used for Qualitative Real-Time PCR (qPCR)

gene	association no.	forward primer	reverse primer
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	XM_001473623.1	5′-TGA AGC AGG CAT CTG AGG G-3′	5′-CGA AGG TGG AAG AGT GGG AG-3′
cyclooxygenase-2 (COX-2)	NM_011198.3	5′-TGC CTG GTC TGA TGA TGT ATG CCA-3′	5′-AGT AGT CGC ACA CTC TGT TGT GCT-3′
inducible nitric oxide synthase 2 (iNOS)	NM_010927.2	5′-CCT GGT ACG GGC ATT GCT-3′	5′-GCT CAT GCG GCC TCC TTT-3′
tumor necrosis factor-alpha (TNF-α)	NM_013693	5′-TCT CAT GCA CCA CCA TCA AGG ACT-3′	5′-ACC ACT CTC CCT TTG CAG AAC TCA-3′
interleukin-1 beta (IL-1β)	NM_008361	5′-AAG GGC TGC FTTC CAA ACC TTT GAC-3′	5′-ATA CTG CCT GCC TGA AGC TCT TGT-3′
interleukin-6 (IL-6)	NM_031168	5′-ATC CAG TTG CCT TCT TGG GAC TGA-3′	5′-TAA GCC TCC GAC TTG TGA AGT GGT-3′
hemeoxygenase-1 (HO-1)	NM_010442.1	5′-CCT CAC TGG CAG GAA ATC ATC-3′	5′-CCT CGT GGA GAC GCT TTA CAT A-3′
nuclear factor-erythroid 2-related factor 2 (Nrf2)	NM_010902	5′-TCA CAC GAG ATG AGC TTA GGG CAA-3′	5′-TAC AGT TCT GGG CGG CGA CTT TAT-3′

measurement of the biologically produced nitrite.[29] Briefly, sodium nitrite standards prepared by serial dilution in deionized water were used to quantitate the NO concentrations in the samples. 50 μL of cell culture medium was added to a 96-well plate, and then 10 μL of freshly prepared 2,3-diaminonaphthalene (0.05 mg/mL in 0.62 N HCl) was added. After 10 min of incubation at room temperature in the dark, 5 μL of 2.8 N sodium hydroxide was added to terminate the reaction. The reaction generated 2,3-diaminonaphthotriazole in each of the standards, and samples were measured with excitation at 360 nm and emission at 460 nm with a gain setting of 80% using a microplate fluorescence reader, FLx-800 (Bio-Tek Instruments, Winooski, VT). Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) concentrations of the culture medium were analyzed using the respective enzyme-linked immunosorbent kits (TNF-α ELISA assay kit, Pierce Endogen, Rockford, IL; IL-6 ELISA assay kit, Invitrogen, Carlsbad, CA) according to the manufacturers' protocols.

2.4. mRNA Isolation and Reverse-Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction (qPCR). RNAs from mouse peritoneal macrophages were isolated using Invitrogen RNeasy Mini Kit (Carlsbad, CA), and the mRNA concentrations were then measured using Invitrogen (Carlsbad, CA) Quant-It reagents. Equal amounts of mRNAs (approximately 600 ng) were then converted by reverse-transcription to cDNA using Superscript III First Strand Synthesis System from Invitrogen. SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA) was used for the qPCR analyses on Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). The primers were designed using Primer Quest Oligo Design and Analysis Tool and obtained from Integrated DNA Technologies (Coralville, IA) (see Table 1). GAPDH was used as the endogenous loading control.

2.5. Data Processing and Statistical Analysis. All data are expressed as mean ± SEM and represent at least three independent experiments. The statistical analysis was performed using the one-side Student's *t* test with statistical significance of the mean difference indicated in the figures. The results were considered significant if $p < 0.05$.

3. Results

3.1. DHA/EPA Reduced Protein Expression Levels of COX-2 and iNOS but Induced HO-1 Protein Expression. To investigate the anti-inflammatory effect of DHA/EPA and the role of Nrf2 in these anti-inflammatory effects, protein expression of COX-2 and iNOS experiments were carried out by Western-blot analyses. Figure 1(A) and Figure 1(B) show the protein expressions from the macrophages treated with DHA/EPA at 25 and 50 μM with LPS in both Nrf2 (−/−) and Nrf2 (+/+) mice. The protein expression levels of COX-2 clearly showed induction by LPS, and that this induction was significantly attenuated by DHA at the 50 μM level or EPA at both the 25 μM and 50 μM levels in the Nrf2 (+/+) (Figure 1B) but not in the Nrf2 (−/−) (Figure 1A). For iNOS, the protein level was obviously induced by LPS treatment, although, at 25 μM and 50 μM, iNOS expressions were significantly suppressed by DHA in the Nrf2 (−/−) group, and suppressions were significantly more pronounced in the Nrf2 (+/+) group for both DHA and EPA at 25 μM and 50 μM levels. On the other hand, HO-1, was significantly induced by EPA at 50 μM in the Nrf2 (−/−) group and by DHA at 25 μM and 50 μM and EPA at 50 μM in the Nrf2 (+/+) group.

3.2. DHA Inhibits LPS-Induced Secretion of Nitrite in Nrf2 (+/+) Macrophages More Than That in Nrf2 (−/−) Macrophages. Figure 2 shows the nitrite inhibition based on the nitrite concentrations produced and secreted by macrophages in the cell culture medium. DHA treatment showed significant nitrite inhibition in Nrf2 (+/+) wild type mice as compared with Nrf2 (−/−) mice. EPA treatment did not show such substantial nitrite inhibition under the same conditions (data not shown). This is consistent with earlier report that DHA induces a more effective anti-inflammatory effect than EPA.[30]

3.3. LPS-Induced Secretions of TNF-α and IL-6 Were Inhibited by DHA/EPA in Nrf2 (+/+) Peritoneal Macrophages as Compared to That in Nrf2 (−/−) Peritoneal Macrophages. Figure 3 shows the concentration of TNF-α secreted by the macrophages in the medium using the ELISA

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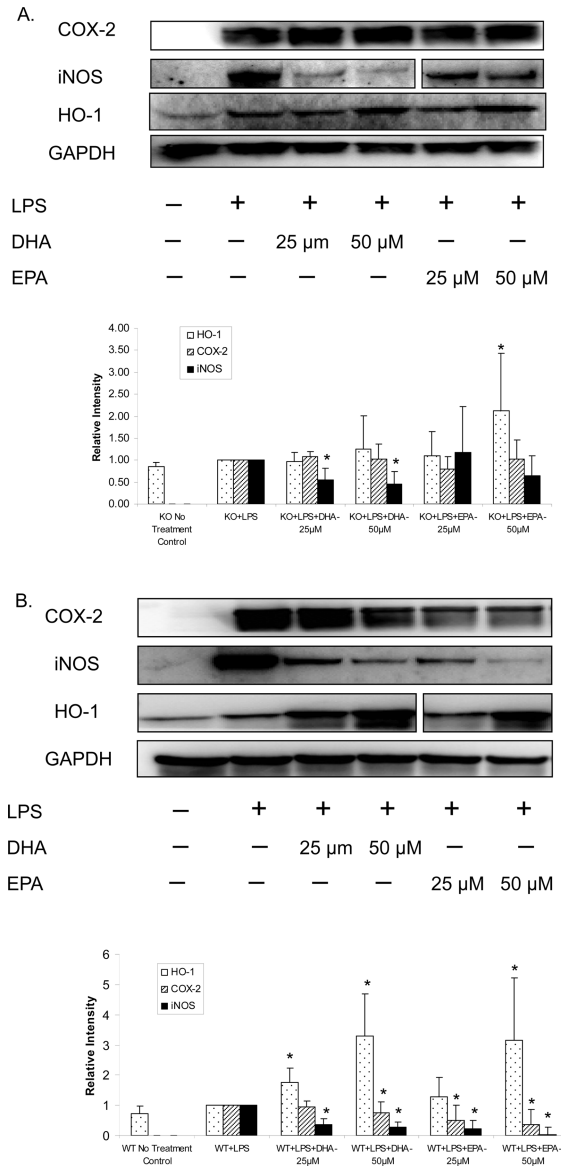

Figure 1. Western blot showing that LPS potently induced COX-2 and iNOS in the macrophages collected from the Nrf2 (−/−) mice (A). The inductions were not suppressed by DHA or EPA for COX-2 and were moderately suppressed by DHA for iNOS, but not by EPA. HO-1 expressions were induced by DHA and by EPA. Western blot figures show that LPS potently induced COX-2 and iNOS in the macrophages collected from the Nrf2 (+/+) mice (B). The inductions are suppressed dose-dependently by DHA and by EPA for COX-2 and iNOS. HO-1 expressions were induced by DHA and by EPA. The results were obtained from at least three analyses of three groups of mice (densitometry $n = 3$).

kit. LPS induced the secretion of TNF-α in both the Nrf2 (+/+) and Nrf2 (−/−) macrophages. In the Nrf2 (−/−) group, DHA or EPA at either 25 μM or 50 μM showed no suppression of TNF-α (Figure 3A). However, in the Nrf2 (+/+) group, DHA at 25 and 50 μM and EPA at 50 μM significantly suppressed TNF-α secretion in a dose-dependent fashion (Figure 3B).

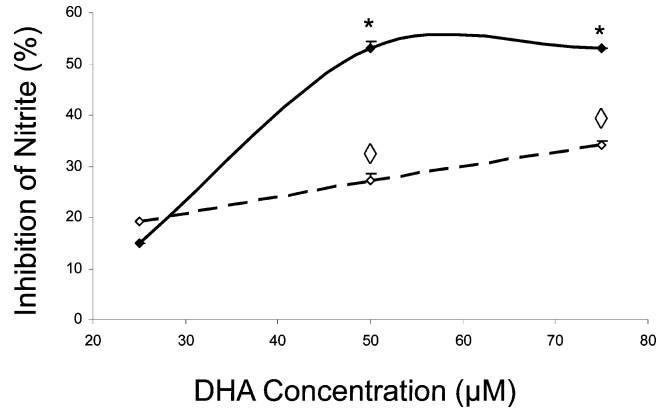

Figure 2. DHA inhibited nitrite secretion more dramatically in the Nrf2 (+/+) wild type (solid line —) than in Nrf2 (−/−) (dashed line – – –) mouse macrophages ($n = 3$). Asterisk (*) indicates significantly different ($p < 0.05$) in Nrf2 (+/+) mouse peritoneal macrophages; diamond (◊) indicates significantly different ($p < 0.05$) between Nrf2 (+/+) and Nrf2 (−/−) mouse peritoneal macrophages. The inhibition by EPA was not substantial (data not shown).

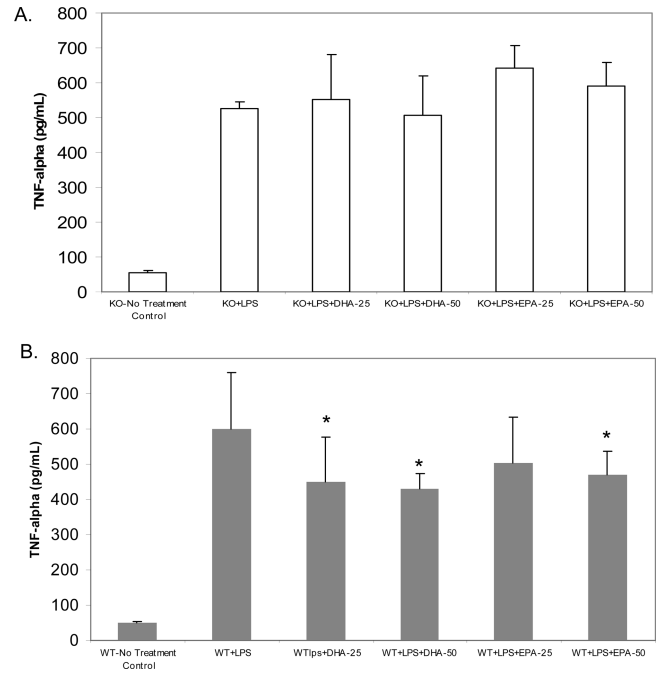

Figure 3. DHA/EPA inhibited secretion of TNF-alpha in Nrf2 (+/+) mice significantly but not in Nrf2 (−/−) mice ($n = 6$).

The concentrations of IL-6 secreted by the macrophages in the medium measured using the ELISA kit are shown in Figure 4. LPS induced the secretion of IL-6 in both the Nrf2 (+/+) and Nrf2 (−/−) macrophages. In the Nrf2 (−/−) group, DHA/EPA (25 or 50 μM) showed no suppression of

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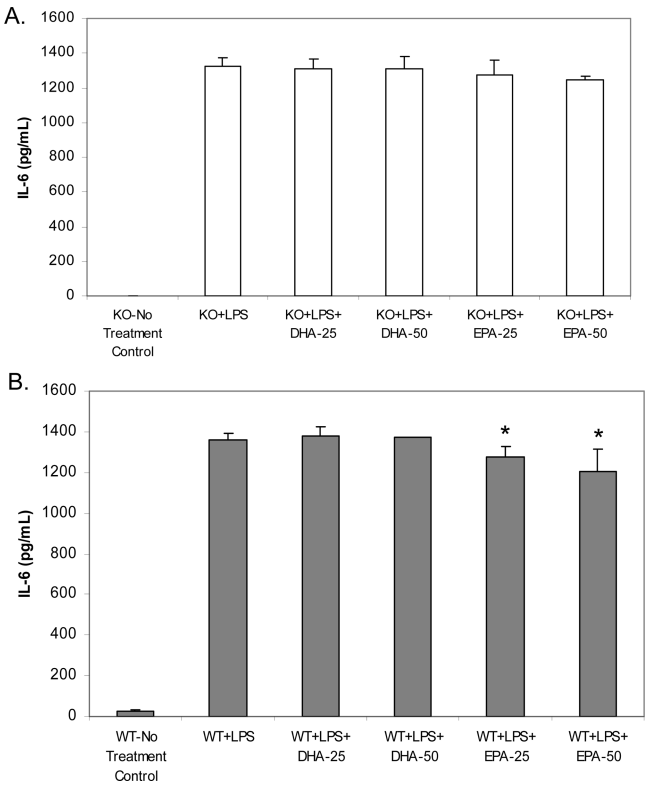

Figure 4. EPA significantly inhibited secretion of IL-6 in Nrf2 (+/+) mice but not in Nrf2 (−/−) mice ($n = 3$).

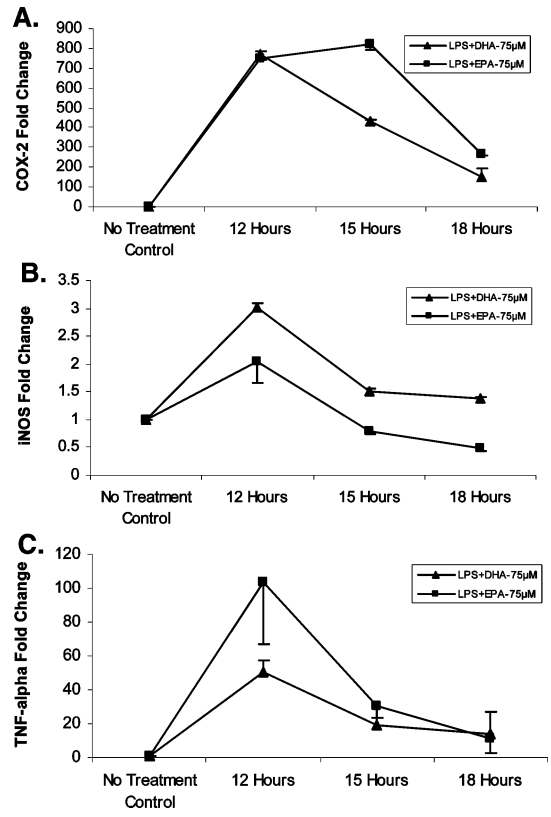

Figure 5. qPCR analysis of mRNA expressions of (A) COX-2, (B) iNOS, and (C) TNF-α at different times after DHA/EPA treatment in Nrf2 (+/+) wild-type mice ($n = 3$). The maximum detection levels were observed at 12 h after the beginning of DHA/EPA treatment (LPS treatment started 6 h after the beginning of DHA/EPA treatment).

IL-6 (Figure 4A), whereas, in the Nrf2 (+/+) group, while DHA did not significantly suppress the IL-6 secretion, EPA at either 25 or 50 μM significantly suppressed the IL-6 secretion dose-dependently (Figure 4B).

3.4. DHA/EPA Inhibited LPS-Induced COX-2, iNOS, IL-1β, IL-6, TNF-α mRNA in Nrf2 (+/+) Peritoneal Macrophages but Not in Nrf2 (−/−) Peritoneal Macrophages. The role of Nrf2 in suppression of LPS-stimulated inflammation in macrophages by DHA/EPA was investigated by pretreating primary peritoneal macrophages of both Nrf2 knockout and wild-type mice with DHA/EPA. After 6 h of DHA/EPA treatment, the medium containing DHA/EPA was replaced with medium containing LPS (1 μg/mL) to challenge/stimulate the macrophages for 8 h. To test the time course of induction of inflammatory markers after LPS treatments, Figures 5A, 5B, and 5C show that RNA obtained 12 h after DHA/EPA treatment (6 h of DHA/EPA treatment followed by 6 h of LPS treatment) display the highest COX-2, iNOS and TNF-α mRNA expression, and thus the time point of 6 h of DHA/EPA treatment followed by 6−8 h of LPS only treatment was selected for all subsequent LPS treatments and followed by RNA collections. This experiment determined the optimal time of LPS treatment and thus prepared for the subsequent experiments to determine DHA/EPA's effects on the mRNA expressions in the macrophages.

Figures 6(A) to 6(E) show that, in both Nrf2 (+/+) and Nrf2 (−/−) groups, LPS induced the expression of COX-2, iNOS, IL-1β, IL-6, TNF-α mRNA measured by qPCR. DHA/EPA selectively inhibited LPS-induced mRNA expression dose-dependently in macrophages only from Nrf2 (+/+) mice, but the inhibition was attenuated in macrophages from Nrf2 (−/−) mice, particularly COX-2 (Figure 6A), iNOS (Figure 6B), and IL-6 (Figure 6D). DHA was more potent in inhibiting these inflammatory markers than EPA.

To investigate the antioxidative stress effect of PUFA, it was observed that the HO-1 expression (one of the target genes of Nrf2) was induced by LPS treatment, and further enhancement by DHA/EPA was observed in both Nrf2 (−/−) and Nrf2 (+/+), particularly in the Nrf2 (+/+) with 75 μM DHA treatment that was statistically different (Figure 6F). DHA induced Nrf2 expression more substantially at higher doses than EPA, although not statistically different (Figure 6G).

4. Discussion

A recent phase I pharmacokinetic study on DHA/EPA with 48 subjects consuming fish 1−2 times a month showed the plasma concentrations of DHA and EPA of 182 μM and 33 μM respectively.[31] This *in vivo* DHA concentration in human is much higher than the DHA concentrations that we utilized

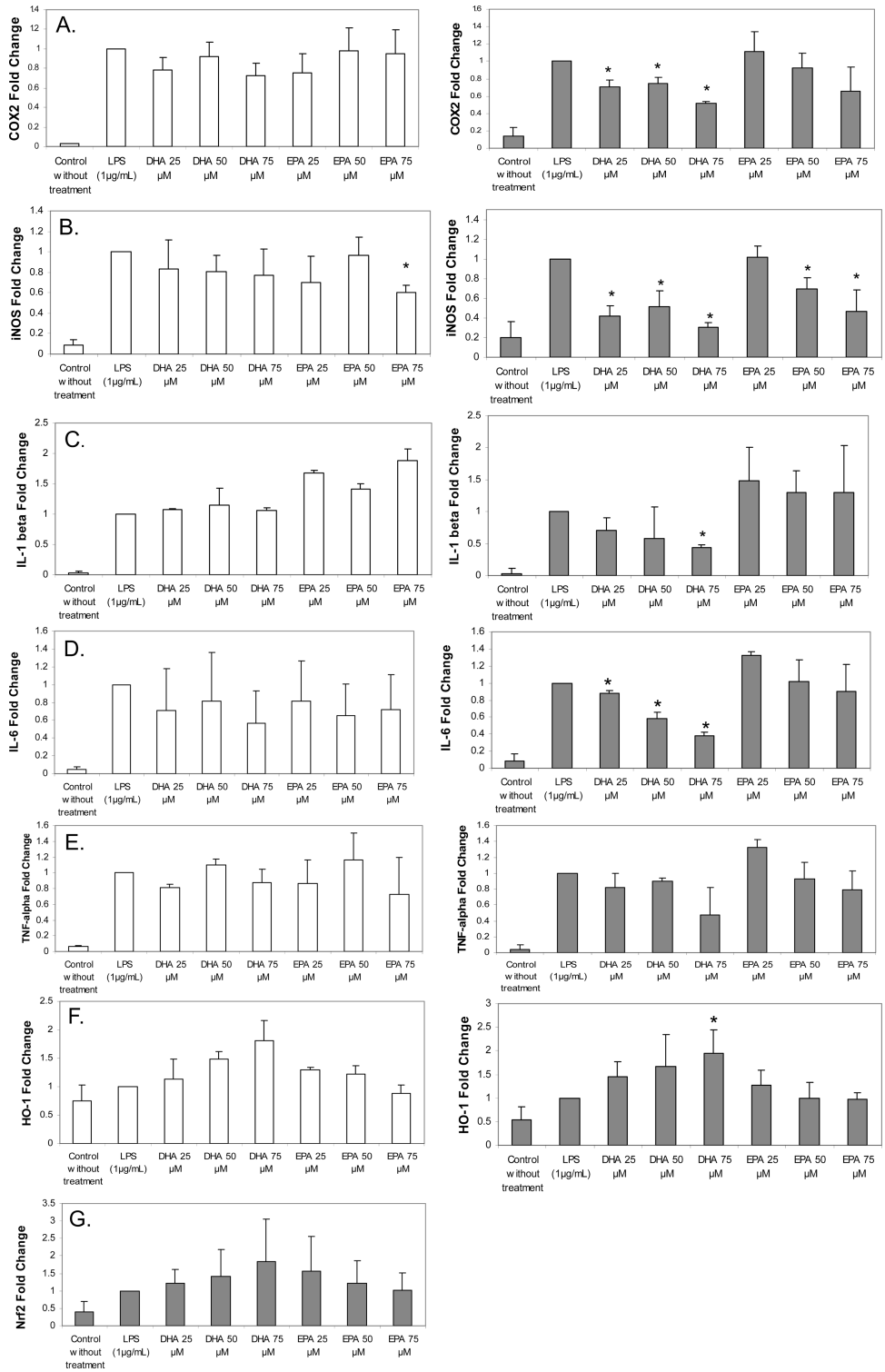

Figure 6. qPCR analyses show that LPS potently induced (A) cyclooxygenase-2 (COX-2), (B) inducible nitric oxide synthase (iNOS), (C) interleukin-1 beta (IL-1β), (D) interleukin-6 (IL-6), and (E) tumor necrosis factor-alpha (TNF-α) in the macrophages collected from both Nrf2 (+/+) wild-type (filled bars) and Nrf2 (−/−) knockout (unfilled bars) mice. DHA and EPA inhibited LPS-induced COX-2, iNOS, IL-1β, IL-6, or TNF-α in Nrf2 (+/+) wild-type but not in Nrf2 (−/−) knockout mice with one exception for iNOS when treated with EPA at 75 μM in Nrf2 (−/−) group. DHA and EPA increased (F) heme-oxygenase (HO-1) expression in both wild-type and knockout mouse macrophages and (G) Nrf2 expression in wild-type mouse macrophages in different dose-dependency. The results were obtained from at least three analyses of three groups of mice ($n = 4$).

in our current study, while the EPA level is within the range of our current study. Therefore, the concentration range in our current study provided reasonable physiologically relevant levels of DHA/EPA and also would allow direct

comparison of DHA and EPA for their anti-inflammatory effects via the Nrf2-dependent signaling pathway.

DHA and EPA's anti-inflammatory effects are shown in many previous studies. Gao et al. reported that EPA and DHA are subjected to an *in vitro* free radical oxidation process that could model *in vivo* conditions.[32] Oxidized omega-3 fatty acids reacted directly with Keap1, the negative regulator of Nrf2, initiating Nrf2 dissociation from Keap1, thereby inducing Nrf2-directed gene expression.[32] However, the role of Nrf2 in mediating the anti-inflammatory responses of DHA and EPA has not been investigated in detail.

In the present study, we hypothesized that DHA/EPA could exert their anti-inflammatory activities via activation of transcription factor Nrf2 in the mouse peritoneal macrophages. The protein expression as measured by Western blot and ELISA demonstrated that Nrf2 plays an important role in DHA/EPA's anti-inflammatory effects. In agreement with the results from protein expression, inhibitions of mRNA expression of COX-2 and iNOS by DHA and iNOS by EPA were significant in Nrf2 (+/+) mouse peritoneal macrophages. Our results also show inhibitions of mRNA expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α in the primary peritoneal macrophages from Nrf2 (+/+) mice as compared to those from Nrf2 (−/−) mice. Comparing mRNA and protein expressions, when treating the macrophages with DHA, it appears that, for COX-2 and iNOS, the protein expression regulation might be at the transcription level. For EPA, both COX-2 and iNOS mRNA did not show significant suppression from those induced by LPS in Nrf2 (+/+) group, however, the protein expressions were significantly attenuated from the LPS induced proinflammatory mediator expressions when treated with EPA, where the transcription regulation might be at a post-transcriptional level.

NF-κB is a transcription factor binding to DNA, plays an essential role in activating proinflammatory genes, such as iNOS and COX-2, and is involved in acute inflammation.[33] LPS is known to generate reactive oxygen species (ROS), which is involved in the inflammatory processes. ROS generation by LPS activates NF-κB and increases iNOS and COX-2 mRNA and protein levels. It was previously shown that although activation of inflammatory cells is a common defense mechanism in response to exogenously derived oxidative stress, activation of the inflammatory response can itself serves as a source of further oxidative stress.[34] Nonetheless, this study shows that NF-κB-target cytokines, IL-1β and IL-6, were induced by LPS. As reported previously, Nrf2 suppressed inflammation by inhibiting NF-κB activation through the regulation of redox balance,[26] as in a recent study that sulforaphane has been shown to decrease the effects of inflammatory response through Nrf2 pathway.[28] This is also consistent with the study published by Woods et al. using mouse macrophages in studying Nrf2-mediated adaptive response and related stress response to hypochlorous acid.[34]

Mullen et al. reported that, in their ELISA analyses, DHA was more potent than EPA in reducing the secretion of IL-1β and IL-6, whereas EPA appeared to be more effective at reducing TNF-α.[12] Weldon et al. also reported that DHA induces an anti-inflammatory profile in LPS-stimulated human THP-1 macrophages more effectively than EPA.[30] Our current results suggest that DHA is more potent in suppressing the mRNA expression of IL-1β, IL-6, and TNF-α. In addition, as shown in Figures 5(A), 5(B), and 5(C), DHA and EPA show different time−response profile in the anti-inflammatory biomarker expression, similar to that reported by Mullen et al.[12]

The protein and mRNA expressions of HO-1 were induced by the treatment of LPS and further enhanced by DHA and EPA (Figures 1A, 1B, and 6F). However, in the Nrf2 (−/−) group, the HO-1 induction was less substantial than that in Nrf2 (+/+) group, and higher doses of DHA but not EPA induced HO-1 more substantially. A similar pattern of induction of Nrf2 mRNA in the Nrf2 (+/+) group was observed (Figure 6G). Early studies show that oxidative stress could induce HO-1 and activator protein-1 (AP-1), and AP-1 could upregulate HO-1[35] and conversely HO-1 could also upregulate AP-1.[36] Ashino et al. reported negative feedback of LPS-induced iNOS expression by HO-1 in mouse macrophages.[37] Our current results are consistent with previous findings in iNOS expression and HO-1 induction. While our current anti-inflammatory results indicate that Nrf2 plays an important role in DHA/EPA's effects, it appears that NF-κB and AP-1 may also be involved in the induction of HO-1 by DHA/EPA. Blockade of AP-1 by DHA/EPA is also possible since AP-1 is an alternate known LPS-inducing

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proinflammatory transcription factor in the peritoneal macrophages that could regulate gene expression in response to a variety of stimuli, including LPS, as reported by Park et al.[38]

NO produced by iNOS in macrophages and some other cells in response to inflammatory mediators can act as double-edge sword, exerting either beneficial (e.g., bactericidal) or deleterious (e.g., DNA damage and protein oxidation) effects.[39] These beneficial or deleterious effects depend on both local and spatial concentrations of NO and the intracellular microenvironment.[40,41] In this study, while EPA did not show inhibitory effects on the production of NO, DHA did inhibit the production of NO in either Nrf2 (+/+) or Nrf2 (−/−) mouse macrophages. However, more substantial inhibition of NO in the Nrf2 (+/+) macrophages at the higher DHA doses (50 μM and 75 μM) is consistent with the iNOS mRNA and its protein expression levels.

Our ELISA results of TNF-α and IL-6 showed that, in the Nrf2 (−/−) group, DHA or EPA had no effect in suppressing these LPS-induced proinflammatory proteins. However, in the Nrf2 (+/+) group, DHA at 25 and 50 μM and EPA at 50 μM significantly suppressed TNF-α, and EPA at 25 and 50 μM significantly suppressed LPS-induced IL-6 expression. These results show that Nrf2 would play a role in DHA/EPA's anti-inflammation effects at the protein level.

In summary, our present findings utilizing Western blotting, ELISA and qPCR approaches coupled with Nrf2 (−/−) macrophages directly and clearly show that Nrf2 plays a role in the anti-inflammatory effects elicited with the selected physiologically relevant doses of DHA/EPA. They provided direct evidence that Nrf2-dependent signaling plays an important role in DHA/EPA's suppression of proinflammatory mediators (e.g., iNOS, COX-2) and proinflammatory cytokines (e.g., IL-1β, IL-6, TNF-α). Furthermore, DHA and EPA could also induce Nrf2 and Nrf2-target gene HO-1. These findings offer new insights into the potential mechanisms of action of PUFA in mediating the many beneficial health effects in human, and future clinical studies would be warranted.

Abbreviations Used

ARE, antioxidant response element; COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, hemeoxygenase-1; IL-1β, interleukin-1 beta; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa-B; Nrf2, nuclear factor-erythroid 2-related factor 2; PUFA, polyunsaturated fatty acids; q-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha.

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