Docosahexaenoic Acid-Enriched Fish Oil Attenuates Kidney Disease and Prolongs Median and Maximal Life Span of Autoimmune Lupus-Prone Mice

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Docosahexaenoic Acid-Enriched Fish Oil Attenuates Kidney Disease and Prolongs Median and Maximal Life Span of Autoimmune Lupus-Prone Mice

Ganesh V. Halade,*1 Md Mizanur Rahman,*1 Arunabh Bhattacharya,*† Jeffrey L. Barnes,*‡ Bysani Chandrasekar,*‡ and Gabriel Fernandes*

The therapeutic efficacy of individual components of fish oils (FOs) in various human inflammatory diseases still remains unresolved, possibly due to low levels of n-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) or lower ratio of DHA to EPA. Because FO enriched with DHA (FO-DHA) or EPA (FO-EPA) has become available recently, we investigated their efficacy on survival and inflammatory kidney disease in a well-established animal model of human systemic lupus erythematosus. Results show for the first time that FO-DHA dramatically extends both the median (658 d) and maximal (848 d) life span of (NZB × NZW)F1 (B × W) mice. In contrast, FO-EPA fed mice had a median and maximal life span of ~384 and 500 d, respectively. Investigations into possible survival mechanisms revealed that FO-DHA (versus FO-EPA) lowers serum anti-dsDNA Abs, IgG deposition in kidneys, and proteinuria. Further, FO-DHA lowered LPS-mediated increases in serum IL-18 levels and caspase-1-dependent cleavage of pro-IL-18 to mature IL-18 in kidneys. Moreover, FO-DHA suppressed LPS-mediated PI3K, Akt, and NF-κB activations in kidney. These data indicate that DHA, but not EPA, is the most potent n-3 fatty acid that suppresses glomerulonephritis and extends life span of systemic lupus erythematosus-prone short-lived B × W mice, possibly via inhibition of IL-18 induction and IL-18-dependent signaling. The Journal of Immunology, 2010, 184: 5280–5286.

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by heterogeneous clinical manifestations, including skin rashes, joint pain, glomerulonephritis, thrombocytopenia, hemolytic anemia, atherosclerosis, and CNS damage (1, 2). Autoantibody production is the major pathogenic mediator in SLE (3), and a hallmark of the disease is the elevation in serum IgG antinuclear Abs (4). The heterogeneous clinical manifestations in SLE appear to be associated with the production of different pathogenic autoantibodies, particularly to nuclear Ags and by an abnormal production of proinflammatory cytokines (5).

SLE is an autoimmune and chronic inflammatory disease. IL-18 is a proinflammatory cytokine, and is synthesized as a nonglycosylated inactive precursor and converted to its biologically active form after cleavage by the cysteine protease caspase-1. Released mature IL-18 exerts its effects on binding to its cognate receptor (IL-18R), a heterodimer comprised of α and β subunit. Its levels are increased in both human and animal models of SLE (5–9). In MRL/lpr mice, a positive correlation has been shown between elevated systemic and kidney IL-18 levels to disease severity (10, 11). This is further confirmed in MRL/lpr mice deficient in IL-18Rα. These mice had reduced levels of anti-dsDNA Abs and no leukocyte infiltration in kidneys and lungs. Importantly, these mice failed to develop autoimmune kidney disease (12), suggesting that IL-18 plays a critical role in glomerulonephritis, and thus a potential therapeutic target.

Dietary interventions with long-chain polyunsaturated fatty acids (PUFAs) profoundly influence both physiological processes as well as inflammatory diseases (13, 14). The ω-3 (n-3) FAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) exert anti-inflammatory effects in various diseases, including inflammatory bowel disease and rheumatoid arthritis (13). We previously showed that dietary supplementation with Menhaden fish oil (FO) (20–22% n-3 FAs) delays the onset of renal disease and extends life span of (NZB × NZW)F1 (B × W) mice (15–17). Interestingly, dietary supplementation of FO to SLE patients showed only a moderate beneficial effect on disease severity (18, 19). The FO used in those studies contained both EPA and DHA, but at lower levels. However, Robinson et al. demonstrated that feeding EPA prolongs survival (20), and in synergy with DHA exerts anti-inflammatory effects, and alleviates renal disease in B × W mice (21). But the mechanisms still remain elusive. Because FO enriched with EPA or DHA has become available, we investigated for the first time the prolonged effects of FO enriched with DHA (FO-DHA) or EPA (FO-EPA) on kidney disease, and median and maximal life span of short-lived SLE-prone B × W mice.

Materials and Methods

Animals and experimental diets

Weanling (NZB × NZW)F1 (B × W) female mice were purchased from The Jackson Laboratory, Bar Harbor, ME. At 2 mo of age, mice were switched to semipurified diets containing 10% corn oil (CO; MP Biologicals, Irvine, CA) as control oil and FOs enriched in either EPA or DHA: 1) FO-18/12, 2) 55/5 FO-EPA, and 3) 5/60 FO-DHA (Ocean Nutrition, Nova Scotia, Canada). FO composition of diets is given in Table I.
The study was carried out in two phases. In the first phase, survival, systemic anti-dsDNA Abs, IgG deposition in kidneys, and proteinuria were studied. In the second phase, to emphasize the mechanisms of improved survival by DHA and LPS-evoked IL-18 signaling (22, 23), 5-mo-old mice were challenged with LPS (5 mg/kg body weight; i.p.). PBS served as a vehicle control. Both serum and kidneys were collected after 4 h, and analyzed for immunologic, biochemical, and molecular changes. All studies were approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center, San Antonio, TX.

Serum FA analysis
FA composition was analyzed by gas chromatography as described previously (24). Briefly, 100 μl serum were subjected to lipid extraction. FA methyl esters were derived by heating at 75°C for 1 h in 5% hydrochloric acid–methanol reagent. FA methyl esters were analyzed by gas chromatography using a fully automated HP5890A series II system equipped with a flame-ionization detector. Peaks of resolved FAs were identified by comparison with FA standards (Matreya, Pleasant Gap, PA), and area percentage for all resolved peaks was analyzed by using a HP 3396 series II integrator.

IgG deposition in kidneys
Kidney tissues were snap-frozen in Optimal Cutting Temperature Compound (Miles Scientific, Naperville, IL) and sectioned (4 μm thick). To examine IgG deposits within renal glomeruli, the sections were incubated with FITC-conjugated goat anti-mouse IgG Ab (Serotec, Oxford, U.K.). Fluorescence intensity within glomerular capillary walls was scored on a scale of 0–3 (0, none; 1, weak; 2, moderate; 3, strong). At least 10 glomeruli per section were analyzed by two independent investigators in a blinded fashion, and scored.

Proteinuria
Proteinuria was assessed using chemstrips (Roche Diagnostic, Indianapolis, IN). In this semiquantitative system, trace corresponds to <30 mg/ml, 1+ to 30–100 mg/dl, 2+ to 100–500 mg/dl, and 3+ to >500 mg/dl. Consistent with the criteria applied in previous studies of murine lupus, proteinuria >100 mg/dl (≥2+) was interpreted as an evidence of significant end-stage renal disease.

Anti-dsDNA Abs
Serum anti-dsDNA Ab titers were assessed as previously described using a solid-phase ELISA (16).

Serum IL-18 levels
Serum IL-18 levels were quantified by ELISA (Bender MedSystems, Burlingame, CA). The sensitivity of the assay is 10.0 pg/ml.

Caspase-1 activity
Caspase-1 activity in kidney homogenates was determined by the caspase-1/ICE colorimetric Protease assay kit (BioVision Research Products, Mountain View, CA). The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate YVAD-pNA. The pNA light emission was quantified spectrophotometrically at 405 nm, and the results were expressed in fold-increase from controls.

Pro– and mature IL-18 levels
IL-18 protein levels were quantified by Western blotting using Abs specific for pro (R&D Systems, Minneapolis, MN) and mature (Santa Cruz Biotechnology, Santa Cruz, CA) forms of IL-18.

Measurement of PI3-kinase
PI3K lipid kinase assays were performed as described previously (25) using p85 immunoprecipitates.

Akt levels and Akt kinase activity
We used two independent but complimentary methods to quantify activation of Akt: immunoblotting using whole cell homogenates and activation-specific Abs, and immune-complex kinase assays using a commercially available nonradioactive Akt kinase assay kit (Cell Signaling Technology, Danvers, MA). The assay is based on Akt-induced phosphorylation (Ser21/9) of glycogen synthase kinase-3.

NF-κB activation
NF-κB DNA binding activity was analyzed by EMSA using nuclear protein extracts and double stranded consensus (sense, 5'-AGT GGA GGC TTT CCC AGG C-3') or mutant (sense, 5'-AGT GGA GGC GAG TTT CCC AGG C-3') NF-κB oligonucleotides (Santa Cruz Biotechnology). Nuclear p65 levels were quantified by Western blotting (Cell Signaling Technology). Actin served as a loading control.

Statistical analysis
Data are expressed as mean ± SEM. Results were analyzed by ANOVA, followed by Newman-Keuls test using Graph Prism 4 software (GraphPad, San Diego, CA) and p < 0.05 was considered statistically significant. Survival data were analyzed by Logrank, followed by χ² test.

Results
Delayed onset of kidney disease and maximal life span in FO-DHA mice
We have previously demonstrated that Menhaden FO attenuates kidney disease and moderately extends life span of B × W mice (26). We now investigated whether enriching FO with DHA (60% DHA, 5% EPA) or EPA (5% DHA, 55% EPA) will further extend life span and delay progression of renal disease (Fig. 1). Female B × W mice were fed regular FO (18% EPA, 12% DHA; FO-18/12), FO-DHA, and FO-EPA. CO that contains neither DHA nor EPA served as a control (Table I). Results show that the median life span of CO-fed control animals was 372 d, and FO-18/12 moderately extended median life span to 414 d. In contrast, FO-DHA significantly increased median life span to 658 d. Interestingly, FO-EPA had minimal effect on life span (384 d), and was comparable to that of CO-fed mice. Similarly, maximal life span was significantly higher nearly doubled in FO-DHA fed mice (848 d) compared with FO-EPA (500 d), FO-18/12 (539 d), and CO-fed (444 d) mice. These results indicate that DHA, but not

![Figure 1](http://www.jimmunol.org/)
EPA-enriched FO, significantly extends both median and maximal life span of the short-lived B × W mice.

Serum FA profile

To verify whether dietary oils influence serum FA profile, we analyzed serum for PUFA by gas chromatography (24). FO-fed mice exhibited higher levels of n-3 FA as compared with CO-fed mice. Although there was no difference in 20:5n-3 (EPA) levels analyzed serum for PUFA by gas chromatography (24). FO-fed mice. To verify whether dietary oils influence serum FA profile, we

Serum anti-dsDNA Abs are reduced in FO-DHA fed mice

Anti-dsDNA Abs are implicated in the pathogenesis of SLE. FO-DHA fed mice compared with CO-fed mice (Fig. 2A). These effects were more pronounced in FO-DHA fed mice. In contrast, FO-EPA failed to significantly modulate anti-dsDNA Ab titer, and the levels were comparable to that seen in CO-fed mice, indicating that DHA, but not EPA, significantly lowers systemic anti-dsDNA Abs in SLE-prone B × W mice (Fig. 2A).

IgG deposition and proteinuria are decreased in kidneys of FO-DHA fed mice

Elevated proteinuria and deposition of IgG in glomeruli are characteristic features of renal disease in B × W mice (27). When compared with CO-fed mice, histological evaluation of IgG deposition in kidneys (Fig. 2B) and proteinuria levels (Table III) were both significantly decreased in FO-18/12 and FO-DHA fed mice, but to a greater extent in the latter group. There was, however, no difference in IgG deposition between CO and FO-EPA fed mice, indicating that DHA, but not EPA, potentially downregulates IgG deposition in kidneys of SLE-prone B × W mice (Fig. 2C).

Serum IL-18 levels are lowered in FO-DHA fed mice

IL-18 plays a causal role in SLE (5), and LPS is a potent inducer of IL-18 (22). Therefore, we investigated serum IL-18 levels after LPS challenge. Results in Fig. 3A show that FO-18/12 and FO-DHA both significantly lowered LPS-induced IL-18 in serum as compared with CO-fed mice. Once again, FO-EPA failed to modulate LPS-mediated IL-18 expression, indicating that FO-DHA significantly lowers proinflammatory IL-18 levels in serum of SLE-prone B × W mice (Fig. 3A).

Mature, but not pro-IL-18 expression, is lowered in kidneys of FO-DHA-fed mice

IL-18 is synthesized as a proform, and is cleaved by caspase-1 to a mature biologically active 18 kDa secreted form. Because FO-DHA significantly attenuated serum IL-18 levels (Fig. 3A), we investigated whether decreased serum IL-18 levels reflect reduced IL-18 expression in the kidneys. Western blot analysis of kidney homogenates revealed detectable levels of pro-IL-18, and LPS administration failed to significantly modulate its expression. In contrast, mature IL-18 was detected at low levels in vehicle (PBS)-treated animals, and was increased in all groups after LPS administration. However, LPS-mediated increase in mature IL-18 expression was attenuated in both FO-18/12 and FO-DHA groups, and once again, FO-DHA was the most potent. Because caspase-1 cleaves pro-IL-18 to mature IL-18, we further investigated whether decreased levels of mature IL-18 were due to reduced

Table I. Composition of semipurified CO and FO diets enriched with EPA or DHA

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>CO</th>
<th>FO-18/12</th>
<th>FO-EPA</th>
<th>FO-DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
<td>14.00</td>
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<tr>
<td>Corn starch</td>
<td>42.43</td>
<td>42.43</td>
<td>42.43</td>
<td>42.43</td>
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<tr>
<td>Dextrose corn starch</td>
<td>14.50</td>
<td>14.50</td>
<td>14.50</td>
<td>14.50</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.00</td>
<td>9.00</td>
<td>9.00</td>
<td>9.00</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>AIN-93 mineral mix</td>
<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>AIN-93 vitamin mix</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>t-cystine</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>TBHQ</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>CO</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FO</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

*All diet ingredients were purchased from MP Biomedicals.

FO enriched in either EPA or DHA: 1) FO-18/12, 2) 55/5 FO-EPA, and 3) 5/60 FO-DHA (Ocean Nutrition).

CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil.

Table II. Profiles of PUFA n-6 and n-3 FAs in serum of (NZB × NZW)F1 mice fed with specialized CO and FO diets enriched with EPA or DHA

<table>
<thead>
<tr>
<th>FAs</th>
<th>CO</th>
<th>FO-18/12</th>
<th>FO-EPA</th>
<th>FO-DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2n-6</td>
<td>30.33 ± 0.70a</td>
<td>13.69 ± 0.31b</td>
<td>9.79 ± 0.49b</td>
<td>8.85 ± 0.37b</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.28 ± 0.03</td>
<td>0.27 ± 0.00</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>19.52 ± 0.36c</td>
<td>9.59 ± 0.25c</td>
<td>12.51 ± 0.48b</td>
<td>5.21 ± 0.22a</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>ND</td>
<td>20.17 ± 1.33</td>
<td>22.69 ± 1.17</td>
<td>20.76 ± 0.41</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.34 ± 0.12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>ND</td>
<td>0.39 ± 0.02d</td>
<td>0.96 ± 0.15b</td>
<td>0.70 ± 0.07b</td>
</tr>
<tr>
<td>PUFA</td>
<td>53.79 ± 0.74</td>
<td>56.48 ± 0.56</td>
<td>55.84 ± 0.92</td>
<td>59.40 ± 0.62</td>
</tr>
<tr>
<td>n-6 FA</td>
<td>33.33 ± 0.40c</td>
<td>33.11 ± 1.20b</td>
<td>33.54 ± 1.01b</td>
<td>45.34 ± 0.66a</td>
</tr>
<tr>
<td>n-6 FA</td>
<td>50.46 ± 0.48a</td>
<td>23.37 ± 0.64b</td>
<td>22.30 ± 0.24b</td>
<td>14.06 ± 0.57a</td>
</tr>
<tr>
<td>n-6/3 FAs</td>
<td>15.85 ± 1.90</td>
<td>0.71 ± 0.05</td>
<td>0.67 ± 0.03</td>
<td>0.31 ± 0.02</td>
</tr>
</tbody>
</table>

Total lipids of (NZB × NZW)F1 mice serum were extracted, methylated and subjected to analysis by gas chromatography. The values (% of total FAs) are means of three independent measurements ± SEM. n = 5. Significant difference (p < 0.05) is indicated with different superscripted letters analyzed by ANOVA, followed by Newman-Keuls test. Ratio of n-6/n-3 fatty acids is expressed as (18:2n-6 + 20:3n-6 + 20:4n-6 + 22:5n-6)/(20:5n-3 + 22:5n-3 + 22:6n-3).

CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FO, fish oil; ND, not detected; PUFA, polyunsaturated fatty acids.
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subunit nuclear translocation was observed in FO-18/12 fed mice and it was even higher in DHA-enriched FO-fed mice as compared with CO-fed mice. There was, however, no inhibition in EPA-enriched FO fed mice (Fig. 5A, 5B).

Discussion

We previously reported that n-3 FA (20–25% EPA and DHA) present in Menhaden FO extends median and maximal life span of short-lived lupus-prone B × W mice compared with a diet supplemented with n-6 FA-rich CO (15, 16, 28). Our subsequent studies demonstrated that a combination of n-3 FA and caloric restriction (CR) further extends the life span of B × W mice even more than n-6 FA fed ad libitum or with CR (29), suggesting that source of dietary fat (n-3 FA versus n-6 FA) was an important determinant of disease progression and severity in B × W mice. Although these findings are encouraging, there are obvious concerns that a dietary regimen of 30–40% CR may be impractical for SLE patients, and that FO with low EPA/DHA content would have only moderate beneficial effects in the same patients. Because EPA and DHA are the principal biologically active FAs in FO, and as their levels/ratio affect the anti-inflammatory effects, in the current study we investigated the efficacy of FO enriched with DHA (60% DHA/5% EPA; FO-DHA) or EPA (5% DHA/55% EPA; FO-EPA) on disease severity and longevity in B × W mice. Our results demonstrate for the first time that DHA is a potent inhibitor of autoantibody production, IL-18 expression and kidney disease, and that DHA significantly extends life span of short-lived B × W mice.

FO-DHA significantly attenuated serum and kidney IL-18 expression. IL-18 is a proinflammatory cytokine and its systemic levels are significantly elevated in SLE patients (5, 10). Similarly, SLE-prone MRL/lpr mice also express high levels of serum IL-18 (10). Further, B × W mice repeatedly exposed to LPS develop an early and accelerated form of lupus nephritis (23), with enhanced polyclonal B cell activation and persistence of exacerbated nephritis, even after LPS clearance (30). In the current study, we found that LPS treatment significantly increased serum IL-18 levels in both CO and FO-EPA fed mice. However, a significant inhibition in serum and kidney IL-18 levels was observed in FO-DHA and FO-18/12 fed mice (inhibitory activity: FO-DHA > FO-18/12 > FO-EPA). Because caspase-1 cleaves pro–IL-18 to mature IL-18, and as caspase-1 activity was lower in the LPS-treated FO-DHA fed mice, our results suggest that lower levels of mature IL-18 in serum and kidneys were due to the reduced cleavage of pro–IL-18 to mature IL-18.

Because PI3 kinase plays a critical role in IL-18 induction as well as in IL-18 signaling (31, 32), we next analyzed the activation status of PI3K in kidneys after LPS treatment. Compared with vehicle-treated mice, a robust increase in PI3K activation was noted in kidneys from LPS-injected CO and FO-EPA fed mice. In contrast, FO-DHA and FO-18/12 fed mice showed lower levels of PI3K-dependent PI3P levels. Of note, PI3Kγ is an important target for inhibition of glomerulonephritis and extension of life span in MRL/lpr mice (33). Because FO-DHA attenuated PI3K activation, and because inhibition of PI3Kγ was shown to prolong life span and decrease glomerulonephritis, our results also suggest that dietary supplementation of FO-DHA is a viable therapeutic strategy to ameliorate chronic inflammation (33). Because the serine/threonine kinase Akt/protein kinase B is one of the major downstream targets of PI3K, we also analyzed total and phospho-Akt (Ser473) levels in kidney homogenates. Our results indicate that although total Akt remained similar in the kidneys of all four groups, phospho-Akt levels were reduced in FO-DHA and FO-18/12 fed mice. Once again, FO-DHA was more potent in inhibiting caspase-1 activity (Fig. 3B). Indeed, FO-DHA was more potent in inhibiting LPS-mediated increase in caspase-1 activity. These results indicate that FO-DHA attenuates IL-18 expression by inhibiting caspase-1 activity and caspase-1–dependent pro–IL-18 to IL-18 processing (Fig. 3C, 3D).

FO-DHA inhibits LPS-mediated PI3K, Akt, and NF-κB activations in kidneys

Because LPS signals via PI3K, we measured PI3P in kidneys of PBS and LPS-injected mice. Although there was no difference in basal levels of PI3P, FO-18/12 and to a higher extent DHA-enriched FO prevented LPS-stimulated PI3P activation status as compared with CO feeding. There was no difference in PI3P activation between CO-fed and EPA-enriched FO-fed mice (Fig. 4A).

Akt is downstream of PI3K and its phosphorylation at Ser473 denotes activation. Similar to its inhibition of PI3K, a significant inhibition of phospho-Akt levels (Fig. 4B) as well as Akt kinase activity (Fig. 4C) were observed in FO-18/12 fed mice, and to a much higher extent in DHA-enriched FO-fed mice as compared with CO-fed mice. No inhibition was observed in EPA-enriched FO fed mice.

Because NF-κB is involved in IL-18 induction, and is downstream of PI3K and Akt, we next analyzed NF-κB DNA binding activity by EMSA using kidney nuclear extracts. We also analyzed nuclear translocation of NF-κB p65 by immunoblotting. Significant inhibition of NF-κB DNA binding activity and NF-κB p65

FIGURE 2. Effect of CO, FO-18/12, FO-EPA, and FO-DHA diets on serum anti-dsDNA Abs and IgG deposition in kidneys of (NZB × NZW)F1 female mice. At 2 mo of age, female (NZB × NZW)F1 mice were fed semipurified diets containing 10% CO (control), and FOs enriched in EPA or DHA: 1) FO-18/12, 2) 55/5 FO-EPA, and 3) 5/60 FO-DHA. At 5 mo of age, mice were challenged with LPS i.p., and serum anti-dsDNA Abs were quantified by ELISA. Histological evaluation of IgG deposition was scored on a scale of 0–3 (0, none; 1, weak; 2, moderate; 3, strong) based on fluorescence intensity within glomerular capillary walls (10 glomeruli/mouse and 6 mice/group). A, FO-DHA fed mice exhibit significantly lower anti-dsDNA Abs in serum (p < 0.01, ANOVA). B, Representative photomicrographs of immunofluorescent stained (original magnification ×100) kidneys indicate lower IgG deposition in FO-DHA fed mice compared with FO-EPA and CO fed mice. C, Histological evaluation of kidney demonstrates significantly decreased IgG deposition in FO-DHA fed mice compared with CO, FO-18/12, and FO-EPA fed mice. Different signs (+, †, and ‡) indicates significant differences (p < 0.05, ANOVA, followed by Newman-Keuls test).
Akt activation. These results were further confirmed by immune-complex kinase assays, which revealed reduced Akt kinase activity in FO-DHA fed mice, suggesting that FO-DHA potently inhibits Akt activation in kidneys in vivo. Although DHA has been shown to inhibit LPS-induced Akt phosphorylation in RAW264.7 macrophages in vitro (34), our studies are the first to show that DHA, but not EPA, inhibits PI3K/Akt signaling in vivo in the kidneys of SLE-prone mice.

NF-κB is a ubiquitous stress-responsive transcription factor and plays a role in inflammation. Because NF-κB is a downstream mediator of PI3K and Akt pathways, and plays a role in IL-18 induction and signaling (31, 32), we analyzed NF-κB activation by EMSA and nuclear translocation of p65 by immunoblotting in kidneys after LPS treatment. Our results show a significant inhibition in LPS-mediated NF-κB DNA-binding activity in FO-DHA fed mice, followed by FO-18/12 fed mice. These results corroborate previous studies demonstrating inhibition of NF-κB activation by n-3 FAs both in vivo and in vitro (35–38). Our results also show that reduced levels of nuclear NF-κBp65 in kidneys of FO-DHA fed mice, suggesting that FO-DHA inhibits NF-κB activation by inhibiting nuclear translocation of p65. This finding is in agreement with a recent study, which showed that DHA attenuates LPS-induced nuclear p65 levels in cultured human THP-1 macrophages, whereas EPA had no effect (36). Our study provides the first in vivo evidence that DHA is a potent inhibitor of NF-κB activation, and suggests that reduced NF-κB activation might be a contributing factor in the inhibition of LPS-induced renal disease in FO-DHA fed mice.

Activations of PI3K, Akt, and NF-κB play a role in both IL-18 induction and IL-18–dependent signaling. Importantly, our results show that FO-DHA is a potent inhibitor of these three critical players of inflammation, thus inhibiting perpetuation of inflammatory signaling during SLE. Although we demonstrated that FO-DHA inhibits LPS-mediated NF-κBp65 nuclear translocation, recently it has also been shown that IκB degradation contributes to LPS-mediated NF-κB activation and IL-18 signaling (22, 39, 40), suggesting that the observed p65 nuclear translocation follows IκB degradation, and FO-DHA might activate NF-κB via classic IκB degradation and NF-κB activation in LPS-treated mice. Further, P3K, which is now considered a potential therapeutic target in SLE (33, 41), has also been shown to be a target of DHA in neuronal cells, thus its inhibition improves their survival (42). Because, FO-DHA fed mice exhibited reduced levels of LPS-induced NF-κB activation, and serum and kidney IL-18 expression, it is reasonable to speculate that FO-DHA inhibits IL-18 expression by attenuating LPS-mediated PI3K/Akt-dependent NF-κB activation. We will investigate this possibility in the future in isolated mesangial cells treated with DHA and LPS. In addition, we will also investigate the origin of IL-18 in kidneys using in situ hybridization and immunohistochemistry.

Circulating autoantibodies to DNA is one of the hallmarks of SLE in humans (1) and B × W mice (3, 4). Anti-dsDNA Abs form immune complexes and their deposition results in arthritis and nephritis. B × W mice on CO and FO-EPA diets exhibited higher anti-dsDNA Ab levels in serum and higher IgG deposition in the kidneys. In correlation with their survival data, FO-DHA fed mice had lower levels of serum anti-dsDNA Ab and kidney IgG deposition. It was previously reported that MRL/lpr mice show higher serum IgG1 and IgG2 anti-dsDNA Abs and higher IgG deposition in kidneys after IL-18 administration (10). In contrast, lower total IgG and IgGa anti-dsDNA Abs were reported in serum of MRL/lpr mice deficient in IL-18 receptor (12). These mice also exhibited lower glomerular deposition of IgG, thus supporting the role of IL-18 as an important mediator of renal disease in FO-DHA fed mice. Indeed, our results are in agreement with a recent study, which showed that DHA attenuates LPS-induced nuclear p65 levels in cultured human THP-1 macrophages, whereas EPA had no effect (36). Our study provides the first in vivo evidence that DHA is a potent inhibitor of NF-κB activation, and suggests that reduced NF-κB activation might be a contributing factor in the inhibition of LPS-induced renal disease in FO-DHA fed mice.

**Table III.** Proteinuria of (NZB x NZW)F1 mice fed with specialized CO and FO diets enriched with EPA or DHA

<table>
<thead>
<tr>
<th>Groups (Diet)</th>
<th>Age (mo)</th>
<th>No. of Mice</th>
<th>Trace (&lt;30 mg/dl)</th>
<th>++ (30–100 mg/dl)</th>
<th>+++ (100–500 mg/dl)</th>
<th>++++ (&gt;500 mg/dl)</th>
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*At 2 mo of age, mice were switched to semipurified diets containing 10% CO (control), and FOs enriched in EPA or DHA: 1) FO-18/12, 2) 55/5 FO-EPA, and 3) 5/60 FO-DHA.

*Proteinuria was determined using Chemstrips (Roche Diagnostic) (n = 15).

*Significant difference in proteinuria levels of FO-18/12 and FO-EPA fed mice compared with CO fed mice measured by ANOVA.

*Significant difference in proteinuria levels of FO-DHA fed mice compared with CO fed mice measured by ANOVA.

CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; FO, fish oil; FO-DHA, FO enriched with DHA; FO-EPA, FO enriched with EPA.
SLE-prone mice, and inhibition of IL-18 and its signaling by DHA may alleviate clinical features of SLE. It should be pointed out that FO doses used in this study are relatively higher compared with human consumption via food or dietary supplementation. Our recent findings, using prescribed FO (Lovaza) 1% as a human equivalent dose in B × W mice, also revealed significant decrease in proteinuria and improved survival compared with 1% placebo (unpublished data). Future clinical trials with purified DHA enriched FO or Lovaza in lupus patients is warranted.

In summary, our studies demonstrate for the first time that FO enriched in DHA attenuates glomerulonephritis and significantly extends life span of short-lived SLE-prone B × W mice, compared with EPA enriched FO or FO with lower EPA+ DHA levels. This beneficial effect of DHA may be attributed to its anti-inflammatory activity through inhibition of IL-18 expression and IL-18–dependent signaling.

FIGURE 5. Effect of CO, FO-18/12, FO-EPA, and FO-DHA diets on NF-κB activation. Female (NZB × NZW)F1 mice were fed with CO, FO-18/12, FO-EPA, and FO-DHA for 3 mo, and then challenged with LPS (5 mg/kg body weight, i.p.) for 4 h. Kidneys were harvested and analyzed for NF-κB DNA binding activity by EMSA using nuclear protein extracts (A). Activation of NF-κB was confirmed by analyzing nuclear p65 levels immunoblotting. Immunoblotting (B). Actin served as a loading control. The results show significant inhibition of LPS-mediated NF-κB DNA binding activity (A) and nuclear p65 translocation (B) in FO-DHA fed mice.
Disclosures
The authors have no financial conflicts of interest.

References