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Attenuation of mycotoxin-induced IgA nephropathy by eicosapentaenoic acid in the mouse: dose response and relation to IL-6 expression

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Abstract

Clinical trials have revealed that progression of immunoglobulin A nephropathy (IgAN), the most common form of human glomerulonephritis, is inhibited by dietary (n-3) polyunsaturated fatty acid (PUFA) supplementation. The early stages of IgAN can be mimicked by feeding mice the mycotoxin deoxynivalenol (DON). Here, the effects of consuming the (n-3) PUFA eicosapentaenoic acid (EPA) on DON-induced IgAN were assessed relative to dose dependency and to expression of interleukin (IL-6). In the dose-response study, weight gain and feed intake did not differ among mice consuming 20 ppm DON supplemented with 0%, 0.1%, 0.5% and 3% EPA for 16 weeks. Mice fed the two highest EPA concentrations exhibited markedly increased splenic EPA, docosapentaenoic acid and docosahexaenoic acid, whereas arachidonic acid was decreased in all three EPA fed groups. Deoxynivalenol consumption significantly increased serum IgA and IgA immune complexes as well as kidney mesangial IgA deposition. All three IgAN markers were attenuated in mice fed 3% EPA diet but not in those fed 0.1% or 0.5% EPA. Elevated IgA production was observed in spleen and Peyer's patch (PP) cell cultures derived from mice fed DON in control diets, but this was reduced in cultures from mice fed 0.1%, 0.5% and 3% EPA. Acute DON exposure increased serum levels of IL-6, a cytokine that drives differentiation of IgA-committed B cells to IgA secretion. Relatedly, expression of IL-6 mRNA and IL-6 heteronuclear RNA, a marker of IL-6 transcription, was increased in spleen and PP. All three indicators of IL-6 expression were suppressed in mice consuming 3% EPA. Suppressed IL-6 corresponded to decreased binding activity of two factors that regulate transcription of this cytokine, cyclic AMP response element-binding protein and activator protein-1. The results indicate that a threshold existed for EPA relative to suppression of experimental IgAN and that the threshold dose was effective at inhibiting IL-6 transcription.

Keywords: n-3 PUFA; IgA nephropathy; Deoxynivalenol (DON); Interleukin-6; Fish oil

1. Introduction

Immunoglobulin A nephropathy (IgAN), the most common human glomerulonephritis, is defined by predominant or codominant deposits of IgA within the mesangial regions of the kidney glomerulus [1]. Immunoglobulin A nephropathy accounts for 5% to 10%, 20% to 35% and up to 50% of glomerulonephropathies in North America, Europe and Japan, respectively [2,3]. Approximately 150 000 people in the United States have been diagnosed with IgAN with 4000 new cases occurring each year [4]. Between 20% and 40% of these patients will develop progressive renal failure with 1% to 2% of adult patients entering end-stage renal failure yearly [5]. The fundamental abnormality in IgAN lies within the IgA system and not the kidney because IgA deposition in IgAN patients recurs after renal transplantation [6]. An overly robust IgA response to mucosal infections and dietary antigens in terms of quantity, size (primarily polymeric), glycosylation status and IgA immune complex (IgA-IC) formation is suspected to contribute to IgAN [3,7]. Resultant IgA-IC deposition in glomeruli likely triggers mesangial cell proliferation, matrix secretion and inflammation in which proinflammatory cytokines play important roles. Although no consistent infectious or

Abbreviations: DON, deoxynivalenol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IgA-IC, immunoglobulin A immune complex; IgAN, immunoglobulin A nephropathy; IL, interleukin; PUFA, polyunsaturated fatty acid.

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environmental agent has been identified to cause dysregulation of the IgA antibody response, the onset of IgA nephropathy is often associated with upper respiratory tract infection [3]. Therapeutic strategies for IgAN remain elusive, partly because of its poorly defined etiology.

Dietary supplementation with (n-3) polyunsaturated fatty acids (PUFAs) found in fish oil, particularly [22:6(n-3)] docosahexaenoic acid (DHA) and [20:5(n-3)] eicosapentaenoic acid (EPA), has potential human health benefits relative to inflammatory diseases [8,9]. (n-3) PUFAs suppress inflammatory responses through eicosanoid-dependent or eicosanoid-independent mechanisms [10]. Case-control studies reveal that dietary (n-3) PUFAs are negatively associated with the risk of IgAN [11], whereas high intake of (n-6) PUFAs is positively associated with increased risk [12]. Holman et al. [13] demonstrated that some IgAN patients are deficient in α-linolenic acid [18:3(n-3)], a precursor of DHA and EPA, and that supplementation with EPA and DHA suppresses arachidonic acid (AA) synthesis, decreases proteinuria and improves glomerular filtration rate in this group. Several elegant clinical trials by Donadio and Grande [14] have now demonstrated that (n-3) PUFA retards late-stage renal disease progression in IgAN patients by reducing inflammation and glomerulosclerosis.

The trichothecene mycotoxin deoxynivalenol (DON) is a secondary metabolite produced by members of the fungus *Fusarium* and is frequently found in dietary staples such as wheat, barley, corn, rice and oats [15]. It is recalcitrant to inactivation during milling and processing, and frequently enters finished food products. Prolonged feeding of DON causes a dramatic elevation in total serum IgA in mice with IgA becoming the major serum isotype [16]. This co-occurs with marked elevation of serum IgA-IC, kidney mesangial IgA accumulation, electron dense mesangial deposits and hematuria [17,18], which are hallmarks of the early stages of human IgAN [35]. Interleukin-6 (IL-6) is critical to mucosal IgA immunity based both on its differentiative effects on IgA-committed B cells and its production in the gut by macrophages, T cells and other cells [19]. Deoxynivalenol up-regulates IL-6 expression in vivo and in vitro, and this has been linked to increased IgA production [20,21]. Interleukin-6-deficient mice are refractory to DON-induced dysregulation of IgA production and development of IgAN [22]. Thus, IL-6 is pivotal to DON-induced IgAN.

Fish oil as well as the (n-3) PUFAs DHA and EPA suppress DON-induced IgAN [23–25]. This preclinical model enables exploration in how n-3 PUFAs attenuate aberrant IgA responses associated with IgAN and as well as gain insight into therapeutic strategies for this disease. Key issues concerning use of fish oil for treating IgAN and other inflammatory diseases relate to most efficacious types of n-3 PUFAs, optimal doses required to achieve ameliorative effects and underlying molecular mechanisms. In particular, clarification is needed on reported differential effects of DHA or EPA relative to biological efficacy and disease prevention [26–31]. Previously, our laboratory established that dietary DHA concentrations between 1% and 3% were necessary to ameliorate DON-induced IgAN [24,25]. The goals of this research were to (1) evaluate dose-response effects of EPA-enriched oils on DON-induced IgAN and (2) determine effects of the optimal EPA dose on IL-6 mRNA expression and transcription factor binding activities.

2. Methods and materials

2.1. Materials

All chemicals were reagent grade or better and were purchased from Sigma (St. Louis, MO) unless otherwise noted. Deoxynivalenol was produced in *Fusarium graminearum* R6576 cultures and purified chromatographically [32]. Deoxynivalenol was added to powdered diets as detailed previously [16]. Deoxynivalenol-contaminated labware was detoxified by soaking for >1 h in 10% (v/v) sodium hypochlorite [33].

2.2. Animals

Female B6C7F1 mice (7 weeks, 17–22 g) were obtained from Charles River Laboratories (Portage, MI). Housing, handling and sample collection procedures conformed to the policies and recommendations of the Michigan State University Laboratory Animal Research Committee and were in accordance with guidelines established by the National Institutes for Health. Mice were housed three per cage in a humidity (45–55%)- and temperature (23–25°C)-controlled university animal care facility room with a 12-h light and dark cycle. Mice were acclimated for 1 week prior to experiment initiation.

2.3. Experimental design

For the dose-response study, experimental diets were based on the AIN-93G formulation of Reeves et al. [34] and consisted of the following ingredients (per kilogram): 10 g of AIN-93G mineral mix, 10 g of AIN-93 vitamin mix, 200 g of casein, 397.5 g of cornstarch, 132 g of Dyetrose (dextrinized cornstarch), 50 g of cellulose, 3 g of L-cysteine, 2.5 g of choline bitartrate, 14 mg of TBHQ and 100 g of sucrose (Dyets, Bethlehem, PA). Corn oil (Dyets), high oleic acid safflower oil containing 75% oleic acid (Hain Pure Foods, Melville, NY) and MEG-3 EPA-Rich Oil containing 49.9% EPA, 6.7% DHA and 1.2% docosapentaenoic acid (DPA) (Ocean Nutrition Canada, Dartmouth, Nova Scotia) were used to amend basal AIN-93G diet to yield five experimental groups ($n=9$): control, control+DON, 0.1% EPA+DON, 0.5% EPA+DON and 3% EPA+DON (Table 1). This range of doses was selected to be equivalent, respectively, to 0.2×, 1× and 5× the maximum recommended level of n-3 PUFA consumption by FDA. A DON concentration of 20 mg/kg was selected based on its previously observed efficacy in inducing IgAN in B6C3F1 mice [35].

Table 1
Experimental groups of mice for assessing the effects of different dosage of EPA on DON-induced IgA[1]

Experimental group	DON (mg/kg diet)	Corn oil (g/kg diet)	Oleic acid[2] (g/kg diet)	EPA-enriched oil[3] (g/kg diet)	Total EPA (g/kg diet)	Total (n-3)[2]	Total (n-6) (g/kg diet)	n-6/n-3 ratio
Control	0	10	60	0	0	0.1	6.0	146:1
Control+DON	20	10	60	0	0	0.1	6.0	146:1
0.1% EPA+DON	20	10	58	2	1.0	1.3	6.0	11:1
0.5% EPA+DON	20	10	50	10	5.0	5.9	6.2	2.3:1
3.0% EPA+DON	20	10	0	60	29.9	34.8	7.4	1:4.7

[1] All diets were adjusted with oleic acid to have final oil concentrations of 70 g/kg.
[2] Refers to high oleic safflower oil which contains 75% oleic acid and 14% linoleic acid.
[3] Refers to concentrated fish oil that is enriched for EPA. This oil was 49.9% EPA and 6.7% DHA.

Diets were prepared every 2 weeks, stored in aliquots at −20°C and fed to mice fresh daily. Blood was collected from saphenous vein [10] with Microvettes (Aktiengesellschaft and Co., Germany) at 4-week intervals for serum IgA-IC measurement. After 16 weeks, mice were euthanized and spleens and Peyer's patches (PPs) aseptically removed. One half of the spleens and the entire PP pool were used for cell culture. The remaining half of spleen was used for fatty acid analysis. Kidneys were removed and frozen at −80°C for measurement of IgA deposition.

For IL-6 expression studies, B6C3F1 mice were fed control or 3% EPA diet ($n=9$) for 4 weeks. Prior to experiment termination, mice were gavaged with 25 mg/kg DON or vehicle. After 3 h, mice were euthanized and one half of spleen and pooled PP were subjected to real-time PCR analysis. The remaining spleen portion was frozen at −80°C for fatty acid analysis. The same approach was used for transcription factor studies except that tissues were harvested after 30 min based on an optimal time point for DON induction reported previously [36]. Nuclear proteins from spleen cell suspensions were extracted and analyzed by electrophoretic mobility shift assay (EMSA) for transcription factor binding activity.

2.4. Fatty acid analysis

Fatty acids were analyzed by gas chromatography (GC) utilizing a GC-2010 Gas Chromatograph (Shimadzu Scientific Instruments, Chicago, IL) and standard fatty acid methyl ester (Nu-Check Prep, Elysian, MN) by the protocol of Hasler et al. [37].

2.5. Cell cultures

Spleens and PP were teased apart in harvest buffer consisting of 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 2% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), passed through a sterile 100-mesh stainless steel screen and resuspended in the same buffer. Cells were centrifuged at 400×*g* for 10 min and erythrocytes lysed for 3 min at 25°C in 20 mM Tris (pH 7.65) containing 0.14 M ammonium chloride. Cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), and counted using a hemacytometer. Cells (1×10^5) from individual mice were cultured in flat-bottomed 24-well tissue culture plates at 37°C under a 6% CO_2 in a humidified incubator. Supernatants were collected after 5 days and analyzed by ELISA to determine IgA secretion.

2.6. IgA and IgA-IC measurement

Serum IgA was measured by ELISA [17] using mouse reference immunoglobulin serum (Bethyl Laboratories, Montgomery, TX), goat antimouse IgA and peroxidase-conjugated goat antimouse IgA (heavy chain specific) (Organon Teknika, West Chester, PA). Immunoglobulin A was quantified by measuring enzyme end product absorbance using a Vmax Kinetic Microplate Reader and Softmax program from Molecular Devices (Menlo Park, CA). For the detection of IgA-IC, diluted serum samples were first precipitated by 70% (w/v) polyethylene glycol (PEG 6000, Sigma) [18] and quantified by IgA ELISA [17].

2.7. Assessment of kidney mesangial IgA deposition

Kidney sections were prepared and analyzed for IgA deposition according to Pestka et al. [16]. Briefly, removed kidneys were frozen in liquid nitrogen, sectioned to 7 mm with a cryostat (Reichert-Jung; Cambridge Instruments, Buffalo, NY) and stained with fluorescein isothiocyanate-labeled goat antimouse IgA (Sigma). Immunoglobulin A immunofluorescence was assessed with a Nikon Labophot Microscope (Mager Scientific, Dexter, MI) equipped with a Kodak DC290 digital camera (Kodak, Rochester, NY). Mean fluorescence intensity of 10 consecutively arranged glomeruli from each section was determined using UTHSCSA Image Tool Software V 1.2 [25]. Pixels were measured on a grayness scale ranging from 0 (black) to 255 (white).

2.8. Interleukin-6 mRNA and heteronuclear RNA analysis

Total RNA from spleens and PPs was isolated using the Trizol (Sigma) according to the manufacturer's protocol. Resultant RNA was dissolved in 50 μl of RNAse-free water and stored at −80°C until analysis. Probe and primers for mouse IL-6 mRNA and 18S RNA (endogenous control) were purchased as TaqMan assay reagents (PE Applied Biosystems). PCR reactions for IL-6 mRNA and 18S RNA quantification were performed on an ABI PRISM 7700 Sequence Detector System using TaqMan

Table 2
Food consumption and body weight gain of mice in different diet groups*

Experimental group	Mean daily food consumption (g)	Mean body weight gain (g)
Control	3.6±0.48[a]	12.94±1.68[a]
Control+DON	2.94±0.66[b]	2.76±0.84[b]
0.1% EPA+DON	2.65±0.79[b]	3.01±0.48[b]
0.5% EPA+DON	2.71±0.70[b]	2.26±0.55[b]
3.0% EPA+DON	2.37±0.59[b]	2.91±0.54[b]

* Values are means±S.E.M., $n=9$. Means in a column without a common letter differ ($P<.05$).

One-Step RT-PCR Master Mix Reagents Kit according to the manufacturer's protocol (PE Applied Biosystems) [25]. SYBER Green PCR Master Mix (PE Applied Biosystems) was used to detect IL-6 heteronuclear RNA (hnRNA) using primers (forward: gtccaactgtgctatcgctcact; backward: agaaggcaactggatggaagtct). Ct values were related to RNA concentrations using the standard curves derived from serial dilutions of total RNA (ranging 1.37–1000 ng per well) and normalized by dividing the IL-6 RNA value by the 18S RNA value.

2.9. Electrophoretic mobility shift assay

Spleen cells were dissociated, passed through a 100-mesh stainless steel screen and pooled. Cells were suspended in Dulbecco's PBS. Erythrocytes were lysed for 2 min at 25°C in 10 mM $KHCO_3$ containing 0.14 M NH_4Cl. Nuclear extracts were prepared based on the method of Zhou et al [38]. Briefly, cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol) with phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride), Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) and 0.7% (v/v) Nonidet P-40. Nuclei were pelleted by centrifugation and suspended in hypertonic buffer containing 20 mM HEPES, pH 7.9, 0.4 M KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 10% (w/v) glycerol and phosphatase and protease inhibitors. After 30 min on the ice, supernatant was collected by centrifugation at 14,000×g for 10 min. Resultant extracts were analyzed with a Bio-Rad Protein Assay kit (Melville, NY), aliquoted and stored at −80°C for EMSA.

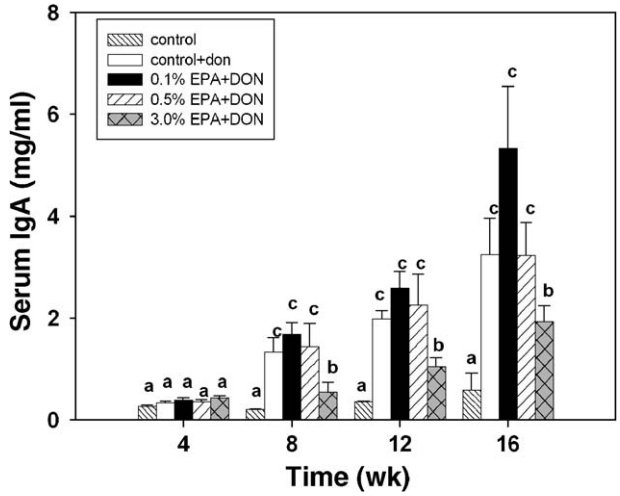

Fig. 1. Eicosapentaenoic acid-enriched fish oil consumption suppresses DON-induced serum IgA elevation in mice. Mice were fed 20 ppm DON containing 0%, 0.1%, 0.5% and 3% EPA, and serum IgA was measured by ELISA at 4, 8, 12 and 16 weeks. Data are means±S.E.M. ($n=8$). Means at a specific time point without a common letter differ ($P<.05$).

Double-stranded consensus probes for cyclic AMP response element-binding protein (CREB), activator protein-1 (AP-1), nuclear factor kappa B (NF-κB) and C/EBPβ (Santa Cruz Biotech, Santa Cruz, CA) were radiolabeled with [γ-^{32}P]ATP using Ready to Go Polynucleotide Kinase Kit (Pharmacia Biotech, Piscataway, NJ). Nuclear protein (10 μg) was added to DNA-binding reaction buffer consisting of 20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 0.5 mM DTT and 2 mg of poly(dI-dC), and preincubated on ice for 15 min to block the nonspecific binding. After the addition of 1 ml of ^{32}P-labeled probe containing 30,000 cpm, the incubation was continued for 30 min at room temperature to promote

Table 3
Fatty acid composition of spleen phospholipids in mice fed EPA-enriched oil with and without DON for 16 weeks*,[1]

Fatty acid	% of fatty acids				
	Control	Control+DON	0.1% EPA+DON	0.5% EPA+DON	3.0% EPA+DON
C14:0	1.8±0.5[a]	2.1±0.4[a]	2.3±0.4[a]	1.9±0.4[a]	2.6±0.5[a]
C16:0	21.1±1.5[a]	24.5±0.8[a]	23.3±0.6[a]	25.1±3.4[a]	21.6±1.7[a]
C16:1	1.9±0.6[a]	3.3±0.7[a]	2.1±0.5[a]	1.7±0.7[a]	1.7±0.5[a]
C18:0	26.2±1.3[a]	26.2±2.8[a]	28.0±1.4[a]	24.0±2.5[a]	25.7±1.6[a]
C18:1	23.4±1.7[a]	22.5±2.5[a]	23.5±1.7[a]	20.4±2.9[a]	18.9±1.9[a]
C18:2(n-6)	3.8±0.3[a]	4.4±0.4[a]	3.8±0.5[a]	4.7±0.6[a]	4.5±0.3[a]
C20:4(n-6)	16.8±2.1[a]	13.4±3.0[a]	8.3±1.1[b]	5.7±1.0[b]	5.6±1.0[b]
C20:5(n-3)	ND[a,2]	ND[a]	0.4±0.2[a]	3.2±1.2[b]	5.6±0.6[b]
C22:5(n-3)	2.1±0.6[a]	1.0±0.3[a]	3.2±0.6[a,b]	5.8±1.2[b]	7.9±1.5[b]
C22:6(n-3)	3.0±0.5[a]	2.7±0.8[a]	5.3±1.0[a,b]	7.5±1.4[b]	6.1±1.2[b]

* Values are means±S.E.M., $n=7$. Means in a row without a common letter differ ($P<.05$).
[1] Only the major fatty acids are shown.
[2] ND, not detectable.

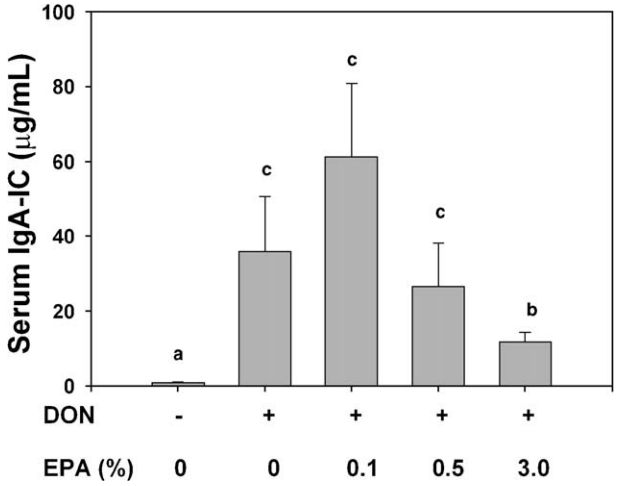

Fig. 2. Eicosapentaenoic acid-enriched fish oil consumption attenuates DON-induced serum IgA-IC elevation in mice. Mice were treated as described in Fig. 1. Sera were collected at 16 weeks and analyzed by IgA after polyethylene glycol precipitation. Data are means±S.E.M. ($n=8$). Means without a common letter differ ($P<.05$).

the formation of nucleoprotein complexes. Resultant nucleoprotein complexes were separated on 4% (w/v) native polyacrylamide gels, dried and visualized by autoradiography [38].

2.10. Statistics

Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Serum IgA, IgA-IC and IgA deposition data were not normally distributed and were therefore subjected to Kruskal–Wallis ANOVA on ranks and pairwise comparisons made by Dunn's or Student–Newman–Keuls methods. All other data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student–Newman–Keuls methods. Differences were considered significant at $P<.05$.

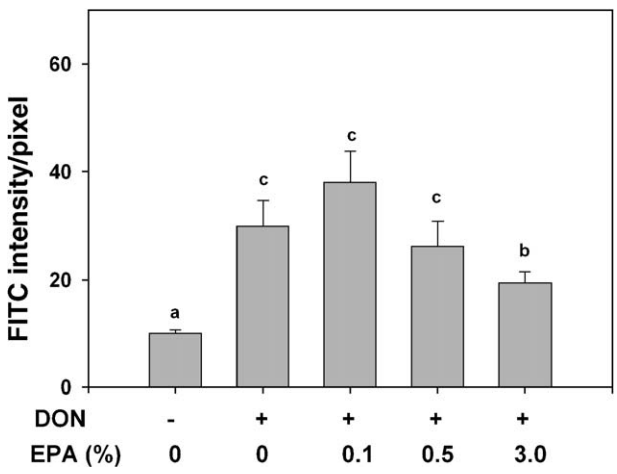

Fig. 3. Eicosapentaenoic acid-enriched fish oil consumption inhibits DON-induced mesangial IgA deposition in mice. Mice were treated as described in Fig. 1. Relative mesangial IgA quantitation after 16 weeks was assessed by measuring immunofluorescence by image analysis. Data are means ±S.E.M. ($n=8$). Means without a common letter differ ($P<.05$).

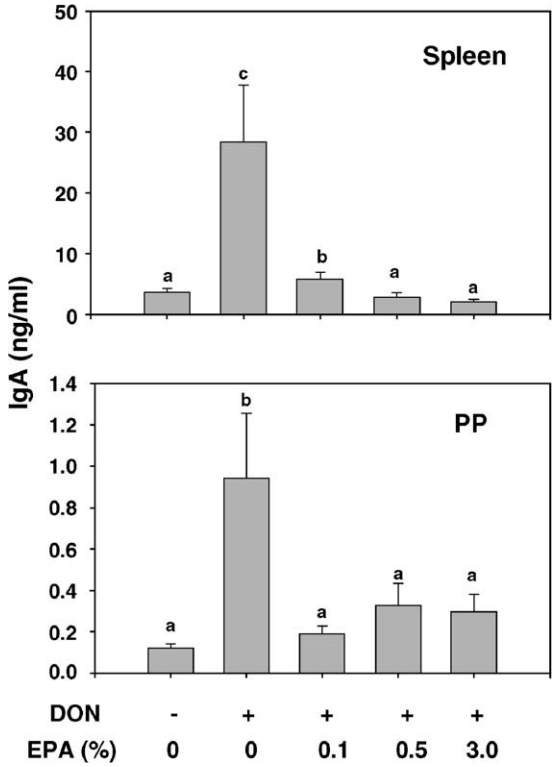

Fig. 4. Deoxynivalenol-induced ex vivo IgA secretion is suppressed by PP and spleen cell cultures from mice fed EPA-enriched fish oil for 16 weeks. Data are means±S.E.M. ($n=8$). Means without a common letter differ ($P<.05$).

3. Results

The capacity of DON to induce early markers of IgAN was compared in mice fed diets containing 0%, 0.1%, 0.5% and 3% EPA. Consistent with its reported anorexic effects [15], consumption of 20 mg/kg DON in diet reduced feed

Table 4
Fatty acid composition of spleen phospholipids in mice fed control or 3% EPA diet for 4 weeks*,[1]

Fatty acid	% of fatty acids	
	Control	3.0% EPA
C14:0	2.2±0.5[a]	2.3±0.4[a]
C16:0	20.1±0.8[a]	23.6±1.0[a]
C16:1	2.5±0.4[a]	1.9±0.6[a]
C18:0	25.8±2.3[a]	22.3±1.8[a]
C18:1	24.2±2.2[a]	23.5±2.1[a]
C18:2(n-6)	4.1±0.3[a]	5.2±0.4[a]
C20:4(n-6)	18.4±1.8[a]	5.7±1.4[b]
C20:5(n-3)	ND[a,2]	5.2±0.6[b]
C22:5(n-3)	1.9±0.5[a]	6.4±0.9[b]
C22:6(n-3)	2.5±0.3[a]	6.8±1.4[b]

* Values are means±S.E.M., $n=3$. Means in a row without a common letter differ ($P<.05$).

[1] Only the major fatty acids are shown.

[2] ND, not detectable.

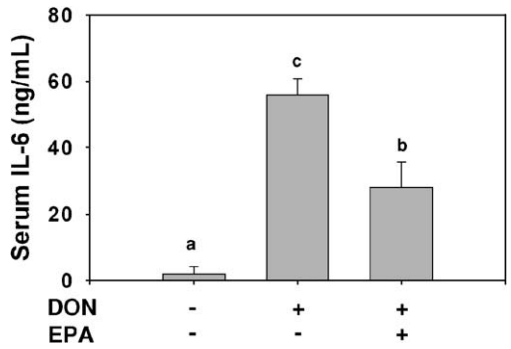

Fig. 5. Induction of serum IL-6 by acute DON exposure is attenuated in EPA-fed mice. Mice were fed experimental diets for 4 weeks and then gavaged with 25 mg/kg body weight DON or vehicle. Data are means ± S.E.M. ($n=5$). Means without a common letter differ ($P<.05$).

intake and weight gain in all groups (Table 2). However, no differences existed among control and EPA-fed mice in either of these parameters. When fatty acid content in splenic phospholipid fractions were assessed by GC, concentrations of AA [20:4(n-6)] in control mice were two to three times higher than in those of mice fed with 0.1% to 3% EPA (Table 3). Eicosapentaenoic acid content was 0%, 0.4%, 3.2% and 5.6% and DHA content 2.7%, 5.3%, 7.5% and 6.1% in mice fed 0%, 0.1%, 0.5% and 3.0% EPA diets, respectively. In addition, DPA, an intermediate in the conversion of EPA to DHA, was also markedly elevated in mice fed the two highest EPA concentrations. Thus, EPA-containing diets markedly decreased the ratio of (n-6) to (n-3) PUFAs in lymphoid tissue.

Serum IgA, serum Ig-IC and kidney mesangial IgA were used as markers for the early stages of IgAN in DON-fed mice. Deoxynivalenol exposure significantly increased serum IgA concentrations in mice fed control diet beginning at 8 weeks, reaching 10 times the control value after 16 weeks (Fig. 1). Serum IgA accumulation was significantly suppressed in mice fed 3% EPA at 8, 12 and 16 weeks, whereas the 0.1% and 0.5% EPA diets had no effect. Deoxynivalenol induced increases in serum Ig-IC at week 16, and this was also suppressed by the 3% but not the 0.1% or 0.5% EPA diets (Fig. 2). When glomerular mesangial IgA deposition was measured at week 16 by immunofluorescence, DON was found to induce IgA deposition (Fig. 3). Again, this elevation was inhibited only by the 3% EPA diet, but not lower EPA concentrations.

Deoxynivalenol-induced IgAN has been previously shown to correlate with increased IgA secretion ex vivo in lymphoid tissue cultures derived from the mucosal and systemic compartments [35]. The effects of EPA consumption on ex vivo IgA production were therefore compared. Inclusion of DON in basal control diet significantly increased IgA production in spleen and PP cell cultures as compared to control group (Fig. 4). However, IgA secretion was markedly suppressed in cultures from all three EPA groups.

Jia et al. [24,25] demonstrated that DHA consumption dose dependently suppresses DON-induced IL-6 expression in spleen and that this correlates with reduced IL-6 mRNA transcription. To assess DON's acute effects on IL-6, we fed the mice 0% or 3% EPA diet for 4 weeks prior to acute DON

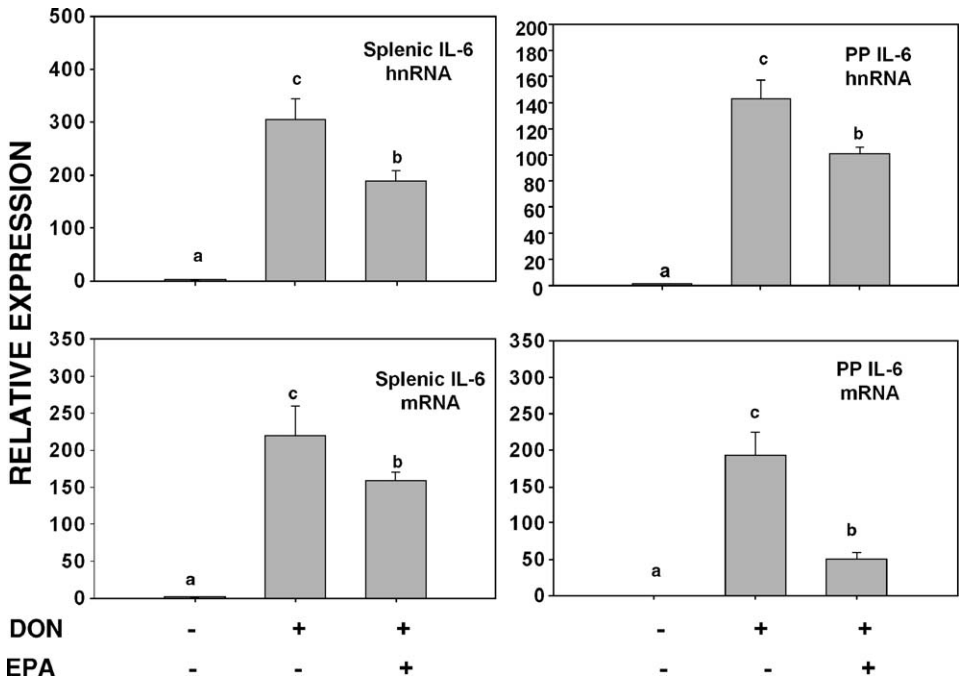

Fig. 6. Eicosapentaenoic acid-enriched fish oil consumption suppresses induction of IL-6 hnRNA and mRNA after acute DON exposure in spleens and PPs of B6C3F1 mice. Mice were fed control or 3% EPA diet for 4 weeks and then gavaged with 25 mg/kg DON or vehicle. RNA was extracted from organs after 3 h and analyzed by real-time PCR. Data are means±S.E.M. ($n=5$). Means without a common letter differ ($P<.05$).

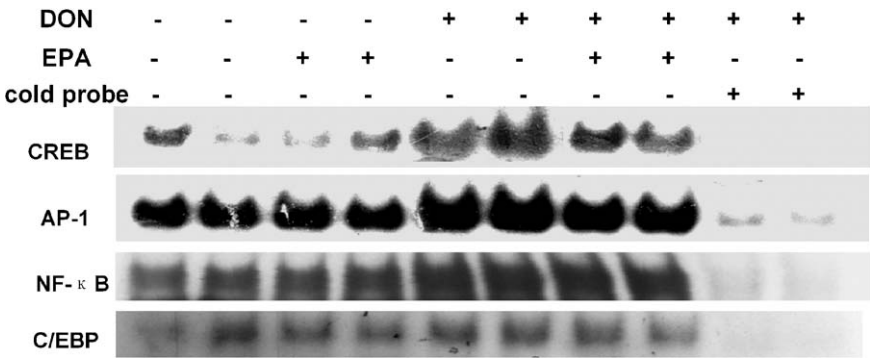

Fig. 7. Eicosapentaenoic acid-enriched fish oil consumption suppresses DON-induced splenic transcription factor binding activity. Mice were fed control or 3% EPA diet for 4 weeks and then gavaged with 25 mg/kg DON or vehicle. Splenic nuclei were isolated after 30 min and then analyzed for transcription factor binding activity by EMSA using double-stranded consensus probes for CREB, AP-1 protein, NF-κB and C/EBPβ radiolabeled with [γ-^{32}P]ATP. Cold probe was incubated in 100-fold excess.

challenge. This feeding period was sufficient to lower splenic AA and concurrently raise EPA, DHA and DPA to levels observed after 16 weeks (Table 4). In control mice, acute DON exposure was found to markedly induce serum IL-6 (Fig. 5) as well as expression of IL-6 mRNA and hnRNA, a marker of IL-6 transcription, in spleen and PP (Fig. 6). Prior consumption of 3% EPA suppressed all three end points suggesting that this fatty acid impaired IL-6 transcription. Consistent with these findings, EMSA revealed that CREB and AP-1 binding activities in splenic nuclear extracts were significantly increased by acute DON exposure, but that consumption of 3% EPA diet suppressed this induction (Fig. 7). Eicosapentaenoic acid did not affect DON-induced NF-κB activation. Neither DON nor EPA affected C/EBP binding.

4. Discussion

Although (n-3) PUFA supplementation has been suggested to be efficacious in the treatment of late-stage IgAN [14], the potential preventative benefits of (n-3) PUFAs in early stages of the disease are unknown. Aberrant serum IgA production is an important early etiological factor for IgAN, and resultant IgA-IC likely binds receptors on mesangial cells, thereby inducing proliferation and cytokine production [39]. Polymeric IgA deposition might also activate complement via the alternative pathway, causing glomerular damage. Because DON-induced dysregulation of IgA production and aberrant IgA accumulation mimic the early stages of human IgAN [24,35], the results presented herein suggest that EPA consumption might have possible prophylactic value in suppressing early elevation of IgA and nephritogenic IgA-IC among individuals who have been diagnosed with IgAN or who have genetic predisposition for this disease.

We observed here that 3% but not 0.1% or 0.5% EPA in the diet suppressed serum IgA and IgA-IC elevation and glomerular IgA deposition. Jia et al [24] previously found that 1% EPA in diet could also suppress these parameters. Taking these studies together, dietary EPA concentrations of 1% to 3% appear to be the prophylactic threshold in this model. These findings are consistent with prior studies showing the same efficacious doses for DHA-enriched oil [24,25]. Because both EPA- and DHA-enriched oils seem to be similarly effective and inhibiting IgA up-regulation, consumption of fish oils containing EPA+DHA might be more cost effective to achieve the IgA endpoint than oils processed to selectively contain either of the two (n-3) PUFAs.

A 1% to 3% EPA dose range provides 2.2% to 6.7% of daily kilocalorie consumed in the mouse, which can be compared to 1.5% daily kilocalorie for (n-3) PUFAs in supplements [14] recommended for therapy in patients. Here we employed the 3% diet as a strong positive control to discern underlying mechanisms for inhibition of IgA up-regulation. A limitation of this approach is that this dose would be equivalent to human consumption at 15 g/day, which is five times that of the recommended FDA maximum of 3.0 g/day. Nevertheless, it should be noted that ex vivo cultures from all three EPA concentrations showed suppressed IgA production. This suggests that ongoing IgA production at experiment termination (16 weeks) might have indeed been suppressed even at the lowest (0.1%) EPA concentration later in the feeding study. Thus, a key future question relates to whether prefeeding (n-3) PUFAs at low concentrations for an extended period before DON exposure might attenuate IgA dysregulation.

Although EPA content in spleen (0.4–5.6%) was highly dependent on the concentration of this (n-3) PUFA in experimental diet, DHA content was similar (5.3–7.5%) among the experimental groups. Splenic DHA could arise from two sources. First, although the EPA-enriched fish oil employed here contained predominantly EPA (49%), it also contained a smaller amount of DHA (6.7%) that could accumulate in the splenic pool. Second, EPA can be transformed to DHA in mammalian tissue via intermediate DPA [40,41]. Accumulation of DPA (3.2–7.9%) in EPA-fed mice suggests that such a conversion was ongoing and thus could be another likely DHA source.

A critical observation was that EPA consumption significantly reduced serum IL-6 and splenic IL-6 mRNA induced by acute DON exposure. The mucosal immune system is a primary target of DON [35] where it disrupts oral tolerance and promotes production of IgA capable of binding antigens found in food and commensal bacteria [42,43]. Deoxynivalenol up-regulates expression of proinflammatory genes, notably IL-6, both in vivo and in vitro [44,45]. IL-6 is produced by activated monocytes and macrophages, and also by endothelial cells, T cells and keratinocytes [46]. Interleukin-6 drives IgA-committed B cell to terminally differentiate to IgA-secreting plasma cells [19]. Differentiated IgA-secreting B cells can migrate to distal mucosal and systemic sites, survive for prolonged periods and produce IgA. Interleukin-6's role in DON-induced IgAN is supported by the ex vivo antibody neutralization studies [20,21] and by the observation that IL-6-deficient mice resist DON-induced serum IgA elevation and mesangial IgA deposition [22].

Because hnRNA is a precursor species observed in cells prior to RNA splicing to mRNA, its abundance can be used as a surrogate for the run-on assay in detection of gene transcriptional activity [47]. Eicosapentaenoic acid-enriched oil consumption significantly blocked accumulation of IL-6 hnRNA as well as IL-6 mRNA, indicating that (n-3) PUFA attenuation IL-6 gene expression occurred in part at the transcriptional level. This contention is further supported by our previous observation that mice consuming 3% DHA for 4 weeks exhibit markedly less induction of IL-6 mRNA and IL-6 hnRNA expression following acute DON exposure [25]. Diminished IL-6 transcription and ultimately, IL-6 expression, might attenuate IL-6 production by affecting transcription factor binding to the response element in IL-6 promoter. Cyclic AMP response element-binding protein, AP-1 NF-κB and C/EBPβ have all been associated with binding and transactivation of the IL-6 promoter [48,49]. Of these, suppression of DON-induced IgAN corresponded to decreased induction of CREB and AP-1 binding activity. These two factors might thus be important in diminished IL-6 transcription.

n-3 PUFAs potentially influence functional activities of cells of the immune system via several mechanisms. The ameliorative effects of the experimental diet in this study were likely mediated by dramatic decreases in AA and corresponding increases in splenic (n-3) PUFAs. Deoxynivalenol-induced IL-6 expression is mediated in part through increased COX-2 levels and PGE2 production [50–52]. Cyclooxygenase and lipoxygenase products produced from EPA are much less potent as inflammatory mediators than are products generated from AA [10]. Thus, decreased AA tissue concentration might reduce IL-6 gene expression and attenuate overall IgA production in DON-exposed mice. Inhibition of IL-6 expression by (n-3) PUFAs could also involve deregulation of key signal transduction pathways. Deoxynivalenol does not act through a known receptor but rather acts via the ribotoxic stress response and activation of mitogen-activated protein kinases (MAPKs) that drive expression of IL-6 and other inflammatory genes [53]. Consumption of fish oil or (n-3) PUFAs can cause modest suppression of MAPK activation [25,54].

Taken together, the data presented here suggest, for the first time, that a threshold exists for EPA relative to suppression of experimental IgAN and that the threshold EPA dose for IgA inhibition was effective at suppressing IL-6 transcription. Relevant to our findings, two clinical trials reported that IL-6 production by peripheral blood mononuclear cells is decreased in persons who consume (n-3) PUFA, and this co-occurs with increased plasma and cell membrane (n-3) PUFA incorporation [55,56]. (n-3) PUFA suppression of IL-6 production might have additional clinical relevance because this cytokine is believed to contribute to kidney injury [1 /id}[57,58] in human IgAN [59]. Further study at the preclinical level is needed on the relationship between dietary (n-3) PUFA and tissue phospholipid concentrations needed for optimal, efficacious prophylactic and therapeutic treatment for IgAN and other immune-related diseases. The strategy described here offers one animal model for such preclinical testing.

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