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Deoxynivalenol-induced mitogen-activated protein kinase phosphorylation and IL-6 expression in mice suppressed by fish oil

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Abstract

The trichothecene mycotoxin deoxynivalenol (DON) induces IgA hyperelevation and mesangial IgA deposition in mice that mimics the early stages of human IgA nephropathy (IgAN). Among potential mediators of this disease, interleukin-6 (IL-6) is likely to play a particularly critical role in IgA elevation and disease exacerbation. Based on previous findings that dietary fish oil (FO) suppresses DON-induced IgAN, we hypothesized that FO inhibits the induction of IL-6 expression by this mycotoxin in vivo and in vitro. Mice were fed modified AIN 93G diet amended with 7% corn oil (CO) or with 1% corn oil plus 6% menhaden fish oil (FO) for up to 8 weeks and then exposed acutely to DON by oral gavage. DON-induced plasma IL-6 and splenic mRNA elevation in FO-fed mice were significantly suppressed after 8 weeks when compared to the CO-fed group. The effects of FO on phosphorylation of mitogen-activated protein kinases (MAPKs), critical upstream transducers of IL-6 up-regulation, were also assessed. DON-induced phosphorylation of extracellular signal regulated protein kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinases 1 and 2 (JNK1/2) was significantly suppressed in spleens of mice fed with FO, whereas p38 was not. Splenic COX-2 mRNA expression, which has been previously shown to enhance DON-induced IL-6, was also significantly decreased by FO, whereas plasma levels of the COX-2 metabolite, prostaglandin $E_{2,}$ were not affected. To confirm in vivo findings, the effects of pretreatment with the two primary n-3 PUFAs in FO, eicosapentaenoic acid (20:5[n-3]; EPA) and docosahexaenoic acid, (22:6[n-3]; DHA), on DON-induced IL-6 expression were assessed in LPS-treated RAW 264.7 macrophage cells. Consistent with the in vivo findings, both EPA and DHA significantly suppressed IL-6 superinduction by DON, as well as impaired DON-induced ERK1/2 and JNK1/2 phosphorylation. In contrast, the n-6 PUFA arachidonic acid (20:4[n-3]) had markedly less effects on these MAPKs. Taken together, the capacity of FO and its component n-3 PUFAs to suppress IL-6 expression as well as ERK 1/2 and JNK 1/2 activation might explain, in part, the reported suppressive effects of these lipids on DON-induced IgA nephropathy.

Keywords: Mycotoxin; Cytokine; n-3 PUFA; IgA Nephropathy; Signal transduction; Kinase

1. Introduction

Consumption of (n-3) polyunsaturated fatty acids (PUFAs) in fish oil (FO)-containing diets has been associated with modulation of a diverse array of immunological events that include suppression of lymphocyte proliferation, antigen presentation, MHC II expression, cytotoxic T lymphocyte activity, NK cell activity, macrophage-mediated cytotoxicity, and neutrophil/monocyte chemotaxis [1–3]. These functional effects have been the rationale for experimental and clinical attenuation of acute inflammatory, delayed-type hypersensitivity, autoimmune and transplant rejection responses by (n-3) PUFA [4–7]. The mechanisms for these effects may be related to the capacity of (n-3) PUFAs to modulate inflammatory mediator profiles and concentrations as well as immune cell populations by altering apoptotic signaling, cell-to-cell communication and signal transduction [8–10]

Human IgA nephropathy (IgAN), the most common type of glomerulonephritis in the world is characterized by the presence of prominent IgA deposits in the kidney mesan-

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gium region as discerned by immunofluorescence microscopy [11]. Etiological factors that have been associated with this disease include prior mucosal infection, genetic predisposition, and diet. Dietary supplementation with fish oil has been suggested to benefit patients with immune-related renal diseases including IgAN, lupus nephritis and cyclosporine-induced nephrotoxicity [12]. Despite supportive data from several randomized clinical trials evaluating efficacy of fish oil in treating IgAN [13–15], there have been conflicting findings [16, 17]. To clarify these issues, animal models might potentially be used to establish mechanisms by which FO or its component (n-3) PUFAs interferes with the development and progression of IgAN. Several animal models for IgAN have been developed using approaches that include injection with IgA immune complex, mucosal immunization, viral infection and use of genetically modified mice prone to mesangial IgA deposition [18]. Notably, dietary exposure to the mycotoxin deoxynivalenol (DON or vomitoxin) has been found to cause clinical signs in mice that are analogous to early stages of human IgAN [19, 20].

DON, a trichothecene produced by *Fusarium graminearum* and *F. culmorum*, is frequently found in grain-based agricultural commodities from the Midwestern United States such as wheat, barley, and corn [21]. The toxin is a potent inhibitor of protein synthesis that can impact actively dividing tissues with the immune system being particularly susceptible [22]. Acute exposure to high doses of DON can induce rapid diminution of lymphoid tissues via apoptosis and lymphopenia in experimental animals. Paradoxically, low dose or chronic DON exposure can be immunostimulatory as evidenced by its capacity to induce cytokine gene expression in vivo [23, 24]. Dietary DON exposure induces elevation of serum IgA, mesangial kidney IgA deposition and hematuria in mice; these are hallmarks of IgAN [19, 20]. Recently, we have shown that FO-containing diets impair these immunopathologic endpoints [25].

Aberrant IgA production following DON ingestion appears to be mediated in part by superinduction of cytokine gene expression in mononuclear phagocytes [26, 27] and T helper cells [28]. Among these cytokines, interleukin-6 (IL-6) is recognized to be particularly important to the modulation of mucosal B cell differentiation to IgA-secreting plasma cells [29]. In addition to effects of IL-6 on IgA production, this cytokine might also promote pathogenic processes in IgAN by enhancing mesangial cell proliferation and producing proinflammatory lipid mediators and superoxide anion [30, 31]. Therefore, IL-6 induction by DON might be critical to both early stage IgA production and the later disease progression in IgAN. In support of the former possibility, genetic disruption of IL-6 in mice markedly reduces DON-induced increases in serum IgA as well as kidney IgA deposition in mice [32]. The purpose of this investigation was to test the hypothesis that dietary FO impairs DON-induced IL-6 gene expression. The results demonstrated that FO and (n-3) PUFAs impair up-regulation of IL-6 by DON both in vivo and in vitro. Furthermore, these ameliorative effects were associated with decreased activation of mitogen-activated protein kinases (MAPKs).

2. Methods and materials

2.1. Animal studies

All animal handling was conducted according to the guidelines established by the NIH. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory Animal Committee. Male $B6C3F_1$ mice (6 to 7 week old) were obtained from Charles River (Portage, MI) and acclimated for one week in a humidity- and temperature-controlled room with an alternating 12 hr light and dark cycle. Mice were housed singly in the environmentally protected cages that consisted of a transparent polycarbonate body with a filter bonnet, stainless steel wire lid and a layer of heat-treated hardwood chips.

All dietary constituents were purchased from Dyets (Bethlehem, PA). Experimental diets were based on the AIN-93G formulation of Reeves et al. , [33] and consisted of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.49 g cornstarch, 132 g Dyetrose (dextranized cornstarch), 100 g sucrose, 50 g cellulose, 14 mg t-butylhydroquinone (TBHQ), 3 g L-cystine, and 2.5 g choline bitartrate. Corn oil (CO) and menhaden fish oil (FO), each of which contained 200 mg/kg of tert-butyl hydroquinone (TBHQ), were then added to yield two kinds of experimental diets containing the following (per kg): (*1*) 70 g CO (CO group); and (*2*) 10 g CO plus 60 g FO (FO group). The basal 1% level of CO was included to ensure that minimal (n-6) PUFA requirements were met. Relative amounts of (n-3) and (n-6) PUFAs are shown in Table 1. Equivalent amounts of TBHQ were added to both stock oils by the manufacturer to prevent confounding effects from this antioxidant.

Mice were randomly assigned to experimental groups. Feed was provided ad libitum and changed daily. To prevent rancidity, FO diets were prepared weekly and stored at −20°C in freezer bass in aliquots sufficient to provide enough feed for 1 day. Water was provided ad libitum and changed every 2 to 3 days. Mice were fed experimental diets for 2, 4, and 8 weeks and then orally gavaged with DON (25 mg/kg bw). Following DON treatment, animals were held for 30 min or 3 hr and then bled retroorbitally under metaflurane anesthesia for plasma IL-6 and PGE_2 quantitation. Mice were immediately euthanized by cervical dislocation and spleens removed for Western blot analysis and mRNA measurement.

2.2. Cell culture studies

RAW 264.7 murine macrophage cells (American Type Culture Collection, Rockville, MD) were used as a model

Table 1
Fatty acid composition of oils used for experimental diets[1]

Fatty Acid	Corn Oil Diet (%)[2]	Fish Oil Diet (%)[3]
	g/100 g total fatty acids	
14:0	—	7.7
16:0	10.8	16.2
16:1(n17)	—	10.8
16:2(n-4)	—	1.5
16:3(n-3)	—	1.5
16:4(n-1)	—	1.5
18:0	2.1	2.7
18:1(n-9)	26.5	13.5
18:2(n-6)	60.0	9.7
18:3(n-3)	—	1.5
18:4(n-3)	—	3.0
20:1(n-9)	—	1.4
20:4(n-3)	—	1.2
20:4(n-6)	—	0.8
20:5(n-3)	—	13.3
21:5(n-3)	—	0.7
22:5(n-3)	—	2.1
22:6(n-3)	—	7.8
Σ SFA	12.9	26.7
Σ MUFA	26.5	25.6
Σ PUFA	60.0	44.5
Σ (n-6) PUFA	60.0	10.5
Σ (N-3) PUFA	0.6	26.6

[1] Only the major fatty acids are shown. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[2] 1000 g/L corn oil.
[3] 857 g/L fish oil and 143 g/L corn oil.

for studying effects of fatty acids on DON-induced IL-6 expression cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals Inc, Norcross, GA), 100 unit/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma, St. Louis, MO) in a 5% CO_2 humidified incubator at 37°C. Macrophage cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hematocytometer [34].

Supplementation of media with fatty acids was based on previously published methods [35]. Briefly, fatty acid-free bovine serum albumin (BSA, Roche, Indianapolis, IN) and fatty acid (eicosapentaenoic acid 20:5[n-3], EPA); or docosahexaenoic acid, 22:6[n-3], DHA; or arachidonic acid, 20:4[n-6], AA) (Sigma Chemical Co., St. Louis, MO) were mixed in Ca^{2+}, Mg^{2+} free PBS at a 3:1 molar ratio under nitrogen on a rocking shaker for 24 hr at 37°C. These mixtures were then diluted with serum-free DMEM media to desired concentration of fatty acid. Media were freshly prepared before each experiment. Before adding fatty acid-amended media, adherent RAW 264.7 cells (2.5×10^5/mL) were incubated in serum free DMEM medium for 18 hr. Cells were then cultured with serum-free fatty acid-amended medium for 24 hr. For IL-6 and COX-2 studies, cells were cultured with or without DON and/or LPS for an additional 24 hr after replacing with fresh DMEM medium supplemented with 10% heat-inactivated FBS. For MAPK studies, cells were incubated for 48 hr with serum-free fatty acid-amended media and then exposed to DON or vehicle for 30 min.

2.3. Interleukin 6 (IL-6) ELISA

Plasma or cell culture supernatant were diluted in 0.1M phosphate buffered saline (PBS, pH 7.2) and incubated for 1 hr at 37°C in Immunolon IV removawell microtiter strips (Dynatech Laboratories, Chantilly, VA) which were coated with 1 μg/mL purified rat anti-mouse IL-6 (Pharmingen, San Diego, CA) diluted in coating buffer (0.84% [w/v] sodium bicarbonate, pH 8.2). After washing 4 times with PBS containing 0.05% (v/v) Tween 20 (PBST), wells were incubated with 100 μL 1.5 μg/mL biotinylated rat anti-mouse-IL-6 (Pharmingen, San Diego, CA) for 1 hr at room temperature. Wells were washed 6 times and incubated for 1 hr with 100 μL 1.5 μg/mL horseradish peroxidase (HRP)-conjugated Streptoavidin (Sigma, St. Louis, MO) in PBST at room temperature. After washing 8 times with PBST, substrate (100 μl) consisting of 3′, 3′, 5′, 5′- tetramethyl benzidine (100 μg/mL, Fluka Chemical Co. Ronkonkoma, NY) in 0.1 M citric phosphate buffer (pH 5.5) and 0.003% (w/v) hydrogen peroxide was added to each well and incubated for 10 min at room temperature for color development. The reaction was terminated with 100 μl 6N sulfuric acid. Absorbance was read at 450 nm with Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) and IL-6 was quantified using the manufacturer's software.

2.4. Detection of IL-6 and COX-2 mRNA by RT-competitive PCR

Total RNA from murine tissue was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA (100 ng) from each sample was converted to cDNA by reverse-transcriptase [36] and resultant cDNA was amplified competitively with truncated internal standard of cDNA constructed by the bridging-deletion method [37]. Amplification was performed in a 9600 Perkin Elmer Cycler (Perkin-Elmer Corp., Norwalk, CT) using the following parameters: 30 cycles of reactions of denaturation at 94°C for 30 s, annealing at 55°C for 45 s (IL-6) or 56°C for 45 s (COX-2), and elongation at 72°C for 45 s. An aliquot of each PCR product was subjected to 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers were synthesized at Michigan State University Molecular Structure facility. The 5′ forward (F) and 3′ reverse (R)-complement PCR primers for amplification of mouse COX-2 and IL-6 cDNA were ACACTCTATCACTGGCATCC (COX-2 F), GAAGGGACACCCTTTCACAT (COX-2 R), TTCA-CAAGTCCGGAGAGGAG (IL-6 F), and TGGTCTTG-GTCCTTAGCCAC (IL-6 R) respectively. The final end-

product of amplified cDNAs were 584 bp of COX-2 , 500 bp of COX-2 internal standard cDNA, 488 bp of IL-6, and 400 bp of IL-6 internal standard cDNA. Densitometric ratios of native cDNA to each internal standard cDNA were used to make each standard curve for calculating cDNA concentrations in RT reaction products.

2.5. PGE_2 assay

Blood was collected in vacutainers containing 1.0 mM EDTA, and 10 μM indomethacin and centrifuged at 4000*g* for 5 min. Plasma supernatant was diluted to 1:5 in 0.1 M phosphate buffer solution (pH 7.2). Samples were cleaned up with a PGE_2 affinity sorbent to remove interfering substances. PGE_2 affinity sorbent (50 mL, Cayman Chemical CO., Ann Arbor, MI) was added to 1 mL of the prepared samples, and the suspension was gently mixed for 1 hr on the rocker at 25°C. Samples were briefly centrifuged at 1500*g* to sediment the sorbent and the sediment was then washed once with 1 mL of 0.1 M phosphate buffer. Sorbent pellets were resuspended in 0.5 mL elution solution (95% [v/v] ethanol solution), mixed by vortexing, and centrifuged briefly at 1500*g*. The supernatant was evaporated, and residue in dissolved in EIA assay buffer. The solution was then analyzed by ELISA (Cayman Chemical Co., Ann Arbor, MI) according to manufacturer's instructions.

2.6. Western blot analysis

At the time of harvest, cells were washed with ice-cold phosphate buffer, boiled in lysis buffer (1% (w/v) SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris pH 7.4), and sonicated for 5 s. For spleen samples, frozen tissue was homogenized in the boiled lysis buffer and sonicated for 10 s. Protein was measured by the Lowry method using Dc protein assay reagent (Bio-Rad, Cambridge, MA). Extracts (10 μg) were mixed with Laemmli sample buffer (Bio-Rad), and boiled for 5 min before resolving on a 10% (w/v) acrylamide gel. Resolved proteins were transferred to PVDF membrane and blocked with Tris-buffered saline (10 mM Tris-HCl pH 7.5, 100 mM NaCl) containing 0.1% (v/v) Tween-20 and 1% (w/v) BSA (TBST-BSA). The membrane was incubated for 1 hr with MAPK antibodies (rabbit IgG, New England Biolab, Beverly, MA) at a 1:1000 dilution in TBST-BSA, and, then was washed three times with TBST. Specificities of the antibodies were as follows: P-ERK 1/2 (p42, p44), Thr202/Tyr204 phosphorylation; P-JNK 1/2 (p46, p54), Thr183/Tyr185 phosphorylation; and P-p38, Thr180/Tyr182 phosphorylation. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA). Blots were washed four times and developed using an ECL chemiluminescent detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

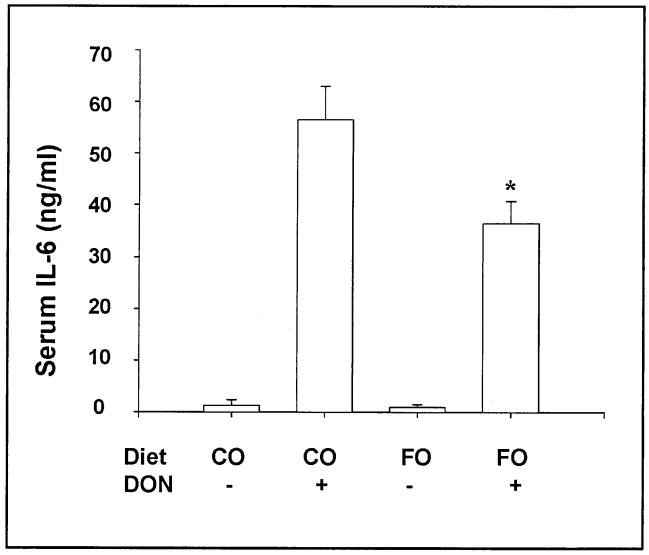

Fig. 1. Effect of FO diet on serum IL-6 production in mice acutely exposed to DON or vehicle. CO and FO diets were fed to male $B6C3F_1$ mice for 8 weeks and 25 mg/kg DON or vehicle was administered via gavage. After 3 hr of the toxin treatment, blood was collected for IL-6 ELISA analysis. Asterisk indicates significant difference ($P < 0.05$) of FO/DON group from CO/DON group.

2.7. Statistics

Data were analyzed by test using the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). For comparison of two groups of data, Student's T test was performed. For comparison of multiple groups, data were subjected to one way analysis of variance (ANOVA) and pairwise comparisons made by Student-Newman-Keuls (SNK) method. Data not meeting normality assumption were subjected to Kruskal-Wallace ANOVA on Ranks and the pairwise comparisons made by SNK method. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

The effects of feeding FO containing high concentrations of (n-3) PUFAs for different lengths of time on induction of IL-6 by acute DON exposure were assessed based on the known relationship established between this cytokine and up-regulation of IgA production [32]. The FO diet employed here has been previously used to ameliorate DON-induced IgAN and does not affect feed consumption or weight gain [25]. At 2 and 4 weeks, no significant differences were observed in DON-induced plasma IL-6 between CO-fed and FO-fed mice (data not shown). However, mice ingesting FO diet for 8 weeks showed a significantly reduced plasma IL-6 response ($P < 0.05$) to DON as compared with CO-fed mice (Fig. 1). Consistent with the plasma findings, induction of IL-6 mRNA by DON in spleens of FO-fed mice was also significantly suppressed ($P < 0.05$)

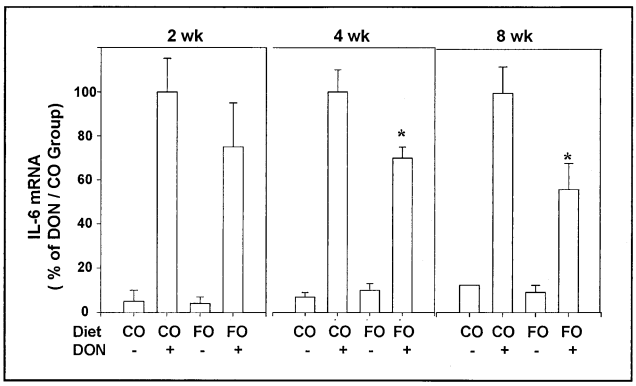

Fig. 2. Effects of FO diet on splenic IL-6 mRNAs of mice exposed to DON or vehicle (VH). CO and FO diets were fed to male B6C3F$_1$ mice for 2, 4, and 8 weeks and then vehicle or 25 mg/kg DON or VH were administered. After 3 hr of the toxin treatment, spleens were analyzed for IL-6 mRNA levels using RT-competitive PCR. Asterisk indicates significant difference ($P < 0.05$) of FO/DON group from CO/DON group at corresponding exposure times.

relative to that in CO-fed mice after 4 and 8 weeks of feeding (Fig. 2). Splenic IL-6 mRNA levels in FO- or CO-fed mice were maximal at 3 hr after DON gavage which was corresponded with IL-6 mRNA kinetics previously described for mice exposed to this mycotoxin [26].

COX-2 is induced by DON [38] and has been shown, in part, to mediate IL-6 up-regulation in vitro and in vivo [39]. In addition, (n-3) PUFAs displace arachidonic acid in the cell membrane and reduces PGE_2 production [40]. Thus, expression of cyclooxygenase-2 (COX-2) and production of its metabolite PGE_2 might be involved in FO-mediated suppression of the IL-6 response. DON-induced COX-2 mRNA levels in FO-fed mice were significantly decreased as compared to CO-fed mice (Fig. 3A). Although, there was a trend toward less DON-induced PGE_2 in FO-fed mice, it was not significant (Fig. 3B). Thus, the IL-6 effects observed in FO-fed mice only weakly corresponded to COX-2 expression but not production of its metabolite PGE_2.

Trichothecene mycotoxins, including DON, activate MAPKs in macrophages and T cells via a mechanism known as the ribotoxic stress response, which has been further, associated with toxin-induced proinflammatory gene expression and apoptosis, depending on the dose regimen employed [41–43]. Since MAPK activation is likely to be involved in the transcriptional or post-transcriptional regulation of IL-6 gene expression [44–45], FO feeding might affect DON-induced IL-6 expression by modulating activity of this kinase family. The effects of FO feeding for 8 weeks on MAPK phosphorylation were therefore assessed in spleen 0.5 or 3 hr after oral exposure to DON (Fig. 4). FO diet significantly suppressed DON-induced ERK1/2 and JNK1/2 activation whereas there was a slight increase in p38 phosphorylation in FO-fed mice. JNK1/2 activation was not detectable 3 hr after DON exposure, however, suppression of DON-induced ERK 1/2 phosphorylation was persistent in FO-fed mice. Slightly increased p38 phosphorylation was also observed 3 hr after DON exposure. Thus, it is possible that FO feeding can affect DON-induced IL-6 expression by modulating MAPK signaling pathways.

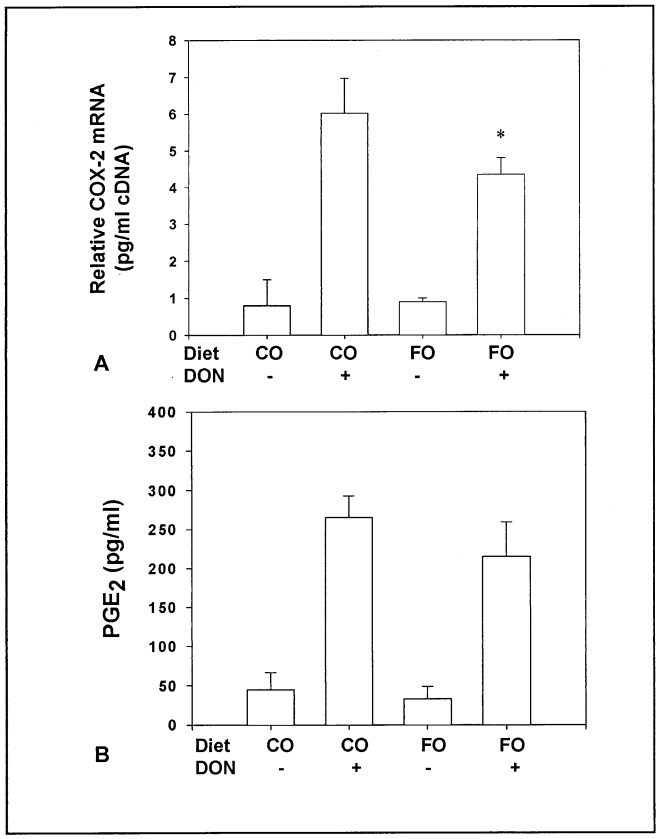

Fig. 3. Effect of FO diet on COX-2 metabolism in mice acutely exposed to DON or vehicle (VH). CO or FO diet was fed to male B6C3F$_1$ mice for 8 weeks and 25 mg/kg DON or VH was administered via gavage. Three hr after toxin treatment, spleens were analyzed for COX-2 mRNA levels (A) using RT-competitive PCR and for PGE_2 (s) measurement by ELISA. Asterisk indicates significant difference ($P < 0.05$) of FO/DON group from CO/DON group at each exposure time.

DON-induced IgA elevation is driven by macrophage-derived IL-6 [26, 27]. Therefore, to confirm results of feeding studies in vitro, the effects of specific (n-3) PUFAs on DON-induced IL-6 production were evaluated in macrophage cells, a primary source of this proinflammatory cytokine. RAW 264.7 cells were used as based on our previous findings that DON induces IL-6 expression in this murine macrophage model [57, 58] as well as the wide use of this cell line as a surrogate for peritoneal macrophages. Although DON alone does not directly induce IL-6 production in macrophages, the toxin can superinduce gene expression of this cytokine in the presence of lipopolysaccharide (LPS). This superinduction model was therefore employed to assess the effects of (n-3) PUFA on DON-induced IL-6 production in vitro using the RAW 264.7 macrophage cell line. Although PUFAs are known to induce programmed cell death in several systems [48, 49] the PUFA concentrations employed here were not significantly cytotoxic to the cells. Cellular viability was maintained over

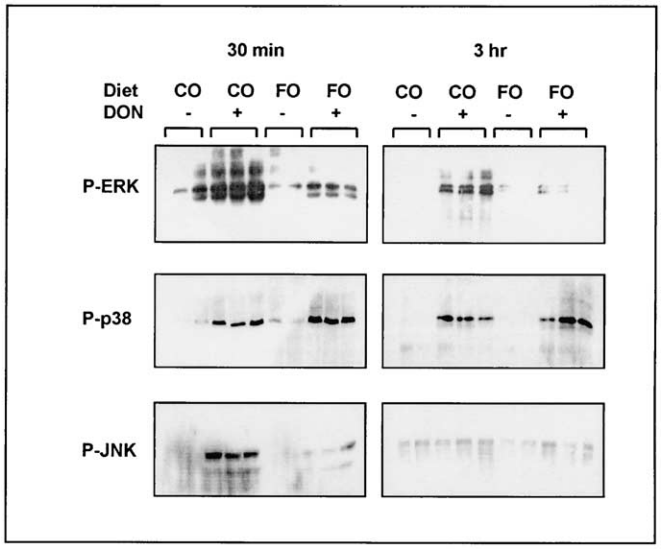

Fig. 4. Effect of FO diet on MAPK phosphorylation in spleens of mice exposed to DON or vehicle (VH). Male B6C3F$_1$ mice were fed with oil-containing diet for 8 weeks. DON 25 mg/kg BW) or VH was administered to mice. Spleens were removed 0.5 and 3.0 hr later and analyzed for MAPK phosphorylation by Western blotting.

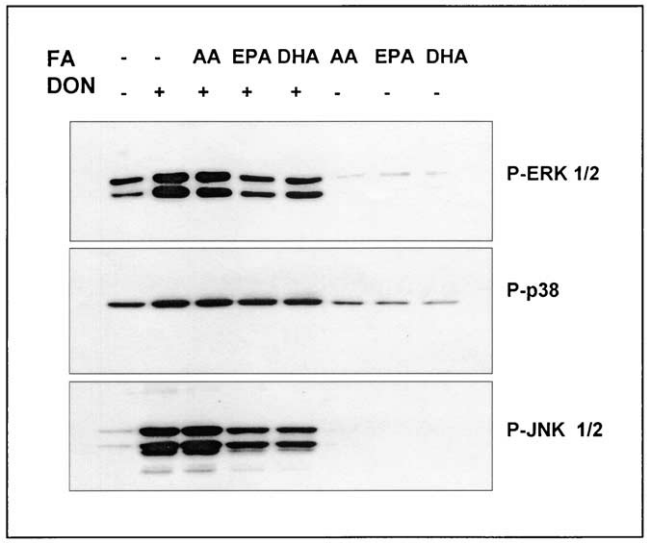

Fig. 6. Effect of PUFAs on DON-induced MAPK phosphorylation. Phosphorylated MAP kinases of macrophage cells. RAW 264.7 cells were incubated in serum free DMEM medium for 18 hr. Cells were then incubated with 110 μM fatty acid AA, EPA, or DHA-containing serum-free DMEM medium for 48 hr and then exposed to DON (250 ng/mL) or vehicle (VH) for 30 min. Cellular lysate was analyzed by Western blotting.

90% with all the treatment of chemical combinations in the study (data not shown). Prior to the IL-6 superinduction with DON and LPS, macrophages were cultured with BSA pre-treated with EPA, DHA, AA or vehicle. Culture with EPA or DHA at 3 and 30 μM retarded DON-mediated IL-6 superinduction and the increased with increased concentration of (n-3) PUFA (Fig. 5). In contrast, AA at 1 μg/mL to 3 μM had no effect whereas 10 μg/mL to 30 μM was mildly inhibitory. Taken together, pre-exposure to (n-3) PUFA found in FO effectively decreased DON-mediated IL-6 production.

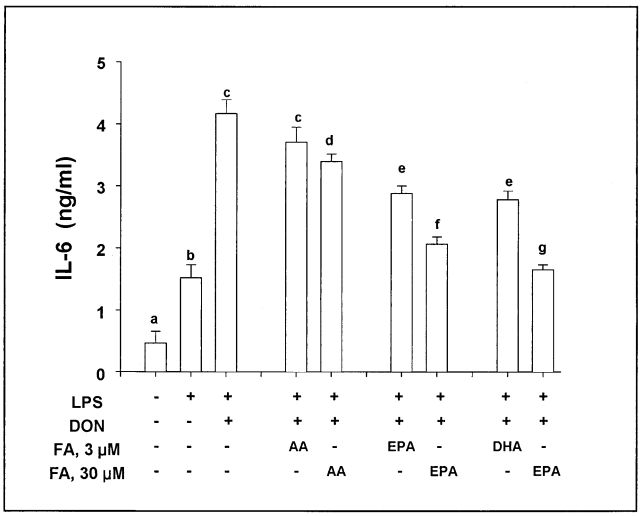

Fig. 5. Effect of PUFAs on DON-induced IL-6 production in murine macrophage cells. RAW 264.7 cells were incubated in serum free DMEM medium for 18 hr and then exposed to the fatty acid AA, EPA, or DHA)-containing serum free DMEM medium for 24 hr. Cells were further incubated for 24 hr in the presence of DON (100 ng/mL) and/or LPS (200 ng/mL) with fresh DMEM medium supplemented with 10% (v/v) heat-inactivated FBS . IL-6 production in supernatant was measured by ELISA. Bars with different letters are significantly different ($P < 0.05$).

The direct effects of (n-3) PUFA on phosphorylation of DON-activated MAPK were also analyzed in vitro. RAW 264.7 cells were preincubated with each fatty acid for 48 hr and then exposed to 250 ng/mL DON for 30 min. As consistent with a previous study [42], DON activated the three major MAPKs (Fig 6). Moreover, pretreatment with EPA or DHA resulted in less ERK1/2 and JNK1/2 activation by DON than vehicle or AA-treated cells. These in vitro results concur with MAPK suppression observed upon FO feeding in DON-exposed mice.

4. Discussion

Whereas high concentrations of trichothecenes completely shut down translation and induce apoptosis, sub-inhibitory concentrations of these mycotoxins can up-regulate cytokine production by elevating transcription factor activity [50] and increasing mRNA stability [47, 51]. Of several cytokines upregulated by DON in vivo, IL-6 appears to play a pivotal role in promoting polyclonal IgA secretion based on: (*1*) prolonged kinetics and magnitude of the IL-6 response relative to other cytokines [23, 43]; (*2*) reduced IgA production by neutralization with anti-IL-6 antibody in ex vivo Peyer's patch cultures [26]; and (*3*) the resistance of IL-6 knockout mice to DON-induced IgAN [32]. Moreover, induction of IL-6 might be further associated with the cy-

totoxic and shock-like responses that occur during acute DON intoxication in mice [24]. Given the importance of IL-6 production in the DON-induced IgAN model, suppression of this cytokine appears to be a viable strategy for preventing elevation of IgA as well as IgA-immune complex at level of both plasma and kidney mesangium.

Dietary fish oil containing (n-3) PUFA has been linked to the amelioration of IL-6-mediated immunopathogenic sequelae and inflammation including autoimmune nephritis and cardiovascular diseases [52–54]. FO-feeding also retards DON-induced IgA nephropathy in the mouse model [25]. The data presented here suggest that dietary fish oil suppresses DON-induced IL-6 in vivo. The observations that IL-6 superinduction is suppressed by (n-3) PUFA pretreatment in the cell culture model is supportive of the in vivo studies. There are several possible explanations for the decreased IL-6 response to DON by FO-feeding or pretreatment with (n-3) PUFA.

ERK1/2 and JNK1/2 are crucial signaling mediators of IL-6 induction [44] and fish oil components such as DHA and EPA inhibit ERK 1/2 activity in human T cells, macrophages and tumor cells [35, 55, 56]. FO diets suppressed response in the ERK1/2 and JNK1/2 to DON in this study and similar effects were found in vitro. Thus one possible explanation for depression of DON-induced IL-6 is that altered MAPK signaling by FO or (n-3) PUFA can affect DON-mediated IL-6 gene expression. A limitation of this interpretation is that exposure to 3 to 30 μM (n-3) PUFA was sufficient to get the significant reduction in IL-6 expression in vitro whereas higher n-3 PUFA concentrations (110 mM) were needed to discern robust DON-induced MAPK activation. To establish a link between IL-6 and MAPK effects, further investigation is needed on the relationship of PUFA concentration, timing and incorporation to these two endpoints.

Fatty acids are known to modulate activity of signaling molecules such as phospholipase C and protein kinase C (PKC) [57, 58]. The molecular mechanism for inhibitory action of (n-3) PUFAs on ERK 1/2 has been previously investigated in relation to PKC [55, 59]. Notably, DHA and EPA inhibit PKC α and ϵ, which are known to be located at the upstream of MAP kinase cascade [60]. (n-3) PUFAs inhibit the enzymatic activity of theses PKC isoforms either via direct interaction with phosphatidylserine binding site of PKC [61] indirectly by producing diacylglycerol-containing PUFA [62]. Thus DON-induced MAPK phosphorylation could be suppressed by (n-3) PUFA-mediated PKC inhibition. It should be noted that (n-3) PUFA have been recently shown to suppress ERK 1/2 activity through PKC-independent pathway [56].

Relative to the MAPKs, it should be noted that the p38 signaling pathway is another possible modulator of promoter activity and mRNA stability of IL-6 gene expression [45, 63]. Interestingly, p38 MAPK activation by DON was not reduced but rather was slightly activated in FO-fed mice. This observation might explain the incomplete reduction of IL-6 production in FO-fed mice. Relatedly, DHA is known to activate p38 MAPK in vascular smooth muscle to mediate apoptosis [64]. Furthermore, p38 MAPK plays a critical role in transcription and mRNA stability of COX-2 DON-induced COX-2 [42], which might explain why FO had little effect on PGE_2 induced by DON and partial reduction in DON-induced COX-2 mRNA by dietary FO.

A second possible explanation for impairment of DON-induced IL-6 expression is that (n-3) PUFA might act as ligands for peroxisome proliferator-activated receptor (PPAR), the steroid-thyroid superfamily of nuclear receptor. Notably, (n-3) PUFA bind to or modulate PPARα and PPARγ, which regulate specific gene transcription by binding to PP response element (PPRE) via two zinc finger motifs in DNA-binding domain of PPAR [65]. Although PPAR(does not mediate the negative regulation of IL-6 [66], PUFA-PPARα signaling may play an inhibitory role in the proinflammatory gene expression [67, 68]. However, (n-6) PUFA can also effect PPAR-mediated signaling. Indeed, high concentrations of AA did slightly suppress DON-induced IL-6 superinduction in vitro. Thus, further studies are needed on structure function effects of (n-3) and (n-6) PUFA in suppressing IL-6 gene expression.

Cross talk between PPAR and MAPK signaling pathways can also occur [69–71]. Activated MAPKs modulate phosphorylation of PPAR, which contributes to the reduction in promoter activation by exogenous ligands. Therefore, ERK1/2 and JNK1/2 suppressed by (n-3) PUFA-containing fish oil might be related to more active PPAR-mediated function and, thus, reduced DON-induced IL-6 transcription.

A third explanation for the (n-3) PUFA effects is alteration in profile of cyclooxygenase (COX) metabolites. It is well established that (n-3) PUFAs lower the level of PGE_2, LTB_4, and TXB_2 by changing the substrate pool of cyclooxygenase metabolism [40, 54]. Dietary (n-3) PUFA such as EPA and DHA can be metabolized to form prostaglandins (e.g., PGE_3) and thromboxanes (e.g., TXA_3) that counterbalance the effects of proinflammatory products (PGE_2 or TXA_2) derived from arachidonic acid. Although DHA also suppresses LPS-induced COX-2 expression in macrophage cells [72], thus gene is upregulated via a feedback pathway upon longer exposure to EPA [73]. A positive association exists between endogenous COX-2 metabolites (particularly PGE_2) and IL-6 synthesis in vitro and in vivo models of several inflammatory diseases [74, 75]. It is thus possible that FO-mediated changes in COX-2 metabolism might lead to less of IL-6 production. Recently, DON was reported to induce COX-2 expression in macrophage cells [42] and DON-induced IL-6 is partially dependent on COX-2 metabolites [39]. Consistent with those findings, 8 weeks FO feeding here inhibited COX-2 mRNA and caused inhibitory trends in PGE_2 in DON-treated mice. Longer feeding might possibly be required to demonstrate a more suppressive effect of FO on DON-elicited COX-2 activity. In addition, analysis of PGE_2 in tissues will be desirable in this model

because actions of sum of circulating eicosanoids metabolized by COX-2 might be critical in DON-induced IgA nephropathy. The capacity for FO and (n-3) PUFAs to modulate DON-induced IL-6 via altered eicasanoid production as well as by modified pools of endogenous lipid metabolites therefore requires additional investigation.

The finding that n-3 PUFAs inhibited transient DON activation of JNK was notable and suggest that these fatty acids may impact events downstream of this kinase family. There is no substantive evidence for a link between activation of JNK in the macrophage and IL-6 expression in the DON-exposed animals or cells or relative to other inducers. However, tricothecene-mediated JNK activation has been strongly associated with the toxin-induced apoptosis at their cytoxic doses [41] Therefore, JNK retardation in FO-fed mice may contribute to the decreased cytotoxic stress by the toxins.

One observation that may be of clinical significance is that, although statistically significant, amelioration of IL-6 expression by FO was partial. One possible explanation for this finding is that multiple signaling pathways might be required for DON-induced IL-6 and that FO only affects part to of these. This could be clarified by improved understanding of the signaling mechanisms evoked by DON and how these are affected by (n-3) PUFAs. A second explanation may relate to the use of high dose DON acute exposure that was employed here to evoke a strong IL-6 response and then probe the effects of prior FO feeding. It is possible that this high dose overcame the capacity of the FO to more fully suppress IL-6 expression. The actual effects of chronic dietary DON on IL-6 might be more subtle and recurrent over repeated feedings. Thus, further investigation on the effects FO feeding on IL-6 induction by lower acute DON doses and by chronic DON feeding is warranted.

Macrophages were used here as an in vitro model to study the effects of fatty acids based on the known capacity of DON to induce IL-6 is this leukocyte phenotype. However, it should be noted that T cells could be a major source of IL-6. Indeed, we have observed that DON up-regulates IL-6 expression in concanavalin A-treated murine CD4+ cells [28] and in PMA-stimulated EL-4 cells [77]. Thus, since an in vivo role for T-cell derived IL-6 cannot be excluded for DON-induced IgA production, it will be worthwhile to examine the effect of fatty acids on this cell population in the future.

In conclusion, FO-feeding diminished IL-6 production in mice exposed to DON, and this correlated with suppression of DON-activated ERK1/2 and JNK1/2. Similarly, (n-3) PUFA, such as DHA or EPA, reduced DON-induced IL-6 as well as ERK 1/2 and JNK 1/2 phosphorylation in LPS-driven macrophage cells. It will be of further interest to investigate the direct dietary effect of purified (n-3) PUFA on IgA nephropathy and IL-6 production in vivo. Moreover, studies on the effects of extended FO feeding as well as the kinetics of DON-induced gene expression in FO fed mice will clarify questions about (n-3) PUFAs on DON-induced eicosanoid production as well as MAPK-mediated IL-6 induction [46, 76].

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