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Truncated deoxynivalenol-induced splenic immediate early gene response in mice consuming (n-3) polyunsaturated fatty acids☆

Shawn Kinser[a], Maioxing Li[a,b], Qunshan Jia[a,b], James J. Pestka[a,b,c,*]

[a]*Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224, USA*
[b]*Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824-1224, USA*
[c]*Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824-1224, USA*

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Abstract

Expression profiling has previously revealed that acute exposure to the common foodborne mycotoxin deoxynivalenol (DON) induces a large number of immediate early genes in murine lymphoid tissues that potentially affect immune function. The purpose of this study was to test the hypothesis that consumption of (n-3) polyunsaturated fatty acids (PUFAs) found in fish oil interferes with DON-induced immediate early gene expression. Mice were fed AIN-93G diet containing 1% corn oil (CO) plus 6% oleic acid (control) or a diet containing 1% CO, 2% fish oil enriched in the (n-3)-PUFAs docosahexaenoic and eicosapentaenoic acid and 4% oleic acid. After 12 weeks, the mice were gavaged orally with 25 mg/kg DON and the kinetics of immediate early gene expression in spleen monitored over 8 h by real-time polymerase chain reaction (PCR). Deoxynivalenol was found to readily induce expression of cytokines (IL-1α, IL-1β, and IL-6 and IL-11), chemokines (MCP-1, MCP-3, CINC-1 and MIP-2), components of the activator protein-1 (AP-1) transcription factor complex (c-Fos, Fra-2, c-Jun and JunB), as well as two hydrolases (MKP1, CnAβ). Expression of these genes was transient, peaking within 2–4 h and declining thereafter, with the single exception being IL-11 that was elevated at 8 h. (n-3)-PUFA consumption significantly suppressed DON-induced expression of IL-1α, IL-6, IL-11, MCP-1, MCP-3, MIP-2 and Fra-2 at 8 h. In contrast, mice fed (n-3)-PUFA exhibited significant increases in MKP1 and CnAβ expression. Taken together, these data suggest that dietary supplementation with (n-3)-PUFAs prematurely truncated cytokine, chemokine and transcription factor expression responses to DON that may impact its previously described capacity to disrupt immune function including immunoglobulin A (IgA) production. Since expression of many of these genes has been linked to mitogen-activated protein kinase (MAPK) activation, enhanced expression of MKP1, a negative MAPK regulator in (n-3)-PUFA-fed mice might contribute to this suppression.

Keywords: Mycotoxin; Fish oil; Inflammation; Cytokine; Chemokine; Kinase

1. Introduction

Consumption of (n-3) polyunsaturated fatty acids (PUFAs) found in fish oil suppresses immune and inflammatory responses in experimental animals, and these properties have been applied to prophylaxis and therapy of human autoimmune and inflammatory diseases [1]. For example, fish oil reduces severity of several human disorders such as rheumatoid arthritis and immune-related skin disorders. Particularly noteworthy is the potential of (n-3)-PUFA supplementation in the treatment of immunoglobulin A nephropathy (IgAN), the most common primary human glomerulonephritis. Holman et al. [2] demonstrated that some IgAN patients were deficient in alpha-linolenic acid [18:3 (n-3)], a precursor of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and that EPA and DHA suppressed arachidonic acid synthesis, decreased proteinuria, and improved glomerular filtration rate in IgAN. Several clinical trials have demonstrated that fish oil retarded renal disease progression in IgAN patients by reducing inflammation and glomerulosclerosis [3].

Deoxynivalenol (DON), a trichothecene mycotoxin, is a common contaminant of food crops worldwide. It is a

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* Corresponding author. Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224, USA. Tel.: +1 517 353 1709; fax: +1 517 353 8963.

E-mail address: pestka@msu.edu (J.J. Pestka).

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metabolite produced by the fungus *Fusarium graminearum*, which grows readily on field crops such as corn and wheat [4]. Deoxynivalenol is persistent in the environment and survives most food processing procedures [5], and this makes it a human health concern [6]. The capacity of this toxin to modulate immune function is of particular importance [7]. Both in vivo and in vitro gene profiling studies have revealed that DON up-regulates genes clustered in five different categories: cytokines, chemokines, transcription factors, hydrolases/phosphatases and signaling proteins [8–10]. Most of these are considered to be immediate early genes, which can potentially impact leukocyte function, immunity and inflammation. Mechanisms for increased immediate gene expression by DON include increased transcription [11–14] and mRNA transcript stabilization [14–16]. Activation of mitogen-activated protein kinases (MAPKs) appears to precede and contribute to up-regulation of many of these genes [17].

Mice fed DON develop early characteristic features of IgAN including polyclonal activation of IgA-secreting cells, polyreactive IgA autoantibody secretion, elevated serum IgA and circulating IgA immune complexes and kidney mesangial IgA deposits [7]. Thus, this mycotoxin can be used as a probe to study early stages of IgAN. The ability of DON to induce polyclonal expansion of IgA-secreting cells and IgAN in mice appears to be mediated by both macrophages [18] and T cells [19] as well as their cytokines [11,15,18,20].

Recently, we have observed that consumption of menhaden fish oil or DHA- and EPA-enriched oils attenuates DON-induced serum IgA elevation and IgA deposition in mice [21,22]. These preclinical findings are valuable because they suggest that consumption of (n-3)-PUFAs might also have potential value for early intervention and prophylaxis in persons with a familial history of IgAN or in patients diagnosed to be at an early stage of IgAN. The mechanisms by which n-3 PUFAs alter immune function are not completely clear but relate in part to alterations in cytokine and inflammatory mediator profiles [1]. Relatedly, Moon and Pestka [23] recently observed that mice fed fish oil diets exhibit reduced DON-induced COX-2 and IL-6 expression as well as impaired MAPK activation [23]. The purpose of this study was to test the hypothesis that consumption of (n-3)-PUFAs interferes with DON-induced immediate early gene expression. Accordingly, we compared expression kinetics of selected cytokines, chemokine, transcription factor and phosphatase genes in spleen following acute oral DON exposure in mice fed high and low ratios of (n-6)/(n-3)-PUFA.

2. Materials and methods

2.1. Materials

All chemicals (reagent grade or better) were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Deoxynivalenol was produced in *F. graminearum* R6576 culture and purity verified as described by Clifford et al. [24].

2.2. Animals and diet

Male B6C3F1 mice from Charles River (Portage, MI) were housed singly in humidity- and temperature-controlled room with a 12-h light and dark cycle. All animals were handled in accordance with guidelines established by the National Institutes for Health. Basal experimental diets were based on the AIN-93G formulation of Reeves et al. [25] and consisted the following ingredients purchased from Dyets (Bethlehem, PA) per kilogram: 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397 g cornstarch, 100 g sucrose, 50 g cellulose, 14 mg *t*-butylhydroquinone (TBHQ), 3 g L-cystine, 2.5 g choline bitartrate and 10 g corn oil (CO) (which contained 200 mg/kg TBHQ). Mice ($n=30$) were fed basal AIN-93G diet supplemented with either 60 g/kg oleic acid (control group) or 23 g/kg MEG fish oil enriched in DHA and EPA (Ocean Nutrition, Bedford, Nova Scotia) plus 37 g/kg oleic acid [(n-3)-PUFA group] for 12 weeks (Table 1). The resultant control and (n-3)-PUFA diets contained (n-6)/(n-3) ratios of 16:1 and 1:18, respectively. This (n-3)-PUFA diet has been previously shown to be effective in increasing DHA in liver phospholipids from 9% to 23% and EPA from 0% to 12% while decreasing arachidonic acid from 23% to 6% [22].

After 12 weeks of feeding experimental diets, groups of mice ($n=5$) were then fasted for 2 h and given either DON (25 mg/kg po) in 0.1 ml water or the vehicle alone (Table 2). After 2, 4 and 8 h of DON treatment, mice were sacrificed by cervical dislocation and spleens immediately removed for total RNA isolation.

Table 1
Experimental groups for assessing effects of (n-3)-PUFA on DON-induced gene expression[a]

Group	Animal no.	DON[b] (mg/kg BW)	CO (g/kg)	Oleic acid (g/kg)	(n-3)-enriched oil[c] (g/kg)	Total (n-3) (g/kg)	(n-6)/(n-3) ratio
Control	15	0	10	60	0	0.6	16:1
Control+DON	15	25	10	60	0	0.6	16:1
(n-3)-PUFA	15	0	10	37	23	17.2	1:1.8
(n-3)-PUFA+DON	15	25	10	37	23	17.2	1:1.8

[a] Concentrations are relative to total diet. All diets were adjusted with oleic acid to have final oil content of 70 g/kg.
[b] A single acute oral gavage with DON was used just prior to experiment termination.
[c] DHA/EPA is fish oil enriched with DHA and EPA at an approximate 1:1 ratio.

Table 2
Fatty acid composition of study diets for assessing effects of (n-3)-PUFA on DON-induced gene expression

Fatty acid	Control	(n-3)-PUFA
	g/100 g total fatty acids	
14:0	0.29	0.31
16:0	6.45	5.75
16:1	0.16	0.21
18:0	4.41	3.29
18:1	72.5	50.1
18:1	<0.10	0.85
18:2 (n-6)	13.6	12.1
20:0	0.34	0.44
18:3 (n-6)	<0.10	<0.10
20:1	0.2	0.82
18:3 (n-3)	0.32	0.53
20:2 (n-6)	<0.10	0.15
22:0	0.89	0.64
22:1	<0.10	<0.10
20:3 (n-3)	<0.10	0.11
20:4 (n-6)	<0.10	0.44
24:0	0.22	<0.10
20:5 (n-3)	0.28	9.31
24:1	<0.10	<0.10
22:5 (n-3)	<0.10	1.64
22:6 (n-3)	0.26	10.9
Total (n-3)	0.9	21.0
Total (n-6)	13.6	13.0
(n-6)/(n-3)	16:1	1:1.8

2.3. RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from spleens using Rneasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. DNA was eliminated using on-column Dnase digestion. RNA quality was verified by electrophoresis on a denaturing agarose gel. Real-time polymerase chain reaction (PCR) was performed on selected genes for all replicates in treatment and control groups ($n=5$) to further quantify expression of total mRNA. Reverse transcriptase reaction cDNA was produced from mRNA using Superscript II reverse transcriptase (Invitrogen, San Diego, CA) primed with oligo (dT) 12–15. Reverse and forward oligonucleotide primers, specific to the chosen genes, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) (Table 3). SYBR Green PCR [26] was performed in a 384-well plate using a 7900 ABI prism sequence detection system (Applied Biosystems). 18S rRNA expression levels were used as normalizing endogenous controls [27]. The average 18S rRNA Ct value for all treatments was subtracted by the 18S rRNA Ct value of each sample creating a normalization term. This normalization term was subtracted from the corresponding target gene Ct value. The normalized target gene Ct value was then converted to copy number per microgam of total RNA using a standard curve created with serial dilutions of purified PCR product from the gene of interest. Gene mRNA expression levels were expressed as fold change that was calculated by dividing each gene quantity value by the average value for the control values.

2.4. Statistics

Data were compared by analysis of variance (ANOVA) using NCSS 2000 Statistical Software package (Keysville, UT). Post hoc tests were performed using Fisher's Least Squared Difference Multiple Comparison Test. Results were considered significantly different if $P<.05$.

3. Results

Deoxynivalenol significantly induced IL-1α (8- to 11-fold), IL-1β (13- to 18-fold) and IL-6 (80- to 160-fold) in spleens of mice fed control diet (Fig. 1). Peak expression for IL-1α and IL-1β occurred from 2 to 4 h, whereas IL-6 expression was elevated from 2 to 8 h. In contrast, DON-induced IL-11 expression increased (24-fold) only at 8 h. Both DON-induced IL-1α and IL-1β expressions were slightly increased by (n-3)-PUFA feeding at 4 h, whereas after 8 h, splenic IL-1α, IL-6 and IL-11 mRNA levels were significantly reduced by (n-3)-PUFAs. IL-1β mRNA followed a similar trend.

Chemokine expression in spleens of mice fed control diets was also markedly up-regulated by DON treatment (Fig. 2). At 2, 4 and 8 h, MCP-1, MCP-3 and MIP-2

Table 3
Primers for real-time PCR quantitation

Category	Gene	Forward primer	Reverse primer
Cytokine	IL-1α	atgaagctcgtcaggcagaag	gagatagtgtttgtccacatcctgat
Cytokine	IL-1β	ggacccatatgagctgaaagct	tgtcgttgcttggttctcctt
Cytokine	IL-6	tgggactgatgctggtgaca	tgggagtggtatcctctgtgaa
Cytokine	IL-11	ctgcacagatgagagacaaattcc	gaagctgcaaagatcccaatg
Chemokine	CINC-1	cactgcacccaaaccgaagt	agcatcttttggacaattttctgaac
Chemokine	MIP-2	ggctgttgtggccagtgaa	ctcaagctctggatgttcttgaag
Chemokine	MCP-1	aactgcatctgccctaaggtctt	tgcttgaggtggttgtggaa
Chemokine	MCP-3	aagatccccaagaggaatctcaag	cagacttccatgcccttctttg
Transcription factor	c-Fos	aatccgaagggaacggaataa	tccgcttggagtgtatctgtca
Transcription factor	Fra-2	cagcagaagttccgggtagatatg	gtcctggctggtggtgatg
Transcription factor	c-Jun	gctacagtaaccctaagatcctaaaacag	ggaggtgcggcttcagact
Transcription factor	JunB	accatcagctacctcccacatg	tacggtctgcggttcctctt
Hydrolase	CnAβ	gcgtcgtcaaagaccaa	ctgttcctacccgcccttctttt
Hydrolase	MKP1	tgcctgacagtgcagaatcc	tccttccgagaagcgtgatag

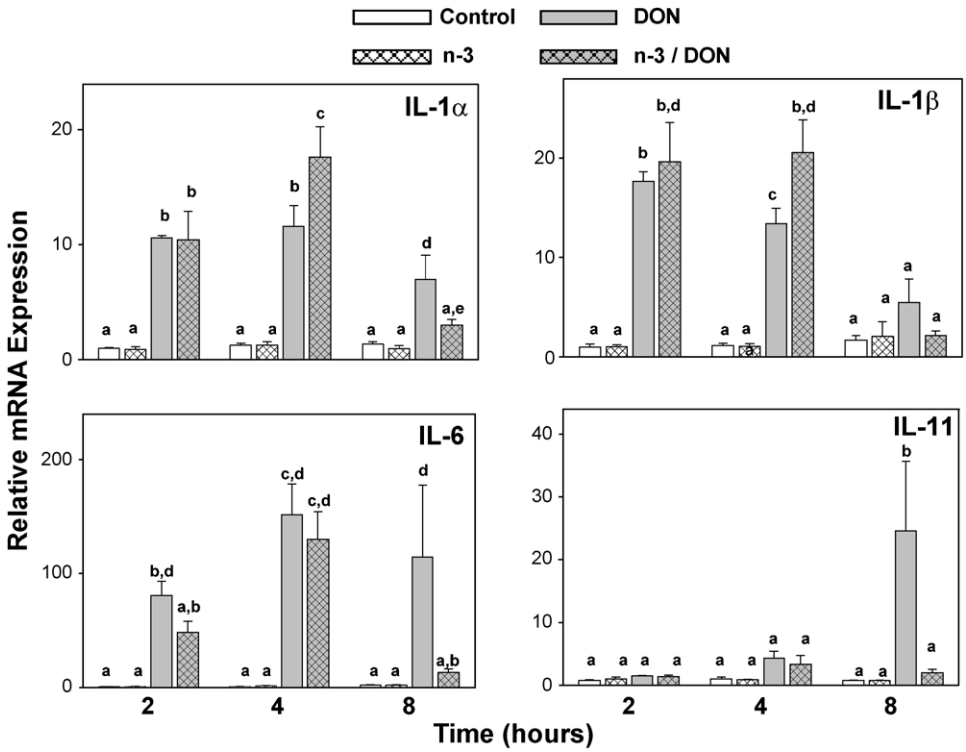

Fig. 1. Effect of (n-3)-PUFA consumption on DON-induced cytokine mRNA expression in spleen. Mice were fed (n-3)-PUFA or control diets for 12 weeks and exposed to a single oral dose of DON (25 mg/kg). After 2, 4 and 8 h after administration of DON, mice were euthanized and splenic mRNA expression quantitated using real-time PCR. Expression levels were normalized to 18S rRNA expression level. Data are means±S.E. ($n=5$). Bars without the same letter are significantly different ($P<.05$).

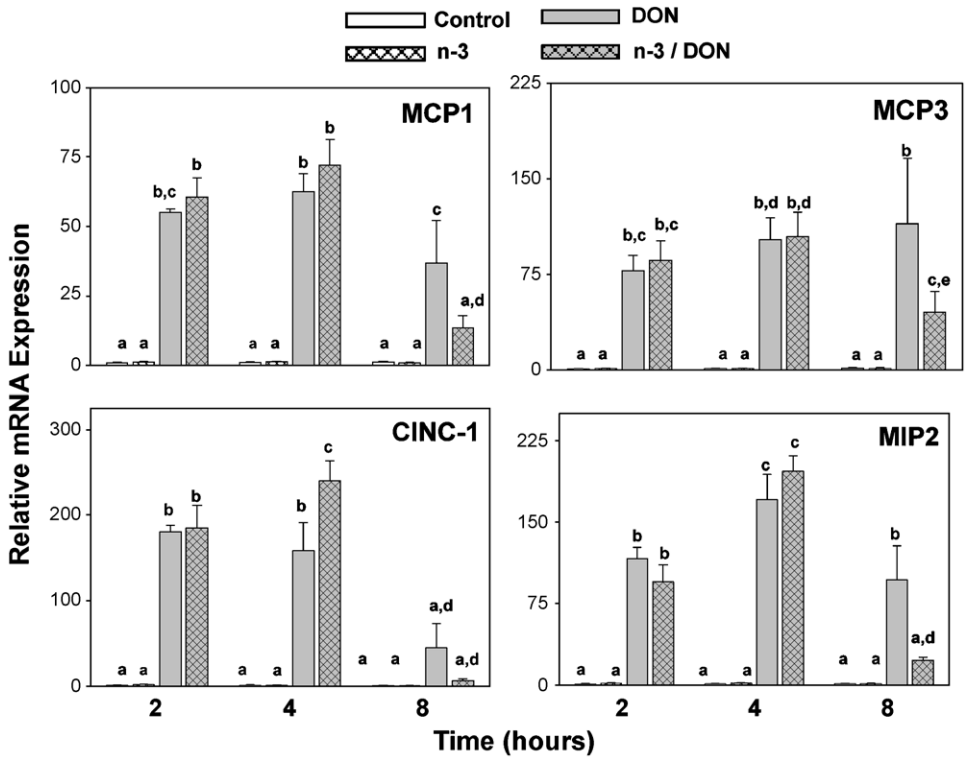

Fig. 2. Effect of (n-3)-PUFA consumption on DON-induced chemokine mRNA expression in spleen. Splenic mRNA expression was quantitated as described in Fig. 1 legend. Data are means±S.E. ($n=5$). Bars without the same letter are significantly different ($P<.05$).

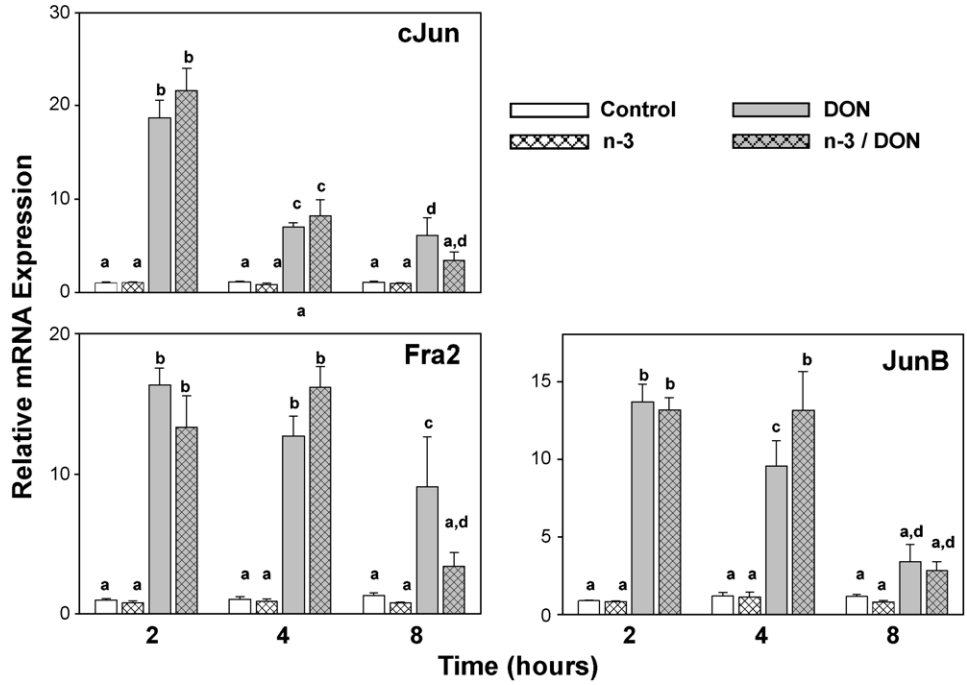

Fig. 3. Effect of (n-3)-PUFA consumption on DON-induced transcription factor mRNA expression in spleen. Splenic mRNA expression was quantitated as described in Fig. 1 legend. Data are means±S.E. ($n=5$). Bars without the same letter are significantly different ($P<.05$).

concentrations were increased 35- to 60-fold, 75- to 100-fold and 75- to 160-fold, respectively, whereas CINC-1 was significantly elevated (175- to 180-fold) at 2 and 4 h. (n-3)-PUFA feeding slightly increased in MCP-1 and CINC-1 mRNA levels at 2 h but suppressed expression of MCP-1, MCP-3 and MIP-2 at 8 h. CINC-1 mRNA followed a similar trend.

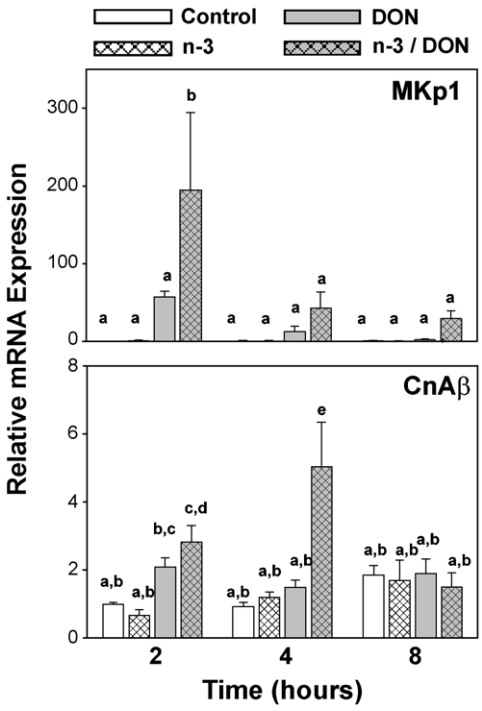

Fig. 4. Effect of (n-3)-PUFA consumption on DON-induced phosphatase/ mRNA expression in spleen. mRNA expression in spleen following acute DON exposure. Splenic mRNA expression was quantitated as described in Fig. 1 legend. Data are means±S.E. ($n=5$). Bars without the same letter are significantly different ($P<.05$).

Deoxynivalenol induced expression of components of the activator protein-1 (AP-1) transcription factor complex in mice fed control diet (Fig. 3). c-Jun mRNA was elevated by 1.7-fold over control at 2 h and declined but remained elevated (6- to 8-fold) at 4 and 8 h. Deoxynivalenol induced expression of JunB by 10- to 13-fold at 2 and 4 h and of Fra-2 by 9- to 16-fold at 2 to 8 h. (n-3)-PUFA ingestion increased JunB expression at 4 h and significantly decreased Fra-2 expression at 8 h.

Mitogen-activated protein kinase phosphatase 1 (MKP1) mRNA was increased approximately 50-fold by DON at 2 h and declined to control levels at later time points (Fig. 4). (n-3)-PUFA consumption markedly up-regulated MKP1 expression at 2 h with similar trends found at 4 and 8 h. Modest (2- to 3-fold) but significant increases were found for protein phosphatase 3, catalytic subunit, and beta isoform (CnAβ) at 2 h but not 4 and 8 h. Deoxynivalenol-induced CnAβ was significantly increased (5-fold) in (n-3)-PUFA-fed mice at 4 h.

4. Discussion

The results presented here indicate that DON induced the transient expression of several important immediate early genes. With the exception of MKP1, (n-3)-PUFA consumption decreased expression of many of these genes at the latest time point (8 h), suggesting that these lipids generally

decreased the duration of the immediate early gene response either by inhibiting transcription or mRNA stability. These findings might have relevance to decreasing the immunotoxic effects of DON, and specifically, the induction of experimental IgAN by this toxin.

Marked up-regulation of IL-6 by DON is consistent with previous findings for this gene [20,28,29]. IL-6 has both proinflammatory and antiinflammatory properties and modulates plasma cell development, bone resorption and hematopoiesis [30]. Of particular importance, IL-6 is an essential contributor to the development of DON-induced IgAN [31]. The capacity of (n-3)-PUFA consumption to suppress DON-induced IL-6 confirms our prior observations for fish oil [23] and suggests a possible mechanism for suppression of DON-induced IgA dysregulation and IgAN.

IL-1α and IL-1β are two other inflammatory cytokines that were up-regulated by DON. Although there was a slight capacity of (n-3)-PUFAs to potentiate this response at 4 h, the potent suppression of these genes following (n-3)-PUFA consumption is consistent with the similar impairment of IL-1 responses in the context of other inflammagenic stimuli [32].

As previously observed [10], acute DON exposure induced IL-11. This cytokine inhibits the expression of the cytokines IL-12 and IFN-γ, which are associated with T helper 1 polarization and can stimulate T-cell-dependent development of immunoglobulin-producing B-cells [33]. Increased ability to stimulate T-cell-dependent development of immunoglobulin-producing B-cells may be an important step in the development of DON-induced IgAN. Thus, the capacity of (n-3)-PUFAs to suppress expression of this cytokine might be another important factor in their ability to attenuate DON-induced IgA dysregulation.

Deoxynivalenol was a remarkably potent inducer of chemokines. These are a group of small, structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of transmembrane, G-protein-coupled receptors [34]. MIP-2 (or cxcl2), CINC-1 (or cxcl1) and Crg-2 (or cxcl10) are all members of the CXC subfamily of chemokines. Chemokines, in conjunction with tissue-specific adhesion molecules, coordinate the migration of antibody-secreting cells (ASCs) from their sites of antigen-driven differentiation in lymphoid tissues to target effector tissues. Developing ASCs down-regulate the expression of receptors for lymphoid tissue chemokines and selectively up-regulate the expression of chemokine receptors that might target the migration of IgA ASCs to mucosal surfaces, IgG ASCs to sites of tissue inflammation and both types of ASC to the bone marrow, an important site for serum antibody production. By directing plasma-cell homing, chemokines can define the character and efficiency of mucosal, inflammatory and systemic antibody responses. Since DON-induced IgAN is associated with increased numbers of IgA-secreting cells in the systemic compartment [7], it might be speculated that a generalized increase in multiple chemokines is a factor in diverting the homing of IgA-committed cells from the mucosal to systemic compartments in DON-exposed animals. Decreased duration of the DON-induced chemokine response might thus be an additional contributory mechanism for suppression of IgAN by (n-3)-PUFAs.

The AP-1 family of transcription factors is involved in the up-regulation of many immune and inflammatory genes [34]. Deoxynivalenol-induced toxicity has previously been suggested to involve AP-1 [13,16,17,35,36]. c-Jun, JunB, Fra-2 and other related protein subunits heterodimerize to form AP-1. The prolonged expression of these subunit genes following DON exposure might be one mechanism for up-regulating AP-1 activity, whereas the trend toward their suppression by (n-3)-PUFA might down-regulate this response.

Deoxynivalenol's marked induction of MKP1 corresponds to rapid up-regulation of MAPK activation in vivo [16] and in vitro [14,23,37] by the mycotoxin. MKP1 has specificity for both tyrosine and threonine and specifically inactivates MAPKs [38]. Induction of MKP1 serves to down-regulate both MAPK activation and consequent downstream gene expression, thereby ensuring a transient, nonphlogistic response. The capacity of (n-3)-PUFA to further up-regulate MKP1 might potentiate MAPK down-regulation. In support of this contention, Jia et al. [39] recently observed that (n-3)-PUFA inhibition of DON-induced IL-6 in the mouse spleen correlated with a reduction of phosphorylation of p38, extracellular signal-regulated protein kinases (ERKs) 1/2 and c-Jun N-terminal kinases (JNKs) 1/2 in the spleen.

CnAβ is a member of the calcineurin family. This gene and its downstream transcriptional effector NFAT are important regulators of gene expression in multiple immune cell types and are particularly critical for mediating lymphocyte activation [40]. It might be speculated that enhanced CnAβ expression following (n-3)-PUFA ingestion modulates T-cell signaling in a manner that reduces the DON-induced IgA response.

In conclusion, DON was effective at inducing transient expression of multiple immediate early genes that likely contribute to its immune and inflammatory effects. The capacity of (n-3)-PUFA consumption to prematurely truncate many of these responses likely mediates the protective effects of this lipid class in DON-induced immune dysregulation and IgA nephropathy. The role of MKPI induction in this process requires further study. It will also be desirable to assess whether these findings can be confirmed relative to protein expression and also extended to other inflammagenic stimuli. We chose to use 25 mg/kg DON based on it having maximal inductive effects for proinflammatory cytokines in previous studies [28,29]. It would be of interest to further assess the ameliorative effects at suboptimal DON dosing. Finally, because this study focused on the entire spleen, it was not possible to identify effects on specific phenotypes. Future application of this technique to homogeneous cell populations in vitro will facilitate the understanding of how specific leukocyte

phenotypes within the spleen and other lymphoid tissues are affected in vivo by (n-3)-PUFA.

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