MECHANISMS OF (N-3) POLYUNSATURATED FATTY ACID INHIBITION ON EXPERIMENTAL IMMUNOGLOBULIN A NEPHROPATHY

By

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ABSTRACT

MECHANISMS OF (N-3) POLYUNSATURATED FATTY ACID INHIBITION ON EXPERIMENTAL IMMUNOGLOBULIN A NEPHROPATHY

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IgA nephropathy (IgAN) is the most common form of human glomerulonephritis in the world. Clinical studies have demonstrated that (n-3) polyunsaturated fatty acids (PUFAs) found in fish oil are beneficial in treating inflammatory diseases including IgAN. Consumption of mycotoxin deoxynivalenol (DON) elevates serum IgA, IgA immune-complex and IgA deposition in the kidney thus mimicking the early stages of IgAN. The goal of this study was to elucidate the molecular mechanisms by which (n-3) PUFAs attenuate DON-induced IgAN. Initially, it was determined that fish oil significantly decreased serum IgA, serum immune complex and kidney mesangial IgA deposition. Two major components of fish oil, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were found to retard DON-induced IgAN with DHA enriched oil (60g/kg diet) being most efficacious. DHA's effects were dose-dependent with 60 g/kg DHA enriched oil in diet determined to be optimal. Both cyclooxygenase-2 (COX-2) and IL-6 genes expression were inhibited significantly by DHA. However, COX-2 knockout mice and COX-2 specific inhibitor did not support a role for this enzyme in DON-induced IgAN. IL-6, which has been previously shown to play an essential role in DON-induced IgA elevation, might be down-regulated by DHA both at transcriptional level or post-transcription level. Dietary DHA did not affect IL-6 mRNA degradation in thioglycollate-elicited peritoneal macrophages, but significantly inhibited

IL-6 gene transcription by blocking cAMP response element binding protein (CREB) phosphorylation and its binding to the IL-6 promoter in vivo. Although mitogen-activated protein kinases (MAPKs) p38 and ERK are the most important kinases for CREB phosphorylation, DON-induced phosphorylation of these two kinases were not affected in macrophages from mice fed DHA, nor were their downstream kinases MSK1 and p90RSK, which mainly mediate CREB phosphorylation. Taken together, the results suggest that the (n-3) PUFA, DHA potentially retards development of DON-induced IgAN by blocking IL-6 gene expression at the transcriptional stage with CREB being most important target.

To my grandma in law.

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ABBREVIATIONS

AP-1	Activate protein 1
C/EBP	CCAAT/enhancer binding protein
CaMKII	Camodulin dependent kinase II
cAMP	cyclic adenosine monophosphate
COX-2	Cyclooxygnease-2
CRE	CREB response element
CREB	cAMP response element binding protein
DON	Deoxynivalenol
ERK	Extracellular signal-regulated kinase
IgAN	Immunoglobulin A nephropathy
IL-6	Interleukin-6
JNK	N-terminal kinase of c-Jun
LPS	Lipopolysaccharide
LTB_4	Leukotrienes B_4
MAPK	Mitogen activated protein kinase
MSK1	mitogen- and stress-activated protein kinase 1
NFκB	Nuclear factor kappa B
p90RSK	ribosomal S6 kinase with MW 90 kDa
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PKC	Protein kinase C

PLA	Phospholipase A
PDGF	Platelet derived growth factor receptor
PAF	Platelet activated factor
PPAR	Proliferator activated receptor
INF	Interferon
STAT	Signal transducer and activator of transcription
JAK	Janus kinase
bZIP	Dimmeric basic region–leucine zipper
ATF	Activating transcription factor
TXA	Thromboxane

INTRODUCTION

Primary IgA nephropathy (IgAN) is an immune complex disease, which mainly affects children and young adults (Endo, 1997). Elevation of serum IgA is an important etiological factor for the development of IgAN (Feehally, 1997; Endo, 1997), which can deposit in the kidney and induce cytokine production, complement activation and ultimately causing renal injury (Wyatt and Julian, 1988; Ibels and Gyory, 1994; Feehally, 1997; Endo 1997). Diet supplementation with (n-3) polyunsaturated fatty acids (PUFAs) might be a promising treatment for IgAN due to their multiple effects on inflammation and safety record (Ng, 2003). Epidemiological studies reveal that dietary (n-3) PUFAs are negatively associated with the risk of IgAN (Wakai *et al.*, 1999) whereas high (n-6) PUFA intake is associated with increased risk of IgAN (Wakai *et al.*, 2002). Consistent with these findings, several clinical trials have shown that fish oil might be safe and beneficial for long-term treatment of progressive IgAN (Grande *et al.*, 2001). Benefits of (n-3) PUFAs on IgAN might be explained by their inhibition on inflammatory gene expression (Endres *et al.*, 1995; Watanabe *et al.*, 2000).

Deoxynivalenol is a type B trichothecene mycotoxin that is produced by the fungi of *Fusarium* genus, and that is very stable in the environment and detrimental to both livestock and human health (Rotter *et al.*, 1996). Immunotoxicological studies have shown that chronic exposure to DON can upregulate IgA production in mice which mimics the early stages of human IgAN (Pestka *et al.*, 1989; Dong *et al.*, 1991; Dong and Pestka, 1993; Pestka, 2003). DON might destroy mucosal tolerance by induction of macrophage and T cell cytokines, most notably, IL-6, that promote IgA production and

contribute to the systemic compartment polymeric IgA elevation and ultimately IgAN (Pestka, 2003).

DON-induced IgAN provides a unique model to investigate potential therapeutic mechanisms of (n-3) PUFA. In vitro and in vivo experiments indicate that IL-6 is essential for IgA production (Bertolini and Benson, 1990; Kono *et al.*, 1991; Xu-Amano *et al.*, 1992). Ex vivo cell reconstitution (Yan *et al.*, 1998), antibody neutralization (Yan *et al.*, 1997) and IL-6 deficient (Pestka and Zhou, 2000) mice studies indicate that IL-6 is also required in DON-induced IgAN. The aims of this research were to verify and optimize the regimen of (n-3) PUFAs for prevention of DON induced IgAN and uncover molecular mechanisms by which (n-3) PUFAs inhibit IL-6 gene expression.

Chapter 1 is a review of the key literature pertinent to this thesis. Chapter 2 confirms the inhibitory effects of fish oil on DON-induced IgAN in mice. Chapter 3 compares the effects of the two main components of fish oil, DHA and EPA, on DON-induced IgAN. Chapter 4 assesses the optimal DHA feeding regimen and the potentential mechanisms for DHA suppression of DON-induced IgAN. In chapter 5, both COX-2 knockout mice and COX-2 specific inhibitors (VIOXX) were employed to assess the role of COX-2 gene in DON-induced IgAN model. Finally, Chapter 6 provides insight into possible mechanisms of transcriptional regulation of DON-induced IL-6 by DHA.

CHAPTER 1

Literature Review

Deoxynivalenol. Deoxynivalenol (DON, Vomitoxin, dehydronivalenol, RD-Toxin, Figure 1.1) [12,13-epoxy-3, 4,15-trihydroxytrichotec-9en-8-one, $C_{15}H_{20}O_6$, MW 296.32] is a type B trichothecene produced by the fungi of *Fusarium* genus, i.e. *Fusarium culmorum* and *Fusarium graminearum*. These fungi are important plant pathogens and cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize. DON is very stable in the environment and detrimental to both livestock and human health (Rotter *et al.*, 1996). DON can bind readily to eukaryotic 60S ribosomal subunit and prevent polypeptide initiation and elongation. An acute dose of DON can induce vomiting in pigs, while chronic exposure to it will cause anorexia, weight loss and nutrient utilization problems. Immunotoxicological studies have shown that DON can be immunosuppressive or immunostimulatory (Pestka, 2003). On the one side, it can depress antibody plaque-forming and delayed hypersensitivity responses and suppresses normal immune response to pathogens. On the other side, DON upregulates IgA production, which is similar to human immunoglobulin A nephropathy (IgAN) by superinduction of cytokines from T helper cells and macrophages.

The mitogen-activated protein kinase (MAPK) is a family of serine/threonine protein kinases, which transduces extracellular stimuli into intracellular post-translational and transcriptional responses. The involvement of MAPKs in DON-induced proinflammatory gene expression in vivo has been documented extensively. As little as 5 mg/kg BW DON induces phosphorylation of extracellular signal regulated kinase (ERK), p38 and N-terminal kinase of c-Jun (JNK) in mice spleen within 15 min and these effects can be reconstituted in vitro in macrophage (Zhou *et al.*, 2003; Moon and Pestka, 2003).

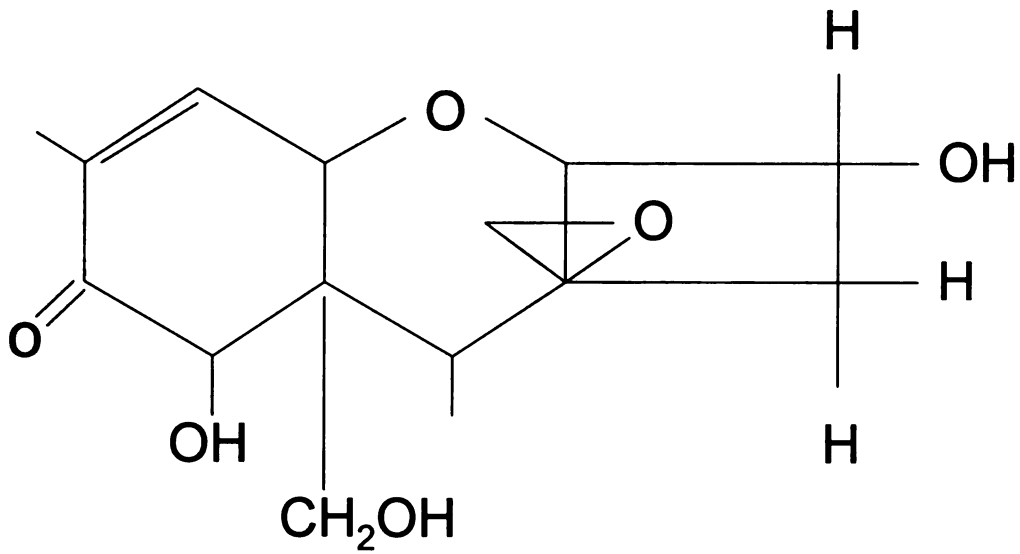

Figure 1.1 The structure of deoxynivalenol ($C_{15}H_{20}O_6$, MW 296.32).

Two possible DON targets upstream of the MAPKs have been identified. One is an Src family kinase called hematopoietic cell kinase (Hck), and the other is double stranded RNA-dependent protein kinase (PKR) (Zhou *et al.*, 2003; Pestka *et al*, 2004). DON can activate these two kinases as early as 5 min in RAW267.4 macrophage cells. Inclusion of Hck and PKR inhibitors impairs DON-induced MAPK phosphorylation. Activated MAPKs mediate phosphorylation of different transcription factors such as activated protein 1(AP-1), CCAAT enhancer binding protein (C/EBPβ), NFκB and cAMP response element binding protein (CREB) which, in turn, upregulate expression of proinflammatory genes (Wong *et al.*, 2001; Zhou *et al.*, 2003; Sugita-Konishi and Pestka, 2001).

IgA nephropathy. IgAN is the most common form of glomerular nephritis worldwide. It mainly affects children and young adults of which 20-40 % of which will develop the end stage of renal disease (Endo, 1997). Clinical diagnosis of IgAN is based on the presence of mesangial IgA deposition, which can induce renal injuries varying from segmental or diffused glomerular hypercellularity, tubular atrophy and interstitial fibrosis to renal failure (Ibels and Gyory, 1994).

Although a high serum IgA concentration alone is not sufficient to induce IgAN (Kilgore *et al.*, 1985), it can be an important etiological factor (Feehally, 1997; Endo 1997). IgA can exist in polymeric and monomeric forms. Polymeric IgA is believed to originate from the mucosal compartments and is secreted into the gut, while monomeric IgA is released into the serum by bone marrow (Floege and Feehally, 2000). Polymeric IgA production by the mucosal immune system is decreased in patients with IgA nephropathy, whereas polymeric IgA production in the bone marrow was increased

(Feehally, 1997). The observations that IgAN is frequently associated with upper respiratory tract infections and that increased permeability of the gastrointestinal mucosa will increase IgA production in IgAN patients suggests that IgA nephropathy is related to a hypetactivated mucosal immune system (Suzuki *et al.*, 2000; Kovacs *et al.*, 1996).

Aberrant IgA structure is another etiological factor for IgAN. In some human IgAN patients, the IgA1 hinge region O-link glycan is deficient of galactose, which makes it difficult to be cleared through the liver IgA receptor (ASGP-R) (Rifai and Mannik, 1984). Galactose-deficient IgA1 is also prone to form self-aggregates and immune complexes with anti -glycan IgG and glomerular deposits (Novak and Julian, 2001). Mice have only one isotype of IgA, which was similar to the IgA2 in human, however, the Th2 cytokines (IL-4 and IL-5) can decrease the galactosylation of IgA in BALB/c mice and may facilitate the IgA deposition (Chintalacharuvu *et al.*, 2001). Elevated levels of IgA and IgA immune complexes can bind to receptors on mesangial cells and induce proliferation and cytokine production. Dimeric and polymeric IgA can activate complement via the alternative pathway and cause glomerular damage (Wyatt and Julian, 1988).

At the genetic level, IgAN has been related to race, family, HLA polymorphisms, cytokine (IL-1, IL-6) polymorphisms, renin-angiotensin system and TCR polymorphisms. However cause-effect relationship remains ill-defined (Galla *et al.*, 2001).

Treatment of IgAN is still not disease-specific. The role of immunosuppressive therapy in IgAN remains controversial (Harmankaya *et al.*, 2002). Treatments being investigated include angiogenesis inhibition, glucocorticoids, cyclophosphamide,

tonsillectomy and (n-3) PUFA (Julian *et al,* 1999). Future therapies will likely focus on 1) decreasing IgA-IC level, thus limiting the binding of IgA to mesangial cells; 2) antagonizing the effects of platelet-derived growth factors (PDGF) and transforming growth factors beta (TGF-β); 3) reducing noxious glomerular injuries due to infiltrating neutrophils (Lai *et al.*, 2002); and 4) reducing inflammatory mediators including thromboxanes, leukotrienes and platelet-activating factors (PAF) (Wardle *et al.*, 2000). Due to its multiple effects on inflammation and safety record (n-3) PUFA might be a promising treatment for IgAN (Ng, 2003).

Multiple IgAN animal models have been developed in the past. Rifai *et al.*, (1979) reported a model in mice with injection of immune-complex of IgA and bovine serum albumin (BSA) conjugated with DNP (DNP-BSA). A spontaneous glomerulonephritis resembling human IgA nephritis was developed in ddY (Wakui *et al.*, 1989). Uteroglobin (UG) is a potent endogenous immunomodulatory and anti-inflammatory protein. UG knockout mice also represent a new model for IgAN (Pouria and Challacombe, 2000). CD89 is a human IgA receptor, which can form complexes with mouse IgA. CD89 transgenic mice can develop IgAN in six-month (Launay *et al.*, 2000)

DON consumption induces IgAN in mice and this might result from stimulation of the mucosal immune response (Pestka, 2003). It has been shown that mice fed 25 mg/kg DON for 12 wk develop IgAN with high concentrations of serum polymeric IgA (pIgA) (Pestka *et a/.*, 1989; Dong *et al.*, 1991; Dong and Pestka 1993; Pestka, 2003). Although the gut acts as a portal of entry to a vast array of foreign antigens in food, lymphocytes in the gut-associated lymphoid tissues (GALT) are seldom activated because of mucosal tolerance. DON might destroy this tolerance by induction of

macrophage and T cell cytokines, most notably, IL-6, that promote IgA production and contribute to the systemic compartment polymeric IgA elevation and ultimately IgAN (Pestka, 2003).

(n-3) PUFA and IgAN. Mammals lack the ability to synthesize PUFA with double bonds distal to the ninth carbon atom. Therefore, linoleic acid and α-linolenic acid are two essential PUFAs in diet. Linoleic acid (C18: 2 n-6) is the precursor of n-6 PUFA found in corn, soy and safflower oil. Once consumed, linoleic acid is elongated and desaturated to yield arachidonic acid (C20: 4 n-6), the usual precursor of the 2 and 4 series eicosanoids, synthesized by the cyclooxygenase pathway and 5-lipoxygenase pathway (Donadio and Grande 2004; Figure 1.2, 1.3). α-Linolenic acid (C18:3 n-3) is another essential fatty acid, which is mainly found in the chloroplasts of green leafy vegetables, plant oils (canola, flaxseed, and soy), and nuts (walnut oil and walnuts) (Donadio and Grande, 2004). In mammals, once consumed, α-linolenic acid only slowly elongates and desaturates to eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3),, the parent of 3 and 5 series eicosanoids. EPA and DHA compete with arachidonic acid as substrate for cyclooxygenase, for the sn-2 position in membrane phospholipids and for elongase and desaturase enzymes, thereby reducing synthesis of arachidonic acid from linoleic acid (Donadio and Grande, 2004). DHA and EPA are the (n-3) PUFA with most biological activities. However, conversion of α-linolenic acid by the human to the more active longer-chain metabolites is inefficient: < 5-10% for EPA and 2-5% for DHA (Davis and Kris-Etherton, 2003). Since

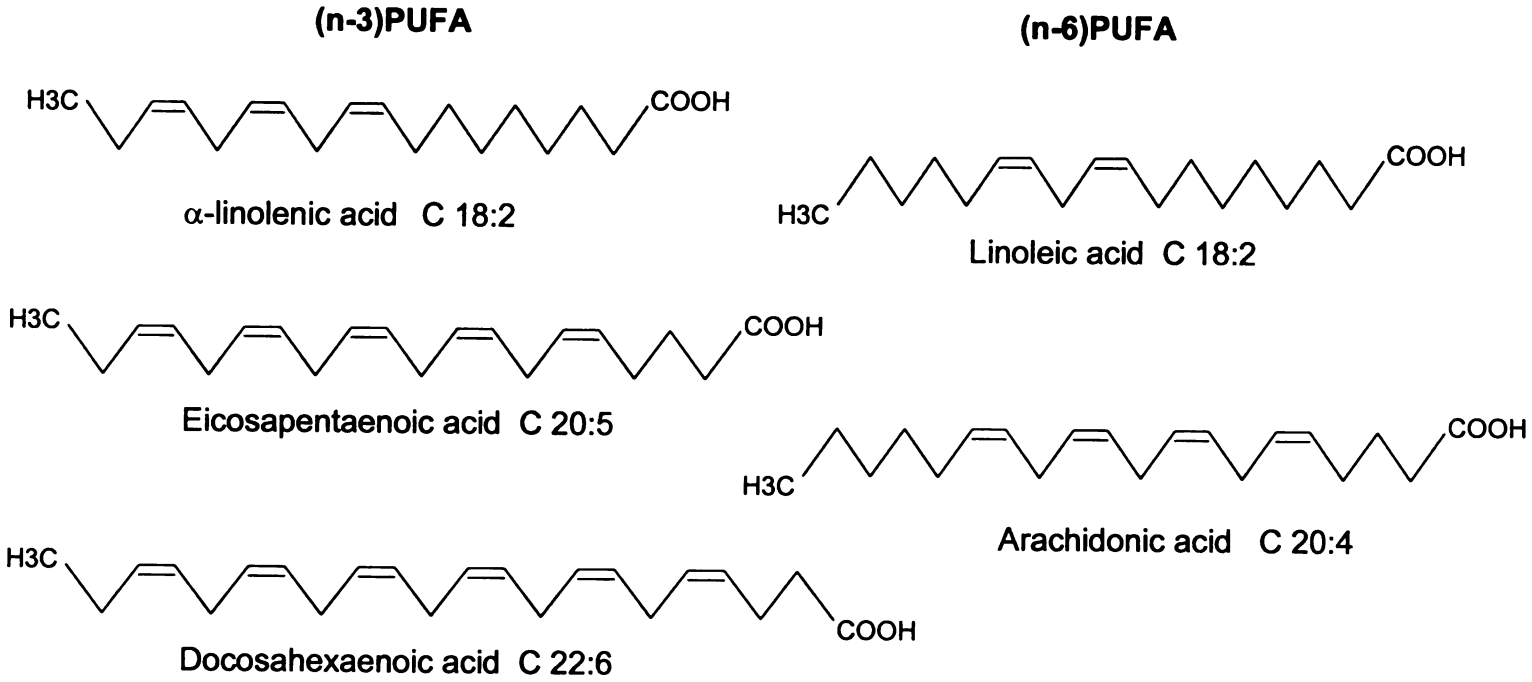

Figure 1.2 The structure of (n-3) and (n-6) PUFAs.

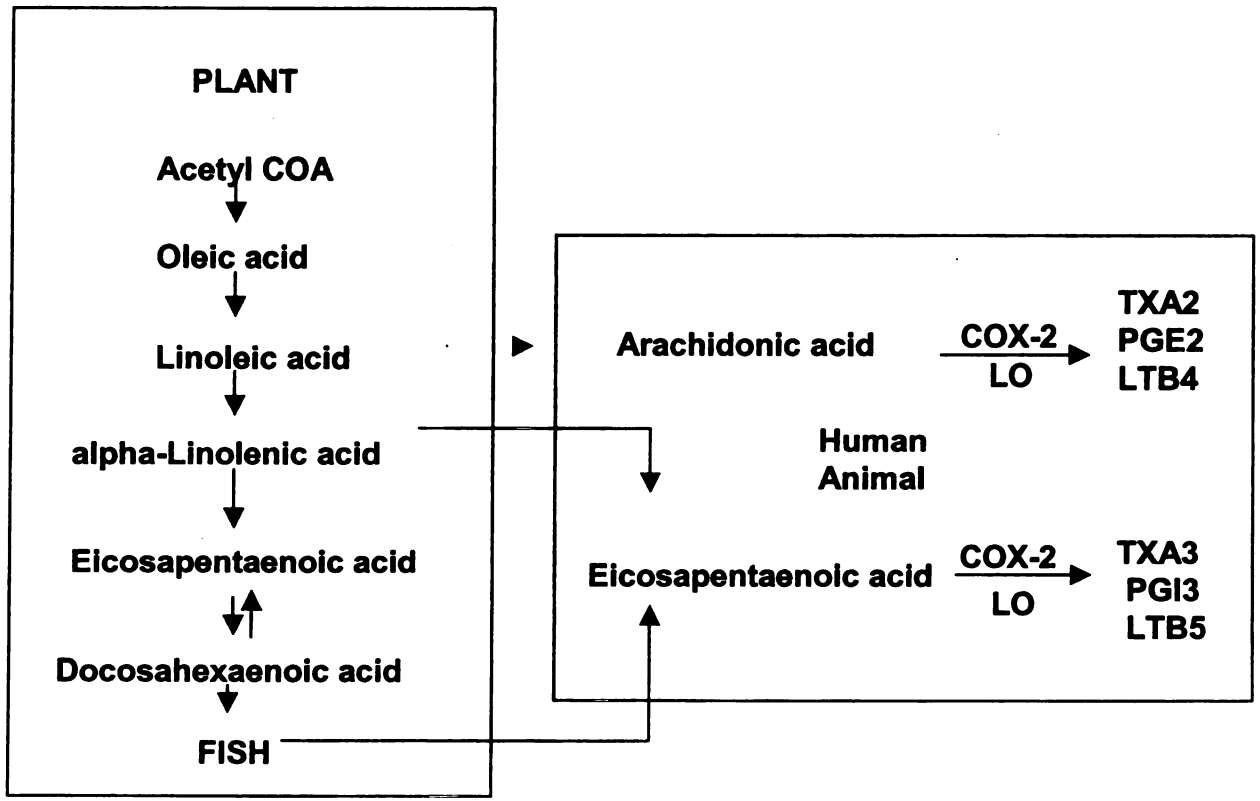

Figure 1.3 De novo synthesis pathways of PUFAs in plant and their metabolism in human and animal. Plants and fish oil are idea foods for human to get (n-3) PUFAs.

marine algae are rich in and marine fish can bioaccumulate the (n-3) PUFA, fish oil is an ideal food for humans to obtain DHA and EPA.

During the past 30 years, the ratio of (n-6) to (n-3) PUFA in diets has increased in industrialized societies because of (1) increased consumption of vegetable oils rich in (n-6) PUFA, (2) increased use of intensive, cereal-based livestock, and (3) reduced consumption of foods rich in (n-3) PUFA (Sanders, 2000). In the United States, the intake of (n-3) PUFA is 1.4 g for linolenic acid (ALA; 18:3) and 0.1–0.2 g for DHA and EPA. The recent estimated ratio of (n-6) to (n-3) in the diet is about 9.8:1, higher than that suggested to be optimized (i.e., 2.3:1), thus a four-fold increase in fish consumption has been recommended (Kris-Etherton *et al*, 2000). The Food and Nutrition Board raised the acceptable range for alpha-linolenic acid from 0.6% to 1.2% of energy, or 1.3–2.7 g per day on the basis of a 2000 calorie diet for healthy people (Donadio and Grande, 2004). (n-3) PUFA beneficial effects in IgAN are based on epidemiologic studies and randomized clinical trials. Epidemiologic studies reveal that dietary (n-3) PUFAs are negatively associated the risk of IgAN (Wakai *et al.*, 1999) and high intake of (n-6) PUFAs was associated with increased risk of IgAN (Wakai *et al.*, 2002). Holman *et al*, (1994) also found that some IgAN patients were deficient in α-linolenic acid [18:3 (n-3)], and supplementation with EPA and DHA will decrease proteinuria and improved glomerular filtration rate in these persons. Consistent with these results, several clinical trials have shown that fish oil might be safe and beneficial for long-term treatment of progressive IgAN (Grande *et al.*, 2001). The largest long-term clinical trial in high-risk patients with IgAN has shown that fish oil could retard renal disease progression by reducing inflammation and glomerulosclerosis (Donadio, 2001a). Two 4-year prospective

studies have been conducted in the United States to test the high dose (n-3) PUFA effects on IgAN (Donadio, 2000). The results show that both high dose (EPA 3.76g, DHA 2.94g /day) and low dose (EPA 1.88g,DHA 1.47g/day) have the same benefits on IgAN.

(n-3) PUFA benefits in IgAN might depend on their downregulation of multiple inflammatory genes. Kaminski *et al.* (1993) showed that consumption of 7g/day of 85% pure (n-3) PUFA for 7 wk increases (n-3) PUFA content in human monocyte phospholipids and down-regulates PDGF-A and PDGF-B expression by 70%. Intake of 18 g/day (n-3) PUFA by healthy volunteers without altering their normal Western diet for 6 wk suppressed IL-1 and TNF-α production by stimulated peripheral-blood mononuclear cells in vitro. These effects persist more than 10 wk after (n-3) PUFA supplementation (Endres *et al.*, 1995). LPS-induced spleen IL-1β mRNA expression was inhibited in mice fed a diet containing 4% DHA but not EPA (Watanabe *et al.*, 2000). (n-3) PUFA also inhibited inflammation by downregulating endothelial cell activation. Preincubation of endothelial cells with DHA (1-25 μmol) reduces endothelial expression of vascular cell adhesion molecule 1(VCAM-1), E-selectin, intercellular adhesion molecule 1(ICAM-1), IL-6 and IL-8 in response to IL-1, IL-4, TNF or bacterial endotoxin (De Caterina *et al.*, 2000).

(n-3) PUFA might affect gene transcription through cell membrane phospholipid modification and altered cell signaling. Dietary supplementation with (n-3) PUFA increases DHA and EPA while decreasing the AA level in the cell membrane phospholipids (Palombo *et al.*, 1994). This modification reduces prostaglandin E_2 (PGE2), leukotriene B4 (LTB_4) and platelet activated factor (PAF) production by decreasing the ratio of (n-6)/(n-3) in cell membrane (Calder 2002; Simopoulos, 2002;

Whelan, 1996). Reduced membrane arachidonic acid level can further reduce Ras protein activation (Sermon *et al.*, 1996). Lipid rafts, membrane microdomains enriched in cholesterol and glycosphingolipids, are central to cell signaling and have been implicated in processes as diverse as signal transduction, endocytosis and cholesterol trafficking (Pike, 2004). Both in vivo and in vitro studies have shown that sphingomyelin, which facilitates raft formation, is significantly decreased in T cells by (n-3) PUFA (Fan *et al.*, 2003). This modification might block protein tyrosine phosphorylation and calcium response in T cells and thus contribute to the inhibitory effects of (n-3) PUFA on cell signaling (Stulnig *et al.*, 2001). In addition, Src family protein-tyrosine kinases are highly concentrated in lipid rafts due to post-translational palmitoylation. It has been shown that (n-3) PUFA can selectively displace signaling proteins such as Src family kinases from lipid rafts, which are generally attached to the cytoplasmic membrane lipid leaflet by means of acyl moieties under physiological conditions (Stulnig *et al.*, 2001).

Several studies have reported that (n-3) PUFAs inhibit MAPK activation in vitro (Denys *et al., 2001*), which are important for IL-6 gene expression (Koranteng *et al.*, 2004; Kim *et al.*, 2004). With an anti-thymocyte (ATS) model of mesangial proliferative glomerulonephritis, it was recently demonstrated that DHA, but not EPA, decreased ERK activation by 30%. (Yusufi *et al.*, 2003). In the human pulmonary microvascular endothelial cells (HPMECs), EPA suppressed IL-1 stimulated p38 phosphorylation (Ait-Said *et al.*, 2003). When treated with a sterile, commercially available, pharmaceutical grade (n-3) PUFA emulsion, LPS-induced ERK and JNK phosphorylation in the RAW 264.7 macrophage is inhibited, however, p38 remains unchanged. Also, (n-3) PUFA can bind directly to the different recombinant activation domains of PKC, CaMKII and PKA

and inhibit their activation in vitro (Mirnikjoo *et al.*, 2001). Phosphorylation of ERK, JNK and p38 are the immediate prior steps in AP-1, CREB, and NFκB activation (Cho *et al.*, 2004; Wadgaonkar *et al.*, 2004; Jang *et al.*, 2004). Attenuated transcriptional factor activation and subsequent depression of proinflammatory cytokine gene expression would thus be anticipated after inhibition of these MAPKs (Babcock *et al.*, 2004).

Peroxisomal proliferator activated receptor αlpha (PPARα) and gamma (PPARγ) might be important transcriptional factors that can mediate (n-3) PUFA effects on gene transcription (Jump *et al.*, 2004; Jump *et al.*, 1997). When PPARs bind to their ligands, they physically interfere with transcriptional factors binding to the cis-element and thus downregulating proinflammatory gene expression (Ren *et al.*, 1996; Figure 1.4). For example, interferon gamma (IFN-γ), IL-6 and TNF-α production are impaired in spleens from mice fed the PPARα ligand, WY14, 643 (Cunard *et al.*, 2002). When WY14, 643 binds to PPARα, it can sharply reduce IL-6 and COX-2 gene expression by physically interfering with p65, c-jun and CBP interaction with DNA in vitro (Delerive *et al.*, 1999). The PPARγ ligands, troglitazone, pioglitazone and 15-deoxy-Delta (12,14)-prostaglandin J (2) also inhibit IL-1β-induced IL-6 expression at transcriptional level in vascular smooth muscle cells by interfering NFκB and C/EBP binding to the DNA (Takata *et al.*, 2002). Arachidonic acid and LTB4 can also bind to the PPARα and γ, but the binding will elicit their degradation by increasing β-oxidation of PUFA (Devchand *et al.*, 1996). Thus, PPARs are important negative feedback molecules in vivo for control of inflammation responses (Delerive *et al.*, 1999; Pointer and Daynes, 1998; Berger and Moller, 2002; Delerive *et al.*, 2000; Clark, 2002). Since DHA and EPA are now recognized to be natural ligands for PPARα and PPARγ, DHA and/or EPA might inhibit

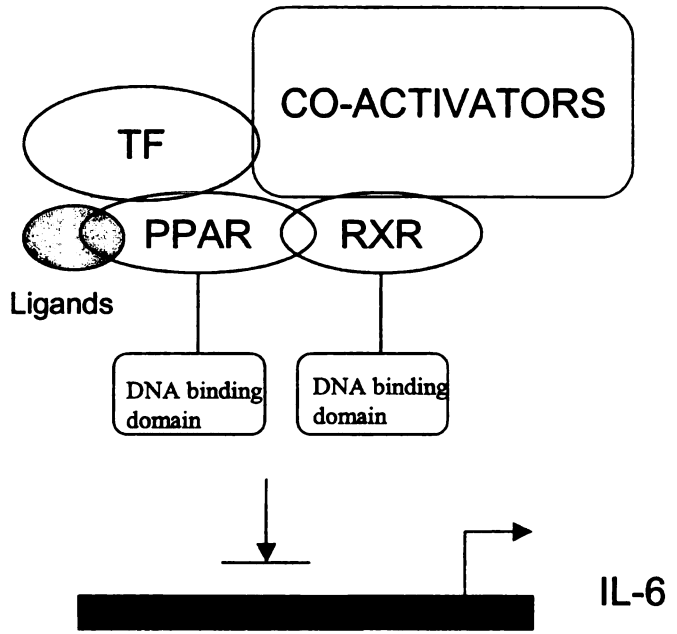

Figure 1.4 The mechanism by which PPARs block gene expression. After binding to their ligands, PPARs will recruit many transcriptional factors, block them binding to IL-6 promoter and shut down gene expression.
------Philippe Delerive et al. (1999) J Biol Chem, 274(45)

IL-6 gene expression by blocking transcription factors p65, c-jun binding to the IL-6 promoter.

IL-6 gene expression. IL-6 is a pleiotropic cytokine produced by monocytes/macrophages, fibroblasts, endothelial cells and astrocytes in response to infections and toxins (Horn *et al.,* 2000). As a major mediator of acute phase responses, IL-6 is involved in the regulation of differentiation, proliferation and survival of lymphocytes, astrocytes, endothelial and hematopoietic cells, bone marrow turnover and liver regeneration. Both in vitro and in vivo experiments suggest that IL-6 is an important factor for the development of IgA producing B cells. IL-6 was found to induce IgA production in Epstein Barr virus (EBV) transformed B lymphoblasts (Bertolini and Benson, 1990). Addition of human rIL-6 to peripheral blood mononuclear cell (PBMC) increases IgA-subclass spot-forming cell (SFC) responses (Kono *et al.*, 1991) and IL-6 produced by Th2-type cells has been shown to induce Peyer's Patches sIgA+ B cells to secrete IgA (Xu-Amano *et al.*, 1992). Anti IL-6 antibody can block B cell differentiation, immunoglobulin isotype class-switch and antibodies production induced by anti-CD40 antibodies (Labara *et al.*, 1990). IL-6 deficient mice show defects in inflammatory and immune responses, impaired macrophage and neutrophile functions and are resistant to experimental autoimmune encephalomyelitis (Friedmann *et al.,* 2000). Ex vivo cell reconstitution, antibody neutralization (Yan *et al.*, 1997; 1998) and IL-6 deficient mice studies (Pestka and Zhou, 2000) indicate that IL-6 is essential in DON-induced IgAN.

IL-6 binds to receptor glycoprotein 80 and the IL-6/gp80 complex will then interact with the transmembrane protein gp130 to trigger gp130 dimerization (Montero-Julian, 2001). This recruits and activates the non-receptor cytoplasmic protein tyrosine

kinase Janus kinases (JAK) via phosphorylation. JAK1/2 activate transcription factors C/EBPβ and δ. The *cis*-elements for these two factors have been found on the promoter region of immunoglobulin light and heavy chain, suggesting that IL-6 signal might be directly involved in immunoglobulin production. Another unique transcription factor activated by JAK1/2 is signal transducer and activator of transcription (STAT3), which also plays a crucial role in cell proliferation, differentiation and antibody production (Friedmann *et al.*, 2000).

IL-6 can act synergistically with CD40. Cross-linking CD40 with CD40 ligand (CD40L) will promote B cell proliferation, immunoglobulin class-switching and prevent B apoptosis in germinal centers. STAT3 also mediates CD40 signal transduction. So IL-6/JAK1/2/STAT3 and CD40/JAK3/STAT3 signaling pathways are important for B cell survival and antibody production. Two lineages of murine B cells mediate immune defense at mucosal surfaces, designated as B1 and B2 cells, identified by their origins, anatomical distribution, cell surface markers, antibody repertoire. Both contribute to the IgA plasma cells found in the intestine. The majority of intestinal IgA plasma cells derive from B2 cell precursors originating from Peyer's patches (Bao *et al.*, 1998; Husband *et al.*, 1978; Su *et al.*, 2000). In B1 B cells, which contribute almost half of the IgA production in the mucosal compartments, STAT3 is constitutively activated (Feehally, 1997), which might explain why IgA production in B cell from the peritoneal cavity is IL-6-independent (Beagley *et al.*, 1995).

Normally IL-6 transcription is tightly controlled despite its potent induction during acute phase responses. Multiple transcriptional factors are involved in IL-6 transcription (Figure 1.5). Electrophoresis mobility shift assay (EMSA) and point

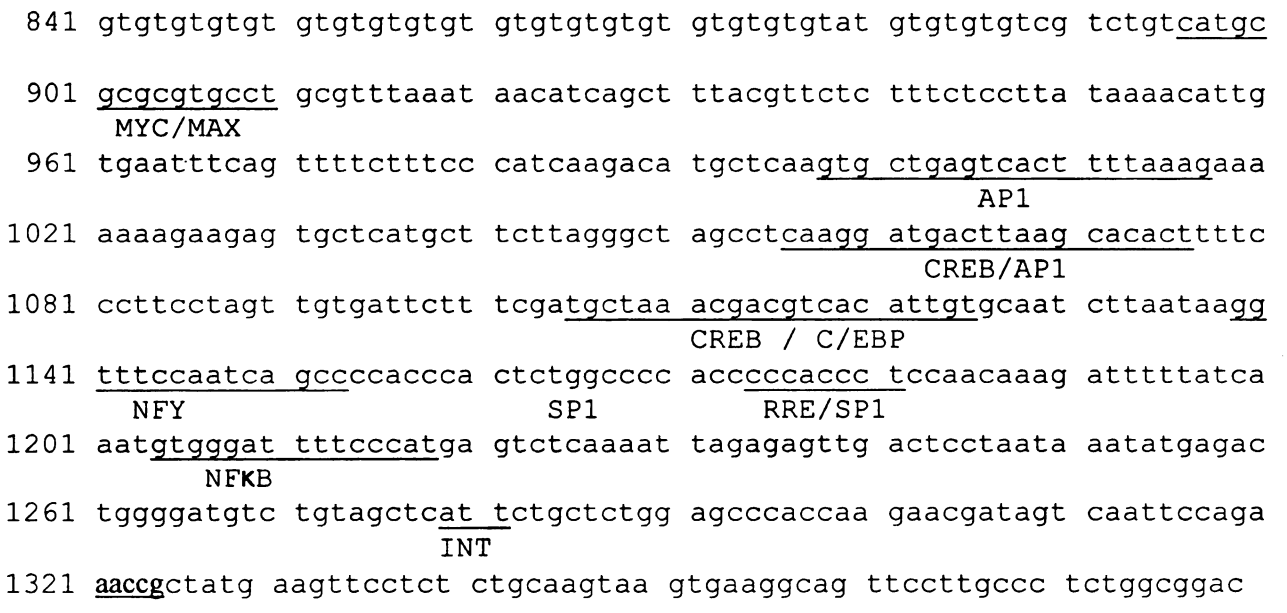

Figure 1.5 Mouse IL-6 promoter region

mutation analysis revealed that AP1, CREB, C/EBPβ and NFκB are important for IL-6 transcription regulation (Matsusaka *et al.*, 1993; Robb *et al.*, 2002). Generally, after AP1, C/EBPβ, CREB and NFκB are sequentially arranged along the promoter region of IL-6, CBP/P300 will be recruited onto the multiple-protein complex called enhancesome by protein-protein interaction, which will then interact with the general transcription factors and RNA polymerase (RNP) II to turn on IL-6 expression. However, the exact mechanism of IL-6 regulation is different between different cell lines and different kinds of stimulation.

The AP-1 transcription factor family is dimeric basic region–leucine zipper (bZIP) proteins, which belong to Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf, and ATF subfamilies. AP-1 recognizes 5'-TGAG/CTCA-3'or cAMP response element (CRE, 5'- TGACGTCA-3'). c-Jun has the most potential transacting activity in Jun family (Shaulian and Karin, 2001). AP-1 can mediate the induction of pro-inflammatory cytokines triggered by MAPKs. Activated JNK can phosphorylate c-jun, which then will bind to the AP-1 *cis*-element and drive gene expression (Yoon *et al.*, 2004). p38 can directly activate ATF2, monocyte specific enhancer binding factors 2c (MEF2C) and ternary complex factors (TCFs) by phosphorylation. Activated ERK translocates into the nucleus and activates Fra1 and/or Fra2, which enhance c-Jun DNA binding. AP-1 plays an important role in cell proliferation, transformation, cell survival and death (Shaulian and Karin, 2001, 2002). In B cells AP-1 family (c-Jun, JunB, JunD, and c-Fos) are involved in the IL-6 expression via CD40 ligation (Mann *et al.*, 2002).

The C/EBP proteins, including α,β,γ,δ,ε,ζ, belong to the basic leucine zipper class transcription factor family, all of which contain a conserved bZIP domain at C-terminus

which can interact with CCAAT box motif. The transactivation properties of C/EBP members might come from its recruitment of co-activators. C/EBPβ can form dimmers with p50, CREB/ATF, AP1 and retinoblastoma protein. Different heterodimmers might have different transactivation potentials. In P388 murine B-lymphocytes, C/EBP bZIP region can confer LPS-inducible IL-6 expression and this effect is completely dependent on NFκB binding sites, suggesting an interaction between C/EBP and NFκB (Hu *et al.*, 2000). In the human enterocyte Caco-2 cell line, C/EBP beta and delta are important for IL-6 expression induced by IL-10 (Robb *et al,* 2002; Hungness *et al.*, 2002).

CREB belongs to the bZIP superfamily. The C-terminal domain of CREB mediates DNA binding and the leucine zipper domain facilitates its dimmerization. Usually, CREB will bind to the palindromic *cis*-regulatory elements (CRE): 5'-TGACGTCA-3' and organize the transcription machine (Bonnie *et al.*, 2002). Deletion and mutation studies within IL-6 promoter region have revealed that cAMP response element (CRE) plays a crucial role in the regulation of IL-6 transcription (Tokunou *et al.*, 2001). p38-activated MSK1, Ca/CAMKII and PKA all can phosphorylate CREB at serine 133, which is required for CREB transactivation (Shaywitz and Greenberg, 1999; Rosenberg *et al.*, 2002).

NFκB contains several subunits including p50, p65 (RelA), c-Rel, p52, and RelB. All of these proteins share a conserved Rel homology region (RHR), which is responsible for dimerization, DNA binding and interaction with IκB. These form homodimers or heterodimers composed of several of combination of members. In mammalian cells, the p65 homodimer is the most potent and abundant transcriptional activator found in most cell types (Dixit and Mak, 2002). NFκB stays in an inactivated form in cytoplasm by

interaction with the protein IκBs. TNFα, LPS and IL-1, potent activators of NFkB, rapidly induces phosphorylation of IκBs and degradation by 26S proteasome (Dixit and Mak, 2002). The p50/p65 will then translocate into the nucleus and turn on a large number of genes, which are involved in the immune and inflammation responses. During the development and in response to injury and infection, IL-1 and TNF-α are considered the most potent factors for NFκB activation in vivo (Bowie and O'Neill, 2000). It has been firmly established that IκB phosphorylation at Ser32 and Ser36 are critical for NFκB activation. Protein kinases specific for IκBα and β are called IκB kinases (IKK). These are considered to be key convergence points for many NFκB signaling pathways. Three IKKs (α, β, γ) have been identified of which IKKβ is the most critical for inflammation. Termination of NFκB activation is complicated and is thought to involve new synthesis of IκBα, which enable NFκB to be transported back into the cytoplasm. In murine monocytic PU5-1.8 cells, U937 and HeLa cell lines, NFκB is critical for LPS-induced IL-6 expression (Endorser *et al.*, 1994).

An acute dose of DON (25 mg/kg BW) induces serum IL-6 and IL-6 mRNA in spleen and Peyer's patches of mice (Moon and Pestka, 2003). In RAW 264.7 macrophages, DON can increase DNA binding of junD, junB, phosphorylated c-Jun, c-Fos, Fra-2, the NFκB family of p50, c-Rel subunits, and C/EBPβ (Wong *et al.*, 2002). EMSA has also revealed that DON could also induce AP-1, CREB, C/EBPβ and NFκB binding in mouse spleen (Zhou *et al.*, 2003). One or more of these transcription factors might be involved in DON induced the IL-6 gene regulation.

Cyclooxygenase-2. Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids after arachidonic acid is released from

membrane phospholipids by phospholipase A_2 (PLA_2). There are two isoforms of COX. COX-1 is a constitutive enzyme and is associated with the endoplasmic reticulum (ER). Prostaglandins (PG) synthesized by COX-1 mediate cell homeostasis functions. In contrast, COX-2, expressed by macrophages and monocytes, is an inducible, immediate-early gene product and primarily responsible for increased PG production during inflammation, reproduction and carcinogenesis (Ristimaki, 2004). Expression of COX-2 is low or nondetectable in most tissues, but can be readily induced in response to cell activation by cytokines, growth factors and chemicals. The COX-2 products, PGs, are generally considered to be proinflammatory agents, but in vitro studies show that they play anti-inflammatory roles during certain situations (Takayama *et al.*, 2002). For example, PGE_2 can behave as a pro- or an anti-inflammatory lipid depends on which PGE2 receptor (EP) subtype is stimulated.

Four subtypes of PGE_2 receptor (EP), designated EP1, EP2, EP3, and EP4, have been found (Nataraj *et al.*, 2001). EP1 is coupled with the Gq protein, which signals through the phospholipase C (PLC) pathway, increasing intracellular Ca^{2+} concentration. EP2 and EP4 are coupled with the Gs protein and activate adenylyl cyclase, increasing cyclic adenosine monophosphate (cAMP) levels and signaling through the protein kinase A (PKA) pathway. EP3 α and β decrease cAMP by inhibiting adenylyl cyclase and increase intracellular Ca^{2+} concentration via Gi-mediated PLC activation, whereas EP3 γ signaling increases cAMP via the Gs protein (Akaogi *et al.*, 2004). PGE_2 and EP4/2 agonists can modulate macrophage IL-6 production via EP2 or EP4 receptor. (Akaogi *et al.*, 2004). EP4/EP2-mediated IL-6 induction was PKA-dependent in an early human T cell line, whereas PKC and p38 MAPK dependence is reported in human astroglioma

cells suggesting that different signaling pathways mediated IL-6 induction. EPs may regulate IL-6 differentially depending on the mode of stimulation, species, cell types (Akaogi *et al.*, 2004).

DON-induced ERK and p38 activation mediates COX-2 transcription, while p38 mediate COX-2 mRNA stability (Moon and Pestka, 2002). Furthermore, DON upregulation of LPS-induced IL-6 production is significantly reduced by the COX-2 inhibitors indomethacin and NS-398. COX-2 knockout mice also produce significantly reduced splenic IL-6 mRNA and serum IL-6 responses to oral DON exposure compared to their parental wild type (Moon and Pestka, 2003). These in vitro and in vivo data suggest that DON-induced COX-2 gene expression and resultant COX-2 metabolites contribute, in part, to subsequent upregulation of IL-6 gene expression (Moon and Pestka, 2003). Recent studies suggest that (n-3) PUFA might reduce the concentrations of 2-series PG and increase the synthesis of 3-series PG by competing with arachidonic acid for the COX-2 (Bagga *et al.*, 2003; Obata *et al.*, 1999; Dommels *et al.*, 2003).

Rationale. To summarize, primary IgA nephropathy is an immue complex disease, which mainly affects young adults, of which 20-40% will develop the end stage renal disease. The etiology of IgAN is still not clear and different animal models have been constructed to study the development and treatment of this disease. Mice exposed to the mycotoxin DON develop high serum IgA and kidney mesangial IgA deposition that mimics the early stages of IgAN and provides a unique IgAN model to investigate potential therapeutic mechanisms. Treatment of IgAN is still not disease-specific, but consumption of (n-3) PUFA holds promise for retarding the disease progression (Donadio, 2001a; Donadio *et al.*, 2001; Prokopiuk *et al.*, 2001; Grande and Donadio,

1998; Donadio *et al.*, 1994; Pettersson *et al.*, 1994) by reducing renal inflammation. Although (n-3) PUFAs inhibit inflammation, the mechanisms remain unclear. In this thesis, the effects of (n-3) PUFA on DON-induced IgAN will be confirmed and an optimized regimen determined. Next (n-3) PUFA effects on regulation of IL-6 gene expression will be investigated. A long term outcome of this work will be to uncover new cellular regulatory pathways that may be exploited in the control of IgAN.

This research is important for the following reasons. First, it will provide the basis and regimen for the prevention and treatment of IgAN with (n-3) PUFA. Second, this research will reveal specific intervention targets for treatment of IgAN and potentially other inflammatory diseases. Third, this work will uncover the molecular basis of (n-3) PUFA on IL-6 gene regulation, which may help to understand (n-3) PUFA anti-inflammatory effects. Fourth, this study will provide insight into different signal transduction pathways, which regulate upstream IL-6 gene expression and how these might be affected by (n-3) PUFA in diet.

CHAPTER 2

Dietary Fish Oil Suppresses Experimental Immunoglobulin A Nephropathy in Mice

ABSTRACT

Dietary fish oil supplementation reportedly retards the progression of renal disease in patients with immunoglobulin A nephropathy (IgAN), the most common glomerulonephritis worldwide. Here the effects of fish oil were assessed with mycotoxin deoxynivalenol (DON) induced experimental IgAN. DON significantly increased serum IgA, serum IgA immune complexes and kidney mesangial IgA deposition compared with the control group, whereas all three variables were significantly attenuated in mice fed DON and fish oil. In addition, spleen cell cultures from the DON plus fish oil group produced markedly less IgA than those cultures from mice fed DON plus corn oil. Taken together, the results suggested that diets containing fish oil might impair early immunopathogenesis in DON-induced IgAN.

INTRODUCTION

Human IgA nephropathy (IgAN) is a primary autoimmune disease with diffuse mesangial IgA deposition in the kidney glomerulus, which accounts for up to 50% glomerulopathies in Japan (Hunley and Kon, 1999; Emancipator and Lamm, 1989). Approximately 150,000 people in the United States have been diagnosed with IgAN with 4000 new cases occurring each year (Hellegers *et al.*, 1993) and 30% of them will develop progressive renal failure over a 25 y period (Donadio *et al*, 1994; Harper *et al*, 1993). Mucosal infections (D'Amico, 1987), genetic predisposition (Schena *et al.*, 2001), diet (Coppo *et al.*, 1986) and environmental agents such as mycotoxins (Coppo *et al.*, 1988; Hinoshita *et al.*, 1997) have been related to with IgAN. Currently no disease specific treatments are available for IgAN.

Human studies have demonstrated that fish oil consumption was correlated with low incidence of autoimmune and inflammatory disorders (Kromhout *et al.*, 1985). In several mouse models fish oil also showed potential to reduce inflammation (Cathcart *et al.*, 1991; Robinson *et al.*, 1986; Robinson *et al.*, 1993; Empey *et al.*, 1991). Several studies have proposed that fish oil may benefit patients with immune-related renal diseases including IgAN, lupus nephritis and cyclosporine-induced nephrotoxicity (Donadio, 1991; Holman *et al.*, 1994; Wakai *et al.*, 1999; Hamazaki *et al.*, 1984; Donadio *et al.*, 2001). A randomized trial of fish oil in IgAN (Donadio *et al.*, 1994; Donadio *et al.*, 1999) also reported that fish oil can significantly retard the development of renal failure. Since dietary fish oil supplementation appears to be a promising therapeutic regimen for IgAN, further research is required both to establish the mechanistic basis for these effects and to determine the optimal dosing regimens of fish oil.

It was found that mice exposed experimental diets containing mycotoxin deoxynivalenol (DON) develops the early characteristic features of human IgAN including elevated serum polymeric IgA and IgA-IC as well as mesangial IgA deposition (Pestka *et al.*, 1989) which last for up to 3 months after removal of DON from diet (Dong and Pestka, 1993). DON-exposed mice also exhibits increased numbers of membrane IgA^+ cells and IgA-secreting cells in Peyer's patches and spleens (Pestka *et al.*, 1990a, b; Bondy and Pestka, 1991). The mechanisms underlying this model appear to involve dysregulation of cytokine gene expression, which promote differentiation of IgA-secreting cells and systemic over production of IgA (Pestka, 2003).

The purpose of this study was to test the hypothesis that fish can suppress serum IgA, serum IgA-IC elevation and kidney mesangial IgA deposition in DON induced experimental IgAN in mice. The results suggest that diets containing fish oil attenuated IgA production in this model.

MATERIALS AND METHODS

Materials. All chemicals (reagent grade or better) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. The DON used in this study was produced in *Fusarium graminearum* R6576 cultures and purified by the water-saturated silica gel chromatography method of Witt *et al.*, (1985). Purity of DON was verified by a single HPLC peak occurring at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware that was contaminated with mycotoxin was detoxified by soaking for >1 h in 100 mL/L sodium hypochlorite (Thompson & Wannemacher, 1986). Purified DON was added to powdered diets as described previously (Dong *et al.*, 1991).

Animals. Male B6C3F1 mice (7 wk old) were obtained from Charles River (Portage, MI). Mice were housed singly in environmentally protected cages, which consisted of a transparent polycarbonate body with a filter bonnet, stainless steel wire lid and a layer of heat-treated hardwood chips. The mice were allowed to acclimate for at least 7 d to their new housing, regulated temperature (25°C), feed, 12-h light:dark cycle and to a negative-pressure ventilated area before feeding regimens began. All animal handling was conducted in accordance with guidelines established by the National Institutes of Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and were approved by Michigan State University Laboratory Animal Research Committee.

Diets and experimental design. The diet was based on the AIN-93G formulation (Reeves *et al.*, 1993) and consisted of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.5 g cornstarch, 132 g Dyetrose (dextrinized cornstarch), 50 g cellulose, 3 g L-cystine, 2.5 g choline bitartrate, 14 mg

TBHQ, which were purchased from Dyets, and 100 g sucrose, which was obtained from a local commercial source. Control and menhaden fish oil (each already containing 200 mg/kg of TBHQ) from Dyets, were used to amend the basal diet to yield three experimental diet groups containing the following (per kg): 1) 70 g corn oil; 2) 70 g corn oil and 10 mg DON; and 3) 10 g corn oil, 60 g fish oil and 10 mg DON. The PUFA compositions of these corn oil and fish oil-containing diets are shown in Table 2.1. Mice were fed for 20 wk and body weight were measured every wk. Animals were bled at 4 wk intervals. Serum was analyzed for IgA, IgG and IgM. After bleeding at wk 20, mice were killed by cervical dislocation. Spleens and Peyer's patches were removed aseptically for preparing cell cultures. Kidneys of each mouse were removed for immunohistochemical examination.

Measurement of immunoglobulin and IgA-IC. Serum IgA, IgG and IgM were measured by capture ELISA (Dong *et al*, 1991) using mouse immunoglobulin reference serum (Bethyl Laboratories, Montgomery, TX), goat anti-mouse IgA, G and M (heavy chain specific) and peroxidase-conjugated goat IgG fraction to mouse IgA, IgG, IgM (Organon Teknika, West Chester, PA). For detection of IgA-IC, diluted serum samples were precipitated using 70 g/L polyethylene glycol (PEG 6000; Sigma) (Imai *et al.*, 1987). IgA in precipitate was redissolved in PBS and quantified by ELISA.

Assessment of kidney mesangial IgA deposition. At experiment termination, kidneys of each euthanized mouse were removed, cut in half and immediately frozen in liquid nitrogen. Each kidney was sectioned to 7 μm with a cryostat (Reichert-Jung, Cambridge Instruments, Buffalo, NY) and stained for IgA deposition with fluorescein

Table 2.1 Fatty acid composition of oils used for experimental diets

Fatty acid	Corn oil (g/100g total fat)	Fish oil (g/100g total fat)
16:0	10.8	16.2
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
20:4(n-6)	-	0.8
20:5(n-3)	-	13.3
22:5(n-3)	-	2.1
22:6(n-3)	-	7.8

Only the major fatty acids are shown. The diet contained 70g oil/kg.

isthiocyanate–labeled goat anti-mouse IgA (Sigma) as previously described (Valenzuela & Deodhar 1980). Sections from each animal were viewed under a Nikon Labophot epifluorescence microscope through a Sony (Tokyo, Japan) imaging system consisting of CCD Video Camera DXC-151A and a PVM 13442Q Trinitron Video Monitor. Ten glomeruli from each section were randomly selected and the image captured on a microcomputer using a Snappy Video System (Play Incorporated, Rancho Cordova, CA). Mean fluorescence intensity was determined in polygons encircling the glomeruli using UTHSCSA Image Tool Software V 1.2 (available via anonymous FTP at ftp://maxrad6.uthscsa.edu). This system generates a quantitative value from an encircled immunofluorescent stained glomerulus in a frozen frame and calculates the average brightness for the circled area based on each pixel of the screen included in the circle. The pixels in the circled area were measured on a grayness scale that ranged from 0 (black) to 255 (white).

Cell culture. Spleen and Peyer's patches were teased apart in harvest buffer consisting of 0.01 mol/L PBS, pH 7.4 containing 20 mL/L heat inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY), 1 x 10^5 U/L penicillin and 100 mg/L streptomycin. Tissues were passed through a sterile 100-mesh stainless screen in the same buffer and cell suspensions held on ice for 10 min to allow settling of tissue particles. Supernatant was removed following centrifugation at 450 x g for 10 min. Erythrocytes were lysed for 3 min at room temperature in 0.02 mol/L Tris buffer (pH 7.65) containing 0.14 mol/L ammonium chloride. Cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 100 mL/L FBS, 1 mmol/L sodium pyruvate, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, 0.1 mmol/L nonessential amino acid and 50 μmol/L 2-

mercaptoethanol and then counted using a hemacytometer (American Optical, Buffalo, NY). Cells (2 x 10^8/L) from individual mice were cultured separately in 1 mL of medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific, Corning, NY) at 37°C under 70% CO_2 in a humidified incubator. Supernatants were collected at 5 d and stored in aliquots at -20°C until analysis for IgA.

Statistics. Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. If data did not meet the normality assumption, they were subjected to Kruskal-Wallace ANOVA on Ranks and pairwise comparisons made by Dunn's or Student-Newman Student-Newman-Keuls methods. Differences were considered significant at $P < 0.05$.

RESULTS

Dietary DON retarded the weight gain and fish oil did not alter the inhibition on body weight gain induced by DON (Figure 2.1). DON induced IgA elevation at wk 4, 8, 12, 16 and 20 from 0.3 to 15 fold, respectively (Figure 2.2). Serum IgA increased only from 0.7 to 6-fold, respectively in mice exposed to fish oil and DON. Serum IgA in the DON and fish oil + DON groups did not differ until after 12 wks (Figure 2.2). Serum IgA-IC followed similar trends with IgA-IC concentrations in the DON group being consistently ($p = 0.05$) higher than fish oil plus DON group (Figure 2.3).

IgA production ex vivo by spleen and Peyer's cells cultured was assessed. Spleen and Peyer's patch cultures from DON groups produced more IgA than did their corresponding control groups (Figure 2.4). Supernatant IgA levels in spleen cultures from the fish oil + DON group were also higher than control group but were lower than DON group. Supernatant IgA levels in Peyer's patch cultures from the fish oil + DON group were not significantly different from the control group or the control + DON group.

Immunofluorescence microscopy revealed strong mesangial deposition of IgA in mice fed DON compared with mice fed corn oil only (Figure 2.5). IgA deposition was lower in mice fed fish oil and DON. Mean glomerular fluorescent intensities for DON and fish oil + DON groups were 3 and 1.3 fold greater than that of the control group ($P < 0.05$). DON did not alter serum IgG (Figure 2.6) and IgM (Figure 2.7) concentration at wk 4, 8, 16 and 20. The IgG and IgM level in fish oil + DON groups were also not different from that of DON group.

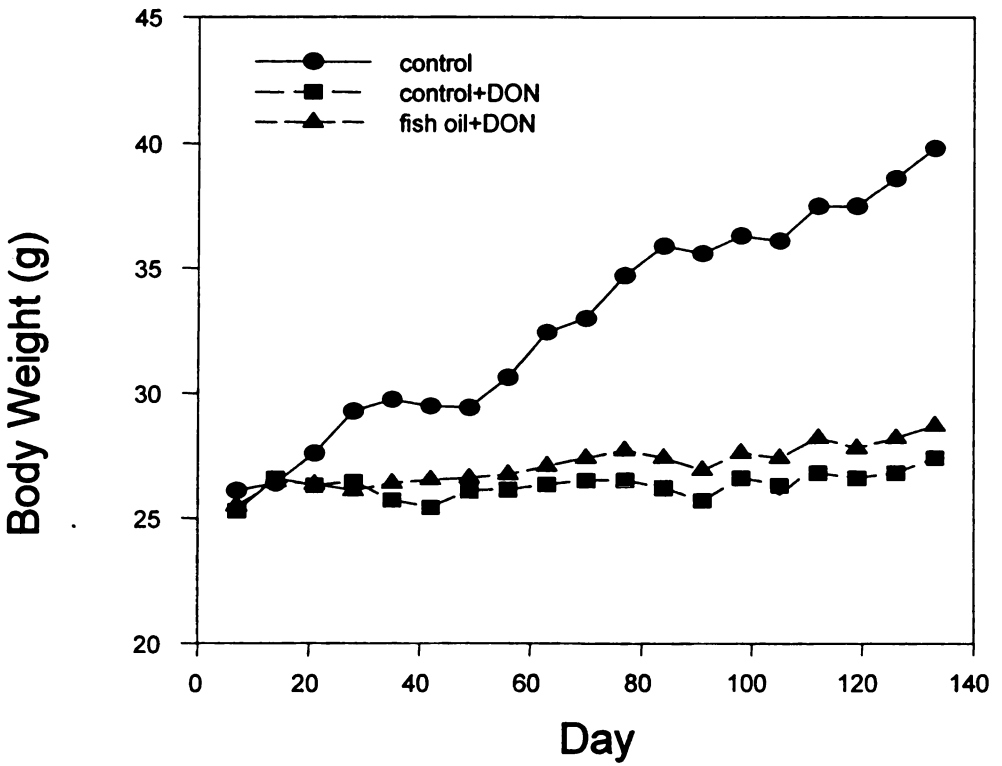

Figure 2.1 Body weight changes in mice in control, control+DON and fish oil +DON. Kinetics of boy weight response over 20 wk feeding period. Values are mean of each group at different time point, n=8.

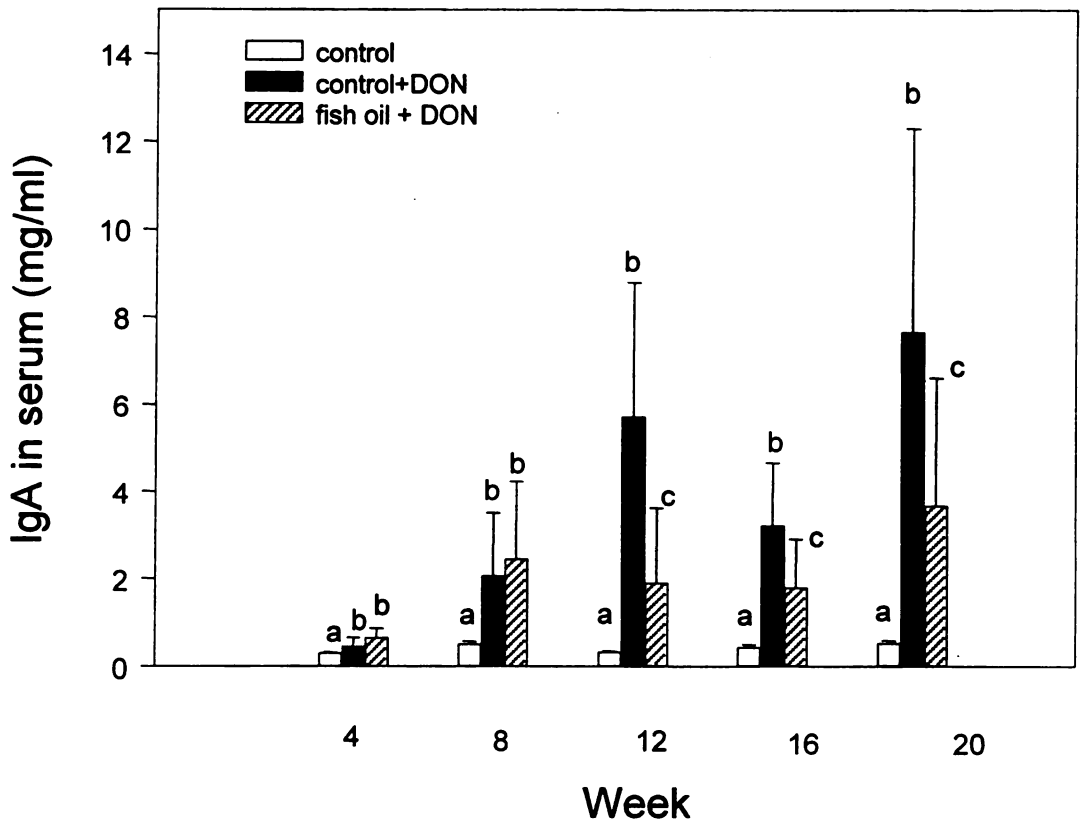

Figure 2.2 Serum IgA levels in mice in control, control +DON and fish oil +DON group. Serum were collected at wk 4, 8,12,16 and 20. IgA was measured by ELISA. Values (mg/ml) are mean ± SEM, n=8. Bars marked with different letter differ, ($P<0.05$).

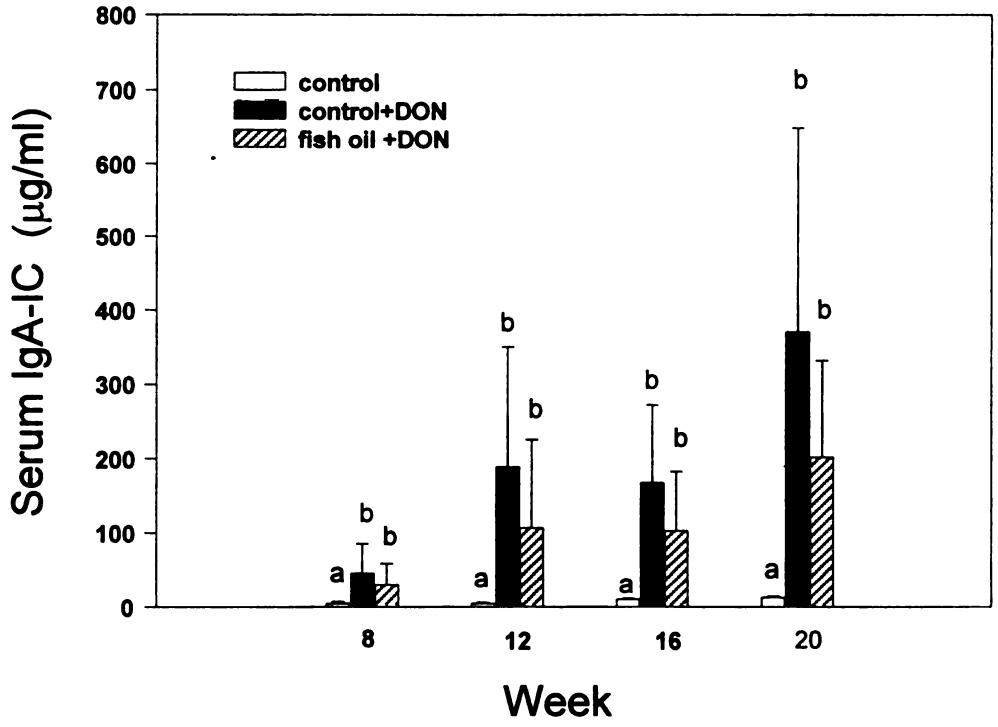

Figure 2.3 Serum IgA-IC levels in mice in control, control +DON and fish oil +DON group. Serum were collected at wk 4,8,12 and 20, subjected to polyethylene glycol fractionation and precipitate analyzed for IgA-IC was measured by ELISA. Values (:g/ml) are mean ± SEM, n=8. Bars marked with different letter differ, ($P<0.05$).

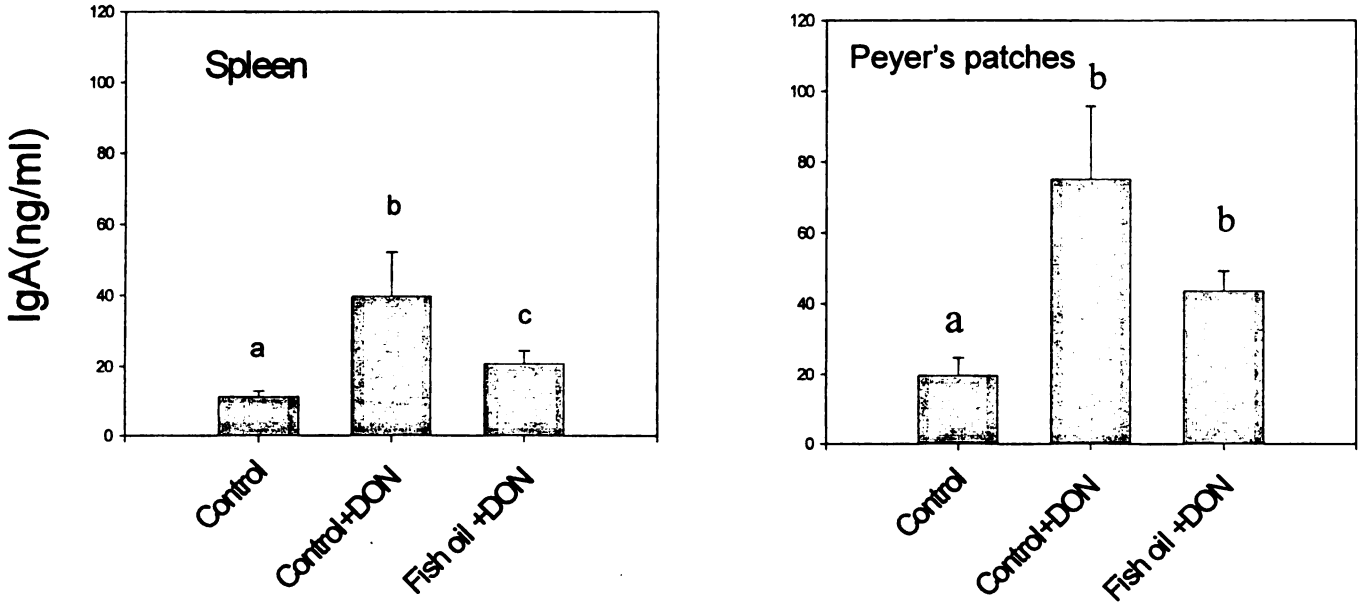

Figure 2.4 Cell culture supernatant IgA levels in mice in control, control +DON and fish oil +DON group. Mice were sacrificed at 20 wk and spleen and peyer's patch cells (2×10^8/L) were cultured for 5 d in 24-well plates. IgA was measured by ELISA. Values (ng/ml) are mean ± SEM, (n=8). Bars marked with different letter differ, ($P<0.05$).

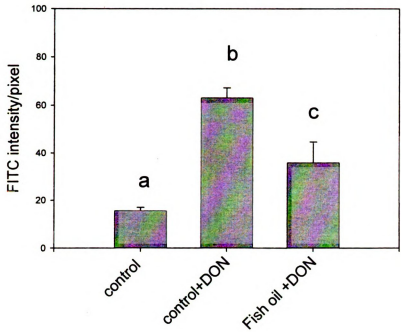

Figure 2.5 Mean glomerular immunofluorescence intensity of kidney sections taken from mice in control, control +DON and fish oil +DON group. Kidneys of mice fed experimental diets for 20 wk were cryostat sectioned and stained with fluorescein isthiocyanate–labeled anti-mouse IgA. Mesangial IgA quantitation was performed by image analysis. Values are mean ± SEM (n = 8). Bars that do not have the same letter differ, $P < (0.05)$.

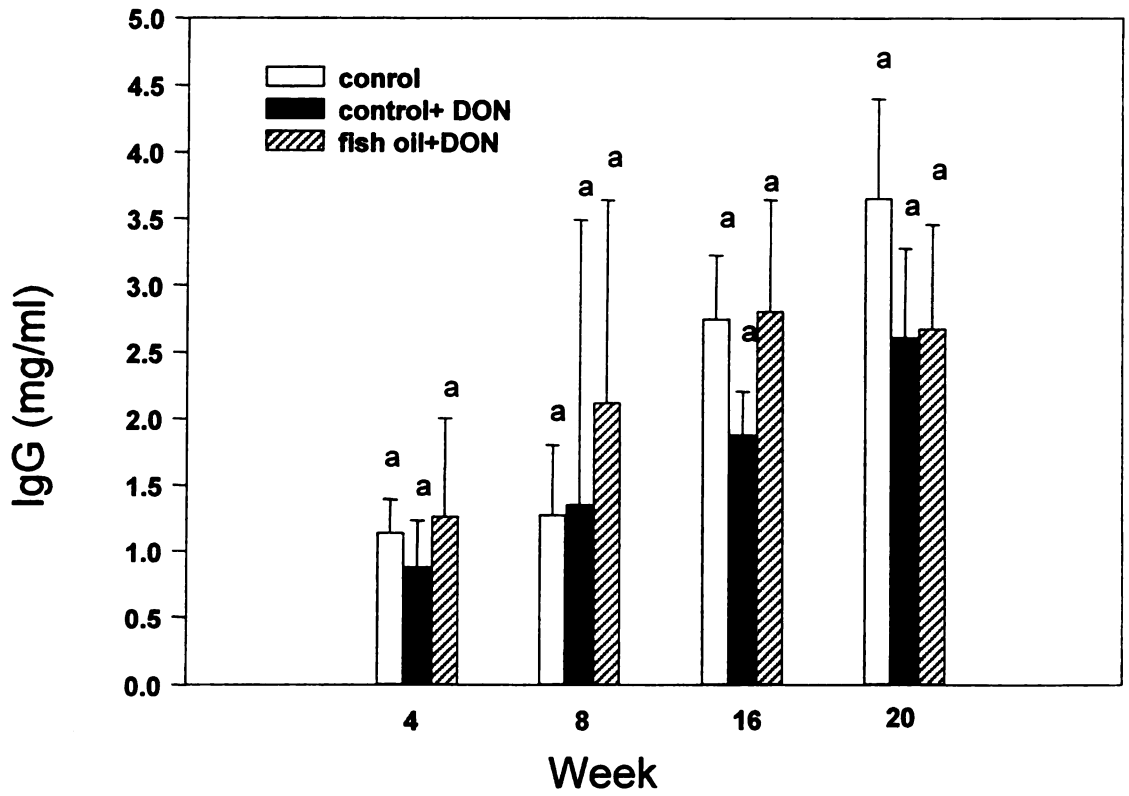

Figure 2.6 Serum IgG levels in mice in control, control +DON and fish oil +DON group. Serum were collected at wk 4, 8,16 and 20. IgG was measured by ELISA. Values(mg/ml) are mean ± SEM, n=8. Bars marked with different letter differ, ($P<0.05$).

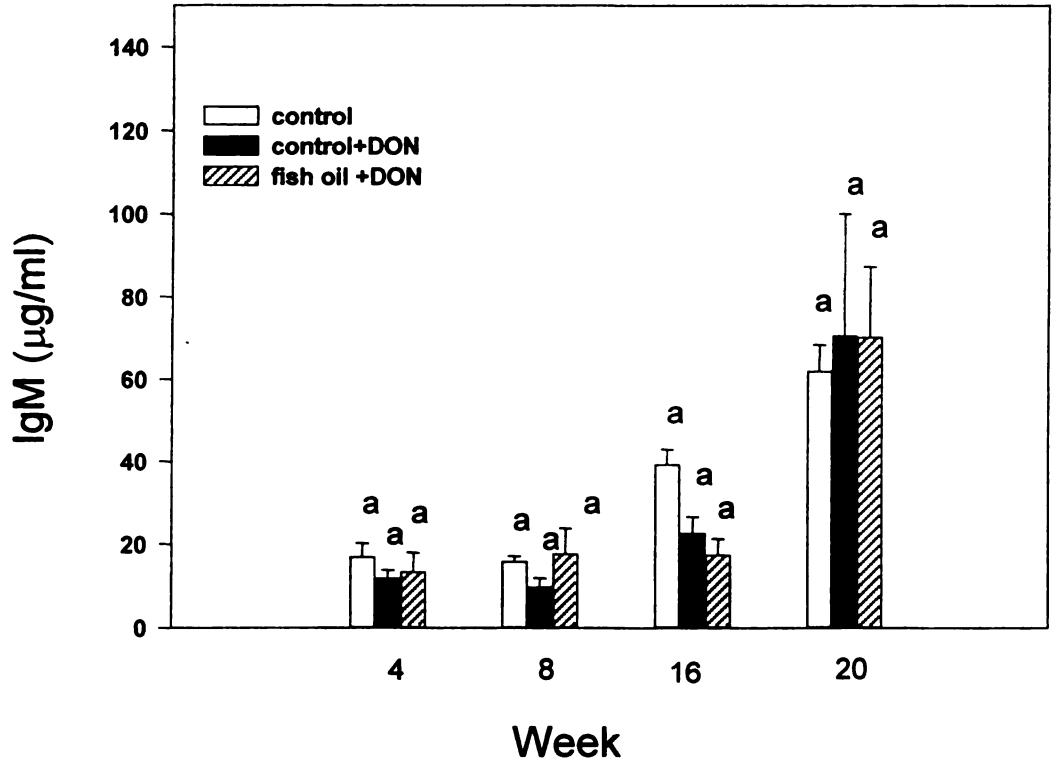

Figure 2.7 Serum IgM levels in mice in control, control +DON and fish oil +DON group. Serum were collected at wks 4, 8,16 and 20 . IgM was measured by ELISA. Values(mg/ml) are mean ± SEM, n=8. Bars marked with different letter differ, ($P<0.05$).

DISCUSSION

It has been previously shown that mice fed DON exhibited a high level of polyclonal serum IgA, IgA-IC, mesangial IgA deposition, systemic and mucosal compartment IgA-bearing and IgA-secreting lymphocytes (Pestka *et al.*, 1989; Dong *et al.*, 1991; Dong & Pestka, 1993; Greene et *al.*, 1994b; Rasooly & Pestka, 1994), which mimic the early stage of human IgAN. Our data here were consistent with previous results. At the same time, diet containing fish oil attenuated DON-induced IgA elevation, mesangial IgA deposition and ex vivo IgA production. These results provided a unique model for understanding the development of IgA nephropathy and how (n-3) might affect early development of IgAN.

It should be noted that this model only mimics the early stage of IgAN with high serum IgA and kidney IgA deposition without hematuria and proteinuria during the 20-wk period. Although fish oil has been used for IgAN patients due to its multiple anti-inflammatory effects during the later stage of the disease (Donadio *et al.*, 1999), our results nevertheless suggested that fish oil might also block IgA production. As the fundamental abnormality in IgAN lies within the IgA system (Harper *et al.*, 1996), the observation that fish oil inhibited overactive IgA production, IgA-IC development and mesangial IgA deposition indicated that this nutritional regimen could have prophylactic value for IgAN at the onset of very early clinical signs.

DON retarded weight gain throughout the feeding study, which confirmed the previous results of DON-feeding studies (Rotter *et al.*, 1996). The effects are likely to be related to DON-induced inhibition of protein translation (Rotter *et al.*, 1996) and

depressed nutrient absorption (Maresca, 2002). Fish oil treatment did not alter DON effect on body weight loss.

Previous studies indicate that DON specifically elevates IgA concentration but decreases or has no effect on serum IgG and IgM concentration and kidney deposition in different feeding studies (Banotai *et al.*, 1999; Greene *et al.*, 1994b; Rasooly *et al.*, 1992; Dong *et al.*, 1993). This suggests that DON's effects are isotype-specific (Pestka *et al.*, 1990b). In this study, the serum IgG level was not changed and IgM level was decreased at 16 wk, which is consistent with previous data. Since IgA is mainly formed in the mucosal compartment, DON might target IgA production at the level of the intestinal tract (Pestka, 2003).

IL-6 plays a critical role in B cell terminal differentiation, antibody-secreting cell accumulation and IgA production in the gut (Bao *et al,* 1998). Recently, ex vivo cell reconstitution (Yan *et al.*, 1988), antibody neutralization (Yan *et al.*, 1997) and IL-6 deficient mice study indicated that IL-6 appears to be the most important cytokine in DON-induced IgAN (Pestka & Zhou, 2000). In the future, fish oil effects on DON-induced IL-6 gene expression should be examined.

CHAPTER 3

Docosahexaenoic Acid and Eicosapentaenoic Suppress Deoxynivalenol Induced Experimental Immunoglobulin A Nephropathy in Mice

ABSTRACT

Primary IgA nephropathy (IgAN) is an immune complex disease with elevated serum IgA and kidney mesangial IgA deposition as its hallmaks. (n-3) polyunsaturated fatty acids (PUFAs) can retard the progression of IgAN in humans and in a mouse model induced by trichothecene mycotoxin deoxynivalenol (DON). In order to assess the efficiency of two major (n-3) PUFAs found in fish oil, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) effects on DON-induced IgAN were evaluated. Mice were fed for 18 wk with AIN-93G diets containing: 1) 10 g/kg corn oil plus 60 g/kg oleic acid (control); 2) 10 g/kg corn oil plus 35 g/kg oleic acid and 25 g/kg DHA-enriched fish oil (DHA); 3) 10 g/kg corn oil plus 33 g/kg oleic acid and 27 g/kg EPA-enriched fish oil (EPA); and 4) 10 g/kg corn oil plus 37 g/kg oleic acid and 23 g/kg DHA + EPA (1:1) enriched fish oil (DHA + EPA). DON significantly increased serum IgA, IgA immune complexes and kidney mesangial IgA deposition. DHA, EPA and DHA/EPA significantly attenuated all three immunopathological parameters as well as IgA secretion by spleen cells. Pre-feeding of DHA/EPA significantly reduced serum interleukin 6 (IL-6) induced by acute oral exposure to DON. Taken together, both DHA and EPA were effective at ameliorating DON-induced IgAN and IL-6 gene expression, which is required for IgA production.

INTRODUCTION

Primary IgA nephropathy (IgAN) is an autoimmune disease, which mainly affects children and young adults. About 20-40 % of IgAN patients develop end stage renal disease. High serum IgA and IgA immune complexes (IgA-IC) are considered to be important etiologic factors for IgAN (Endoh *et al.*, 1984 Layward *et al.*, 1993; Suga 1985; van den Wall Bake *et al.*, 1989). Serum IgA-IC, dimeric and polymeric IgA might deposit in the kidney and cause inflammation by activating complement via the alternative pathway (Floege and Feehally, 2000). Renal injury in IgAN ranges from glomerular hypercellularity, tubular atrophy, and interstitial fibrosis to renal failure in the end.

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by the fungus of *Fusarium* genus, which can bind readily to eukaryotic 60S ribosome and prevent polypeptide initiation and elongation (Rotter *et al.*, 1996). Chronic exposure to DON can induce IgAN in mice, characterized by high serum IgA, IgA-IC and IgA deposition in the kidney (Greene *et al.*, 1994a,b; Rasooly *et al.*, 1994; Yan *et al.*, 1998). Antigenic stimulation of the gut mucosal immune system apparently contributes to DON induced IgA dysregulation (Pestka, 2003). Although the gut acts as an entry to a vast array of foreign antigens, antibody responses are seldom induced due to the mucosal tolerance. Acute oral exposure of DON can induce proinflammatory cytokines IL-1, IL-6 and TNF-α (Dong *et al.*, 1994; Wong *et al.*, 2001; Moon and Pestka 2003), which might overide the mucosal tolerance and promote IgA production (Pestka, 2003).

Therapeutic targets for IgAN include decreasing the synthesis of IgA-IC, inhibition of IgA binding to the mesangial cell, antagonizing the effects of platelet

derived growth factor receptor (PDGF) and transform growth factor (TGF)β, reducing infiltrating neutrophiles (Lai *et al.*, 2002) and blocking the production of lipid inflammation mediators (Wardle *et al.*, 2000). Based on these considerations (n-3) PUFA might be a promising treatment because of their anti-inflammatory effects (Cheng *et al.*, 1990; Donadio, 1991; Pettersson *et al.*, 1994; Endres *et al.*, 1995; Whelan 1996). Clinical trials in high-risk patients with IgAN have shown that fish oil retards the IgAN progression by reducing the inflammation and glomerulosclerosis (Donadio, 2001a,b). Recently, feeding studies in our lab have revealed that supplementation with fish oil (4-6g/100g diet) can also block IgA dysregulation and deposition in the kidney of DON-fed mice (Pestka *et al.*, 2002).

DHA and EPA are primary (n-3) PUFAs found in fish oil. Here, it was hypothesized that both DHA and EPA can inhibit DON-induced IgAN. This hypothesis was confirmed and correlated with inhibition of cytokine IL-6 expression in vivo in response to DON stimulation.

MATERIALS AND METHODS

Materials. All chemicals (reagent grade or better) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. The DON used in this study was produced in *Fusarium graminearum* R6576 cultures and purified by the water-saturated silica gel chromatography method of Witt *et al.* (1985). Purity of DON was verified by a single HPLC peak occurring at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware that was contaminated with mycotoxin was detoxified by soaking for >1 h in 100 mL/L sodium hypochlorite. Purified DON was added to powdered diets as described previously (Dong *et al.*, 1991).

Animals. Male B6C3F1 mice (7 wk) were obtained from Charles River (Portage, MI). Mice were housed singly in environmentally protected cages, which consisted of a transparent polycarbonate body with a filter bonnet, stainless steel wire lid and a layer of heat-treated hardwood chips. The mice were allowed to acclimate for at least 7 d to their new housing, regulated temperature (25°C), feed, 12-h light: dark cycle and to a negative-pressure ventilated area before feeding regimens began. All animal handling was conducted in accordance with guidelines established by the National Institutes of Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and were approved by Michigan State University Laboratory Animal Research Committee.

Diets and experimental design. Experimental diets were based upon purified AIN-93G formulation (Reeves *et al.*, 1993), which consisted of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.5 g

cornstarch, 132 g dyetrose (dextrinized cornstarch), 50 g cellulose, 3 g L-cystine, 2.5 g chlorine bitartrate, 14 mg TBHQ, 100 g sucrose and 70 g oil.

In study 1, corn oil (Dyets), oleic acids (Dyets), DHA-enriched oil (containing 604 g/kg DHA, 71 g/kg EPA, Ocean Nutrition Canada Ltd), EPA-enriched oil (540 g/kg EPA, 71 g/kg DHA, Ocean Nutrition Canada Ltd.) and DHA/EPA lipid mix (402 g/kg DHA, 341 g/kg EPA, Ocean Nutrition Canada Ltd) were used to amend the basal diet to yield five experimental groups: control, control+DON, DHA+DON, EPA+DON and DHA/EPA + DON (Table 3.1). Diets were prepared every two wk, stored at -20°C and provided fresh to the mice each day. Final lipid composition of experimental diets is shown in table 3.2. Mice (n=10) were fed diets for 18 wk. Food intake was measured daily and body weight was monitored weekly. Animals were bled at 4 wk intervals and blood samples were used for plasma IgA, IgA-IC measurement. At week 18, mice were anesthetized with methoxyfluorane and killed by cervical dislocation. Spleens and Peyer's patches were removed aseptically for preparing cell cultures. Kidneys from each mouse were removed for immunohistochemical examination.

Study 2, corn oil, oleic acids and DHA/EPA lipid mix enriched (n-3) PUFA were used to amend the basal diet to yield 2 diet groups (n=10) (Table 3.3): control and DHA/EPA. Mice (n=5) were fed control or DHA/EPA lipid mix for 8 wk. At experiment termination, one half of the mice were exposed to DON (25mg/kg. body weight) by gavage and the other half was exposed to vehicle. After 3 hrs mice were anesthetized, bled and their spleen and Peyer's patches were removed. Serum IL-6 and IL-6 mRNA in spleen and Peyer's patches were measured.

Lipid extraction and fatty acids analysis. To confirm tissue incorporation of (n-3) PUFA after 18 wk of feeding the experimental diets, liver phospholipid contents were measured by a modification of the method of Hasler *et al.* (1991) with the assistance of Dr M Bennink (Michigan State University) and Sherry Shi (Michigan State University). This organ was selected as a tissue surrogate because the two immune organs of concern, spleen and Peyer's patches, were completely utilized for cell culture studies. Briefly, mouse livers were homogenized with a chloroform:methanol (2:1) solution. Total phospholipids were extracted, separated, and collected using a silica column. Phospholipid samples were dried and esterified with methanol:acetonitrile:boron trifluoride (11:4:5, by vol). The resulting fatty acid methyl ester (FAME) was extracted with hexane. After centrifugation at 1200 x g for 5 min, the hexane supernatant was decanted, dried, and redissolved in chloroform and then analyzed by GC utilizing a Varian 3700 GLC. Fatty acids profiles were identified by comparing the retention times with those of appropriate standard FAME (Nu-Check-Prep).

IgA and IgA-IC measurement. IgA was measured in serum by ELISA (Dong et al, 1991) using mouse reference Ig serum (Bethyl Laboratories), goat anti-mouse IgA (heavy chain–specific), and peroxidase-conjugated goat IgG fraction to mouse IgA (Organon Teknika). For detection of IgA-IC, diluted serum samples were first precipitated by 70 g/L polyethylene glycol (PEG 6000; Sigma) (Imai *et al*, 1987) and quantified by IgA ELISA (Dong *et al*, 1991).

Interleukin 6 (IL-6) ELISA. Mouse sera were analyzed for IL-6 as previously described by Moon and Pestka (2003). Briefly, mouse serum was incubated for 1 h at 37°C in Immunolon IV removawell microtiter strips (Dynatech Laboratories, Chantilly,

Table 3.1. Experimental groups for assessing DHA and EPA on DON-induced IgAN (Study 1)

Group	Animal No.	DON (g/kg)	Corn oil (g/kg)	Oleic acid (g/kg)	DHA/EPA enriched oil(g/kg)	Total n-3 (g/kg)	n-6/n-3 ratio
Control	10	0	10	60	0	0.6	16/1
Control+DON	10	0.01	10	60	0	0.6	16/1
DHA+DON	10	0.01	10	35	25	17.0	1/1.8
EPA+DON	10	0.01	10	33	27	16.6	1/1.8
EPA/DHA + DON	10	0.01	10	37	23	17.2	1/1.8

• All diets will be adjusted with oleic acid to have final oil content of 7%.
•DHA and EPA is fish oil enriched with DHA and EPA; The EPA/DHA ratio in DHA enriched oil is 1/9 and in EPA enriched oil is 8/1.

Table 3.2. Fatty acid composition of the diets in feeding study 1

	g/100g total diet			
	Corn oil and oleic acid	DHA	EPA	DHA/EPA
Saturated Fat	0.87	0.63	0.7	0.65
Monounsaturated Fat	5.01	3.09	3.25	3.29
polyunsaturated Fat	1.32	2.72	2.62	2.73
Total fat	7.2	6.44	6.56	6.67
	g/100g total lipid			
C14:0 Myristic	0.29	0.34	0.36	0.31
C16:0 Palmitic	6.45	5.87	5.5	5.75
C16:1 Palmitoleic	0.16	0.36	0.23	0.21
C18:0 Stearic	4.41	3.1	4.37	3.29
C18:1 C Oleic	72.5	48	49.2	50.1
C18:1 C Vaccenic	<0.10	0.8	1.77	0.85
C18:2 Linoleic (b)	13.6	13.2	11.7	12.1
C20:0 Arachidic	0.34	0.29	0.4	0.44
C18:3 Gamma Linolenic (b)	<0.10	<0.10	<0.10	<0.10
C20:1 Eicosenoic	0.2	0.32	0.9	0.82
C18:3 Linolenic (a)	0.32	0.4	0.75	0.53
C20:2 Eicosadienoic(b)	<0.10	<0.10	0.21	0.15
C22:0 Behenic	0.89	0.76	0.49	0.64
C22:1 Erucic	<0.10	0.23	<0.16	<0.10
C20:3 Eicosatrienoic(a)	<0.10	0.2	<0.100	0.11
C20:4 Arachidonic acid(b)	<0.10	0.12	0.94	0.44
C24:0 Lignoceric	0.22	<0.10	<0.10	<0.10
C20:5 Eicosapentaenoic(a)	0.28	2.7	17.7	9.31
C24:1 Nervonic	<0.10	1.06	<0.10	<0.10
C22:5 Docosapentaenoic(a)	<0.10	2.95	0.32	1.64
C22:6 Docosahexaenoic(a)	0.26	17.1	2.19	10.9
Total n-3	0.86	23.35	20.96	21.01
Total n-6	13.6	13.32	13.2	13.01
n-6/n-3	17	1.75	1.58	1.59

Only the major fatty acids are shown. Letter "a" stands for n-3 PUFA; "b" for n-6 PUFA

Table 3.3 Experimental groups for assessing effects of DHA /EPA on DON induced IL-6 production (Study 2)

Group	Animal No.	DON (mg/kg.BW)	Corn oil (g/kg)	Oleic acid (g/kg)	DHA/EPA Enriched oil(g/kg)	Total n-3 (g/kg)	n-6/n-3 ratio
Control	5	0	10	60	0	0.6	16/1
Control+DON	5	25	10	60	0	0.6	16/1
DHA/EPA	5	0	10	37	23	14	1/1.8
DHA/EPA + DON	5	25	10	37	23	14	1/1.8

•All diets will be adjusted with oleic acid to have final oil content of 7%.
•DHA/EPA is fish oil enriched with DHA and EPA.
•DON is gavaged at the end of experiment.

VA) that were coated with 100 µl of 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 four times with PBS containing 0.05 % (v/v) Tween-20 (PBST), wells were incubated with 100 µL of 1.5 µg/ml biotinylated rat anti-mouse-IL-6 (Pharmingen, San Diego, CA) for 1 h at room temperature. Wells were washed six times and incubated for 1 h with 100 µL of 1.5 µg/ml HRP-conjugated streptavidin (Sigma) in PBST at room temperature. After washing eight times with PBST, substrate (100 µl) consisting of 3',3',5',5'-tetramethyl benzidine (100 µg/ml; Fluka Chemical, 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 6 N sulfuric acid. Absorbance was read at 450 nm with a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) and IL-6 was quantified using the manufacturer's software.

Assessment of kidney mesangial IgA deposition. Kidney sections were prepared and analyzed for IgA deposition according to the previously described procedure of Pestka *et al.*, (2002). Kidneys were removed, sectioned to 7 µm with a cryostat (Reichert-Jung, Cambridge Instruments, Buffalo, NY) and stained for IgA deposition with fluorescein isthiocyanate–labeled goat anti-mouse IgA (Sigma). IgA immunofluorescence was recorded by microscope(Nikon, LABOPHOT and HB-10101AF) and Kodak DC290 digital camera. Mean fluorescence intensity of 10 randomly selected glomeruli from each section was determined in polygons encircling the glomeruli using UTHSCSA Image Tool Software V 1.2 (http://www.scioncorp.com/). The pixels in the circled area were measured on a grayness scale that ranged from 0 (black) to 255 (white).

Cell culture. Spleen and Peyer's patch cell culture followed the procedure previously described. Tissues were passed through a sterile 100-mesh stainless screen in harvest buffer. Erythrocytes from spleen were lyses. Cells (1 x 10^9/L) from individual mice were cultured separately in 1 ml of RPMI1640 medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific, Corning, NY) at 37°C under 7% CO_2 in a humidified incubator. Supernatants were collected after 5 days and stored in aliquots at -20°C until analysis for IgA.

IL-6 mRNA measurement by competitive RT-PCR. RNA was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA from each sample was co-reverse transcribed to cDNA with a truncated IL-6 RNA internal standard constructed by the RT-PCR Competitor Construction Kit (Ambion). The cDNA was amplified in a 9600 Perkin Elmer Cycler (Perkin-Elmer Corp., Norwalk, CT) using the following parameters: 35 cycles of reactions of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s. An aliquot of each PCR product was subjected to 1.5% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers were synthesized at Michigan State University's Molecular Structure facility. The 5' forward and 3' reverse-complement PCR primers for amplification of mouse IL-6 cDNA were AAG AAA GAC AAA GCC AGA and TTC GTA GAG AAC AAC ATA A respectively. Sizes of amplified IL-6 cDNA and its internal standard cDNA were 329 and 270 base pairs (bp), respectively. The densitometric ratio of IL-6 cDNA/IL-6 internal standard was used to construct a standard curve to calculate IL-6 transcript concentration in RT reaction products.

Statistics. Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. If data did not meet the normality assumption, they were subjected to Kruskal-Wallace ANOVA on Ranks and pairwise comparisons made by Dunn's or Student-Newman-Keuls methods. Differences were considered significant at $P < 0.05$.

RESULTS

Study 1. As has been previously described (Pestka *et al.*, 2001), DON was found to impair food intake (Figure 3.1) and weight gain (Figure 3.2). Inclusion of (n-3) PUFA had no effects on DON- induced change in these parameters.

To assess tissue incorporation of (n-3) PUFA, liver phospholipid contents were measured by gas chromatography. DON itself did not significantly affect fatty acids profile compared to the control group. DHA [22:6(n-3)] accumulated to 26.1%, 23.1% , 23.1% and EPA [20:5(n-3)] accumulated to 9.0%, 10.4%, 11.7% respectively in mice livers fed with DHA, EPA or DHA/EPA lipid mix, higher than that of control group and control+DON group ($P < 0.05$) (Table 3.4). Mice in control group had an arachidonic acid [n-6 (20:4)] 3 to 6 times higher than that of mouse fed with (n-3) PUFA ($P < 0.05$) (Table 3.4). Linoleic acid [n-6 (18:2)] level was higher in control group mice than groups fed DHA, EPA and DHA/EPA.

DON 10 mg/kg in diet significantly induced serum IgA elevation in mice fed control diet beginning at wk 9 and reached 6 times control value at wk 18 (Figure 3.3). At wk 13, DON-induced serum IgA elevation was significantly attenuated in groups fed with DHA, EPA and DHA/EPA. No statistical differences within DHA, EPA and DHA/EPA group were observed. DON feeding significantly induced serum IgA-IC in control mice at wk 13 and 18 (Figure 3.4). DHA, EPA and DHA/EPA significantly suppressed these effects at wk 18. Suppressive effects of various (n-3) PUFA on serum IgA-IC were not significantly different at wk 18.

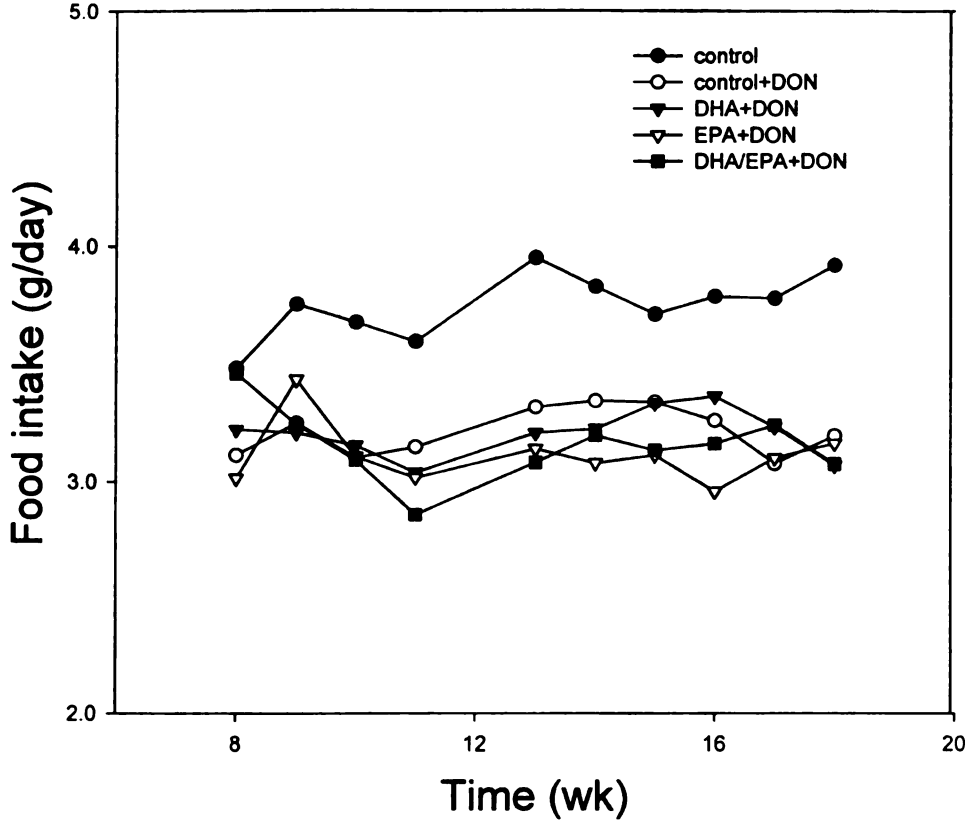

Figure 3.1 Food intake in male B6C3F1 mice fed modified AIN93G diets containing DON (10ppm) and (n-3) PUFA from 8-18 wk. Food intake was measured everyday for each mouse. Values are mean for a group (n=10).

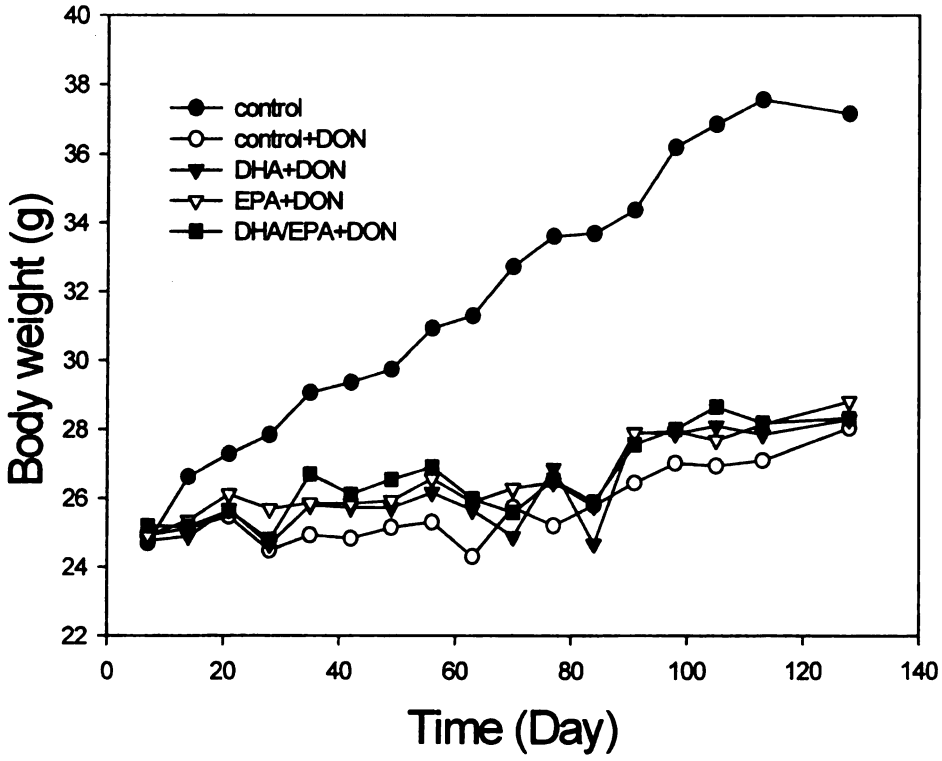

Figure. 3.2 Body weight changes in male B6C3F1 mice fed modified AIN-93G diets. Kinetics of body weight response of different groups over 18 wk feeding period. Values are mean for a group (n=10).

TABLE 3.4 Liver phospholipid composition (g/100 g total phospholipid fatty acids)[1, 2] in mice fed various (n-3) PUFA with and without DON.

	Control	Control + DON	DHA + DON	EPA + DON	(DHA + EPA) + DON
16:00	18.4 ± 1.0^{a}	21.8 ± 0.7^{b}	25.0 ± 0.9^{b}	22.9 ± 1.2^{b}	23.2 ± 0.9^{b}
18:00	22.3 ± 0.9^{d}	19.5 ± 0.9^{c}	15.5 ± 0.4^{a}	15.3 ± 0.4^{a}	16.8 ± 0.3^{b}
18:01	18.3 ± 0.8^{b}	14.6 ± 0.5^{a}	13.1 ± 0.4^{a}	12.1 ± 0.6^{a}	13.6 ± 0.5^{a}
18:2(n-6)	7.5 ± 0.5^{a}	12.6 ± 0.5^{c}	8.0 ± 0.4^{b}	8.4 ± 0.4^{b}	9.5 ± 0.4^{b}
20:4(n-6)	24.0 ± 2.3^{b}	22.6 ± 1.5^{b}	4.1 ± 0.7^{a}	8.0 ± 0.2^{a}	6.2 ± 0.3^{a}
20:5(n-3)	ND[3]	ND	9.0 ± 3.4^{a}	10.4 ± 0.3^{b}	11.7 ± 0.4^{c}
22:6(n-3)	9.9 ± 0.9^{a}	8.6 ± 0.9^{a}	26.1 ± 1.8^{c}	23.1 ± 0.73^{b}	23.1 ± 5.9^{b}

[1]Values are means ± SEM, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.
[2] Only the major fatty acids are shown.
[3] ND, not detectable

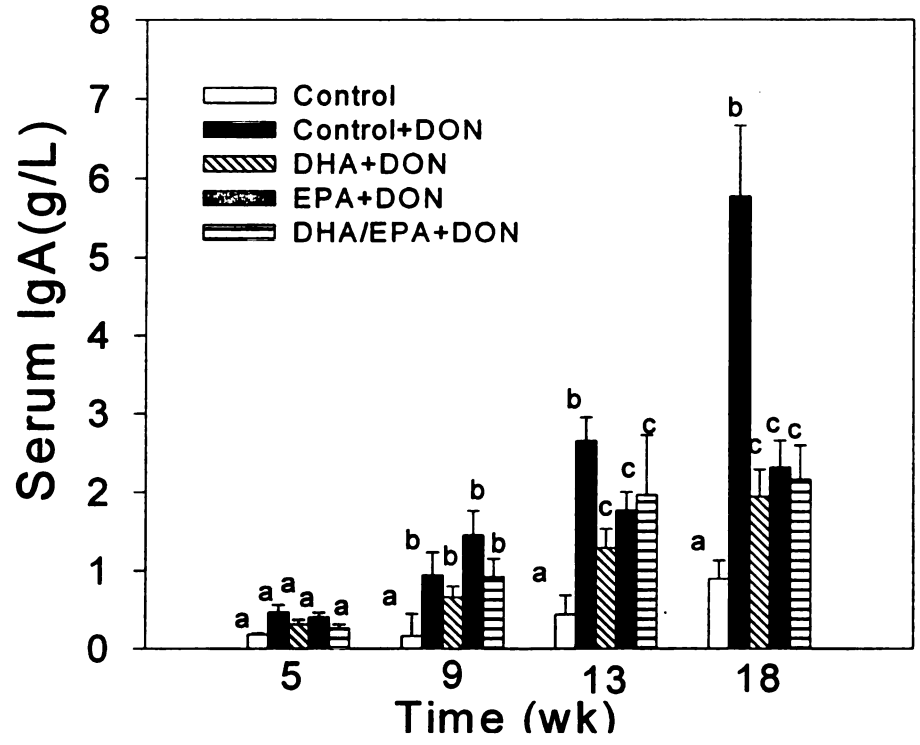

Figure. 3.3 Serum IgA level in male B6C3F1 mice fed AIN93G diets containing DON and different kinds of (n-3) PUFA. Serum were collected at 5, 9,13 and18 wk. IgA were measured by ELISA. Values are mean ± SEM, n=10. Bars marked with different letter differ, ($p<0.05$).

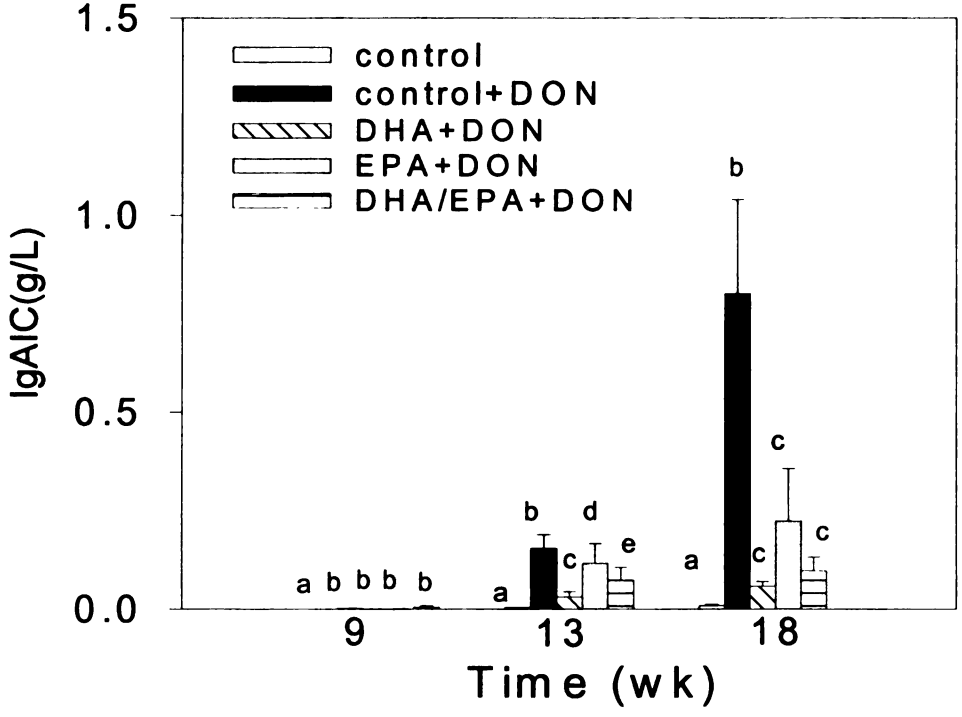

Figure 3.4. Serum IgA-IC level in male B6C3F1mice fed AIN93G diets containing DON and different kinds of (n-3) PUFA. Serum were collected at 9,13 and18 wk. IgA-IC was precipitated by polyethelene glycol (PEG) first and measured by IgA ELISA. Values are mean ± SEM, n=10. Bars marked with different letter differ, ($p<0.05$).

The effects of feeding (n-3) PUFAs on spleen and Peyer's patch cell IgA secretion were also compared (Figure 3.5). Supernatant IgA concentrations in spleen cell cultures from mice fed control + DON were significantly higher than control concentrations, indicating that the mycotoxin was inducing terminal differentiation of IgA-secreting cells. DON-enhanced IgA production was significantly suppressed in spleen cell cultures from DHA, EPA, and (DHA + EPA) fed mice. IgA concentration within groups fed the various (n-3) PUFAs did not differ. Similarly, IgA production tended to increase in Peyer's patch cultures from DON-fed mice (P = 0.065) and with downward trends for DHA (p = 0.259), DHA + EPA (P = 0.179), and EPA (p = 0.390).

Kidney mesangial IgA deposition was measured by immunofluorescence. Image analysis of the fluorescence revealed that DHA, EPA, and DHA + EPA significantly inhibited DON-induced IgA deposition in the kidney (Figure 3.6). DHA feeding more strongly inhibited IgA deposition than did EPA and DHA + EPA.

Study 2. Study 1 indicated that both DHA and EPA diet could attenuate induction of DON-induced IgAN. Since cytokine IL-6 is required for DON induced IgAN (Pestka and Zhou, 2000) and acute oral exposure to DON can induce IL-6 (Wong *et al.*, 2001; Moon and Pestka, 2003), a second study was conducted to verify the effects of feeding DHA/EPA lipid mix on DON-induced proinflammatory IL-6 expression. An acute dose of DON upregulated serum IL-6 within 3 hr and this effect was significantly ablated by the prior feeding of DHA /EPA. Serum IL-6 concentration in control and DHA/EPA groups were below the limit of detection (Figure 3.7).

Acute oral exposure of DON also induced a significant increase in spleen and Peyer's patches IL-6 mRNA in control-fed mice. Prior feeding with DHA/EPA impaired

this increase, which correlated with serum IL-6 concentration. IL-6 mRNA in control and DHA/EPA group was below detection limit for competitive RT-PCR (Figure 3.8).

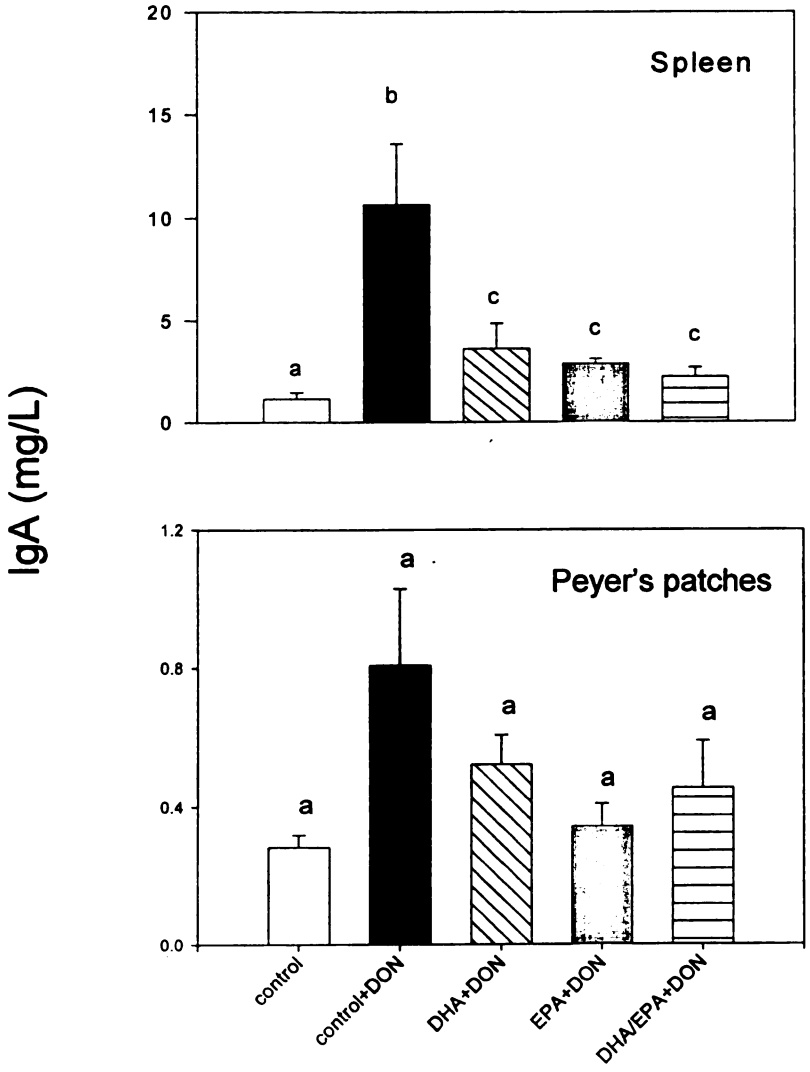

Figure 3.5. Supernantant IgA concentrations from cultures of spleen and Peyer's patch cells from male B6C3F1 mice fed modified AIN-93G diets containing DON and (n-3) PUFA. Mice were killed at 18 wk and spleen (5 x 10^9/L) and Peyer's patch cells (1 x 10^9/L) were cultured for 5 d. IgA was measured by ELISA. Values are means ± SEM (n = 10). Bars that do not have the same letter differ, (P < 0.05).

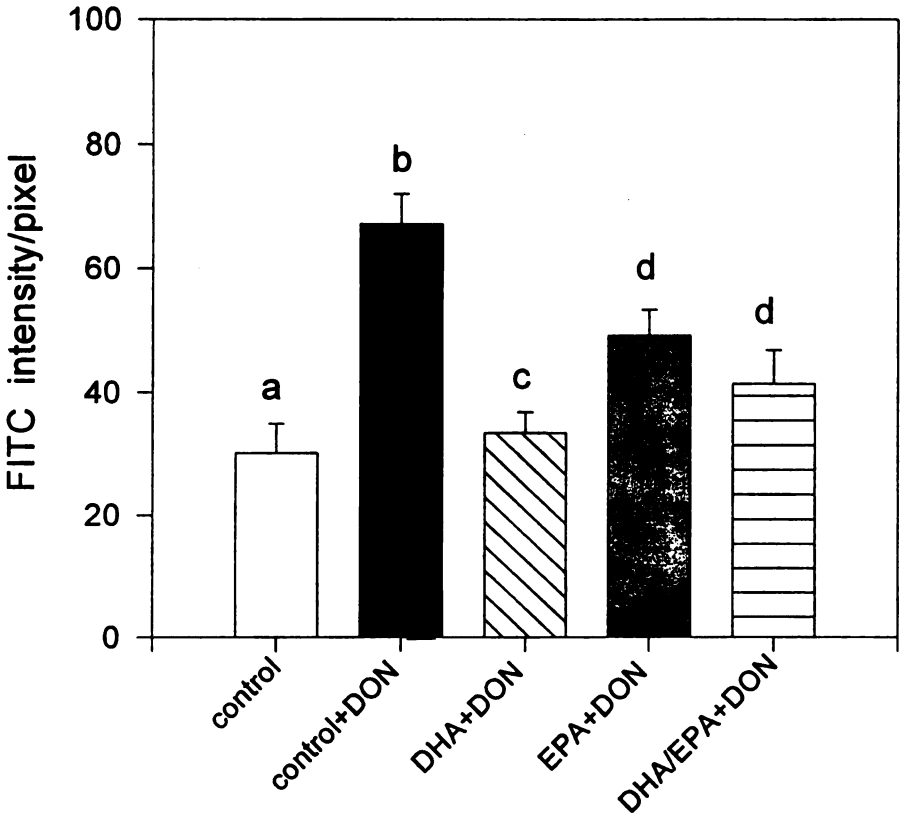

Figure 3.6 Mesangial IgA deposition in male B6C3F1 mice fed modified AIN-93G diets containing DON and (n-3) PUFA. Kidneys of mice fed experimental diets for 18 wk were cryostat sectioned and stained with fluorescein isthiocyanate–labeled anti-mouse IgA. Mesangial IgA quantitation was performed by image analysis. Values are mean ± SEM (n = 10). Bars that do not have the same letter differ, $P < (0.05)$.

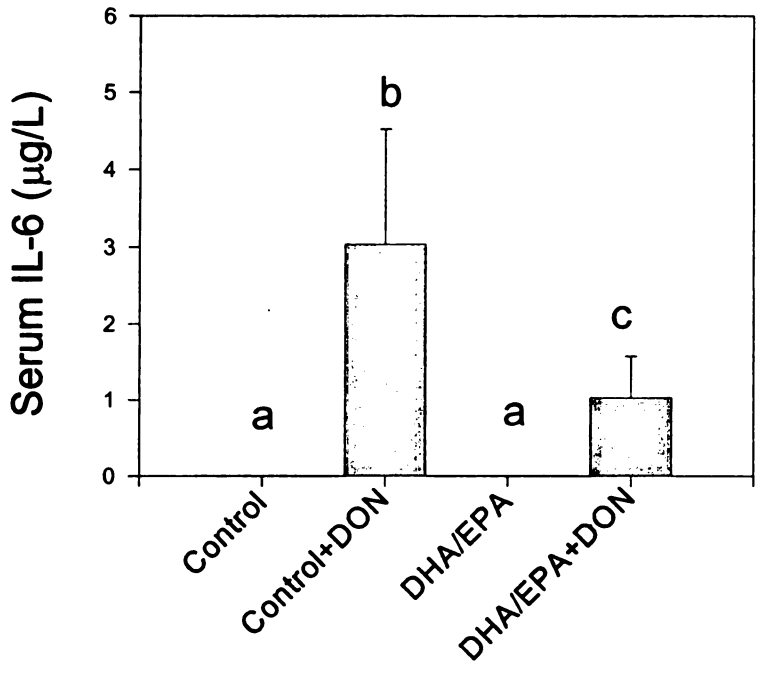

Figure 3.7 DHA/EPA effects on DON induced serum IL-6. B6C3F1 female mice were kept on diet containing 23g /kg DHA/EPA enriched fish oil for 8 wk. Mice in DON group was gavaged 25 mg/kg.BW DON at the end of the experiment. Serum was collected 3 h later and serum IL-6 was measured by ELISA. Values are mean ± SEM, n=5. Bars marked with different letter differ, ($p<0.05$).

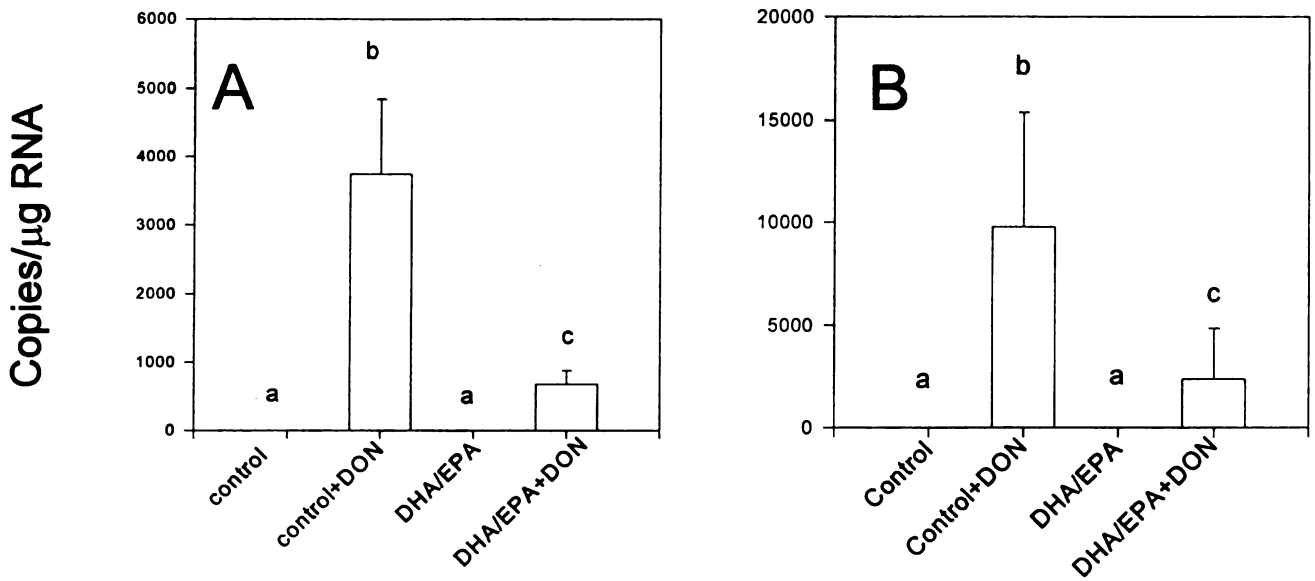

Figure 3.8. IL-6 mRNA expression in female B6C3F1 mice fed modified AIN-93G diets containing DHA and EPA lipid mix (Study 2). After 8 wk pretreatment, DON (25mg/Kg.Body.Weight) was gavaged and 3 h later total RNA were extracted from spleen and Peyer's patches. IL-6 mRNA level was measured by compeititive RT-PCR with truncated RNA internal standard. An aliquot PCR product was subjected to 1.5% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide (A) for spleen and (B) for Peyer's patches. The densitometric ratio of IL-6 cDNA/IL-6 internal standard was used to construct a standard curve to calculate mRNA concentration Values are mean ± SEM, n=5. Bars marked with different letter differ, ($p<0.05$).

DISCUSSION

Several clinical studies have shown that dietary fish oil has distinct beneficial effects in IgAN (Sethi *et al.*, 2002; Donadio 2001b; Grande and Donadio, 1998; Donadio *et al.*, 1994; Cheng *et al.*, 1990). The aim of this research was to test the effects of primary components of fish oil, DHA, EPA on the pathogenesis of DON-induced IgAN in mice.

DON-induced IgAN in mice provides a unique window for studying the mechanisms of IgAN and its treatments. In this study, serum IgA and IgA-IC were significantly increased after 9 wks DON treatment instead of 4 wks compared with the previous studies (Pestka *et al.*, 2002). The corn oil content in this research was reduced to 1% from 6%, which kept (n-6) PUFA on a lower but consist level in different experimental groups. Corn oil contains about 60% α-linoleic acid, the precursor of arachidonic acid, which can be metabolized into prostaglandin E_2 (PGE_2) in vivo. Reducing the corn oil concentration in the diet may result in a lower level of PGE_2, which may affect mucosal inflammation in the intestinal duct (Wiercinska-Drapalo *et al.*, 2001) and be responsible for the observed delayed increase of serum IgA. Nevertheless, significant DON-induced IgA and IgA-IC elevation was observed in both studies.

DON significantly retarded the weight gain and lowered the food intake throughout the feeding study, which confirmed the well-documented DON effects on food intake and weight gain. These effects are likely to be related to DON's effects on protein translation, proinflammatory cytokine superinduction (Pestka, 2003) and nutrient utilization (Maresca, 2002). Importantly, it was found that (n-3) PUFA did not attenuate or potentiate these effects.

The mechanism by which DON induces IgA dysregulation is still not clear. The gut mucosal compartment may be a primary target of DON (Pestka, 2003). Antigenic specificity studies had revealed that DON-induced serum IgA can react with DNA, sphingomyelin, thyroglobulin, collagen, casein and cardiolipin as well as bovine serum albumin conjugates of phosphorylcholine, insulin and trinitrophenol which collectively represent self and non-self antigens (Rasooly *et al.*, 1994; Rasooly and Pestka 1994). More than 80% of the monoclonal IgA derived from hybridomas of Peyer's patches from DON-fed mice bound to at least some diet antigens (Rasooly and Pestka, 1994), which suggests that dietary DON may promote the polyclonal activation of IgA-secreting B cell (Rasooly *et al.*, 1994; Rasooly and Pestka, 1992). Furthermore, serum IgA shifted from monomeric to primary polymeric IgA after DON treatment (Pestka *et al.*, 1989), which predominates in the gut. It is possible that DON impairs mucosal tolerance and promotes IgA production in response to food and self antigens (Pestka, 2003). DON might also distribute into the lymphoid tissues and promote IgA secretion directly by activating lymphocytes. As previously found, lymphocytes from both spleen and Peyer's patches secrete more IgA than that from control group, which were consistent with prior experiments (Greene *et al.*, 1994a,b; Pestka *et al.*, 1990b; 1989).

The diet used in this study was recommended by the American Institute of Nutrition for an optimized balance of essential nutrients for long-term studies with laboratory rodents (Reeves *et al.*, 1993). All diets in different groups contained 7% total lipid with the same percentage of n-6 and caloric density. During the past 30 years, the ratio of n-6 to (n-3) PUFA in diets has increased in industrialized societies because of the increased consumption of vegetable oils rich in (n-6) PUFA (Sanders, 2000). The recent

estimated ratio of (n-6) to (n-3) in diet is about 9.8:1, much higher than that deemed optimal (i.e., 2.3:1). A four-fold increase in fish consumption has thus been recommended (Kris-Etherton *et al*, 2000). Therefore the ratio of (n-6)/(n-3) for control and (n-3) PUFA treatment group were adjusted to 16/1 and 1/1.8 respectively in this study. We found that both DHA and EPA enriched oil blocked DON-induced serum IgA elevation and IgA deposition, suggesting (n-3) PUFA can block the progress of IgAN at an earlier stage.

IL-6 is required for DON-induced IgA dysregulation (Pestka and Zhou, 2000). It thus was a critical finding that pretreatment with DHA and EPA lipid mix for 8 wk significantly impaired DON-induced serum IL-6 and IL-6 mRNA level both in spleen and Peyer's patches. Blocking IL-6 production will block B cell proliferation in response to antigen stimulation and thus block the antibody production. So the inhibition of IL-6 by DHA and EPA may play an important role for its attenuation on IgAN induced by DON. The inhibition of IgA and IL-6 is (n-3) PUFA specific because (n-6) precursor, α-linoleic acid, in control plus DON group did not show any protective effects although it was converted into arachidonic acid efficiently in vivo.

Supplementation with (n-3) PUFA in the diet will modify the (n-6)/(n-3) in the membrane of lymphocytes (Calder *et al*, 2002). The modification of membrane phospholipids will change the eicosanoid profile such as the prostaglandin E2 (PGE2), leukotrienes B4 (LTB4) and secondary message molecules, diacylglycerol (DAG), ceramide, Ca2+, production in response to stimulations, which then can affect intracellular kinase activation including phospholipase A (PLA), PKC, and mitogen activated protein kinases (MAPKs). Macrophages from mice fed fish oil produce less PGE_2, thromboxane B (2) (TXB_2), and IL-6 in response to lipopolysaccharide (LPS)

stimulation (Yaqoob and Calder, 1995). DHA and EPA treatment can also prevent the recruitment of Ras protein to the membrane by changing the membrane structure and thus blocks the MAPK activation (Collett *et al.*, 2001). DHA can also affect protein palmitalation and prevent the recruitment of Src family kinases to the cell membrane, which generally cluster in a lipid raft through glycosylphosphatidylinositol and are essential for T cell activation (Webb *et al.*, 2000). Thus, cell membrane modification might play an important role for (n-3) PUFA effects on the immune system activation and IgA production.

DHA caused a significantly stronger inhibition on IgA deposition than that of EPA and DHA/EPA with a similar trend being found for serum IgA-IC. The difference between DHA and EPA may due to their differences in structure and the metabolism. The release of DHA from plasma membrane was almost negligible compared to AA and EPA under the action of cPLA2 and thus reduces arachidonic acid (AA) and platelet-activating factors (PAF) production in response to stimulations (Shikano *et al.*, 1994).

Taken together, the results suggest that both DHA and EPA ameliorated the DON-induced IgA dysregulation and these effects might be related to a reduced capacity for IL-6 production. Further insight is required into the molecular mode of action of (n-3) PUFAs in blocking IgA dysregulation as well as the role of IL-6 and other cytokines.

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CHAPTER 4

Dose-Dependent Suppression of Experimental Immunoglobulin A Nephropathy by Docosahexaenoic Acid: Relation to Interleukin-6 Expression, Cyclooxygenase-2 and Mitogen-Activated Protein Kinase Phosphorylation

ABSTRACT

The purpose of this investigation was to test the hypotheses that docosahexaenoic acid (DHA) dose-dependently attenuates induction of IgA nephropathy (IgAN) in mice by the mycotoxin deoxynivalenol (DON) and that DHA's effects correspond to suppressed proinflammatory gene expression and mitogen-activated protein kinase (MAPK) activation. Consumption of a modified AIN-93G diet containing 2,10 and 60 g/kg DHA-enriched oil resulted in dose-dependent increases of DHA in liver phospholipids with concomitant decreases in arachidonic acid (AA) as compared to control diets. DHA dose-dependently inhibited increases in serum IgA and IgA immune complexes (IC) as well as IgA deposition in the kidney in DON-fed mice with the 60 g/kg DHA diet having the earliest detectable effects and maximal efficacy. Both splenic interleukin-6 (IL-6) mRNA and heteronuclear nuclear RNA (hnRNA), an indicator of IL-6 transcriptional activity, were significantly reduced in DON-fed mice that consumed 10 and 60 g/kg DHA with a similar trend being observed for cyclooxygenase (COX-2) mRNA. In a subsequent study, acute DON exposure (25 mg/kg body weight) was found to induce splenic IL-6 mRNA and hnRNA as well as COX-2 mRNA in mice fed control diet while induction of both RNA species was significantly inhibited in mice fed 60 g/kg DHA. These latter inhibitory effects corresponded with a reduction of DON-induced phosphorylation of p38, extracellular signal regulated protein kinases (ERK) 1/2 and c-Jun N-terminal kinases (JNK) 1/2 in the spleen. Taken together, the results indicate that DHA dose-dependently inhibited DON-induced IgAN, and that impairment of MAPK activation and expression of COX-2 and IL-6 are potential critical upstream mechanisms for attenuation by DHA.

INTRODUCTION

Immunoglobulin A nephropathy (IgAN), the most common form of human primary glomerulonephritis, has marked kidney mesangial IgA deposition as its diagnostic hallmark (Donadio and Grande, 2002). Children and young adults are mainly affected by IgAN (Galla, 1995) with 20-40% developing end stage renal disease (Endo, 1997). High serum IgA and IgA immune complex (IgA-IC) concentration are potential early contributory factors for IgAN (Endo, 1997; Feehally, 1997) and can induce proliferation and cytokine production when they bind to receptors on mesangial cells (Monteiro and Van De Winkel, 2003). Deposition of dimeric and polymeric IgA might also activate complement via the alternative pathway, causing glomerular damage (Floege and Feehally, 2000).

The ratio of (n-6) to (n-3) polyunsaturated fatty acids (PUFAs) present in diet has increased in industrialized countries over the last three decades and has been suggested to contribute etiologically to increased risk of many chronic diseases (Sanders, 2000 and Simopoulo, 2003). Notably, the recent estimated ratio of (n-6) to (n-3) consumption in the United States is about 9.8:1, which is much higher than recommended (2.3:1) (Kris-Etherton *et al*, 2000). Dietary supplementation with (n-3) PUFAs, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), might have potential human health benefits particularly with regard to inflammatory diseases (Calder, 2003; Gil, 2002). Wakai *et al.* (1999) reported in a Japanese case control study that dietary (n-3) PUFAs were negatively associated with the risk of IgAN. In a follow-up study, this research group found that high intake of (n-6) PUFAs was associated with increased risk of IgAN (Wakai *et al.*, 2002). Holman *et al.* (1994) further demonstrated that some IgAN

patients were deficient in α-linolenic acid [18:3 (n-3)], a precursor of DHA and EPA, and, that supplementation with EPA and DHA suppressed arachidonic acid (AA) synthesis and decreased proteinuria and improved glomerular filtration rate in these persons. Consistent with these findings, several clinical trials have demonstrated that (n-3) PUFAs from fish oil retarded renal disease progression in IgAN patients by reducing inflammation and glomerulosclerosis (Grande and, 2001a; Donadio, 2000; Donadio, 2001b).

Deoxynivalenol is a trichothecene mycotoxin produced by *Fusarium graminearum* that is often encountered in cereal grains (Pestka and Smolinski, 2005). Trichothecenes bind to eukaryotic ribosomes, which inhibits protein translation as well as induces multiple stress signaling pathways that involve the mitogen-activated protein kinases (MAPKs). Immunotoxicological studies have revealed that mice chronically exposed to 2 to 25 mg/kg DON in diet have elevated serum IgA and IgA-IC concurrently with mesangial IgA deposition in kidneys, thus mimicking the early stages of human IgAN (Pestka *et al.*, 1989; Dong *et al.*, 1991; Dong and Pestka, 1993).

The mucosal immune system appears to be a primary target of DON (Pestka, 2003). DON disrupts mucosal tolerance and promotes production of IgA in response to antigens found in food and commensal bacteria (Rasooly and Pestka, 1992). DON upregulates proinflammatory gene expression, most notably IL-6, both in vivo and in vitro (Azcona-Olivera *et al.*, 1995; Zhou *et al.*, 1997; Zhou *et al.*, 1999; Zhou *et al.*, 2003; Sugita-Konishi and Pestka, 2001). A plausible role for IL-6 in DON-induced IgAN is supported by ex vivo studies (Yan *et al.*, 1997; 1998) and by the observation that IL-6 deficient mice resist DON-induced serum IgA elevation and mesangial IgA deposition

(Pestka and Zhou, 2000). Cyclooxygenase-2 (COX-2) potentially modulates mucosal immunity by catalyzing conversion of AA to prostaglandins (PGs), which, in turn, induce IL-6 expression in macrophages (Kim *et al.*, 2001; Bagga *et al.*, 2003). Since DON induces COX-2 (Moon and Pestka, 2002), this enzyme might also contribute to induction of IgAN by promoting expression of IL-6 and other proinflammatory cytokines. In support of this contention, we have recently verified that DON-induced COX-2 expression and prostaglandin E_2 (PGE_2) contributes, in part, to IL-6 induction by this mycotoxin (Moon and Pestka, 2003 a).

Our laboratory has reported that consumption of menhaden fish oil attenuates induction of IgAN in mice fed DON (Pestka *et al.*, 2002) as well as induction of IL-6 following acute DON exposure (Moon and Pestka, 2003b). In the latter study, fish oil ingestion also suppressed DON-induced phosphorylation of extracellular signal regulated protein kinases (ERK) 1/2 and c-Jun N-terminal kinases (JNK) 1/2, which are critical upstream MAPK regulators of IL-6 expression. Recently, Jia *et al.* (2004) observed that consumption of DHA-enriched fish oil and, to a lesser extent, EPA-enriched fish oil at 20 g/kg significantly impaired DON-induced serum IgA elevation and IgA deposition, whereas high α-linolenic acid-containing flax seed oil did not. The purpose of this study was to test the hypotheses that DHA dose-dependently attenuates DON-induced IgAN in mice and that DHA's effects correspond to attenuation of IL-6 and COX-2 gene expression as well as MAPK activation.

MATERIALS AND METHODS

Materials. All chemicals (reagent grade or better) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. DON was produced in *Fusarium graminearum* R6576 cultures and purified by silica gel chromatography (Clifford *et al.*, 2003). Purity of DON was verified by a single HPLC peak occurring at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware contaminated with the mycotoxin were detoxified by soaking for at least 1 h in 100 ml/L sodium hypochlorite. Purified DON was added to powdered diets as detailed by Pestka *et al.* (1989).

Animals. Female B6C3F1 mice (7 wk old), weighing between 20 to 25 g were obtained from Charles River (Portage, MI). Mice were housed in environmentally protected transparent polypropylene cages with stainless steel wire tops for 1 wk prior to introduction of different treatments. Mice were given free access to water and food. Cages were filter-bonneted and kept in a laminar flow cage rack under negative pressure. Experimental diets were placed in feed jars designed to minimize spillage. The environmental conditions included 23 to 25 °C, 45 to 55% relative humidity, and 12:12 h artificial photoperiod. Housing, handling and sample collection procedures conformed to the policies and recommendations of the Michigan State University All University Committee on Animal Research and were in accordance with guidelines established by the National Institute of Health.

Diets and experimental design. Experimental diets were derived from the purified AIN-93G formulation of Reeves *et al.* (1993). The basal diet consists of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix,

200 g casein, 397.5 g cornstarch, 132 g dyetrose (dextrinized cornstarch), 50 g cellulose, 3 g L-cysteine, 2.5 g choline bitartrate, 14 mg TBHQ, 100 g sucrose (Dyets Inc., Bethlehem, PA) and 70 g oil.

In Study 1, corn oil (Dyets Inc), oleic acid (Dyets Inc) and DHA enriched oil (containing DHA 483 g/kg and 113 g/kg EPA)(Ocean Nutrition, Bedford, Nova Scotia) were used to amend the basal diet to yield five experimental diet groups (n=9): Control, Control+DON, 2 g/kg DHA+DON, 10 g/kg DHA+DON and 60 g/kg DHA+DON (Table 4.1). Diets were prepared every 2 wk, stored at -20 °C and provided fresh to mice each day. Mice were fed diet for 16 wk. Body weight and food intake were monitored weekly. Blood was collected every 4 wk from the saphenous leg vein (Hem *et al*, 1998) and analyzed for IgA and IgA-IC. At wk 16, mice were anesthetized with methoxyfluorane and killed by cervical dislocation. Peyer's patches were removed aseptically and used to prepare cell cultures. Spleens were removed for total RNA extraction and IL-6 mRNA, hnRNA and COX-2 mRNA determination. Kidneys of each mouse were removed for immunofluorescence examination. The liver was used as a surrogate for assessing (n-3) PUFA incorporation in cellular phospholipids.

In Study 2, DHA-enriched fish oil was used to amend AIN-93G basal diet to yield 3 experimental diet groups (n=3): Control, Control+DON and 60 g/kg DHA+DON (Table 4.2). Mice were fed DHA enriched oil for 4 wk and then orally gavaged with a single DON dose (25 mg/kg body weight) at the end of experiment. After 3 h, mice were anesthetized with methoxyfluorane, killed by cervical dislocation, and splenic IL-6 mRNA, hnRNA and COX-2 mRNA expression were quantified.

Table 4.1. Experimental groups for assessing DHA enriched oil on DON-induced IgAN (Study 1)

Group	Animal No.	DON (g/kg)	Corn oil (g/kg)	Oleic acid (g/kg)	DHA enriched oil[2] (g/kg)	n-6 (g/kg)	Total n-3 (g/kg)	n-6/n-3
Control	9	0	10	60	0	8.3	0.18	46/1
Control+DON	9	0.02	10	60	0	8.3	0.18	46/1
0.2% DHA+DON	9	0.02	10	58	2	8.3	1.5	5.5/1
1%DHA+DON	9	0.02	10	50	10	7.9	6.9	1/1.1
6%DHA+DON	9	0.02	10	0	60	7.9	36.1	4.5/1

[1] All diets will be adjusted with oleic acid to have final oil content of 70 g/kg.
[2]DHA is fish oil enriched with DHA, containing DHA 483 g/kg and EPA 113 g/kg.

Table 4.2. Experimental groups for assessing DHA enriched oil on DON-induced IgAN (Study 2)

Group	Animal No.	Corn oil (g/kg)	Oleic acid (g/kg)	DHA enriched oil (g/kg)	n-6 (g/kg)	n-3 (g/kg)	n-6/n-3
Control	3	10	60	0	8.3	0.18	46/1
Control+DON	3	10	60	0	8.3	0.18	46/1
6%DHA+DON	3	10	0	60	7.9	36.1	1/4.5

[1] All diets will be adjusted with oleic acid to have final oil content of 70 g/kg.
[2]DHA is fish oil enriched with DHA, containing DHA 483 g/kg and EPA 113 g/kg.

In Study 3, the experimental design for feeding and DON exposure were identical to Study 2, except that each group contained 5 mice. Mice were euthanized and spleen was removed 30 min after DON gavage as described by Zhou *et al.* (2003). Splenic MAPKs were analyzed by Western blotting.

Measurement of IgA and IgA-IC. Serum IgA was measured by ELISA (Dong *et al.*, 1991) using mouse reference immunoglobulin serum (Bethyl Laboratories, Montgomery, TX), goat anti-mouse IgA (heavy chain specific) and peroxidase-conjugated goat IgG fraction to mouse IgA (Organon Teknika, West Chester, PA). To quantify IgA-IC, diluted plasma samples were first precipitated by 70g/L polyethylene glycol (PEG 6000; Sigma) (Imai *et al.*, 1987) and quantified by IgA ELISA (Dong *et al.*, 1991). Absorbance at 450 nm was measured with a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) and analyte concentrations calculated using SoftMax (Molecular Devices).

Assessment of kidney mesangial IgA deposition. Kidney sections were prepared and analyzed for IgA deposition according to Pestka *et al.* (1989). Briefly, kidneys were frozen, sectioned to 7 μm with a cryostat (Reichert-Jung Cambridge Instruments, Buffalo, NY) and stained for IgA deposition with fluorescein isothiocyanate-labeled goat anti-mouse IgA (Sigma). IgA immunofluorescence was assessed with a Nikon Labophot microscope (Melville, NY) equipped with a digital camera. Three sections were analyzed per mouse. Three glomeruli from each section were randomly selected and mean fluorescence intensity was determined using UTHSCSA Image Tool Software V 1.2. (UTH SCSA Image Tool, 2004). Pixels in the designated areas were measured on a scale from 0 (black) to 255 (white).

Cell culture. Peyer's patch cell cultures were prepared as described previously (Pestka *et al.*, 2002). Briefly, tissues were passed though a sterile 100-mesh stainless screen in harvest buffer. Cells (1 x 10^9/L) from individual mice were cultured separately in 1 ml of medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific, Corning, NY) at 37 C under 7% CO_2 in a humidified incubator. Supernatants were collected after 5 d and stored in aliquots at -20 °C for IgA analysis.

Real-time polymerase chain reaction. Total RNA was extracted from mouse spleens using Trizol reagent (Life Technologies, Gaithersburg, MD) and RNease Min Elute Cleanup Kit (Qiagen, Valencia, CA). IL-6 mRNA, IL-6 hnRNA and COX-2 mRNA expression were measured by Real Time PCR. Probe and primers for mouse IL-6 mRNA and endogenous control (18S RNA) were purchased as TaqMan assay reagents (PE Applied Biosystems, Foster City, CA). Taqman Universal PCR Master Mix (PE Applied Biosystems) was used to quantify IL-6 and 18S RNA following manufacturer's instructions on an ABI Prism 7700 (PE Applied Biosystems). Real-time Polymerase Chain Reaction Primer Express software (PE Applied Biosystems, Foster City, CA) was employed to design primer pairs for mouse IL-6 hnRNA (forward primer: gtcc aac tgt gct atc tgc tca ct; backward primer: aga agg caa ctgg atg gaa gtc t) and COX-2 mRNA (forward primer: cag aac cgc att gcc tct g; backward primer: agc tgta ctc ctg gtc ttc aat gtt). SYBER Green PCR Master Mix (PE Applied Biosystems) was used to detect IL-6 hnRNA and COX-2 mRNA. 18S RNA was used to normalize target gene expression. Target gene expression levels were calculated relative to the control group.

Lipid extraction and analysis. Fatty acids in liver phosphospholipids were analyzed by a modification of the method of Hasler *et al.* (1991) as described by Jia *et*

al. (2004) using a GC-2010 Gas Chromatograph (Shimadzu Scientific Instruments, Chicago, IL). Fatty acid profiles were identified by comparing the retention times with those of the appropriate standard fatty acid methyl ester (Nu-Check Prep. Inc, Elysian, MN).

Western analysis. Proteins were fractionated by SDS-PAGE using a 10% (w/v) acrylamide separation gel, and then analyzed by Western blotting using antisera specific for p42/44 (ERK 1/2), phospho-p42/44, p46/54 (c-jun N-terminal kinase, JNK 1/2), phospho p46/54, p38 and phospho-p38 (Cell Signaling, Beverly, MA) in conjunction with an Enhanced Chemiluminescence Kit (from Amersham Biosciences, Piscataway, NJ) as described by Zhou *et al.* (2003). Relative phosphorylation was measured with Kodak ID Image Analysis Software (New Haven, CT) and normalized against expression of non-phosphorylated forms of these MAPK families.

Statistics. Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. If data were not normally distributed, they were subjected to Kruskal-Wallace ANOVA on Ranks and pairwise comparisons made by Dunn's or Student-Newman-Keuls methods. Differences were considered significant at $P < 0.05$.

RESULTS

Study 1. The effects of feeding different concentrations of DHA-enriched oil on DON-induced IgAN were assessed. As found previously (Pestka *et al.*, 2002), DON at 20 mg/kg in diet significantly reduced daily food intake (Figure.4.1) and impaired body weight gain (Figure.4.2) compared to control group over the 16 wk period. Consumption of DHA-enriched oil did not significantly modulate these effects.

The liver was used as a surrogate to determine how tissue phospholipid composition was modified by consumption of the DHA-enriched oils for 16 wk. Results from gas chromatography revealed that DON alone had no effects on concentration of AA, DHA, EPA or other fatty acids analyzed. In livers of mice fed diets containing 2, 10 and 60 g/kg DHA-enriched oil, DHA concentration increased to 5.0, 6.5, and 9.7 g/100g phospholipid respectively, as compared to 1.9 g/100g in control group ($p<0.05$) (Table 4.3). EPA concentrations were 0.22, 1.6 and 4.1 g/100g lipid respectively, compared to 0.1 g/100g in control group ($p<0.05$). AA concentrations decreased to 7.1, 4.0. 2.4 g/100g lipid respectively, compared to 9.9 g/100g in control group ($p<0.05$). Linoleic acid [(18:2) (n-6)] concentrations were also decreased in 60 g/kg DHA group. Oleic acid [18:1] concentrations were lower in the DHA-enriched oil groups than control group ($p<0.05$), which is likely a reflection of the reduced concentrations of this monosaturated fatty acid fed to DHA groups. Palmitic acid [16:0] dose-dependently increased with increasing DHA-enriched oil concentration. Thus, all three DHA diets were effective at increasing (n-3) PUFA content in tissue while simultaneously decreasing AA.

Serum IgA in control + DON group rose significantly at wk 8 and thereafter was increased 9-fold over control group at wk 16 ($p<0.05$) (Figure 4.3). DHA- enriched oil at

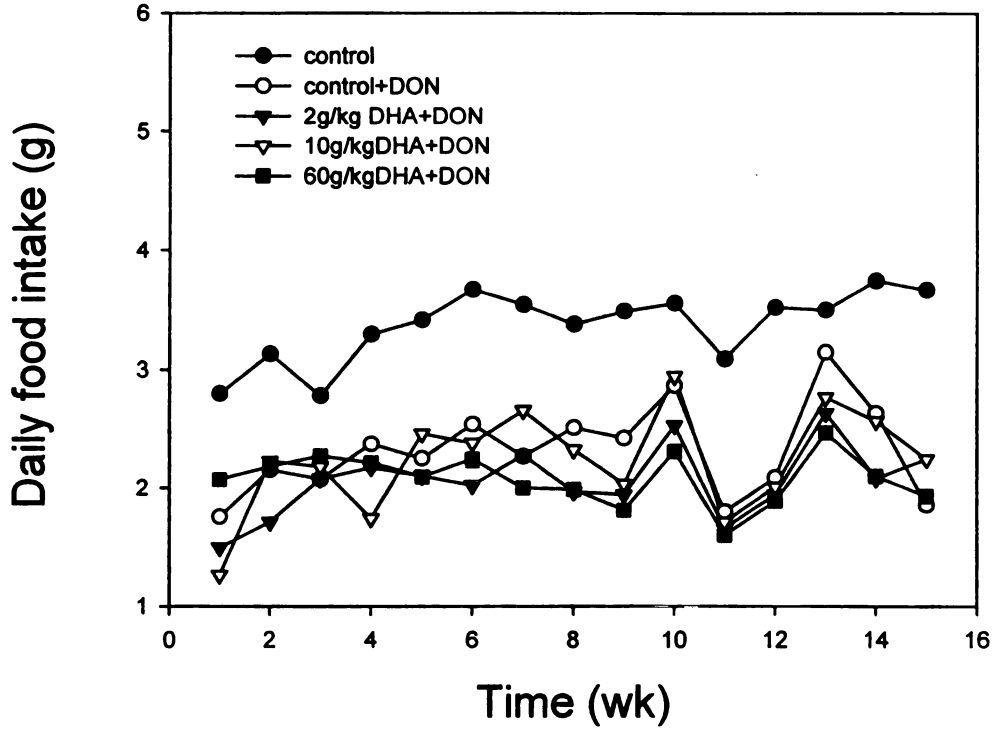


Figure 4.1 Effects of dietary DHA on food intake changes in mice induced by AIN93G diets containing DON (20 mg /kg) over 16 wk (Study 1). Food intake was measured once per week for each cage, 3 mice per cage. Values are mean for a group (n=3).

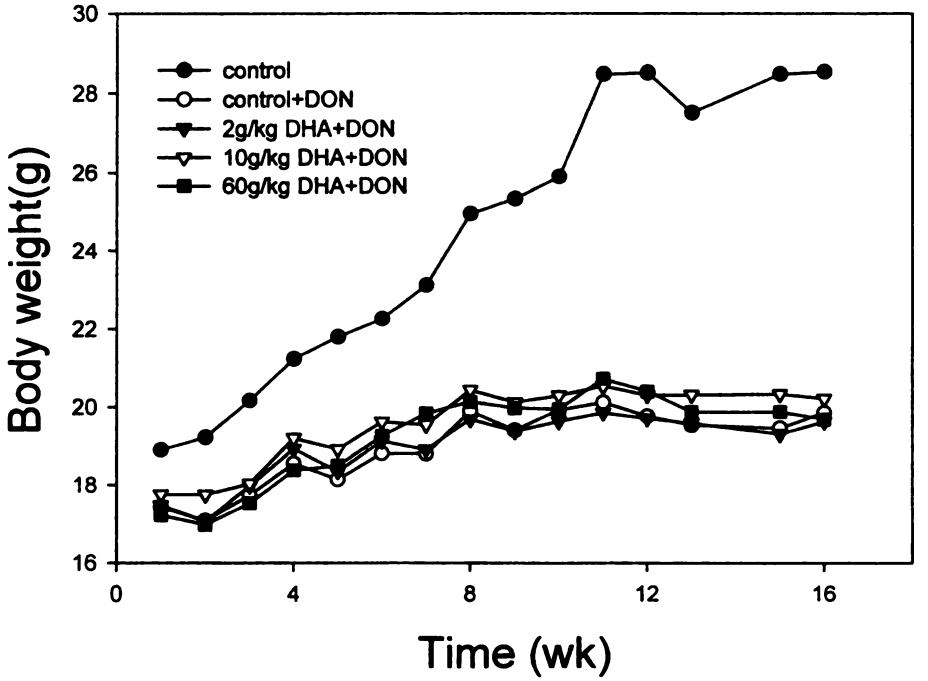


Figure. 4.2 Effects of dietary DHA on body weight changes in mice induced by AIN-93G diets containing DON (20 mg/kg) over 16 wk (Study 1). Body weight was measured once per week. Values are mean for a group (n=9).

Table 4.3 . Fatty acid composition of liver phospholipid in study 1(n=6).

	AA	EPA	DHA	C 16:0	C 18:0	C 18:1	C 18:2
Control	9.93±1.70^{a}	0.09±0.06^{a}	1.90±1.04^{a}	26.80±1.15^{a}	14.86±0.37^{a}	22.13±1.01^{a}	5.93±0.42^{a}
Control +DON	9.55±1.60^{a}	0.17±0.07^{a}	1.68±0.75^{a}	25.50±0.80^{a}	14.70±0.38^{a}	21.30±0.80^{a}	6.38±0.37^{a}
2 g/kg DHA+ DON	7.06±1.08^{b}	0.22±0.07^{a}	5.04±0.63^{b}	32.40±0.70^{b}	16.70±0.65^{b}	17.00±0.42^{b}	6.52±0.45^{a}
10g/kg DHA+DON	3.95±0.49^{c}	1.60±0.26^{b}	6.45±0.79^{c}	32.0±1.33^{b}	15.90±0.35^{a}	17.37±0.48^{b}	5.72±0.30^{a}
60 g/kg DHA + DON	2.37±0.38^{d}	4.10±0.49^{c}	9.67±0.66^{d}	36.80±0.59^{c}	19.10±0.59^{c}	9.62±0.40^{c}	4.62±0.25^{b}

Only the major fatty acids are shown. The value stands for the percentage of total phospholipid extracted from liver. Different letter within a column indicates significant difference, $P<0.05$. DHA docosahexaenoic acid; EPA eicosapentaenoic acid; AA arachidonic acid.

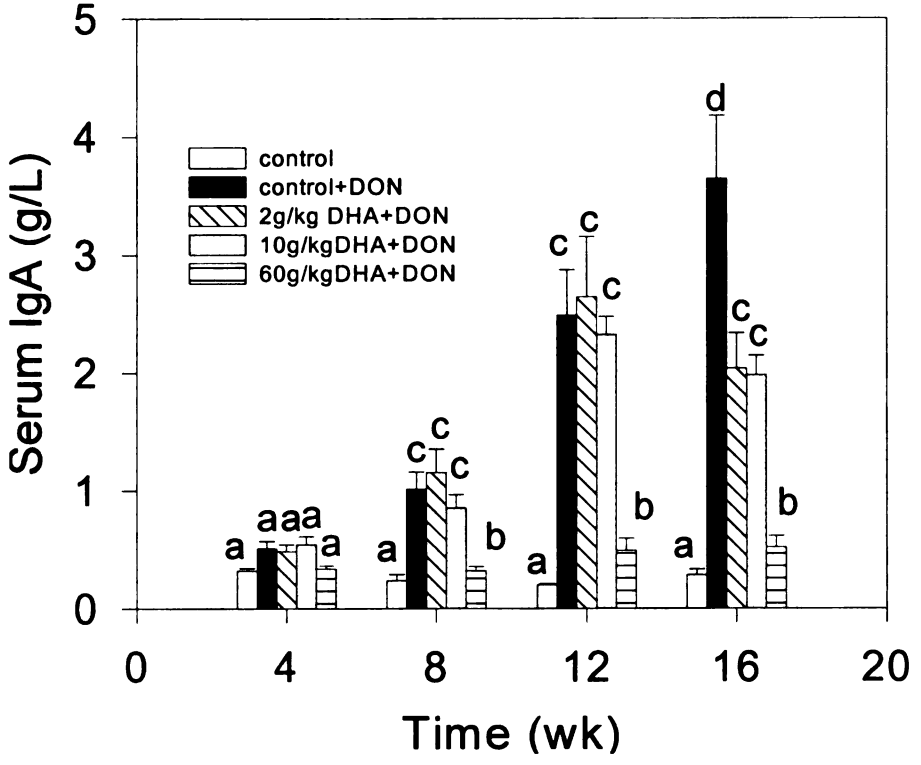


Figure. 4.3 Effects of dietary DHA on serum IgA in mice fed AIN-93G diets containing DHA and DON (20 mg/kg) over 16 wk (Study 1). Serum IgA was measured by ELISA; Values are means ± SEM (n = 9). Bars that do not have the same letter differ, ($P < 0.05$).

60 g/kg significantly attenuated DON-induced serum IgA elevation as early as 8 wk, whereas suppression by diets containing 2 g and 10 g/kg DHA were first detectable at wk 16 ($p<0.05$). DHA-enriched oil at 60g/kg also significantly suppressed serum IgA-IC elevation after 16 wk, but 2 and 10g/kg DHA had no effect (Figure 4.4).

Peyer's patches play important roles in IgA responses to mucosal antigens and respond to DON feeding with increased total IgA secretion (Pestka, 2003). The effects of DHA-enriched oil on ex vivo IgA secretion from Peyer's patches in DON-fed mice were therefore assessed. Supernatant IgA concentrations in cultures from DON-fed mice tended to be higher than that of controls although were not significantly different ($p=0.44$) (Figure 4.5). Feeding mice with DHA-enriched oil at 60 g/kg significantly reduced ex vivo IgA secretion as compared to the control plus DON group ($p<0.05$). Mice fed 10 g/kg DHA-enriched oil showed a strong trend for reduced IgA secretion when compared to control plus DON group ($p=0.06$).

When kidney sections were analyzed by immunofluorescence, image analysis revealed that DON significantly induced IgA deposition compared to control ($p<0.05$) (Figure 4.6). As with IgA-ICs, DHA-enriched oil at 60 g/kg significantly blocked IgA deposition ($p<0.05$) and DHA-enriched oil at 10 g/kg exhibited a similar trend ($p=0.13$). Consumption of 2 g/kg DHA had no effect as compared to control plus DON group.

The effects of DHA on expression of IL-6 and COX-2 mRNA were also determined. Relative IL-6 mRNA expression in spleens of the DON-fed control group was not significantly different from control group (Figure 4.7A). However, IL-6 mRNA was significantly reduced in mice fed DON with 10 or 60 g/kg of DHA-enriched oil as compared to DON-fed control mice ($p<0.05$). Analogous effects were found for IL-6

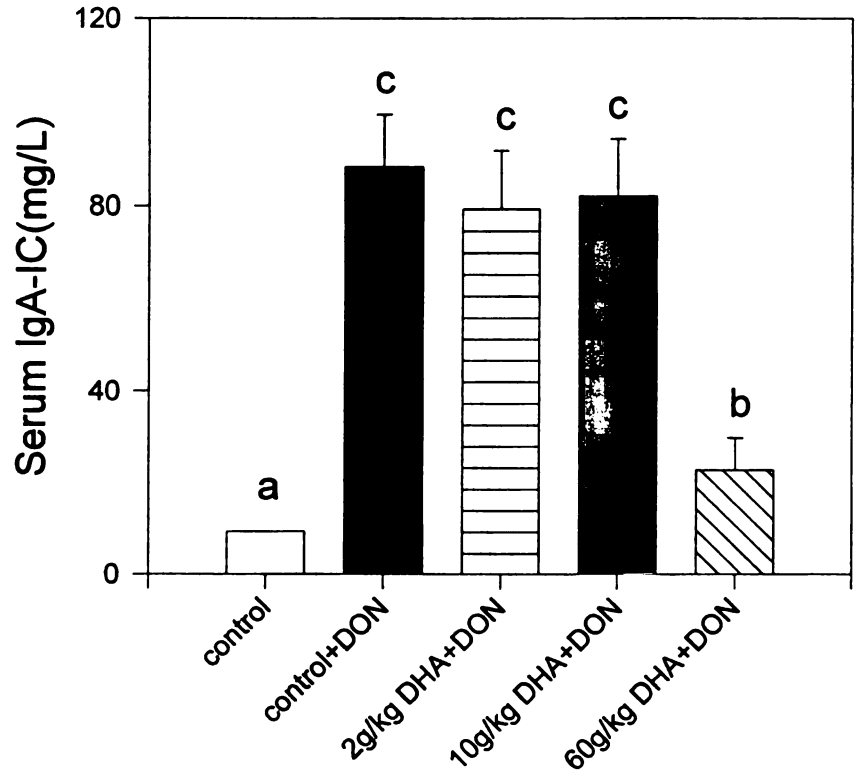


Figure. 4.4 Effect of dietary DHA on serum IgA-IC in mice fed modified AIN-93G diets containing DHA and DON (20 mg/kg) over 16 wk (Study1). Mice were bleed at 16 wk. Serum IgA-IC was precipitated by PEG and measured by IgA ELISA. Values are means ± SEM (n = 9). Bars that do not have the same letter differ, ($P < 0.05$).

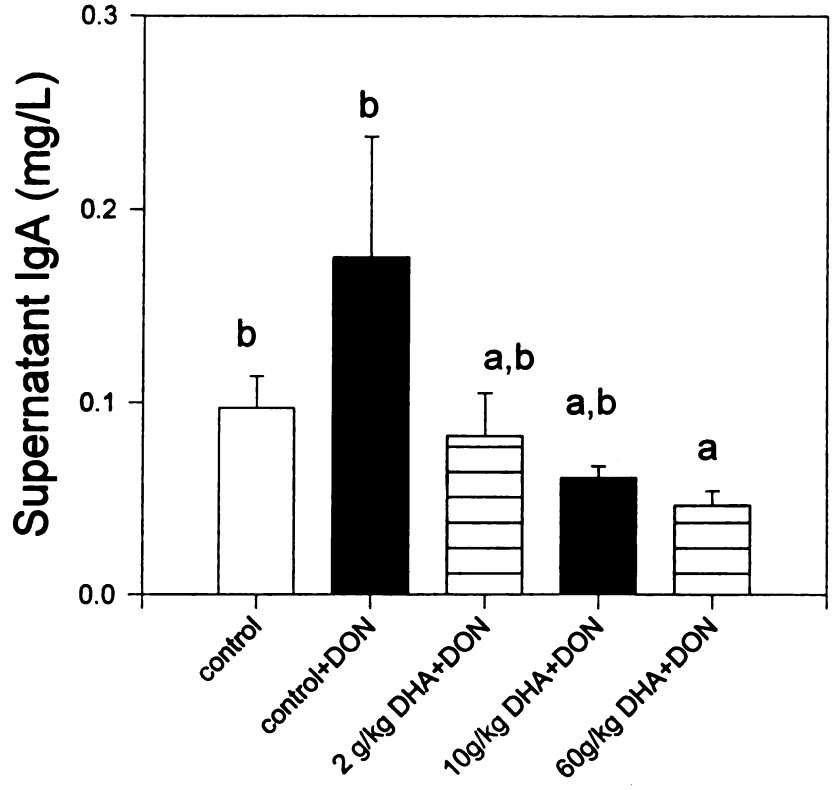


Figure. 4.5 Effects of dietary DHA on ex vivo IgA production in mice fed modified AIN-93G diets containing DHA and DON (20 mg/kg) over 16 wk (study1). Peyer's patch cells (1 x 10^9/L) were cultured for 5 day. IgA was measured by ELISA. Values are means ± SEM (n = 9). Bars that do not have the same letter differ, ($P < 0.05$).

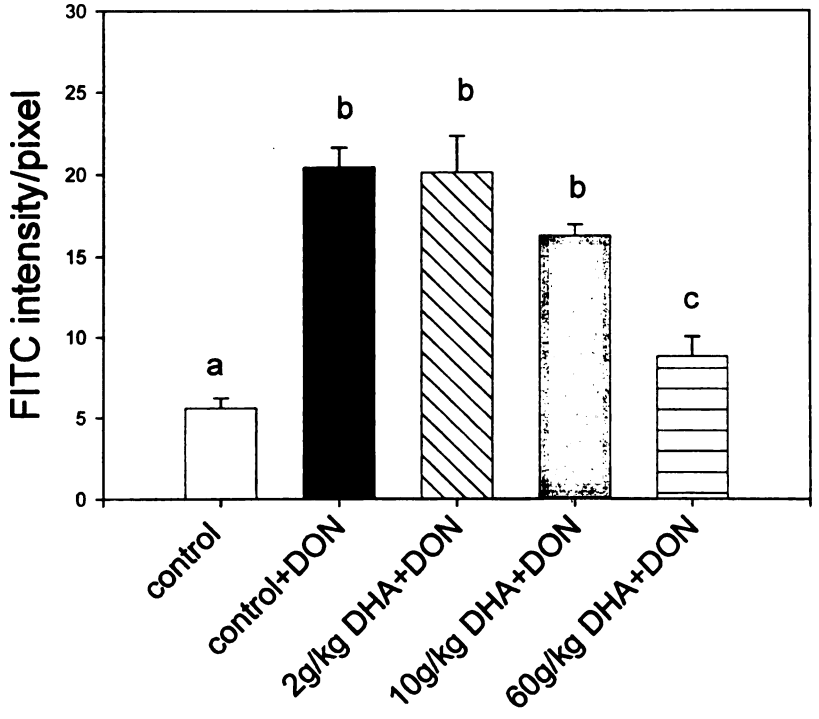


Figure. 4.6 Effects of dietary DHA on mesangial IgA deposition in mice fed modified AIN-93G diets containing DON (20 mg/kg) over 16 wk (Study1). Kidneys were cryostat sectioned and stained with fluorescein isthiocyanate–labeled anti-mouse IgA. Mesangial IgA quantitation was performed by image analysis. Values are mean ± SEM (n =9). Bars that do not have the same letter differ, ($P < 0.05$).

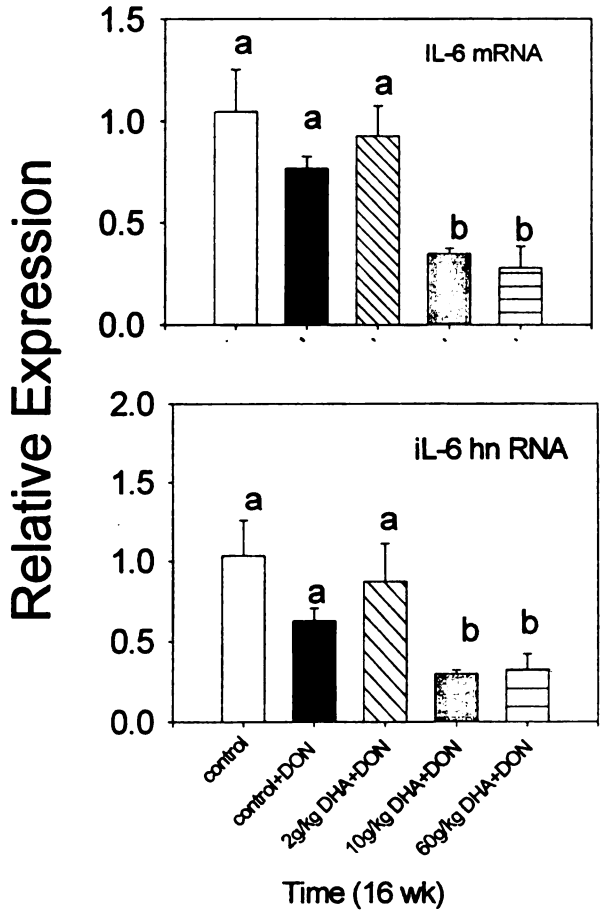


Figure 4.7 Effects of dietary DHA on spleen IL-6 mRNA and hnRNA expression in mice fed with AIN93G diet containing DHA and DON (20 mg/kg) over 16 wk (Study 1). At experiment termination, spleen RNA was extracted and analyzed by real-time PCR . Data were normalized against 18S RNA and expressed relative to control value. Values are mean ± SEM, n=9. Bars with different letters differ, ($p<0.05$).

hnRNA , an indicator of gene transcription activity (Figure 4.7B). There were also trends toward increased splenic COX-2 mRNA expression in DON-fed control mice ($p= 0.12$) compared to control mice as well as suppression by 60 g/kg DHA (Figure 4.8). COX-2 mRNA expression in the 60 g/kg DHA group was also significantly lower than the 2 and 10 g/kg groups ($p<0.05$).

Study 2. To further evaluate effects of the optimized DHA-enriched oil feeding regimen (60 g/kg) on DON-induced IL-6 and COX-2 expression, mice were fed control diet or 60 g/kg DHA for 4 wk and then treated with a single oral dose of DON (25 mg/kg body weight). When liver phospholipid profiles were measured to verify DHA incorporation into the plasma membrane after 4 wk exposure (Table 4.4), the data confirmed that 60 g/kg DHA significantly reduced AA and increased DHA and EPA content in similar fashion to that observed in mice fed DHA for 16 wk.

Acute DON exposure significantly induced IL-6 mRNA and hnRNA in spleen after 3 h ($p<0.05$) (Figure 4.9). Feeding 60 g/kg DHA-enriched oil significantly suppressed induction of IL-6 mRNA and hnRNA in spleen by 54% and 71% respectively ($p<0.05$). DHA's effects on COX-2 mRNA level were also assessed. As with IL-6, COX-2 mRNA was markedly induced 3 h after DON treatment ($p<0.05$) (Figure 4.10.). Consumption of DHA (60g/kg) for 4 wk inhibited DON-induced COX-2 mRNA expression by 64% ($p<0.05$).

Study 3 Western analysis was employed to determine if DHA feeding impairs DON-induced phosphorylation of ERK, JNK and p38 (Figure 4.11). The results indicated that 30 min after acute DON treatment, phosphorylation of p38, ERK 1/2, and JNK 1/2

was significantly induced ($p<0.05$). Consumption of 60 g/kg DHA significantly suppressed DON-induced activation of all three MAPK families ($p<0.05$).

Figure. 4.8 Effects of dietary DHA on spleen COX-2 mRNA expression in mice fed with AIN93G diet containing DHA and DON (20 mg/kg) over 16 wk (Study 1). At experiment termination spleen RNA were extracted and analyzed by real-time PCR. Relative expression was determined following normalization with 18S RNA internal standard. Data were expressed relative to control group. Values are mean ± SEM, n=6. Bars with different letters differ, ($p<0.05$).

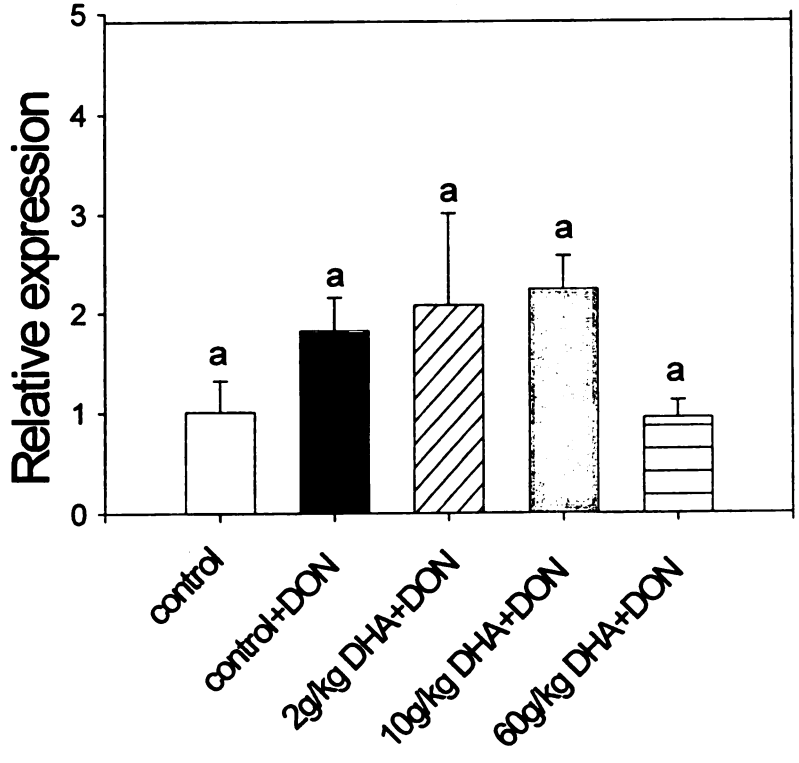
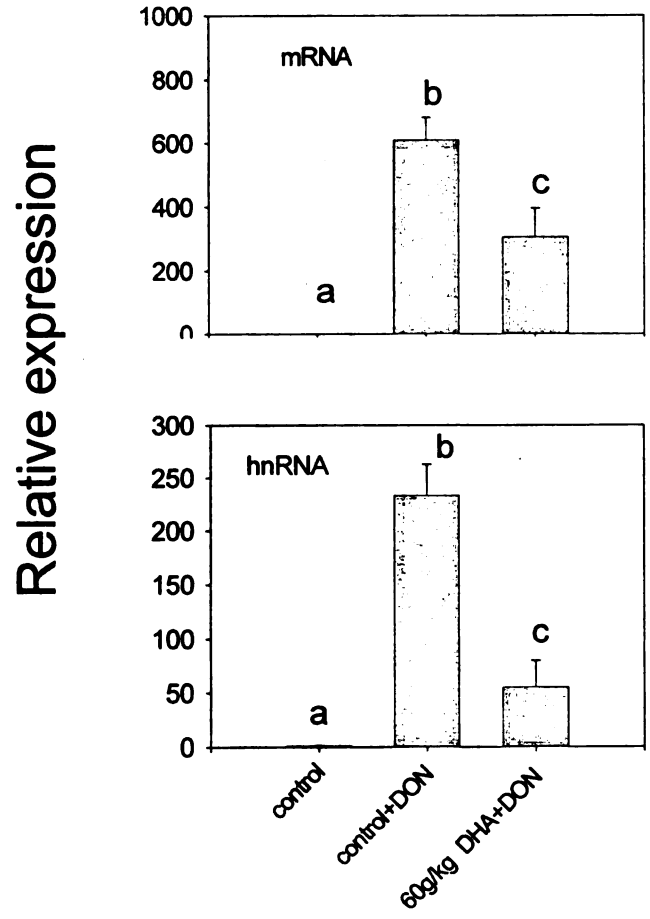


Table 4.4 . Fatty acid composition of liver phospholipid in study 2 (n=3).

	AA	EPA	DHA	C 16:0	C 18:0	C 18:1	C 18:2
Control +DON	12.53±1.15[a]	0.11±0.05[a]	3.17±0.29[a]	24.43±0.49[a]	14.23±0.50[a]	21.93±0.29[a]	6.70±0.51[a]
6o g/kg DHA + DON	2.97±0.06[b]	4.83±0.06[b]	11.46±0.33[b]	37.03±0.44[b]	18.13±0.33[a]	8.74±0.26[b]	5.42±0.34[a]

Only the major fatty acids are shown. The value stands for the percentage of total phospholipid extracted from liver. Different letter within a column indicates significant difference, $P<0.05$.
DHA docosahexaenoic acid; EPA eicosapentaenoic acid; AA arachidonic acid.

Figure 4.9 Effects of dietary DHA on IL-6 mRNA and hnRNA expression in spleen of mice subjected to DHA and DON. Mice were fed diet containing DHA at 60 g/kg for 4 wks (Study 2). At experiment cessation, mice were gavaged with DON (25mg/kg body weight). After 3 h, total RNA was extracted and analyzed by real-time PCR. The 18S RNA was used as internal standard. Results are reported relative to control group expression. Values are mean ± SEM, n=3. Bars with different letters differ, ($p<0.05$).



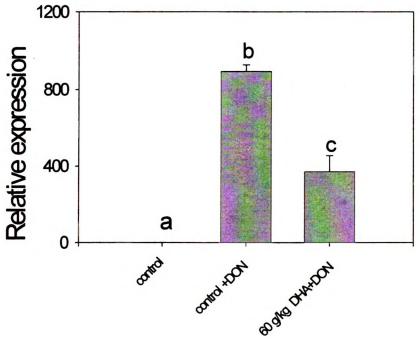


Figure. 4.10 Effect of DHA on COX-2 mRNA expression in spleen induced by acute oral exposure to DON (Study 2). Mice were fed diet containing DHA at 60 g/kg in diet for 4 wk. At the experiment cessation, mice were gavaged 25 mg/kg body weight DON. After 3 h, total RNA was extracted and analyzed by real-time PCR. Results are reported relative to the control group. Values are mean ± SEM, n=3. Bars with different letters differ, ($p<0.05$).

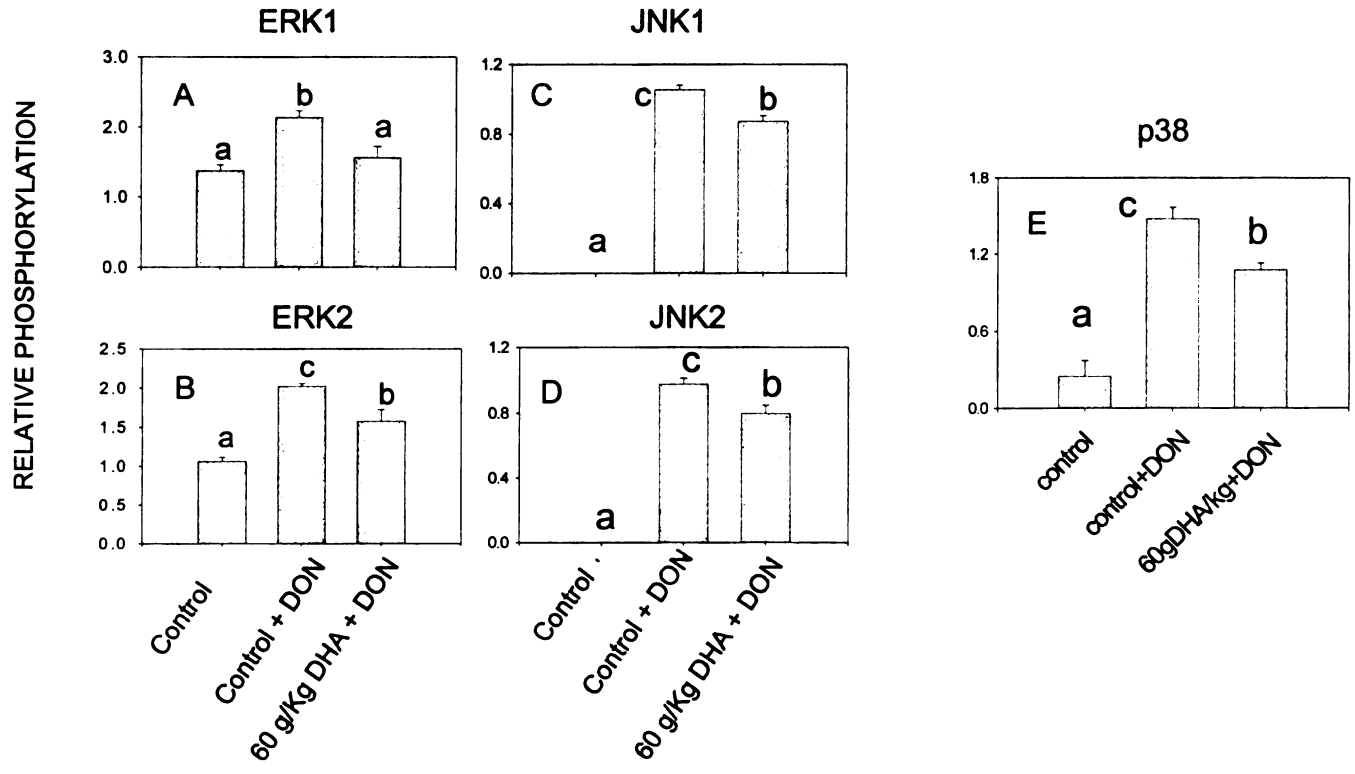


Figure 4.11 Effects of dietary DHA on MAPK phosphorylation in spleen induced by acute oral exposure to DON (Study 3). Half hour later, spleen total protein was extracted, Western blot was performed and band density was measured. Statistic analysis was conducted and bar with different letter differ, $p<0.05$ (n=5).

DISCUSSION

Potential effects of fish oil and (n-3) PUFA supplementation for human IgAN patients have been demonstrated by clinical studies which report marked retarded renal disease progression in association with reduced inflammation and glomerulosclerosis (Grande and Donadio, 2001; Donadio, 2001b). As shown here and previously (Pestka *et al.*, 2002; Jia *et al.*, 2004), (n–3) PUFAs impair production and accumulation of serum IgA potentially in a chemical-induced IgAN model. These preclinical findings are valuable because they suggest that consumption (n-3) PUFAs might also have potential benefits 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 results presented here indicate that DHA-enriched oil at 60 g/kg completely abrogated increases in serum IgA as early as 4 wk, whereas, at 2 and 10g /kg DHA, efficacy was detectable only at wk 16. The highest DHA concentration used here was also the most effective at inhibiting serum IgA-IC elevation and mesangial IgA deposition. In our prior study (Jia *et al.*, 2004), DHA-enriched oil at 20 g/kg, partially inhibited DON-induced IgAN beginning at 12 wk of toxin exposure. Thus, a correlation exists between the dietary concentration of DHA and its inhibitory effects on DON-induced IgAN.

The cytokine IL-6 drives IgA-committed B cells to terminally differentiate to IgA-secreting plasma cells (Beagley *et al.*, 1989). Differentiated IgA-secreting B cells can migrate to distal mucosal and systemic sites, survive for prolonged periods and produce IgA. IL-6 deficient mice are resistant to DON-induced IgA elevation (. Pestka *et al.*, 2000). Ex vivo studies also implicate a role for IL-6 in DON-induced IgAN (Yan *et*

al., 1997; 1998). A critical observation made here was that consumption of 10 and 60 g/kg DHA diet significantly reduced splenic IL-6 mRNA levels to below those of mice fed control or control + DON diets. Since 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 (Johnson *et al.*, 2003). The finding that DHA-enriched oil consumption significantly blocked accumulation of IL-6 hnRNA as well as IL-6 mRNA suggests that DHA blocks IL-6 gene expression at the transcriptional level. This contention is further supported by the observation that mice consuming 60 g/kg DHA for 4 wk exhibited markedly less induction of IL-6 mRNA and hnRNA expression following acute DON exposure. These results have potential clinical significance because: IL-6 is believed to play a contributory role in human IgAN (Donadio and Grande, 2002) and because IL-6 production by peripheral blood mononuclear cells is decreased in persons consuming (n-3) PUFA which corresponds to increased plasma and cell membrane (n-3) PUFA incorporation (Trebble *et al.*, 2003).

Differences between IL-6 mRNA expression following chronic and acute DON exposure (ie. no induction vs. induction) might arise from two factors. First, DON-fed mice (20 mg/kg of diet) ingest approximately 2 g/d of food, an exposure level of 3 mg/kg body weight DON per d can be estimated. This is much lower than the acute dose (25 mg/kg body weight) used. Second, in this study, experiments were terminated in the morning. However, mice typically will eat most food when daily light period ends and then consume smaller amounts sporadically throughout the day. Since IL-6 is an early response gene with rapid turnover (Ross, 1995), its induction at the local sites in DON-fed mice might have escaped detection in our model but was, nevertheless, sufficient to

chronically promote B cell differentiation to IgA secretion with attendant cumulative effects. These possibilities are consistent with our previous observations that DON enhances immunoglobulin response to commensal and self antigens (Rasooly and Pestka, 1992; Pestka *et al.*, 1990a) and that IgA-secreting cells, serum IgA, serum IgA-IC and mesangial IgA remain elevated for at least 4 months after withdrawal of DON from diet (Dong and Pestka, 1993; Pestka *et al.*, 1990b).

The effects of DHA-enriched oil on IL-6 expression might be partially mitigated by attenuated expression of COX-2, an essential enzyme known to mediate functions of (n-3) and (n-6) fatty acids (Lee *et al.*, 2003a). Moon and Pestka (2003a) found that COX-2-deficient mice have significantly reduced capacity to respond to DON with increased IL-6 mRNA and serum IL-6 expression. The acute DON exposure data presented here suggest that DHA can reduce COX-2 mRNA expression with similar trends observed following DON feeding. DHA's capacity to suppress COX-2 gene expression might thus be one upstream mechanism by which IL-6 expression is impaired and, ultimately, IgA production is attenuated.

Another finding of this study was that AA concentrations in liver phospholipid were significantly depressed by DHA consumption. AA is considered to be a risk factor in IgAN (Holman *et al.*, 1994; Lefkowith and Klahr, 1996). Decreasing AA concentration corresponded to reduction in serum IgA, serum IgA-IC, mesangial IgA and expression of IL-6 and COX-2. Reduced AA in phospholipids might decrease generation of inflammatory mediators such as PGE_2 (Trebble *et al.*, 2003) thereby attenuating mucosal inflammation and IgA production. In support of this contention, Bagga *et al.*, (2003) demonstrated that (n-3) PUFAs impair PGE_2 release but enhance PGE_3 release by

macrophages in vitro. PGE_3 is less efficient in inducing COX-2 and IL-6 gene expression compared to PGE_2. Since DON induces PGE_2 production in mice (Moon and Pestka, 2003a), it is reasonable to suggest that decreased AA tissue concentration could reduce IL-6 gene expression and attenuate overall IgA production in DON-exposed mice. This suppression could be synergistically enhanced by reduced COX-2 expression.

The DHA-enriched oil used here also contained EPA (113 g/kg) and it thus is not surprising that the tissue phospholipids from mice fed this oil also contained elevated EPA concentrations. Since the inhibitory effect of this (n-3) PUFA on COX-2 enzyme activity is reportedly greater than DHA (Ringbom *et al.*, 2001), EPA might be another key factor in suppression of DON-induced IgA dysregulation. However, this interpretation is tempered by previous observations in our model that DHA-enriched oil was somewhat more effective than EPA-enriched oil attenuating plasma IgA elevation (Jia *et al.*, 2004a).

Although it is not yet clear how DHA impairs IL-6 and COX-2 expression, in vitro studies have provided evidence that (n-3) PUFAs inhibit kinase activities in several cell phenotypes (Denys *et al.*, 2001). Particularly noteworthy are the MAPKs, which couple cell-surface receptors to critical regulatory targets and transcription of genes including COX-2 and IL-6 (Hedges *et al.*, 2000). Yusufi *et al.* (2003) found that DHA, but not EPA, decreased ERK activation in mesangial cells, whereas JNK activity was increased and p38 activity was not significantly affected. DON can activate ERK, JNK and p38 both in macrophages and mice and this contributes to transcriptional and post-transcriptional up-regulation of proinflammatory genes (Zhou *et al.*, 2003; Moon and Pestka, 2002; Fan *et al.*, 2000). Recently, Moon and Pestka (2003b) found that

consumption of 60 g/kg menhaden fish oil by mice suppressed DON-induced ERK 1/2 and JNK 1/2 phosphorylation but not p38 in spleen. In contrast, results presented herein demonstrated that prior consumption of 60 g/kg DHA for 4 wk not only impaired DON-induced ERK 1/2 and JNK 1/2 phosphorylation but p38 phosphorylation as well. There is one difference between these two investigations. Since mice here consumed 36 g/kg (n-3) PUFA in DHA-enriched oil whereas the previous study's mice consumed 16 g/kg (n-3) in menhaden oil PUFA (Moon and Pestka, 2003b), it is possible that the lack of significant p38 effect in the latter study might be dose-related. Further investigation of these possibilities should involve assessing effect of DHA dose on DON-induced p38 activation as well as the effects of concurrent ingestion of AA or other (n-6) PUFA.

It was interesting to note that while DHA consumption suppressed DON-induced phosphorylation of all three MAPK families, these inhibitions were quite modest relative to rather extensive IL-6 inhibition. Two considerations are preeminent here. First, Western analysis provides a qualitative picture of total MAPK phosphorylation status in a tissue. In reality, MAPK signaling modules interact via a series of sequential binary interactions to create protein kinase cascade (Morrison and Davis, 2003). These modules are likely to be associated with scaffold proteins which can allow sorting of relevant and irrelevant stimuli and provide spatial and temporal control of MAPK signaling. Thus, DHA might affect one or more specific modules that control induction of IL-6 by DON but this cannot be resolved by Western analysis from other less relevant or irrelevant modules. Second, the analysis conducted here represents the entire spleen cell population. While we speculate DHA's effects on MAPKs and IL-6 are mediated in macrophages and dendritic cells, Western analysis does not allow discrimination of such effects from

those of B cells, T cells, epithelial and endothelial cells. Thus, future studies of DHA's effects in this model require consideration of the spatio-temporal context of MAPK targets as well as cell phenotypes affected.

Other mechanisms besides interference with MAPK activation might explain DHA inhibition of IL-6 and COX-2 gene expression. For example, a recent study suggests that DHA inhibits COX-2 mRNA expression though toll-like receptors (Lee *et al.*, 2003b). Also, lipid rafts are important signaling platforms for T cell activation. Sphingomyelin, which facilitates raft formation, is significantly decreased in T cells from (n-3) PUFA-fed mice (Fan *et al.*, 2003). In vitro studies using the Jurkat T cell line also indicate that (n-3) PUFAs selectively modify lipid rafts and suppress signal transduction. Therefore, DHA might alter surface receptor protein function and lymphocyte signal transduction by altering raft phospholipid composition. These and other signaling mechanisms require further exploration.

Typical (n-3) PUFA intake recommendations for healthy people are 0.3 to 0.5 g/d for DHA plus EPA along with 0.8 to 1.1 g/d for α-linolenic acid (Institute of Medicine, 2002). However, consumption of more DHA and EPA might be required for disease prophylaxis and therapy. The combined DHA and EPA concentrations in the diets employed here ranged from 1 to 36 g/kg, which would account for 0.2 to 7.2% of total caloric intake. Upon extrapolation, a human consuming 2000 kCal/day would need to ingest 0.5 to16 g/d DHA to correlate with that amount consumed by the mouse per d per kg body weight in this experiment.

Relative to prophylaxis and therapy for immune diseases, the upper level of safe intake of (n-3) PUFA is a critical consideration. Although the FDA has ruled that intakes

of up to 3 g/d of marine (n-3) PUFAs in diet are generally recognized as safe (Department of Health and Human Services, 1997), higher doses have been used in clinical trials. Two 4-year prospective studies have demonstrated that high dose (n-3) PUFA (3.76g EPA plus 2.94g DHA per d) and low dose (1.88g EPA, 1.47g DHA per d) are equivalent in treating IgAN (Donadio *et al.*, 2001). In another fish oil therapy study, doses of (n-3) PUFA (4.3g EPA, 2.8 g DHA/d) were used for 5 years without any observed side effects (Ng, 2003). In a 6-month trial, 6.9 g/d EPA plus DHA was provided to 275 patients, no side effects were found (Leaf *et al.*, 1994). No adverse effects were reported when subjects consumed up to 1.8 g/d of EPA and DHA and 9.0 g/d ALA over a 4 wk period (Mantzioris *et al.*, 2000). Further study is needed to determine the requisite amounts of (n-3) PUFA in diet to achieve sufficient tissue phospholipid concentrations for optimal, efficacious prevention and treatment of IgAN and other immune-related diseases. The approach described herein offers one animal model for obtaining such preclinical data.

In summary, the results presented here suggest that consumption of diets containing DHA-enriched oil significantly inhibited DON-induced IgAN in dose-dependent fashion. These effects correlated with impairment of IL-6 and COX-2 expression as well as MAPK activation. It might be speculated that DHA consumption will have prophylactic value in suppressing elevation of IgA and IgA-IC among persons with genetic predisposition for IgAN or who have been diagnosed with the disease. Future perspectives should include improved understanding of the mechanistic basis for DHA's effects in this IgAN model and determination of the required intake to establish

prophylactic and therapeutic tissue concentrations that modulate critical molecular targets.

CHAPTER 5

Role of Cyclooxygenase-2 Gene In Deoxynivalenol-Induced Immunoglobulin A Nephropathy

ABSTRACT

Ingestion of the trechothecene mycotoxin deoxynivalnenol (DON) induces serum IgA elevation and kidney mesangial deposition in a manner that mimics human IgA nephrophathy (IgAN). Previous studies indicate that interleukin-6 (IL-6) is crucial for DON-IgAN and that DON-induced cyclooxygenase-2 (COX-2) might contribute to IL-6 upregulation. (N-3) Polyunsaturated fatty acids (PUFAs) inhibit IL-6 and COX-2 gene expression as well as DON-induced IgAN. It was therefore hypothesized that COX-2 and its metabolites thus are essential for DON-IgAN. In this study, COX-2 knockout mice and COX-2 specific inhibitor, VIOXX, were employed in order to test the contribution of COX-2 to this model. The results demonstrated that neither COX-2 deficiency nor dietary exposure to COX-2 enzyme activity inhibitor, VIOXX, could block DON-induced serum IgA, IgA-IC accumulation, IgA kidney deposition and spleen IgA secretion. Rather, these treatments promoted DON-induced serum IgA elevation. These results suggest that COX-2 might not be required for DON-induced IgAN and further, that COX-2 inhibitor, VIOXX, would be contraindicated for the prevention of early stages of IgAN.

INTRODUCTION

Cyclooxygenases (COX) convert arachidonic acid (AA) released from membrane phospholipids to prostanoids. COX-1 is a constitutive enzyme, which mediates cell homeostasis. COX-2 is an inducible, immediate-early gene product, which is primarily responsible for increased prostaglandin (PG) production during inflammation, reproduction and carcinogenesis. Expression of COX-2 is low in most tissues, but can be readily induced by cytokines, growth factors and chemicals (Ristimaki, 2004).

The trichothecene mycotoxin, deoxynivalenol (DON), induces COX-2 gene expression by promoting transcriptional activity and mRNA stability via mitogen activated protein kinase (MAPK) signaling pathways in vitro and in vivo (Moon and Pestka, 2002; Moon *et al.*, 2003). The observations that DON upregulation of IL-6 production is significantly reduced by COX-2 inhibitors indomethacin and NS-398 (Moon and Pestka, 2003) and that COX-2 knockout mice significantly reduced splenic IL-6 mRNA and serum IL-6 level in response to oral DON exposure (Moon and Pestka, 2003) suggest that DON-induced COX-2 gene expression and resultant COX-2 metabolites might contribute, in part, to subsequent upregulation of IL-6 gene expression.

IgA nephropathy (IgAN) is the most common form of glomerular nephritis, which mainly affects children and young adults (Endo, 1997). Clinical diagnosis of IgAN is based on the presence of kidney mesangial IgA deposition (Ibels and Gyory, 1994). Serum IgA concentration is considered to be an important etiological factor for IgAN (Feehally *et al.*,1997; Endo, 1997). It has been shown that mice exposed to a diet containing DON developed IgAN with high serum IgA (Pestka *et al.*, 1989; Dong *et al.*, 1991; Dong and Pestka, 1993 and Pestka, 2003). IL-6 gene plays an important role in the

development of IgAN induced by DON (Pestka and Zhou, 2000). DON might destroy mucosal tolerance by induction of IL-6 and promote IgA production, theraby contributing to systemic compartment IgA elevation (Pestka, 2003). Since (n-3) polyunsaturated fatty acids (PUFAs), such as DHA, inhibit DON-induced IgAN as well as COX-2 and IL-6 gene expression (Jia *et al*, 2004), it was hypothesized that direct suppression of COX-2 by (n-3) PUFAs might contribute to their inhibition on IL-6 gene expression and IgAN. Therefore, COX-2 knockout mice and COX-2 enzyme inhibitor, VIOXX, were employed in this study to assess the role of COX-2 in DON-induced experimental IgAN. The results indicated that both COX-2 knockout mice and mice fed with COX-2 specific inhibitors, VIOXX, were not resistant to DON-induced IgAN but rather exhibited even higher serum IgA levels than mice fed DON alone.

MATERIALS AND METHODS

Materials. All chemicals (reagent grade or better) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. VIOXX (Merck & Co Inc, Whitehouse Station, NJ) was obtained through Michigan State University Clinical Center. The DON used in this study was produced in *Fusarium graminearum* R6576 cultures and purified by the water-saturated silica gel chromatography method of Clifford *et al.* (2003). Purity of DON was verified by a single HPLC peak occurring at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware that was contaminated with mycotoxin was detoxified by soaking for >1 h in 100 mL/L sodium hypochlorite. Purified DON was added to powdered diets as described previously (Dong and Pestka, 1993).

Animals B6,129P2-*Ptgs2*^{tm1Smi} (002181-M; COX-2 knockout) mice those are homozygous for a targeted disruption of COX-2 gene (Morham *et al*, 1995), and B6, 129P2-*Ptgs2*^{tm1Smi} (002181-W) mice which served as wild-type controls, were obtained at 7 wk of age from Taconic (Germantown, NY USA). Mice were housed in environmentally protected cages, which consisted of a transparent polycarbonate body with a filter bonnet, stainless steel wire lid and a layer of heat-treated hardwood chips. The mice were allowed to acclimate for at least 7 d to their new housing, regulated temperature (25°C), feed, 12-h light and dark cycle and to a negative-pressure ventilated area before feeding regimens began. All animal handling was conducted in accordance with guidelines established by the National Institutes of Health. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and were approved by Michigan State University Laboratory Animal Research Committee.

Diets and experimental design. For studies with COX-2 knockout mice, diet was based on the AIN-93G formulation of Reeves *et al.* (1993) which consisted of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.5 g cornstarch, 132 g dyetrose (dextrinized cornstarch), 50 g cellulose, 3 g L-cystine, 2.5 g choline bitartrate, 14 mg TBHQ, 100 g sucrose, 10 g corn oil (Dyets) and 60g high oleic acid sunflower oil (Hain pure food, Garden City NY). DON (10 mg/kg) was used to amend the basal diet to yield 3 experimental groups (n=5): wild type (WT) control, wild type (wt) + DON, mutant type (KO) + DON.

For studies with COX-2 inhibitor, VIOXX was mixed in the above diet (0.0075% w/w) (Hegazi *et al.*, 2003) and the B6C3F1 female mice were divided into three groups (n=6): control, control+DON and VIOXX + DON. Mice were fed for 16 wk, bled at 8 wk intervals and serum analyzed for IgA and IgA-IC. At wk 16, mice were killed by cervical dislocation. Spleens and Peyer's patches were removed aseptically for preparing cell cultures.

Detection of IgA and IgA-IC. IgA and IgA-IC was measured in serum by capture ELISA (Dong and Pestka, 1993) using mouse immunoglobin reference serum (Bethyl Laboratories, Montgomery, TX), goat anti-mouse IgA (heavy chain specific) and peroxidase-conjugated goat IgG fraction to mouse IgA (Organon Teknika, West Chester, PA). For detection of IgA-IC, diluted serum samples were precipitated using 70 g/L polyethylene glycol (PEG 6000; Sigma) (Imai *et al.*, 1987) and IgA in precipitate redissolved in PBS and quantified by IgA ELISA.

Assessment of kidney IgA deposition. At experiment termination, kidneys of each euthanized mouse were removed, cut in half and immediately frozen in liquid nitrogen.

Each kidney was sectioned to 7 μm with a cryostat (Reichert-Jung, Cambridge Instruments, Buffalo, NY) and stained for IgA deposition with fluorescein isthiocyanate–labeled goat anti-mouse IgA (Sigma) as previously described (Jia *et al.*, 2004). IgA immunofluorescence was assessed with a Nikon Labophot microscope (Mager Scientific, Dexter, MI). Three glomeruli from each section were randomly selected and mean fluorescence intensity was determined in polygons encircling the glomeruli using UTHSCSA Image Tool Software V 1.2. (http://www.scioncorp.com/frames/fr_download_now.htm). Pixels in the circled area were measured on a grayness scale that ranged from 0 (black) to 255 (white).

Cell culture. Spleen and Peyer's patches were teased apart in harvest buffer consisting of 0.01 mol/L PBS, pH 7.4 containing 20 ml/L heat inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY), $1x10^5$ U/L penicillin and 100 mg/L streptomycin. Tissues were passed through a sterile 100-mesh stainless screen in the same buffer and cell suspensions held on ice for 10 min to allow settling of tissue particles. Supernatant was removed following centrifugation at 450 g for 10 min. Erythrocytes were lysed for 3 min at room temperature in 0.02 mol/L Tris buffer (pH 7.65) containing 0.14 mol/L ammonium chloride. Cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 100 mL/L FBS, 1 mmol/L sodium pyruvate, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, 0.1 mmol/L nonessential amino acid and 50 μmol/L 2-mercaptoethanol and then counted using a hemacytometer (American Optical, Buffalo, NY). Cells ($2x10^8$/L) from individual mice were cultured separately in 1 mL of medium in flat-bottomed 24-well tissue culture plates (Fisher Scientific, Corning, NY) at 37°C

under 70 g/L CO_2 in a humidified incubator. Supernatants were collected after 5 d and stored in aliquots at -20°C until analysis for IgA.

Statistics. Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. If data were not normally distributed, they were subjected to Kruskal-Wallace ANOVA on Ranks and pairwise comparisons made by Dunn's or Student-Newman-Keuls methods. Differences were considered significant at $P < 0.05$.

RESULTS

In the first study, dietary DON retarded weight gain in both wild type and COX-2 knockout mice (Figure 5.1). Mice fed DON exhibited significant elevation of serum IgA. COX-2 deficiency did not impair but rather instead promoted IgA concentration (Figure 5.2). DON also significantly induced serum IgA-IC elevation in mice, which was not suppressed in COX-2 knockout mice (Figure 5.3). DON treatment significantly induced IgA secretion from spleen (Figure 5.4) while COX-2 knockout mice are not resistant to DON-induced spleen IgA production.

Significant mesangial IgA deposition was also observed in kidney from DON-fed wild type mice as compared to wild type control (Figure 5.5). Mesangial IgA deposition in DON-fed COX-2 knockout mice was also higher than wild type fed control diet but was at the same level with DON-fed wild-type mice. Thus COX-2 deficiency in mice could not attenuate induction of DON-induced IgAN.

A second study was conducted in which the effects of specific COX-2 inhibition were evaluated in DON-induced IgAN. Similar with first study, DON impaired body weight increase (Figure 5.6). No differences in terminate body weight were found between DON and VIOXX plus DON group after 16 wk. At wk 16, DON also caused serum IgA to increase significantly (Figure 5.7). VIOXX did not inhibit but rather promoted DON-induced IgA elevation. DON also significantly induced serum IgA-IC, IgA deposition and spleen IgA secretion, but VIOXX treatment did not reduce these effects (Figure 5.8, 5.9, 5.10). Thus, mice treated with COX-2 inhibitor behaved similarly to COX-2 knockout mice in their lack of resistance to DON-induced IgAN.

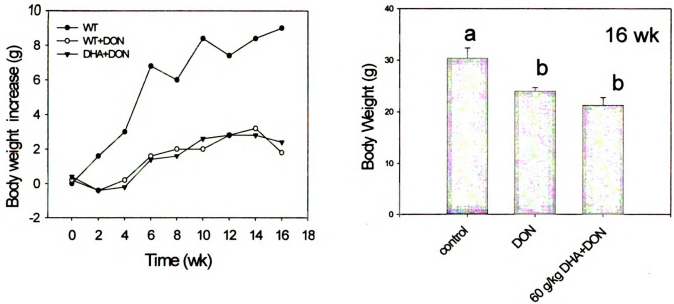


Figure 5.1. Kinetics of body weight increase in COX-2 knockout mice (mt) and wild-type(wt) mice fed modified AIN-93G diets (Study 1) over 16-wk feeding period. (A)Values are increase of mean body weight of each group at different time. (B) Body weight at the end of experiment.

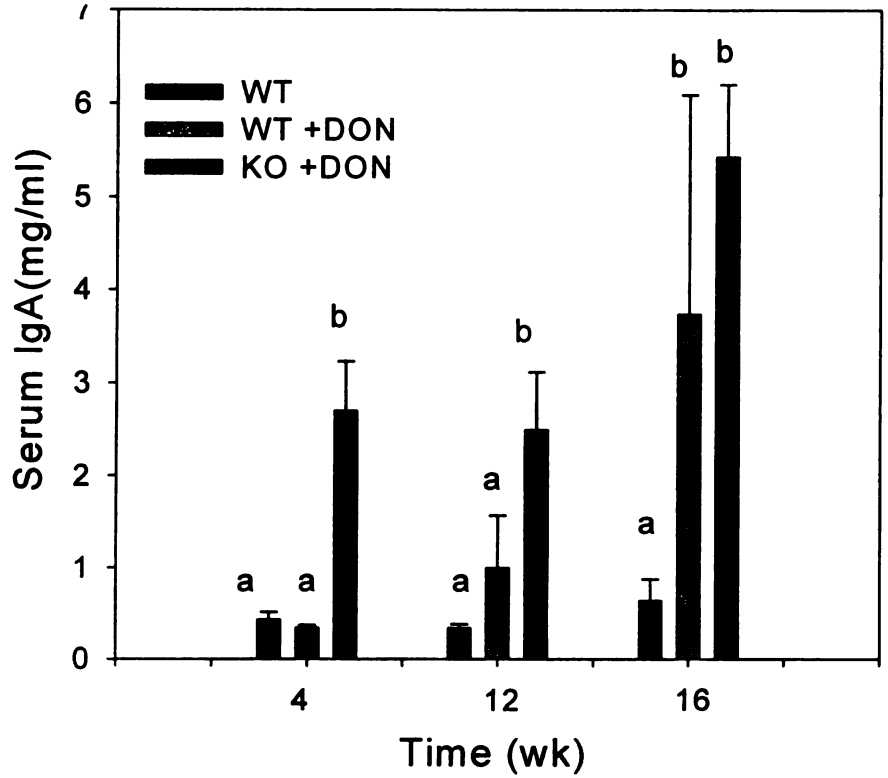


Figure 5.2 Effects of DON (10 mg/kg) on serum IgA in COX-2 knockout mice (KO) and wild type (WT) over 16 wk. Serum IgA was measured by ELISA. Values are means ± SEM (n = 3-6). Bars that do not have the same letter differ, ($P < 0.05$).

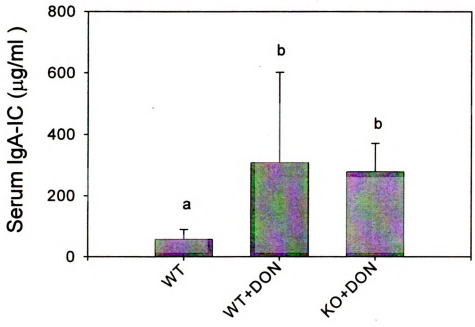


Figure 5.3 Effects of DON (10ppm) on serum IgA-IC in COX-2 knockout mice(KO) and wild type(WT) over 16 wk . Mice were bled at 16 wk. Serum IgA-IC was precipitated by polyethylene glycol (PEG) and measured by IgA ELISA. Values are means ± SEM (n = 3-6). Bars that do not have the same letter differ, ($P < 0.05$).

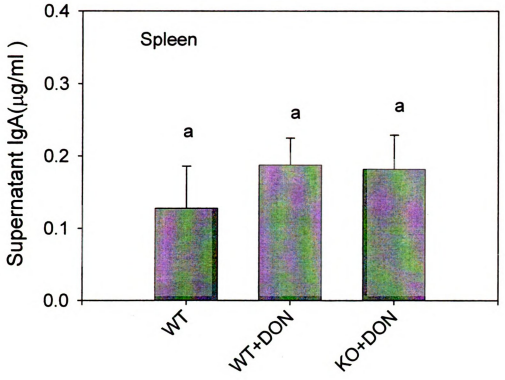


Figure 5.4 Effects of dietary DON (10ppm) on mesangial IgA deposition in COX-2 knockout mice (KO) and wild type (WT) mice after 16 wk. Kidneys were cryostat sectioned and stained with fluorescein isthiocyanate–labeled anti-mouse IgA. Mesangial IgA quantitation was performed by image analysis. Values are mean ± SEM (n =3-6). Bars that do not have the same letter differ, ($P < 0.05$).

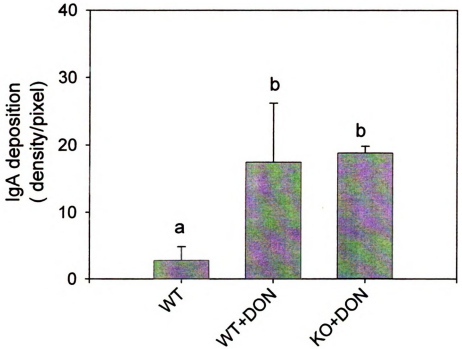


Figure 5.5 Effects of DON (10ppm) on ex vivo IgA production in COX-2 knockout mice(KO) and wild type (WT) mice. Spleen cells (1×10^9 /L) were cultured for 5 d. IgA was measured by ELISA. Values are means ± SEM (n=3-6). Bars that do not have the same letter differ, ($P < 0.05$).

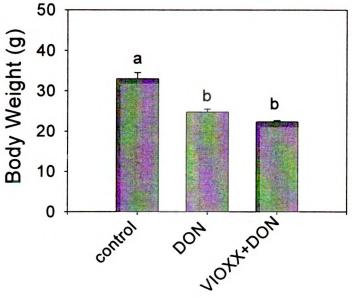


Figure 5.6 Body weight in B6C3F1 female mice fed with modified AIN-93G diets containing DON (25 mg/kg) and VIOXX (0.0075% w/w) for 16 wk (Study 2). Values are means ± SEM (n=3-6). Bars that do not have the same letter differ, ($P < 0.05$).

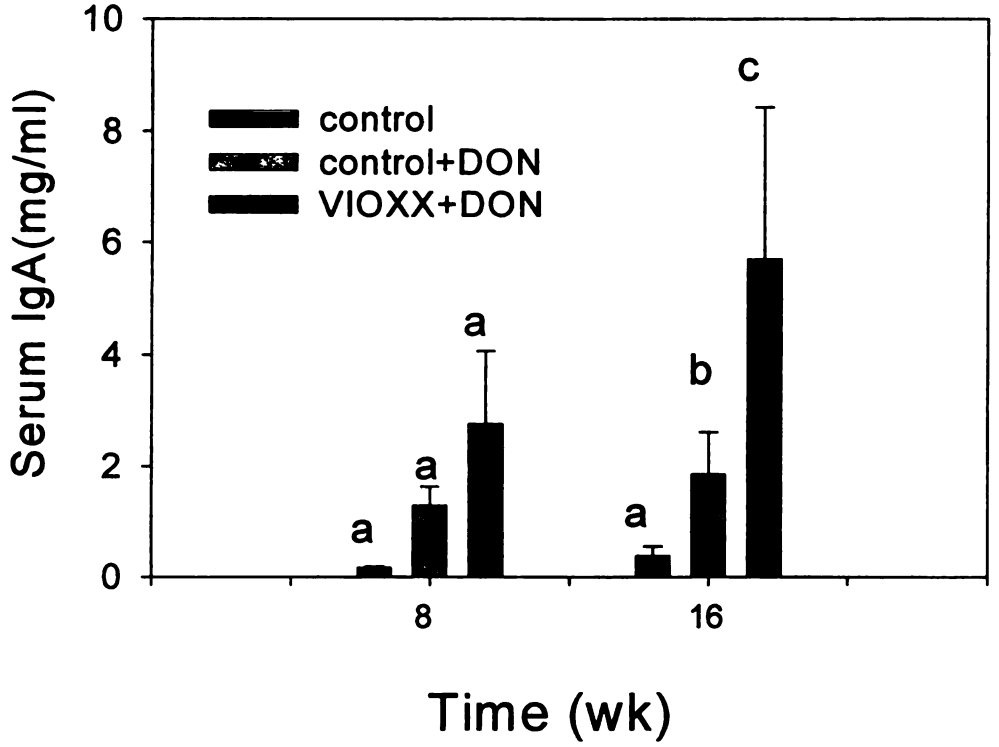


Figure 5.7 .Serum IgA in B6C3F1 female mice fed with modified AIN-93G diets containing DON (25 mg/kg) and VIOXX (0.0075% w/w) over16 wk. Serum IgA were measured by ELISA; Values are means ± SEM (n = 3-6). Bars that do not have the same letter differ, ($P < 0.05$).

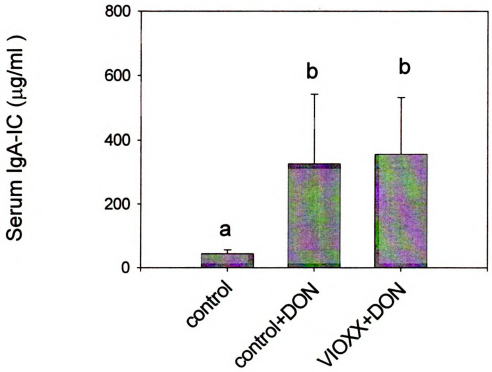


Figure 5.8 Serum IgA-IC in B6C3F1 female mice fed with DON (25 mg/kg) and VIOXX (0.0075% w/w) over16 wk. Mice were bled at 16 wk. Serum IgA-IC was precipitated by polyethylene glycol (PEG) and measured by IgA ELISA. Values are means ± SEM (n = 3-6). Bars that do not have the same letter differ, ($P < 0.05$).

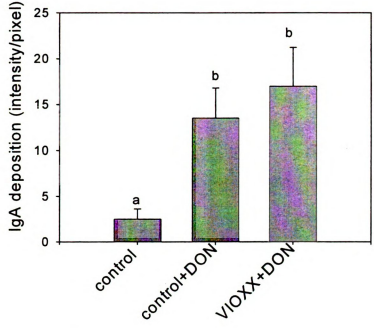


Figure 5.9 Effects of dietary DON (20 mg/kg) on mesangial IgA deposition in B6C3F1 female mice fed with VIOXX (0.0075% w/w) over1 6 wk. Kidneys were cryostat sectioned and stained with fluorescein isthiocyanate–labeled anti-mouse IgA. Mesangial IgA quantitation was performed by image analysis. Values are mean ± SEM (n =3-6). Bars that do not have the same letter differ, ($P <$ 0.05).

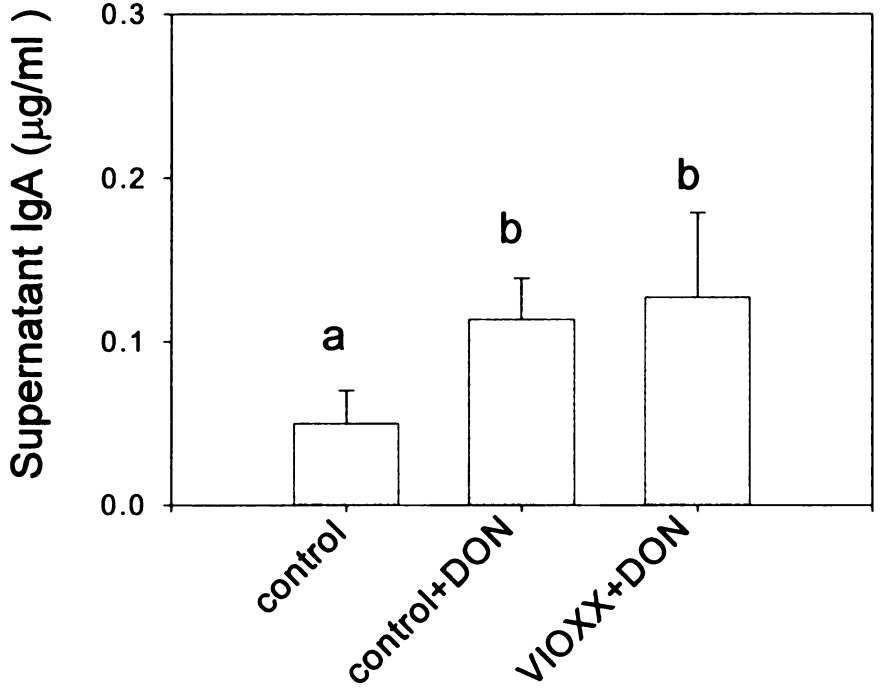


Figure 5.10 Effects of DON (25 mg/kg) on ex vivo IgA production in B6C3F1 female mice fed with VIOXX (0.0075% w/w) over16 wk. Spleen cells (1 x 10^9/L) were cultured for 5 d. IgA was measured by ELISA. Values are means ± SEM (n=3-6). Bars that do not have the same letter differ, ($P < 0.05$).

DISCUSSION

COX-2 is the rate-limiting enzyme in the conversion of AA to prostanoids after AA is released from membrane phospholipids by phospholipase A_2 (PLA_2) (Ristimaki, 2004). The COX-2 products, PGs, are generally considered to be proinflammatory agents, but in vitro studies show that they could also play anti-inflammatory roles during certain situations (Takayama *et al.*, 2000). Since high levels of IL-6 accompanies over-expression of COX-2 and non-steroidal anti-inflammatory agent (NSAIDS), COX-2 inhibitors, might be useful in treating chronic inflammatory conditions in which IL-6 is abnormally elevated (Joy and Emily, 1997). IL-6 is a pleiotropic cytokine produced by monocytes/macrophages, fibroblasts, endothelial cells and astrocytes in response to infections and toxins. Since IL-6 plays an important role in DON-induced IgAN (Pestka and Zhou, 2000) and COX-2 gene deficiency partially blocks induction of IL-6 gene expression by acute DON exposure both in vitro and in vivo (Moon and Pestka, 2003) suggest COX-2 might mediate DON-induced IgAN by enhancing IL-6 gene expression (Moon and Pestka, 2003). However, the data presented here demonstrated that interference with COX-2 function did not prevent DON-induced IgAN but rather enhanced DON's capacity to promote IgA elevation.

COX-2 knockout mice completely lack normal lipopolysaccharide (LPS) induction of COX-2 mRNA and protein (Morham *et al*, 1995). Here we found that COX-2 knockout mice could not resist DON-induced serum IgA, IgA-immune complex (IC) elevation, spleen IgA secretion and IgA deposition. As described above, COX-2 initiates the conversion of AA into PGs, whereas 5-lipoxygenase (5-LO) generates leukotrienes (LT) from AA. Knocking out COX-2 gene might disturb AA metabolism by reducing

PGs but could result in increased LTB_4 production (Tuo *et al.*, 2004). Since LTB_4 is also a potential inducer of IL-6 (Stankova and Rola-Pleszczynski, 1992), knocking out COX-2 must be insufficient to remove IL-6 production completely (Moon and Pestka, 2003). Such a disturbance in AA metabolism might account for the higher serum IgA in COX-2 knockout mice. Further studies on DON's effects on 5-LO gene expression and LTB_4 production were therefore warranted.

VIOXX is an NSAID that selectively inhibits COX-2 enzyme activity but not COX-2 gene transcription (Evans, 2003). Although selective COX-2 inhibitors have anti-inflammatory activity and can reduce proteinuria in experimental membranous glomerulonephritis, they also possess several side effects such as 1) impairing glomerular capillary repair in rat anti-Thy 1.1-induced glomerulonephritis (Kitahara *et al.*, 2002), 2) increasing the risk for serious cardiac and/or cerebrovascular events (Zhao *et al.*, 2001) and 3) causing acute renal failure (Brater, 1999). In the DON-induced IgAN model, VOIXX did not reduce serum IgA, IgA-IC, IgA deposition and spleen IgA secretion but rather, promoted DON-induced serum IgA which is an early pathogenic factor for the development of IgAN (Feehally *et al.*,1997). These data are consistent with that of the COX-2 knockout study described above. Thus blocking COX-2 enzyme activity might not be effective reducing IL-6 gene expression and ultimately serum IgA production in mouse chronically exposed to DON. It should be therefore be further noted that VIOXX might cause damage of intestine duct (Leite *et al.*, 2004), which could promote IgA production by exposing lymphoid tissues to food antigens.

Since high serum IgA is an early etiologic factor for the development of IgAN, the observation that COX-2 gene knockout and VIOXX treatment did not reduce but

promote DON-induced IgA production suggested that COX-2 gene is not required for the development of DON-induced IgAN. Furthermore, regardless of mechanism, these preclinical data suggest that the COX-2 inhibitor, VIOXX, might not be good for the prophylaxis of IgAN due to its induction on IgA production.

CHAPTER 6

Docosahexaenoic Acid Inhibits CREB Activation and Interleukin-6 Gene Transcription Induced By the Ribotoxic Stressor Deoxynivalenol In Macrophage

ABSTRACT

The trichothecene mycotoxin, deoxynivalenol (DON), induces experimental IgA nephropathy (IgAN) in mice by upregulating interleukin-6 (IL-6) gene expression. Consumption of (n-3) polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA), retards DON-induced IL-6 and IgAN. The purpose of this study was to determine the effects of DHA consumption on DON-induced IL-6 mRNA transcription. DON significantly induced binding of cAMP response element binding protein (CREB), active protein (AP-1), but not nuclear factor κ B (NFκB) and CCAAT/enhancer binding protein (C/EBPβ) to their respective consensus sequences in thioglycollate-elicited peritoneal macrophages and these effects were suppressed in macrophages similarly elicited from DHA-fed mice. These findings were consistent with the results found in splenic nuclear extracts. Chromosome immunoprecipitation (ChIP) assay confirmed that DON increased CREB binding to the cis element of the IL-6 promoter in macrophages and that this was inhibited by DHA feeding. DON also induced phosphorylation of p38, extracellular signal-regulated kinase (ERK1/2) and their downstream kinases, mitogen and stress-activated protein kinase 1 (MSK1), ribosomal S6 kinase (p90RSK) which are important for CREB activation. DON-induced CREB phosphorylation was blocked in macrophage form DHA-fed mice, but analogous effects were not observed for p38, ERK, MSK1 and p90RSK. At the post-transcriptional level, DON enhanced IL-6 mRNA stability in macrophages from control mice but this was not affected in macrophages from DHA fed mice. Taken together, these data suggest that DHA might suppress transcriptional activation of IL-6 gene by blocking CREB phosphorylation and subsequently binding to the IL-6 promoter.

INTRODUCTION

Deoxynivalenol (DON) induced IgA nephropathy (IgAN) in mice provides a unique preclinical window for studying prophylaxis and treatment of IgAN at its early stages (Jia *et al.*, 2004a,b). Ex vivo reconstitution (Yan *et al.*, 1998), antibody neutralization (Yan *et al.*, 1997) and interleukin-6 (IL-6) knockout mice studies (Pestka and Zhou, 2000) have revealed induction of IL-6 gene expression to be crucial to DON-induced IgAN. (n-3) Polyunsaturated fatty acids (PUFAs) were efficacious in treatment of IgAN in several clinical studies (Donadio, 2001b). In our previous studies, consumption of DHA, EPA was found to retard the progress of IgAN in mouse and this correlated with its inhibition on IL-6 gene expression and MAPK activation (Jia *et al*, 2004b). However the mechanisms by which DHA downregulates IL-6 gene expression and the upstream signal transduction remain unclear.

Regulation of IL-6 gene expression can potentially involve several signal transduction pathways and multiple transcriptional factors (Hershko *et al.*, 2002; Mainiero *et al.*, 2003). cAMP Response element binding protein (CREB), activating protein-1 (AP-1), CCAAT/enhancer binding protein beta (C/EBPβ) and nuclear factor κ B (NFκB) all contribute to IL-6 transcriptional upregulation based on electrophoresis mobility shift assay (EMSA) and point mutation analyses (Matsusaka *et al.*1993; Robb *et al.* 2002). Acute oral exposure to DON induces serum IL-6 as well as IL-6 mRNA expression in spleen and Peyer's patches and this correlates with both MAPK activation and increased AP-1, CREB, C/EBPβ and NFκB binding activity both in mice and in RAW 264.7 macrophage cells (Zhou *et al.*, 2003; Moon and Pestka, 2003).

The purpose of this study was to identify molecular mechanisms by which DHA inhibits IL-6 gene expression. Multiple transcriptional factors involved in IL-6 regulation and their relative upstream pathways were examined. It was found that DON significantly induced transcriptional factor CREB and AP-1 activation and CREB binding to the IL-6 promoter. DHA antagonized DON-induced CREB phoshorylation and subsequent binding to IL-6 gene promoter both in vitro and iv vivo.

MATERIALS AND METHODS

Materials. All chemicals (reagent grade or better) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Fluo-3, F125, ionomycin, SB203580, SP600125, PD98059, MDL-12330A, KN-62, KT5720 were purchased form EMD Biosciences, Inc (San Diego, CA). DON used in the cell culture was purchased from Sigma Chemical (St. Louis). DON used in feeding study was produced in *Fusarium graminearum* R6576 cultures and purified by the improved water-saturated silica gel chromatography method (Clifford *et al.*, 2003). Purity of DON was verified by a single HPLC peak occurring at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware contaminated with mycotoxin were detoxified by soaking for at least 1 hr in 100 ml/L sodium hypochlorite. Purified DON was added to powdered diets as detailed by Pestka *et al.* (1989).

Animals. Female B6C3F1 mice (7 wk old), weighing between 20 to 25 g were obtained from Charles River (Portage, MI). Mice were housed in environmentally protected transparent polypropylene cages with stainless steel wire tops for 1 wk prior to introduction of different treatments. Mice were given free access to water and food. Experimental diets were placed in feed jars containing stainless steel top and mesh cylinder to minimize spillage. The environmental conditions included 23–25°C, 45 to 55% relative humidity, and 12:12 hr artificial photoperiod. Housing, handling and sample collection procedures conformed to the policies and recommendations of Michigan State University. All University Committees on Animals were in accordance with guidelines established by the National Institute of Health.

Diets and experimental design. Experimental diets were derived from a purified AIN-93G formulation (Reeves *et al.*, 1993). The basal diet consists of the following ingredients (per kg): 10 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.5 g cornstarch, 132 g dyetrose, 50 g cellulose, 3 g L-cystine, 2.5 g choline bitartrate, 14 mg TBHQ, and 100 g sucrose (Dyets Inc, Bethelehem, PA). Corn oil (Dyets Inc), oleic acids (Dyets Inc) and MEG-3^{TM} DHA enriched oil (containing DHA 483g/kg and 113g/kg EPA), a gift from Ocean Nutrition Canada Ltd, were used to amend to yield two experimental diets: Control (10 g corn oil and 60 oleic acid/ kg diet) and DHA (10 g corn oil and 60 g DHA enriched oil/ kg diet). Diets were prepared every 2 wk, stored at -20°C and provided to mice daily. Previous studies demonstrated that consumption of this DHA diet increased liver DHA from 3% to 11% and EPA from 0.11 to 4.8% of total phospholipid within 4 wk (Jia *et al.*, 2004b). Mice were kept on diets for 4-5 wk before experiments. Macrophage were collected as described below. For spleen nuclear protein extraction, mice were anesthetized with methoxyfluorane and killed by cervical dislocation. Spleen were removed for nuclear protein extraction.

Peritoneal macrophage cultures. Macrophages were harvested as described by (Conrad, 1981). Briefly, after kept on diets for 4 wk, mice were injected (ip) with 1 ml 9% thioglycollate. After 3 d, macrophages were collected by peritoneal lavage with cold Hanks buffer (Invitrogen Corporation, CA) and pelleted at 1200 rpm for 5 min. Cells were resuspended in DMEM (Invitrogen Corporation) containing 10% heat inactivated FBS, and 0.025% Penicillin-Streptomycin solution (Sigma) and settled down at 37°C under 7% CO_2 in a humidified incubator for 24 h before addition of DON.

Lipid extraction and analysis. Fatty acids were analyzed by a modification of the method of Hasler *et al.* (1991) by gas chromatography (GC) utilizing GC-2010 gas chromatograph (Shimadzu Scientific Instruments, Inc, IL) and standard fatty acid methyl ester (Nu-Check Prep.Inc. Elysian, MN).

IL-6 gene expression. DON (250 ng/ml) was added to the macrophage cell culture (1×10^6/ml) for 24 hrs. Cell supernatant was analyzed for IL-6 by ELISA (Moon and Pestka, 2003) using purified rat anti-mouse IL-6 (Pharmingen, San Diego, CA) and biotinylated rat anti-mouse-IL-6 (Pharmingen, San Diego, CA). IL-6 hnRNA and mRNA were measured by Real-time PCR as described by Jia *et al.* (2004b). 18S RNA was used to normalize target gene expression. Target gene expression levels were calculated relative to the control group (PE Applied Biosystems user bulletin number 2). Probe and primers for mouse IL-6 mRNA and endogenous control (18S RNA) were purchased as TaqMan assay reagents (PE Applied Biosystems, Foster City, CA). Real-time Polymerase Chain Reaction Primer Express software (PE Applied Biosystems, Foster City, CA) was employed to design primer pairs for mouse IL-6 hnRNA (forward primer: GTC CAA CTG TGC TAT CTG CTC ACT; backward primer: AGA AGG CAA CTGG ATG GAA GTC T).

Nuclear extraction. After keeping mice on diets for 4 wk, macrophages were prepared as described above. Cells were treated with DON (250 ng/ml) for 30 min before nuclear protein extraction. For splenic nuclear extracts, mice were gavaged with DON (25 mg/kg BW), 30 min later spleen cells were prepared as described previously (Pestka *et al.*, 2002). Nuclear extracts were prepared by lysing 1×10^7 cells in buffer A (20 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, containing 1.5

microgram/ml aprotinin, pepstatin, leupeptin and chymostatin) for 15 min on ice. After the addition of Nonidet P-40 (0.5% final concentration), cell lysates were centrifuged at 1000×g for 10 min at 4 C. Pelleted material was incubated with 20 mM HEPES pH 7.9, 400mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, containing 1.5 microgram/ml aprotinin, pepstatin, leupeptin and chymostatin for 60 min on ice. Insoluble material was removed by centrifugation at 14 000 ×g for 15 min at 4 °C. The protein concentration of the supernatant was determined using a Bio-Rad protein assay (Bio-Rad Laboratories Inc, Melville, NY).

Electrophoretic mobility shift assay (EMSA). EMSAs (Zhou *et al*, 2003) were performed as using NFκB consensus sequence 5' AGT TGA GGG GAC TTT CCC AGG C 3', mutant 5'AGT TGA GGC GAC TTT CCC AGG C 3'; C/EBP consensus sequence 5' TGC AGA TTG CGC AAT CTG CA 3', mutant 5' TGC AGA GAC TAG TCT CTG CA 3'; AP-1 consensus sequence 5' CGC TTG ATG ACT CAG CCG GAA 3', mutant 5' CGC TTG ATG ACT TGG CCG GAA 3' and CRE consensus sequence 5' AGA GAT TGC CTG ACG TCA GAC AGC TAG 3', mutant 5'-AGA GAT TGC CTG TGG TCA GAG AGC TAG-3' from Santa Cruz Laboratories (Santa Cruz, CA). Probes were end-labeled with [γ-^{32}P] ATP using polynucleotide kinase T4 (GIBCO BRL, Grand Island, NY) and purified from unincorporated [γ-^{32}P] ATP using a purification column (Bio-Rad Laboratories). After purification using NucTrap probe column (Stratagene, CA), binding was achieved by incubating 20 μg of cell nuclear extracts with 2 μl of labeled fragment at room temperature for 30 min. For competition experiments, synthetic wild type and mutant type oligonucleotides were used in 150-fold molar excess and incubated with nuclear extract for 15 min before the radiolabelled probe was loaded. Samples were then

subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 30 mA using Tris borate-glycine buffer (0.45 M Tris borate, 0.001 M EDTA, pH 8.3). Blots were dried at 80°C for 3 h and analyzed by autoradiography.

Chromosome immunoprecipitation (ChIP) assay. ChIP-IT Kit (Active motif, Carlsbad, California).was used according to manufacturer's instruction. Briefly, peritoneal macrophage cells (2×10^7) were fixed using 1% (v/v) formaldehyde to cross link protein and DNA, 30 min after DON (250 ng/ml) stimulation. DNA was sonicated into small uniform fragments form 200-500 bp. DNA/protein complexes were immunoprecipitated with rabbit polyclonal antibodies directed against the phospho-CREB (Cell signaling), c-jun, C/EBP and p65 (Santa Cruz Biotech, CA). Following immunoprecipitation, cross-linking was reversed, proteins were removed by proteinase K treatment and the DNA purified on column provided. The DNA fragments were then screened by SYBR green Real-time PCR to determine which transcriptional factor was bound to the IL-6 promoter. Primers that flanked the different transcriptional factors binding sites were designed using Primer express software (AppliedBiosystems, Inc) and were listed as follows: CREB binding site: Forward: GGC TAG CCT CAA GGA TGA CTT AAG Backward: TTG CAC AAT GTG ACG TCG TTT; AP-1 binding site : Forward : CCT TAT AAA ACA TTG TGA ATT TCA GTT TTC , Backward : CAT GAG CAC TCT TCT TTT TTT CTT TAA A; NFκB : Forward: CTT TCG ATG CTA AAC GAC GTC ACA Backward : AAA TCT TTG TTG GAG GGT GGG (Figure 6.1).

Western analysis. Macrophages were washed with ice-cold phosphate buffer, lysed in 1% (w/v) SDS buffer containing 1.0 mM sodium ortho-vanadate and 10 mM Tris (pH 7.4) and sonicated for 10 s. After protein concentration determination, equal amounts

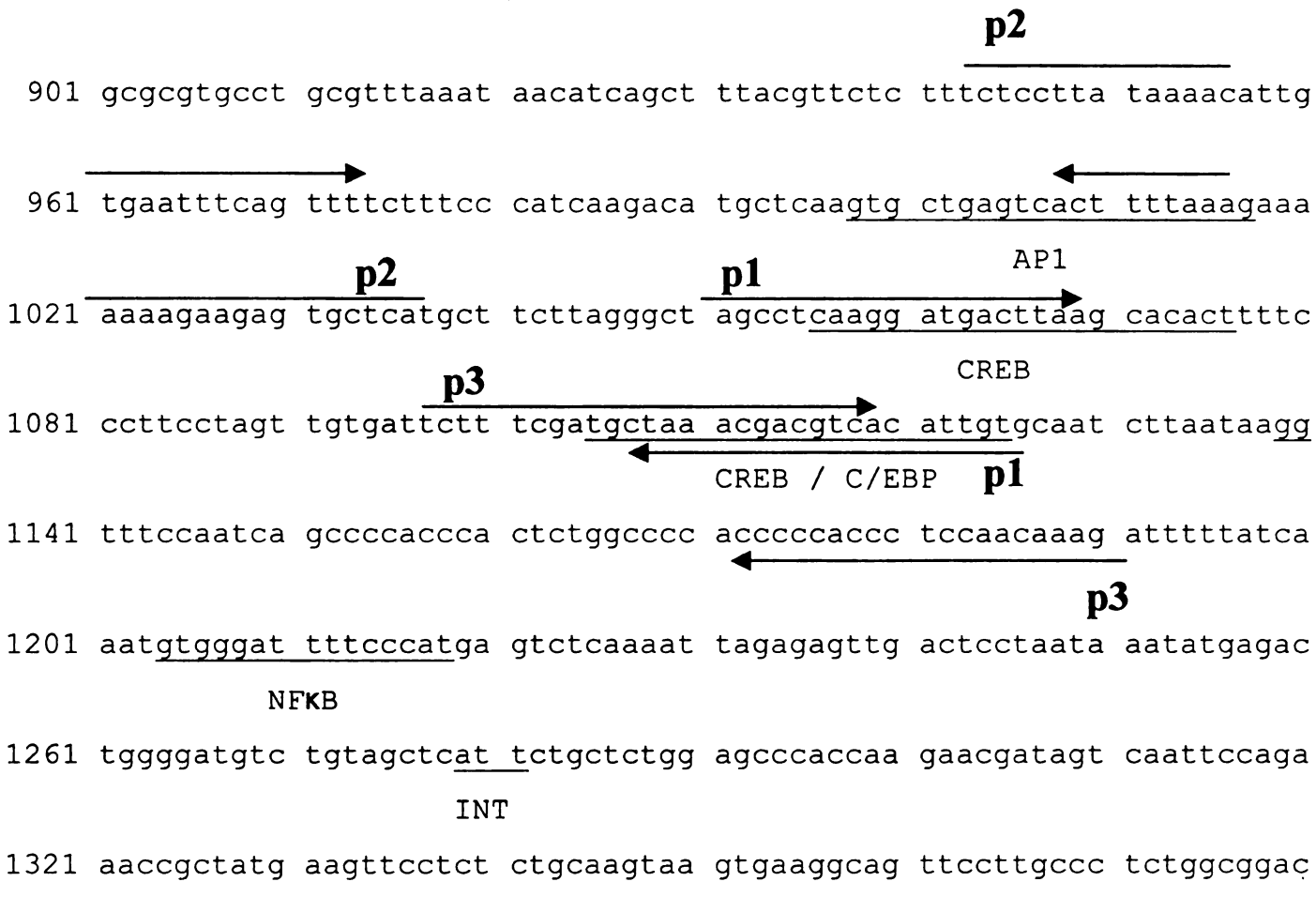


Figure 6.1 The position of the primers in ChIP assay. p1 for CREB and C/EBP; p2 for AP-1; p3 for NFκB.

of protein were fractionated by SDS-PAGE with 10%(w/v) acrylamide separation gel, and subjected to Western analysis using specific antibodies for p38, phospho-p38, ERK, phospho-ERK, JNK, phospho-JNK antibodies (New England Biolabs, Beverly, MA), phospho-CREB, CREB (cell signalling CA), phospho c-jun, c-jun, phospho p65, p65, phospho C/EBPβ and C/EBPβ, phospho MSK1 and phospho p90RSK (Santa Cruz, CA).

Inhibitor studies. Macrophages were harvested and cultured in 1 ml of DMEM in 6 well plate (1×10^6 cells /well) at 37 °C under 7% CO_2 in a humidified incubator for 24 h as described above. Inhibitors for different kinases, PKA inhibitor (KT5720, 1μM), CaMkII inhibitor (KN62, 1μM), adenylyl cyclase inhibitor (MDL13322, 1 μM), p38 inhibitor (SB-203580, 20 μM), ERK inhibitor (PD-98059, 100 μM) and JNK inhibitor (SP600125, 10 μM) were added to the medium for 30 min before DON (250 ng/ml) stimulation. Three hours after DON treatment, total RNA was extracted and IL-6 mRNA measured. Following treatment described in inhibitor assay, cytotoxicity was measured by MTT assay. Briefly, 20 μl of a 5 mg/ml solution of 3-(4,5 dimethylthiazol-2-ul)-2,5-diphenyl tetrazolium bromide (MTT) in 0.01 M PBS was added to each well for the final 3 h incubation period (Uzarski *et al*, 2003). Microtiter plates were centrifuged at 450×*g* for 20 min and media was aspirated to minimize formazan crystal loss. Resultant crystals were dissolved in 150 μl DMSO for 15 min. Optical density was measured using a Vmax Microplate Reader (Molecular Devices, Menlo Park, CA) at dual wavelengths, 570 and 690 nm. Percent control response was calculated as follows: ([1-absorbance of treatment/absorbance of control]×100).

Measurement of $[Ca^{2+}]$. Intracellular $[Ca^{2+}]$ was measured as described by McCormack and Cobbold (1991). Macrophages (1×10^7) were suspended in 0.75 ml

incubation media (DMEM containing 2% [w/v] BSA) and combined with 0.25 ml of loading buffer (containing 0.025% fluronic F127 and 20 μM Fluo-3AM in incubation buffer) for 30 min at 35°C water bath. Cells were then washed three times with incubation media without BSA and diluted to a concentration of 2×10^6 /ml. Cells (150 μl/ well) were aliquoted into Fluoronunc 96 well plates (Fisher Scientific, Pittsburgh, PA) Background fluorescence was recorded (485 nm excitation, 534 nm emission) using a CytoFluor II microwell fluorescence reader and the CytofluorII software Version 2.0 (Biosearch Incorporated, Bedford, MA). When baseline fluorescence stabilized, ionomycin (positive control, 1μM), DON (250 ng/ml) were added and fluorescence intensity was recorded at several time points.

Measurement of cAMP. Intracellular cAMP was measured by Cyclic AMP (low pH) Immunoassay Kit (R&D Systems, Inc, Minneapolis, MN) according to manufacturer's instruction. Briefly, peritoneal macrophage cells (1×10^6/ well) were lysed with 0.1N HCl for 30 min at 37 °C. The cell lysate (100 μl) was added to the microplate stripes provided in the kit. The cAMP present in a sample competes with a fixed amount of alkaline phosphatase-labeled cAMP for sites on a rabbit polyclonal antibody. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The color development is stopped and the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of cAMP in the sample.

Statistics. Data were analyzed using the Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Data were subjected to one-way ANOVA and pairwise comparisons made by Bonferroni or Student-Newman-Keuls methods. If data did not

meet the normality assumption, they were subjected to Kruskal-Wallace ANOVA on Ranks and pairwise comparisons made by Dunn's or Student-Newman-Keuls methods. Differences were considered significant at $P < 0.05$.

RESULTS

The capacity for DHA to modify the cellular phospholipid profile in thioglycollate elicited peritoneal macrophage was confirmed by GC. DHA increased DHA percentage form 0.9% to 2.9 %, decreased arachidonic acid level form 4 to 2% in the percentage of phosholipid whereas EPA concentrations were not changed (Table 6.1).

Previous studies suggest that DON enhances IL-6 production in vivo by increasing transcriptional activity and DHA can impair DON-induced IL-6 production (Jia *et al*, 2004b). As we have been documented, macrophage cells play an important role in DON-induced IL-6 production (Yan *et al.*, 1998), but it is not known whether DON can induce IL-6 gene expression in primary macrophage cells. Treatment of thioglycollate elicited peritoneal macrophage with the DON (250 ng/ml) significantly induced IL-6 mRNA, hnRNA expression after 3 hr (Figure 6.2A, B) and IL-6 protein production in supernatant after 24 h (Figure 6.2C). The three parameters were suppressed in macrophages from DHA fed mice by 90,77 and 98% respectively.

DON can increase IL-6 mRNA concentration by enhancing mRNA stability (Wong *et al.*, 2001). To test if DHA can reduce IL-6 mRNA stability, the transcriptional inhibitor, 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB, 100 μM) was used to block gene transcription 2h after LPS stimulation, and remaining IL-6 mRNA was measured. DRB inhibited LPS-induced IL-6 mRNA, (Figure 6.3A) and DHA did not alter DON-induced mRNA stability (Figure 6.3B).

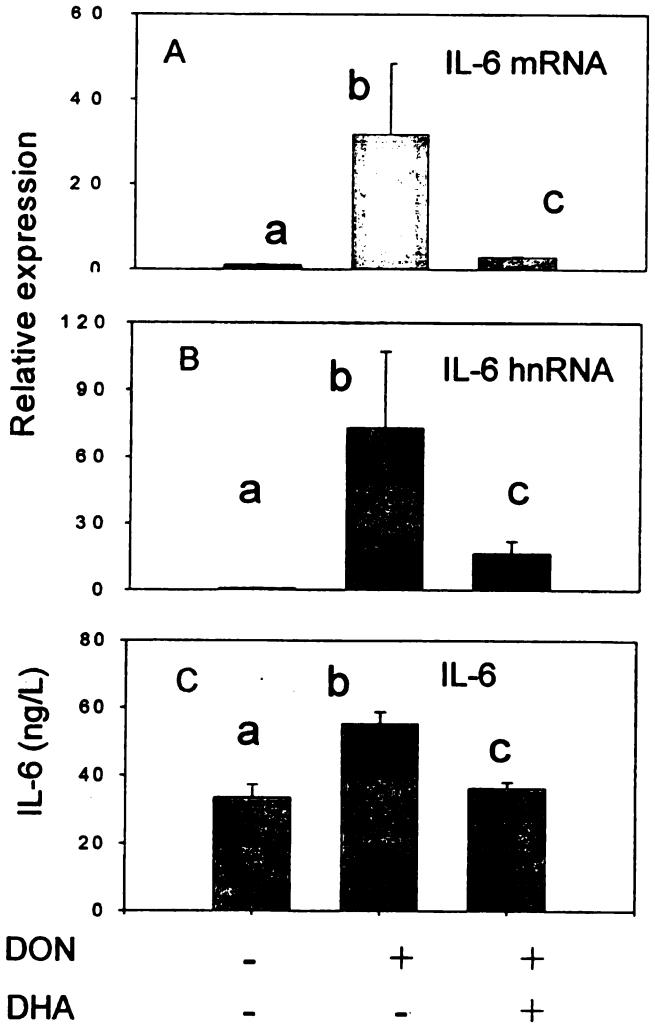
Because the results described above suggested that DHA might influence IL-6 mRNA level at transcriptional stage. DHA effects on transcriptional factor activation

Table 6.1 .Fatty acid composition of macrophage phospholipid in study 1(n=3).

		control	6%DHA
Stearic Acid	18: 0	16.1±3.5[a]	17.0±0.6[a]
Oleic Acid	18: 1	11.9±1.9[a]	13.0±2.5[a]
linoleic Acid	18: 2	2.18±0.3[a]	2.3±0.06[a]
Arachidonic Acid	20: 4	4.2±0.2[b]	2.4±0.2[a]
Eicosapentaenoic Acid	20: 5	1.2±0.2[a]	1.3±0.4[a]
Docosahexaenoic Acid	22: 6	0.9±0.21[a]	2.9±0.4[b]

The value stands for the percentage of total phospholipid extracted from macrophage. Different letter mean significant difference compared to the control group. DHA docosahexaenoic acid; EPA eicosapentaenoic acid: AA arachidonic acid.

Figure 6.2 Effects of DHA on DON-induced IL-6 gene expression in thioglycollate elicited peritoneal macrophages. Mice were fed with AIN93G diet containing DHA for 4 wk. At experiment termination, thioglycollate elicited peritoneal macrophages were harvested and cultured in DMEM. Cells were treated with DON (250 ng/ml) for 3h. RNA was extracted and analyzed by real-time PCR for IL-6 mRNA (A) and hnRNA(B). Data were normalized against 18S RNA and expressed relative to control value. (C) In another experiments cells were treated with DON (250 ng/ml) for 24h. Supernatant IL-6 was analyzed by ELISA. Results are means ± SE; n=3. Bars with different letters differ, ($p<0.05$).



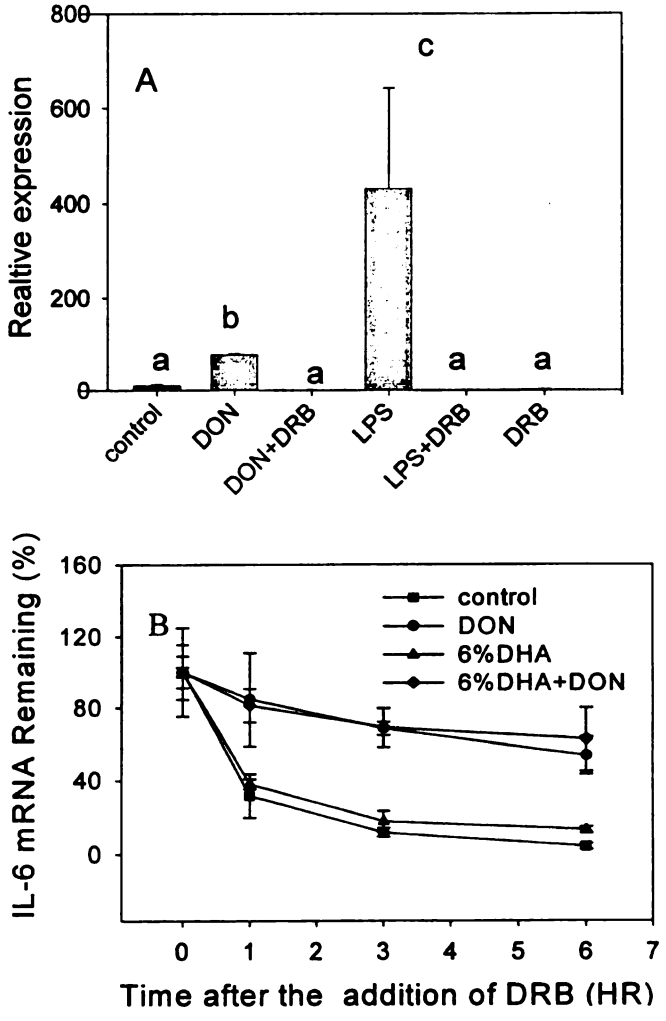


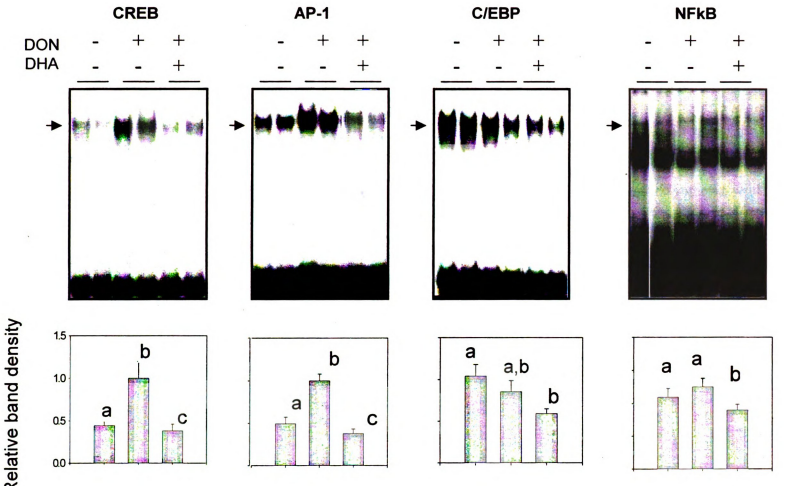
Figure 6.3 Effect of DHA on DON-induced IL-6 mRNA stability in LPS-treated thioglycollate elicited macrophage. (A) Cells were treated with 100ng/ml LPS and DRB (100 μM) for 2 h RNA was extracted and analyzed by real-time PCR for IL-6 mRNA. (B) Cells were pre-treated with 100ng/ml LPS for 2 h, medium was then removed and fresh medium containing DON (250 ng/ml) and/or DRB (100 μM) was added. RNA was extracted at 0, 1, 2, 3 and 6h and analyzed by real-time PCR for IL-6 mRNA. Data were normalized against 18S RNA and expressed relative to control value. Results are means ± SE; n=3. Bars with different letters differ, ($p<0.05$).

were tested ex vivo and in vivo. In macrophages, DON induced nuclear protein specific binding both to CREB and to AP-1 consensus sequence and this binding was inhibited in macrophages coming form mice fed DHA enriched oil (Figure 6.4 A). Although DON did not induce C/EBP and NFκB binding, DHA inhibited C/EBP binding and had no effects on NFκB binding (Figure 6.4 A). Competitive experiment with excess wild type and mutant consensus sequences indicated that the analyzed binding was specific (Figure 6.4 B). In spleen, acute DON exposure increased nuclear protein binding to CREB, AP-1 and NFκB consensus sequences but not to C/EBP consensus sequence. Nuclear protein form DHA fed mice showed less binding activity to all 4 consensus sequences (Figure 6.3 C).

To investigate whether EMSA binding reflects the intranuclear binding within nuclear, ChIP was performed in conjunction with SYBR PCR. DON was found to elevate phospho-CREB binding to the promoter region of IL-6 in macrophage from mice fed control diet and this interaction was magnificently inhibited in macrophages from DHA fed mice (Figure 6.5). In contrast, p65, c-jun and C/EBPβ binding did not differ within control, DON and DHA plus DON groups.

To test whether DHA inhibited transcriptional factor binding by changing their protein level or by phosphorylation modification, Western blots were performed on whole cell protein lysate. The results showed that DON-induced phosphorylation of CREB, ATF-1, c-jun and p65 but this was suppressed in macrophages from mice fed DHA (Figure 6.6). The non-phosphorylated forms of these transcriptional factors were constant within these groups. Phosphorylation of C/EBPβ was not affected by DON and DHA.

Figure 6.4 A Characterization of effects of DHA on DON-induced transcriptional factor binding to their consensus sequence in thioglycollate elicited peritoneal macrophage. EMSA analysis was conducted with labeled CREB, AP-1, C/EBP and NFκB consensus sequenceS and 20μg of macrophage nuclear extract. The band density was scanned and two experimental results were combined together. Data were expressed relative to DON group value. Results are means ± SE; n=6. Bars with different letters differ, ($p<0.05$).


CREB
AP-1
C/EBP
NFkB
DON - + + - + + - + + - + +
DHA - - + - - + - - + - - +
Relative band density
1.5
1.0
0.5
0.0
a
b
c
a
b
c
a
a,b
b
a
a
b

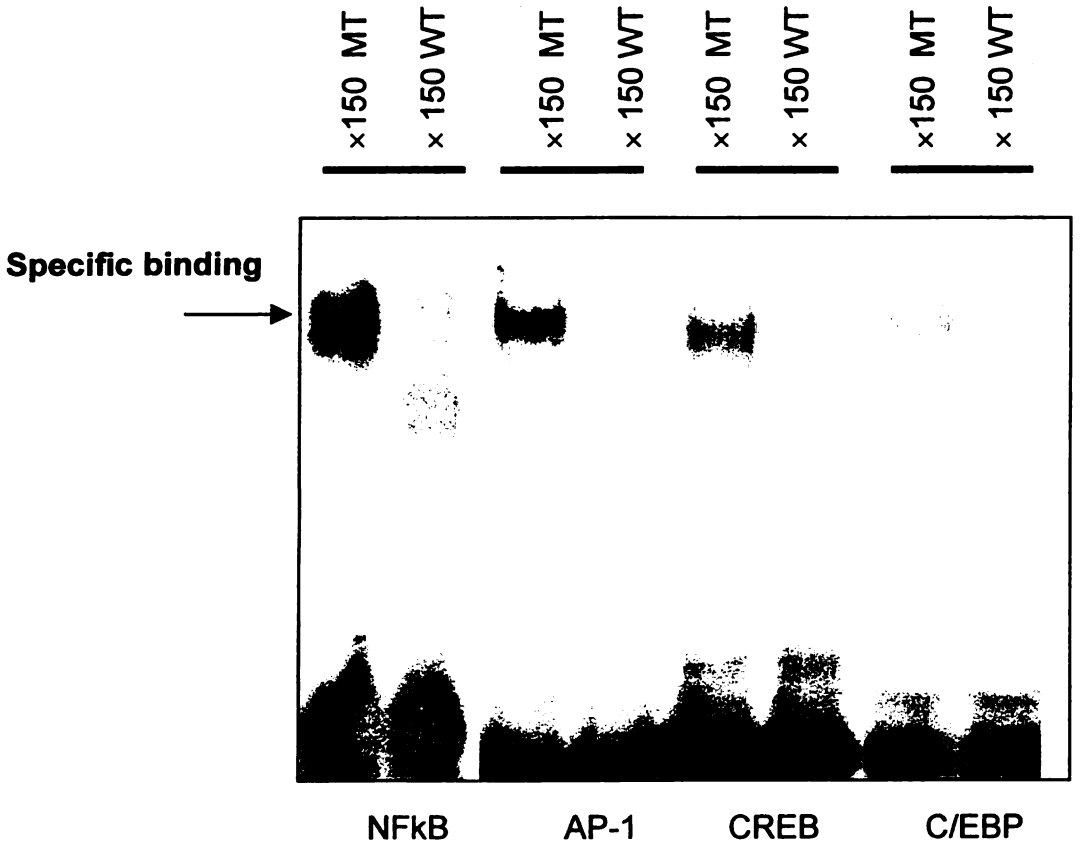


Figure 6.4 B EMSA competition experiments to identify the specific binding. Synthetic wild type and mutant type consensus sequences for CREB, AP-1.C/EBP and NFkB were used in 150-fold molar excess and incubated with 20µg macrophage nuclear extract for 15 min before the radiolabelled probe was loaded and EMSA analysis was conducted as described above.

Figure 6.4 C Characterization of effects of DHA on DON-induced transcriptional factor binding to their consensus sequence in splenic nuclear extract. EMSA analysis was conducted with labeled CREB, AP-1.C/EBP and NFkB consensus sequences and 20μg of splenic nuclear extract. The band density was scanned. Data were expressed relative to DON group value. Results are means ± SE; n=3. Bars with different letters differ, $p<0.05$.

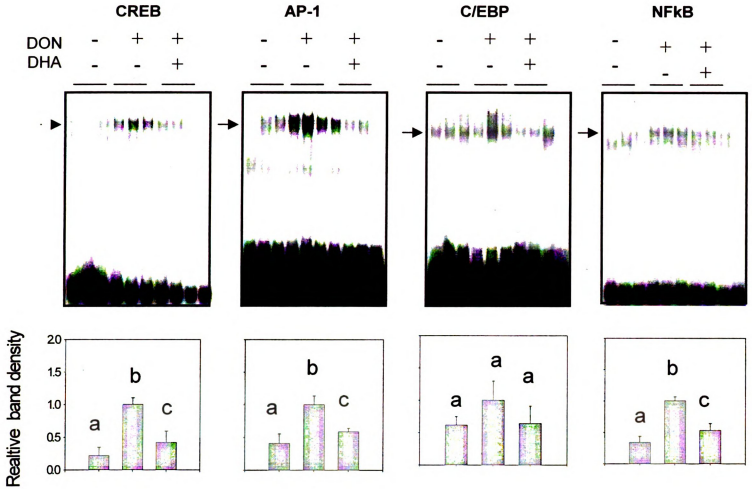

CREB
AP-1
C/EBP
NFkB
DON - + + - + + - + + - + +
DHA - - + - - + - - + - - +
Realtive band density
2.0
1.5
1.0
0.5
0.0
a
b
c
a
b
c
a
a
a
b
c

Figure 6.5 ChIP analysis of DHA effects on DON-induced transcriptional factor binding to IL-6 promter in thioglycollate elicited peritoneal macrophage in vivo. ChIP was performed using anti phospho-CREB, c-jun, C/EBPβ and p65 antibody as described in method. The enriched DNA fragment was quantified by SYBR green real time PCR with primers to amplify a 82-bp segment CREB and C/EBPβ binding site (-149 to –231); a 97-bp segment AP-1 binding site (-240 to –332) and a 97-bp segment NFκB binding site (-240 to –332) upstream the initiation site of IL-6 gene. The negative IgG enriched DNA was also analyzed as a negative control. The data was normalized to their input control. To minimize the variance all data were expressed relative to the value of DON group, and 3-4 independent experiments results were combined for transcriptional factor data and 2-3 experiments results were combined for negative control IgG data. Results are means ± SE; n=3. Bars with different letters differ, $p<0.05$.

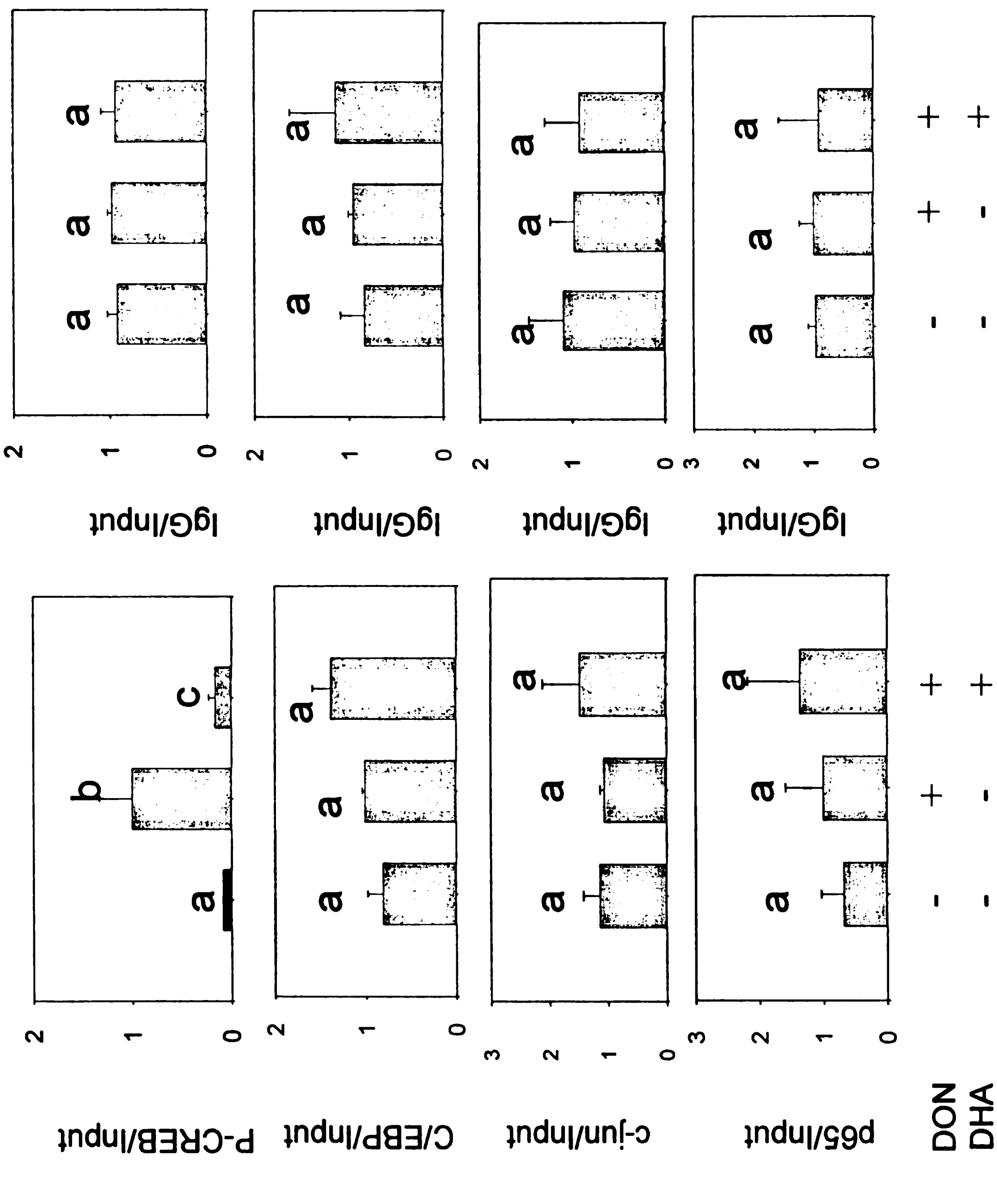
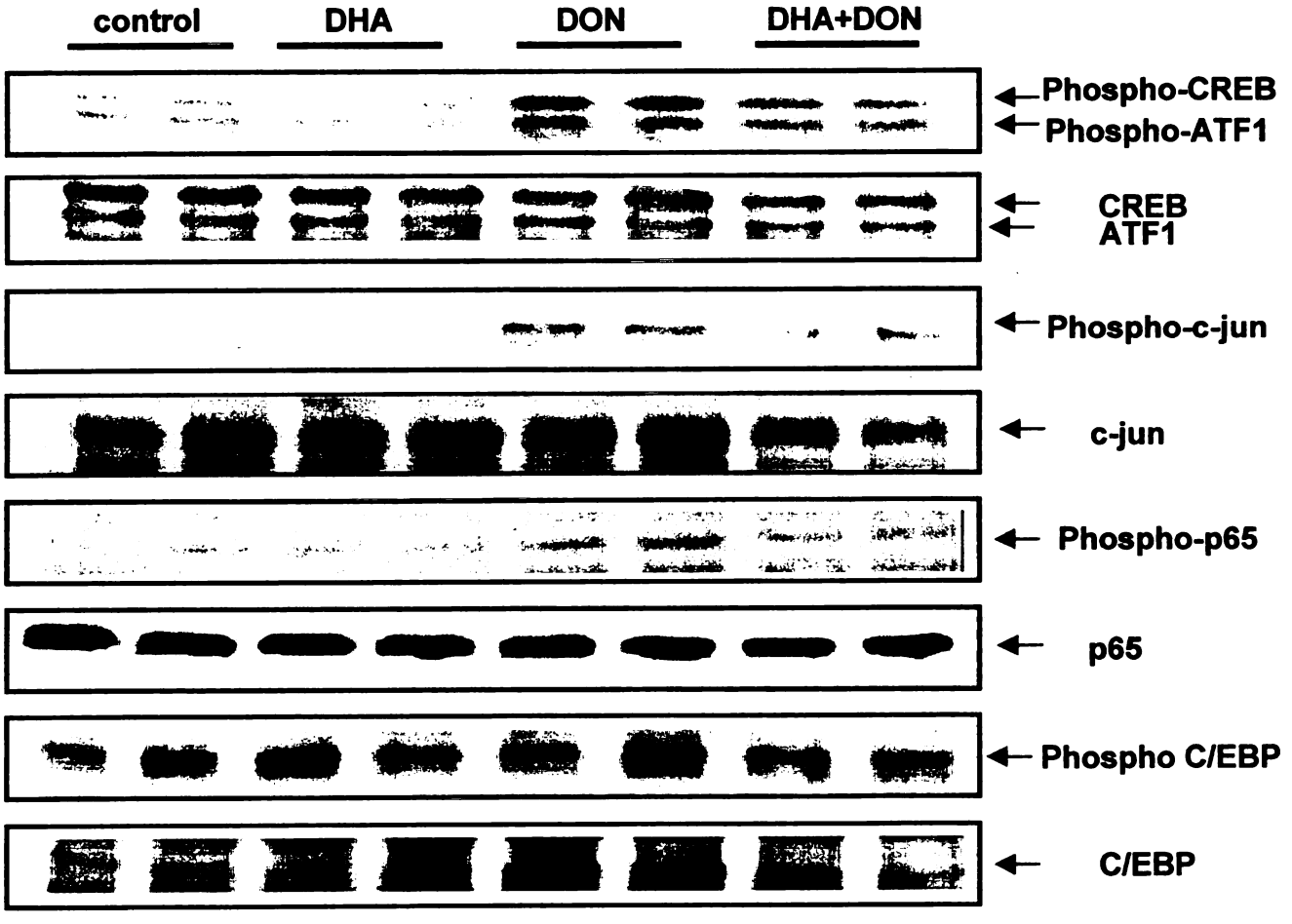

P-CREB/Input
C/EBP/Input
c-jun/Input
p65/Input
IgG/Input
DON
DHA
a
b
c

Figure 6.6 DHA effects on DON-induced transcriptional factor phosphorylation. Cells were treated with 250 ng/ml of DON for 30 min. Whole cell lysates were subjected to Western blotting using antibodies specific for phosphorylated and non phosphorylated forms of CREB , c-jun, p65 and C/EBP. Data are representative of two independent experiments.


control
DHA
DON
DHA+DON
Phospho-CREB
Phospho-ATF1
CREB
ATF1
Phospho-c-jun
c-jun
Phospho-p65
p65
Phospho C/EBP
C/EBP

CREB mediated IL-6 gene expression might be activated by several kinases including MAPKs, PKA and calcium camodulin protein kinase (CAMKII). The results indicated p38 inhibitor caused the maximum inhibition on IL-6 mRNA expression whereas JNK inhibitor had no effects (Figure 6.7). The ERK inhibitor, CaMKII and PKA inhibitor inhibit DON-induced IL-6 mRNA by 75, 63 and 10%, respectively, while adenylyl cyclase inhibitor did not inhibit DON-induced IL-6 mRNA. MTT assay indicated that these inhibitors did not cause cell toxicity compared to control group (Data are not shown).

Since CREB phosphorylation and activation might be mediated by MAPKs, DHA effects on DON-induced p38, JNK and ERK phosphorylation were examined. DON stimulated p38, ERK and JNK phosphorylation after 30 min. Phosphorylation of JNK but not p38 and ERK was suppressed in macrophage form mice fed DHA (Figure 6.8). Two downstream kinases of p38 and ERK: MSK1 and p90RSK were also phosphorylated but were not affected by DHA treatment (Figure 6.9).

Since CaMKII and PKA inhibitor can partially inhibit DON-induced IL-6 mRNA expression, the [Ca^{2+}] and cAMP was then measured right after DON stimulation. The data indicated that ionomycin increased [Ca^{2+}] in macrophages both from mice fed control diet and DHA diet. DON did not induce [Ca^{2+}] increase as reflected by fluorescence increase (Figure 6.10). DON did not induce intracellar cAMP elevation (Figure 6.11).

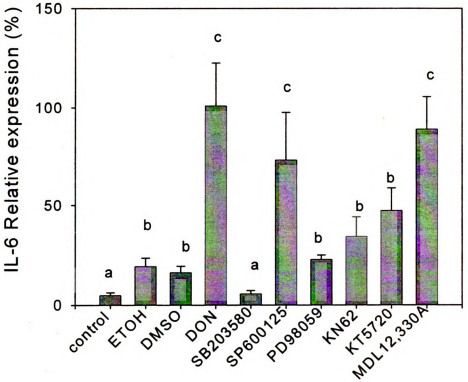
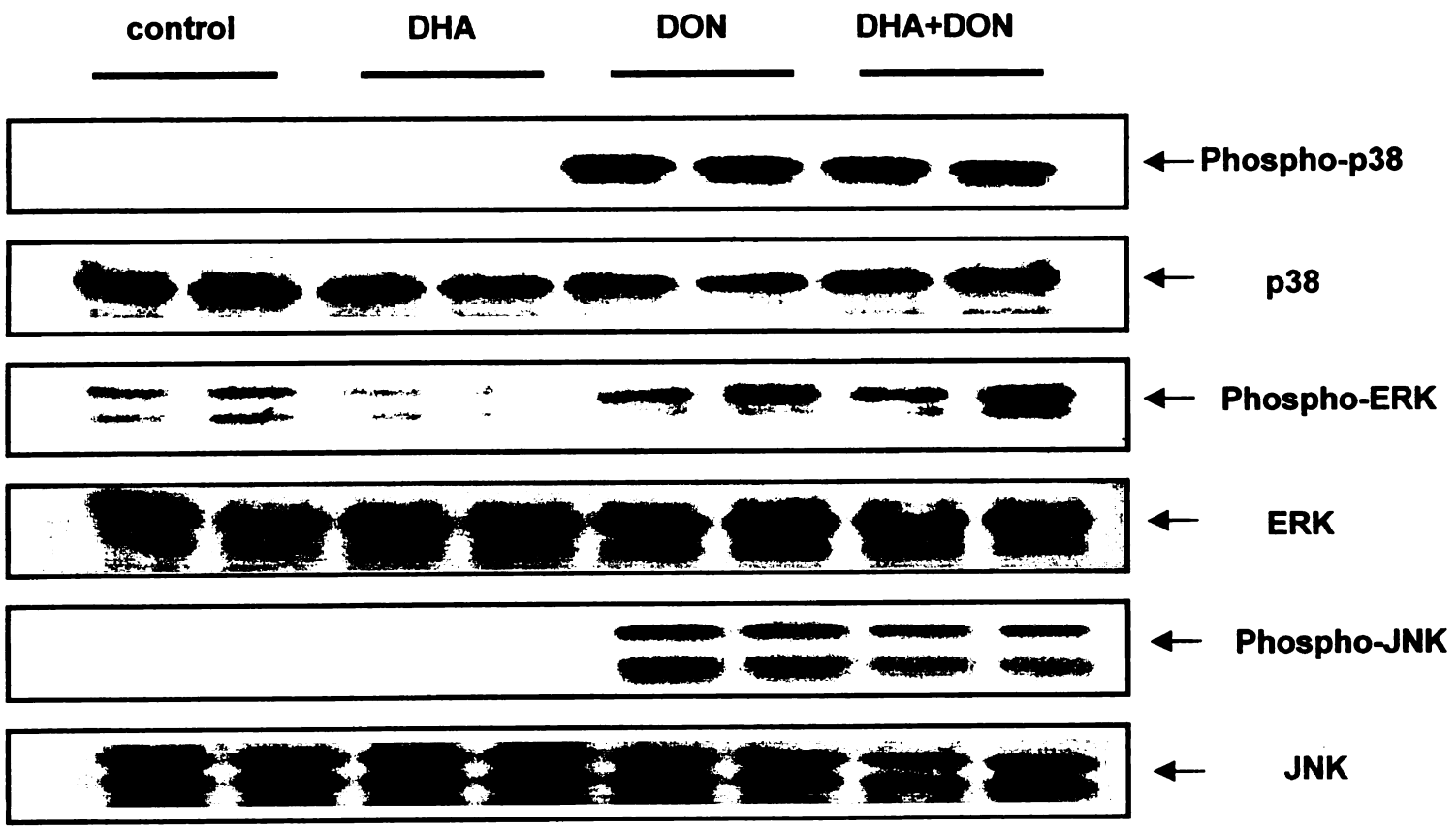


Figure 6.7 Multiple inhibitors on DON-induced IL-6 gene expression in thioglycollate elicited peritoneal macrophage. Macrophages were harvested and cultured in 1 ml of DMEM in 6 well plate (1×10^6 cells /well) at 37 °C under 7% CO_2 in a humidified incubator for 24 h as described above. Inhibitors for PKA (KT5720, 1μM), CaMkII (KN62, 1μM), adenyl cyclase (MDL13322, 1 μM), p38 (SB-203580, 20 μM), ERK (PD-98059, 100 μM) and JNK (SP600125, 10 μM) were added to the medium for 30 min before DON (250 ng/ml) stimulation. Three hours after DON treatment, total RNA was extracted and IL-6 mRNA was measured by real time PCR as described above. Results are means ± SE; n=3-6. Bars with different letters differ, $p<0.05$.

Figure 6.8 DHA effects on DON induced MAPK phosphorylation .Thioglycollate elicited peritoneal macrophage were treated with 250 ng/ml of DON for 30 min, whole cell lysates were subjected to Western blotting using antibodies specific for phosphorylated and non phosphorylated forms of p38 , JNK and ERK. Data are representative of two independent experiments.


control
DHA
DON
DHA+DON
Phospho-p38
p38
Phospho-ERK
ERK
Phospho-JNK
JNK

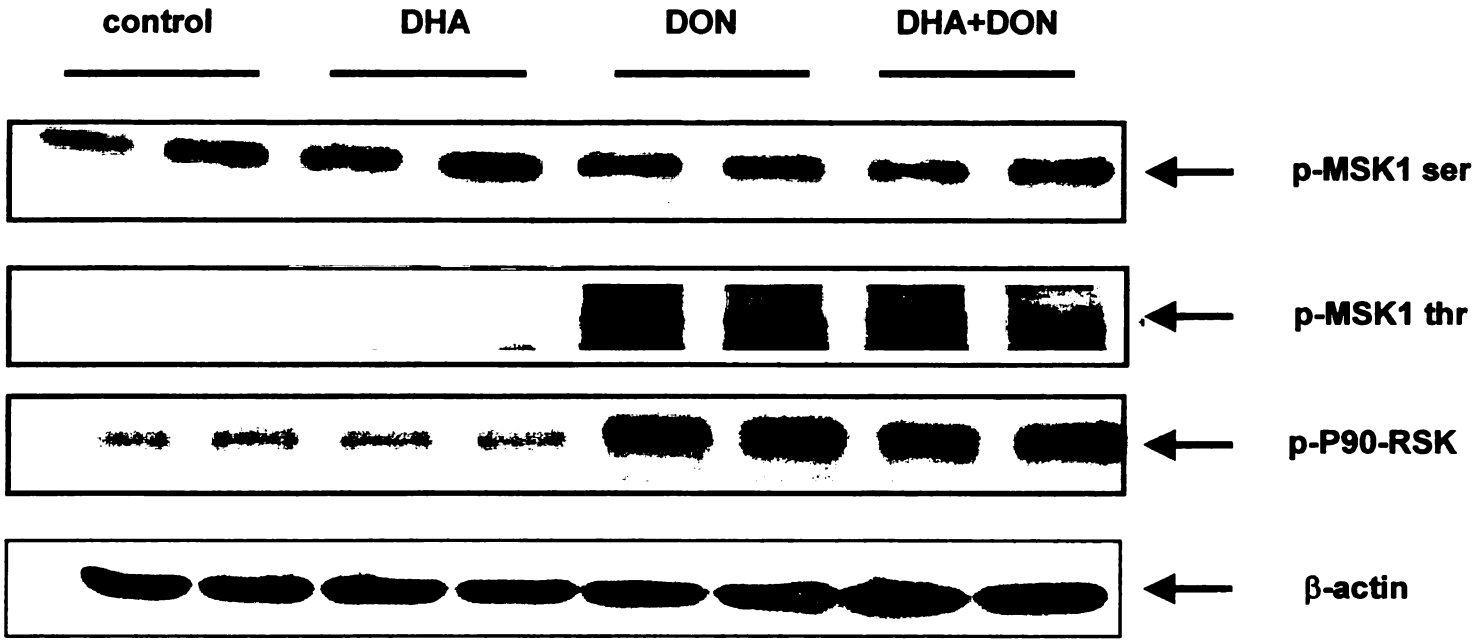
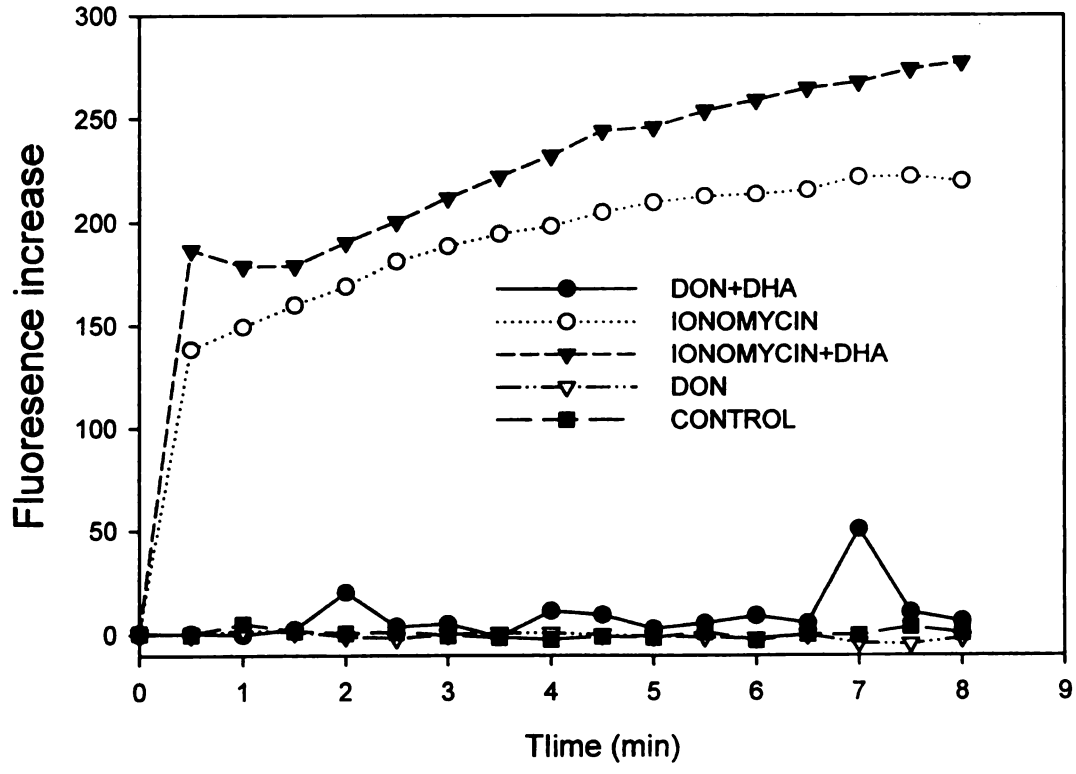


Figure 6.9 DHA effects on DON induced MSK1 and p90RSK phosphorylation .Thioglycollate elicited cells were treated with 250 ng/ml of DON for 30 min. Whole cell lysates were subjected to Western blotting using antibodies specific for phosphorylated form of MSK1 and p90RSK, β-actin was used as loading control. Data are representative of two independent experiments.

Figure 6.10 Intracellular [Ca^{2+}] modulation by DON. Thioglycollate elicited peritoneal macrophages were loaded with Fluo-3 AM as described in Material and Methods. When background was stable, ionomycin (1μM, positive control), DON (250 ng/ml) were added. Fluorescence intensity was monitored using a Cytofluor II microwell reader. Results are means at each time point, n=3.



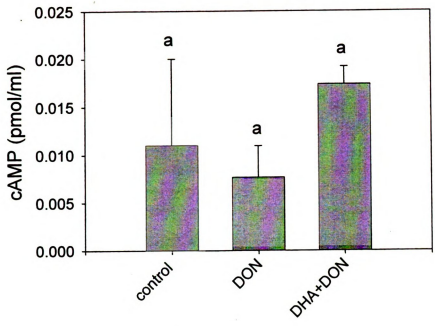


Figure 6.11 Intracellular cAMP modulation by DON. Thioglycollate elicited peritoneal macrophages were stimulated with DON (250 ng/ml) for 30 min. Cell were lysed by 0.1N HCl and cAMP was measured following the Kit's instruction. Results are means, n=3. Bars with different letters differ, $p<0.05$.

DISCUSSION

(n-3) PUFA effects on proinflammatory gene expression (Endres *et al*, 1995) and transcriptional factor, AP-1, NFκB and C/EBP, activation have been previously described (Babcock *et al.*, 2003; Zhao *et al.*, 2004; Weber *et al.*, 1995; Bousserouel *et al.*, 2003). Although the interaction between the (n-3) PUFA and the CREB has been proposed by Logan (2003), this is the first study to report that DHA potentially inhibit expression of the proinflammatory gene IL-6 by blocking transcriptional factor CREB activation and its binding to the IL-6 promoter.

Changes in macrophage phospholipid profile might modulate to IL-6 gene expression. Here, in macrophages DHA was increased from 0.9 to 3.9%, and arachidonic acid was decreased from 4.2% to 2%. These modifications can potentially reduce prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and platelet activated factor (PAF) production (Calder, 2002; Simopoulos, 2002;Whelan, 1996) as well as disrupt lipid raft on cell membrane, which are important for cell signaling (Stulnig *et al.*, 2001). Kaminski *et al.* (1993), in a human study, reported that consumption of 7g/day of 85% pure (n-3) PUFA for 7 wk increases (n-3) PUFA content in human monocyte phospholipids and down-regulates by 70% both PDGF-A and PDGF-B expression. Jia *et al.* (2004b) previously found that DHA consumption significantly increase DHA percentage from 3 to 11% while decreasing arachidonic acid from 12 to 3% in mouse liver after 4 wk DHA consumption of 60 g/kg DHA enriched oil. However, since the liver acts a storage organ for phospholipid of DHA and EPA (Sekine, 1995), the phospholipid profile in liver can not be directly compared to concentrations of these fatty acids in lymphoid tissue after DHA feeding.

Levels of hnRNA, the precursor of mRNA, can be utilized as a substitute method for run-on assay (Johnson *et al.*, 2003), to measure gene transcriptional activity. Because DON induces both IL-6 mRNA and hnRNA in macrophages and because DON-induced IL-6 mRNA expression is blocked by transcriptional inhibitor DRB, DON appeared to activate IL-6 gene transcription in the thioglycollate elicited macrophages. The observation that DHA significantly reduced IL-6 protein, IL-6 hnRNA and mRNA level in DON-treated macrophage, was highly consistent with the previous in vivo study using spleens of DHA-fed mice and exposed acutely to DON (Jia *et al.*, 2004b).

Control of mRNA stability facilitates a rapid adjustment of mRNA levels at posttranscriptional stage. p38 mediates the stability of mRNA with clusters of the AUUUA motif in their 3' untranslated regions (Saklatvala *et al.*, 2003). Wong *et al* (2001) found that post-transcriptional control via enhancement of mRNA stability was likely to contribute to IL-6 superinduction in RAW 264.7 macrophages by DON. DON-induced p38 and ERK activation contributes to transcriptional upregulation of IL-6 whereas p38 plays a role in increasing mRNA stability in cloned macrophage cells (Chung *et al.*, 2003). It had been found that DON could superinduce IL-2 by enhancing mRNA stability (Li *et al.*, 1997). Moon *et al* (2003) showed that DON enhanced both COX-2 transcriptional activation and mRNA stabilization with luciferase reporter vectors. As found previously, addition of DON did enhance IL-6 mRNA stability in peritoneal macrophage. The observation that DHA did not affect IL-6 mRNA degradation suggests that DHA acts primarily by impairing DON-induced IL-6 gene transcriptional activity.

DON induced IL-6 expression correlated with significantly increased CREB, AP-1 binding activities 30 min after stimulation both in spleen and in macrophage revealed by EMSA experiments. NFκB binding activity was also detected in spleen but not in macrophage and C/EBP binding activities were only weakly induced by DON. These results suggested among the transcriptional factors, CREB and AP-1 were initially and potently induced by DON both in thioglycollate-elicited peritoneal macrophages and in spleen.

The DNA in cell nucleus is condensed and packaged by being wrapped around histone octamers called nucleosomes which suppress transcription by imposing a barrier to the access of transcription factors and basal transcriptional machinery to DNA. Chromatin structural remodeling plays fundamental roles in eukaryotic gene regulation (Kadonaga, 1998.). Thus EMSA assay does not always reflect in vivo interaction between transcriptional factors and DNA. However, this endpoint can be measured with ChIP, which revealed that DON significantly induced phosphorylated-CREB binding to the native IL-6 promoter, and this was blocked by DHA treatment. Thus, ChIP was indeed consisted with EMSA results, suggesting CREB might be the most important transcriptional factor involved in DON-induced IL-6 transactivation in elicited peritoneal macrophage, and, furthermore, that DHA consumption blocks CREB binding to IL-6 promoter in vivo. CREB is a nuclear target of signaling pathways activated by multiple stimuli. Evidence suggested that CREB binding to adapter protein CBP that can recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box. CBP possesses an intrinsic histone acetyltransferase (HAT) activity and contributes to

chromatin structure modification, which can make the DNA template more accessible to the transcriptional machinery (Shaywitz and Greenberg, 1999).

DON has been previously proved to drive phosphorylation of multiple transcriptional factors (Zhou *et al.*, 2003). Phosphorylation of CREB, c-Jun, NFκB, will then enhance their affinity to recruit CBP to the IL-6 promoter thus enhancing their transactivtion (Theresia, 2002; Quinn, 2002; Shaywitz and Greenberg 1999). DON induces CREB, c-jun and p65 phosphorylation 30 min after stimulation, which was impaired by the DHA treatment. The finding that, phosphorylation of CREB was most affected by DON and impaired by DHA was consistent with EMSA and ChIP data, suggesting that DHA only affected the signal transduction elicited by DON.

ChIP data did not support involvement of c-jun, C/EBP and NFκB in intranuclear IL-6 promoter. It should be noted here that the inability to detect the interaction between c-jun, C/EBP, p65 and IL-6 promoter region might be because the antibodies used in this experiment were not suitable for ChIP assay. Formadehyde fixation might change the epitope of the protein or the protein complex formed in vivo might also block the antibody-binding site (Kuo and Allis, 1999).

PKA, CaMKII, p38 and ERK all reportly can contribute phosphorylation of CREB at serine 133 (Ser133) which is required for CREB-mediated gene transcription (Shaywitz and Greenberg, 1999; Figure 6.10). The p38 inhibitor, SB202190 and the inhibitor of the upstream activator of ERK1/2, MEK (ERK kinase), PD 98059 showed the most potent inhibition on IL-6 gene expression whereas JNK inhibitor, SP-600125, showed no effect. The failure of JNK inhibitor, SP-600124, on IL-6 gene expression can be explained by the fact that it stimulates CREB phosphorylation (Vaishnav *et al.*, 2003).

Since dominant negative-JNK do not block DON induced PGE2 and COX-2 in macrophage cells (Moon and Pestka, 2003), JNK is likely to be less important than p38 and ERK in IL-6 production. The observation that PKA and CaMKII inhibitor also contributed partially to DON-induced IL-6 mRNA while adenylyl cyclase did not suggests the [Ca2+] and cAMP might be induced by DON. However, we failed to detected DON effects on [Ca2+] and cAMP (Data are not shown). Thus the role of PKA and CaMKII in DON-induced IL-6 gene expression need to be further investigated

Although DHA blocked DON-induced CREB phosphorylation, it failed to suppress p38 and ERK phosphorylation. MSK1 is widely distributed in mammalian cells and can be activated by growth factors, cell-damaging stimuli and proinflammatory cytokines through both ERKs and p38 pathway while p90 RSK (also called MAPKAP-K1) can only be activated through ERK pathway. Both MSK1 and p90RSK can phoshorylate CREB at serine 133 (Song *et al*, 2003). MSK1 knockout mouse showed that it was required for the stress-induced phosphorylation of transcription factors CREB and ATF1 in primary embryonic fibroblasts (Wiggin *et al.*, 2002). The activation of MSK1 and CREB by LPS, adenosine, membrane disruption, angiotensin II, TNFα, lysophosphatidic acid can be similiarly prevented by preincubating the cells with PD 98059 and/or SB 203580 (Caivano and Cohen, 2000; Nemeth *et al.*, 2003; Togo, 2004; Cammarota *et al.*, 2001; Gustin *et al.*, 2004; Lee *et al.*, 2003c; Vaishnav *et al.*, 2003). However, DHA failed to impair DON induced MSK1 and p90RSK phosphorylation suggesting other mechanisms might mediate DHA inhibition on CREB phosphorylation and, ultimately IL-6 gene expression. Similar results were also found in a recent study in which DHA failed to block phorbol 12-tetradecanoate

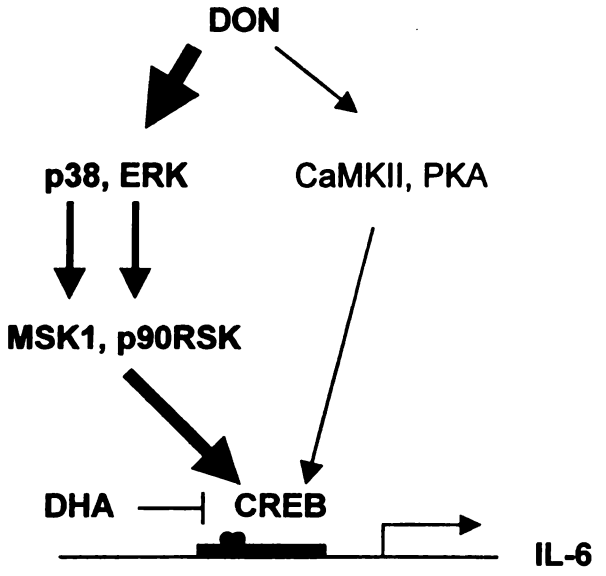


Figure 6.12 Possible pathways involved in DON induced IL-6 gene expression. p38 and ERK might be the most important kinases which mediate DON-induced signal transduction down to the CREB phosphorylation and then IL-6 gene transcription. DHA can significantly reduce IL-6 gene transcription by blocking CREB phosphorylation while has little effects on p38 and MSK1 and P90rsk phosphorylation.

13-acetate (TPA) or epidermal growth factor (EGF) -induced phosphorylation of ERK or p38 kinase and JNK kinase activity in JB6 Cl 41cells (Liu *et al.*, 2001).

The JNK signal is crucial for AP-1 transcription factor activation in cells (Ventura *et al.*, 2003). Neff *et al.*, (2001) found that cells lack JNK exhibited decreased c-Jun, phosphorylation of c-Jun and AP-1 DNA binding activity. Since AP-1 activation is associated with the production of IL-6 (Cuschieri *et al.*, 2004), mice deficient in the JNK pathway had decreased serum levels of IL-6 in response to LPS compared (Morse *et al.*, 2003). Furthermore DN-c-Jun, DN-JNK as well as AP-1 inhibitor, curcumin, can significantly impeded TGF-beta1 induced IL-6 (Park *et al.*, 2003) and JNK/AP1 pathway has also been approved to play an important role in IL-6 promoter activation. (An *et al.*, 2003). So DHA's inhibition on JNK phosphorylation might contribute to its inhibition on c-jun phosphorylation and IL-6 gene expression.

In summary, DON activated multiple transcriptional factors, especially CREB, through p38 and ERK kinase pathways. DHA treatment likely inhibited IL-6 gene transcription by blocking multiple transcription factor activation, among which CREB was likely to be most important. The failure of DHA to inhibit phophorylation of p38 and ERK or their down stream kinases MSK1 and p90RSK, suggests that other mechanisms might exist for DHA inhibition on CREB phosphorylation and IL-6 gene transcription or CREB might also be a target for prophylaxis against IgAN.

CHAPTER 7

Summary and Conclusions

IgA nephropathy (IgAN) is the most common form of nephritis but treatment of this disease remains non-specific. Based on clinical studies, (n-3) polyunsaturated fatty acids (PUFAs) have been showing promising beneficial effects due to its anti-inflammatory effects and safety. The capacity of DON to induce the early stage of IgAN provides a unique window for testing our guiding hypothesis that (n-3) PUFAs might retard development of IgAN by impairing the proinflammatory cytokine production.

The first investigation for this dissertation was to determine if fish oil could attenuate DON-induced IgAN. DON significantly increased serum IgA, serum IgA immune complexes and kidney mesangial IgA deposition compared with the control group, whereas all three variables were significantly attenuated in mice fed 60 g/kg fish oil. In addition, spleen cell cultures from the DON +fish oil group produced markedly less IgA than those cultures from mice fed DON and fish oil. The results confirmed that diets containing fish oil might impair early immunopathogenesis in DON-induced IgAN.

DHA and EPA are two major components of (n-3) PUFAs in fish oil. The second investigation compared their efficacy in preventing DON-induced IgAN. Mice were fed for 18 wk with AIN-93G diets containing EPA or DHA enriched oil. It was found that while DHA and EPA significantly attenuated serum IgA, serum IgA immune complexes and kidney mesangial IgA deposition as well as IgA secretion by spleen cells. DHA was slightly more efficacious than EPA. Pre-feeding of DHA/EPA also significantly reduced serum IL-6 concentration induced by acute oral exposure to DON, which is required for DON induced IgAN.

In the third investigation, the dose response effects of DHA on DON-induced IgAN were measured. DHA dose-dependently inhibited elevated serum IgA and IgA

immune complex (IC) as well as IgA deposition in the kidney. At the same time, spleen IL-6 mRNA and heterogeneous nuclear RNA (hnRNA) concentrations were significantly reduced by DHA. In an acute study, DHA consumption inhibited DON-induced COX-2 mRNA, IL-6 mRNA and hnRNA expression. Phosphorylation of p38, ERK and JNK was also attenuated by DHA. Together, the results indicated that DHA enriched oil at 60 g/kg significantly inhibited DON-induced IgAN and this correlated with impaired IL-6, cyclooxygenase-2 (COX-2) gene expression and mitogen active protein kinase (MAPK) activation. The inhibition of IL-6 hnRNA suggested that DHA might inhibit IL-6 gene expression at transcriptional stage.

To clarify the role of COX-2 gene in development of IgAN, both COX-2 knockout mice and COX-2 specific inhibitor, VIOXX were employed in the fourth investigation. The results demonstrated that knocking out COX-2 and VIOXX treatment failed to block DON induced IgAN, but rather enhanced DON induced serum IgA elevation. These results suggested that COX-2 was not required for DON induced IgAN and the COX-2 inhibitor, VIOXX, might not be suitable for prophylaxis of IgAN.

Finally, IL-6 mRNA regulation at post transcriptional and transcriptional stages were investigated in thioglycollate-elicited macrophages from mice fed control and DHA diet. It was found that dietary DHA did not affect IL-6 mRNA stability but significantly inhibited IL-6 gene transcription. Binding of cAMP response element binding protein (CREB), active protein-1 (AP-1), nuclear factor κ B (NFκB) and CCAAT/enhancer binding protein (C/EBP) in vitro and in vivo was inhibited by DHA consumption. Subsequent demonstration of inhibition on in vivo interaction between CREB and IL-6

promoter by DHA suggested CREB to be particularly critical in DON-induced IL-6 gene transcription.

Taken together, these studies confirmed prophylactic effects of (n-3) PUFA in this chemical-induced IgAN and provided the insight into molecular mechanisms of DHA on proinflammatory gene, IL-6, expression, which can be exploited for disease prevention. The following experiments might be considered in the future to elucidate mechanisms by which DHA blocks IL-6 gene expression and CREB activation.

1) In vitro study to measure DHA effects on MSK1 enzyme activity.
2) In vitro study to detect if DHA can bind CREB and block its phosphorylation
3) ChIP study to investigate DHA effects on chromatin acetylation.
4) Immunostaining to detect if DHA can block transcription factor nuclear translocation.
5) Immunoprecipitation.study to detect if DHA can bind to PPARs and block IL-6 gene expression.

APPENDIX A

DHA EFFECTS ON DON INDUCED PPAR BINDING ACIVITIES

Peroxisomal proliferator activated receptor αlpha (PPARα) and gamma (PPARγ) might be important transcriptional factors that can mediate (n-3) PUFA effects on gene transcription (Jump *et al.*, 2004; Jump *et al.*, 1997). PPARs can bind to their ligands and physically interfere with transcriptional factors binding to their cis-element and thus downregulating proinflammatory gene expression (Ren *et al.*, 1996). For example, interferon gamma (IFN-γ), IL-6 and TNF-α production are impaired in spleen from mice fed with PPARα ligand, WY14, 643 (Cunard *et al.*, 2002). When WY14, 643 binds to PPARα, it can sharply reduce IL-6 and COX-2 gene expression by physically interfering with p65, c-jun and CBP interaction with DNA in vitro (Delerive *et al.*, 1999). The PPARγ ligands, troglitazone, pioglitazone and 15-deoxy-Delta (12,14)-prostaglandin J (2) also inhibit IL-1β-induced IL-6 expression at transcriptional level in vascular smooth muscle cells by interfering NFκB and C/EBP binding to the DNA (Takata *et al.*, 2002). Arachidonic acid and LTB4 can also bind to the PPARα and γ, but the binding will elicit their degradation by increasing β-oxidation of PUFA (Devchand *et al.*, 1996). Thus, PPARs are important negative feedback molecules in vivo for control of inflammation responses (Delerive *et al.*, 1999; Pointer and Daynes, 1998; Berger and Moller, 2002; Delerive *et al.*, 2000; Clark, 2002). Since DHA and EPA are now recognized to be natural ligands for PPARα and PPARγ, DHA effects on PPAR binding activities were tested with EMSA with consensus sequence AG GTC AAA GGT CA (Figure AA1, 2).

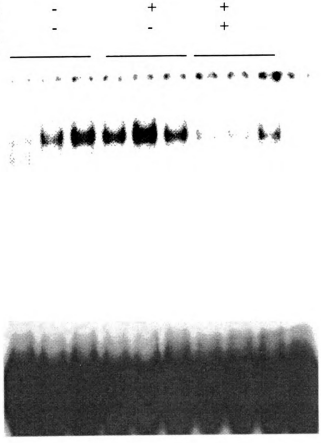


Figure AA1. Characterization of effects of DHA on DON-induced transcriptional factor binding to their consensus sequence in splenic nuclear extract. Mice were fed DHA(60 g/kg) containing diet for 4 wk and gavaged with DON (25mg/kg) 30 min before spleens were removed and nuclear protein extracted. EMSA analysis was conducted with labeled PPAR consensus sequence and 20μg of splenic nuclear protein.

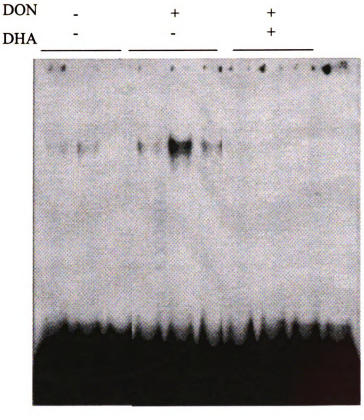


Figure AA2. Characterization of effects of DHA on DON-induced transcriptional factor binding to their consensus sequence in splenic nuclear extract. Mice were fed DHA(60 g/kg) containing diet for 4 wk. Thioglycollate elicited peritoneal macrophage were harvested and treated with DON (250 ng/ml) 30 min before nuclear protein was extracted. EMSA analysis was conducted with labeled PPAR consensus sequence and 20μg of splenic nuclear protein.

APPENDIX B

INDUCTION OF HEAMATURIA BY CD89

As a specific human IgA receptor (FcaR), CD89 proteins are expressed on blood myeloid cells, characterized as heavily glycosylated molecules (M, *55* to 100 kDa) with a protein core of 32 kDa (Patry *et al.*, 1996). Since CD89 transgenetic mice develop IgA nephropathy and this is related with the formation of CD89-IgA complex and its deposition in the kidney (Launay *et al.*, 2000), CD89 protein was cloned and expressed in a eukaryotic expression system, EasySelect. *Pichia* Expression Kit (Invitrogen Corporation, Carlsbad, CA). The expressed protein is going to be injected into the mice with high serum DON-induced IgA to induce heamaturia.

Step 1 Construct the vector. U937 cells were stimulated with PMA (10^{-7} M) for 12 hours. Total RNA was extracted and reverse transcription was conducted. The cDNA was amplified with a pair of primers of forward: GGG CTC GAG AAG AGA GAG GCT GAA GCA CAG GAA GGG GAC T and backward: GGG GGG GGG GGC TCT AGA TTG CAG ACA CTT GGT in the condition of 94 ^{o}C 30s, 57 ^{o}C 1 min and 72 ^{o}C 1min for 35 cycle. A 848 bp PCR product was identified (Figure AB1).

The PCR product was cloned into pGEM-T (Promega Incorporation, Madison WI) and confirmed by sequencing (Figure AB2). The CD89 insert was cut by restriction enzyme Xba I (Invitrogen) and Xho I (Invitrogen) and extracted by gel purification. After ligation with pPICZA provided in the kit, ligation mixtures were transformed into *E. coli,* (DH5α) and selected on Low Salt LB medium with Zeocin. Transformants are isolated and analyzed for the presence and orientation of insert. The CD89 construct link region was confirmed by sequencing again to make sure the protein is in frame.

Step 2 Transformation and Integration. Linearize 5-10 μg of CD89 construct with the restriction enzyme *Sac* I (209 bp) which cuts one time in the 5′ *AOX1* region. Electroporation transformation of the yeast GS115 with plasmid DNA linearized. Transformants were plated on YPDS plates containing100-500 μg/ml Zeocin to isolate Zeocin.-resistant clones. Six positive clones of recombinants were tested because the site of recombination may affect expression. The CD89 construct in the clones selected were confirmed by PCR with the 5'AOX and 3'AOX primers provided in the kit (Figure AB3)

The transformants in GS115 should be Mut+. Mut+ phenotype was confirmed by the growth on both Minimal Dextrose with histidine (MDH) agar plate and Minimal Methanol with histidine (MMH) agar plates.

Protocal for electroporation

1 Grow cells to an OD 600 of between 0.5-0.8 in a 50 ml culture

2 Pellet and resuspend in 10 ml of ice cold 100 μM tris , 10 μM EDTA buffer with 200 μM DTT

3 Incubate for 15 minutes at 30°C with shaking at 100 rpm

4 Wash cells twice with ice-cold sterile water (ddH2O) and once with 1M sorbitol

5 Resuspend in 100 microliter of 1M sorbitol to a final volume of 200 microliter

6 Electroporation 80 microliter of competent cells with 2-6 μg DNA.

7 Valtage 1.5 KV (0.2CM), Field strength 7.5 kv/cm, Capacitor 25 micro F , Resistor 200-400 ohms, time constant about 9 msec.

8 Immediately (, < 30 sec) add 1ml ice-cold 1M sorbitol.

9 Recover overnight at room temperature and plate on YPD containing Zeocin (100-500 μg/ml).

Step 3 Expression of Recombinant Pichia Strains Two positive clones were selected and expressed following protocol. The results indicated the expressed protein at different time both in supernatant and in cell lysate (Figure AB4). Once expression is optimized, it was scaled up. This may be done by increasing the culture volume using larger baffled flasks. Cells can be processed immediately or stored at -80°C until ready for use. The glycosylation was determine by treatment the cell lysate with the Peptide:N-Glycosidase F (Glyko Inc, Cambridge, MA) and analyzed by Western (Figure AB5).

Protocol for recombinant protein expression.

1 Using a single clone, inoculate 25 ml of BMGY in a 250 ml baffled flask. Grow at 28-30°C in a shaking incubator (250-300 rpm) until culture reaches an OD600 = 2-6 (approximately 16-18 hours). The cells will be in log-phasegrowth.

2. Harvest the cells by centrifuging at 1500-3000 x g for 5 minutes at room temperature. Decant supernatant and resuspend cell pellet to an OD600 of 1.0 in MMH medium to induce expression (approximately 100-200 ml).

3. Place culture in a 1 liter baffled flask. Cover the flask with 2 layers of sterile gauze or cheesecloth and return to incubator to continue growth.

4. Add 100% methanol to a final concentration of 0.5% methanol every 24 hours to maintain induction.

5. At each of the time point: 0, 8h, 24 (h), 48 (h), 72 (h), transfer 1 ml of the expression culture to a1.5 ml microcentrifuge tube. These samples will be used to analyze expression levels and determine the optimal time post-induction to harvest. Centrifuge at maximum speed in a tabletop microcentrifuge for 2-3 minutes at room temperature.

6.After centrifugation, transfer the supernatant to a separate tube.. Freeze supernatant and cell pellets quickly in a dry ice/alcohol bath and store the at -80°C until ready to assay.

7. Analyze the supernatants and cell pellets for protein expression by Western blot with anti c-myc antibody.

Step 4 Induction of hematuria by CD89 crude extract. Express your protein using the optimal conditions(Figure AB6). Harvest the cells and store them at -80°C until you are ready to purify your fusion protein or inject mice. The mice were kept on diet containing deoxynivalenol (DON) for 1 year. Serum IgA and IgA-IC were measured before injection (Figure AB7). Once the recombinant protein was identified in the cell lysate (Figure AB6), it is ready for injection. The hematuria and proteinuria were checked both by Multistix and microscope (Table AB1 and Figure AB8).

Protocol for intracellular CD89 extraction from yeast.

1.Wash cells once in breaking buffer (Invitrogen Instruction) and centrifuging 5-10 minutes at 3000 x g at +4°C.

2. Resuspend the cells to an OD600 of 50-100 in BB.

3. Add an equal volume of acid-washed glass beads (0.5 mm). Estimate volume by displacement.

4. Vortex the mixture for 30 seconds, then incubate on ice for 30 seconds. Repeat 7 more times. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of your protein.

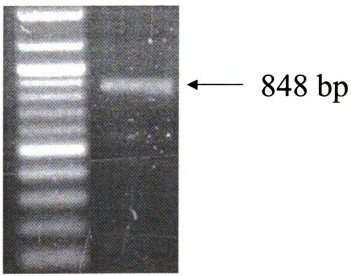
5. Centrifuge the sample at +4°C for 5-10 minutes at 12,000 x g.

6 The supernatant was harvest, filter sterilized and injected in the mice 1ml /mouse (About 0.5 liter cultured yeast cells / per mouse) through tail vein.

7 Collect urine over night after injection.

8 The urine was test by Multistix for blood and protein. After centrifugation at 500g for 10 min, the RBCs in sediments were also checked under the microscope.

Figure AB1. PCR the transcript of CD89 cDNA following the reverse transcription. Human U937 cells were stimulated with PMA (10^{-7} M) for 12 hr. RNA was extracted and reverse transcription was conducted. The cDNAs were then used as templates to amplify CD89. The PCR product was separated on 1.2% agarose gel and the CD89 was identified as the band with 848 bp length.



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  1    caggaag gggactttcc catgcctttc atatctgcca aatcgagtcc tgtgattccc
 58  ttggatggat ctgtgaaaat ccagtgccag gccattcgtg aagcttacct gacccagctg
118 atgatcataa aaaactccac gtaccgagag ataggcagaa gactgaagtt ttggaatgag
178 actgatcctg agttcgtcat tgaccacatg gacgcaaaca aggcagggcg ctatcagtgc
238 caatatagga tagggcacta cagattccgg tacagtgaca ccctggagct ggtagtgaca
298 ggcttgtatg gcaaaccctt cctctctgca gatcggggtc tggtgttgat gccaggagag
358 aatatttccc tcacgtgcag ctcagcacac atcccatttg atagattttc actggccaag
418 gagggagaac tttctctgcc acagcaccaa agtggggaac acccggccaa cttctctttg
478 ggtcctgtgg acctcaatgt ctcagggatc tacaggtgct acggttggta caacaggagc
538 ccctacctgt ggtccttccc cagtaatgcc ttggagcttg tggtcacaga ctccatccac
598 caagattaca cgacgcagaa cttgatccgc atggccgtgg caggactggt cctcgtggct
658 ctcttggcca tactggttga aaattggcac agccatacgg cactgaacaa ggaagcctcg
718 gcagatgtgg ctgaaccgag ctggagccaa cagatgtgtc agccaggatt gacctttgca
778 cgaacaccaa gtgtctgcaa gtaa
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Figure AB2. The CD89 gene with its signal peptide deleted was sequenced and confirmed.

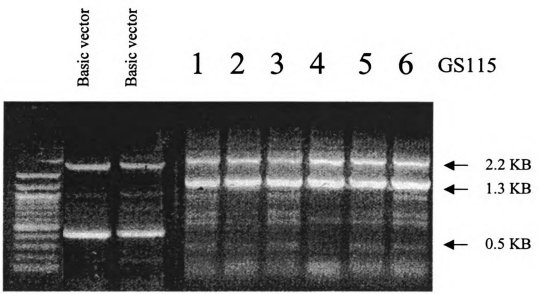

Figure AB3 PCR screening of GS115 Pichia clones. The yeast genomic DNA was extracted following the protocal in the kit. A pair of 5'AOX and 3' AOX primers were used to amplify the positive GS115 clones.Two major bands were found. The 2.2 kb band corresponds AOX1 gene and the 1.3 kb corresponds to the CD89 insert.

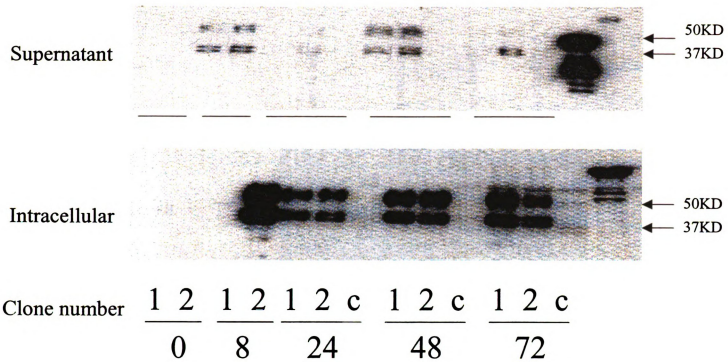

Figure AB4. Optimization of recombinant Pichia strain expression. Two positive clones(1,2) and a clones with basic vector (c) were selected and expressed under the condition described in the kit. The expressed protein both in supernatant and in cell lysate were identified by SDS-PAGE with the antibody specific for c-myc. The time points include 0, 8, 24, 48 and 72 h.

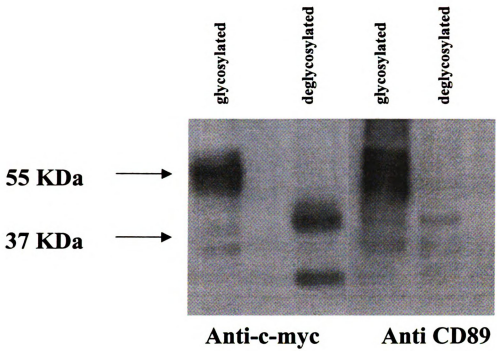

Figure AB5 Indentifaction of CD 89 and its glycosylation. After the protein extract was treated with N-Glycosidase F overnight to remove carbohydrate, the extracts were separated on SDS-PAGE and identified by Western Blot with antibody specific for human CD89(Research Diagnostic Inc, Flanders, NJ) and c-myc.

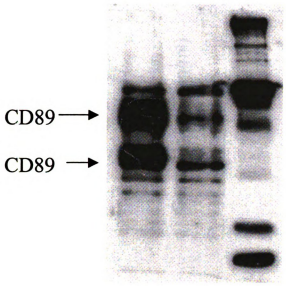

Figure AB6. The expressed protein in the cell lysate injected into the mice. The protein expression was scaled up and time optimized at day 1 and day2. Then it was injected in to the mice with high serum IgA through tail vein.

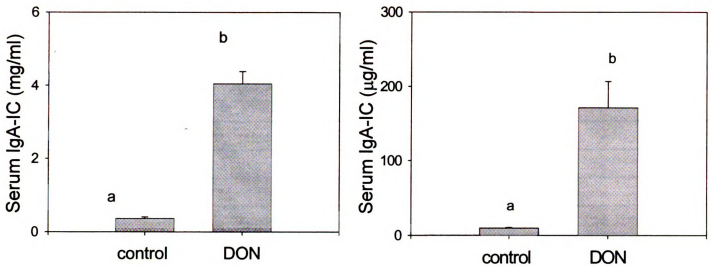

Figure AB7 Serum IgA and IgA-IC level in male B6C3F1 mice. Mice were fed AIN93G diets containing DON for 1 year. Serum were collected and IgA were measured by ELISA. Values are mean ± SEM, n=5-6. Bars marked with different letter differ, ($p<0.05$).

Table AB1 The hematuria and proteinuria induced by CD89 crude extract.

	24 h			48 h		
	Microscope Red Blood Cell	Multistix hematuria	Protein urine	Microscope Red Blood Cell	Multistix hematuria	Protein urine
Basic vector	-	-	-	-	-	-
Basic vector	-	-	-	-	-	-
CD89	0	-	-	-	-	-
CD89	10*	+++	+	2	+	-
CD89	9*	++	+	-	-	-

After high serum IgA and IgA-IC were induced in mice, two mice were injected with protein extracts from yeast with basic vector as controls and three mice were injected with protein extract form yeast with CD89 construct. Urine were collected in metabolite cages 24 and 48 h after injection. Hematuria was checked with both microscope and multistix. The proteinuria was checked with Multistix. * mean the number of RBC per low power field (× 100). "-" means negative, "+" means hematuria.

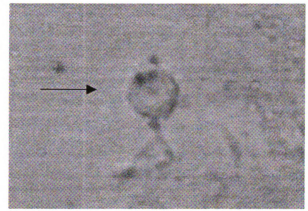

Figure AB8 The red blood cell in urine under the high power microscope field (× 400) 18 h after injection of CD89 crude extract.

INDUCTION OF HEMATURIA BY TNP-BSA AND IGG ANTI IGA

Female B6C3F1 and BALB/c mice were fed ANN93 G diet containing 10 and 20 ppm DON for 12 wk. The B6C3F1 mice were injected with TNP-BSA (1mg/mouse) for 3 consecutive days and the BALB/C mice were injected with IgG anti IgA (50μg/mouse) for 3 consecutive days. Urine was collected at the 5th day after the first injection. The urine sample was centrifuged at 500g for 10 min. The sediments were used to check hematuria (Table 1). Multistix was also employed to check hematuria and proteinuria but the results for all sample are negative.

Table AB2 The hematuria induced by TNP-BSA in B6C3F1 and in BALB/C induced by IgG anti IgA

	B6C3F1			BALB/C		
	4day	5 day	7 day	5 day	6 day	7 day
control	2	7	13	1	11	17
control	9	-	29	3	13	12
control	5	-	19	3	-	-
10 ppm DON	50	22	55	18	42	66
10 ppm DON	11	23	63	14	-	34
10 ppm DON	10	-	-	-	-	50
20 ppm DON	15	15	50	37	-	31
20 ppm DON	12	12	66	41	-	32
20 ppm DON	30	30	40	-	-	-

The mice were kept on diet containing DON (10, 20 ppm) for 12 wk. B6C3F1 mice were injected with TNP-BSA (1mg/ml) and BALB/C mice were injected with IgG anti IgA (50 μg / per mouse). Urine was collected in metabolic cage at different point. The red blood cells were counted as every 10 high power fields (400). The "-" means no urine was collected.

APPENDIX C

PROTOCOL FOR CHIP ASSAY

Preparing chromatin

1. Culture 5 $\times 10^6$ thioglycollate-elicited macrophages in 10 cm plates for 48 hours and four dish cells are good for one treatment group.

2 Prepare fresh fixation solution, ice-cold 1X PBS, glycine stop-fix solution and cell scraping solution as follows:

a.Fixation solution: Add 1.62 ml of 37% formaldehyde to 60 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.

b.1X PBS: Add 7 ml 10X PBS to 63 ml dH2O, mix and place on ice.

c.Glycine stop-fix solution: Combine 3 ml 10X glycine buffer, 3 ml 10X PBS and 24 ml dH2O.

d. Cell scraping solution: Add 600 µl 10X PBS to 5.4 ml dH2 O, mix and place on ice. just before use (in step 7 below) add 30 µl 100 mM PMSF.

3. Pour medium off the plates and add 10 ml fixation solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

4. Pour fixation solution off the plates and wash by adding 10 ml ice-cold PBS to each plate, rocking the plate for 5 seconds and then pouring off the PBS.

5. Stop the fixation reaction by adding 5 ml glycine stop-fix solution to each of the plates, swirling to cover and then rocking at room temperature for 5 minutes.

6. Wash each plate by pouring off the glycine stop-fix solution and adding 5 ml ice-cold PBS, rocking the plate for 5 seconds, and then pouring off the PBS.

7. Add 1 ml ice cold cell scraping solution to each of the plates and scrape cells with a rubber policeman. Hold the plate at an angle and scrape the cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 15 ml conical tube on ice. Do the same for the other 3 plates and pool cells from the three plates in one 10 ml conical tube.
8. Pellet the pooled cells by centrifugation for 10 minutes at 2500 rpm (720 RCF) at 4°C.
9. Remove the supernatant. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C. When you are ready, continue with step 10.
10. Thaw pellet (if necessary) and resuspend cells in 1.5 ml ice-cold Lysis Buffer (supplemented with 7.5 µl PIC + 7.5 µl PMSF). Incubate on ice for 30 minutes.
11. Transfer the cells to an ice-cold dounce homogenizer. Gently dounce on ice with 10 strokes to aid in nuclei release. Transfer cells to a 15 ml conical tube and centrifuge at 5000 rpm (approximately RCF 2400) for 10 minutes at 4°C to pellet the nuclei.
12. Carefully remove the supernatant. Resuspend the nuclei pellet in 1.0 ml shearing buffer (supplemented with 5 µl PIC) and aliquot into 2 1.7 ml microcentrifuge tubes. Each aliquot should be approximately 400 µl.
13. Shear the three aliquots of DNA using your optimized conditions 25% of power 30 second pulse with 30 s rest on ice and repeat for 5 times.
14. Centrifuge the sheared DNA samples at 10,000 to 15,000 rpm in a 4°C microcentrifuge for 12 minutes. Pool the supernatants by transferring each to the same fresh tube. This contains the sheared chromatin. It can be used right away or aliquoted and stored at -80°C. Remove 25 µl for use in checking the DNA shearing

efficiency and DNA concentration. Aliquot the remainder into 2 equal aliquots (usually about 200 µl each). Each aliquot can then be used for 4 ChIP reactions (i.e. each aliquot can be tested with four different antibodies).

Pre-clearing of Chromatin (Day 2)

Chromatin is pre-cleared with Protein G beads to reduce non-specific background. Normally, each chromatin preparation will be used for several ChIPs (e.g. a negative control ChIP, a positive control ChIP and ChIP with antibody of interest). In these cases, the chromatin for the ChIPs can be pre-cleared in the same tube (see example below). The volumes below assume that the chromatin was prepared as described in the preceding sections. Reagent µl for one ChIP rxn µl for 3 ChIP rxns; Chromatin 50 µl 150 µl; Resuspend Protein G beads 100 µl 300 µl; ChIP IP Buffer 59 µl 177 µl ; PIC 1 µl 3 µl; Total Volume 210 µl 630 µl

1. Use the above table to calculate the amount of chromatin, resuspended Protein G beads, ChIP IP Buffer and PIC required for pre-clearing reactions. Combine the reagents in a 1.7 ml microcentrifuge tube.
2. Rotate the tube at 4°C for 1 to 2 hours.
3. Place tube in a microcentrifuge for 2 minutes at 4,000 rpm.
4. After centrifugation is complete, place tube on ice for 2 minutes to let the beads settle.
5. Transfer the supernatant (chromatin) to a fresh tube. Do not disturb the beads.
6. Repeat steps 3 through 5 to ensure that all beads are removed from the chromatin.

Addition of Antibodies to Pre-cleared Chromatin

Note: Some volume (~15%) is lost during pre-clearing. Assume that 170 µl of pre-cleared chromatin is available for each ChIP reaction.

1. Transfer 10 µl of the pre-cleared chromatin to a microcentrifuge tube and store at -20°C. This sample is the "Input DNA" and will be used in PCR analysis. It will be treated to remove cross-links at a later stage (see Section H. Reverse Cross-links and Remove RNA).
2. Perform the antibody incubations in the provided 0.65 ml siliconized tubes. To begin, label the appropriate number of tubes.
3. Add 170 µl pre-blocked chromatin to each of the labeled 0.65 ml tubes.
4. Add the appropriate antibody to each of the labeled tubes. We recommend using between 1 and 3 µg of antibody for each ChIP reaction. The kit's Negative Control IgG should be used at 9 µl (1.8 µg) per ChIP reaction.(3 µl for p-CREB)
5. Incubate the tubes overnight on a rotator at 4°C (sensitivity may be improved by overnight incubation)

Addition of Protein G to Antibody/Chromatin Mixture (Day 3)

1. Resuspend the blocked Protein G beads fully by inverting the tubes several times.
2. Aliquot 100 µl of the fully resuspended beads into each of the antibody/chromatin incubations performed in the preceding step.
3. Incubate the tubes on a rotator for 1.5 hours at 4°C.
4. During this incubation, prepare the ChIP IP and Wash Buffers as described below. Each ChIP reaction will be washed once with ChIP IP Buffer + PIC, four times with

Wash Buffer 1 + PIC, once with Wash Buffer 2 + PIC and twice with Wash Buffer 3. The quantities listed below are sufficient for one ChIP reaction.

ChIP IP Buffer + PIC: Add 2 µl Protease Inhibitor Cocktail (PIC) to 400 µl ChIP IP Buffer, mix and place on ice.

Wash Buffer 1 + PIC: Add 1.6 µl PIC to 1.6 ml Wash Buffer 1, mix and place on ice.

Wash Buffer 2 + PIC: Add 0.4 µl PIC to 400 µl Wash Buffer 2, mix and place on ice.

Wash Buffer 3: Place on ice (supplied ready-to-use).

Washing ChIP Reactions

1. Following incubation of the beads with the antibody/chromatin mixture, pellet the beads by centrifuging each ChIP reaction for 2 minutes at 4000 rpm. Place tubes in a rack and allow 30 seconds for the beads to fully settle.

   Note: All subsequent bead pelleting steps should be performed in this manner.

2. Remove the supernatant. Use a 200 µl pipette to withdraw 200 µl twice (discard supernatants). Avoid disturbing the beads.

3. Add 400 µl ChIP IP Buffer + PIC to each tube of beads and cap the tubes. Flick tubes to fully resuspend beads and incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.

4. Add 400 µl Wash Buffer 1 and cap tubes. Resuspend beads and incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.

5. Repeat Step 4 three times.

6. Add 400 µl Wash Buffer 2 + PIC and cap tubes. Resuspend beads and incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.

7. Add 400 µl Wash Buffer 3 and cap tubes. Resuspend beads and incubate on rotator for 1-3 minutes. Pellet beads and remove supernatant.

8. Repeat Step 7 once. This is the final wash. Remove as much buffer as possible without disturbing the bead

DNA Elution from Protein G

In this section, immunoprecipitated DNA will be collected from the washed Protein G beads using two elutions with 50 µl ChIP Elution Buffer.

1. Freshly prepare 105 µl of ChIP Elution Buffer for each ChIP reaction by adding 5 µl 1M NaHCO3 to 100 µl 1% SDS and mixing thoroughly.

2. Add 50 µl ChIP Elution Buffer to each of the washed Protein G bead pellets in the 0.65 ml tubes. Cap tubes, vortex briefly and incubate for 15 minutes at room temperature with gentle rotation.

3. Centrifuge tubes for 2 minutes at 4000 rpm to pellet beads. Remove tubes from centrifuge, place in tube holder in vertical position and wait several seconds for the beads to settle completely.

4. Use a 200 µl pipette to transfer each supernatant to an appropriately labeled 1.5 or 1.7 ml microcentrifuge tube.

5. Repeat steps 2 to 4 and pool the appropriate elutions.

Reverse Cross-links and Remove RNA

Note: The reserved Input DNA (from Step 1 of Section D on page 13) must also be taken through the following steps. Remove the reserved Input DNA from the freezer and add

90 µl dH2 O to bring the volume to 100 µl and treat this sample along with the ChIP elutions below.

1. Add 4 µl 5 M NaCl and 1 µl RNase A to each ChIP elution and to the sample of Input DNA.
2. Vortex to mix completely and centrifuge the tubes briefly to remove liquid from the sides of the tubes. Place tubes in a 65°C incubator or water bath for 4 hours to overnight. (The experiment can be stopped here and tubes stored at -20°C until use.)

Treat with Proteinase K (Day 4)

1. Remove tubes from 65°C incubator and centrifuge for 1 minute to collect liquid from the sides of the tubes.
2. Add the following three components to each tube: 2 µl 0.5 M EDTA, 2 µl 1 M Tris-Cl pH 6.5 and 2 µl Proteinase K solution.
3. Vortex to mix, centrifuge briefly to collect liquid from the sides of the tubes and incubate at 42°C for 1.5 to 2 hours to digest the proteins. During this incubation, prepare the reagents that will be needed for DNA purification in the next section.

Purify Eluted DNA

1 Label and place the required number of DNA purification mini-columns in their provided collection tubes in a rack. The DNA will be eluted from the columns into microcentrifuge tubes (1.5 ml or 1.7 ml). Label and set aside the appropriate number of tubes

2. Remove the Proteinase K-treated samples from the 42°C incubator and centrifuge briefly to collect the liquid condensed on the sides of the tubes.

3. Add 500 µl of DNA Binding Buffer to each DNA sample and vortex to mix completely. Transfer each sample into a labeled DNA purification mini-column and centrifuge for 30 seconds at 10,000 to 15,000 rpm.

4. Remove the mini-column from the collection tube, discard the flow-through and replace the mini-column in the tube.

5. Add 600 µl of DNA Wash Buffer to each mini-column.

6. Centrifuge for 30 seconds at 10,000 to 15,000 rpm.

7. Remove the mini-column from the collection tube, discard the flow-through and replace the mini-column in the tube.

8. Add 300 µl of DNA Wash Buffer to each mini-column.

9. Centrifuge for 2 minutes at 10,000 to 15,000 rpm.

10. Place each dry mini-column into the appropriately labeled 1.5 ml or 1.7 ml tube prepared in Step 2. Add 50 µl dH2 O directly to the resin at the bottom of each mini-column. Incubate for 3 minutes at room temperature.

11. Spin for 1 minute at 10,000 to 15,000 rpm.

12. Remove the mini-columns and collection tubes from the centrifuge and place in a tube rack. Repeat the DNA elution by adding 50 µl dH2 O directly to the resin, incubating 3 minutes at room temperature and then centrifuge for 1 minute at 10,000 to 15,000 rpm.

13. The eluted DNA can be used immediately in PCR or stored at -20°C.

PCR Analysis

Syber Green PCR analysis (5 µl DNA elution per reaction)

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