MECHANISMS OF N-3 POLYUNSATURATED FATTY ACID INHIBITION OF MYCOTOXIN DEOXYNIVALENOL-INDUCED IMMUNE RESPONSE

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ABSTRACT

MECHANISMS OF N-3 POLYUNSATURATED FATTY ACID INHIBITION OF MYCOTOXIN DEOXYNIVALENOL-INDUCED IMMUNE RESPONSE

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Consumption of deoxynivalenol (DON), a type B trichothecene mycotoxin produced by the *Fusarium sp.*, has been recognized to affect immune function and induce immunoglobulin A nephropathy (IgAN) in the mouse. Clinical trials have shown that dietary n-3 polyunsaturated fatty acid (PUFA) supplementation is beneficial for patients with progressing IgAN. The purpose of this dissertation was to determine the mechanisms by which n-3 PUFAs suppressed pathogenesis of IgAN and interleukin-6 (IL-6) upregulation induced by DON. DON consumption in the mouse model induced hallmarks of IgAN, such as elevated serum IgA, IgA immune-complexes and IgA deposition in the kidney. However, dietary supplementation of eicosapentaenoic acid (EPA), an n-3 PUFA, alleviated these markers. Proinflammatory cytokine IL-6 has been shown to be critical in the development of DON-induced IgAN. EPA consumption decreased IL-6 gene expression by inhibiting the transcription factor from binding to the IL-6 gene promoter. Therefore we proposed that n-3 PUFAs ameliorate DON-induced IgAN through suppression of IL-6. Using peritoneal macrophages as a model, we elucidated part of the signal transduction pathway through which DON activated transcription factor cAMP response element-binding protein (CREB) and induced IL-6 expression. Double-stranded RNA-activated protein kinase (PKR) is a very important

upstream protein kinase in this signal pathway. Inhibition of PKR suppressed phosphorylation of CREB and its upstream kinases, Akt1, MSK1 and RSK1, and abolished DON induced IL-6 expression. Consumption of docosahexaenoic acid (DHA), another n-3 PUFA, suppressed IL-6 expression by inhibiting PKR, CREB kinases and CREB activation. This inhibition was found not due to upregulation of protein phosphatase 1 and 2A activities. We also investigated the role of endoplasmic reticulum (ER) stress in the upregulation of IL-6. DON degraded BiP, an ER chaperone, and induced an ER stress-like response in peritoneal macrophages. The degradation appeared to be cathepsin/calpain-related. In addition, activating transcription factor 6 (ATF6) upregulation due to ER stress was indicated to be involved in IL-6 gene expression. Taken together, these data suggest that n-3 PUFA consumption suppresses DON-induced IgAN-like disease in the mouse by interfering with signal transduction involved in IL-6 gene expression. Upregulation of IL-6 might involve multiple pathways in DON-treated peritoneal macrophages.

This work is dedicated to my dearest parents and sister.

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KEY TO ABBREVIATIONS

AA	Arachidonic acid
ALA	α-linolenic acid
AP-1	Activating protein-1
ATF6	Activating transcription factor 6
BiP	Immunoglobulin binding protein
C/EBP	CCAAT/enhancer binding protein
COX	Cyclooxygenase
CRE	cAMP response element
CREB	cAMP response element-binding protein
DHA	Docosahexaenoic acid
DON	Deoxynivalenol
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
GalNAc	N-acetylgalactosamine
IC	Immune complexes
IgAN	Immunoglobulin A nephropathy
IL-6	Interleukin-6
IL-10	Interleukin-10
IRE1	Inositol requiring enzyme 1

LA	Linoleic acid
LOX	Lipoxygenase
LT	Leukotriene
MSK1	Mitogen/ stress- activated protein kinase 1
NeuAc	N-acetylneuraminic acid
NF-κB	Nuclear factor-kappa B
OA	Oleic acid
PERK	PKR-like endoplasmic reticulum kinase
PG	Prostaglandin
PKC	Protein kinase C
PKR	Double-stranded RNA-activated protein kinase
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PUFA	Polyunsaturated fatty acid
RSK1	Ribosome S6 kinase 1
TX	Thromboxane
UPR	Unfolded protein response
XBP1	X-box binding protein 1

INTRODUCTION

Deoxynivalenol (DON) is a type B trichothecene mycotoxin produced by the fungi *Fusarium* species. Dietary exposure to DON is common due to its frequent detection in cereal-based food (Pestka and Smolinski 2005) (Rotter *et al.* 1996). In mice, consumption of DON induces an IgA nephropathy (IgAN)-like disease (Pestka *et al.* 2004) (Pestka *et al.* 1989) (Dong *et al.* 1991). DON-induced murine IgAN provides us a unique model to study the pathogenesis, prevention and treatment of this disease.

Overproduction of interleukin (IL)-6 is involved in the polyclonal B cell activation and autoantibody production (Ishihara and Hirano 2002) (Nishimoto and Kishimoto 2004) (Choy 2004). IL-6 can also promote mesangial cell proliferation and extracellular matrix synthesis in the kidney. Furthermore, elevated circulating IL-6 has been observed in patients with IgAN (Lim *et al.* 2003) (Harada *et al.* 2002). Accordingly, IL-6 is considered a chief player in IgAN. In addition, IL-6 has been demonstrated to be critical in the DON-induced IgAN in our mouse model (Yan *et al.* 1997) (Pestka and Zhou 2000).

There is no consensus on how to best treat human IgAN due to the complexity of this disease (Strippoli *et al.* 2003). Current treatments only alleviate its symptoms (Barratt and Feehally 2006). However, epidemiological studies suggest that the risk of IgAN is negatively correlated to n-3 polyunsaturated fatty acid (PUFA) tissue levels (Wakai *et al.* 1999). Therefore n-3 PUFA consumption seems to be promising to treat this disease. Although clinical trials have demonstrated beneficial effects of n-3 PUFA consumption on IgAN (Donadio and Grande 2004), the mechanisms are still not completely known.

In addition, previous study in out laboratory showed that DON treatment decreased endoplasmic reticulum (ER) chaperone BiP (immunoglobulin binding protein) in the EL-4 thymoma cell line (Yang *et al.* 2000). BiP is a key regulator of ER stress response that has been related to inflammation and cytokine production (Hung *et al.* 2004) (Zhang *et al.* 2006) (Iwakoshi *et al.* 2003b). Therefore, we proposed that DON-induced ER stress-like response was also involved in the IL-6 gene expression.

The goal of this research was to: (1) confirm the effiacy of dietary n-3 PUFAs on DON-induced mouse IgAN in a dose-response manner; (2) verify that n-3 PUFAs suppressed IgAN by inhibiting the IL-6 gene expression; (the DON-induced activation of transcription factor cAMP response element-binding protein (CREB) pathway in peritoneal macrophages was studied) (3) relate DON-induced ER stress-like response to the IL-6 gene expression.

CHAPTER 1

Literature Review

Deoxynivalenol. Deoxynivalenol, also known as DON, vomitoxin, 4-deoxynivalenol or 12, 13-epoxy-3, 4, 15-trihydroxytrichothec-9-en-8-one (Figure 1.1), is a type B trichothecene mycotoxin produced by the fungi *Fusarium graminearum* and *F. culmorum*. There have been concerns over this toxin because of its frequent detection in cereal grains and potential for causing adverse effects in human beings and livestock (Rotter *et al.* 1996).

DON was first isolated by Japanese workers in 1972 (Yoshizawa et al., 1973). The production of DON is associated with ear rot in corn and scab in wheat and barley as a result of low temperature and high humidity (Rotter *et al.* 1996). Economic losses due to DON contamination are hundreds of millions of dollars in the United States annually (Pestka and Smolinski 2005). In order to control food quality for human health, the U.S. Food and Drug Administration has established an advisory level of 1 ppm of DON on finished wheat products.

Although DON is not as toxic as other trichothecenes, such as T-2 toxin, satratoxin and roridin, acute exposure to high doses of DON is lethal. Depending on species and route of exposure, the LD50 of DON ranges from 27 to 140 mg/kg bodyweight (Rotter *et al.* 1996). At lower doses, it has been found to affect the digestive, reproductive and immune systems in animals (Pestka and Bondy 1990) (Pestka *et al.* 2004) (Pestka and Smolinski 2005). In regard to immune function modulation, DON exposure impairs mitogen-activated lymphocyte proliferation (Tryphonas *et al.* 1984) (Forsell and Pestka 1985) and pathogen clearance (Pestka *et al.* 1987) (Tryphonas *et al.* 1986). DON consumption also promotes proinflammatory cytokine production (Zhou *et al.* 2003a) and selectively upregulates immunoglobulin A (Pestka 2003).

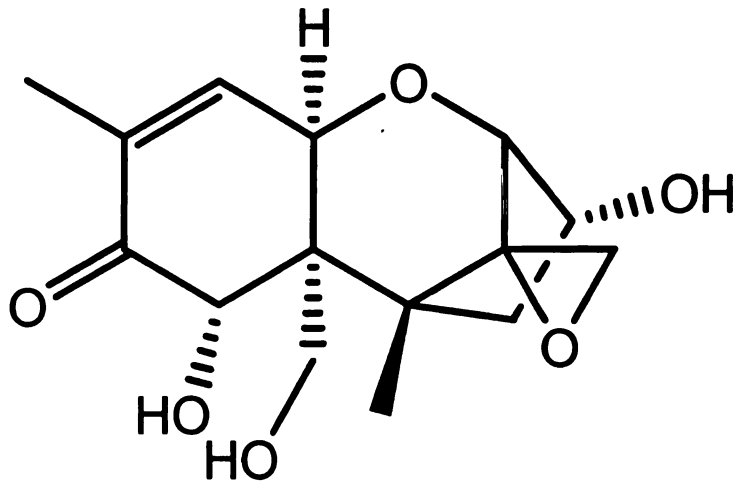


Figure 1.1 The structure of deoxynivalenol ($C_{15}H_{20}O_6$, MW 296.32).

DON-induced IgA nephropathy-like disease. Mouse consumption of DON induces a marked increase of serum IgA and IgA immune complexes, a shift of IgA molecule from monomeric to polymeric form, hematuria and IgA accumulation in the kidney mesangium, all of which mimic human IgA nephropathy (IgAN)(Pestka *et al.* 1989) (Dong *et al.* 1991). Consumption of DON has been found to increase the percentage of IgA+ and CD4+ cells in Peyer's patches and spleen (Pestka *et al.* 1990b). Increased IgA production from Peyer's patch and spleen cell cultures was also observed in mice fed DON (Pestka *et al.* 1989) (Pestka *et al.* 1990a). These IgA molecules were shown to be polyclonal and polyspecific to many different antigens such as casein, inulin as well as autoantigens such as phosphorylcholine, sphingomyelin and cardiolipin (Rasooly and Pestka 1994). The increased IgA production has been related to the superinduction of several cytokines, esp. interleukin-6 (Pestka and Zhou 2000) (Pestka and Smolinski 2005) (Yan *et al.* 1997). It has also been shown that macrophages play a key role in the elevation of IgA production and IgAN pathogenesis in DON-exposed mice (Yan *et al.* 1998).

IgA nephropathy. IgAN is the most common primary glomerulonephritis worldwide. It is defined by the presence of predominant deposits of IgA within the mesangial regions of glomeruli. Depending on different stages of this disease, the kidney may have a wide variety of proliferative, inflammatory, and sclerosing lesions. Histopathologic features include glomerular hypercellularity, the presence of interstitial inflammatory infiltrates, excessive matrix deposition, and vascular sclerosis (Barratt *et al.* 2004) (Donadio and Grande 2004). Although the kidney is primarily affected, IgAN has various clinical presentations. Current evidence indicates that IgAN is not a single

disease but rather a final common response to different causative and pathogenic processes (Barratt and Feehally 2005).

The pathogenesis of IgAN remains unclear. Abnormalities in IgA production, structure, and/or catabolism are suggested to facilitate renal deposition of immune complexes containing IgA (Chintalacharuvu and Emancipator 1997). Many studies have demonstrated that serum IgA levels are elevated in patients with IgAN (Barratt *et al.* 2007b). In addition, numerous molecular changes within IgA itself have been found in IgAN patients. These changes include an altered glycosylation profile of IgA molecule (Moldoveanu *et al.* 2007) (Yan *et al.* 2006) (Baharaki *et al.* 1996) (Suzuki *et al.* 2008), increased anionic charge, λ light chain usage and polymerization (Barratt *et al.* 2007b).

Due to the complexity of the characteristics of IgAN itself, many animal models have been established via various means for studying this disease. ddY mice develop IgAN spontaneously after the age of 40 wk (Imai *et al.* 1985); genetic knockout of β-1, 4-galactosyltransferase (β4GalT)-I or uteroglobin in mouse could upregulate IgA production and induce mesangial IgA deposition and matrix expansion (Kobayashi *et al.* 2002) (Marquina *et al.* 2004) (Kim *et al.* 2001); inoculation with Sendai virus (Yamashita *et al.* 2007) or Coxsackie B4 virus (Kawasaki *et al.* 2006) or injection of IgA immune complexes (Rifai *et al.* 1979) (Chao *et al.* 2006), Haemophilus parainfluenzae antigens (Yamamoto *et al.* 2002) staphylococcal enterotoxin B (Jia *et al.* 2007) or even ovalbumin (Kurihara *et al.* 2005) could also generate symptoms mimic human IgAN.

Treatment of IgAN. IgAN accounts for up to 5 to 10% of glomerulopathies in North America. Approximately 150,000 people in the U.S. have been diagnosed with IgAN with 4000 new cases occurring each year (Hellegers 1993). IgAN is a progressive

disease and needs proper treatment. Natural resolution of urinary abnormalities among IgAN patients is less than 10% (Barratt and Feehally 2006). Between 20 to 40% of IgAN patients develop renal failure with 1-2% of adult patients entering end-stage renal failure each year (Donadio, Jr. *et al.* 1994) (Donadio, Jr. *et al.* 1999).

Currently, there is no consensus on how to best treat this disease (Strippoli *et al.* 2003). No treatment has been found to modify mesangial IgA deposition and those available treatments are extrapolated from the management of symptoms of chronic glomerulonephritis, such as proteinuria, hypertension and reduced glomerular filtration rate (Barratt and Feehally 2006). Analysis of the effects of steroids (Lai *et al.* 1986) (Pozzi *et al.* 1999) (Shoji *et al.* 2000) and cytotoxic agents (Walker *et al.* 1990) (Woo *et al.* 1987) on IgAN have shown controversal results and their use is limited due to severe side effects. Although angiotensin-converting enzyme inhibitor (ACEI) showed some beneficial effects on hypertension in patients with IgAN, there is no controlled clinical trial showing that blocking the renin-angiotensin system decreases the risk of progression of IgAN (Pozzi *et al.* 2006).

It is important to note that epidemiological studies reveal a negative correlation between n-3 polyunsaturated fatty acid (PUFA) tissue levels and IgAN (Wakai *et al.* 1999), whereas a positive correlation exists with n-6 PUFAs (Wakai *et al.* 2002). Clinical trials have also demonstrated beneficial effects of n-3 PUFA consumption on IgAN by inhibiting renal inflammation and mesangial proliferation. However the mechanisms are still under investigation (Calder and Grimble 2002) (Donadio and Grande 2004).

n-3 polyunsaturated fatty acids. A fatty acid is a hydrocarbon chain with a carboxyl group at one end and a methyl group at the other end. Naturally occurring fatty

acids have chains containing 2 to 30 (even number) of carbon atoms or more. Based on the number (n) of double bonds in the carbon chain, fatty acids could be saturated (n=0), monounsaturated (n=1) or polyunsaturated (n≥2). According to the position of the first double bond from the methyl group, PUFA can be classified as n-6 or n-3. The simplest member of n-6 or n-3 classes are linoleic acid (LA; 18:2) and α-linolenic acid (ALA; 18:3) respectively (Calder 2005) (Calder and Grimble 2002). LA and ALA are essential to the human diet because neither of them is synthesized endogenously by humans, and the n-3/n-6 families cannot be interconverted. Plant oils are the major food source of LA (corn oil, cottonseed oil, sunflower oil, et al.) and ALA (flaxseed oil, canola oil, et al.) (Lerman 2006). Docosahexaenoic acid (DHA; 22:6) and eicosapentaenoic acid (EPA; 20:5), two main components of fish oil, are synthesized from the n-3 precursor ALA, whereas long chain n-6 PUFAs such as arachidonic acid (AA; 20:4) are synthesized from the precursor LA. An outline of the pathways of biosynthesis and metabolism of polyunsaturated fatty aids is shown in figure 1.2 (Young and Nicholls 2006).

It has long been observed that several diseases are influenced by the type and amount of fat consumed (Yu *et al.* 1995). Observational and clinical evidence suggest that n-3 PUFAs have beneficial effects on atherosclerosis (Anand *et al.* 2008) (Hansen and Harris 2007), diabetes (Storlien *et al.* 1987) (Peyron-Caso *et al.* 2002), cancer (Cave, Jr. 1991) (Xia *et al.* 2006), and other inflammatory diseases(Ruxton *et al.* 2004) (Grimm *et al.* 2002). Therefore immunomodulating functions of n-3 PUFAs have gained increasing attention.

Fatty acids are crucial components of cell membranes. The exact proportion of fatty acids in human immune cells varies according to cell type and the lipid fraction

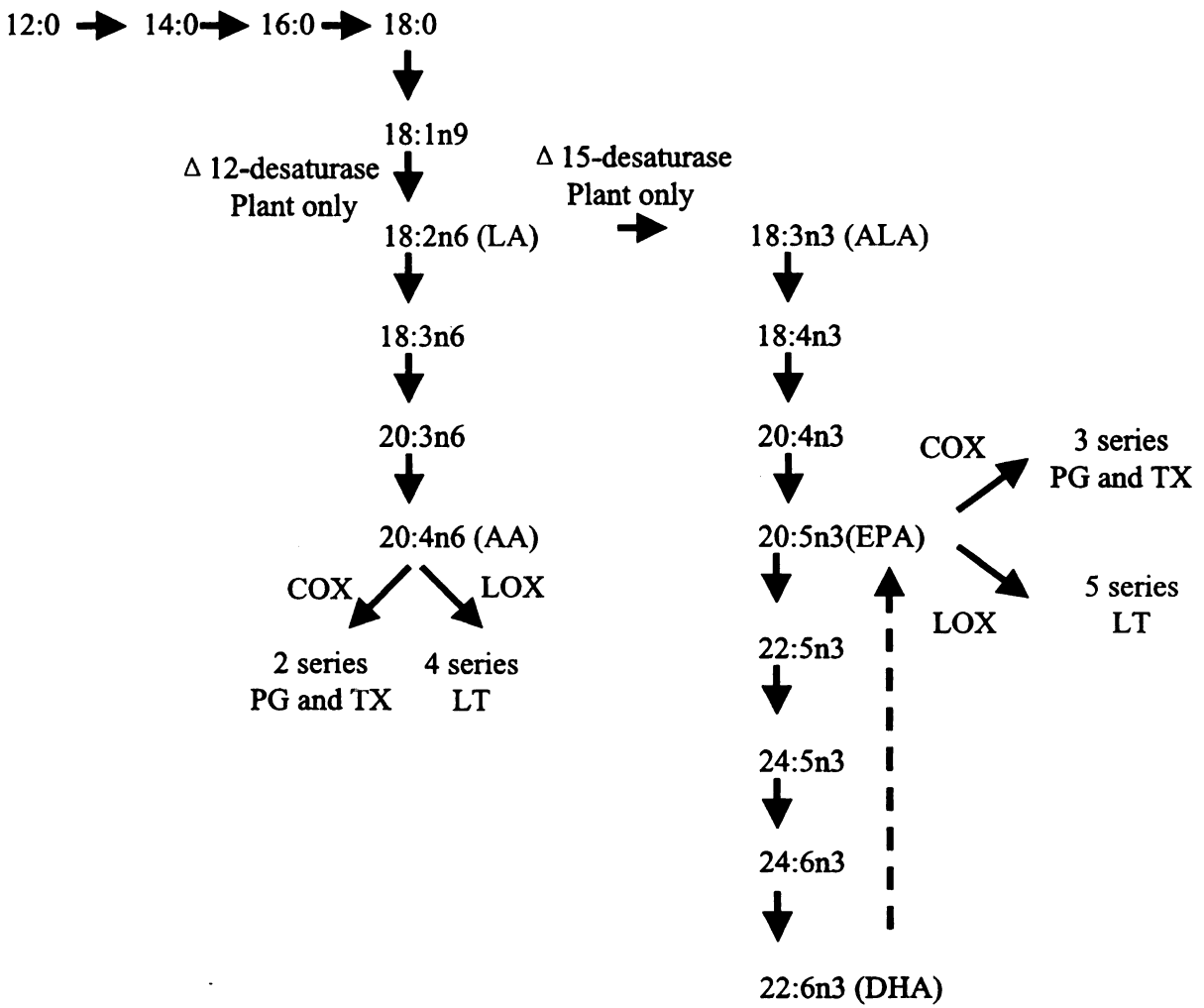


Figure 1.2. Biosynthesis and metabolism of fatty acids.

examined. As precursors of many inflammatory mediators, n-6 PUFAs are more prominent than n-3 PUFAs. The phospholipids of human mononuclear cells contain 6-10% LA and 15-25% AA. n-3 PUFAs are low: ALA is rare; EPA and DHA comprise only 0.1 to 0.8% and 2 to 4% respectively (Calder and Grimble 2002). Although ALA can be converted to EPA and DHA, humans still need an exogenous supply of these long chain PUFAs. The reason exists in that biosynthetic pathways of both the n-3 and the n-6 families share the same enzyme called Δ-6-desaturase. Δ-6-desaturase is vital for the conversion of LA to AA, and ALA to EPA and DHA. High levels of plasma LA due to high n-6 PUFA intake shift its actions towards the n-6 pathway. Furthermore, the conversion of ALA to DHA is inefficient (Ruxton *et al.* 2004). Lacking EPA and DHA in the diet could make the body more susceptible to some diseases.

The important link between fatty acids and inflammation is a family of inflammatory mediators termed eicosanoids. They are generated from 20-carbon PUFAs (AA and EPA). Both AA and EPA liberated from cell-membrane phospholipids by phospholipase A_2 can be metabolized to the eicosanoid family that include prostaglandins (PG), leukotrienes (LT) and thromboxanes (TX). AA metabolized by cyclooxygenase-2 (COX-2) gives rise to the 2-series PG and TX, metabolized by 5-lipoxygenase (5-LOX) gives rise to 4-series LT (Calder 2005). EPA also acts as a substrate for COX and LOX enzymes, and gives rise to different eicosanoids: the 3-series PGs and TXs, and 5-series of LTs (Calder and Grimble 2002). Eicosanoids are involved in modulating the intensity and duration of inflammatory and immune response. Those eicosanoids produced from AA are more potent than those derived from EPA (Ruxton *et al.* 2007). Increased consumption of fish oil results in increased proportions of EPA and DHA in inflammatory

cell phospholipids, and decreased arachidonic acid as compensation. Fish oil supplementation has been shown to result in decreased production of PGE_2 (Trebble *et al.* 2003b), TXB_2 (Caughey *et al.* 1996), LTB_4 (Sperling *et al.* 1993) and LTE_4 (Grimminger *et al.* 1997) because of decreased substrate level.

There are also eicosanoid-independent immune effects that play critical roles in n-3 PUFA suppression of inflammation. n-3 PUFAs may directly (1) affect transcription factor activity or abundance and thus modulate gene expression (Duplus and Forest 2002). Non-esterified fatty acids and their metabolites are able to bind transcription factors such as peroxisome proliferator-activated receptors (PPARs) as ligands to activate them (Kliewer *et al.* 1997) (Murakami *et al.* 1999) (Keller *et al.* 1993) (Yu *et al.* 1995). The monounsaturated fatty acid oleic acid and PUFAs have been demonstrated to decrease transcription factor sterol regulatory element-binding protein (SREBP) in the liver and consequently change expression of enzymes for lipogenesis (Worgall *et al.* 1998). (2) interfere with signal transduction. Fatty acids can either bind to receptors, such as G-protein-coupled receptors 40 (GPR40) (Itoh *et al.* 2003), or change activities of signal molecules such as JNK (Hirosumi *et al.* 2002), NF-κB (Rolph *et al.* 2006), (3) change lipid/lipid raft composition and membrane fluidity (Calder *et al.* 1990) (Clandinin *et al.* 1991) (Spector and Yorek 1985) (Chen *et al.* 2007) (Schley *et al.* 2007) thus alter the function of membrane receptors and enzymes. Potent anti-inflammatory functions of E- and D-series resolvins derived from EPA and DHA respectively as well as protectins derived from DHA have also been noticed (Serhan *et al.* 2008).

Through both eicosanoid-dependent and eicosanoid-independent mechanisms, n-3

PUFAs have been shown to inhibit proinflammatory gene expression. Fish oil feeding decreased *ex vivo* production of TNF-α, IL-1β and IL-6 by rodent macrophages and splenocytes (Sears and Nevins 2002) (Patel and Mohan 2005) (Rathmell *et al.* 2003) (Donadio and Grande 2004). In addition, supplementation of the diet for human volunteers with fish oil has been shown to decrease production of TNF-α and IL-6 by mononuclear cells (Bene and Faure 1988) (Taniguchi *et al.* 1996) (Moore *et al.* 2001).

Interleukin-6. IL-6 is an important proinflammatory cytokine that is produced by various types of lymphoid and non-lymphoid cells, such as macrophages, monocytes, T cells, B cells, fibroblasts, endothelial cells, adipocytes, mesangial cells, and several tumor cells. IL-6 binds to its receptor to exert its functions. There are two components of IL-6 receptor (IL-6R), an 80-kDa IL-6 binding protein (α chain) and a 130-kDa signal transducer known as gp130 (β chain). IL-6 binds to IL-6Rα which induces the homodimerization of gp130 and generates the high-affinity complex of IL-6/IL-6Rα/gp130 that will induce downstream signal transduction(Naka *et al.* 2002).

IL-6 was originally identified as a B-cell differentiation factor, but now it is known to be a multi-functional cytokine that regulates the immune response, hematopoiesis and the acute phase response (Kishimoto 2006) (Song and Kellum 2005). In regard to inflammation, IL-6 plays a critical role in the initiation of the reaction and transformation from acute to chronic phase. Moreover, sustained IL-6 upregulation is required to maintain chronic inflammation. Due to its function in eliciting T cell activation, promoting end-stage B cell differentiation and immunoglobulin secretion, dysregulation of IL-6 causes various clinical symptoms and abnormal laboratory findings

(Gabay 2006). Specifically, overproduction of IL-6 is involved in the polyclonal B cell activation and autoantibody production (Ishihara and Hirano 2002) (Nishimoto and Kishimoto 2004) (Choy 2004). It has been shown that circulating IL-6 levels are elevated in several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and psoriasis, and correlate with markers of disease activity (Ishihara and Hirano 2002). Accordingly, IL-6 is considered a chief player in autoimmune diseases. Therapies that target IL-6, such as specific antibody to IL-6 receptor has been shown to be beneficial (Nishimoto and Kishimoto 2004) (Choy 2004).

Elevated IL-6 also promotes proliferation of mesangial cells and synthesis of extracellular matrix macromolecules in IgAN. Increased renal expression and urinary excretion of interleukin-6 correlate with the degree of IgA deposition in the kidney, extent of renal damage and disease progression in patients with IgAN (Lim *et al.* 2003) (Harada *et al.* 2002) (Bene and Faure 1988) (Taniguchi *et al.* 1996).

In our IgAN mouse model, IL-6 has been demonstrated to be critical in the upregulation of IgA. Anti-IL-6 antibody decreased IgA levels to background in DON-exposed Peyer's patch or spleen cell cultures (Yan *et al.* 1997). IL-6 knockout mice have also been shown to be refractory to DON-induced IgAN (Pestka and Zhou 2000). These data suggest that IL-6 is a requisite cytokine for DON-induced IgA production and resultant IgA.

IL-6 gene transcription: Transcription factor and coactivator binding to their recognition sequences on the IL-6 promoter and enhancer regions is important in IL-6 transcriptional regulation. In macrophages, IL-6 gene expression is induced by activation of at least 4 transcription factors: cAMP responsive element-binding protein (CREB),

activating protein-1 (AP-1), nuclear factor-kappa B (NF-κB), and CCAAT/enhancer binding protein (C/EBP) (Dendorfer 1996) (Matsusaka *et al.* 1993).

The CREB protein contains several functional domains. The C-terminal basic domain facilitates DNA binding (conserved sequence: TGACGTCA) and the leucine zipper domain facilitates dimerization with CREB or other members of the CREB family such as cAMP response element modulator (CREM) and activating transcription factor 1 (ATF-1). Another important domain is the kinase inducible domain (KID) that contains the critical serine-133 amino acid residue (Pandey 2004). According to different stimuli, this residue can be phosphorylated by Akt1 (Shaywitz and Greenberg 1999), cAMP-dependent protein kinase A (PKA) (Hagiwara *et al.* 1993), Ca^{2+}/calmodulin- dependent protein kinases II (CaMK II) (Dash *et al.* 1991), ribosomal S6 kinase 1 (RSK1) (Ginty *et al.* 1994) and mitogen /stress-activated protein kinase 1 (MSK1) (Shaywitz and Greenberg 1999). The phosphorylation status of CREB is also modulated via dephosphorylation by activation of several serine/ threonine phosphatases including protein phosphatase 1 (PP1) (Alberts *et al.* 1994) and protein phosphatase 2A (PP2A) (Choe *et al.* 2004). Phosphorylated CREB (p-CREB) could bind to cAMP-response element (CRE) that is an eight-base-pair sequence, TGACGTCA, located within 100 base pairs of the TATA box (Mayr and Montminy 2001) and promote recruitment of transcriptional coactivator CREB binding protein (CBP) and p300 for gene expression (Shankar and Sakamoto 2004) (Johannessen *et al.* 2004).

NF-κB is composed of the Rel family of proteins including p65 (RelA), c-Rel, RelB, p50, and p52. They form homo- or hetero-dimeric complexes. These proteins share

a 300 amino acid region, designated the Rel homology domain, which mediates dimerization and DNA binding to κB elements in the enhancer regions of target genes (Matt 2002). NF-κB is sequestered in the cytoplasm through associating with inhibitory proteins, the IκBs. When cells are exposed to activation signals, the IκBs are phosphorylated by the IκB kinase (IKK) complexes and undergo degradation by the ubiquitin-proteasome system. Degradation of the IκBs exposed nuclear translocation signal of NF-κB and results in moving of the free NF-κB into the nucleus (Karin and Ben Neriah 2000) (Baldwin, Jr. 1996).

Both AP1 and C/EBP contain a highly conserved, basic-leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding. AP1 are homodimers or heterodimers composed of protein Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), and activating transcription factor (ATF2, ATF3, B-ATF) (Wagner 2002). The C/EBP family has at least six members isolated and characterized to date (C/EBPα - C/EBPζ) (Ramji and Foka 2002). Phosphorylation plays a key role in the modulation of both AP1 and C/EBP function.

Among the aforementioned transcription factors, we found previously that consumption of n-3 PUFAs inhibited CREB, AP-1 and NF-κB binding activity. CREB exhibited marked inhibition in both electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (CHIP) assay experiments (Jia *et al.* 2004b) (Jia *et al.* 2006) (Shi and Pestka 2006). How CREB is inhibited by n-3 PUFAs is still under investigation. It has been shown that three possible CREB kinases, Akt1, MSK1, and RSK1, are activated by DON (Jia *et al.* 2006).

Akt, MSK and RSK: The protein kinases Akt1, MSK1 and RSK1 phosphorylate CREB in the murine peritoneal macrophages upon DON treatment (Jia *et al.* 2006).

Akt/PKB has been extensively studied because alteration of Akt activity has been associated with several human diseases, such as tumor (Creighton 2008) (Campbell *et al.* 2008), cardiovascular disease (Adya *et al.* 2008) (Bagli *et al.* 2004) (Fernandez-Hernando *et al.* 2007), neuronal degeneration (Greggio and Singleton 2007) (Burke 2007) ,diabetes (Kobayashi *et al.* 2004) (Ono *et al.* 2001), and so on. Akt is a highly conserved protein and the amino acid identity between mouse, rat and human is more than 95%. This conservation makes them a feasible choice to study human Akt functions using murine models (Hanada *et al.* 2004).

Three Akt isoforms have been identified. Although encoded by distinct genes localized on different chromosomes, these three isoforms have approximately 80% amino acid identity and similar domain structures. All three Akt isoforms consist of a conserved domain structure: an amino terminal pleckstrin homology (PH) domain which interacts with membrane lipid products such as phosphatidyl inositol (3, 4, 5) triphosphate (PIP_3) produced by phosphatidyl inositol 3-kinase (PI3-kinase) and a carboxyl-terminal hydrophobic regulatory domain. The kinase domain of Akt has a high similarity with other PKA, PKG, and PKC related kinases (AGC kinases) (Yang *et al.* 2004) (Song *et al.* 2005).

In mouse tissues, the Akt1 isoform is ubiquitously expressed, while Akt2 is expressed predominantly in insulin targeted tissues, such as adipose tissues, liver and skeletal muscle. Akt3 is highly expressed within the brain and testis (Hanada *et al.* 2004). Of these isoforms, Akt1 is most relevant to lymphocyte and immune function (Patel and

Mohan 2005). *Akt1* mutant mice were reported born smaller than their wild-type litter mates and had increased spontaneous apoptosis in the thymus (Hanada *et al.* 2004). Mice with high Akt1 expression showed accumulation of peripheral lymphocytes, particularly $CD4^+$ and $B220^+$ cells, as well as resistance to Fas-mediated apoptosis in splenocytes. Other features of systemic autoimmunity were also present, including lymphocyte infiltration in various organs and increased IgA levels and deposition in tissues (Patel and Mohan 2005). Due to different functions these Akt isoforms play, we will focus on Akt1 regulation.

The 90 kDa ribosomal S6 family of serine/threonine kinases (p^{90}RSK) mediates cellular signaling downstream of the mitogen-activated protein kinase (MAPK) cascade. The RSK family consists of four members, RSK*1–4,* that are encoded by distinct genes and show 75-80% amino acid identity (Kohn *et al.* 2003) (Chung *et al.* 1991). p^{90}RSK is a unique serine-threonine kinase in that it contains two functional kinase domains which include a N-terminal kinase domain that phosphorylates the substrates of p^{90}RSK and a C-terminal kinase domain involved in the activation of p^{90}RSK itself (Frodin and Gammeltoft 1999). As for signalling pathways, RSK is located downstream of the Raf-MEK-ERK protein kinase cascade. ERK activates the C-terminal kinase of RSK, which leads to activation of the N-terminal kinase by autophosphorylation. Thus, RSK represents a continuation of the ERK. p^{90}RSK has a broad range of substrates including the transcription factors CREB, NF-κB and c-Fos (Chung *et al.* 1991).

MSK is an RSK-related kinase family and is about 40% structurally identical to RSK1. MSK has two isoforms: MSK1 and MSK2. Both of them contain two protein kinase domains in a single polypeptide typical of the RSK family. MSK is found to be the substrate of ERK and p38 MAPKs. They are predominantly located in the nucleus and phosphorylate transcription factors such as CREB, ATF-1 and AP-1 (Frodin and Gammeltoft 1999) (De Cesare *et al.* 1998).

Protein phosphatases and protein dephosphorylation. The function of one-third of all proteins in eukaryotic cells are controlled by phosphorylation of specific serine(Ser), threonine(Thr), and/or tyrosine(Tyr) residues (Ceulemans and Bollen 2004). The status of protein phosphorylation reflects the balance of the activities of protein kinases and protein phosphatases. Mammalian cells have about 400 protein Ser/Thr kinases, and less than 30 protein Ser/Thr phosphatases (Plowman *et al.* 1999). Based on different functions and structures, protein Ser/Thr phosphatases are currently divided into the protein phosphatase family P (PPP) and the protein phosphatase family M (PPM). Protein phosphatase 1 (PP1), PP2A and PP2B (also known as calcineurin) are major Ser/Thr protein phosphatases (Barford *et al.* 1998).

Both CREB and its kinases, Akt1, MSK1 and RSK1, are regulated by phosphorylation at Ser and/or Thr residues. Major phosphatases that dephosphorylate those residues include PP1 and PP2A (Alberts *et al.* 1994) (Wadzinski *et al.* 1993) (Choe *et al.* 2004) (Li *et al.* 2003) (Resjo *et al.* 2002) (Garcia *et al.* 2003). PP1 is one of the most conserved eukaryotic proteins. This enzyme consists of multimeric structures composed of a catalytic subunit complexed to a number of regulatory subunits. These regulators include primary regulators such as inhibitor-2, NIPP1 and Sds22 that have

PP1-binding sites and secondary regulators such as AKAP149, Nek2 and Bcl2 that lack PP1-binding sites (Ceulemans and Bollen 2004). The core structure of PP2A consists of a 36 kDa catalytic subunit ($PP2A_c$) and a 65 kDa regulatory subunit ($PP2A_a$) as a scaffolding component. A third regulatory B subunit can associate with this core enzyme and direct the enzyme to various intracellular locations and also provides substrate specificity (Janssens *et al.* 2005) (Van Hoof and Goris 2003) (Yu *et al.* 2004). PP1 and PP2A have overlapping substrate specificities (Oliver and Shenolikar 1998).

BiP and ER stress. The endoplasmic reticulum (ER) is a cytoplasmic compartment within cells where membrane and secretory proteins are synthesized and modified. The ER provides an environment for the folding of these proteins to be transported to the cell surface, lysosomes and Golgi apparatus. Due to the presence of high concentrations of proteins, protein chaperones exist in the ER to bind newly-synthesized proteins and prevent folding intermediates from aggregation (Faitova *et al.* 2006).

In the presence of a range of cytotoxic conditions, such as calcium depletion from the ER lumen, RNA virus infection, glucose depletion and protein overexpression, the accumulation of nascent, unfolded or misfolded polypeptides in the ER leads to ER stress. In response to the excessive protein loading, the cells start the process called ER stress response or the unfolded protein response (UPR) to overcome the situation. UPR includes transient attenuation of protein translation, ER-associated degradation (ERAD) of malfolded proteins and the induction of molecular chaperones and folding enzymes to augment the ER capacity of protein folding and degradation. If the stress cannot be

relieved, apoptotic pathways will be activated (Ma and Hendershot 2001).

BiP (Immunoglobulin binding protein, also known as glucose-regulated protein 78/GRP 78) is the best characterized ER chaperone. It belongs to the heat shock protein 70 family. This protein consists of an N-terminal ATPase and a C-terminal substrate-binding domain. By recognizing and binding to a hydrophobic domain of unfolded proteins, BiP stabilizes unfolded protein for further modification. BiP also serves as a master regulator of the ER stress response and plays a key role in activating ER stress sensors (Zhang and Kaufman 2006). In both yeast and mammalian cells, overexpression of BIP down-regulates the UPR, while reduction of BiP is sufficient to induce the UPR (Kaufman 1999).

There are three major UPR pathways transduced by ER transmembrane sensors including activating transcription factor 6 (ATF6), the inositol requiring kinase 1 (IRE1) and the double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK). BiP interacts directly with all the ER stress sensors and maintains them in the inactive forms. Accumulated misfolded proteins titrate BiP away and release those sensors. Free ATF6, IRE1 and PERK are activated and transduce signals cross the ER membrane to the cytosol and nucleus (Ni and Lee 2007) (Schroder and Kaufman 2005). However, in certain cells, different stresses or physiologic conditions can selectively activate only one or two of the ER stress sensors. It is still not clear how a general BiP repression mechanism activates individual components of the UPR (Zhang and Kaufman 2006).

In mammalian cells, ATF6 is a 90 kDa constitutively expressed protein. It is a transmembrane glycoprotein embedded in the ER membrane with the N-terminal

fragment facing the cytoplasm. After dissociating from BiP, the full-length ATF6 translocates to the Golgi complex and gets cleaved by a serine protease, site-1 protease (S1P) and a metalloprotease, site-2 protease (S2P) to yield a cytosolic soluble ATF6 (50 kDa). The p50ATF6 translocates into the nucleus, binds to the ATF/cAMP response element and to the ER stress response element and acts as a transcription factor (Yoshida *et al.* 1998) (Haze *et al.* 1999).

IRE1 in yeast encodes a 1115-amino-acid polypeptide which has an amino-terminal domain localized to the ER lumen, a short transmembrane domain spanning the ER membrane and a carboxy-terminal domain. IRE1 in mammals has two homologs named IRE1α (110 kDa), which is expressed ubiquitously and IRE1β, which is expressed predominantly in the gut epithelium (Ma and Hendershot 2001). The carboxyl terminus has homology to the ribonuclease domain of RNaseL and thus has endoribonuclease activity upon activation by autophosphorylation (Kaufman 1999). The mRNA of X-box binding protein (XBP1) is cleaved by IRE1 and a 26-nucleotide intron is removed. The spliced mRNA has a translational frame-shift and generates a bigger translational product (from 33 kDa to 54 kDa) (Calfon *et al.* 2002). (Figure 1.3) The translational product of the spliced XBP1 has increased transcriptional activity. XBP1 is a transcription factor of the ATF/CREB family (Shen *et al.* 2001).

PERK is an eIF2α kinase localized in the ER with an intralumenal "stress sensor" domain that shares some homology with the IRE1 intralumenal domain and a cytosolic eukaryotic translation initiation factor 2 α (eIF2α) kinase domain. Activated PERK undergoes homodimerization and phosphorylates eIF2α (Kaufman 1999).When eIF2α is

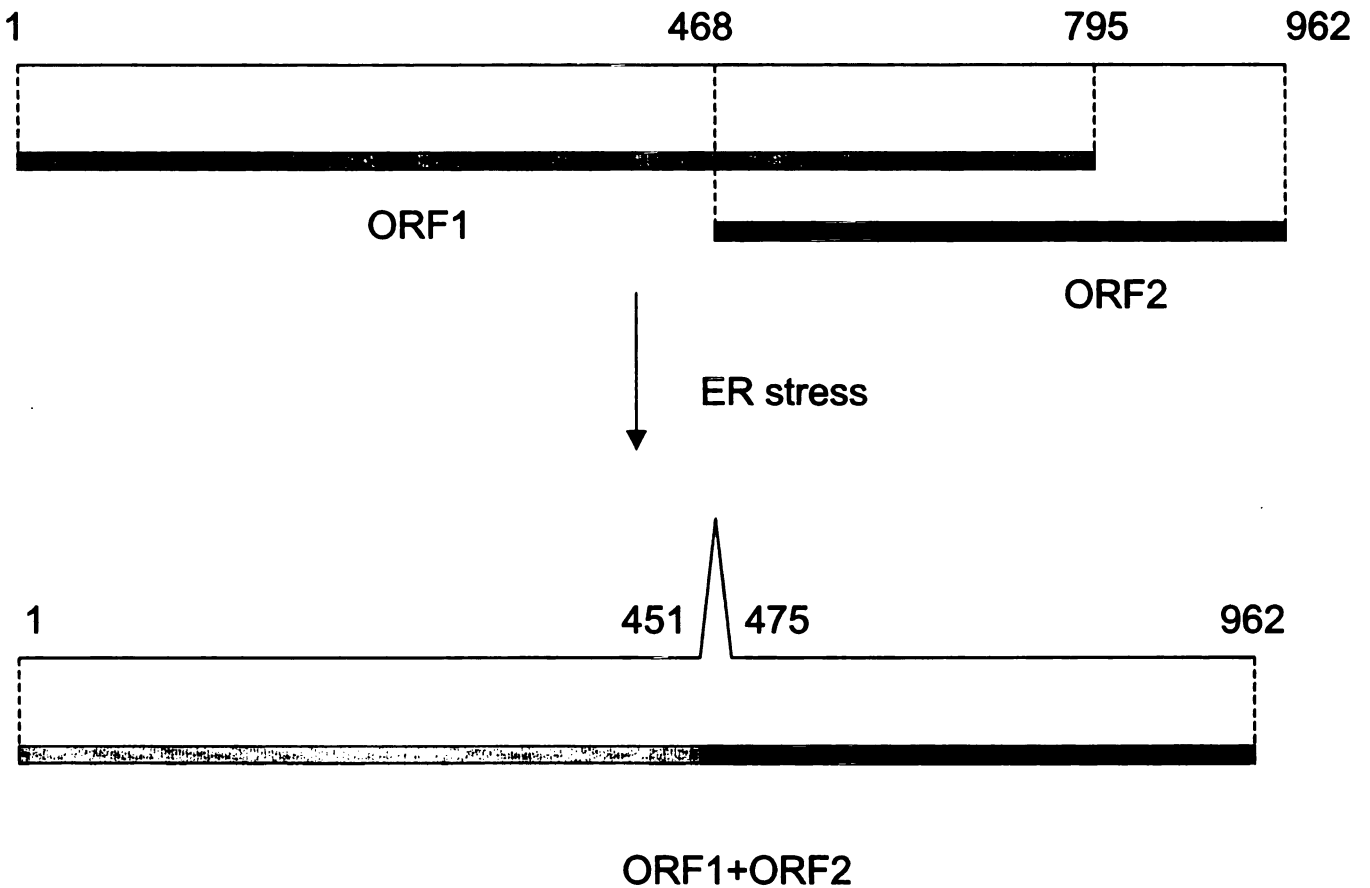


Figure 1.3. Schematic representation of XBP1 transcripts splicing before and after ER stress.

phosphorylated, the formation of the ternary translation initiation complex eIF2/GTP/Met-tRNA$_i^{Met}$ is prevented, leading to attenuation of the global translation. At the same time, phosphorylated eIF2α selectively stimulates translation of a specific subset of mRNAs by allowing translation of the open reading frame (ORF) in response to stress (Zhang and Kaufman 2006).

Classical UPR maintains ER homeostasis by enhancing transcription of resident ER proteins, such as BiP, protein disulfide isomerase (PDI), calreticulin and CHOP, etc, which facilitate protein maturation, secretion and degradation (Kaufman 1999) (Zhang and Kaufman 2006). UPR is critical in differentiation and function of specialized cells. During immune response, activated XBP1 (Reimold *et al.* 2001) and ATF6 (Gass *et al.* 2002) (Gunn *et al.* 2004) are required for plasma cell differentiation and secretion of high levels of immunoglobulins. XBP1 is also involved in the induction of IL-6 gene expression (Iwakoshi *et al.* 2003a) (Iwakoshi *et al.* 2003b).

Protein degradation. Eukaryotic cells have two major avenues for protein degradation, the ubiquitin-proteasome and autophagy-lysosomal pathways (Cecarini *et al.* 2007) (Yorimitsu and Klionsky 2005).

Protein ubiquitination and proteasome-mediated protein degradation is an important pathway responsible for misfolded and short-lived intracellular proteins (Guerrero *et al.* 2006) (Rubinsztein 2006). The 26S proteasome is a 2.5 MDa complex composed of two multisubunit subcomplexes: one is a 20S core particle and the other a 19S regulatory particle. (Demartino and Gillette 2007) The 20S core particle is a cylinder-shaped structure composed of four stacked rings, each containing seven subunits

($\alpha_7\beta_7\beta_7\alpha_7$). The two outer rings have seven different α-subunits that regulate the opening of the proteasome to proteins. The core particle possesses three proteolytic activities: chymotrypsin-like (subunit β5), trypsin-like (subunit β2) and peptidylglutamyl peptide-hydrolyzing activities (subunit β1). The regulatory particle is composed of 20 different subunits, which is able to bind to the external rings to form the 26S proteasome and therefore regulate proteolytic activity. Degradation of proteins by the proteasome requires covalent attachment of ubiquitins to the substrates for recognition by the proteolytic complex. Other enzymes are able to remove ubiquitins from proteins to be degraded and allow them to be recycled (Cuervo and Dice 1998).

Another process named autophagy is also an important mechanism for the degradation of cytoplasmic components, from single macromolecules (proteins, lipids and nucleic acids) to whole organelles. Autophagy is the process conserved from yeast to mammalian cells for degradation by lysosomes. There are two main steps in this process including delivery of the substrates to the lysosomal lumen and breakdown of these substrates by resident enzymes. Depending on how the substrates are delivered for degradation, autophagy is classified as either macro-, micro- or chaperone-mediated autophagy (CMA). Both macro- and microautophagy sequester a region of the cytosol for non-selective complete degradation, while CMA only selectively degrades cytosolic proteins that are targeted and translocated to lysosomes by lysosomal receptor binding and chaperone unfolding (Massey *et al.* 2004; Yorimitsu and Klionsky 2005). Once inside the lysosomal system, substrates are degraded by different acidic hydrolases for protein turnover (Grinyer *et al.* 2007) (Cecarini *et al.* 2007). Lysosomes contain a powerful

mixture of more than 80 types of proteases, peptidases and other hydrolases and all the proteases inside lysosomes are called cathepsins (Cuervo and Dice 1998).

Calpains are a family of Ca^{2+}-regulated cysteine proteases that mediate cleavage of specific substrates involved in cell differentiation, life and death (Demarchi and Schneider 2007). Depending on the Ca^{2+} concentration necessary for their activation, calpains are classified into two isozymes, μ- and m-calpains. The proteins cleaved by calpains include cytoskeletal and associated proteins, kinases and phosphatases, membrane receptors and transporters, etc. Activated calpains can also compromise the integrity of lysosomal membranes and liberate cathepsins from lysosomes (Yamashima 2004).

Immunoglobulin A and IgAN. IgA is the most abundantly produced immunoglobulin and its production occurs in both mucosal and systemic compartments. Based on structural difference, human IgA is classified into two different isotypes, IgA1 and IgA2. IgA1, but not IgA2, possesses a hinge region which is composed of 13 amino acids (Figure 1. 4). The majority of IgA molecules produced in the mucosal compartment are polymeric (mostly dimeric) IgA1 or IgA2. The main function of secretory IgA is to bind and neutralize potential mucosal pathogens and toxins and prevent them from entering into the human body. Systemic IgA is produced in the bone marrow and is the source of circulating IgA. Circulating IgA molecules are mostly monomeric with the ratio of IgA1 to IgA2 9:1. They are cleared from the circulation by the hepatocytes and leucocytes and their function is still not understood (Barratt *et al.* 2007b) (Yoo and Morrison 2005).

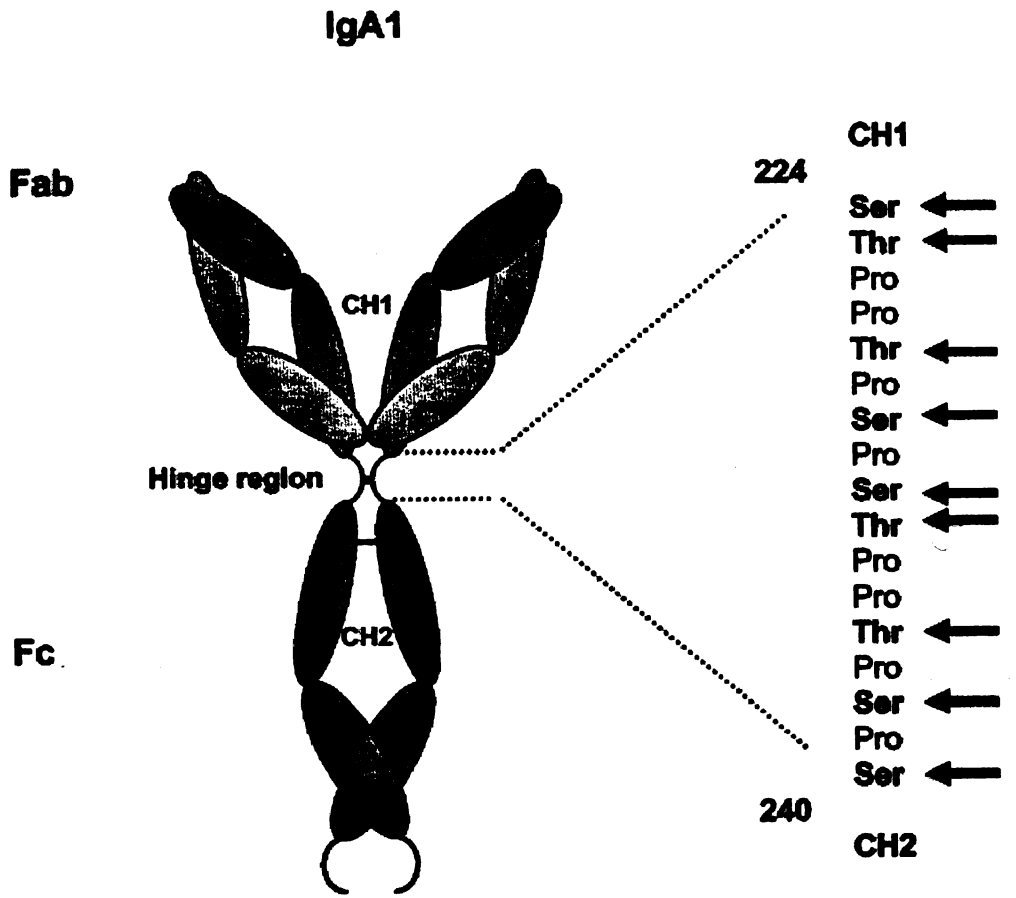


Figure 1.4. The structure of human IgA1. (Barratt *et al.* 2007b)

The IgA molecule is heavily glycosylated by both N-linked and O-linked glycosylation. The N-linked carbohydrates are linked to asparagine and are the most common form. The O-linked carbohydrates are linked to serine or threonine in the hinge region (Barratt *et al.* 2007a). The glycosylation of IgA molecules is isotype specific: IgA1 has both O-linked glycosylation in the hinge region and N-linked glycosylation in the FC region, while IgA2 has only N-linked glycosylation due to absence of the hinge region (Yoo and Morrison 2005) (Baenziger and Kornfeld 1974a) (Baenziger and Kornfeld 1974b). Each human IgA1 heavy chain hinge region has 3 to 5 O-linked glycans which include a core GalNAc linked to serine and/or threonine residues in the hinge region and extended with galactose. This structure is covered by sialic acid N-acetylneuraminic acid (NeuAc) linked to GalNAc or galactose (Suzuki *et al.* 2008).

IgAN is a disease characterized by mesangial deposition of polymeric IgA1 that has reduced galactosylation in its *O*-linked glycans. These IgA molecules have reduced galactose and/or sialic acid content with increased exposure of the *N*-acetylgalactosamine (GalNAc). The structurally abnormal IgA tends to bind a wide variety of molecules (antigens) by carbohydrate interactions, or form IgG-IgA, IgA-IgA immune complexes. The hypoglycosylated IgA and its immune complexes are more likely to deposit in the kidney mesangial area and cause IgAN (Coppo and Amore 2004). The exposed GalNAc can be detected by binding of GalNAc-specific lectins and other more complicated methods such as "fluorophore-assisted carbohydrate electrophoresis" (FACE) (Allen *et al.* 1999) and "matrix-assisted laser desorption ionization time of flight mass spectrometry" (MALDI-TOFMS) (Hiki *et al.* 2001) (Coppo and Amore 2004).

There are some concerns about using animal models to study the pathogenesis of

human IgAN because of the structural difference between human IgA and mouse IgA: (1) human IgA has two isotypes, while mouse IgA has only one isotype; (2) comparison of the sequence of IgA between human and mouse suggests that mouse IgA gene may be more similar to human IgA2 than IgA1; (3) human IgA1 is glycosylated by both *O*-linked and *N*-linked glycans, mouse IgA does not have *O*-glycosylation sites in its hinge region. Despite these differences, mouse IgA and human IgA1 share some characteristics: (1) mouse IgA has a hinge region (Phillips-Quagliata 2002); (2) it also has two *N*-glycosylation sites in its CH1 and CH3 regions (Nishie *et al.* 2007b). Although many studies on human IgAN have focused on *O*-linked glycosylation of IgA1, aberrant N-linked glycosylation has also been reported (Amore *et al.* 2001) (Barratt *et al.* 2007b). These studies suggest that hypoglycosylation on either *O*-glycans or *N*-glycans might be involved in the pathogenesis of IgAN and mouse models can be used to study IgAN despite difference between species (Allen *et al.* 1995).

CHAPTER 2

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

ABSTRACT

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

IL-6 transcription.

INTRODUCTION

Immunoglobulin A nephropathy (IgAN), the most common human glomerulonephritis, is defined by predominant deposits of IgA within the mesangial regions of the kidney glomerulus (Feehally 1997). Immunoglobulin A nephropathy accounts for 5% to 10%, 20% to 35% and up to 50% of glomerulonephropathies in North America, Europe and Japan, respectively (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). Between 20% and 40% of these patients will develop progressive renal failure with 1% to 2% of adult patients entering end-stage renal failure yearly (Donadio, Jr. *et al.* 1999). The fundamental abnormality in IgAN lies within the IgA system and not the kidney because IgA deposition in IgAN patients recurs after renal transplantation (Harper *et al.* 1996). An overly robust IgA response to mucosal infections and dietary antigens in terms of quantity, size (primarily polymeric), glycosylation status and IgA immune complex (IgA-IC) formation is suspected to contribute to IgAN (Emancipator and Lamm 1989) (Montinaro *et al.* 1999). Resultant IgA-IC deposition in glomeruli likely triggers mesangial cell proliferation, matrix secretion and inflammation in which proinflammatory cytokines play important roles. Although no consistent infectious or environmental agent has been identified to cause dysregulation of the IgA antibody response, the onset of IgA nephropathy is often associated with upper respiratory tract infection (Emancipator and Lamm 1989). Therapeutic strategies for IgAN remain elusive, partly because of its poorly defined etiology.

Dietary supplementation with n-3 PUFAs found in fish oil, particularly (22:6n-3) docosahexaenoic acid (DHA) and (20:5n-3) eicosapentaenoic acid (EPA), has potential human health benefits relative to inflammatory diseases (Calder 2003) (Gil 2002). n-3 PUFAs suppress inflammatory responses through eicosanoid-dependent or eicosanoid-independent mechanisms (Calder 2006b). Case-control studies reveal that dietary n-3 PUFAs are negatively associated with the risk of IgAN (Wakai *et al.* 1999), whereas high intake of n-6 PUFAs is positively associated with increased risk (Wakai *et al.* 2002). Holman et al. (Holman *et al.* 1994) demonstrated that some IgAN patients are deficient in α-linolenic acid (18:3n-3), a precursor of DHA and EPA, and that supplementation with EPA and DHA suppresses arachidonic acid (AA) synthesis, decreases proteinuria and improves glomerular filtration rate in this group. Several elegant clinical trials by Donadio and Grande (Donadio and Grande 2004) have now demonstrated that n-3 PUFA retards late-stage renal disease progression in IgAN patients by reducing inflammation and glomerulosclerosis.

The trichothecene mycotoxin deoxynivalenol (DON) is a secondary metabolite produced by members of the fungus *Fusarium* and is frequently found in dietary staples such as wheat, barley, corn, rice and oats (Pestka and Smolinski 2005). It is recalcitrant to inactivation during milling and processing, and frequently enters finished food products. Prolonged feeding of DON causes a dramatic elevation in total serum IgA in mice with IgA becoming the major serum isotype (Pestka *et al.* 1989). This co-occurs with marked elevation of serum IgA-IC, kidney mesangial IgA accumulation, electron dense mesangial deposits and hematuria (Dong and Pestka 1993) (Dong *et al.* 1991) which are hallmarks of the early stages of human IgAN (Pestka 2003). IL-6 is critical to mucosal

IgA immunity based both on its differentiative effects on IgA-committed B cells and its production in the gut by macrophages, T cells and other cells (Beagley *et al.* 1989). Deoxynivalenol up-regulates IL-6 expression in vivo and in vitro, and this has been linked to increased IgA production. (Yan *et al.* 1998) (Yan *et al.* 1997) Interleukin-6-deficient mice are refractory to DON-induced dysregulation of IgA production and development of IgAN (Pestka and Zhou 2000). Thus, IL-6 is pivotal to DON-induced IgAN.

Fish oil as well as the (n-3) PUFAs DHA and EPA suppress DON-induced IgAN (Pestka *et al.* 2002) (Jia *et al.* 2004a) (Jia *et al.* 2004b). This preclinical model enables exploration of how n-3 PUFAs attenuate aberrant IgA responses associated with IgAN and as well as gain insight into therapeutic strategies for this disease. Key issues concerning use of fish oil for treating IgAN and other inflammatory diseases relate to the most efficacious types of n-3 PUFAs, optimal doses required to achieve ameliorative effects and underlying molecular mechanisms. In particular, clarification is needed on reported differential effects of DHA or EPA relative to biological efficacy and disease prevention (Seo *et al.* 2005) (Yusufi *et al.* 2003) (Obajimi *et al.* 2005) (Egashira *et al.* 2004) (Woodman *et al.* 2003) (Leigh-Firbank *et al.* 2002). Previously, our laboratory established that dietary DHA concentrations between 1% and 3% were necessary to ameliorate DON-induced IgAN (Jia *et al.* 2004a) (Jia *et al.* 2004b). The goals of this research were to (1) evaluate dose-response effects of EPA-enriched oils on DON-induced IgAN and (2) determine effects of the optimal EPA dose on IL-6 mRNA expression and transcription factor binding activities.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade or better and were purchased from Sigma (St. Louis, MO) unless otherwise noted. DON was produced in *Fusarium graminearum* R6576 cultures and purified chromatographically (Clifford *et al.* 2003). DON was added to powdered diets as detailed previously (Pestka *et al.* 1989). DON-contaminated labware was detoxified by soaking for >1 h in 10% (v/v) sodium hypochlorite (Thompson and Wannemacher, Jr. 1986).

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

Experimental design. For the dose-response study, experimental diets were based on the AIN-93G formulation of Reeves *et al.* (Reeves *et al.* 1993) and consisted of the following ingredients (per kilogram): 10 g of AIN-93G mineral mix, 10 g of AIN-93 vitamin mix, 200 g of casein, 397.5 g of cornstarch, 132 g of Dyetrose (dextrinized cornstarch), 50 g of cellulose, 3 g of l-cysteine, 2.5 g of choline bitartrate, 14 mg of TBHQ and 100 g of sucrose (Dyets, Bethlehem, PA). Corn oil (Dyets), high oleic acid safflower oil containing 75% oleic acid (Hain Pure Foods, Melville, NY) and MEG-3 EPA-Rich Oil containing 49.9% EPA, 6.7% DHA and 1.2% docosapentaenoic acid (DPA)

(Ocean Nutrition Canada, Dartmouth, Nova Scotia) were used to amend the basal AIN-93G diet to yield five experimental groups (n=9): control, control+DON, 0.1% EPA+DON, 0.5% EPA+DON and 3% EPA+DON (Table 2.1). This range of doses was selected to be equivalent, respectively, to 0.2×, 1× and 5× the maximum recommended level of n-3 PUFA consumption by FDA. A DON concentration of 20 mg/kg was selected based on its previously observed efficacy in inducing IgAN in B6C3F1 mice (Pestka 2003).

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

For IL-6 expression studies, B6C3F1 mice were fed control or 3% EPA diet (n=9) for 4 weeks. Prior to experiment termination, mice were gavaged with 25 mg/kg DON or vehicle. After 3 h, mice were euthanized and one half of the spleen and pooled PP were subjected to real-time PCR analysis. The remaining spleen portion was frozen at −80°C for fatty acid analysis. The same approach was used for transcription factor studies except that tissues were harvested after 30 min based on an optimal time point for DON induction reported previously (Zhou *et al.* 2005b). Nuclear proteins from spleen cell suspensions were extracted and analyzed by electrophoretic mobility shift assay (EMSA) for transcription factor binding activity.

Experimental group	DON (mg/kg diet)	Corn oil (g/kg diet)	Oleic acid (g/kg diet)	EPA-enriched oil* (g/kg diet)	Total EPA (g/kg diet)	Total (n-3) (g/kg diet)	Total (n-6) (g/kg diet)	n-6/n-3 ratio
Control	0	10	60	0	0	0.1	6.0	60:1
Control+DON	20	10	60	0	0	0.1	6.0	60:1
0.1%EPA+DON	20	10	58	2	1.0	1.3	6.0	4.6:1
0.5%EPA+DON	20	10	50	10	5.0	5.9	6.2	1.1:1
3.0%EPA+DON	20	10	0	60	29.9	34.8	7.4	1:4.7

*Refers to concentrated fish oil that is enriched for EPA. This oil has 49.9% EPA and 6.7% DHA.

Tables 2. 1. Experimental groups of mice for assessing the effects of different dosage of EPA on DON-induced IgA. (All diets were adjusted with oleic acid to have final oil concentrations of 70 g/kg.)

Fatty acid analysis. Fatty acids were analyzed by gas chromatography (GC) utilizing a GC-2010 Gas Chromatograph (Shimadzu Scientific Instruments, Chicago, IL) and standard fatty acid methyl ester (Nu-Check Prep, Elysian, MN) by the protocol of Hasler *et al* (Hasler *et al.* 1991).

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

IgA and IgA-IC measurement. Serum IgA was measured by ELISA (Dong and Pestka 1993) using mouse reference immunoglobulin serum (Bethyl Laboratories, Montgomery, TX), goat antimouse IgA and peroxidase-conjugated goat antimouse IgA (heavy chain specific) (Organon Teknika, West Chester, PA). Immunoglobulin A was quantified by measuring enzyme end product absorbance using a Vmax Kinetic Microplate Reader and Softmax program from Molecular Devices (Menlo Park, CA). For

the detection of IgA-IC, diluted serum samples were first precipitated by 70% (w/v) polyethylene glycol (PEG 6000, Sigma) (Dong *et al.* 1991) and quantified by IgA ELISA (Dong and Pestka 1993).

Assessment of kidney mesangial IgA deposition. Kidney sections were prepared and analyzed for IgA deposition according to Pestka *et al.*. (Pestka *et al.* 1989) Briefly, removed kidneys were frozen in liquid nitrogen, sectioned to 7 mm with a cryostat (Reichert-Jung; Cambridge Instruments, Buffalo, NY) and stained with fluorescein isothiocyanate-labeled goat antimouse IgA (Sigma). Immunoglobulin A immunofluorescence was assessed with a Nikon Labophot Microscope (Mager Scientific, Dexter, MI) equipped with a Kodak DC290 digital camera (Kodak, Rochester, NY). Mean fluorescence intensity of 10 consecutively arranged glomeruli from each section was determined using UTHSCSA Image Tool Software V 1.2. (Jia *et al.* 2004b) Pixels were measured on a grayness scale ranging from 0 (black) to 255 (white).

Interleukin-6 mRNA and heteronuclear RNA analysis. Total RNA from spleens and PPs was isolated using the Trizol (Sigma) according to the manufacturer's protocol. Resultant RNA was dissolved in 50 μl of RNAse-free water and stored at −80°C until analysis. Probe and primers for mouse IL-6 mRNA and 18S RNA (endogenous control) were purchased as TaqMan assay reagents (PE Applied Biosystems). PCR reactions for IL-6 mRNA and 18S RNA quantification were performed on an ABI PRISM 7700 Sequence Detector System using a TaqMan One-Step RT-PCR Master Mix Reagents Kit according to the manufacturer's protocol (PE Applied Biosystems) (Jia *et al.* 2004b). SYBER Green PCR Master Mix (PE Applied Biosystems) was used to detect IL-6 heteronuclear RNA (hnRNA) using primers (forward: gtccaactgtgctatcgctcact; backward:

agaaggcaactggatggaagtct). Ct values were related to RNA concentrations using the standard curves derived from serial dilutions of total RNA (ranging 1.37–1000 ng per well) and normalized by dividing the IL-6 RNA value by the 18S RNA value.

Electrophoretic mobility shift assay. Spleen cells were dissociated, passed through a 100-mesh stainless steel screen and pooled. Cells were suspended in Dulbecco's PBS. Erythrocytes were lysed for 2 min at 25°C in 10 mM $KHCO_3$ containing 0.14 M NH_4Cl. Nuclear extracts were prepared based on the method of Zhou *et al* (Zhou *et al.* 2003a). Briefly, cells were lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM dithiothreitol) with phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride), Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) and 0.7% (v/v) Nonidet P-40. Nuclei were pelleted by centrifugation and suspended in hypertonic buffer containing 20 mM HEPES, pH 7.9, 0.4 M KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 10% (w/v) glycerol and phosphatase and protease inhibitors. After 30 min on the ice, a supernatant was collected by centrifugation at 14,000×*g* for 10 min. Resultant extracts were analyzed with a Bio-Rad Protein Assay kit (Melville, NY), aliquoted and stored at −80°C for EMSA.

Double-stranded consensus probes for cyclic AMP response element-binding protein (CREB), activator protein-1 (AP-1), nuclear factor kappa B (NF-κB) and C/EBPβ (Santa Cruz Biotech, Santa Cruz, CA) were radiolabeled with [γ-^{32}P]ATP using a Ready to Go Polynucleotide Kinase Kit (Pharmacia Biotech, Piscataway, NJ). Nuclear protein (10 μg) was added to DNA-binding reaction buffer consisting of 20 mM HEPES, pH 7.9,

60 mM KCl, 1 mM EDTA, 0.5 mM DTT and 2 mg of poly(dI-dC), and preincubated on ice for 15 min to block the nonspecific binding. After the addition of 1 ml of ^{32}P-labeled probe containing 30,000 cpm, the incubation was continued for 30 min at room temperature to promote the formation of nucleoprotein complexes. Resultant nucleoprotein complexes were separated on 4% (w/v) native polyacrylamide gels, dried and visualized by autoradiography (Zhou *et al.* 2003a).

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

RESULTS

The capacity of DON to induce early markers of IgAN was compared in mice fed diets containing 0%, 0.1%, 0.5% and 3% EPA. Consistent with its reported anorexic effects (Pestka and Smolinski 2005), consumption of 20 mg/kg DON in the diet reduced feed intake and weight gain in all groups (Table 2.2). However, no difference existed among control and EPA-fed mice in either of these parameters. When fatty acid content in splenic phospholipid fractions were assessed by GC, concentrations of AA [20:4(n-6)] in control mice were two to three times higher than in those of mice fed with 0.1% to 3% EPA (Table 2.3). Eicosapentaenoic acid content was 0%, 0.4%, 3.2% and 5.6% and DHA content 2.7%, 5.3%, 7.5% and 6.1% in mice fed 0%, 0.1%, 0.5% and 3.0% EPA diets, respectively. In addition, DPA, an intermediate in the conversion of EPA to DHA, was also markedly elevated in mice fed the two highest EPA concentrations. Thus, EPA-containing diets markedly decreased the ratio of (n-6) to (n-3) PUFAs in lymphoid tissue.

Serum IgA, serum IgA-IC and kidney mesangial IgA were used as markers for the early stages of IgAN in DON-fed mice. Deoxynivalenol exposure significantly increased serum IgA concentrations in mice fed control diet beginning at 8 weeks, reaching 10 times the control value after 16 weeks (Figure 2.1). Serum IgA accumulation was significantly suppressed in mice fed 3% EPA at 8, 12 and 16 weeks, whereas the 0.1% and 0.5% EPA diets had no effect. Deoxynivalenol induced increases in serum IgA-IC at week 16, and this was also suppressed by the 3% but not the 0.1% or 0.5% EPA diets (Figure 2.2). When glomerular mesangial IgA deposition was measured at week 16 by immunofluorescence, DON was found to induce IgA deposition (Figure 2.3). Again, this elevation was inhibited only by the 3% EPA diet, but not lower EPA concentrations.

Experimental group	Mean daily food consumption (g)	Mean body weight gain (g)
Control	3.61 ± 0.48^{a}	12.94 ± 1.68^{a}
Control + DON	2.94 ± 0.66^{b}	2.76 ± 0.84^{b}
0.1% EPA + DON	2.65 ± 0.79^{b}	3.01 ± 0.48^{b}
0.5% EPA + DON	2.71 ± 0.70^{b}	2.26 ± 0.55^{b}
3.0% EPA+ DON	2.37 ± 0.59^{b}	2.91 ± 0.54^{b}

Values are means ± S.E.M, n=9.
Means in a column without a common letter differ ($P<0.05$).

Table 2.2. Food consumption and body weight gain of mice in different diet groups.

Fatty acid	Control	Control + DON	0.1% EPA + DON	0.5% EPA + DON	3.0% EPA + DON
	% in phospholipids				
C14:0	1.8 ± 0.5 [a]	2.1 ± 0.4 [a]	2.3 ± 0.4 [a]	1.9 ± 0.4 [a]	2.6 ± 0.5 [a]
C16:0	21.1 ± 1.5 [a]	24.5 ± 0.8 [a]	23.2 ± 0.6 [a]	25.1 ± 3.4 [a]	21.7 ± 1.7 [a]
C16:1	1.9 ± 0.6 [a]	3.3 ± 0.7 [a]	2.1 ± 0.5 [a]	1.7 ± 0.7 [a]	1.7 ± 0.5 [a]
C18:0	26.2 ± 1.3 [a]	26.2 ± 2.8 [a]	28.0 ± 1.4 [a]	24.0 ± 2.5 [a]	25.7 ± 1.6 [a]
C18:1	23.4 ± 1.7 [a]	22.5 ± 2.5 [a]	23.5 ± 1.7 [a]	20.4 ± 2.9 [a]	18.9 ± 1.9 [a]
C18:2 (n6)	3.8 ± 0.3 [a]	4.4 ± 0.4 [a]	3.8 ± 0.5 [a]	4.7 ± 0.6 [a]	4.5 ± 0.3 [a]
C18:3 (n3)	16.8 ± 2.1 [a]	13.4 ± 3.0 [a]	8.3 ± 1.1 [b]	5.7 ± 1.0 [b]	5.6 ± 1.0 [b]
C20:5 (n3)	ND [a]	ND [a]	0.4 ± 0.2 [a]	3.2 ± 1.2 [b]	5.6 ± 0.6 [b]
C22:5 (n3)	2.1 ± 0.6 [a]	1.0 ± 0.3 [a]	3.2 ± 0.6 [ab]	5.8 ± 1.2 [b]	7.9 ± 1.5 [b]
C22:6 (n3)	3.0 ± 0.5 [a]	2.7 ± 0.8 [a]	5.3 ± 1.0 [ab]	7.5 ± 1.4 [b]	6.1 ± 1.2 [b]

Values are means ± S.E.M, N=7. Means in a row without a common letter differ ($P<0.05$).
Only the major fatty acids are shown.
ND: not detectable.

Table 2.3. Fatty acid composition of spleen phospholipids in mice fed different diets for 16 wk.

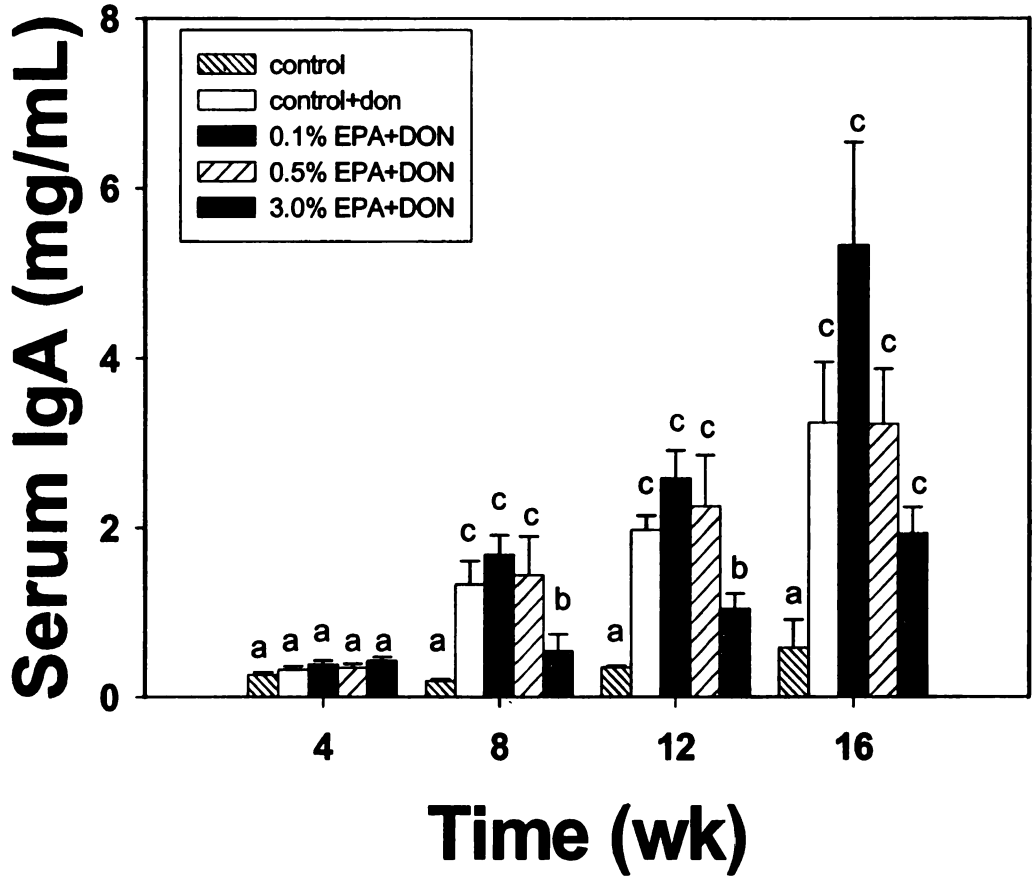


Figure 2.1. EPA-enriched fish oil consumption suppresses DON-induced serum IgA elevation in mice. Mice were fed control or 20 ppm DON containing 0%, 0.1%, 0.5% or 3% EPA, and serum IgA was measured by ELISA at 4, 8, 12, and 16 wk. Data are means ± S.E.M. (n=8). Means at a specific time point without a common letter differ ($P<0.05$).

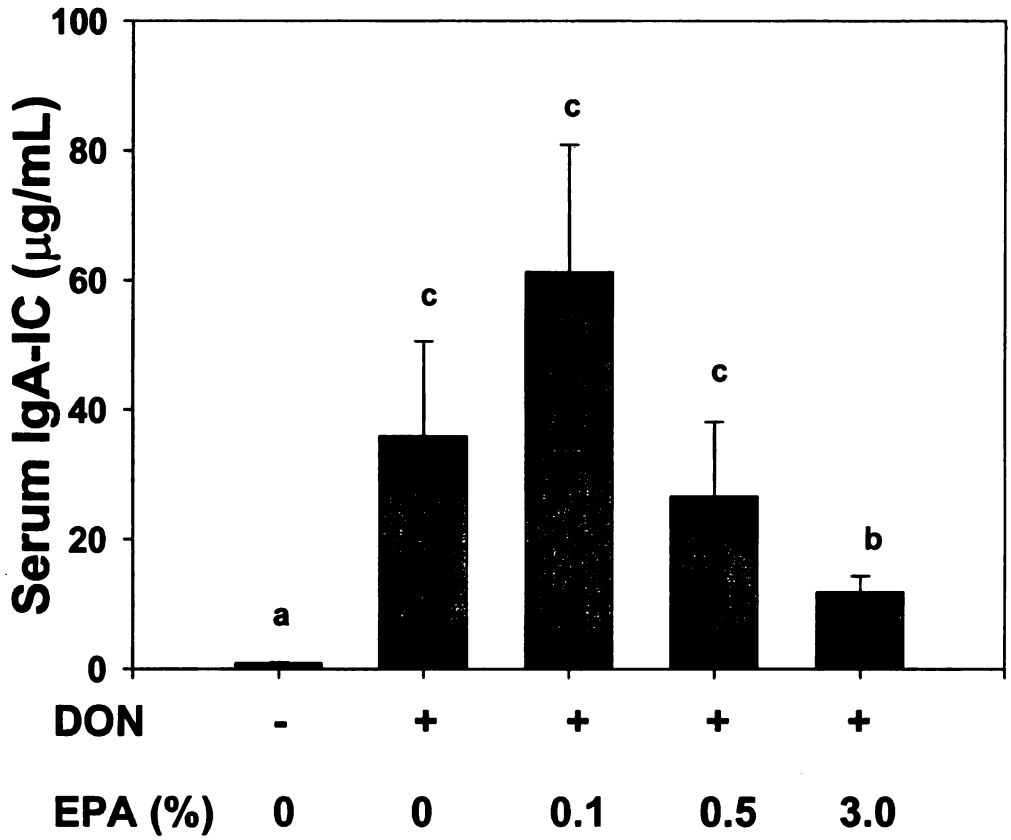


Figure 2.2. EPA-enriched fish oil consumption attenuates DON-induced serum IgA-IC elevation in mice. Mice were treated as described in figure 2.1. Sera were collected at 16 wk and analyzed by ELISA after polyethylene glycol precipitation. Data are means ± S.E.M. (n=8). Means without a common letter differ ($P<0.05$).

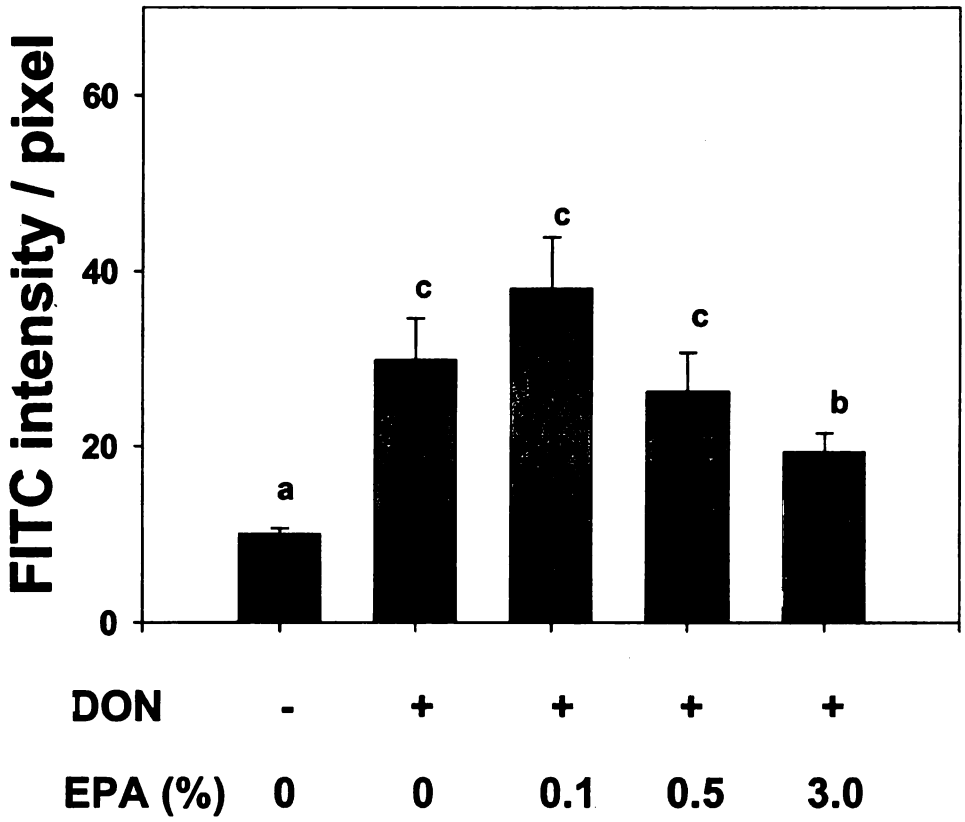


Figure 2.3. EPA-enriched fish oil consumption inhibits DON-induced mesangial IgA deposition in mice. Mice were treated as described in figure 2.1. Relative mesangial IgA quantitation at 16 wk was assessed by measuring immunofluorescence by image analysis. Data are means ± S.E.M. (n=8). Means without a common letter differ ($P<0.05$).

Deoxynivalenol-induced IgAN has been previously shown to correlate with increased IgA secretion ex vivo in lymphoid tissue cultures derived from the mucosal and systemic compartments (Pestka 2003). The effects of EPA consumption on ex vivo IgA production were therefore compared. Inclusion of DON in basal control diet significantly increased IgA production in spleen and PP cell cultures as compared to control group (Figure 2.4). However, IgA secretion was markedly suppressed in cultures from all three EPA groups.

Jia *et al.* (Jia *et al.* 2004a) (Jia *et al.* 2004b) demonstrated that DHA consumption dose dependently suppresses DON-induced IL-6 expression in spleen and that this correlates with reduced IL-6 mRNA transcription. To assess DON's acute effects on IL-6, we fed the mice 0% or 3% EPA diet for 4 weeks prior to acute DON challenge. This feeding period was sufficient to lower splenic AA and concurrently raise EPA, DHA and DPA to levels observed after 16 weeks (Table 2.4). In control mice, acute DON exposure was found to markedly induce serum IL-6 (Figure 2.5) as well as expression of IL-6 mRNA and hnRNA, a marker of IL-6 transcription, in spleen and PP (Figure 2.6). Prior consumption of 3% EPA suppressed all three end points suggesting that this fatty acid impaired IL-6 transcription. Consistent with these findings, EMSA revealed that CREB and AP-1 binding activities in splenic nuclear extracts were significantly increased by acute DON exposure, but that consumption of 3% EPA diet suppressed this induction (Figure 2.7). Eicosapentaenoic acid did not affect DON-induced NF-κB activation. Neither DON nor EPA affected C/EBP binding.

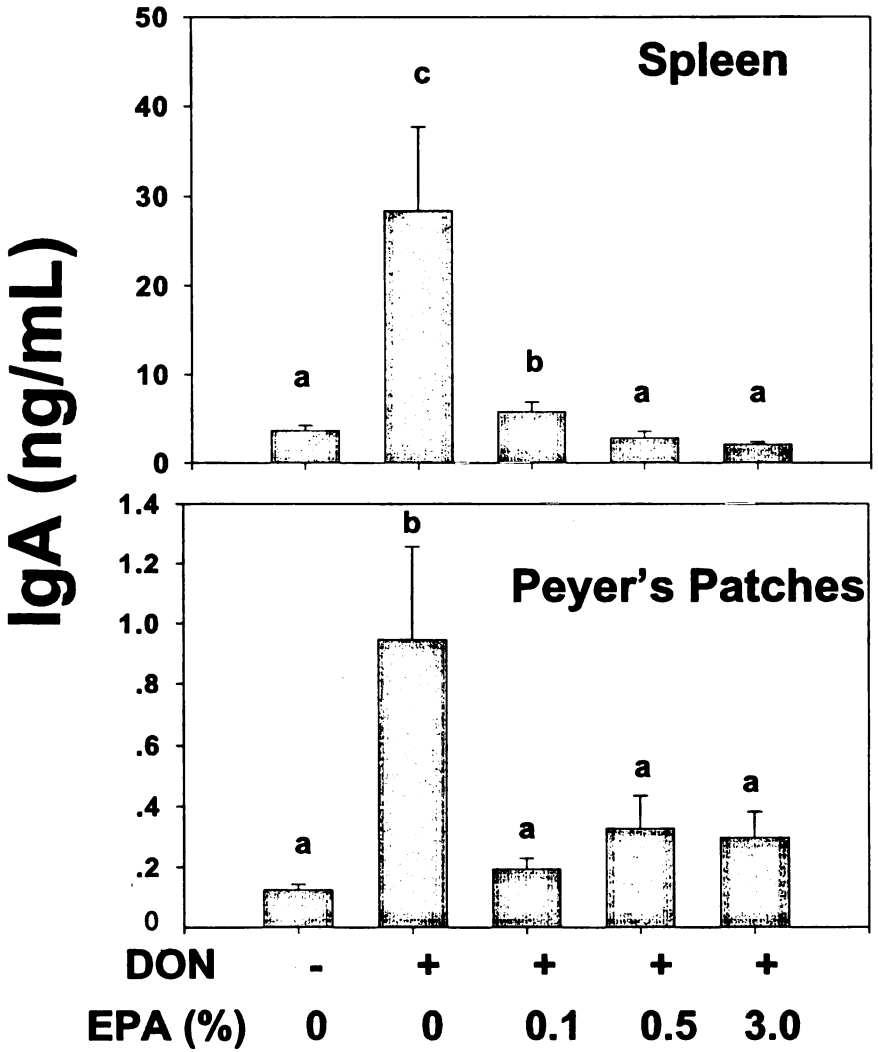


Figure 2.4. DON-induced ex vivo IgA secretion is suppressed in PP and spleen cell cultures from mice fed EPA-enriched fish oil for 16 wk. Data are means ± S.E.M. (n=8). Means without a common letter differ ($P<0.05$).

Fatty acid	Control	3% EPA + DON
	% in phospholipids	
C14:0	2.2 ± 0.5 [a]	2.3 ± 0.4 [a]
C16:0	20.1 ± 0.8 [a]	23.6 ± 1.0 [a]
C16:1	2.5 ± 0.4 [a]	1.9 ± 0.6 [a]
C18:0	25.8 ± 2.3 [a]	22.3 ± 1.8 [a]
C18:1	24.2 ± 2.2 [a]	23.5 ± 2.1 [a]
C18:2	4.1 ± 0.3 [a]	5.2 ± 0.4 [a]
C18:3	18.4 ± 1.8 [a]	5.7 ± 1.4 [b]
C20:5	ND [a]	5.2 ± 0.6 [b]
C22:5	1.9 ± 0.5 [a]	6.4 ± 0.9 [b]
C22:6	2.5 ± 0.3 [a]	6.8 ± 1.4 [b]

Values are means ± S.E.M, N=3. Means in a row without a common letter differ ($P<0.05$).
Only the major fatty acids are shown.
ND: not detectable.

Table 2.4. Fatty acid composition of spleen phospholipids in mice fed control or 3% EPA diet for 4 wk.

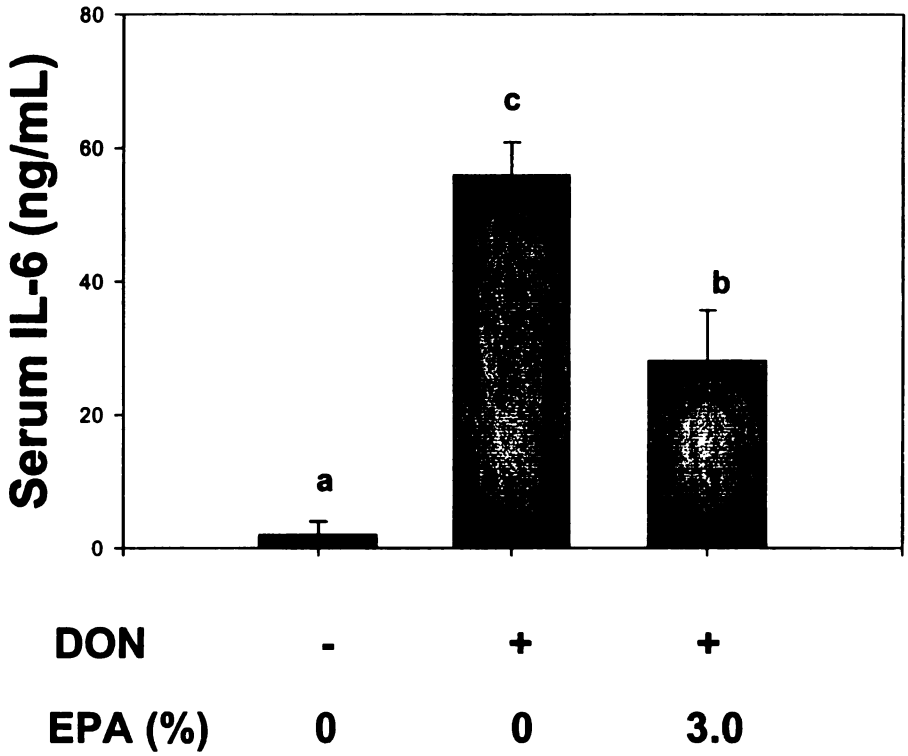
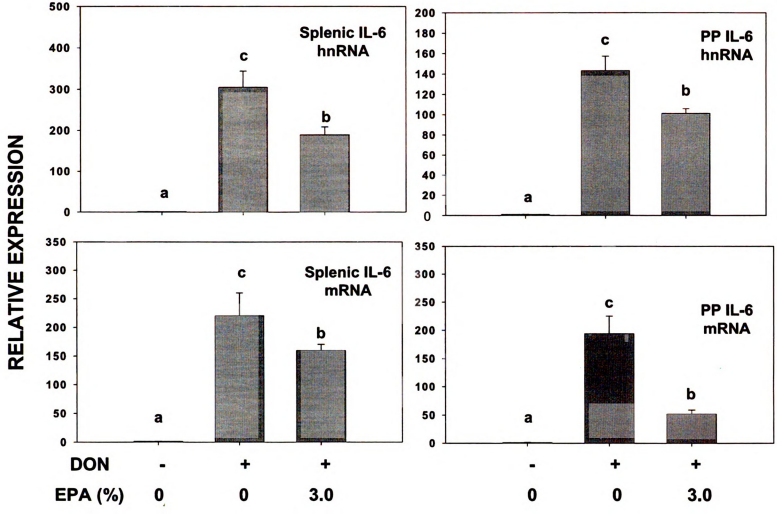


Figure 2.5. Induction of serum IL-6 by acute DON exposure is attenuated in EPA-fed mice. Mice were fed experimental diets for 4 wk and then gavaged with 25 mg/kg body weight DON or vehicle. Data are means ± S.E.M. (n=5). Means without a common letter differ ($P<0.05$).

Figure 2.6. EPA-enriched fish oil consumption suppresses induction of IL-6 hnRNA and mRNA after acute DON exposure in spleen and Peyer's patches from B6C3F1 mice. Mice were fed control or 3% EPA diet for 4 wk and gavaged with 25 mg/kg DON or vehicle. RNA was extracted from organs after 3 h and analyzed by real-time PCR. Data are means ± S.E.M. (n=5). Means without a common letter differ ($P<0.05$).


RELATIVE EXPRESSION
Splenic IL-6 hnRNA
PP IL-6 hnRNA
Splenic IL-6 mRNA
PP IL-6 mRNA
a
b
c
DON - + + - + +
EPA (%) 0 0 3.0 0 0 3.0

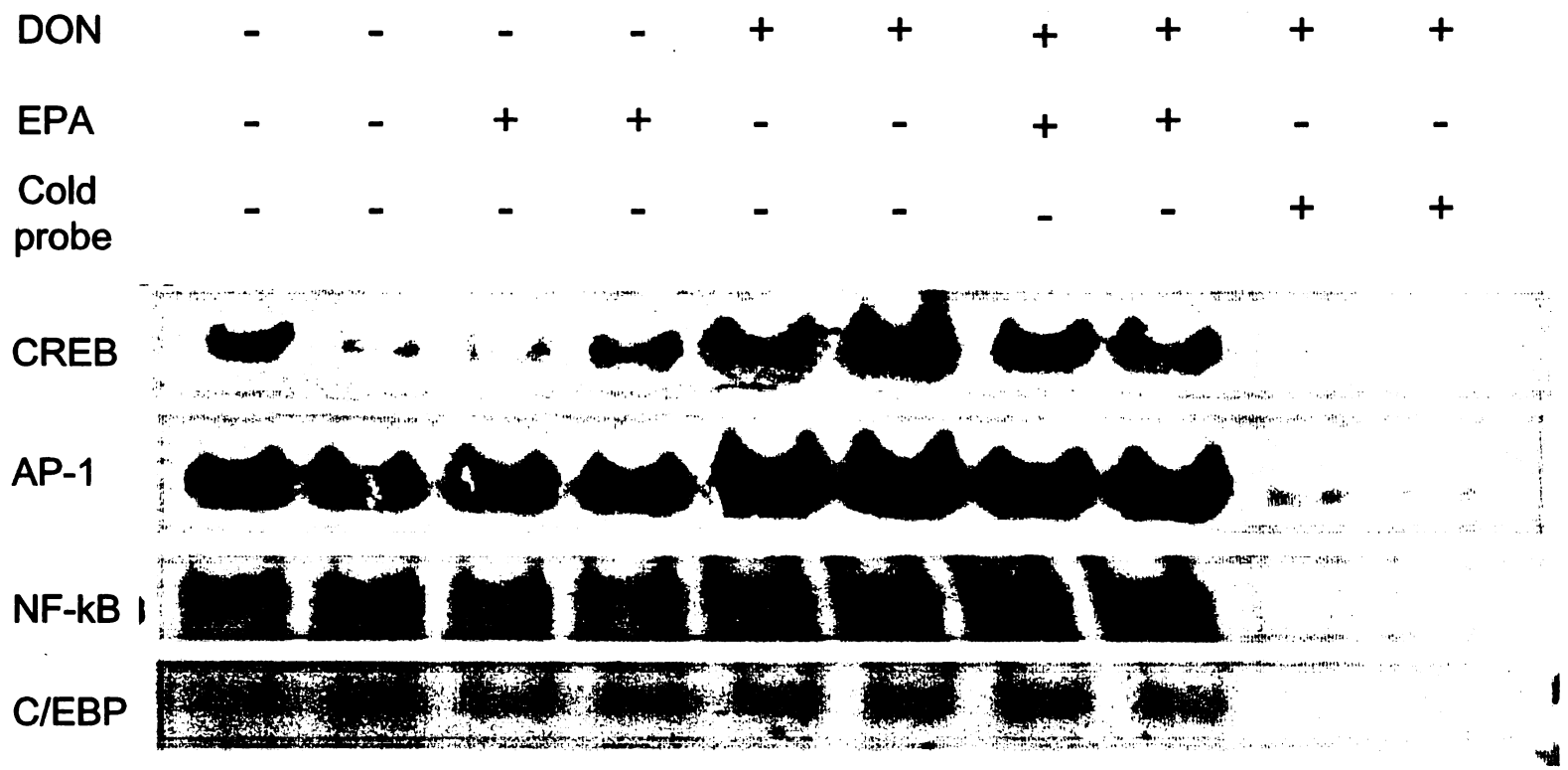


Figure 2.7. EPA-enriched fish oil consumption suppresses DON-induced splenic transcription factor binding activity. Mice were fed control or 3% EPA diet for 4 wk and gavaged with 25 mg/ml DON or vehicle. Splenic nuclei were isolated after 30 min and then analyzed or transcription factor binding activity by EMSA using double-stranded consensus probes for CREB, AP-1, NF-κB and C/EBPβ radiolabeled with [γ-^{32}P]ATP. Cold probe was incubated in 100 fold excess.

DISCUSSION

Although (n-3) PUFA supplementation has been suggested to be efficacious in the treatment of late-stage IgAN (Donadio and Grande 2004), the potential preventative benefits of (n-3) PUFAs in early stages of the disease are unknown. Aberrant serum IgA production is an important early etiological factor for IgAN, and resultant IgA-IC likely binds receptors on mesangial cells, thereby inducing proliferation and cytokine production (Barratt *et al.* 2004). Polymeric IgA deposition might also activate complement via the alternative pathway, causing glomerular damage. Because DON-induced dysregulation of IgA production and aberrant IgA accumulation mimic the early stages of human IgAN (Jia *et al.* 2004a) (Pestka 2003), the results presented herein suggest that EPA consumption might have possible prophylactic value in suppressing early elevation of IgA and nephritogenic IgA-IC among individuals who have been diagnosed with IgAN or who have genetic predisposition for this disease.

We observed here that 3% but not 0.1% or 0.5% EPA in the diet suppressed serum IgA and IgA-IC elevation and glomerular IgA deposition. Jia *et al.* (Jia *et al.* 2004a) previously found that 1% EPA in diet could also suppress these parameters. Taking these studies together, dietary EPA concentrations of 1% to 3% appear to be the prophylactic threshold in this model. These findings are consistent with prior studies showing the same efficacious doses for DHA-enriched oil (Jia *et al.* 2004a) (Jia *et al.* 2004b). Because both EPA- and DHA-enriched oils seem to be similarly effective and inhibiting IgA up-regulation, consumption of fish oils containing EPA+DHA might be more cost effective to achieve the IgA endpoint than oils processed to selectively contain either of the two (n-3) PUFAs.

A 1% to 3% EPA dose range provides 2.2% to 6.7% of the daily kilocalorie consumed in the mouse, which can be compared to 1.5% daily kilocalorie for (n-3) PUFAs in supplements (Donadio and Grande 2004) recommended for therapy in patients. Here we employed the 3% diet as a strong positive control to discern underlying mechanisms for inhibition of IgA up-regulation. A limitation of this approach is that this dose would be equivalent to human consumption at 15 g/day, which is five times that of the recommended FDA maximum of 3.0 g/day. Nevertheless, it should be noted that ex vivo cultures from all three EPA concentrations showed suppressed IgA production. This suggests that ongoing IgA production at experiment termination (16 weeks) might have indeed been suppressed even at the lowest (0.1%) EPA concentration later in the feeding study. Thus, a key future question relates to whether prefeeding (n-3) PUFAs at low concentrations for an extended period before DON exposure might attenuate IgA dysregulation.

Although EPA content in the spleen (0.4–5.6%) was highly dependent on the concentration of this (n-3) PUFA in the experimental diet, DHA content was similar (5.3–7.5%) among the experimental groups. Splenic DHA could arise from two sources. First, although the EPA-enriched fish oil employed here contained predominantly EPA (49%), it also contained a smaller amount of DHA (6.7%) that could accumulate in the splenic pool. Second, EPA can be transformed to DHA in mammalian tissue via intermediate DPA (Fyfe and Abbey 2000) (Kanayasu-Toyoda *et al.* 1993). Accumulation of DPA (3.2–7.9%) in EPA-fed mice suggests that such a conversion was ongoing and thus could be another likely DHA source.

A critical observation was that EPA consumption significantly reduced serum IL-

6 and splenic IL-6 mRNA induced by acute DON exposure. The mucosal immune system is a primary target of DON (Pestka 2003) where it disrupts oral tolerance and promotes production of IgA capable of binding antigens found in food and commensal bacteria (Rasooly and Pestka 1994) (Rasooly *et al.* 1994). Deoxynivalenol up-regulates expression of proinflammatory genes, notably IL-6, both in vivo and in vitro (Zhou *et al.* 1997) (Wong *et al.* 1998). IL-6 is produced by activated monocytes and macrophages, and also by endothelial cells, T cells and keratinocytes (Kanda and Takahashi 2004). Interleukin-6 drives IgA-committed B cell 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. Interleukin-6's role in DON-induced IgAN is supported by the ex vivo antibody neutralization studies (Yan *et al.* 1998) (Yan *et al.* 1997) and by the observation that IL-6-deficient mice resist DON-induced serum IgA elevation and mesangial IgA deposition (Pestka and Zhou 2000).

Because hnRNA is a precursor species observed in cells prior to RNA splicing to mRNA, its abundance can be used as a surrogate for the run-on assay in detection of gene transcriptional activity (Elferink and Reiners, Jr. 1996). Eicosapentaenoic acid-enriched oil consumption significantly blocked accumulation of IL-6 hnRNA as well as IL-6 mRNA, indicating that (n-3) PUFA attenuation IL-6 gene expression occurred in part at the transcriptional level. This contention is further supported by our previous observation that mice consuming 3% DHA for 4 weeks exhibit markedly less induction of IL-6 mRNA and IL-6 hnRNA expression following acute DON exposure (Jia *et al.* 2004b). Diminished IL-6 transcription and ultimately, IL-6 expression, might attenuate IL-6

production by affecting transcription factor binding to the response element in the IL-6 promoter. Cyclic AMP response element-binding protein, AP-1, NF-κB and C/EBPβ have all been associated with binding and transactivation of the IL-6 promoter (Dendorfer *et al.* 1994) and (Tokunou *et al.* 2001). Of these, suppression of DON-induced IgAN corresponded to decreased induction of CREB and AP-1 binding activity. These two factors might thus be important in diminished IL-6 transcription.

n-3 PUFAs potentially influence functional activities of cells of the immune system via several mechanisms. The ameliorative effects of the experimental diet in this study were likely mediated by dramatic decreases in AA and corresponding increases in splenic (n-3) PUFAs. Deoxynivalenol-induced IL-6 expression is mediated in part through increased COX-2 levels and PGE2 production (Moon *et al.* 2003) (Moon and Pestka 2003a) (Moon and Pestka 2002). Cyclooxygenase and lipoxygenase products produced from EPA are much less potent as inflammatory mediators than are products generated from AA (Calder 2005). Thus, decreased AA tissue concentration might reduce IL-6 gene expression and attenuate overall IgA production in DON-exposed mice. Inhibition of IL-6 expression by (n-3) PUFAs could also involve deregulation of key signal transduction pathways. Deoxynivalenol does not act through a known receptor but rather acts via the ribotoxic stress response and activation of mitogen-activated protein kinases (MAPKs) that drive expression of IL-6 and other inflammatory genes (Moyad 2005). Consumption of fish oil or (n-3) PUFAs can cause modest suppression of MAPK activation (Jia *et al.* 2004b) (Moon and Pestka 2003b).

Taken together, the data presented here suggest, for the first time, that a threshold exists for EPA relative to suppression of experimental IgAN and that the threshold EPA

dose for IgA inhibition was effective at suppressing IL-6 transcription. Relevant to our findings, two clinical trials reported that IL-6 production by peripheral blood mononuclear cells is decreased in persons who consume (n-3) PUFA, and this co-occurs with increased plasma and cell membrane (n-3) PUFA incorporation (Trebble *et al.* 2003a) (Wallace *et al.* 2003). (n-3) PUFA suppression of IL-6 production might have additional clinical relevance because this cytokine is believed to contribute to kidney injury (Taniguchi *et al.* 1996) (Bagheri *et al.* 1997) in human IgAN (Donadio and Grande 2002). Further study at the preclinical level is needed on the relationship between dietary (n-3) PUFA and tissue phospholipid concentrations needed for optimal, efficacious prophylactic and therapeutic treatment for IgAN and other immune-related diseases. The strategy described here offers one animal model for such preclinical testing.

CHAPTER 3

Mechanisms for Suppression of Interleukin-6 Expression in Peritoneal Macrophages from Docosahexaenoic Acid-Fed Mice

ABSTRACT

Consumption of the trichothecene mycotoxin deoxynivalenol (DON) induces interleukin-6 (IL-6)-dependent IgA nephropathy (IgAN) in mice. This effect can be prevented by feeding long chain n-3 polyunsaturated fatty acids (PUFAs) found in fish oil. The purpose of this study was to identify the signal transduction pathways by which DON upregulates IL-6 in the peritoneal macrophage and how consumption of fish oil enriched with the n-3 PUFA, docosahexaenoic acid (DHA), suppresses these processes. Incubation with DON induced IL-6 expression in naïve macrophages maximally at 3 h. Knockdown of the transcription factor cAMP response element-binding protein (CREB) or pharmacologic inhibition of the CREB kinases, Akt1/2, MSK1 and RSK1, downregulated this expression. Inhibition of double-stranded RNA-activated protein kinase (PKR) suppressed not only IL-6 expression but also phosphorylation of CREB and its upstream kinases, Akt1, MSK1 and RSK1. Phosphorylation of PKR, CREB kinases and CREB was markedly impaired in peritoneal macrophages isolated from mice that consumed DHA-enriched fish oil for 6 to 8 wk. DHA's effects were not explainable by increased activity of protein phosphatase 1 and 2A since both were suppressed in mice consuming the DHA diet. Although cells cultured directly with DHA expressed less IL-6 compared to cells cultured with arachidonic acid (AA), neither fatty acid treatment affected DON-induced protein phosphorylation. Furthermore, DHA and AA similarly inhibited cell-free protein kinase activity. These data suggest that DON-induced IL-6 expression is CREB-mediated and PKR-dependent and that requisite kinase activities for these pathways were suppressed in macrophages from mice fed DHA for an extended

period.

INTRODUCTION

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by *Fusarium* that is frequently encountered in cereal-based foods and that potentially evoke adverse effects on human health. DON can induce both proinflammatory cytokine expression and apoptosis in mononuclear phagocytes depending on exposure frequency and dose (Pestka *et al.* 2004). Dietary exposure to DON selectively promotes polyclonal activation and expansion of immunoglobulin A (IgA)-secreting B cells by activating macrophages and T cells. Production of autoreactive IgA and its deposition in the mouse kidney mimic the early stages of human IgA nephropathy (IgAN) (Pestka 2003) (Pestka *et al.* 1989). DON-induced interleukin-6 (IL-6) expression in macrophages plays a critical role in IgA upregulation (Yan *et al.* 1997) (Pestka and Zhou 2000). The upstream mechanisms by which DON induces IL-6 production in macrophages remain unclear but appear to be mediated both transcriptionally and post-transcriptionally (Caravatta *et al.* 2008) (Suzuki *et al.* 2008) (Nishie *et al.* 2007a).

IL-6 plays a critical role in inflammation initiation and maintenance of chronic inflammatory states. IL-6 also elicits T cell activation, end-stage B cell differentiation and immunoglobulin secretion. Notably, circulating IL-6 levels are elevated in several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and psoriasis, and correlate with markers of disease activity (Gabay 2006) (Kishimoto 2006) (Ishihara and Hirano 2002). IL-6 has also been related to the degree of IgA deposition in the kidney and disease progression in patients with IgAN (Lim *et al.* 2003) (Harada *et al.* 2002).

Consumption of the n-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic

acid (DHA) or eicosapentaenoic acid (EPA), suppresses DON-induced IgAN in mice (Jia *et al.* 2004b) (Shi and Pestka 2006), which concurs with the proposed anti-inflammatory action of these fatty acids. These results are consistent with randomized clinical trials demonstrating that fish oil consumption retards the renal function loss in IgAN patients (Donadio, Jr. *et al.* 1994) (Donadio, Jr. *et al.* 1999) (Donadio, Jr. *et al.* 2001) (Donadio and Grande 2004).

Given the potential importance of IL-6 in the pathogenesis of IgAN and other autoimmune diseases, it is important to understand how DON induces IL-6 overexpression in macrophages and how n-3 PUFA consumption ameliorates these effects. DON-induced phosphorylation of cAMP response element-binding protein (CREB), a transcription factor associated with IL-6 expression, and its subsequent binding to the IL-6 promoter have recently been shown to be inhibited in mice fed DHA or EPA (Jia *et al.* 2006) (Shi and Pestka 2006). The purpose of this study was to (1) verify that CREB activation is critical for DON-induced IL-6 expression and (2) identify upstream signaling pathways by which DHA suppresses DON-induced CREB activation.

MATERIALS AND METHODS

Materials. All chemicals including DON and cell culture components were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) unless otherwise noted. DON contaminated labware and cell culture media were detoxified by sodium hypochlorite. All kinase and phosphatase inhibitors were purchased from Calbiochem, Inc. (San Diego, CA).

Animals and diet. Female B6C3F1 mice (5 wk old) weighing 16 to 18 g were obtained from Charles River Laboratories, Inc (Wilmington, MA) or Harlan (Indianapolis, IA). Housing, handling, and sample collection procedures conformed to the policies of the Michigan State University All-University Committee on Animal Use and Care in accordance with NIH guidelines. Mice were fed Harlan Teklad 22/5 Rodent chow or fat-amended diets prepared as described in previous studies (Jia *et al.* 2006) (Shi and Pestka 2006). Briefly, corn oil (Dyets, Bethlehem, PA), high oleic acid safflower oil (Hain Celestial Group, Inc., Melville, NY) and MEG-3^{TM} DHA-enriched fish oil (containing DHA 483 g/kg and 113 g/kg EPA) (Ocean Nutrition Canada, Dartmouth, Nova Scotia) were added to AIN 93G basal diet (Dyets) to generate a control diet (10 g corn oil and 60 g safflower oil/kg diet) and a DHA diet containing 30 g DHA/kg diet (10 g corn oil and 60 g DHA enriched oil/kg diet), respectively. Mice were fed one of the diets for 6 to 8 wk before peritoneal macrophage harvest. The DHA concentration was selected based on previous work (Jia *et al.* 2004b) and the time period was chosen based on its efficacy in preliminary studies to consistently suppress DON-induced IL-6 expression.

Peritoneal macrophage cultures. Mice were injected ip with 1.5 ml of sterile 3% (w/v) thioglycollate broth. After 4 d, mice were euthanized and macrophages collected by

peritoneal lavage with ice-cold Hank's BSS (Invitrogen Corporation, Carlsbad, CA). Cells were pelleted by centrifugation at 1,100 × g for 5 min. Cells were washed with BSS once and resuspended in RPMI-1640 containing 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C under 6% CO_2 in a humidified incubator for 24 h before treatment.

Macrophages were incubated with or without DON for various time periods and analyzed for mRNA expression by real-time PCR or protein phosphorylation by Western analysis. DON was dissolved in PBS first to make a 250 μg/ml stock solution and then added to cell culture media as a 1:1000 (v/v) dilution to generate a 250 ng/ml working solution. Two milliliters of cell suspension (1×10^6 /ml) were incubated in each well of 6-well cell culture plates (Corning Life Sciences, Lowell, MA) for experiments requiring RNA isolation. For protein collection, 10 ml cell suspensions (1×10^6 /ml) were incubated in 100 mm-diameter cell culture dishes (Corning Life Sciences).

For protein kinase studies, inhibitors of MSK1/RSK1 (Ro31-8220) and Akt1/2 (Akt inhibitor IV, V and VIII) were dissolved in DMSO and added to cultures 1 h before DON treatment. DMSO alone was used at the vehicle control. PKR inhibitor C16 and its negative control were added to cultures 45 min before DON treatment.

For protein phosphatase studies, calyculin A (20 nM), an inhibitor to type 1 and 2A protein phosphatase, was added to cell cultures 1 or 2 h prior to DON treatment. None of the inhibitors at the indicated concentrations affected cell viability, as verified by

trypan blue staining, or induced morphological changes, as verified by phase contrast microscopy.

Real-time PCR. RNA was extracted using RNeasy Mini (Promega, Madison, WI) and analyzed by real-time PCR for IL-6 mRNA expression (Shi and Pestka 2006). TaqMan primers and probes were purchased from Applied Biosystems (Foster City, CA). β-2 microglobulin RNA expression is not affected by DON treatment and thus was used as endogenous control to normalize target gene expression.

Western analysis. For protein phosphorylation studies, macrophages were washed with ice-cold PBS, lysed in Tris buffer (10 mM, pH 7.4) containing 1% (w/v) SDS and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), boiled and sonicated. After centrifugation at 18,000 × g for 15 min, extracts were subjected to Western analysis using specific antibodies to CREB, phospho-CREB, phospho-Akt1, phospho-RSK1, phospho-MSK1 (Cell Signaling Technology, Inc., Danvers, MA), PKR (Millipore, Billerica, MA), phospho-PKR (Calbiochem) and β-actin (Sigma-Aldrich). Alexa Fluor 680 goat-anti rabbit and IRDye® 800 goat-anti mouse secondary antibodies were purchased from Invitrogen Corporation and Rockland Immunochemicals, Inc. (Gilbertsville, PA) respectively. Infrared fluorescence was directly detected by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

siRNA transfection. An siRNA cocktail targeting mouse CREB and a comparable scrambled siRNA were purchased from Dharmacon (Lafayette, CO). siRNA transfection was performed by electroporation using an Amaxa Nucleofector (Gaithersburg, MD). Briefly, 2×10^6 cells were suspended in 100 µl electroporation buffer (mixture of 40 µl of

buffer 1 [20% ATP-disodium and 12% $MgCl_2$-$6H_2O$] and 2 ml of buffer 2 [1.2% K_2HPO_4, 0.12% $NaHCO_3$ and 0.04% glucose]) and mixed with 10 μM siRNA. Electroporation was performed using program D032 for macrophages according to the manufacturer's protocol. Transfection efficacy was verified by assessing loss of CREB protein by Western blot 48 h after transfection. IL-6 expression induced by DON after transfection was analyzed by real-time PCR.

Akt1 assay. Akt1 activity in immunoprecipitates was measured by Western analysis. For immunoprecipitation, media were removed by centrifugation and adherent cells were washed twice with ice-cold PBS. After PBS was aspirated, 0.5 ml lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% [v/v] Triton X-100, 2.5 mM sodium pyrophosphate, phosphatase inhibitor cocktail and protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]) were added. Following incubation on ice for 5 min, cells were scraped off the plates and transferred to microcentrifuge tubes, sonicated 4 × 5 sec and clarified by centrifugation at 18,000 × g for 10 min. The supernatants (40 μl) were incubated with 1 μg anti-Akt1 antibody with gentle rocking for 2 h at 4 °C. Protein A-Sepharose beads (20 μl of 50% slurry) were then added and incubated for 30 min. Beads were pelleted at 18,000 × g for 30 sec, washed twice with lysis buffer and kinase assay buffer (25 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol [DTT], 10 mM $MgCl_2$, phosphatase inhibitor cocktail and protease inhibitor cocktail) respectively. Akt1-specific CREB kinase activity in the immunoprecipitate was assessed at 30°C for 30 min using 10 μM glutathione *S*-transferase (GST)-CREB (Upstate, Lake Placid, NY) and 30 μM ATP as substrate. Assays were terminated by adding 2% (w/v)

SDS buffer, and CREB phosphorylation was detected by Western analysis.

Protein phosphatase assay. Phosphatase activity of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) in immunoprecipitates was determined using para-nitrophenyl phosphate (pNPP) as substrate. For immunoprecipitation, cells were rinsed twice with ice-cold Hank's BSS and incubated with lysis buffer (50 mM HEPES [pH 7.4], 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 1 mM DTT and protease inhibitor cocktail) on ice. After 5 min, cells were scraped off the plates and transferred to microcentrifuge tubes, sonicated and clarified by centrifugation at 18,000 × g for 10 min. PP1 and PP2A were immunoprecipitated using mouse IgG2b antibodies specific to C subunit of PP1α or PP2A respectively (Upstate). Briefly, a supernatant containing 500 μg protein was incubated with 4 μg specific antibody or mouse IgG2b and 40 μl protein A-Sepharose beads (50% slurry) at 4°C with gentle rocking for 2 h. The beads were pelleted at 18,000 × g for 30 sec and washed 3 times with lysis buffer and phosphatase assay buffer (50 mM HEPES [pH 7.2], 10 mM $MnCl_2$, 2 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail) respectively. The pellet was reconstituted in phosphatase assay buffer, and pNPP was added to make a final concentration of 20 mM. The reaction mixture was incubated with agitation at 30°C for 1 h. Phosphatase activity was measured by reading the absorbance of supernatant at 405 nm. Phosphatase activity was expressed as relative values compared to control group at time 0.

Fatty acid treatment of cell cultures. DHA, arachidonic acid (AA) and oleic acid (OA) were prepared as 200 mM stock solutions in ethanol and stored under nitrogen in the dark at -20 °C until needed. Fatty acid-bovine serum albumin (BSA) complexes were

made based on published methods (Moon and Pestka 2003b) with some modifications. Briefly, fatty acids in ethanol and BSA (fatty acid free) (Serologicals proteins Inc., Kankakee, IL) were mixed in PBS at a 3:1 molar ratio under nitrogen on a rocking shaker at 37 °C for 24 h. This ratio was previously shown to suppress DON-induced IL-6 in the RAW 264.7 macrophage cell line(Moon and Pestka 2003b). These mixtures were then diluted with RPMI-1640 which was supplemented with 0.25% (v/v) FBS. Fresh media were prepared for each experiment. Prior to adding fatty acid-amended media, naïve peritoneal macrophages were incubated in RPMI-1640 medium with 0.25% (v/v) FBS for 18 h to elicit fatty acid deprivation. Cells were then cultured with media amended with 50 μM fatty acids for 24 h before DON (250 ng/ml) was added. Total RNA was collected after 3 h and IL-6 mRNA was detected by real-time PCR. To measure phosphorylation of CREB and its upstream kinases, cells were incubated with vehicle DON for 20 min after fatty acid incubation. Protein was extracted and analyzed by Western blot analysis.

Fatty acid treatment of CREB kinases. The effects of fatty acids on CREB kinases in a cell-free system were tested over a range of concentrations (0, 12.5, 25, 50 and 100 μM) using a constant ethanol concentration. Active Akt1, MSK1 or RSK1 (1 ng) (Upstate) was incubated with or without free fatty acids for 1 min in kinase assay buffer according to the protocol from Upstate. CREB (30 μM) and ATP (100 μM) were then added. The reaction was incubated at 30°C for 10 min and then was terminated with 2% SDS buffer. CREB phosphorylation was analyzed by Western analysis.

Statistics. All data were analyzed with SigmaStat v 3.1 (Jandel Scientific, San Rafael, CA) with the criterion for significance set at $p<0.05$. Student's t-test was used for comparison of two groups of data. One-way ANOVA was performed for comparison of

multiple groups. Holm-Sidak (if normality test passed) or Dunnett's (ANOVA on ranks if normality test failed) tests were used as post-hoc analysis.

RESULTS

Incubation of naïve peritoneal macrophages with DON (250 ng/ml) induced IL-6 mRNA expression within 1 h (Figure 3.1). Increased expression was detectable up to 7 h with maximum induction being observed at 3 h. Based on this finding, a 3 h incubation was chosen to study mechanisms for DON-induced IL-6 expression and how these are affected by DHA.

To verify the role played by CREB in DON-induced IL-6 expression, this transcription factor was knocked down by electroporating with a specific siRNA cocktail. As revealed by Western blotting, CREB protein was knocked down by 47% at 48 h after transfection. (Figure 3.2A) Correspondingly, DON-induced IL-6 mRNA expression was decreased by 42%. (Figure 3.2B) These data confirm that CREB is likely to be a critical transcription factor in DON-induced IL-6 expression in the macrophage.

DON induces phosphorylation of several protein kinases that are capable of phosphorylating and activating CREB (Jia *et al.* 2006). Specific inhibitors were thus employed to investigate the relationship between IL-6 mRNA expression and CREB kinases. When naïve macrophages were preincubated with the MSK1/RSK1 inhibitor Ro31-8220 at 1000 nM (Figure 3.3A), DON-induced IL-6 expression was markedly inhibited. The role of another CREB kinase family, Akt 1 and 2, in DON-induced IL-6 was also assessed with three inhibitors. Akt Inhibitor IV is an ATP-competitive inhibitor of a kinase upstream of Akt, but downstream of PI-3 K while Akt Inhibitor V targets an Akt effector molecule other than PI-3 K or PDK1. Akt Inhibitor VIII selectively inhibits Akt1 and Akt 2 and appears to be pleckstrin homology (PH) domain-dependent. This

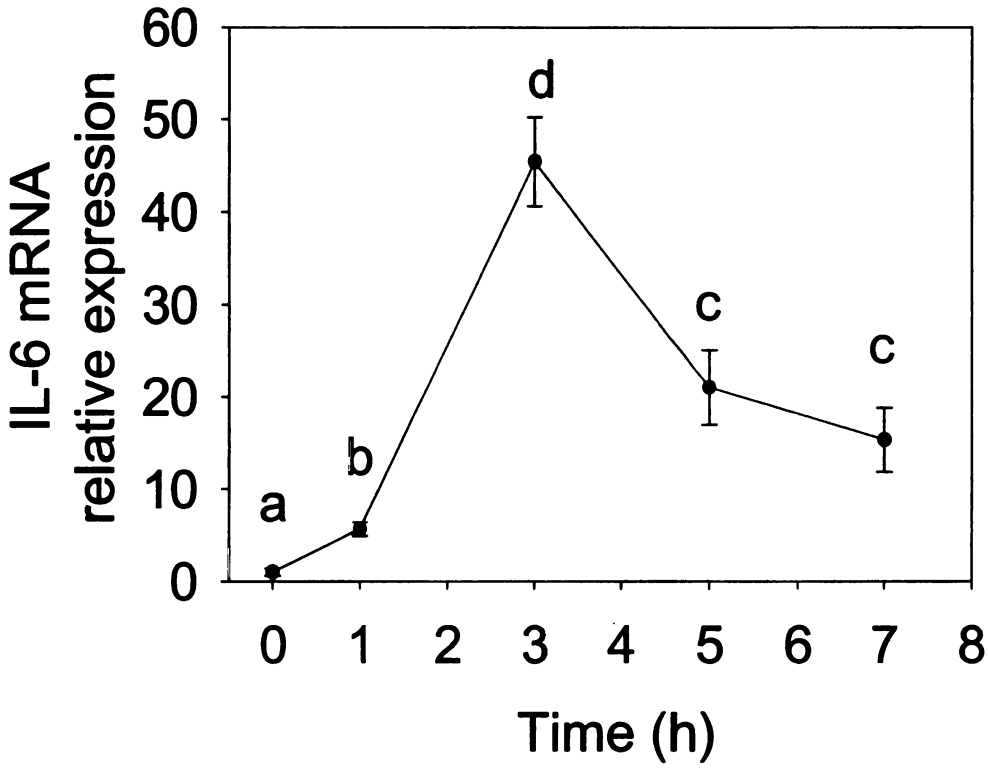
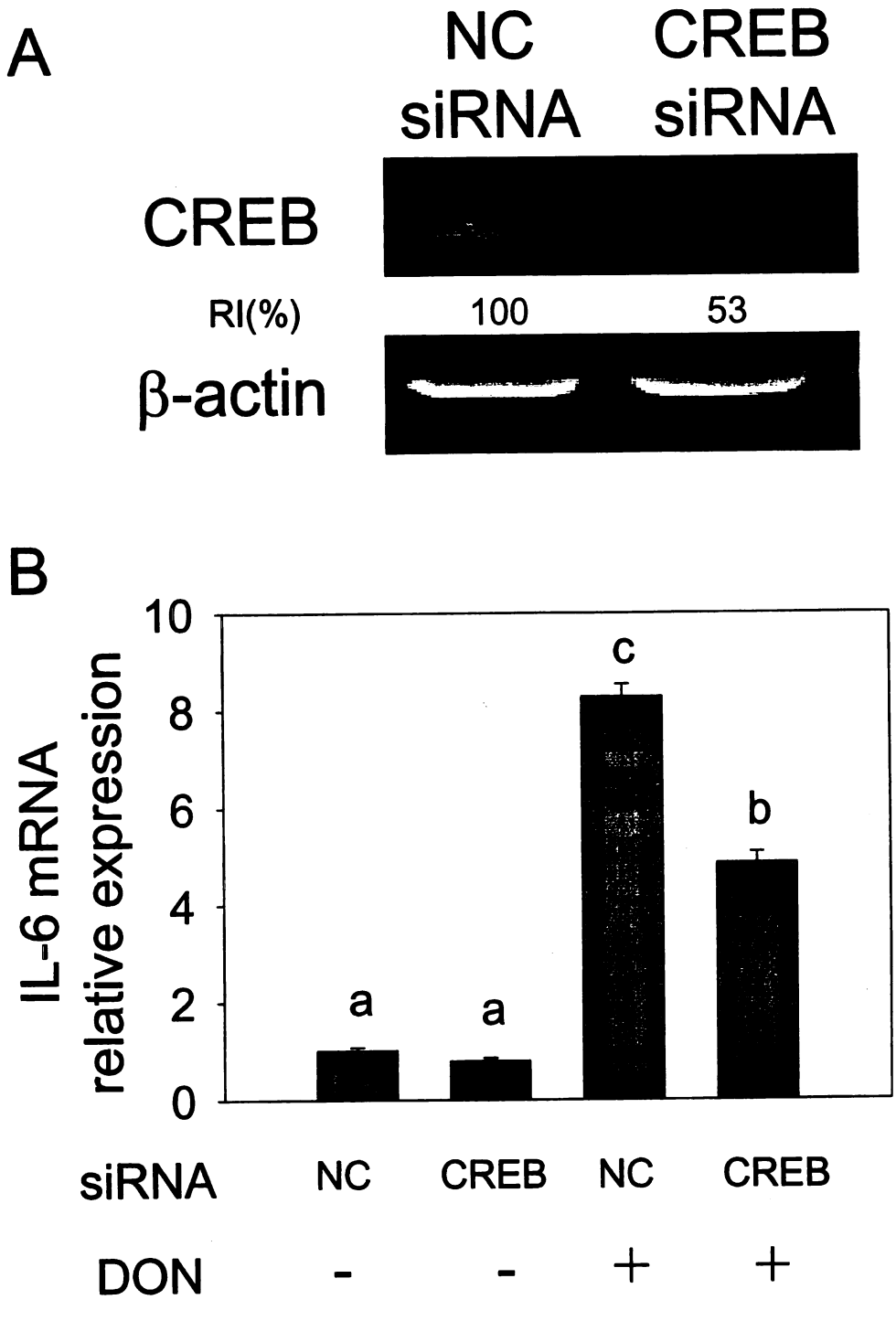


Figure 3.1. Kinetics of DON-induced IL-6 mRNA expression in peritoneal macrophages. Naïve peritoneal macrophages were cultured with DON (250 ng/ml) for different time periods. Total RNA was extracted and IL-6 mRNA was analyzed by real-time PCR. Data are means ± SEM. Data points with different letters differ ($p<0.05$). All data were normalized against b2-microglobulin and expressed relative to the value at time 0. Results are representative of two independent experiments.

Figure 3.2. Transcription factor CREB knockdown inhibits IL-6 mRNA expression induced by DON. siRNA specific to CREB or scrambled siRNA (NC) was transfected by electroporation into naïve peritoneal macrophages. (A) To evaluate CREB knockdown efficiency, total protein was collected after 48 h and CREB measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) To detect the role of CREB on IL-6 mRNA expression, cells were treated with DON 48 h after transfection. Total RNA was collected and IL-6 mRNA was analyzed by real-time PCR. Data are means $\pm$ SEM. Bars with different letters differ ($p<0.05$). Results are representative of two independent experiments.


A
NC siRNA
CREB siRNA
CREB
RI(%)
100
53
β-actin
B
IL-6 mRNA relative expression
10
8
6
4
2
0
a
a
c
b
siRNA
NC
CREB
NC
CREB
DON
-
-
+
+

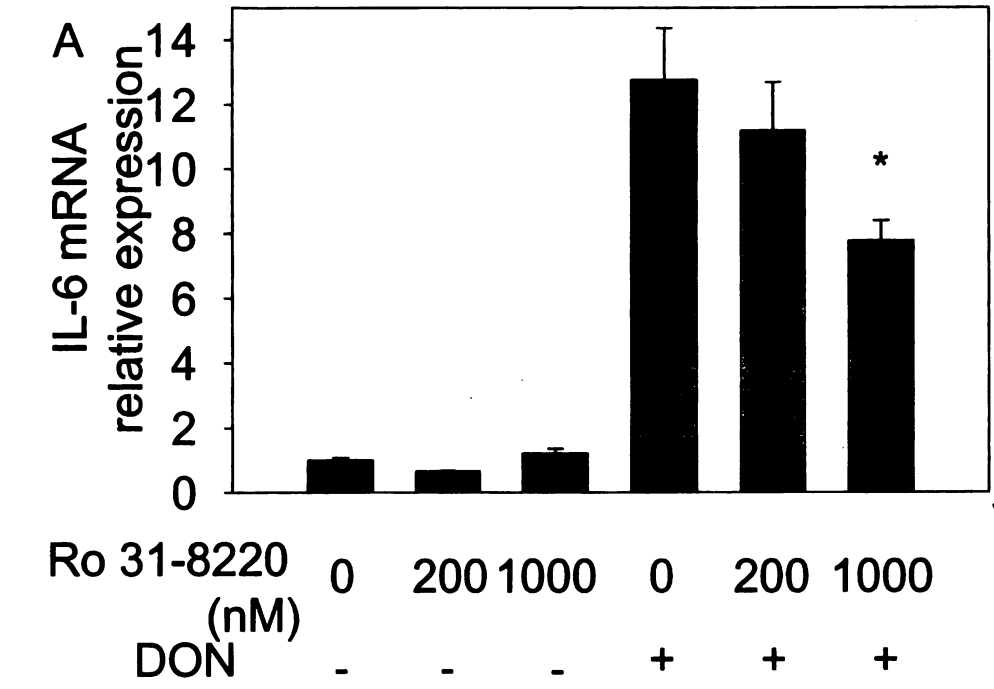
inhibitor has no activity against PH domain-lacking Akts, or other closely related AGC family kinases, PKA, PKC, and SG Incubation of naïve macrophages with each of these inhibitors for 1 h prior toxin treatment, suppressed DON-induced IL-6 mRNA expression (Figure 3.3B-D) suggesting the involvement of Akt 1 and 2.

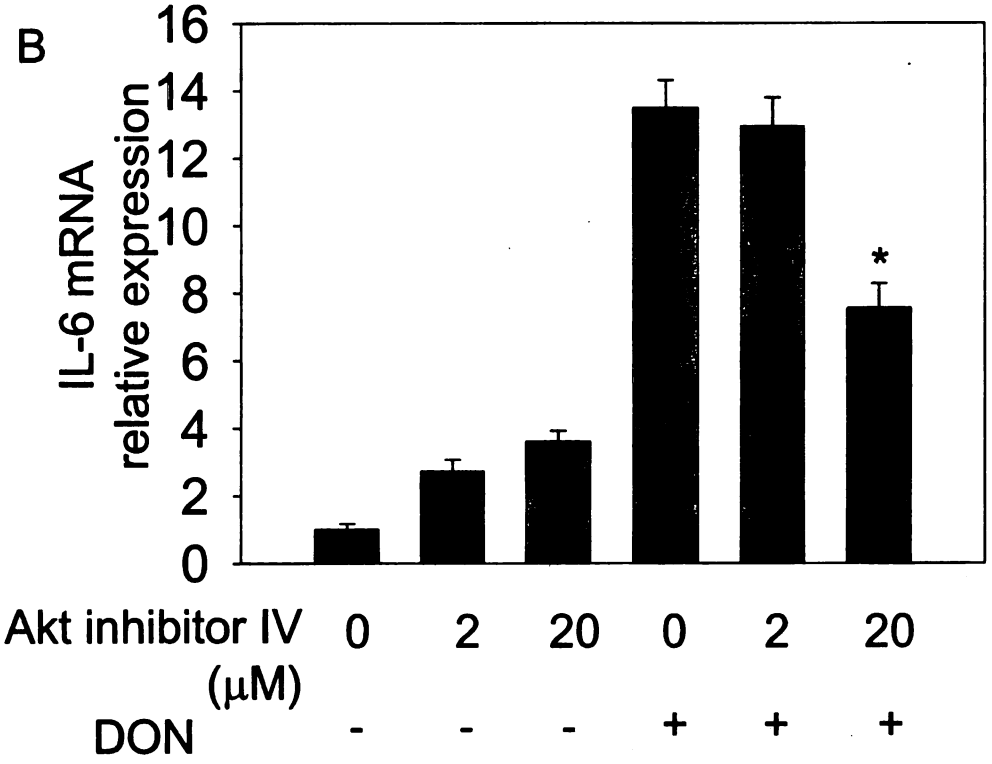
Double-stranded RNA-activated protein kinase (PKR) has been previously shown to be a critical upstream mediator of DON-induced ribotoxic stress response (Zhou *et al.* 2003b) (Yang *et al.* 2000). Incubation with a specific PKR inhibitor was found to markedly inhibit DON-induced IL-6 mRNA expression (Figure 3.4A). Suppressed IL-6 expression appeared to correlate with impaired phosphorylation of CREB, Akt1, RSK1 and to a lesser extent, MSK1 by PKR inhibitor (Figure 3.4B).

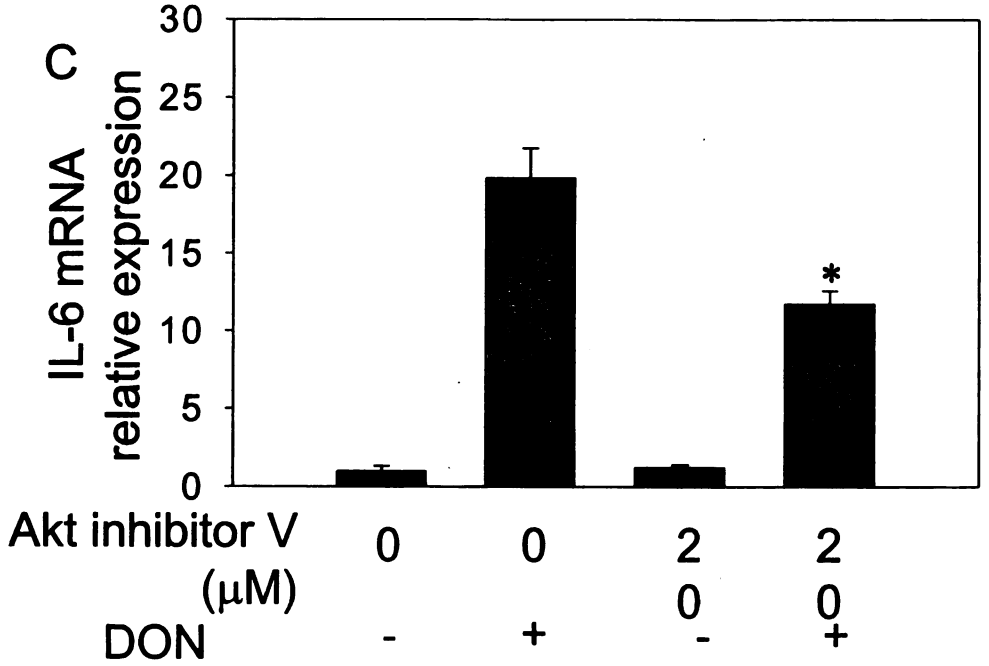
Peritoneal macrophages from mice fed control or DHA diet were compared relative to their ability to phosphorylate CREB kinases and CREB following DON exposure. DON induced Akt1, MSK1 and RSK1 phosphorylation as early as 1 min after treatment and these effects were maximal between 5 to 15 min (Figure 3.5A, B). CREB phosphorylation was maximal at 30 min but decreased dramatically after 60 min. DHA consumption suppressed DON-induced phosphorylation of CREB kinases and CREB at most of these time points.

The capacity of DHA feeding to modify DON-induced CREB kinase activity was assessed in peritoneal macrophages using Akt1 as a model. Specifically, immunoprecipitated Akt1 was pulled down from extract of DON-treated macrophages from mice fed DHA or control diets and then assessed for its ability to phosphorylate CREB (Figure 3.6). CREB kinase activity was highest at 30 min after DON treatment.

Figure 3.3. Inhibition of CREB kinases suppresses DON-induced IL-6 expression. Naïve peritoneal macrophages were cultured for 1 h with CREB kinase inhibitors dissolved in DMSO or the DMSO vehicle, incubated with DON (250 ng/ml) for 3 h and then IL-6 mRNA measured by real-time PCR.. Compounds used were (A) MSK1/RSK1 inhibitor Ro 31-8220, (B) Akt inhibitor IV, (C) Akt inhibitor V and (D) Akt inhibitor VIII. Data are means $\pm$ SEM. Bar with asterisk differs from that of DON treatment without inhibitor ($p<0.05$). Results are representative of at least two independent experiments.


A
IL-6 mRNA relative expression
0
2
4
6
8
10
12
14
*
Ro 31-8220 (nM)
0 200 1000 0 200 1000
DON
- - - + + +


B
IL-6 mRNA relative expression
0
2
4
6
8
10
12
14
16
*
Akt inhibitor IV (μM)
0 2 20 0 2 20
DON
- - - + + +



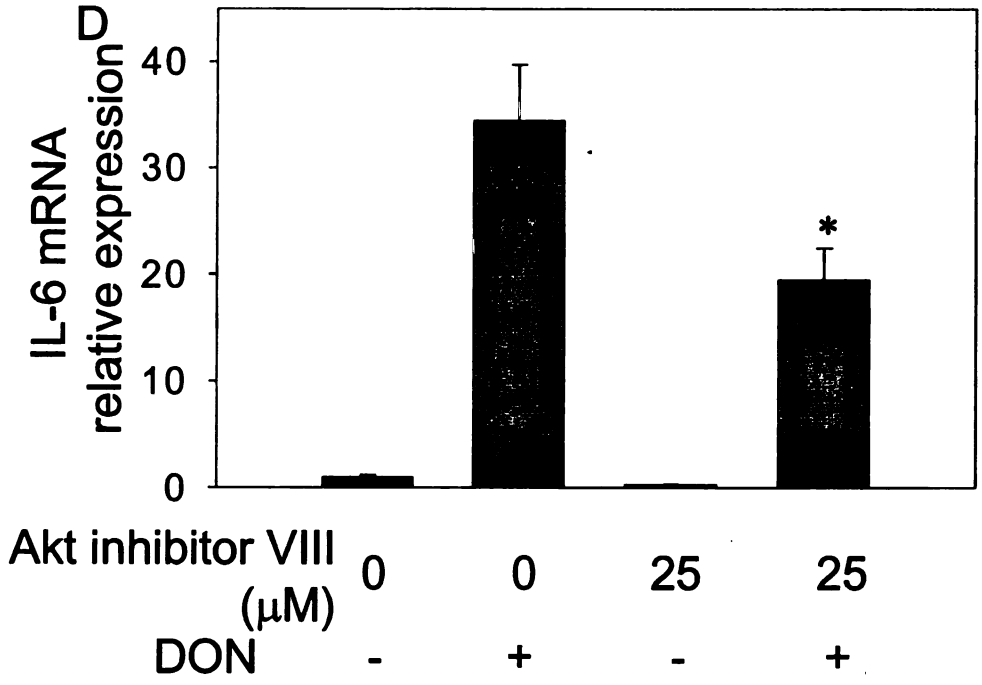


Figure 3.3 continued.

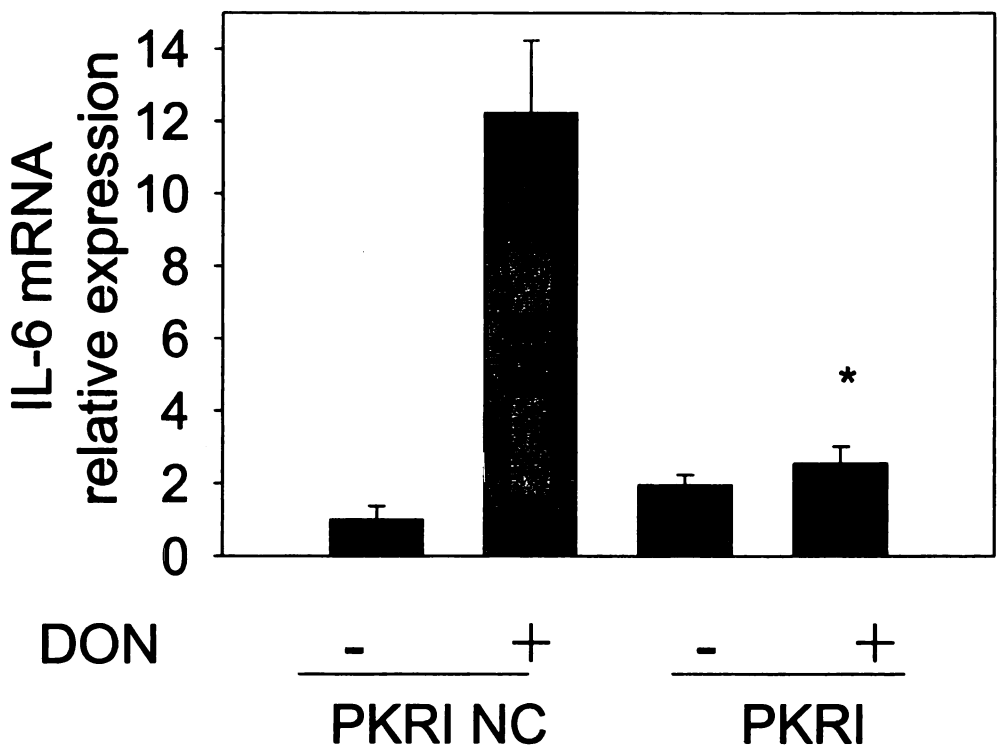
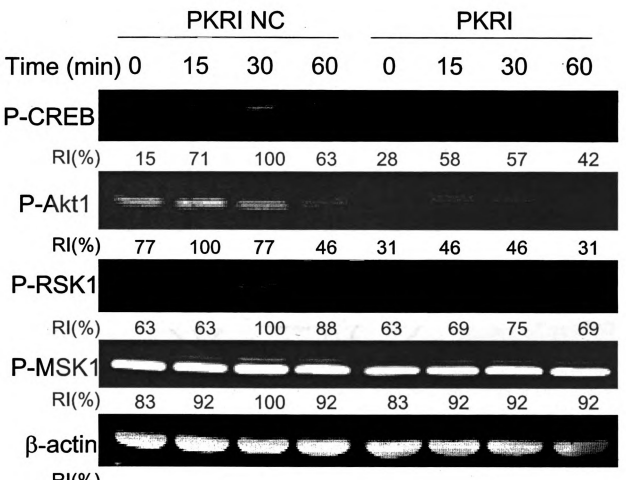


Figure 3.4 A. PKR inhibition blocks DON-induced IL-6 expression. Naïve peritoneal macrophages were cultured for 45 min with PKR inhibitor (PKRI) or PKR inhibitor negative control (PKRI NC) and then with DON 250 ng/ml for 3 h. IL-6 mRNA was measured by real-time PCR. Data are means ± SEM. Bar with asterisk differs from that of DON treatment without inhibitor ($p<0.05$).

Figure 3.4B. PKR inhibition blocks DON-induced protein phosphorylation. To detect the role of PKR activation in CREB phosphorylation, naïve macrophages were cultured for 45 min with PKR inhibitor (PKRI) or PKR inhibitor negative control (PKRI NC) and then with DON (250 ng/ml) for different periods. Phosphorylation of CREB and its upstream kinases was measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. Results are representative of two independent experiments.


PKRI NC
PKRI
Time (min) 0 15 30 60 0 15 30 60
P-CREB
RI(%) 15 71 100 63 28 58 57 42
P-Akt1
RI(%) 77 100 77 46 31 46 46 31
P-RSK1
RI(%) 63 63 100 88 63 69 75 69
P-MSK1
RI(%) 83 92 100 92 83 92 92 92
β-actin
RI(%)

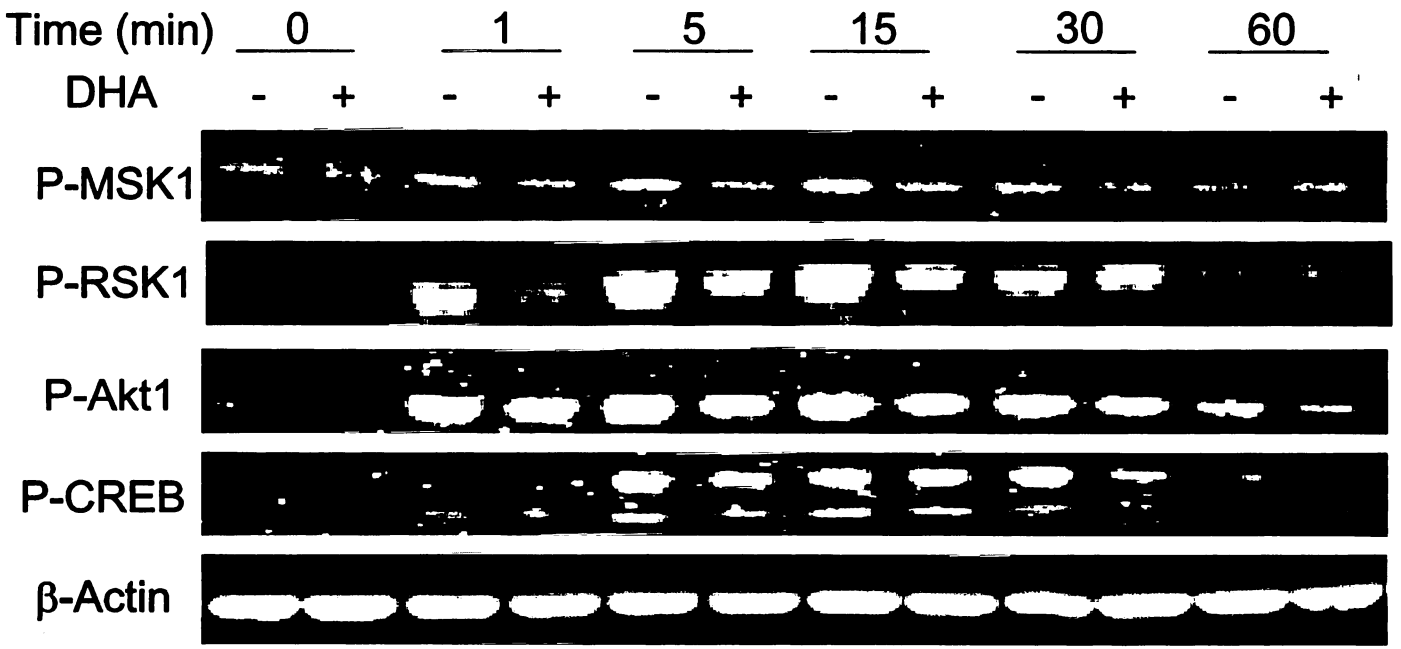


Figure 3.5A. DON-induced phosphorylation of CREB, Akt1, MSK1 and RSK1 is suppressed in macrophages from DHA-fed mice. Peritoneal macrophages from mice fed control or DHA diet were incubated with DON (250 ng/ml) for indicated time periods. Cell lysates were subjected to Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

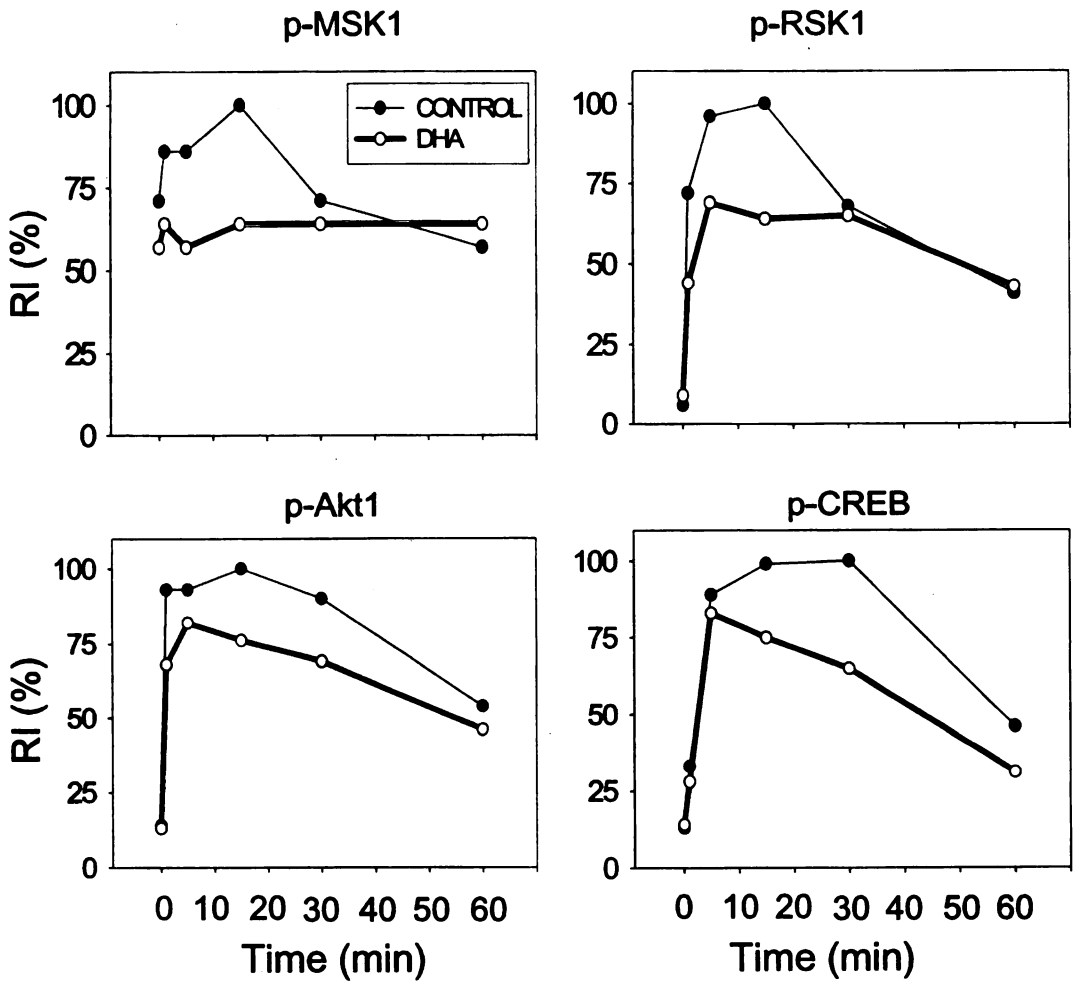


Figure 3.5 B. Plots of figure 3.5A.

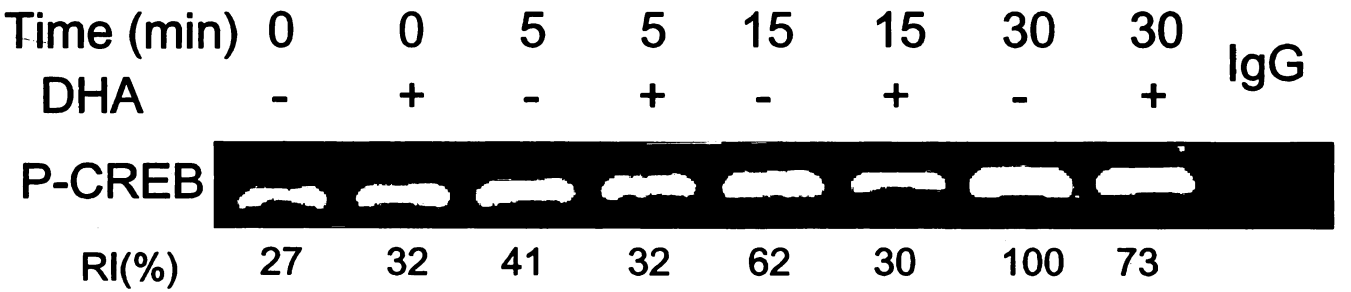


Figure 3.6. DON-induced Akt1activation is suppressed in macrophages from DHA-fed mice. Peritoneal macrophages from mice fed control or DHA diet for were incubated with DON (250 ng/ml) for indicated time points. Following cell lysis, Akt1 was immunoprecipitated and incubated with CREB and ATP. Phosphorylation of CREB was analyzed by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

DHA consumption suppressed Akt1 kinase activity at all the time points.

Peritoneal macrophages from mice fed control or DHA diet were treated with DON for 0, 15, 30 or 60 min and PKR phosphorylation measured. DON treatment moderately upregulated PKR phosphorylation at 15 and 30 min (Figure 3.7). In contrast, PKR phosphorylation appeared to be suppressed in macrophages from DHA-fed mice at the initiation of the experiment as well as at 15 and 30 min.

The possibility exists that DHA consumption inhibits phosphorylation of CREB and CREB kinases by upregulating protein phosphatase activities. Therefore, the activities of PP1 and PP2A were measured. DON treatment slightly induced phosphatase activities in macrophages from mice fed control diet (Figure 3.8A). However, both phosphatase activities in macrophages from DHA-fed mice were decreased as compared to control diet regardless of whether they were treated with DON or not.

To further assess possible roles of phosphatases in DHA-suppressed protein phosphorylation, peritoneal macrophages from mice fed control or DHA diet were incubated with the general protein phosphatase inhibitor calyculin A prior to DON treatment. Calyculin A did not abolish the inhibition of CREB and Akt1 phosphorylation by DHA consumption. (Figure 3.8B)

To test the direct effects of fatty acid treatments on responses of macrophages to DON, naïve peritoneal macrophages were incubated with different fatty acids complexed with BSA. Both DHA and AA increased IL-6 expression compared to monounsaturated fatty acid oleic acid (OA). DON induced IL-6 expression in all three groups according to the rank order: AA>DHA>OA. (Figure 3.9A) When the effects of *in vitro* fatty acid treatment on IL-6 expression were related to protein phosphorylation, fatty acid

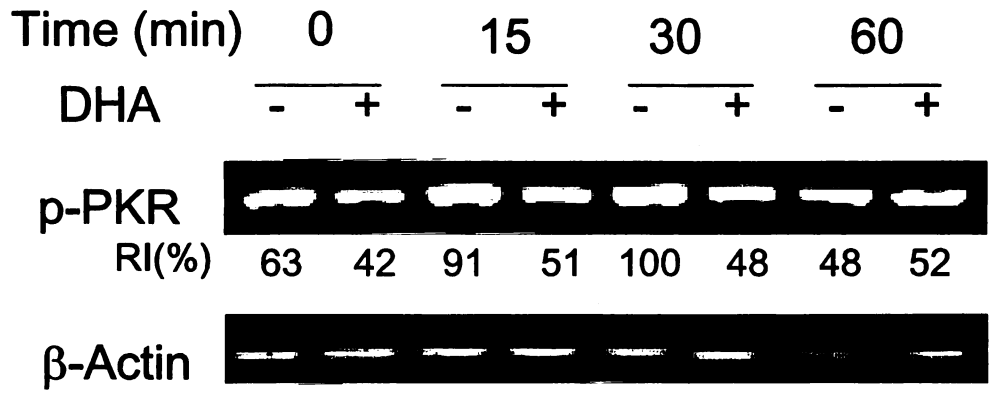


Figure 3.7. Phosphorylation of PKR in the peritoneal macrophage is inhibited by DHA consumption. Peritoneal macrophages from mice fed control or DHA diet were treated with DON (250 ng/ml) for indicated time periods. Cell lysates were subjected to Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

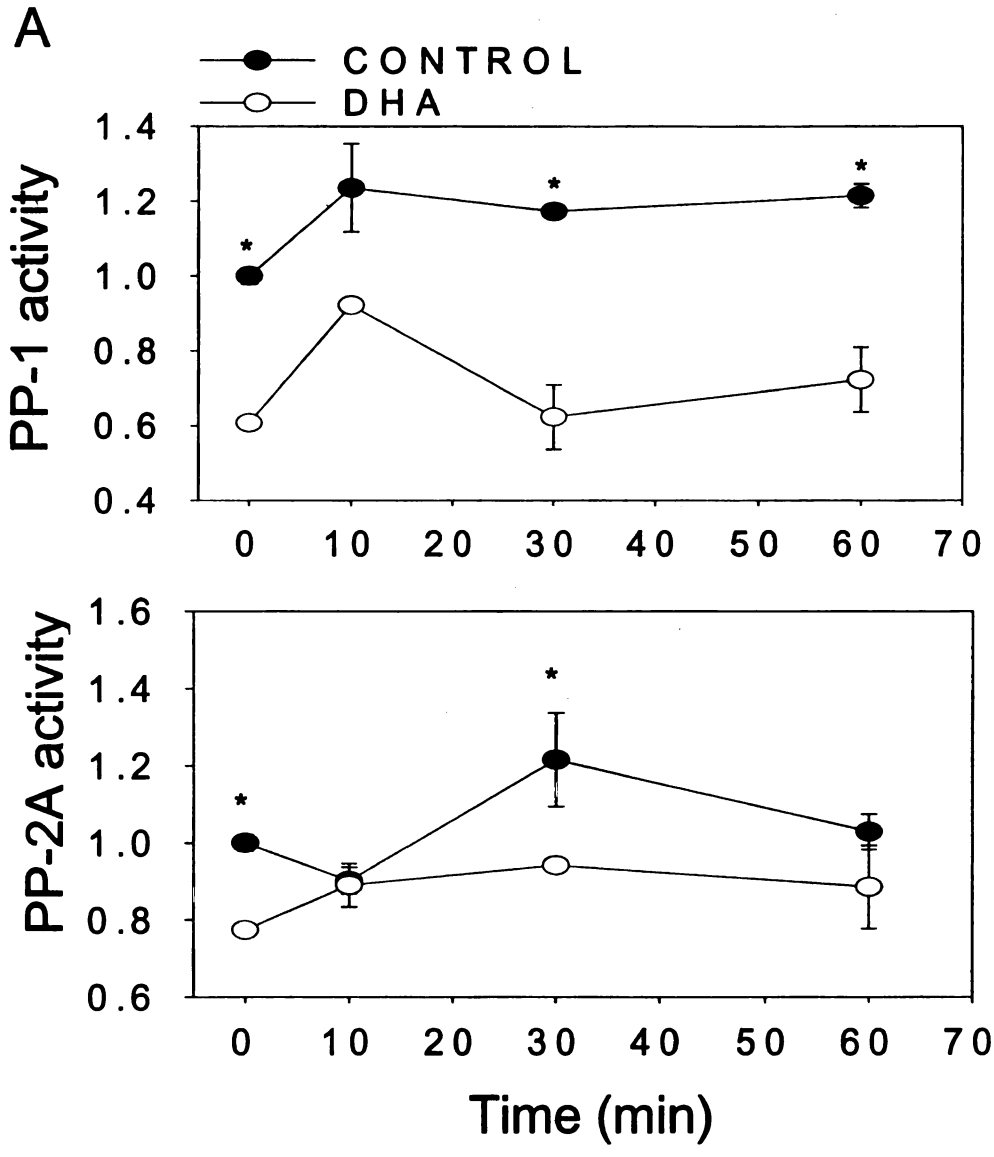


Figure 3.8A. PP1 and PP2A phosphatase activities are not increased in macrophages from DHA-fed mice. Peritoneal macrophage from control- or DHA-fed mice were treated with DON (250 ng/ml) for 0, 10, 30, or 60 min. PP1 and PP2A in the cell lysates were immunoprecipitated and analyzed respectively for phosphatase activities. Values were expressed relative to control at time 0. Data are means ± SEM. Points with asterisk differ from those of corresponding DHA group ($p<0.05$).

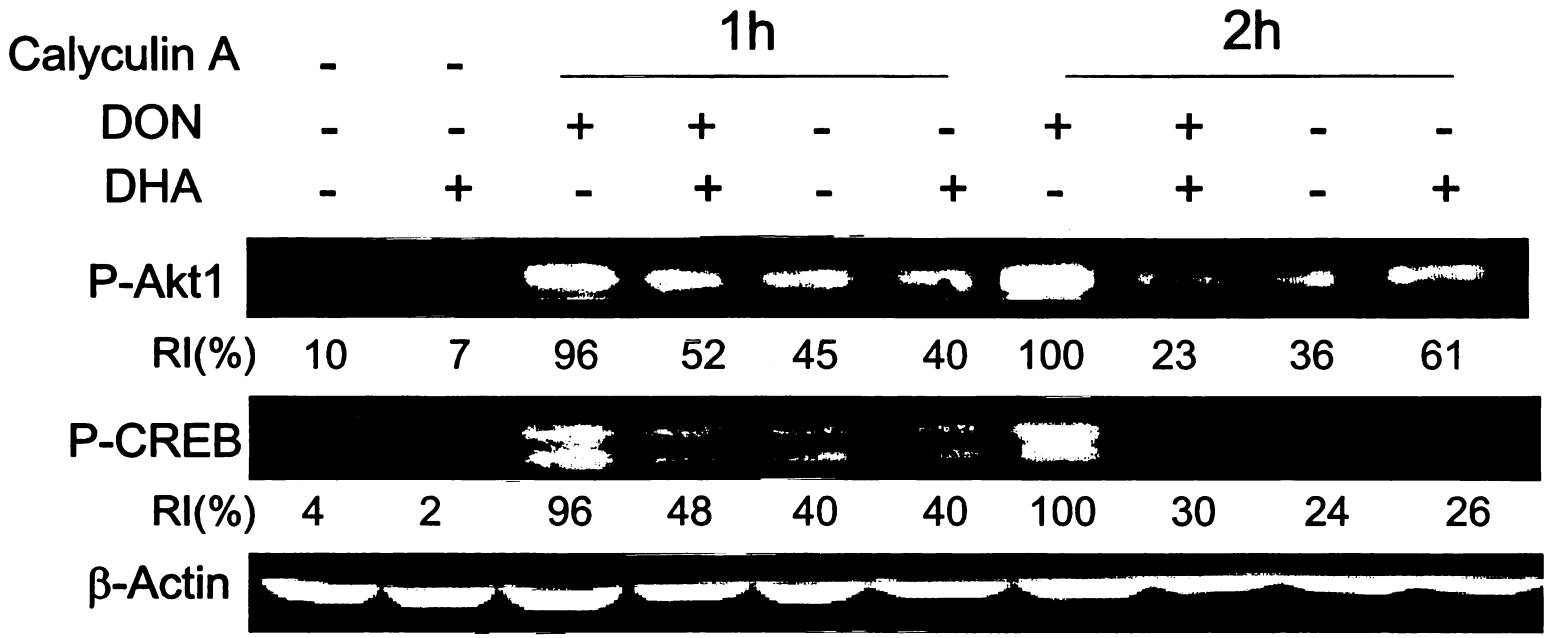


Figure 3.8 B. To evaluate effects of PP1 and PP2A on protein phosphorylation, peritoneal macrophages from mice fed control or DHA diet were pretreated with vehicle or calyculin A (20 nM) for 1 or 2 h and then incubated with DON for 30 min. Cell lysates were subjected to Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

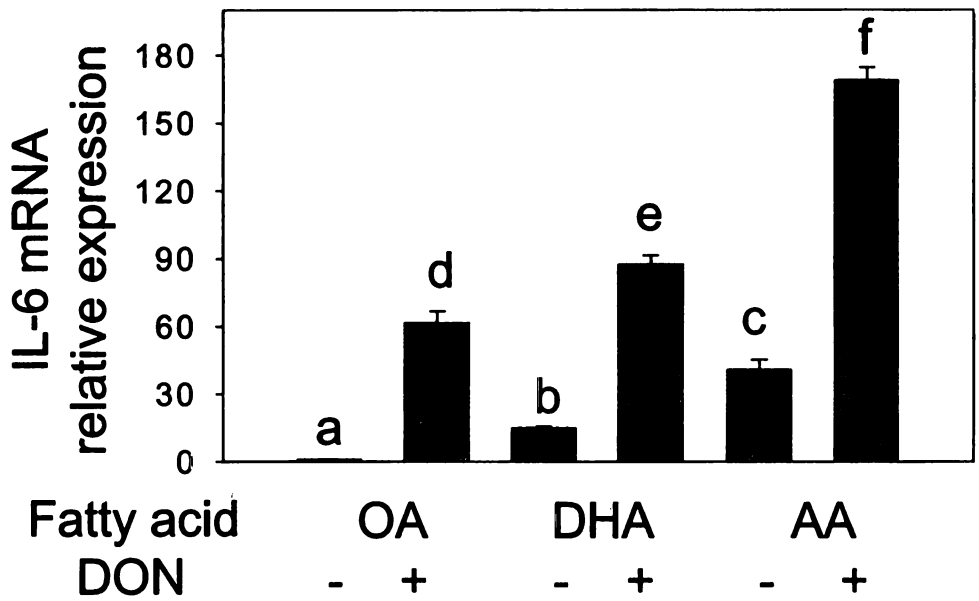


Figure 3.9 A. Fatty acids differentially affect DON-induced IL-6 mRNA expression in peritoneal macrophages. Serum-deprived peritoneal macrophages were treated with 50 μM fatty acid complexed with BSA for 24 h. For IL-6 measurement, RNA was extracted after 3-h DON (250 ng/ml) treatment and analyzed by real-time PCR. Data are means ± SEM. Bars with different letters differ ($p<0.05$).

treatments did not affect phosphorylation of CREB, Akt1, MSK1 or RSK1 induced by DON compared to control group. (Figure 3.9B)

The effects of direct incubation of free fatty acids on Akt1, MSK1 and RSK1 activity was also assessed using CREB as substrate. Both DHA and AA similarly inhibited AKT1 (≥50 μM), RSK1 (≥25 μM) and MSK1 (≥50 μM) activity to a much greater extent than OA. (Figure 3.10)

Figure 3.9 B. Fatty acids do not affect protein phosphorylation in peritoneal macrophages. Serum-deprived peritoneal macrophages were treated with 50 μM fatty acid complexed with BSA for 24 h. To detect protein phosphorylation affected by different fatty acids, cells were incubated with DON for 20 min, and total protein was analyzed by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

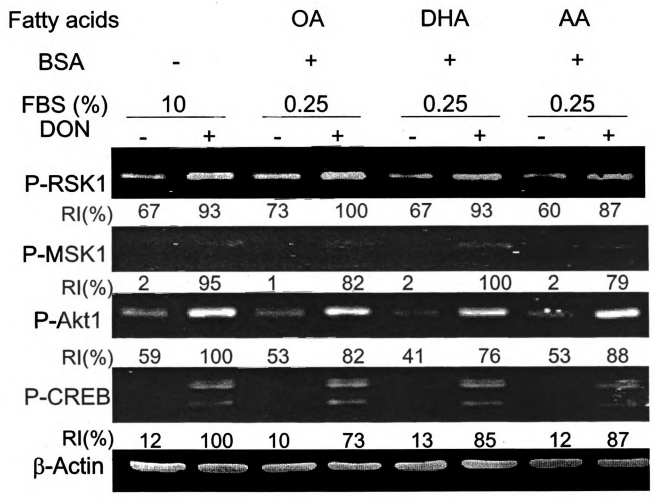
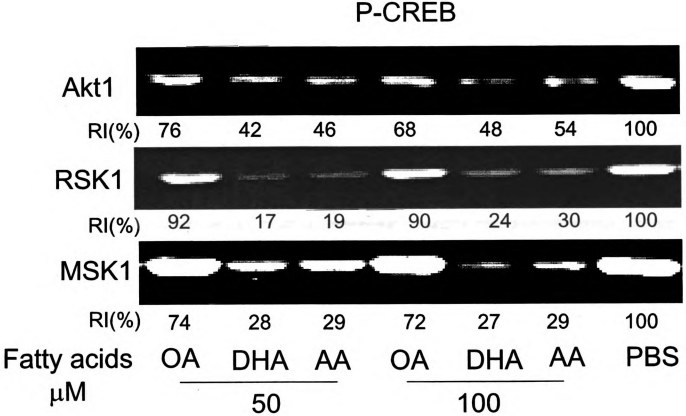

Fatty acids
OA
DHA
AA
BSA
-
+
+
+
FBS (%)
10
0.25
0.25
0.25
DON
- + - + - + - +
P-RSK1
RI(%) 67 93 73 100 67 93 60 87
P-MSK1
RI(%) 2 95 1 82 2 100 2 79
P-Akt1
RI(%) 59 100 53 82 41 76 53 88
P-CREB
RI(%) 12 100 10 73 13 85 12 87
β-Actin

Figure 3.10. AA and DHA similarly decrease CREB kinase activities in the cell-free system. Protein kinases (Akt1, RSK1 and MSK1) were incubated with 12.5, 25, 50 and 100 μM fatty acids or with PBS vehicle before CREB and ATP were added. CREB phosphorylation was analyzed by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence (for PBS vehicle control, far right) in the same row.


P-CREB
Akt1
RI(%) 76 42 46 68 48 54 100
RSK1
RI(%) 92 17 19 90 24 30 100
MSK1
RI(%) 74 28 29 72 27 29 100
Fatty acids OA DHA AA OA DHA AA PBS
μM
50
100

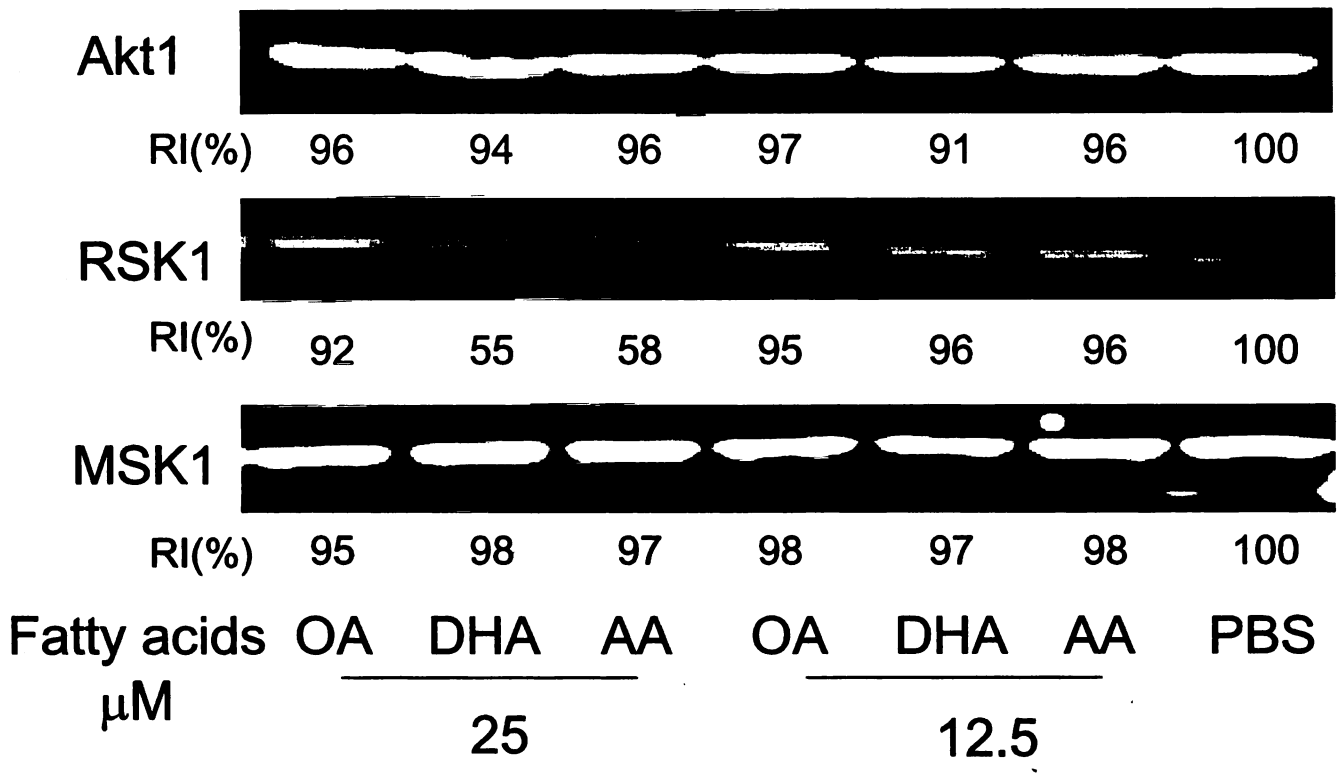


Figure 3.10 continued.

DISCUSSION

Clinical studies suggest that consumption of n-3 PUFAs is efficacious for prophylaxis and treatment of chronic inflammatory diseases that impact millions of people in the U.S. and contribute extensively to morbidity, mortality and health care costs. Our laboratory has focused on the mechanisms by which n-3 PUFAs suppress IgAN, the most common primary glomerulonephritis worldwide, using an experimental mouse model. Consumption of n-3 PUFAs has been determined to attenuate DON-induced IgAN and that correlates with impairment of both systemic IgA hyperproduction and IL-6 gene expression (Jia *et al.* 2004a) (Shi and Pestka 2006) (Jia *et al.* 2004b).

Since IL-6 is a proinflammatory cytokine that plays a role in numerous inflammatory and autoimmune diseases (Gabay 2006) (Ishihara and Hirano 2002), the capacity of n-3 PUFAs to reduce its transcription is of fundamental importance. The transcription factor CREB which binds to the promoter region of the IL-6 gene and regulates its expression contains several functional domains. The C-terminal basic domain facilitates DNA binding (conserved sequence: TGACGTCA) and the leucine zipper domain facilitates dimerization with CREB or other members of the CREB family such as cAMP response element modulator (CREM) and activating transcription factor 1 (ATF-1). Most importantly, CREB has a kinase inducible domain (KID) that contains the critical serine-133 amino acid residue. Exposure to DON results in phosphorylation of this residue through the action of Akt1, ribosomal S6 kinase 1 (RSK1) and mitogen /stress-activated protein kinase 1 (MSK1) (Jia *et al.* 2006). Since knockdown of CREB by siRNA and pharmacologic inhibition of CREB kinases suppressed IL-6 expression, we conclude that this transcription factor and its upstream kinases, Akt1, MSK1 and RSK1,

are likely to be critical for DON-induced IL-6 production.

A key question relates to the molecular mechanism by which DON-induced stress upregulates IL-6 expression. The ribotoxic stress response is a mechanism by which a number of translational inhibitors, such as DON, act on cells and induce activation of mitogen-activated protein kinases (MAPK), proinflammatory cytokine production and apoptosis (Zhou *et al.* 2005a). It has been previously shown that PKR is a critical early mediator of DON-induced ribotoxic stress response (Zhou *et al.* 2003b). PKR is a ubiquitously expressed serine/ threonine protein kinase that is activated by double-stranded RNA, interferon, cytokines and stress signals. It is an essential signal transducer and integrator for immune cells to respond to different stresses. Upon activation, PKR inhibits translation initiation by phosphorylating eIF2α which leads to selective protein synthesis inhibition and regulates several signal transduction pathways such as activation of MAPK and NF-κB (Garcia *et al.* 2006) (Taylor *et al.* 2005). The results presented here confirm that, in peritoneal macrophages, PKR is also an essential upstream regulator of DON-induced IL-6 expression and CREB activation. It should be noted that while inhibition of PKR almost completely abolished IL-6 expression, weak CREB activation was still evident. Since DON also activates other transcription factors via PKR such as NF-κB and AP-1 that can contribute to IL-6 expression, suppression of their activation by PKR inhibition might synergistically contribute to IL-6 suppression.

Inhibition of CREB activation by n-3 PUFAs can be caused by decreased CREB kinase activity (Caravatta *et al.* 2008) (Kato *et al.* 2007) (Chepurny *et al.* 2002) (Arthur *et al.* 2004) (Zhang *et al.* 2002). In this study, we compared kinetic changes of protein phosphorylation and kinase activity induced by DON in macrophages from mice fed

control or DHA diet. Phosphorylation of Akt1, MSK1 and RSK1 occurred earlier than that of CREB and all such phosphorylations were suppressed in macrophages from DHA-fed mice. The results presented here suggest that suppression of PKR activation contributed to reduced CREB kinase and CREB phosphorylation.

An alternative explanation for DHA's inhibitory effects is that it interrupts CREB activation by increasing serine/threonine protein phosphatase activities in macrophages. Phosphorylation of serine and/or threonine is important for activation of CREB, Akt1, MSK1 and RSK1. Phosphorylation can be fine-tuned by competing dephosphorylations carried out by protein phosphatases. The primary phosphatases that dephosphorylate these residues are PP1 and PP2A (Alberts *et al.* 1994) (Comerford *et al.* 2006) (Wadzinski *et al.* 1993) (Katsiari *et al.* 2005). PP1 and PP2A consist of multimeric structures including a catalytic subunit complexed to a number of accessory subunits that are able to regulate the activity of the catalytic subunit. Here, activities of the phosphatases were measured rather than protein amounts of catalytic subunits. The results showed that prior DHA consumption decreased both basal and DON-induced PP1 and PP2A activities in peritoneal macrophages, suggesting the n-3 PUFAs do not suppress protein phosphorylation by upregulating phosphatase activities. This conclusion was further supported by studies employing calyculin A, a potent PP1 and PP2A inhibitor, which did not restore the reduced phosphorylation of CREB and Akt1 observed in macrophages from DHA-fed mice.

A further possibility was that DHA suppressed CREB phosphorylation by direct interaction with macrophages. We thus examined the direct effects of fatty acids on IL-6 expression and protein phosphorylation in naïve peritoneal macrophages. The

concentrations of total non-esterified fatty acids (NEFAs) in plasma range from 0.2 to 1.7 mM and the individual concentrations of the major fatty acids can range from 30 to 130μM. Most (>99%) NEFAs bind with albumin to make complexes with the remainder existing as unbound free fatty acid (Lloyd *et al.* 2006) (Calder *et al.* 1990) (Itoh *et al.* 2003) (Richieri and Kleinfeld 1995). Therefore the concentrations of fatty acid-BSA complexes used in our *in vitro* experiments were in a physiological range.

The *in vitro* experiments showed that although macrophages secreted more DON-induced IL-6 following treatment with AA than with OA or DHA, there was no marked difference in DON-induced phosphorylation of CREB or CREB kinases among the three different fatty acid treatments. Thus n-3 PUFA effects *in vitro* did not mimic those seen *ex vivo*. One explanation for these differences might relate to the use of primary macrophages which are central to innate immunity and are crucial for initiating, maintaining and resolving an adaptive immune response. Macrophages are not a homogeneous cell population, but rather encompass different phenotypes, which exhibit a wide range of pro- and anti-inflammatory activities depending on their stage of differentiation and activation. Fatty acid consumption could suppress inflammation by differentially modulating expression of genes related to proinflammatory responses such as colony-stimulating factor-1 (CSF-1) and PU.1, or anti-inflammatory responses such as adenosine A3 receptor, CD1d, and IL-1 receptor II (Ehrchen *et al.* 2006) (Desnues *et al.* 2006) (Hume 2006) (Shi and Simon 2006). It might be speculated that the DHA effects observed herein represent a cumulative change in macrophage phenotypes resulting from subchronic n-3 PUFA consumption.

Another explanation for the differences between ex vivo and in vivo responses is

that DHA is a precursor to some more potent anti-inflammatory mediators such as resolvins and protectins (Ariel and Serhan 2007) (Hong *et al.* 2003). These mediators are produced by epithelial cells, neutrophils and glial cells in intact animals and can have anti-inflammatory effects on macrophages. Their effects might not be immediately detectable in purified macrophage cell culture treated with DHA for a short period. A further possible reason for the difference is that arachidonic acid depletion upon DHA incorporation might result in less PGE_2 production. Since DHA can be incorporated into the cell membrane relatively rapidly, the latter might be a greater factor in suppressing IL-6 expression *in vitro* than *ex vivo* (Calder 2006a).

After cell entry, free fatty acids bind to fatty acid binding proteins, which facilitate their transportation, storage and metabolism (Rolph *et al.* 2006) (Makowski and Hotamisligil 2004). These fatty acids can directly interact with proteins and modulate their activities. It has been reported that unsaturated fatty acids are ligands not only for nuclear (Kliewer *et al.* 1997) (Murakami *et al.* 1999) and membrane receptors (Itoh *et al.* 2003), but also for protein kinases (Lopez-Nicolas *et al.* 2006) (Eitsuka *et al.* 2005). A cell-free system was therefore used to assess direct interactions among three molecules: kinase, substrate and fatty acid. Although AA and DHA inhibited CREB kinase activities at 100 and 50 μM compared to OA, these n-6 and n-3 PUFAs did not differ in the extent of inhibition. It should be further noted that although direct effects of fatty acids on protein kinases were observed, the concentrations employed were relatively high. The total unbound intracellular fatty acids and FA-CoA levels reported previously are lower than 10 μM (Gossett *et al.* 1996) (Jump and Clarke 1999). Since no inhibition of kinase activity by fatty acids was observed at 12.5 μM, the effects of PUFA on Akt1, RSK1 and

MSK1 at high concentrations in peritoneal macrophages might not be physiologically relevant.

In summary, the data presented here suggest that IL-6 expression induced by DON is PKR-dependent and mediated, in part, by the transcription factor CREB. DHA consumption appears to suppress these pathways in macrophages rendering them less capable of CREB activation and thus IL-6 transcription. (Figure 3.11) Suppression of IL-6 expression by DHA might have general importance to human health relative to the prevention and treatment of inflammatory and autoimmune diseases mediated by this proinflammatory cytokine.

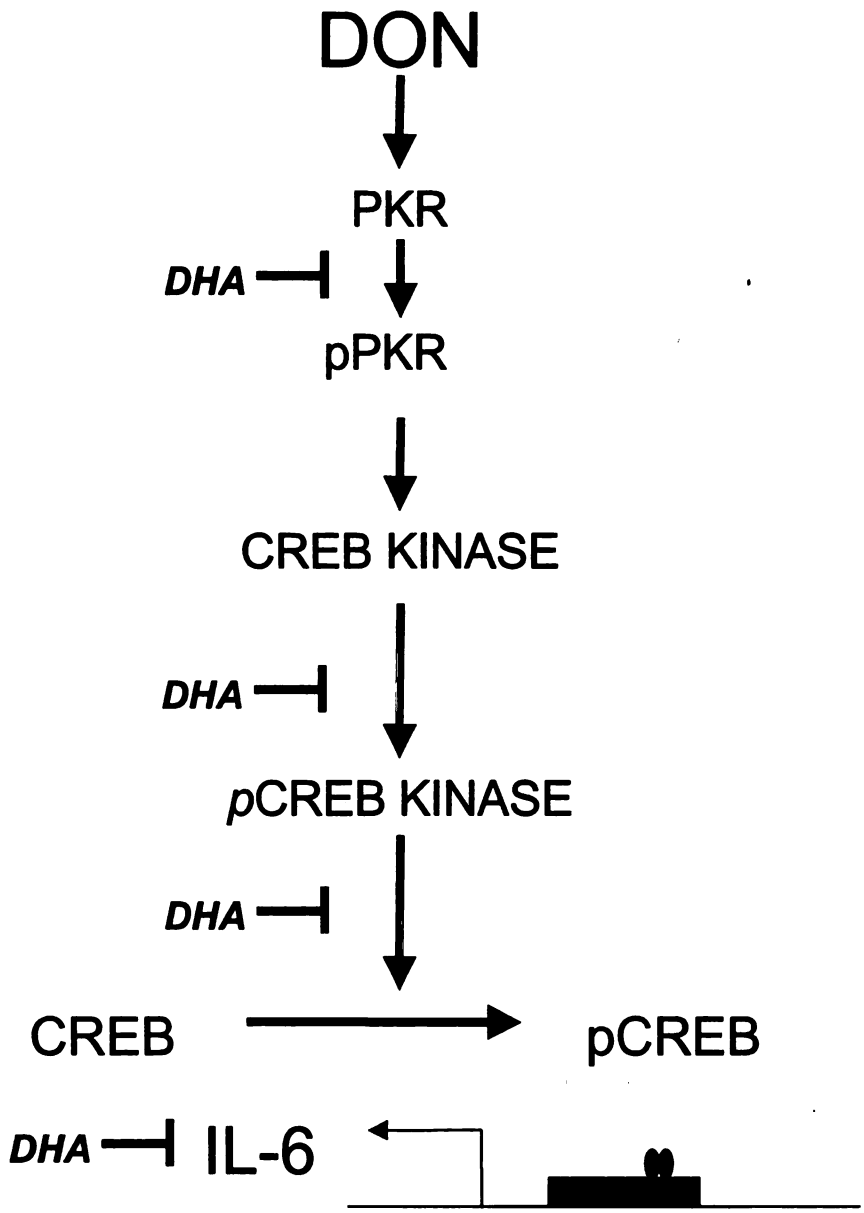


Figure 3.11. Effects of DHA consumption on signal transduction pathways mediating DON-induced IL-6 expression in peritoneal macrophages ex vivo. Possible CREB kinases inhibited by DHA feeding include AKT, RSK1 and MSK1. The symbol ⊣ on the left indicates inhibition of the pathway step on the right.

CHAPTER 4

Role of ER stress in deoxynivalenol-induced interleukin-6 expression in peritoneal macrophages

ABSTRACT

Oral exposure to the trichothecene deoxynivalenol (DON) in mice induces aberrant systemic expression of the proinflammatory cytokine interleukin-6 (IL-6). The purpose of this study was to relate DON-induced IL-6 expression to the endoplasmic reticulum (ER) stress response in mouse peritoneal macrophages. BiP, an ER chaperone, was markedly decreased upon incubation with DON (500 ng/ml) for 1 h. As little as 100 ng/ml of DON was found to decrease Bip within 6 h. In contrast, BiP mRNA was not affected by DON suggesting BiP loss resulted from protein degradation. DON-induced BiP degradation was suppressed by cathepsin/calpain inhibitors. DON was also found to increase protein expression of ER stress sensor the inositol requiring kinase 1α (IRE1α) and two transcription factors, X-box binding protein (XBP1) and activating transcription factor 6 (ATF6), as well as XBP1 mRNA splicing. Knockdown of ATF6 with siRNA partially decreased DON-induced IL-6 expression in peritoneal macrophages; while knockdown of BiP induced IL-6 gene expression directly. These data suggest that DON exposure induces BiP degradation and evokes an ER stress-like response that is likely to contribute in part to DON-induced IL-6 gene expression.

INTRODUCTION

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by *Fusarium spp.* that is prevalent worldwide in cereal-based foods (Rotter *et al.* 1996) (Pestka and Smolinski 2005). Chronic exposure generates concern about DON. By selectively promoting polyclonal activation and expansion of immunoglobulin A (IgA)-secreting B cells, dietary exposure to DON causes a dramatic elevation in serum IgA, serum IgA immune complexes (IC) and IgA deposition in the mouse kidney, which mimic the early stages of human IgA nephropathy (IgAN) (Pestka *et al.* 1989) (Dong *et al.* 1991) (Rasooly and Pestka 1994). Our laboratory has observed that DON-induced interleukin-6 (IL-6) upregulation plays a critical role in this mouse model of IgAN (Pestka and Zhou 2000) (Yan *et al.* 1997) (Yan *et al.* 1998).

Membrane and secretory proteins synthesized in the endoplasmic reticulum (ER) must be folded properly with the assistance of chaperones and folding enzymes (Yoshida 2007). Under conditions of Cytotoxicity or nutrient starvation, unfolded or misfolded proteins can accumulate and cause ER stress (Ma and Hendershot 2001). As a results, cells can activate a series of self-defense mechanisms referred to as the "ER stress response" or "unfolded protein response" (UPR) (Zhang and Kaufman 2006).

ER stress is involved in several human diseases including neurodegenerative diseases, diabetes mellitus, heart diseases, kidney diseases and inflammation (Yoshida 2007). It has been shown that proinflammatory cytokines (Oliver *et al.* 2005) (Nowis *et al.* 2007) and lipopolysaccharide (Endo *et al.* 2006) (Endo *et al.* 2005) induce ER stress leading to expression of acute response proteins. ER stress is also related to some autoimmune diseases, such as rheumatoid arthritis (Purcell *et al.* 2003), autoimmune

myositis (Nagaraju *et al.* 2005) and collagen-induced arthritis (Gao *et al.* 2008).

BiP (immunoglobulin binding protein, also known as glucose-regulated protein 78/GRP 78) is the one of the most characterized ER chaperones. BiP serves as a master regulator in ER stress response and plays a key role in activating ER stress effectors that consist of activating transcription factor 6 (ATF6), the inositol requiring kinase 1α (IRE1 α), and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK). IRE1α can activated transcription factor X-box binding protein (XBP1) (Zhang and Kaufman 2006) (Ni and Lee 2007) (Schroder and Kaufman 2005).

We have previously shown that the transcription factor cAMP response element-binding protein (CREB) promotes IL-6 mRNA transcription by binding to cAMP response element (CRE) (Jia *et al.* 2006) (Shi and Pestka 2006). Both ATF6 and XBP1 are transcription factors belonging to CREB/ATF family and could regulate gene expression by binding to CRE (Hai and Hartman 2001) (Kanemoto *et al.* 2005) (Schroder and Kaufman 2005). Treatment to the EL-4 thymoma cell line with DON decreases BiP mRNA and protein (Yang *et al.* 2000). The downregulation of BiP could adversely affect protein folding and modification, thus lead to the ER stress-like response, which could predominantly activate XBP1 and ATF6. XBP1 and ATF6 might thus play important roles in the DON-induced upregulation of interleukin-6.

The purpose of our research was to test the hypothesis that BiP dysregulation was a modulator in DON-induced IL-6 upregulation in the macrophage.

MATERIALS AND METHODS

Materials. All chemicals including DON and cell culture components were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) unless otherwise noted. DON contaminated labware and cell culture media were detoxified by sodium hypochlorite. All inhibitors were purchased from Calbiochem, Inc. (San Diego, CA).

Animals and diet. Female B6C3F1 mice (7-wk old) weighing around 25 g were obtained from Charles River Laboratories, Inc (Wilmington, MA). Housing, handling, and sample collection procedures conformed to the policies of the Michigan State University All-University Committee on Animal Use and Care in accordance with NIH guidelines. Mice were provided free access to food and water.

Peritoneal macrophage cultures. Mice were injected ip with 1.5 ml of sterile 3% (w/v) thioglycollate broth. After 3 d, mice were euthanized and macrophages collected by peritoneal lavage with ice-cold Hank's BSS (Invitrogen Corporation, Carlsbad, CA). Cells were pelleted by centrifugation at 1,100 × g for 5 min, washed with BSS once and resuspended in RPMI-1640 containing 10% (v/v) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin. These cells were cultured at 37°C under 6% CO_2 in a humidified incubator for 24 h before treatment.

Macrophages were incubated with or without DON for various time periods and analyzed for mRNA expression by real-time PCR or protein amount by Western blot analysis. DON was dissolved in PBS first to make a 500 μg/ml stock solution and then added to cell culture media to generate different working concentration. Two milliliters of

cell suspension (1×10^6 /ml) were incubated in each well of 6-well cell culture plates (Corning Life Sciences, Lowell, MA) for experiments requiring RNA isolation. For protein collection, 10 ml cell suspensions (1×10^6 /ml) were incubated in 100 mm-diameter cell culture dishes (Corning Life Sciences).

Western blot analysis. For protein detection studies, macrophages were lysed in Tris buffer (10 mM, pH 7.4) containing 2% (w/v) SDS, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), heated to 100 °C for 5 min and sonicated. After centrifugation at 18,000 × g for 15 min, supernatants were subjected to Western analysis using specific antibodies to BiP or IRE1α (rabbit anti-mouse antibody, Cell Signaling Technology, Inc., Danvers, MA), XBP1, ATF6 (rabbit anti-mouse antibody, Santa Cruz Biotechnology, Inc.) and β-actin (mouse anti-mouse antibody, Sigma-Aldrich). Alexa Fluor 680 goat-anti rabbit and IRDye® 800 goat-anti mouse secondary antibodies were purchased from Invitrogen Corporation and Rockland Immunochemicals, Inc. (Gilbertsville, PA) respectively. Infrared fluorescence was directly detected by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Real-time PCR. Total RNA of peritoneal macrophages was extracted using RNeasy Mini (Promega, Madison, WI) and analyzed by real-time PCR for mRNA expression. TaqMan primers and probes for IL-6 mRNA were purchased from Applied Biosystems (Foster City, CA). The primer sequence for unspliced (u) and spliced XBP1 (s) were designed as follows: (forward) 5'- tgg ccg ggt ctg ctg agt ccg-3' (u), 5'-ctg agt ccg cag cag gtg cag -3' (s); (reverse) 5'-gtc cat ggg aag atg ttc tgg-3' (u and s). SYBER

Green PCR Master Mix (Applied Biosystems) was used for real time PCR to detect unspliced and spliced XBP1 mRNA. Beta-2 microglobulin RNA expression was not affected by DON treatment and thus was used as endogenous control to normalize target gene expression. Target gene expression levels were calculated relative to the control group.

siRNA transfection. siRNA cocktail targeting mouse BiP, XBP1, ATF6 or a comparable scrambled siRNA were purchased from Dharmacon (Lafayette, CO). siRNA transfection was performed by electroporation using an Amaxa Nucleofector (Amaxa Inc., Gaithersburg, MD). Briefly, 2×10^6 cells were suspended in 100 μl electroporation buffer (Amaxa Inc.) and mixed with 10 μM siRNA. Electroporation was performed using program D032 for macrophages according to the manufacturer's protocol. Transfection efficacy was verified by assessing loss of BiP, XBP1 or ATF6 protein by Western blot 48 h after transfection. IL-6 expression induced by DON after transfection was analyzed by real-time PCR.

Statistics. All data were analyzed with SigmaStat v 3.1 (Jandel Scientific, San Rafael, CA) with the criterion for significance set at $p<0.05$. Student's t-test was used for comparison of two groups of data; and one-way ANOVA was performed for comparison of multiple groups. Holm-Sidak (if normality test passed) or Dunn (ANOVA on ranks if normality test failed) tests were used as post-hoc analysis.

RESULTS

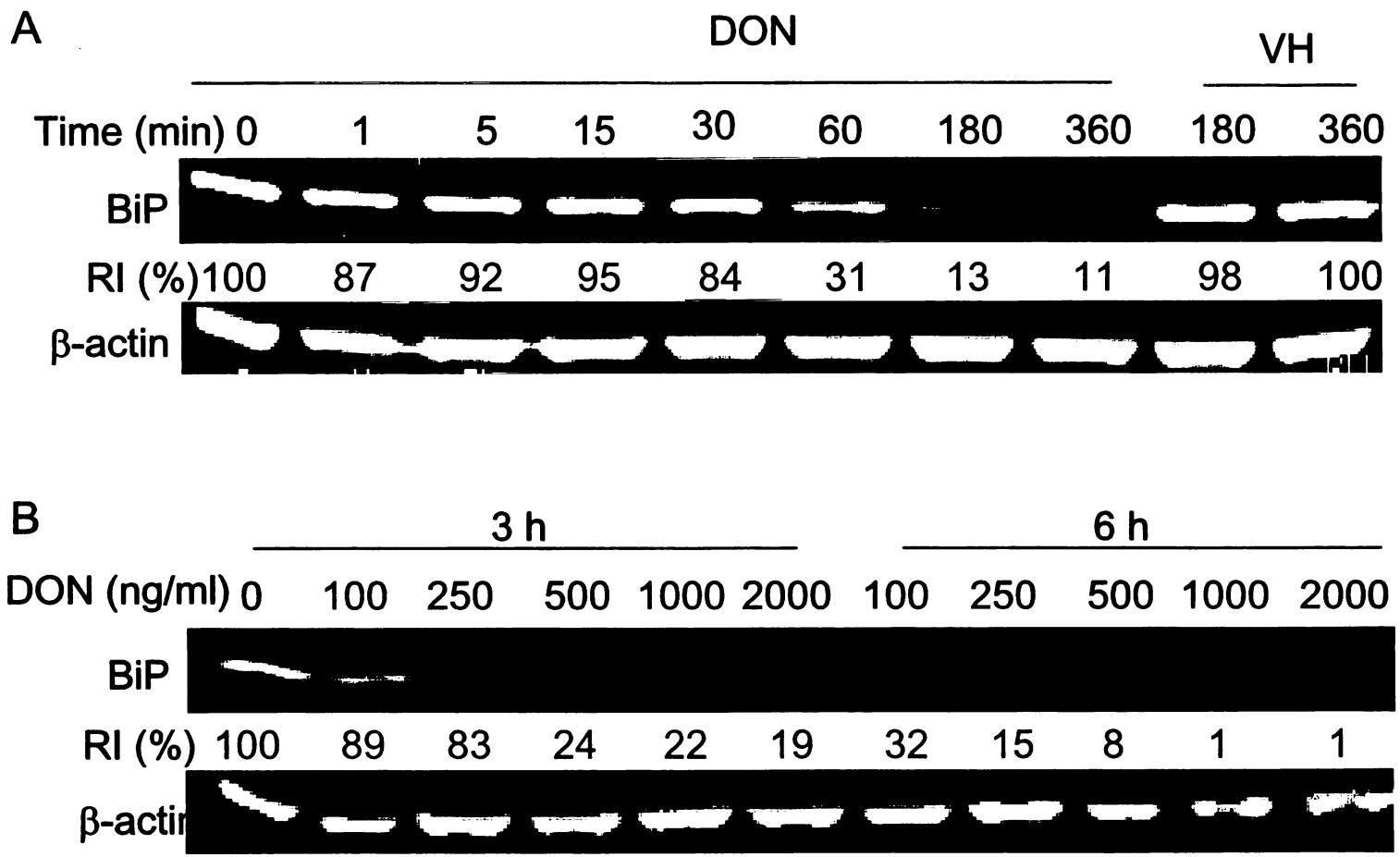
The effects of incubating peritoneal macrophages with DON (500 ng/ml) on BiP protein was assessed (Figure 4.1A). As compared with vehicle treatment, BiP was markedly decreased with 60-min DON treatment and no longer detectable after 6 h. The effects of different DON concentrations on BiP degradation were determined (Figure 4.1B). As little as 100 ng/ml DON decreased BiP protein dramatically at 3- and 6-h treatment, DON at 500 ng/ml caused marked reduction of BiP after 3 h. Based on these data, a concentration of 500 ng/ml was used in the following studies.

To determine if the DON-induced decrease in BiP was due to downregulated BiP gene expression, the effects of DON on BiP protein and mRNA in peritoneal macrophages were compared (Figure 4.2). BiP mRNA was not affected upon 12-h incubation whereas BiP protein was undetectable at 6 and 12 h. From these data we concluded that decreased BiP protein amount was not related to BiP gene expression.

Specific inhibitors were used to ascertain whether DON-induced BiP degradation is proteasome-dependent (Figure 4.3A). There was no inhibition by the specific proteasome inhibitor epoxomicin. However, DON-induced BiP degradation was inhibited by the inhibitor ALLN which inhibits the activities of both cathepsins and calpains. Cathepsins and calpains inhibitors were used to confirm these results (Figure 4.3B). Both cathepsin inhibitor I (CATI-I) and calpain inhibitor III (CALI-III) inhibited DON-induced BiP degradation.

To detect if DON-induced degradation of the ER chaperone BiP coincided with ER stress-like response, treated macrophages were analyzed for changes in inositol

Figure 4.1. Kinetics of DON-induced BiP decrease in peritoneal macrophages. (A) Time-course response of DON-induced BiP decrease. Peritoneal macrophages were cultured with DON (500 ng/ml) for different time periods. Total protein was extracted and BiP was analyzed by Western Blot. (B) Dose-course response of DON-induced BiP degradation. Peritoneal macrophages were cultured with DON (0, 100, 250, 500, 1000 or 2000 ng/ml) for 3 or 6 h. Total protein was extracted and BiP was analyzed by Western Blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.


A
DON
VH
Time (min) 0 1 5 15 30 60 180 360 180 360
BiP
RI (%) 100 87 92 95 84 31 13 11 98 100
β-actin
B
3 h
6 h
DON (ng/ml) 0 100 250 500 1000 2000 100 250 500 1000 2000
BiP
RI (%) 100 89 83 24 22 19 32 15 8 1 1
β-actin

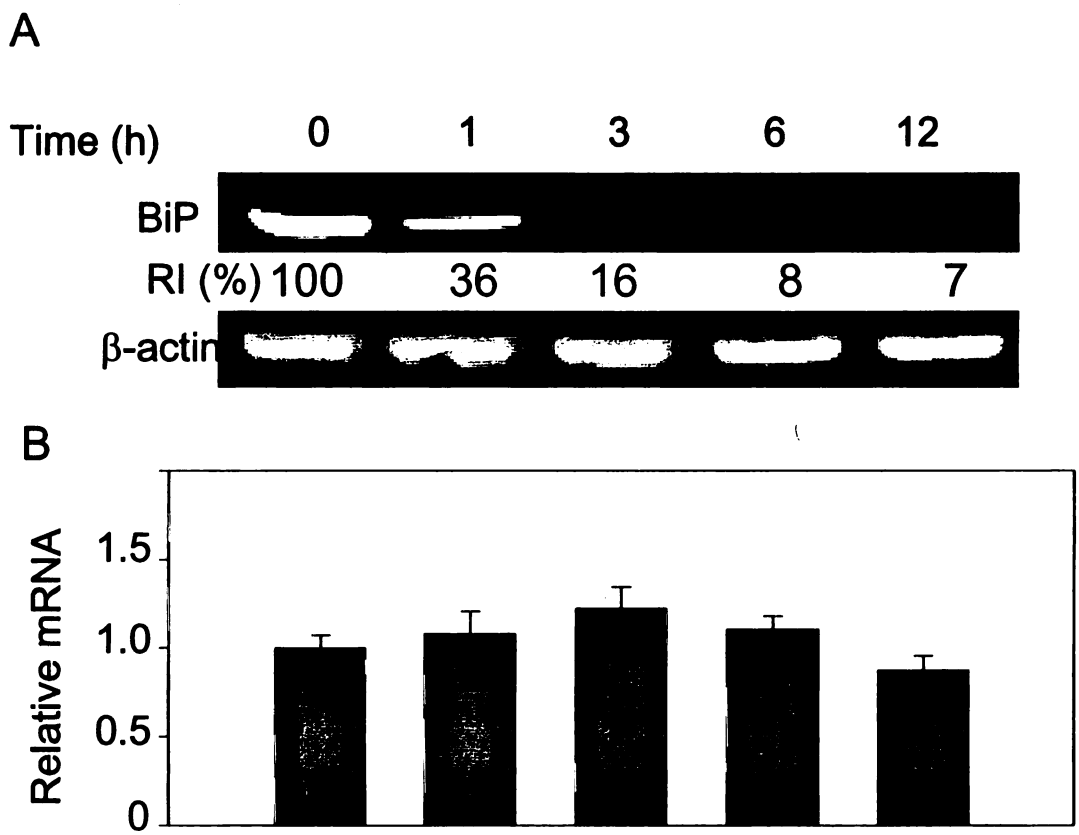
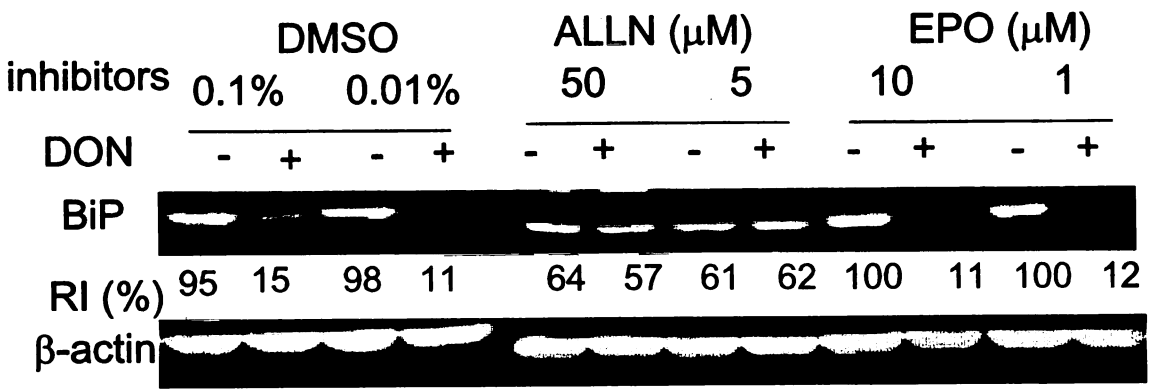


Figure 4.2. DON treatment does not change BiP gene expression in peritoneal macrophages. Peritoneal macrophages were treated with DON (500ng/ml) for 0, 1, 3, 6, or 12 h. BiP protein was detected by Western Blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. Total RNA was collected and BiP mRNA was analyzed by real-time PCR. Data are means ± SEM.

Figure 4.3. DON-induced BiP degradation is cathepsin/calpain-dependent. (A) Peritoneal macrophages were incubated with vehicle (DMSO), ALLN or epoxomicin (EPO) for 1 h, and DON (500ng/ml) was added to cell culture media. After 3 h, total protein was extracted and BiP was analyzed by Western Blot. (B) Peritoneal macrophages were incubated with inhibitors to cathepsins (CATI-I) (50 μM) or calpains (CALI-III) (25 μM) for 1 h, and then treated with DON (500 ng/ml) for 3 h. Total protein was extracted and BiP was analyzed by Western Blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lanl.

A


DMSO
ALLN (μM)
EPO (μM)
inhibitors
0.1%
0.01%
50
5
10
1
DON
- + - + - + - + - + - +
BiP
RI (%)
95 15 98 11 64 57 61 62 100 11 100 12
β-actin

B

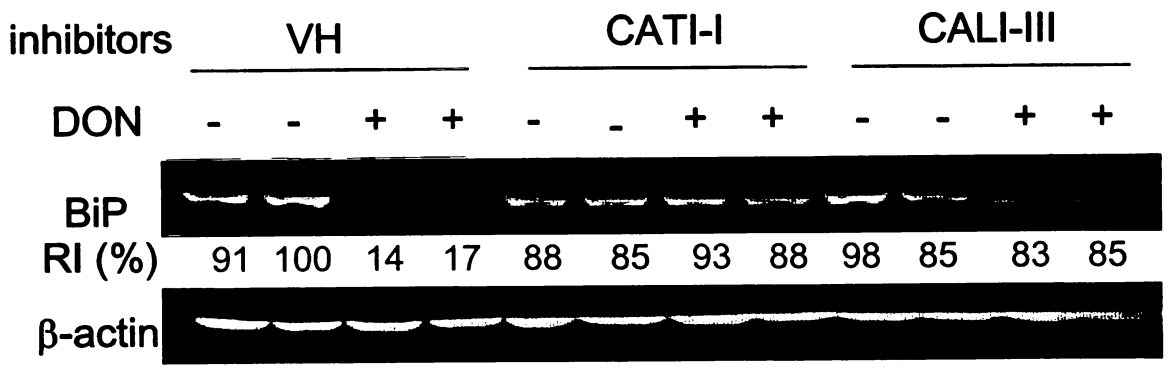


Figure 4.3 continued.

requiring enzyme (IRE)-1α, an ER stress sensor activated upon dissociation from BiP, and transcription factors XBP1 and ATF6 (Figure 4.4A and B). Western analysis revealed that DON upregulated IRE1 α , XBP1 (54 kDa, spliced mRNA product) and ATF6 (50 kDa, activated form).

Since XBP1 mRNA is spliced in response to ER stress, both unspliced and spliced XBP1 mRNA after DON treatment was detected by real-time PCR (Figure 4.5A and B). Consistent with ER stress response, the spliced but not the unspliced form was upregulated by DON.

XBP1 and/or ATF6 were knocked down by specific siRNA transfection to determine if DON-induced IL-6 gene expression is related to ER stress-like response (Figure 4.6A). The protein level of XBP1 and ATF6 was decreased markedly 48 hr after siRNA transfection compared with negative control. Knockdown of transcription factor ATF6 decreased DON-induced IL-6 expression, while there was no effect observed with transcription factor XBP1 knockdown (Figure 4.6B).

To further confirm the relationship between DON-induced BiP degradation and IL-6 gene expression, we knocked down BiP via siRNA transfection (Figure 4.7A). BiP protein was downregulated 48 h after transfection, which induced IL-6 mRNA expression (Figure 4.7B).

In order to check if BiP degradation was a general effect induced by different toxins, peritoneal macrophages were treated with different toxins including roridin A, satratoxin G, ricin, and T-2 toxin. Tunicamycin is a classic ER stress inducer thus used as a positive control. BiP protein in macrophages was measured after toxin incubation

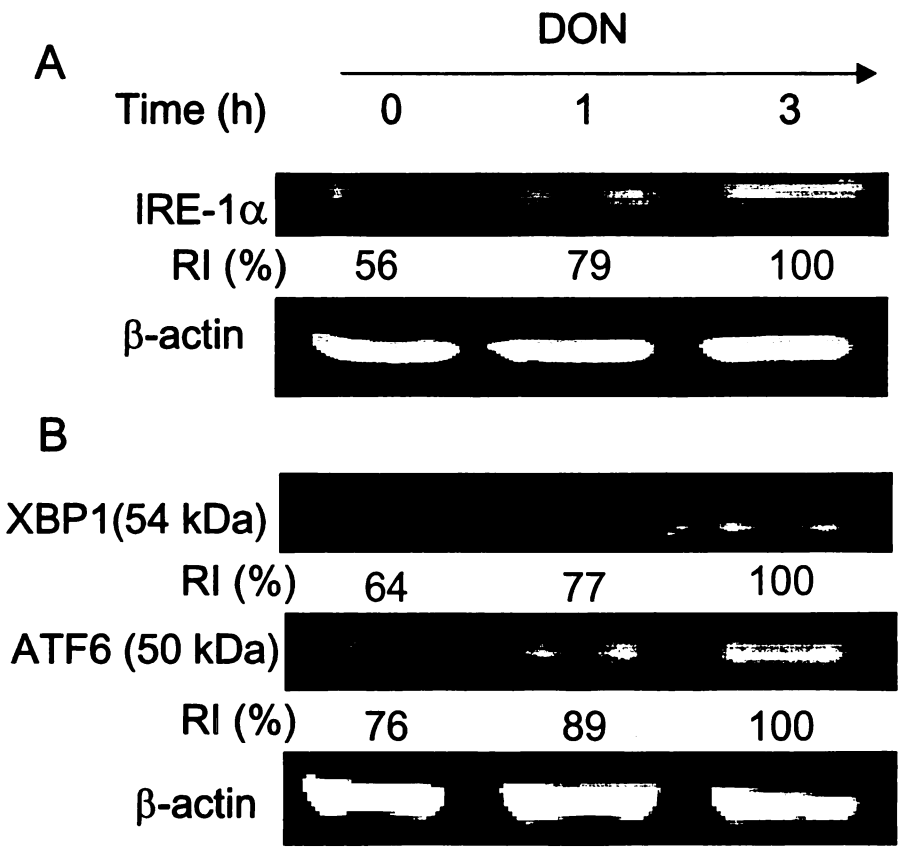


Figure 4.4. DON treatment upregulates IRE1α , XBP1 and ATF6 in peritoneal macrophages. Peritoneal macrophages were cultured with DON (500 ng/ml) for different time periods. Total protein was extracted; IRE1α (A), XBP1 and ATF6 (B) were analyzed by Western Blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

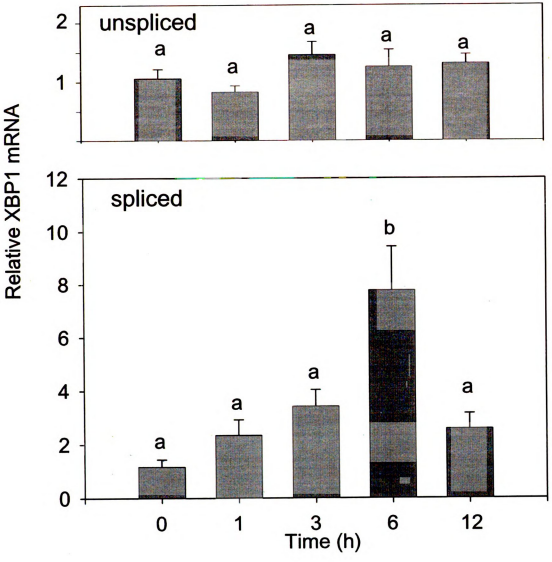
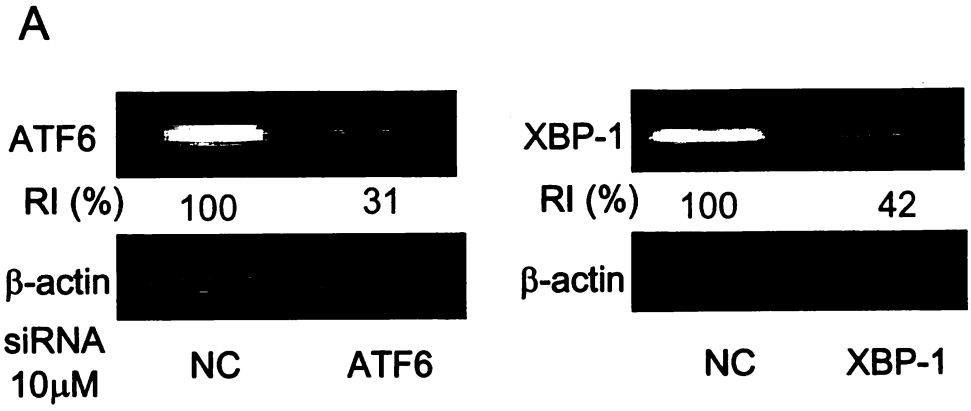
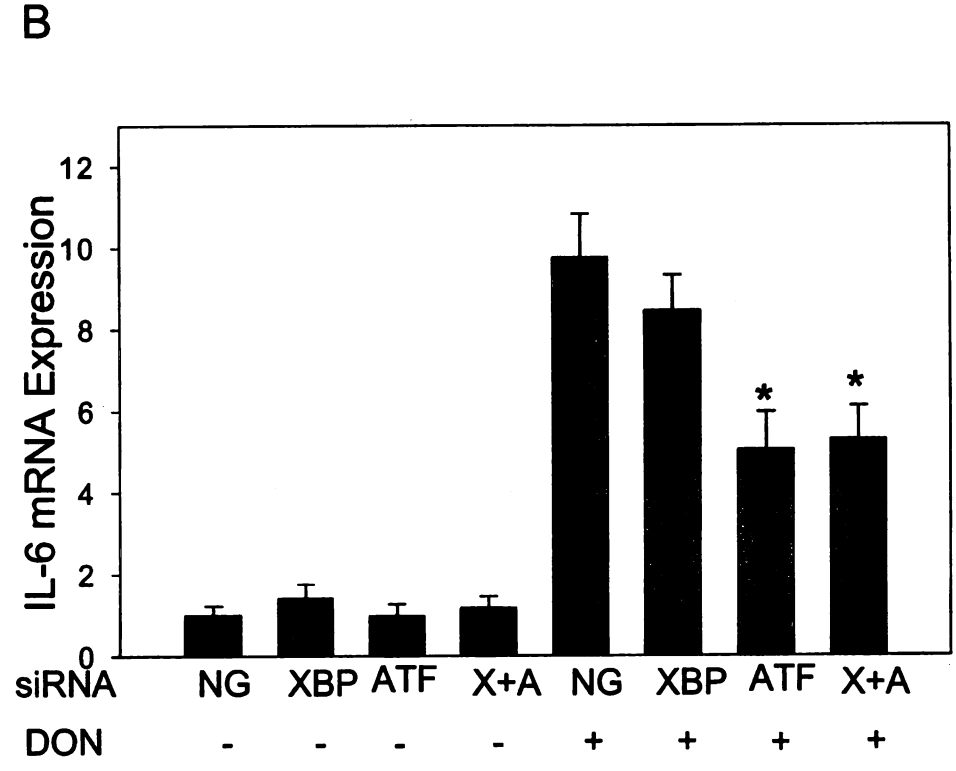


Figure 4.5. DON treatment induced splicing of XBP1 mRNA. To detect splicing of XBP1 mRNA, peritoneal macrophages were cultured with DON (500 ng/ml) for different time periods. Total RNA was extracted for detection of unspliced and spliced XBP1 mRNA by real-time PCR. Data are means ± SEM. Bars with different letters differ ($p<0.05$).

Figure 4.6. DON-induced IL-6 gene expression is related to ATF6 activation. siRNA specific to ATF6, XBP1 or scrambled siRNA was transfected by electroporation into peritoneal macrophages. (A) To evaluate ATF6 and XBP1 knockdown efficiency, total protein was collected after 48 h. ATF6 and XBP1 were measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) To detect the role of ATF6 and XBP1 on IL-6 mRNA expression, cells were treated with DON after transfection with siRNA specific to XBP1 (XBP), ATF6 (ATF) or both (X+A). Total RNA was collected 3 h later and IL-6 mRNA was analyzed by real-time PCR. Data are means ± SEM. Bars with asterisk differ from NG+DON group ($p<0.05$).


A
ATF6
RI (%)
100
31
β-actin
siRNA
10μM
NC
ATF6
XBP-1
RI (%)
100
42
β-actin
NC
XBP-1


B
IL-6 mRNA Expression
0
2
4
6
8
10
12
*
*
siRNA
NG
XBP
ATF
X+A
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ATF
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DON
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-
-
+
+
+
+

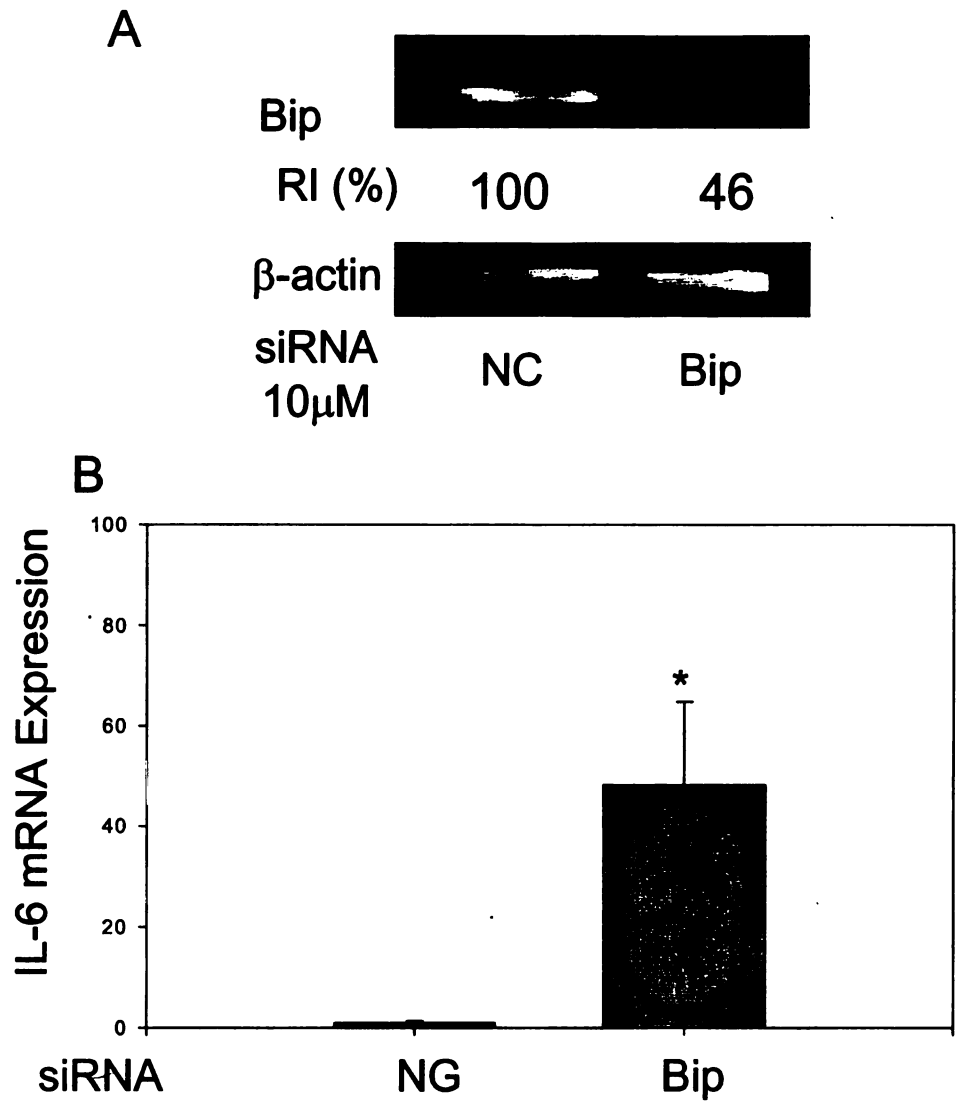

Figure 4.7. Bip knockdown induces IL-6 gene expression. siRNA specific to mouse Bip or scrambled siRNA was transfected by electroporation into peritoneal macrophages. (A) To evaluate knockdown efficiency, total protein was collected after 48 h. Bip were measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) To detect the correlation of Bip knockdown and IL-6 gene expression, total RNA was extracted and IL-6 mRNA was analyzed by real-time PCR. Data are means ± SEM. Bar with asterisk differs statistically from NG group ($p<0.05$).

(Figure 4.8). As a positive control, tunicamycin upregulated BiP dramatically at 6 h; while all the toxin treatments induced BiP degradation at 3 and 6 h. This observation may give some insight into the mechanisms by which these toxins function inside the cells.

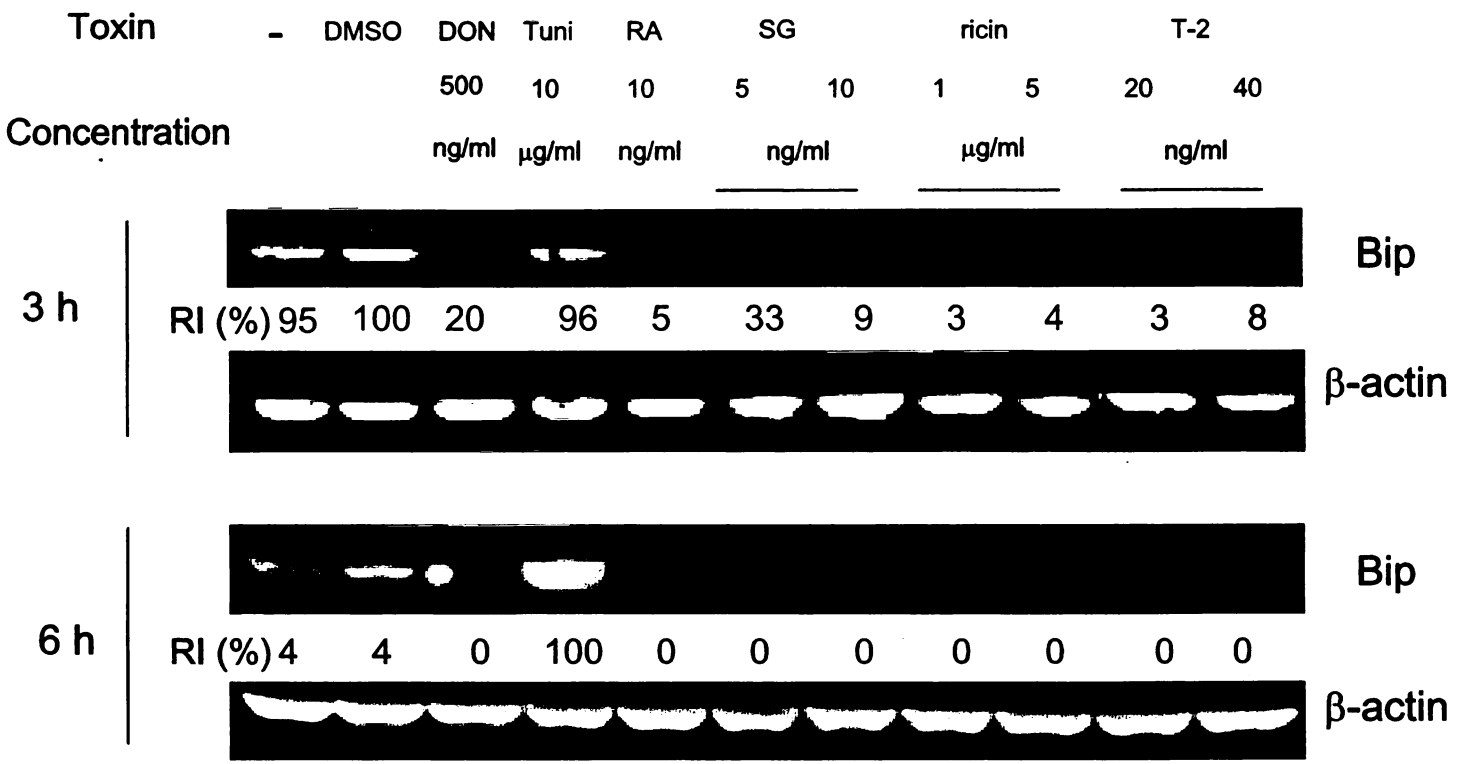

Figure 4.8. BiP degradation is induced by different toxins. Peritoneal macrophages were treated with DON, tunicamycin (Tuni), roridin A (RA), satratoxin G (SG), ricin, and T-2 toxin for 3 or 6 h. BiP protein was measured by Western Blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

DISCUSSION

The classical ER stress response is evoked by accumulation and aggregation of nascent, unfolded or misfolded polypeptides in the ER. In order to maintain ER homeostasis, the cells initiate a process that includes (1) transient attenuation of protein translation, (2) ER-associated degradation (ERAD) of malfolded proteins and (3) the induction of molecular chaperones and folding enzymes to augment the ER's capacity for protein folding and degradation. If the stress cannot be relieved, apoptotic pathways might be activated in the damaged cells (Ma and Hendershot 2001).

BiP is an ER chaperone and belongs to the heat shock protein 70 family which serves as a master regulator of ER stress response (Zhang and Kaufman 2006). By recognizing and binding to the hydrophobic domains of unfolded proteins, BiP stabilizes unfolded proteins for further modification. BiP also interacts directly with ER stress sensors such as ATF6 and IRE1 α to maintain them in inactive forms. When unfolded proteins accumulate in the ER lumen, BiP is titrated away and thus releasing ATF6 and IRE1 α (Kaufman 1999) (Zhang *et al.* 2006) (Zhang and Kaufman 2006). ATF6 can migrate from ER to the Golgi where it is cleaved (from 90 kDa to 50 kDa) by two proteases, S1P and S2P to become an active transcription factor (Shen *et al.* 2002). Activated IRE1 α has RNase activity enabling it to cleave XBP1 mRNA and generate spliced XBP1. The resultant gene product is another active transcription factor that can contribute to ER stress-related gene expression (Ron and Hubbard 2008).

The results presented herein indicate that DON induces BiP degradation in the peritoneal macrophages and consequently, IRE1 α and ATF6 were released and activated.

Another ER stress sensor, PERK was not detectable in our study. Upregulation of the ER stress sensors and transcription factors after DON treatment suggests an ER stress-like response. Downregulation of BiP has also been seen in the leukemia cells treated with a multikinase inhibitor (Rahmani *et al.* 2007), kidney Vero cells treated with subtilase cytotoxin (SubAB) (produced by Shiga-toxin producing *E. coli* (Morinaga *et al.* 2008) and tumor cells treated with deoxyverrucosidin (produced by *Penicillium* sp.) and prunustatin A (produced by *Streptomyces* sp.) (Choo *et al.* 2005) (Umeda *et al.* 2005).

A question relates to how BiP is degraded after DON treatment. Eukaryotic cells have two major pathways for protein degradation, the ubiquitin-proteasome and autophagy-lysosomal pathways (Cecarini *et al.* 2007) (Yorimitsu and Klionsky 2005). Protein ubiquitination and proteasome-mediated protein degradation is an important pathway responsible for misfolded and short-lived intracellular protein (Guerrero *et al.* 2006) (Rubinsztein 2006). The 26S proteasome is a 2.5 MDa complex composed of two multisubunit subcomplexes: one is a 20S core particle and the other a 19S regulatory particle (Demartino and Gillette 2007). The core particle is characterized by three proteolytic activities: chymotrypsin-like, trypsin-like and peptidylglutamyl peptide-hydrolyzing activities (Cuervo and Dice 1998). Another process named autophagy is also an important mechanism for the degradation of cytoplasmic components, from single macromolecules (proteins, lipids and nucleic acids) to whole organelles (Yorimitsu and Klionsky 2005). Once inside the lysosomal system, substrates are degraded by a mixture of more than 80 types of proteases, peptidases and other hydrolases, which are all called cathepsins(Grinyer *et al.* 2007) (Cecarini *et al.* 2007) (Cuervo and Dice 1998). Calpains are a family of Ca^{2+}-regulated cysteine proteases that mediate cleavage of specific

substrates involved in cell differentiation, life and death (Demarchi and Schneider 2007). The proteins cleaved by calpains include cytoskeletal and associated proteins, kinases and phosphatases, membrane receptors and transporters, etc. Activated calpains can also compromise the integrity of lysosomal membranes and liberate cathepsins from lysosomes (Yamashima 2004)

Specific inhibitors were used here to study the mechanisms by which BiP was degraded upon DON treatment. Epoxomicin is a potent, highly specific and irreversible inhibitor of chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing activities of the proteasome. ALLN is an inhibitor of cathepsins, calpains and at higher concentration proteasome. Epoxomicin did not affect DON-induced degradation of BiP; while ALLN almost abolished these effects completely. In order to specify the results, we used inhibitors to cathepsins and calpains. The results showed DON-induced BiP degradation was cathepsin/calpain dependent. So the lysosomal pathway might be important in this BiP degradation.

ER stress response has been shown to be involved in many immune processes. For example, activation of XBP1 and ATF6 is required for B lymphocytes differentiation into plasma cells and antibody production (Iwakoshi *et al.* 2003b) (Gass *et al.* 2002). ER stress response can also activates transcription factor NF-κB and cyclic AMP response element binding protein H (CREBH) which drives expression of inflammatory genes such as TNF-α, C-reactive protein and COX-2. (Deng *et al.* 2004) (Hung *et al.* 2004) (Hu *et al.* 2006) (Zhang *et al.* 2006). Transcription factor XBP1 has also been shown to be upstream of IL-6 gene expression (Iwakoshi *et al.* 2003b). In our study, we observed that another ER stress activated transcription factor ATF6 might be upstream of IL-6

expression (Figure 4.9). Our results also showed that BiP knockdown directly induced IL-6 upregulation, which confirms that BiP degradation induced ER stress-like response is related to IL-6 gene expression.

Based on these data, we conclude that DON induces ER stress-like response by degrading ER chaperone BiP. Cathepsins and calpains are involved in the BiP degradation. ER stress-like response is probably involved in DON-induced IL-6 expression in the peritoneal macrophage.

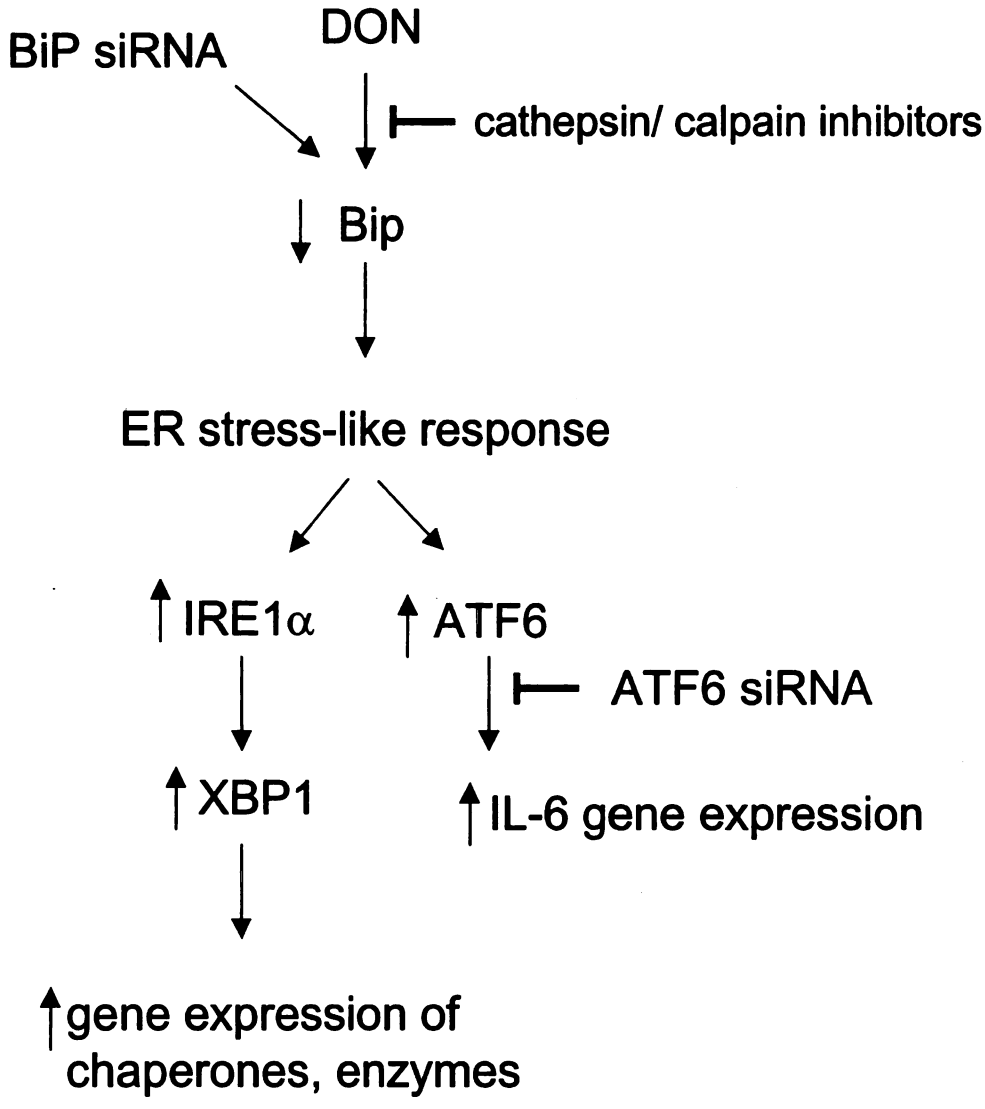

Figure 4.9. Summary of DON-induced ER stress-like response and IL-6 gene expression.

CHAPTER 5

Summary and Perspectives

Clinical trials have shown that progression of human IgAN is inhibited by dietary n-3 PUFA supplementation. The early stages of IgAN can be mimicked by feeding B6C3F1 female mice the mycotoxin DON. Therefore, we used this animal model to study the mechanisms by which n-3 PUFA suppressed the pathogenesis of IgAN.

Consumption of 20 ppm DON significantly increased mouse serum IgA, IgA immune complexes and kidney mesangial IgA deposition compared with mice taking control diet. Effects of EPA on DON-induced IgAN were assessed relative to dose dependency. Mice were fed control diet or diets with 20 ppm DON supplemented with 0%, 0.1%, 0.5% and 3% EPA for 16 weeks. The two highest EPA concentrations markedly increased splenic EPA, docosapentaenoic acid (DPA) and DHA, whereas arachidonic acid was decreased in all three EPA fed groups. All three IgAN markers were attenuated in mice fed 3% EPA diet but not in those fed 0.1% or 0.5% EPA. Elevated IgA production induced by DON in spleen and Peyer's patch (PP) cell cultures was reduced by feeding 0.1%, 0.5% and 3% EPA. Increased expression of IL-6 in serum, spleen and PP were also suppressed in mice consuming 3% EPA. Suppressed IL-6 corresponded to decreased binding activity of two transcription factors CREB and AP-1. The results suggest that the n-3 PUFA EPA consumption could impair pathogenesis of IgAN by suppressing IL-6 gene expression.

In the next study, we identified part of the signal transduction pathway through which DON upregulated IL-6 in peritoneal macrophages and investigated how consumption of DHA, suppressed this pathway. Incubation with DON induced IL-6 gene expression in peritoneal macrophages. Knockdown of the transcription factor CREB or

pharmacologic inhibition of the CREB kinases, Akt1/2, MSK1 and RSK1, downregulated IL-6 expression. Inhibition of PKR suppressed not only IL-6 expression but also phosphorylation of CREB and its upstream kinases. Phosphorylation of PKR, CREB kinases and CREB was markedly impaired in peritoneal macrophages isolated from mice that consumed DHA-enriched fish oil for 6 to 8 wk. DHA's effects were not explainable by increased activity of protein phosphatase 1 and 2A since both were suppressed in mice consuming the DHA diet. We concluded from these data that DON-induced IL-6 expression is CREB-mediated and PKR-dependent and the requisite kinase activities for this pathway were suppressed in macrophages from mice fed DHA.

The third investigation in my research was to explore a possibly alternative pathway of DON-induced IL-6 gene upregulation. The ER chaperone BiP was markedly decreased in peritoneal macrophages following incubation with DON. However, BiP mRNA was not changed. Inhibitor studies showed that DON-induced BiP degradation was cathepsin- and calpain-dependent. BiP degradation caused ER stress-like response that includes increased IRE1α, XBP1 and ATF6 protein as well as XBP1 mRNA splicing. Knockdown of ATF6 partially decreased DON-induced IL-6 expression, while knockdown of BiP itself increased IL-6 mRNA expression in peritoneal macrophages. These data suggest that DON treatment decreases BiP protein and evokes ER stress-like response. Furthermore, transcription factor ATF6 activation as a result of ER stress-like response is likely to contribute to DON-induced IL-6 gene expression. Consumption of DHA-enriched fish oil did not affect DON-induced BiP degradation, suggesting that n-3 PUFAs do not affect the DON-induced ER stress-like response.

Taken together, these studies indicate that n-3 PUFAs have beneficial effects on DON-induced IgAN. The data also give an insight into the mechanisms by which n-3 PUFAs suppress IL-6 gene expression. A summary of the proposed mechanisms by which n-3 PUFAs inhibit DON-induced IL-6 gene expression and IgAN in the mouse model is shown in Figure 5.1. Since DON-induced IL-6 gene expression may involve multiple pathways, further study is needed to determine how these pathways interact with each other.

The following experiments are suggested for future study:

1. Detect the populations of different macrophage phenotypes from mice fed control or n-3 PUFA-supplemented diet. (Markers for inflammatory phenotype in the mouse may include CCR2, CD62L [also known as L-selectin] and CX_3C-chemokine receptor 1 [CX_3CR1]). Since n-3 PUFA consumption might change the phenotypes of macrophages and thus render them less proinflammatory or even anti-inflammatory, measuring their presence will provide insight into how long-term n-3 PUFA feeding modulates the immune system.
2. Analyze resolvins and protectins in different tissues. Resolvins and protectins are potent anti-inflammatory mediators that are upregulated by n-3 PUFA consumption and may be important for the anti-inflammatory effects provided by long-term n-3 PUFA consumption. Analysis of resolvins and protectins in the whole blood and different tissues in mice fed the n-3 PUFA supplemented diet and of the effects of treatment with

resolvins and protectins on IL-6 expression will help determine whether this is another n-3 suppression of DON-induced inflammation.

3. Measure fatty acid composition of different organelles inside the cell. Fatty acids are important building blocks of cell membranous structures. The changes of fatty acid components will affect the functions of membrane-associated proteins and signal transductions in the cell. Detection of fatty acid composition of different membranous organelles, such as the endoplasmic reticulum, Golgi complex, mitochondria and lysosomes from mice fed control or n-3 PUFA supplemented diet will give important clues to relate the structural changes to the functional changes in the immune cells.
4. Relate DON-induced ribotoxic stress to the ER stress-like response. DON is a trichothecene mycotoxin that acts by inducing the ribotoxic stress in the cell and it also evokes ER stress-like response. Since ribosomes and the ER are structural- and functional- related organelles inside the cell, it will be important to relate ribotoxic stress to the ER stress-like response for better understanding the mechanisms of DON's function.

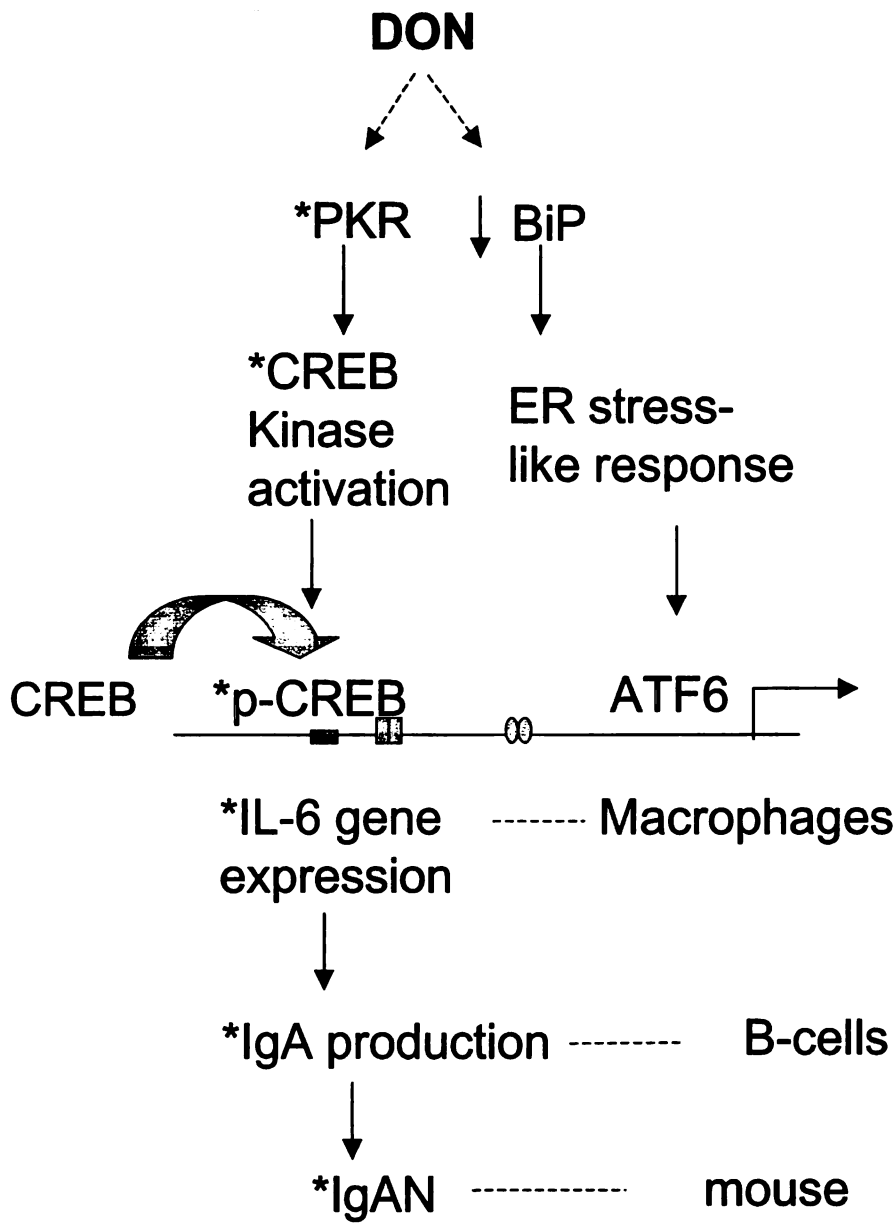

Figure 5.1. Summary of mechanisms by which DON induces IL-6 gene expression and IgAN. Asterisks indicate steps at which n-3 PUFAs cause suppression.

Appendix A

Effects of n-3 PUFA α-linolenic acid on DON-induced IgAN

The n-3 PUFA, ALA, is an essential fatty acid for humans and is derived mainly from terrestrial plant consumption. It is the principal precursor for EPA and DHA. In human and animal studies, ALA was shown to be as beneficial to cardiovascular health as EPA and DHA from marine and fish oils (Lanzmann-Petithory 2001). In respect of attenuating inflammation, ALA showed conflicting results in publications (Marion-Letellier *et al.* 2008) (Nelson *et al.* 2007) (Yoneyama *et al.* 2007) (Ren and Chung 2007) (Calder 2006a). Furthermore, the potential effects of ALA on IgAN remain unknown.

Here, we conducted an experiment using diet supplemented with different concentrations of flaxseed oil that contains high ALA to study the effects of ALA on DON-induced IgAN. Corn oil, oleic acid, and flaxseed oil containing 570 g/kg ALA (Dyets) were used to modify the AIN93G basal diet to yield 4 diet groups (n = 10): control, control + DON (0.020 g/kg), 30 g/kg flaxseed oil + DON and 60 g/kg flaxseed oil + DON (0.020 g/kg) (Table AA.1). Approximate fatty acid compositions of the experimental diets are shown in Table AA.2. Diets were prepared every 2 wk, stored in aliquots at –20°C, and provided fresh to female B6C3F1 mice each day. Mice were housed 2–3 per cage and fed the diet for 18 wk. Mice were bled every 4 wk and serum IgA was analyzed by ELISA.

DON significantly increased serum IgA beginning at 8 wk until 16 wk (Figure AA.1). However, diets containing 30 or 60 g/kg flaxseed oil did not affect the elevation in serum IgA. Because of this lack of effect, further analyses of downstream effects (IgA-IC and mesangial IgA elevation) were not conducted. These data suggest that ALA did not reduce the aberrant IgA production induced by DON in mice. Among the n-3 PUFAs,

Group	n	DON	Corn oil	Oleic acid	Flaxseed oil	Total n3	n6:n3 ratio
		g/kg					
Control	10	0	10	60	0	0.6	16:1
Control + DON	10	0.02	10	60	0	0.6	16:1
3% flaxseed oil + DON	10	0.02	10	30	30	17	1:1.8
6% flaxseed oil + DON	10	0.02	10	0	60	34	1:3.6

Table AA.1. Experimental groups of mice for assessing the effects of α-linolenic acid on DON-induced IgAN.

	Corn oil and oleic acid	3% flaxseed oil	6% flaxseed oil
		g/100g total fat	
Type of fat			
Saturated	0.6	0.63	0.66
Monounsaturated	5.24	3.29	1.34
Polyunsaturated	0.85	2.92	5
Total fat	7	7	7
		g/100g fatty acids	
Fatty acid			
C16:0	4.6	5.1	5.7
C18:0	4.0	3.8	3.7
C18:1	74.9	47	19.1
C18:2 (n6)	11.9	17.2	22.5
C18:3 (n3)	0.26	24.6	49.0
C20:5 (n3)	0	0	0
C22:6 (n3)	0	0	0
Σ n3	0.26	24.6	49.0
Σ n6	11.9	17.2	22.5
n6:n3	46:1	1:1.4	1:2.2

Only the major fatty acids are shown.

Table AA.2. Fatty acid composition of different diets for assessing the effects of α-linolenic acid on DON-induced IgAN.

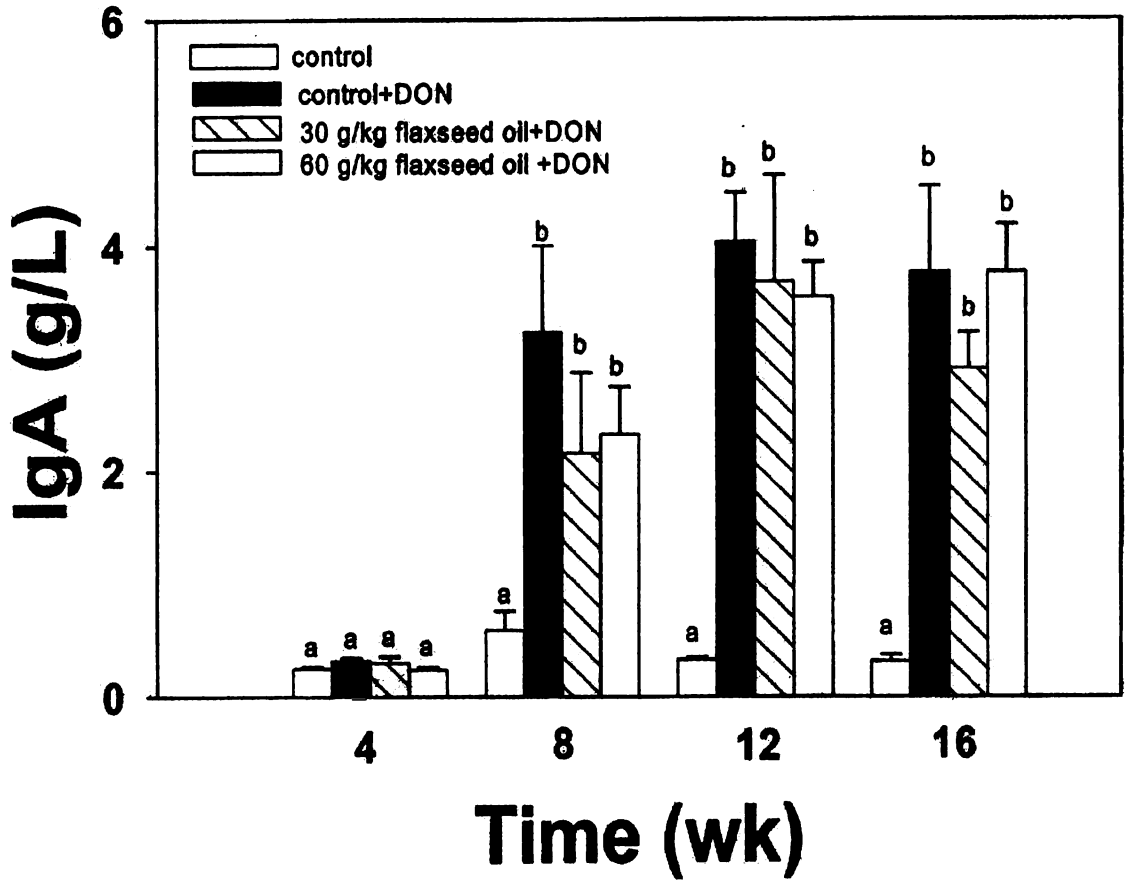

Figure AA.1. Effects of flaxseed oil consumption on DON-induced serum IgA elevation in B6C3F1 mice. Values are means ± SEM, $n = 10$. Means without a common letter differ, $P < 0.05$.

EPA and DHA are more biologically potent than ALA in preventing experimental IgAN.

Appendix B

Protein kinase C and DON-induced IL-6 upregulation

Protein kinase C (PKC) is a family of protein kinases that are expressed broadly and regulate various cellular functions. They have been shown to be mediators of different immune responses such as signal transductions initiated by T-cell receptor and B-cell receptor activation (Hayashi and Altman 2007) (Saijo *et al.* 2003). PKC family has at least ten isoenzymes and these isoenzymes might have distinct roles in the immune system (Tan and Parker 2003).

DON treatment induced multiple protein kinase activation. Protein kinase Akt1, MSK1 and RSK1 belong to the protein kinase AGC family. In this experiment, we studied if DHA consumption suppresses PKC (an important member of AGC family) activation and if PKC is involved in DON-induced IL-6 gene expression.

Peritoneal macrophages from mice fed control or 3% DHA diet for 6-8 wk were treated with DON (250 ng/ml) for indicated time points and cell lysates were subjected to Western blotting. (Figure AB.1) DON treatment induced very weak phosphorylation of PKC isoenzymes, DHA consumption inhibited the induction.

In order to test if PKC is involved in DON-induced IL-6 gene expression, peritoneal macrophages were treated with 10 μM PKC inhibitor (PKC inhibitor Peptide 19-36) (Calbiochem) for 30 min. Then 250 ng/ml DON was added to cell cultures. Total RNA was extracted for IL-6 expression 3 h later. Figure AB. 2 showed that PKC inhibitor did not inhibit IL-6 gene expression upon DON stimulation.

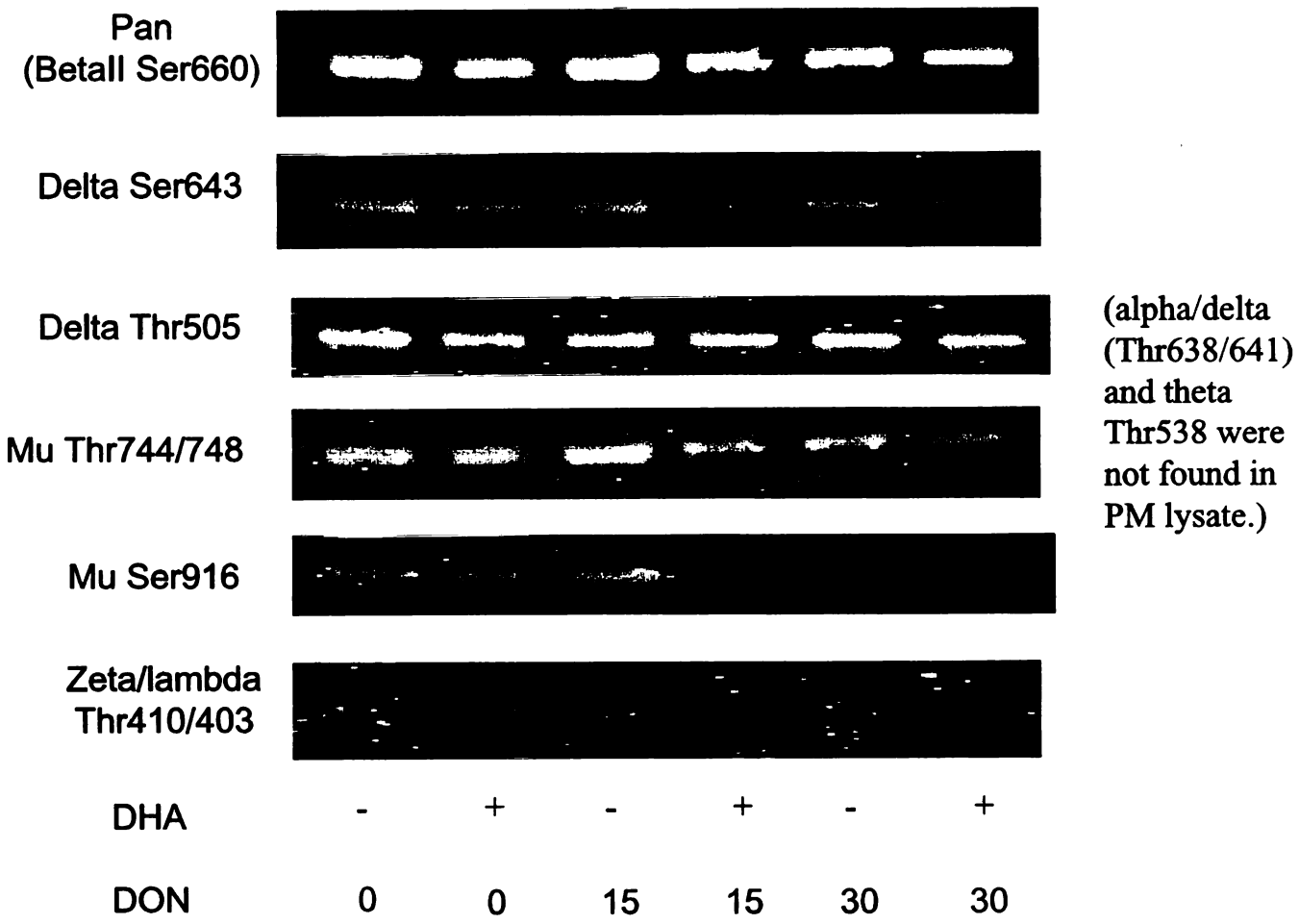

Figure AB.1. Effects of DHA on DON-induced PKC phosphorylation.

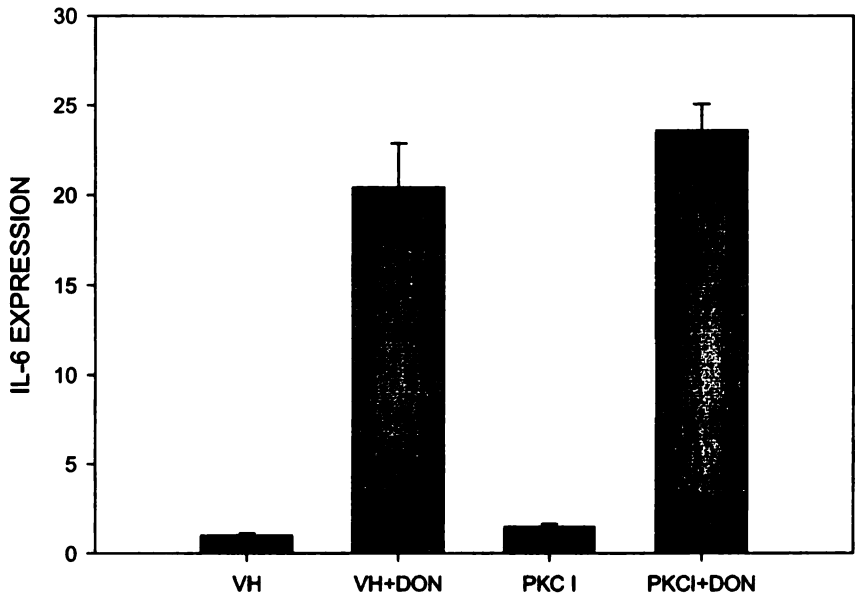

Figure AB. 2. Effects of PKC inhibitor on DON-induced IL-6 gene expression.

Appendix C

Role of interleukin-10 in DON-induced protein phosphorylation

Interleukin-10 (IL-10) is a pleiotropic cytokine produced by a variety of cells such as T cells, B cells, monocytes, and macrophages (Moore *et al.* 2001). IL-10 has been shown to strongly decrease the production of not only inflammatory cytokines such as IL-6, IL-1, IL-8, TNF-α but also reactive oxygen and nitrogen species from macrophages (Adorini 2003).

Clinical trials showed IL-10 could be beneficial to treat some autoimmune diseases such as rheumatoid arthritis (Maini and Taylor 2000), Crohn's disease (van Deventer *et al.* 1997), and psoriasis (Adorini 2003). In research conducted by Matsumoto et al., human recombinant IL-10 inhibited the spontaneous as well as the LPS-stimulated cytokine secretion from peripheral blood monocytes in patients with IgAN (Matsumoto 1996).

Since IL-10 is important for inflammation regulation, we used protein phosphorylation as a marker to study if knockout of IL-10 will affect DON-induced macrophage activation and if DHA consumption will affect the results.

Peritoneal macrophages from wild type or IL-10 KO mice fed control or DHA diet were taken and treated with DON (250 ng/ml). Total protein was subjected to Western blot analysis. As shown in figure AC.1, IL-10 KO increased both total and phosphorylated CREB and Akt1. DHA diet attenuated protein phosphorylation in macrophages from both wild type and IL-10 KO mice.

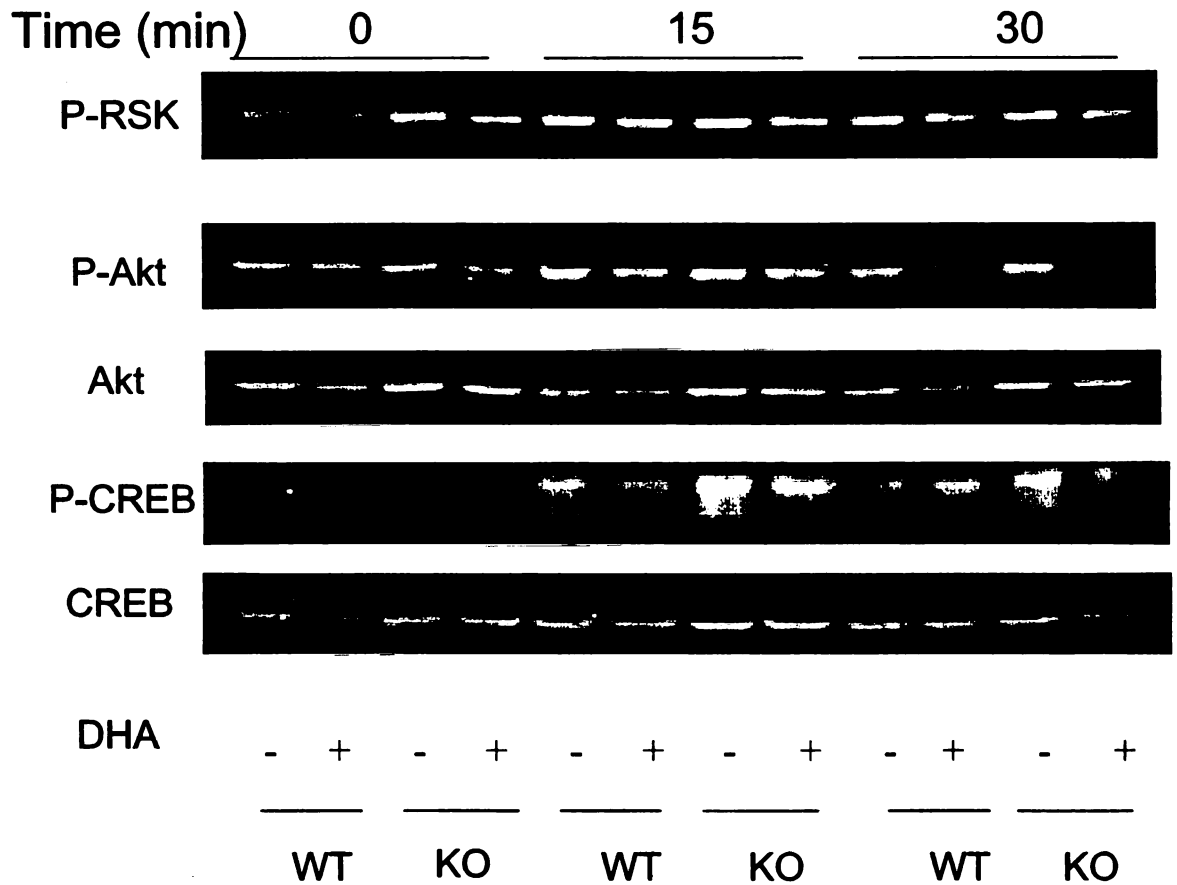

Figure AC.1. Effects of IL-10 KO and DHA consumption on DON-induced protein phosphorylation in macrophages. Peritoneal macrophages were taken from mice fed control or DHA diets and were treated with DON (250 ng/ml) for indicated time points. Cell lysates were subjected to WB.

Appendix D

Effects of DON on IgA glycosylation

As mentioned in the literature review, IgA hypoglycosylation might be involved in the pathogenesis of IgAN. ER is an important organelle for protein modification including glycosylation. DON induces chaperone BiP degradation, which might affect IgA glycosylation and result in IgAN in the mouse model.

To test the glycosylation of IgA affected by DON, we conducted an 8-wk feeding study. Female B6C3F1 mice were fed diet supplemented with 20 ppm DON. Serum IgA and its hypoglycosylated form were detected by ELISA.

The results showed that DON consumption upregulated IgA in the serum. Hypoglycosylated IgA was also increased by DON treatment (Figure AD.1). The higher hypoglycosylated IgA level might be a risk factor for the pathogenesis of IgAN.

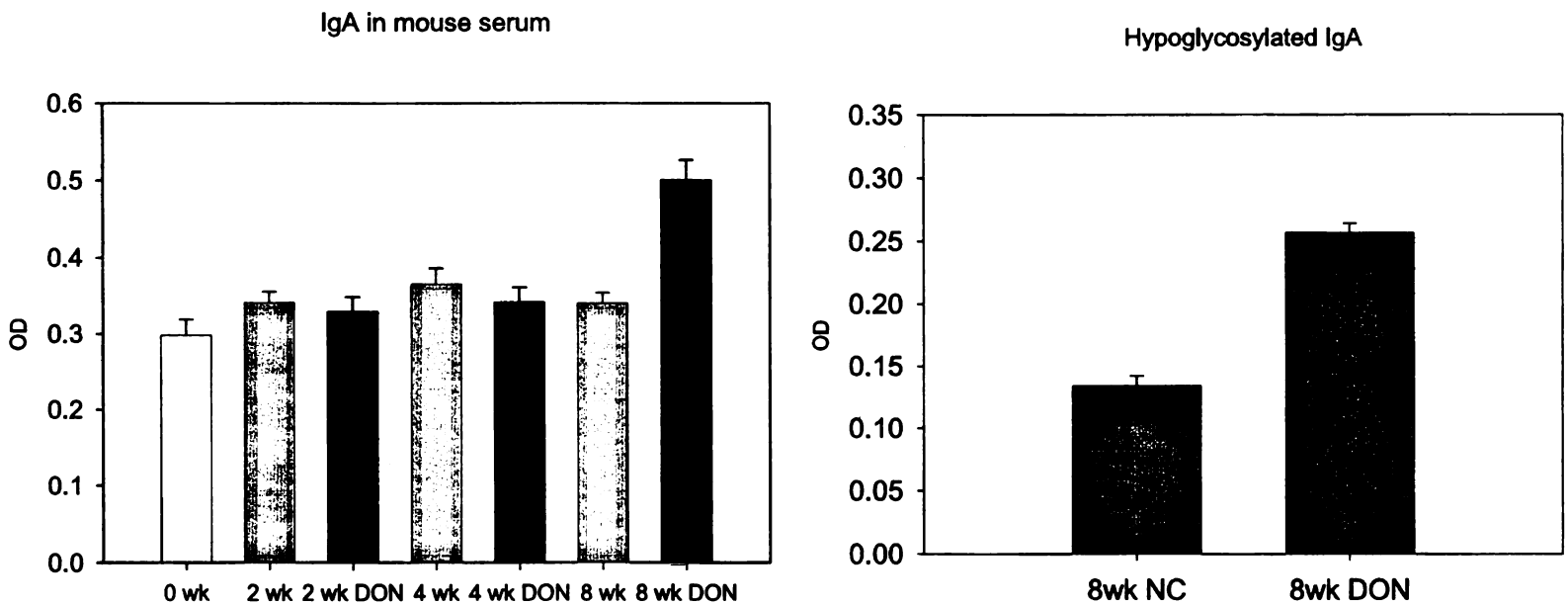

Figure AD.1. Effects of DON consumption on serum IgA and hypoglycosylated IgA.

Method to detect serum hypoglycosylated IgA

Reagents

1. Carbonate Coating Buffer

   Na_2CO_3 1.59g

   $NaHCO_3$ 2.93g

   dd-H_2O 1000ml

   pH = 9.6

2. Dilution buffer: 1% BSA-PBS
3. Phosphate Buffered Saline (PBS)

   NaH_2PO_4 6.62g

   Na_2HPO_4 49.98g

   NaCl 11.80g

   dd-H_2O 4.0L

   pH = 7.5

4. PBS Tween 0.02%

   To 4.0L PBS add 0.8ml of Tween-20

5. 0.01 M sodium acetate buffer, PH = 5.
6. Substrate: TMB substrate
7. Stopping reagent: 2N H_2SO_4

Procedure:

1. Coat goat anti-mouse IgA onto plate with 50µl solution of protein in coating buffer. For affinity purified anti IgA use 1:1000 (1 µg/ml).

10ml carbonate buffer + 10µl anti-mouse IgA=1 plate

2. Cover plate with parafilm and aluminum foil to prevent evaporation and store overnight at 37 °C.
3. Wash plate 3 times with PBS-Tween using plate washer.
4. Block all wells with 1% BSA-PBS; add 300 µl/well and incubate 2 h at 37 °C; this binds nonspecific sites in well.
5. Wash plate 5 times as before.
6. Add sample.
7. Wrap in aluminum foil and incubate overnight at 4 °C; seal tightly.
8. Wash 5times as before.
9. Add 50 µl 10µM neuraminidase in sodium acetate buffer and incubate for 3 h at 37 °C.
10. Add 50µl of a 1/500 solution of HAA-HRP and incubate for 3 h at 37C.
11. Wash 6 times with PBST.
12. Then wash with distilled water twice to clean plate.
13. Add TMB substrate at 100µl/well.
14. Wrap in aluminum foil to protect substrate from light.
15. Develop color at RT for about 30 to 60 min.
16. Stop with 100 µl/well of stopping reagent.
17. Read on ELISA reader at 450nm.
18. Calculate using SOFTMAX computer program.

Appendix E

Effect of DHA on BiP degradation

DHA has been shown to inhibit DON-induced signal pathways involved in IL-6 gene expression in peritoneal macrophages. We found that DON treatment induced BiP degradation which was also related to IL-6 gene expression. Here, we investigated if DHA consumption could affect BiP degradation and result in suppressed gene expression.

Peritoneal macrophages taken from B6C3F1 female mice fed 8-wk control or DHA diet were treated with DON (500 ng/ml) for indicated period (Figure AE.1). DON treatment induced BiP degradation, while there was no difference between control and DHA group.

Based on these data, we conclude that although BiP degradation is involved in DON-induced IL-6 gene expression, DHA feeding might not suppress IL-6 by affecting this pathway.

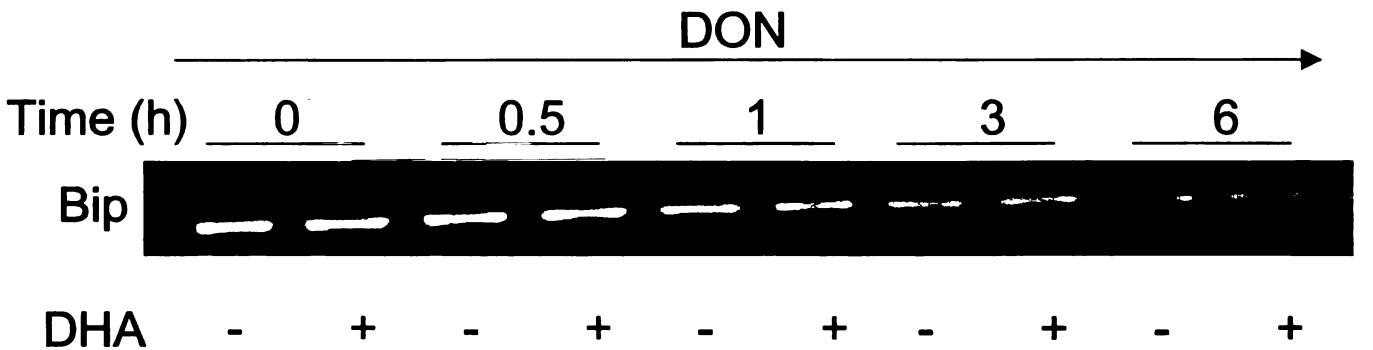

Figure AE.1. Effect of DHA consumption on DON-induced BiP degradation.

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