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ROLE OF CYCLOOXYGENASE-2 IN
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ROLE OF CYCLOOXYGENASE-2 IN DEOXYNIVALENOL-INDUCED IMMUNOTOXICITY

By

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ABSTRACT

ROLE OF CYCLOOXYGENASE-2 IN DEOXYNIVALENOL-INDUCED IMMUNOTOXICITY

BY

Yuseok Moon

Deoxynivalenol (DON, vomitoxin or VT), one of the trichothecene mycotoxins, has been recognized to cause immune stimulation or immune suppression in the experimental animals dependently on dose regimes. The purpose of this thesis was to determine whether DON affects cyclooxygenase-2 (COX-2) expression and its possible contribution to DON-induced immune dysfunctions. COX-2 was strongly induced in mouse spleens and Peyer's patches by the acute exposure to DON. Using the macrophage as a model for COX-2 production, the gene induction mechanism was investigated. Elevated COX-2 production was found to be due to enhanced transcriptional activation and mRNA stability by DON. DON-activated extracellular signal regulated protein kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) played an evident role in enhancing transcriptional activity of COX-2 gene. Moreover, p38 MAPK was strongly involved in the DON-mediated COX-2 mRNA stabilization as well as promoter activation. Type B trichothecenes other than DON, namely, 15-acetyl DON, 3-acetyl DON and fusarenon-X were also potent inducers of COX-2 expression whereas Type A and Type D trichothecenes had less effect on COX-2 expression. Type B trichothecene-induced COX-2 was also regulated via transcriptional and post-transcriptional control of the gene.

Interleukin-6 (IL-6) is known to play a pivotal role in DON-mediated IgA nephropathy (IgAN) and the toxin-induced COX-2 was assessed for its effect on IL-6 production. Blocking with COX-2 inhibitors or genetic ablation using COX-2 knockout mice retarded IL-6 induction by DON. Therefore, DON-mediated IL-6 production was, in part, due to COX-2 induction and its metabolites.

From a therapeutic perspective, dietary fish oil (FO) suppresses DON-induced IgAN in the animal model. To determine the pro-inflammatory gene responses and the signaling molecules related to the disease amelioration by FO, mice were fed with corn oil or fish oil for 8 weeks and then were acutely exposed to DON. Fish oil-fed mice demonstrated significantly reduced IL-6, ERK1/2 and JNK1/2 responses to DON as compared to corn oil-fed mice. However, there was little significant effect of FO on DON-induced COX-2 activity. Mainly present in fish oil, (n-3) polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5(n-3), EPA) or docosahexaenoic acid (22:6(n-3), DHA) strongly decreased DON-mediated IL-6 superinduction in presence of lipopolysaccharide in vitro using RAW 264.7 cells. Moreover, DON-activated ERK1/2 and JNK1/2 were suppressed by pretreatment of the cells with (n-3) PUFA. It is thus assumed that fish oil might retard DON-induced IL-6 production by regulating MAPK signaling pathway.

DEDICATION

"This dissertation is dedicated to the memory of my late father"

ACKNOWLEDGMENTS

I really appreciate Dr. James Pestka's contribution to this work. He encouraged my sprits in adverse times and helped me to finish this precious accomplishment in my life. Furthermore, I thank Dr. John Linz, Dr. Norbert Kaminski, Dr. Jay Shroeder and Dr. Kate Claycombe so much for their academic advice and concern. It is also my pleasure to share this God's grateful present with my lovely mom and sister's family who supported my persistent standing.

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ABBREVIATIONS

AA: arachidonic acid

Act-D: actinomycin-D

ARE: AU-rich element

C/EBP: CCAAAT/enhancer-binding protein

CO: corn oil

COX: cyclooxygenase

CREB: cAMP response element binding protein

DHA: docosahexaenoic acid (22:6(n-3))

DON: deoxynivalenol

EET: epoxyeicosatrienoic acid

EPA: eicosapentaenoic acid (20:5(n-3))

ERK: extracellular signal regulated protein kinases

FO: fish oil

IC: immune complex

IgAN: IgA nephropathy

IκB: inhibitory kappa B

IKK: IκB kinase

IL-6: interleukin 6

IND: indomethacin

JNK: c-Jun N-terminal kinases

KO: knockout mice

LOX: lipoxygenase

LPS: lipopolysaccharide

LTB: leukotriene B

MAPK: mitogen-activated protein kinases

MHC: major histocompatibility complex

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

MUFA: momo-unsaturated fatty acids

NF-κB: Nuclear Factor kappa B

NIV: nivalenol

NK: natural killer

PDTC: pyrrolidine dithiocarbamate

PGE: prostaglandin E

PKR: double-stranded RNA-dependent protein kinase

PPAR: proxisome proliferator activating factor

PUFA:poly-unsaturated fatty acids

SFA: saturated fatty acids

TXA: thromboxane A

UTR: untranslated region

VH: vehicle

VT: vomitoxin

CHAPTER 1

LITERATURE REVIEW

A. Trichothecenes and their mycotoxicoses The trichothecenes are a group of 182 structurally related sesquiterpenoid metabolites produced by *Fusarium, Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium, and Stachybotrys* growing on food, feed stuff and the environment (Ueno, 1985). Trichothecene contamination in agricultural commodities such as wheat, barley, and corn during fungal colonization has become a serious problem due to changing climatic patterns or agricultural practice (Peraica *et al.*, 1999). The trichothecene family is divided into four groups (A through D) based on modifications of the parent trichothecene ring system (Ueno, 1985). Three of these subtypes, Type A, B, and D, have great potential risk to human and animal health and therefore, will be the focus of this study. Type A trichothecenes are characterized by a hydroxyl or acyl moiety at R_1, R_2, R_3, or R_5 position (Table 1). Type B family possesses a ketone group at R_5 in addition to the derivation of Type A trichothecene. Macrocyclic Type D trichothecenes contain a diester or triester ring structure bridging between R_2 and R_3 positions (Jarvis, 1991).

Acute exposure to trichothecenes causes dehydration with gastrointestinal (GI) lesions, hematopoietic and immunosuppressive radiomimetic syndromes in experimental animals (Ueno *et al.*, 1984; Ueno, 1985). Notably, leukopenia, granulopenia, and progressive lymphocytosis can occur in the highly susceptible immune system. CNS toxicity

includes anorexia, lassitude, and nausea and acute vascular effects lead to hypotension and shock. Longer chronic mycotoxicoses exhibit the petechial rash, intensive ulceration, gangrenous processes, and high susceptibility to various secondary infections. However, trichothecene mycotoxins demonstrate neither mutagenic activity nor the capacity to damage DNA (Busby, *et al.*, 1981).

Trichothecenes have been etiologically related to several human diseases. One such disease is alimentary toxic aleukia (ATA), which was fatal toxicosis associated with overwintered moldy millet, wheat, and barley (Joffe and Yagen, 1977). Cotton lung disease is caused by inhalation of cotton dust contaminated with verrucarin-producing *Myrothecium verrucaria* (Jarvis, 1991). Red mold disease is a mycotoxicosis caused by eating Fusarium-infected wheat, rice and barley contaminated with nivalenol, deoxynivalenol, and fusarenon-X (Li *et al.*, 1999). Finally, attentions have focused on the contribution of molds to indoor air illness (Elidemir *et al.*, 1999; Scheel *et al.*, 2001; Trout *et al.*, 2001) and specifically, *Stachybotrys* has been associated with water-damaged building syndrome and was isolated from the bronchoalveolar lavage fluid of one infant with hemosiderosis. The involvement of Type D trichothecenes in this syndrome was not determined. However, Type A (T-2 toxin, HT-2 toxin, and diacetoxyscirpenol) and Type D toxins (satratoxins and verrucarins) have been identified in indoor air samples in which respiratory disease has been reported (Smoragiewicz *et al.*, 1993; Johanning *et al.*, 1996; Tuomi *et al.*, 1998; Mahmoudi and Gershwin, 2000).

B. Immunotoxicity of deoxynivalenol DON can be either immunosuppressive or immunostimulatory, depending on the dose and exposure regime (Bondy and Pestka, 2000).

B.1 Immune suppression. Dietary exposure to DON at concentration as low as 2 ppm for several weeks and 5 ppm for 1 wk as well as in vitro exposure to DON depress mitogen-induced proliferation responses of murine lymphocytes (Forsell *et al.*, 1985; Tryphonas *et al.*, 1986; Robbana-Barnat *et al.*, 1988). Deoxynivalenol also is cytotoxic to macrophages at high dose levels (Yang *et al.*, 2000a). Humoral immunity also can be impaired by DON. Exposure to DON in diet at a 10 ppm threshold or by gavage at 0.75 mg/kg body weight impairs the murine splenic plaque-forming cell (PFC) response to the sheep red blood cells (SRBC) (Pestka *et al.*, 1987; Robbana-Barnat *et al.*, 1988).

It is apparent that high doses of trichothecene promote rapid onset of leukocyte apoptosis and this will be undoubtedly manifested as immunosuppression (Ihara *et al.*, 1997; Shinozuka *et al.*, 1997; Islam *et al.*, 1998a; Islam *et al.*, 1998b; Shinozuka *et al.*, 1998). Moreover, the tissue damage becomes synergistically severe in presence of endotoxin, lipopolysaccharide, which have been known to be associated with elevation in pro-inflammatory cytokines and glucocorticoids (Zhou *et al.*, 1999; Zhou *et al.*, 2000; Islam *et al.*, 2002).

At the molecular level, apoptotic signals can be transduced through caspase-cascades by trichothecenes and elevation in calcium also has been implicated (Yoshino *et al.*, 1996; Nagase *et al.*, 2001). Ribotoxic stresses such as alterations in 28S rRNA by protein synthesis inhibition by DON are postulated to be an initiation signal for activation of MAPK such as JNK1/2 and p38 MAPK which have been known to be associated with apoptotic cell death (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). Interestingly, ERK1/2 is considered to be an anti-apoptotic signal in DON-mediated programmed cell death (Yang *et al.*, 2000a).

B.2 Immune stimulation. Both single gavage and multiple gavage of mice with

DON enhance resistance to the mastitic pathogen, which of immunostimulatory effect may have been mediated by altered macrophage and T regulatory cell activity (Corrier, 1991). When isotype-specific effects of DON on the immunoglobulin response are analyzed, there are dramatic elevations in total serum IgA, IgA immune complexes and polymeric IgA with concurrent decreases in total IgM and IgG (Pestka *et al.*, 1990a; Pestka *et al.*, 1990b). In addition to IgA hyperelevation, DON-exposed mice exhibit kidney mesangial IgA deposition and hematuria, which are hallmarks of human IgA nephropathy (IgAN) (Dong *et al.*, 1991).

B.3 DON-induced IgAN. Human IgAN is the most common glomerulonephritis worldwide. Approximately, 150,000 people in the United States have been diagnosed with IgAN with nearly 4000 new cases occurring each year (Hellegers, 1993). Between 20-40% of these will develop progressive renal failure over a 25 year period from the time of initial diagnosis, with 1-2 % of adult patients entering end-stage renal failure yearly and requiring hemodialysis or kidney transplantation (Emancipator and Lamm, 1989). Several lines of evidence support the possibility that IgAN is an immune complex (IC) disease. These include the following: a) the correlation between circulating IgA-IC and disease activities in IgAN patients; b) high-molecular weight IgA from serum and renal eluates of IgAN patients that contain dimeric IgA and activated complement; and c) experimental rodent studies in which IgAN is induced with injected IgA-IC (Emancipator and Lamm, 1989; Montinaro *et al.*, 1999). An overly robust IgA response to mucosal infections and dietary antigens in terms of quantity, size (primarily polymeric) and glycosylation status is believed to contribute to the amount and pathogenicity of IgA-IC in IgAN. Recurrent accumulation can recruit a battery of inflammatory events such as activation of alternative complement system, leading later to glumerulonephritis and kidney failure (Rantala *et al.*, 2001).

DON-induced IgA nephropathy has also other several common traits with the human Berger's disease such as male susceptibility to the disease and polyclonal autoreactive IgA production as well as mesangial IgA deposition (Greene *et al.*, 1994; Rasooly *et al.*, 1994; Rasooly and Pestka, 1994; Greene *et al.*, 1995). It is considered that cytokines, prostaglandins, and growth factors play the central modulatory role in producing defectively modified IgA, reactive T cell, and disease progresses in human IgAN (Rantala *et al.*, 2001).

Whereas DON has little inducible effect on B cells to produce IgA , T cells have been implicated to affect switching in B cells to produce IgA via a diverse array of cytokines (Minervini *et al.*, 1993; Warner *et al.*, 1994). Moreover, exposure to low levels of deoxynivalenol appears to promote expression of several cytokines in vivo and in vitro (Dong *et al.*, 1994; Zhou *et al.*, 1997) and macrophages are also considered as another important component to produce cytokines affecting the humoral IgA production (Wong *et al.*, 1998; Yan *et al.*, 1998). Deoxynivalenol-induced aberrant IgA production may be mediated through an over-expression of cytokine genes in T helper cells and the mononuclear phagocyte system transcriptionally or post-transcriptionally (Ouyang *et al.*, 1996; Li *et al.*, 1997; Wong *et al.*, 1998; Wong *et al.*, 2001). Among these cytokines, interleukin-6 (IL-6) is widely recognized to be particularly critical to regulation of mucosal B cell differentiation to IgA secreting plasma cells (Beagley *et al.*, 1989; Beagley *et al.*, 1991; Fujihashi *et al.*, 1992). Genetic disruption of IL-6 in mice markedly reduces DON-induced increases in serum IgA as well as kidney IgA deposition in mice suggesting a strong correlation between IL-6 and these IgA effects (Pestka and Zhou, 2000). In addition to the role of IL-6 on IgA nephropathy, induction or superinduction of the cytokine is likely to be associated with the shock-like and cytotoxic responses that occur during acute DON

intoxication in mice, synergistically acting with lipopolyssaccharide (LPS) (Zhou *et al.*, 1997; Zhou *et al.*, 1999).

The pattern of cytokine induction by deoxynivalenol is so diverse that it may also contribute to other abnormal pathogenesis such as allergic inflammation or autoimmune dysfunctions (Rasooly *et al.*, 1994; Rasooly and Pestka, 1994). Relatedly, prolonged deoxynivalenol ingestion (6 months) can induce progressive dysregulation of IgE production in addition to the previously described increase in IgA (Pestka and Dong, 1994).

C. Cellular mode of action of trichothecenes

C.1 Target organelles. Although the specific receptors have not yet been identified, ribotoxic stress and oxidative stress are considered to be the primary factors in the trichothecene cytotoxicity (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). Trichothecenes are very heat-stable molecules that inhibit protein synthesis by specific and high affinity binding to ribosome. Notably, the primary target, both in vivo and in vitro, of a variety of trichothecenes is 28S ribosomal RNA, where the peptidyl-transferase activity of the complex is inhibited by the toxins (Shifrin and Anderson, 1999). These translation-inhibiting toxins are cytotoxic to the rapidly dividing cells and thus target the immune system and epithelial tissues. In addition to inhibition of the ribosomal protein synthesis, DON may affect protein processing such as folding and secretion because the toxin down-regulates endoplasmic reticulum chaperone (GRP78/BiP) and co-chaperone P58 which have been known to play critical roles in regulating post-translational modification such as glycosylation and cellular homeostasis (Yang *et al.*, 2000b). As another target of the toxins, some trichothecenes can cause damage cellular membranes. The lipid peroxidation is increased in liver, spleen,

intoxication in mice, synergistically acting with lipopolyssaccharide (LPS) (Zhou *et al.*, 1997; Zhou *et al.*, 1999).

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kidney, thymus, and bone marrow of rat exposed to the trichothecenes (Suneja *et al.*, 1989). Trichothecene-stimulated alteration in mitochondrial membranes contributes to the interference with cellular ATP production from the organelle via damaged electron transport and high chance of production of free radicals.

C.2 Ribotoxic stress and mitogen-activated protein kinases (MAPKs). The ribotoxic stress response is the capacity of the ribosomes to sense cellular stress. The capacity of cellular reaction is notably reflected on MAPK activation by toxicants that perturb the functioning of the 3'-end of the large 28S rRNA such as aminoacyl-tRNA binding, peptidyl transferase activity or ribosomal translocation (Iordanov *et al.*, 1997). Through the inhibition of peptidyltransferase activity, a variety of trichothecenes can cause the ribotoxic stresses in the cells, which then stimulate mitogen-activated protein kinases (MAPKs). MAPKs are evolutionally conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. They also respond to chemical and physical stresses, thereby controlling cell survival and adaptation. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) (Chang and Karin, 2001). Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38a/b/g) and ERK5, that are activated by specific MAPKs : MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and the diversity.

This process can lead to the expression of genes important in cellular homeostasis as well as in the control of cell survival, proliferation and differentiation (Laskin *et al.*, 2002).

Interestingly, MAPK signals such as p38 and JNK can mediate trichothecene-induced apoptotic cell death at high concentrations (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). By and large, activation of ERK1/2 has been linked to cell survival, whereas JNK and p38 are to induction of apoptosis (Xia *et al.*, 1995).

As shown in the dose regimes of the immunotoxicity by trichothecenes, the compounds at sub-lethal concentrations can potentiate expression of cytokines by elevating transcription and increasing mRNA stability whereas higher concentrations of trichothecenes completely shut down translation and induce apoptosis (Ouyang *et al.*, 1996; Wong *et al.*, 2001; Wong *et al.*, 2002). This paradoxical gene expression can be investigated in terms of ribotoxic stress events. As mentioned before, the ribotoxic stress-mediated MAPKs can modulate gene expression transcriptionally or post-transcriptionally and cell survival pathway (Chang and Karin, 2001; Laskin *et al.*, 2002).

D. Cyclooxygenase. As inducible targets by MAPK signaling cascades, eicosanoid-producing enzyme systems was investigated in this thesis because eicosanoids have been known to be involved in diverse inflammatory diseases as well as trichothecne toxicosis (Shohami and Feuerstein, 1986; Shohami *et al.*, 1987; Naseem *et al.*, 1989).

D.1 Production and action of its metabolites : Orthodox process. Products derived from arachidonic acid metabolism affect a variety of biologic processes including inflammation and homeostasis (Vane *et al.*, 1998). They can be thought of as short range hormones that act locally at the site of generation and then rapidly spontaneously decay, or are enzymatically destroyed. Arachidonic acid is a 20-carbon polyunsaturated fatty acid (4 double bonds) derived primarily from dietary linoleic acid and present in the body only in

the esterified form as a component of cell membrane. It is released via cellular phospholipases (PLA2, PLC and PLD) from the membrane and further metabolized by three enzymatic pathways: Cyclooxygenase (COX), Lipoxygenase (LOX), or Cytochrome P450 monooxygenase (Epoxygenases) (Vane *et al.*, 1998). Final products include prostaglandins/thromboxanes, leucotrienes/lipoxin, and epoxyeicosatrienoic acid (EET), respectively (Fig. 1). Prostaglandin production is catalyzed by Prostaglandin Synthetase (PGS) from arachidonic acid via a two-step process involving distinct cyclooxygenase to produce PGG2 (rate-limiting) and peroxidase activities to PGH2 (Smith *et al.*, 2000). Two isoforms of COX have been identified and characterized. COX-1 is constitutively expressed in many tissues and cell types and is presumed to be involved in maintaining cell homeostasis such as hemodynamics and renal function whereas COX-2 is transiently induced by mitogenic or inflammatory stimuli in most tissues (Table 1). COX-2 metabolites may be thus involved in inflammatory responses (Harris *et al.*, 2002). However, COX-2 is constitutively expressed in the brain and kideny of the rodent. Although COX-1 and COX-2 are products of different genes and their amino acid identity is only about 61 % overall, their three-dimensional x-ray crystal structures are virtually superimposable (Serhan and Oliw, 2001). Prostaglandins are ligands for receptor-mediated pathways that may contribute to prolonged activation of biologic responses such as cell growth and differentiation (Harris *et al.*, 2002; Neurath *et al.*, 2002). The rapid metabolic breakdown of prostaglandins suggests that they act in transient autocrine or paracrine fashion to modulate the action and synthesis of other secondary messengers. Prostaglandin E2 (PGE2) exerts its action by binding to one (or a combination) of its four receptors subtypes, EP1, EP2, EP3 or EP4. These receptors are coupled to different G proteins and use several messenger signaling pathways.EP1 is

coupled to Gq/p and ligand binding stimulates protein synthesis through both extracellular and intracellular Ca^{2+} mobilization. EP2 and EP4 are coupled to Gs proteins and induce the elevation of cAMP, which lead to gene expression. EP3 are primarily coupled to Gi and is most often inhibitory to cAMP. In addition, some COX-2 metabolites such as cyclopentanone prostaglandins can bind to a nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-γ, thus impacting proinflammatory gene transcription (Ricote *et al.*, 1998; Thieringer *et al.*, 2000).

D.2 Production and action of its metabolites : Unorthodox routes. There are still uncovered pathways leading to the formation of bioactive prostannoids via COX or non-enzymatic chemical transformation (Serhan and Oliw, 2001). One of these is prostanoids found in seminal fluid such as 19R-hydroxy-PGE compounds which is assumed to be produced by cytochrome P450 such as CYP4F8. Polyunsaturated fatty acids (PUFAs) can also undergo autooxidation and p450 epoxidation in vivo, generating a complex mixture of hydroperoxides, epoxides, and cyclic peroxides. Of particular medical interest are the isoprostanes, which serves as a marker of in vivo lipid peroxidation (Morrow, 2000; Roberts and Morrow, 2000). Aspirin has been known to block COX-mediated prostanoid production. However, it was recently reported that aspirin triggers formation of novel series of lipid mediators, the aspirin-triggered lipoxins (ATLs) (Serhan and Oliw, 2001). Whereas S-configured lipoxins are generated by initial lipoxygenation by 15-LOX followed by 5-LOX, R-configured 15-hydroxyeicosatetraenoic (15-HETE) is produced by aspirin-triggered acetylated COX-2 followed by 5-LOX to present ATL. ATLs analogous to lipoxin are involved in promoting resolution of inflammation by inhibiting neutrophil recruitment and chemokine production (Levy *et al.*, 2001; Levy and Serhan, 2002).

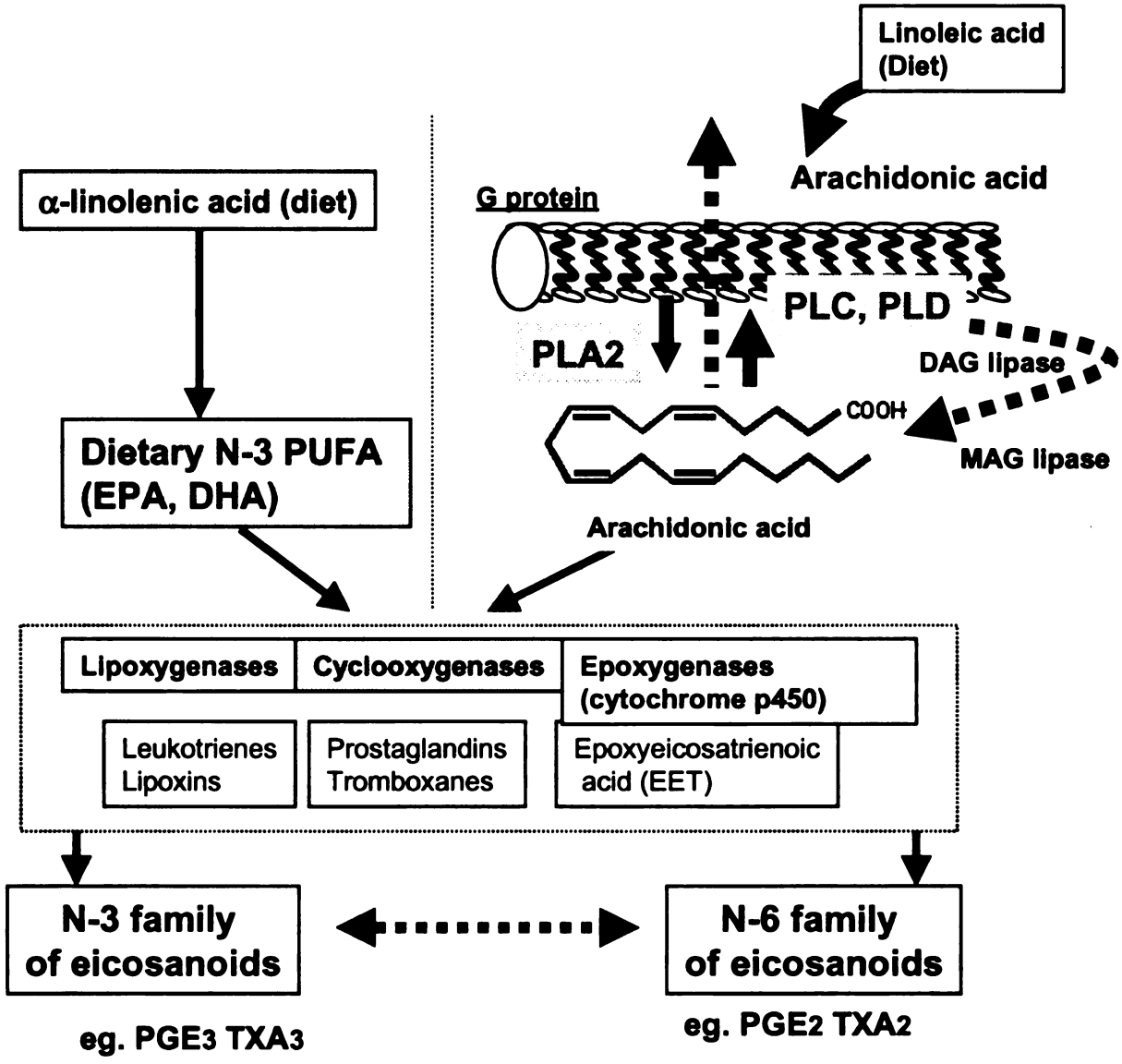

Fig. 1. Metabolism of arachidonic acid and essential fatty acids.

	COX-1	COX-2
Gene locus	9q32-q33.3	1q25.2-q25.3
Size of gene	22 kb	8.3 kb
# of exon	11	10
Size of mRNA	2.8 kb (stable)	4.6 kb (unstable)
Coding nucleotides	1797 bp	1812 bp
# of amino acids	576 aa	604 aa
5'-flanking region	No TATA box, GC rich (Human: AP2, Myb, GATA, Sp1, NF-IL6)	Human: Sp1, NF-kB, AP2, NF-IL6, CRE, E box, TATA
3'-UTR	0.7 kb	2 kb AUUUA rich elements (Conserved region in 100 bp is critical for mRNA stability)
Protein locus	ER and nuclear membrane	Mainly nuclear membrane
Aspirin treatment	No metabolites	15R-HETE
Expression	constitutive in most tissue Inducible in endothelial, platelet, renal collecting tubules, seminal vesicle, gallbladder.	Inducible Constitutive in renal cortex, thyroid tissue, brain.
Regulators	endogenously unknown	PPAR ligands, glucocorticoids

Table 1. Comparison between human COX-1 and COX-2

D.3 MAPK involvement in COX-2 induction signals Several inflammatory stimuli which induce COX-2 gene expression also activate the mitogen-activated protein kinases (MAPKs). Among the MAPKs, c-Jun N-terminal kinase 1 and 2 (JNK1/2), extracellular signal regulated protein kinase 1 and 2 (ERK1/2), and p38 MAPK have been extensively studied relative to COX-2 gene expression (Xie and Herschman, 1995; Guan *et al.*, 1998). The transcriptional activity of COX-2 gene expression is redundantly modulated by MAPKs (Wadleigh *et al.*, 2000; Mestre *et al.*, 2001). Its promoter contains Notably, p38 MAPK plays an important role in the signaling pathway for the stability of COX-2 mRNA, which is dependent on regulatory elements contained within the 3'-UTR of the message (Dean *et al.*, 1999; Lasa *et al.*, 2000). Post-transcriptional regulation is not limited to changes in message stability. Sequences within the 3'-UTR of mRNAs have also been shown to be important for enhancing message translation as well as for translational silencing (Di Liegro *et al.*, 2000; Mbella *et al.*, 2000; Piecyk *et al.*, 2000).

D.4 Involvement of Nuclear Factor kappa B (NF-κB)/Rel-related signaling pathway in COX-2 induction signals The NF-κB/Rel-related signaling pathway has also been implicated in the induction of COX-2 gene expression in various types of cells (Hwang *et al.*, 1997; Inoue and Tanabe, 1998; Smith *et al.*, 2000). Most NF-κB/Rel dimers are bound to IkBs and retained in the cytoplasm. Phosphorylation of IκB by IκB kinase (IKK) complex lead to the ubiquitination of IκB via a non-lysosomal ATP-dependent 26S proteolytic complex composed of a 700 kDa proteasome and allow the release of NF-κB/Rel dimers which translocate to the nucleus for transcriptional regulation of COX-2 gene (Perkins, 2000). The most characterized NF-κB inhibitor is IκBα which binds avidly to the p65 (RelA). It is rapidly and completely degraded following inducible phosphorylation but is

quickly resynthesized in an NF-κB dependent manner (Jobin and Sartor, 2000). In contrast, IκBβ is slowly degraded and lead to persistent activation of NF-κB.

E. N-3 PUFA and its immunomodulation. When assessed as nutraceutical candidates for modifying eicosanoid metabolism, n-3 polyunsaturated fatty acids (n-3 PUFAs) like docosahexaenoic acid (DHA 22:6n-3) and eicosapentaenoic acid (EPA 20:5n-3), which are plentiful in fish oil (FO) and metabolized from a-linolenic acid, have been known to down-regulate inflammatory and autoimmune diseases in human clinical trials. One mechanism is the formation of prostaglandins (PGE3) and thromboxane (TXA3) that counterbalance the effects of often more inflammatory products (PGE2 or TXA2) derived from arachidonic acid (Figure 1). N-3 PUFAs can reduce clotting as well as suppress pain and inflammation.

The spectrum of (n-3) PUFA activities is broad and includes suppression of lymphocyte proliferation, cytotoxic T lymphocyte activity, natural killer cell activity, macrophage-mediated cytotoxicity, neutrophil/monocyte chemotaxis, major histocompatibility complex II expression and antigen presentation. Evidence that these specific cellular effects indeed affect immune function in vivo is reflected in the experimental attenuation of acute inflammation, delayed-type hypersensitivity, graft rejection, and autoimmune responses (Wu and Meydani, 1998; Kehn and Fernandes, 2001). The mechanistic basis for the immune effects of (n-3) PUFA may relate to their capacity to modulate cytokine and inflammatory mediators, cell-to-cell communication, and cellular population via apoptosis (Meydani *et al.*, 1991; Purasiri *et al.*, 1994; von Schacky, 1996; Jolly *et al.*, 2001). Speaking of their cellular events, production of diverse immune mediators is attenuated extensively in tissues from animals and humans fed with (n-3) PUFA.

Fish oil ingestion decreases *ex vivo* pro-inflammatory cytokine production in human mononuclear cells (Caughey *et al.*, 1996), rat blood mononuclear cells (Grimm *et al.*, 1994), mouse thioglycolate-elicited macrophages (Yaqoob and Calder, 1995), and rat kupfer cells (Billiar *et al.*, 1988). In addition to macrophage cytokines, n-3 PUFA also can modulate the differential activation of T cell subsets and their cytokines (Arrington *et al.*, 2001; Jolly *et al.*, 2001). Finally, FO can regulate cellular viability of autoreactive lymphocytes via apoptosis (Fernandes *et al.*, 1998; Jolly *et al.*, 2001; McCarty, 2001). FO consumption also suppress proliferation of carcinoma by apoptotic regulation such as leukemia and colon cancer (Narayanan *et al.*, 2001; Siddiqui *et al.*, 2001).

E.1 Effect on IgA Nephropathy. The observation that (n-3) PUFA attenuate untoward immune responses has lead to consideration of their use as therapeutic agents for human autoimmune and inflammatory disease. Clinically and experimentally, fish oil reduces the severity of rheumatoid arthritis (Kremer *et al.*, 1990; Ergas *et al.*, 2002), immune-related skin disease (Mayser *et al.*, 1998) and systemic lupus erythmatosous (Clark *et al.*, 1989). One of the promising targets of this nutritional therapy can be human IgA nephropathy as discussed previously. As an nutritional therapeutic method against this disease, Donadio proposed that dietary supplementation with fish oil may benefit patients with immune-related renal diseases including IgAN, lupus nephritis and cyclosporine-induced nephrotoxicity (Donadio, 1991). In spite of positive data from many randomized clinical trials evaluating efficacy of fish oil in treating IgAN (Hamazaki *et al.*, 1984; Donadio *et al.*, 1999; Donadio *et al.*, 2001), there are still controversies over these findings. Some clinical trials could not demonstrate definitely alleviating effects of FO or (n-3) PUFA on progress of the IgAN (Bennett *et al.*, 1989; Pettersson *et al.*, 1994). The lack of effect might

relate to the inherent problems with population size, time of follow-up, or stage of disease (Donadio *et al.*, 1999). Thus, although dietary FO supplementation appears to be a promising therapeutics for IgA nephropathy, further research is warranted both to establish the mechanistic basis for the effects and to determine the optimum dosing regimens for EPA and DHA. DON-induced nephropathy in mice is ameliorated with fish oil diet (Pestka *et al.*, 2002) and the cellular mechanism was investigated in this research.

E.2 Cellular mechanisms of (n-3) PUFA. Fish oil has been proposed to act by several mechanisms. First, it may prevent conversion of arachidonic acid to proinflammatory eicosanoids by changing endogenous pool of substrates for lipid metabolic enzymes such as COX or LOX, leading to anti-inflammatory eicosanoids (Serhan and Oliw, 2001). As another pathway of (n-3) PUFAs metabolism than orthodox routes, (n-3) PUFA can be converted to 18R- or 15R-hydroxy-eicosapentaenoic acid (HEPE) by acetylated COX-2 in the presence of aspirin, which can be further metabolized to15R-lipoxin series by 5-LOX, countering pro-inflammatory signals (Serhan *et al.*, 2002).

DHA and EPA inhibit ERK 1/2 activity in human T cells, macrophages and tumor cells (Lo *et al.*, 2000; Denys *et al.*, 2001a; Denys *et al.*, 2001b; Denys *et al.*, 2002). Fatty acids are known to modulate activity of signaling molecules such as phospholipase C and protein kinase C (PKC) (May *et al.*, 1993; Hwang *et al.*, 1996). The molecular mechanism for inhibitory action of (n-3) PUFAs on ERK 1/2 has been previously investigated in relation to PKC (Denys *et al.*, 2001a; Denys *et al.*, 2001b; Denys *et al.*, 2002). Notably, DHA and EPA inhibit PKC α and ε, which are known to be located at the upstream of MAP kinase cascade (Clark and Murray, 1995). (n-3) PUFAs inhibit the enzymatic activity of theses PKC isoforms either via direct interaction with phosphatidylserine binding site of PKC

(Nishizuka, 1995) or indirectly by producing diacylglycerol-containing PUFA (Marignani *et al.*, 1996). It should be also noted that (n-3) PUFA have been recently shown to suppress ERK 1/2 activity through PKC-independent pathway (Denys *et al.*, 2002).

RESEARCH RATIONALE

DON is an unavoidable food toxicant frequently found in usual U.S. human diet and causes acute and chronic inflammatory diseases such as circulatory shock-like syndrome and IgA nephropathy in the animal model. The molecular mechanisms about the inflammation-causing mediators need to be investigated in order to support better risk assessment of DON. At the cellular level, a variety of trichothecene mycotoxins are known to activate MAPK cascades via ribotoxic stress by inhibiting peptidyltransferase activity of 3'-end of 28S rRNA. A question started in order to address the involvement of MAPK activation by DON in the toxin-induced immunotoxicity. Altered MAPK signaling by ribotoxic stress could modulate proinflammatory gene induction as well as cell death in response to chemical insults. Particularly, this study has focused on MAPK-mediated COX-2 gene induction which is expected to contribute to trichothecene-mediated inflammation. COX-2 is a rate limiting enzyme in the inducible synthesis of prostaglandin endoperoxides. These lipid mediators are critical components of the inflammation response such as acute shock and chronic nephritis.

Previous studies demonstrated the central role of pro-inflammatory IL-6 in DON-induced shock and in IgAN. This thesis assessed the relationship between of DON-induced COX-2 and IL-6 production. A positive association exists between endogenous COX-2 metabolites and IL-6 synthesis in vitro and in vivo in models of several inflammatory diseases (Dendorfer *et al.*, 1994; Meyer *et al.*, 1994; Meyer *et al.*, 1995; Anderson *et al.*, 1996; Hinson *et al.*, 1996; Williams and Shacter, 1997; Zeng *et al.*, 1998; Williams *et al.*, 2000). Moreover, PGE2 has been considered as one of the pivotal driving factors in the Th2-

dominant immune response by polarizing Th2 cytokines(Betz and Fox, 1991; Kozawa *et al.*, 1998; Kuroda *et al.*, 2000).

As an experimental probe, Fish oil was applied to test the hypotheses since FO has been shown to alter MAPK signaling as well as COX-2 metabolism in several inflammatory models. In addition, FO as a therapeutic agent is recently known to specifically suppress DON-induced IgA nephropathy (Pestka *et al.*, 2002).

The guiding hypothesis for this thesis is that ribotoxic stress-mediated MAPK can induce COX-2 gene which contributes to immunotoxicity of DON. Four specific aims were proposed based on the hypotheses.

Aim 1. To test the hypothesis that DON modulates COX-2 gene expression via ribotoxic-stress-mediated MAPK (Chapter 2).

Aim 2. To test hypothesis that other trichothecene families have similar inducible effects on COX-2 gene in a global way (Chapter 3).

Aim 3. To test the hypothesis that COX-2 metabolites affect DON-mediated IL-6 production (Chapter 4).

Aim 4. To test the hypothesis that fish oil ameliorates COX-2 and IL-6 responses to DON via MAPK signaling (Chapter 5).

CHAPTER 2.

DON-INDUCED COX-2 EXPRESSION VIA MAPK PATHWAY [1]

[1]This work was published in the title "Vomitoxin-Induced Cyclooxygenase-2 Gene Expression in Macrophages Mediated by Activation of ERK and p38 but Not JNK Mitogen-Activated Protein Kinases" (Moon and Pestka, 2002) and highlighted in the same issue, "The ribotoxic stress response as a potential mechanism for MAP kinase activation in xenobiotic toxicity" (Laskin *et al.*, 2002).

ABSTRACT

Deoxynivalenol and other trichothecene mycotoxins mediate a broad range of immunotoxic effects via the induction of inflammation-associated genes in leukocytes. The purpose of this study was to test the hypothesis that DON induces cyclooxygenase-2 (COX-2) gene expression in macrophages and that this is regulated at the level of mitogen-activated protein kinases (MAPKs). Exposure of the murine macrophage cell line RAW 264.7 to 50 to 250 ng/ml DON for 24 hr markedly enhanced the production of prostaglandin E2 (PGE2), a major COX-2 metabolite. PGE2 elevation was preceded by increases in COX-2 mRNA (2 hr) and COX-2 protein (15 hr) in DON-treated cells. DON induced rapid (15 min) and persistent (up to 240 min) phosphorylation of extracellular signal regulated protein kinases 1 and 2 (ERK1/2) and p38 MAPK as well as a rapid (15 min) but transient (up to 60 min) phosphorylation of c-Jun N-terminal kinases 1 and 2 (JNK1/2). The ERK inhibitor PD98059 and p38 inhibitor SB203580 suppressed DON-induced PGE2 and COX-2 protein expression, whereas impairment of JNK function by transient transfection with a dominant negative(dn) JNK vector had no effect of COX-2 protein expression. Relatedly, in cells transfected with a COX-2 promoter-luciferase construct, PD98059 and SB203580 but not dnJNK treatment suppressed DON-induced luciferase transcription. DON also increased COX-2 mRNA stability and this was inhibited by PD98059 but not SB203580. Taken together, these results indicate that DON induced PGE2 production and COX-2 expression by elevating transcriptional activity and mRNA stability. Enhanced transcriptional activity was modulated by ERK and p38 signaling pathways whereas mRNA stability was promoted exclusively by

DON-activated p38 phosphorylation. These data provide insight into possible general mechanisms by which DON and other trichothecenes up-regulate proinflammatory genes and impart immunotoxicity.

INTRODUCTION

The trichothecene mycotoxins are a group of sesquiterpenoid fungal metabolites that include some of the most potent eukaryotic protein synthesis inhibitors known (Ueno, 1983). Interest in the trichothecene mycotoxins arises from their widespread contamination of agricultural commodities (Kotsonis *et al.*, 1996), their recalcitrance to degradation during milling or processing (Scott, 1990) and their adverse effects on human and animal health (Bhat *et al.*, 1989; Peraica *et al.*, 1999). Deoxynivalenol, a trichothecene mycotoxin produced by *Fusarium graminearum* and *F. culmorum,* is frequently found in grain-based agricultural products, particularly in wheat and barley grown in the Midwestern United States (Abouzied *et al.*, 1991).

Animals exhibit feed refusal and weight loss upon chronic exposure to low dietary DON concentrations, whereas acute high level exposure to DON can cause nausea, vomiting, and leukocytosis in experimental animals (Rotter *et al.*, 1996). The immune system is particularly susceptible to DON with macrophages, B cell, and T cells being highly sensitive to the toxin (Bondy and Pestka, 2000). Notably, mice exposed to DON develop clinical signs that mimic human IgA nephropathy (Dong *et al.*, 1991). The broad spectrum of toxicity found for DON and other trichothecenes is likely to relate, in part, to their capacity to evoke production of proinflammatory mediators (Azcona-Olivera *et al.*, 1995a; Azcona-Olivera *et al.*, 1995b; Zhou *et al.*, 1997; Ji *et al.*, 1998; Wong *et al.*, 1998; Zhou *et al.*, 1999; Wong *et al.*, 2001).

Metabolites of arachidonic acid are known to play key roles in the proinflammatory

responses (Smith *et al.*, 2000). Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the oxygenation of arachidonic acid to prostaglandin endoperoxides. These metabolites are converted enzymatically into prostaglandins and thromboxane A_2 which play both physiologic and pathologic roles in a diverse array of inflammatory sequelae (Vane *et al.*, 1998; Smith *et al.*, 2000). Two distinct isoforms of COX have been identified.COX-1 is constitutively expressed at low levels in most tissues and may be related with housekeeping function. In contrast, COX-2, a 70 kd protein, is strongly induced by mitogenic and proinflammatory stimuli, superinduced by protein synthesis inhibitors, and can be regulated at both transcriptional and posttanscriptional levels (Fletcher *et al.*, 1992; Newton *et al.*, 1997a; Newton *et al.*, 1997b; Newton *et al.*, 1998; Dixon *et al.*, 2000; Wadleigh *et al.*, 2000).

Several inflammatory stimuli which induce COX-2 gene expression also activate the mitogen-activated protein kinases (MAPKs). Among the MAPKs, c-Jun N-terminal kinases 1 and 2 (JNK1/2), extracellular signal regulated protein kinases 1 and 2 (ERK1/2), and p38 MAPK have been extensively studied relative to their regulation of COX-2 gene expression(Xie and Herschman, 1995; Guan *et al.*, 1998; Ridley *et al.*, 1998; Scherle *et al.*, 1998). Upon exposure to the prototypic inflammagen lipopolysaccharide (LPS), transcriptional regulation of COX-2 gene expression is redundantly modulated by the three MAPK families (Wadleigh *et al.*, 2000; Mestre *et al.*, 2001) whereas p38 plays an important role in the signaling pathway for the stability of COX-2 mRNA (Dean *et al.*, 1999; Lasa *et al.*, 2000).

Several investigations have suggested that trichothecenes can increase arachidonic acid metabolism and upregulate prostaglandin production (Shohami and Feuerstein, 1986;

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Naseem *et al.*, 1989). Recently, we have determined that a single acute exposure to DON induces COX-2 gene expression in the murine spleen (Islam *et al.*, 2002). Furthermore, trichothecenes have been suggested to activate MAPKs via a ribotoxic stress response (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). Based on these observations, we hypothesized that DON induces COX-2 expression in macrophages and that this was regulated at the level of MAPKs. The aim of this study was to (1) assess the effects of DON on COX-2 mRNA and protein expression in the RAW 264.7 macrophage model and to (2) relate DON-induced MAPK activation to transcriptional and post-transcriptional regulation of COX-2 gene expression. The results indicate that DON induces COX-2 expression and that MAPKs play critical roles in both transcriptional and post-transcriptional regulation of this gene response.

MATERIALS AND METHODS

Cell culture. RAW 264.7 murine macrophage cells (American Type Culture Collection, Rockville, MD) (2.5 x 10^5 per ml) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals Inc, Norcross, GA), 100 Unit/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in a 5 % CO_2 humidified incubator at 37°C. Macrophage cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hematocytometer as described by Strober (1991).

Prostaglandin E2 (PGE2) assay. PGE2 was measured using an EIA kit (Cayman Chemical Co., Ann Arbor, MI). Cell culture supernatants were collected 18 hr after DON treatment. The aliquots were diluted 10-fold in fresh culture medium and the assays were conducted according to the instructions of the supplier.

Western blot analysis. At the time of harvest, cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1% [w/v] SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris pH 7.4), and sonicated for 5 seconds. The amount of protein was assayed by Lowry method using Dc protein assay reagent (Bio-Rad, Cambridge, MA). Extracts (10 μg) were mixed with Laemmli sample buffer (Bio-Rad), and boiled for 5 min before resolving on a 10 % (w/v) acrylamide gel. Resolved proteins were transferred to PVDF membrane and blocked with Tris-buffered saline (10 mM Tris-HCl pH 7.5, 100 mM

NaCl) containing 0.1 % (v/v) Tween-20 and 1% (w/v) BSA (TBST-BSA). The membrane was incubated for 1 hr with MAPK antibodies (rabbit IgG, New England Biolab, Beverly, MA) at 1:1000 dilution or COX-2 antibody (mouse IgG1, Transduction Laboratories, Lexington, KY) at 1:250 dilution in TBST-BSA, and then were washed three times with TBST. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA) at 1:5000 dilution in TBST-BSA for MAPK detection or with HRP-conjugated anti-mouse IgG (Sigma, St. Louis, MO) at 1:10,000 dilution for COX-2 detection. After washing three times with TBST, bound HRP-conjugated antibody was detected with Enhanced Chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions.

Reverse transcription-competitive polymerase chain reaction (RT-cPCR). RNA was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA (100 ng) from each sample was transcribed to cDNA by reverse-transcriptase (Riedy *et al.*, 1995). COX-2 cDNA was amplified competitively with a truncated COX-2 cDNA internal standard constructed by the bridging-deletion method (Hall *et al.*, 1998). The amplification was performed in a 9600 Perkin Elmer Cycler (Perkin-Elmer Corp., Norwalk, CT) using the following parameters: 30 cycles of reactions of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.5 % (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers were synthesized at Michigan State University Molecular Structure facility. The 5' forward and 3' reverse-complement PCR primers for amplification of mouse COX-2 cDNA were ACACTCTATCACTGGCATCC

and GAAGGGACACCCTTTCACAT, respectively. Sizes of amplified COX-2 cDNA internal standard cDNA were 584bp and 500 bp, respectively. The densitometric ratio of COX-2 cDNA / COX-2 internal standard was used to construct a standard curve to calculate COX-2 cDNA concentrations in RT reaction products.

Plasmids and transfections. The 5' upstream segment (-724/+7) of the mouse COX-2 gene from spleen chromosomal DNA was cloned into pXP2 (ATCC, Manasas, VA) at Hind III/Xho I restriction sites to construct a mouse COX-2 promoter-luciferase plasmid (pXP-5COX2). An expression vector for the dominant negative JNK1 (dnJNK) was kindly provided by Roger Davis (University of Massachusetts Medical School, Worcester, MA). All plasmids were purified with Endofree Plasmid Prep Kit (Qiagen, Valencia, CA).

For transfections, RAW 264.7 cells (4x 10^5/ml) were washed twice in serum-free DMEM and then incubated for 3 hr with a premixed complex of plasmid and lipopectamine (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. The cells were then replaced with fresh serum-containing DMEM and incubated for 24 hr prior to DON exposure. pCMV-β-gal (1 μg) (BD Biosciences Clontech, Palo Alto, CA) was also co-transfected with 1 μg promoter-luciferase construct to standardize transfection efficiency.

JNK activity assay. For determination of JNK activity, cells (5x10^5) were washed once with ice cold PBS and incubated for 5 minutes with 0.5 ml ice-cold lysis buffer (20 mM Tris [pH 7.4] containing 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). After incubation, cells were harvested, sonicated, centrifuged at 14,000 rpm

in a microcentrifuge for 10 min and the supernatant was collected for the assay. c-Jun fusion protein beads (20 μg, Cell signaling, Beverly, MA) were added to the supernatant (250 μl) and the mixture was incubated with gently rocking overnight at 4°C. The bead complex was collected, suspended and incubated with 100 μM ATP in kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM $Na_3 VO_4$, 10 mM $MgCl_2$) for 30 min at 30°C. The reaction was terminated by Laemmli sample buffer and centrifuged. The supernatant was analyzed by Western immunoblotting with phospho-c-Jun rabbit antibody (Cell Signaling, Beverly, MA).

Luciferase and β-galactosidase assays. Cells were washed with cold PBS, lysed with lysis buffer (25 mM Tris-H_3PO_4, pH 7.8, 2 mM EDTA, 2 mM DTT, 10 % [v/v] glycerol, 1% [v/v] Triton X-100) and then centrifuged at 12,000 xg for 2 min. Resultant supernatant was stored at -80°C until assessment of luciferase activity and b-galactosidase. Luciferase activity was measured with a luminometer (Model 20e, Turner Designs Co., Sunnyvale, CA) after briefly mixing the supernatant with an equal volume Luciferase Assay Systems substrate solution (Promega, Madison, WI). β-galactosidase activity was measured with β-galactosidase Enzyme Assay Kit (Promega, Madison, WI). Luciferase activity was normalized against β-galactosidase activity using the following formula: luciferase activity / β-galactosidase activity.

Statistics. Data were analyzed using Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). For comparisons of two groups of data, Student's t test was performed. For comparisons of multiple groups of data a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. Differences were considered significant if $p < 0.05$. Quantitative results were expressed as mean ± SEM.

RESULTS

Induction of COX-2 activity and gene expression by DON in RAW 264.7 cells.

Since DON previously has been shown to induce proinflammatory cytokines as well as nitric oxide and hydrogen peroxide in the RAW 264.7 macrophage cell line (Ji *et al.*, 1998), it seemed possible that COX-2 might be similarly induced. The effects of DON on COX-2 activity and expression were therefore assessed in RAW 264.7 cells. Exposure to 50 to 250 ng/ml DON for 24 hr markedly enhanced the production of PGE2, one of the major COX-2 metabolites (Fig. 2A). The veracity of the assay was confirmed using LPS which is well known to induce COX-2 in macrophage cells including RAW 264.7 cells. To explain PGE2 elevation by DON, protein and mRNA levels of COX-2 were measured using Western blot analysis and RT-competitive PCR, respectively. Treatment with 100 and 250 ng/ml DON for 15 hr caused dose-dependent increases of COX-2 protein (Fig. 2B). COX-2 mRNA was also affected by 2 hr DON treatment (Fig 2C), the time point at which the mRNA level was maximal (data not shown). Ratios of upper band (native cDNA) to lower band (internal standard cDNA) corresponding to COX-2 mRNA were also increased by 100 and 250 ng/ml DON treatment (Fig. 2C). Thus, DON markedly up-regulated COX-2 gene expression and enzyme activity in RAW 264.7 cells.

Relationship of MAPK activation to DON-induced COX-2 expression.

MAPKs are known to be important signaling modulators in COX-2 expression and these have been recently shown to be activated by trichothecene mycotoxins (Shifrin and

Anderson, 1999; Yang *et al.*, 2000c). Therefore, the involvement of MAPK signaling pathways in DON-induced COX-2 expression was investigated. When the effects of DON on phosphorylation of ERK, p38, JNK were analyzed by Western blot analysis, the toxin was found to enhance phosphorylation of all three MAPKs, with maximal effects being observed 15 to 30 min after 250 ng/ml toxin exposure (Fig. 3). ERK and p38 were persistently activated (up to 240 min) whereas JNK phosphorylation was transient and not detectable after 60 min. The levels non-phosphorylated MAPKs were unaffected by DON treatment.

Treatment with MAPK signaling inhibitors, PD98059 (an MEK1/2 inhibitor which inhibits ERK activation) or SB203580 (a p38 MAPK inhibitor), significantly reduced DON-induced PGE2 production (Fig. 4A). DON-enhanced COX-2 protein expression was also down-regulated in the presence of these MAPK inhibitors (Fig.4B). Both PD98059 and SB203580 inhibited ERK and p38 activities, respectively, at the concentrations employed without having cytotoxic effects (data not shown). COX inhibitors such as indomethacin and NS-398 suppressed PGE2 production to vehicle levels (Fig. 4A). In contrast, indomethacin did not decrease but rather enhanced COX-2 protein (Fig. 4B) which is consistent with stabilization effects previously attributed to NSAIDS (Pang and Hoult, 1996; Callejas *et al.*, 1999). As expected, the positive control LPS induced PGE2 and COX-2 expression.

JNK is also an important MAPK family that has been shown to influence COX-2 expression in several systems (Subbaramaiah *et al.*, 1998; Wadleigh *et al.*, 2000; Yang *et al.*, 2000c). Inhibition with a dominant negative JNK expression vector (dnJNK) did not reduce DON-induced COX-2 expression (Fig. 5). The effectiveness of treatment with dominant negative JNK was verified by observing decreased phosphorylation of substrate c-Jun.

In total, these data suggest that activation of ERK and p38 MAPK but not JNK were

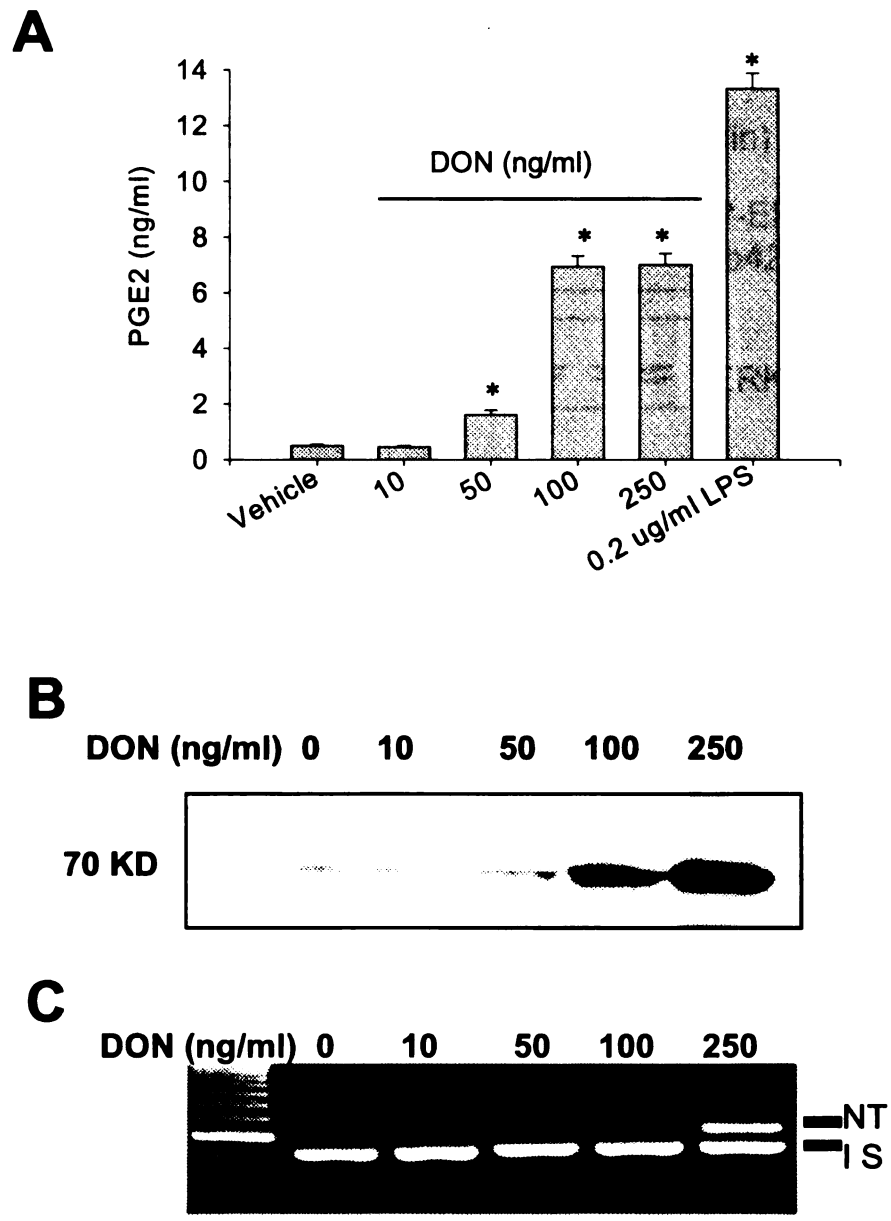


Figure 2. Dose-response effects of DON on PGE2 and COX-2 gene expression in RAW 264.7 cells. Cells (2.5 x10^5/ml) were incubated for 24 hr (A), 15 hr (B), or 2 hr (C) and analyzed by ELISA (A), Western blot analysis (B), or RT-competitive PCR (C). ELISA data are mean +/- SE (n=5). NT and IS refer to native transcript and internal standard, respectively. Asterisk indicates significant difference ($p<0.05$) from vehicle group. Results are representative of at least three experiments.

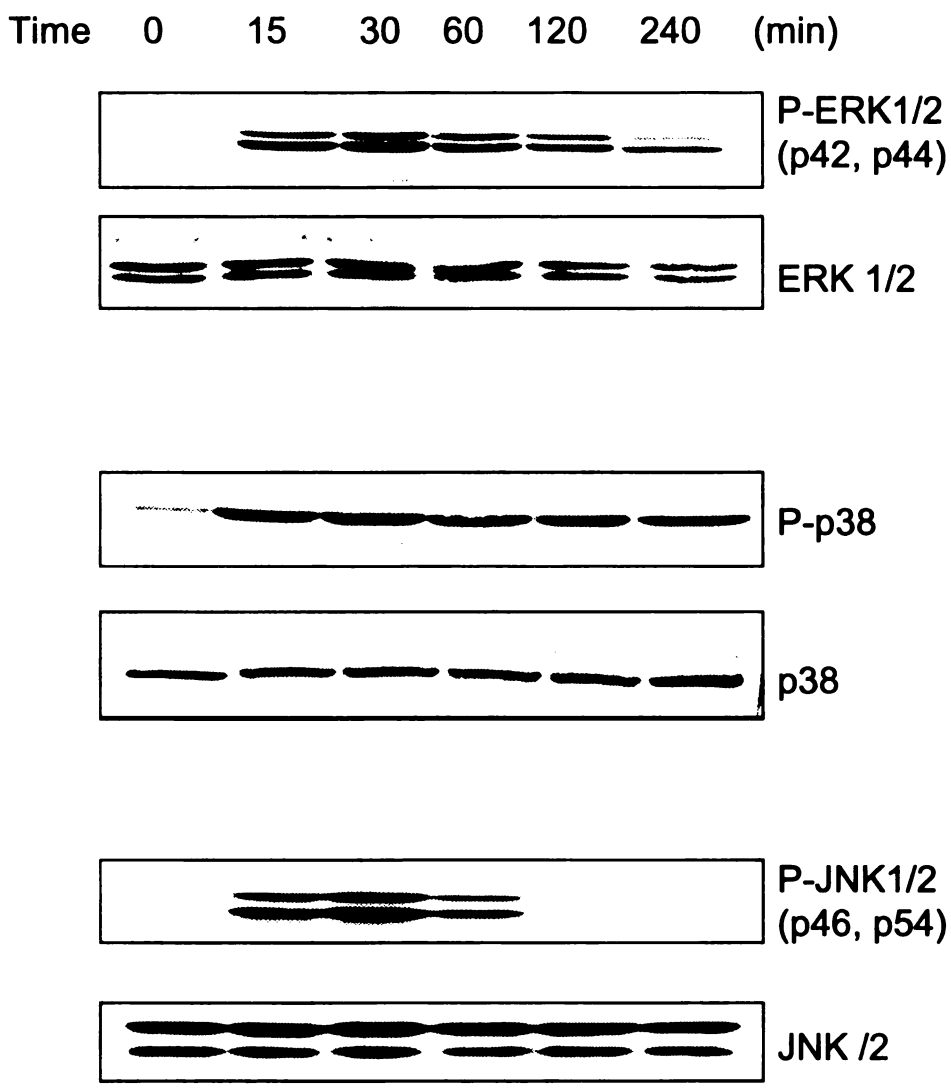


Figure 3. Activation of MAPKs by DON in RAW 264.7 cells. Cells (2.5 x10^5/ml) were incubated for each time point (0, 15, 30, 60, 120, and 240 min) with 250 ng/ml DON. Cellular lysates were analyzed by Western blot analysis. Results are representative of three experiments.

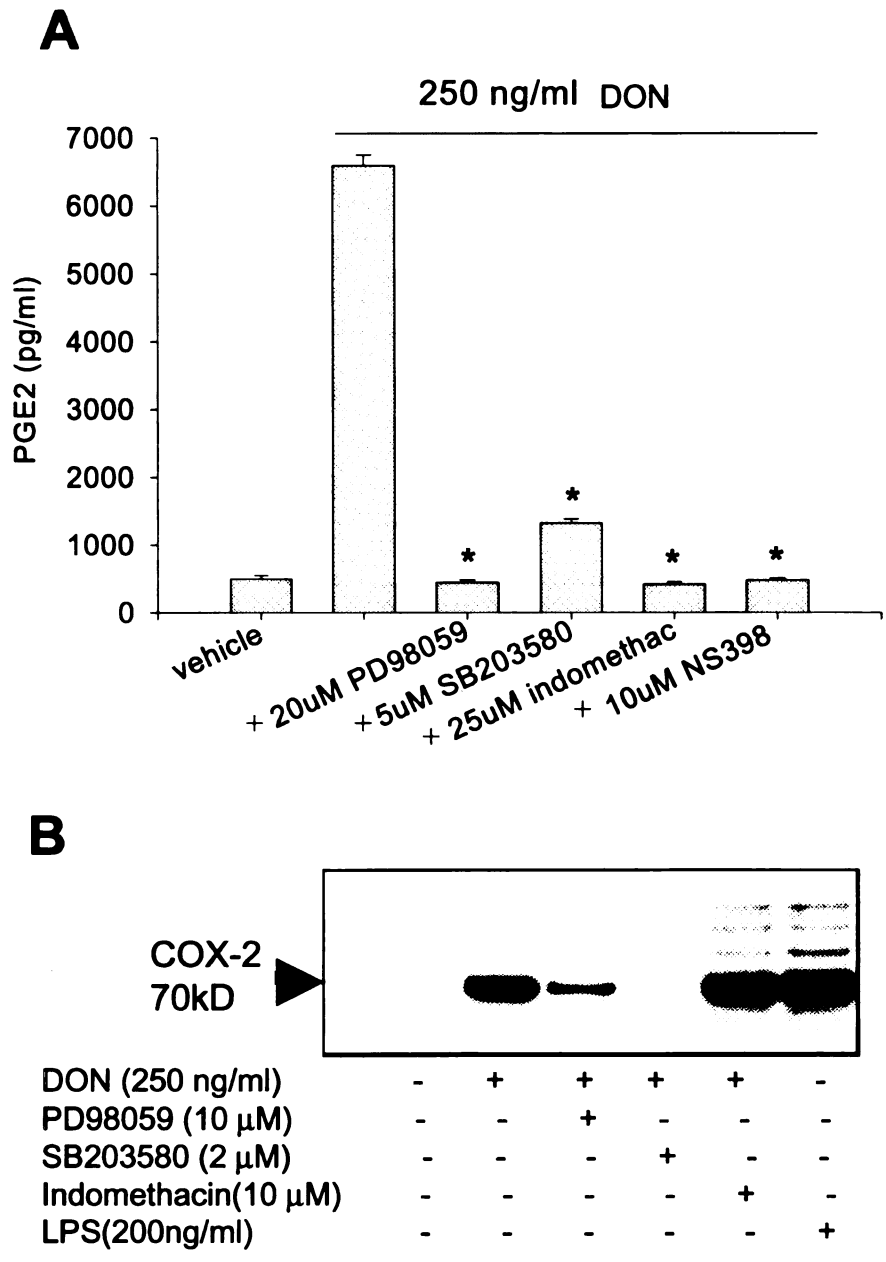


Figure 4. Involvement of MAPK (ERK and p38) in DON-induced COX-2 expression. (A) cells (2.5 x10^5/ml) were treated with each chemical combination for 24 hr and PGE2 was measured from the culture supernatant.(B) cells (2.5 x10^5/ml) incubated with each chemical combination was collected for Western blot analysis of cellular lysate. ELISA data are mean +/- SE (n=5). Asterisk indicates significant difference ($p<0.05$) from vehicle group. Results are representative of three experiments.

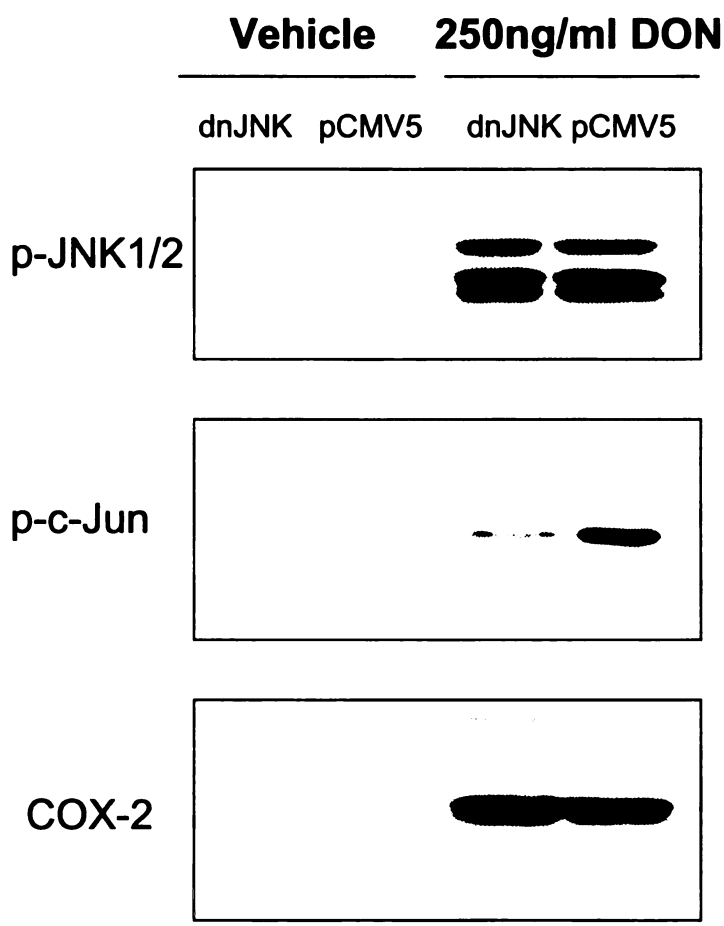


Figure 5. Role of JNK in DON-induced COX-2 expression. After cells (2.5 x10^5/ml) were transiently transfected with empty vector (pCMV5) or dnJNK-containing pCMV5. Cells were incubated with 250 ng/ml DON for 30 min for p-JNK and p-c-Jun and 15 hr for COX-2. Cellular lysate was analyzed for p-JNK1/2 and COX-2 by Western blot analysis. For assessment of JNK activity, the capacity of lysates to catalyze phosphorylation of c-Jun was assessed by immunoprecipitation in conjunction with Western blot analysis. Results are representative of three experiments.

critical in DON-induced upregulation of COX-2 gene expression. The specific roles of these MAPKs on COX-2 transcription and mRNA stabilization were therefore examined further.

Role of MAPKs in DON-induced transcriptional activation of COX-2 expression.

To analyze the effect of DON on COX-2 promoter activity, the 5' COX-2 promoter region was cloned into a luciferase reporter vector (pXP2) system and the resultant plasmid (pXP-5COX2) was transiently transfected into RAW 264.7 cells. DON at concentrations of 10 to 250 ng/ml significantly increased luciferase expression dose-dependently in the transfected cells at 12 hr (Fig. 6). LPS (200 ng/ml) was used as the positive control because it has also been shown to activate COX-2 promoter activity in RAW 264.7 cells (Paul *et al.*, 1999; Wadleigh *et al.*, 2000). In contrast, DON had no effect on luciferase activity in cells transfected with the empty vector, pCMV5 (data not shown).

The involvement of MAPKs in DON-induced transcriptional activation was investigated. The MAPK inhibitors (SB203580 or PD98059) significantly suppressed luciferase reporter induction in DON-treated transfected cells (Fig. 7A and 7B). However, JNK inhibition by dnJNK had no effect on elevated luciferase reporter expression by DON (Fig. 7C), which was consistent with the above-described lack of effect on COX-2 protein expression (Fig. 5).

Taken together, the results indicate that ERK and p38 MAPK activation by DON contributed to transcriptional activation of the COX-2 gene, but JNK did not appear to be involved in the COX–2 promoter activation by DON.

Role of p38 MAPK in DON-induced post-transcriptional regulation of COX-2 expression.

Stability of mRNA is also a potentially important factor in COX-2 gene regulation.

The direct effect of DON on the COX-2 mRNA stability was analyzed by measuring the kinetics of mRNA decay after induction with 200 ng/ml LPS. Cells were collected at intervals following transcriptional arrest by actinomycin-D (Act-D) with or without DON 3 hr after LPS induction (Fig. 8). Marked decay of COX-2 mRNA was observed after transcriptional arrest in control cultures, whereas DON markedly delayed COX-2 mRNA degradation (Fig. 8).

To specifically address the involvement of MAPK in the DON-enhanced mRNA stability, the effects of MAPK inhibitors (PD98059 and SB203580) were evaluated when COX-2 induction by DON was maximal (2 hr after DON exposure). After the maximal induction, cells were arrested with Act-D in absence or presence of MAPK inhibitors. Inhibition of ERK had no effect on COX-2 mRNA stability with observed half-lives exceeding 8 hr (Fig. 9A). In contrast, COX-2 mRNA half-life was reduced to only 35 min in the presence of p38 inhibitor SB 203580 (Fig. 9B). These results suggest that the p38 MAPK signaling pathway may be critical to COX-2 mRNA stability observed in DON-treated RAW 264.7 cells.

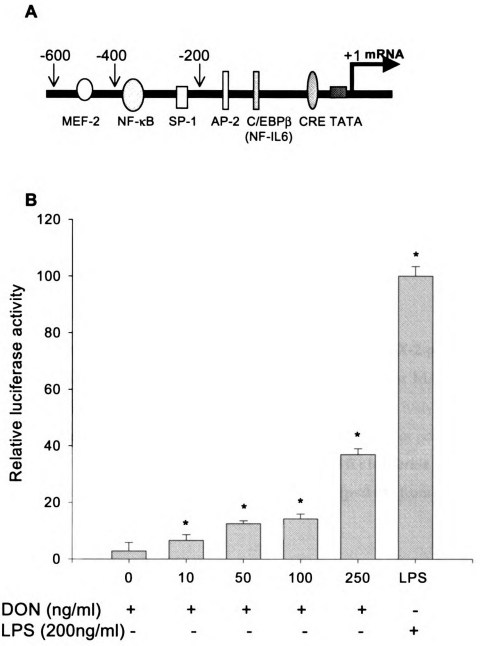
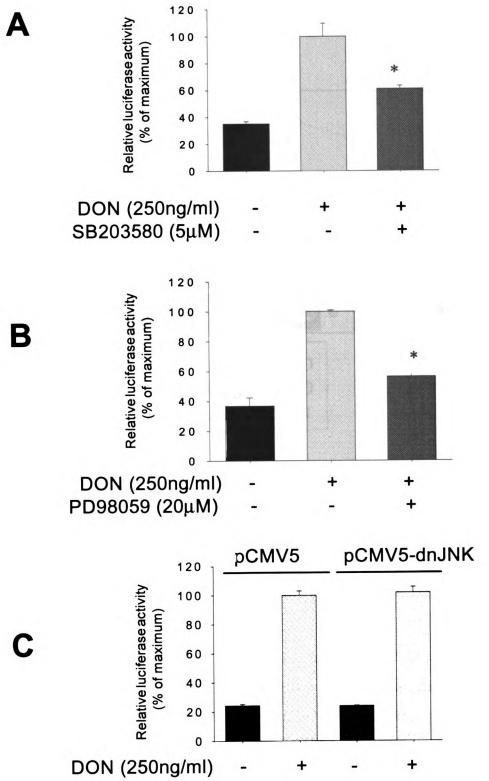


Figure 6. Effect of DON on COX-2 promoter activity. (A) Gross map of murine COX-2 promoter. (B) pXP-5COX2 transfected cells were treated with vehicle or DON for 12 hr and cell lysates were analyzed for luciferase assay. Data are mean +/- SE (n= 3). Asterisk indicates significant difference ($p<0.05$) from vehicle group. Results are representative of three experiments.

Figure 7. Involvement of MAPK in DON-induced activation of COX-2 promoter. Cells transfected with pXP-5COX2 were treated with or without DON or MAPK inhibitors (SB203580 [5 μM] or PD98059 [15 μM]) for 12 hr (A and B), respectively. For dominant negative JNK expression, cells were also co-transfected with pCMV5 or pdnJNK and then treated with DON for 12 hr (C). All cell lysates were analyzed for luciferase assay. Data are mean +/- SE (n=3). Asterisk indicates significant difference ($p<0.05$) from vehicle group. Results are representative of three experiments.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SB203580 (5μM) - - +
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Relative luciferase activity (% of maximum)
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*
DON (250ng/ml) - + +
PD98059 (20μM) - - +
C
pCMV5
pCMV5-dnJNK
Relative luciferase activity (% of maximum)
120
100
80
60
40
20
0
DON (250ng/ml) - + - +

Figure 7

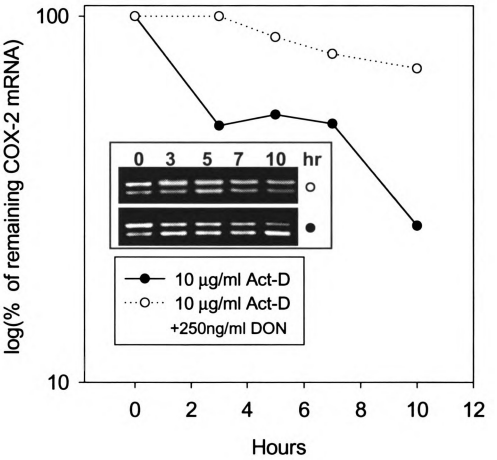


Figure 8. Effect of DON on COX-2 mRNA stability following LPS induction. Cells (2.5 x10^5/ml) were treated with 200 ng/ml LPS and then transcription was arrested with 10 µM actinomycin D in absence or presence of 250 ng/ml DON for specific periods (0, 2, 4, 6, 8, or 10 hr). After RT-competitive PCR with cellular RNA, cDNA was separated by agarose electrophoresis and DNA bands were quantified by image analysis. Results are representative of three experiments.

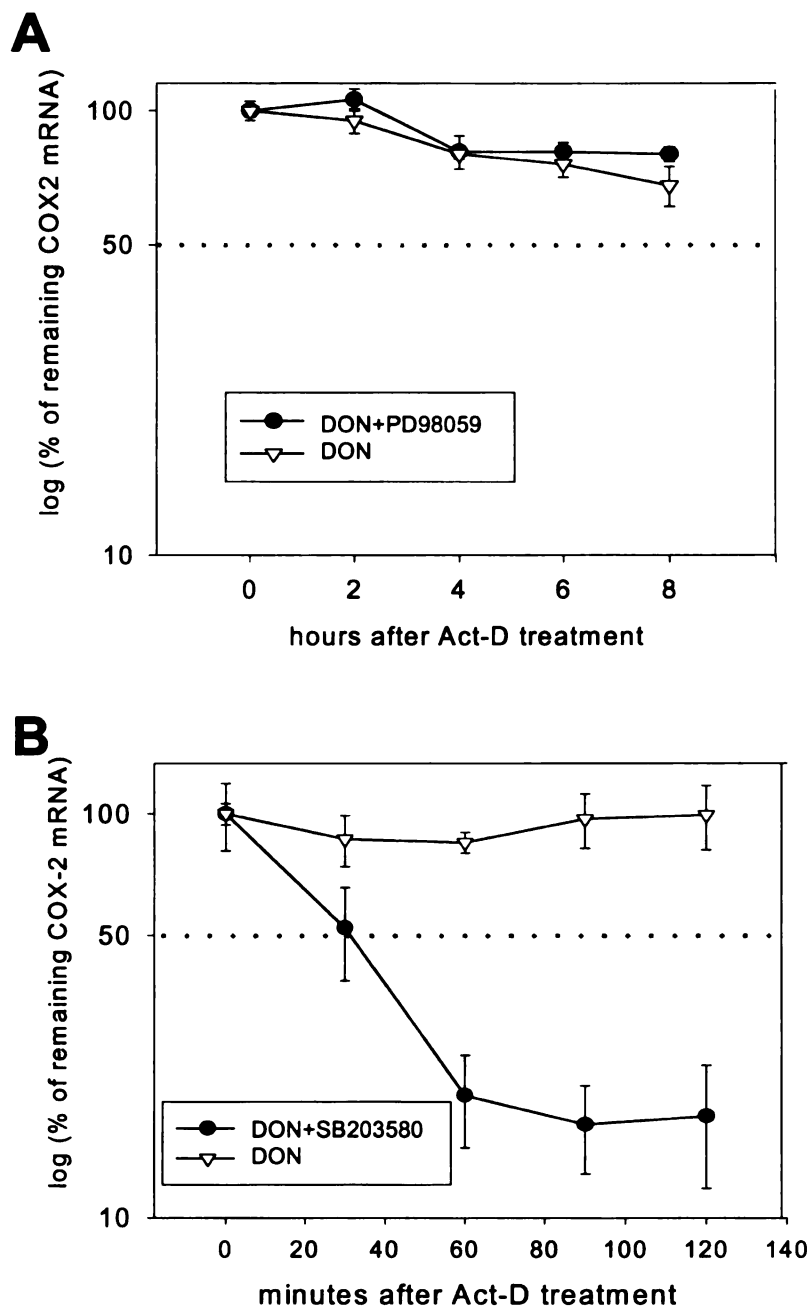


Figure 9. Role of MAPK in DON-induced COX-2 mRNA stability. Cells (2.5 x10^5/ml) were activated with 250 ng/ml DON for 2 hr and then transcription was arrested with 10 μM actinomycin D in absence or presence of (A) MEK inhibitor PD98059 (15 μM) or (B) p38 inhibitor SB203580 (5 μM). Samples were analyzed as described in Fig. 8 legend. Results are representative of two experiments.

DISCUSSION

This is the first report that a trichothecene can directly induce COX-2 gene expression in macrophages. This result is significant because induction of COX-2 metabolites including the prostaglandins and thromboxanes are well-known mediators of various inflammatory manifestations as well as immunological disorders such as hypersensitivity and autoimmune diseases (Myers *et al.*, 2000; Pang, 2001; Samad *et al.*, 2001). Our findings are consistent with previous reports that several kinds of COX-2 products such as PGE2, TXB2 and 6-keto-FGF1a are elevated following treatment with the trichothecene T-2 toxin, and that these effects are associated clinical responses which resembled bacterial endotoxemia (Shohami and Feuerstein, 1986; Shohami *et al.*, 1987). Further critical finding in this study was the establishment of a definitive link between DON-activated MAPK and DON-induced COX-2 expression at the transcriptional and post-transcriptional levels (Fig. 30). These results are partially consistent with the observations that, in LPS-stimulated macrophages, MAPKs can affect COX-2 gene expression by altering both transcriptional activity and mRNA stability (Smith *et al.*, 2000; Vogel, 2000).

The DON concentrations used here to alter COX-2 or luciferase expression are consistent with levels of the toxin found in plasma and tissues of mice that had been treated orally with sufficient DON to upregulate COX-2 expression (Islam *et al.*, 2002) or proinflammatory cytokine expression in the spleen (Azcona-Olivera *et al.*, 1995a; Zhou *et al.*, 1997). In the latter study, concentrations of DON equivalents in spleen 0.5 to 8 hr after exposure to 5 mg DON/kg body weight were 680 to 106 ng/ml, respectively. Thus, the

effective DON concentrations observed here (10 to 250 ng/ml) are physiologically relevant to previous studies in mice. Since DON carry-over into plasma in people exposed to the toxin has not been studied to date, it is not possible to directly relate the in vitro concentrations employed herein to humans. It should be further noted that although DON is a translational inhibitor, protein synthesis is only partially inhibited by DON at 100-250 ng/ml (Yang *et al.*, 2000a). Thus it appears that the increase in COX-2 mRNA pool is sufficient to drive increased COX-2 protein translation even in the face of overall depression in protein translation.

The dose response study on PGE2 production after 24 hr suggests that the effects were non-linear in that levels of this metabolite were identical at 100 and 250 ng/ml DON (Fig. 1). This contrasts with the 15 hr COX-2 protein and 2 hr COX-2 mRNA data which both increased as the DON dose increased from 50 to 250 ng/ml. The PGE2 threshold effect may relate to the fact that supernatant PGE2 reflects not the only sum total of COX-2 mRNA expression and translational efficiency but also substrate availability, receptor binding and metabolism by the cells. Another possibility is that this effect is a reflection of DON cytotoxicity. Using the 3-(4,5-di-methylthizol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay, we have found after 24 hr that while 100 ng/ml of DON or less had no effect, 250 ng/ml typically inhibits the maximal response by 5 to 20 percent (data not shown). The capacity of DON to partially inhibit cell proliferation and/or reduce viability at the highest concentration is consistent with this toxin's inhibitory effects on translation. Thus, it is possible that even if the amount of COX-2 protein per cell was higher in the 250 ng/ml DON treatment, the decreased cell number may contribute to the lack of change in PGE2 compared to the lower toxin dose. This effect might not have been observed in COX-2

protein and mRNA measurements because these employed equivalent concentrations of the respective extracted macromolecules rather than represent a culture aliquot as does the MTT assay.

Several intestinal tumor studies have suggested mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)1/2 and its downstream ERK signaling pathway are essential for both increased transcription and stability of COX-2 mRNA of K-Ras-induced COX-2 (Zhang *et al.*, 2000; Sheng *et al.*, 2001). ERK 1/2 activation by DON was clearly involved in the transcriptional regulation of COX-2 expression but did not contribute to the mRNA stability. DON-activated ERK 1/2 may be critical upstream signals regulating transcription factors rather than contributing to mRNA stability. Notably, ERK1/2 are crucial upstream kinases of CCAAAT/enhancer-binding protein-beta (C/EBPβ) and cAMP response element binding protein (CREB), which are major transcriptional factors involved in murine COX-2 expression (Davis *et al.*, 2000; Hu *et al.*, 2001). Consistent with such activity, we have recently observed that C/EBPβ binding activities in RAW 264.7 cells was increased 2 and 8 hr after DON exposure (Wong *et al.*, 2002). It is thus possible that DON-induced ERK activity contributes to increased activation of C/EBPβ and other transcription factors.

p38 is also known to mediate xenobiotic- or endogenous factor-induced COX-2 expression by transcriptional and post-transcriptional mechanisms (Fiebich *et al.*, 2000; Vogel, 2000; Yan *et al.*, 2000). As shown here, p38 contributed to DON-induced COX-2 expression by both mechanisms. DON-induced transactivation of the COX-2 gene may result from p38-mediated activation of NF-κB and AP-1. Activation of these transcription factors by p38 has been described in LPS-exposed macrophages (Chen *et al.*, 1999). This possibility

is supported by our recent observation that DON exposure also increases AP-1 and NF-κB activation in RAW 264.7 cells (Wong *et al.*, 2002).

Relative to p38 and post-transcriptional mechanisms, the presence of multiple copies of AUUUA pentamer in the 3'-untranslated region (UTR) of COX-2 mRNA suggests the involvement of mRNA stability in the DON-induced upregulation of the gene. Some proteins such as AUF1, HuR, or tristetraprolin can differentially regulate COX-2 or cytokine mRNA stability by binding to the regulatory AU-rich elements (Sirenko *et al.*, 1997; Dixon *et al.*, 2000; Dixon *et al.*, 2001; Nabors *et al.*, 2001; Zhu *et al.*, 2001). Notably, binding of tristetraprolin, a member of a family of zinc finger proteins, is suppressed by p38-mediated phosphorylation (Zhu *et al.*, 2001). The observation that COX-2 mRNA stabilization via DON-activated p38 may be an important clue to global mechanisms of trichothecene-induced proinflammatory gene expression.

DON also activated JNK, which was transient relative to the other two MAPK families. Macrophage cells require the activation of JNK/MEKK1 in LPS-induced COX-2 transcription (Wadleigh *et al.*, 2000) and, more specifically, blocking JNK impairs both NF-κB- and C/EBPβ-mediated transcription of COX-2 (Mestre *et al.*, 2001). In contrast, our results showed that blocking JNK activity with dnJNK did not affect DON-induced COX-2 expression. It is possible that, in the case of DON, the activated MAPK network functions redundantly in upregulating COX-2 gene transcription. In this case, impairment of JNK, might be overcome by alternate pathways involving ERK and p38.

Interestingly, trichothecenes are also thought to induce leukocyte apoptosis via the p38 and JNK signaling pathways (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). The DON concentrations employed here to induce COX-2 expression were non-cytotoxic to

weakly cytotoxic. The possibility exists that trichothecene concentration may selectively dictate which MAPKs are activated and to what degree. The resultant effects may ultimately determine whether a cell generates a proinflammatory gene response or undergoes apoptosis. Further evaluation of how trichothecenes differentially regulate these two responses is warranted.

The potential exists that eicosanoid production may contribute to acute and chronic toxic effects associated with acute exposure to DON and other trichothecenes. Sub-chronic feeding of DON to mice results in a spectrum of immunologic effects including some manifestations that mimic human IgA nephropathy, an immune complex disease (Dong *et al.*, 1991). Relative to the latter, DON enhances polyclonal autoreactive immunoglobulin A which deposit in the kidney mesangium (Rasooly *et al.*, 1994; Rasooly and Pestka, 1994). Of critical importance is the capacity of DON elevating IL-6 expression, which is a critical mediator in the DON-induced IgA production (Zhou *et al.*, 1999; Pestka and Zhou, 2000). PGE2 is known to enhance IL-6 in several inflammatory models such as endotoxemia, airway inflammation, and autoimmune arthritis (Meyer *et al.*, 1995; Anderson *et al.*, 1996; Tavakoli *et al.*, 2001). In contrast, PGE2 selectively impairs the production of IFNγ and Th1 immune function in both human and murine T cell models (Betz and Fox, 1991; Snijdewint *et al.*, 1993). PGE2 suppresses interleukin-12 (IL-12) p70 heterodimer, a major Th1-driving cytokine, whereas it participates in the induction of IL-12 p40, which can function as antagonist of biologically active IL-12p70 thus favoring a Th2 response (Kalinski *et al.*, 2001). Interestingly, we have previously shown that DON induces IL-12p40 but not IL-12 p35 which would be inherently required for an increase in functionally active IL-12p70 (Zhou *et al.*, 1997). Thus, it is feasible that DON-induced PGE2 production might alter an

optimal balance between Th1 and Th2 and drive increased IgA production that is observed in the sub-chronic feeding models.

Although COX-2 induction can be related to adverse effects such as tissue damage, COX-2 also can play protective role in gastrointestinal inflammation (Langenbach *et al.*, 1999a; Langenbach *et al.*, 1999b; Morteau, 1999; Morteau *et al.*, 2000). It has been reported that COX-2-dependent arachidonic acid metabolites are essential modulators of the intestinal immune response to dietary antigens by promoting oral tolerance (Morteau, 1999; Newberry *et al.*, 1999). Moreover, COX-2 products can be very important in the resolution of late stage inflammation and may specifically involve an alternate set of prostaglandins such as those of the cyclopentenone family (Morteau, 1999). Consistent with this observation, COX-2 knockout mice exhibit increased susceptibility to chemical-induced colitis (Morteau *et al.*, 2000). Therefore, the up-regulation of COX-2 and its metabolites by DON as described herein might be interpretable as a protective defensive mechanism or compensatory stress response.

The mechanisms by which DON induces MAPK phosphorylation are unknown. Trichothecenes (Shifrin and Anderson, 1999; Yang *et al.*, 2000a) and other translational inhibitors (Iordanov *et al.*, 1997) which bind to eukaryotic ribosomes have been previously shown to activate MAPKs. Iordanov observed that anisomycin and other antibiotics that bind to 28S rRNA are potent activators of JNK (Iordanov *et al.*, 1997). Furthermore, two ribotoxic enzymes, ricin A chain and alpha-sarcin, both of which catalyze sequence-specific RNA damage in the 28S rRNA, are strong agonists of JNK1 and of its activator SEK1/MKK4. Anisomycin and the ribotoxic enzymes initiate signal transduction from the damaged 28S rRNA to JNK in active ribosomes but not inactive ribosomes.

These investigators described this capacity of the ribosomes to sense cellular stress as the "ribotoxic stress response". The possibility exists that DON's capacity to activate MAPKs reflects a ribotoxic stress response and that this is manifested in transcriptional and post-transcriptional upregulation of COX-2 mRNA.

In conclusion, the results presented herein revealed that DON induced PGE2 production and COX-2 expression by elevating transcriptional activity and mRNA stability. Enhanced transcriptional activity was modulated by ERK and p38 signaling pathways whereas mRNA stability was promoted exclusively by DON-activated p38 phosphorylation. Future studies will be directed toward identifying MAPK-regulated transcription factors and stabilizing factors binding to 3'-UTR of DON-induced COX-2 gene. Additional investigation is needed at the *in vivo* level relative to the role of COX-2 products in DON-induced immunopathogenic and physiological sequelae.

CHAPTER 3.

RELATIONSHIP OF TRICHOTHECENE STRUCTURE TO COX-2 INDUCTION : SELECTIVE ACTION OF TYPE B (8-KETO) TRICHOTHECENES [1]

[1] Most part of his work is in press for publication in of the title "Relationship of Trichothecene Structure to COX-2 Induction in the Macrophage: Selective Action of Type B (8-keto) Trichothecenes" by Moon, Y., Uzarski, R., and Pestka, J. J. in *Journal of Toxicology and Environmental Health* (2003).

ABSTRACT

The mycotoxin deoxynivalenol (DON, vomitoxin) induces COX-2 expression by promoting transcriptional activity and mRNA stability via mitogen activated protein kinase (MAPK) signaling pathways. Based on this model, representative members of the thee major trichothecene families (A, B, and D) were compared for COX-2 induction using the murine macrophage RAW 264.7 cell line. When treated with concentrations inhibiting viability response using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by 20% (ID_{20}), Type B trichothecenes including DON, 15-acetyl DON, 3-acetyl DON and fusarenon-X were very effective inducers of COX-2 mRNA expression whereas Type A and Type D trichothecenes had, markedly less effect. To compare transcriptional effects on COX 2 expression, luciferase reporter vectors containing 5' promoter or 3'-untranslated regions of the gene, respectively, were transfected into RAW 264.7 cells and the effects of various trichothecenes on luciferase activities were measured. At equitoxic concentrations, Type B but not Type A or D toxins typically enhanced luciferase activities indicating preferential COX-2 transcriptional activation and mRNA stabilization by this subset. Type B trichothecenes also activated the three major MAPK families At ID_{20} concentrations whereas type A and D did not. Blocking ERK and p38 with chemical inhibitors significantly suppressed Type B-induced COX-2 expression. Although JNK has been well known to contribute to COX-2 expression in the other models, transfection with the dominant negative JNK vector did not diminish COX-2 expression. Additionally, PDTC (pyrrolidine dithiocarbamate), an inhibitor of nuclear factor-κB (NF-κB) activation, completely

suppressed COX-2 induction, suggesting the involvement of the NF-κB signaling pathway in the trichothecene-induced COX-2 expression. Taken together, the Type B trichothecenes were strong inducers of inflammatory COX-2 gene and this induction was likely to be due to both enhanced transcription and stabilization of the gene. Furthermore, the ERK 1/2 and p38 signaling pathway, as well as NF-κB activation appeared to mediate the trichothecene-induced COX-2 expression. Selective action on COX-2 is likely to contribute to Type B trichothecene-mediated immunotoxicity.

INTRODUCTION

The trichothecene mycotoxins are a group of over 180 sesquiterpenoid fungal metabolites produced by various species of *Fusarium, Myrotecium, Cephalosporium, Verticimonosporium*, and *Stachybotrys* , which include some of the most potent eukaryotic protein synthesis inhibitors known (Ueno, 1985). These fungal metabolites are characterized by a tetracyclic scirpenol ring system (Table 2) and categorized into four subtypes based on the presence or absence of specific functional groups (Ueno, 1983). Three of these subtypes, Type A, B, and D, have great potential risk to human and animal health and therefore, will be the focus of this study. Type A trichothecenes are characterized by a hydroxyl or acyl moiety at R_1, R_2, R_3 or R_5 position (Table 2). Type B family possesses a ketone group at R_5 in addition to the derivation of Type A trichothecene. Macrocyclic Type D trichothecenes contain a diester or triester ring structure bridging between R_2 and R_3 positions (Jarvis, 1991).

The structure-activity relationship of trichothecenes has been broadly investigated in terms of their toxicity. It is generally considered that Type A trichothecenes tend to be more toxic than type B with decreasing order of toxicity from isovaleryl > hydrogen > hydroxyl related to the substitution of the R_5 (Rotter *et al.*, 1996). The translational inhibitory activity of trichothecenes requires the presence of an intact 9,10-double bond and the C-12,13 epoxide and substitution of both R_1 and R_2 predominantly inhibit polypeptide chain initiation (Ehrlich and Daigle, 1987). Moreover, the rank of potency for in vitro inhibition of lymphocyte proliferation is macrocyclic type D > type A group > type B group, and the

degree of acylation in substituents determines the lymphotoxicity of the Type A and B (Bondy and Pestka, 2000). Interest in the trichothecene mycotoxins arises from their widespread contamination of agricultural commodities (Kotsonis et al., 1996), association with water-damaged buildings (Sudakin, 1998), recalcitrance to degradation (Jackson and Bullerman, 1999), and adverse effects on human and animal health (Peraica *et al.*, 1999). This family of mycotoxins causes multiorgan effects including emesis and diarrhea, weight loss, nervous disorders, cardiovascular alterations, immunodepression, hemostatic derangements, skin toxicity, decreased reproductive capacity, and leukocytosis (Ueno *et al.*, 1984; Ueno, 1985; Peraica *et al.*, 1999). Experimentally, acute oral exposure to trichothecene mycotoxins causes severe damage to actively dividing cells in bone marrows, lymph nodes, spleen, thymus, and intestinal mucosa. Thus, the immune system might be a primary target for trichothecenes (Bondy and Pestka, 2000). In general, high doses of trichothecenes induce apoptotic cell death in lymphoid tissues whereas proinflammatory mediators such as cytokine, chemokine, and eicosanoids can be up-regulated by low doses of toxins (Marin *et al.*, 1996; Shifrin and Anderson, 1999; Yang *et al.*, 2000a; Sugita-Konishi and Pestka, 2001; Islam *et al.*, 2002).

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the oxygenation of arachidonic acid to prostaglandin endoperoxides. These are converted enzymatically into prostaglandins and thromboxane A_2 which play both physiologic and pathologic roles in diverse inflammatory sites (Vane *et al.*, 1998; Smith *et al.*, 2000). Two distinct isoforms of COX have been identified. COX-1 is constitutively expressed at low levels in most tissues and may be related with housekeeping function. In contrast, COX-2 resembles an early response gene. It is strongly induced by mitogenic and proinflammatory stimuli,

superinduced by protein synthesis inhibitors, and regulated at both transcriptional and post-transcriptional levels (Fletcher *et al.*, 1992; Newton *et al.*, 1997a; Newton *et al.*, 1997b; Newton *et al.*, 1998; Dixon *et al.*, 2000; Wadleigh *et al.*, 2000).

Several inflammatory stimuli which induce COX-2 gene expression also activate the mitogen-activated protein kinases (MAPKs). Among the MAPKs, c-Jun N-terminal kinase 1 and 2 (JNK1/2), extracellular signal regulated protein kinase 1 and 2 (ERK1/2), and p38 MAPK have been extensively studied for COX-2 gene expression (Xie and Herschman, 1995; Guan *et al.*, 1998). The transcriptional activity of COX-2 gene expression is redundantly modulated by MAPK (Wadleigh *et al.*, 2000; Mestre *et al.*, 2001) and typically p38 MAPK plays an important role in the signaling pathway for the stability of COX-2 mRNA (Dean *et al.*, 1999; Lasa *et al.*, 2000).

Nuclear Factor kappa B (NF-κB)/Rel-related signaling pathway also has been implicated in the induction of COX-2 gene expression in various types of cells (Hwang *et al.*, 1997; Inoue and Tanabe, 1998; Smith *et al.*, 2000). Most NF-κB/Rel dimers are bound to IκBs and retained in the cytoplasm. Phosphorylation of IκB by IκB kinase (IKK) complex lead to the ubiquitination of IκB and release the NF-κB/Rel dimers which translocate to the nucleus for transcriptional regulation of COX-2 gene (Perkins, 2000). Deoxynivalenol is known to activate NF-κB transcription factors, which may contribute to cytokine gene regulation in T cells (Ouyang *et al.*, 1996; Wong *et al.*, 2002).

Recently, we determined that the trichothecene deoxynivalenol (vomitioxin, DON) can induce COX-2 expression and that this was related to activation of MAPKs (Moon and Pestka, 2002). The purpose of this study was to relate the structure of representative members of the Type A, B and D trichothecene families to the capacity to induce COX-2

gene expression transcriptionally and post-transcriptionally. Furthermore, MAPK and NF-κB/Rel signaling pathways were related to potential upstream regulation of COX-2 gene expression.

MATERIALS AND METHODS

Trichothecenes. Satratoxins F,G and H were obtained from Dr. Bruce Jarvis (University of Maryland). Acetyl-T-2, HT-2, 3'-OH T-2, and 3'-OH HT-2 were kindly supplied by Dr. Takumi Yoshizawa (Kagawa University, Japan). All other trichothecenes were obtained from Sigma Chemical Co. (St. Louis, MO). Personnel handling concentrated trichothecenes wore gloves and face masks. Contaminated labware and benchtops were detoxified in dilute sodium hypochlorite (Thompson and Wannemacher, 1986). Waste materials were disposed through the toxic waste unit of the MSU Office of Radiation, Chemical and Biological Safety.

Cell culture. The murine macrophage cells, RAW 264.7 cells (American Type Culture Collection, Rockville, MD) (2.5×10^5 per ml) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals Inc, Norcross, GA), 100 unit/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in 5% CO_2 incubator at 37°C. Macrophage cell numbers and viabilities were assessed by trypan blue (Sigma) dye exclusion using a hematocytometer (American Optical, Buffalo, NY) as described by Strober (1991).

Cytotoxicity assay (MTT assay). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a pale yellow substrate that is cleaved by living cells to yield

a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT. Decrease in MTT reduction reflects the loss of cell viability. Cells ($2x10^5$/ml) were treated with each dose of chemicals for 18 hours in 96 well plates (200 ml medium/well) and then 20 ml of 5 mg/ml MTT was added to each well. After 3 hour incubation at 37°C, plate was centrifuged at 600 rpm for 10 min, and the culture medium was removed from the wells. The remaining crystals was dissolved with 150 µl DMSO and then incubated on the shaker for 5 min to thoroughly solubilize the formazan product. The absorbance of each well was measured at 540 nm using a plate reader. ID_{20} was calculated based on MTT assay, corresponding to the trichothecene dose causing 20% decrease in MTT reduction.

DNA fragmentation analysis Cells ($2x10^6$) were harvested at 500 xg for 5 min at 4 °C. Pellet was suspended in 100 µl hypotonic lysis buffer (10mM Tris-Cl, 10 mM EDTA, and 0.5% Triton X-100 at pH8) and incubated 10 min at 4°C. The resultant lysate was centrifuged at 13,000 xg for 30 min at 4°C and supernatant was digested for 1 hr at 37 °C with 0.4 mg/ml DNase-free RNase (Boehringer Mannheim, Indianapolis, IN). The mixture was then incubated an additional hour at the same temperature with 0.4 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN). DNA was precipitated in 50 % (v/v) isopropanol in 0.5 M NaCl at -20°C overnight. The precipitate was centrifuged at 13,000 xg for 30 min at 4 °C. The resultant pellet was air-dried and resuspended in 10 mM Tris, pH 7.4, and 1 mM EDTA, pH8.0. An aliquot equivalent to $2x10^6$ cells was electrophoresed at 70 V for 2 hr in 2% (w/v)agarose gel in 90 mM Tris-borate buffer containing 2 mM EDTA, pH 8.0. After electrophoresis, the gel was stained with 0.5 mg/ml ethidium bromide, and the nucleic acid

were visualized with a UV-transilluminator. A 100 bp DNA ladder (GIBCO-BRL, Rockville, MD) was used for size marker (SM).

Western blot analysis. At the time of harvest, cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1% (w/v) SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris pH 7.4), and sonicated for 5 seconds. The amount of protein was assayed by Lowry method using Dc protein assay reagent (Bio-Rad, Cambridge, MA). Extracts were mixed with Laemmli sample buffer (Bio-Rad, Cambridge, MA) and boiled for 5 min before resolving on a 10 % (w/v) acrylamide gel. Resolved proteins were transferred to PVDF membrane and blocked with Tris-buffered saline with Tween-20 (TBST, 10 mM Tris-HCl pH 7.5, 100mM NaCl, 0.1 % (v/v) Tween-20) containing 1% (w/v) BSA. The membrane was incubated for 1 hour with each MAPK antibody (Rabbit IgG, New England Biolab, Beverly, MA) at 1:1000 dilution or COX-2 antibody (mouse IgG1, Transduction Laboratories, Lexington, KY) at 1:250 dilution in TBST containing 1% (w/v) BSA, and then were washed three times with TBST. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA) at 1:5000 dilution in TBST containing 1% (w/v) BSA for MAPK detection or with HRP-conjugated anti-mouse IgG (Sigma, St. Louis, MO) at 1:10,000 dilution for COX-2 detection. After washing three times with TBST, bound HRP-conjugated antibody was detected with Enhanced Chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions.

Plasmids. The 5' upstream segment (-724/+7) of the mouse COX-2 gene from spleen

chromosomal DNA was cloned into pXP2 vector at Hind III/Xho I restriction sites to construct a mouse COX-2 promoter-luciferase plasmid (pXP-5COX2). The 3' untranslated regions (3'-UTR, 1/1558, 1/338 and 339/1558 from coding terminal sequence) of COX-2 gene were cloned into pGL3 control vector (pGL3c) at XbaI site to construct pGL3COX2a, pGL3COX2b, and pGL3COX2c, respectively. An expression vector for the dominant negative JNK1 (pdnJNK) was kindly provided by Roger Davis (Unversity of Massachusetts Medical School, Worcester, MA). All plasmids were purified with Endofree Plasmid Prep Kit (Qiagen, Valencia, CA).

Transient transfection. Raw 264.7 cells (4x 10^5/ml) were washed twice in serum-free DMEM and then incubated for 3 hours with a premixed complex of plasmid, plus reagent and lipopectamine plus (Life Technologies, Gaithersburg, MD) according to manufacturer's instructions. The cells were then replaced with fresh serum-containing DMEM and incubated for 24 hours and an additional time of DON exposure. pCMV-β-gal (1 μg) as internal standard was also co-transfected with 1 μg promoter-luciferase construct to standardize transfection efficiency.

Luciferase assay and β-galactosidase assay. Cells were washed with cold PBS, lysed with lysis buffer (25mM Tris-H_3PO_4, pH 7.8, 2 mM CDTA, 2 mM DTT, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100) and then centrifuged at 12,000 xg for 2 min. The supernatant was collected isolated and stored at -80°C until assessment of luciferase activity and b-galactosidase. Luciferase activity was measured with luminometer (Model 20e, Turner Designs Co., Sunnyvale, CA) after briefly mixing the supernatant with luciferase assay

substrate solution (Promega, Madison, WI). For β-galactosidase assay, the supernatant was mixed with same volume of 2x assay buffer (Promega, Madison, WI) and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 1 M sodium carbonate (the equal volume with the assay buffer) and the absorbance of the mixuture was read at 420 nm. The luciferase activity was normalized against β-galactosidase activity using the following formula: luciferase activity / β-galactosidase activity.

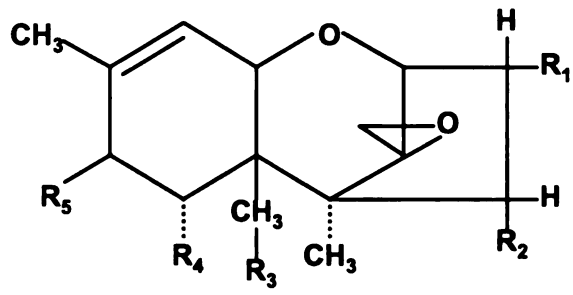
Statistics. Data were analyzed by Student's T test using the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). A p value of less than 0.05 was considered statistically significant.

RESULTS

Normalized concentration of each trichothecene were used to compare their effects on COX-2 induction. Each toxin was applied at the dose which does not interfere with significant cellular viability and we determined each 20 % inhibitory dose (ID_{20}) of trichothecenes, based on MTT conversion assay for cytotoxicity (Table 2). Consistent with previous blastogenesis studies employing ^{3}4-thymidine (Pestka and Forsell, 1988), Type D macrocyclic trichothecenes were most cytotoxic and Type B toxins were relatively less cytotoxic than other two groups of toxins here. A comparable study reports that the cytotoxicity of trichothecenes in macrophages occurs through apoptosis process with the similar rank order among trichothecene groups (Yang *et al.*, 2000a). In contrast to apoptosis or cytoxicity at high concentration, exposure to low levels of trichothecenes appears to promote expression of a diverse array of cytokines in vivo and in vitro (Bondy and Pestka, 2000). In the case of a variety spectrum of deoxynivalenol, it started to induce apoptotic ladder, which corresponds to about 80 % MTT viability (Fig. 10A and B). Therefore, this dose criteria might be a critical point to cellular death signals. The ID_{20} values was subsequently used to prevent the wide-scale cytotoxicity or apoptosis on the macrophage cultures.

Comparative effects of trichothecenes on COX-2 protein expression .

As found previously in vitro and in vivo (Islam *et al.*, 2002; Moon and Pestka, 2002) DON effectively induced COX-2 protein (Fig. 11). All Type B trichothecenes with the exception of nivalenol induced COX-2 gene expression.



Trichothecene	Abbreviation	R_1	R_2	R_3	R_4	R_5	ID_{20} by MTT (ng/ml)
Type A							
T-2	A1	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$	1.5
HT-2	A2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$	2.32
Diacetoxyscirpenol	A3	OH	OAc	OAc	H	H	4.6
Acetyl T-2	A4	OAc	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$	20.0
3'-OH T-2	A5	OH	OAc	OAc	H	$OCOCH_2C(OH)(CH_3)_2$	17.6
3'-OH HT-2	A6	OH	OH	OAc	H	$OCOCH_2C(OH)(CH_3)_2$	7.48
Type B							
DON (VT)	B1	OH	H	OH	OH	=O	225
Fusarenon-X	B2	OH	OAc	OH	OH	=O	208
Nivalenol (NIV)	B3	OH	OH	OH	OH	=O	150
15-acetyl DON	B4	OH	H	OAc	OH	=O	155
3-acetyl DON	B5	OAc	H	OH	OH	=O	1511
4,15-diacetyl NIV	B6	OH	OAc	OAc	OH	=O	51.7
Type D							
Verrucarin	D1	H	-O-R'-O-		H	H	12.8
Satratoxin G	D2	H	-O-R'-O-		H	H	0.6
Satratoxin H	D3	H	-O-R'-O-		H	H	0.92
Satratoxin F	D4	H	-O-R'-O-		H	H	3.14
Roridin A	D5	H	-O-R'-O-		H	H	1.8

OAc : $-OCOCH_3$
R' : macrocyclic ester or ester-ether bridge at the R_2 and R_3 positions

Table 2. Comparative cytotoxicity of trichothecenes. Cells ($2.5x10^5$/ml) were incubated with each specific trichothecene for 18 hs. Cell proliferation was measured with MTT assay. ID_{20} was defined as the dose at which 20% of cellular proliferation was inhibited by each toxin. Results indicative of mean (n=8) and were representative of three separate experiments.

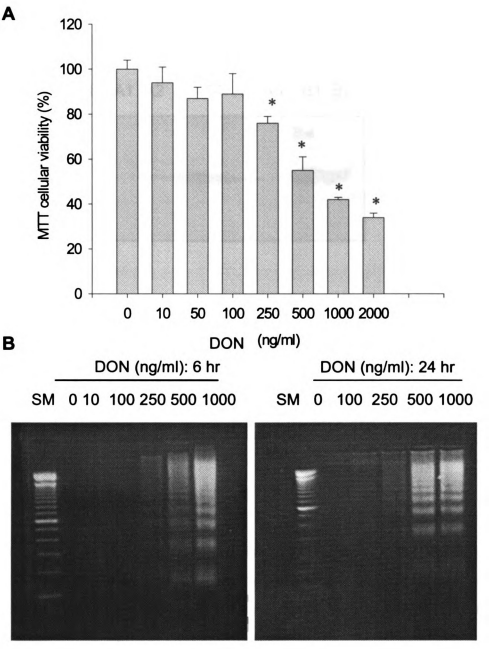


Figure 10. Dose response of DON-mediated cytotoxicity in macrophage cells (A) Cellular viability with increasing dose of DON in RAW 264.7 cells. Cells ($2x10^5$/ml) were incubated with each dose of DON for 18 hr and viability was analyzed using MTT assay. (B) Cells ($2.5x10^5$/ml) were exposed to DON for 6 or 24 hr and nuclear fragmentation was assessed on the agarose gels.

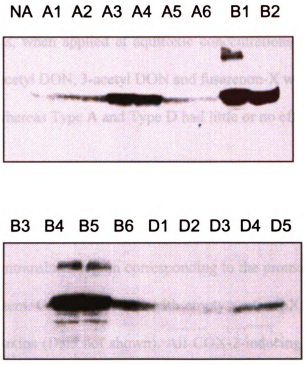


Figure 11. Comparative effects of trichothecenes on COX-2 expression. Cells ($2.5x10^5$/ml) were incubated with ID_{20} of each specific trichothecene for 18 h. COX-2 was detected with anti-mouse COX-2 antibody by Western blot analysis. Trichothecenes are identified in Table 2 legend. Results representative of three experiments.

In particular, fusarenon-X and acetyl DON derivatives including DON strongly enhanced COX-2 protein levels. Among the Type A trichothecenes, only diacetoxyscirpenol and acetyl T-2 toxin induced COX-2. The five macrocyclic Type D toxins had no inductive effect on COX-2 protein. Thus, when applied at aquitoxic concentrations Type B trichothecenes including DON, 15-acetyl DON, 3-acetyl DON and fusarenon-X were effective inducers of COX-2 expression whereas Type A and Type D had little or no effect.

Comparative effects of trichothecenes COX-2 transcriptional activity.

To analyze the effect of COX-2-inducing trichothecenes on COX-2 promoter activity, a 0.7KB COX-2 5'-untranslated region corresponding to the promoter was inserted into a luciferase vector system. Cells transfected with empty vector (pXP5) did not respond to trichothecene mycotoxins (Data not shown). All COX-2-inducing type B trichothecenes significantly promoted transcriptional activity of COX-2 gene in the constructed vector-transfected cells (Fig. 12). Representative type A (diacetoxyscirpenol and acetyl T-2 toxin) and Type D (Satratoxin F and roridin A) did not increase COX-2 promoter activity when present at equitoxic concentrations.

Comparative effects of trichothecenes COX-2 mRNA stabilization through 3'-UTR.

Besides promoter-driven effects, DON has been previously shown to regulate COX-2 mRNA stability via p38 MAP kinase (Moon and Pestka, 2002). Modulation of mRNA stability might be explained by the presence of multiple copies of AUUUA motif in the 3'-untranslated region (3'-UTR) of COX-2 mRNA since AU-rich element (ARE) in the 3'-UTR of an mRNA targets a transcript for rapid degradation (Dixon *et al.*, 2000).

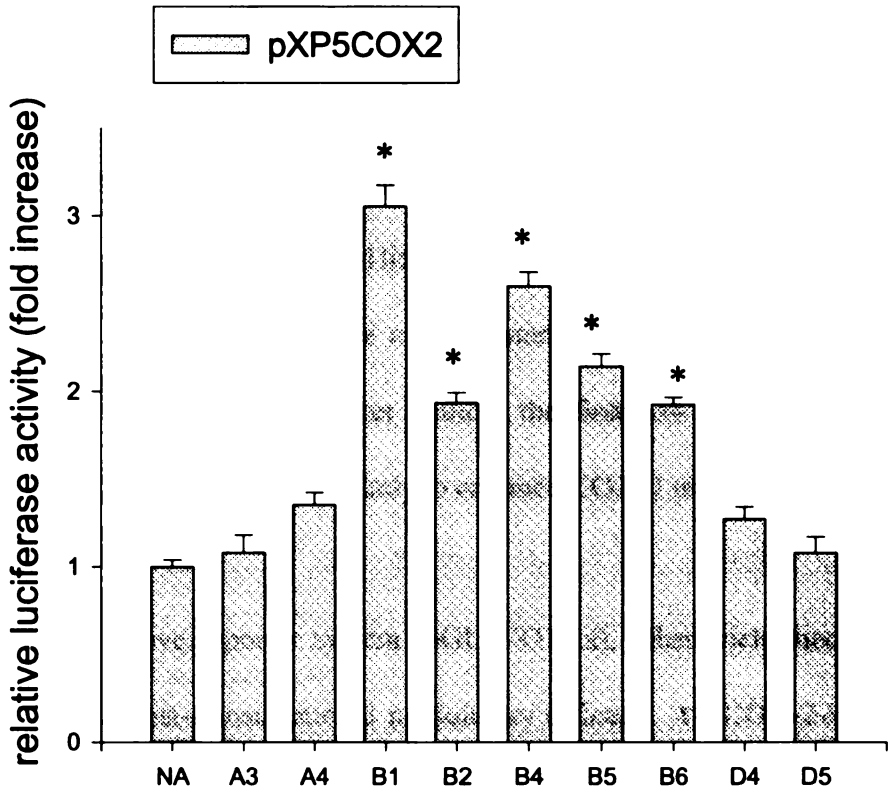


Figure 12. Effects of trichothecenes-mediated COX-2 promoter activity. Transfected cells with promoter-containing luciferase reporter vector (pXP5COX2) were incubated with $1/2ID_{20}$ of each specific trichothecene for 12 h. Cell lysates were analyzed for luciferase activity. Trichothecenes are identified in Table 2 legend. Data are mean $\pm$ SEM (n=4). Asterisk indicates significantly different from vehicle ($p<0.05$). Results are representative of three separate experiments.

To test the hypothesis in terms of DON-mediated mRNA stabilization, we constitutively expressed SV40 promoter-luciferase reporter gene, which was ligated with three different 3'-UTR sections (1/1558, 1/338 and 339/1558 from coding terminal sequence) of COX-2 gene (Fig. 13A). When reporter-expressing cells were treated with DON to analyze the effect of the toxin on stability of the reporter transcript via 3'-UTR, the toxin enhanced the products of pGL3COX2a, pGL3COX2b, but had little effect on reporters of pGL3c and pGL3COX2c (Fig. 13B). Most AUUUA motifs are concentrated in 60 base pairs from the terminal sequence and, as expected, the reporter without the first 338 base pair 3'-UTR did not respond to DON. Therefore, DON is likely to enhance COX-2 mRNA stability via AU-rich elements.

Using the above reporter system (pGL3COX2a), other trichothecenes were also compared for their post-transcriptional modulatory efficacy. All COX-2-inducing type B trichothecenes showed significantly increased levels of reporter, which can be related to the stabilization of mRNA transcripts via 3'-UTR (Fig. 14). In contrast, representative Type A and D trichothecenes had no effect. The regulation of 3'-UTR including AU-rich repeats might be thus involved in COX-2 mRNA stabilization by COX-2-inducing Type B trichothecenes. Taken together, when at equitoxic concentrations, Type B but not Type A or D toxins enhanced luciferase activities reporting COX-2 transcriptional activation and mRNA stabilization.

Figure 13. Involvement of 3'-UTR in DON-induced COX-2 mRNA stabilization (A) Constructs of luciferase reporter systems (pGL3COX2a, pGL3COX2b, and pGL3COX2c) containing COX-2 3'-untranslated regions of 1-1558, 1-338, and 339-1558 base pairs respectively from termination codon sequence. # represents the site of AUUUA motif. (B) Effects of DON on each reporter induction in the RAW 264.7 cells. Transfected cells with each constructed reporter were incubated with doses of DON for 12 h. Cell lysates were analyzed with luciferase assay. Data are mean ± SEM (n=3). Asterisk indicates significantly different from vehicle ($p<0.05$). Results are representative of three separate experiments.

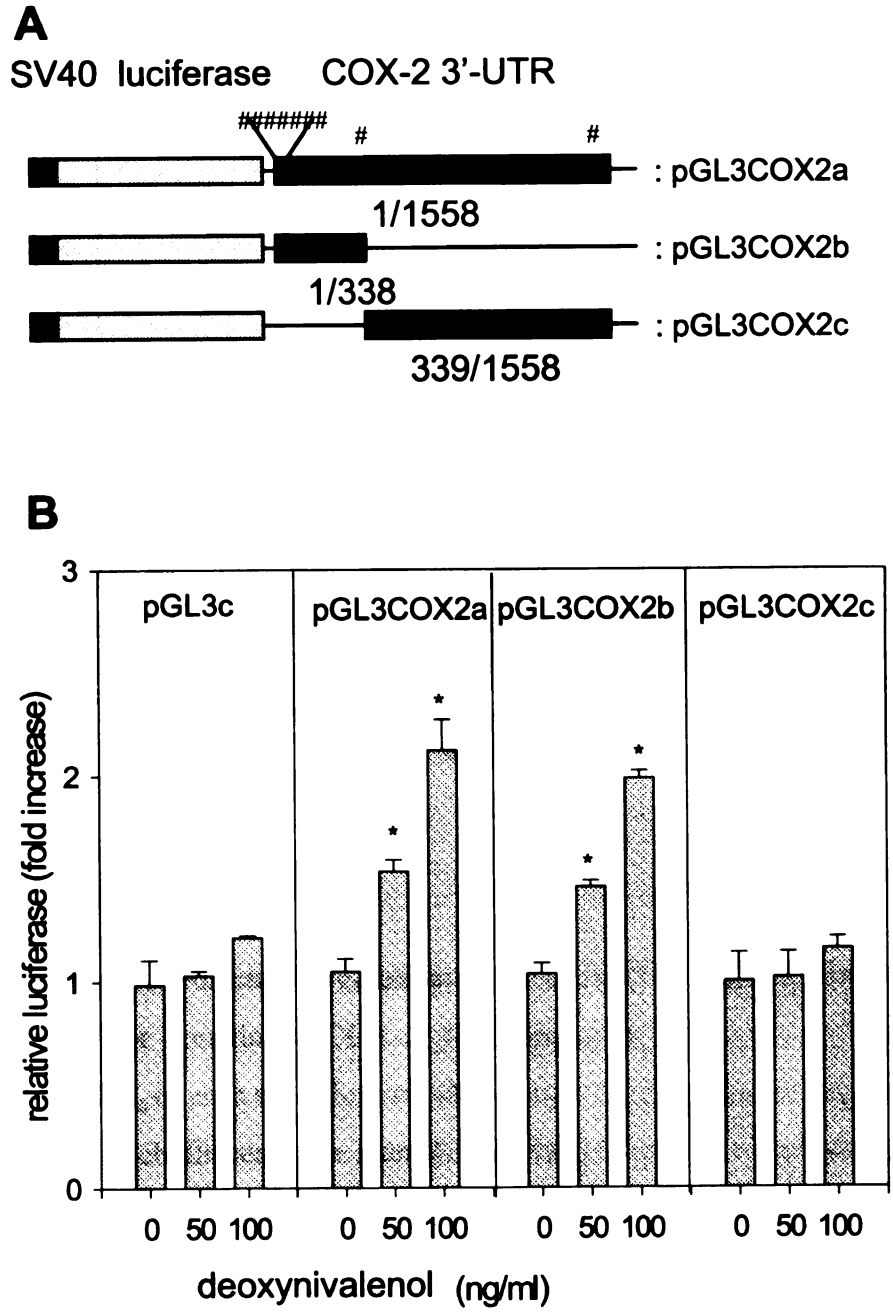

A
SV40 luciferase
COX-2 3'-UTR
#######
#
#
: pGL3COX2a
1/1558
: pGL3COX2b
1/338
: pGL3COX2c
339/1558
B
pGL3c
pGL3COX2a
pGL3COX2b
pGL3COX2c
relative luciferase (fold increase)
3
2
1
0
0 50 100
0 50 100
0 50 100
0 50 100
deoxynivalenol (ng/ml)

Figure 13

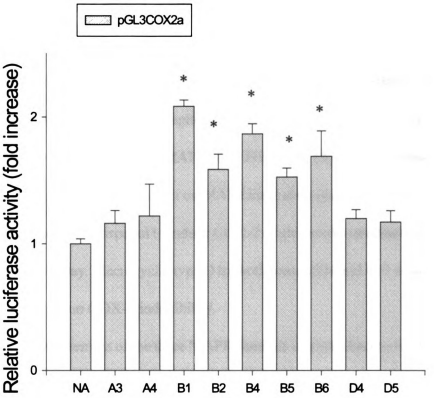


Figure 14. Effects of trichothecene mycotoxins on COX-2 mRNA stability. Transfected cells with luciferase vector containing COX-2 3'-untranslated region (pGL3COX2a) were incubated with 1/2 ID_{20} of each specific trichothecene for 12 h. Cell lysate were analyzed for luciferase activity. Trichothecenes are identified in Table 2 legend. Data are mean ± SEM (n=4). Asterisk indicates significantly different from vehicle ($p<0.05$). Results are representative of three separate experiments.

Type B trichothecenes preferentially activate MAPK.

Trichothecenes and protein synthesis inhibitors have been previously shown to activate MAP kinases and cause apoptosis via a ribotoxic stress response (Shifrin and Anderson, 1999; Yang *et al.*, 2000). Therefore, we examined the effects of equitoxic concentrations of trichothecenes on phosphorylation of MAPKs. All COX-2-inducing Type B trichothecenes activated ERK, p38 MAPK, and JNK (Fig. 15). COX-2-inducing type A trichothecenes (A3 and A4) had no effect on MAP kinase activation. Therefore, the capacity of acetyl T-2 and diacetoxyscirpenol to induce COX-2 might involve mechanisms other than MAPK signaling pathway. Macrocyclic type D trichothecenes (D4 and D5) weakly activated p38 although they had no COX-2 inducibility.

To assess involvement of the three MAPK families in trichothecene-induced COX-2 expression, COX-2 induction was analyzed after blocking MAPK signals with specific ERK and p38 inhibitors or dominant negative JNK. Inhibition with ERK with PD98059 or p38 with SB203580 markedly abrogated Type B trichothecene-induced COX-2 expression (Fig. 16A). However, dominant JNK had no suppressive effect on COX-2 production indicating that did not contribute to Type B toxin-induced COX-2 expression (Fig. 16B).

Nuclear factor-κB (NF-κB) activation is also involved in Type B trichothecene-induced COX-2 expression.

NF-κB can be activated by ERK1/2 and p38 and this transcription factor enhances COX-2 transcription (Smith *et al.*, 2000). Thus NF-κB might be a possible signaling mediator that is involved in trichothecene-induced COX-2 production. Pyrrolidine dithiocarbamate (PDTC) used here has been known to prevent degradation of IκBα and thus

allow inactivation of nuclear factor-κB (NF-κB) (Liu *et al.*, 1999). PDTC-mediated blocking of NF-κB signaling pathway completely ablated COX-2 gene expression. This suggests that the NF-κB signaling pathway is critical to Type B trichothecene-induced COX-2 expression (Fig. 17).

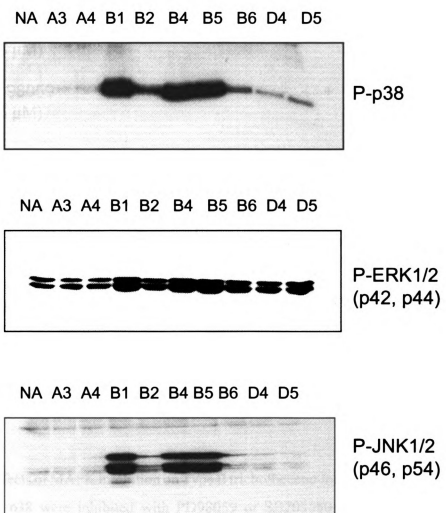


Figure 15. Activation of MAPK by trichothecene mycotoxins. RAW 264.7 cells ($2.5x10^5$/ml)were incubated with ID_{20} of each specific trichothecenes for 30 min. Cellular lysate were blotted with each anti-phosphorylated MAPK (JNK, ERK, and p38) antibodies. Results are representative of three separate experiments.

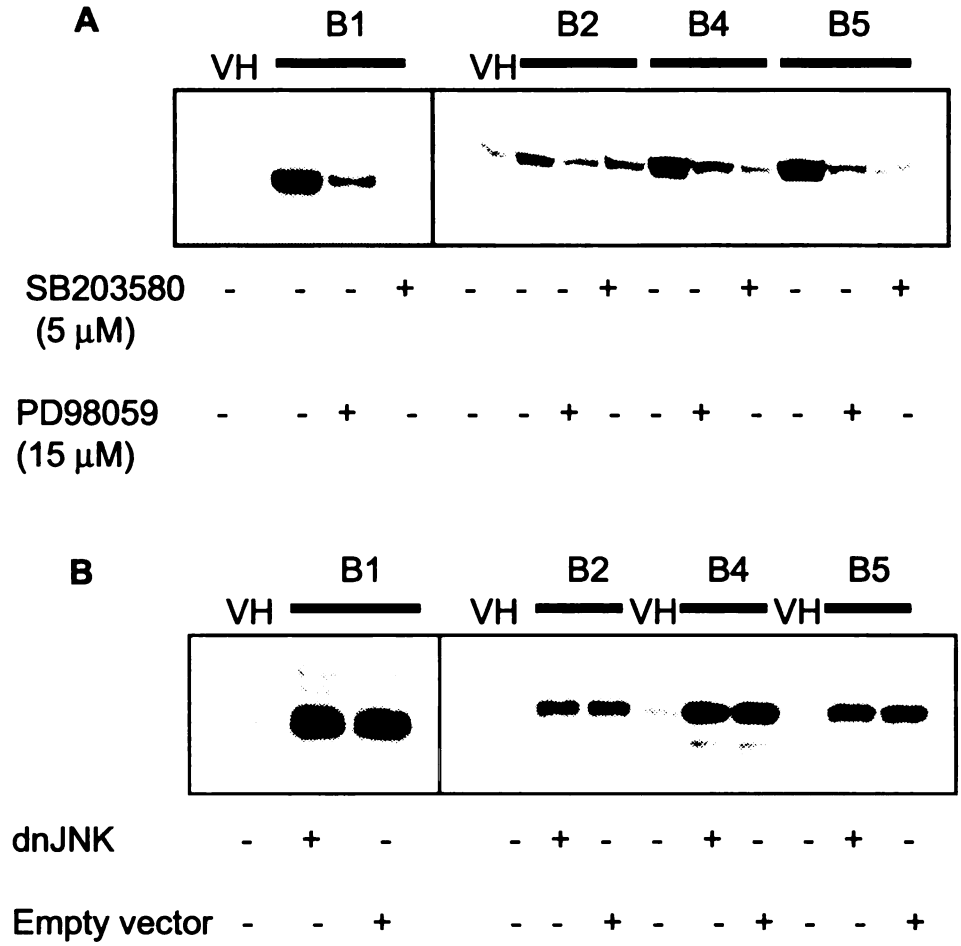


Figure 16. Effects of MAPK inhibition on Type B trichothecene-induced COX-2 expression. (A) ERK or p38 were inhibited with PD98059 or SB203580, respectively. Cells (2.5 x10^5/ml) were treated with each chemical combination for 15 h. VH stands for the vehicle. (B) JNK was inhibited by expression of dominant negative JNK (dnJNK). Cells transfected either with dominant negative JNK vector (pdnJNK) or empty vector (pCMV5) was incubated with ID_{20} of each trichothecene for 15 h. Cellular lysate was analyzed with anti-mouse COX-2 antibody. Trichothecenes are identified in Table 2 legend. Results are representative of three separate experiments.

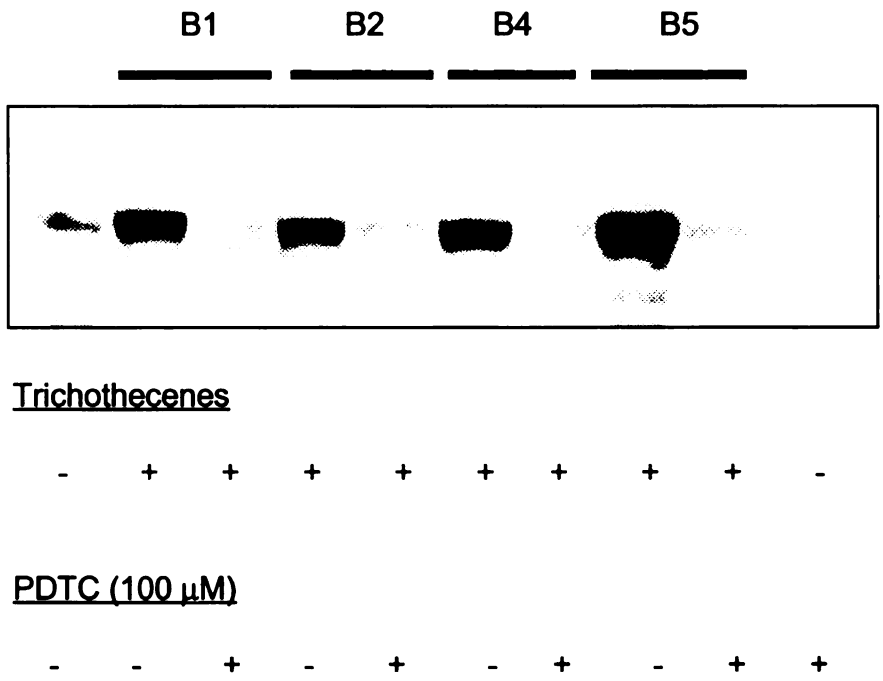


Figure 17. Involvement of NF-κB signaling in Type B trichothecene-induced COX-2 expression. Cells ($2.5x10^5$/ml) were incubated with trichothecene with or without 100 μM Pyrrolidine dithiocarbamate (PDTC) for 15 h. COX-2 was blotted by anti-mouse COX-2 antibody with Western blot analysis. Trichothecenes are identified in Table 2 legend. Results are representative of three separate experiments.

DISCUSSION

This study is novel because it is the first to report a selective biochemical mode of action for the Type B trichothecene subset. Specifically, these data indicated that, when cells are exposed to concentrations of equivalent cytotoxicity, the Type B trichothecenes preferentially target COX-2 gene expression as compared to the Type A and Type D trichothecenes. Notably, Type B trichothecenes strongly induced COX-2 gene expression by enhancing promoter activity and 3'-UTR-mediated mRNA stability of the gene and, furthermore, this involved MAPK and NF-κB/Rel signaling pathways. Thus unique action on COX-2 might result in a different inflammatory spectrum and pathologic outcome resulting from trichothecene mycotoxin exposure and must be taken into account in hazard assessments for these toxins.

Trichothecene mycotoxins have been previously shown to activate MAPK in T cells and macrophages via a putative ribotoxic stress response, and these findings were further associated with toxin-induced apoptosis depending on the dose regimen employed (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). A critical finding in this study is the further establishment of a definitive link between Type B trichothecene-activated MAPK and the toxin-induced COX-2 expression at benign doses to the cells.

Type B trichothecenes including DON, its derivatives and fusarenon-X, where present at equitoxic concentrations were the most effective inducers of inflammatory COX-2 gene and the induction was due to both enhanced transcription and UTR-mediated stabilization of the gene. Furthermore, MAPK signaling pathway and NF-κB activation

mediate the trichothecene-induced COX-2 expression. COX-2 induction may contribute to the Type B trichothecene-mediated immunotoxicity. Induction levels of COX-2 expression by DON and DON-derivatives agree with the degree of reporter expression in transcription and mRNA stability, and MAPK activation. However, because levels of MAPK activation, transcription and post-transcription in fusarenon-X are relevantly low, fusarenon-X-induced COX-2 expression may involve other mechanisms such as translation level of COX-2. Nivalenol has very similar chemical structure to DON and is capable of inducing and superinducing proinflammatory cytokines such as IL-6 and TNF-α and IL-8 at concentrations similar to that of DON (Sugita-Konishi and Pestka, 2001). Surprisingly, nivalenol did not induce COX-2 expression. Taken together, COX-2 induction by trichothecenes and MAPK involvement appears to be most affected by DON and DON-derivatives.

T-2 toxin also has been known to synthesize and release inflammatory eicosanoids in the circulating blood, alveolar macrophages and brain slices (Shohami *et al.*, 1987; Naseem *et al.*, 1989). In support of these findings, acetyl T-2 and diacetoxyscirpenol also weakly induced COX-2 protein. However, they had little effect on COX-2 promoter activity and 3'-UTR-mediated mRNA stability. This suggests that the mechanism for COX-2 induction for Type A toxins may differ from Type B toxins. It is possible that although only weak and nonsignificant trends were observed for the transcriptional and post-transcriptional assays employed here, the combination of both regulatory pathways might be necessary to observe effects on COX-2 protein. Eicosanoids can be evoked by a variety of inducers such as cytokines, growth factors (TGF-β), hormones (serotonin), reactive oxygen species, and nitric oxide which are elicited by T-2 toxin (Plasencia and Rosenstein, 1990; Ouyang *et al.*, 1995; Wang *et al.*, 1998; Albarenque *et al.*, 2000; Smith *et al.*, 2000). Therefore, as a second

possible explanation, modest changes in COX-2 protein expression could be due to indirect effect of Type A trichothecenes mediated by eicasanoid-inducing mediators, whereas Type B toxins induce COX-2 directly by transcriptional and post-transcriptional mechanisms.

Type A trichothecenes did not activate MAPK in the model described here, which was inconsistent with the previous reports that Type A trichothecenes such as T-2 toxin, HT-2, diacetoxyscirpenol, and acetyl T-2 can activate MAPK activity as a prelude to apoptosis (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). The critical differences between this study and the previous reports were the treatment times and toxin doses that were employed. Shifrin and Anderson (1999) used 10 μM of trichothecenes for 2 h and Yang *et al* (2001) treated RAW 264.7 cells with 10 ng/ml toxin for 4 h. Compared with these previous reports, the doses in this study were normalized as ID_{20} based on MTT cytotoxicity assay and the toxin incubation time was 30 min for MAPK activation. Therefore, longer incubation time and/or higher concentration of Type A toxins may have driven MAPK activation and, ultimately, apoptosis. Further exploration of the comparative concentration-dependent effects of trichothecenes on MAPK induction and downstream sequelae is warranted.

The Type D verrucarin A greatly enhances IL-2 and IL-4 mRNA in the murine T cells even at a concentration as low as 25 pg/ml whereas there is no increase of the secreted cytokines in the supernatant (Ouyang *et al.*, 1995). The capacity of macrocyclic Type D trichothecenes such as satratoxin F and roridin A to activate MAPKs without affecting COX-2 expression were consistent with previous studies, (Shifrin and Anderson, 1999; Yang *et al.*, 2000). The reason for the lack of COX-2 response may relate to the relatively weak MAPK responses compared to Type B trichothecenes. MAPK-mediated gene induction might not be enough to overcome the strong global inhibition of translation by Type D

trichothecenes. Alternatively, Type D toxin-activated MAPKs may be associated with different downstream signaling sets or different MAPK scaffolding complexes from those shown in Type B group.

Several intestinal tumor studies have suggested MEK and its downstream ERK signaling pathway are essential for both increased transcription and stability of mRNA of K-Ras-induced COX-2 (Zhang *et al.*, 2000; Sheng *et al.*, 2001). Type B trichothecene-activated ERK 1/2 might be also critical upstream signals for regulating transcription factors. In support of this contention, ERK 1/2 can activate CCAAAT/enhancer-binding protein-beta (C/EBPβ) and cAMP response element binding protein (CREB), which are major transcriptional factors involved in murine COX-2 expression (Fig. 6A) (Davis *et al.*, 2000; Hu *et al.*, 2001). Consistent with such activity, we have recently observed that C/EBPβ binding activities in RAW 264.7 cells was increased 2 and 8 h after DON exposure (Wong *et al.*, 2002). It is thus possible that Type B-induced ERK activity contributes to increased COX-2 transcription.

p38 is known to mediate xenobiotic- or endogenous factor-induced COX-2 expression by regulating transcription or mRNA stability of the gene (Fiebich *et al.*, 2000; Vogel, 2000; Yan *et al.*, 2000). The findings here that p38 was involved both in transcriptional activity and mRNA stability of COX-2 gene are consistent with previous findings for DON (Moon and Pestka, 2002).

It has long been recognized that transcripts that contain AU-rich elements (AREs) are selectively targeted for rapid degradation (Chen and Shyu, 1995). Most AREs are clustered within 60 base pairs from the coding terminal sequence and DON was found to enhance ARE-containing reporter stabilization (Fig. 13). Therefore, it is possible that DON and other

Type B trichothecenes mediate mRNA stabilization signals by targeting AREs at 3'-UTR. Relatedly, p38 is known to mediate COX-2 mRNA stabilization through AREs (Lasa *et al.*, 2000) and this is thought to be controlled by ARE-binding proteins. Specifically proteins such as ARE/poly-(U)-binding/ degradation factor 1 (AUF1), HuR (a member of the ELAV family of RNA binding proteins), tristetraprolin, Y box binding factor 1 (YB-1), TIAR, and CarG box-binding factor-A (CBF-A) can differentially regulate COX-2 or cytokine mRNA stability by binding to the regulatory AU-rich elements (Sirenko *et al.*, 1997; Dixon *et al.*, 2001; Nabors *et al.*, 2001; Zhu *et al.*, 2001; Dean *et al.*, 2002). Tristetraprolin, a member of a family of zinc finger proteins was reported to play a crucial role in maintaining gene quiescence, and this quenching effect on transcription can be released by p38-mediated phosphorylation of tristetraprolin (Zhu *et al.*, 2001). The presence of multiple copies of AUUUA pentamer in the 3'-untranslated region (3'-UTR) of COX-2 mRNA further suggests the mRNA stability in the Type B trichothecene-induced up-regulation of the gene can be modulated through p38 MAPK signaling pathway.

Type B trichothecenes also activated JNK, which did not appear to contribute to COX-2 induction. Macrophage cells require the activation of JNK/MEKK1 in LPS-induced COX-2 transcription and, more specifically, blocking JNK impairs both NF-κB- and C/EBPβ-mediated transcription of COX-2 (Wadleigh *et al.*, 2000). In contrast, our results differed and showed that blocking JNK activity with dnJNK did not affect DON-induced COX-2 expression. It is possible that, in the case of Type B toxins, the activated MAPK network functions redundantly in upregulating COX-2 gene transcription. In this case, impairment of JNK, may be overcome by alternate pathways involving ERK and p38 (Mestre *et al.*, 2001). Interestingly, trichothecenes are thought to induce leukocyte apoptosis via the

p38 and JNK signaling pathways (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). The trichothecene concentrations employed here to induce COX-2 expression were non-cytotoxic to weakly cytotoxic. The possibility exists that trichothecene concentration may selectively dictate which MAPKs are activated and to what degree and that these effects ultimately determine whether a cell generates a proinflammatory gene response or undergoes apoptosis. Furthermore, evaluation of how trichothecenes differentially regulate these two responses is warranted.

Our data also implicated NF-κB/Rel signaling in Type B trichothecene-induced COX-2 expression. COX-2 promoter contains two consensus sequences for the cis-acting elements recognized by the NF-κB/Rel transcription factor family and it is thus expected that NF-κB/Rel might modulate COX-2 transcription. DON elevates NF-κB/Rel binding activity and may be involved mechanistically in DON-induced cytokine expression in both cloned (EL-4) and primary (CD4+) murine T cells (Ouyang *et al.*, 1996). Moreover, DON decreases IκBa levels which bind NF-κB dimers and restrict the dimer from translocating to the nucleus. Type B trichothecene-induced COX-2 expression might be explained by NF-κB/Rel signaling pathway as well as MAPK signal transduction. Because multiple signaling pathways exist for activation of IKK complex that include members of the MAPK kinase (MAPKK) family such as MAPK/ERK kinase kinase1 (MEKK1), 2 and 3 (Perkins, 2000) and sustained JNK is suppressed by NF-κB signaling (Tang *et al.*, 2001), there could be crosstalk among MAPK and NF-κB/Rel signaling pathways.

In summary, Type B trichothecenes that include DON and its closely related derivatives preferentially induced COX-2 gene expression when compared to equitoxic concentrations of Type A and Type B trichothecenes. These effects were via altered

transcriptional and post-transcriptional regulation. Both MAPK and NF-κB/Rel signaling pathway were involved in the COX-2 induction by Type B trichothecenes. These data are particularly critical because they suggest that Type B trichothecenes which are found in grain-based food may cause a different spectrum of toxicity than other trichothecenes found in food (Type A) or in contaminated indoor air (Type D).

CHAPTER 4.

ROLE OF COX-2 IN DON-MEDIATED IL-6 INDUCTION [1]

[1] This work was published in the title of "Cyclooxygenase-2 Mediates Interleukin-6 Upregulation by Vomitoxin (Deoxynivalenol) In Vitro and In Vivo" (Moon and Pestka, 2003).

ABSTRACT

Interleukin-6 (IL-6) is a central mediator of immunotoxicity that is associated with exposure to the trichothecene deoxynivalenol (DON). The purpose of this investigation was to test the hypothesis that the inducible cyclooxygenase-2 (COX-2) and its metabolites contribute to DON-induced IL-6 upregulation. DON at 100 to 250 ng/ml readily induced COX-2 protein expression in the RAW 264.7 murine macrophage cell line. Superinduction of LPS-mediated IL-6 production by DON in these cells was significantly reduced by the COX inhibitors indomethacin and NS-398, whereas the inhibitors did not affect direct induction of IL-6 by LPS alone. Mice that had been gavaged orally with 5 and 25 mg/kg DON exhibited elevated COX-2 mRNA expression in Peyer's patches and spleen with peak induction occurring 2 hr after DON exposure. IL-6 mRNA was also induced by DON in vivo, however, peak expression occurred from 2 to 4 hr after toxin exposure suggesting that maximal COX-2 gene upregulation preceded or was concurrent with that for IL-6. Also consistent with a putative contributory role for COX-2 was the finding that both induction of splenic IL-6 mRNA and serum IL-6 by DON were significantly reduced by pretreating mice with the COX inhibitors indomethacin or NS-398. Finally, COX-2 knockout mice showed significantly reduced splenic IL-6 mRNA and serum IL-6 responses to oral DON exposure as compared to their parental wild-type. Taken together, these in vitro and in vivo data suggest that DON-induced COX-2 gene expression and resultant COX-2 metabolites contributed, in part, to subsequent upregulation of IL-6 gene expression which has been previously shown to be a hallmark of DON-mediated immunotoxicity.

INTRODUCTION

The trichothecenes are sesquiterpenoid fungal mycotoxins that are potent inhibitors of eukaryotic protein synthesis and have a wide spectrum of toxic effects. Trichothecenes are a safety concern because they (1) widely contaminate agricultural commodities (Abouzied *et al.*, 1991), (2) are resistant to degradation during milling or processing (Scott, 1990) and (3) can adversely affect humans and animals (Ueno *et al.*, 1984; Ueno, 1985; Bhat *et al.*, 1989). Of particular importance is the trichothecene deoxynivalenol which is produced by *Fusarium graminearum* and *F. culmorum,* and is frequently found in grain-based agricultural commodities such as wheat, barley, and corn (Rotter *et al.*, 1996).

In experimental animals, acute exposure to high doses of DON causes nausea, emesis, leukocyte apoptosis, and circulatory shock, whereas effects of low dose DON exposure include anorexia, decreased nutritional efficiency, and immunotoxicity (Rotter *et al.*, 1996). Relative to the latter, our laboratory has observed that, in the mouse, chronic dietary DON exposure induces elevation of serum IgA, mesangial kidney IgA deposition and hematuria, all of which are hallmarks of human IgA nephropathy (Forsell *et al.*, 1986; Dong *et al.*, 1991; Dong and Pestka, 1993). Aberrant IgA production appears to be mediated through superinduction of cytokine gene expression in mononuclear phagocytes (Yan *et al.*, 1997; Yan *et al.*, 1998) and T helper cells (Warner *et al.*, 1994). One of these cytokines, interleukin-6 (IL-6), is widely recognized to be critical to regulation of mucosal B cell differentiation to IgA-secreting plasma cells (Beagley *et al.*, 1989; Beagley *et al.*, 1991; Fujihashi *et al.*, 1992). Genetic disruption of IL-6 in mice markedly reduces DON-induced

increases in serum IgA as well as kidney IgA deposition in mice suggesting a strong correlation between IL-6 and these IgA effects (Pestka and Zhou, 2000). In addition to the effects on IgA, superinduction of IL-6 by DON in the presence of endotoxin is likely to be associated with the shock-like and cytotoxic responses that occur during acute DON intoxication in mice (Zhou *et al.*, 1999).

The cyclooxygenases (COX) are rate-limiting enzymes that catalyze oxygenation of arachidonic acid to prostaglandin endoperoxides which are subsequently converted enzymatically into prostaglandins and thromboxane A_2 (Smith *et al.*, 2000). These metabolites play important physiologic and pathologic roles in diverse inflammatory sites (Vane *et al.*, 1998). Two distinct isoforms of COX have been identified. COX-1 is constitutively expressed at low levels in most tissues and may be related to homeostatic function. In contrast, COX-2 resembles an early response gene in that it is strongly induced by mitogenic and proinflammatory stimuli, and superinduced by protein synthesis inhibitors (Fletcher *et al.*, 1992; Newton *et al.*, 1997a; Newton *et al.*, 1997b; Newton *et al.*, 1998). DON has recently been shown by our laboratory to induce COX-2 gene expression in vitro and in vivo and to enhance the production in vitro of prostaglandin E2 (PGE2), a major metabolite of COX-2. A positive association exists between endogenous COX-2 metabolites and IL-6 synthesis in vitro and in vivo in models of several inflammatory diseases (Dendorfer *et al.*, 1994; Meyer *et al.*, 1994; Meyer *et al.*, 1995; Anderson *et al.*, 1996; Hinson *et al.*, 1996; Williams and Shacter, 1997; Zeng *et al.*, 1998; Williams *et al.*, 2000). PGE2 regulates production of macrophage and T cell cytokines which impact cell-mediated responses as well as immunoglobulin induction by B cells (Snijdewint *et al.*, 1993; Fedyk *et al.*, 1997). In some inflammatory response models, PGE2 inhibits Th1 cytokine expression, whereas

production of Th2 cytokines is unaffected or even enhanced by PGE2 (Betz and Fox, 1991; Kozawa *et al.*, 1998; Kuroda *et al.*, 2000). Macrophages are a major source of PGE2 during inflammation and also have receptors for eicosanoids. Macrophage-generated PGE2 is now recognized to modulate proinflammatory cytokines such as IL-6 in an autocrine or paracrine fashion (Arakawa *et al.*, 1996; Williams and Shacter, 1997; Williams *et al.*, 2000).

The aim of this study was to test the hypothesis that COX-2 and its metabolites contribute to IL-6 induction by DON in vitro and in vivo. The results suggest that DON-induced COX-2 and COX-2 metabolites mediated up-regulation of IL-6 expression in both macrophage culture and in mice. COX-2 might, therefore, play a momentous role in immunological and inflammatory events associated with DON exposure.

MATERIALS AND METHODS

Cell culture studies. All cell culture reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The RAW 264.7 murine macrophage cell line (American Type Culture Collection, Rockville, MD) was cultured at 2.5 x 10^5 cells per ml in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals Inc, Norcross, GA), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5 % CO_2 at 37°C. Macrophage cell numbers and viabilities were assessed prior to culturing by trypan blue dye exclusion using a hematocytometer (American Optical, Buffalo, NY). Cells were incubated with DON at concentrations ranging from 0 to 250 ng/ml in the presence and absence of selective inhibitors. and then analyzed for COX-2 and IL-6 induction. To assess the effects of DON and these inhibitors, the 3-(4,5-di-methylthizol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Ji *et al.*, 1998) was used.

Mouse studies. All animal handling was conducted according to the guidelines established by the NIH. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory Animal Committee. For most studies, male B6C3F1 mice (7 wk old) were obtained from Charles River (Portage, MI) and acclimated for one week in a humidity- and temperature-controlled room with an alternating 12 hr light and dark cycle. Mice were fed AIN-76A diet (ICN Nutritional Biochemical, Cleveland, OH) and water *ad lib.*

For COX-2 deficiency studies, COX-2 knockout (KO, homozygous 129P2-*ptgs2*^{tm1}) and parental wild type(WT, C57BL/6) (Langenbach *et al.*, 1999a) were obtained from Taconic (Germantown, NY) and treated as described for B6C3F1 mice. Blood was obtained from retroorbital plexus under metaflurane anesthesia and mice were immediately euthanized by cervical dislocation. Peyer's patches and spleen were removed and total RNA extracted for measurements of gene expression.

Detection of COX-2 protein expression. Cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1%[w/v] SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris pH 7.4), and sonicated for 5 sec. Resultant extract was then analyzed for COX-2 protein by Western blot. Cell lysates were mixed with Laemmli sample buffer (Bio-Rad, Cambridge, MA) and boiled for 5 min before electrophoretic separation on a 10 % (w/v) acrylamide gel. Resolved proteins were transferred to PVDF membrane and blocked with Tris-buffered saline with Tween-20 (TBST; 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 % [v/v] Tween-20) containing 5% (w/v) BSA. The membrane was incubated for 1 hr with COX-2 antibody (mouse IgG1, Transduction Laboratories, Lexington, KY) 1:300 dilution in TBST containing 5% (w/v) BSA, and then was washed three times with TBST. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma, St. Louis, MO) (1:10,000 dilution) for COX-2 detection. After washing three times with TBST, bound HRP-conjugated antibody was detected with an Enhanced Chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to manufacturer's instructions.

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

Detection of COX-2 and IL-6 mRNA by competitive RT-PCR. Total RNA from murine tissue was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA (100 ng) from each sample was converted to cDNA by reverse-transcriptase (Riedy *et al.*, 1995) and resultant cDNA was amplified competitively with truncated internal standard of cDNA constructed by the bridging-deletion method (Hall *et al.*, 1998). Amplification was performed in a 9600 Perkin Elmer Cycler (Perkin-Elmer Corp., Norwalk, CT) using the following parameters: 30 cycles of reactions

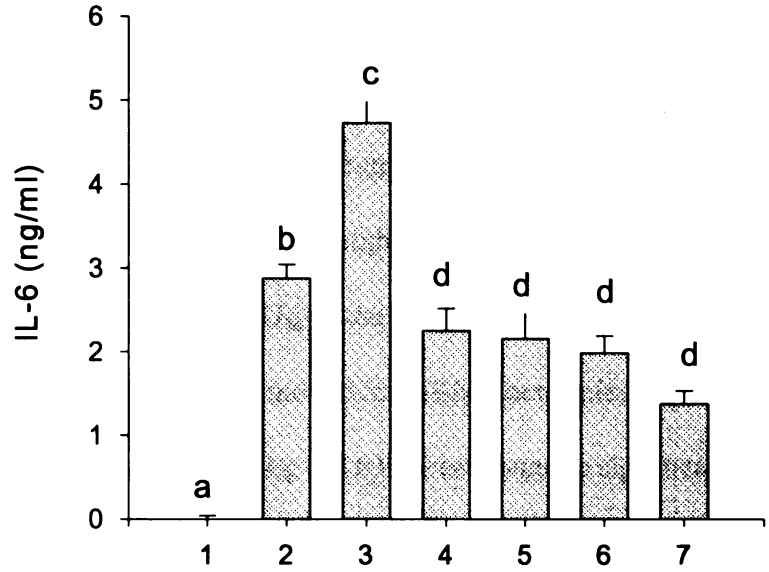
of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s (IL-6) or 56 °C for 45 s (COX-2), and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.5 % agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers were synthesized at Michigan State University Molecular Structure facility. The 5' forward (F) and 3' reverse (R)-complement PCR primers for amplification of mouse COX-2 and IL-6 cDNA were ACACTCTATCACTGGCATCC (COX-2 F), GAAGGGACACCCTTTCACAT (COX-2 R), TTCACAAGTCCGGAGAGGAG (IL-6 F), and TGGTCTTGGTCCTTAGCCAC (IL-6 R), respectively. The final end-product of amplified cDNAs were 584 bp of COX-2 , 500 bp of COX-2 internal standard cDNA, 488 bp of IL-6, and 400 bp of IL-6 internal standard cDNA. Densitometric ratios of native to internal standard cDNA were used to make standard curves for COX-2 and IL-6 to calculate cDNA concentrations in RT reaction products.

Statistics. Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student's T test was performed. For comparison of multiple groups, data were subjected to one way analysis of variance (ANOVA) and pairwise comparisons made by Student-Newman-Keuls (SNK) method. Data not meeting normality assumption were subjected to Kruskal-Wallace ANOVA on Ranks and then pairwise comparisons made by SNK method.

RESULTS

COX-2 contributes to IL-6 superinduction by DON in RAW 264.7 cells.

Since COX-2 is abundantly expressed in macrophage cells during tissue inflammation, this enzyme might, through its metabolites, drive autocrine and paracrine effects on IL-6 expression. Using RAW 264.7 cells as a macrophage model, the interaction between COX-2 metabolism and DON-induced IL-6 production was investigated. Consistent with an earlier study (Moon and Pestka, 2002), DON at concentrations of 100 and 250 ng/ml induced COX-2 protein expression (Fig. 2). Although DON does not directly induce IL-6 in RAW 264.7 cells, the toxin can superinduce IL-6 production in the presence of lipopolysaccharide (LPS) (Wong *et al.*, 1998; Wong *et al.*, 2001). Here, LPS at 200 ng/ml significantly induced IL-6 production and this response was superinduced by co-treatment with 100 ng/ml DON (Fig. 18). Co-exposure to the COX inhibitors indomethacin or NS-398 significantly depressed the superinduced IL-6 response in DON + LPS-treated cells. LPS-mediated IL-6 production was not significantly affected by COX-2 inhibitors (data not shown). These results suggested that IL-6 superinduction may be mediated by DON-induced COX-2 metabolites. Since the in vivo response to DON is likely to be much more complicated than that for the macrophage cell model, subsequent studies were conducted in intact mice.



Treatment	1	2	3	4	5	6	7
LPS (200ng/ml)	-	+	+	+	+	+	+
DON (100ng/ml)	-	-	+	+	+	+	+
Indomethacin (2μM)	-	-	-	+	-	-	-
(20μM)	-	-	-	-	+	-	-
NS-398 (1μM)	-	-	-	-	-	+	-
(10μM)	-	-	-	-	-	-	+

Figure 18. Down-regulation of DON-mediated IL-6 superinduction in RAW 264.7 cells by COX-2 inhibitors, indomethacin and NS-398. Cells (2.5x10 5 /ml) were treated with DON (100 ng/ml), LPS (200 ng/ml) and indomethacin (2 or 20μM) or NS-398 (1 or 10μM) for 24 hr and supernatant was analyzed for IL-6 by ELISA. Data are mean ± SD (n=5). Groups with different letters are significantly different ($p<0.05$). Results are representative of three experiments.

DON induces COX-2 mRNA expression in mouse lymphoid tissues.

COX-2 gene expression was assessed in lymphoid tissues of mice exposed acutely to DON. Food and water were withdrawn 3 hr before DON administration. DON, dissolved in 0.25 ml of endotoxin-free tissue culture-grade water, was administered to B6C3F1 mice at doses of 0, 1, 5, or 25 mg/kg body weight via oral gavage. The 25 mg/kg DON dose represents approximately 1/3 the LD_{50} in the B6C3F1 mouse (Forsell *et al.*, 1987). When spleens and Peyer's patches were removed for mRNA determination to assess COX-2 gene expression treatment, 5 and 25 mg/kg of DON were found to significantly enhance COX-2 mRNA levels in both organs, while 1 mg/kg DON had no effect (Fig. 19).

COX-2 mRNA expression precedes or is concurrent with IL-6 mRNA expression in lymphoid tissue of mice exposed to DON.

To assess the possible relationship between DON-induced COX-2 and IL-6 expression, the kinetics of COX-2 mRNA appearance was compared with that for IL-6 after exposure to 25 mg/kg DON for 1, 2, 4, 6 and 12 hr. COX-2 mRNA expression was maximal in both Peyer's patches (Fig. 20A) and spleen (Fig. 20B) at 2 hr. In Peyer's patches and spleen, IL-6 mRNA was also induced by DON, however, peak expression occurred 2 to 4 hr after toxin exposure. These data indicate that maximal COX-2 gene upregulation preceded or was concurrent with that for IL-6 in vivo.

***COX inhibition ablates DON-induced IL-6 mRNA expression in splee*n.**

To evaluate the effect of COX metabolism on IL-6 production by DON, mice were pretreated intraperitoneally with the COX inhibitors indomethacin or NS-398 30 min before

DON gavage. Mice pretreated with idomethacin (Fig. 22A) or NS-398 (Fig. 22B) showed significantly lower DON-induced IL-6 mRNA responses in spleen than did vehicle-pretreated mice. Furthermore, DON (12.5 and 25 mg/kg) elevated serum IL-6 at 3 hr and this enhancement was significantly suppressed by pretreatment with the COX inhibitors (Fig. 21). Indomethacin or NS-398 controls had no effect on IL-6 mRNA expression (data not shown). These results suggest a role for COX-2 metabolites in elevated IL-6 mRNA and protein expression in DON-exposed mice.

COX-2 deficiency ablates DON-induced IL-6 expression in vivo

To further establish a role for COX-2 in DON-induced IL-6 expression, induction of IL-6 mRNA and protein following oral DON exposure was compared between COX-2 KO and COX-2 WT mice. Splenic IL-6 mRNA levels from DON-treated KO mice were 6 times lower than that for the DON-treated wild type (Fig. 23A). Stimulation indices (DON/vehicle) for WT and KO mice were 17.7 and 8.3, respectively, indicating that the response of the WT to DON was at least twice that for the KO. Relatedly, DON-treated COX-2 KO mice exhibited one-half of the serum IL-6 response of the DON-treated WT (Fig. 23B). These data support the contention that COX-2 is a important mediator in DON-induced IL-6 expression and further suggest that DON can also induce IL-6 by a Cox-2 independent pathway.

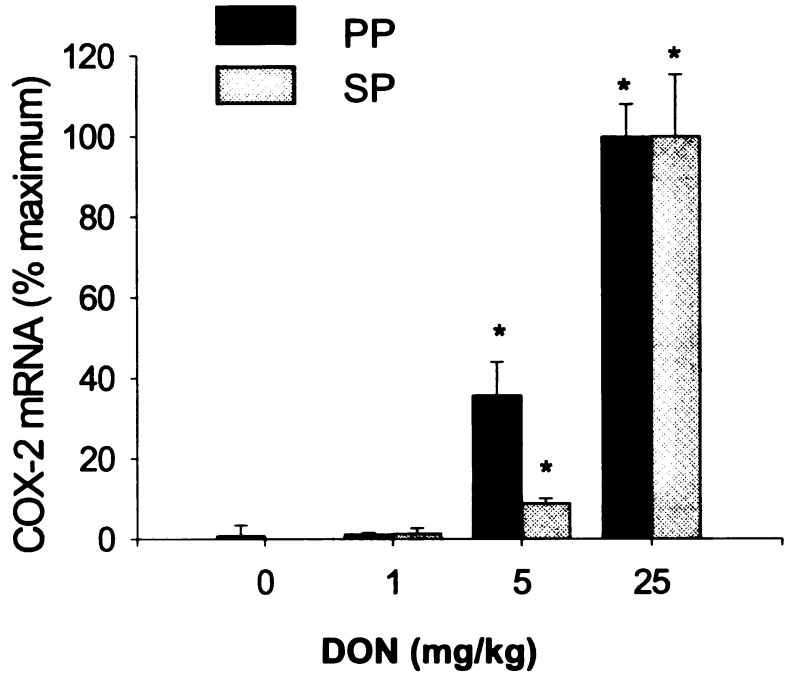


Figure 19. Dose response of DON-induced COX-2 mRNA expression in murine Peyer's patches and spleen. DON was administered via oral gavage and mRNA from spleen or Peyer's patches was analyzed 2 hr later by competitive RT-PCR. Data are mean ± SD (n=3). The symbol * indicates significant difference ($p<0.05$) from vehicle (0 mg/kg DON). Results are representative of two experiments.

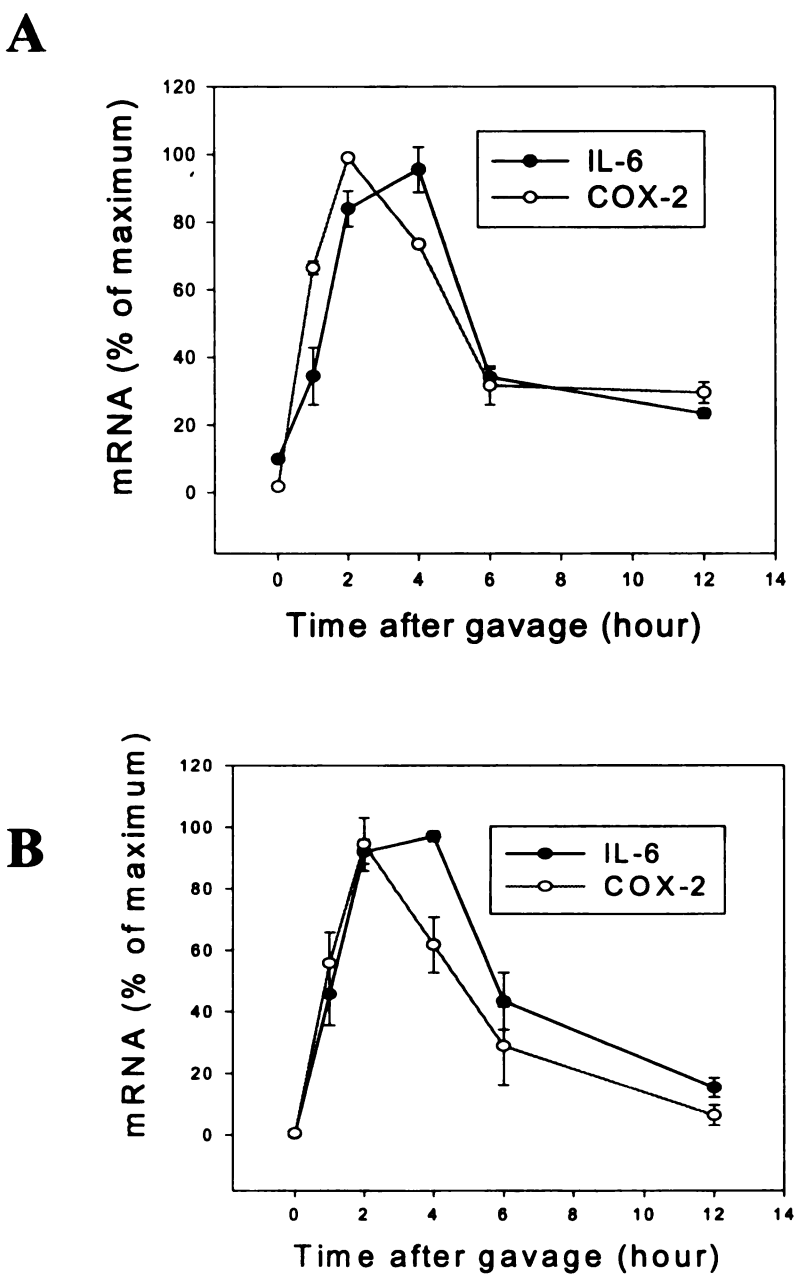
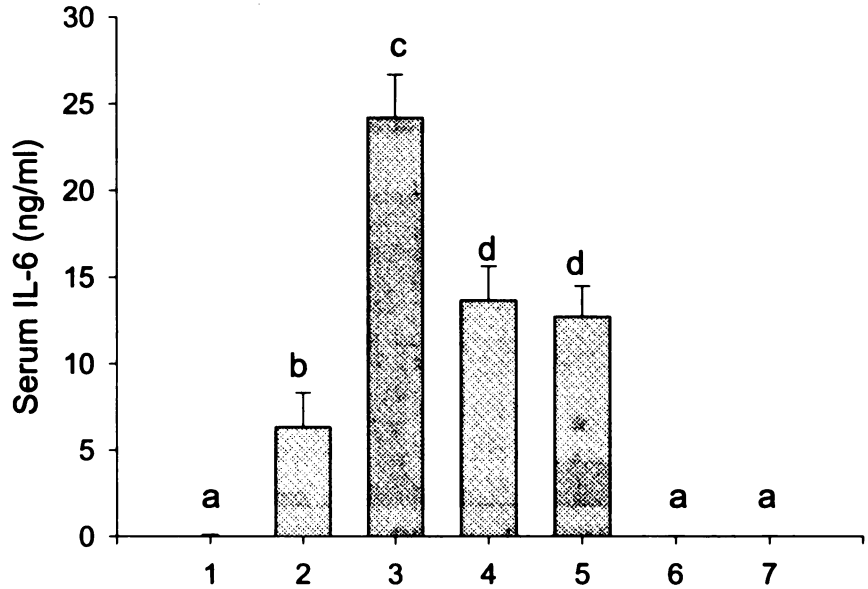


Figure 20. Time response of DON-induced COX-2 and IL-6 mRNA expression in murine Peyer's patches and spleen. DON (25 mg/kg) was administered via oral gavage and mRNA from Peyer's patches (A) or spleen (B) was analyzed 2 hr later by competitive RT-PCR at various time intervals. Data are mean ± SD (n=3). Results are representative of two experiments.



Treatment	1	2	3	4	5	6	7
DON (12.5mg/kg)	-	+	-	-	-	-	-
(25 mg/kg)	-	-	+	+	+	-	-
Indomethacin (20mg/kg)	-	-	-	+	-	+	-
NS-398 (5mg/kg)	-	-	-	-	+	-	+

Figure 21. Suppression of DON-induced serum IL-6 response in mice by COX-2 inhibitors indomethacin and NS-398. Mice were treated as described in Materials and Methods, and serum analyzed 3 hr later. Data are mean ± SD (n=4). Groups marked with different letters are significantly different ($p<0.05$). Results are representative of two experiments.

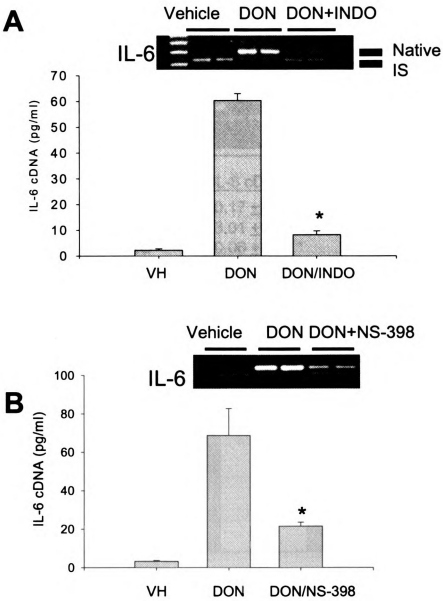


Figure 22. Suppression of DON-induced IL-6 mRNA response in spleen by COX-2 inhibitors, indomethacin and NS-398 suppresses. Mice were pre-exposed to 20 mg/kg indomethacin (A) or 5 mg/kg NS398 (B) min and then given 25 mg/kg of DON by oral gavage. Splenic mRNA was isolated 3 hr later and analyzed by RT-competitive-PCR. Data are mean ± SD (n=4). The * indicates significant difference ($p<0.05$) from DON treatment group. Results are representative of two experiments.

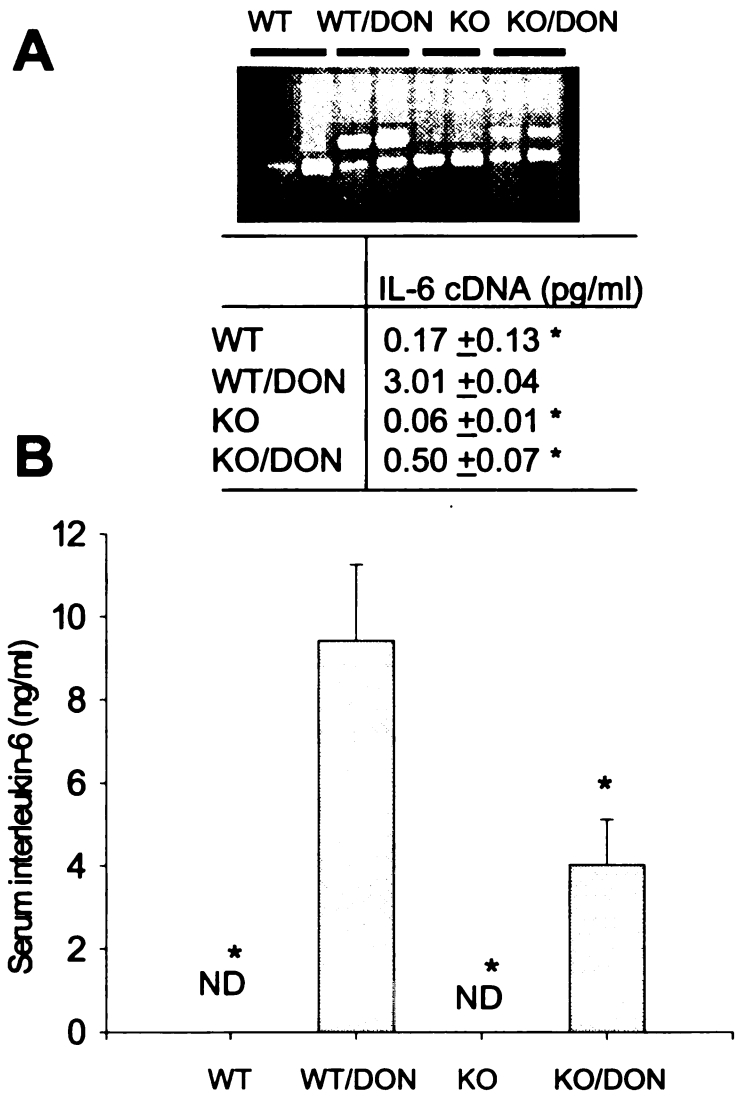


Figure 23. Impaired response of splenic IL-6 mRNA and serum IL-6 to DON in COX-2 knockout mice. COX-2 knockout (KO) and wild type mice (WT) were exposed orally to 25 mg/kg DON. After 3 hr, splenic IL-6 mRNA (A) was analyzed by competitive RT-PCR and serum IL-6 (B) was measured by ELISA. Data are mean ± SD (n=5). The * indicates significant difference ($p<0.05$) from DON/WT treatment group. ND indicates not detectable.

DISCUSSION

Th1/Th2 cell balance and level of proinflammatory cytokine response are key determinative factors in the pathogenic outcomes and the severity of inflammatory diseases (Ebihara *et al.*, 2001; Lim *et al.*, 2001). PGE2 plays an important Th2-driving role (Harris *et al.*, 2002). Notably, PGE2 is known to enhance IL-6, which is considered to be a Th2 cytokine in endotoxemia, airway inflammation, and autoimmune arthritis (Meyer *et al.*, 1995; Anderson *et al.*, 1996; Tavakoli *et al.*, 2001). The results presented herein suggest that COX-2 metabolism might similarly be involved in upregulating IL-6 in DON-exposed animals and, thus, contribute to resultant immunotoxic effects.

IL-6 plays a critical role in the DON-induced pathogenesis, particularly relative to experimental IgA hyperproduction and IgA nephropathy. DON ingestion induces polyclonal autoreactive IgA production which forms immune complexes and subsequently deposits in mesangial regions of the kidney glomerulus (Rasooly *et al.*, 1994; Rasooly and Pestka, 1994). IL-6 is widely recognized to promote terminal differentiation of Peyer's patch B cells to IgA-secreting plasma cells (Beagley *et al.*, 1989; Beagley *et al.*, 1991; McGhee *et al.*, 1991) and several lines of evidence suggest IL-6 contributes to DON-induced IgA hyperelevation. First, oral DON exposure rapidly induces IL-6 in spleens and Peyers patches of mice (Zhou *et al.*, 1997). Second, IL-6-deficient mice are refractory to DON-induced dysregulation of IgA production (Pestka and Zhou, 2000). Third, Yan observed that IgA and IL-6 production are increased in PP cultures from mice orally exposed to DON at 2 hr prior to termination and, furthermore, addition of anti-IL-6 to these cultures completely ablated

IgA production (Yan *et al.*, 1997). IgA and IL-6 production in PP cultures derived from DON-exposed mice are also abrogated when cultures are depleted of macrophages but are restored upon co-culture with peritoneal macrophage from mice exposed to DON in vivo (Yan *et al.*, 1998). This latter evidence suggests that macrophages may be a primary source of IL-6 in DON-induced IgA dysregulation.

Understanding how trichothecenes dysregulate IL-6 is very important because aberrant serum IgA accumulation may be specifically deleterious to human health. Persistently elevated polyclonal serum IgA and IgA immune complexes, increased IgA-bearing lymphocytes, mesangial IgA accumulation, hematuria and increased susceptibility of males that is found after DON ingestion by mice. (Pestka *et al.*, 1989; Dong *et al.*, 1991; Dong and Pestka, 1993; Greene *et al.*, 1994; Greene *et al.*, 1995) are identical manifestations to those associated with the human IgA nephropathy (Clarkson *et al.*, 1987). Although the etiology of this disease remains unknown, GI/respiratory tract infections (Emancipator and Lamm, 1989) as well as grain-containing diets (Coppo *et al.*, 1986) have been suggested to play roles. IgA nephropathy accounts for 20-40% of all primary glomerular disease worldwide (D'Amico, 1987). Approximately 150,000 persons in the U.S. exhibit the clinical features of IgAN with between 2800 to 4200 entering into end-stage renal failure each year (Hellegars, 1993). The potential exists for dietary DON to contribute to this disease. In addition, the novel capacity of chronic oral DON exposure to induce aberrant elevation of serum IgA and mesangial IgA accumulation provides a model for resolving contributory factors and potential mechanisms associated with IgAN as well as therapeutic regimens. In addition to IL-6's effects on IgA production, this cytokine might also promote pathogenic processes in IgA nephropathy by enhancing mesangial cell proliferation and producing

proinflammatory lipid mediators and superoxide anion (Chen *et al.*, 1995; Taniguchi *et al.*, 1999; Harada *et al.*, 2002).

The mechanisms by which DON induces COX-2 expression have recently been studied in the RAW 264.7 macrophage models (Moon and Pestka, 2002). DON was found to increase COX-2 mRNA levels by elevating both transcriptional activity and mRNA stability. Since DON exposure can result in the phosphorylation of the mitogen activated protein kinase (MAPKs) ERK 1/2, p38 and JNK 1/2, the role of these kinases in COX-2 expression have also been explored. Enhanced transcriptional activity was modulated by ERK and p38 signaling pathways whereas mRNA stability was promoted exclusively by DON-activated p38 phosphorylation. In contrast, JNK was not involved in increased COX-2 transcript and protein expression. The specific upstream mechanisms by which DON enhances MAPK phosphorylation remain unresolved but appear to involve interaction with the ribosome via a "ribotoxic stress response" (Laskin *et al.*, 2002).

The means by which PGE2 , one of representative COX-2 metabolites, modulates cytokine expression have been well-described (Harris *et al.*, 2002). Briefly, PGE2 exerts its action by binding to one (or a combination) of its four receptors subtypes, EP_1, EP_2, EP_3 or EP_4. These receptors are coupled to different G proteins and use several messenger signaling pathways. EP_1 is coupled to $G_{q/p}$ and ligand binding stimulates IL-6 synthesis through both extracellular and intracellular Ca^{2+} mobilization. EP_2 and EP_4 are coupled to Gs proteins and induce the expression of cAMP, which lead to IL-6 expression. EP_3 are primarily coupled to Gi and is most often inhibitory to cAMP. In addition, some COX-2 metabolites such as cyclopentanone prostaglandins can bind to a nuclear receptor, peroxisome proliferator-activated receptor (thus impacting proinflammatory gene transcription (Ricote *et al.*, 1998).

Since COX-2 metabolites can either potentiate or attenuate proinflammatory cytokine production, further research on how DON might differentially affect these two modulatory routes is warranted. PGE_2 also selectively impairs IFN-γ production and directly promotes the production of Th2 cytokines in murine and human Th clones (Betz and Fox, 1991; Snijdewint *et al.*, 1993). It is notable that interleukin-12 (IL-12)p70 heterodimer, a major Th1-driving cytokine, is suppressed by PGE2 , whereas PGE2 participates in the induction of IL-12p40 which can function as an antagonist of biologically active IL-12p70 (Kalinski *et al.*, 2001). The capacity of DON to drive IL-12 p40 but not IL-12 p35 expression in vivo (Zhou *et al.*, 1997) may be related to COX-2 and PGE2.

Proinflammatory cytokines also have been related to the cellular injury like apoptosis during chemical toxicosis such as alcohol and CCl_4 (Luster *et al.*, 2001; Hong *et al.*, 2002). DON in the presence of endotoxin synergistically enhance proinflammatory cytokines such as IL-6 and cause marked cell death with characteristic features of apoptosis (Zhou *et al.*, 1999). In some aspect, IL-6 production by chemical irritants could be rather one of critical endogenous defensive mechanism because it rescues tissue from the apoptotic injuries and promotes the wound healing processes (Rollwagen *et al.*, 1998; Gallucci *et al.*, 2000). COX-2 metabolites also can play protective role in the gastrointestinal inflammation (Langenbach *et al.*, 1999a; Langenbach *et al.*, 1999b; Morteau, 1999; Morteau *et al.*, 2000). COX-2 knockout mice developed increased susceptibility to chemical-induced colitis. Moreover, some COX-2 products might be very important resolving agents during the late stage of inflammation with alternate set of prostaglandins such as those of the cyclopentenone family (Morteau, 1999). Therefore, the body exposed to DON has the defensive strategies with the COX-2 and IL-6 enhanced to some degree. It should be noted that NS-398 did not completely inhibit IL-6 gene expression and appeared to be equipotent to indomethacin

which is a comparatively weaker COX inhibitor. There may be several reasons for this observation. First, the 5 mg/kg NS-398 dose might not be optimal for complete inhibition of COX-2 expression. Previous studies reported that NS-398 selectively inhibits COX-2 and suppresses PGE2 production in inflamed tissue to the level seen in noninflamed tissues at 10-30 mg/kg in vivo (Arai *et al.*, 1993; Futaki *et al.*, 1993; Futaki *et al.*, 1994; Futaki *et al.*, 1997).Second, although both indomethacin and NS-398 block COX-2 activity, each can have different production inhibition efficiency such as diverse prostaglandins and thromboxanes (Tegeder *et al.*, 2000). If specific COX-2 products are important in IL-6 gene regulation, a differential alteration of COX-2 products by INDO or NS-398 might affect IL-6 production in different ways. The third and most likely possibility is that a COX-2 independent pathway was also involved in DON-mediated IL-6 induction. For this reason, COX-2 KO mice were adopted in order to confirm the positive regulation of IL-6 gene expression by COX-2 metabolites. Although the IL-6 response to DON was significantly ablated compared to wild-type mice, COX-2 deficient mice were indeed capable of mounting a significant IL-6 response thus suggesting an COX-2-independent pathway existed in tandem with COX-2 dependent pathway.

Taken together, DON was found to induce COX-2 expression in vitro and in vivo and this mediated induction of IL-6 gene expression. The capacity of COX-2 metabolites to induce IL-6 production may be a crucial mechanism for DON-induced IgA nephropathy and other diverse immunotoxic effects such as shock and defensive role. Further research should be directed understanding mechanisms for DON-induced COX-2 expression as well as the relationship between trichothecene structure and such inductive effects.

CHAPTER 5.

EFFECT OF FISH OIL/(N-3) PUFA ON DON-INDUCED IL-6 AND COX-2 PRODUCTION [1]

[1] Most part of this work has been submitted for publication in the title "Deoxynivalenol-Induced Mitogen-Activated Protein Kinase Phosphorylation and IL-6 Expression in Mice Suppressed by Fish Oil" by Moon, Y.,and Pestka, J. J. in *Journal of Nutritional Biochemistry.*

ABSTRACT

Deoxynivalenol (DON)-induced IgA hyperelevation and mesangial IgA deposition in mice mimics the early stages of human IgA nephropathy (IgAN). Among potential mediators in this disease, interleukin-6 (IL-6) is likely to play a particularly critical role in IgA elevation and disease exacerbation. Based on previous findings that dietary fish oil (FO) suppresses DON-induced IgAN, we hypothesized that FO inhibits induction of IL-6 expression by this mycotoxin in vivo and in vitro. Mice were fed modified AIN 93G diet amended with 7% corn oil (CO) or 1% corn oil plus 6% menhaden fish oil (FO) for up to 8 wk and then exposed acutely to DON by oral gavage. DON-induced plasma IL-6 and splenic mRNA elevation in FO-fed mice were significantly suppressed when compared to the CO-fed group after 8 wk. The effects of FO on phosphorylation of mitogen-activated protein kinases (MAPKs), critical upstream transducers of IL-6 upregulation, were also assessed. DON-induced phosphorylation of extracellular signal regulated protein kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinases 1 and 2 (JNK1/2) by DON was significantly suppressed in spleens of mice fed with FO, whereas p38 was not. Splenic COX-2 mRNA expression, which has been previously shown to enhance DON-induced IL-6, was also significantly decreased by FO, however, plasma levels of the COX-2 metabolite, prostaglandin E_2, were not affected. To confirm in vivo findings, the effects of pretreatment with the two primary n-3 PUFAs in FO, eicosapentaenoic acid (20:5[n-3]; EPA) and docosahexaenoic acid, (22:6[n-3]; DHA), on DON-induced IL-6 expression were assessed in LPS-treated RAW 264.7 macrophage cells. Consistent with the in vivo findings, both EPA

and DHA significantly suppressed DON-induced IL-6 superinduction by DON, as well as impaired DON-induced ERK1/2 and JNK1/2 phosphorylation. In contrast, the n-6 PUFA arachidonic acid (20:4[n-3]) had markedly less effect. Taken together, the capacity of FO and its component n-3 PUFAs to suppress IL-6 expression as well as ERK 1/2 and JNK 1/2 activation might explain, in part, the reported suppressive effects of these lipids on DON-induced IgA nephropathy.

INTRODUCTION

Consumption of (n-3) polyunsaturated fatty acids (PUFAs) in fish oil (FO)-containing diets has been associated with modulation of a diverse array of immunological events that include suppression of lymphocyte proliferation, antigen presentation, MHC II expression, cytotoxic T lymphocyte activity, NK cell activity, macrophage-mediated cytotoxicity, and neutrophil/monocyte chemotaxis (Wu and Meydani, 1998; Kehn and Fernandes, 2001; Weiss *et al.*, 2002). These functional effects have been the rationale for experimental and clinical attenuation of acute inflammatory, delayed-type hypersensitivity, autoimmune and transplant rejection responses by (n-3) PUFA (Otto *et al.*, 1990; Taki *et al.*, 1992; Harbige and Fisher, 2001; Ergas *et al.*, 2002; Venkatraman and Meksawan, 2002). The mechanisms for these effects may be related to the capacity of (n-3) PUFAs to modulate inflammatory mediator profiles and concentrations, immune cell populations by apoptotic signaling and cell-to-cell communication (Meydani *et al.*, 1991; Purasiri *et al.*, 1994; von Schacky, 1996; Jolly *et al.*, 2001).

Human IgA nephropathy (IgAN), the most common type of glomerulonephritis in the world, is characterized by the presence of prominent IgA deposits in the mesangial region as discerned by immunofluorescence microscopy (D'Amico, 1987; D'Amico, 2000). Etiological factors that have been associated with this disease include prior mucosal infection, genetic predisposition and diet. Dietary supplementation with fish oil has been suggested to benefit patients with immune-related renal diseases including IgAN, lupus nephritis and cyclosporine-induced nephrotoxicity (Donadio, 1991). Despite supportive data

from several randomized clinical trials evaluating efficacy of fish oil in treating IgAN (Hamazaki *et al.*, 1984; Donadio *et al.*, 1999; Donadio *et al.*, 2001), there have been conflicting findings (Bennett *et al.*, 1989; Pettersson *et al.*, 1994). To clarify these issues, animal models might potentially be used to elucidate mechanisms for intervention in IgAN with FO or its component (n-3) PUFAs. Several animal models for IgAN have been developed using approaches that include injection with IgA immune complex, mucosal immunization, viral infection and use of genetically modified mice prone to mesangial IgA deposition (Montinaro *et al.*, 1999). Notably, dietary exposure to the mycotoxin deoxynivalenol (DON or vomitoxin) has been found to cause clinical signs analogous to early stages of human IgAN (Pestka *et al.*, 1989; Dong *et al.*, 1991). DON, a trichothecene produced by *Fusarium graminearum* and *F. culmorum,* is frequently found in grain-based agricultural commodities from the Midwestern United States such as wheat, barley and corn (Rotter *et al.*, 1996). The toxin is an potent inhibitor of protein synthesis that can impact actively dividing tissues with the immune system being particularly susceptible (Rotter *et al.*, 1996; Bondy and Pestka, 2000). Acute exposure to high doses of DON can induce rapid diminution of lymphoid tissues via apoptosis and lymphopenia in experimental animals. Paradoxically, low dose or chronic DON exposure can be immunostimulatory as evidenced by its capacity to induce cytokine gene expression in vivo (Zhou *et al.*, 1997; Zhou *et al.*, 1999). Dietary DON exposure induces elevation of serum IgA, mesangial kidney IgA deposition and hematuria in the mice which are hallmarks of IgAN (Pestka *et al.*, 1989; Dong *et al.*, 1991). Recently, we have shown that FO-containing diets impair these immunopathologic endpoints (Pestka *et al.*, 2002).

Aberrant IgA production upon DON ingestion appears to be mediated in part by

superinduction of cytokine gene expression in mononuclear phagocytes (Yan *et al.*, 1997; Yan *et al.*, 1998) and T helper cells (Warner *et al.*, 1994). Among these cytokines, interleukin-6 (IL-6) is recognized to be particularly important to the modulation of mucosal B cell differentiation to IgA-secreting plasma cells (Beagley *et al.*, 1989; McGhee *et al.*, 1991; Fujihashi *et al.*, 1992; Ramsay *et al.*, 1994). In addition to effects of IL-6 on IgA production, this cytokine might also promote pathogenic progresses in IgA nephropathy by enhancing mesangial cell proliferation and producing proinflammatory lipid mediators and superoxide anion (Chen *et al.*, 1995; Taniguchi *et al.*, 1999; Harada *et al.*, 2002). Therefore, IL-6 induction by DON might be critical to both early stage IgA production and the later disease severity of IgA nephropathy. In support of the former possibility, genetic disruption of IL-6 in mice markedly reduces DON-induced increases in serum IgA as well as kidney IgA deposition in mice (Pestka and Zhou, 2000). The purpose of this investigation was to test the hypothesis that dietary FO impairs DON-induced IL-6 gene expression. The results demonstrated that FO and (n-3) PUFAs impair upregulation of IL-6 by DON both in vivo and in vitro. Furthermore, these ameliorative effects were associated with decreased activation of mitogen-activated protein kinases (MAPKs).

MATERIALS AND METHODS

Animal studies. All animal handling was conducted according to the guidelines established by the NIH. Experiments were designed to minimize numbers of animals required to adequately test the proposed hypothesis and approved by Michigan State University Laboratory Animal Committee. For most studies, male B6C3F$_1$ mice (6 to 7 week old) were used. These were obtained from Charles River (Portage, MI) and acclimated for one week in a humidity- and temperature-controlled room with an alternating 12 hr light and dark cycle. Mice were housed singly in the environmentally protected cages which consisted of a transparent polycarbonate body with a filter bonnet, stainless steel wire lid and a layer of heat-treated hardwood chips.

All dietary constituents were purchased from Dyets (Bethlehem, PA). Experimental diets were based on the AIN-93G formulation (Reeves *et al.*, 1993) and consisted of the following ingredients (per kg): 35 g AIN-93G mineral mix, 10 g AIN-93 vitamin mix, 200 g casein, 397.49 g cornstarch, 132 g Dyetrose (dextranized cornstarch), 100 g sucrose, 50 g cellulose, 14 mg t-butylhydroquinone (TBHQ), 3 g L-cystine, and 2.5 g choline bitartrate. Corn oil (CO) and menhaden fish oil (FO), each of which contained 200 mg/kg of test-butyl hydroquinone (TBHQ), were used to amend the basal diet to yield two kinds of experimental diets containing the following (per kg): (1) 70 g CO (CO group); and (2) 10 g CO plus 60 g FO (FO group). The basal 1% level of CO was included to ensure that minimal (n-6) PUFA requirements were met. Relative amounts of (n-3) and (n-6) PUFAs are shown in Table 3. Equivalent amounts of TBHQ were added to both stock oils to prevent confounding effects

from this antioxidant.

Mice were fed experimental diets for 2, 4, and 8 wk and then orally gavaged with DON (25mg/kg bw) Following DON treatment, animals were held for 30 min or 3 hr and then bled retroorbitally under metaflurane anesthesia for plasma IL-6 and PGE2quantitation. Mice were immediately euthanized by cervical dislocation and spleens removed for Western blot analysis and mRNA measurement.

Cell culture studies. RAW 264.7 murine macrophage cells(American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals Inc, Norcross, GA), 100 unit/ml penicillin (Sigma), and 100 mg/ml streptomycin (Sigma, St. Louis, MO) in a 5 % CO_2 humidified incubator at 37°C. Macrophage cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hematocytometer (Strober, 1991).

Supplementation of media with fatty acids was based on previously published methods (Lo *et al.*, 1999; Lo *et al.*, 2000). Briefly, fatty acid-free bovine serum albumin (BSA, Roche, Indianapolis, IN) and fatty acid (eicosapentaenoic acid 20:5[n-3], EPA); or docosahexaenoic acid, 22:6[n-3], DHA; or arachidonic acid, 20:4[n-6], AA) (Sigma Chemical Co., St. Louis, MO) were mixed in Ca^{2+}, Mg^{2+} free PBS at a 3:1 molar ratio under nitrogen on a rocking shaker for 24 hr at 37 °C. These mixtures were then diluted with serum-free DMEM media to desired concentration of fatty acid. Media were freshly prepared before each experiment. Before adding fatty acid-amended media, adherent RAW 264.7 cells (2.5×10^5/ml) were incubated in serum free DMEM medium for 18 hr. Cells were then

cultured with serum-free fatty acid-amended medium for 24 hr. For IL-6 and COX-2 studies, cells were cultured with or without DON and/or LPS for an additional 24 hr after replacing with fresh DMEM medium supplemented with 10 % heat-inactivated FBS. For MAPK studies, cells were incubated for 48 hr with serum-free fatty acid-amended media and then exposed to DON or vehicle for 30 min.

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

Detection of IL-6 and COX-2 mRNA by RT-competitive PCR. Total RNA from murine tissue was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. RNA (100 ng) from each sample was converted to cDNA by reverse-transcriptase (Riedy *et al.*, 1995) and resultant cDNA was amplified competitively with truncated internal standard of cDNA constructed by the bridging-deletion method (Hall *et al.*, 1998). Amplification was performed in a 9600 Perkin Elmer Cycler (Perkin-Elmer Corp., Norwalk, CT) using the following parameters: 30 cycles of reactions of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s (IL-6) or 56 °C for 45 s (COX-2), and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.5 % agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers were synthesized at Michigan State University Molecular Structure facility. The 5' forward (F) and 3' reverse (R)-complement PCR primers for amplification of mouse COX-2 and IL-6 cDNA were ACACTCTATCACTGGCATCC (COX-2F), GAAGGGACACCCTTTCACAT (COX-2R), TTCACAAGTCCGGAGAGGAG (IL-6F), and TGGTCTTGGTCCTTAGCCAC (IL-6R) respectively. The final end-product of amplified cDNAs were 584 bp of COX-2 , 500 bp of COX-2 internal standard cDNA, 488 bp of IL-6, and 400 bp of IL-6 internal standard cDNA. Densitometric ratios of native cDNA to each internal standard cDNA were used to make each standard curve for calculating cDNA concentrations in RT reaction products.

PGE2 assay. Blood was collected in vacutainers containing 1 mM EDTA and 10 μM indomethacin, and centrifuged at 4000 xg for 5min. Plasma supernatant was diluted to 1:5 in 0.1 M phosphate buffer solution. Samples were cleaned up with a PGE2 affinity sorbent

to remove interfering substances. PGE2 affinity sorbent (50 ml, Cayman Chemical CO., Ann Arbor, MI) was added to 1 ml of the prepared samples, and the suspension was gently mixed for 1 hr on the rocker at 25°C. Samples were briefly centrifuged at 1500 x g to sediment the sorbent and the sediment was then washed once with 1 ml of 0.1 M phosphate buffer. Sorbent pellets were resuspended in 0.5 ml elution solution (95 % ethanol solution), mixed by vortexing, and centrifuged briefly at 1500 x g. The supernatant was evaporated, and residue in dissolved in EIA assay buffer. The solution was then analyzed by ELISA (Cayman Chemical Co., Ann Arbor, MI) according to manufacturer's instructions.

Western blot analysis. At the time of harvest, cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1% (w/v) SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris pH 7.4), and sonicated for 5 seconds. For spleen samples, frozen tissue was homogenized in the boiled lysis buffer and sonicated for 10 seconds. Protein was measured by the Lowry method using Dc protein assay reagent (Bio-Rad, Cambridge, MA). Extracts (10 μg) were mixed with Laemmli sample buffer (Bio-Rad), and boiled for 5 min before resolving on a 10 % (w/v) acrylamide gel. Resolved proteins were transferred to PVDF membrane and blocked with Tris-buffered saline (10 mM Tris-HCl pH 7.5, 100 mM NaCl) containing 0.1 % (v/v) Tween-20 and 1% (w/v) BSA (TBST-BSA). The membrane was incubated for 1 hr with MAPK antibodies (rabbit IgG, New England Biolab, Beverly, MA) at 1:1000 dilution in TBST-BSA, and then were washed three times with TBST. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA).

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

Table 3. Fatty acid composition of oils used for experimental diets[1].

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

[1] Only the major fatty acids are shown. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[2] 1000 g/L corn oil.

[3] 857 g/L fish oil and 143 g/L corn oil.

RESULTS

Fish oil composition was shown in Table 3 based on grouping categories of fatty acids. Prominent difference between CO and FO was the composition of (n-6) and (n-3) PUFA. FO contains high amount of (n-3) PUFA, which has been well known to suppress diverse immune-related diseases and inflammations. Other than (n-3) PUFA with ameliorating action on IgAN model (Chandrasekar and Fernandes, 1994), antioxidant effect by added TBHQ was excluded because FO and CO contained the same amount of this antioxidant. In the diet plan, we also strengthened the basic level of CO to ensure optimal (n-6) fatty acid intake.

Fish oil feeding suppressed IL-6 production in mice exposed to DON

After feeding with oil-containing diets for 2, 4, and 8 weeks, mice were acutely exposed to vehicle (water) or 25 mg/kg DON. DON modulates bunches of cytokine array including proinflammatory mediators such as IL-6 and TNF-α (Zhou *et al.*, 1997; Zhou *et al.*, 1999). At 2 and 4 wk, the difference in the DON-enhanced plasma IL-6 between CO-fed and FO-fed mice was not significant (Data not shown), but FO-fed mice for 8 weeks showed reduced IL-6 response to the toxin compared to CO-fed mice (Fig. 24). Furthermore, the induction of IL-6 transcript by DON (25 mg/kg) in the spleen of FO-fed mice was also lower than that in CO-fed mice and this reduced response was significant from 4 wk feeding (Fig. 25). The IL-6 mRNA level in FO- or CO-fed mice was almost maximal around 3 hr after

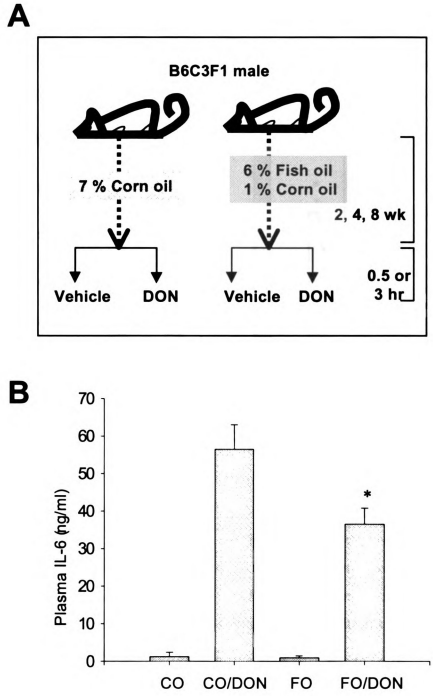


Figure 24. A. Feeding plan with corn oil or fish oil in B6C3F1 male mice. Details are described in methods. B. Effect of FO diet on plasma IL-6 production in mice acutely exposed to DON or vehicle. CO and FO diets were fed to male B6C3F$_1$ mice for 8 wk and 25 mg/kg DON or vehicle was administered via gavage. After 3 hr of the toxin treatment, blood was collected for IL-6 ELISA analysis. Asterisk indicates significant difference ($P<0.05$) of FO/DON group from CO/DON group.

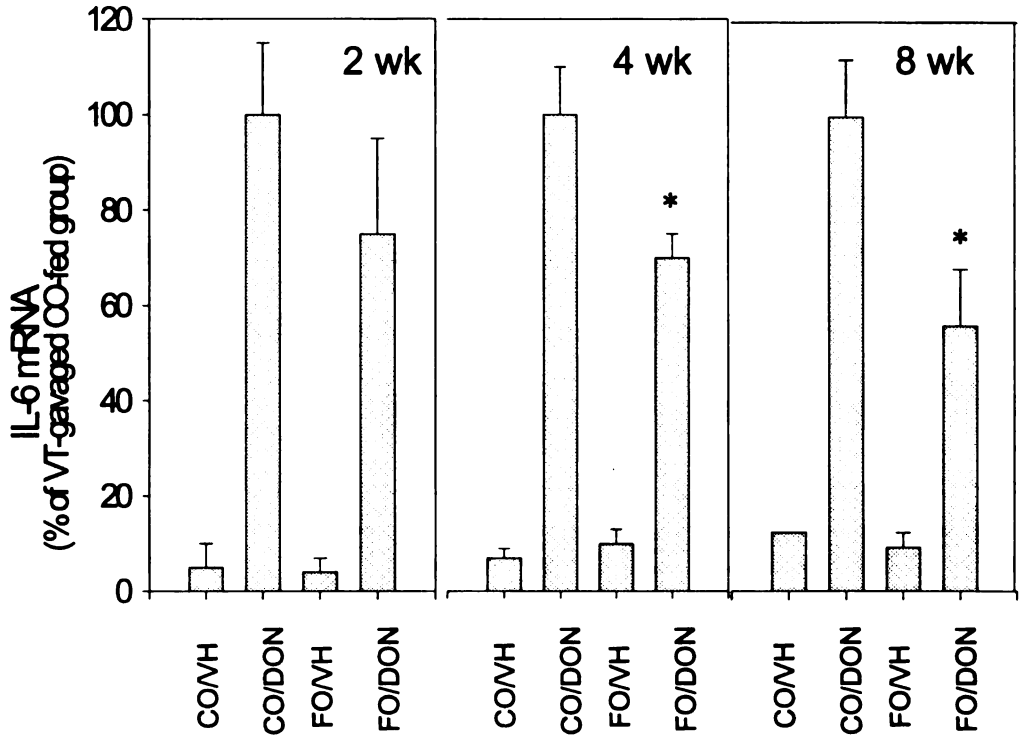


Figure 25. Effects of FO diet on splenic IL-6 mRNAs of mice exposed to DON or vehicle (VH). CO and FO diets were fed to male B6C3F$_1$ mice for 2, 4, and 8 wk and 25 mg/kg DON or VH was administered via gavage. After 3 hr of the toxin treatment, spleens were analyzed for IL-6 mRNA levels using RT-competitive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

DON gavage which was corresponding with IL-6 mRNA kinetics in normal mice exposed to DON (Zhou *et al.*, 1997).

Fish oil feeding had suppressive effect on DON-induced COX-2 mRNA level but not on COX-2 activity

As another important proinflammatory mediator, prostaglandin E2 (PGE2) was measured because (n-3) PUFA displaces membranous arachidonic acid and reduce PGE2 production (Lo *et al.*, 1999; Tanaka *et al.*, 1999). Moreover, cycloooxygenase-2 (COX-2) is induced by DON (Moon and Pestka, 2002) and fatty acid feeding can change the pool of the enzyme substrate. Therefore, PGE2 production by DON is expected to be modulated by the change of fatty acid composition from different oil diet. However, there was little significant difference in PGE2 production by DON between CO- and FO-fed mice although the dietary FO have a trend of efficacy (Fig. 26A). DON-induced COX-2 mRNA level in FO-fed mice has decreased significantly from the level in CO-fed mice which was exposed to DON (Fig. 26B).

Dietary FO weakened the MAPK response to DON in mice.

Among diverse signaling pathways, trichothecene mycotoxins including DON have been previously shown to activate MAPK in T cells and macrophages via a ribotoxic stress response or oxidative stress response, and these findings were further associated with toxin-induced apoptosis depending on the dose regimen employed (Shifrin and Anderson, 1999; Yang *et al.*, 2000a). A critical finding in Figure 27 is a definitive link between DON-activated MAPK and FO-feeding.

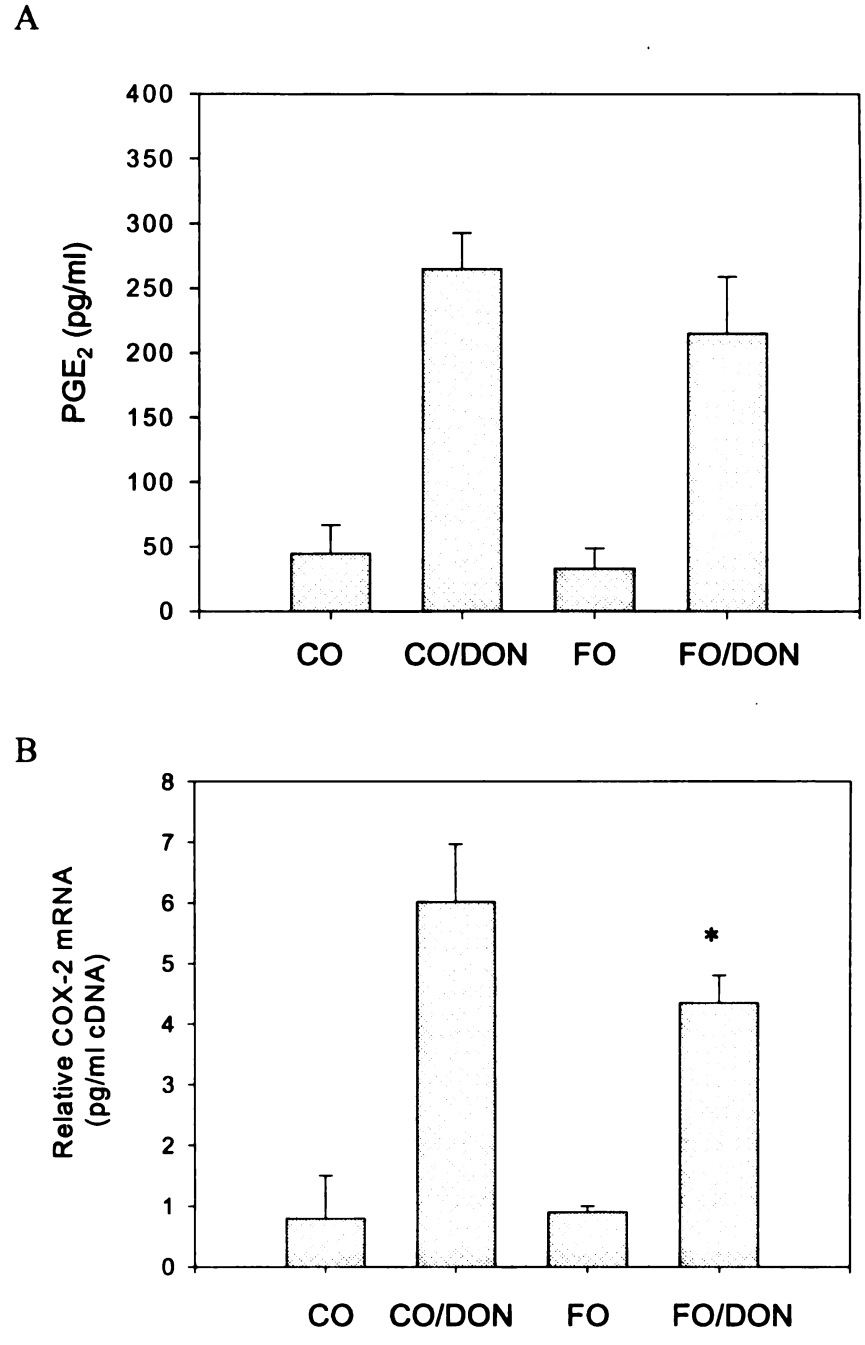


Figure 26. Effect of FO diet on COX-2 metabolism in mice acutely exposed to DON or vehicle (VH). CO or FO diet was fed to male B6C3F$_1$ mice for 8 wk and 25 mg/kg DON or VH was administered via gavage. Three hr after toxin treatment, spleens were analyzed for COX-2 mRNA levels (A) using RT-competitive PCR and for PGE2 measurement by ELISA. Asterisk indicates significant difference ($p<0.05$) of FO/DON group from CO/DON group at each exposure time.

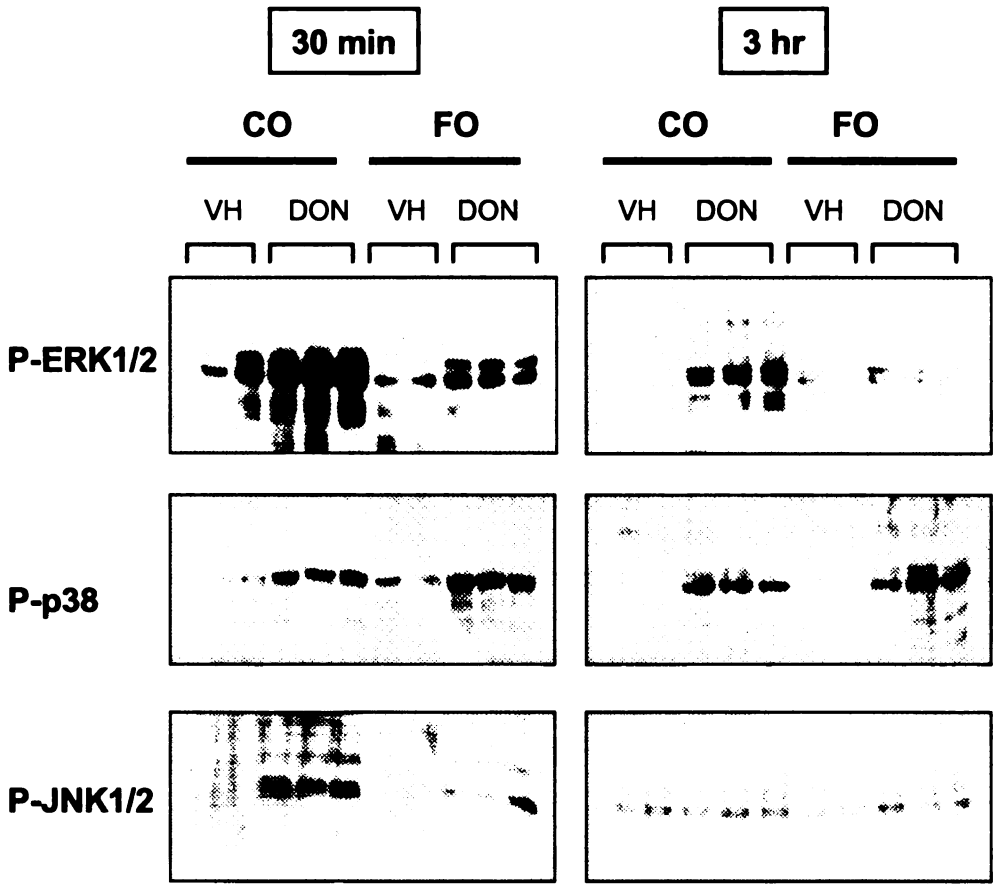


Figure 27. Effect of FO diet on MAPK phosphorylation in spleens of mice exposed to DON (25mg/kg) or vehicle (VH). Male B6C3F$_1$ mice were fed with oil-containing diet for 8 wk and DON or VH was administered to mice. Spleens were removed 0.5 and 3.0 hr later and analyzed for MAPK phosphorylation by Western blotting.

Mice fed with CO- or FO-containing diets for 8 weeks were exposed to 25 mg/kg DON for 0.5 or 3 hr and whole spleen was collected for Western blot analysis (Fig. 27). When mice were exposed to DON for 30 min, FO-feeding significantly suppressed DON-induced ERK1/2 and JNK1/2 activation, but there was no suppression in DON-activated p38 MAPK in fish oil fed mice, which showed even a little increased activation. After 3 hr exposure to DON, the effects of MAPK activation by DON were weakened and JNK1/2 activation almost disappeared. However, the suppressive effect of FO on DON-activated ERK1/2 was persistent and p38 level showed similar trend to 30 min exposure. The activated MAPK is also involved in the transcriptional or post-transcriptional regulation of IL-6 gene expression (Beyaert *et al.*, 1996; Leonard *et al.*, 1999; Tuyt *et al.*, 1999; Winzen *et al.*, 1999), and thus FO feeding can affect DON-induced IL-6 expression by regulating MAPK signaling pathway.

IL-6 superinduction by DON was retarded by (n-3) PUFA preincubation in macrophage cells

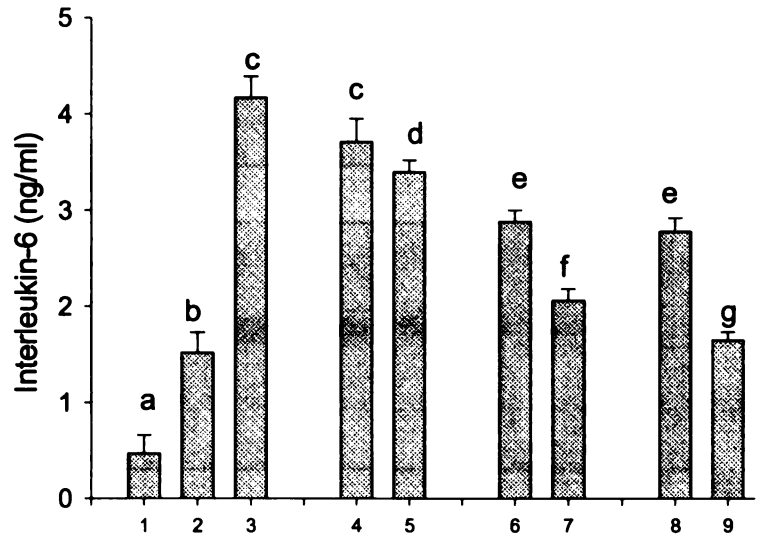
To confirm the results from feeding study, specific (n-3) PUFAs were administered to check their suppressive action on DON-induced IL-6 production in cell culture model. DON itself does not induce IL-6 production in macrophage cells which is supposed to be a main source of this proinflammatory cytokine. However, DON can superinduce IL-6 in the presence of lipopolysaccharide (LPS) in macrophage cells (RAW 264.7 cells) (Wong *et al.*, 1998; Wong *et al.*, 2001) and this superinduction model was applied for assessing the effects of (n-3) PUFA on DON-induced IL-6 production in vitro. Before the IL-6 superinduction with DON and LPS in RAW 264.7 cells, cells were pre-treated with (n-3) PUFA-bovine

serum albumin (BSA) complexes, arachidonic acid (AA)-BSA complexes, or vehicle(PBS)-BSA complexes for 24 hr. Arachidonic acid, one of (n-6) PUFA, was compared with (n-3) PUFA, eicosapentaenoic acid (20:5(n-3), EPA) or docosahexaenoic acid (22:6(n-3), DHA). Pre-treatment with EPA or DHA retarded DON-mediated IL-6 superinduction and the decrease in IL-6 was positively related with the increased concentration of (n-3) PUFA (Fig. 28). PUFA also has been known to induce programmed cell death in several systems (Latham *et al.*, 1999; Chen and Istfan, 2000; Fyfe and Abbey, 2000; Arita *et al.*, 2001; Latham *et al.*, 2001) but the PUFA concentrations used here were not significantly cytotoxic to the cells. Cellular viability was maintained over 85 % with all the treatment of chemical combinations in the study (Data not shown). Endotoxin-mediated IL-6 production is known to be down-regulated by dietary FO (Sadeghi *et al.*, 1999). Therefore, the suppressed IL-6 superinduction by (n-3) PUFA might be also due to its effect on LPS action to some degree. Taken together, (n-3) PUFA as crucial components of fish oil decreased DON-mediated IL-6 production in vitro.

MAPK activation by DON was suppressed in (n-3) PUFA-pretreated macrophage cells

As with the dietary oil feeding study, the direct effect of (n-3) PUFA on phosphorylation of DON-activated MAPK was analyzed because MAPKs are evidently involved in IL-6 production. RAW 264.7 cells were preincubated with 100μM of each fatty acid for 48 hrs and then exposed to 250 ng/ml DON for 30 min. As consistent with a previous study (Moon and Pestka, 2002), DON activated three major MAPKs (Fig 29). Moreover, pretreatment with EPA or DHA caused less ERK1/2 and JNK1/2 activation by DON than that of vehicle or arachidonic acid. These in vitro results agreed with the MAPK suppression by FO feeding in DON-exposed mouse model (Fig. 27). Therefore, the reduced

levels of ERK1/2 and JNK1/2 activation by (n-3) PUFA might contribute to less IL-6 production in DON-exposed cells.



	1	2	3	4	5	6	7	8	9
LPS (200ng/ml)	-	+	+	+	+	+	+	+	+
DON (100ng/ml)	-	-	+	+	+	+	+	+	+
AA (1μg/ml)	-	-	-	+	-	-	-	-	-
AA (10μg/ml)	-	-	-	-	+	-	-	-	-
EPA (1μg/ml)	-	-	-	-	-	+	-	-	-
EPA (10μg/ml)	-	-	-	-	-	-	+	-	-
DHA (1μg/ml)	-	-	-	-	-	-	-	+	-
DHA (10μg/ml)	-	-	-	-	-	-	-	-	+

Figure 28. PUFAs on DON-induced IL-6 production in murine macrophage cells. RAW 264.7 cells were incubated in serum free DMEM medium for 18 hr and then exposed to the fatty acid AA, EPA, or DHA)-containing serum free DMEM medium for 24 hr. Cells were further incubated in the presence of DON and/or LPS with fresh DMEM medium supplemented with 10% (v/v) heat-inactivated FBS combination. IL-6 production in supernatant was measured by ELISA. Bars with different letters are significantly different ($p<0.05$).

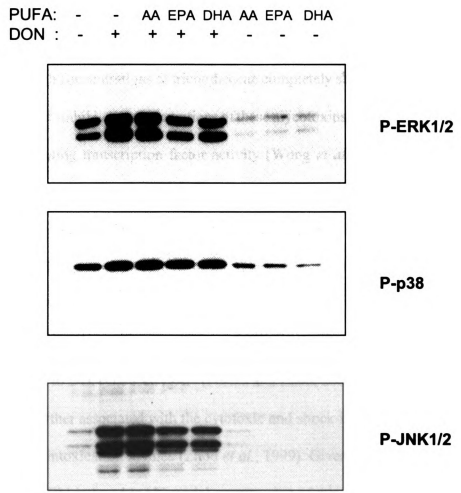


Figure 29. Effect of PUFAs on DON-induced MAPK phosphorylation in macrophage cells. RAW 264.7 cells were incubated in serum free DMEM medium for 18 hr. Cells were then incubated with 110 μM fatty acid AA, EPA, or DHA-containing serum free DMEM medium for 48 hr and then exposed to 250 ng/ml DON or vehicle (VH) for 30 min. Cellular lysate was analyzed for Western blotting.

DISCUSSION

Whereas high concentrations of trichothecene completely shut down translation and induce apoptosis, sub-inhibitory concentrations of these mycotoxins can upregulate cytokine production by elevating transcription factor activity (Wong *et al.*, 2002) and increasing mRNA stability (Li *et al.*, 1997; Wong *et al.*, 2001). Of several cytokines upregulated by DON in vivo, IL-6 appears to play a pivotal role in promoting polyclonal IgA secretion based on: (1) prolonged kinetics and magnitude of the IL-6 response relative to other cytokines (Zhou *et al.*, 1997; Zhou *et al.*, 1999) 2) reduced IgA production by neutralization with anti-IL-6 antibody in ex vivo Peyer's patch cultures (Yan *et al.*, 1997); and (3) the resistance of IL-6 knockout mice to DON-induced IgAN (Pestka and Zhou, 2000). Moreover, induction of IL-6 might be further associated with the cytotoxic and shock-like responses that occur during acute DON intoxication in mice (Zhou *et al.*, 1999). Given the importance of IL-6 production in the DON-induced IgAN model, suppression of this cytokine appears to be a viable strategy for preventing elevation of IgA as well as IgA-immune complex at serum level and kidney mesangium.

Dietary fish oil containing (n-3) PUFA has been linked to the amelioration of IL-6-mediated immunopathogenic sequelae and inflammation including autoimmune nephritis and cardiovascular diseases (Chandrasekar and Fernandes, 1994; McCarty, 1999; Venkatraman and Chu, 1999). DON-induced IgA nephropathy is also retarded by FO-feeding in the mouse model (Pestka *et al.*, 2002). The data presented here suggest that dietary fish oil suppresses DON-induced IL-6 in vivo. The observations that IL-6 superinduction is suppressed by (n-3)

PUFA pre-treatment in the cell culture model is supportive of the in vivo studies. There are several possible explanations for the decreased IL-6 response to DON by FO-feeding or pretreatment with (n-3) PUFA.

ERK1/2 and JNK1/2 are known to be crucial signaling mediators of IL-6 induction (Leonard *et al.*, 1999; Tuyt *et al.*, 1999) and fish oil components such as DHA and EPA inhibit ERK 1/2 activity in human T cells, macrophages and tumor cells (Lo *et al.*, 2000; Denys *et al.*, 2001b; Denys *et al.*, 2002). FO diets suppressed response in the ERK1/2 and JNK1/2 to DON in this study and similar effects were found in vitro. Therefore, (n-3) PUFA-modulated MAPK suppression might contribute to the reduced IL-6 response. Thus one possible explanation for depression of DON-induced IL-6 is that altered MAPK signaling by FO or (n-3) PUFA can affect DON-mediated IL-6 gene expression.

Fatty acids are known to modulate activity of signaling molecules such as phospholipase C and protein kinase C (PKC) (May *et al.*, 1993; Hwang *et al.*, 1996). The molecular mechanism for inhibitory action of (n-3) PUFAs on ERK 1/2 has been previously investigated in relation to PKC (Denys *et al.*, 2001a; Denys *et al.*, 2001b). Notably, DHA and EPA inhibit PKC α and ε, which are known to be located upstream of MAP kinase cascade (Clark and Murray, 1995). (n-3) PUFAs inhibit the enzymatic activity of theses PKC isoforms either via direct interaction with phosphatidylserine binding site of PKC (Nishizuka, 1995) or indirectly by producing diacylglycerol-containing PUFA (Marignani *et al.*, 1996). It is thus possible that DON-activated MAPK could be suppressed by PKC inhibition by (n-3) PUFA. It should be noted that (n-3) PUFA have been recently shown to suppress ERK 1/2 activity through PKC-independent pathway (Denys *et al.*, 2002).

The p38 signaling pathway is another possible modulator of promoter activity and

mRNA stability of IL-6 gene expression (Beyaert *et al.*, 1996; Winzen *et al.*, 1999). Interestingly, p38 MAPK activation by DON was not reduced but rather was lightly activated in FO-fed mice. This observation might explain the incomplete reduction of IL-6 production in FO-fed mice. Relatedly, DHA is known to activate p38 MAP kinase in vascular smooth muscle to mediate apoptosis (Diep *et al.*, 2000). Furthermore, p38 MAP kinase plays a critical role in transcription and mRNA stability of COX-2 DON-induced COX-2 (Moon and Pestka, 2002), which might explain why FO had little effect on PGE2 produced by DON and partial reduction in DON-induced COX-2 mRNA by dietary FO.

A second possible explanation for impaired DON-induced IL-6 expression is that (n-3) PUFA might act as ligands for peroxisome proliferator-activated receptor (PPAR), the steroid-thyroid superfamily of nuclear receptor. Notably, (n-3) PUFA bind to or modulate PPARα and PPARγ, which regulate specific gene transcription by binding to PP response element (PPRE) via two zinc finger mitifs in DNA-binding domain of PPAR (Krey *et al.*, 1997; Hihi *et al.*, 2002). Although PPARγ does not mediate the negative regulation of IL-6 (Thieringer *et al.*, 2000), PUFA-PPARα signaling may play a inhibitory role in the proinflammatory gene expression (Staels *et al.*, 1998; Yu *et al.*, 2002). However, (n-6) PUFA can also effect PPAR-mediated signaling. Indeed, high concentrations of AA did slightly suppress DON-induced IL-6 superinduction in vitro. Thus, further studies are needed on structure function effects of (n-3) and (n-6) PUFA in suppressing IL-6 gene expression.

Cross-talk between PPAR and MAPK signaling pathways can also occur (Camp and Tafuri, 1997; Camp *et al.*, 1999; Roberts, 2002). Activated MAPKs modulate phosphorylation of PPAR phosphoprotein, which contribute to the reduction in promoter

activation by exogenous ligands. Therefore, ERK1/2 and JNK1/2 suppressed by (n-3) PUFA-containing fish oil might be related to more active PPAR-mediated function and, thus, reduced DON-induced IL-6 transcription.

A third explanation for the (n-3) PUFA effects is alteration in profile of cyclooxygenase (COX) metabolites. It is well established that (n-3) PUFAs lower the level of PGE2, LTB4, and TXB2 by changing the substrate pool of cyclooxygenase metabolism (Tanaka *et al.*, 1999; Venkatraman and Chu, 1999). Dietary (n-3) PUFA such as EPA and DHA can be metabolized to form prostaglandins (eg PGE3) and thromboxanes (eg TXA3) that counterbalance the effects of proinflammatory products (PGE2 or TXA2) derived from arachidonic acid. Although DHA also suppresses LPS-induced COX-2 expression in macrophage cells (Lee *et al.*, 2001), thus gene is upregulated via a feedback pathway upon longer exposure to EPA (Lo *et al.*, 1999). A positive association exists between endogenous COX-2 metabolites (particularly PGE2) and IL-6 synthesis in vitro and in vivo models of several inflammatory diseases (Dendorfer *et al.*, 1994; Meyer *et al.*, 1994; Meyer *et al.*, 1995; Anderson *et al.*, 1996; Hinson *et al.*, 1996; Williams and Shacter, 1997; Zeng *et al.*, 1998; Williams *et al.*, 2000). It is thus possible that FO-mediated changes in COX-2 metabolism might lead to less IL-6 production. Recently, DON was reported to induce COX-2 expression in macrophage cells (Moon and Pestka, 2002) and DON-induced IL-6 is partially dependent on COX-2 metabolites (Moon *et al.*, 2001). Consistent with these findings, in this study, 8 wk FO feeding inhibited COX-2 mRNA and caused inhibitory trends in PGE2 in DON-treated mice. Longer feeding might possibly be required to demonstrate a more suppressive effect of FO on DON-elicited expression. Therefore, the capacity for FO and (n -3) PUFAs to modulate DON-induced IL-6 via altered eicasanoid production as well as by

modified pools of endogenous lipid metabolites requires additional investigation.

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

CHAPTER 6

SUMMARY AND PERSPECTIVES

Based on all results of the thesis and previous research, one conclusive model can be derived as the following cartoon about the mechanism of DON-mediated immunotoxicity (Fig. 30). Trichothecenes binds to 28S rRNA and inhibits its peptidyltransferase activity. The ribotoxic stress from the arrested translation can activate MAPK cascades of JNK1/2, p38 and ERK1/2. Stress-induced MAPK signals can be very important modulators in gene expression as well as cellular death signals. Notably, activated p38 and ERK1/2 had strong inducible effects on COX-2 transcriptional activity. Moreover, p38 was very critical modulator in the DON-mediated COX-2 mRNA stability. Differential responses via MAPK over DON dose regime could determine cellular fates. Sub-lethal dose of the toxin elicited pro-inflammatory genes, namely COX-2 and IL-6. These mediators have been also known to have mitogenic effects on cells after chemical insults as a survival response. By and large, during these inflammatory and mitogenic process, cells are under anti-apoptotic controls (Tang *et al.*, 2001). However, MAPK activation with high doses of DON is considered to be related to apoptotic cell death by stimulating unknown death factors. Yang *et al.* (2000) also observed extremely high dose of DON caused MAPK-involved apoptosis.

IL-6 is known to play a pivotal role in DON-mediated IgA nephropathy (IgAN) as well as circulatory shock by the toxin. From the results of the thesis, DON-mediated IL-6

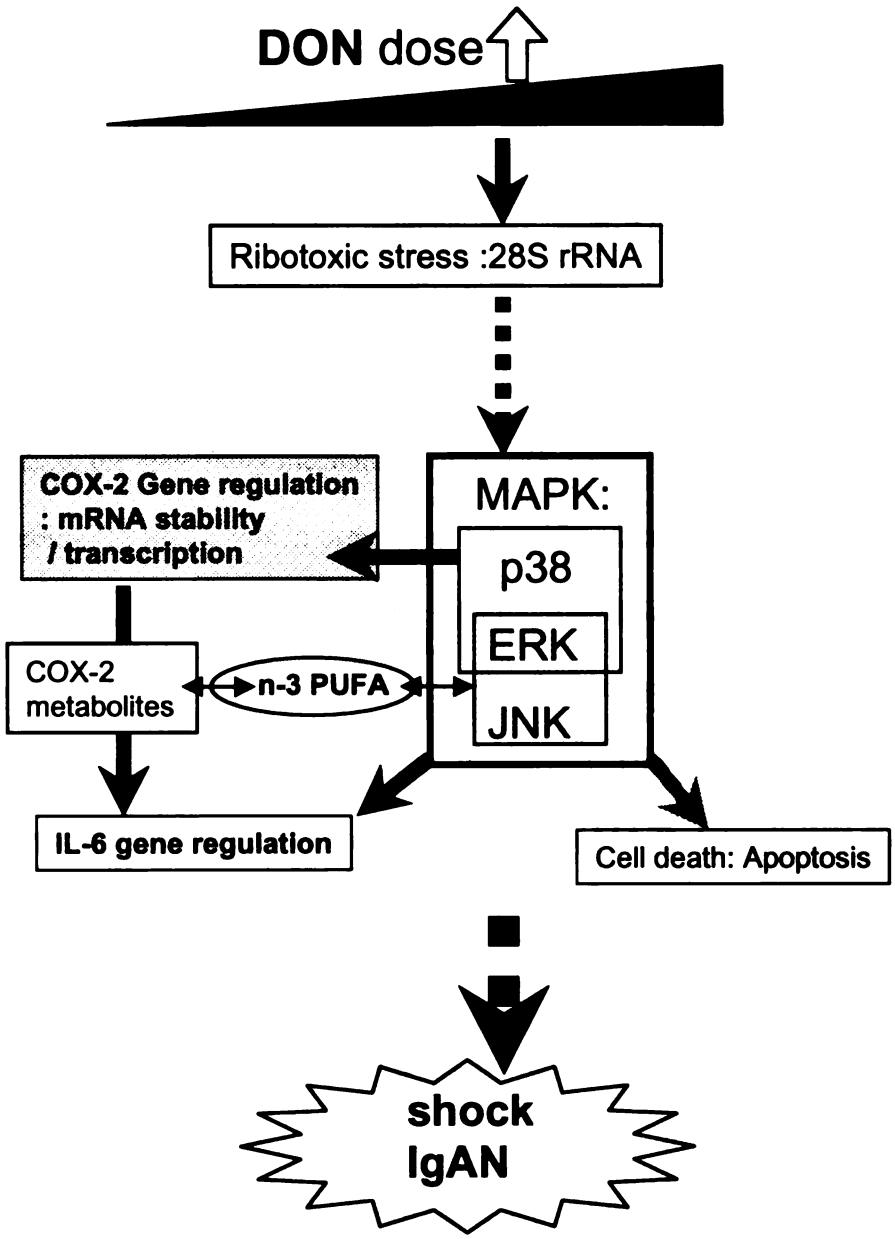


Figure 30. Proposed mode of COX-2 involvement in DON-induced immunotoxicity

production was, in part, due to COX-2 induction and its metabolites. Moreover, fish oil was demonstrated to suppress ERK and JNK, which might contribute to decreased response of IL-6 to DON.

There are possible perspectives after this research. First, it is necessary to analyze the specificity of MAPK signals determining either proinflammatory gene induction or apoptosis over the broad range of DON dose. Different spectrum of toxic results should be analyzed depending on particular MAPK cascades. Sophisticated mechanisms can be considered about the specificity mechanism of MAPK cascades (Chang and Karin, 2001). Scaffold proteins can play a role in organizing a specific cascade and thus can bring about different toxic effects via a typical MAPK signaling depending on dose regimes. There could be a specific DON-regulated stakeholder to determine the specific toxic event by organizing particular sets of MAPK cascades. In addition to this, life evolved its sequential physical interactions between protein members in a given MAPK cascade (Xia *et al.*, 1998). Both mechanisms may operate in parallel, leading to differential activation of MAPKs in response to distinct stimuli.

Second, we need to confirm the outcome of the final toxicological symptoms in vivo by suppressing activity or expression of COX-2. Only the intermediating factor such as IL-6 was measured to assess the involvement of COX-2 in DON toxicity. COX-2 metabolites partially contributed to IL-6 production which has been well known to be critically related to DON-immunotoxicity (Zhou *et al.*, 1999; Pestka and Zhou, 2000; Zhou *et al.*, 2000). Using therapeutic COX-2 inhibitors or COX-2 knockout model, the effect on DON-induced IgA nephropathy and tissue damage need to be assessed. As another aspect, DON-induced COX-2 expression have a defensive meaning in response to the toxic insult and thus it is also

possible that the symptoms can be deteriorated by retarding COX-2 metabolism (Langenbach *et al.*, 1999a; Langenbach *et al.*, 1999b; Morteau, 1999; Morteau *et al.*, 2000). Additionally, it is also necessary to identify COX-2 metabolites specifically contributing to DON-induced immune dysfunction. The final toxic events could be thus ameliorated by blocking the specific array of eicosanoids.

Third, there is an important missing bridge between ribotoxic stress and MAPK activation. Iordanov *et al.* (1997) hypothesized that there must be as yet unidentified intermediate signal transduction steps, presumably mediated by proteins. A number of proteins bind to the site where trichothecene damage occurs in the ribosome, in particular, various elongation factors. Some of them are known to be related to MAPK signals (Knebel *et al.*, 2001; Patel *et al.*, 2002). Elongation factor alpha 2 is known to be phosphorylated by a kinase (the interferon-inducible and double-stranded RNA-dependent protein kinase, PKR) which must be displaced from the ribosome in order to become activated (Raine *et al.*, 1998; Vattem *et al.*, 2001). Moreover, PKR is known to activate p38 and JNK1/2, and LPS-induced proinflammatory cytokine production is impaired in PKR-null mice (Williams, 1999; Goh *et al.*, 2000; Iordanov *et al.*, 2000). PKR is also essential for mRNA stability of ARE-containing transcripts during stress (Zhao *et al.*, 2002). Therefore, it can be assumed that PKR can work both on translational arrest and turning on the proinflammatory cytokines via transcriptional and post-transcriptional control (Iordanov *et al.*, 2000). However, it is not yet known whether the elongation factors themselves, or other related ribosomal binding factors, interact with MAPK signaling cascade to mediate the trichothecene-induced ribotoxic stress response.

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