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By

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ABSTRACT

REGULATION OF INTERLEUKIN-8 EXPRESSION BY DEOXYNIVALENOL IN U937 MONOCYTES

By

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Deoxynivalenol (DON, vomitoxin) is a worldwide contaminant of a variety of grain crops. Since humans and animals are exposed to DON through these contaminated grains, health agencies across the world have set maximal tolerable levels for exposure. In vitro models can provide insight into DON's effects on human cells. Based on observations that monocytes are the primary responders in DON-treated human peripheral blood mononuclear cells, U937 monocytes were chosen here to investigate mechanisms responsible for DON-induced IL-8 expression. Elevated levels of IL-8, a neutrophil chemoattractant, have been linked to a variety of inflammatory conditions.

DON was found to induce marked mitogen-activated protein kinase (MAPK) activation in the U937 cells. p38 inhibition completely suppressed IL-8 protein and mRNA expression whereas ERK and JNK inhibition partially inhibited IL-8 mRNA but had little effect on IL-8 protein levels. As DON has previously been found to increase expression of immune molecules through an activation of transcription, studies were conducted on the effect of this toxin on the expression of an IL-8 promoter-driven luciferase construct. Mutations in five transcription factors previously implicated in IL-8 expression, AP-1, C/EBPβ, Oct-1, NF-κB, and NRF, revealed that only an intact NF-κB site was necessary for DON-induced IL-8 promoter-driven luciferase. Examination of NF-κB binding, utilizing a transcription factor ELISA, indicated that only p65 binding

was increased in the presence of DON. Two other NF-κB subunits, p50 and p52, were present in U937 nuclear protein. Levels of p50 were equivalent in untreated and DON-treated samples, however, DON treatment decreased p52 binding. These findings were further confirmed by the observation that an NF-κB inhibitor, caffenic acid phenyl ester (CAPE) could inhibit both DON-induced IL-8 protein and mRNA. When CAPE was further used to assess DON's post-transcriptional effects, the toxin was not found to increase IL-8 mRNA stability.

Double-stranded RNA protein kinase R (PKR) has been linked to DON-induced MAPK activation and cytokine expression. PKR can mediate MAPK activation as well as NF-κB activation. Investigation into the role of PKR in U937 cells revealed that this kinase was essential for DON-induced IL-8 protein expression. Since protein synthesis inhibitors other than DON are also capable of activating MAPKs and inducing IL-8 protein, two of these, Shiga toxin 1 (Stx1) and ricin, were investigated for a common reliance on PKR as an upstream activator of IL-8 expression. Both Stx1- and ricin-induced IL-8 protein production in U937 cells was found here to be PKR-dependent. In summary, DON-induced IL-8 expression in U937 monocytes was regulated primarily at the transcriptional level and was p38- and NF-κB-dependent. DON-induced IL-8 protein was PKR-dependent with similar effects being observed for the translational inhibitors, Stx1 and ricin suggesting that these toxins might share a common mechanism for IL-8 expression in the U937 cell line. Increased knowledge of the mechanisms triggered by these three toxins might aid in dealing with accidental or deliberate intoxication by these agents.

DEDICATION

Without the patience and support of my family, especially my parents William and Susan Gray and my grandparents Edward and Ardis Drabik none of this would have been possible. Grandpa, I'm sure you'll want to discuss this when you finish reading it – just let me know.

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LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
ActD	actinomycin D
Ad7	Adenovius serotype 7
ANOVA	one way analysis of variance
AP-1	activator protein-1
ARE	AU rich region
C/EBPβ	CCAAT/enhancer binding protein β
CAPE	caffenic acid phenyl ester
CAT	chloramphenical acetyltransferase
CBP	CREB binding protein
COX-2	cyclooxygenase-2
CPPD	calcium pyrophosphate dihydrate
CREB	cAMP response element binding protein
CYP	cytochrome P450
DON	deoxynivalenol
DRB	dichlorobenzimidazole riboside
EHEC	enterohemorrhagic *E. coli*
ELISA	enzyme-linked immunosorbant assay
EMSA	electrophoretic mobility gel shift assay
ERK	extracellular signal-regulated kinases

FDA	Food and Drug Administration
FLS	fibroblast-like synoviocytes
FMF	familial Mediterranean fever
Hck	hematopoietic cell kinase
HDAC	histone deacetylase
HI FBS	Heat-inactivated fetal bovine serum
hnRNA	heteronuclear RNA
HRP	horseradish peroxidase
IgG	immunoglobulin G
IKK	IκB kinase
IL-8	interleukin-8
IPF	idiopathic pulmonary fibrosis
IVIG	intravenous immunoglobulin
IκB	inhibitor of NF-κB
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinases
LPS	lipopolysaccharide
Luc	luciferase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated or ERK kinase
mIL-8Rh KO	mice that lacked the murine homologue of the IL-8 receptor
MIP-2	macrophage-inflammatory protein-2
MKK	MAPK kinase
mRNA	messenger RNA

MSU	monosodium urate monohydrate
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NAC	N-acetyl-cysteine
NE	neutrophil elastase
NF-κB	nuclear factor- κB
NIK	NF-κB inducing kinase
NRF	NF-κB repressing factor
Oct-1	octamer-1
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDAR	Pre-Developed Assay Reagents
PGE2	prostaglandin E2
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKR	double-stranded RNA protein kinase R
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear cells
POU	Pit-Oct-Unc
SDS	sodium dodecyl sulfate
sICAM	soluble intercellular adhesion molecule
Stx1	Shiga toxin 1
TAF	TBP-associated factors
TBP	TATA-binding protein

TDI	Tolerable Daily Intake
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor- α
TSA	trichostatin A
VIP	vasoactive intestinal peptide
WHO	World Health Organization
β-gal	β-galactosidase

INTRODUCTION

Deoxynivalenol (DON; vomotoxin) is a member of the trichothecene mycotoxins, which are commonly found in grain species worldwide. DON is produced by *Fusarium graminearum,* which is the causative agent of head blight affecting maize, wheat, barley, and other grain crops. DON exposure through the ingestion of contaminated grains has been seen worldwide, due in part to the toxin's resistance to high temperatures, such as those used in food processing and cooking. This makes it a common threat to animal and human health. Additionally, DON contamination has been projected to potentially cause annual losses up to $637 million for food for human consumption, $18 million for feed and $2 million for livestock (1).

During the past decade, there have been several surveys on the presence of naturally occurring foodborne contaminants in a variety of foods. One of these surveys collected 11,022 samples from 12 countries and found 57% of the samples were positive for DON (2). DON was found primarily in cereal grains and grain products, with wheat, wheat flour, and bread concluded to be primarily responsible for DON intake by humans. Two countries, France and Germany, were concluded to have human intake levels above the Tolerable Daily Intake (TDI; 1 μg/kg body weight) as set by the World Health Organization (WHO). In another survey published in 2004, Tritscher and Page (3) determined that of 5 regions tested, Africa, Europe, Latin America, the Middle East, and the Far East, 4 exceeded the TDI set by the WHO and estimated the DON intake for the USA to be approximately 51 μg/person/day. In a more recent survey of the French diet,

Leblanc et al. (4) determined that 5% or less of adults, children, or vegetarians exceeded the TDI for DON with cereal products consumption responsible for DON exposure.

Based on the persistence of DON in foods, it is not surprising that DON has been linked to numerous instances of human toxicosis in the last several decades. For example, gastroenteritis outbreaks that have occurred in the past several decades in China and India, have been linked to elevated levels of DON in food and feed. Reported symptoms in those outbreaks were similar to symptoms observed with acute toxicity in animal studies. In the United States, recent outbreaks (October 1997 to October 1998) that occurred in schools were linked to burrito consumption. Of note, DON contaminated wheat was found in the tortillas used for the burritos, although the levels of DON were below the Food and Drug Administration (FDA) advisory level (5). Along with potential effects from acute toxicity, there are also symptoms associated with chronic toxicity have been observed in various animal studies. These symptoms, including altered nutritional efficiency, anorexia, and decreased weight gain, are not as severe as those seen with one large dose of DON, can also be detrimental to food animal health (6).

Due to the concern over human health, various regulatory agencies around the world have set limits on foods and feeds to reduce human and animal exposure to DON. Advisory levels in the USA, set by the FDA, are 1 ppm for finished wheat products, 10 ppm for grains used for cattle older than 4 months and chickens, and 5 ppm for feed for swine and all other animals (7). Health Canada has a slightly higher guideline of 2 ppm for uncleaned wheat and 1.2 ppm for wheat flour for adults, but a 0.6 ppm level for wheat flour for infants (7). The European Union has set an even lower level than Health Canada of 0.75 ppm for raw flour and 0.5 ppm for retail stage cereal products (7).

The symptoms of DON intoxication could, in part, be caused by cytokine and chemokine dysregulation. One of the immune molecules that DON can induce is interleukin (IL)-8 (8). IL-8 is a chemokine involved in the inflammatory process. Its main function is to attract neutrophils to sites of inflammation. In addition, secondary roles include a number of other processes, including angiogenesis, obesity, cardiovascular disease, and contribute to the severity of autoimmune diseases. The overproduction of IL-8 as a result of DON exposure could contribute to chronic inflammation (for example, exacerbate arthritis) or exacerbate other preexisting conditions.

Over the last fifty years, many aspects of trichothecenes' effect on cells have been investigated ranging from interaction with the ribosome and inhibition of protein synthesis to increase the proinflammatory gene expression (9). Figure I.1 provides an overview of trichothecene effects. The figure starts by depicting the toxin interacting with the ribosome and/or interaction with upstream kinases, potentially PKR and/or Hck, which causes inhibition of protein synthesis and an activation of signaling cascades upstream of mitogen-activated protein kinase (MAPK) pathways. Those upstream kinases can, in turn, activate MAPKs. By causing an increase in MAPKs, increases in transcription, mRNA stability, and apoptosis can occur in cells and lead to a variety of effects in both cell lines and animal models.

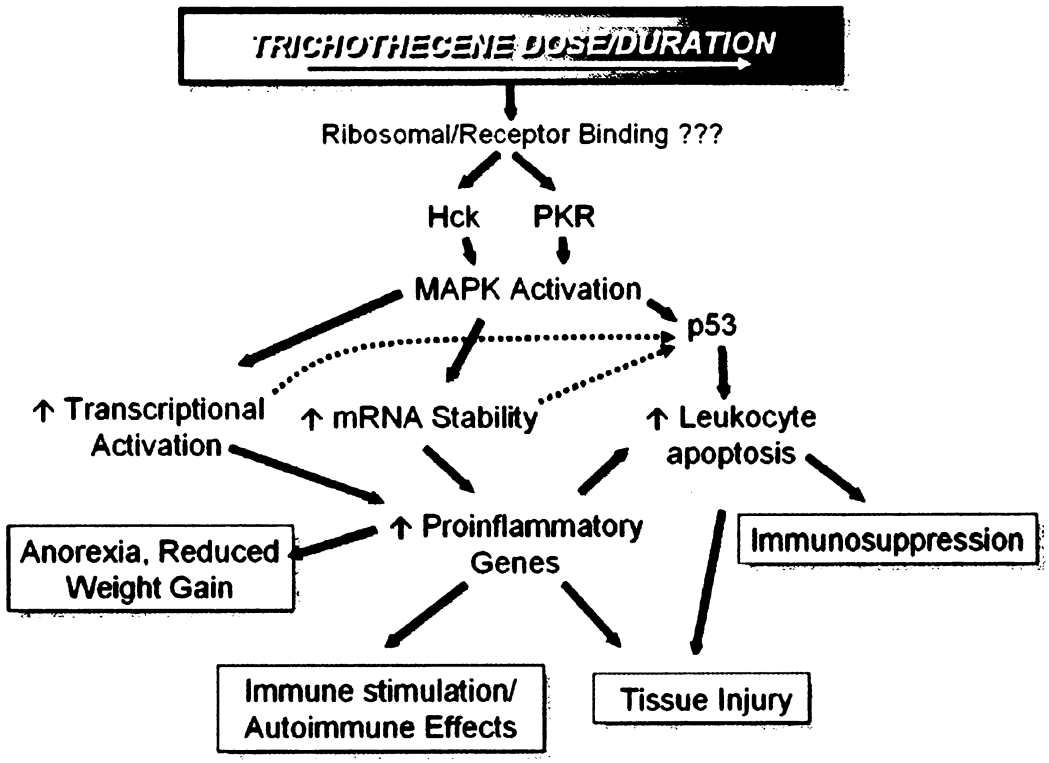

Figure I.1: General model for the mechanism of action of DON and other trichothecene. (9)

Overall hypothesis

In light of the ubiquitous role of IL-8 in many human conditions and its increased levels in the presence of DON, the overall goal of this dissertation was to determine the mechanisms by which IL-8 is induced in U937 cells, a human monocyte line. The guiding hypothesis is that DON induces increases in both transcription and mRNA stability, through the activation of signaling cascades, which are responsible for the increase in IL-8 protein and IL-8 mRNA. The specific aims in this dissertation were (1) to determine if DON-induced IL-8 protein and IL-8 RNA in undifferentiated U937 cells was mediated through the mitogen-activated protein kinases (MAPKs), (2) to determine if transcriptional alteration occurs due to exposure to DON, and if so which transcription factors are involved, (3) to determine the contribution that post-transcriptional stability has on elevated mRNA levels, and (4) to determine the role of an upstream kinase in the elevation of IL-8 levels.

Chapter summaries and hypotheses

Chapter 1 provides background information relevant to the research presented in this dissertation. This includes the effect that DON has on eukaryotic cells, primarily murine and human macrophages, IL-8, the regulation of IL-8, a brief overview of mitogen-activated protein kinases (MAPKs), and U937 cells. General characteristics of IL-8 are given and the relationship of IL-8 and various human diseases are also explored. IL-8 expression is elevated in many human inflammatory conditions and is a chemokine

of importance because DON has been shown to elevate various inflammatory molecules in vivo and in vitro. The mechanisms of IL-8 expression are presented for both mRNA stability and transcription. Transcription is of particular importance because it involves a variety of proteins necessary for the process both before the actual start of transcription and during transcription. Eukaryotic transcription is a complex process involving individualized proteins specific to the molecule that will be expressed and general proteins ubiquitous to the process. The process of transcription occurs at the promoter, which is the region of the DNA that is 5' to the coding sequence. This area contains many binding sites for proteins known as transcription factors along with binding sites for basal transcription machinery. RNA polymerase II, which is responsible for mRNA synthesis, starts as a preinitiation complex consisting of the polymerase along with a variety of TFII proteins, such as TFIID (TATA-binding protein (TBP) and TBP-associated factors (TAFs)). (10) After the polypeptide complex forms, of approximately 40 proteins, it binds to the promoter along with transcription factors specific to an individual promoter, which is collectively known as the initiation complex. After the actual transcription starts, preinitiation factors are replaced by elongation factors. (11) The elongation factors function to speed up the transcription. Once the transcript is produced the multi-protein complex dissociates from the DNA.

The specific transcription factors for each promoter are subject to activation as a response to a variety of stimuli. The IL-8 promoter has binding sites for 5 main transcription factors (AP-1, C/EBPβ, Oct-1, NF-κB, and NRF) that have been found to play a role in IL-8 expression under different conditions. Along with alterations in transcription factor activation, IL-8 expression has also been linked to histone alteration,

which controls the accessibility of the promoter to the transcription machinery. Both transcription factor activation and alteration of the transcription machinery and histones can be due to multiple signaling cascades. The role of upstream signaling cascades, MAPKs in particular, will also be discussed in relation to IL-8 expression in this chapter. Upstream signaling cascades are important due to the involvement of MAPKs pathways in transmission of a response to a specific stimulus.

In the last section, information regarding U937 cells will be presented. U937 cells are a human monocyte line that was first isolated in 1976 (12). They were chosen as the model for this research for several reasons. The most important is that DON targets actively dividing cells, such as immune cells (9). The second reason is that U937 cells, macrophage differentiated, have already been reported to have elevated IL-8 in response to DON exposure. Finally, U937 cells were chosen as a model for this research is because this cell line has been widely used for a variety of studies and has well characterized signaling pathways.

Chapter 2 examines the role that MAPKs might play in the observed alteration of the normal expression of IL-8. The hypothesis is that one, if not more, of the MAPKs families are partly responsible for the increase in IL-8 expression resulting from DON exposure. MAPK phosphorylation was assessed using Western Blot and three MAPKs inhibitors, one each for the p38, ERK, and JNK pathways, to determine the relative contribution of each pathway for IL-8 expression in U937 cells. Data included in this chapter were published, the complete paper can be found at http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WXH-4HVDYJ2-2&_user=1111158&_coverDate=06%2F15%2F2006&_rdoc=1&_fmt=&_orig=search&_

sort=d&view=c&_acct=C000051676&_version=1&_urlVersion=0&_userid=1111158&md5=781ac266c645297d87cef874a617138c , and provided in vitro support to ex vivo experiments with cells isolated from human blood.

Chapter 3 explores the hypothesis that DON-induced IL-8 expression is due to increases at both the transcriptional and post-transcriptional levels. The transcriptional level is examined using an IL-8 promoter-driven luciferase construct with a wild-type sequence or containing mutations in the binding sites of five main transcription factors for IL-8 expression. Additionally, results obtained with the luciferase construct were confirmed with a transcription factor ELISA to measure the binding activity of the transcription factor found to be primarily responsible for IL-8 expression.

Chapter 3 also investigates the role of increased mRNA stability in DON-induced IL-8 expression. Several immune molecules have been shown to have a DON-induced increase in mRNA stability. IL-8 mRNA was measured during a time course after U937 cells were exposed to a transcriptional inhibitor to determine if DON causes an increase in the stability of the IL-8 transcript.

Chapter 4 is a summary chapter integrating how research presented in this dissertation fits into the model of DON response mechanisms.

There are also five Appendices (A, B, C, D, and E). Appendix A presents additional IL-8 protein induction data in differentiated U937 cells as well as two additional cell lines, THP-1 and HEK-293. Differentiated U937 cells were originally considered as the primary cell line for this research. However, they were replaced with undifferentiated U937 cells after the differentiation process was determined to be inconsistent and subject to variability in excess of what can be reasonably expected. The

two additional cell lines are both human and had been considered as replacement cell lines for the U937 cells. Appendix B contains data collected while optimizing the transfection experiments. Experiments were conducted in both RAW 264.7 cells and U937 cells with three different reporter genes, different plasmids concentrations, and different times for both recovery time after the electroporation and exposure time to DON or LPS. Appendix C contains data obtained while optimizing the mRNA stability experiments. Originally mRNA timecourses were conducted using general transcription inhibitors. However, during the course of those experiments inconsistent and inconclusive data were obtained. It was determined that the general transcription inhibitors were inappropriate for the cell line used (possibly because of metabolism) and utilization of more specific inhibitors were explored. Appendix C contains the data obtained while selecting an inhibitor and optimizing both dose and treatment conditions. Appendix D contains the sequences of the mutated IL-8 promoter used for the luciferase constructs and presents data obtained from using two different web-based transcription factor binding site recognition programs. Appendix E contains a complete journal article containing much of the data in Chapter 3 as originally published in Toxicology and Applied Pharmacology (2006).

Significance

Due to the fact that DON is a common contaminant of grains worldwide, and that it can adversely affect both animal and human immune systems at low concentrations, it is important to obtain further information on DON's effects on the human immune system. DON exposure at low doses can induce expression of cytokines, including IL-8

in human cells. Because aberrant regulation of IL-8 and other cytokines can adversely impact human and animal health, it is necessary to understand if DON interferes with the regulation of IL-8. This dissertation investigates the mechanisms behind the DON-induced elevation of IL-8.

CHAPTER 1: LITERATURE REVIEW

Deoxynivalenol

Deoxynivalenol (DON, vomitoxin) is a fungal secondary metabolite that is produced primarily by *Fusarium graminearum* or *Fusarium culmorum* and classified as a trichothecene. It is one of the most common mycotoxins found in cereal grains (6) and is a frequent contaminant of wheat, corn, and barley worldwide (13). Since DON is a very stable compound, it is capable of withstanding the high temperatures used in processing and cooking of foods (13). Because of this, DON is present in processed foods and be consumed by humans and animals.

DON inhibits protein synthesis by binding to ribosomes and interfering with chain elongation (13). The unsaturated bond at C9-C10 and an epoxide at C12-C13 are responsible for biological activity (13). (Figure 1.1) Actively dividing cells, such as immune cells, are very susceptible to the effects of DON. Immune system effects in response to DON exposure include impairment of human and murine lymphocyte proliferation, decreased host resistance to bacterial infection in mice, increased total serum immunoglobulin A production in mice, and increased expression of cytokines in mice and in cell lines (14) (13).

General immune system effects of DON

DON has been found to be immunosuppressive and immunostimulatory in experimental animals, depending on dose and duration of exposure (15). At high doses,

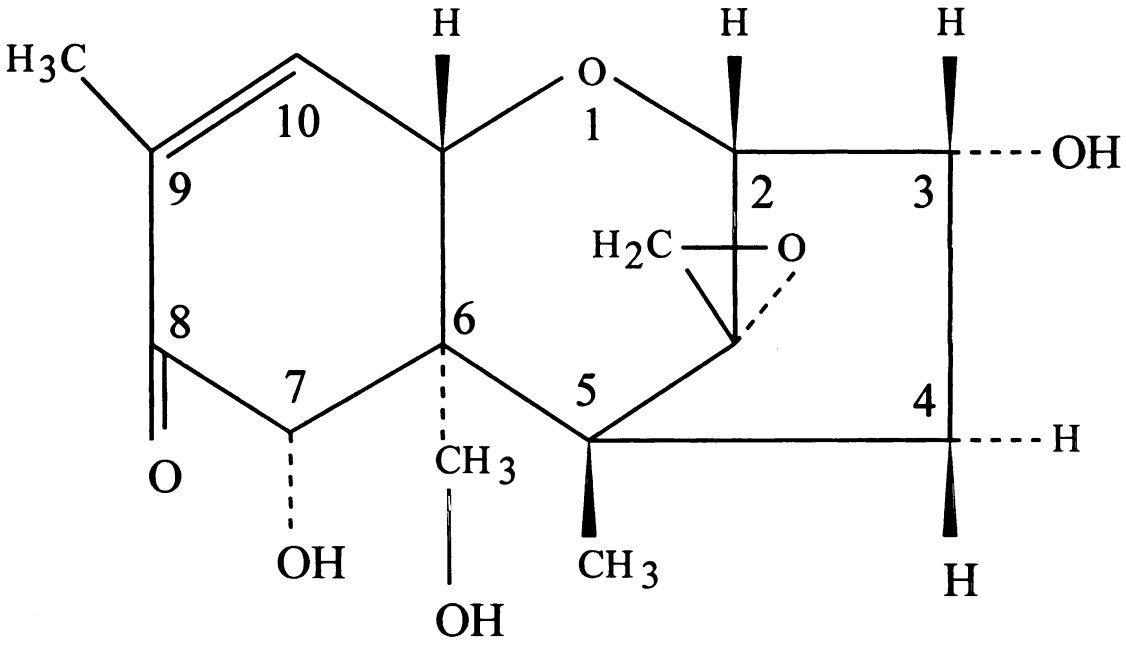

Figure 1.1: Structure of DON. The epoxide and double bond at C9-C10 are responsible for biological activity of this molecule.

DON exhibits immunosuppressive effects. These effects could be mediated by several mechanisms including the inhibition of translation and increased apoptosis (13). Acute poisoning, from extremely high doses of DON, has also been observed in both humans and animals (6). The poisoning presents as a multi-system shock-like syndrome with symptoms of dermal irritation, nausea, vomiting, diarrhea, hemorrhage, and hematological lesions (13).

The immunostimulatory effects of DON, seen at lower doses (16) include the production of cytokines *in vitro* and *in vivo* that apparently results from interference with normal gene regulation. Previous laboratory data have shown that interleukin (IL)-6 protein levels are superinduced in RAW 264.7 murine macrophage cells when activated with lipopolysaccharide (LPS) (17). Levels of both IL-6 protein and mRNA are elevated after DON exposure in B6C3F1 mice (18). In EL-4 human thymoma cells, after exposure to DON, levels of IL-2 protein and mRNA are elevated (19). IL-2 protein is also induced by DON, along with the chemokine IL-8, in Jurkat human T cells (6). In the U937 cell line, a human monocyte cell line that can be differentiated into macrophage cells, DON induces IL-6 and IL-8 protein (8).

Underlying mechanisms for DON's induction of immune molecules

Increased transcription and/or post-transcriptional stability are likely to be responsible, in part, for the DON-stimulated cytokine expression. Previously, DON has been shown to alter transcription factor binding. NF-κB (20) and AP-1 (21) binding are increased in EL-4 cells after DON exposure. Similar effects are seen in RAW 264.7 murine macrophage cells after exposure to DON as evidenced by increased binding, to

consensus sequences, for NF-κB, AP-1, and C/EBPβ (22). Increased binding of CREB and AP-1 were also observed in murine peritoneal macrophages (23). Additionally, cyclooxygenase-2 (COX-2) also seems to be induced by DON at the transcriptional level. Moon et al. (24) observed that a COX-2 promoter-driven luciferase construct was inducible by DON in RAW 264.7 cells.

DON has also been shown to increase post-transcriptional stability of certain mRNA transcripts. Reports have shown an increase of TNF-α and IL-6 mRNA stability in RAW 264.7 cells as a result of DON exposure (25, 26). Increased IL-6 mRNA stability was also identified in murine peritoneal macrophages treated with DON (23). IL-2, another cytokine increased in the presence of DON, was found to have increased mRNA stability in DON-exposed EL-4 thymoma cells (19, 27). Additionally, the stability of luciferase transcripts was increased in a construct including the COX-2 3' untranslated region (UTR) in RAW 264.7 cells (24).

Taken together, DON is capable of inducing a variety of immune molecules, both in cell culture and in whole animals. The induction has been attributed to alterations at both the transcriptional level, through increased activation of transcription factors, and the post-transcriptional level, by increasing mRNA stability.

Modulation of intracellular signaling by DON

DON modulates intracellular signaling cascades. Yang et al. (28) showed that mitogen-activated protein kinases (MAPKs) were activated by trichothecenes in RAW

264.7 cells. Additionally, Zhou et al. (29) determined that along with increased activation of MAPKs in U937 cells, double-stranded RNA-activated protein kinase R (PKR) was also involved in DON's effect on cells. PKR is a kinase activated by extracellular stimuli, such as exposure to double-stranded RNA, which has been linked to activation of NF-κB through the IκB kinase (IKK) complex (30, 31). PKR has also been found to be an upstream element in the "ribotoxic stress response". The ribotoxic stress response is a pathway of signal transduction whereby damage to the 28S rRNA by a chemical triggers activation of a downstream signaling pathway, such as those mediated by MAPKs (32). In U937 cells transfected with a PKR antisense vector, reduced phosphorylation was detected for all three MAPK pathways as well as a decrease in apoptosis following DON exposure (29).

Interleukin (IL)-8

Chemokines, a subgroup of cytokines, affect chemotaxis and other aspects of leukocyte behavior including the extravasation process important in the inflammatory cascade. Chemokines are broken down into 2 families based on the position of conserved cysteines. One family is the CC chemokines and the other is the CXC chemokines, which includes IL-8. Along with the distinctive CXC sequence, IL-8 protein has sequences specific for neutrophil chemotaxis (33) and for angiogenesis (34).

IL-8 can be produced in almost all nucleated cells (35) under a variety of conditions. Cell types that produce IL-8 include monocytes (36-38), neutrophils (39, 40), T lymphocytes (41-43), natural killer cells (44), fibroblasts (45, 46), macrophages (8, 47),

endothelial cells (48-51) and epithelial cells (52, 53). Stimuli for IL-8 production can act on a variety of cells or may be highly selective (54). Stimuli include tumor necrosis factor (TNF)-α, IL-1β, H_2O_2, as well as viral and bacterial infections.

IL-8 induces adherence of neutrophils to vascular endothelium and triggers extravasation into tissues (55). Other functions of IL-8 include initiation of the acute inflammatory response (56) and cell type specific functions such as chemotaxis and shape change in neutrophils, induction of histamine and leukotriene release in basophils (44). The chromosomal location for IL-8 gene in humans is at 4q12-21 and this gene consists of 4 exons with 3 introns (54, 57) and results in a precursor composed of 99 amino acids. After cleavage of a 20 amino acid leader sequence, the functional IL-8 is secreted (35). This protein is resistant to inactivation by plasma peptidases, heat, pH extremes, and other denaturing treatments. Activity of the IL-8 protein is lost, however, if the disulfide bonds holding the cysteines together are broken (35).

Correlation of IL-8 levels with disease

Elevated IL-8 levels have been seen in serum, urine, and at sites of inflammation in a variety of conditions (58-61). Due to its varied functions, IL-8 has been implicated in many diseases. Much damage can be done during disease states by the presence of IL-8 drawing neutrophils to specific areas and triggering activation of an inflammatory response. Table 1.1 presents a list of the various conditions in which IL-8 plays a role.

Several studies have focused on identifying gene products and mediators that might be responsible for symptoms observed in inflammatory conditions. Elevated levels of multiple proinflammatory molecules, including IL-8, are expressed in psoriatic skin

Table 1.1: Conditions with IL-8 involvement.

Condition	Reference
Anti-IL-8 treatment can lessen disease	
acute immune complex-type glomerulonephritis (i.e. IgA nephropathy); LPS/IL-1 induced arthritis; LPS-induced dermatitis; lung perfusion injury	(61)
Elevated IL-8 levels implicated in disease	
psoriasis	(62, 63)
poststreptococcal glomerulonephritis	(64)
Familial Mediterranean fever	(65)
ovarian hyperstimulation syndrome	(66)
pulmonary fibrosis	(67)
alcoholic hepatitis	(68, 69)
IgA nephropathy	(70)
joint inflammatory disease (rheumatoid arthritis and gout)	(45, 46, 71)
cystic fibrosis	(72, 73)
hyperoxia	(74, 75)
inflammatory bowel disease (Crohn's disease)	(76, 77)
bacterial toxin exposure or bacterial infections	(78-81)
Kawasaki disease	(82)

models when compared to normal controls (63). Besbas et al. (64) found elevated levels of IL-8 in urine and serum of children in the acute phase of poststreptococcal glomerulonephritis compared to the resolution phase and the controls. Familial Mediterranean fever (FMF) is another condition with elevated IL-8 (65). FMF has recurrent attacks of inflammation in the membranes that line the abdominal cavity and lungs, arthritis, and fever. Elevated IL-8 and soluble intercellular adhesion molecule (sICAM)-1 was seen in patients with FMF, both during the active attacks and while in remission, compared to patients without FMF. The elevated IL-8 and sICAM-1 levels lead the authors to suggest that neutrophil activation and adhesion is increased in FMF.

While the abovementioned studies have not reported a statistical correlation between increased IL-8 to events leading to inflammation or severity of the condition, several studies have. Most commonly, elevated IL-8 levels have correlated with an increased presence of a cell type necessary for symptoms of the condition. Elevated levels of IL-6 and IL-8 significantly correlated with biological characteristics of ovarian hyperstimulation syndrome (66). A similar trend was seen with IL-8 and psoriasis. An increased density of mast cells, thought to have a role in inflammation in psoriasis, as well as increased density of IL-8 producing keratinocytes in the psoriatic skin with lesions when compared to the psoriatic skin without lesions and normal skin was observed (62). There was a correlation between the elevated IL-8 levels produced by the keratinocytes and increased density of mast cells. In idiopathic pulmonary fibrosis (IPF), Beeh et al. (67) presented data that correlated increased sputum IL-8 levels with increased sputum neutrophil levels. Accumulation of neutrophils and other inflammatory cells in the alveolar space is a characteristic seen in IPF. The increased sputum

neutrophil levels also correlated with impairment of lung function. The authors concluded that neutrophils were responsible for the inflammation observed with IPF.

In patients with alcoholic hepatitis, Bird (68) found a correlation between increased plasma and liver IL-8 levels and increased infiltration of neutrophils into the liver. This study also reported that the highest IL-8 levels were seen in patients that died within four weeks of hospital admission. Huang et al. (69) also observed a correlation between patient death and the high IL-8 levels in alcoholic hepatitis. A correlation was also seen between elevated IL-8 levels and the severity of liver injury. A similar trend was seen in a study conducted by Huang et al. (70) on IgA nephropathy. IL-8 levels were measured in patients with IgA nephropathy, a type of glomerulonephritis. There was a significant correlation between elevated IL-8 levels and increases in disease activity as measured by urinary protein and urinary casts. The authors also found a significant correlation between increased IL-8 levels and increased tubular damage in the kidneys.

Harada et al. (61) summarized data on the effects of a neutralizing antibody against IL-8 in LPS-induced dermatitis, LPS+IL-1-induced arthritis, lung reperfusion injury, and acute immune complex-type glomerulonephritis. The authors found that neutrophil infiltration is inhibited with anti-IL-8 injections in all models examined. Anti-IL-8 is able to prevent skin redness in the dermatitis, destruction of the pulmonary architecture in lung reperfusion, and brought the urinary levels of protein and albumin back to normal in the acute immune complex-type glomerulonephritis. These data led the authors to conclude that IL-8 is involved in the activation and infiltration of neutrophils in the model inflammatory conditions and that by preventing normal neutrophil function, tissue damage could be avoided in these conditions.

Mechanisms of IL-8 induction in disease

In contrast to the above studies, which noted elevated IL-8 alone or correlated with disease markers, other studies have focused on discovering potential pathways that lead to IL-8 production in inflammatory conditions. Mechanisms of IL-8 production in rheumatoid arthritis were investigated by Georganas et al. (45). Fibroblast-like synoviocytes (FLS), obtained from patients with rheumatoid arthritis, produced IL-8 after exposure to IL-1β, mediated by NF-κB activation. AP-1 and C/EBPβ were not involved in IL-8 expression by FLS. Suzuki et al. (46) investigated the role of p38 in IL-8 production in rheumatoid synovial fibroblasts (RSF) isolated from tissue biopsy samples of patients with active rheumatoid arthritis. A p38 inhibitor was able to suppress TNF-α- or IL-1β-induced IL-8 protein, but was unable to inhibit IL-8 mRNA or block p65 translocation. The authors concluded that while the p38 pathway was not involved in the NF-κB cascade, an inhibitor of p38 may be useful in addition to conventional anti-rheumatic drugs that do act through the NF-κB pathway.

In gout, another inflammatory condition that affects joints, IL-8 expression in the joint space is a necessary event in the pathogenesis of the disease. A model of gout was investigated with THP-1 monocytes exposed to monosodium urate monohydrate (MSU) or calcium pyrophosphate dihydrate (CPPD) (71). MSU and CPPD triggered an increase in ERK, p38, and JNK activation. Additionally, both an ERK inhibitor and a NF-κB inhibitor were able to reduce IL-8 mRNA levels in THP-1 cells stimulated with either MSU or CPPD. A p38 inhibitor was able to suppress the level of CPPD-induced IL-8

mRNA but not MSU-induced IL-8 mRNA. Both MSU and CPPD increased the binding of AP-1 and NF-κB to the IL-8 promoter. The ERK inhibitor was able to block the increased binding of AP-1 and reduce the binding of NF-κB for both MSU and CPPD treatments, but the p38 inhibitor was only able to block the increased AP-1 binding. Using an IL-8 promoter-driven luciferase construct, mutations in the NF-κB and AP-1 sites reduced MSU- and CPPD-induced luciferase. Mutation in the C/EBPβ site only partially reduced CPPD-induced luciferase but did not reduce MSU-induced levels. The authors concluded that these data suggested potential targets for treatment of gout (MSU deposition) and CPPD crystal deposition disease.

In lungs of patients with cystic fibrosis, inflammation is primarily caused by neutrophils and their protein secretions, including neutrophil elastase (NE) (73). Devaney et al. (72) examined NE-induced IL-8 production in human bronchial epithelial cells. The authors found that an anti-Toll-like receptor (TLR) was able to inhibit NE-induced IL-8 production significantly. Identification of events involved in NE-induced inflammation provides potential targets for treatments that may be more effective than the standard anti-protease therapies.

Deaton et al. (75) examined the effects of hyperoxia in U937 cells. Oxygen toxicity occurs in patients that require high oxygen concentrations. Infiltration of neutrophils and alveolar damage can be seen in these patients. Both alveolar macrophages and U937 cells had hyperoxia-induced IL-8 levels. The hyperoxia-induced IL-8 expression in U937 cells could be inhibited by treatment with dexamethasone, a corticosteroid. Chemotactic activity of the alveolar macrophages and U937 cells was reduced when an antibody to IL-8 was added. These results indicate that IL-8 is

produced after exposure to high levels of oxygen and that the IL-8 production may play a role in neutrophil infiltration. The authors concluded that agents that suppress IL-8, such as dexamethasone, may be useful treatments to help prevent lung injury induced by oxygen toxicity if additional evidence can link IL-8 to this condition. A later study by Allen et al. (74) indicated that IL-8 is important for high oxygen level-induced lung injury.

Elevated levels of IL-8 and other chemokines occur in Crohn's disease, a chronic inflammatory bowel disease. Banks et al. (77) correlated increased chemokine expression with increased activity of the disease, characterized by local inflammation and tissue damage. Alzoghaibi et al. (76) investigated the role of dietary fatty acids in stimulation of IL-8 in patients with Crohn's disease. Intestinal smooth muscle cells from patients with Crohn's disease exhibited a higher level of IL-8 production than cells from healthy patients. In the cells from patients with Crohn's disease, linoleic acid, but not oleic acid, increased IL-8 production, leading the authors to suggest that a diet with oleic acid, rather than linoleic acid may help to decrease the inflammation that occurs with this disease.

Several other studies have focused on IL-8 involvement in disease conditions triggered by bacteria or bacterial toxins. Hang et al. (80) examined a model urinary tract infection using mice that lacked the murine homologue of the IL-8 receptor (mIL-8Rh KO). Neutrophils in these mice could not respond to IL-8-like stimuli, but retained other normal functions. mIL-8Rh KO mice had elevated MIP-2 (a molecule in mice that has a similar role to IL-8 in humans) levels in response to an intravesical *Escherichia coli* infection that continued to increase in the mIL-8Rh KO mice after control mice returned

to normal. While neutrophils in mIL-8h KO mice were able to migrate to the site of the *E. coli* infection, they were unable to clear the infections and, rather, caused kidney damage due to aberrant, uncontrolled responses.

In another study with a different bacterial toxin, Warny et al. (81) treated THP-1 monocytes with *Clostridium difficile* toxin A, which is enterotoxic and causes acute inflammation with neutrophil infiltration. This toxin induced IL-8 production and this induction was significantly reduced by an inhibitor of ERK or p38. Using an IL-8 promoter-driven luciferase construct, it was observed that dominant negative inhibition of the upstream kinases of the p38 pathway blocked luciferase expression. While oral treatment with the p38 inhibitor did not prevent intestinal inflammation, direct injection of the chemical into the intestinal lumen significantly reduced the severity of the inflammation, including reduction of neutrophil infiltration. These data implicate p38 in the neutrophil recruitment seen in the enteritis as a result of toxin A exposure.

In a later study of *C. difficile* toxin A, Johal et al. (79) used T84 epithelial cells to determine the effect of different toxin concentrations on expression of IL-8 and TGF-β, a protective cytokine. Toxin A concentrations of 10 ng/ml or less increased TGF-β levels but did not alter IL-8 levels. Concentrations of toxin A greater than 10 ng/ml increased IL-8 but not TGF-β levels. The authors concluded that the development and severity of the inflammation as a result of *C. difficile* infection may depend on the exposure of the epithelial cells to the toxin and the expression of proinflammatory or protective molecules.

Dahan et al. (78) examined the mechanisms behind IL-8 production in T84 intestinal epithelial cells as a result of infection with enterohemorrhagic *E. coli* (EHEC).

EHEC causes gastroenteritis and hemorrhagic colitis. Activation of both AP-1 and NF-κB resulted from EHEC infection in T84 cells. Also activated were p38, ERK, and JNK. Inhibitors for ERK, p38, and NF-κB significantly inhibited IL-8 levels, individually, and had an even greater inhibition when used in combination. These results may help develop treatments to lessen the severity of EHEC infections.

Although several of the above-mentioned studies related the host response to IL-8 with the severity of the inflammatory condition, one study utilized IL-8's chemotactic ability to reduce potential complications from an inflammatory condition. Asano et al. (82) determined that mononuclear cells and neutrophils had elevated IL-8 protein and mRNA along with decreased chemotactic activity of the neutrophils in patients with Kawasaki disease (acute multisystem vasculitis) when treated with intravenous immunoglobulin (IVIG). The authors concluded that the increased IL-8 in mononuclear cells, neutrophils, and plasma may help to prevent neutrophils from accumulating at inflammation sites, thereby reducing the risk of an aneurysm.

Regulation of IL-8 expression

Since IL-8 is a biologically potent molecule, it is very important that its production be strictly regulated by cells. Regulation of IL-8 can occur on both the transcriptional and post-transcriptional level. (Table 1.2) On the transcriptional level, silencing is done to suppress basal transcription (83, 84). Various transcription factors act in combination to suppress transcription of a gene. Genes, such as the cytokine genes,

require multiple steps in order to activate gene expression and there are numerous sites and levels where negative regulation could occur (19, 83).

IL-8 expression due to multiple transcription factors

Transcription factor binding sites located in the IL-8 promoter include those for activator protein-1 (AP-1), CCAAT/enhancer binding protein β (C/EBPβ), octamer-1 (Oct-1), nuclear factor- κB (NF-κB), and NF-κB repressing factor (NRF) (Figure 1.2).

AP-1 is a dimer composed of Fos (c-Fos, Fra-1, Fra-2, FosB and FosB2) and Jun (c-Jun, JunB, and JunD) family members (85). Fos proteins can only dimerize with Jun proteins, while Jun proteins can form dimers with Fos proteins or other Jun family members, through a leucine zipper region. AP-1 proteins are also able to form complexes with NF-κB and interact with the TATA-binding protein (TBP) or CBP/p300.

C/EBPβ is a member of the leucine zipper family of transcription factors. Other proteins in this family are C/EBPα, C/EBPγ, and C/EBPδ. These proteins are capable of dimerizing with themselves and each other. C/EBPβ can act cooperatively with the NF-κB site through protein-protein interactions (42, 86-89).

Oct-1 is in the POU family of transcription factors. The Pit-Oct-Unc (POU) family members can interact with other POU members or other transcription factors through protein-protein interactions (90).

The NF-κB family contains five members (91). Three of the members, p65 (RelA), cRel, and RelB, contain a transactivation domain and are able to activate transcription. The other two family members, p50 and p52, can form inactive homodimers or active heterodimers with p65, cRel, or RelB. Saccani et al. (91) found that

Table 1.2: Summary studies investigating regulation of IL-8

Stimulus	Cells	Cell type	Activated transcription factors	mRNA stability occurring	Reference
Staphylococcus α toxin	ECV304	epithelial cells	NF-κB	no	(52)
Shiga toxin *E. coli*	HCT-8	epithelial cells (colon)	ND	yes	(92)
IL-1β	HCT-8	epithelial cells (colon)	ND	no	(92)
TNF-α	H292	epithelial cells (lung)	AP-1, NF-κB	yes	(53)
LPS	THP-1	monocytes	NF-κB	yes	(38)
Staphylococcus α toxin	THP-1	monocytes	NF-κB	no	(52)
IFN-γ	U937	monocytes	ND	yes	(93)
LPS	human whole blood	whole blood	ND	yes	(94)
C5a	RAW 264.7	macrophages (mouse)	AP-1, NF-κB	ND[a]	(47)
TNF-α	HMEC-1	endothelial cells	NF-κB (RelA)	ND	(95)
IL-1	HeLa	epithelial cells	NRF, NF-κB	ND	(96)
IL-1β	Caco-2, HepG2	epithelial cells (colon, liver)	NF-κB, C/EBPβ, Oct-1	ND	(56)
H_2O_2	A549	epithelial cells (lung)	AP-1 (JunD, cFos)	ND	(95)
TNF-α	A549	epithelial cells (lung)	JunD, cFos, RelA	ND	(95)
TNF-α + IL-1β	EAhy926	endothelial cells	NF-κB, C/EBPβ	ND	(50)
Helicobacter pylori	AGS (human gastric cancer cells)	epithelial cells (stomach)	NF-κB, C/EBPβ	ND	(97)

Table 1.2 continued: Summary studies investigating regulation of IL-8

Stimulus	Cells	Cell type	Activated transcription factors	mRNA stability occurring	Reference
LPS	human monocytes	monocytes	NF-κB	ND	(37)
LPS	THP-1	monocytes	NF-κB	ND	(98)
TNF-α	U937	monocytes	NF-κB (RelA)	ND	(99)
LPS	U937 (macrophage differentiated)	macrophages	NF-κB	ND	(100)
LPS, LPS-hypoxia	U937	monocytes	NF-κB (p65, p50), AP-1	ND	(101)
LPS	THP-1	monocytes	NF-κB (p65, p50), AP-1	ND	(101)
TNF-α, LPS, PMA	U937	monocytes	NF-κB	ND	(102)
human T cell leukemia virus type I Tax	Jurkat	T cells	AP-1, NF-κB	ND	(41)
PMA	Jurkat	T cells	NF-κB (RelA), C/EBPβ	ND	(42)
rotavirus	HT-29, primary epithelial colon cells	epithelial cells	AP-1, NF-κB, C/EBPβ	ND	(103)

a: ND = not determined

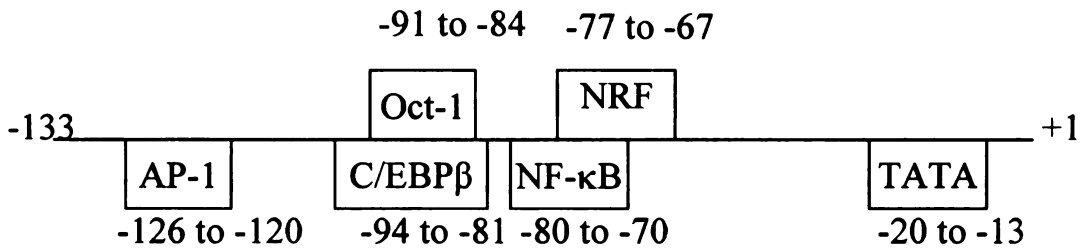

Figure 1.2: Representation of the IL-8 promoter with major regulatory elements.

the NF-κB site in the IL-8 promoter was selective for the p65 subunit. Kunsch and Rosen (43) also observed that the NF-κB site in the IL-8 promoter could bind selected NF-κB family members: p65, cRel, RelB or p50. The p65 subunit of NF-κB can also form complexes with CBP or HDAC-1, depending on its phosphorylation state (104). The NF-κB subunit p50 associated with HDAC-1 binds to DNA to repress transcription. Only phosphorylated p65 can associate with CBP/p300, which can then activate transcription. NRF is a novel transcription factor that acts as a repressor of NF-κB. It binds to a specific site on the IL-8 promoter and prevents transcription by protein-protein interaction (105).

Various studies have found activation of the aforementioned transcription factors to be responsible for IL-8 expression. Many stimuli can activate several transcription factors concurrently. A combination, which varies according to cell type and stimuli present, of two or three transcription factors, is required to obtain the highest level of transcription. The three transcription factors that usually coordinate for IL-8 expression are AP-1, C/EBPβ, and NF-κB (54, 56). Investigations in multiple cell types have revealed that IL-8 expression was the result from NF-κB activation along with either AP-1 or C/EBPβ activation.

Lakshminarayanan et al. (95) found activation of both AP-1 and NF-κB when investigating H_2O_2- and TNF-α-induced IL-8 in HMEC-1 endothelial and A549 lung epithelial cells. H_2O_2 exposure increased AP-1 binding in A549 whereas there was constitutive AP-1 binding in HMEC-1 cells. Both cell lines had no change in NF-κB binding as compared to untreated samples. After TNF-α exposure, both A549 and HMEC-1 cells exhibit increased NF-κB binding activity, while only A549 cells show

increased AP-1 binding. AP-1 complexes were composed of c-Fos and JunD for both H_2O_2- and TNF-α- treated A549 cells while the only detectable component of the NF-κB complex was p65 for TNF-α-treated A549 and HMEC-1 cells. The role of AP-1 in IL-8 expression in HMEC-1 endothelial cells was unclear due to the constitutive activation of AP-1.

Mori et al. (41) found that the Tax protein from the human T cell leukemia virus could induce IL-8 in Jurkat cells and other T cell lines. This induction was determined to be at the transcriptional level through the AP-1 and NF-κB sites. Examination of the nuclear protein, of HUT-102 T cells, revealed increases in AP-1 and NF-κB binding complexes. The authors determine the composition of the AP-1 complex to include JunD while the NF-κB complex was found to contain p50 and cRel.

Hsu et al. (47) also found an increase of both AP-1 and NF-κB binding when using human peripheral blood mononuclear cells (PBMCs) to investigate mechanisms for C5a-induced IL-8 gene expression. The C5a-induced IL-8 protein and mRNA in PBMCs were concluded to be a response of transcriptional activation, as determined by treatment with translational and transcriptional inhibitors. Using RAW 264.7 mouse macrophage cells for transient transfections with an IL-8 promoter-driven chloramphenical acetyltransferase (CAT) construct, the authors observed that the NF-κB site was necessary for C5a-induced IL-8 expression.

Other studies have found that NF-κB and C/EBPβ activation are responsible for the increase in IL-8 production. Kunsch et al. (42) used Jurkat T cells to investigate PMA-induced IL-8 expression. The NF-κB and C/EBPβ binding sites were shown to be necessary for an IL-8 promoter-driven CAT construct. Using EMSA, the authors

determined that both the RelA subunit of NF-κB and C/EBPβ had increased binding and that they cooperatively act to form a ternary complex.

A study conducted in a different cell line with a different stimulator of IL-8 found similar results to the one above. Sharma et al. (97) investigated the effects of *Helicobacter pylori* on IL-8 gene expression in AGS, epithelial gastric cancer cells. The authors determined that *H. pylori* activated IL-8 gene transcription required the NF-κB and C/EBPβ binding sites and that binding of NF-κB, composed of RelA and p50, increased in the presence of *H. pylori* while constitutive binding of C/EBPβ was observed.

Galley et al. (50) observed that, in TNF-α + IL-1β-treated EAhy926 endothelial cells, increased IL-8 levels were produced by increased NF-κB and C/EBPβ binding activity. However, in the same cell line treated with another inducer of IL-8, low levels of ciprofloxacin, no effect on NF-κB and AP-1 binding activities were seen, although there was a decrease in C/EBPβ binding activity. The authors suggested that the ciprofloxacin-induced IL-8 levels could be a due to mechanisms not involving transcription factor activation. Mechanisms other than transcription factor activation will be addressed later in this chapter.

All three of the transcription factors, AP-1, NF-κB, and C/EBPβ, were found to be activated in an epithelial cell line, HT-29, when stimulated by rotavirus (103). The authors determined that the rotavirus-induced IL-8 production was controlled primarily at the transcriptional level. Further investigation showed that AP-1, NF-κB, and C/EBPβ binding sites were involved in the transcriptional activation. Increased AP-1 (composed

of JunD and c-Fos) and NF-κB (composed of RelA and p50) binding was observed after rotavirus infection.

Taken together, some studies that found activation of NF-κB and AP-1 was responsible for increased IL-8 in multiple cell types (T, endothelial, and epithelial cells), whereas other studies related IL-8 expression to increased activation of NF-κB and C/EBPβ. Such differences in transcription factor activation might be caused by the nature of the stimuli or by specific characteristics of cell lines.

Along with binding regions for positive transcription factors (AP-1, C/EBPβ, and NF-κB) the IL-8 promoter has two negative regulatory elements (NRE). Oct-1 acts as a NRE for IL-8 by repressing basal and induced levels of transcription when bound to a site within the same area of the promoter as the C/EBPβ site (54, 56). The octamer binding element is located on the complementary strand and opposite direction as the C/EBPβ binding site. Oct-1 is replaced with NF-κB and C/EBPβ when IL-8 gene expression is induced (106). Wu et al. (56) examined the role of Oct-1 in IL-1β-induced IL-8 expression in Caco-2 epithelial cells. It was determined that C/EBPβ and Oct-1 bind to the promoter in control cells and upon IL-1β stimulation, Oct-1 binding is reduced and NF-κB binds to a site adjacent to C/EBPβ. Thus, authors concluded that C/EBPβ might act as an activator while Oct-1 acts as a repressor of basal transcription in both Caco-2 and HepG2 epithelial cells.

The other negative regulatory factor for IL-8 is the NRF. The role of NRF in IL-1-induced IL-8 expression was investigated in HeLa epithelial cells (96). Mutation of the NRF site produces an increase of IL-8 promoter-driven luciferase in untreated HeLa cells as well as preventing IL-1 stimulated luciferase levels. The increase seen in untreated

cells with the NRF mutation suggests that NRF binding inhibits basal transcription. However, in IL-1-stimulated cells, it is a required factor along with AP-1 and NF-κB to induce IL-8 gene expression. Even though the NRF and NF-κB sites overlap, the NRF mutation did not interfere with IL-1-induced binding of the p65 subunit of NF-κB.

NF-κB as the only transcription factor necessary for IL-8 expression

Along with investigations that discovered activation of multiple transcription factors was responsible for IL-8 production, other studies have demonstrated that the only transcription factor needed was NF-κB. In epithelial ECV304 cells, *Staphylococcus* α-toxin triggered nuclear translocation of p65, which correlated with the observation that the NF-κB site was important in IL-8 promoter-driven luciferase expression in this model (52). *Staphylococcus* α-toxin similarly caused an increase in binding of the p65 subunit of NF-κB in THP-1 monocyte cells (52). Delgado et al. (37) found that in primary human monocytes, LPS- or TNF-α-induced IL-8 protein and mRNA can be inhibited by vasoactive intestinal peptide (VIP). Further investigation (98), using THP-1 monocytes, determined that LPS-induced IL-8 could be inhibited by VIP at the transcriptional level. Using mutations of binding sites in the IL-8 promoter, the authors determined that VIP inhibition required the NF-κB binding site. Subunits present in the NF-κB complex were determined to be p65, p50, and cRel. Additional factors, CREB-binding protein (CBP) and TATA-binding protein (TBP), induced by LPS exposure, were also inhibited by VIP treatment.

In a study by Sugano et al. (100), the inhibitory effects of nicotine on LPS-induced IL-1, IL-8, and PGE2 expression in macrophage-differentiated U937 cells were

examined. Nicotine inhibited IL-1, IL-8, and PGE2 at both the protein and mRNA levels. Using an EMSA, the author observed that nicotine inhibited LPS-induced NF-κB binding. Undifferentiated U937 monocytes were also used to investigate inhibition of TNF-α, LPS, and PMA stimulated IL-8 production by hymenialdisine, a natural product isolated from marine sponges (102). Hymenialdisine inhibited expression of an IL-8 promoter-driven luciferase construct as well as IL-8 protein and mRNA expression. This inhibition was determined to be due to reduction of NF-κB binding, but did not involve C/EBPβ or AP-1 binding.

Vlahopoulos et al. (99) examined the role of antioxidants in TNF-α-induced IL-8 expression in U937 cells. The authors found that mutation of the NF-κB site completely prevented IL-8 promoter-driven luciferase expression after stimulation with TNF-α. The AP-1 and C/EBPβ site were found to be unnecessary for TNF-α inducibility. Further investigation revealed that TNF-α-induced an increase in NF-κB binding, caused by an increase in p65 activation. Pretreatment with an antioxidant, dimethyl sulfoxide (DMSO), can prevent TNF-α-induced IL-8 expression, both the IL-8 protein and luciferase levels with an IL-8 promoter-driven luciferase construct. Additional testing indicated that DMSO was able to interfere with NF-κB transcriptional activation, though not affecting IκBα proteolysis, RelA translocation, or NF-κB binding activity. Other antioxidants, NAC and vitamin C, when tested, produced similar effects to DMSO. The authors concluded that the antioxidants altered NF-κB via a different pathway than the nuclear translocation pathway necessary for inducible DNA binding.

D'Angio et al. (101) investigated the role of NF-κB, C/EBPβ, and AP-1 in LPS- and hyperoxia-induced IL-8 production in U937 and THP-1 cells. Stimulation with LPS

and hyperoxia triggered IL-8 protein and mRNA production in both cell lines, but only an additive effect could be seen in U937 cells. Further examination, using an IL-8 promoter-driven luciferase construct, revealed that only the mutation in the NF-κB site completely prevented LPS and/or hyperoxia stimulated expression of luciferase. Mutation of the C/EBPβ site did not alter the induction of luciferase, while a mutation in the AP-1 site had reduced levels of luciferase compared to the wild-type promoter. Using an EMSA, LPS was found to increase binding of NF-κB, but no difference was observed with either AP-1 or C/EBPβ in both U937 and THP-1 cells. No increases in binding were observed in any of the three transcription factors after cells were exposed to hyperoxic conditions.

Although the abovementioned studies used different stimuli and either monocytes or epithelial cells, there was a consensus in the importance of NF-κB in IL-8 expression. These studies also agreed that there was very little or no involvement of either AP-1 or C/EBPβ in the models investigated.

IL-8 expression is also regulated post-transcriptionally

IL-8 gene expression is also subject to post-transcriptional regulation. Many cytokines have AU rich elements (ARE) in their 3' untranslated region (UTR). These AREs are thought to be binding sites for proteins that stabilize the mRNA transcript and protect it from degradation. AREs are present in the IL-8 3' UTR (107). A 357 base pair element within the IL-8 3'UTR has been determined to destabilize mRNA from a CAT expression vector. However, Yu and Chandee (107) concluded that while the ARE, located in the 357 base pair element, was not the only destabilization element, it was

necessary for maximum suppression of the transcript. Additional factors were speculated to be involved in the destabilization of IL-8 transcripts.

Several studies have found that the increase in IL-8 mRNA stability was primarily responsible for the increase in IL-8 expression. In THP-1 monocyte cells, Haas et al. (38) observed that the proteosome inhibitor PSI increased the IL-8 mRNA stability (15 minutes with LPS alone to 40 minutes with LPS + PSI) even though PSI, at high concentrations, inhibited IL-8 transcription as seen with an IL-8 promoter luciferase construct. In U937 monocyte cells, Bosco et al. (93) determined that increased IL-8 mRNA stability was responsible for interferon (IFN)-γ-induced IL-8. Stability was increased from 40 minutes to 120 minutes with INF-γ treatment. The authors did not observe an increase in transcription.

IL-8 mRNA stability was found to be stimuli-dependent in a study conducted by Thorpe et al. (92). Shiga toxin 1 (Stx1) was found to induce IL-8 protein and mRNA in epithelial HCT-8 cells resulting from an increase in IL-8 mRNA stability. With a different stimulus, IL-1β, the authors determined that the increase in IL-8 expression was not caused by increased IL-8 mRNA stability in HCT-8 cells.

Multiple mechanisms trigger IL-8 expression

Other studies have found that both transcriptional activation and increased mRNA stability coordinate to increase IL-8 expression levels. Roger et al. (53) observed that in epithelial H292 cells had both increased transcriptional activity and increased IL-8 mRNA stability when treated with cyclohexamide + TNF-α. Specifically, the combination treatment increased both AP-1 and NF-κB binding activity and also

increased the stability of the IL-8 transcript from 40 minutes, when treated with TNF-α alone, to more than 4 h with the cyclohexamide + TNF-α treatment.

Villarete et al. (94) determined that, in LPS-treated human whole blood, increased IL-8 levels were a result of increased transcription as well as increased IL-8 mRNA stability. IL-8 and TNF-α gene transcription were induced, as measured by nuclear run-on transcription assays. Though there was an initial increase, transcription decreased after 26 h for TNF-α, but not for IL-8 (levels at 26 h were equivalent to 2 h levels). IL-8 mRNA was found to be twice as stable as TNF-α mRNA. Interestingly, after a 23 h exposure to LPS in the presence of actinomycin D (ActD), TNF-α mRNA was not detectable while the half-life of IL-8 mRNA increased to more than 10 h. This was more than double the half-life of IL-8 mRNA after a 2 h LPS exposure.

Involvement of chromosome remodeling or basal transcription machinery in the expression of IL-8

Mechanisms other than transcription factor activation or increased mRNA stability have also been linked to IL-8 expression. Histone alteration has been found in several studies to be the mechanism behind IL-8 expression in several epithelial cell lines. Tomita et al. (108) determined that H_2O_2- and trichostatin A (TSA)-induced IL-8 expression in BEAS-2S epithelial cells. This expression was attributed to hyperacetylation of histone H4. Hoshimoto et al. (109) examined the inhibitory effect of caprylic acid on IL-1β-induced IL-8 production in Caco-2 epithelial cells. Caco-2 cells had constitutive binding of AP-1 and C/EBPβ. IL-1β stimulation was only able to increase NF-κB binding, but none of the transcription factors were altered by caprylic

acid even though caprylic acid was able to inhibit luciferase expression from an IL-1β stimulated IL-8 promoter-driven luciferase construct. Trichostatin A, an inhibitor of histone deacetylase, decreased IL-1β-induced IL-8 mRNA levels without altering transcription factor binding. However, TSA was shown to induce rapid histone H4 acetylation, while caprylic acid did not, leaving the authors to conclude that while TSA causes chromatin remodeling through histone acetylation; however the inhibitory mechanism of caprylic acid remains unclear.

Yamamoto et al. (110) found, using HeLa epithelial cells, that TNF-α-induced IL-8 production was dependent on IKK-α, p65, and CBP association with the IL-8 promoter. This interaction was found to mediate phosphorylation and acetylation of histone H3. In another report of histone alteration, Ashburner et al. (111) found that, in HeLa epithelial and HEK-293 cells, p65 interacts with HDAC-1 (and HDAC-2 indirectly through HDAC-1). Inhibition of HDAC activity, with exposure to TSA, increased expression of TNF-α-induced IL-8 mRNA. TSA treatment also increased basal levels of IL-8 resulting from hyperacetylation of the IL-8 promoter region. The previous studies primarily found that, through histone modification, IL-8 expression could be induced without activation of transcription factors.

Other studies have found that IL-8 expression required many different molecules to be activated. Hoffmann et al. (112) reported on the mechanisms by which AP-1 regulates the IL-8 promoter in KB epidermal carcinoma cells, primary fibroblasts, and HeLa epithelial cells. IL-1-induced IL-8 protein and mRNA were most strongly inhibited by an ERK pathway inhibitor compared to inhibition using p38 and JNK inhibitors. The ERK pathway inhibitor also inhibited IL-1-induced c-Fos and Fra-1 mRNA. Using

HEK-293IL-1R cells for transfections of IL-8 promoter-driven luciferase constructs with constitutive c-Fos or constitutive Fra-1, the authors determined that c-Fos induced IL-8 transcription while Fra-1 suppressed IL-8 transcription induced by c-Fos or c-Fos + p65, a subunit of NF-κB. Fra-1 was found to interact with HDAC1, a histone deacetylase that can interact with p65. Unstimulated KB cells were found to have high levels of H3 and H4 acetylation within the IL-8 promoter region along with constitutive binding of HDAC1. The authors suggested that strong RNA polymerase II recruitment requires binding of c-Fos, Fra-1, p65, and TBP. In contrast to the strong induction of IL-8 by IL-1, exposure to epidermal growth protein (EGF) only causes a weak induction of IL-8 expression. The authors attributed this difference to the fact that EGF acts through the AP-1 site whereas IL-1 acts through both the AP-1 and NF-κB sites.

Carter and Hunninghake (113) used LPS-stimulated THP-1 monocytes to investigate the MEK/ERK pathway and its role on p38 activity. Previously, this group observed a link between p38 activity and NF-κB dependent gene expression through activation of TBP (114). In the subsequent study, the MEK/ERK pathway was found to inhibit p38 activity. Inhibition of the MEK/ERK pathway did not alter NF-κB translocation and binding or phosphorylation of p65 in LPS-treated THP-1 cells, but constitutively active MEK1 prevented TBP activation. The possibility that constitutively active MEK1 could inhibit IL-8 expression was confirmed with an IL-8 promoter-driven CAT construct. These results also indicated that LPS-induced IL-8 production was dependent on NF-κB.

Activation of MAPKs pathways are closely linked with activation of transcription factors and increased IL-8 mRNA stability. These studies will be discussed in the next section.

Mitogen activated protein kinases (MAPKs)

MAPKs are important messengers of signal transduction (115). The three main families of MAPKs are p38, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs). Once MAPKs are activated by phosphorylation, they translocate to the nucleus where they activate, primarily by phosphorylation, other proteins such as transcription factors (116). Figure 1.3 summarizes some well-known MAPK signaling pathways.

Linkage of MAPKs pathways to IL-8 expression

Several studies have linked IL-8 expression to activation of MAPKs pathways, particularly the p38 pathway (summarized in Table 1.3.). While these studies did not investigate the exact mechanism by which MAPKs upregulate IL-8 expression, they linked MAPK inhibition to reduced or completely suppressed IL-8 expression. Arbabi et al. (117) examined the inhibition of LPS-induced IL-8 levels in primary human mononuclear cells by ethanol. Ethanol inhibited LPS-induced p38 activation as well as the LPS-induced IL-8 protein and mRNA expression. The pattern of inhibition that ethanol mimicked that also seen with a p38 specific inhibitor, SB202190.

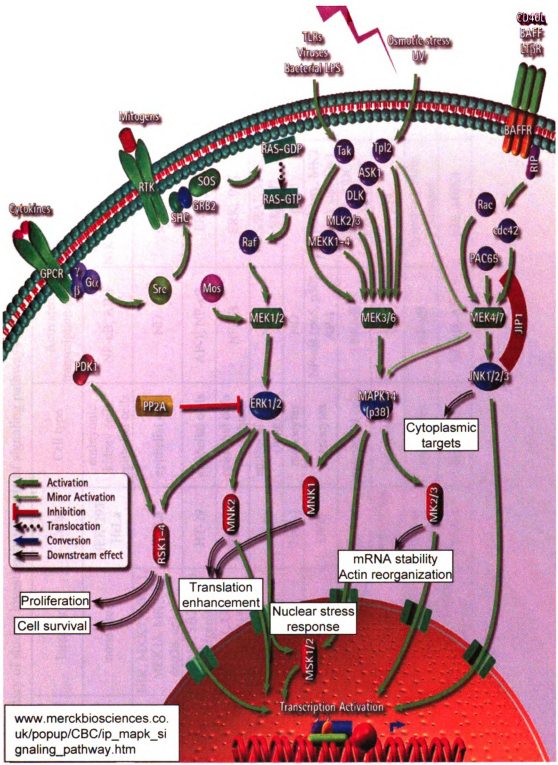

Figure 1.3: General MAPK pathway. (This image is in color.)

Table 1.3: Summary of studies investigating the role of signaling pathways in IL-8 expression.

Stimulus	Inhibitor[b]	Cells	Cell type	Activated transcription factors	Activated signaling pathways	mRNA stability occurring	Reference
none	none used	HEK-293, HeLa	embryonic kidney cells, epithelial cells	NF-κB, AP-1	JNK, p38, active NIK, MKK7, MKK6, MEKK1	yes	(118)
IL-1	NIK, MKK7, MKK1, MEK1δ, MKK6, dnp38, dnMK2,	HeLa	epithelial cells	ND	p38, MK2	yes	(119)
TNF-α	PD98059, SB203580, apigenin	HT-29	epithelial cells	AP-1, NF-κB	ERK, p38	yes	(120)
TNF-α	dnMKK7, U0126, SB202190	16HBE10o-	epithelial cells (bronchial)	NF-κB	ERK, JNK	yes	(121)
Adenovirus type 7	PD98059, U0126, dnRAS	A549	epithelial cells (lung)	ND	RAS/RAF/MAK/ ERK 1/2	no	(122)
LPS	SB203580	THP-1	monocytes	ND	p38	yes	(123)
H_2O_2	SB203580	U937	monocytes	NF-κB (p65, p50), AP-1	p38 pathway, MK2 (mRNA stability)	yes	(124)
LPS	*C. difficile* toxin B-10463, SB202190	HUVEC	endothelial cells	NF-κB	RHO A, CDC 42/RAC1, p38	ND[a]	(51)
LPS	ethanol	mononuclear cells	monocytes	ND	p38	ND	(117)
Mycobacterium tuberculosis	genistein, PD98059, SB203580	human monocytes	monocytes	NF-κB	Tyrosine protein kinases	ND	(125)
PMA/LPS	radicicol, PD98059, SB203580	THP-1	monocytes	NF-κB, AP-1	p38, MEK1	ND	(126)

Table 1.3 continued: Summary of studies investigating the role of signaling pathways in IL-8 expression.

Stimulus	Inhibitor	Cells	Cell type	Activated transcription factors	Activated signaling pathways	mRNA stability occurring	Reference
H_2O extract of *H. pylori*	none	human peripheral blood monocytes	monocytes	ND	ERK1/2, JNK, p38	ND	(127)
H_2O extract of *H. pylori*	U0126, SB203580, dnMEK1, dnMKK6, dnMKK7	THP-1	monocytes	NF-κB (p65, p50), AP-1 (c-Fos, c-Jun)	ERK1/2, JNK, p38	ND	(127)
zymosan	gliotoxin, SB203580	monocyte	monocytes	NF-κB	p38	ND	(128)
nitric oxide	SB203580	U937	monocytes	ND	p38	ND	(129)
Mycoplasma fermentans lipid-associated membrane proteins (LAMPf)	CAPE, PD98059, SB203580	THP-1, PMN	monocytes, neutrophils	NF-κB	ERK, p38	ND	(130)
Type III group B *Streptococcus*	SB203580	neutrophils	neutrophils	ND	p38	ND	(39)
IL-1β or TNF-α	SC-514, dnIKKs	HEK-293, HeLa	embryonic kidney cells, epithelial cells	NF-κB (p65)	IKK, IκB	ND	(131)

a: ND = not determined

b: dn = dominant negative; others are constitutive overexpression

Marie et al. (130) found that *Mycoplasma fermentans* lipid-associated membrane proteins (LAMPf) triggered production of IL-8 in primary human monocytes and polymorphonuclear cells (PMN). Using chemical inhibitors, protein tyrosine kinases were found to be necessary for both monocytes and PMN while ERK activation had a larger role in PMNs than monocytes. When THP-1 monocyte cells were transfected with a NF-κB or AP-1 luciferase constructs, LAMPf was able to increase expression of both constructs. A NF-κB inhibitor was able to prevent IL-8 production in both monocytes and PMNs.

Albanyan et al. (39) examined the role of ERK and p38 in type III group B *Streptococcus*-induced IL-8 in primary human neutrophils. In contrast to the previous study linking ERK activation to IL-8 expression, an ERK inhibitor was only able to partially reduce IL-8 protein levels in *Streptococcus*-treated primary human neutrophils. Another MAPK family, p38, was found to be responsible for the induction of IL-8 protein as determined with a p38 inhibitor. This difference in the degree of importance of the ERK and p38 pathways could be due to the fact that the cells were isolated from different individuals or use of different stimuli.

MAPKs activate transcription factors

MAPKs can also play a role in both transcriptional induction and in post-transcriptional stability. Several studies have linked the three MAPKs families to activation of transcription factors involved in IL-8 expression. Alcorn et al. (122) also found that the ERK pathway had a role in IL-8 production in A549 epithelial cells.

Adenovius serotype 7 (Ad7) increased IL-8 production through an activation of transcription. Ad7 was found to increase ERK activation, and with an inhibitor of ERK, IL-8 production was inhibited at the transcriptional level, though the specific transcription factors were not determined.

Li et al. (121) used bronchial epithelial cells, 16HBE14o-, to examine the role of the MAPKs pathways in TNF-α-induced IL-8 expression. Increased NF-κB binding, composed of p65 and p50 subunits, occurred as a result of TNF-α exposure. With an IL-8 promoter-driven luciferase construct, the authors determined that NF-κB was required for TNF-α-induced transcription. Inhibition of ERK or JNK prevented transcription, while inhibition of p38 and ERK significantly inhibited only IL-8 protein. JNK was found to be important for NF-κB activation, while ERK was found to be important for AP-1 activation. The AP-1 site also was critical for basal transcription as a mutation reduced basal transcription.

Using U937 monocytes, Josse et al. (124) found that H_2O_2 causes an increase in IL-8 mRNA. Using IL-8 promoter-driven luciferase constructs, the authors determined that an increase in NF-κB, specifically p65, p50, c-Rel was responsible for transcriptional activation. AP-1 was also found to contribute to activation of the IL-8 promoter, although a mutation in the AP-1 binding site had only half the decrease in luciferase levels than that seen with a mutation in the NF-κB binding site. Examination of the p38 and ERK pathway revealed that p38 was necessary for increased IL-8 mRNA stability while ERK was not.

Na et al. (126) determined that radicicol, an anti-fungal antibiotic, is able to inhibit both IL-8 protein and mRNA expression in PMA + LPS stimulated THP-1

monocytes. Both NF-κB and AP-1 are necessary for PMA + LPS-induced IL-8, with increased binding observed after treatment. C/EBPβ was not involved in either the PMA + LPS-induced IL-8 production or the inhibition by radicicol. The authors found, however, that THP-1 cells had a strong constitutive binding of C/EBPβ. PMA + LPS treatment also activated ERK, p38, and JNK, with inhibition of ERK and p38 occurring after radicicol treatment. Additional inhibitors for ERK and p38 were also able to inhibit PMA + LPS-induced IL-8 mRNA levels. Further examination of the ERK pathway revealed that binding of both PMA + LPS-induced AP-1 and NF-κB were inhibited after addition of an ERK inhibitor. Addition of a p38 inhibitor did not alter NF-κB binding and only partially inhibited AP-1 binding.

Bhattacharyya et al. (127) used both primary human monocytes and THP-1 monocytes to determine the role of the MAPKs pathways in *Helicobacter pylori*-induced IL-8. *H. pylori* activated ERK, p38, and JNK in human monocytes and THP-1 cells. Both an ERK and p38 phosphorylation was prevented by kinase inhibitors. The upstream signaling molecules of ERK (MEK1), p38 (MKK6 and MKK3), and JNK (MKK7) were found to be necessary for *H. pylori* activation of the MAPKs pathways. NF-κB binding was increased, though not due to activation of the MAPKs pathways, while increased AP-1 binding was attributed to the activation of the ERK and JNK pathways. The NF-κB binding site was also necessary for *H. pylori*-induced luciferase in THP-1 cells transfected with an IL-8 promoter-driven luciferase construct. However, although increased AP-1 binding was seen, a mutation in the AP-1 binding site only partially reduced luciferase levels.

Friedland et al. (128) examined IL-8 production after phagocytosis of zymosan in THP-1 monocyte cells. Zymosan treatment induced NF-κB binding. PKA, PKC, and tyrosine kinase inhibitors were unable to prevent both the increase in NF-κB binding and the IL-8 mRNA production. Significant inhibition of zymosan-induced IL-8 production was seen after inhibition of the p38 pathway while only partial inhibition was observed with an ERK inhibitor. An interesting aspect of their study was that the number of NF-κB binding complexes changed depending on the clone of THP-1 cell used for the experiment. The authors noted the reason for this difference was unknown, as all cells used behaved in a similar manner.

MAPKs also mediate post-transcriptional mechanisms

MAPKs pathways also play a role in increasing mRNA stability. A substrate of p38, MK2, is involved in stabilization of mRNAs containing AU rich elements (132), including TNF-α, in murine cells. In another study, Ridley et al. (133) found that blocking p38 increased the decay of COX-2 mRNA. An increase in p38 activation leads to increased stability of IL-8 mRNA in several cell types.

Winzen et al. (119) found that, in HeLa epithelial cells, constitutive expression of MEKK1 was capable of increasing mRNA stability of IL-8 mRNA from less than 15 minutes to around 110 minutes. Since MEKK1 can be involved in multiple pathways, the effects of constitutive expression of MKK7, NIK, and MKK6 were also checked. It was determined that only MKK6, an upstream signaling molecule in the p38 pathway, was involved in stabilization of IL-8 mRNA.

Frevel et al. (123) similarly found involvement of the p38 pathway in increased IL-8 mRNA stability. Here, a 43.5 fold increase in IL-8 mRNA levels was observed in TNF-α-treated THP-1 monocytes. With the addition of SB203580, a p38 inhibitor, the half-life of the IL-8 transcript decreased to 17 minutes; almost 3 times lower than in cells without the inhibitor thus implicating a role for p38 in the increased half-life. Another study (120), investigated the role both the ERK and p38 pathways have in stabilization of the IL-8 mRNA in HT-29 epithelial cells. The ERK and p38 pathways both were determined to have a role in stabilization of TNF-α-induced IL-8 expression. Both an ERK inhibitor, PD98059, and a p38 inhibitor, SB203580, significantly reduce TNF-α-induced IL-8 protein levels by greater than 40% and about 30%, respectively. The authors concluded that the inhibition of IL-8 seen with the two inhibitors was not due to a difference in AP-1 or NF-κB binding, but to a destabilization of IL-8 mRNA.

MAPKs acts in tandem for both transcription and post-transcriptional mechanisms

MAPKs pathways have also been found to cause increases in IL-8 by stimulating a signaling cascade that can end in activation of transcription and an increase in mRNA stability. Holtmann et al. (118) used KB epithelial and HEK-293 cells to determine the kinases involved in IL-8 transcription and IL-8 mRNA stability. Cells were transfected with constitutive expression vectors and IL-8 protein was measured. The authors determined that MKK7, SAPK/JNK pathway, NIK, and MEKK1 were important for IL-8 expression. Mutations of the AP-1 and NF-κB site caused a decrease in basal activity and prevented induction by NIK or MKK7. Increased IL-8 mRNA stability was also seen. It was determined that MKK6 increased IL-8 mRNA stability.

Other kinases implicated in IL-8 expression

Additional kinases have also been implicated in IL-8 expression. Muhl et al. (129) found that inhibition of protein tyrosine kinases or p38 was able to prevent nitric oxide-induced IL-8 protein in U937 cells. Ameixa and Friedland (125) also found that protein tyrosine kinases were important for *Mycobacterium tuberculosis*-induced IL-8 expression. They examined the role of ERK, p38, and protein tyrosine kinases in *M. tuberculosis*-induced IL-8 expression in primary human monocytes. Using inhibitors, the authors found that ERK was not involved in *M. tuberculosis*-induced IL-8, though necessary for LPS-induced IL-8. The p38 inhibitor did not reduce either *M. tuberculosis*- or LPS-induced IL-8. A broad spectrum protein tyrosine inhibitor inhibited both the *M. tuberculosis*- and LPS-induced IL-8 protein and mRNA.

PKR has also been linked to IL-8 expression via NF-κB, either by activating NF-κB inducible kinase (NIK) (31) or by interacting with the IKK complex (30, 31). Rho proteins were also found to have a role in IL-8 expression. Hippenstiel et al. (51) determined that inhibition of small GTP-binding Rho proteins prevented LPS-induced IL-8 expression in human umbilical vein endothelial cells (HUVECs). Additionally, inhibition of the p38 pathway also reduced IL-8 expression. Both the p38 and Rho inhibitors prevented LPS-induced activation of a NF-κB dependent luciferase reporter construct. The authors concluded that different pathways, one involving Rho proteins and the other consisting of the p38 pathway, both lead to NF-κB activation necessary for IL-8 expression. Buss et al. (131) found that, in HeLa epithelial and HEK-293 cells phosphorylation of p65 was induced by IL-1β or TNF-α. The activation of p65 was not

altered by ERK, JNK, or p38 inhibitors; however a proteosome inhibitor, that prevented IκB degradation, increased basal activation but prevented inducible activation. As shown by these previous studies, stimuli can activate multiple signaling pathways, other than the MAPK pathways, that result in activation of downstream proteins and drive IL-8 expression.

U937 cells

U937 cells are promonocytes that were isolated from the pleural effusion of an individual with diffuse histiocytic lymphoma (12). These cells can be differentiated to form neutrophils, with exposure to retinoic acid, or macrophages, with exposure to PMA. U937 cells can produce a variety of cytokines and chemokines, including IL-8, both in the undifferentiated monocytic form and as a macrophage. Using cytogenetic methods four different sublines of U937 cells have been discovered: U937-1, U937-2, U937-3, and U937-4 (134, 135). This cell line has between 40 and 60 chromosomes, double to triple the normal complement in human cells (134, 136). Examination of U937-1 with whole chromosome painting and comparative genomic hybridization has provided evidence that chromosome 4, the location of the IL-8 gene, has losses of genomic material at 4q and an extra copy of 4p added to chromosome 16p (137). In a separate cytogenetic study of U937 cells, obtained directly from American Type Culture Collection, a translocation from 4p to 16p was observed. However, losses of genomic material on chromosome 4 were not observed with comparative genomic hybridization

(138). This difference could be a reflection of differences in the cells or could be due to the different sources of DNA used in the comparative genomic hybridization.

Another alteration in U937 cells is the lack of production of p53, a tumor suppressor that can induce apoptosis and cell cycle arrest, protein or mRNA (139). In an additional study published in 1992, Sugimoto et al. (140) observed that 46 bases were missing from an exon of the p53 mRNA as a result of an abnormal splicing event. In an interesting connection, mutations of the p53 gene have been linked to both constitutive NF-κB activation and high basal IL-8 protein production (141). The high basal expression was decreased in cells transfected with an expression vector for normal p53. Transfection with normal p53 also reduced NF-κB transcriptional activity, measured with a NF-κB-driven luciferase construct, but not NF-κB DNA binding as seen in an EMSA. Experiments conducted in an earlier study observed that mutations in either p65, a subunit of NF-κB, or p53 result in an increase in transcriptional activity of p53 or p65, respectively (142).

U937 cells were chosen for the research in this dissertation for a variety of reasons. First, actively dividing cells, such as immune cells are very susceptible to the effects of DON (9, 13). Secondly, as DON can be found in plasma of mice, given an oral exposure, within 30 minutes it is likely that blood monocytes are in contact with DON (143). Third, U937 cells, in a macrophage differentiated form, have already been shown to produce IL-8 and cytokines as a result of DON exposure (8). Fourth, U937 cells have been utilized in numerous studies (75, 99, 101, 102, 144) and produce robust levels of IL-8.

CHAPTER 2: ROLE OF MITOGEN-ACTIVATED PROTEIN KINASES IN DEOXYNIVALENOL-INDUCED INTERLEUKIN-8 EXPRESSION

Part of the data in this chapter has been published in Islam, Z., J.S. Gray, and J.J. Pestka, *p38 Mitogen-activated protein kinase mediates IL-8 induction by the ribotoxin deoxynivalenol in human monocytes.* Toxicol Appl Pharmacol, 2006. **213**(3): p. 235-44.

Abstract

Deoxynivalenol (DON), a trichothecene mycotoxin, is a common contaminant of grain crops worldwide. At low concentrations, DON is capable of stimulating production of immune proteins in a variety of cells. Previous reports have determined that mitogen-activated protein kinase pathways (MAPKs) are also activated upon exposure to DON and these kinases might be upstream elements in the expression of immune mediators. The hypothesis tested in this study was that MAPKs play a central role in DON-induced IL-8 expression in the U937 human monocyte model. Activation of three major pathways, p38, ERK, and JNK, was involved in the DON-induced IL-8 expression. Of these, the p38 pathway contributed to the greatest extent as reflected by increased IL-8 protein, mRNA, and hnRNA levels and the extent of inhibition with selective MAPK inhibitors.

Introduction

Deoxynivalenol (DON, vomitoxin) is a fungal secondary metabolite, classified as a trichothecene, which is produced by *Fusarium graminearum* or *Fusarium culmorum*. It is one of the most common mycotoxins found in cereal grains (6) and is a frequent contaminant of wheat, corn, and barley worldwide (13). DON is a very stable compound that is capable of withstanding the high temperatures used in processing and cooking of foods (13). Because of this, DON that is present in the unprocessed grains can still be present in processed foods and be consumed by humans and animals.

DON is both immunosuppressive and immunostimulatory in experimental animals, depending on dose (15). The immunostimulatory effects of DON, seen at lower doses (16), includes the induction of cytokines *in vitro* and *in vivo*. Interference with normal mechanisms of gene regulation appears to drive cytokine production. In macrophage-differentiated U937 cells, DON can induce secretion of IL-6 and IL-8 (8). As a prerequisite of the induction of cytokines, DON activates signaling cascades in cells. Yang et al. (28) showed that mitogen-activated protein kinases (MAPKs) were activated by trichothecenes in RAW 264.7 cells. Additionally, Zhou et al. (29) determined that, prior to inducing MAPK activation in U937 cells, DON activates double stranded RNA activated protein kinase (PKR).

IL-8 is a biologically potent molecule that is produced by a wide variety of cell types, including monocytes (36-38), neutrophils (39, 40), T lymphocytes (41, 42), natural killer cells (44), fibroblasts (44-46), macrophages (8, 47) and endothelial cells (49-51). IL-8 is classified as a CXC chemokine containing sequences specific for neutrophil chemotaxis (33) and for angiogenesis (34). Additional functions of IL-8 include

initiation of the acute inflammatory response (56) and cell specific functions such as neutrophil shape change as well as histamine induction and leukotriene release in basophils (44).

IL-8's varied actions have been implicated in several diseases. Extensive tissue damage can occur following IL-8-mediated recruitment of neutrophils to specific areas and subsequent inflammation. Elevated IL-8 levels have been seen in serum from patients with severe ovarian hyperstimulation (58) and respiratory syncytial virus bronchiolitis (59). Elevated urinary IL-8 is seen in patients with glomerulonephritis (61). IL-8 is found in inflammatory sites in patients with psoriatic scales, rheumatoid arthritis, cystic fibrosis (60), gouty arthritis, and adult respiratory distress syndrome (61). In support of IL-8's role in disease, administration of an IL-8 antibody lessens disease severity in several animal models, including lung perfusion injury and lipopolysaccaride (LPS)/IL-1-induced arthritis (61).

Regulation of IL-8 is critical and can occur at both the transcriptional and post-transcriptional levels. Transcription factor binding sites located in this promoter include AP-1, C/EBPβ, nuclear factor- κB (NF-κB), Octamer-1 (Oct-1), and NF-κB repressing factor (NRF). Combinations of two or three transcription factors, which varies according to cell type, are required to obtain the highest level of transcription. The three transcription factors that usually are present, in such complexes, are AP-1, C/EBPβ, and NF-κB (54, 56, 145).

One way that transcription factors are activated is via the MAPK signaling cascade. Once MAPKs are activated by phosphorylation, they translocate to the nucleus where they are capable of activating, primarily by phosphorylation, other proteins such as

transcription factors (116). The three main families of MAPKs are p38, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) (115). p38 has been correlated to IL-8 transcription with cells stimulated by *Clostridium difficile* toxin A (81), *Mycoplasma* fermentans lipid-associated membrane protein (LAMPf) (130), urate crystals (71), and phorbol-12-myristate-13-acetate (PMA)/LPS (126). ERK (120, 121, 130) and JNK (121, 127) have also been linked to IL-8 transcription.

Besides transcriptional induction, MAPKs can also modulate in mRNA stability. A substrate of p38, MK2, has been found to be involved in stabilization of mRNA containing AU rich elements (132). Ridley et al. (133) found that blocking p38 increases the decay of COX-2 mRNA. p38 also increases the stability of TNF-induced IL-8 transcripts (120, 121), in cells transfected with constitutively active MEK1, which is upstream of p38, ERK, and JNK, and constitutively active MKK (MAPK kinase) 6, upstream of p38 (118). In the same study, constitutively active MKK7, a kinase capable of activating JNK, also could induce IL-8 expression.

Our laboratory has previously shown that DON induces IL-8 protein production in macrophage-differentiated U937 cells (8). The purpose of this study was to test the hypothesis that MAPKs play a central role on DON-induced IL-8 expression in U937 cells, a human monocyte model. The results indicate that all three major MAPKs families were involved in the DON-induced expression of IL-8, although there was a difference in the relative contributions of p38, ERK, and JNK. Notably, p38 played the largest role in DON-induced IL-8 expression, with ERK and JNK having lesser roles.

Materials and Methods

Cell culture

U937 cells, obtained from American Type Culture Collection (ATCC; Manassas, VA), were isolated from the pleural effusion of an individual with diffuse histiocytic lymphoma (Sundstrom and Nilsson 1976). U937 cells were grown in RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL; Rockville, MD). Cells were incubated at 37°C with 6% CO_2. Fresh growth media (RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin) was added as necessary to passage the cells. DON and/or the MAPKs inhibitors were incubated with U937 cells ($1x10^6$ cells/ml) for 3 h. The MAPK inhibitors SP600125 (JNK), PD98059 (ERK), and SB203580 (p38) were obtained from Calbiochem (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO). All other reagents are from Sigma (St. Louis, MO) unless otherwise noted.

Western Blot

Total protein was isolated from U937 cells after a 30 minute exposure to 500 ng/ml DON. Briefly, cells were lysed with hot 1% (w/v) SDS buffer (1 mM sodium ortho-vanadate in 10 mM Tris, pH 7.4) collected, boiled for 5 minutes, sonicated for 30 sec, and centrifuged at 12,000 x g for 15 minutes. The supernatant was collected and the protein was quantitated with the DC Protein Quantitation kit (BioRad; Hercules, CA). Total protein (10 μg) was loaded on a 10% SDS-PAGE gel and run at 100 V. The gel was electrotransferred to a PDVF membrane (Amersham; Piscataway, NJ) overnight at

4°C. The membrane was then blocked in 5% (w/v) bovine serum albumin in TBST (20 mM Tris-HCl, pH 8 and 137 mM NaCl containing 0.1% Tween 20), washed and incubated with a primary antibody for phospho-p38, phospho-ERK, or phospho-JNK (rabbit IgG) (Cell Signaling; Beverly, MA) at room temperature for 1 h. The membrane was then washed and incubated with an anti-rabbit whole IgG (Cell Signaling) antibody conjugated with horseradish peroxidase for 1 h at room temperature. Bands were visualized with the ECL detection system (Amersham). Membranes were stripped, blocked, and incubated with nonphospho p38, ERK, or JNK (anti-rabbit IgG) (Cell Signaling) overnight at 4°C. The next day, the membrane was then washed and incubated with an anti-rabbit whole IgG (Cell Signaling) antibody conjugated with HRP for 1 h at room temperature. Bands were visualized with the ECL detection system (Amersham).

MTT viability assay

Cells were plated (200 µl/well) in 96 well plates at $1x10^6$ cells/ml. DON, dissolved in RPMI-1640, and 25 µl of 5 mg/ml of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) dissolved in Dulbecco's phosphate buffered saline (PBS) were added and cells were incubated at 37°C with 6% CO_2 for 3 h. Plates were centrifuged at 300 x g for 10 minutes and the supernatant was removed. DMSO (125 µl) was added and the absorbance was read at 690-570 nm on a Vmax Kinetic Microplate Reader (Molecular Devices; Menlo Park, CA). Percent of the control was calculated for each well.

IL-8 protein determination

After treatment, cell culture plates were centrifuged for 10 minutes at 300 x g and the supernatant collected and stored at -20°C. An OptELISA IL-8 kit (Pharmingen; San Diego, CA) was used according to manufacturer's instructions with two modifications. First, the highest standard utilized was 1600 pg/ml, instead of 400 pg/ml. Second, to economize on reagents 50 µl of antibody dilutions and samples were used per well instead of 100µl. All samples were read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices).

RNA isolation

U937 cells were treated with DON for the 3 h and then the plates were centrifuged for 10 minutes at 300 x g, the supernatant removed, and the RNaqeous kit (Ambion; Austin, TX) was used to isolate the RNA. Samples were treated with DNA-free (Ambion) to remove gross DNA contamination. The manufacturer's instructions were followed for both kits.

Reverse transcription real-time PCR

Reverse transcription real-time PCR was performed using One-Step PCR Master Mix (Applied Biosystems; Foster City, CA) and IL-8 Pre-Developed Assay Reagents (PDAR; Applied Biosystems) multiplexed with the 18S PDAR (Applied Biosystems). For the hnRNA, Primer Express software v 1.5 (Applied Biosystems) was used to make primer (base numbers 3113351-3113369 and 3113431-3113452) and probe (base

numbers 3113411-3113429) selections from the NM_006216 (Genbank) sequence (IL-8 DNA). The primer/probe set was selected to include an exon-intron junction. Fold change was determined using the relative quantitation method. First, standard curves are created using dilutions of total RNA from LPS-treated U937 cell total RNA. An equation, for the trend line of the standard values, is used to convert the Ct values obtained in the assay to nanogram amounts of the target. The amounts were normalized by dividing the IL-8 value by the 18S (the endogenous control) value. Relative expression is obtained by dividing all normalized values by the average of the control normalized value. Reaction conditions and PCR program were all following the manufacturer's instructions using an ABI 7700 (96 wells) or 7900HT (384 wells), both at the Michigan State University's Genomics Technology and Support Facility, depending on the number of samples to be analyzed.

Statistics

Data were analyzed with SigmaStat v 1.0 (Jandel Scientific; San Rafael, CA). IL-8 protein and RNA data were analyzed using one way analysis of variance (ANOVA) with Student-Newman-Keuls Method for pairwise comparisons. Treatment groups that have a p-value of < 0.05 were considered significant.

Results

DON activated p38 and JNK in U937 cells

To confirm that MAPKs are activated by exposure to DON, U937 cells were treated with 500 ng/ml of DON for 30 minutes. Phospho-p38 levels were increased in the DON group as compared to untreated cells (Figure 2.1A). Nonphospho-p38 levels were approximately equivalent in all samples. A similar increase was observed for phospho-JNK samples even though the nonphospho-JNK bands was less abundent in DON-treated samples as compared to the control samples (Figure 2.1B). There were no apparent differences in the phospho-ERK levels in the DON-treated and untreated cells (Figure 2.1C). Basal ERK activation in control samples was apparent. This was possibly due to the fact that the U937 cells were in suspension and required centrifugation to isolate the total protein, which might result in artifactual activation. Phospho-ERK levels were also examined at a 3 h timepoint, with no difference between the control and DON-treated samples. However, the overall activation level was reduced compared to the 30 minute samples (data not shown).

Cell viability was not affected by the MAPKs inhibitors

An MTT assay was conducted on the U937 cells to determine if the MAPK inhibitors alter the viability of the treated cells using concentrations employed previously by other investigators (25, 39, 127, 128). If the inhibitors were toxic, any observed inhibition may be the caused by fewer cells producing less IL-8 rather than an actual inhibition of IL-8 expression. When cells were treated with 2 μM SB203580, 10 μM PD98059, 1 μM SP600125 or 500 ng/ml DON (Figure 2.2), none of the three MAPK

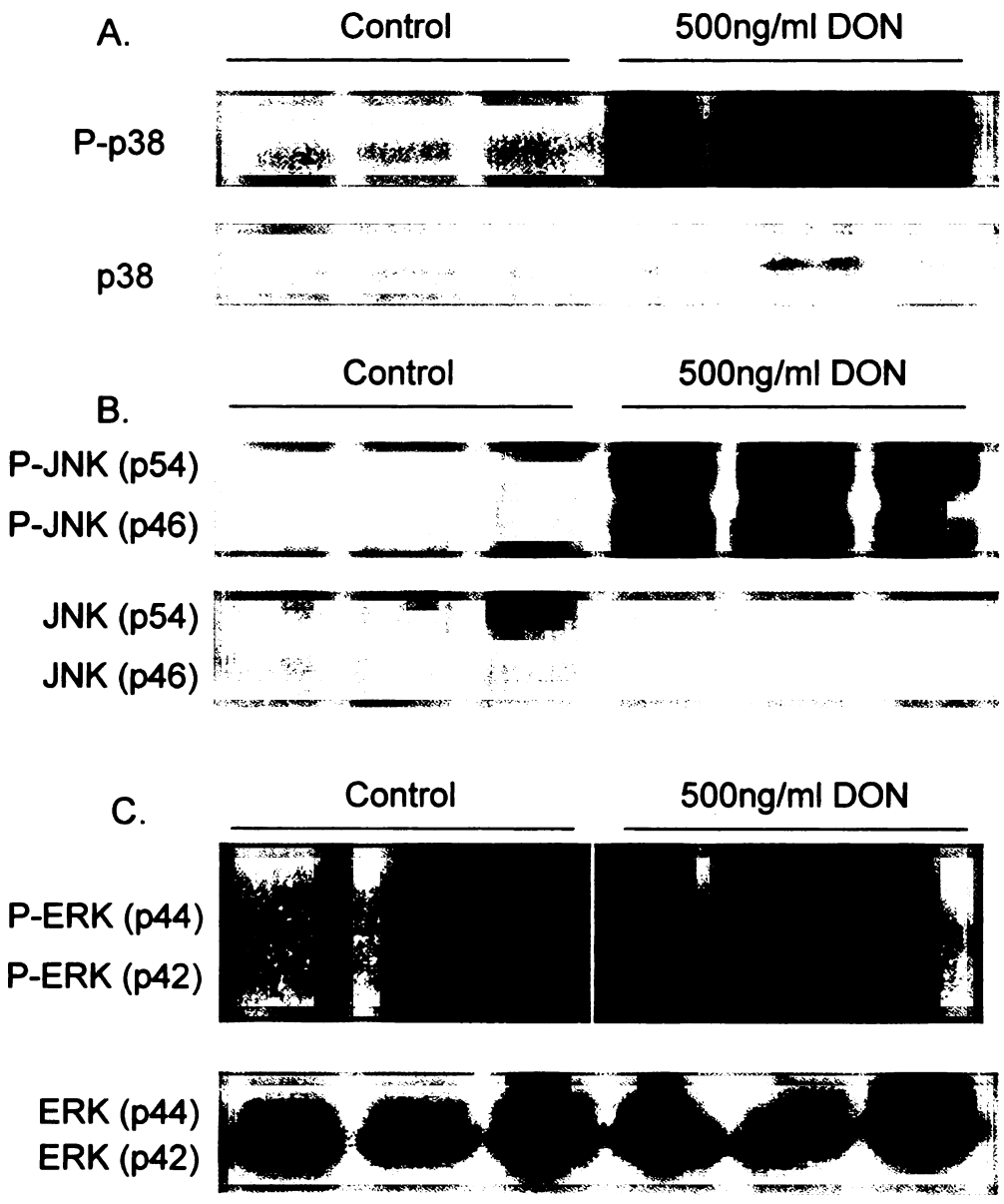

Figure 2.1: Exposure to DON increases phosphorylation of p38, JNK, and ERK in U937 cells. Western blot of U937 total protein samples isolated after a 30 minute exposure to 500 ng/ml DON. (A) Phospho-p38 and nonphospho-p38 (B) Phospho-JNK and nonphospho-JNK (C) Phospho-ERK and nonphospho-ERK. (n = 3) These data are representative of 2 independent experiments.

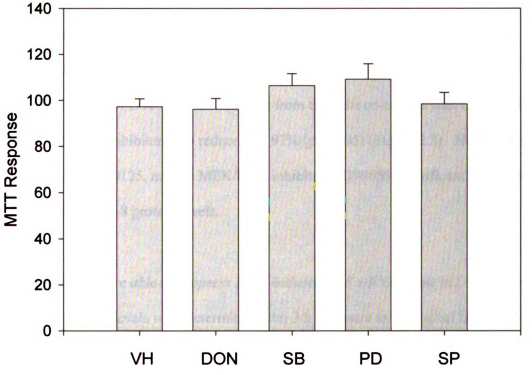

Figure 2.2: Viability of U937 cells does not decrease with exposure to MAPK inhibitors. MTT with U937 cells after a 3 hour exposure to 500 ng/ml DON, 2.0 μM SB203580, 10 μM PD98059, or 1.0 μM SP600125. Data are the mean ± SEM (n = 3). These data are representative of 2 independent experiments.

inhibitors significantly altered the viability of the cells. At 3 h, viabilities of the cells treated with 500 ng/ml DON was not significantly different than from non-treated cells.

MAPK inhibitors were able to suppress DON-induced IL-8 protein levels in U937cells

Since 500 ng/ml DON strongly induced IL-8 (Figure 2.3), it was used in all subsequent experiments. IL-8 concentration in culture media was measured after 3 h exposure to 500 ng/ml DON and/or MAPKs inhibitors (2 μM SB203580, 1 μM SP600125, or 10 μM PD98059). IL-8 protein from the cells co-treated with DON and SB203580, the p38 inhibitor, was reduced by 97% ($p < 0.05$) (Figure 2.3). Neither the JNK inhibitor, SP600125, nor the MEK/ERK inhibitor, PD98059, significantly inhibited the DON-induced IL-8 protein levels.

MAPK inhibitors were able to suppress DON-induced IL-8 mRNA levels in U937 cells

IL-8 mRNA levels were determined after 3 h exposure to 500 ng/ml DON and/or 2 μM SB203580, 1 μM SP600125, or 10 μM PD98059 (Figure 2.4). All three of the inhibitors significantly inhibited IL-8 mRNA levels ($p < 0.05$). SB203580 inhibited approximately 90% of the DON-induced IL-8 mRNA levels while SP600125 and PD98059 caused approximately 50% inhibition. Marked SB203580 inhibition of the IL-8 mRNA was similar to that seen at the protein level.

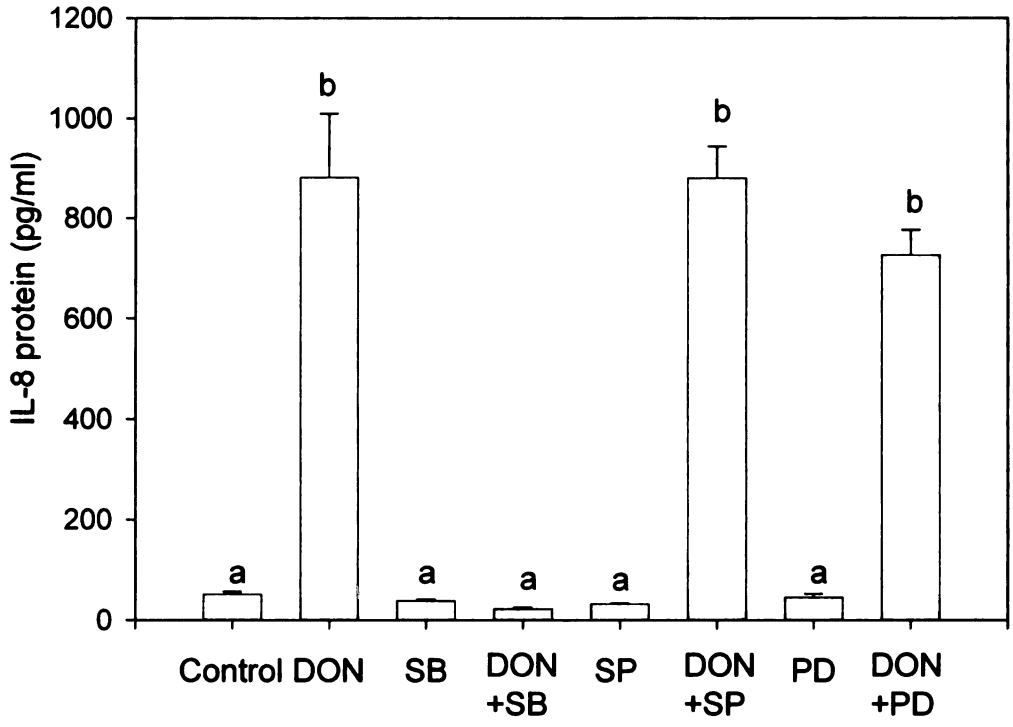

Figure 2.3: DON-induced IL-8 protein levels are suppressed by a p38 inhibitor. IL-8 protein levels in U937 culture supernatant after 3 hours exposure to 500 ng/ml DON, and/or 2.0 μM SB203580, 10 μM PD98059, or 1.0 μM SP600125. Bars without the same letter are statistically different ($p < 0.05$). Data are the mean ± SEM ($n = 5$). These data are representative of 3 independent experiments.

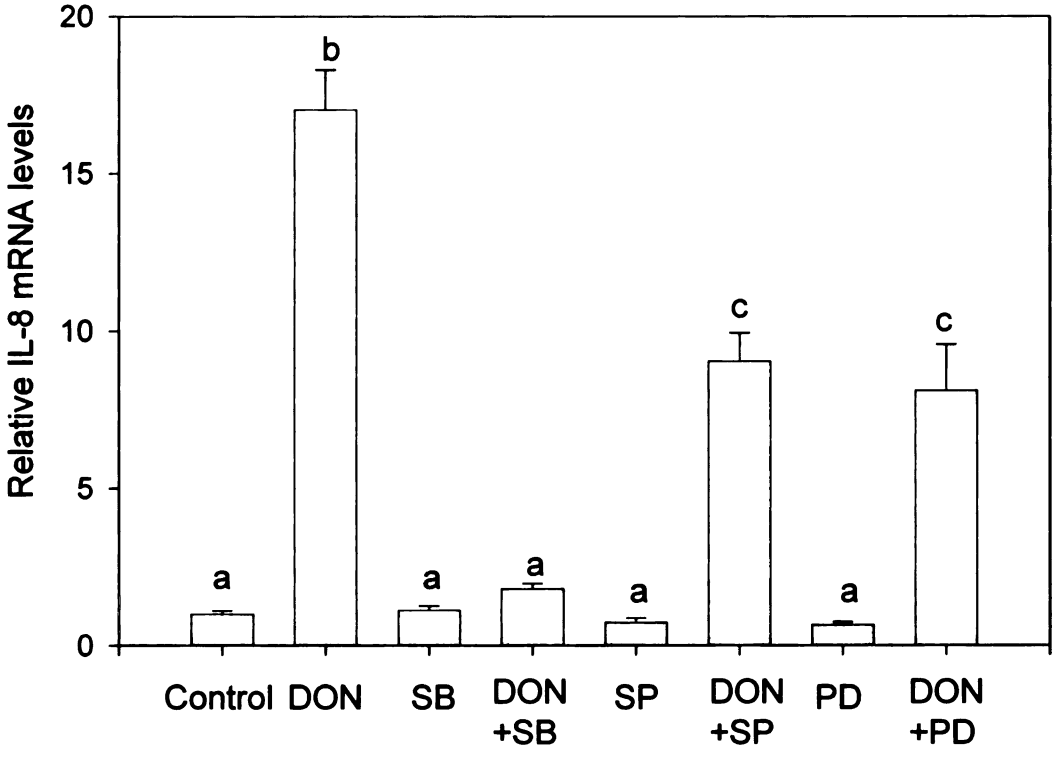

Figure 2.4: DON-induced IL-8 mRNA levels are suppressed by MAPKs inhibitors. IL-8 mRNA levels in U937 after a 3 hour exposure to 500 ng/ml DON, and/or 2.0 μM SB203580, 10 μM PD98059, or 1.0 μM SP600125. Bars without the same letter are statistically different ($p < 0.05$). Data are the mean ± SEM ($n = 11$). These data were collected from 4 separate experiments.

MAPK inhibitors were able to suppress DON-induced IL-8 hnRNA levels in U937 cells

Levels of IL-8 heteronuclear RNA (hnRNA) were determined after 3 h exposure to DON with or without SB203580, SP600125, or PD98059 (Figure 2.5). hnRNA levels can be used to measure transcription as hnRNA is pre-processed mRNA. The levels of the IL-8 hnRNA are equivalent to the levels of IL-8 mRNA in all treatment groups. All inhibition was statistically significant ($p < 0.05$) although, as with the mRNA levels, SP600125 and PD98059 only inhibit approximately half of the induction by DON while SB203580 provides an almost complete inhibition (~90%).

Discussion

The results presented here indicate that p38 and JNK were activated in U937 cells after exposure to DON and that this activation, primarily p38, contributed to the signaling cascades leading to IL-8 expression. SB203580, a p38 inhibitor, PD98059, a MEK/ERK inhibitor and SP600125, a JNK inhibitor, were used to determine if specific MAPK families are involved in the DON-induction of IL-8. Neither the MAPK inhibitors nor the DON concentration used (500 ng/ml) altered the cells' viability, indicating that the differences seen in IL-8 protein and mRNA with the MAPKs inhibitors were not due to reduced cell viability.

Increased phosphorylation of p38 and JNK was found in U937 cells after a 30 minute DON exposure. Similar results have been obtained in RAW 264.7 cells (28).

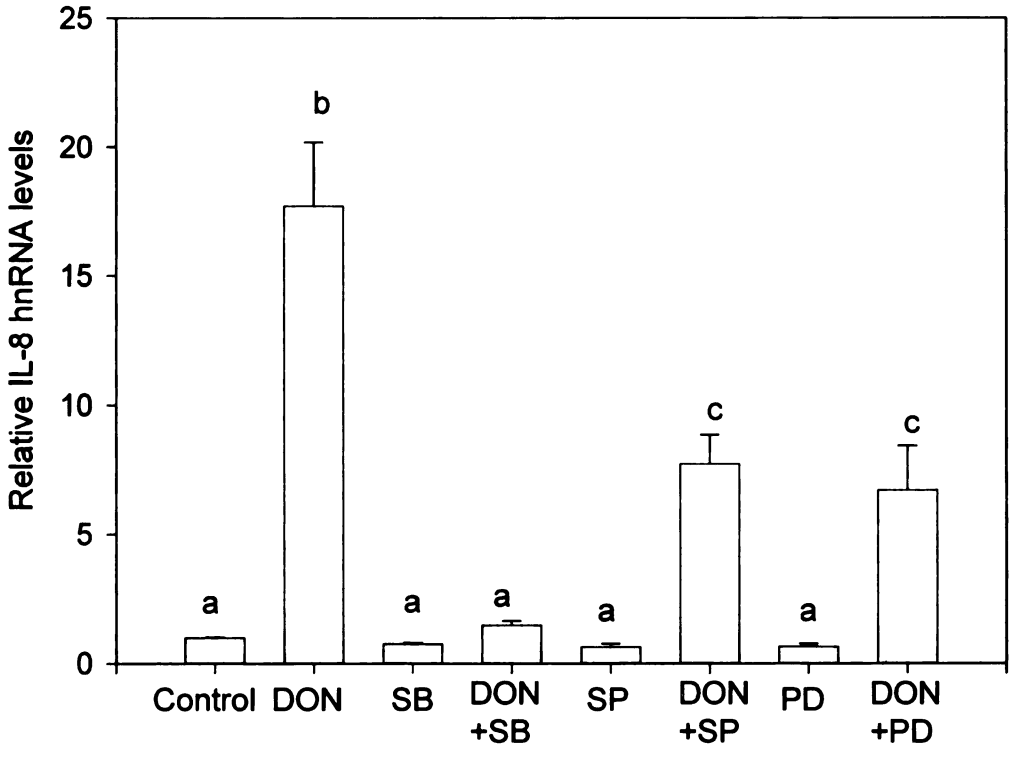

Figure 2.5: DON-induced IL-8 hnRNA is suppressed by MAPKs inhibitors. IL-8 hnRNA levels in U937 after a 3 hour exposure to 500 ng/ml DON, and/or 2.0 μM SB203580, 10 μM PD98059, or 1.0 μM SP600125. Bars without the same letter are statistically different ($p < 0.05$). Data are the mean ± SEM ($n = 11$). These data were collected from 4 separate experiments.

ERK phosphorylation was equivalent between the untreated and DON-treated U937 cells. Constitutive activation of ERK has been found in several cell lines, though has not been seen in U937 cells as reported by Ajenjo et al. (146). The possibility exists that there were alterations to the specific U937 cells utilized for these experiments that may have resulted in constitutive activation of ERK. The high levels of phosphorylation of ERK in the untreated U937 cell may also be an artifact of the sample preparation method. U937 cells are in suspension and require centrifugation and removal of the supernatant before lysis of the cells. ERK may have been activated during that procedure.

SB203580, a p38 inhibitor, was able to completely inhibit DON-induced IL-8 protein and RNA levels. Similar inhibition, with a p38 inhibitor, was previously observed with DON-induced TNF-α (25) and COX-2 (24) with both of those studies found p38 responsible for increased stability of the transcripts investigated. Activation of p38 has been linked to both an increase in IL-8 transcription (78, 147, 148) and in IL-8 mRNA stability (120, 149). More specifically on the transcriptional level, inhibition of p38 has been shown to reduce NF-κB activity (51, 150, 151). There is a NF-κB binding site on the IL-8 promoter and increases in NF-κB activation have been shown to be responsible for increasing IL-8 expression in numerous models (38, 52, 95, 125).

In agreement with results for U937 cells, p38 was also found to be important in human PBMC's response to DON (152). DON induced IL-8 protein in PBMCs isolated from seven different individuals. In all cases, IL-8 protein production was found to be p38-dependent. Furthermore, in individuals with a greater number of CD14+ monocytes, there was an increased sensitivity to DON. This suggests that the monocyte population is

responding to DON exposure. These data provides additional corroboration for the relevance of U937 cells as a model of primary human cells.

Both the JNK and ERK inhibitors were unable to inhibit IL-8 protein and only able to partially inhibit IL-8 RNA. This difference can be seen clearly by comparing fold induction of IL-8 protein, mRNA, and hnRNA. (Figure 2.6) DON induced equivalent levels of all three of the IL-8 forms measured. Several possible mechanisms might explain the discrepancy between the IL-8 RNA and protein amounts in the presence of the ERK and JNK inhibitors. A feedback mechanism might be involved in these differences. With a large amount of transcript, translation could be slowed in response to a certain amount of IL-8 protein released into the culture supernatant. With the ERK and JNK inhibitors, the amount of transcript is reduced and accumulation of the IL-8 protein in the culture supernatant might not have reached the level necessary to trigger a reduction in protein production due to an autocrine effect. IL-8 protein is a fairly stable protein, being resistant to inactivation by plasma peptidases, heat, pH extremes, and other denaturing treatments (35), thus allowing for an accumulation in the media. IL-8 protein might need to be measured after a longer time of exposure (12 h to 24 h) to determine if the protein remains high or is reduced, with levels closer to the RNA levels. Other reasons for the difference between protein and mRNA expression might include decreased degradation of the IL-8 protein in the presence of the ERK or JNK inhibitors or the possibility that the ERK and JNK contribute in a reduced manner to IL-8 transcription, relative to the p38 contribution. Other studies have shown that ERK and/or JNK are necessary for IL-8 expression (121, 122, 126)

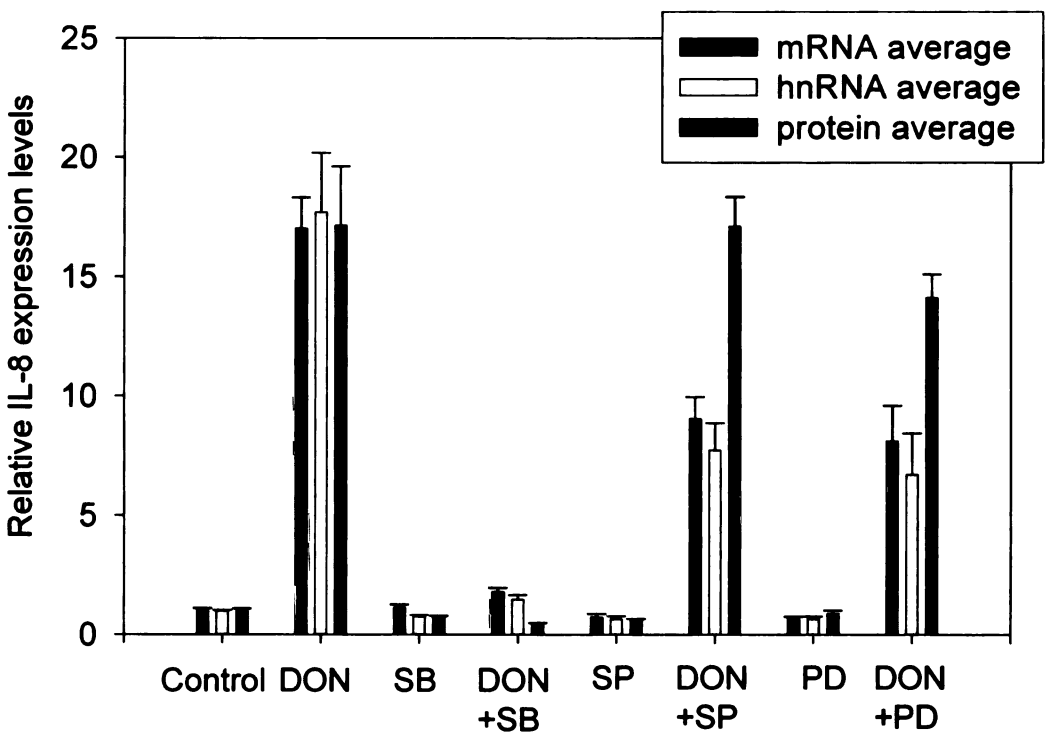

Figure 2.6: Comparison of relative levels of IL-8 protein, mRNA, and hnRNA expression in U937 cells exposed to 500 ng/ml DON, and/or 2.0 μM SB203580, 10 μM PD98059, or 1.0 μM SP600125. (see Figures 2.3, 2.4, and 2.5)

To summarize, activation of the MAPK pathways was important for increased transcriptional expression of DON-induced IL-8. Of the three examined, p38 was the most important in this model. This might be due to interaction of the MAPKs with transcription factors or due to increased mRNA stability. Future studies should focus on downstream events resulting from the activation of the MAPK pathways that mediate IL-8 expression.

CHAPTER 3: DON-INDUCED IL-8 EXPRESSION IN U937 CELLS IS NF-κB DEPENDENT

Abstract

Deoxynivalenol (DON) is a common mycotoxin present in contaminated grains worldwide. DON is a translational inhibitor that has been shown to induce expression of numerous immune proteins, including IL-8, a chemokine that primarily functions as a neutrophil chemoattractant. Previous data have shown that IL-8 protein and mRNA production are increased as a result of DON exposure in U937 human monocytes. The purpose of this investigation was to test the hypothesis that DON modulates transcriptional and post-transcriptional regulation of IL-8 expression in the U937 cell model. Reporter studies revealed that while a mutation in the NF-κB binding site impaired DON-induced IL-8 promoter-driven luciferase expression, mutations in AP-1, C/EBPβ, Oct-1, or NRF binding sites did not. Using an ELISA for transcription factor binding, DON was found to increase p65 binding to a specific IL-8 probe. Furthermore, caffeic acid phenethyl ester (CAPE), an inhibitor of NF-κB, completely inhibited both DON-induced IL-8 protein and mRNA. Since CAPE was able to suppress IL-8 mRNA transcription, this inhibitor was employed to assess whether DON evoked IL-8 mRNA stabilization. However, DON had no effect on the stability of IL-8 mRNA. Inhibition of double-stranded RNA protein kinase R (PKR), a kinase previously shown to mediate DON-induced intracellular signaling, was found to suppress IL-8 production. Two other translational inhibitors also capable of causing ribotoxic stress, Shiga toxin 1 and ricin,

were also found to increase IL-8 protein in U937 cells and that increase in IL-8 was PKR-dependent. These data indicate that DON-induced IL-8 expression is NF-κB-dependent and that production of IL-8 protein after DON, Shiga toxin 1, and ricin treatments share a common reliance on PKR.

Introduction

Deoxynivalenol (DON, vomitoxin) is a common mycotoxin that contaminates grain crops worldwide. DON can either suppress or stimulate immune function, depending on dose and duration of exposure (6). High dose DON exposure causes immunosuppressive effects that include apoptosis as well as inhibition of protein synthesis and can even lead to a shock-like syndrome that can be lethal (13). Additionally, high doses of DON can cause decreased resistance to pathogens (15) and inhibition of antibody responses to antigens (153). Lower dose DON exposures increase gene expression of a variety of immune molecules, including IL-6 (8, 18, 23, 26, 154-157), IL-2 (19, 156, 157), TNF-α (8, 18, 25, 26, 154, 155), COX-2(24), MIP-2 (158), and IL-8 (8, 152).

Increases in IL-6 (23, 26), IL-2 (19), TNF-α (25), and COX-2 (24) have been linked to increased transcription and/or an increased stability of transcripts. DON might increase transcription by increasing binding of several transcription factors, including NF-κB (20), AP-1 (21), and CREB (23). MAPK activation has been shown to lead to transcription factor activation in numerous models. Transfection with a dominant negative plasmid for raf-1, a component of the ERK pathway, reduced NF-κB and AP-1

activation in THP-1 monocytes (71). Similarly, transfection of constitutively active MEK1, upstream of ERK, increased AP-1 activity in U937 monocytes (159). Use of a p38 inhibitor reduced NF-κB activation in fibrosarcoma (150) and endothelial cells (51). p38 appears to play a role in the transactivation of NF-κB, by phosphorylating the p65 (RelA) subunit (150, 151).

Increased mRNA stability (119, 133, 160, 161) has also been linked to increased activation of one or more of the MAPK pathways. Increased phosphorylation of p38, ERK, and JNK has been reported in cells after exposure to DON (24, 25, 29, 152, 162-164). DON activation of double-stranded RNA protein kinase R (PKR) occurs upstream of MAPK phosphorylation. Zhou et al. (29) determined that PKR was phosphorylated in RAW 264.7 cells after exposure to DON or another protein synthesis inhibitor, anisomycin. U937 cells constitutively expressing antisense PKR did not exhibit DON-induced phosphorylation of MAPKs or apoptosis as seen in control cells (29).

MAPK activation by translational inhibitors has been termed the "ribotoxic stress response" (32, 165), and has been observed for trichothecenes as well as other biological toxins such as ricin and Shiga toxin 1 (Stx1) (32, 166). Both ricin and Stx1 induce production of IL-8 protein in human cells (92, 166-168). Notably, increased IL-8 production is mediated through the p38 pathway for 28SC monocytes (167) and HCT-8 epithelial cells (169).

The capacity of DON and other ribotoxic stressors to induce IL-8 is potentially an important toxicological mechanism. IL-8, a CXC chemokine primarily functioning as a neutrophil chemoattractant, is tightly controlled as elevated levels have been implicated in a variety of inflammatory diseases, including inflammatory bowel disease (76, 77) and

inflammatory joint conditions such as gout or rheumatoid arthritis (45, 46, 71). Although IL-8 protein (8, 152) and mRNA (152) increases in monocytes and macrophages as a result of DON exposure, the mechanism(s) by which this occurs is unclear. Elevated phosphorylation of MAPKs, p38 in particular, has been shown in U937 cells and a p38 inhibitor is able to completely repress both IL-8 protein and mRNA (152). However, increased expression of IL-8 can occur through elevated transcription (50, 127, 131), an increase in mRNA stability (92-94), or both together (118, 120, 121, 124).

Regulation of IL-8 at the transcriptional level can be controlled by several different transcription factors. The IL-8 promoter has binding sites for AP-1, C/EBPβ, Oct-1, NF-κB, and NRF that are, depending on stimuli and cell type, important for expression of IL-8. In some models, several of these transcription factors, working together, are necessary for IL-8 expression (47, 50, 56, 97, 120, 126). However, studies of other models have determined that the only transcription factor involved in IL-8 expression is NF-κB (38, 51, 52, 121, 125).

Human monocytes have been identified as targets for DON in human PBMC cultures and data obtained in that study revealed a shared p38-dependence, in both primary human cells and the U937 line, for DON-induced IL-8 (152). The purpose of this chapter was to test the hypothesis that DON-induced IL-8 in monocytes is mediated transcriptionally, through activation of multiple transcription factors, and post-transcriptionally, by increasing IL-8 mRNA stability, and that these effects are mediated by PKR. Experiments were carried out in U937 cells to determine the involvement of transcriptional and post-transcriptional regulation in DON-induced IL-8 expression. U937 monocytes were selected because they were used previously shown to produce IL-8

in response to DON exposure (8, 152). In addition, the role of PKR in the induction of IL-8 protein by DON as well as ricin and Stx1 was examined to determine if PKR was an upstream component of this response.

Materials and Methods

Cell culture

U937 cells, isolated from the pleural effusion of an individual with diffuse histiocytic lymphoma (Sundstrom and Nilsson 1976), were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL; Rockville, MD). Cells were incubated at 37°C with 6% CO_2. Fresh media was added as necessary to passage the cells. All reagents are from Sigma (St. Louis, MO) unless otherwise noted.

U937 cells (1 x 10^6 cells/ml) were treated with 100 μg/ml of caffeic acid phenethyl ester (CAPE; Calbiochem, San Diego, CA), dissolved in ethanol, 2 h prior to addition of DON or LPS where indicated. PKR inhibitor (Calbiochem) or PKR inhibitor negative control (Calbiochem), both dissolved in DMSO, were added to U937 cells (1 x 10^6 cells/ml) at a concentration of 2.5 μM 45 minutes prior to addition of toxins (DON, LPS, Stx1 (Toxin Technology, Inc, Sarasota, FL), or ricin (Vector, Burlingame, CA)). DON, LPS, and Stx1 were dissolved in RPMI-1640 without antibiotics or HI FBS and ricin was dissolved in PBS. Cells were incubated with toxins for various time intervals before RNA isolation (15 minutes to 3 h), nuclear protein isolation (3 h) and supernatant

collection (12 h) for ELISA.

Site-directed mutagenesis

The method used was based on Stratagene's QuikChange kit. Primers were made using the program available at Stratagene's http://www.stratagene.com/tradeshows/feature.aspx?fpId=118 website based on the IL-8 promoter sequence reported by Mukaida et al. (170) (Table 3.1). Reactions consisted of 1x Pfx reaction buffer (Invitrogen, Carlsbad, CA), 50 ng of the -162/+44 IL-8 LUC promoter construct (a gift from Dr. Antonella Casola, Division of Child Health Research Center, University of Texas; (103)), 0.35 μM of each primer, 10 mM dNTP, and RNase- and DNase-free water to a total volume of 50 μl. Pfx (2.5 U; Invitrogen) was added and the reactions were run at 95°C for 2 minutes with 15 repetitions of 95°C for 30 seconds, 55°C for 1 minute, and 68°C for 12 minutes. Then, the reactions were placed on ice to cool. DpnI (20 U; New England Biolabs; Beverly, MA) was added and the reactions were incubated for 2 h at 37°C. An aliquot of 2 μl was used to transformed JM109 *E. coli* cells. Clones were selected and plasmids were isolated (Wizard MiniPrep from Promega, Madison, WI) for sequencing at Michigan State University's Research Technology and Support Facility. Once the mutations were confirmed, plasmids were amplified in JM109 *E. coli* cells and isolated using the EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA).

Table 3.1: Primers used for site-directed mutagenesis of the IL-8 promoter. Mutations are indicated in lower case bold font.

Site	Primer sequences for mutations
AP-1	5'-GAACAAATAGGAAGTGTGAT**at**CTCAGGTTTGCCCTGAGG-3' and 5'-CCTCAGGGCAAACCTGAG**at**ATCACACTTCCTATTTGTTC-3'
C/EBPβ	5'-GGGGATGGGCCATCAG**c**T**a**C**g**A**g**TCGTGGAATTTCCTCTGAC-3' and 5'-GTCAGAGGAAATTCCACGA**c**T**c**G**t**A**g**CTGATGGCCCATCCCC-3'
Oct-1	5'-CTGAGGGGATGGGCCATC**ttg**TGCAAATCGTGGAATTTCCTC-3' and 5'-GAGGAAATTCCACGATTTGCA**caa**GATGGCCCATCCCCTCAG-3'
NF-κB	5'-CATCAGTTGCAAATCGTGGA**a**TAATTTCCTCTGACATAATGAAA-3' and 5'-TTTCATTATGTCAGAGGAAATTA**t**TCCACGATTTGCAACTGATG-3'
NRF	5'-GCAAATCGTGGAATTTCC**c**C**c**GACATAATGAAAAGATGAGGG-3' and 5'-CCCTCATCTTTTCATTATGTC**g**G**g**GGAAATTCCACGATTTGC-3'

Transient transfection of U937 cells

U937 cells (10^7 cells/210 µl of RPMI-1640 without FBS or antibiotics) were electroporated with 15 µg of plasmid containing the IL-8 promoter (-162/+44 IL-8 LUC or one of the 5 mutated constructs all having a firefly luciferase reporter) and 0.5 µg pRL SV40 (containing a *Renilla* luciferase reporter gene; Promega) for normalization using a Bio-Rad (Hercules, CA) Gene Pulser at 280 V and 960 µF in a 0.4 cm gap cuvette (Bio-Rad). Cells were then diluted in 12 ml growth media, allowed to rest for 1 h, then treated with 0, 1 µg/ml DON, or 1 µg/ml LPS and plated at 1 ml/well. After 11 h, the Dual-Luciferase Reporter Assay (Promega) was used to ascertain both firefly and *Renilla* luciferase levels according to manufacturer's instructions. A Turner 20e Luminometer (Turner Designs; Sunnyvale, CA) was used for all luminometer measurements. Firefly luciferase levels were normalized with *Renilla* luciferase levels from the same sample and all treatments were relative to the control for each IL-8 promoter construct.

Assessment of NF-κB binding

Nuclear proteins were isolated using the Nuclear Extraction Kit (Active Motif, Carlsbad CA) according to the manufacturer's instructions. Separation of cytoplasmic and nuclear proteins was done by using a hypotonic buffer and detergent to break the cytoplasmic membrane, then pelleting and lysing the nuclei. Cell morphology was checked using a phase contrast microscope at each step. Nuclear protein was quantitated with the DC Protein Quantitation Kit (Bio-Rad) and diluted to 2.5 µg/µl. Binding of NF-κB family members was assessed with the TransAM NF-κB Family Flexi kit (Active Motif) using IL-8 promoter specific probe sequences (Table 3.2). Nuclear protein (20

μg) was incubated with a duplex, biotin labeled IL-8 specific probe, then added to a strepavidin coated 96-well plate. After washing, primary antibodies, for each NF-κB subunit, were added to individual wells and incubated. After washing, primary antibodies were detected with an anti-rabbit HRP-conjugated antibody. After developing, color development was stopped, and plates were read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices; Menlo Park, CA). The wild-type IL-8 probe was biotin labeled with the Biotin 3' End DNA Labeling Kit (Pierce, Rockford, IL) following the manufacturer's instructions. Both probes were duplexed by incubating 1 pmol/μl of each oligonucleotide together at 95°C for 5 minutes and ramping back to 4°C by decreasing 1°C per minute.

IL-8 protein determination

After treatment, the cell culture plates were centrifuged for 10 minutes at 300 x g and the supernatant collected and stored at -20°C. OptELISA IL-8 kit (Pharmingen; San Diego, CA) was used according to manufacturer's instructions with two modifications. First, the highest standard utilized was 1600 pg/ml, instead of 400 pg/ml. Second, to economize on reagents 50 μl of antibody dilutions and samples were used per well instead of 100μl. All samples were read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices).

Table 3.2: TransAM ELISA probes. Mutations are in lower case bold font.

Probe	Sequence
Wild-type IL-8 (NF-κB, NRF, Oct-1 and C/EBPβ sites)	5' - GATCCATCAGTTGCAAATCGTGGAATTTCCT CTA - 3' and 3' - GTAGTCAACGTTTAGCACCTTAAAGGAGATC TAG - 5'
Mutant NF-κB	5' - GATCCATCAGTTGCAAATCGT**ta**A**c**TTTCCTCT A - 3' and 3' - GTAGTCAACGTTTAGCA**at**T**g**AAAGGAGATCT AG - 5'

RNA isolation and reverse transcription real-time PCR

After treatment, plates were centrifuged for 10 minutes at 300 x g, the supernatant removed, and the RNaqeous kit (Ambion; Austin, TX) was used to isolate the RNA. Briefly, the cells are lysed, nucleic acids precipitated with ethanol, then RNA is trapped in a glass fiber filter, and after washing the RNA is eluted from the filter. Samples were treated with Turbo DNA-free (Ambion) to remove gross DNA contamination. Turbo DNase was added to eluted RNA, along with a buffer, incubated, then the enzyme was precipitated with an inactivation reagent.

Reverse transcription real-time PCR was performed using One-Step PCR Master Mix (Applied Biosystems; Foster City, CA) and IL-8 Pre-Developed Assay Reagents (PDAR) (Applied Biosystems) multiplexed with the 18S PDAR (Applied Biosystems). Fold change was determined using the relative quantitation method (171). First, standard curves are created using dilutions of total RNA from LPS-treated U937 cell total RNA. An equation, for the trend line of the standard values, is used to convert the Ct values obtained in the assay to nanogram amounts of the target. The amounts were normalized by dividing the IL-8 value by the 18S (the endogenous control) value. Relative expression is obtained by dividing all normalized values by the average of the control normalized value. Reaction conditions and PCR program followed the manufacturer's instructions using an ABI 7900HT (384 wells), at the Michigan State University's Research Technology and Support Facility.

Statistics

Data were analyzed with SigmaStat v 3.1 (Jandel Scientific; San Rafael, CA). IL-8 protein and RNA data were analyzed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls Method for pairwise comparisons unless otherwise noted. All samples treated with an inhibitor were compared to the vehicle control using a t test. A t-test was used for the luciferase experiments by comparing the mutated promoter construct to the IL-8 wild-type promoter in the same treatment group and in the NF-κB binding assay comparing DON- and LPS-treated to the control group. Treatment groups that have a p-value of < 0.05 are considered significant.

Results

DON-induced IL-8 transcription requires NF-κB

To determine the action of DON on IL-8 transcription, the effects of DON (1 μg/ml) and LPS (1 μg/ml) in U937 cells transfected with an IL-8 promoter construct were assessed. Incubation with treated DON or LPS significantly ($p<0.05$) induced IL-8 promoter-driven luciferase compared to vehicle treated cells (Figure 3.1). These results suggest that that increased IL-8 transcription occurs as a result of DON exposure.

To identify the transcription factors important for DON-induced IL-8 transcription, the –162/+44 IL-8 LUC plasmid was mutated using site-directed mutagenesis in transcription binding sites of five transcription factors previously reported as important for IL-8 transcription (AP-1, C/EBPβ, Oct-1, NF-κB, and NRF). The mutations of the IL-8 promoter were assessed using two bioinformatics programs that

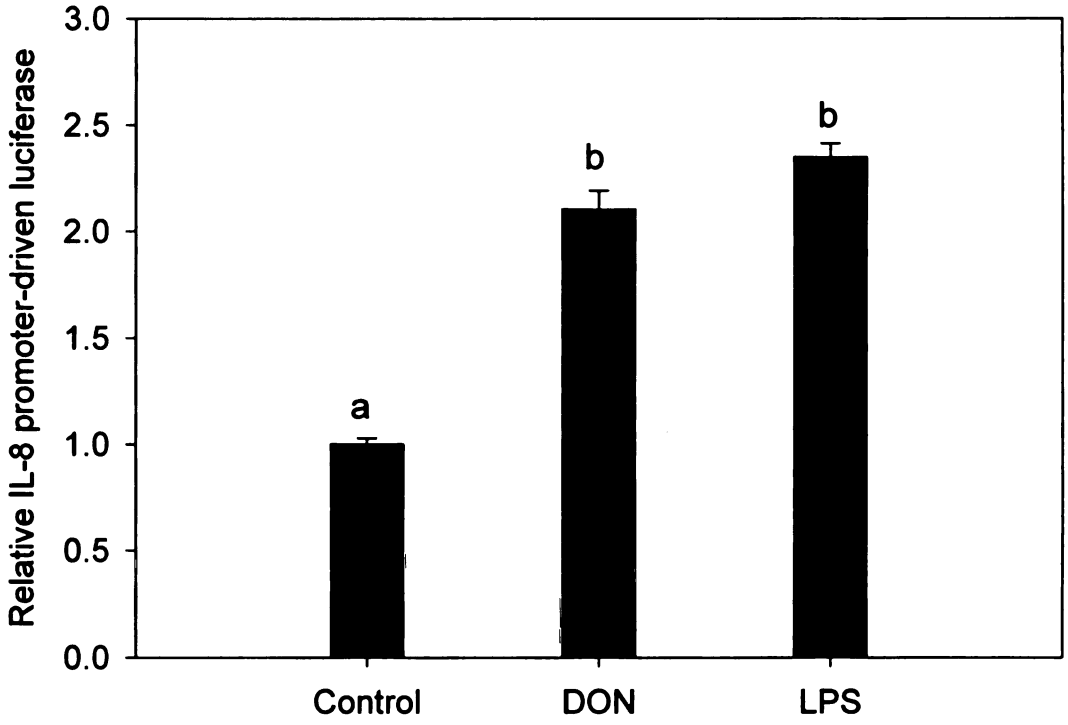

Figure 3.1: DON induces IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with a wild-type IL-8 promoter luciferase (-162/+44 IL-8 LUC) construct incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

predict transcription factor binding sites, PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (172, 173) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) (174) (see Appendix D for additional information). Using the available data, both programs predicted that the mutations for AP-1 and NF-κB would abolish the respective binding sites. Based on limited information in the databases available for C/EBPβ and Oct-1, one of the programs, PROMO, verified that the mutations for C/EBPβ and Oct-1 would also prevent binding. No information was available for NRF.

U937 cells were transfected with each mutated IL-8 promoter plasmid. Relative luciferase levels for cells transfected with the wild-type IL-8 promoter were compared to cells transfected with an IL-8 promoter containing a mutation. Mutation of the NF-κB site was able to significantly ($p<0.05$) reduce both DON- and LPS-induced luciferase as compared to the wild-type promoter (Figure 3.2). In contrast, a mutation in the AP-1 site significantly ($p<0.05$) increased both DON- and LPS-induced IL-8 promoter-driven luciferase compared to the wild-type IL-8 promoter (Figure 3.3).

Mutations in the C/EBPβ (Figure 3.4), Oct-1 (Figure 3.5), or NRF (Figure 3.6) sites did not alter the DON-induced luciferase expression as compared to the wild-type IL-8 promoter construct. While mutation in the C/EBPβ site similarly did not alter LPS-induced luciferase as compared to luciferase expression from the IL-8 wild-type promoter (Figure 3.4), LPS-induced luciferase expression was increased for the Oct-1 mutation (Figure 3.5) and decreased for the NRF mutation (Figure 3.6).

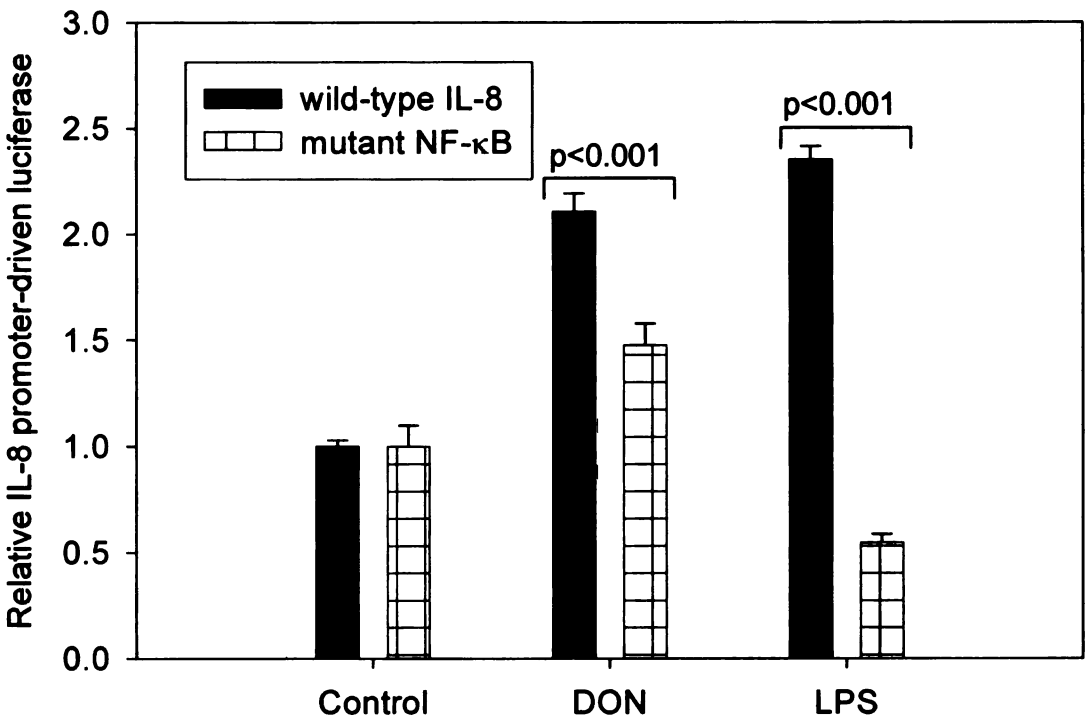

Figure 3.2: The NF-κB site is required for DON-induced IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with either a wild-type IL-8 promoter luciferase construct or NF-κB-mutated IL-8 promoter construct, incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

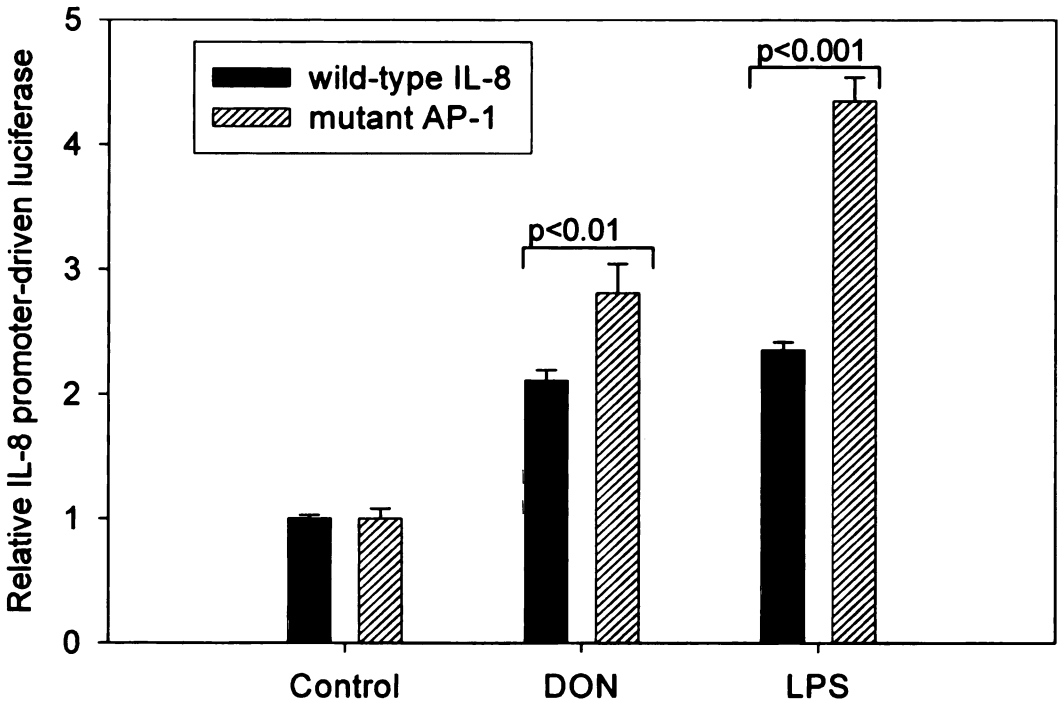

Figure 3.3: The AP-1 site is not required for DON-induced IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with either a wild-type IL-8 promoter luciferase construct or AP-1-mutated IL-8 promoter construct, incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

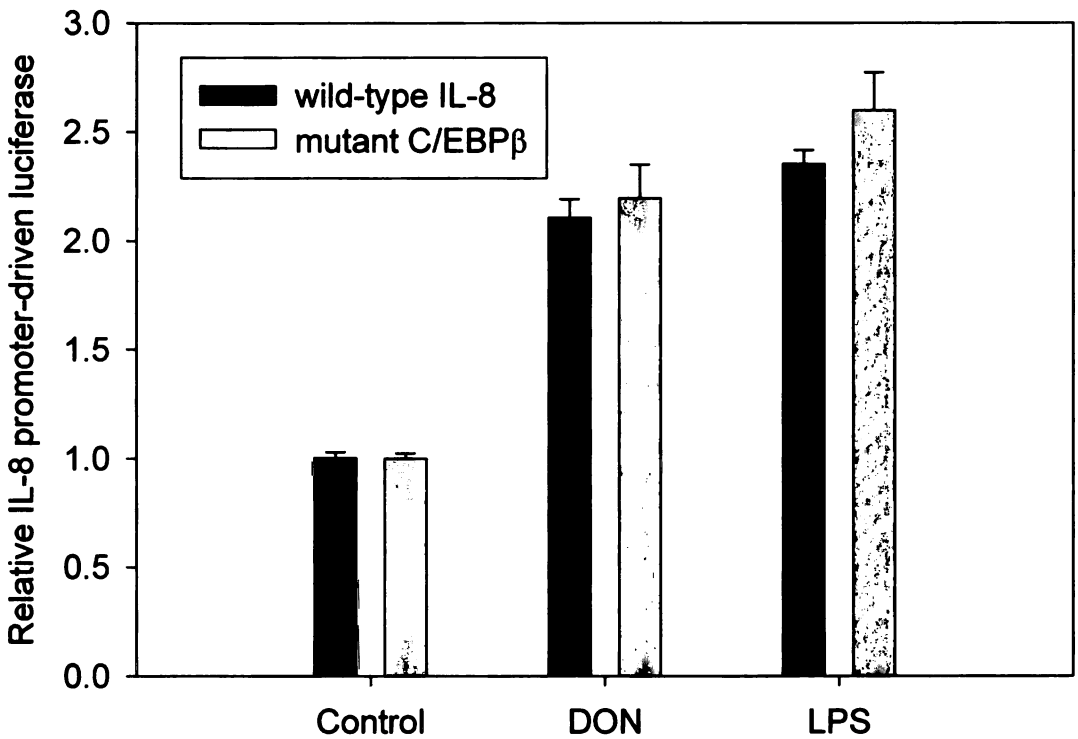

Figure 3.4: The C/EBPβ site is not required for DON-induced IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with either a wild-type IL-8 promoter luciferase construct or C/EBPβ-mutated IL-8 promoter construct, incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

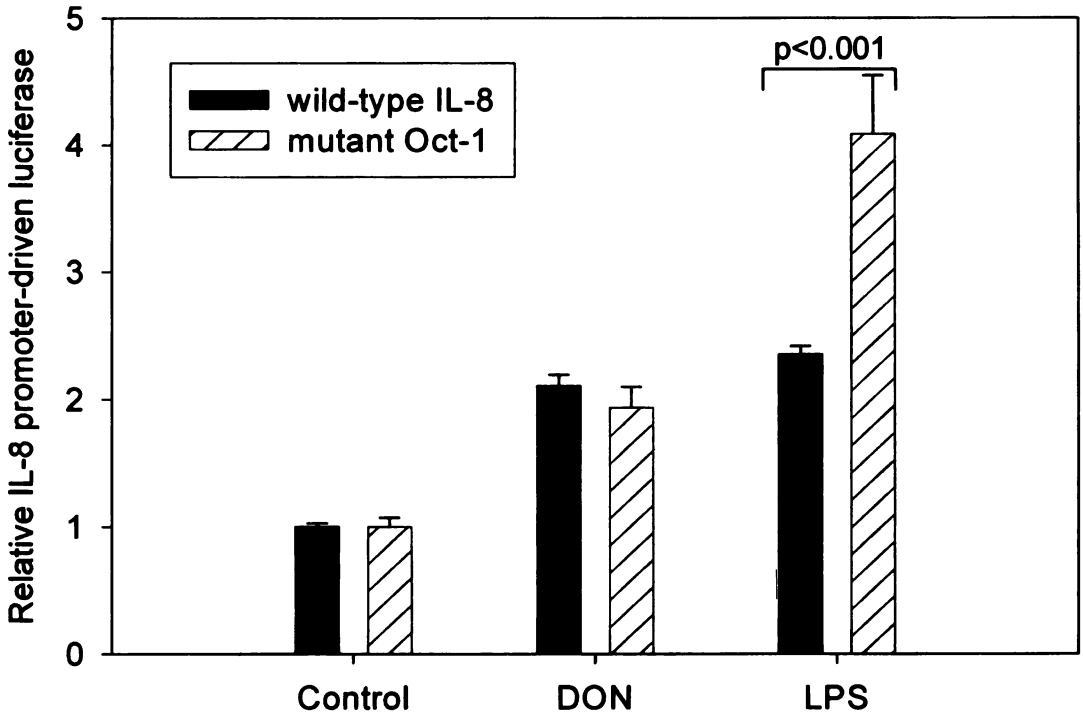

Figure 3.5: The Oct-1 site is not required for DON-induced IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with either a wild-type IL-8 promoter luciferase construct or Oct-1-mutated IL-8 promoter construct, incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

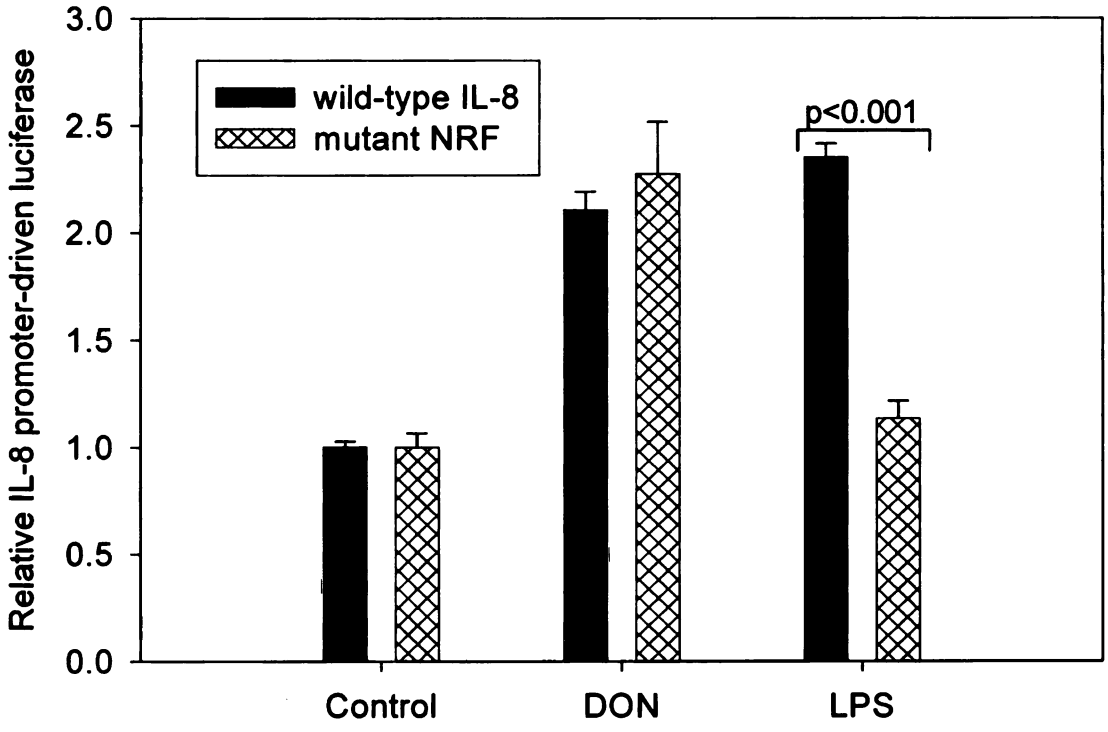

Figure 3.6: The NRF site is not required for DON-induced IL-8 promoter driven luciferase in U937 cells. U937 cells were transfected with either a wild-type IL-8 promoter luciferase construct or NRF-mutated IL-8 promoter construct, incubated for 1 h, then treated with 0, 1 μg/ml DON, or 1 μg/ml LPS for 11 h. Data are mean ± SEM combined from two or more independent experiments. ($n \geq 12$)

Based on the observation that the NF-κB site mutation specifically reduced DON-induced luciferase, further investigations into the effects of DON on NF-κB binding were carried out.

DON increases binding activity of p65

An increase in transcription often is due to increased DNA binding of transcription factors in promoter regions. The effects of DON on binding of five NF-κB subunits (p65/RelA, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2) were assessed using an ELISA-based binding assay. Two of the five subunits, RelB and c-Rel, were undetectable in all U937 cell samples tested (data not shown) while levels of p65, p50, and p52 were measurable. Additionally, specificity of the IL-8 wild-type probe was assessed by incubating with unlabeled wild-type or mutant probe. Unlabeled mutant probe did not reduce the binding, while competition with the unlabeled wild-type probe reduced the binding measured by at least 80% (data not shown). Significantly ($p<0.05$) increased binding of p65 was observed for both the DON- and LPS-treated U937 cells (Figure 3.7A). Although, p50 binding was equivalent in untreated and DON-treated samples, there was a small, but significant ($p<0.05$) increase in p50 binding in LPS-treated U937 cells (Figure 3.7B). The binding of p52 was quite different from the p65 and p50 binding. While in untreated and LPS-treated samples, binding of p52 was equivalent, there was a reduction ($p=0.056$) of p52 binding in the DON-treated samples (Figure 3.7C). Taken together DON treatment affected NF-κB binding by decreasing p52 and significantly increasing p65 binding.

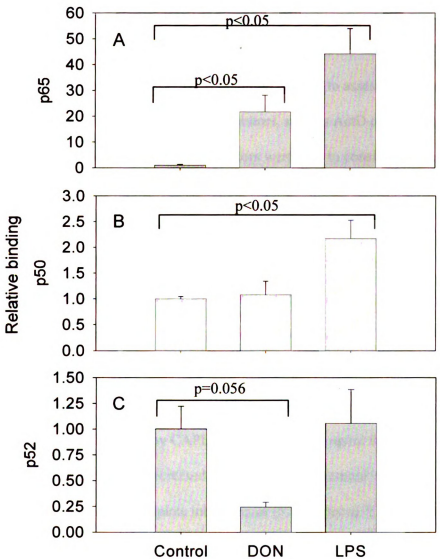

Figure 3.7: DON alters the binding of three NF-κB subunits, p65 (A), p50 (B), and p52 (C), in U937 cells. U937 cells were treated with 0, 1 μg/ml DON, or LPS for 3 h, nuclear protein was isolated, and then NF-κB binding was measured (20 μg per binding reaction). Data are mean ± SEM and pooled from two independent experiments. (n = 6)

DON-induced IL-8 is not due to increased mRNA stability in U937 cells

In addition to transcriptional effects, DON could potentially upregulate IL-8 mRNA expression by increasing the mRNA stability as has been shown for IL-6 (23, 26), TNF-α (25, 26), and COX-2 (24). A common method to assess mRNA stability involves the use of pluripotent transcriptional inhibitors, such as ActD or DRB. However, in numerous trials neither of the two inhibitors were able to consistently shut down transcription in U937 cells (see Appendix C for additional information). Since the aforementioned transcription studies indicated that NF-κB was important for DON-induced IL-8 mRNA, several NF-κB inhibitors were screened for use in mRNA stability studies. Based on its efficacy, CAPE was selected for use in these experiments.

DON-induced IL-8 protein production was completely inhibited (~98%) in U937 cells pretreated for 2 h with 100 μg/ml CAPE (Figure 3.8). CAPE was also similarly able to suppress LPS-induced IL-8 protein (data not shown). DON- and LPS-induced IL-8 mRNA was also inhibited by CAPE pretreatment (100 μg/ml for 2 h) (Figure 3.9). This inhibition was not due to decreased cell viability as measured by a MTT viability assay (data not shown). The complete inhibition of DON-induced IL-8 protein and mRNA expression suggested that CAPE might be a suitable inhibitor for assessing DON's effects on IL-8 mRNA stability.

U937 cells were treated with 100 μg/ml CAPE for 2 h prior to addition of 0, 250, 500 ng/ml DON or 5 ng/ml LPS. RNA was isolated every 15 minutes for 2 h. The lower LPS concentration used for this experiment, as compared to aforementioned experiments, was selected because it induced comparable amounts of IL-8 mRNA to the DON concentrations utilized. Half-life of the IL-8 transcript was approximately 40 minutes in

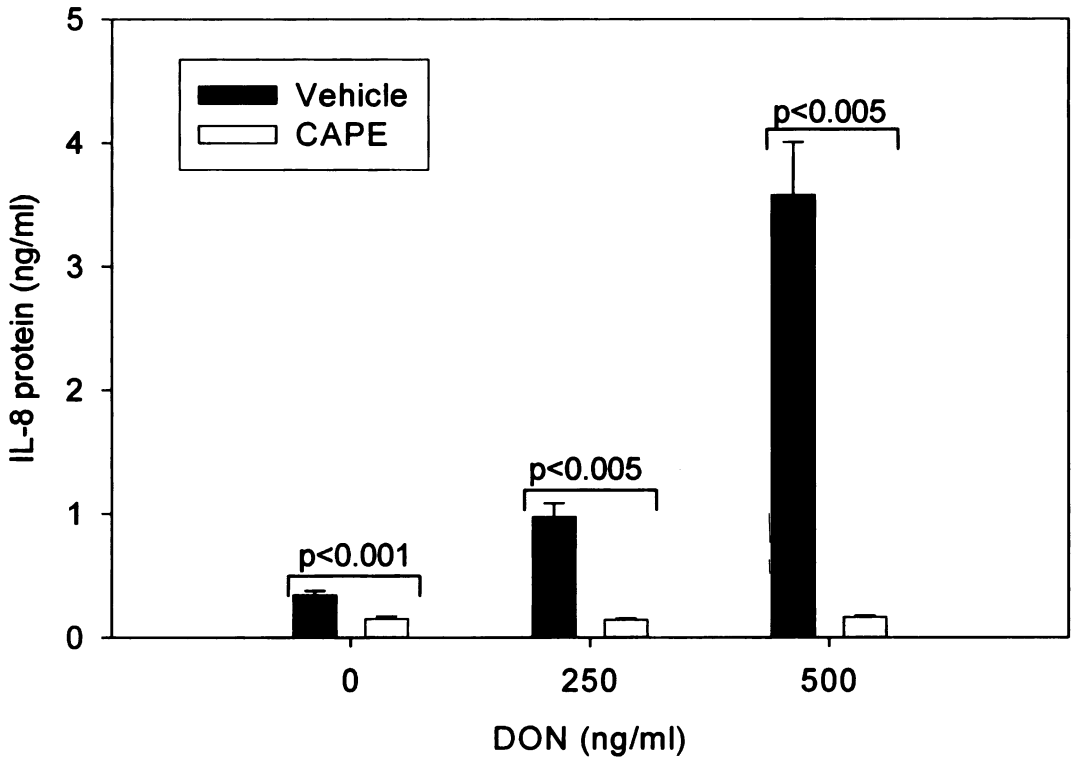

Figure 3.8: CAPE inhibits DON-IL-8 protein in U937 cells. Cells were pretreated for 2 h with either 0 or 100 μg/ml CAPE then treated for 12 h with 0, 250, 500, or 1000 ng/ml DON. Data are mean ± SEM and representative of two independent experiments (n=3)

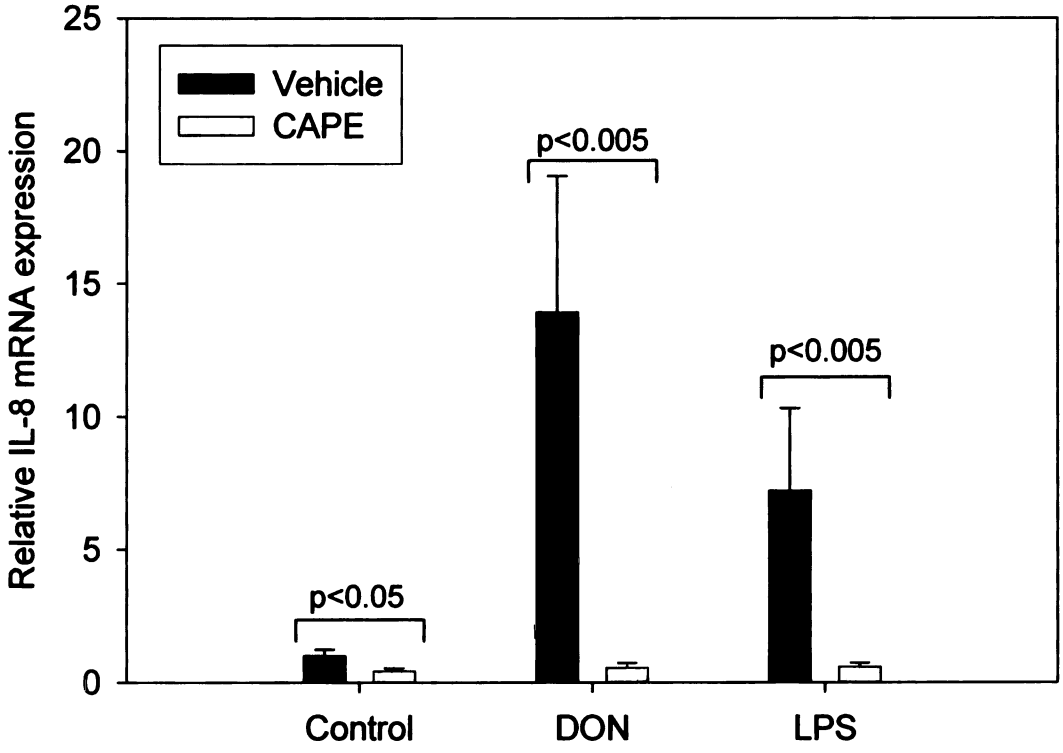

Figure 3.9: CAPE inhibits IL-8 mRNA in U937 cells. Cells were pretreated for 2 h with either 0 or 100 μg/ml CAPE then treated for 3 h with 0, 500 ng/ml DON, or 5 ng/ml LPS. Data are mean ± SEM and representative of two independent experiments. (n = 3)

all treatment groups. Addition of DON did not alter the stability of IL-8 mRNA as compared to the control group (Figure 3.10). Since there was no increase in IL-8 mRNA stability, DON appears to induce IL-8 primarily by increased transcription, specifically by activating NF-κB.

DON-induced IL-8 requires PKR

Our laboratory has recently established that p38 MAPK activation is necessary for DON-induced IL-8 expression in cloned and primary human monocytes (152). Furthermore, PKR has been previously shown to be critical for activation of p38 in U937 cells (29). Therefore, the role of PKR in DON-induced IL-8 production was assessed in the U937 model. Cells were pretreated with 2.5 μM specific PKR inhibitor, 2.5 μM negative control for the PKR inhibitor, or no inhibitor for 45 minutes before addition of DON (0, 500, or 1000 ng/ml). The PKR inhibitor completely (~98%) inhibited ($p<0.05$) DON-induced IL-8 protein production whereas the negative control, a non-functional commercial chemical analog of the specific PKR inhibitor, did not cause a significant decrease in IL-8 protein levels (Figure 3.11).

IL-8 induction by the translational inhibitors, Stx1 and ricin, is also suppressed by a specific PKR inhibitor in U937 cells

Ongoing research in our lab has determined that PKR is bound to the ribosome and that it is necessary to mediate the DON-induced ribotoxic stress response. Thus, it is of interest to determine whether other ribotoxic stressors might upregulate IL-8 production via a PKR mediated mechanism. Stx1, at 500 and 1000 ng/ml, was able to

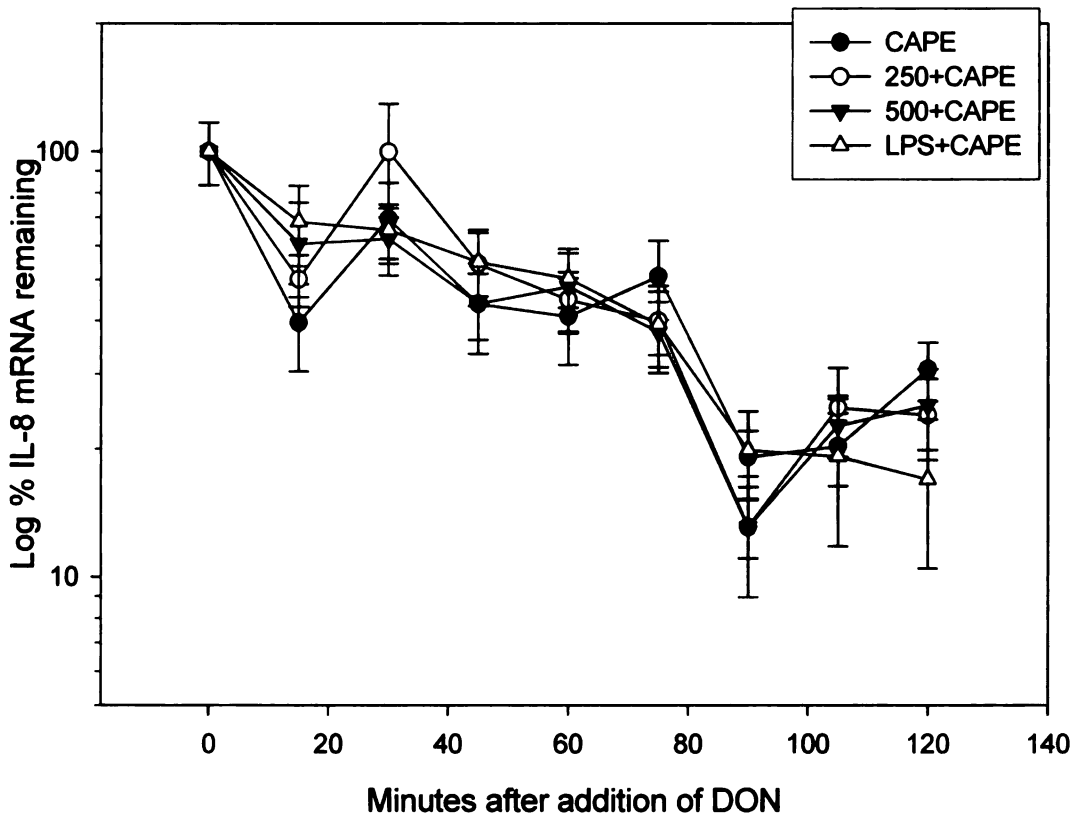

Figure 3.10: DON does not stabilize IL-8 mRNA. U937 cells were pretreated for 2 h with 100 μg/ml CAPE then treated with 0, 250, 500 ng/ml DON, or 5 ng/ml LPS at the timepoints indicated. Data are mean ± SEM pooled from two independent experiments. (n = 6)

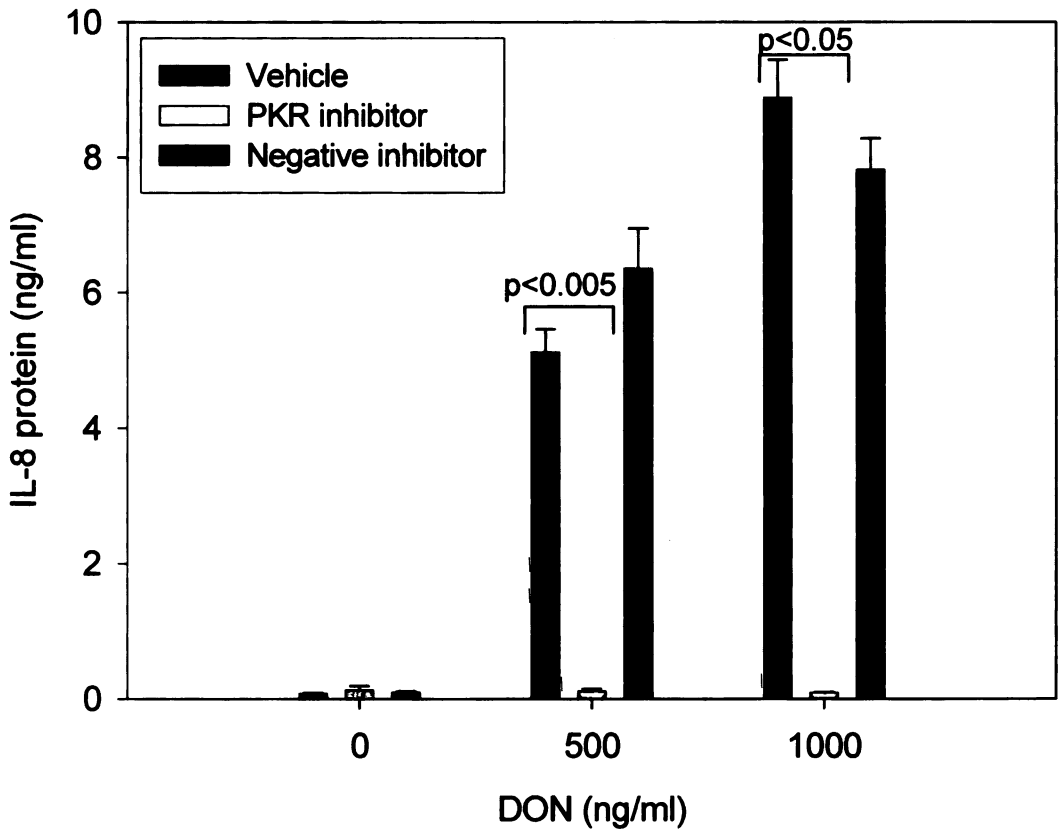

Figure 3.11: A PKR inhibitor suppresses DON-induced IL-8 protein (~98%). U937 cells were pretreated with or without PKR inhibitor (2.5 μM) or a Negative control inhibitor (2.5 μM) for 45 minutes before addition of 0, 500, or 1000 ng/ml DON. Culture supernatant was collected 12 h after addition of DON and IL-8 protein was assessed by ELISA. Data is mean ± SEM. Representative of 3 independent experiments. (n = 3)

significantly ($p<0.05$) induce IL-8 protein production after a 12 h treatment (Figure 3.12). Pretreatment (45 minutes) with the specific PKR inhibitor (2.5 μM) significantly reduced IL-8 protein ($p<0.05$) by approximately 80%. Ricin (0, 10, 50, 500, and 1000 ng/ml), at all concentrations except the 10 ng/ml, significantly increased IL-8 protein production ($p<0.05$) in U937 cells after a 12 h exposure (Figure 3.13). IL-8 protein was significantly reduced, between 30 and 60% ($p<0.05$), after pretreatment with the specific PKR inhibitor (2.5 μM). Pretreatment with the PKR inhibitor negative control did not significantly reduce IL-8 protein levels with either Stx1 or ricin treatment. Thus, PKR is a necessary component in induction of IL-8 for DON, Stx1, and ricin.

Discussion

Our lab has previously shown that DON increases both mRNA stability (24-26) and transcription (21, 23, 162) for IL-6, TNF, and COX-2. Although DON has been shown to induce both IL-8 protein and mRNA in primary human monocytes and U937 cells (8, 152), the mechanism(s) behind that induction were unclear. The data presented here suggest that DON induces IL-8 expression primarily at the transcriptional level, through activation of NF-κB.

An IL-8 promoter-driven luciferase construct was transfected into U937 cells to determine if DON was capable of increasing IL-8 transcription. A comparison of DON-induced wild-type IL-8 promoter luciferase and IL-8 protein revealed that IL-8 promoter-driven luciferase was much lower than IL-8 protein levels, which had an induction of approximately 46-fold. The difference in induction levels could be result from the

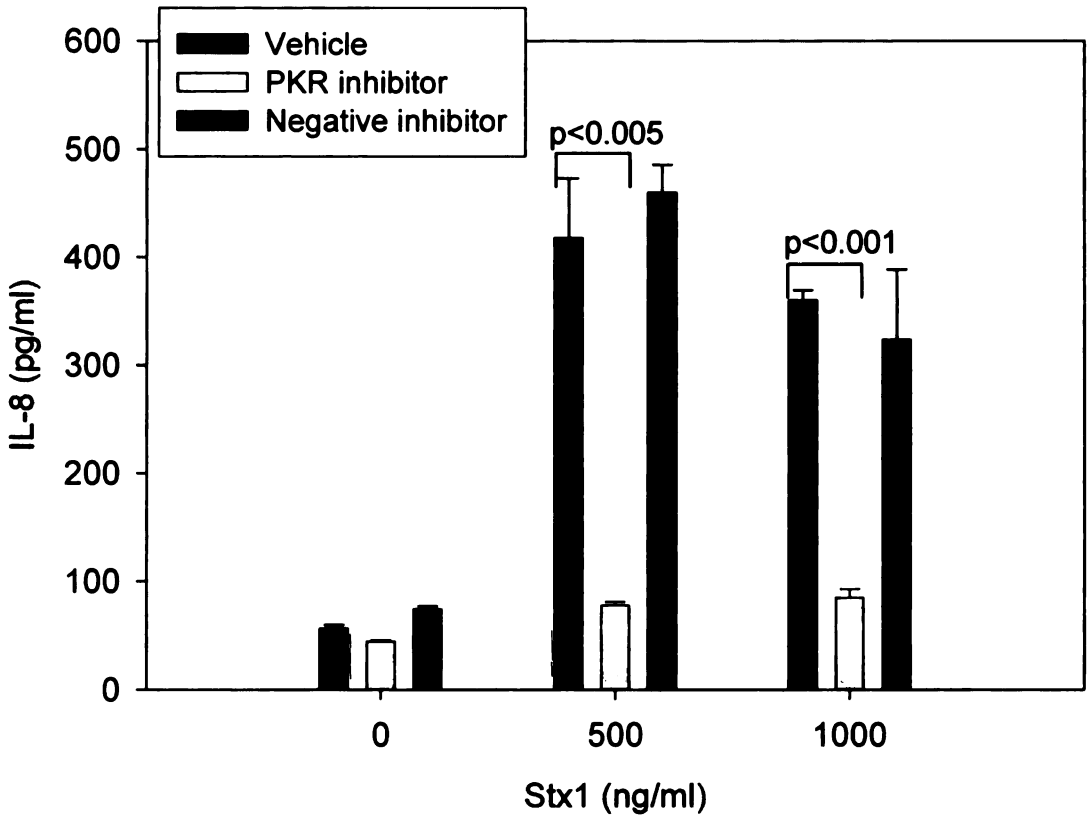

Figure 3.12: A PKR inhibitor suppresses Stx1-induced IL-8 protein (~80%). U937 cells were pretreated with or without PKR inhibitor (2.5 μM) or a Negative control inhibitor (2.5 μM) for 45 minutes before addition of 0, 500, or 1000 ng/ml Stx1. Culture supernatant was collected 12 h after addition of Stx1 and IL-8 protein was assessed by ELISA. Data is mean ± SEM. Representative of two independent experiments. (n = 3)

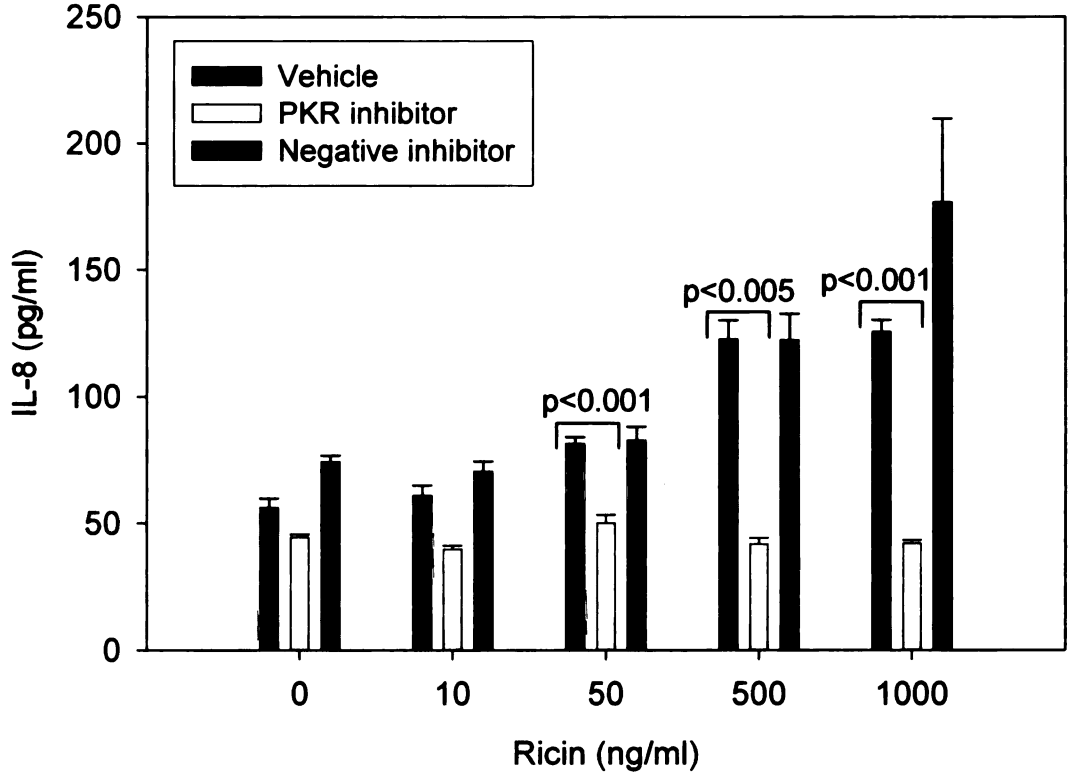

Figure 3.13: A PKR inhibitor suppresses ricin-induced IL-8 protein (~30-60%). U937 cells were pretreated with or without PKR inhibitor (2.5 μM) or a PKR inhibitor negative control (2.5 μM) for 45 minutes before addition of 0, 10, 50, 500, or 1000 ng/ml ricin. Culture supernatent was collected 12 h after addition of ricin and IL-8 protein was assessed by ELISA. Data is mean ± SEM. Representative of 2 independent experiments. (n = 3)

transfection itself or from relatively poor luciferase expression inside of the cells. Several investigations have reported that transfection efficiency for U937 cells is inherently low (175, 176) with one study even stating that transfection results were unreliable in this cell line (177). Malone et al. (178) similarly reported poor levels of expression of a reporter construct in U937 cells.

Another possibility for the lower levels of IL-8 promoter-driven luciferase as compared to IL-8 protein is that increased IL-8 expression depends on alterations of the chromatin along with activation of NF-κB. Several studies have found that alteration of histones was necessary for IL-8 expression. In Caco-2 epithelial cells, trichostatin A (TSA), an inhibitor of histone deacetylase, decreased IL-1β-induced IL-8 mRNA levels without altering transcription factor binding (109). Histone deacetylase has also been shown to be required for IL-8 expression. Ashburner et al. (111) found that, in HeLa epithelial and HEK-293 cells exposure to TSA increased expression of TNF-α-induced IL-8 mRNA. Since promoter constructs lack the structure present in chromatin, additional investigation should be done to determine if chromatin remodeling is occurring in U937 cells after DON exposure.

The observed role for NF-κB in DON-induced IL-8 expression is consistent with the previous observation of increased NF-κB binding in DON-treated murine T cells and macrophages (20, 22). It is not surprising that AP-1 and C/EBPβ were observed to be unnecessary for DON-induced IL-8 promoter-driven luciferase. Even though Kunsch et al. (42), Wu et al. (56), and Matsuaka et al. (89) found that C/EBPβ as well as NF-κB is necessary for IL-8 transcription, Mori et al. (41), Na et al. (126), Nourbakhsh et al. (96), and Holtmann et al. (118) found that C/EBPβ was unnecessary. Similar data have been

reported for AP-1. It is thus possible that NF-κB is the primary transcription factor responsible for DON-induced IL-8 expression in U937 cells.

It should be noted that mutation in the AP-1 site significantly increased IL-8 promoter-driven luciferase expression. Using PROMO, a web-based transcription factor binding site recognition program, an increase in the percent similarity of the binding site in the IL-8 promoter to a consensus binding site for a basal transcription factor, TFIIB, was predicted for the mutated AP-1 IL-8 promoter as compared to the wild-type promoter. This increase in similarity was only approximately 6%, but provides a plausible explanation for the increase in luciferase seen with the mutant AP-1 construct as compared to the wild-type.

While there was no difference between the wild-type IL-8 promoter and the mutant Oct-1 or mutant NRF with DON exposure, the Oct-1 mutation increased the LPS-induced luciferase and the NRF mutation decreased the LPS-induced luciferase. Both Oct-1 and NRF have been reported to act as negative regulatory elements (56, 96) in the IL-8 promoter. The increase in luciferase seen with the Oct-1 mutation might be explained if the Oct-1 site acts as a standard negative regulatory element when U937 cells are treated with LPS but is not involved in any capacity with DON-induced IL-8 regulation. Previously, the function of NRF has varied, being observed to be a negative regulatory element for one stimulus but a positive element necessary for transcription with a different stimulus (96). The results suggest that NRF was not necessary for DON-induced transcription but was a requisite for LPS-induced transcription.

Mukaida et al. (179) found that activation of NF-κB is the most important step for IL-8 gene transcription in T98G glioblastoma cells. Similarly, Delgado et al. (98),

Makarov et al. (180), and Haas et al. (38) all determined that NF-κB was the only transcription factor required for LPS-induced IL-8 in THP-1 cells. Several studies have specifically implicated the p65 (RelA) subunit of NF-κB to be responsible for IL-8 expression (42, 95, 99, 101, 124, 127, 131). Taken together, these data correspond to the results described herein.

Of the three NF-κB subunits detectable in U937 cells, p65 is the only one to contain a transactivation domain (181). The other two NF-κB subunits, p50 and p52, only contain a DNA binding domain and cannot activate transcription. Based on the NF-κB binding data, there appears to be a shift in the composition of NF-κB. Untreated cells are likely to contain a higher proportion of p50 and p52 either as homodimers or heterodimers. Dimers of NF-κB consisting of p50 and p52 have been found in several cell types including HepG2 (182), C33A epithelial (183), and JB6 epithelial (184) cells. Upon exposure to DON, p65 binding significantly increases, p52 binding decreases, and p50 remains equivalent to the control. Treated cells are thus likely to contain a higher proportion of heterodimers composed of p65 and p50. Therefore, NF-κB, composed of p65 and p50, appears to be responsible for DON-induced IL-8 expression. The observation that p50 levels do not change in the DON-treated group compared to the untreated can be explained by a shift in the subunits composing NF-κB from dimers containing p50 and p52 to dimers composed of p65 and p50. Hsu et al. (184) similarly found that in JB6 murine epithelial cells, levels of p50 were the same in untreated and in samples treated with a tumor promoter capable of increasing levels of p65 and p52.

One major problem encountered in this study was the inefficient action of general transcription inhibitors (ActD and DRB). This issue might be explained by the fact that

U937 cells are resistant to chemotherapeutic chemicals. These cells contain multiple members of the cytochrome (CYP) 450 family of enzymes. CYP450 isozymes are responsible for xenobiotic metabolism in cells (185). Wang et al. (186) determined that expression of one CYP450 isozyme correlated with increased resistance to daunorubicin. Additional resistance to several chemotherapeutic chemicals was discovered in U937 cells in a study investigating a phase II drug metabolizing enzyme (177).

CAPE has been previously shown to prevent nuclear translocation of NF-κB while not altering binding of two other transcription factors with binding sites in the IL-8 promoter, AP-1 and Oct-1, or the basal transcription factor TFIID in U937 cells (187). Use of CAPE in this study was valuable for several reasons. First, the complete inhibition of DON-induced IL-8 protein and mRNA by CAPE provides additional support for the role of NF-κB in DON-induced IL-8 expression. Second, CAPE's effective inhibition of IL-8 in U937 cells facilitated investigation of potential DON effects on IL-8 mRNA stability. As shown here, no increase in IL-8 mRNA stability was found after treatment with DON.

Enhanced IL-8 mRNA stability depends on stimulus and cell type. THP-1 cells treated with urate crystals (71) or *Staphylococcus* α toxin (52) show no evidence for enhanced mRNA stabilization, while those exposed to LPS (38, 123) do have an increase in stability. HEK-293 cells treated with neutrophil elastase did not exhibit increased IL-8 mRNA stability (72), whereas transfection with constitutively active MEKK1 and MKK6 increases IL-8 mRNA stability (118). Thus, even though DON has been shown to increase stability of TNF-α and IL-6 in RAW 264.7 (25, 26), IL-2 in cells EL-4 thymoma cells (19), and COX-2 in RAW 264.7 (24), DON did not increase the stability of IL-8

transcripts in U937 cells. Apparently, IL-8 expression in U937 cells does not share this common DON-induced post-transcriptional mechanism with the aforementioned genes.

Previously, the MAPK p38 was found to be essential for IL-8 expression in U937 cells (152). Since the DON-induced activation of MAPKs, including p38 has also been linked to PKR (29), the role of PKR in DON-induced IL-8 was examined. The capacity of a specific PKR inhibitor completely inhibit IL-8 production raises the possibility that PKR impaired NF-κB activation. Another inhibitor to PKR, 2-aminopurine, was found to inhibit NF-κB binding in rat microglia after treatment with LPS (188). Indeed, PKR has been previously found to activate NF-κB by either interacting with the IKK complex (31) or by directly phosphorylating IκB (189). Activation of NF-κB first requires that an inhibitory protein, IκB, is phosphorylated, primarily by the IKK complex, and degraded in the cytoplasm before translocation of active NF-κB to the nucleus (181). PKR is known to associate and activate NIK, one potential IKK complex member (31). Thus, it might be speculated that NF-κB activation is PKR-dependent in this model.

Two other translational inhibitors that are potent toxins, Stx1and ricin, induced PKR-dependent IL-8 protein expression in this study. Since previous studies have found Stx1- and ricin-induced IL-8 expression is p38-dependent (167, 169), the further involvement of PKR might be suggestive of a common mechanism for all three toxins, DON, Stx1, and ricin. One scenario is, that after entrance into the cell, PKR is activated leading to activation of multiple MAPKs, which results in IL-8 expression. Future studies should clarify whether IL-8 expression is similarly affected in other cell lines and if NF-κB is also involved in Stx1- and ricin-induced expression in U937 cells.

In conclusion, DON-induced IL-8 expression is highly NF-κB-dependent and DON-, Stx1-, and ricin-induced IL-8 protein is PKR-dependent in U937 cells. It is plausible that DON-induced PKR and p38 activation leads to the induction of IL-8 observed in the U937 cells and that Stx1 and ricin might share this mechanism. This might provide possible therapeutic targets in cases of intoxication by DON and other ribotoxic stressors.

CHAPTER 4: SUMMARY AND CONCLUSIONS

Overview

DON, a common contaminant of grain crops worldwide, can induce the rapid expression of numerous immune-related proteins both in rodents as well as primary and cloned cell lines. Increased transcription factor activation and mRNA stabilization are likely to be responsible for the DON-induced increases in expression of IL-6 (23), IL-2 (19), TNF-α (25), and COX-2 (24). In many of these cases, increased mRNA stability and transcription factor activation have been shown to be mediated through MAPK signaling (9, 25, 162, 163). IL-8, a DON-inducible human chemokine, primarily functions as a neutrophil chemoattractant. Elevated levels of IL-8 occur in a variety of inflammatory conditions in which tissue damage has been linked to aberrant neutrophil function. Based on its potential to mediate injury, cells tightly regulate IL-8 expression by both controlling the transcription and the mRNA stability. Multiple stimuli have been shown to increase mRNA stability of IL-8, increase transcription, or increase both mRNA stability and transcription. The research presented herein provides the first report into molecular mechanisms by which DON induces IL-8 expression.

Summary of key findings

Three human cell lines were screened as candidates for studying how DON induces IL-8 (Appendix A). This is the first report, to my knowledge, that DON is capable of inducing IL-8 in THP-1 monocytes and HEK-293 cells. Based on the

expression levels and data that DON targets primary human monocytes, U937 cells were chosen as the model cell line. However, use of these cells was, at times, quite challenging and necessitated extensive optimization studies.

As presented in Chapter 2, increased phosphorylation of p38 and JNK occurs in U937 cells after treatment with DON. Also, undifferentiated U937 cells responded to DON with increased IL-8 protein production albeit at lower levels than previously seen with differentiated U937 cells (8). An increase in IL-8 mRNA and hnRNA was also observed after exposure to DON. Chemical inhibitors were used to determine the role of three MAPK families, p38, ERK, and JNK, in DON-induced IL-8 expression in U937 cells. Inhibitors to ERK and JNK only partially reduce IL-8 expression, while the p38 inhibitor was able to completely inhibit both the IL-8 protein and mRNA induced by DON. Corresponding studies in human peripheral blood mononuclear cells in our lab indicated that IL-8 expression was also p38-dependent and that the monocyte population was primarily responsible for the response to DON (152). This correlation between the results obtained from the primary human cells and U937 cells, confirmed that the latter was a relevant model for investigation into DON's effects in humans.

Based on previously published studies and the involvement of the MAPKs pathways, it was hypothesized (see Chapter 3) that DON-induced IL-8 was due to both an activation of various transcription factors necessary for IL-8 transcription and increased mRNA stability. However, before assessment of IL-8 promoter constructs could be carried out, optimization was required for normalization of transfection with DON treatment of U937 cells (Appendix B). Typically, cells are transfected with two plasmids, one with an inducible luciferase reporter and one with a constitutive β-

galactosidase reporter to normalize the for the plasmid amount taken up into the cell. DON, a protein synthesis inhibitor, appeared to inhibit the inducible luciferase reporter expression to a greater degree than the constitutive β-galactosidase reporter expression first utilized for transfection normalization. Assessment of a real-time PCR method to measure both luciferase DNA (plasmid for normalization) and luciferase mRNA (induced by the promoter) was carried out. However, large variation was obtained with the samples and this approach was abandoned. An alternative two-plasmid system was evaluated that employed a constitutive *Renilla* luciferase reporter along with the inducible firefly luciferase reporter gene. Expression of both luciferases was inhibited to the same extent by DON treatment. This approach was optimized for timing of the transfection recovery and DON treatment. Using these parameters, this transfection system was successfully utilized in U937 cells to demonstrate DON-induced IL-8 transcription.

The above approach was used to compare a wild-type IL-8 promoter construct and various mutant constructs were assessed to determine the relative importance of several transcription factors for IL-8 expression. Site-directed mutagenesis was used to make mutants that prevented binding in the AP-1, C/EBPβ, Oct-1, NRF, and NF-κB sites. Only an intact NF-κB site was found to be necessary for IL-8 promoter-driven luciferase expression, which is the first reported instance that DON-driven gene expression is solely due to NF-κB.

The importance of NF-κB was further confirmed using an ELISA that measured the binding of different NF-κB subunits. Increased p65 binding was seen in DON-treated samples as compared to untreated controls. Two subunits, p50 and p52, unable to

activate transcription, were also detected in U937 cell nuclear protein samples. DON appeared to decrease p52 binding but did not alter p50 binding, suggesting hat the toxin might induce a shift in the composition of NF-κB, favoring dimers composed of p65 and p50.

Before IL-8 mRNA stability could be assessed, optimization was required to successfully shut down transcription (Appendix C). Repeated trials indicate that U937 cells were resistant to the commonly used general transcriptional inhibitors, ActD and DRB. Several NF-κB inhibitors were therefore screened for use as a specific IL-8 inhibitor. CAPE was found to be more effective in inhibiting both DON-induced IL-8 protein and mRNA expression. CAPE inhibitory action provides additional evidence for the importance of NF-κB in DON-induced IL-8 protein and mRNA.

Investigation into the effect of DON on IL-8 mRNA stability revealed that DON-treated cells did not have increased IL-8 mRNA stability. These data represent the first instance in using CAPE to assess IL-8 mRNA stability as well as the first demonstration that DON-induced gene expression occurred without increased mRNA stability.

DON-induced MAPK activation, as reported in Chapter 2, might be responsible for the DON-induced activation of NF-κB. Several studies have shown that various kinases in the MAPK pathway are able to activate components of the NF-κB pathway (51, 149-151). Additionally, a previously reported kinase that is activated by DON, PKR (29), has also been linked to activation of NF-κB through interaction with the IKK complex and phosphorylation of IκB. (31, 188, 190).

A specific PKR inhibitor was therefore used to determine if DON-induced IL-8 was PKR-dependent. Pretreatment with the specific PKR inhibitor significantly inhibited

DON-induced IL-8 expression. Interestly, two other toxins that activate MAPKs, Stx1 and ricin, also caused a PKR-dependent increase in IL-8 protein, as demonstrated in Chapter 3. Previous studies have shown that Stx1- and ricin-induced IL-8 protein is p38-dependent in HCT-8 human epithelial cells (169) and 28SC human monocytes (167), respectively. Based on the data in this dissertation and presented in previous studies it is plausible to conclude that PKR might be a common mediator for upregulation of cytokines by DON and other translational inhibitors.

Proposed model for IL-8 induction

Based on the data obtained in this dissertation and previous studies, a putative model for DON-induced IL-8 expression can be proposed (Figure 4.1). Initially, DON rapidly enters the cell by diffusion and interacts with the ribosome or kinases associated with the ribosome, such as PKR (HK Bae, unpublished). Once PKR is activated, it mediates activation of the p38 and other MAPKs (29). PKR and p38 have both been implicated in NF-κB activation (150, 151) (31). Inactive NF-κB is sequestered in the cytoplasm by an inhibitory protein IκB. Phosphorylation and degradation of IκB is necessary for release and activation of NF-κB (181). PKR can activate NF-κB by either activation of the IKK complex (31), which then phosphorylates IκB, or by direct phosphorylation of IκB (191). p38 activates NF-κB in a different manner, through phosphorylation of p65 NF-κB (150, 151) causing an increase in the transactivation of this transcription factor. DON potentially triggers both of these events, PKR-induced

Figure 4.1: Model for DON-induced IL-8 expression. Dotted lines indicate potential pathways in the U937 cell model. Solid lines indicate observed pathways or associations in the U937 cell model. DON diffuses into the cell and triggers activation of PKR in U937 cells (29), either by direct activation of PKR or by an indirect activation mediated by the ribosome. PKR is directly associated with ribosomal proteins in U937 cells (HK Bae, unpublished). Activated PKR, in turn, activates p38 in this cell line (29), again either through phosphorylation of p38 directly or mediated through association between p38 and the ribosome (HK Bae, unpublished). NF-κB is sequestered in the cytoplasm by IκB. The IKK complex typically phosphorylates IκB, causing degradation of IκB and release of NF-κB. Both activated PKR and p38 have been linked to the activation of NF-κB. PKR is able to trigger the release of NF-κB either by activating the IKK complex (31) or directly phosphorylating IκB (191). p38 can increases NF-κB's ability to activate transcription, by phosphorylating the p65 subunit of NF-κB (150, 151). Since DON-induced IL-8 expression is both PKR- and p38-dependent, as seen in Chapter 3 and 2 respectively, both of those pathways may responsible for the NF-κB-dependent increase in transcription and DNA binding, as seen in Chapter 3.

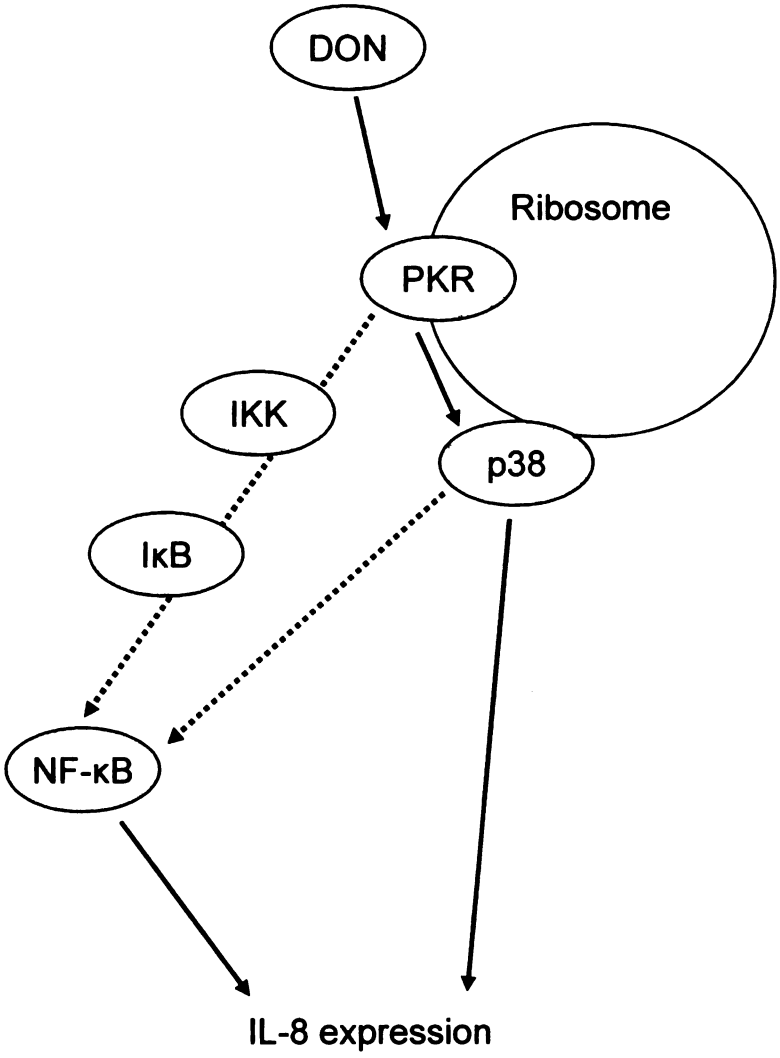

Figure 4.1

release of NF-κB and p38-dependent increase in NF-κB transactivation as, in U937 cells DON-induced IL-8 expression is PKR-, p38- and NF-κB-dependent.

Significance and future studies

The findings of this dissertation indicate that DON-induced IL-8 expression in U937 cells is p38- and NF-κB-dependent and that DON-, Stx1-, and ricin-induced IL-8 protein production share a common reliance on PKR. IL-8 induction is solely regulated on the transcriptional level. This contrasts with previous findings that indicate both an increase of transcription and in mRNA stability are responsible for upregulation of several immune proteins after exposure to DON. It is unclear at this time whether that difference is specifically due to the target investigated, IL-8, or a characteristic of the cell line used, U937 monocytes. Additional investigation into DON-induced IL-8 in human primary cells or alternative monocyte lines might clarify this issue.

The potential mechanism of DON-induced expression might provide insight into chronic inflammatory conditions with IL-8 dysregulation, such as rheumatoid arthritis, psoriasis, or inflammatory bowel disease. Additionally, both elevated serum IL-8 and endothelial damage due to increase neutrophil activation is observed in human patients with hemolytic uremic syndrome (HUS), which can lead to renal failure (192). Stx1 and ricin are able to cause HUS in a mouse model (193, 194). Insight into potential mechanisms leading to IL-8 expression might assist in developing additional therapeutics to minimize damage seen in HUS and increase patient survival. p38 inhibitors have already been suggested to minimize the damage seen in HUS (193) and research in this

dissertation potentially supports that finding if indeed DON, Stx1, and ricin share a common mechanism.

Further investigation into DON-induction of IL-8 is warranted, if a common mechanism for IL-8 induction is shared with other translational inhibitors. For one, clarification of the mechanism leading to NF-κB activation needs to be examined. It is notable that, in these cells, NF-κB is extremely important compared to its relative unimportance in DON induced gene expression in a variety of cells. Several possible mechanisms leading to NF-κB activation are present in the U937 model system. Both PKR and p38 have been observed to activate NF-κB, but the mechanisms by which they do this are quite different. PKR is involved with phosphorylation and degradation of IκB while p38 increases the transactivation of NF-κB through phosphorylation of the p65 subunit.

The common reliance on PKR for DON-, Stx1-, and ricin-induced IL-8 protein production might indicate a larger shared mechanism among translational inhibitors for IL-8 expression. DON and other trichothecenes are still a potential foodborne danger to many countries around the world (3). A common mechanism, if it exists, among DON, Stx1, and ricin could possibly provide common preventative and therapeutic targets for humans and animals exposed to these compounds.

APPENDIX A: IL-8 PRODUCTION IN MULTIPLE HUMAN CELL LINES

Abstract

Initially, the aims of this dissertation were to investigate both DON-induced IL-6 and IL-8 in differentiated U937 cells. However, due to an unknown confounding variable, the DON induction of IL-6 protein could not be replicated. Since IL-8 became the sole focus of this study, differentiation of the U937 cells was reevaluated. Macrophage differentiation of the U937 cells consisted of treating them with phorbol 12-myristate 13-acetate and then allowing sufficient time for the cells to completely differentiate. Differentiation increased the variability in IL-8 production and was further found to be unnecessary for investigating DON-induced IL-8 production. In addition, two other cell lines, THP-1 and HEK-293, were examined for DON-induced IL-8 production as alternatives to the U937 cells. These lines were found to produce less IL-8 protein than the undifferentiated U937 cells. It was therefore concluded that undifferentiated U937 cells were the most suitable cell line model for the various experiments included in this dissertation.

Introduction

Originally, the research conducted in this dissertation was based on initial data obtained by Dr. Y. Sugita-Konishi (8). Macrophage-differentiated U937 cells were exposed to DON and other trichothecenes to determine their response, as measured by

various inflammatory molecules. IL-8, IL-6, and TNF-α protein levels were all found to be induced as a result of DON exposure. U937 cells were selected for that study because of the high induction of cytokines produced after exposure to the various mycotoxins and because a primary purpose of that study was to determine if, in human tissue, there was a similarity to the cytokine upregulation seen in mouse cells. Additionally, the cells were macrophage differentiated so that they would produce several different cytokines, such as IL-6 (195, 196).

While in early experiments, DON could induce IL-6 production, in later experiments macrophage differentiated U937 cells appeared incapable of producing IL-6. This might be potentially due to an alteration in the cell line, PMA, or media components, such as fetal bovine serum. THP-1 cells are a human monocyte line. This monocyte line was established and characterized in 1980 (197). Since the establishment of this cell line, a number of different studies have utilized it to investigate IL-8 expression (52, 81, 101, 126, 127, 198). This line was selected as a potential alternative for U937 cells due to their expression of IL-8, the number of past studies that have used THP-1 cells for investigating IL-8 expression, and because they are a cell type similar to U937 cells. Both U937 cells and THP-1 cells have been classed as belonging to the monocyte differentiation lineage (199) although these two cell lines have been shown to have several different hematopoietic differentiation markers.

HEK-293 cells are human embryonic kidney cells transformed with an adenovirus (200). These cells easily take up plasmids and several transfection methods have proven effective with this cell line. HEK-293 cells have been shown to produce IL-8 protein (72, 201). HEK-293 cells do not express the various forms of the Toll-like receptors (TLRs)

(202). These cells can express up to approximately 20 transcripts of certain TLRs, but that is insignificant compared to the tens or hundreds of thousands of copies that other cells can express (202). TLRs are involved in a cell's response to microbial products. This deficiency in the HEK-293 cells is of particular interest since is can be used to discern the role of TLRs in response to diverse stimuli.

Since the study refocused on the mechanisms DON-induced IL-8, experiments were conducted to determine whether undifferentiated U937 cells were suitable for investigating IL-8 expression. During the course of these experiments, two additional cell lines, THP-1 and HEK-293, were selected and the effect of DON on IL-8 expression was also measured. The principal findings from this comparative study were that the THP-1 and HEK-293 cell lines produced much lower levels of IL-8 protein than either differentiated or undifferentiated U937 cells. Since IL-8 was to be the only immune molecule investigated, it was concluded that macrophage differentiation was unnecessary and thus was an extra unnecessary variable to the study.

Materials and Methods

Cell culture

U937 cells, isolated from the pleural effusion of an individual with diffuse histiocytic lymphoma (12), and THP-1 cells (197) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Both U937 and THP-1 cell types were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL;

Rockville, MD). HEK-293 cells, a gift from Dr. Norbert Kaminski (Michigan State University), were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL), 1x MEM non-essential amino acids, and 1 mM sodium pyruvate. Cells were incubated at 37°C with 6% CO_2. Fresh media was added as necessary to passage the cells. All reagents are from Sigma (St. Louis, MO) unless otherwise noted. Macrophage differentiation of U937 cells was carried out by exposing $4.3x10^5$ cells/ml to 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) overnight. Cells were centrifuged and the PMA-containing medium was replaced with fresh medium. Cells were plated in a 48-well plate (800 μl/well) and allowed to rest for 48 h before exposure to DON. The medium was changed every 24 h.

IL-8 protein determination

After incubation with various concentrations of DON for 12 h, cell culture plates were centrifuged for 10 minutes at 300 x g and the supernatant collected and stored at -20°C. An OptELISA IL-8 kit (Pharmingen; San Diego, CA) was used according to manufacturer's instructions with two modifications. First, the highest standard utilized was 1600 pg/ml, instead of 400 pg/ml. Second, to economize on reagents 50 μl of antibody dilutions and samples were used per well instead of 100μl. All samples were read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices; Menlo Park, CA).

MTT assay for cell viability

Cells were plated (200 µl/well) in 96 well plates at $1x10^6$ cells/ml. DON, dissolved in RPMI-1640, and 25 µl of 5 mg/ml of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), dissolved in Dulbecco's phosphate buffered saline (PBS), were added and cells were incubated at 37°C with 6% CO_2 for 3 h. Plates were centrifuged at 300 x g for 10 minutes and the supernatant was removed. 125 µl of DMSO were added and the absorbance was read at 690-570 nm on a Vmax Kinetic Microplate Reader (Molecular Devices). Percent of the control was calculated for each well.

RNA isolation

U937 cells (1 x 10^6 cells/ml in a 24 well plate with 1 ml/well) were treated with DON at various timepoints and then the plates were centrifuged for 10 minutes at 300 x g, the supernatant removed, and the RNaqeous kit (Ambion; Austin, TX) was used to isolate the RNA. Samples were treated with DNA-free (Ambion) to remove gross DNA contamination. The manufacturer's instructions were followed for both kits.

Reverse transcription real-time PCR

Reverse transcription real-time PCR was performed using One-Step PCR Master Mix (Applied Biosystems; Foster City, CA) and IL-8 Pre-Developed Assay Reagents (PDAR) (Applied Biosystems) multiplexed with the 18S PDAR (Applied Biosystems). For the hnRNA, Primer Express software v 1.5 (Applied Biosystems) was used to make primers (base numbers 3113351-3113369 and 3113431-3113452) and probe (base

numbers 3113411-3113429) selections from the NM_006216 (Genbank) sequence (IL-8 DNA). The primer/probe set was selected to include an exon-intron junction (Figure A.1). Fold change was determined using the relative quantitation method. First, standard curves are created using dilutions of total RNA from LPS-treated U937 cell total RNA. An equation, for the trend line of the standard values, is used to convert the Ct values obtained in the assay to nanogram amounts of the target. The amounts were normalized by dividing the IL-8 value by the 18S (the endogenous control) value. Relative expression is obtained by dividing all normalized values by the average of the control normalized value. Reaction conditions and PCR program were all following the manufacturer's instructions using an ABI 7700 (96 wells) or 7900HT (384 wells), both at the Michigan State University's Genomics Technology and Support Facility, depending on the number of samples to be analyzed.

Statistics

Data were analyzed with SigmaStat v 1.0 (Jandel Scientific; San Rafael, CA). IL-8 protein and RNA data were analyzed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls Method for pairwise comparisons. Treatment groups that have a P-value of < 0.05 are considered significant.

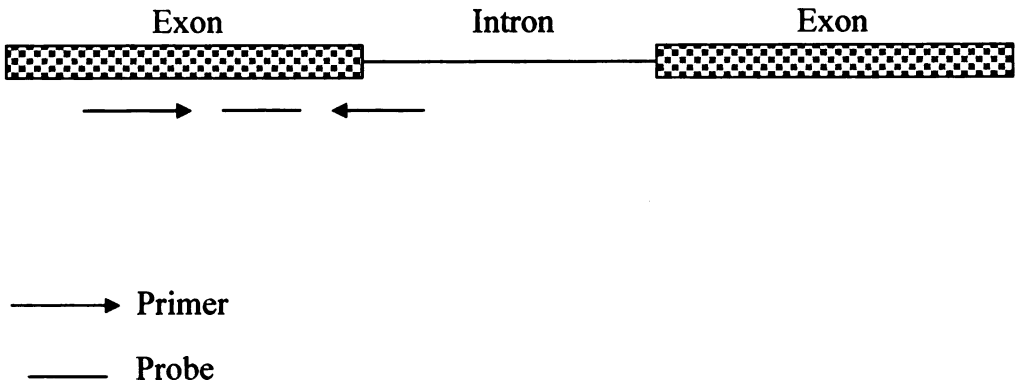

Figure A.1: Diagram of hnRNA primers and probe based on the IL-8 DNA sequence (Genbank sequence NM_006216).

Results and Discussion

DON induces IL-8 protein in several human cell lines

DON caused an increase in IL-8 protein in macrophage-differentiated U937 cells after an exposure time of 12 h. (Figure A.2) Undifferentiated U937 cells also responded to DON by producing IL-8 protein. (Figure A.3) IL-8 protein was reproducibly induced, in the undifferentiated U937 cells, as early as 3 h after exposure to DON (data not shown). Although both macrophage-differentiated and undifferentiated U937 cells produced IL-8 protein as a result of exposure to DON, much higher levels of IL-8 protein were secreted from the differentiated cells.

Two other human cell lines were also examined for DON-induced IL-8 protein production. THP-1, a monocyte cell line, displayed concentration-dependent DON-induced IL-8 protein production, though at lower levels than U937 cells. (Figure A.4) The difference in the IL-8 expression level is not surprising because, although THP-1 and U937 cells are classed as monocytes, they are at different stages of differentiation (199). The difference in IL-8 protein levels seen could also be due to the random mutations that each cell line possesses simply by being cancer cells.

The second cell line HEK-293, embryonic kidney cells, also demonstrated DON-induced IL-8 protein production. (Figure A.5) The induction in the HEK-293 cells, however, was not in a concentration-dependent fashion nor was it as high as the IL-8 protein levels seen with the two monocytic lines. This difference could simply be a result of cell type differences.

Based on robust IL-8 production and simplicity of experimental set-up, undifferentiated U937 cells were chosen for all further studies. An MTT assay was run to

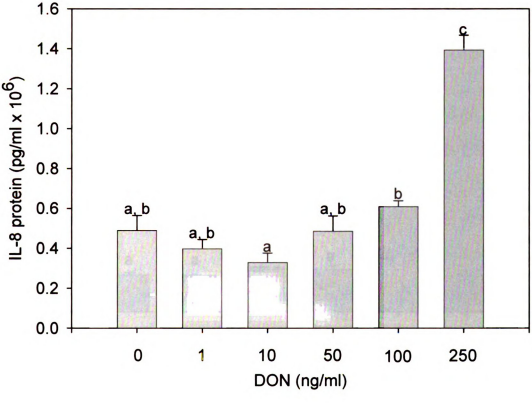

Figure A.2: IL-8 protein production by macrophage differentiated U937 cells after a 12 h exposure to DON. U937 cells were differentiated with PMA and allowed to rest for 48 h after removal of the PMA. Data are mean ± SEM (n = 3). This is representative of 2 independent experiments. Bars without the same letter are statistically different ($p < 0.05$).

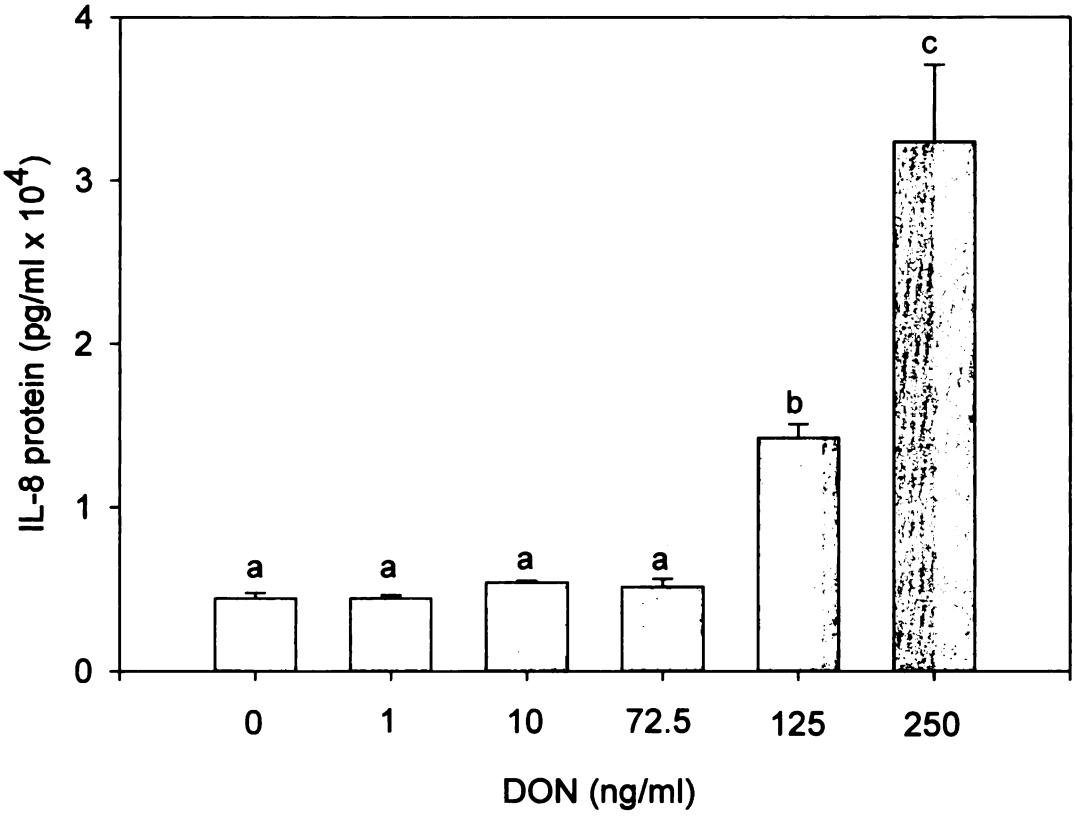

Figure A.3: IL-8 protein production from undifferentiated U937 cells after a 12 h exposure to DON. Data are mean ± SEM (n = 3). This is representative of 2 independent experiments. Bars without the same letter are statistically different ($p < 0.05$).

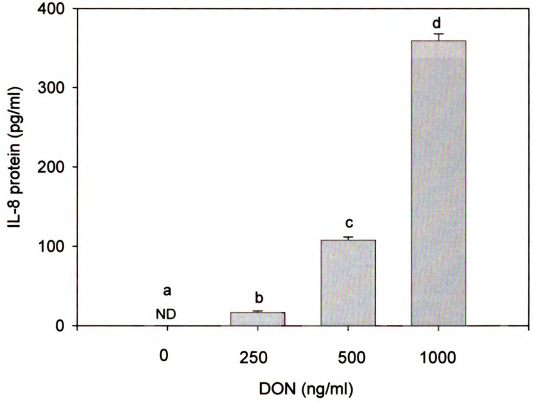

Figure A.4: IL-8 protein production from THP-1 cells after a 12 h exposure to DON. Data are mean ± SEM (n = 3). This is representative of 2 independent experiments. Bars without the same letter are statistically different ($p < 0.05$).

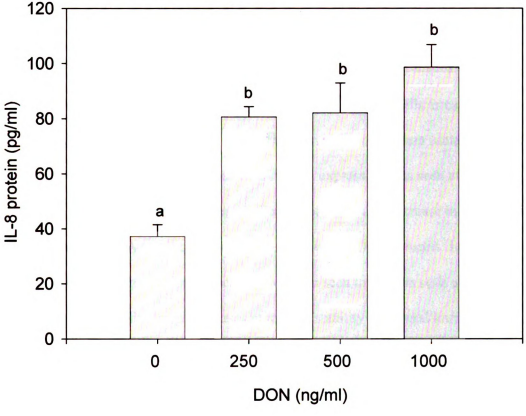

Figure A.5: IL-8 protein production from HEK-293 cells after a 12 h exposure to DON. Data are mean ± SEM (n = 6). This is an average of 2 independent experiments. Bars without the same letter are statistically different ($p < 0.05$).

determine if cell viability was affected after a 3 h exposure to various concentrations of DON. (Figure A.6) There were no statistical differences among the treatment groups, indicating that 3 h exposure to DON did not reduce cell viability. A 3 h timepoint was chosen to examine cell viability because that was the timepoint that was selected in order to measure IL-8 RNA levels.

DON induces IL-8 RNA in undifferentiated U937 cells

DON produced an increase in IL-8 mRNA levels in U937 cells as early as 1 h after exposure although the induction was not concentration dependent (data not shown). Concentration-dependent DON-induced IL-8 mRNA expression was seen after 3 h (Figure A.7). An increase in mRNA can indicate that there is an increase in transcription or an increase in mRNA stability. Either is possible with DON exposure. Increases in activity of a variety of transcription factors have been seen in various cells after exposure to DON (20, 21, 25). Similarly, increases in mRNA stability of several immune molecules have also been seen with exposure to DON (19, 25, 26). Data were obtained to provide circumstantial evidence for an increase in transcription is the heteronuclear RNA (hnRNA) levels.

hnRNA, lacking the processing seen in mRNA, was used to observe whether transcription was affected. Concentration-dependent DON induction of IL-8 hnRNA levels was also seen in U937 cells (Figure A.8). IL-8 hnRNA and mRNA levels were approximately equivalent. Increased IL-8 hnRNA levels could also be seen as early as 1 h after exposure with DON (data not shown).

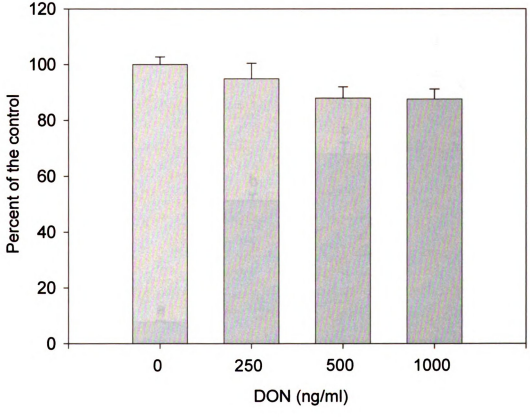

Figure A.6: MTT of U937 cells with a 3 h DON exposure. There is no statistical difference among the treatment groups. Data are mean ± SEM (n = 6). This is an average of 2 independent experiments.

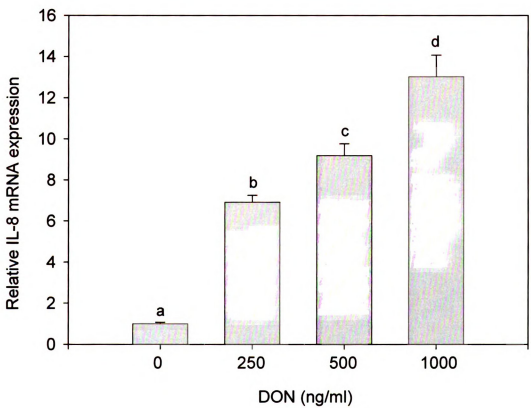

Figure A.7: IL-8 mRNA levels in undifferentiated U937 cells after 3 h exposure to DON. Data are mean ± SEM (n = 6). This is an average of 2 separate experiments. Bars without the same letter are statistically different ($p < 0.05$).

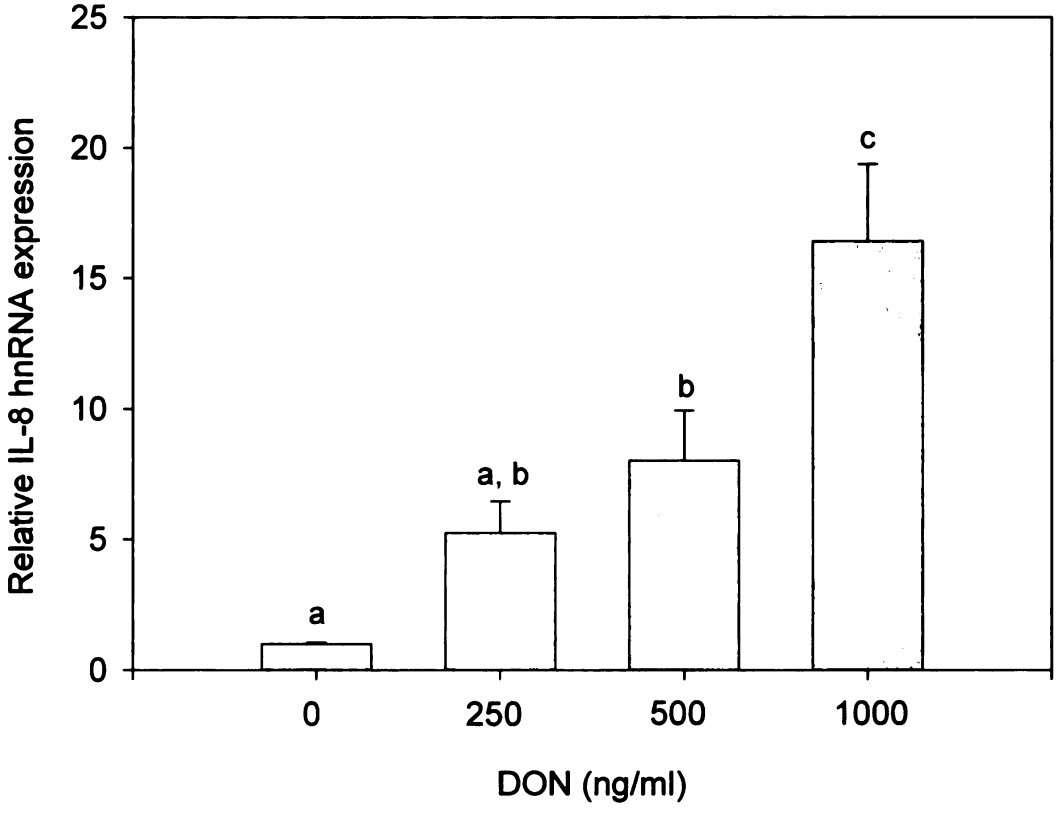

Figure A.8: IL-8 hnRNA levels in undifferentiated U937 cells after a 3 h exposure to DON. Data are mean ± SEM (n = 6). This is an average of 2 separate experiments. Bars without the same letter are statistically different ($p < 0.05$).

Conclusions

DON was able to induce IL-8 protein in all three cell lines tested, differentiated and undifferentiated U937, THP-1, and HEK-293, though in differening amounts. The differences observed in the levels of IL-8 production may be due to cell type differences or even the maturity of the cells. Even though differentiated U937 cells produce higher levels of IL-8 protein, undifferentiated U937 cells have both DON-induced IL-8 protein and RNA. Because of its simplicity, this model was chosen for experiments in this dissertation.

APPENDIX B: OPTIMIZATION OF TRANSFECTIONS USING THE IL-8 PROMOTER-DRIVEN LUCIFERASE CONSTRUCT

Abstract

This appendix describes the optimization experiments for transfections with an IL-8 promoter construct in both U937 and RAW 264.7 cells. Initially, β-galactosidase was used as the reporter gene for transfection normalization. However, this reporter gene was found to be unsuitable. Real-time PCR was then used to measure luciferase for both expression (luciferase mRNA) and normalization (luciferase DNA, plasmid). This technique also was found to be problematic, possibly due to variability problems with U937 cells. Subsequent experiments were attempted in RAW 264.7 murine macrophage cells, which were also found to be unsuitable for studying DON stimulation of an IL-8 promoter-driven luciferase construct. Finally, U937 cell transfections were assessed using the *Renilla* luciferase reporter construct for normalization. This appears to be an accurate and reproducible way to normalize transfections with DON treatment.

Introduction

Many studies use reporter constructs to examine transcriptional regulation triggered by various stimuli in a variety of cell types. These experiments provide information purported to be more reflective of the cell's actual function rather than protein binding differences obtained with Electrophoretic Mobility Gel Shift Assays (EMSA). EMSA data can vary depending on the conditions of the binding reactions and

to probe sequences. Additionally, EMSAs can only provide information about binding differences to a relatively short and specific oligonucleotide sequence. Reporter assays rely upon functioning transcription machinery within the cells along with revealing alterations in transcription factors.

However, there are several concerns that arise from the use of reporter assays. One concern is that the method used for transfection of the reporter construct into specific cells can impact the results. There are several transfection methods that can be used, which include electroporation, lipid reagents, and calcium phosphate. Each method might have different efficiencies and consistency relative to moving the plasmid into the cells as well as inherent toxicity and activation of the target cell (203). A second concern for reporter assays is the choice of cell line.

Different cell lines vary widely relative to transfection efficiency, with some cell lines being particularly problematic. U937 cells are found to be difficult to transfect, partly because of the transfection method (176) and partly a result of the response of the cells to foreign DNA (175). When undifferentiated U937 cells were compared to differentiated U937 cells and to other human monocyte lines (176) using either a lipid reagent or electroporation, it was found that the transfection efficiency for the undifferentiated cells lines was below 10% whereas the differentiated cells could not be transfected. The authors concluded that the lipid reagent had the advantage of using a lower amount of plasmid and that fewer cells were necessary to obtain similar results as to electroporation (176). As well as having low transfection efficiencies, U937 cells were also found to undergo apoptosis after the foreign DNA was electroporated into the cells (175). Apoptosis was found to be unique to electroporation and did not occur with the

two lipid reagents tested. Another problem seen in U937 cells is poor expression of the reporter gene (178).

An additional concern with reporter assays is normalization of the transfection. Efficiency of the transfection and consistency among the samples are typically assayed using a second plasmid that has constitutive expression of a reporter gene. Use of the second plasmid to normalize the transfection depends on the assumption that a reporter that is not altered by the conditions in the experiment. Several studies have reported conditions in which the internal control has been altered by the treatments (204-206) as well as ways to compensate for internal control alterations (207-209).

Other variables that can influence the success of reporter assays are the concentration of plasmid utilized and recovery time. The latter item refers to the amount of time cells are allowed to recover from the transfection itself and the amount of time cells are allowed to express the reporter gene. Expression of the reporter gene is usually assessed by measuring the amount of protein present. In the case of protein synthesis inhibitors, such as DON, protein levels may be altered by exposure of the chemical of interest.

The goal of this study was to optimize transfection conditions so that, when using an IL-8 promoter-driven luciferase construct, the luciferase levels would more accurately reflect IL-8 expression levels in the U937 cells. This appendix summarizes extensive experiments over several years that were undertaken to correct for alteration of the internal control, as a result of exposure to DON, in order to provide more consistent results. Variables assessed include the use of several reporter genes, β-galactosidase, firefly luciferase and *Renilla* luciferase in two different cell lines, RAW 264.7 and U937

cells. Conditions were optimized for the recovery time after electroporation, plasmid amount, treatment time, molecule measured (protein or mRNA), and DON concentration.

Materials and Methods

Transformation of E. coli and isolation of plasmids

Plasmids employed in these studies are summarized in Table B.1. Plasmids (50 ng) were incubated, for 10 minutes, with chemically competent JM109 *E. coli* (Promega, Madison, WI) on ice before incubation at 37°C for 5 minutes. The mixture was immediately placed back on ice and 1 ml of LB broth was added and incubated at 37°C for 1 h with shaking (250 rpm). After incubation, 75 µl of JM109 cells were plated on LB + ampicilin (100 µg/ml) and incubated overnight at 37°C. A single colony was picked for growth in a 3 ml LB + ampicillin culture and incubated overnight at 37°C with shaking (250 rpm). A 0.2 ml or 1 ml aliquot of bacterial culture was added to 100 or 500 ml LB + ampicillin culture, respectively, and incubated overnight at 37°C with shaking (250 rpm). Cells were pelleted in 50 ml tubes (Corning, Corning, NY) at 2000 x g for 15 minutes and pellet wet weight was determined. Pellets were stored at -20°C until isolation with either an EndoFree Plasmid Maxi or Mega kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Cell culture

U937 cells, obtained from American Type Culture Collection (ATCC; Manassas, VA), were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (HI FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL; Rockville, MD).

RAW 264.7 cells (ATCC) were grown in DMEM supplemented with 10% HI FBS and 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37°C with 6% CO_2.

Electroporation of cells and reporter assays

U937 cells (10^7 cells/210 μl of RPMI-1640) were electroporated using a Bio-Rad (Hercules, CA) Gene Pulser at 280 V and 960 μF in a 0.4 cm gap cuvette (Bio-Rad) with various amounts of plasmids. See Table B.1 for the plasmids utilized for these studies. RAW 264.7 cells (5 x 10^6 cells/210 μl of RPMI-1640) were electroporated with a Bio-Rad (Hercules, CA) Gene Pulser at 300 V and 960 μF in a 0.4 cm gap cuvette (Bio-Rad) with various amounts of plasmids. After electroporation, cells were diluted in 12 ml growth media and allowed to rest for various times. Cells were then treated with 250, 500, or 1000 ng/ml DON or 1000 ng/ml LPS and plated at 1 ml/well in a 24 well plate. After the selected incubation times, the cells were lysed with 100 μl of Reporter Lysis Buffer (Promega) or 100 μl of Passive Lysis Buffer for the Dual-Luciferase Assay Kit (Promega).

The Bright-Glo Luciferase assay kit (Promega) and the Beta-Glo Assay kit (Promega) were used to ascertain luciferase and β-galactosidase levels when cells were

Table B.1: Plasmids used for optimization of U937 cell transfection.

Name of Plasmid[a]	Promoter	Reporter gene
pGL2 Control	SV40 (constitutive)	Firefly luciferase
pSV40 β-gal	SV40 (constitutive)	β-galactosidase
-162/+44 IL-8 Luc	IL-8 (inducible)	Firefly luciferase
pRL SV40	SV40 (constitutive)	Renilla luciferase

a = All plasmids except -162/+44 IL-8 Luc were from Promega. The -162/+44 IL-8 Luc plasmid was a gift from Dr. Antonella Casola, Division of Child Health Research Center, University of Texas; (103)

co-transfected with luciferase and β-galactosidase reporter constructs. Normalization of transfections was accomplished by dividing luciferase levels with β-galactosidase levels from the same cell lysate. For cells co-transfected with firefly luciferase and *Renilla* luciferase reporter constructs, luciferase levels were measured with the Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Firefly luciferase levels were normalized against *Renilla* luciferase levels, in the same aliquot of cell lysate. A Turner 20e Luminometer (Turner Designs; Sunnyvale, CA) was used for all luminometer measurements.

Luciferase real-time PCR

Due to difficulties with the β-galactosidase reporter gene, the possibility of measuring both the luciferase mRNA (for expression) and luciferase DNA (plasmid for normalization) was explored. Cells (either U937 cells or RAW 264.7) were electroporated and treated with DON. RNA and DNA were originally isolated with TRI reagent (Ambion) following the manufacturer's protocol. The optional linear acrylamide was used to aid recovery of DNA. However, because of poor RNA recovery, subsequent studies employed the RNAqueous Kit (Ambion) to isolate RNA from half the sample, while the TRI reagent DNA isolation protocol was used on the other half of the sample.

Primers for luciferase (forward primer 5'-GCACATATCGAGGTGAACATCAC-3' and reverse primer 5'-TGCCAACCGAACGGACAT-3') were selected using Primer Express software (Applied Biosystems). cDNA was synthesized from the RNA using the Taqman Reverse Transcription Kit (Applied Biosystems) using random hexamers provided with the kit according to the manufacturer's instructions. SYBR Green Master

Mix (Applied Biosystems) was used according to the manufacturer's instructions. Additionally, cDNA was synthesized using luciferase specific primers and the 18S PDAR (Applied Biosystems) was utilized to normalize the sample amount, both alterations were to reduce variability.

Results and Discussion

Luciferase levels in U937 cells normalized with β-galactosidase

Figure B.1 shows the basic experimental set-up for all transfection experiments conducted. Experiments were conducted using constitutively expressed reporter genes. β-galactosidase, a reporter that is commonly used to normalize transfection experiments, was chosen to use for normalization of transfection due to the ease of measurement and the compatibility of reagents with luciferase assays. Several lipid reagents were screened (Invitrogene's Lipofectamine and DMRIE-C and Roche's FuGENE 6) and were found to be ineffective for transfection of U937 cells (data not shown). Electroporation was chosen as a reproducible means of transfecting the U937 cells.

U937 cells were electroporated with a total of 20 μg plasmid (15 μg of pGL2 Control and 5 μg of pSV40 β-galactosidase). Cells were allowed to recover from the electroporation for 6 h, and then were exposed to 0 or 500 ng/ml DON for 12 h. By comparing the pGL2 Control, which contains a luciferase reporter gene under the control of the constitutive SV40 promoter, to the pSV40 β-galactosidase plasmid, which has a SV40 promoter controlling expression of a β-galactosidase reporter, the effects of DON on both luciferase and β-galactosidase could be compared. As Figure B.2 shows, DON

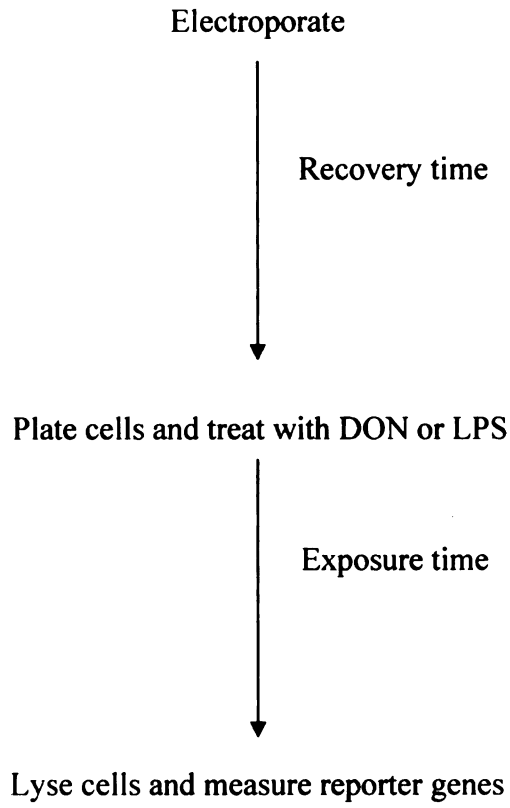

Figure B.1: Basic electroporation scheme for both RAW 264.7 and U937 cells.

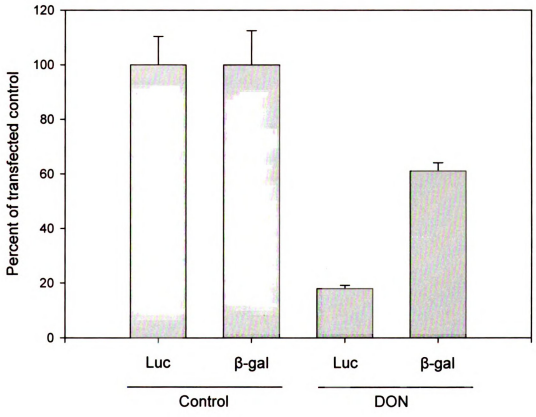

Figure B.2: Unequal suppression of constitutive expression of luciferase (Luc) and β-galactosidase (β-gal) in U937 cells with 500 ng/ml DON. Cells were allowed to rest for 6 h after electroporation (15 μg pGL2 Control and 5 μg pSV40 βgal) and were treated for 12 h with or without DON. (n = 8)

suppressed both luciferase and β-galactosidase, a result not surprising because of DON's capacity to inhibit of protein synthesis. However, β-galactosidase was suppressed approximately 40% while luciferase were suppressed approximately 80%. Thus, reporter expression data using β-galactosidase normalization will not reflect the expression of IL-8 in the U937 cells. Attempts to correct for the differences by measuring total protein in the cell lysate were not successful (data not shown).

Since the differences in the protein levels could not be corrected, the endpoint of the experiments was changed to luciferase mRNA. It seemed reasonable that luciferase mRNA could be measured by real-time PCR and the transfections normalized with the plasmid levels in the cells. The next section discusses these experiments.

Luciferase real-time PCR with U937 or RAW 264.7 cells

Over the course of optimizing the luciferase real-time PCR, concerns over luciferase expression levels in U937 cells prompted a switch to RAW 264.7 cells using a previously described method (210) for assessing luciferase mRNA (Figure B.3A). The method used for these experiments (Figure B.3B) included specific modifications of the Cok and Morrison (210) method (reagents used are detailed in the methods section above). The general strategy of this technique was to use one plasmid and then use the luciferase mRNA to measure expression and the luciferase DNA for normalization. This necessitated isolation of both the DNA and the RNA from a single sample. However, it is possible to have significant DNA contamination of the RNA sample. Based on the amount of plasmid transfected into the cells, approximately 2 μg of plasmid are present

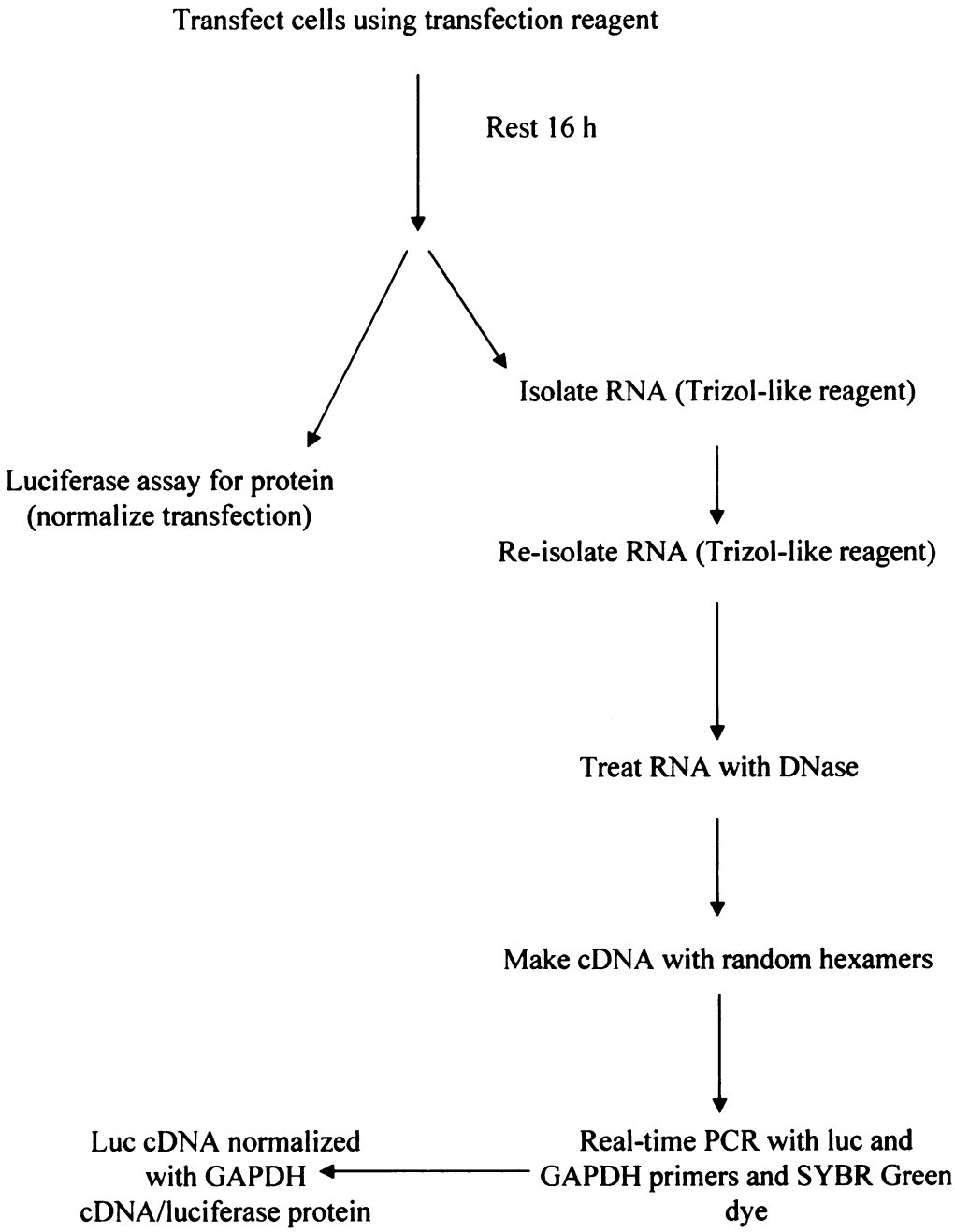

Figure B.3A: Summary of Cok and Morrison (2001) method for measurement of luciferase mRNA using real-time PCR and transfection normalization using using luciferase protein.

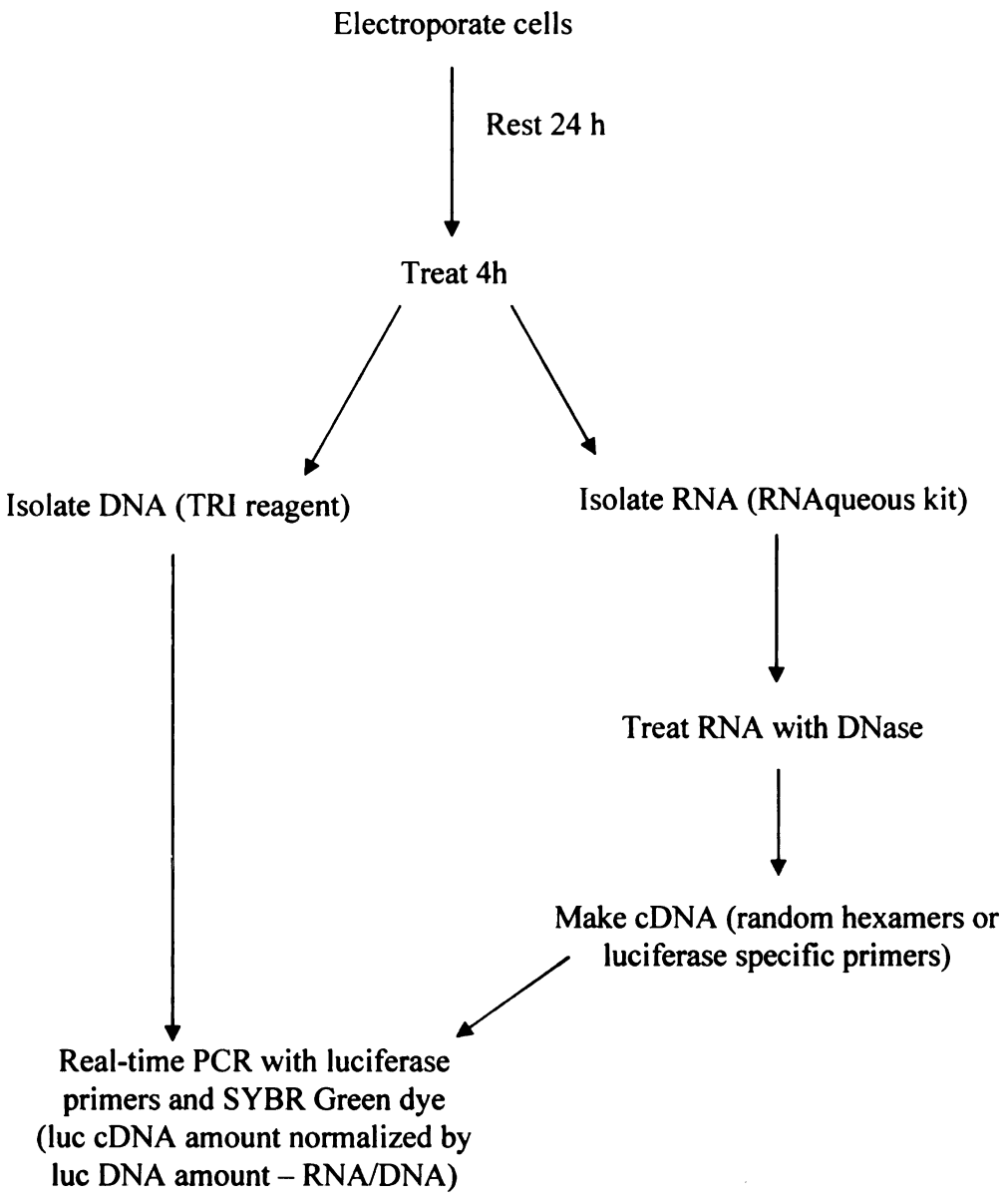

Figure B.3B: Final RNA and DNA isolation scheme for luciferase real-time PCR.

per sample thus necessitating rigorous clean-up of the RNA. In fact, Cok and Morrison re-isolated the RNA in order to remove any gross DNA contamination before DNase treating the RNA sample. Since there is a potential DNA contamination and the low yield of DNA and RNA, several alterations were made to this single isolation technique yielding both DNA and RNA. First, a linear acrylamide carrier was used to increase the yield of DNA. Linear acrylamide was added to the sample and, during the ethanol precipitation step, pulls down a larger amount of DNA than would otherwise precipitate. This step solved the DNA yield difficulties.

Next to be addressed was the RNA yield and DNA contamination issues. Although approximately 1 x 10^6 cells/ml were used, this number did not take into account cell death resulting from the electroporation and/or plasmid uptake. It has been observed that with lower cell number, such as those seen in cell culture experiments, reagents such as the TRI reagent (Ambion) or TRIzol (Invitrogen) are less efficient (unpublished observations). For this reason, the amount of cells in each sample was doubled and half of the sample was used for the DNA isolation and the other half was used for a RNA isolation using the RNAqueous kit (Ambion). This had an additional benefit of providing less DNA contamination of the RNA. RNA samples were treated with a DNase before reverse transcription.

With nucleic acid isolation problems minimized, the reverse transcription was examined. Cok and Morrison (210) used random hexamers to synthesize the cDNA and measured luciferase cDNA, normalized the sample loading with GAPDH amounts, by real-time PCR then normalized the transfection itself with constitutive protein expression from a second plasmid. The experiment presented in Figure B.4 included random

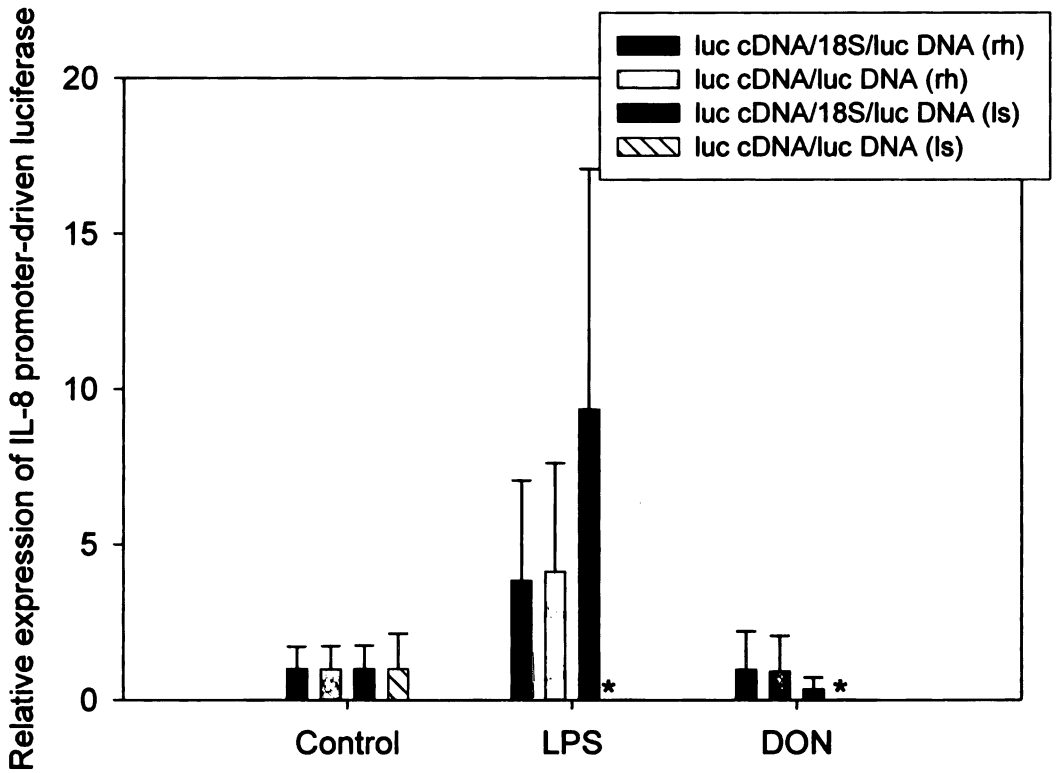

Figure B.4: Preliminary real-time PCR results for luciferase mRNA and DNA in RAW 264.7 cells are highly variable. RNA and DNA, isolated from RAW 264.7 cells electroporated with 20 μg of -162/+44 IL-8 LUC plasmid, were assessed for luciferase. Samples were normalized with either luciferase DNA alone or 18S RNA levels and luciferase DNA. cDNA was made with either random hexamers (rh) or luciferase-specific (ls) primers. * = values that are 0.0002 or lower (n = 2)

hexamers and luciferase specific primers for cDNA synthesis and normalized the sample loading in the real-time PCR by measuring 18S levels.

Transfections were normalized by measuring luciferase DNA (plasmid) with real-time PCR. Additionally, normalization of the real-time PCR was carried out in two different ways. One was calculated by dividing the luciferase cDNA amount by the 18S amount (to normalize for loading) then dividing by the luciferase DNA amount to normalize for the transfection efficiency. This normalization was represented with luc cDNA/18S/luc DON For the other normalization, 18S levels were not considered, represented by luc cDNA/luc DNA in the figure. Neither of the normalization methods reduced the variability seen in the samples. While results obtained using random hexamers were equivalent for both of the calculations, using luciferase specific primers increased the variability observed. Inclusion of 18S values for sample loading in the real-time PCR reaction produced a much larger value (around 10 fold) than the $\leq$0.0002 fold calculated without normalizing for sample loading with 18S. Additionally, all values obtained from DON-treated cells were equal or lower than values from untreated cells.

Due to the extreme variability of these preliminary real-time data, it was concluded that optimization at the protein level must again be considered. This optimization was carried out in RAW 264.7 cells.

Luciferase levels in RAW 264.7 cells (without transfection normalization)

Since there was extremely high variability found in the luciferase real-time PCR data, further optimization was deemed necessary using RAW 264.7 cells. Luciferase protein was used for optimization due to the ease of measurement. The maximum

treatment time of 4 h was chosen based on a study performed by Thompson et al. (203), which determined that the optimal levels of luciferase, induced by a LPS responsive promoter, occurred using a 24 h recovery time followed by a 4 h treatment with 1000 ng/ml LPS. Additional, earlier, timepoints were also examined in order to aid in the selection of a timepoint suitable for RNA isolation to measure luciferase mRNA.

Figure B.5 presents luciferase expression in RAW 264.7 cells electroporated with various (5, 15, or 25 μg of -162/+44 IL-8 Luc plasmid, which contains the wild-type IL-8 promoter driving a firefly luciferase gene). After electroporation, the cells were allowed to recover for 24 h and then the cells were treated with or without 100 ng/ml LPS for 0.5, 2, or 4 h. Luciferase levels were found to be increased in the LPS-treated groups both with increasing plasmids amounts and with increased exposure time. These data indicate that the -162/+44 IL-8 Luc plasmid was LPS responsive, confirming that the plasmid was functioning properly and that increasing amounts of plasmid produced higher luciferase levels.

Based on Figure B.5, 15 μg of plasmid was selected for subsequent experiments as that amount produced high levels of luciferase while limiting the amount of plasmid actually used. Figure B.6 summarizes the different treatment times tested using the 15 μg plasmid amount. RAW 264.7 cells were electroporated and treated as before, with treatment groups of 0, 100, 250 ng/ml DON, 100 ng/ml LPS, or 100 ng/ml DON + 100 ng/ml LPS. The results from this figure indicate that, although the -162/+44 IL-8 Luc plasmid was LPS responsive in RAW 264.7 cells, RAW 264.7 cells transfected with the -162/+44 IL-8 Luc plasmid were not DON responsive. In addition, neither the 100 nor

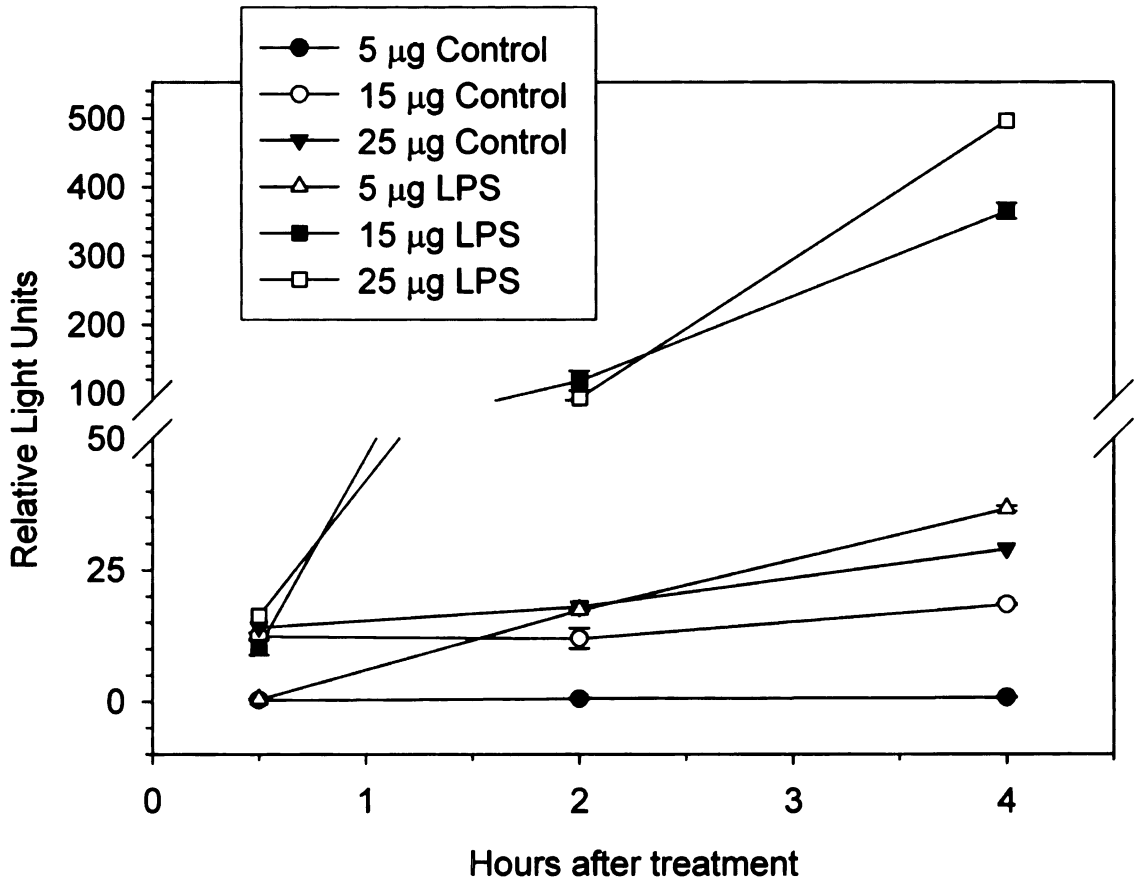

Figure B.5: Increasing the amount of plasmid increases the luciferase protein levels (non-normalized) in LPS-treated RAW 264.7 cells. Cells were electroporated with 5, 15, or 25 µg -162/+44 IL-8 LUC and after a 24 h recovery time were treated for 0.5, 2, or 4 h with or without 1 µg/ml LPS. (n = 2)

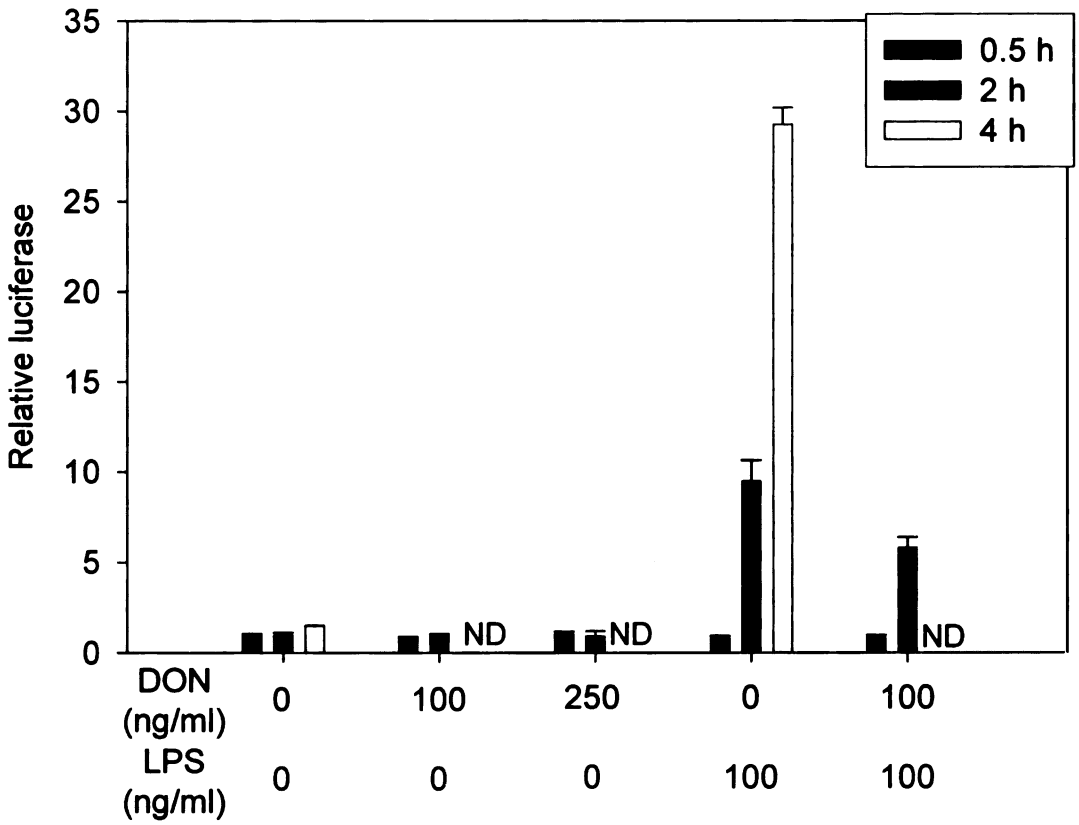

Figure B.6: Longer treatment times have higher luciferase protein levels (non-normalized) in RAW 264.7 cells. Cells were electroporated with 15 μg of -162/+44 IL-8 Luc, allowed to recover for 24 h and treated for 0.5, 2, 4 h with the concentrations of DON and LPS indicated. ND = not determined (n = 2)

250 ng/ml DON concentrations induced luciferase levels. Furthermore, even though the LPS group showed an exposure time dependent increase in luciferase, the co-treatment of DON + LPS did not increase the luciferase levels and in fact slightly reduced the levels in comparison to the LPS alone group.

Other studies in our lab suggested that RAW 264.7 cells were unsuitable for this study. Dr. H-R Zhou determined that RAW 264.7 cells treated with LPS would induce an increase in phosphorylated p65 but DON-treated cells did not have an increase in p65 phosphorylation (data not shown). Thus, this cell line might be unsuitable as DON was unable to activate NF-κB. Experiments were therefore re-initiated in U937 cells.

Constitutive expression of luciferase in U937 cells

Figure B.7 presents data indicate that the shorter the recovery time employed, the higher the luciferase response. U937 cells were electroporated with 0, 5, or 10 μg of the pGL2 Control plasmid. Cells were allowed to recover for either 12 or 24 h. After recovery, cells were incubated an additional 2 or 4 h in order to simulate a treatment time. After the simulated treatment time, cells were lysed and the lysate measured for luciferase. Results indicated that higher luciferase levels occur in cells with the shorter recovery time (12 h recovery versus 24 h). However, differences between the constitutive promoter in the pGL2 Control and the inducible promoter in the IL-8 promoter-driven luciferase construct must be kept in mind. The pGL2 Control plasmid is constantly producing luciferase in fairly large quantities while the IL-8 promoter-driven luciferase construct would produce differing levels depending on the stimulus. Thus, luciferase levels with the IL-8 promoter construct are likely to be dramatically lower. For

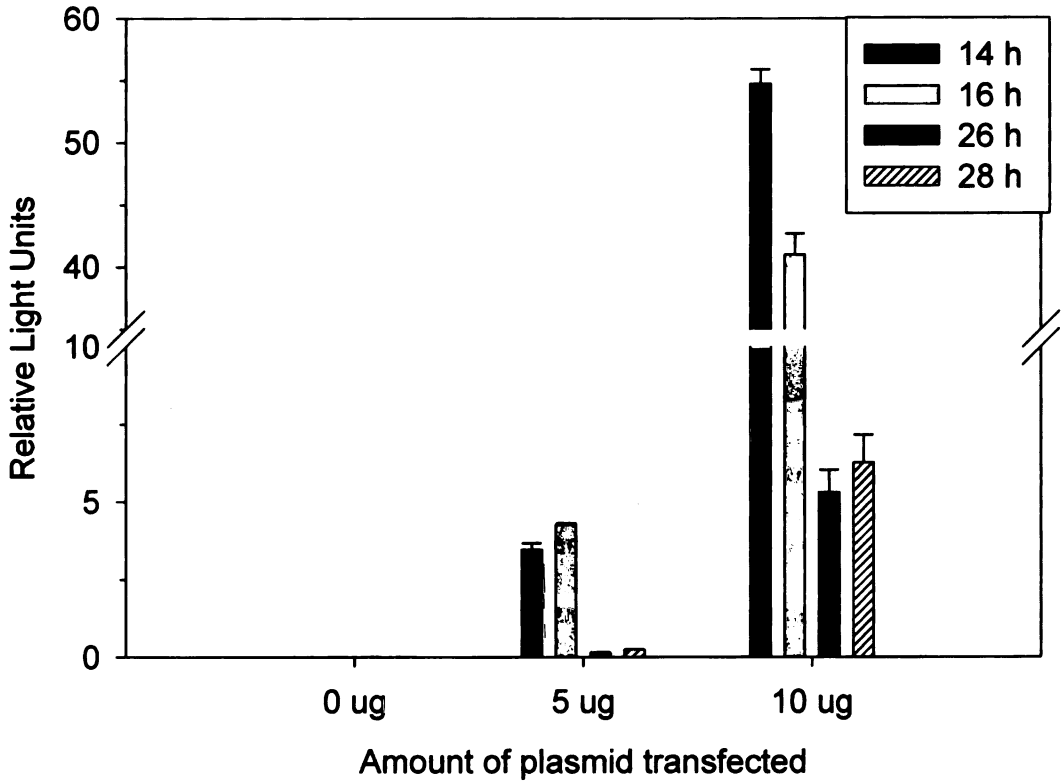

Figure B.7: Shorter recovery times have higher luciferase levels (non-normalized) in U937 cells. Cells were electroporated with 0, 5, or 10 μg of pGL2 control plasmid and allowed to recover for either 12 or 24 h. Luciferase levels were measured after either a 2 (14 h and 26 h) or 4 h (16 h or 28 h) treatment time. (n = 2)

this reason, when continuing to optimize recovery and treatment times for eventual use with the IL-8 promoter construct, a substantially shorter recovery time of 1 h was tested with 2, 4, 6, and 12 h exposure times in order to determine the best luciferase expression time.

Transfection normalization using a constitutively expressed Renilla luciferase gene in U937 cells

The utility of a *Renilla* luciferase plasmid for normalization purposes was also examined. This was done by co-electroporating U937 cells with two SV40 promoter-driven luciferase plasmids, one the pGL2 Control (10 μg) that contained a firefly luciferase reporter and the other the pRL SV40 (1 μg) that contained a *Renilla* luciferase reporter. Cells were allowed to recover for 1 h, and then were treated with 0, 500, or 1000 ng/ml DON. Figure B.8 presents all luciferase levels relative to the untreated, electroporated control luciferase levels. A dramatic decrease in luciferase was observed for both the firefly and *Renilla* luciferase. This decrease was seen at all exposure timepoints and was larger the longer the cells were exposed to DON. However, unlike the unequal suppression of luciferase and β-galactosidase, both firefly and *Renilla* luciferase was inhibited equivalently. Under the same recovery time (1 h) and exposure times to DON (2, 4, 6, or 12 h) the luciferase response to the -162/+44 IL-8 Luc promoter luciferase construct was tested.

For Figure B.9, firefly luciferase (under control of the IL-8 promoter) was divided by the amount of *Renilla* luciferase (under control of a constitutive promoter) for transfection normalization. Figure B.9 presents normalized data, indicating that the

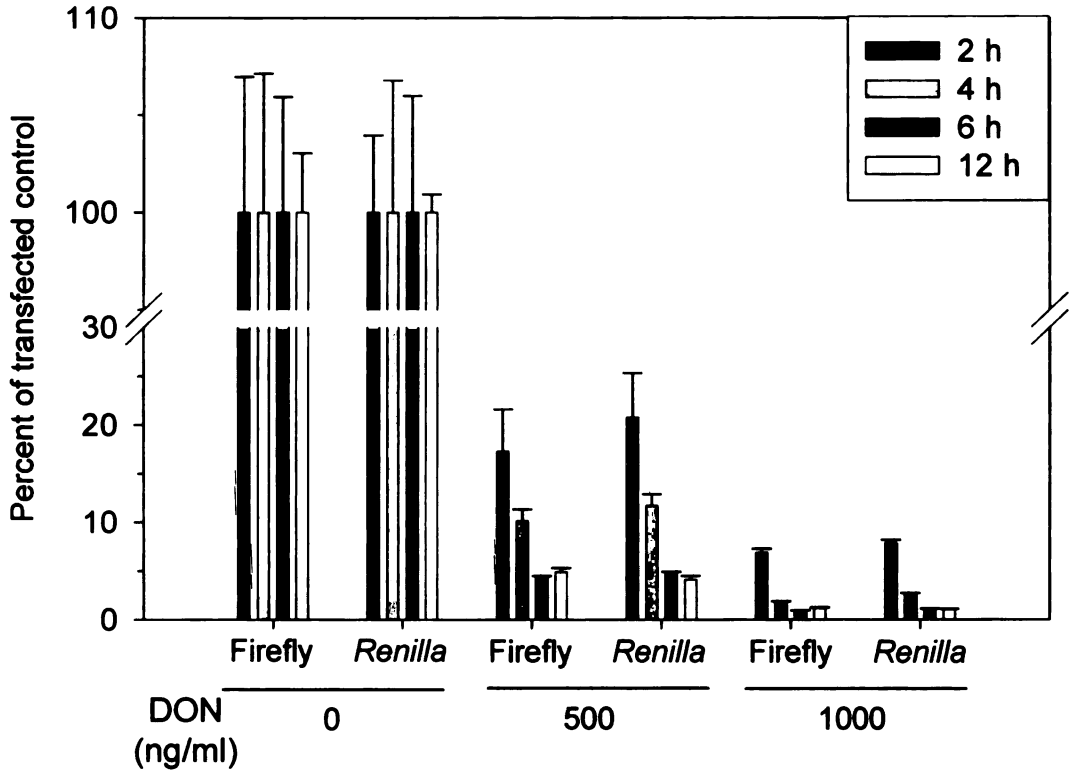

Figure B.8: DON causes approximately equivalent suppression of firefly and Renilla luciferase levels in U937 cells. Cells were electroporated with 10 μg of pGL2 Control and 1 μg pRL SV40 and allowed to recover 1 h before treatment. Cells were treated with 0, 500 or 1000 ng/ml DON for 2, 4, 6, or 12 h. (n = 3)

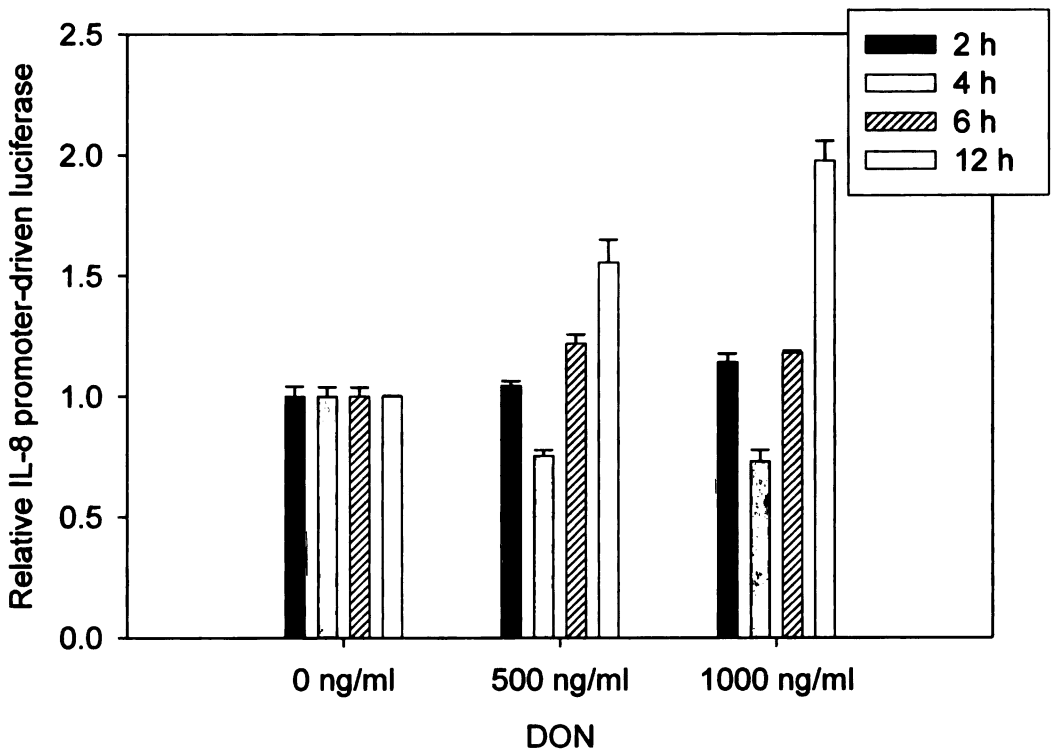

Figure B.9: DON-induction of IL-8 promoter-driven luciferase levels (normalized with *Renilla* luciferase levels) in U937 cells. Cells were electroporated with 15 μg -162/+44 IL-8 LUC and 1 μg pRL SV40, allowed to recover for 1 h and treated with 0, 500, or 1000 ng/ml DON for 2, 4, 6, or 12 h. (n = 3)

luciferase levels for both 500 and 1000 ng/ml DON are equivalent in the earlier timepoints (2, 4, or 6 h), but the later (12 h) timepoint showed a clear increase over the control at both DON concentrations tested. Recovery (1 h) and exposure (12 h) times were selected for further testing. At this point, since there were high levels of *Renilla* luciferase in the control cells the amount of pRL SV40 plasmid was reduced from 1 μg to 0.5 μg.

Confirmation of a successful transfection system in U937 cells

Figure B.10 presents data from several independent experiments, showing the consistency among independent experiments. U937 cells were electroporated with 15 μg -162/+44 IL-8 Luc promoter-driven luciferase construct and 0.5 μg of pRL SV40. Cells were allowed to recover for 1 h and then were treated with 0, 500, 1000 ng/ml DON or 1000 ng/ml LPS for 11 h. Cells were lysed and assayed for luciferase according to the abovementioned methods. Figure B.10 represents a sample size of 20 and there was found to be statistically significant differences between the control and DON or LPS treated groups. Thus, DON induced IL-8 promoter-driven transcription in the U937 cells. The observation that there was only a one-fold increase in luciferase levels was not surprising. In a study conducted by Roux et al. (211) the authors found an approximately 40-fold increase in IL-8 protein induced by LPS in U937 cells but only a 3-fold increase in luciferase levels driven by an IL-8 wild-type promoter also in U937 cells. This optimized experimental set-up was therefore selected to assay mutations in the IL-8 promoter in order to determine the transcription factors that are important in DON-induced IL-8 transcription.

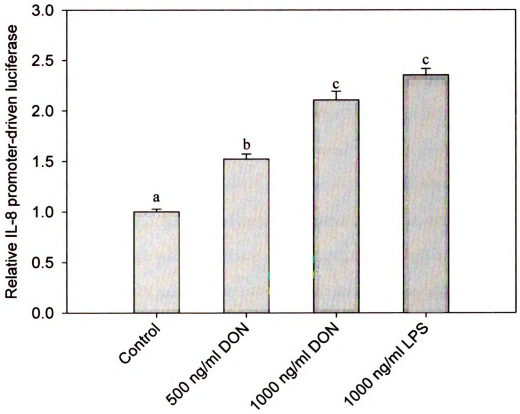

Figure B.10: DON induces IL-8 promoter driven luciferase levels in U937 cells. Cells were electroporated with 15 μg -162/+44 IL-8 LUC and 0.5 μg pRL SV40, allowed to recover for 1 h, and treated for 11 h with 0, 500, 1000 ng/ml DON, or 1000 ng/ml LPS. Representative of several independent experiments. (n = 20)

Conclusions

Since β-galactosidase was found to be unsuitable for normalizing transfection efficiencies after DON exposure, additional methods for normalizing transfections were examined. Utilizing real-time PCR to measure both the inducible luciferase mRNA and the constant luciferase DNA levels had high levels of variation for several reasons. However, use a second plasmid containing a *Renilla* luciferase gene was assessed and was an acceptable method for transfection normalization in U937 cells treated with DON.

APPENDIX C: OPTIMIZATION OF mRNA STABILITY EXPERIMENTS

Abstract

Deoxynivalenol (DON) induces expression of a variety of immune proteins due to, in part, an increase in mRNA stability. The assessment of mRNA stability is generally carried out by using general transcriptional inhibitors to block transcription and then measuring the time the transcript of interest remains. However, assessment of IL-8 mRNA stability in U937 cells using general transcriptional inhibitors provided inconsistent results possibly due to the well-known drug resistance of this cell line. Additional inhibitors, those that are specific to IL-8, were therefore assessed for use in this model system. Caffeic acid phenethyl ester was found to be an effective inhibitor of both IL-8 protein and mRNA and was selected to use in the assessment of IL-8 mRNA stability.

Introduction

Deoxynivalenol (DON) has been found to increase the protein levels of various immune molecules (8, 17, 24-26, 163). One of the ways that DON induces an increase in immune molecules is to increase the stability of the mRNA (25, 26) (19). This allows a larger amount of protein to be translated than if the transcript were degraded quickly.

Conventionally, measurement of mRNA stability is done using a general transcriptional inhibitor to stop transcription and then the amount of the mRNA of

interest is measured over specific time intervals. Two inhibitors that are commonly used are dichlorobenzimidazole riboside (DRB) and actinomycin D (ActD). DRB halts transcription by preventing elongation of the transcript and can also induce p53-dependent apoptosis (212). ActD also inhibits the function of RNA polymerase II, thus preventing mRNA from being synthesized (213). However, there have been reports that ActD can interfere with mRNA degradation making assessment of mRNA stability problematic (119).

Since using general transcription inhibitors did not provide clear results in DON-treated U937 cells, alternative inhibitors were considered. Alternative inhibitors were selected based on their ability to inhibit IL-8 transcription specifically. NF-κB inhibitors were the first group selected for consideration based on preliminary experiments showing the importance of this transcription factor for DON-induced IL-8 as well as previously published studies using a variety of these inhibitors. Three NF-κB inhibitors, BAY 11-7085, caffeic acid phenethyl ester (CAPE), and gliotoxin, were selected to be tested in this model system. BAY 11-7085 was successfully used in U937 (214) and human retinal endothelial cells (215) and resulted in decreased NF-κB binding activity and inhibition of IL-8 protein, respectively. CAPE was used successfully in human retinal endothelial cells (215) to inhibit IL-8 protein and also in microglia (216) to inhibit IL-6 and nitric oxide as well as inhibiting NF-κB binding. Notably, two studies found CAPE to be effective in U937 cells. Nardini et al. (217) found that there was an inhibition of ceramide-induced NF-κB binding. In the other study, CAPE was able to selectively inhibit NF-κB binding in U937 after exposure to a variety of stimuli (187). Table C.1 is a summary of selected studies using these three inhibitors.

Table C.1: Summary of selected studies using NF-κB inhibitors

Inhibitor	Concentration	Pretreatment	Stimulator	Cell type	Results	Reference
BAY 11-7085	5 μM	3h	TPA	U937	inhibit NF-κB binding activity	(214)
BAY 11-7085	40 μM	1h	GHSA	hRPE	inhibited IL-8 protein	(218)
CAPE	25 μg/ml	2h	GHSA	hRPE	inhibited IL-8 protein	(218)
CAPE	50 or 100 μM	9h	Ceramide	U937	inhibited NF-κB binding	(217)
CAPE	25 μg/ml	2h	TNF, PMA, Ceramide, H_2O_2, okadaic acid	U937	inhibited NF-κB binding, no effect on IκBα	(187)
CAPE	2 μM	none	TSA/LPS	microglia	inhibit IL-6 and NO, inhibit binding of NF-κB	(216)
CAPE	0.5 to 2.5 μM	none	arsenite	N-HOS	no difference in IL-8 mRNA levels	(219)
gliotoxin	1 pg/ml	1h	HDM allergen	eosiophils	inhibit NF-κB and IL-8 protein	(220)
gliotoxin	0.1 μg/ml	0.5h	zymosan	PBMC	inhibited IL-8 protein	(128)
gliotoxin	1 μg/ml	2h	IL-1β	U-251 MG	inhibited RANTES, not IL-8	(221)

This appendix presents the experiments using the general transcription inhibitors, DRB and/or ActD and the experiments to select a better inhibitor for determining if DON increases IL-8 mRNA stability. Of the three inhibitors, CAPE was best able to inhibit IL-8 protein and also was found to inhibit IL-8 mRNA. It was therefore used in the mRNA stability experiment to determine of IL-8 mRNA stability is increased by DON.

Materials and Methods

Cell culture

U937 cells, isolated from the pleural effusion of an individual with diffuse histiocytic lymphoma (12) were obtained from American Type Culture Collection (ATCC; Manassas, VA). U937 cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL; Rockville, MD). Cells were incubated at 37°C with 6% CO_2. Fresh media was added as necessary to passage the cells. All reagents are from Sigma (St. Louis, MO) unless otherwise noted.

RNA isolation

U937 cells were treated with DON at various timepoints and then the plates were centrifuged for 10 minutes at 300 x g, the supernatant removed, and the RNaqeous kit (Ambion; Austin, TX) was used to isolate the RNA. Samples were treated with DNA-free

(Ambion) to remove gross DNA contamination. The manufacturer's instructions were followed for both kits.

Reverse transcription real-time PCR

Reverse transcription real-time PCR was performed using One-Step PCR Master Mix (Applied Biosystems; Foster City, CA) and IL-8 Pre-Developed Assay Reagents (PDAR) (Applied Biosystems) multiplexed with the 18S PDAR (Applied Biosystems). For the hnRNA, Primer Express software v 1.5 (Applied Biosystems) was used to make primers (base numbers 3113351-3113369 and 3113431-3113452) and probe (base numbers 3113411-3113429) selections from the NM_006216 (Genbank) sequence (IL-8 DNA). The primer/probe set was selected to include an exon-intron junction (see Figure A.1). Fold change was determined using the relative quantitation method. First, standard curves are created using dilutions of total RNA from LPS-treated U937 cell total RNA. An equation, for the trend line of the standard values, is used to convert the Ct values obtained in the assay to nanogram amounts of the target. The amounts were normalized by dividing the IL-8 value by the 18S (the endogenous control) value. Relative expression is obtained by dividing all normalized values by the average of the control normalized value. Reaction conditions and PCR program were all following the manufacturer's instructions using an ABI 7700 (96 wells) or 7900HT (384 wells), both at the Michigan State University's Genomics Technology and Support Facility, depending on the number of samples to be analyzed.

IL-8 protein determination

U937 cells ($1x10^6$ cells/ml) were treated with various concentrations of BAY 11-7085 (Calbiochem; San Diego, CA), caffeic acid phenethyl ester (CAPE; Calbiochem), or gliotoxin (Sigma; St. Louis, MO) either with a 2 h pretreatment or with no pretreatment. After incubation with various concentrations of DON for 12 h, the cell culture plates were centrifuged for 10 minutes at 300 x g and the supernatant collected and stored at -20°C. OptELISA IL-8 kit (Pharmingen; San Diego, CA) was used according to manufacturer's instructions with two modifications. First, the highest standard utilized was 1600 pg/ml, instead of 400 pg/ml. Second, to economize on reagents 50 µl of antibody dilutions and samples were used per well instead of 100µl. All samples were read at 450 nm in a Vmax Kinetic Microplate Reader (Molecular Devices; Menlo Park, CA).

Results and Discussion

IL-8 stability experiments using general transcription inhibitors

When conventional transcriptional inhibitors were used to examine whether IL-8 mRNA stability is increased after exposure to DON, results obtained were highly variable, either tending to be ambiguous or suggesting that there was no increased IL-8 stability with exposure to DON. Table C.2 is a summary of all of the IL-8 mRNA stability experiments. As indicated in the table, several different pretreatment conditions were attempted along with various concentrations of the two general transcriptional inhibitors. Additionally, heteronuclear (hn) RNA was tested in several instances and

Table C. 2: Summary of IL-8 mRNA stability experiments. Numbers in parentheses indicates the number of experiments where IL-8 hnRNA was also measured.

No pretreatment	**Ambiguous results**	**No observed mRNA stability**
ActD 1 μg/ml	0	1
ActD 10 μg/ml	0	2 (2)
DRB 100 μM	0	3 (2)
DRB 50 μM	1	0
DRB 50 mM + ActD 1 μg/ml	1	0
3h DON pretreatment	**Ambiguous results**	**No observed mRNA stability**
DRB 100 μM	1 (1)	2 (1)
DRB 100 μM + SB 2 μM	0	1 (1)
3h LPS pretreatment	**Ambiguous results**	**No observed mRNA stability**
ActD 1 μg/ml	3	0
DRB 100 μM	1	1
DRB 50 μM	3	1
DRB 50 μM + ActD 1 μg/ml	0	1

these studies indicated that transcription was indeed occurring. Figure C.1A and C.1B provide examples of the inconclusive results. Both figures are IL-8 RNA levels measured by real-time PCR after a co-exposure with 100 μM DRB and 0, 250, 500, or 1000 ng/ml DON. DRB-treated cells show a swift decrease of the IL-8 RNA, with 50% remaining around the 0.5 h timepoint. However, when co-treated with DON the results become quite variable. For Figure C.1A, there is an increase of IL-8 mRNA at either the 0.5 or 1 h timepoints. This initial early increase in IL-8 mRNA could indicate that the DRB is not efficiently shutting down transcription. The possibility that DRB is not shutting down IL-8 transcription is corroborated by the results seen in Figure C.1B with the 500 ng/ml DON co-treated with 100 μM DRB. The IL-8 hnRNA increases at the 1 h timepoint. This increase should not be seen if DRB is actually inhibiting transcription. A possible reason for the lack of effect of the DRB is that the U937 cells are able to convert or remove DRB from inside of the cell.

U937 cells have been shown to contain multiple members of the cytochrome (CYP) P450 family of enzymes that are responsible for metabolizing xenobiotics in cells. U937 cells were found to possess CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A7, CYP2D6, and CYP2E1. Barely detectable expression of CYP3A4 and 3A5 mRNA was also observed (185). Wang et al. (186) corroborated the presence of CYP3A5 in U937 cells and correlated the expression of that enzyme with increased resistance to daunorubicin. Additional resistance to several chemotherapeutic chemicals was discovered in U937 cells in a study investigating a phase II drug metabolizing enzyme (177). Additionally, U937 cells were found to contain a multi-drug resistance transporter found to protect the cells from mercury cytotoxicity (222). Both

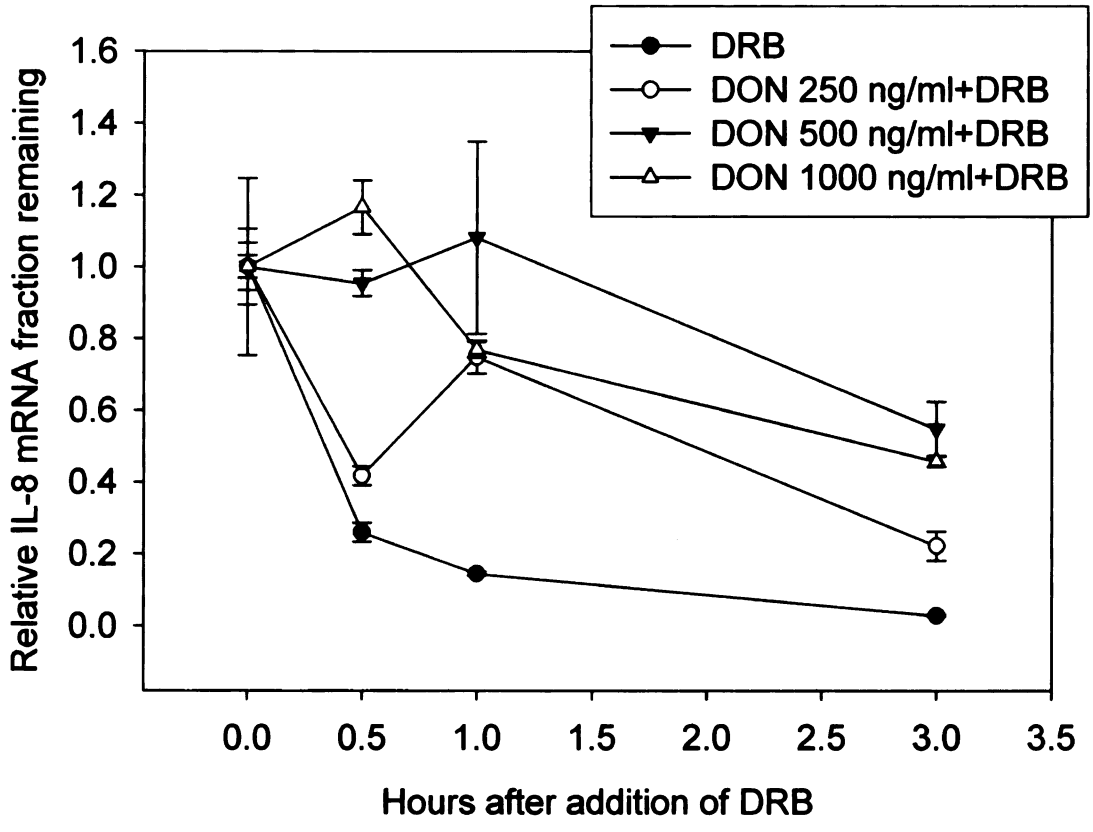

Figure C.1A: IL-8 mRNA in U937 cells after exposure to 100 μM of DRB alone or with 250, 500, or 1000 ng/ml DON. Cells were co-treated with various concentrations of DON and DRB for the specified times, then total RNA was isolated and assessed for IL-8 mRNA. Data are mean ± SEM (n = 3).

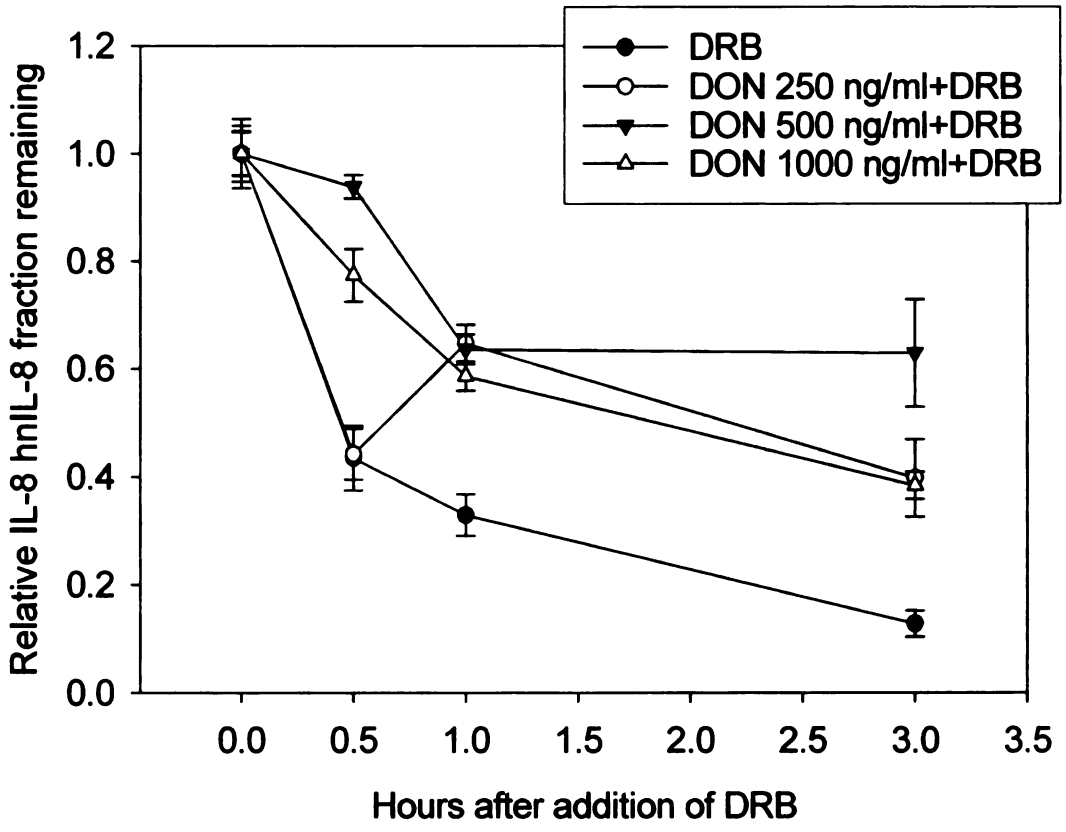

Figure C.1B: IL-8 hnRNA in U937 cells after exposure to 100 μM of DRB alone or with 250, 500, or 1000 ng/ml DON. Cells were co-treated with various concentrations of DON and DRB for the specified times, then total RNA was isolated and assessed for IL-8 hnRNA. Data are mean ± SEM (n = 3).

mechanisms of drug resistance suggest that these cells are capable of neutralizing various chemicals, possibly including those used to inhibit transcription such as DRB or ActD. Due to the inefficiency of the two general transcription inhibitors, additional inhibitors were evaluated in this model.

Assessment and optimization of NF-κB inhibitors

Since NF-κB is critical for DON-induced IL-8 expression (see Chapter 3) the NF-κB inhibitors chosen to screen for inhibition of DON-induced IL-8. Based on the ease of the assay as well as amount of inhibitor and cells required, assessment and optimization was carried out using an ELISA for IL-8 protein. For an ELISA, less than 200 μl of culture supernatant is necessary, which allows for a much smaller amount of both inhibitor and cells required for the assay then would be required for real-time PCR measurement of IL-8 mRNA.

U937 cells were either co-treated with an inhibitor and DON or pretreated for 2 h with each inhibitor before treatment with DON. Once DON was added, cells were incubated for 12 h before the supernatant was collected. Figure C.2 presents the results from cotreating DON and BAY 11-7085. BAY 11-7085, at all three concentrations tested, was unable to inhibit DON-induced IL-8 protein levels and in some samples seemed to increase the IL-8 protein produced by the U937 cells. Co-treatment of CAPE was far more successful (Figure C.3). At 100 μg/ml CAPE, DON-induced IL-8 protein was completely inhibited. Gliotoxin co-treatment was also ineffective, at both concentrations tested, and seemed to also increase IL-8 protein levels in the DON-treated samples (Figure C.4). Along with cotreating the cells with each inhibitor and DON cells

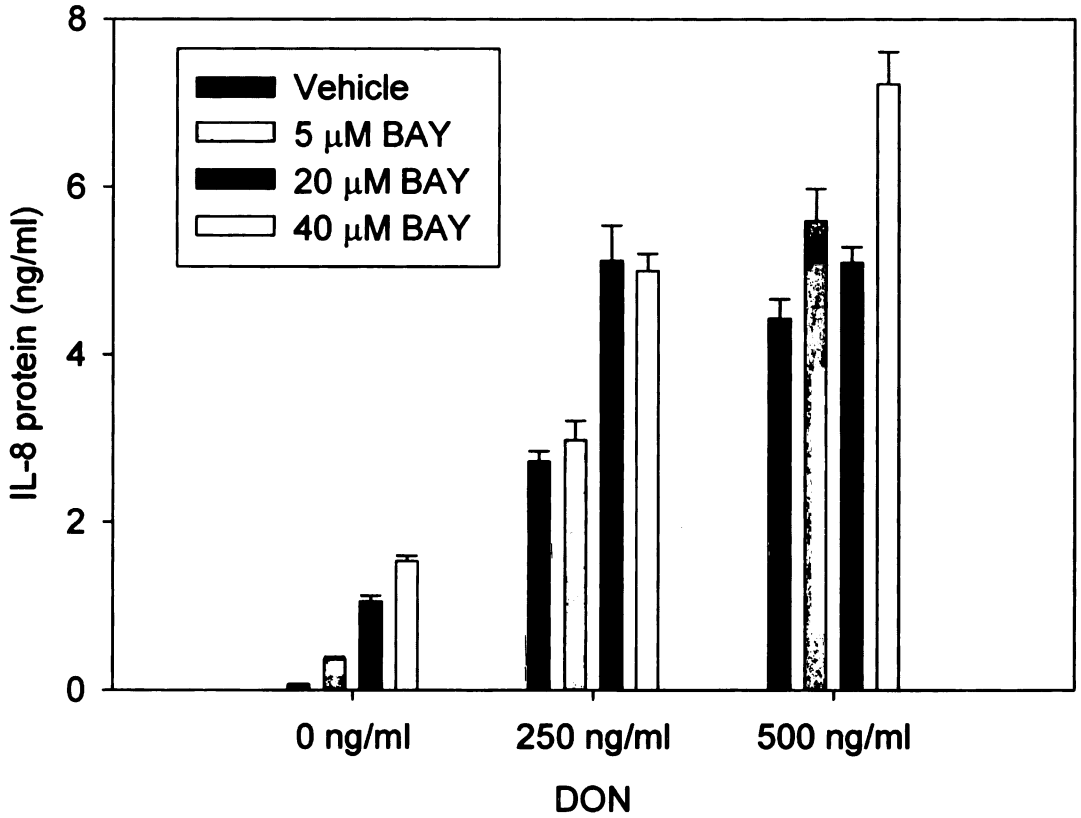

Figure C.2: BAY 11-7085 does not inhibit DON-induced IL-8 protein production. U937 cells were exposed to a 12 h co-treatment of BAY 11-7085 (0, 5, 20, or 40 μM) with DON (0, 250, or 500 ng/ml). Data are mean ± SEM (n = 3).

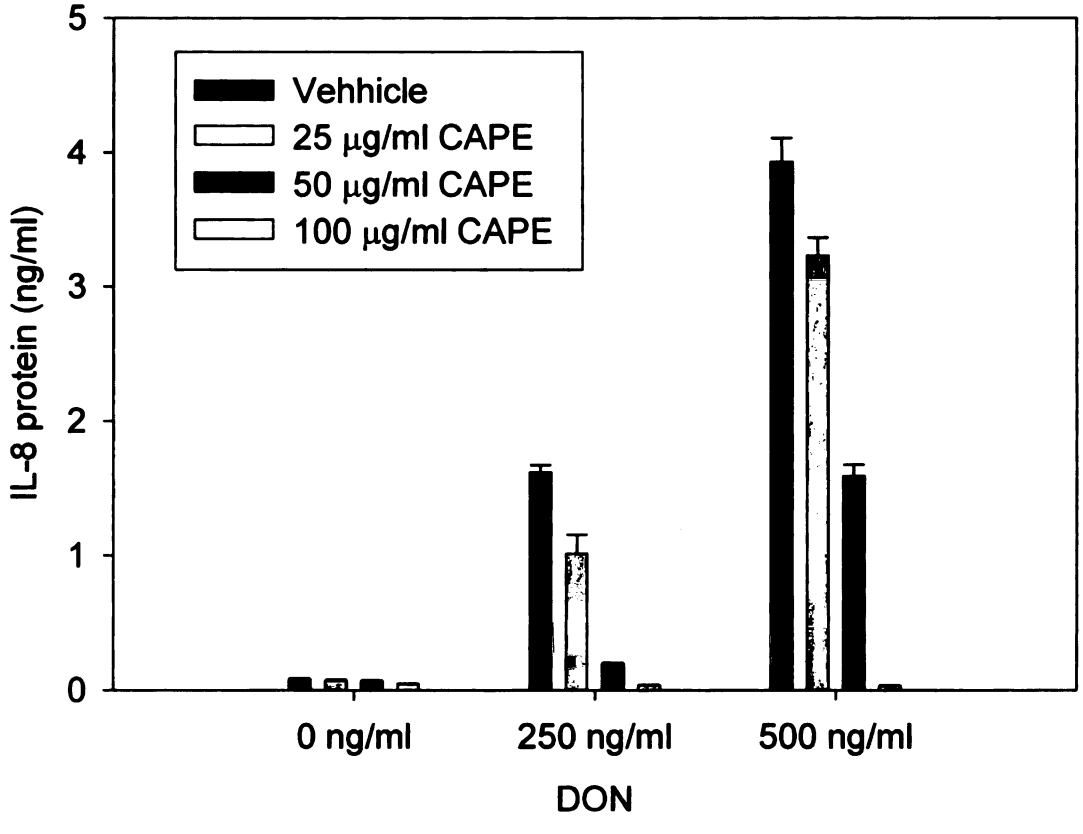

Figure C.3: CAPE inhibits DON-induced IL-8 protein production. U937 cells were co-exposed to CAPE (0, 25, 50, or 100 μg/ml) with DON (0, 250, or 500 ng/ml) for 12 h. Data are mean ± SEM (n = 3).

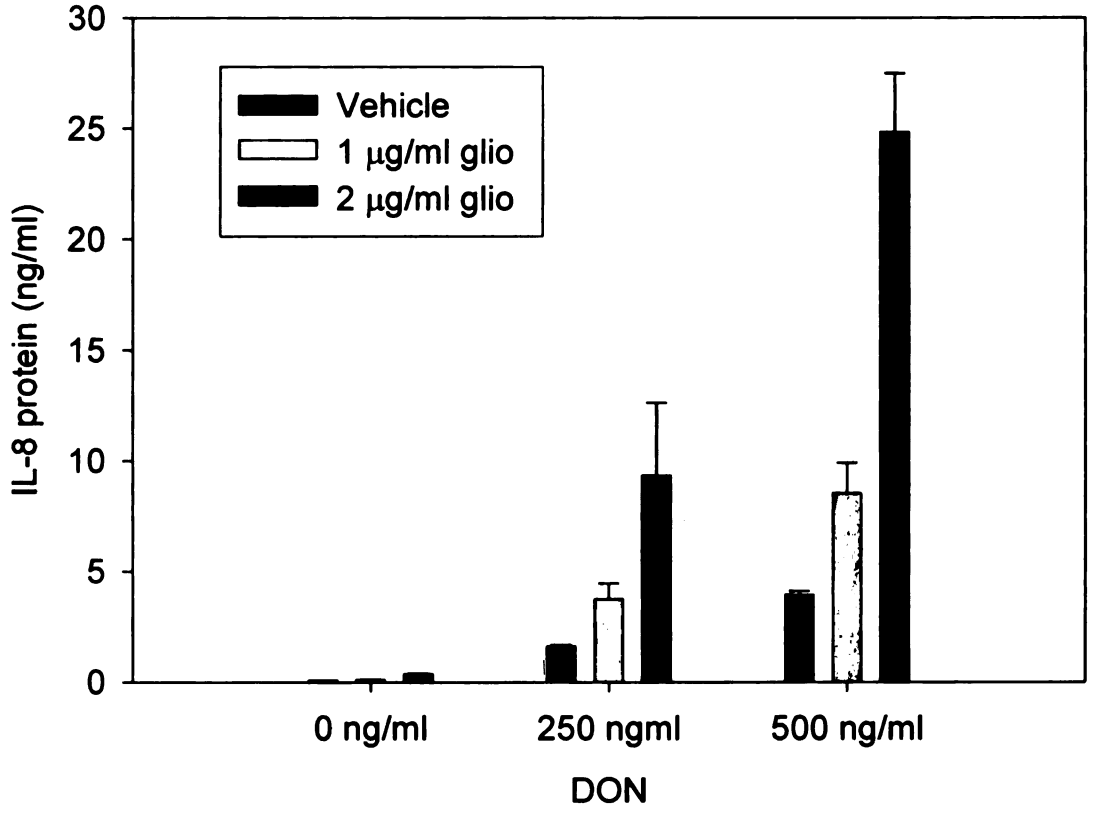

Figure C.4: Gliotoxin does not inhibit IL-8 protein production. U937 cells were co-treated with gliotoxin (0, 1, or 2 μg/ml) with DON (0, 250, or 500 ng/ml) for 12 h. Data are mean ± SEM (n = 3).

were also pretreated with the inhibitors before DON exposure. A 2 h timepoint was chosen based on the studies in Table C.1 (187, 215, 223). Even with the 2 h pretreatment, BAY 11-7085 (Figure C.5) was still unsuccessful in inhibiting DON-induced IL-8 protein. As Figure C.6 shows, CAPE pretreatment produced similar successful results to the CAPE co-treatment, with 100 μg/ml of CAPE completely inhibiting DON-induced IL-8 protein. Gliotoxin remained unsuccessful even with the 2 h pretreatment at both concentrations tested (Figure C.7).

CAPE was the only effective inhibitor of DON-induced IL-8 protein with both the co-treatment and the pretreatment of 100 μg/ml CAPE causing complete inhibition of DON-induced IL-8 protein. The next step was evaluating the effect of CAPE on IL-8 mRNA. For measuring IL-8 mRNA from CAPE- and DON-treated cells, a 2 h pretreatment was used. U937 cells were pretreated with 0, 50, or 100 μg/ml of CAPE and then treated for 3 h with or without 500 ng/ml DON or 5 ng/ml LPS. Both the 50 and 100 μg/ml of CAPE were effective at inhibiting DON-induced IL-8 mRNA, though the 100 μg/ml concentration showed the most inhibition of DON-induced IL-8 mRNA (Figure C.8). The 100 μg/ml CAPE concentration was chosen for the IL-8 mRNA stability experiments due to the complete inhibition seen of both DON-induced IL-8 protein and mRNA.

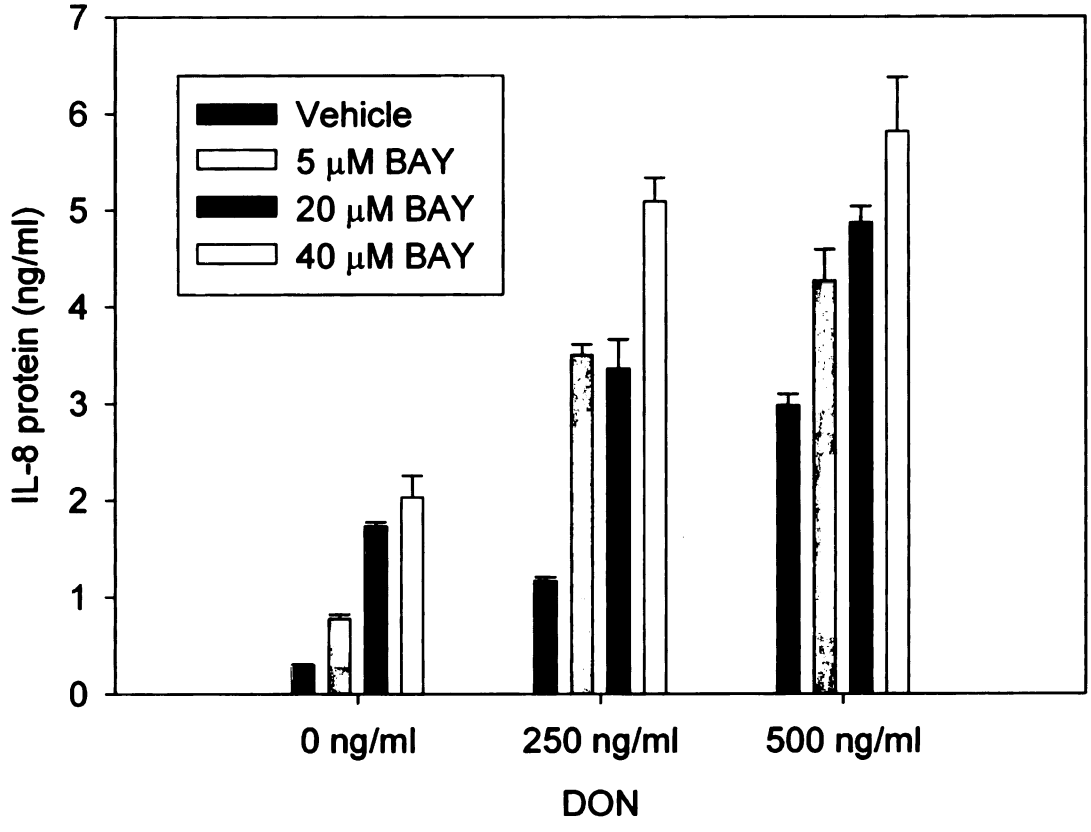

Figure C.5: BAY 11-7085 does not inhibit DON-induced IL-8 protein with a 2 h pretreatment. U937 cells were treated for 2 h with BAY 11-7085 (0, 5, 20, or 40 μM) then a 12 h treatment with DON (0, 250, or 500 ng/ml). Data are mean ± SEM (n = 3).

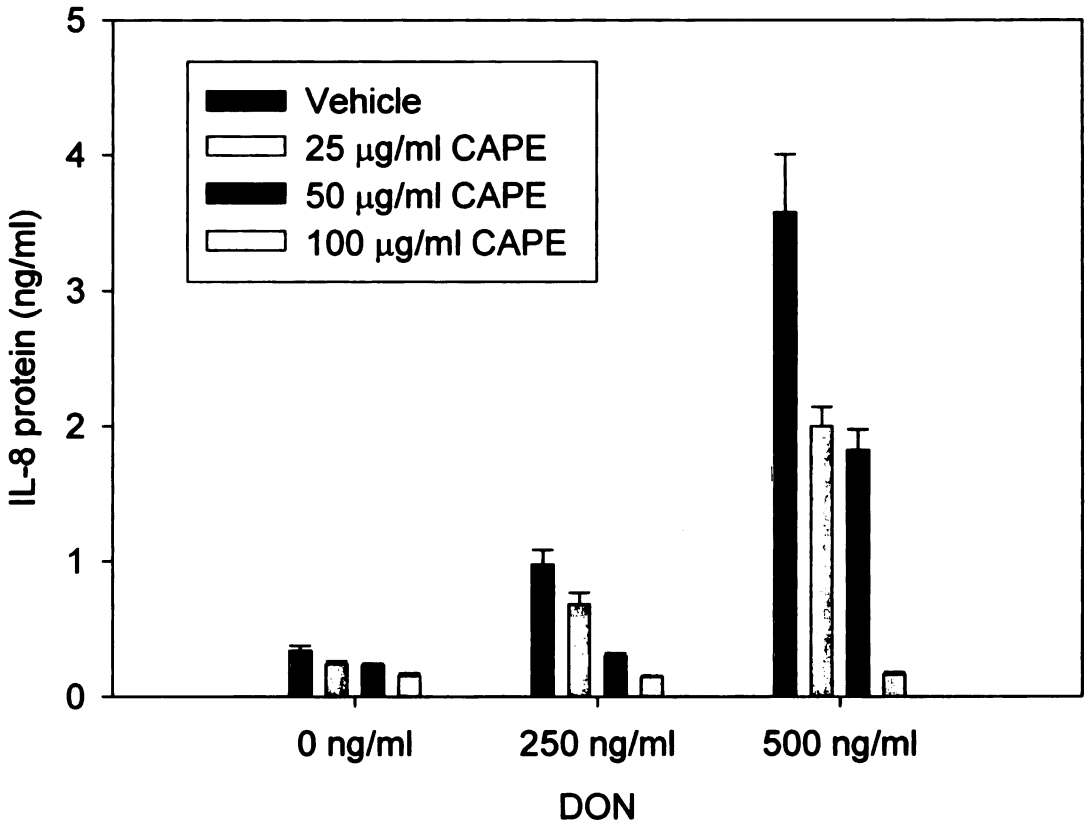

Figure C.6: CAPE inhibits DON-induced IL-8 protein. U937 cells were treated with CAPE (0, 25, 50, or 100 µg/ml) for 2 h then treated for 12 h with DON (0, 250, or 500 ng/ml). Data are mean ± SEM (n = 3).

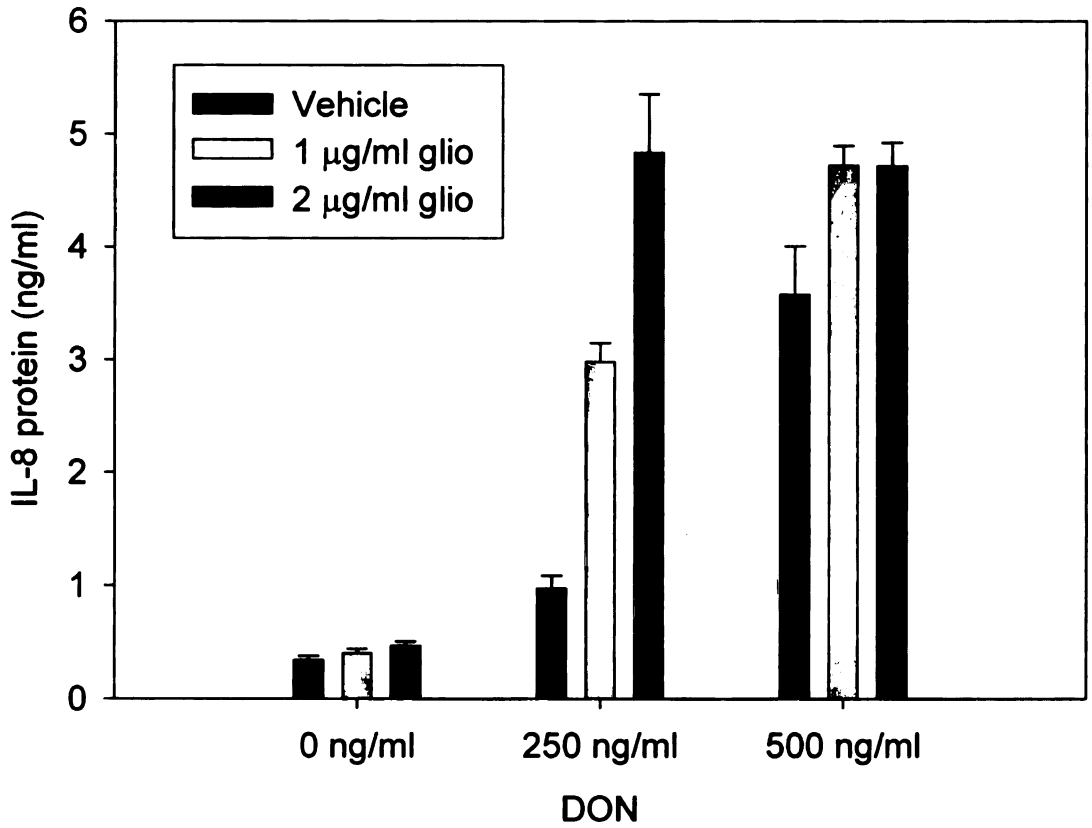

Figure C.7: Gliotoxin does not inhibit DON-induced IL-8 protein. U937 cells were given a 2 h pretreatment of gliotoxin (0, 1, or 2 μg/ml) then a 12 h treatment with DON (0, 250, or 500 ng/ml). Data are mean ± SEM (n = 3).

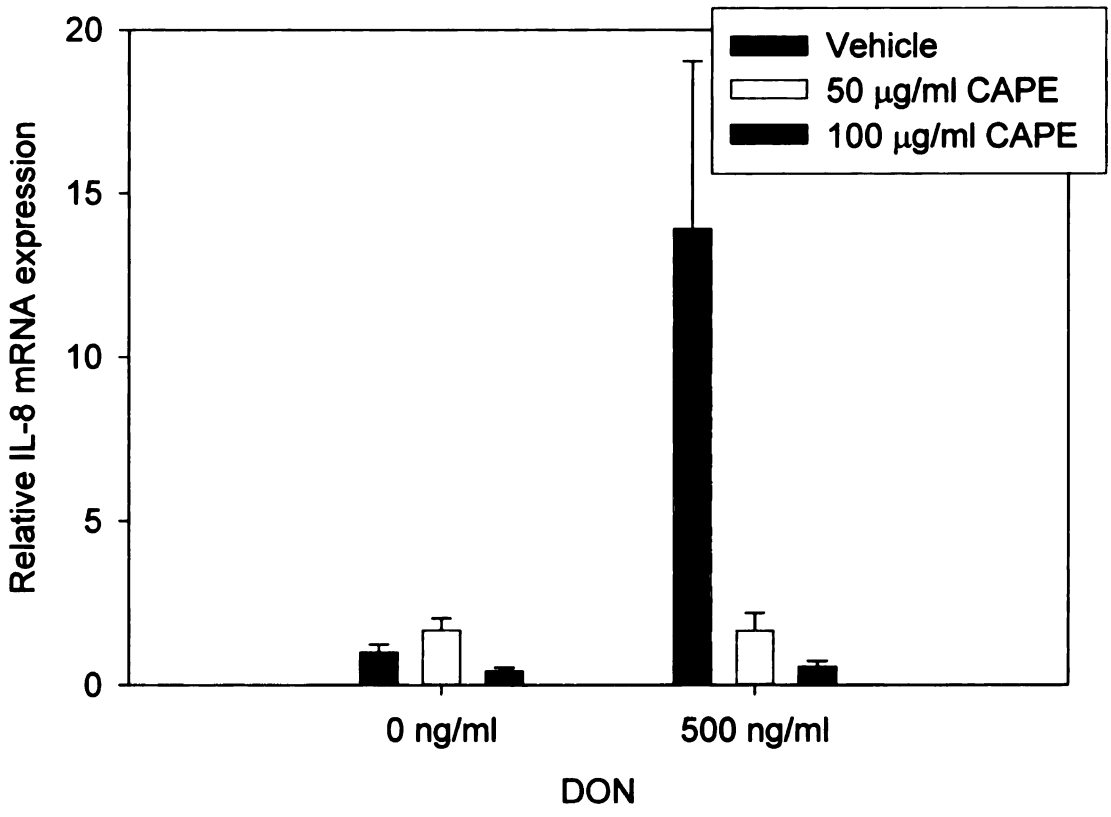

Figure C.8: CAPE inhibits IL-8 mRNA in U937 cells. Cells were pretreated for 2 h with either 0 or 100 µg/ml CAPE then treated for 3 h with 0 or 500 ng/ml DON. Representative of two independent experiments. Data are mean ± SEM (n = 3).

Conclusions

U937 cells did not appear to have transcription suppressed with two general transcriptional inhibitors, DRB and ActD. NF-κB inhibitors were assessed for suppression of IL-8 transcription and one of the inhibitors, CAPE, was found to completely inhibit IL-8 expression. CAPE was selected for use in experiments to determine if DON increased the stability of IL-8 mRNA.

APPENDIX D: IL-8 PROMOTER CONSTRUCT MUTATIONS AND PREDICTIONS OF BINDING SITES USING TRANSCRIPTION FACTOR BINDING SITE RECOGINITION DATABASES

Abstract

Two different web-based transcription factor binding site recognition programs (TFSEARCH and PROMO) were used to determine if the site-directed mutations in transcription factor binding sites, specifically AP-1, C/EBPβ, Oct-1, NF-κB, or NRF, of the IL-8 promoter-driven luciferase constructs would prevent binding of the transcription factors examined. Both web-based programs gave similar results and, in general, predicted that the mutations present in the luciferase constructs would prevent the specific transcription factors of interest from binding. Furthermore, the programs suggested that, except for one potential increase in a basal transcription factor in the construct with the AP-1 mutation, most other potential binding sites would not be altered.

Introduction

Many published studies have used mutations in either promoter constructs or EMSA probes to weigh the involvement of various transcription factors. In the past several decades, web-based programs have been available to determine potential binding sites and provide *in silico* verification that the mutations are actually preventing binding. An additional advantage of using these programs is that transcription factors other than the ones directly examined can be screened for differential binding.

The wild-type sequence for the IL-8 promoter (Genbank Accession number NT_006216.14 base pairs 3113125 to 3113282) contains 5 major transcription factor

binding sites, AP-1, C/EBPβ, Oct-1, NF-κB, and NRF, that are important for transcription of IL-8 in multiple cell types under a wide variety of condition.

In these studies, a wild-type IL-8 promoter-driven luciferase construct was employed and the site-directed mutagenesis was utilized to assess the role of specific transcription factors in DON-induced IL-8 (see Materials and Methods, Chapter 3). Two web-based programs were used to examine the wild-type IL-8 promoter and effects of mutations in the IL-8 promoter.

Methods

Both TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) (174) and PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) (172, 173) employ a version of the TRANSFAC database that compiles transcription factors and binding site sequences. All of the IL-8 promoter sequences, wild-type and all five mutant sequences, were entered individually into each of web-based programs and the results were assessed.

For TFSEARCH, sequences were entered to the site and the vertebrate matrix was selected for comparison. The threshold score was left at the default of 85% similarity, indicating that sites with less than 85% similarity to sites in the IL-8 promoters would be disregarded. The sequence was submitted and the results were examined. The PROMO program had more complex options.

For PROMO, only human factors were considered with binding sites used from eukaryota. The maximum matrix dissimilarity rate was left at the default of 15,

indicating that binding sites would only be predicted if the site similarity was equal to or greater than 85%. The sequences were then submitted and the output was examined.

Results and Discussion

Predicted transcription factor binding sites using TFSEARCH

TFSEARCH was the first program used to assess the mutations of the IL-8 promoter. When the wild-type sequence was searched using the vertebrate sequences AP-1 and NF-κB binding sites were found. NRF was not found at all on the promoter sequence and potential C/EBPβ and Oct-1 binding sites were not found in their correct locations. Use of all possible sequences for these transcription factors did not alter these basic findings. Table D.1 contains a summary of this information.

Mutations of the NRF, C/EBPβ, and Oct-1 sites did not change the potential binding of AP-1 or NF-κB and did not alter the basic score of these two transcription factors. Scores are the percent similarity to the consensus sequences and were approximately 90% for both AP-1 and NF-κB.

When the AP-1 mutation IL-8 promoter was assessed, binding of AP-1 was absent and there were no alterations to scores of other potential transcription factor binding sites in that area. Similar results were obtained with the NF-κB mutated IL-8 promoter. Potential binding of NF-κB was completely absent and other potential

Table D.1: Transcription factor binding site predictions using TFSEARCH.

		Percent similarity according to the TFSEARCH website					
Binding site	Transcription factor	IL-8 wild-type promoter	mAP-1 IL-8 promoter	mC/EBPβ IL-8 promoter	mOct-1 IL-8 promoter	mNF-κB IL-8 promoter	mNRF IL-8 promoter
AP-1	AP-1	89.4%-98.3%	no binding	Identical to wild-type	Identical to wild-type	Identical to wild-type	Identical to wild-type
C/EBP and Oct-1	None found	None found	Identical to wild-type	Identical to wild-type	Identical to wild-type	Identical to wild-type	Identical to wild-type
NF-κB and NRF	NF-κB	87.5%-96%	Identical to wild-type	Identical to wild-type	Identical to wild-type	no binding	87%-96%
	cRel	86%-93.4%				no binding	92.6%-93.4%

transcription factor binding was unaltered. Since TFSEARCH only assessed the binding of AP-1 and NF-κB for the IL-8 promoter a second web-based program, PROMO, was used.

Predicted transcription factor binding sites using PROMO

PROMO was also used to assess potential transcription factor binding sites. As with TFSEARCH, NRF was absent from the assessment. Additionally, the specific factor that was Oct-1 was difficult to identify. This database contains several POU domain entries and different octamer family proteins. Using the wild-type IL-8 promoter, AP-1 and various constituents (such as c-Jun and c-Fos), C/EBPβ, a variety of Oct family factors, and NF-κB potential binding sites were identified in expected locations. All mutated promoters assessed had no alterations except in the area of the mutation. Table D.2 contains a summary of this information.

In the mutated NRF promoter, there was a decrease of the basic NF-κB score and potential binding sites of components of NF-κB (NF-κB1 and RelA) were found. For the mutated NF-κB promoter, no binding was found for NF-κB or members of the NF-κB family. This matches the results obtained using the TFSEARCH program.

With the Oct-1 mutated promoter, several increases to three different potential binding sites occurred. Two of these were C/EBPβ transcription factors and one was a member of the Oct family. There was no change to a majority of the Oct family and POU domain members. This is not alarming because the specific Oct-1 transcription factor that interacts with a binding site on the IL-8 promoter might not be in the databas

Table D.2: Transcription factor binding site predictions (in percent similarity) using PROMO.

Binding site	Transcription factor	IL-8 wild-type promoter	mAP-1 IL-8 promoter	mC/EBPβ IL-8 promoter	mOct-1 IL-8 promoter	mNF-κB IL-8 promoter	mNRF IL-8 promoter
AP-1	c-Jun	100.00%	no binding	Identical to wild-type	Identical to wild-type	Identical to wild-type	Identical to wild-type
	c-Fos (1)	100.00%	no binding				
	JunD	87.17%	no binding				
	AP-1	100.00%	no binding				
	TFIIB	89.48%	95.36%				
C/EBPβ and Oct-1	POU3F2	91.64%	Identical to wild-type	no binding	91.64%	Identical to wild-type	Identical to wild-type
	Oct-B1	87.50%		no binding	87.50%		
	C/EBPβ	98.50%		no binding	99.36%		
	C/EBPβ	98.04%		no binding	100.00%		
	POU2F1	86.36%		no binding	86.36%		
	POU2F2	86.90%		no binding	86.90%		
	POU2F2 (Oct-2.1)	93.64%		no binding	94.43%		
	octamer-binding factor	86.61%		no binding	86.61%		
	POU2F2B	87.50%		no binding	87.50%		
NF-κB and NRF	POU3F2	92.67%	Identical to wild-type	Identical to wild-type	Identical to wild-type	94.04%	92.67%
	p300 (1)	97.30%				97.30%	97.30%
	p300 (2)	94.59%				94.59%	94.59%
	NF-κB	96.42%				no binding	85.78%
	c-Fos (2)	91.71%				91.71%	no binding
	NF-κB1	no binding				no binding	90.56%
	RelA	no binding				no binding	97.80%

or the sequence on the IL-8 promoter may be different enough from the consensus sequences to be unrecognizable with the cut-off of 85% similarity. This percent similarity means that of sequences are more then 15% different they will not even be on the results from the program.

The mutated AP-1 promoter results were the most interesting. Along with no AP-1 binding for the mutated promoter there was an increase in percent similarity of a basal transcription factor, TFIIB. Potential TFIIB binding increased from 89.48% in the wild-type IL-8 promoter to 95.36%. While it is unclear exactly what biological significance of this difference is, it provides a possible reason for the increased IL-8 promoter-driven luciferase expression observed for the mutant AP-1 construct (see Chapter 3). TFIIB is responsible for stabilizing the interaction of TBP with the DNA as well as recruiting the RNA Polymerase II complex and selection of the transcriptional start site (224, 225).

It should be mentioned that both of these web-based programs might not contain specific transcription factors important for IL-8 transcription or that if transcription factors are present in the databases there is some uncertainty about the form of the transcription factor. For example, neither of the programs have entries on NRF and some binding sites are just listed as AP-1 or NF-κB without any mention of the specific dimer composition of the factors. Additionally, experimental reports might show that binding is altered with certain mutations that the programs will not detect due to reliance on consensus sequences.

Conclusions

The wild-type IL-8 promoter and the five promoters with mutations in transcription factor binding sites (AP-1, C/EBPβ, Oct-1, NF-κB, or NRF) were screened using two web-based transcription factor binding site recognition programs, TFSEARCH and PROMO. Overall, results from these two programs indicated that the wild-type IL-8 promoter had the transcription factor binding sites of interest and that the point mutations used should disrupt these binding sites. Additionally, a potential increase in the binding of a basal transcription factor, TFIIB, was found in the mutant AP-1 promoter, which would provide a tentative explanation for the increased luciferase expression results presented in Chapter 3.

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