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MODULATION OF GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR AXIS BY TRICHOTHECENE DEOXYNIVALENOL

By

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ABSTRACT

MODULATION OF GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR AXIS BY TRICHOTHECENE DEOXYNIVALENOL

By

Chidozie Joshua Amuzie

Deoxynivalenol (DON), a fungal metabolite by *Fusarium sp* is one of 217 known trichothecenes. DON is the most commonly detected trichothecene in cereals and processed food for human consumption worldwide. In addition, DON can become airborne in the breathing zones of some agricultural workers. Upon exposure, DON is distributed (≤30 min) to tissues, and induces proinflammatory cytokines. Chronic DON exposure reduces weight gain in many species. However, the underlying mechanisms for this effect are less understood, thus creating uncertainties in regulatory limits and human safety assessment. The central hypothesis of this dissertation is that proinflammatory cytokine upregulation is associated with modulation of the growth hormone (GH)/insulin-like growth factor (IGF) axis in DON-exposed mice. Various models of murine DON exposure were used to test this hypothesis. The effects of oral and intranasal DON exposure were compared relative to proinflammatory cytokine upregulation, as a model for foodborne and airborne DON. Regardless of exposure route, DON concentrations rapidly (15 to 30 min) peaked in plasma and tissues, with subsequent (1 h) cytokine mRNA upregulation. However, DON concentrations were greater (1.5 to 3 times) by nasal exposure, and proinflammatory cytokine mRNAs such as IL-1β, IL-6, and TNF-α were also greater (2 to 10 times) following nasal exposure than oral exposure. Based on the transient nature of DON-induced cytokines, additional studies were done to determine

DON's capacity to induce suppressors of cytokine signaling (SOCS). DON dose-dependently (1 to 12.5 mg/kg bw) induced several SOCS mRNAs in many tissues. Hepatic SOCS3 mRNA was a sensitive indicator of DON exposure and hepatic SOCS3 protein remained upregulated after cytokine decline (5 h). Since SOCS elevation is associated with hepatic GH impairment, DON-induced modulation of the GH/IGF axis was investigated. DON exposure rapidly (2 h) and dose-dependently (0.5 to 12.5 mg/kg bw) suppressed hepatic insulin-like growth factor acid-labile subunit (IGFALS) mRNA by 60-80%, with or without exogenous GH, and with concurrent hepatic SOCS3 mRNA increase. Chronic dietary DON exposure also impaired hepatic IGFALS mRNA by 65% after wk 8. DON exposure also suppressed circulating IGFALS and associated IGF1 by 66 and 26 %, respectively; concurrent with elevation of plasma DON ($\leq$ 63 ng/ml), and weight gain reduction. Additional studies investigated DON's capacity to reduce IGFALS in mice fed high fat diet (HFD), and diet-induced obese (DIO) mice. Again, dietary DON (5 and 10 ppm) suppressed circulating IGFALS by 18 and 30% in lean mice on HFD; and by 20 and 42 % in DIO mice. IGFALS suppression was related to adiposity and weight gain reduction in both models. Taken together, these data indicate that DON sequentially upregulates cytokines and SOCS, but suppresses circulating IGFALS and weight gain in different dietary and physiological conditions. DON also shifted the phenotype of mice on high fat diet towards that of low fat diet. Therefore, circulating IGFALS might be a potential biomarker of DON's effect in mice. Furthermore, the association of IGFALS suppression with weight loss in obese mice suggests that the GH/IGF axis might be a novel target for obesity prevention and control.

DEDICATION

This work would not have been possible without my parents Young and Stella Amuzie, and my grandfather Samuel Uguru who stood beside me at various levels of curricular education over the last 27 years. They taught me the principles of work, shared their unwavering belief in education as the way of personal advancement, and were unflinching in their support.

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
AP-2	Activating protein 2
AUC	Area under the curve
AR	Androgen receptor
bw	Body weight
C/EBP	CCAAT-enhancer binding proteins
CP2	Transcription factor CP2
CIS	Cytokine-inducible SRC homology-domain containing protein
CREB	cAMP response element binding
DIO	Diet-induced obesity
DON	Deoxynivalenol
DR1	Down-regulator of transcription 1
DR4	Death receptor 4
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
E2F1	E2F transcription factor 1
GH	Growth hormone
HFD	High fat diet
HNF4	Hepatocyte nuclear factor 4
IGF1	Insulin-like growth factor 1
IGFALS	Insulin-like growth factor binding protein, acid-labile subunit
IGFBP3	Insulin-like growth factor binding protein 3

IGFBP5	Insulin-like growth factor binding protein 5
IL-1	Interleukin 1
IL-6	Interleukin 6
IPCS	International programme on chemical safety
JAK	Janus kinases
JNK	c-Jun N-terminal kinase
kg	Kilogram
LFD	Low fat diet
LPS	lipopolysaccharide
LYF1	IKAROs family zinc finger 1
MAF	Musculoaponeurotic fibrosarcoma transcription factor
MAPKS	Mitogen-activated protein kinases
mg	Milligram
mRNA	Messenger ribonucleic acid
MSU	Michigan state university
MZF1	Myeloid zinc finger 1
NF1	Nuclear factor 1
NOAEL	No observed adverse effect level
OSHA	Occupational safety and health administration
PAX4	Paired box gene 4
PBX1	Pre-B-cell leukemia homeobox 1
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
ppb	Parts per billion
ppm	Parts per million

PPAR	Peroxisome proliferator-activated receptor
P53	Protein 53
p-38	P38 mitogen activated protein kinases
SH2	Src homology 2
SMAD	SMAD protein
SOCS	Suppressors of cytokine signaling
SP1	Sp1 transcription factor
SRF	Serum response factor
STAT	Signal transducers and activators of transcription protein
TDI	Tolerable daily intake
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor-alpha receptor
T3R	Thyroid hormone receptor
USF	Upstream transcription factor

INTRODUCTION

Toxicologists are often required to determine the risks posed by natural and synthetic compounds that contaminate human food and the environment. The process of risk assessment by its nature is laden with uncertainties, in part because the test organisms and scenarios are often different from real world. Recently, uncertainties in risk assessment of trichothecene (a group of over 200 fungal metabolites in food and the environment) exposures have raised human safety concerns because of their continued presence in human food supply and water-damaged buildings (Pestka *et al.* 2008b). Deoxynivalenol (DON) is the most commonly detected trichothecene in cereal grains around the world and its exposure is unavoidable because its contamination of grains cannot be prevented and it is not destroyed by milling processes (Pestka and Smolinski 2005). In problematic years, DON-related economic losses can be up to $1 billion (CAST 2003), because of rejected and destroyed cereal grains. Determination of human risk(s) associated with low levels of DON in global food supply has been difficult because of uncertainties in mechanism(s) of effects in experimental animals and additional uncertainty in human dose-response determination.

DON is regulated because of concerns for growth retardation and immunotoxicity based on a two-year animal study in mice (Tritscher and Page 2004). However, the mechanism(s) for this growth retardation and immunotoxicity are not sufficiently clear, and evidence for such effects in human populations with low levels of DON in diet is not obvious. The US Food and Drug Administration (FDA) has

established a 1 ppm level of concern for DON in flour that is in concurrence with Japanese and Canadian standards. On the contrary, the European Union has established different regulatory limits for DON (i.e 200 ppb and 750 ppb for infant and adult foods, respectively). These regulatory differences highlight the level of uncertainty in DON risk assessment but also create additional burden for global trade and food sufficiency. Clearly, the knowledge gap in DON mechanisms has led to human safety concerns, regulatory uncertainties and potential trade barriers in many countries.

The International Programme on Chemical Safety (IPCS) has warned that the application of risk assessment paradigm to trichothecenes should consider human health, trade and food sufficiency (Tritscher and Page 2004). There is increasing recognition of the need for reduction of uncertainty in risk assessment, by incorporation of mechanistic knowledge (McClellan 1996; Renwick 2000). Detailed knowledge of the mode of action in experimental animals is a prerequisite for precise use of animal data in determination of human risk. The iterative nature of risk assessment will allow the use of such mechanistic knowledge to (re)define susceptible populations so that human safety decisions can be made with reduced uncertainty, and discrepancies in regulatory limits might be resolved with sufficient scientific evidence.

In addition to the uncertainty in foodborne DON, there is uncertainty regarding the potential risks of airborne DON in agricultural environments. DON has been detected in agricultural dusts (Halstensen *et al.* 2006; Nordby *et al.* 2004b) and agricultural workers who may inhale DON-containing grain dust are thought to have an additional risk of toxin effects (Halstensen *et al.* 2006).One way to reduce these

uncertainties is to identify complementary biomarkers of DON exposure and effect that are measurable in body fluids, which would enhance epidemiological surveillance in the risk assessment process.

Recently, our lab and others have used ELISA and mass spectrometry to measure DON in murine plasma and human urine, respectively (Pestka *et al.* 2008a; Turner *et al.* 2008a). While these efforts might facilitate the measurement of DON in body fluids as a biomarker of exposure, most plasma DON is cleared within a few hours, regardless of exposure route (Amuzie *et al.* 2008). The elimination profile of DON could limit the utility of plasma/urinary DON as a biomarker of exposure, since these will be mostly indicative of exposures within a few hours/days. Therefore, utility of this biomarker of exposure needs to be enhanced by complementary mechanism-related biomarker(s) of effect, which are also in body fluids, but longer lasting. Such biomarkers of effect could also be related to dietary DON concentrations and might enhance epidemiological surveillance of DON effects, thereby reducing uncertainties in risk assessment. Clearly, identification of a mechanism-linked biomarker of effect is a critical gap in our understanding of the risk(s) associated with human DON exposure.

Previous DON studies indicate that proinflammatory cytokine upregulation is a rapid, acute event that occurs in DON exposed murine (Wong *et al.* 1998) and human cells (Islam *et al.* 2006). Furthermore, cytokine upregulation is a central acute event in whole animals across many species (Pestka and Smolinski 2005). However, for subchronic to chronic DON exposure, weight gain reduction, which has also been termed growth retardation occurs in many species (Pestka and Smolinski 2005; Rotter *et al.*

1996). The common occurrence of acute proinflammatory cytokine induction and subchronic weight gain reduction in many species suggest that these events could be linked mechanistically, and that perhaps there might be other common markers on the acute-chronic continuum of DON effects. Previous investigators have suggested anorexia as a mechanism for DON-induced weight gain effects but could not identify any anorexia-linked biomarkers of effect in plasma (Prelusky *et al.* 1997; Prelusky and Trenholm 1993), thereby making the anorexia hypothesis inadequate for uncertainty reduction in risk assessment. In this dissertation, an alternative hypothesis that is based on cytokine signaling and includes a potential biomarker will be proposed, investigated and presented.

Overall Hypothesis

Based on the central nature of cytokine signaling in DON exposed animals, the goal of this dissertation is to use proinflammatory cytokine signaling as a foundation to extend knowledge, reduce uncertainty and identify potential biomarkers of effect in DON exposed animals. The guiding hypothesis is that proinflammatory cytokine induction is a central early event in DON exposed animals that is associated with cellular dysregulation and impairment of the GH/IGF axis in mice. The specific aims are to (1) compare proinflammatory cytokine induction by oral and nasal routes of DON exposure, (2) determine the capacity of DON to induce suppressors of cytokine signaling (SOCS) along with proinflammatory cytokines, (3) determine the capacity of DON exposure to impair hepatic GH signaling and suppress circulating IGFALS and (4) determine the influence of obesity on DON-induced IGFALS impairment.

Chapter summaries and hypothesis

Chapter 1 is a review of literature that is related to the research problems addressed in this dissertation. It includes an introduction of trichothecenes and DON relative to toxicity and potential human exposure scenarios. The anorexia hypothesis of DON-induced weight gain reduction will be presented together with its shortcomings and the central acute nature of proinflammatory cytokine induction in DON-exposed animals. Subsequently, an alternative hypothesis for DON-induced weight gain reduction that is based on cytokine signaling studies will be proposed and the supporting evidence discussed.

Suppressors of cytokine signaling will be introduced and discussed relative to the similarities between cytokine and growth hormone (GH) signaling pathways. Potential checkpoints of SOCS interference on the GH signaling pathway are presented. GH induction of the insulin-like growth (IGF) system will be introduced and discussed in the context of its relevance to postnatal development, potential for SOCS-related impairment, and the inherent complexities on the axis. The evidence that chronic DON effects such as reduction in weight gain are opposite to the effects seen in animals with elevated circulating IGF1 is presented with the contention that DON might act, in part, by opposing the IGF system.

Potential anti-IGF1 effects of DON will be extended relative to the need for additional preventive/therapeutic strategies in the management of overweight and obese patients. Emerging evidence for the role of IGF system in adipocyte differentiation, maturation and maintenance are presented. Finally, the regulatory uncertainties

associated with human risk of DON exposure will be discussed. The need to reduce regulatory uncertainty by identifying biomarkers of effect is the concluding argument of chapter 1.

In Chapter 2, the effects of nasal and oral DON exposure routes will be compared, relative to plasma and tissue DON concentrations as well as proinflammatory cytokine induction in the mice. Data presented in this chapter have been published and can be found at http://www.ncbi.nlm.nih.gov/pubmed/18433975?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum, and indicate that there are route-dependent quantitative differences in tissue DON concentration and proinflammatory cytokine induction. The data indicate that proinflammatory cytokine induction is a rapid (1 h), transient, route-independent acute event in DON-exposed mice

In chapter 3, the capacity of DON to induce suppressors of cytokine signaling (SOCS) in addition to proinflammatory cytokines in murine tissues will be determined. Real-time PCR and immunohistochemistry will be used to demonstrate sustained upregulation (beyond 5 h) of SOCS in hepatocytes. The data will indicate that SOCS are upregulated, concurrent with, or immediately after proinflammatory cytokines in many tissues. The fact that hepatic SOCS3 mRNA and protein upregulation lasted beyond hepatic cytokine upregulation will be noted, with the contention that there might be a functional significance of elevated SOCS in DON-exposed mice.

The capacity of DON exposure to impair GH signaling in a SOCS-associated manner

will be presented in chapter 4. Acute and chronic models of DON exposure will be used to demonstrate that DON exposure acutely (3 h) impairs hepatic GH-induced IGFALS mRNA expression. Chronic dietary DON exposure (8 wk) will reveal that IGFALS mRNA impairment is sustained and associated with a marked reduction of circulating IGFALS and circulating IGF1. The data will relate a potential biomarker of effect (circulating IGFALS) to a biomarker of exposure (plasma DON) and an observed phenotype (weight gain reduction) in DON-exposed mice.

In chapter 5, the hypothesis that DON-induced IGFALS reduction is associated with obesity prevention and attenuation will be investigated and presented. Experimental models of diet-induced obesity (DIO) prevention and therapy were used to demonstrate that DON prevents and attenuates obesity. DON's effects on obese mice will be associated with IGFALS reduction, with the suggestion that IGFALS is a biomarker of effect in different physiological states (lean and obese).

The conclusion will integrate the data presented in this dissertation and outline its relevance to human risk assessment and potential to uncover anti-obesity targets.

Significance

This work is unique because it integrates known acute and chronic events of DON in a manner that identifies new target tissue, involvement of new signaling molecules and pathways, potential mechanism of action and target for disease prevention. Researchers may now use DON in body fluids as a biomarker of exposure to complement circulating IGFALS as a biomarker of effect in other species and ultimately humans. IGFALS suppression might be a screening assay for other environmental chemicals that suppress

weight gain. Overall, identification of IGFALS suppression has the potential to significantly improve risk assessment and prevent human illnesses.

CHAPTER 1: LITERATURE REVIEW

Trichothecenes

Trichothecenes include over 200 compounds (Grove 2007) produced by several toxigenic fungi. Members of this group have a basic 15-carbon skeleton (trichothecene ring), characterized by an unsaturated bond at C9-C10 position and a 12,13-epoxy ring (Grove 2007). Trichothecenes are subdivided into Types A, B, D (macrocyclic) based on additional keto, acetyl, hydroxyl, and cyclic rings that are attached to the basic trichothecene ring. Cellular toxicity among various trichothecenes may vary up to 4 orders of magnitude depending on which functional groups are present and the toxicity outcomes that are evaluated (Rotter *et al.* 1996). Trichothecenes are protein synthesis inhibitors (Ehrlich and Daigle 1987) and are of concern because of (a) unpreventable contamination grains, (b) resistance to milling and processing techniques, (c) economic losses due to crop losses and reduced livestock production efficiency and (d) potential health effects resulting from human exposure (Pestka and Smolinski 2005).

Deoxynivalenol (DON)

DON is a trichothecene with a ketone group in the C8 position and is associated with soft, shriveled, often pink wheat ("scabby wheat") as well as shriveled barley. DON is produced by strains of *Fusarium graminearum* and *Fusarium culmorum*, and was originally called "vomitoxin" because of its emetic effects on swine (Rotter *et al.* 1996). DON, the most commonly detected foodborne trichothecene both in North

America and Europe (Placinta *et al.* 1999), is detectable in cereals (Trucksess *et al.* 1995) and processed foods (Lombaert *et al.* 2003; Wolf-Hall and Schwarz 2002). Economic losses due to scab have been estimated at $ 700 million to as high as $ 1 billion (CAST 2003). To illustrate the extent of this problem, most cereal processors refused Michigan-produced wheat in 1996, 2000 and 2004.

Human DON exposure

Low concentrations of DON are frequently detected in human diets (Lombaert *et al.* 2003; Wolf-Hall and Schwarz 2002) and more recently in human urine (Turner *et al.* 2008b). However, acute human poisoning is sporadic and has been mostly anecdotal. In 1913 and the 1930s, fatal outbreaks of a disease known as alimentary toxic aleukia (ATA) were reported in Siberia and involved vomiting, leucopenia and hemorrhage in people that ate overwintered grains. The involvement of trichothecenes in ATA was proposed after trichothecene producing *Fusarium* were recovered from grain isolates obtained during the outbreaks, many years later (Pestka and Smolinski 2005). In the late 1980s, over 20 gastroenteritis outbreaks were reported in China, involving several thousand people. Wheat samples collected from the Chinese outbreaks contained DON at various concentrations (2-50 ppm). Furthermore, DON-related gastroenteritis outbreaks have been reported in India and USA, albeit with inconclusive evidence (Pestka and Smolinski 2005). The dynamics of DON occurrence in food suggests that most of the global population will be exposed to low levels of DON in cereals and processed food, while high level exposures may occur sporadically in certain countries of the world.

Risk assessment of human DON exposure

The human effects of foodborne DON and the dose(s) at which these effects might occur is a primary area of uncertainty. DON is regulated by governmental food safety agencies worldwide because of its frequent occurrence in food and associated human safety concerns. In toxin risk assessment, risk is the probability that a biological effect will occur when people are exposed to a particular toxin. Animal bioassays and epidemiological studies are used to determine the dose, above which an adverse effect is likely to occur (Tolerable Daily Intake [TDI]). Frequently, TDI is obtained by dividing a safe dose in an animal bioassay (i.e., no observed adverse effect level) by an additional safety factor of 100. This widely used safety factor assumes a 10-fold variation when extrapolating from the most sensitive animal species to humans and another 10-fold variation in sensitivity among human (Renwick 2000). Thus, TDI is a projected safe exposure dose for humans, which is usually 100 times lower than the observed safe dose in experimental animals. In the last decade, TDI for DON has been determined to be 1 μg/kg bw/day based on the absence of growth effects (the most sensitive endpoint) over a 2-year exposure in mice (Tritscher and Page 2004). However, use of default assumptions in TDI determination has been criticized, and increasing use of mechanisms encouraged (McClellan 1996).

Many countries have used the above DON TDI to set limits in grains and flour for human consumption, some of which are shown (Figure 1.1). In many countries, the opinion is that a DON level below 1000 ppb in human food does not pose a significant risk of adverse effects. Some exposure assessments were done in the European

population, and they indicate that human DON exposure across countries and across age groups is below the TDI (Leblanc *et al.* 2005; Turner *et al.* 2008b).

However, one Dutch probabilistic assessment suggested that in some countries, infants in a worst-case scenario (i.e., 95th percentile of wheat consumers) may be exposed to DON levels above the TDI (up to 3 μg/kg bw/day). The authors also used a probabilistic method to predict that growth reduction may occur in infants consuming European food and, therefore, recommended a reduction of DON limits in food to 129 ppb (Pieters *et al.* 2002). This probabilistic assessment became one foundation for the EU to propose a 5-fold reduction of DON limits in infant food to 200 ppb, yet other EU estimates suggest that most people in all age groups, at all wheat consumption levels are not exposed to DON levels above the TDI (Leblanc *et al.* 2005; Schothorst and van Egmond 2004).

It is uncertain that lowering the limits for DON in infant foods will greatly increase food safety, and there has been no clear demonstration of DON-induced effects in infants, in any country, at regular dietary exposure levels. Furthermore, the International Programme on Chemical Safety (IPCS) has warned that the application of risk assessment paradigm to trichothecene mycotoxins should consider human health, trade and food sufficiency" (Tritscher and Page 2004). Such balance may be

Country

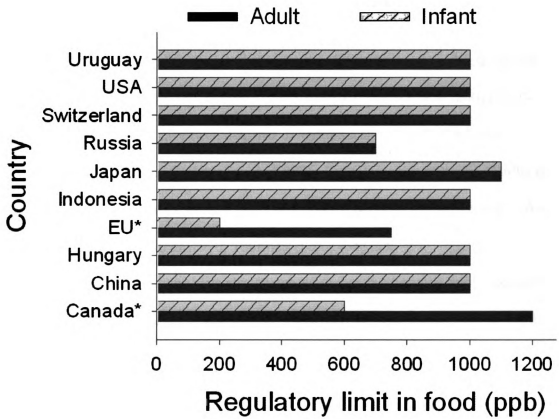

Figure. 1.1 Regulatory limits of DON in selected countries, adapted from (FAO 2003). Asterisk represents countries/regions with disparity in regulatory DON levels of adult and infant food

achieved by identifying biomarkers of exposure and complementary biomarkers of effect that are shared between laboratory animals and humans.

Another area of uncertainty in human risk of DON effects is among agricultural workers who are exposed to dust-borne fungi and its products (Radon and Nowak 2003), which are released into their breathing zones during harvesting, threshing and loading of grains (Hintikka and Nikulin 1998; Krysinska-Traczyk *et al.* 2001). DON and other trichothecenes have been detected in agricultural dusts resulting from handling wheat and barley (Halstensen *et al.* 2006; Lappalainen *et al.* 1996; Nordby *et al.* 2004b), and some studies suggest that inhalation exposure to trichothecenes evokes greater toxicity (Creasia *et al.* 1990; Thurman *et al.* 1988) than other routes. Epidemiological observations have also suggested that trichothecenes from Fusarium and other fungi might cause adverse effects in farmers and their families (Joffe 1978; Nordby *et al.* 2004a). It is not clear whether grain farmers are exposed to DON-related risks at levels higher than the average population, and whether inhalation route plays a major role in increasing DON risk(s).

Biomarkers and their use in reduction of uncertainty

Biomarkers have been defined by the U.S National Institutes of Health as a characteristic that is objectively measured as an indicator of biological processes or pharmacological responses (Lock and Bonventre 2008). Some organ system injuries have been associated with classical biomarkers. For example, elevated circulating alanine transaminase (ALT) indicates hepatotoxic injury, while elevated creatinine kinase indicates muscle damage. In a pharmacological/toxicological context, biomarker

definition may be broadened as parameters of injury or toxicity in animals or patients that help diagnose or monitor a disease, predict outcome or evaluate therapeutic intervention (Lock and Bonventre 2008). For high doses of xenobiotics that are associated with tissue injuries, biomarker identification is less cumbersome. The bigger challenge is to determine the most appropriate biomarkers for chronic low dose exposures in the human population.

Conventional epidemiology tends to use disease outcomes associated with chronic chemical exposure to provide dose-response relationships (Legator 1995). This method has been criticized as insensitive, and the use of biomarkers proposed as a more sensitive alternative (Legator 1995; Olden 1995). An ideal biomarker system for low dose xenobiotic exposure monitoring should include two components of the dose-response curve. First, a biomarker of internal dose (biomarker of exposure), which could be the toxin or its metabolite(s) that indicates body burden of toxin. Second, a complementary biomarker of early effect which represents both the central physiological process associated with the toxin and ultimate biological effect. Such biomarker system will provide more information than conventional environmental sampling and will allow dose-response modeling using internal dose and early biological effect. Therefore, an ideal biomarker system has the capacity to reduce the uncertainties that arise from interspecies extrapolation, delineate susceptible human populations through surveillance and thereby refine the risk assessment process.

Based on the aforementioned definitions, a biomarker should be a molecule on the exposure-effect pathway of a xenobiotic, and its utility in population studies will depend, in part, on the convenience of obtaining and analyzing samples from a large number of

people. Therefore, easily obtained body fluids like plasma or urine are good biomarker reservoirs (Lock and Bonventre 2008) as well as skin and hair when applicable.

For DON risk assessment, an ideal biomarker might be defined as a molecular/macromolecular indicator of DON exposure that is easily measured in urine and/or plasma of experimental animals and man. Identification of such molecule will enhance epidemiological surveillance of DON exposure in humans; enable appropriate dose-response modeling, more precise risk assessment and informed risk management decisions for regulators. Clearly, such task will require a detailed understanding of the mechanism(s) by which DON affects experimental animals.

DON toxicity

DON is rapidly absorbed and can reach peak plasma concentration within 30 mins of oral dosing both in swine (Prelusky *et al.* 1988) and mice (Amuzie *et al.* 2008). It undergoes de-epoxidation by gut micro-flora (He *et al.* 1992; Worrell *et al.* 1989) and glucuronidation (Obol'skii *et al.* 1998) as detoxification reactions. Acute exposure to DON can cause feed refusal and/or vomiting in some sensitive species (Hughes *et al.* 1999; Pestka *et al.* 1987a). In addition, monogastric mammals are sensitive to retardation of growth and weight gain (Arnold *et al.* 1986b; Forsell *et al.* 1986; Iverson *et al.* 1995; Rotter *et al.* 1992) when exposed to DON in a sub-chronic to chronic fashion.

At a high bolus dose (25 mg/kg bw), DON alone or in synergy with bacterial lipopolysaccharide (LPS), can induce lymphocyte apoptosis in mice (Islam *et al.* 2002; Zhou *et al.* 2000). Short term (2 wk) dietary concentrations of DON ($\geq$ 25 ppm) can

impair resistance to *Listeria monocytogenes*, but mice may recover after longer exposure (Pestka *et al.* 1987b). Conversely, low dose DON exposure can enhance murine resistance to *Staphylococcus hyicus* (Atroshi et al. 1994). Thus, DON may be immunostimulatory or immunosuppressive depending on dose and duration of exposure. DON also enhances lymphocyte differentiation to IgA secreting plasma cells in Peyer's patches, thus increasing systemic IgA and impacting systemic immunity (Pestka and Smolinski 2005). IgA-immune complex deposition in the kidney and associated hematuria have been observed in DON-fed mice, and these symptoms are similar to those seen in some idiopathic human IgA nephropathy (Pestka 2003).

In summary, weight gain reduction and immune effects are longer term events mostly associated with chronic relatively low dose DON exposures, whereas vomiting, diarrhea and leukocyte apoptosis are acute events most likely to occur at high doses in DON-exposed sensitive animals. Taken together, dose and duration of exposure are important determinants of the toxic outcomes in DON-exposed animals, and weight gain reduction is predominant at lower doses ($\leq$ 10 ppm).

Cytokine induction by DON

DON can rapidly and transiently upregulate cytokines *in vitro* and *in vivo* (Azcona-Olivera *et al.* 1995a; Dong *et al.* 1994; Zhou *et al.* 1998). In cell culture, DON increases the binding of cytokine-inducing transcription factors such as AP-1 in T-cells (Li *et al.* 2000) and macrophages (Wong *et al.* 2002). In mice, a single oral dose of DON will rapidly (15-30 mins) induce mitogen-activated protein kinases

(MAPKs), which leads to increased binding of transcription factors like AP-1, C/EBP and CREB as well as rapid (1-2 hr) cytokine upregulation (Zhou *et al.* 2003a). MAPK-related cytokine induction has also been reported in DON-exposed human primary monocytes (Islam *et al.* 2006). DON has also been reported to increase the mRNA stability of proinflammatory cytokines such as IL-6 and TNF-α (Pestka *et al.* 2004). The mechanism for DON-related increase in cytokine mRNA stability involves stabilization of 3'-untranslated regions in the proinflammatory cytokine mRNA (Chung *et al.* 2003; Wong *et al.* 2001). An integrated model from *in vitro* and *in vivo* observations predicts that DON first binds to ribosome, and initiates phosphorylation of ribosome-associated MAPKs, leading to selective transcription, increased mRNA stability and increased translation of cytokine mRNA (Bae and Pestka 2008; Zhou *et al.* 2003a; Zhou *et al.* 2003b). Taken together, upregulation of proinflammatory cytokines is a central acute event in DON-exposed animals.

Weight gain reduction by DON

DON causes feed refusal as well as weight gain reduction. The main hypothesis had been that weight gain reduction occurs because of feed refusal (Pestka and Smolinski 2005; Rotter *et al.* 1996). In pursuit of this hypothesis, researchers have proposed the involvement of serotoninergic pathways in DON-exposed animals. In these studies, DON increased concentrations of serotonin in rat brain (Fitzpatrick *et al.* 1988) and its metabolite (5-hydroxyindoleacetic acid) in cerebrospinal fluid (CSF) of swine (Prelusky 1993). Serotonin receptor antagonists have also been used to block DON-induced emesis in swine (Prelusky and Trenholm 1993). However, researchers were unable to demonstrate a DON-related increase in plasma (peripheral)

concentration of serotonin or its metabolites (Prelusky 1994). Furthermore, studies with a combination of an appetite stimulant (cyproheptadine) and DON could not validate the role of feed refusal in DON-induced weight gain reduction, and the authors later concluded that the effect of dietary DON on weight gain appears to be influenced by more than just reduced feed intake (Prelusky 1997). In support of the conclusion from serotonin studies, feeding studies by our laboratory (Forsell *et al.* 1986) and Canadian researchers (Iverson *et al.* 1995) failed to show a strong correlation between DON-induced weight gain reduction and feed refusal especially at lower doses ($\leq$ 10 ppm). Thus, anorexia is considered as a component of DON-induced effects, primarily at higher doses. Clearly, the mechanism(s) by which DON-induced effects result in weight gain reduction in several species is a critical gap in our understanding of DON effects and creates uncertainty in the risk assessment process. This uncertainty arises, in part, because mechanism-linked biomarkers of effect have not been identified and human population at risk cannot be determined.

An alternative hypothesis for DON-induced weight gain reduction

Cytokine induction and weight gain reduction are central, acute and chronic events, respectively, in DON-exposed animals. It is plausible that both events are linked mechanistically. There are several lines of evidence supporting this contention. First, transgenic animals overexpressing proinflammatory cytokines such as IL-6 (De Benedetti *et al.* 1997) and TNF-α (Probert *et al.* 1996) experience reduced weight gain, in comparison to wild type controls. Second, proinflammatory cytokine signaling impairment seen in IL-6 deficient mice (Wallenius *et al.* 2002) and IL-1 receptor knockout mice (Garcia *et al.* 2006) both result in significant increase in weight gain.

Third, TNFR deficient mice show higher food conversion efficiency (increased weight gain per gram food consumed) (Pestka and Zhou 2002). Fourth, increased weight in IL-6 deficient mice is reversible with IL-6 replacement (Wallenius *et al.* 2002). Finally, a 100-fold increase in basal IL-6 occurs during exercise (Glund and Krook 2008; Pedersen and Febbraio 2008), has been suggested to mediate the benefits of human exercise. It is evident from these studies that increase in basal signaling by proinflammatory cytokines is associated with reduced weight gain, whereas a reduction in the signaling of the same cytokines is associated with increased weight gain. Thus, cytokine upregulation in DON-exposed animals might be responsible, in part, for weight gain reduction observed in these animals.

How might DON-induced cytokine upregulation cause weight gain reduction?

Multiple, complementary pathways might exist for cytokine-induced weight gain reduction. Studies on the cytokine-associated JAK-STAT pathway have resulted in the identification of a variety of SH2 domain-containing proteins, all of which negatively regulate cytokine signaling (Endo *et al.* 1997; Naka *et al.* 1997; Starr *et al.* 1997; Yoshimura *et al.* 1995). These cytokine-inducible inhibitors of cytokine signaling, better known as suppressors of cytokine signaling (SOCS), are induced in a tissue-specific manner (Starr *et al.* 1997) to regulate various members of the cytokine receptor superfamily. Members of this group include the well characterized cytokine-inducible SH2 domain protein (CIS), SOCS1, SOCS2, and SOCS3; and the less well characterized SOCS4, SOCS5, SOCS6, and SOCS7. There has been increasing recognition that SOCS family proteins are critically important in the negative regulation of cytokine and growth factor signaling pathways.

Interestingly, growth hormone (GH) receptor (GHR), a member of the cytokine receptor superfamily (Bazan 1989) is susceptible to SOCS-dependent impairment. Several lines of evidence suggest that inflammagens impair the signaling of GH. Treatment with proinflammatory cytokines or the inflammagen lipopolysaccharide has been shown to inhibit GH-induced gene expression in isolated mammalian hepatocytes (Ahmed *et al.* 2007; Bergad *et al.* 2000; Boisclair *et al.* 2000; Shumate *et al.* 2005; Thissen and Verniers 1997; Wolf *et al.* 1996) and whole liver (Mao *et al.* 1999; Yumet *et al.* 2006). Since these effects are SOCS-dependent (Chen *et al.* 2007; Denson *et al.* 2003; Yumet *et al.* 2006), SOCS proteins might mediate a form of crosstalk between proinflammatory cytokine signaling and GH signaling. The phenomenon of inflammagen-induced impairment of GH signaling has been described as GH resistance and appears to involve a reduction in other GH-induced circulating hormones such as insulin-like growth factor 1 (IGF1) (Lang *et al.* 2005). GH-induced IGF1 is also critical for postnatal growth and weight gain (Liu and Leroith 1999). Based on the above studies, DON-induced cytokine upregulation might induce SOCS upregulation, which, in turn, could reduce IGF1 and impair weight gain.

Mechanism(s) of SOCS action

SOCS proteins share a conserved SOCS-box in their carboxy-terminal region and a Src homology 2 (SH2) domain that mediates their interactions with other proteins. Three mechanisms have been proposed to act either independently or in concert to achieve the SOCS-induced negative regulation of cytokine/growth factor signaling. These include (1) direct inhibition of signaling kinases by binding to intra-cytoplasmic domains or the activation loop of kinases on receptors; (2) competition

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with other SH2 domain-containing signaling proteins for binding sites on receptors; and (3) proteasomal degradation of receptors by SOCS box-elongin interactions (O'Sullivan *et al.* 2007). SOCS-induced negative regulation of cytokine/growth factor pathways have been demonstrated *in vivo* and *in vitro*, using many species (Croker *et al.* 2008). Accordingly, there are, at least, three potential levels at which SOCS might affect GH signaling (Figure 1.2). If DON-induced cytokine expression causes SOCS upregulation like other inflammagens, GH signaling impairment might be a consequence of SOCS upregulation that leads to weight gain reduction

Growth hormone and the IGF system

Growth hormone (GH) is a 191-amino acid protein (in humans) released from the anterior pituitary and stimulates many aspects of postnatal growth such as longitudinal bone growth, cellular growth and differentiation, and overall metabolism (Laron 2004; Waxman and O'Connor 2006). GH signals by binding the growth hormone receptor (GHR) to initiate intracellular events.

GH signaling is impaired in a congenital disease (Laron Syndrome) associated with retarded growth, delayed puberty and a marked reduction in circulating IGF1 (a hormone secreted by the liver and other peripheral tissues that mediates the growth promoting effects of GH) (Laron 2004). In many species, GH release is pulsatile in males but continuous in females, and ultimately activates intracellular cascades, resulting in gene transcription (Waxman and O'Connor 2006).

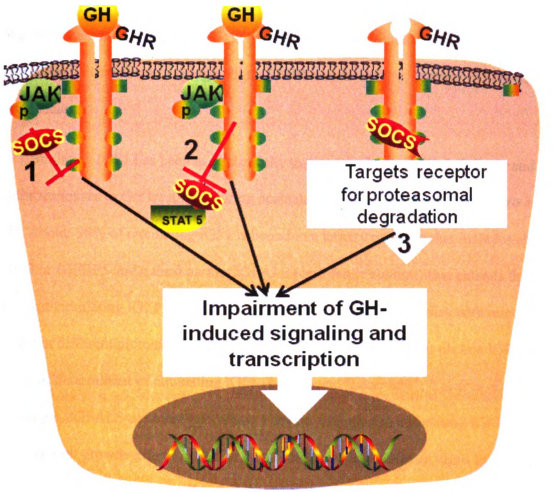

Figure 1.2 Mechanism of SOCS-induced growth hormone receptor impairment. SOCS impairment of growth hormone receptor (GHR) signaling and transcription may be achieved by (1) SOCS impairment of growth hormone (GH) induced kinase (JAK) phosphorylation of GHR tyrosine residue (y), (2) SOCS impairment of STAT phosphorylation by competition for binding, and (3) SOCS-induced proteasomal degradation of GHR. The potential outcome of any or all of these events is a reduction in GH-induced IGF proteins, adapted from (O'Sullivan *et al.* 2007)

One hypothesis proposed for this sexual dimorphism is that GH-free intervals are necessary for gender-specific transcription of some liver enzymes such as cytochrome P450s (Waxman and O'Connor 2006) in male animals. In summary, GH-induced transcriptional events lead to upregulation of IGF1 and its binding partners in both sexes across species.

Circulating IGF1 has been used clinically to diagnose human GH deficiency and to monitor responses to GH treatment during postnatal growth (Bang *et al.* 1990; Pozo *et al.* 2005). About 90% of circulating IGF1 is bound in a ternary complex that includes either IGFBP3 or IGFBP5 and a third partner IGFALS (acid-labile subunit) that extends the half-life of circulating IGF1 (Heath *et al.* 2008; Ueki *et al.* 2009). Studies with mice deficient in different proteins of the IGF1 ternary complex suggest that plasma IGFALS is a critical determinant of circulating IGF1 (Leroith and Yakar 2007; Yakar *et al.* 2005). For example, IGFALS deficient mice show a severe reduction in circulating IGF1 associated with growth reduction (Yakar *et al.* 2005). Recently, human cases of IGFALS mutation have been described and include both a reduction in circulating IGF1, and growth deficit (Domene *et al.* 2004; Heath *et al.* 2008; Ueki *et al.* 2009). IGFALS is known to stabilize IGF1 in the circulation, thereby extending its half-life from 15 min to 15 h (Guler *et al.* 1989). In summary, evidence from IGFALS knock-out mice studies and emerging clinical data suggest that IGFALS is a critical partner in the IGF1 ternary complex, and that its deficit has growth and metabolic consequences. Furthermore, plasma levels of circulating IGF1 during post-natal growth might be regulated at many levels including GH release, post-GHR signaling, and transcription of IGF1-associated binding proteins.

Complexities in the GH/IGF axis

The GH/IGF1 system is complicated. The following lines of evidence highlight such complexity. First, all the members of IGF ternary complex were thought to be under GH control, but recent reports indicate that there might be a GH-independent mechanism of IGF1 transcription involving other hormones like estradiol (Venken *et al.* 2005). Second, there are 19 putative STAT transcription factor binding sites in both human and mouse IGF1 promoters (Eleswarapu *et al.* 2008), whereas only ALSGAS1 (an 8-nucleotide GH-responsive γ-interferon activated-like sequence) is sufficient for IGFALS transcription (Boisclair *et al.* 2000), suggesting that individual proteins of the IGF complex might have different transcription machinery, yet are somewhat under GH control. Third, GH is pleiotropic and can signal through MAPKs , STATs and other proteins to achieve cellular functions (Zhu *et al.* 2001). Finally, GH-STAT signaling is further complicated by the involvement of many STATs and JAK kinase. For example, GH can activate STATs 1,3, directly through JAK phosphorylation; or activate STATs 5a or 5b via the distal tyrosine residues on GHR. Regardless of these complexities, SOCS proteins can inhibit GH signaling (Zhu *et al.* 2001) at various levels as shown (Figure 1.2). The physiological implication of such complexity is that upon SOCS induction, GH induction of a ternary complex protein may be affected more than others, depending on which SOCS is induced and its downstream partners.

Obesity, IGF system and DON

There are 2.1 billion overweight people globally (Baur *et al.* 2006), and an unprecedented increase in obesity and obesity-related morbidity and mortality. In the

U.S., 30% of adults and 15% of young people are obese (Ogden *et al.* 2006). Obesity and its associated metabolic syndrome represent a huge national and global health challenge. Obesity pathogenesis is linked to aberrant energy balance, i.e when energy intake is more than necessary for body functions, the excess is stored as fatty acids and triglycerides; resulting in adipocyte hypertrophy and/or hyperplasia, with a subsequent increase in visceral fat (Gesta *et al.* 2007; Hirsch and Batchelor 1976). Such excess in visceral fat increases circulating fatty acids and causes broader metabolic complications such as type 2 diabetes and cardiovascular diseases (Muoio and Newgard 2006; Raghow *et al.* 2008). In the U.S, health care costs attributable to overweight and obesity is projected to reach $1 trillion by the year 2030 (Wang *et al.* 2008). Obesity is a growing public health challenge that requires intervention.

Weight loss is accepted as the best step to prevent and control obesity-related morbidities and mortalities (Idelevich *et al.* 2009). Unfortunately, behavior-related weight control methods such as exercise and diet control has not been very successful in stemming the obesity pandemic (Idelevich *et al.* 2009). Consequently, some anti-obesity drugs have also been approved for obesity management. These include (a) phentermine (catecholamine stimulant), (b) sibutramine (serotonin reuptake inhibitor), (c) rimonabant (cannabinoid receptor antagonist), and (d) orlistat (lipase inhibitor) (Atkinson 2008). All approved drugs focus on central appetite control except orlistat, which acts on the gastrointestinal tract. However, the efficacy and safety of these pharmacotherapeutic interventions are not optimal (Bray 2008). Therefore, drugs with more efficacious targets are necessary to combat the obesity epidemic.

Chronic exposure to DON reduces weight gain significantly (Pestka and Smolinski 2005; Rotter *et al.* 1996), and weight loss is beneficial in obese states. DON and other related trichothecenes might help identify better targets for the control of obesity pandemic. One potential target for DON effects is the IGF axis. Circulating IGF1 has been shown to positively correlate with obesity and carcinogenesis (Hursting *et al.* 2007). Interestingly, available evidence suggests that DON might have the opposite effect of elevated circulating IGF1 in both disease states. First, impaired weight gain is well recognized in DON exposed animals across species (Iverson *et al.* 1995; Pestka and Smolinski 2005; Rotter *et al.* 1996). Second, mice that were exposed to DON in a 2-year carcinogenicity study had less neoplastic lesions in the liver than age-matched untreated controls (Iverson *et al.* 1995) suggesting that DON exposure may result in some anti-proliferative effects in the liver. It might be speculated that DON acts by opposing IGF1.

IGF1 drives maturation of preadipocytes to adipocytes (Rolland-Cachera *et al.* 1999) and induces glucose and lipid uptake in adipocytes (Sebert *et al.* 2005). IGFI increases have also been associated with diet-induced obesity in pigs (Sebert *et al.* 2005), dogs (Gayet *et al.* 2004), mice (Ogus *et al.* 2003) and people (Rolland-Cachera *et al.* 1999). However some inconsistencies in circulating IGF1 had been reported in human models of obesity, where IGF1 may be increased, reduced or remains unchanged (Maccario *et al.* 2000). The reason for these inconsistencies are unclear but the majority of diet-induced obesity animal models indicate that an increase in circulating IGF1 is associated with obese states (Baur *et al.* 2006; Fenton *et al.* 2009; Yakar *et al.* 2006).

Rationale for identification of biomarker

Human risk assessment of DON has been difficult in part because DON's effects on human beings cannot be systematically evaluated for ethical reasons. Furthermore, the short plasma half-life of DON has made exposure monitoring difficult. Recently, mass spectrometry has been used to measure urinary DON in human subjects (Turner *et al.* 2008b), and our laboratory has measured DON in plasma and tissue matrices using a rapid and convenient immunoassay (Pestka *et al.* 2008a). However, these biomarkers of exposure are inadequate in addressing the uncertainties related to foodborne and airborne DON as outlined above. Therefore, these new biomarkers of DON exposure must be complemented with mechanism-linked biomarker(s) of effect to enhance their utility in human epidemiological surveillance and risk assessment. Changes in such biomarkers of DON effect should precede any adverse effect so that preventive measures can be taken in the population.

Since DON acutely induces proinflammatory cytokines in murine and human cells, it should be expected that proinflammatory cytokines and other proteins associated with their signaling pathways could be likely candidates for biomarker(s) of DON effect. The overall goal of this dissertation is to use proinflammatory cytokine signaling as a foundation to extend knowledge, reduce uncertainty and identify potential biomarkers of effect in DON exposed animals. The guiding hypothesis is that proinflammatory cytokine induction is a central early event that is associated with cellular dysregulation and impairment of the GH/IGF axis, and leads to alterations in physiological states of DON-exposed animals. The specific aims in this dissertation are to (1) compare proinflammatory cytokine induction by oral and nasal routes of DON exposure, (2)

determine the capacity of DON to induce suppressors of cytokine signaling (SOCS) along with proinflammatory cytokines, (3) determine the capacity of DON exposure to impair hepatic GH signaling and suppress circulating IGFALS and (4) determine the influence of obesity on DON-induced IGFALS impairment. It is hoped that these specific aims will reveal biomarker candidates in lean and obese mice, from which the best biomarker of effect candidate will be selected.

Model justification

Mouse models of acute and chronic DON exposure will be used to achieve our specific aims. The decision to use a mouse model of DON exposure was influenced by many reasons. First, the mouse immune system is well characterized. Second, the mouse had been studied for decades relative to DON exposure and there is enormous amount of literature. Finally, the present DON regulatory limit is based on a study done in B6C3F1 mice (Iverson *et al.* 1995), which is the strain of choice in our studies. Similarities in human and murine cellular response to DON lead us to believe that biomarkers of effect identified in this study might be useful in future human risk assessment of DON.

CHAPTER 2: A COMPARISON OF ORAL AND NASAL DEOXYNIVALENOL TISSUE DISTRIBUTION AND PROINFLAMMATORY CYTOKINE INDUCTION

Data in this chapter has been published in Amuzie, C.J., Harkema, J.R. and Pestka J.J. *Tissue distribution and proinflammatory cytokine induction by the trichothecene deoxynivalenol in the mouse: Comparison of nasal vs. oral exposure. Toxicology* Jun 3;**248**(1):39-44.

Abstract

Oral exposure to the trichothecene deoxynivalenol (DON), a common cereal grain contaminant, adversely affects growth and immune function in experimental animals. Besides foodborne exposure, the potential exists for DON to become airborne during the harvest and handling of grains and therefore pose a risk to agricultural workers. The purpose of this study was to compare the effects of oral and intranasal exposure to DON (5 mg/kg bw) on tissue distribution and proinflammatory cytokine induction in the adult female mouse. Competitive direct ELISA revealed that, regardless of exposure route, DON concentrations in plasma, spleen, liver, lung and kidney were maximal within 15 to 30 min and declined by 75 to 90% after 120 min. However, plasma and tissue DON concentrations were 1.5 to 3 times higher following intranasal exposure as compared to oral exposure. The functional significance of elevated DON tissue concentrations was assessed by measuring IL-1β, IL-6, and TNF-α mRNA responses in spleen, liver and lung. Oral exposure to DON induced robust proinflammatory cytokine gene expression after 60 and 120 min. In contrast, induction of IL-1β, IL-6, and TNF-α mRNAs in nasally exposed mice were 2 to 10, 2 to 5 and 2 to 4 times greater, respectively, than those in the

tissues of orally exposed mice. Taken together, these data suggest that DON was more toxic to the mouse when nasally exposed than when orally exposed, and that this might relate to greater tissue burden of the toxin.

Introduction

Deoxynivalenol (DON), a trichothecene associated with *Fusarium* head blight, is of concern because of (1) its frequent, unpreventable contamination of grains and (2) potential health effects resulting from human and animal exposure (Pestka and Smolinski 2005). In mice, ingested DON is rapidly absorbed and reaches peak plasma concentration within 30 min after oral dosing (Pestka and Smolinski 2005). Acute oral exposure to DON can cause vomiting, while subchronic DON ingestion results in anorexia and/or growth retardation depending on the dose.

An integrated model, derived from mouse and human cell culture investigations and studies of acute oral exposure in mice, suggests that DON sequentially activates MAPKs, increases transcription and mRNA stability, as well as translation of proinflammatory cytokines (Pestka and Smolinski 2005). These cytokines include TNF-α, IL-1 β and IL-6, and are widely-recognized to aberrantly affect food intake, growth and immune function (Borish and Steinke 2003; Johnson 1998). Thus, these proinflammatory cytokines might serve as markers to gauge DON's adverse effects in animal models.

Exposure of agricultural workers to dust-borne fungi and their products released into breathing zones during harvesting, threshing and loading of grains is a growing environmental health concern (Hintikka and Nikulin 1998; Krysinska-Traczyk *et al.*

2001; Krysinska-Traczyk *et al.* 1999; Radon and Nowak 2003). Of particular importance, DON and other trichothecenes are detectable in agricultural dust generated from handling wheat and barley (Halstensen *et al.* 2006; Lappalainen *et al.* 1996; Nordby *et al.* 2004b). Previous studies indicate that, when delivered by inhalation, the trichothecene T-2 toxin is 2-, 10- and 20 times more toxic in guinea pigs, mice and rats than when delivered by other parenteral routes (Creasia *et al.* 1987; Creasia *et al.* 1990).

A critical gap exists in the understanding of potential adverse effects associated with DON inhalation. The purpose of this study was to test the hypothesis that tissue distribution and proinflammatory cytokine induction in mice differ following intranasal and oral exposure to an identical DON dose. The results suggest that intranasal exposure to DON resulted in greater plasma and tissue DON concentrations than oral exposure and that these effects were reflected by commensurate differences in proinflammatory cytokine mRNA expression.

Materials and Methods

Laboratory animals

Pathogen-free female B6C3F1 mice (9-10 wk, Charles River, Portage, MI) were randomly assigned to experimental groups ($n \geq 5$) and housed in polycarbonate boxes containing Cell-Sorb Plus bedding (A & W Products, Cincinnati, OH). Boxes were covered with filter bonnets and mice were provided free access to food and water. Room lights were set on a 12-hour light/dark cycle, and temperature and relative humidity were

maintained between 21-24^{0}C and 40-55% humidity, respectively. Mice were maintained according to National Institutes of Health guidelines as overseen by the All University Committee on Animal Use and Care at Michigan State University.

Exposure regimen and tissue collection

Deoxynivalenol was purchased from Sigma Chemical Co. (St. Louis, MO). For each experiment, groups of mice were first anesthetized with 4% isoflurane and 96% oxygen and then instilled intranasally with 5 mg/kg bw DON, dissolved in 20 µl of Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO). Mice receiving DON by the oral route were gavaged with 5 mg/kg bw of DON, using a 22 G intubation needle (Popper and Sons, New Hyde Park, NY). At experiment termination, mice were deeply anesthetized by i.p. injection with 0.1 ml of 12% (w/v) sodium pentobarbital in saline at designated post-exposure time intervals (0, 15, 30, 60 and 120 min). The abdominal cavity was opened and blood was collected via the caudal vena cava, and stored in EDTA-containing tubes. Following blood collection, other tissues were collected. From each mouse, plasma, left lung lobe, cranial half of spleen, lateral lobe of liver and right kidney were collected for DON quantitation by ELISA, while cardiac lobe of lung, caudal half of spleen and medial lobe of liver were collected for real time PCR.

DON quantitation

Prior to DON measurement, plasma was prepared from blood by centrifugation. Organs (40-200mg) were homogenized in PBS (1:10 [w/v]), and the homogenate centrifuged at 15,000 x g for 10 min. The supernatant fraction was first heated at 100EC for 5 min to inactivate endogenous enzymes and precipitate proteins and then centrifuged at 15,000 x g for 10 min. The resultant supernatant was used for DON analysis.

DON was measured in tissues using a Veratox High Sensitivity (HS) ELISA (Neogen, Lansing, MI) according to the manufacturer's instructions with some modifications. Briefly, DON horseradish peroxidase conjugates were diluted (1:7 [v/v]) in 1% (w/v) bovine serum albumin (Sigma) in PBS. One hundred microliter (100 ul) aliquots of DON standards (1-200 ng/ml) or appropriately diluted samples were mixed with 100 µl of diluted enzyme conjugates and then incubated in antibody-coated microtiter wells for 45 min. After incubation, wells were aspirated and washed with distilled water. DON HS substrate (100 ul) was added and further incubated for 20 min. The reaction was terminated by adding 100 µl of stop reagent and plates read at 690 nm on an ELISA plate reader (Molecular Devices, Menlo Park, CA). DON concentrations in samples were determined from standard curve using Softmax software (Molecular Devices). Since this ELISA might detect DON as well as some of its metabolites, data were reported as DON equivalents per ml plasma or per g tissue.

Toxicokinetic analysis

A two-compartment open model (Shargel *et al.* 2004) was employed to calculate pharmacokinetic parameters. DON concentrations in plasma and tissue were fitted to biexponential expression to calculate clearance rates (Li *et al.* 1997). Briefly, using the Trapezoidal rule, plasma area under the curve ($AUC_{0-\infty}$)=$A/\alpha+B/\beta$, where A and B are y-intercepts of distribution and elimination curves respectively; while α and β are slopes for distribution and elimination curves respectively

Quantitative real-time PCR for proinflammatory cytokine mRNAs

Excised tissues for PCR analyses were stored immediately after harvesting in RNA*later*™ (Ambion Inc., Austin, TX). RNA was isolated using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH). Real-time PCR for IL-1β, TNF-α and IL-6 were performed on an ABI PRISM® 7900HT Sequence Detection System, using Taqman One-Step Real-time PCR Master Mix and Assays-on-Demand™ primer/probe gene expression products according to the manufacturer's protocols (Applied Biosystems, Foster City, NY). Relative quantification of proinflammatory cytokine gene expression was carried out using β2-microglobulin RNA control and an arithmetic formula method (Audige *et al.* 2003; Islam and Pestka 2006).

Statistics

All data were analyzed, and differences between groups determined by Analysis of Variance (ANOVA) using SigmaStat v 3.1 (Jandel Scientific; San Rafael, CA) with the criterion for significance set at $p < 0.05$.

Results

Plasma and Tissue DON are greater following nasal than oral exposure

DON plasma and tissue concentrations were compared 15, 30, 60 and 120 min after oral and intranasal exposure to 5 mg/kg of the toxin. DON was rapidly taken up in plasma after oral exposure with peak concentrations of approximately 1 μg/ml being found at 15 and 30 min (Figure 2.1). Plasma DON rapidly declined between 30 and 60 min, and this was followed by slower rate of decline with 78% of peak plasma DON being cleared by 120 min. These distinct rates of decline were suggestive of a two-

compartment kinetics, which has been previously reported for DON in mice (Azcona-Olivera *et al.* 1995a).

DON toxicokinetics in nasally exposed mice were similar to that of the orally exposed group, however, peak plasma concentration at 15 and 30 min was nearly 3 times that observed for oral exposure (Figure 2.1). At 60 and 120 min, plasma DON concentration in mice following intranasal instillation was 2 and 1.5 times that seen in orally gavaged mice, respectively. Approximately 84% of the peak plasma DON concentration was removed by 120 min. The plasma area under the concentration-time curves (AUCs) for oral and nasal exposure regimens were 3.1 and 7.2 μg.h/ml, respectively. Accordingly, nasal instillation resulted in a much greater DON plasma levels than did oral gavage.

Following oral exposure, DON accumulation in the four organs followed similar kinetics to that of plasma with maximum toxin concentrations being detectable at 15 or 30 min (Figure 2.2). Peak DON concentrations in spleen, liver, lung and kidney (Figure 2.2A- D) were 0.77, 1.10, 0.95, and 1.76 μg/g, respectively. By comparison, DON concentrations in these tissues were 1.87, 2.37, 2.20 and 3.73 μg/g, respectively, following nasal exposure. By 120 min, DON concentrations were reduced by 75 to 90% of the peak levels. DON levels in the four tissues following intranasal exposure were significantly higher than those for oral exposure all time points tested. Thus, as seen for plasma, nasal instillation resulted in much greater DON levels in organs than was observable in orally exposed mice.

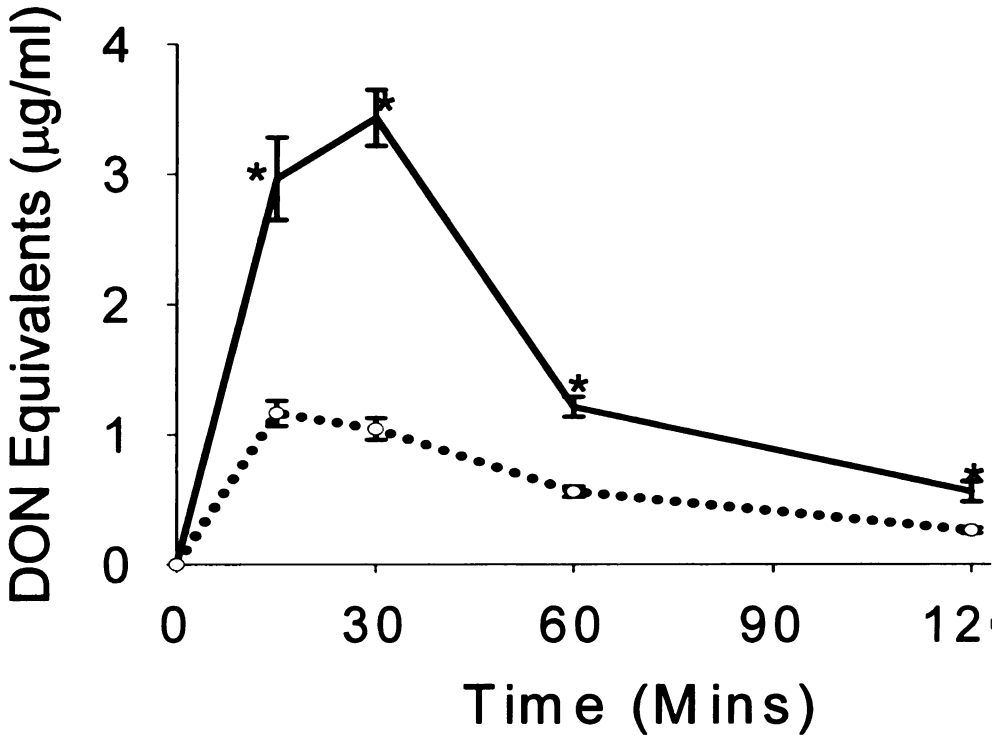

Figure 2.1 Plasma DON concentrations after intranasal and oral DON exposures. Mice were treated with 5 mg/kg DON by intranasal instillation (solid line) or oral gavage (dotted line) and plasma DON analyzed at intervals by ELISA. Data are mean ± SEM ($n \geq 8$). Asterisk indicates significant difference from orally exposed mice ($p<0.05$).

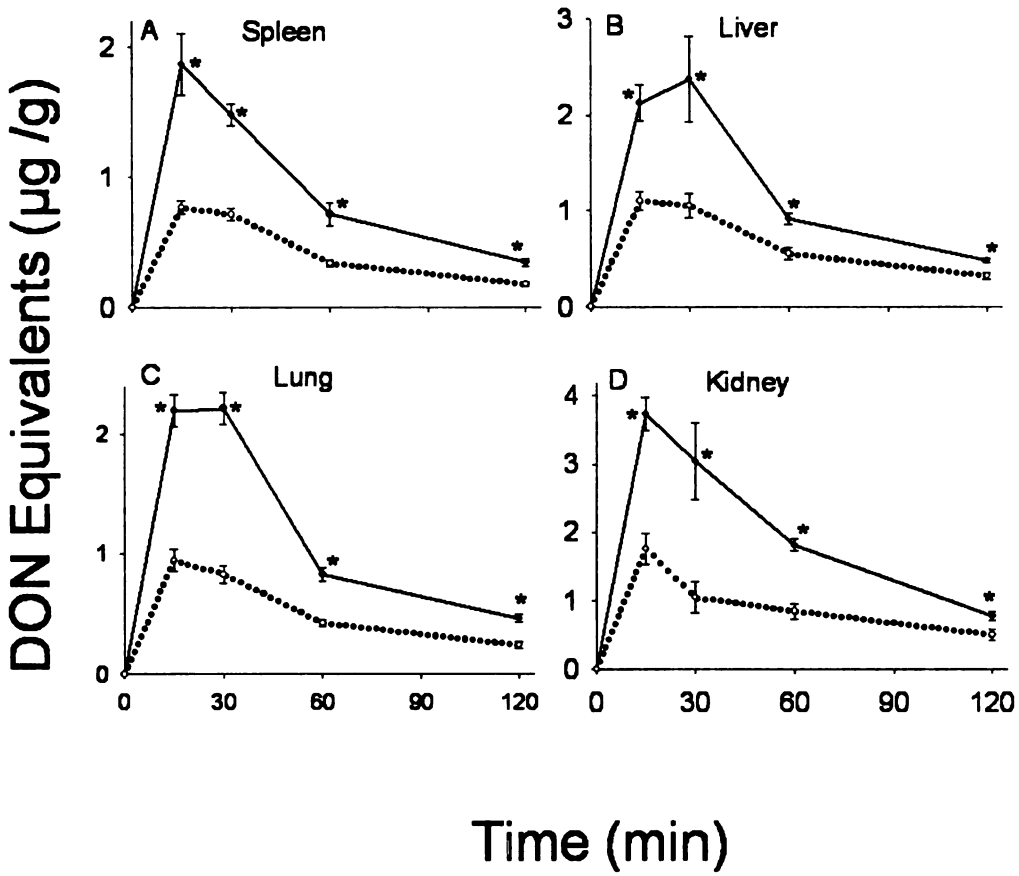

Figure 2.2. Tissue DON after intranasal and oral DON exposures. Mice were treated as described in Figure 2.1 legend and lung (A), spleen (B), liver (C), and kidney (D) analyzed for DON by ELISA. Data are mean ± SEM ($n \geq 8$). Asterisk indicates significant difference from orally exposed mice ($p<0.05$).

Tissue proinflammatory cytokine mRNAs are greater following nasal than oral exposure

The expression of proinflammatory cytokine mRNAs was used as a biomarker of effect to ascertain whether mice were differentially affected by DON exposure routes. Oral DON exposure upregulated IL-1β mRNA in spleens by 10- and 13-fold after 60 and 120 min, respectively (Fig 2.3). In contrast, nasal instillation induced splenic IL-1β 85-fold at 60 min and 29-fold at 120 min. There was also significant upregulation of splenic IL-6 mRNAs expression following oral DON exposure at 60 min (13-fold) and 120 min (60-fold). There was a trend towards higher splenic IL-6 expression following nasal exposure at 60 min (66-fold) and 120 min (162-fold). Splenic TNF-α mRNA expression was significantly upregulated at 60 (4.3-fold) and 120 min (2.5-fold) after oral DON exposure. Nasal instillation with DON resulted in significantly more TNF-α expression at 60 min (13-fold) and a trend toward higher expression at 120 min (5-fold) (Figure 2.3). Overall, nasal instillation with DON resulted in greater splenic proinflammatory cytokine mRNA expression than that observed following oral exposure.

IL-1β was also upregulated in the liver at 60 min after DON exposure following both types of exposure and declined at 120 min (Figure 2.4). Nasal instillation induced significantly higher expression of IL-1β than oral gavage at these two time points. Hepatic IL-6 mRNA and TNF-α mRNA were induced to the same extent by both exposure routes at 60 min followed by decline at 120 min, suggesting that upregulation was transient (Figure 2.4).

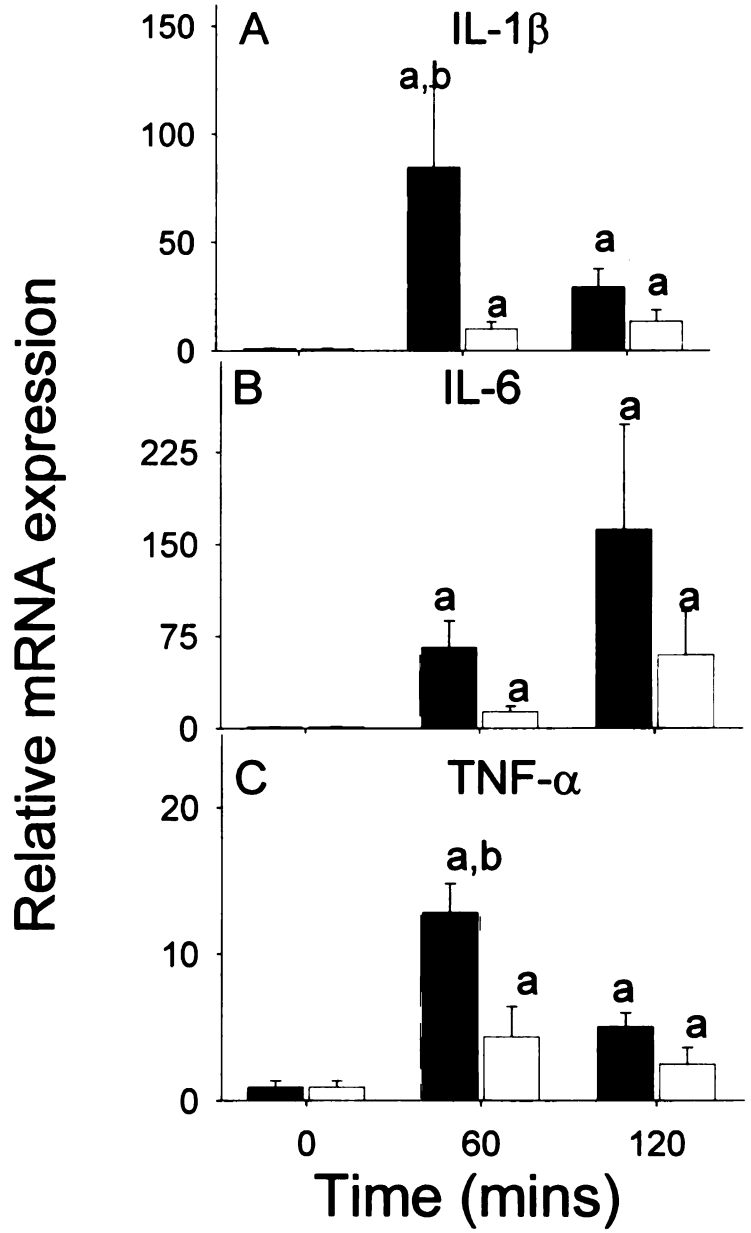

Figure 2.3. Proinflammatory cytokine mRNA in spleen after intranasal and oral DON exposures. Mice were treated as described in Figure 2.1 legend and lung analyzed for IL-1β (A), IL-6 (B) and TNF-α (C) mRNA expression. Data are mean ± SEM (n≥4). Nasal and oral exposures are indicated by dark and white bars respectively. Bars labeled (a) are significantly higher than control (0 min) whereas bars labeled (b) are significantly higher than orally exposed mice at the same time point ($p<0.05$).

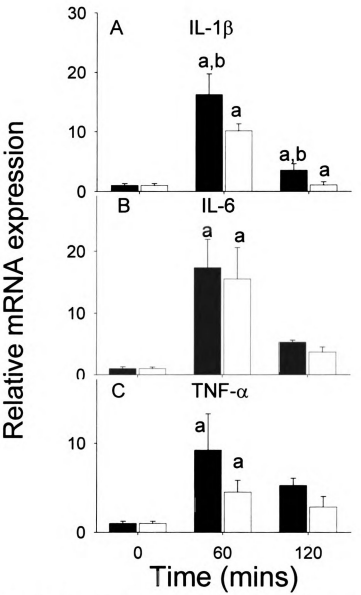

Figure 2.4. Proinflammatory cytokine mRNA in liver after intranasal and oral DON exposures. Mice were treated as described in Figure 2.1 legend and lung analyzed for IL-1β (A), IL-6 (B) and TNF-α (C) mRNA expression. Data are mean ± SEM ($n \geq 4$). Nasal and oral exposures are indicated by dark and white bars respectively. Bars labeled (a) are significantly higher than control (0 min) whereas bars labeled (b) are significantly higher than orally exposed mice at the same time point ($p<0.05$).

Oral exposure to DON induced pulmonary IL-1β mRNA at 60 and 120 min, whereas nasal instillation induced twice as much IL-1β mRNA at the two time points (Figure 2.5). Oral DON exposure induced modest IL-6 expression at 60 and 120 min (Figure 2.5). IL-6 mRNA expression of nasally exposed mice was approximately four times higher than that of orally exposed mice after 120 min. Whereas oral DON exposure did not induce a significant pulmonary TNF-α mRNA expression at either timepoint, mRNA for this cytokine was markedly upregulated at 60 min but not 120 min after nasal exposure (Figure2.5). Overall, nasal instillation resulted in higher expression of proinflammatory cytokines in the lung than oral exposure.

Discussion

Toxicokinetics represents the summation of absorption, distribution, metabolism and excretion of an ingested or inhaled toxicant. The potential exists for different exposure routes to influence DON's toxicokinetics. When DON was delivered by the oral route, it was rapidly absorbed ($\leq$ 30 min) into the plasma compartment and distributed with a similar concentration-time profile in all four organs, characterized by a rapid increase in concentration followed by a fast decrease in concentration. While the profile observed following nasal instillation was comparable, DON concentrations were 1.5 to 3-times higher than by the oral route. Enhanced proinflammatory cytokine expression in spleen and lung of nasally exposed mice corresponded with their elevated tissue DON burden.

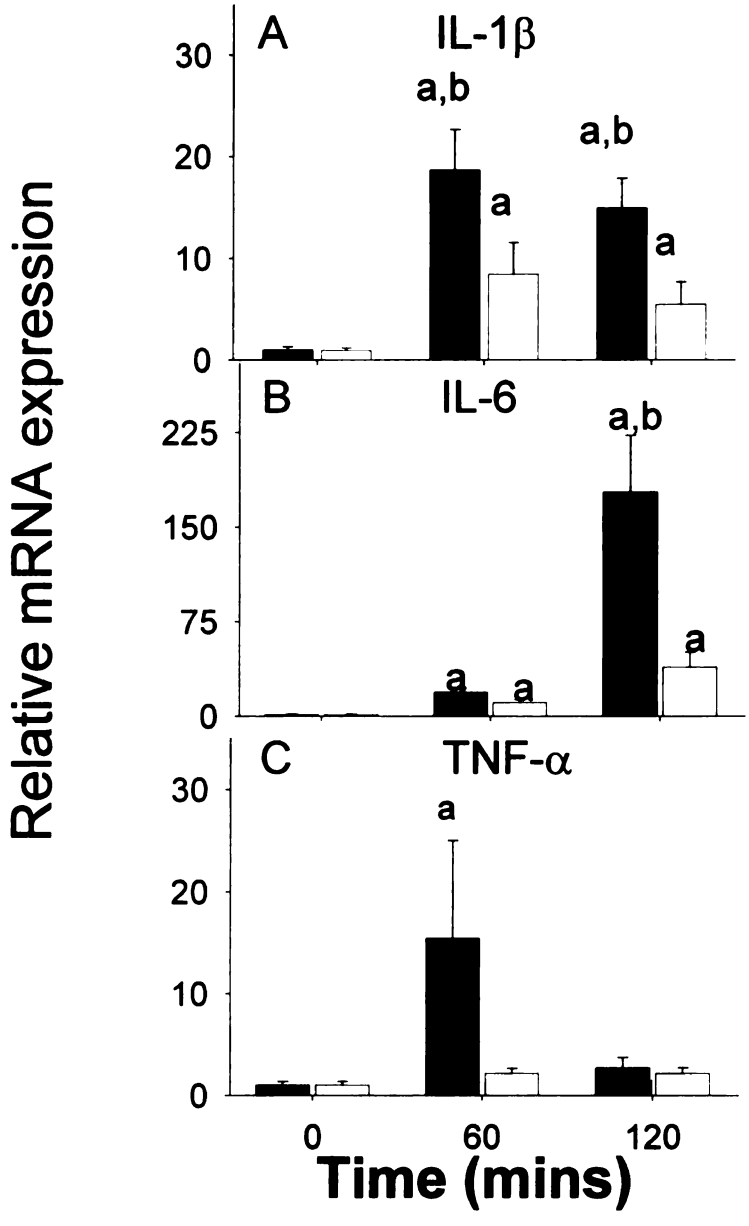

Figure 2.5. Proinflammatory cytokine mRNA in lung after intranasal and oral DON exposures. Mice were treated as described in Figure 2.1 legend and lung analyzed for IL-1β (A), IL-6 (B) and TNF-α (C) mRNA expression. Data are mean ± SEM (n≥4). Nasal and oral exposures are indicated by dark and white bars respectively. Bars labeled (a) are significantly higher than control (0 min) whereas bars labeled (b) are significantly higher than orally exposed mice at the same time point ($p<0.05$).

A DON dose of 5 mg/kg bw employed in this study equals 100 μg of DON for a 20 gram mouse. With high DON contamination of dust, at OSHA permissible grain dust exposure levels, an average person might get about 80 μg of DON during an 8-h work period (Table 2.1). A similar calculation for a mouse, when adjusted by multiplying with 100-fold default safety factors will result in approximately 30 μg of DON. If other trichothecenes in grain dust are given DON toxic equivalency factors, the bolus amount employed in our study might be attained in an 8 h work-shift, in high DON-contamination exposure settings, especially for occupational exposures to grain dust at levels beyond OSHA standards.

^{3}[H]-DON was previously used to assess DON distribution and clearance from 30 min to 24 h in adult mice orally exposed to 5 mg/kg bw of the toxin (Azcona-Olivera et al., 1995). Two-compartment kinetics was observed in that study, with an initial rapid clearance phase and a slower terminal elimination phase. The plasma half-lives for the two phases $t_{1/2}\alpha$ and $t_{1/2}\beta$ were 21.6 min and 7.6 h respectively. The present study similarly revealed two compartment kinetics with initial half-lives of 31.6 min and 41 min being observed for intranasally and orally exposed mice, respectively. DON concentrations observed in plasma at 30, 60 and 120 min (1300, 740 and 370 ng/ml, respectively) in the isotope study were remarkably similar to those found here (Figure 2.1). The plasma AUCs for the 30 to 120 min period in the ^{3}[H]-DON investigation were estimated to be 0.60 μg·h/ml as compared to 0.47 μg·h/ml observed here for the same exposure route and time span.

Table 2.1 Comparison of Inhalable DON in mice and humans

	Mice	Human
Tidal volume (ml)	.15[1]	500[1]
Respiratory rate (breath/min)	175[1]	15[1]
Minute volume (liter)	.0263	7.5
Inhaled dust particles per min at 10 μg/l [2] (μg)	.263	75
Particulates inhaled after 8-hour workday (mg)	.12624	36
Potential DON in Dust at 2200 ppm [3] (μg)	.2777	79.2

[1] Thorne, P. S. 2000. Inhalation toxicology models of endotoxin- and bioaerosol-induced inflammation. *Toxicology* 152:13-23.

[2] U.S Department of Labor, Occupational Safety and Health Administration. Toxic and Hazardous Substances. Occupational Safety and Health Standards 1910.1000 TABLE Z-1(Z).

[3] Halstensen, A. S., K. C. Nordby, S. S. Klemsdal, O. Elen, P. E. Clasen, and W. Eduard. 2006. Toxigenic Fusarium spp. as determinants of trichothecene mycotoxins in settled grain dust. *J.Occup.Environ.Hyg.* 3:651-659.

Similarly DON concentrations at 30, 60 and 120 min determined in the radiolabel study for spleen (680, 210 and 120 ng/g, respectively), liver (1500, 550 and 420 ng/g, respectively) and kidney (1670, 780 and 510 ng/g, respectively) were comparable to the corresponding tissue concentrations observed here (Figures 2.2A-D). Overall, these data support the validity of using ELISA for comparing DON toxicokinetics in experimental animals.

DON rapidly and dose-dependently upregulates TNF-α, IL-6 and IL-1β splenic mRNAs expression in the mouse (Azcona-Olivera *et al.* 1995a; Zhou *et al.* 1997). Since these cytokines evoke anorectic, growth and immunologic effects (Borish and Steinke 2003; Johnson 1998), their expression can be used as surrogates to predict DON toxicity in the mouse. Upregulation of cytokines by DON involves MAPK-driven activation of transcription factors and mRNA stabilization (Pestka *et al.* 2004). In the mouse exposure to DON sequentially evokes (1) JNK 1/2, ERK 1/2 and p38 phosphorylation (15-30 min), (2) activation of transcription factors (1-2 h), and (3) proinflammatory cytokine mRNA expression (1-4 h) in the spleen (Zhou *et al.* 2003a). Here, plasma and spleen DON concentrations reached their peak 15 to 30 min after toxin exposure. This is quite consistent with the kinetics of MAPK activation and proinflammatory cytokine mRNA upregulation seen in this and previous studies. Lastly, the finding that plasma and organ DON concentrations in nasally exposed mice were 1.5 to 3 times that of orally exposed animals correlates well with the observation that proinflammatory gene upregulation was 2 to 10 times in tissues of nasally exposed mice.

The greater DON tissue burden in nasally exposed mice compared to orally exposed ones might result from differences relative to absorption and/or metabolism

between the groups. The nose is a complex organ, designed not only for olfaction but with an inherent capacity to efficiently absorb soluble materials, thus protecting the lower respiratory tract from injurious chemicals (Harkema *et al.* 2006). Absorption is facilitated by the extensive blood supply to the nasal region, which humidifies the airways, absorbs and dilutes toxicants. The lung on the other hand receives all of its blood supply from the heart. With such robust airway perfusion, it is likely that intranasally instilled DON is rapidly and efficiently absorbed into circulation. Upon nasal absorption DON can enter the venous blood via the jugular vein, reach the cranial vena cava, and return to the lung for further reoxygenation. DON might also enter the lung first, mix with oxygenated blood and go to the heart for subsequent systemic circulation. Furthermore, since orally exposed chemicals first reach the liver by portal circulation, one would expect (a) a greater amount of DON in the liver and (b) a greater cytokine induction in the livers of orally-exposed mice. However, the reverse was the case as nasally-exposed mice had higher tissue DON, suggesting that even after proximity of exposure route to target organ is considered, nasal DON exposure still delivers greater tissue burden than oral route. Overall the absorption and distribution of DON via this nasal-pulmonary route might be more efficient than the alimentary route.

In addition to absorption efficiency, DON might be differentially metabolized depending on exposure route. DON undergoes de-epoxidation by gut microflora (He *et al.* 1992); and glucuronidation (Obol'skii *et al.* 1998), at the 3-carbon position (Wu *et al.* 2007) as detoxification reactions. Following oral ingestion, gut microflora can de-epoxidate DON, prior to being absorbed and distributed to the liver. Furthermore, UDP-glucuronosyltransferases (UGTs), which catalyze DON glucuronidation are differentially

expressed in tissues, with 5 isoforms being highly expressed in the gut when compared to 1 isoform highly expressed in the nasal epithelia (Buckley and Klaassen 2007). If there is less detoxification in the murine nose than in the gut, more DON, on an equimolar basis, would reach the systemic compartment following nasal instillation than after oral gavage. Thus, DON greater plasma concentration and tissue distribution in nasally-exposed mice as compared to orally-exposed mice might be a result of less detoxification.

IL-1β is a potent activator of alveolar macrophages, implicated in chronic lung diseases (Barnes 2004). Induction of pulmonary IL-1β observed in this study is consistent with DON-induced IL-1β observed previously in other tissues (Islam and Pestka 2003; Islam and Pestka 2006). At the tested dose, nasally instilled mice evoked almost twice as much IL-1β than orally gavaged mice, thus correlating with higher lung DON concentration. In spleen and liver, IL-1β was higher in nasally instilled mice at 60 min than orally exposed mice, but declined quickly in both tissues. This early wave of IL-1β in tissues fits the phasic model of cytokine-induced inflammation (Fey *et al.* 1994), which considers IL-1β to be among early cytokines that drive later cytokine expression.

Pulmonary IL-6 was about three to four times higher in nasally exposed mice, suggesting a potential to impact the lung. The physiological relevance of IL-6 upregulation in the lungs of nasally instilled mice is unclear, but, in the context of chronic disease, elevated IL-6 has been observed in sputum, bronchoalveolar lavage and exhaled breadth of patients with chronic obstructive pulmonary disease (COPD) (Barnes 2004). Greater IL-6 upregulation at 120 min (spleen and lung) is consistent with pattern for secondary cytokines observed in the phasic model (Fey *et al.* 1994). Interestingly, relative IL-6 expression in liver was lower than other tissues and did not follow the

predicted pattern. This might be related to the role of hepatocytes as responders rather than producers of IL-6 (Parker and Picut 2005).

TNF-α, along with IL-6 and IL-1β, are considered to be among the most important relative to initiation of inflammation (Hopkins 2007). In the present study, TNF-α was modestly induced in lung, spleen and liver, in an early fashion (60 min), consistent with its role as an early cytokine. Early cytokines such as TNF-α can amplify IL-6 induction (Hopkins 2007), and might explain both the kinetics and magnitude of IL-6 induction in our experiment. The observation that TNF-α induction was nearly two times higher, following nasal instillation, when compared to oral gavage and similar trends in other tissues observed in nasally instilled mice are noteworthy.

In summary, the toxicokinetic and functional data presented here suggest that DON is distributed to a greater extent and evokes greater response in target tissues when delivered by the nasal route than the oral route. The potential for enhanced toxicity following nasal DON exposure concurs with a similar observation for T-2 toxin in several species (Creasia *et al.* 1987). Collectively, these findings raise the possibility that DON in grain dust might represent, a hitherto unrecognized human health hazard. Furthermore, the robust induction of proinflammatory cytokines in the lung suggests for the first time that this organ is a potentially important site of DON action. A recent study employing samplers of breathing zones in occupational settings revealed markedly higher particulates in the 3-10 μm range of grain industry workers when compared to other agricultural workers, with about 40% of these particulates being fungal spores (Lee *et al.* 2006). Particles with aerodynamic diameters of ≥ 5 μm are likely to be deposited in the nose (Lippmann *et al.* 1980). Given that DON is soluble in aqueous solutions, it is

reasonable to expect that when dust laden with this mycotoxin is inhaled, nasal deposition would result in rapid diffusion and absorption of DON into the systemic compartment. This scenario underscores the utility of the nasal instillation model in understanding the distribution of highly soluble, rapidly diffusing mycotoxins like DON and other trichothecenes.

CHAPTER 3: INDUCTION OF SUPPRESSORS OF CYTOKINE SIGNALING (SOCS) BY THE TRICHOTHECENE DEOXYNIVALENOL IN THE MOUSE

Data in this chapter have been submitted in Amuzie, C.J., Shinozuka, J. and Pestka J.J., *Induction of Suppressors of cytokine signaling by the trichothecene deoxynivalenol in the mouse* to Toxicological Sciences.

Abstract

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium sp*, is commonly found in grains and cereal-based foods worldwide. Although DON-induced impairment of weight gain has been documented in many species, the underlying mechanisms for this effect are less understood. Upon oral exposure in mammals, DON is rapidly absorbed and distributed (≤30 min), with a subsequent upregulation of proinflammatory cytokine expression. Cytokines are known to induce at least eight suppressors of cytokine signaling (SOCS 1-7, and CIS), some of which impair growth hormone signaling. We hypothesized that an acute oral DON exposure rapidly induces SOCS expression in the mouse. Real-time PCR revealed that DON rapidly (1 h) induced IL-1β and IL-6 mRNA expression, and that SOCS mRNAs were upregulated concurrently (1 h) or thereafter (2 h). Specifically, DON at doses of 1-12.5 mg/kg bw markedly induced SOCS3 mRNAs in muscle, spleen and liver, with CIS1, SOCS1, and SOCS2 occurring to a lesser extent. Hepatic SOCS3 mRNA was a very sensitive indicator of DON exposure. SOCS3 protein remained upregulated in the liver long after the onset of cytokine decline (5 h). Taken together, DON-induced hepatic

SOCS3 has the potential to modulate cytokine and growth hormone signaling in the liver.

Introduction

An integrated model for DON toxicity derived from *in vitro* and *in vivo* observations predicts that DON first binds to ribosomes, initiates phosphorylation of ribosome-associated MAPKs, leading to selective transcription, increased mRNA stability, and increased translation of cytokine mRNA (Bae and Pestka 2008; Chung *et al.* 2003; Zhou *et al.* 2003a; Zhou *et al.* 2005). Accordingly, upregulation of proinflammatory cytokines is a central outcome of DON exposure in mice. Both growth and immune effects in rodents are considered critical end points in DON risk assessments and thus have provided the basis for DON's regulatory standards (Tritscher and Page 2004).

The mechanism(s) for DON-induced growth/weight gain reduction are not well understood, leading to uncertainties in human risk estimation. It has been previously suggested that DON-induced growth effects are caused by feed refusal (Prelusky 1997). The putative role of feed refusal might be questioned for several reasons. First, although feed refusal could result from altered central appetite control following serotonin dysregulation (Fitzpatrick *et al.* 1988; Prelusky 1993; Prelusky and Trenholm 1993), serum serotonin concentrations remain unchanged in animal models of DON exposure (Prelusky 1994), making its systemic relevance less clear. Second, rodent studies using a serotonin antagonist (cyproheptadine) did not support a central role of feed refusal in DON-induced weight gain reduction, leading to the conclusion that DON's weight effects

might be secondary to other pharmacological actions (Prelusky *et al.* 1997) and influenced by factors other than reduced feed intake. Finally, feeding studies by our group (Forsell *et al.* 1986) and Canadian researchers (Iverson *et al.* 1995) failed to demonstrate a strong correlation between DON-induced weight reduction and DON-induced feed refusal, particularly at lower dietary concentrations ($\leq$ 10 ppm), thus corroborating the aforementioned conclusions from serotonin studies.

An alternative hypothesis for DON-induced weight gain reduction can be derived from several human and animal models of proinflammatory cytokine signaling (especially IL-6, IL-1β and TNF-α). First, overexpression of proinflammatory cytokines like IL-6 (De Benedetti *et al.* 1997) and TNF-α (Probert *et al.* 1996) in mice causes a reduction in weight gain. Second, deficiency of IL-6 (Wallenius *et al.* 2002) and IL-1 receptor (Garcia *et al.* 2006) in mice causes increased weight gain, while TNF receptor deficient mice exhibit high food conversion efficiency (increased weight gain per gram food consumed) (Pestka and Zhou 2002). Third, the weight increase in IL-6 deficient mice is reversible with IL-6 replacement (Wallenius *et al.* 2002). Finally, a 100-fold increase in plasma IL-6 observed during human exercise has been suggested to mediate the metabolic benefits of exercise (Pedersen and Febbraio 2008), including weight loss. Taken together, these studies suggest that cytokine upregulation in DON-exposed mice might contribute, in part, to impaired weight gain.

Multiple, complementary pathways might exist for cytokine-induced growth/weight reduction. Cytokine signaling studies have resulted in the identification of a variety of SH2 domain-containing proteins, all of which negatively regulate cytokine signaling (Endo *et al.* 1997; Naka *et al.* 1997; Starr *et al.* 1997; Yoshimura *et al.* 1995). These

cytokine-inducible inhibitors of cytokine signaling, better known as suppressors of cytokine signaling (SOCS), are induced in a tissue-specific manner (Starr *et al.* 1997) to regulate various members of the cytokine receptor superfamily. Members of this group include the well characterized CIS (cytokine-inducible SH2 domain protein), SOCS1, SOCS2, and SOCS3; and the less characterized SOCS4, SOCS5, SOCS6, and SOCS7. Many SOCS proteins impair cytokines and growth factor signaling (O'Sullivan *et al.* 2007).

Interestingly, growth hormone (GH) binds to growth hormone receptor, a member of the cytokine receptor superfamily (Bazan 1989) and may be susceptible to SOCS-dependent impairment. In support of this contention, treatment with proinflammatory cytokines or the inflammagen lipopolysaccharide inhibits growth hormone-induced gene expression in isolated mammalian hepatocytes (Ahmed *et al.* 2007; Bergad *et al.* 2000; Boisclair *et al.* 2000; Shumate *et al.* 2005; Thissen and Verniers 1997; Wolf *et al.* 1996) and whole liver (Mao *et al.* 1999; Yumet *et al.* 2006) in a SOCS-dependent manner (Chen *et al.* 2007; Denson *et al.* 2003; Yumet *et al.* 2006), suggesting that SOCS proteins might mediate crosstalk between proinflammatory cytokine signaling and GH signaling. The phenomenon of inflammagen-induced impairment of GH signaling has been described as GH resistance and appears to involve reduced circulating IGF1 (Lang *et al.* 2005). Based on the aforementioned studies, DON-induced cytokine upregulation could induce SOCS upregulation, which may impair GH signaling and reduce growth (Fig 3.7).

In this study, we hypothesized that acute DON exposure will induce SOCS expression in the mouse. To test this, we exposed female B6C3F1 (3-4 wk) to a single bolus of various doses of DON, sacrificed at various times to investigate SOCS and

cytokine upregulation in murine organs. Our results showed that DON exposure rapidly (1 h) induces cytokines (1L-1β and IL-6) in organs, with concurrent or subsequent CIS, SOCS1, and SOCS2 mRNA, and to a greater extent SOCS3 mRNA and protein upregulation. Since SOCS proteins impair cytokine and growth hormone signaling (O'Sullivan *et al.* 2007), these data suggest upregulation of SOCS in DON-exposed mice might be responsible for cytokine impairment and has the potential to impair GH signaling.

Materials and Methods

Laboratory animals

Pathogen-free female B6C3F1 mice (3-4 wk) (Charles River laboratories, Portage, MI) were randomly assigned to experimental groups ($n \geq 5$) and housed in polycarbonate boxes containing Cell-Sorb Plus bedding (A & W Products, Cincinnati, OH). Boxes were covered with filter bonnets and mice were provided free access to food and water. Room lights were set on a 12-hour light/dark cycle, and temperature and relative humidity were maintained between 21-24^{0}C and 40-55%, respectively. Mice were maintained according to National Institutes of Health guidelines as overseen by the All University Committee on Animal Use and Care at Michigan State University.

Exposure regimen and tissue collection

Deoxynivalenol (DON) was purchased from Sigma Chemical Co. (St. Louis, MO). For each acute exposure experiment, DON was dissolved in Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO) to yield an exposure volume of

100-200 µl per mouse for any of the selected DON doses (0.1, 0.5, 1, 5, and 12.5 mg/kg bw), while equivalent volumes of PBS were used as vehicle control (0 mg/kg bw). Mice were orally gavaged using a 22 G intubation needle (Popper and Sons, New Hyde Park, NY). At experiment termination, mice were deeply anesthetized by i.p. injection with 0.1 ml of 50 mg/kg sodium pentobarbital. The abdominal cavity was opened and then the blood was collected with heparinized syringes via the caudal vena cava, and transferred to centrifuge tubes. Following blood collection, the caudal half of spleen, the caudolateral piece of the lateral lobe of liver and the gastrocnemius muscle were collected from each mouse for real time PCR and/or immunohistochemistry.

Quantitative real-time PCR

Excised tissues for PCR analyses were stored immediately after harvesting in RNA*later*™ (Ambion Inc., Austin, TX). RNA was isolated using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH). Real-time PCR for CIS1, SOCS1, SOCS2, SOCS3 and IL-6 and IL-1β were performed on an ABI PRISM® 7900HT Sequence Detection System, using Taqman One-Step Real-time PCR Master Mix and Assays-on-Demand™ primer/probe gene expression products according to the manufacturer's protocols (Applied Biosystems, Foster City, NY). Fold change of targets was determined using β2-microglobulin RNA control and a relative quantitation method (Smolinski and Pestka 2005).

Immunohistochemistry

Immunohistochemistry for SOCS3 was performed on 10% (v/v) neutral buffered formalin-fixed paraffin-embedded liver sections (5 µm). Briefly, sections were placed in citrate buffer (10mM, pH 6.0) and then placed in a Minichef microwave (Samsung) for

10 min. Microwaved sections were stained with rabbit anti-human CIS3/SOCS3 monoclonal antibody (clone C204, 1:20; Immuno-Biological Laboratories, Inc., Gunma, Japan) as primary antibody, followed by the avidin-biotin peroxidase complex reaction using VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame,California, USA). Positive reactions were visualized after peroxidase-diaminobenzidine (DAB) reaction and counterstaining with hematoxylin.

Statistics

Differences between two groups were determined by Student's t-test, or Mann-Whitney U test when equality of variance failed. Differences among multiple groups were determined by Analysis of Variance (ANOVA) using SigmaStat v 3.1 (Jandel Scientific; San Rafael, CA) combined with Student-Neuman-Keul's post-hoc test; or by Kruskal-Wallis ANOVA on ranks combined with Dunn's test when normality or equality of variance test failed. Grubb's test (www.graphpad.com) was used to isolate significant outliers. The criterion for significance was set at $p < 0.05$.

Results

DON dose-dependently induces SOCS mRNA in the mouse

Real-time PCR was used to assess the effects of exposure to DON at 0.1-12.5 mg/kg bw in spleen, muscle and liver on the expression of four SOCS mRNAs (CIS, SOCS1, SOCS2 and SOCS3). In spleen, CIS, SOCS1, SOCS2 and SOCS3 were upregulated 7-, 25-, 5- and 35-fold, respectively, at the highest DON dose (Figure 3.1). At 1 mg/kg bw DON, CIS, SOCS1, SOC2 and SOCS3 mRNAs were upregulated 1-, 4-, 6- and 6-fold, respectively.

SOCS mRNAs were upregulated in the muscle of DON-treated mice, but at levels lower than that in the spleen and liver. CIS mRNA expression was not detectable in muscles of both control and DON-treated mice. Muscle SOCS1 mRNA levels was modestly elevated only at 0.5 mg/kg bw (Figure 3.2A). SOCS2 and SOCS3 mRNAs were significantly upregulated at 5 and 12.5 mg/kg bw, with SOCS2 reaching 3-fold (Fig 3.2B). As observed in the spleen, there was a robust elevation of SOCS3 in muscle (12-fold) at the two highest doses (Figure 3.2C).

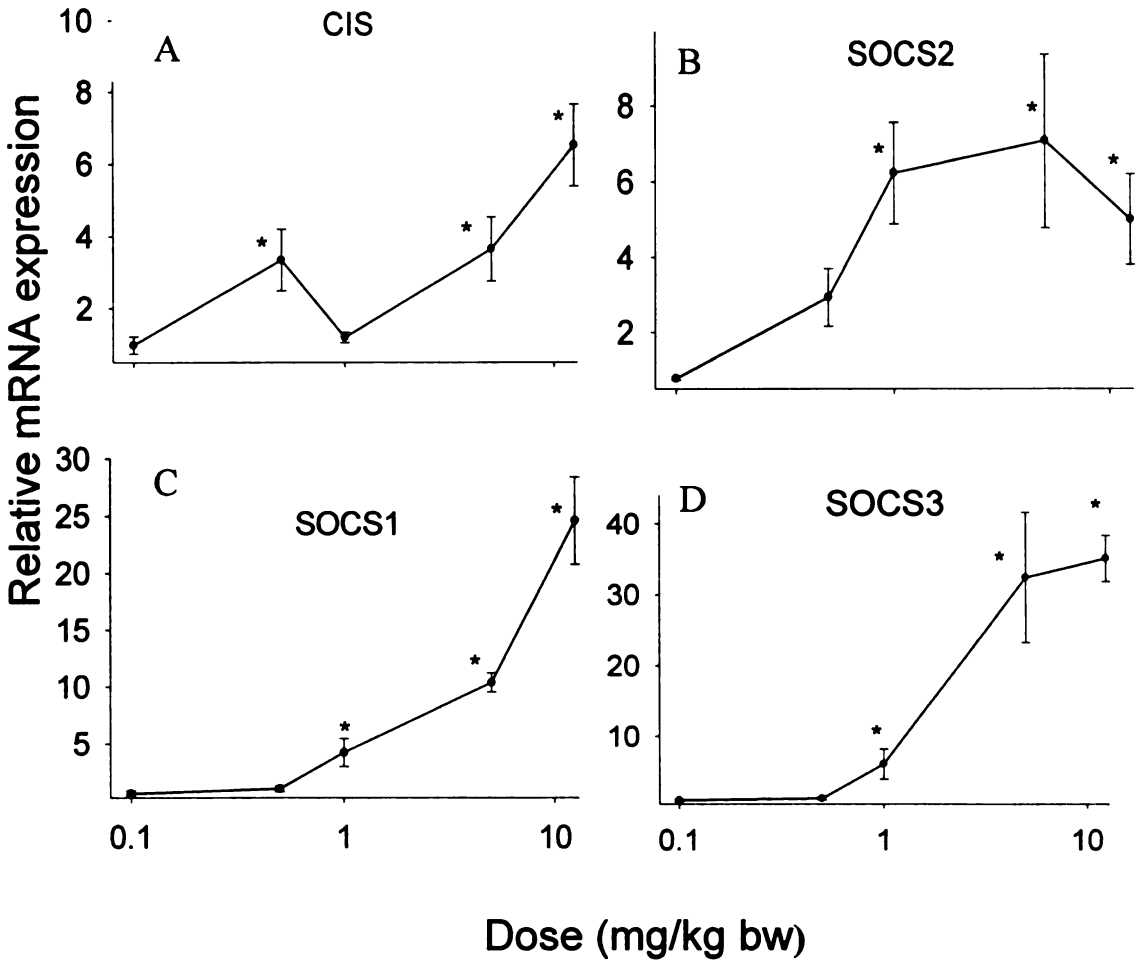

Figure 3.1 DON dose-dependently induces SOCS mRNA expression in the spleen. Mice were treated with DON (0.1-12.5 mg/kg bw) once, sacrificed 2 h later and spleen was analyzed for four SOCS (CIS (A), SOCS2 (B), SOCS1 (C), and SOCS3 (D)) mRNA expression by real-time PCR. Data are mean ± SEM. ($n \leq 5$) of mRNA fold change relative to an untreated (naïve) group (1 fold). Means with asterisks differ from naïve mice ($p < 0.05$).

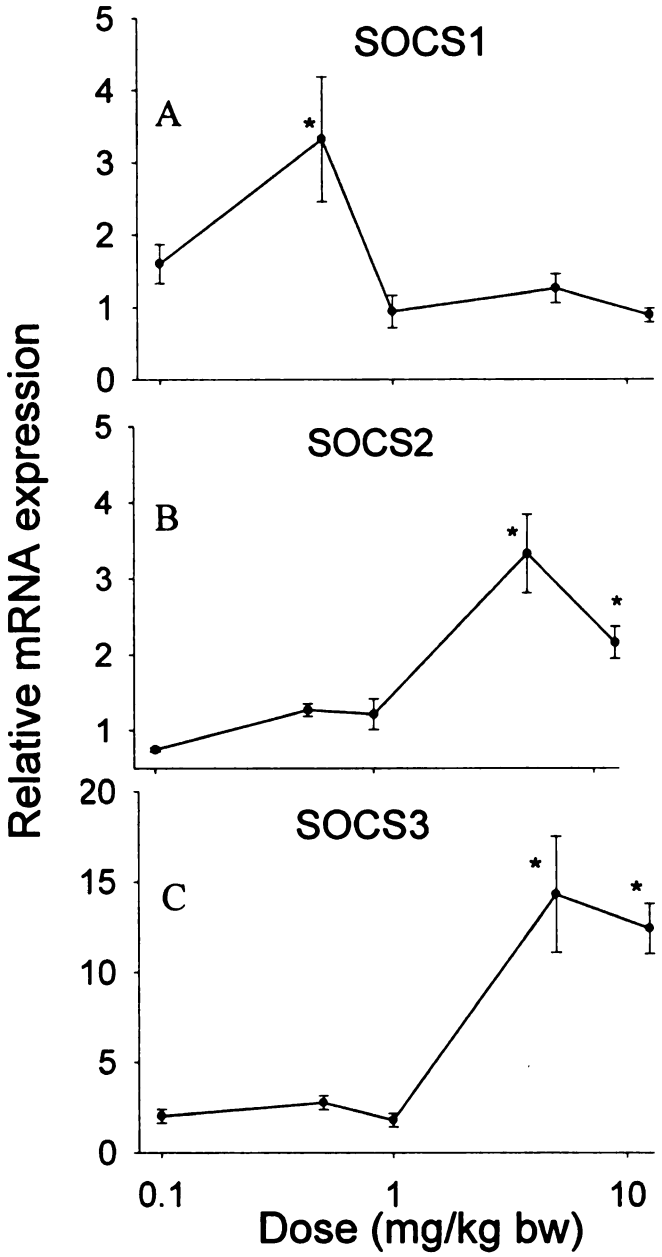

Figure 3.2 DON dose-dependently induces SOCS mRNA expression in the muscle. Mice were treated with DON as in *Figure 3.1*. Real-time PCR was used to analyze gasctrocnemius muscle total mRNA for SOCS (SOCS1 (A), SOCS2 (B), and SOCS3 (C)). Data are mean ± SEM ($n \leq 5$) of mRNA fold change relative to an untreated (naïve) group (1 fold). Means with asterisks differ from naïve mice ($p < 0.05$).

In the liver, there were marked increases of CIS mRNAs in DON treated mice, reaching 14-fold at 5 and 12.5 mg/kg bw (Figure 3.3A). Hepatic SOCS1 expression was not detectable in control and DON-treated mice. All DON doses caused modest SOCS2 mRNA increases (2- to 6- fold) (Figure 3.3B). As in spleen and muscle, SOCS2 in the liver was affected least among all SOCS analyzed. Hepatic SOCS3 upregulation was the highest among all SOCS analyzed, with over a 100-fold change being observed at the highest dose. SOCS3 mRNAs increased with dose, differing significantly from vehicle at 1 mg/kg bw DON (6-fold), 5 mg/kg bw (38-fold), and 12.5 mg/kg bw (108-fold) (Figure 3.3C). Furthermore, hepatic SOCS3 mRNA levels differed significantly among the three highest DON doses. Overall, hepatic SOCS3 mRNA expression appeared to correlate with dose in organs tested.

Kinetics of DON-induced cytokine and SOCS upregulation

The kinetics of DON-induced proinflammatory cytokine mRNA induction was related to SOCS upregulation in the murine spleen and liver. In spleen, DON induced IL-1β mRNA expression rapidly (1 h), reached peak concentrations at 2 h and returned to basal levels at 4 h (Figure 3.4A). IL-6 mRNA was upregulated and reached peak within 2 h but returned to basal level at 4 h after DON exposure (Figure 3.4B). CIS mRNA followed a similar pattern to IL-6 (Figure 3.4C). SOCS3 mRNA was upregulated as early as 1 h, reached 20- to 35-fold at 2-3 h and remained modestly upregulated at 5 h after DON exposure. It was notable that the maximal CIS and SOCS3 mRNA expression (2-3 h) corresponds to the peak and onset of decline for both proinflammatory cytokine mRNAs.

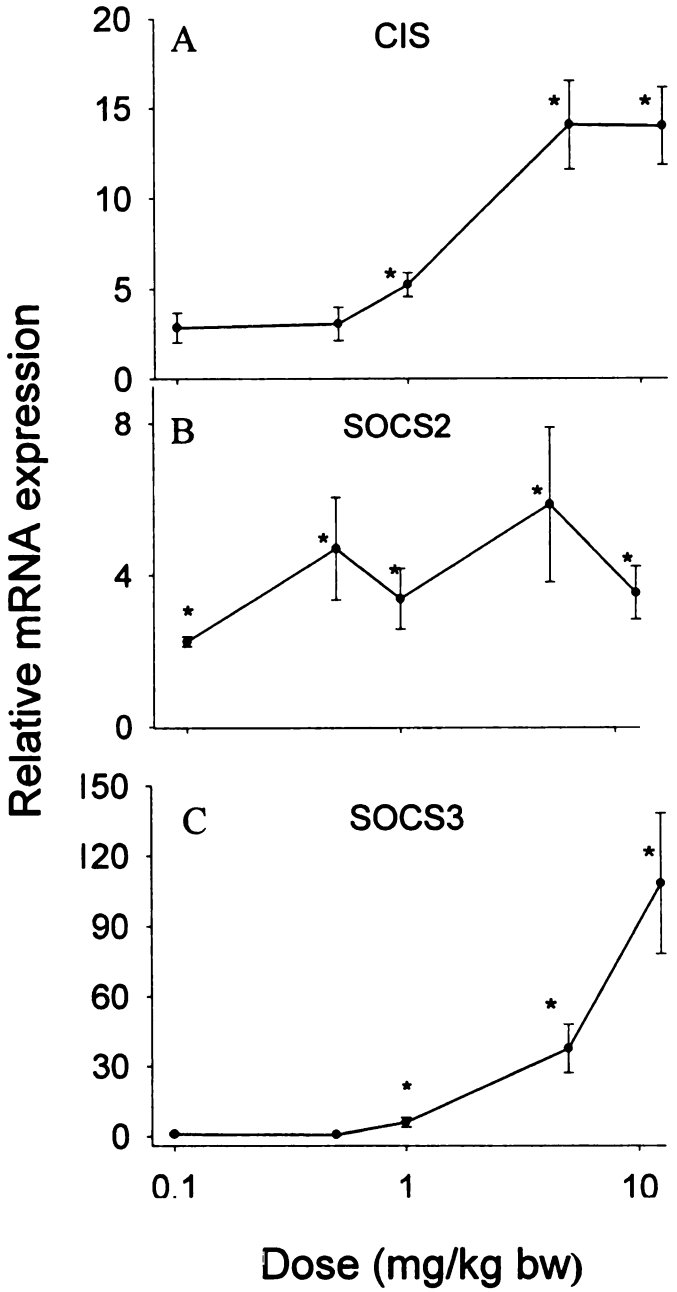

Figure 3.3 DON dose-dependently induces SOCS mRNA expression in the liver. Mice were treated with DON as in *Figure 3.1*. Liver was analyzed by real-time PCR for SOCS (CIS (A), SOCS2 (B), and SOCS3 (C)). Data are mean ± SEM ($n \geq 5$) of mRNA fold change relative to an untreated (naïve) group (1 fold). Means with asterisk differ from naïve mice ($p < 0.05$).

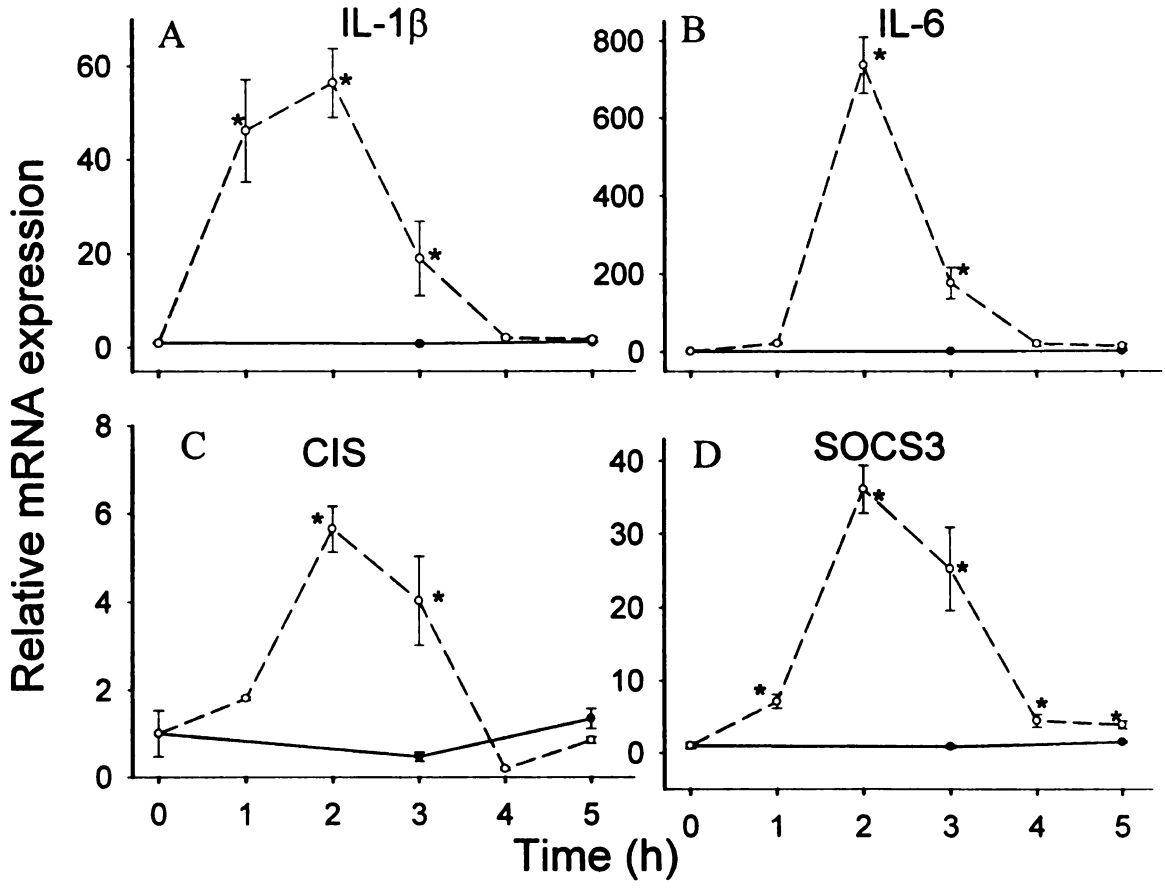

Figure 3.4 Kinetics of DON-induced cytokine and SOCS mRNA expression in the spleen. Mice were orally gavaged with either 12.5 mg/kg bw DON (broken lines) or PBS (solid lines). Tissues were collected 0-5 h after gavage. IL-1β (A), IL-6 (B), CIS (C) and SOCS3 mRNA (D) were analyzed by real-time PCR. Data are mean ± SEM ($n \geq 3$) of mRNA fold change relative to an untreated (naïve) group (1 fold). Means with asterisk differ from naïve $p < 0.05$.

In the liver both IL-1β and IL-6 mRNAs were upregulated within 1 h (Figure 3.5A,B) and both cytokines reached peak expression at 2 h and decline onset thereafter. Hepatic CIS and SOCS3 induction peaked at 2 h and SOCS3 remained elevated beyond 4 h while IL-1β and IL-6 returned to near basal levels. Thus, robust proinflammatory cytokine induction appeared to precede hepatic SOCS induction (2 h) in DON-treated mice. SOCS3 mRNA decline was relatively slower than proinflammatory cytokines.

DON induces hepatic SOCS3 protein expression

DON-induced SOCS3 mRNA was related to expression of SOCS3 protein in liver using immunohistochemistry. Vehicle-exposed mice did not exhibit binding of anti-SOCS3 at 3 h and 5 h (Figure 3.6E-F). DON induced modest SOCS3 immunostaining in the centrilobular area at 3 h (Figure3.6C), however, SOCS3 staining was pronounced at 4 h and remained strong through 5 h (Figure 3.6B,D). DON induced strong SOCS3 staining around the centrilobular areas. Overall, the highest acute dose of DON used in our experiment caused a 108-fold increase in SOCS3 mRNA around 2-3 h post-exposure, and a robust protein increase around 4-5 h, suggesting a sequential increase in transcription and translation of hepatic SOCS3.

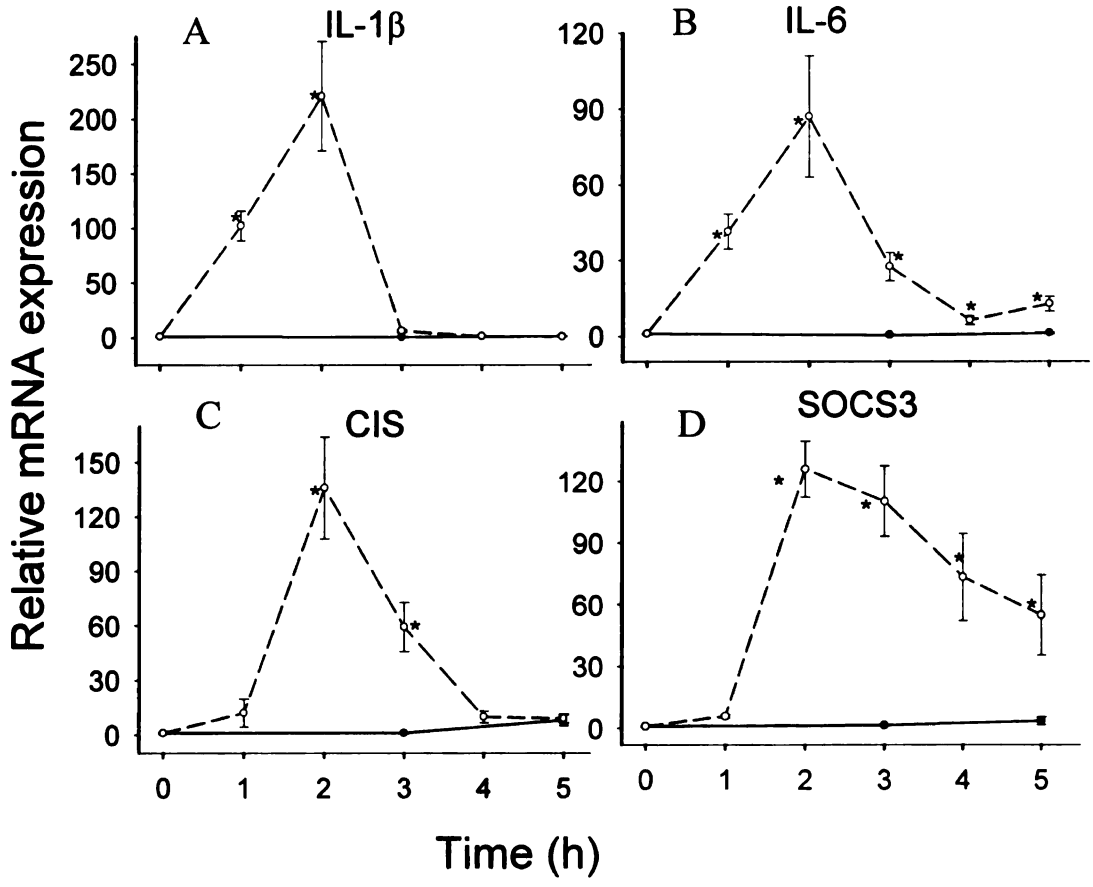

Figure 3.5 Kinetics of DON-induced cytokine and SOCS mRNA expression in the liver. Mice were exposed to DON or PBS as in *Figure* 3.4 above. Tissues were collected 0-5 h after gavage. IL-1β (A), IL-6 (B), CIS (C) and SOCS3 mRNA (D) were analyzed by real-time PCR. Data are mean ± SEM ($n \geq 3$) of mRNA fold change relative to an untreated (naïve) group (1 fold). Means with asterisk differ from naïve $p < 0.05$.

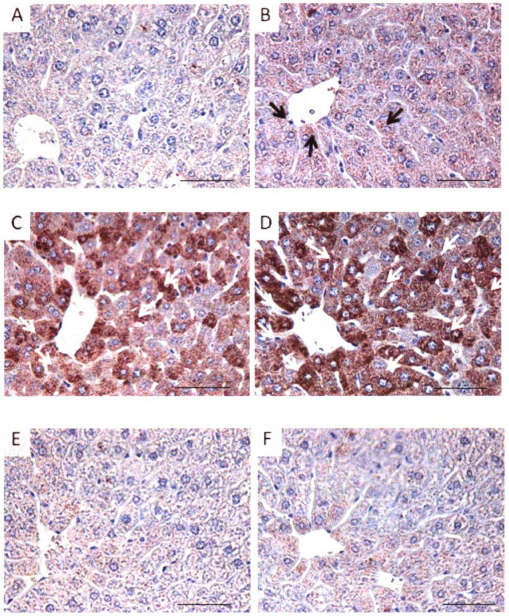

Figure 3.6: Kinetics of DON-induced SOCS3 protein expression in the liver. Mice were exposed to DON and PBS (vehicle), as in *Figure* 3.4 above. Histologic sections of the liver were taken at 2, 3, 4, and 5 h after DON exposure (A, B, C and D, respectively); and 3, 5 h after vehicle exposure (E and F, respectively). Paraffin-embedded sections were incubated with anti-SOCS3 antibody and counterstained with hematoxylin after DAB reaction. Arrows indicate areas of SOCS3 protein staining. Bar = 50 μm

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Discussion

There has been increasing recognition that SOCS family proteins are critically important in the negative regulation of cytokine and growth factor signaling pathways. SOCS proteins share a conserved SOCS-box in their carboxy-terminal region and a Src homology 2 (SH2) domain that mediates their interactions with other proteins. This is the first report of SOCS induction by a mycotoxin and is likely to play a regulatory role in signaling pathways mediated by receptors of the cytokine receptor superfamily such as IL-6 and GH.

Three mechanisms have been proposed to act either independently, or in concert to achieve the SOCS-induced negative regulation of signaling. These include (1) direct inhibition of signaling kinases by binding to intra-cytoplasmic domains of receptors or the activation loop of kinases; (2) competition with other SH2 domain-containing signaling proteins for binding sites on receptors; and (3) proteasomal degradation of receptors by SOCS box-elongin interactions (O'Sullivan *et al.* 2007). SOCS-induced negative regulation of cytokine/growth factor pathways have been demonstrated in many *in vivo* and *in vitro* experiments, using many species (Croker *et al.* 2008).

Among the eight known SOCS proteins, four most well characterized in terms of their physiological roles, are CIS, SOCS1, SOCS2 and SOCS3 (Tan and Rabkin 2005). Consistent with earlier reports (Starr *et al.* 1997), we observed differential tissue expression of SOCS (Figures 3.1-3.5), both relative to basal expression and DON-induced expression.

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CIS was upregulated in the spleen and to a much greater extent in the liver. Notably, CIS inhibits growth hormone signaling *in vitro* (Ram and Waxman 1999), and growth retardation occurs in mice overexpressing CIS (Matsumoto *et al.* 1999). Furthermore, CIS is involved in IL-6 inhibition of hepatic growth hormone signaling (Denson *et al.* 2003). Since DON significantly induces IL-6, the potential exists for CIS upregulation to impair hepatic GH signaling and reduce growth.

The robust induction of SOCS1 in the spleen might modulate immune function. SOCS1 is well-characterized as an inhibitor of IL-4, IL-6, IL-12 and interferon-γ signaling pathways (Ram and Waxman 1999) but not a growth hormone inhibitor. The absence of SOCS1 upregulation in the liver of DON exposed mice suggests that SOCS1 might not play a major role in DON-related hepatic signaling impairment. It was surprising that SOCS1 was non-detectable in the liver of mice, in contrast to another study (Wormald *et al.* 2006). This discrepancy might be a result of one or a combination of the following differences: (1) strain (C57BL/6 versus B6C3F1); (2) PCR method (SYBR green versus Taqman); (3) stimulus (interferon-γ versus DON) and (4) age (7 wk versus 4 wk). Regardless of the cause, it was clear that our method can detect SOCS1 upregulation in the spleen but not in the liver.

SOCS2 was not upregulated in liver or spleen but was significantly upregulated in the muscle at the highest DON doses. This observation is notable because SOCS2-deficient mice have upregulated peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) in their skeletal muscles (Rico-Bautista *et al.* 2006) suggesting that SOCS2 might have a role in the transcription of this metabolic regulator. SOCS2 deficiency in mice causes a gigantism associated with muscle PGC-1α

upregulation, whereas DON exposure causes SOCS2 upregulation and impairment of weight gain; it is possible that DON-induced impairment of weight gain, and associated muscle SOCS2 upregulation will result in PGC-1α reduction. The putative role of PGC-1α in DON-induced growth reduction requires further investigation. The role of SOCS2 in growth hormone signaling is complicated because both SOCS2 overexpressing mice and SOCS2 deficient mice exhibit excess growth phenotype (Tan and Rabkin 2005).

Hepatic SOCS3 upregulation was the most robust indicator of DON exposure among the SOCS analyzed. SOCS3 shares a kinase inhibitory region with SOCS1, and is effective in binding intracellular kinases on the IL-6 family receptors (Tan and Rabkin 2005). SOCS3 inhibits GH signaling *in vitro* (Rico-Bautista et al. 2006) and causes IL-6 impairment of GH signaling *in vivo* (Denson *et al.* 2003). DON induces IL-6 (Pestka and Smolinski 2005) and IL-6 induces hepatic SOCS3 (Denson *et al.* 2003; Wormald *et al.* 2006). Thus, one explanation for the pronounced SOCS3 increase might be a robust induction of IL-6 by DON (Amuzie *et al.* 2008). The IL-6 might originate in liver and exert its response in an autocrine or paracrine manner; or might be produced by distant organs (e.g spleen and lung) and exert its hepatic effects in an endocrine fashion. However, it should be noted that other proinflammatory cytokines such as IL-1β and TNF-α also induce SOCS3 (Alexander 2002), albeit to a lesser degree than IL-6. The redundancy of cytokine signaling networks presents a major challenge in determining the specific cytokine inducer of hepatic SOCS3. Regardless of which cytokine(s) are upstream, SOCS3 upregulation offers an mRNA marker of acute DON exposure that appears to be more persistent than proinflammatory cytokines. In addition to spleen and

other immune organs that have been previously reported, SOCS3 protein staining in hepatocytes confirms that liver is indeed another target organ for DON effects

The reason for centrilobular zonation of DON-induced SOCS3 is not clear, but consistent with SOCS3 expression in another model of SOCS induction in the liver (Ogata *et al.* 2006). Zonation of metabolic enzymes, physiological responses, and oxygen tension occur in the liver (Jungermann and Kietzmann 2000) and might contribute to zonation of toxicant effects. Centrilobular SOCS3 expression suggests that (a) the eliciting cytokines are more concentrated around the central vein or (b) the necessary cytokine receptors are more abundant around the central vein. These possibilities highlight the complexity of hepatocyte responses and need to be resolved in future studies. Overall, sensitive and sustained SOCS3 expression in hepatocytes is consistent with its negative regulatory role and might impair hepatic GH signaling.

SOCS characterization offers an integrative approach to study potential metabolic effects of non-hepatotoxic immunotoxicants, outside the traditional immune organs. For example, previous chronic DON studies (2 months and 2 years) have reported an unexplained but significant reduction in liver weights (Forsell *et al.* 1986; Iverson *et al.* 1995), without overt hepatotoxicity. Since SOCS proteins impair cell proliferation and growth, it is possible that the liver reductions were a result of continuous/episodic hepatic SOCS upregulation, resulting in a proliferative disadvantage. Another evidence supporting anti-proliferative hypothesis is that spontaneous pre-neoplastic liver lesions were reduced in DON-fed mice when compared to age-matched controls after a 2-yr DON feeding (Iverson *et al.* 1995). These studies suggest an unexplored DON effect in the liver, which can be studied, in an integrative manner with our SOCS signaling

hypothesis. SOCS characterization also ties DON effects more closely to specific cellular pathways and reduces the knowledge gap between exposure and effect.

To our knowledge, this is the first report of a robust systemic induction of SOCS mRNA and protein by a natural foodborne toxin. Recombinant proinflammatory cytokines have been shown to induce SOCS proteins in a tissue specific manner (Starr *et al.* 1997). Bacterial lipopolysaccharide can also induce SOCS (Mao *et al.* 1999). The kinetics of DON-induced SOCS expression relative to proinflammatory cytokines indicates that DON induces cytokines in spleen and liver first (1 h) and SOCS3 later (2 h). SOCS3 remains upregulated after cytokines have returned to basal levels. Thus, DON's induction of SOCS extends our knowledge of DON effects and opens new opportunities. First, SOCS-positive cells may be isolated with techniques like laser-capture microdissection for further mechanistic understanding of a toxicant's effect. Second, since SOCS are downstream of cytokines in the JAK-STAT signaling pathway, characterization of systemic xenobiotic response based on all 8 SOCS proteins will be much easier than numerous cytokines, and may be applied to other inflammation-associated xenobiotics. Taken together, SOCS signaling offers the opportunity to integrate previously reported immune effects of DON and other toxins into a metabolic context, involving the liver and other organs, which might offer mechanism(s) of weight gain reduction and lead to identification of novel biomarker(s) of effect for human risk assessment.

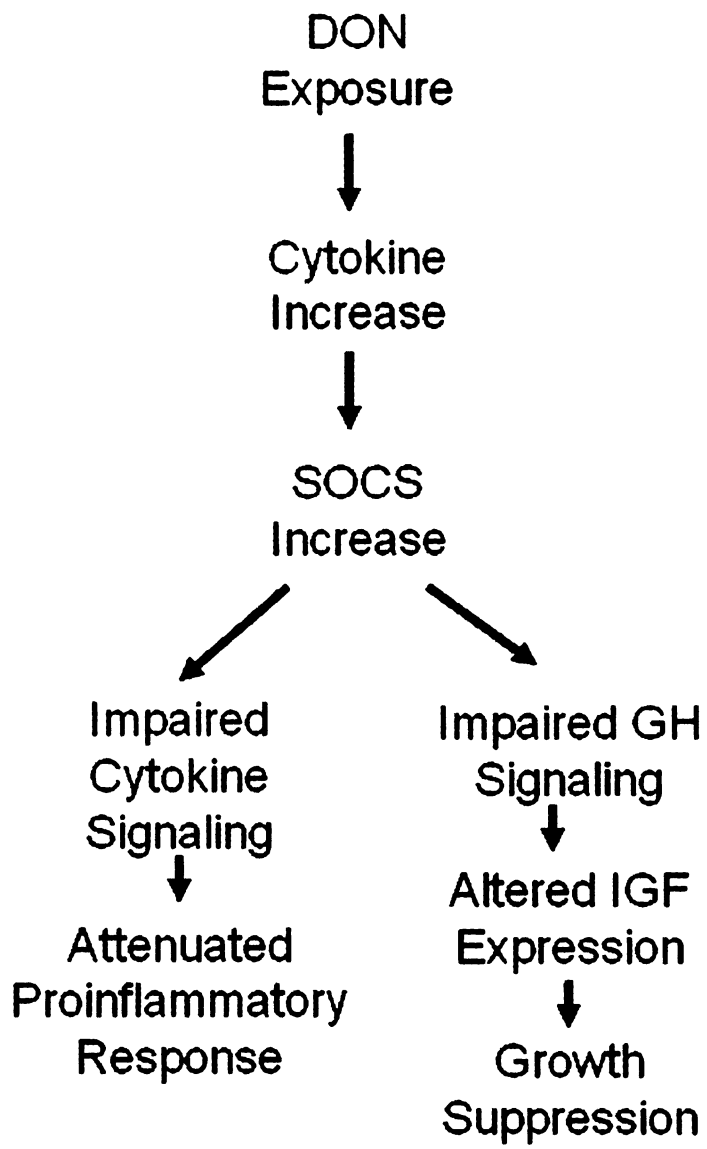

Figure 3.7: Proposed pathway for DON-induced SOCS expression and potential downstream effects. Impairment of cytokine signaling and GH-IGF1 has been demonstrated in other inflammatory models (Denson *et al.* 2003; Lang *et al.* 2005; O'Sullivan *et al.* 2007; Rico-Bautista *et al.* 2006)

CHAPTER 4: TRICHOTHECENE DEOXYNIVALENOL IMPAIRS GROWTH HORMONE SIGNALING AND SUPPRESSES INSULIN-LIKE GROWTH FACTOR ACID-LABILE SUBUNIT IN MICE

Abstract

Deoxynivalenol (DON), a metabolite produced by *Fusarium sp* is in a group of over 200 sesquiterpenoid fungal metabolites called trichothecenes. DON is the most commonly detected trichothecene in cereals and processed foods worldwide. DON reduces weight gain in many species, but the underlying mechanisms for this effect are less understood. After exposure, DON is rapidly distributed (≤30 min) to tissues, induces proinflammatory cytokines and suppressors of cytokine signaling, some of which impair growth hormone signaling. We hypothesized that acute and chronic DON exposure impairs growth hormone (GH) signaling in mice. Real-time PCR revealed that acute oral DON exposure rapidly (within 2 h) and dose-dependently (0.5-12.5 mg/kg bw) suppressed hepatic IGFALS mRNA by about 60-80%, with or without exogenous GH, and with concurrent hepatic SOCS3 upregulation. In addition, chronic dietary DON exposure (20 ppm) progressively impaired hepatic IGFALS mRNA up to 65% by the eighth week. ELISA equally revealed that dietary DON exposure suppresses circulating IGFALS and IGF1 by 66 and 26 %, respectively; concurrent with an increase in plasma DON concentration (≤ 63 ng/ml), and a reduction of weight gain. Taken together, these data suggest that DON might perturb GH axis and suppress clinically relevant growth-related proteins (IGF1 and IGFALS). Thus, IGFALS and IGF1 might be potential biomarkers of effect in future epidemiologic surveillance and human risk assessment of this common mycotoxin.

Introduction

DON is rapidly absorbed into the tissues of animals and can reach peak plasma concentrations within 15-30 min of oral dosing (Amuzie *et al.* 2008; Prelusky *et al.* 1988). Upregulation of proinflammatory cytokines is a central acute outcome of DON exposure *in vitro* and *in vivo* (Amuzie *et al.* 2008; Azcona-Olivera *et al.* 1995b; Dong *et al.* 1994; Zhou *et al.* 1997). Chronic DON exposure also results in weight gain reduction in many species

At high doses, DON induces anorexia in animal species, which has led to the suggestion that weight gain reduction is caused by anorexia in DON-exposed animals. Thus, earlier studies on DON-induced weight reduction focused on central appetite control pathways involving serotonin (Fitzpatrick *et al.* 1988; Prelusky 1993). Although there is apparent involvement of serotonin in the swine brain, serum serotonin concentrations were unchanged (Prelusky 1994) making the peripheral actions and general role of serotonin unclear. Furthermore, rodent studies with a serotonin antagonist (cyproheptadine) do not support a central role of feed refusal in weight gain reduction. Therefore DON's effects on weight might be secondary to other pharmacological actions (Prelusky *et al.* 1997), and might be influenced by factors other than reduced feed intake (Prelusky 1997). Studies by our group (Forsell *et al.* 1986) and Canadian researchers (Iverson *et al.* 1995) did not demonstrate a strong correlation between weight reduction and feed refusal in DON-exposed animals, particularly at lower dietary concentrations (<25 ppm), thus corroborating the conclusions from serotonin studies.

An alternative hypothesis for DON-induced weight reduction related to

proinflammatory cytokine upregulation and its downstream effects has been proposed based on several previous investigations (chapter 3). Proinflammatory cytokines induce a variety of SOCS proteins, in a tissue-specific manner, to negatively regulate several receptors of the cytokine superfamily signaling (Endo *et al.* 1997; Starr *et al.* 1997). Some of these SOCS proteins also inhibit growth factor signaling pathways.

Growth hormone (GH) binds to GH receptor, a member of the cytokine receptor superfamily (Bazan 1989) which mediates upregulation of insulin-like growth factor 1 (IGF1) (Salmon, Jr. and Daughaday 1957) and other binding partners essential for post-natal growth and development (Baker *et al.* 1993). Treatment with proinflammatory cytokines or the inflammagen lipopolysaccharide can inhibit GH-induced gene expression in mammalian hepatocytes (Boisclair *et al.* 2000; Shumate *et al.* 2005; Thissen and Verniers 1997) and whole liver in a SOCS-dependent manner (Mao *et al.* 1999; Yumet *et al.* 2002), suggesting that SOCS proteins might mediate crosstalk between proinflammatory cytokine signaling and GH signaling. Taken together, these studies suggests that perturbations in the GH/cytokine signaling pathways might mediate, in part, the weight gain effects observed in DON-exposed animals.

The pattern of SOCS expression in DON-exposed mice indicates that DON exposure could impact liver function in a manner that might affect GH signaling and hepatic metabolism. Recent experiments have shown that DON induces several SOCS mRNAs in the liver and robust SOCS3 protein in hepatocytes within 4 h of exposure (Chapter 3). SOCS3 upregulation can impair GH signaling in hepatocytes (Boisclair *et al.* 2000). Specifically, SOCS3 severely reduced GH-induced transcription of insulin-like growth factor acid labile subunit (IGFALS), an IGF1 binding partner responsible

for increasing the half-life of circulating IGF1. IGFALS reduction is known to severely reduce circulating IGF1 and growth, both in mouse and humans (Heath *et al.* 2008; Leroith and Yakar 2007; Ueki *et al.* 2009).

Based on the above evidence for potential cytokine-GH signaling crosstalk, and our previous observations of sequential induction of cytokines and SOCS by DON, we hypothesized that DON might dysregulate GH/IGF1 axis via reduction of circulating IGFALS and IGF1. The data presented here indicates that, concurrent with SOCS3 increase, acute DON exposure impairs GH-induced hepatic IGFALS mRNA production. Furthermore, chronic DON exposure resulted in marked reduction of hepatic IGFALS mRNA, circulating IGFALS and IGF1, concurrent with reduced weight gain. Thus, DON-induced weight gain reduction might result from perturbations in the growth axis, with IGFALS and IGF1 being potential biomarkers of the toxin's effects.

Materials and Methods

Laboratory animals

Pathogen-free female B6C3F1 mice (3-4 wk) (Charles River laboratories, Portage, MI) were randomly assigned to experimental groups ($n \geq 4$) and housed in polycarbonate boxes containing Cell-Sorb Plus bedding (A & W Products, Cincinnati, OH). Boxes were covered with filter bonnets and mice were provided free access to food and water. Room lights were set on a 12 h light/dark cycle, and temperature and relative humidity were maintained between 21-24^{0}C and 40-55%, respectively. Mice were maintained according

to National Institutes of Health guidelines as overseen by the All University Committee on Animal Use and Care at Michigan State University.

Exposure regimen and tissue collection

For each acute exposure experiment, DON (Sigma Chemical Co., St. Louis, MO) was dissolved in Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO) to give an exposure volume of 100 to 200 µl per mouse for any of the selected DON doses (0.1-12.5 mg/kg bw), while equivalent volumes of PBS were used as vehicle (0 mg/kg bw). Mice were orally gavaged using a 22 G intubation needle (Popper and Sons, New Hyde Park, NY), and given GH by i.p injection wherever necessary as described below.

At experiment termination, mice were deeply anesthetized by i.p. injection with 0.1 ml of 12% (w/v) sodium pentobarbital. The abdominal cavity was opened and blood was collected in heparinized syringes via the caudal vena cava, and transferred to centrifuge tubes. Following blood collection, the caudolateral piece of the lateral lobe of liver was collected for real time PCR as described below.

For growth hormone (GH) experiments, bovine somatotropin (Monsanto, St. Louis, MO) was kindly supplied by Dr. Gregg Bogosian, and was dissolved in 35 mM $NaHCO_3$ pH 9.5 and administered i.p at a dose of 5 mg/kg bw, once or twice, at various times (0-2 h) after oral DON gavage. Mice were sacrificed at indicated times (1-4 h) after GH exposure, caudolateral piece of liver was collected in and real-time PCR used to determine mRNA expressions.

For the feeding study, DON, purified from *Fusarium graminearum* cultures reported by (Clifford *et al.* 2003), was added at 20 mg/kg of powdered AIN-93 G Purified Rodent Diet 101847 (Dyets Inc, Bethlehem, PA) as previously described (Pestka *et al.* 1989) and fed to the treatment group, while the control mice were fed the purified diet alone. Mouse cages were kept in class II ventilated cabinets for the duration of the experiment. Mice were fed for 8 wk and weighed weekly. Groups of mice from each treatment were sacrificed at 2, 4 and 8 wk. Additional group of mice, acclimated to control diet for one week, was sacrificed immediately prior to initiating the experiment (0 wk). Anesthesia and tissue collection methods were identical to those described above. Plasma was analyzed for DON, IGF1, and IGFALS concentrations using ELISA, while IGFALS and IGF1 mRNAs were also analyzed in the liver.

DON quantitation

DON was measured in plasma using a Veratox High Sensitivity (HS) ELISA (Neogen, Lansing, MI) as previously described (Amuzie *et al.* 2008).

Quantitative real-time PCR

Excised liver for PCR analyses were stored immediately after harvesting in RNA*later*™ (Ambion Inc., Austin, TX). RNA was isolated using Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH). Real-time PCR for IGF1, IGFALS, IGFBP3, and SOCS3 were performed on an ABI PRISM® 7900HT Sequence Detection System, using Taqman One-Step Real-time PCR Master Mix and Assays-on-Demand™ primer/probe gene expression products according to the manufacturer's protocols (Applied Biosystems, Foster City, NY). Fold change of targets was determined using β2-microglobulin RNA control and a relative quantitation method (Smolinski and Pestka 2005).

IGF1 ELISA

Plasma (10 µl) was assayed for IGF1 using a mouse Quantikine® ELISA (MG-100) Kit (R & D systems, Minneapolis, MN) according to manufacturer's instructions. Plates were read at 450 nm on an ELISA plate reader (Molecular Devices, Menlo Park, CA) and sample values determined from a standard curve according to manufacturer's instructions.

IGFALS ELISA

IGFALS ELISA was performed according to method described by (Hwang *et al.* 2008), with modifications. Briefly, 96-well Nunc Immuno™ microwell plates (Cat. #439454, Thermo Fisher Scientific, Rochester, NY) were coated with 100 µl of 1 µg/ml IGFALS monoclonal antibody (MAB 1436, R & D systems) dissolved in PBS (Sigma-Aldrich) and incubated on a shaker, overnight at room temperature. Plates were washed three times with PBST and blocked with IGFALS blocking buffer (PBS, 5% [w/v] sucrose and 0.5% Tween-20 [v/v]) for 1 h at room temperature. Plasma was acidified (1:4, v/v) in 0.2 M glycine-HCL, pH 2.3 for 30 min, and further diluted (1600 times) in IGFALS buffer (50 mM sodium phosphate, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween-20 and 0.2% (v/v) BSA); IGFALS standards were prepared by dissolving recombinant IGFALS protein (rmALS, R & D systems) 0 to 20 ng/ml in IGFALS buffer. Plates were washed three times as above, samples and standards (100 µl) were incubated for 2 h, on a shaker at room temperature. Plates were washed three times, and incubated with 100 ul of 200 ng/ml IGFALS biotinylated antibody (BAF 1436, R & D systems) dissolved in PBS containing 2% (v/v) goat serum (Sigma) and 0.5% (v/v) Tween-20, for 2 h on a shaker at room temperature. Plates were washed three times in PBST and 100 µl of Streptavidin-

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peroxidase polymer, ultrasensitive (Sigma-Aldrich) diluted in PBS (1:200 v/v) was added for 30 minutes. Plates were washed four times and 100 µl of TMB substrate (Neogen) was added for 15 minutes. Reaction was stopped with 2N H_2S0_4 and read at 450 nm on a plate reader (Molecular Devices); and IGFALS values were determined from a 4-parametric standard curve.

Statistics

Differences between two groups was determined with Student's t-test, whereas differences among more than two groups determined by Analysis of Variance (ANOVA) using SigmaStat v 3.1 (Jandel Scientific; San Rafael, CA) with the criterion for significance set at $p < 0.05$. Student-Newman-Keul's post-hoc test was used to isolate significant groups while Grubb's test was used to isolate significant outliers.

Results

Dietary DON consumption elevates plasma DON and impairs weight gain

Mice consuming DON diet (20 ppm) exhibited elevated plasma DON concentration (48 ng/ml) within 2 wk of initiation of feeding (Figure 4.1A). These mice maintained a near steady state concentration of DON between 44 and 63 ng/ml in plasma the feeding period, ranging from for the time points measured. As expected, mice fed control diet (without DON) exhibited no detectable plasma DON.

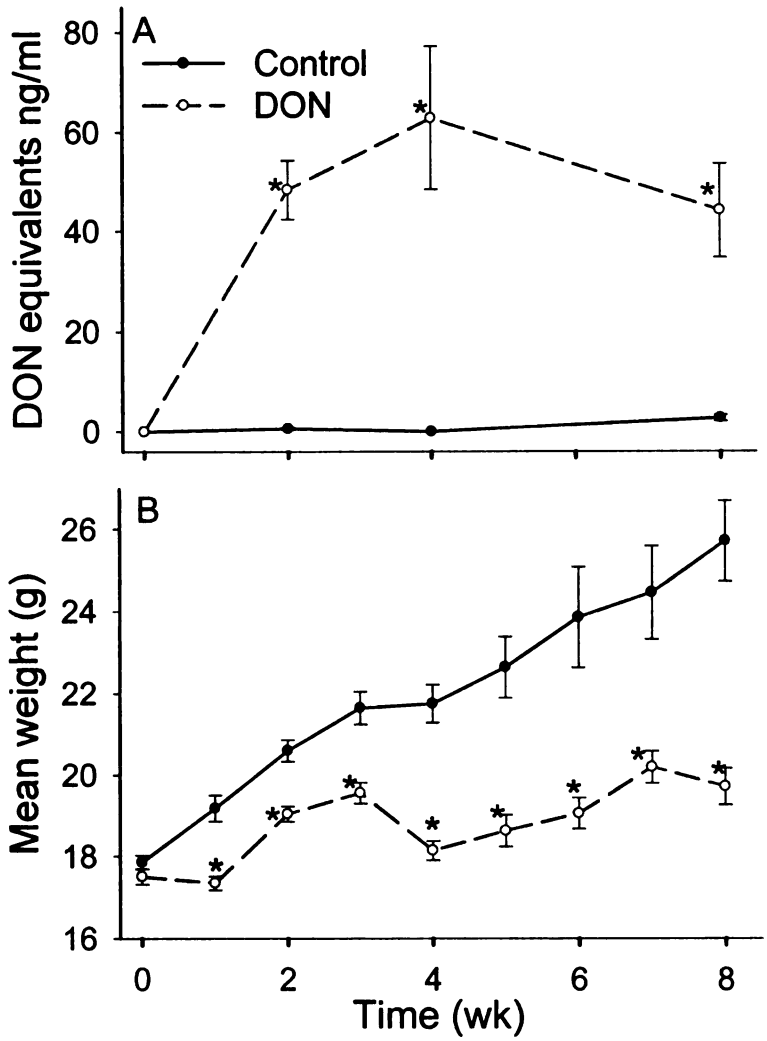

Figure 4.1 DON consumption increases plasma DON and reduces weight gain. Mice were fed diets without DON (Control), or with 20 PPM DON (DON). Groups of mice were sacrificed from both treatments at 0,2,4,6 and 8 wk. (A) Plasma was analyzed for DON by ELISA and (B) mice in different treatment groups were weighed weekly on a weighing scale. Data are mean ± SEM. ($n \geq 6$). Means with asterisks differ from another mean at the same time ($p < 0.05$).

Weekly weights of all mice were used as an index of growth (Figure 4.1B). Mice fed regular diet exhibited marked weight gain, starting at 17.8 g and reaching 25.7 g by the end of experiment. In contrast, mice fed DON only progressed from 17.5 g to 19.7 g within the experimental period suggesting that consumption of the toxin impaired weight gain in mice. Thus, while DON-fed mice did not lose weight, they failed to gain weight in a fashion that was commensurate with mice fed control diet.

Dietary DON consumption reduces circulating IGFALS and IGF1

Rapidly growing mice (4 to 12 wk old) were used to determine the effect of DON on circulating IGF1 and IGFALS, which are indicators of growth efficiency. Mice fed control diet exhibited plasma IGF1 concentrations ranging from 380 to 430 ng/ml during the 8 wk period, with the highest values being observed at experimental onset (Figure 4.2A). However, DON-fed mice had lower circulating IGF1, which was reduced to 74 and 64 percent that of control at 2 and 8 wk respectively.

In mice fed control diet, circulating IGFALS ranged from 18 μg/ml at wk 0 to 16 μg/ml at wk 8. Conversely, DON-fed mice exhibited severely suppressed IGFALS with plasma values ranging from 34 to 40 percent that of control values during the experimental period (Figure 4.2B). Thus, the percentage of IGFALS reduction relative to control mice is greater than that of IGF1. In summary, DON-induced weight gain reduction corresponded with circulating levels of IGF1 and IGFALS.

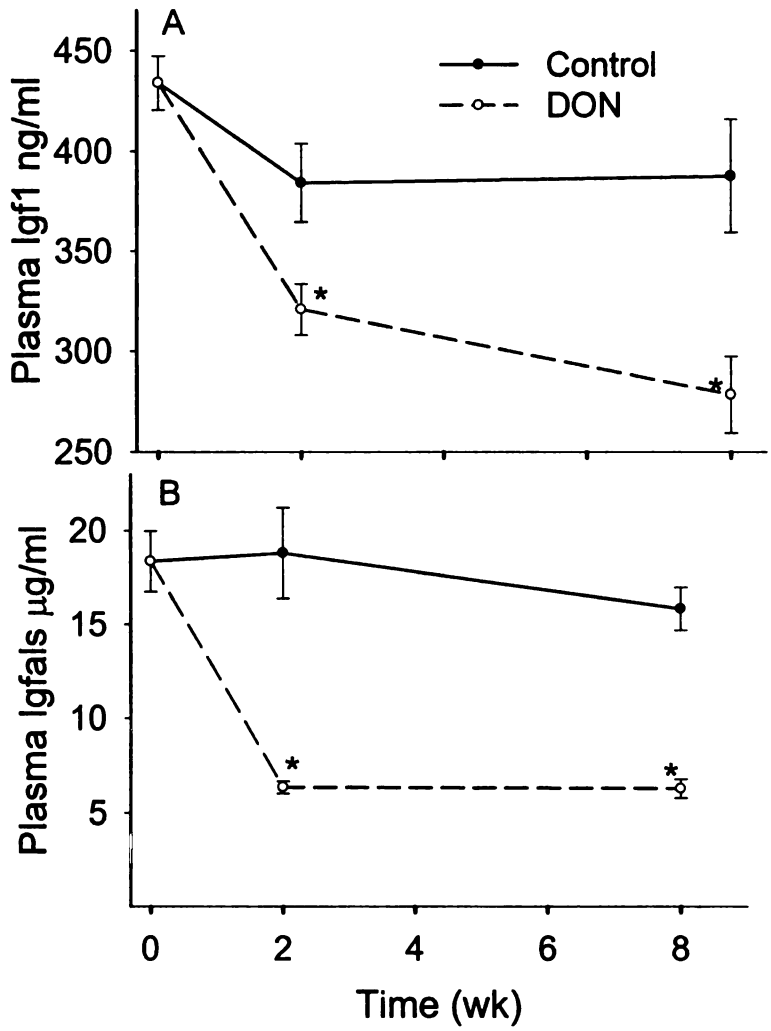

Figure 4.2 DON consumption reduces circulating IGF1 and IGFALS. Mice were fed diets as *in Figure 4.1* above. Groups of mice were sacrificed from both treatments at 0,2, and 8 wk. Plasma was analyzed for (A) IGF1 and (B) IGFALS by ELISA. Data are mean ± SEM. ($n \geq 6$). Means with asterisks differ from another mean at the same time ($p < 0.05$).

Exogenous GH induces hepatic expression of murine IGF ternary complex

Prior to investigating the effects of acute DON exposure on GH signaling, an experiment employing bovine somatotropin was designed to determine the optimal time point for GH-induced mRNA. Three mRNAs representing the IGF1 ternary complex, increased over time, reaching about 200% of the original expression after 4 h of GH exposure (Table 4.1). Specifically, IGF1, IGFALS, and IGFBP3 and increased by additional 125%, 86% and 188% respectively, at 4 h after GH exposure. Based on this study and another evidence (Chen *et al.* 2007), the 4 h time point was considered optimal to investigate potential perturbations on the GH axis.

DON impairs GH-induced IGFALS, but not other ternary complex partners

A GH experiment was designed to investigate DON's effect on GH capacity to induce members of the IGF ternary complex. Here, GH was injected once, 2 h after vehicle- or DON-treatment, a timepoint previously found to represent the peak of SOCS mRNA induction (chapter 3). At 3 and 6 h after DON exposure, DON suppressed GH-induced IGFALS mRNA to 26%, and 20% of vehicle-treated mice, respectively (Figure 4.3A). DON treatment did not suppress IGF1 and IGFBP3, but rather, IGF1 (Figure 4.3B) and IGFBP3 (Figure 4.3C) mRNAs of DON-treated mice at 6 h after exposure were approximately twice that of vehicle-treated, with IGF1 increase being significant. Accordingly, DON treatment suppressed hepatic IGFALS mRNA but increased IGF1 mRNA.

Table 4.1 Relative mRNA expression of IGF ternary complex members

Time (h)	IGFALS mRNA	IGF1 mRNA	IGFBP3 mRNA
0	1.0±0.1	1.0±0.4	1.0±0.1
2	1.34±0.3	1.48±0.2	2.06±0.5
3	1.76±0.3	1.65±0.5	1.3±0.5
4	1.86±0.3	2.25±0.4	2.88±0.6*

Table 4.1 Bovine somatotropin (GH) induces members of IGF1 ternary complex. Mice were treated with GH once, and sacrificed 2, 3, and 4 h later. Naïve mice (0 h) were sacrificed together with treated mice. Liver sections were collected and analyzed for IGFALS, IGF and IGFBP3 mRNA expression by real-time PCR. Data are mean ± SEM (n =5) of fold changes in mRNA relative to an untreated group (1 fold). Means with asterisks differ from untreated group ($p < 0.05$)

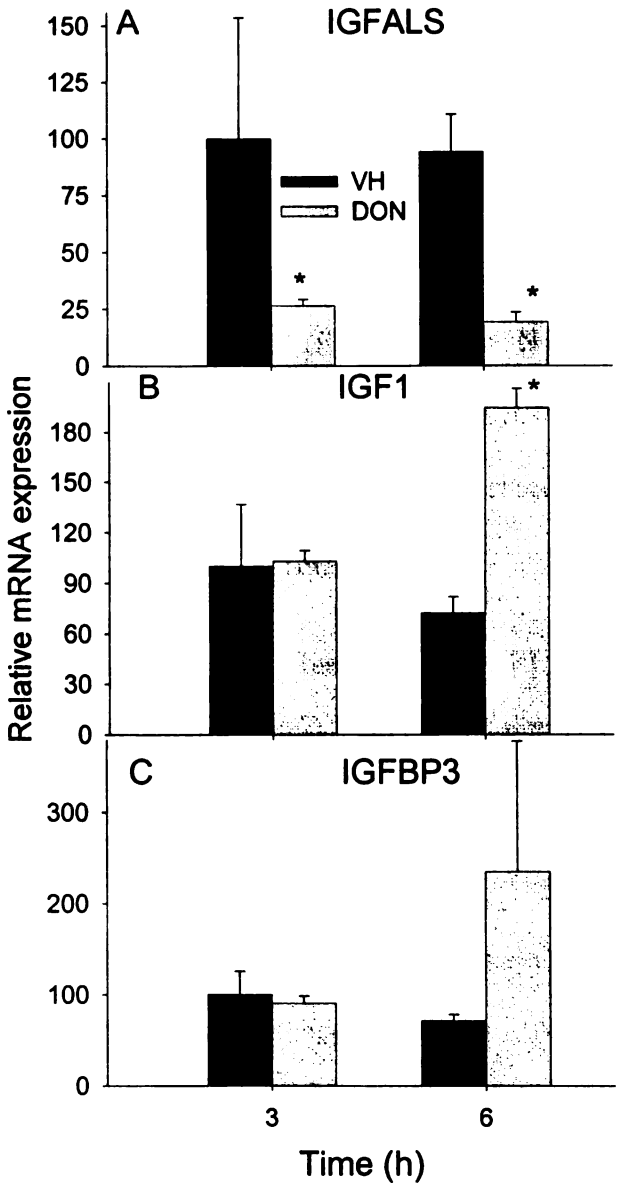

Figure 4.3 DON exposure differentially affects the IGF1 ternary complex partners' mRNA expression. Mice were orally gavaged with 12.5 mg/kg bw DON (DON) or PBS (VH), and treated with growth hormone (GH) i.p at 2 h after DON/VH exposure. Mice were sacrificed at different times (3 and 6 h) after DON exposure and liver sections were collected and analyzed by real-time PCR for IGFALS (A), IGF1 (B), and IGFBP3 (C). Data are mean ± SEM (n =4) of relative mRNA change in target relative to vehicle (VH) treatment (100 fold). Means with asterisks differ from VH at the same time ($p < 0.05$).

DON exposure affects hepatic IGFALS and IGF1 expression differentially, in both GH- and vehicle-treated mice

To confirm DON's differential effects on IGFALS and IGF1, mice that were treated with both exogenous GH and control were assessed. GH induced IGF1 mRNA in mice, with or without DON treatment (Figure 4.4). DON treatment caused 62% and 73% increase in IGF1 mRNA expression in vehicle- and GH-treated mice, respectively. Thus, even though DON feeding reduced circulating IGF1 (Figure 4.2A), acute DON appeared to increase IGF1 mRNA in the liver (Figure 4.4).

IGFALS is a binding partner that stabilizes IGF1 in circulation (Guler *et al.* 1989). Contrary to IGF1, DON exposure suppressed hepatic IGFALS induction, with or without exogenous GH (Figure 4.5). IGFALS mRNA in DON-treated mice was 39 and 33 percent that of vehicle-treated mice, with or without exogenous GH, respectively. Furthermore, in vehicle-treated mice (without DON), exogenous GH elevated IGFALS mRNA by 53% over that of age-matched control. In summary, DON impaired hepatic IGFALS mRNA production capacity, and this impairment could not be relieved by exogenous GH.

DON-induced hepatic IGFALS suppression is associated with SOCS3 mRNA increase

SOCS3 has been previously associated with impairment of GH-induced IGFALS in another model of cytokine-induced growth hormone resistance (Boisclair *et al.* 2000) and we have previously reported a robust expression of SOCS3 in the hepatocytes of

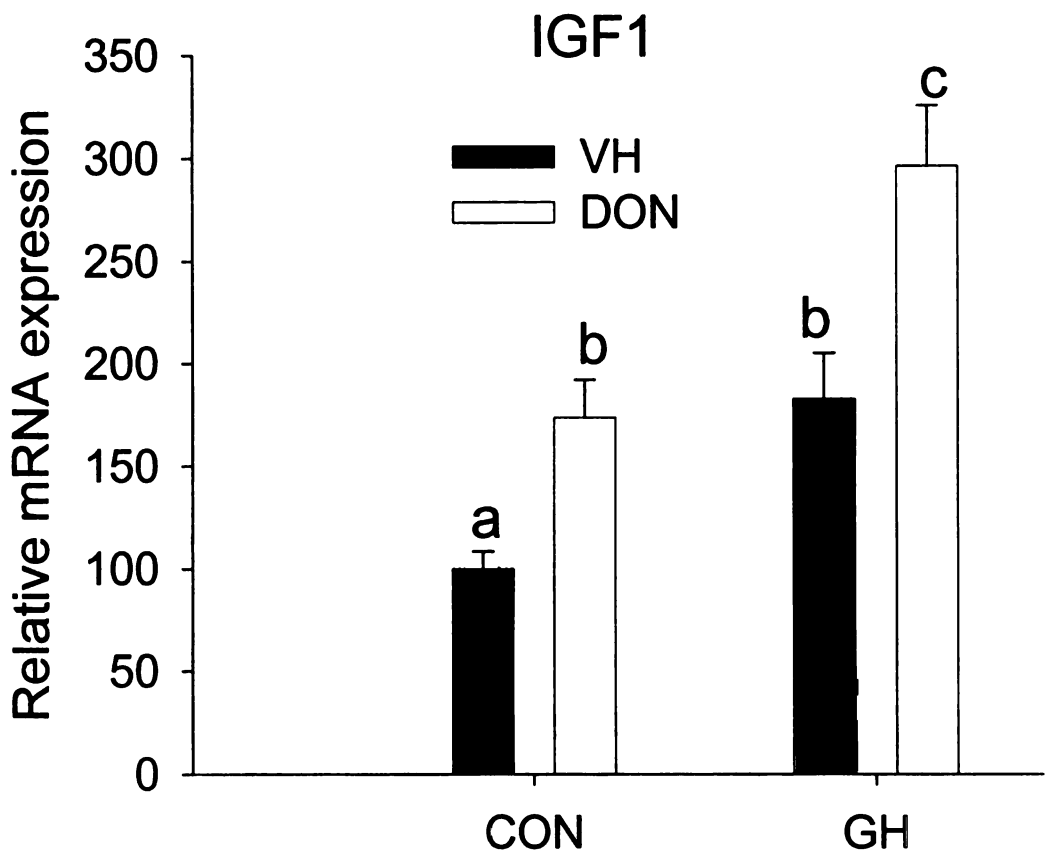

Figure 4.4 DON exposure induces IGF1 mRNA expression in livers of control- and GH-treated mice. Mice were orally gavaged with DON 12.5 mg/kg bw (DON) or PBS (VH); and later treated twice with growth hormone (GH) or $NaHCO_3$ buffer (CON) i.p, at 0.25 and 2 h after DON exposure. Mice were sacrificed after 4 h and liver sections collected and analyzed for IGF1 mRNA expression by real-time PCR. Data are mean ± SEM (n = 4) of mRNA fold change relative to VH/CON group arbitrarily set to 100 fold. Means with different letters differ ($p < 0.05$).

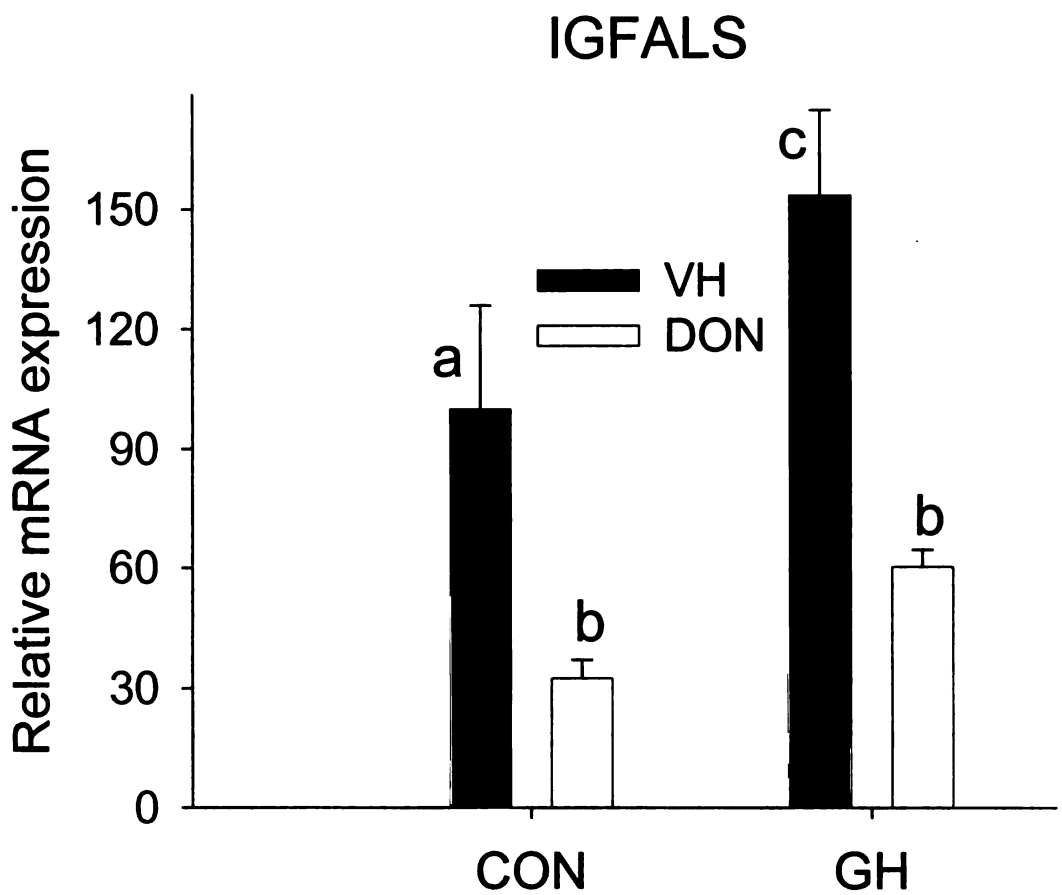

Figure 4.5 DON exposure suppresses IGFALS mRNA in livers of control- and GH-treated mice. Mice were treated and liver collected and analyzed as described in *Figure 4.4* legend. Data are mean ± SEM (n =4) of mRNA fold change relative to VH/CON group arbitrarily set to 100. Means with different letters differ ($p < 0.05$).

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DON-treated mice (Chapter 3). When SOCS3 mRNA was measured in DON-exposed mice, the toxin induced hepatic SOCS3 upregulation and this was not significantly affected by exogenous GH (Figure 4.6). SOCS3 remained upregulated about 6-fold, with or without GH exposure, 4 h after DON exposure. DON-induced SOCS3 increase corresponded to IGFALS reduction, with neither endpoint being influenced by exogenous GH.

DON-induced changes in hepatic IGFALS and IGF1 mRNA are time-dependent

Since GH release is variable in animals, it was necessary to determine the time-dependent effects of DON exposure without exogenous GH. Mice were orally gavaged with DON, and hepatic IGFALS and IGF1 mRNA then measured over 5 h. Consistent with earlier observations, hepatic IGF1 mRNA of DON-treated mice was significantly higher than vehicle-treated mice at 3 and 5 h after DON exposure (Figure 4.7A). In contrast, DON treatment progressively reduced IGFALS mRNA to 68%, 25% and 24% of the naïve mice values at 1, 3 and 5 h after exposure, respectively (Figure 4.7B). In comparison, IGFALS mRNA was unaffected in vehicle-treated mice over the 5 h period. The time course experiment indicated that a single bolus of DON, given orally, progressively suppressed IGFALS mRNA, for at least 5 h.

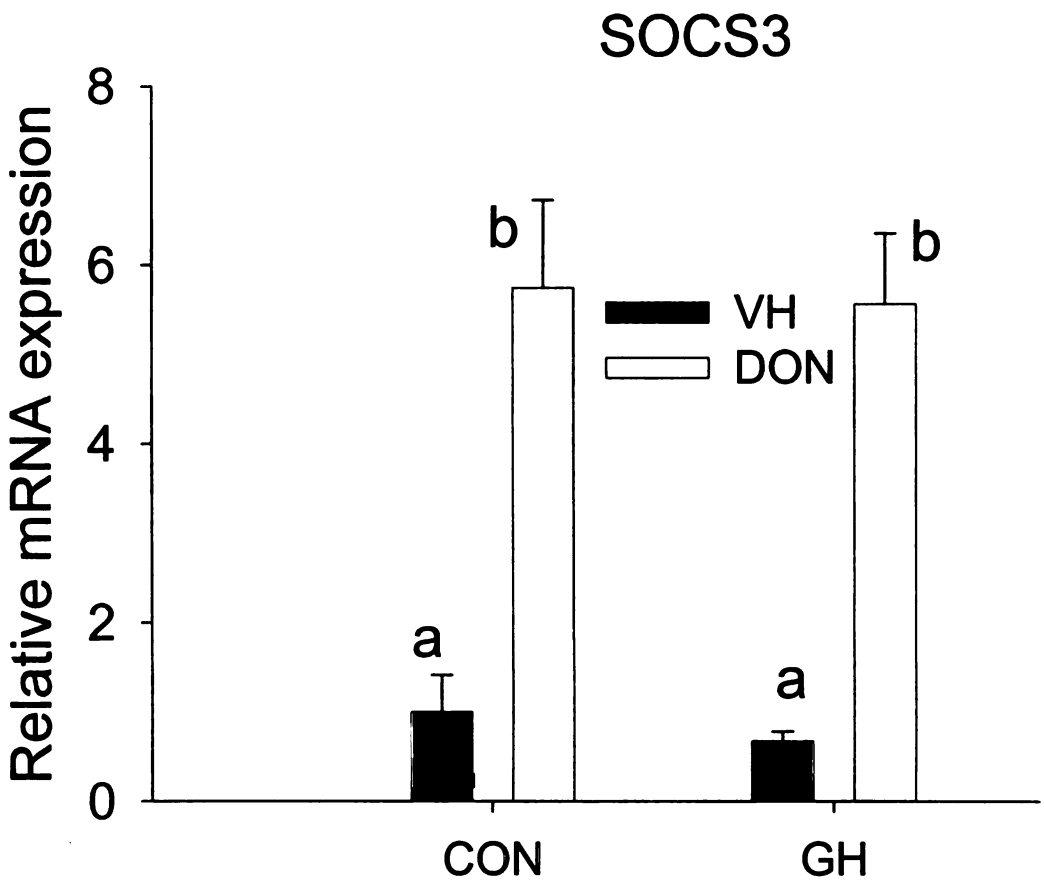

Figure 4.6 DON exposure increases SOCS3 mRNA in livers of control-and GH-treated mice. Mice were treated and liver collected and analyzed as described in *Figure 4.4* legend. Data are mean ± SEM (n =4) of mRNA fold change relative to VH/CON. Means with different letters differ ($p < 0.05$).

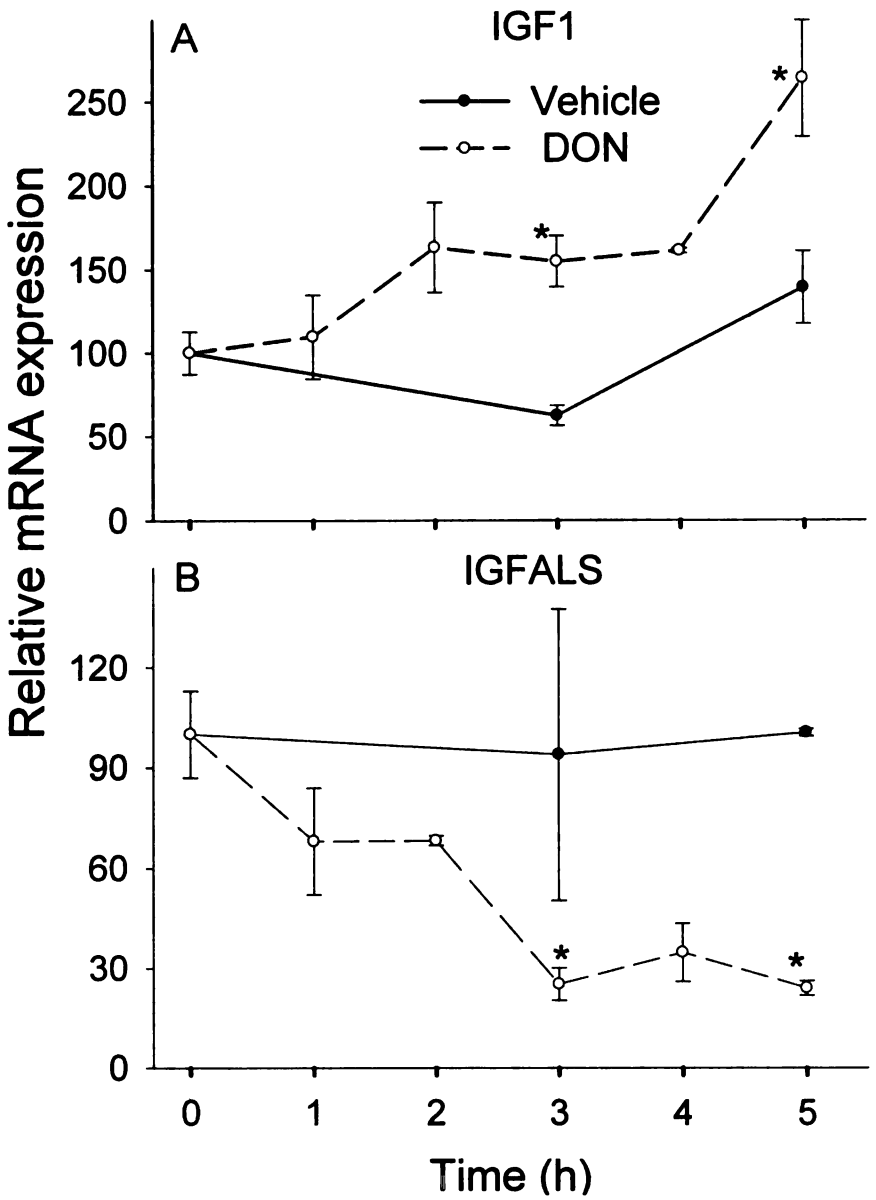

Figure 4.7 Kinetics of DON's effect on hepatic IGF1 and IGFALS mRNA. Mice were orally gavaged with either 12.5 mg/kg bw DON (broken lines) or PBS (solid lines).Livers were collected at intervals after gavage. IGF1 (A) and IGFALS (B) mRNAs were analyzed by real-time PCR. Data are mean ± SEM (n = 4) of mRNA fold change relative to naïve group (0 h) arbitrarily set at 100. Means with asterisk differ from vehicle at the same time ($p < 0.05$).

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DON-induced suppression of hepatic IGFALS mRNA is dose-dependent

A dose-response experiment with DON-gavaged mice revealed that 0.5 mg/kg bw and higher doses of DON (Figure 4.8), given orally significantly impaired IGFALS mRNA production. Although 0.1 mg/kg bw of DON exposure caused a reduction in IGFALS mRNA, this reduction was not significant. DON exposure causes a significant reduction of IGFALS at 0.5 mg/kg bw or higher doses.

Dietary DON consumption results in sustained suppression of hepatic IGFALS mRNA

To determine the relevance of IGFALS reduced hepatic IGFALS mRNA in foodborne DON exposure, hepatic IGFALS mRNA was measured in mice fed DON diet. Consumption of DON-amended diet resulted in a sustained reduction of IGFALS mRNA as compared to mice fed control diet (Figure 4.9). Relative IGFALS mRNA levels were 37% and 35% those of control mice by wk 2 and wk 8, respectively. Taken together, our results indicate that continuous DON exposure caused rapid and sustained suppression of IGFALS mRNA expression.

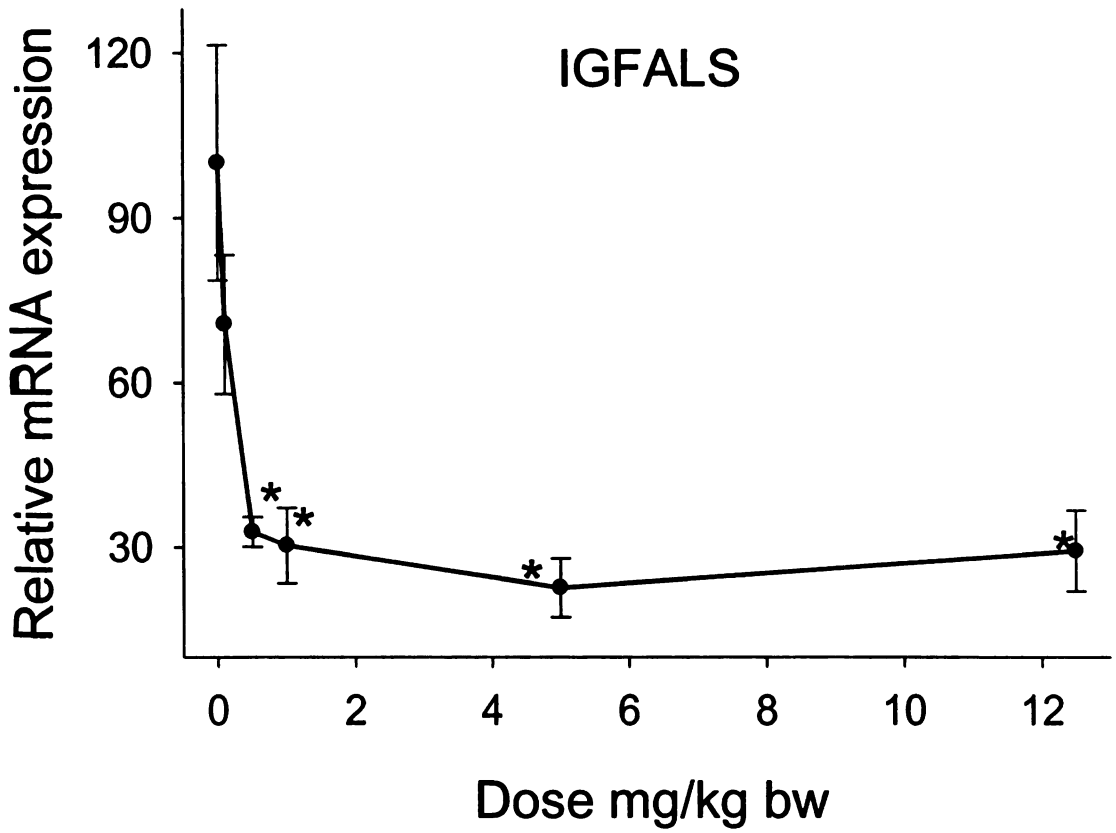

Figure 4.8 DON's suppression of hepatic IGFALS mRNA is dose-dependent. Mice were orally gavaged with various doses of DON (0 (PBS), 0.1-12.5 mg/kg bw) for 2 h, sacrificed and liver sections collected. Total mRNA was isolated and analyzed by real-time PCR. Data are mean ± SEM ($n \leq 5$) of mRNA fold change relative to a vehicle treatment (1 fold). Means with asterisk differ from vehicle ($p < 0.05$).

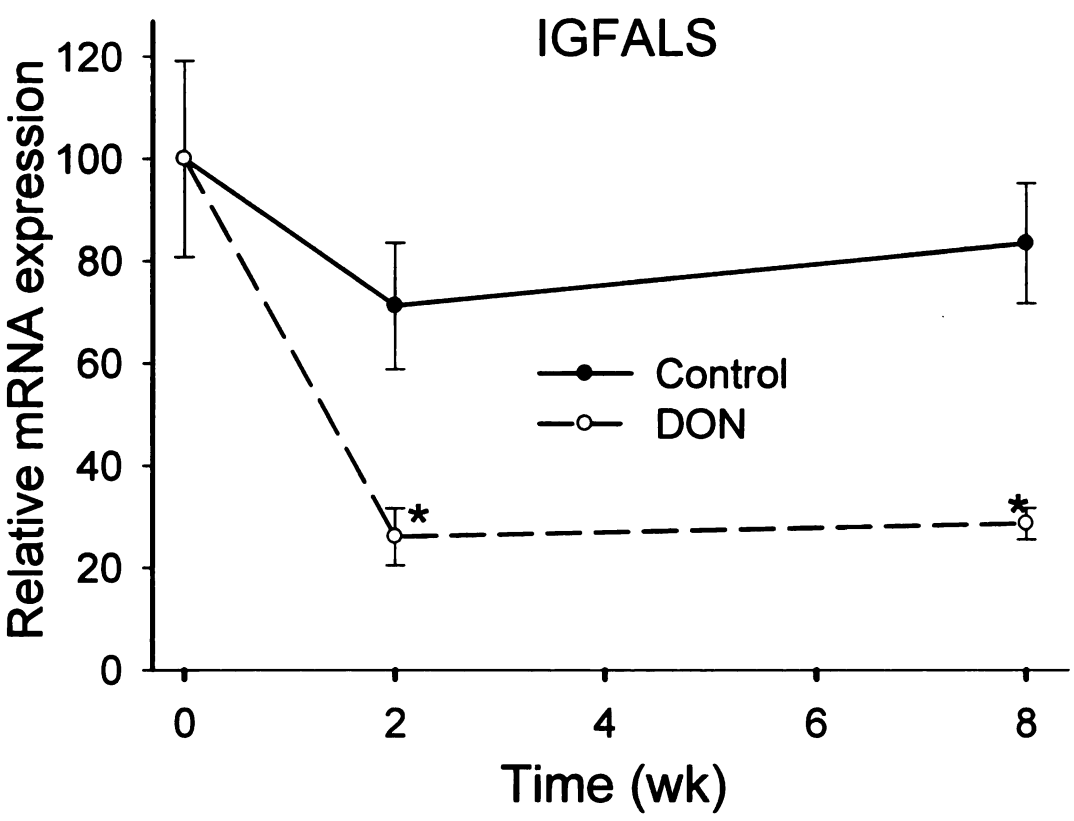

Figure 4.9 Dietary DON consumption reduces hepatic IGFALS mRNA. Mice were fed diets as *in Figure 4.1* above. Groups of mice were sacrificed from both treatments at 0,2, and 8 wk. Total mRNA from sections of liver was analyzed by real time PCR. Data are mean ± SEM ($n \geq 6$) of fold changes relative to a naïve group that is arbitrarily set at 100. Means with asterisks differ from another mean at the same time ($p < 0.05$).

Discussion

DON has long been recognized to impair weight gain, and this effect has been well-described in mice (Forsell *et al.* 1986; Iverson *et al.* 1995), rat (Arnold *et al.* 1986a; Morrissey and Vesonder 1985), and swine (Bergsjo *et al.* 1992; Rotter *et al.* 1992). Although this effect has been referred to as "growth retardation" (Iverson *et al.* 1995; Tritscher and Page 2004), involvement of GH axis has not been systematically evaluated. The Scientific Committee on Food for the European commission noted the "very unspecific" use of growth retardation in a DON opinion paper (EC 2002) and suggested that this might be indicative of a spectrum of events involving central nervous system aberration and feed refusal. The results presented here provide the first reported evidence that DON perturbs the GH/IGF1 axis. In humans, circulating IGF1 has been used as a marker to diagnose GH deficiency and to monitor response to GH treatment during postnatal growth (Bang *et al.* 1990; Pozo *et al.* 2005). DON-induced reduction of circulating IGF1 in growing mice, and the corresponding reduction in weight gain might be linked.

DON fed mice maintained a steady state concentration of 50-60 ng/ml. This is noteworthy for a couple of reasons. First, it suggests that only a small fraction of consumed DON is maintained in circulation after oral exposure, with the assumption that our sensitive ELISA detected most plasma DON. A combination of chronic and acute toxicokinetic data equally supports the low absorption/bioavailability possibility. For example, a 20-gram B6C3F1 mouse consuming 4 g of DON-contaminated food (20 ppm) daily (estimated from [Forsell *et al.* 1986; Iverson *et al.* 1995) would be equivalent to a maintenance dose of 40 mg/kg of daily DON. Assuming that DON is distributed to

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total body fluid (0.6 Liter per kg), and a DON elimination half-life of 0.5 h (Chapter 2), one would expect a steady state concentration of 2000 ng/ml ($C_{pss}=K_a/K_e*V_d$, where C_{pss} equals plasma steady state concentration, K_a equals loading dose, K_e equals elimination constant and V_d= volume of distribution) at the dietary concentration used in this study. However, the measured DON concentrations were much less (approximately 3% of 2000 ng/ml). Within the constraints of these kinetic assumptions and DON measurement method, it is possible that only a small fraction of orally exposed DON is bioavailable. This possibility has also been highlighted in our oral-nasal comparison experiments (Amuzie *et al.* 2008). A second indication from the DON measurement data is that despite rapid DON clearance profile, continuous exposure to relatively low dietary DON concentrations might result in a plasma steady state concentration, which could result in biological activity. In the future, additional studies designed to understand details of DON's kinetic profile in exposed people will be very important for relating DON's exposure to its effects in humans.

DON exposure resulted in 60-80% suppression in IGFALS mRNA and protein, within 2 h, with or without exogenous GH, in different experimental designs. Furthermore, circulating IGFALS reduction was associated with impairment of weight gain in growing mice. DON's capacity to robustly depress circulating IGFALS is a critical indication of the toxin's capacity to impair GH signaling. IGFALS is known to stabilize IGF1 in circulation, thereby extending its half-life from 15 min to 15 h (Guler *et al.* 1989). Evidence from knock-out mice and emerging clinical data suggest that IGFALS is an essential partner in the IGF1 ternary complex, and that its deficit might have growth and metabolic consequences. Circulating IGFALS reduction severely

decreases circulating IGF1, which is associated with growth reduction in IGFALS knockout mice (Yakar *et al.* 2005). Recently, human cases of IGFALS mutation have been described and its features include both a reduction in circulating IGF1, and growth deficit (Domene *et al.* 2004; Heath *et al.* 2008; Ueki *et al.* 2009).

Previous reports indicate the proinflammatory cytokine IL-1β impairs GH-signaling in hepatocytes (Barreca *et al.* 1998). This impairment depended on SOCS3 and was mediated through the IGFALS promoter (Boisclair *et al.* 2000). Thus, it was interesting that a bolus of DON, given to mice orally, increased SOCS3 and reduced IGFALS with or without exogenous GH. Since DON also induces IL-1 β and other proinflammatory cytokines (Amuzie *et al.* 2008), DON-induced hepatic IGFALS suppression shares some features with other models of GH impairment. The shared features include the involvement of proinflammatory cytokines, hepatocytes and SOCS3 upregulation. However, the main difference is that our model used an integrated animal system to demonstrate hepatic IGFALS impairment. Regardless of the model differences, these studies suggest that inflammagen-induced GH impairment is mediated by SOCS3 increase and that IGFALS reduction is a consequence of such impairment.

One unexpected observation is the DON-related differential effects of exogenous GH on IGF ternary complex members. Specifically, when DON-treated mice were exposed to GH, IGF1 mRNA increased, while IGFALS mRNA was suppressed. The question that arises is why does DON affect two GH controlled transcripts differentially? Although all members of IGF ternary complex are thought to be under GH control, some reports indicate that there might be a GH-independent mechanism of IGF1 transcription involving estradiol (Venken *et al.* 2005). In addition, there are 19 putative STAT

transcription factor binding sites in both human and mouse IGF1 promoters (Eleswarapu *et al.* 2008), whereas a single 8-nucleotide sequence (ALSGAS1) is responsible for GH-induced IGFALS transcription (Boisclair *et al.* 2000). These comparative differences suggest that IGF1 and IGFALS might have markedly different transcriptional control mechanisms relative to their responses to GH.

GH is pleiotropic, signaling through MAPKs , STATs and other proteins to achieve cellular functions (Zhu *et al.* 2001). GH-STAT signaling is further complicated by the involvement of many STATs and JAK kinase. Therefore, GH can activate STATs 1and 3, directly through JAK phosphorylation, or activate STATs 5a or 5b via phosphorylation of tyrosine residues on GHR. Different SOCS proteins can inhibit GH signaling at multiple levels (Zhu *et al.* 2001). For example, SOCS1 binds directly to JAK2, SOCS3 inhibits JAK2- induced signaling, while CIS inhibits STAT activation through distal tyrosine residues in GHR (Zhu *et al.* 2001). A potential consequence of this complex GH organization is that in GH-impairment studies, different GH-induced transcripts could be up-or down-regulated by a stimulus, depending on duration, types of upregulated SOCS and identities of impaired STAT. Other investigators have observed differential up- and down-regulation of IGF1 partners in an endotoxin model of growth impairment (Fan *et al.* 1995). Nevertheless, upregulation of SOCS3 is very consistent with the possibility that impairment of a specific STAT like STAT3 might mediate IGFALS reduction. In support of this, SOCS3 has been shown as sufficient for impairment of GH-dependent IGFALS transcription in rat hepatocytes (Boisclair *et al.* 2000). Future experiments need to identify the critical STAT(s) for DON-induced

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IGFALS impairment and determine the relevance of the same STAT(s) for IGF1 transcription.

In addition to differential IGF1 and IGFALS responses to GH, DON treatment induced greater IGF1 mRNA than vehicle-treated mice. This was also unexpected, but might be explained by DON's capacity to increase the stability of some mRNAs, DON increases the mRNA stability of some cytokines (TNF-α, IL-6 and IL-2) through the 3` untranslated region (Chung *et al.* 2003; Wong *et al.* 2001), thus selectively extending the half-lives of some mRNAs and increasing total transcript levels. Acute DON treatment increased IGF1 mRNA, yet this observation is complicated by the reduced circulating IGF1 observed in DON-fed mice. There are at least two possible explanations for the IGF1 paradox: (1) IGF1 mRNA is increased and translated but the protein is degraded rapidly because of IGFALS reduction; and (2) IGF1 mRNA is increased, but selectively not translated. While there is evidence for the first option in other models of circulating IGF1 reduction (Thissen *et al.* 1992), some investigators contend that all IGF1 reduction in inflammation and sepsis models is related to decreased IGF1 synthesis (Lang *et al.* 2005). It is clear in our model that IGFALS mRNA is rapidly suppressed but not IGF1 mRNA, suggesting that circulating IGFALS reduction might precede circulating IGF1 reduction in DON-exposed mice. Models employed to date, including that described herein vary in terms of species, age, gender, organs, methods, stimuli, duration of stimuli and indices measured, making it difficult to make definite conclusions as to whether one or both IGF1 reduction pathways are operational. In the future, it will be essential to fully characterize DON- and other stimuli-induced GH perturbations in terms of GH-

related transcripts (IGF1 and IGFALS), SOCS, STATs and perhaps other IGF1 binding partners, so that subtle differences could be discerned.

Hepatic IGFALS mRNA was markedly affected by dietary DON exposure throughout the experimental period and was consistent with reduced circulating IGF1. IL-6 and SOCS3 were not significantly different throughout the feeding period (not shown) indicating that IGFALS mRNA suppression is exquisitely sensitive to low level dietary DON exposure. Acute studies with bolus doses of DON suggest that DON-induced IL-6 and SOCS3 expression were transient and tend to recover between 3 and 5 h, consistent with the role of SOCS3 as a suppressor of cytokine signaling. It might be speculated that variable feeding patterns of mice and relatively low dietary DON concentrations may have caused episodic spikes of IL-6 and SOCS3 expression that were not detectable at experiment termination. Such episodic increases in SOCS3 could have resulted in a longer lasting IGFALS suppression, which was measurable at sacrifice. Evidence from our acute studies support this position because 2 h DON treatment resulted in a decline in hepatic IGFALS at 0.5 mg/kg bw, whereas increases in hepatic SOCS3 mRNA and circulating IL-6 are not significant until 1 mg/kg bw and 2 mg/kg bw, respectively (Chapter 3, Islam and Pestka 2006). In summary, IGFALS reduction is measurable at mRNA and protein levels, in the liver and plasma; and after acute and chronic DON exposures.

Reduction of circulating IGF1 and IGFALS correlated with increased plasma DON concentrations and reduced weight gain. The elevation of plasma DON, lowering of circulating IGFALS and IGF1 and reduction of growth are important from a biomarker perspective for several reasons. First, DON-induced weight gain effects are now

associated with readily measured, clinically relevant molecules in the GH signaling pathway. Second, a biomarker of exposure (plasma DON) is now associated with a potential biomarker of effect (plasma IGFALS), and an observed phenotype (reduced weight gain), which is remarkable for risk assessment purposes. A limitation of this study is that it was conducted in 4 wk old female mice that were rapidly growing. Further experiments, are necessary to determine the validity of IGFALS/IGF1 as age- and gender-independent biomarkers of DON effect. In addition, the effect(s) of DON on GH receptor has not been sufficiently addressed, relative to identity of kinases and critical tyrosine residues that are involved.

This study opens opportunities for risk assessment scientists to use plasma DON and/or urinary DON (Amuzie *et al.* 2008; Turner *et al.* 2008a) as biomarkers of exposure with IGFALS and IGF1 as biomarkers of effect in other species and, ultimately, humans. Following cross-species validation, these biomarkers of exposure and effect might be used in concert for epidemiological surveillance. Such epidemiological knowledge will be a critical step in translating dose-response of DON in rodents to dose-response in humans and thereby reduce uncertainties that arise from interspecies extrapolation. In the long-term, it might be possible to generate DON-specific safety factors as opposed to default factors.

Taken together, within the constraints outlined above, this study is unique because it integrates DON's capacity to upregulate cytokines with its robust effects on weight gain in a manner that suggests potential mode(s) of action and identifies potential biomarkers for risk assessment. The combination of IGFALS reduction and SOCS induction by a natural toxin opens an area of inquiry on the metabolic effect of low doses

of foodborne contaminants that might be applicable to prevention and treatment of metabolic diseases. This is the first demonstration of sustained reduction of circulating IGFALS by a foodborne toxin and is significant from a food safety perspective. The potential that cytokines, induced by inflammagens can impair GH signaling is a unique area of inquiry that requires more effort. GH impairment by a foodborne toxin represents a major step in our understanding of chronic low dose exposures and holds promise in elucidating mechanisms of environmental illnesses. Overall, our findings suggest that DON-induced weight gain reduction might result from a DON-induced, SOCS-associated impairment of hepatic GH signaling leading to a reduction of circulating IGFALS and IGF1.

CHAPTER 5: PREVENTION AND AMELIORATION OF OBESITY BY THE TRICHOTHECENE DEOXYNIVALENOL IN MICE IS ASSOCIATED WITH INSULIN-LIKE GROWTH FACTOR ACID-LABILE SUBUNIT SUPPRESSION

Abstract

There are 2.1 billion overweight people globally and obesity is an increasing public health challenge. Weight loss is accepted as the best way to stem the obesity pandemic. Since behavior-related weight loss has had limited success, there is need for drug-based interventions. Current approved drugs have limited efficacy, however, and are inadequate for obesity control. Hence, new drugs with efficacious targets are necessary. The trichothecene deoxynivalenol (DON) is a fungal metabolite, present in global food supply that impairs weight gain in growing animals. Recently, we determined that DON also induces suppressors of signaling in many tissues and dysregulates hepatic growth hormone signaling. We hypothesized that dietary DON exposure will prevent and ameliorate diet-induced obesity (DIO) in mice. Lean and obese mice on high fat diet were fed various concentration of DON. In the preventive model, DON-fed mice (10 wk) on high fat diet (HFD) exhibited 15 and 24 % suppression of weight gain at 5 and 10 ppm DON respectively. For the therapeutic model, DIO mice fed same diets for 8 wk exhibited 16 and 23 % weight loss, respectively. In addition, the same diets (5 and 10 ppm) resulted in 50 and 83% suppression of periuterine fat in the preventive model, compared to 0 and 40% periuterine fat loss in the therapeutic model. Furthermore, plasma DON negatively correlated adiposity (Pearson's coefficient=-0.647) in the preventive model. Circulating insulin-like growth factor acid-labile subunit (IGFALS) was assessed in both models using ELISA. Although high fats diets elevated circulating

IGFALS, DON diets suppressed circulating IGFALS by 18 and 30% at 5 and 10 ppm, respectively, in the preventive model. The same diets resulted in 20 and 42 % suppression of IGFALS for the therapeutic model. Taken together, our data indicate that DON exposure prevents and ameliorates weight gain, adiposity and IGFALS elevation in obese mice, and shifts their phenotype towards that of lean mice. To our knowledge, this is the first demonstration of an obesity-attenuating compound that suppresses IGFALS. Future studies on the IGF axis might lead to new targets for obesity prevention and control.

Introduction

There are 2.1 billion overweight people globally (Baur *et al.* 2006), and an unprecedented increase in obesity and obesity-related morbidity and mortality. In the U.S., 30% of adults and 15% of young people are obese (Ogden *et al.* 2006). Obesity and its associated metabolic syndrome represent a huge national and global health challenge. Obesity pathogenesis is mostly linked to increased energy intake, which results in increased storage of fatty acids and triglycerides, adipocyte hypertrophy and/or hyperplasia with a subsequent increase in visceral fat mass (Gesta *et al.* 2007; Hirsch and Batchelor 1976). Excess visceral fat increases circulating fatty acids and can cause broader metabolic complications such as type 2 diabetes and cardiovascular diseases (Muoio and Newgard 2006; Raghow *et al.* 2008). In the U.S, health care costs attributable to being overweight and obese is projected to reach nearly $1 trillion by the year 2030 (Wang *et al.* 2008). Clearly, obesity is a growing public health challenge that requires intervention.

Weight loss is accepted as the best step to prevent and control obesity-related morbidities and mortalities (Idelevich *et al.* 2009). Unfortunately, behavior-related weight control such as exercise and diet control has not been very successful in stemming the obesity epidemic (Idelevich *et al.* 2009). Some anti-obesity drugs have also been approved for obesity management. These include (a) phentermine (catecholamine stimulant), (b) sibutramine (serotonin reuptake inhibitor), (c) rimonabant (cannabinoid receptor antagonist), and (d) orlistat (lipase inhibitor) (Atkinson 2008). All approved drugs focus on central appetite control except orlistat, which acts on the gastrointestinal tract. However, the efficacy and safety of these pharmacotherapeutic interventions are not optimal (Bray 2008), because they have been associated with reduction in fat-soluble vitamins (orlistat), hypertension (sibutramine), insomnia (phentermine) and suicide in extreme cases (rimonabant). Therefore, drugs with more efficacious targets and less toxicity are necessary to combat the obesity problem.

The trichothecene deoxynivalenol (DON) is one of 217 sesquiterpenoid metabolites elaborated by several fungal genera (Grove 2007). DON is produced by *Fusarium sp,* and is a common contaminant of cereal grains and processed food globally (Pestka and Smolinski 2005). Acute high dose exposure to DON can cause vomiting in swine (Pestka *et al.* 1987a), while chronic low dose DON exposure can impair weight gain (Pestka and Smolinski 2005; Rotter *et al.* 1996). Since weight loss is beneficial in obese states, understanding how DON and related trichothecenes impair weight gain might uncover new molecular targets for the control of obesity pandemic.

The mechanisms by which DON impairs weight gain are not completely understood. Upon oral exposure in mice, DON is rapidly (30 min) absorbed and

distributed (Amuzie *et al.* 2008), initiates a subsequent cascade involving MAPK activation, transcription factor activation, increased mRNA stability, and selective mRNA translation ultimately resulting in cytokine upregulation (Pestka *et al.* 2004). An integrated model from *in vitro* and *in vivo* observations predicts that DON rapidly (< 5 min) binds to ribosomes, activates ribosome-associated MAPK, and initiates the cascade (Bae and Pestka 2008). Cytokine induction is a central short-term (2 h) event in DON exposed animals, while weight gain impairment is a central longer term (> 1 wk) outcome in DON exposed animals. Cytokine induction and weight gain impairment in DON-exposed animals might be mechanistically linked.

Recently, we determined that suppressors of cytokine signaling (SOCS) are upregulated in DON-exposed mice (Chapter 3). Many of these cytokine-inducible proteins also impair growth factor signaling by inhibiting intracellular receptor phosphorylation of kinases in the JAK-STAT pathway (Croker *et al.* 2008). Furthermore, we have determined that DON exposure reduces circulating insulin-like growth factor -1 (IGF1) and markedly impairs the hepatic production of its binding partner (IGFALS). Based on previous studies from cytokine/growth factor signaling axis (Chen *et al.* 2007; De Benedetti *et al.* 1997; Lang *et al.* 2005; Wallenius *et al.* 2002), reduction of circulating IGF1 and IGFALS and might contribute to this toxin's effect on weight gain.

Since IGF1 system is important for differentiation of pre-adipocyte to adipocytes (Leroith and Accili 2008; Schaffler *et al.* 2006), it is possible that DON exposure will prevent and ameliorate obesity/adiposity in mice. In this study, we tested this hypothesis. Lean and obese female B6C3F1 mice were exposed to increasing concentrations of dietary DON concurrent with high fat diet (HFD). After various intervals of DON

exposure, weight, adiposity, plasma DON concentrations, IGF1 and IGFALS were measured. Our data indicate that DON dose-dependently prevented weight gain and adiposity in lean mice on HFD. Furthermore, DON reduced weight and adiposity in DIO mice regardless of HFD. The reduction of obesity and adiposity in mice correlated with a reduction of circulating IGFALS and increases in plasma DON concentrations. These data suggest that DON reduces obesity and adiposity in mice, and that IGFALS might be an unexplored peripheral target for obesity prevention and control.

Materials and Methods

Chemicals

All reagent were of reagent-grade quality and purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise described. DON used for feeding study was produced in *F. graminearum* R6576 cultures and purified by silica gel chromatography (Clifford *et al.* 2003). Purity of DON was verified by a single HPLC peak at 224 nm. Concentrated toxin solutions were handled in a fume hood. Labware that was contaminated with mycotoxin was detoxified by soaking for >1 h in 100mL/L sodium hypochlorite.

Laboratory animals

Pathogen-free female B6C3F1 mice (11-12 wk) (Charles River laboratories, Portage, MI) were randomly assigned to experimental groups (n = 6) and housed in polycarbonate boxes containing Cell-Sorb Plus bedding (A & W Products, Cincinnati, OH). Boxes were covered with filter bonnets and mice were provided free access to food and water. Room

lights were set on a 12-hour light/dark cycle, and temperature and relative humidity were maintained between 21-24^{0}C and 40-55%, respectively. Mice were maintained according to National Institutes of Health guidelines as overseen by the All University Committee on Animal Use and Care at Michigan State University.

Diet regimen and tissue collection

DON was incorporated into defined very high fat diet (HFD) (60% kcal from fat, Research Diets, New Brunswick, NJ, D12492), at varyious concentrations (0 to 10 ppm). Dietary DON concentrations were confirmed by High sensitivity DON ELISA kit (Neogen, Lansing) according to manufacturer's instruction.

For the obesity prevention study (preventive model), female B6C3F1 mice (10 to 11 wk) were fed, *ad libitum*, with HFD containing 0, 2, 5, and 10 ppm DON for 10 weeks; while low fat diet (LFD) (Research Diets, 10% kcal from fat, D12450B) was fed to control mice throughout the study.

For the obesity amelioration study (therapeutic model), mice were first fed very high fat diet for 8 wk to induce obesity. Mice were the regrouped to obtain similar weight distribution and then fed various concentrations of DON (0 to 10 ppm) in HFD for an additional 8 weeks. LFD was also fed to control mice throughout the obesity amelioration study.

Mice were weighed weekly. At the end of the experiment, mice were deeply anesthesized with 100 µl of 50 mg/ml sodium pentobarbital, blood was collected with heparinized syringes via the caudal vena cava. Periuterine fat was excised, weighed and their ratios to mouse body weight reported as an index of adiposity. Plasma was separated

from blood, aliquoted and stored at -80°C prior to analysis. Plasma was analyzed for DON, IGF1, and IGFALS concentrations using ELISA.

DON quantitation

DON was measured in plasma using a Veratox High Sensitivity (HS) ELISA (Neogen, Lansing, MI) as previously described (Amuzie *et al.* 2008).

IGF1 ELISA

Plasma (10 µl) was assayed for IGF1 using a mouse Quantikine® ELISA (MG-100) Kit (R & D systems, Minneapolis, MN) according to manufacturer's instructions. Plates were read at 450 nm on an ELISA plate reader (Molecular Devices) and sample values determined from a standard curve according to manufacturer's instructions.

IGFALS ELISA

IGFALS ELISA was performed according to method described by (Hwang *et al.* 2008), with modifications. Briefly, 96-well microtitre plates were coated with 100 µl of 1 µg/ml IGFALS monoclonal antibody (MAB 1436, R & D systems) dissolved in PBS (Sigma-Aldrich) and incubated on a shaker, overnight at room temperature. Plates were washed three times with PBST and blocked with IGFALS blocking buffer (PBS, 5% sucrose and 0.5% tween-20) for 1 h at room temperature. Plasma was acidified (1:4, v/v) in 0.2 M glycine-HCL, pH 2.3 for 30 min, and further diluted (1600 times) in IGFALS buffer (50 mM sodium phosphate, pH 7.6, 150 mM NaCl, 0.1% tween-20 and 0.2% BSA); while IGFALS standards were prepared by dissolving recombinant IGFALS protein (rmALS, R & D systems) 0-20 ng/ml in IGFALS buffer. Plates were washed three times as above, samples and standards (100 µl) were incubated for 2 h, on a shaker

at room temperature. Plates were washed three times, and incubated with 100 ul of 200 ng/ml IGFALS biotinylated antibody (BAF 1436, R & D systems) dissolved in PBS containing 2% goat serum (Sigma) and 0.5% tween-20, for 2 h on a shaker at room temperature. Plates were washed 3 times in PBST and 100 µl of Streptavidin-peroxidase polymer, ultrasensitive (Sigma-Aldrich) diluted in PBS (1:200 v/v) was added for 30 minutes. Plates were washed four times and 100 µl of TMB substrate (Neogen) was added for 15 minutes. Reaction was stopped with 2N H_2S0_4 and read at 450 nm on a plate reader (Molecular devices); and IGFALS values were determined from a 4-parametric standard curve.

Comparative analysis of transcription factor binding sites on IGFALS promoter using rVISTA

rVISTA (Loots *et al.* 2002) was used to compare IGFALS gene in three mammalian genera. IGFALS genomic DNA from mouse (NC_000083.5), human (NC_000016.8), and rat (NC_005109.2) were downloaded from Pubmed in the FASTA format and saved in plain text. Sequences were then submitted to rVISTA database (http://genome.lbl.gov/vista/rvista/submit.shtml) on March 4th, 2009 for genomic comparison using default settings. All eukaryotic transcription factors on rVista database were queried on the conserved sequence (1 to 200 nucleotides) in the mouse and human IGFALS gene with TRANSFAC Professional 9.2 to predict conserved binding sites, using default core similarity values. A default criterion for conserved sequence (70% similarity within a 100 base pairs) was selected. Data are shown as obtained from rVISTA with minor editing.

Statistics

Differences among multiple groups were determined by Analysis of Variance (ANOVA) using SigmaStat v 3.1 (Jandel Scientific; San Rafael, CA) combined with Student-Neuman-Keul's post-hoc test; or by Kruskal-Wallis ANOVA on ranks combined with Dunn's test when normality or equality of variance test failed. Grubb's test (www.graphpad.com) was used to isolate significant outliers. The criterion for significance was set at $p < 0.05$.

Results

DON consumption suppresses weight gain in mice on high fat diet

DON incorporation prevented weight gain in mice on HFD. For the preventive model, mice fed HFD increased their mean weight from 21 to 33 g, while mice on LFD weighed 70 % that of HFD at termination of experiment (Fig 5.1). Furthermore, mice on HFD+2 ppm DON had similar weight gain pattern curve as those fed HFD alone. However, when dietary DON was increased to 5 ppm and 10 ppm in HFD diets, final mean weights were reduced to 85% and 76% respectively, of the mean weight for mice

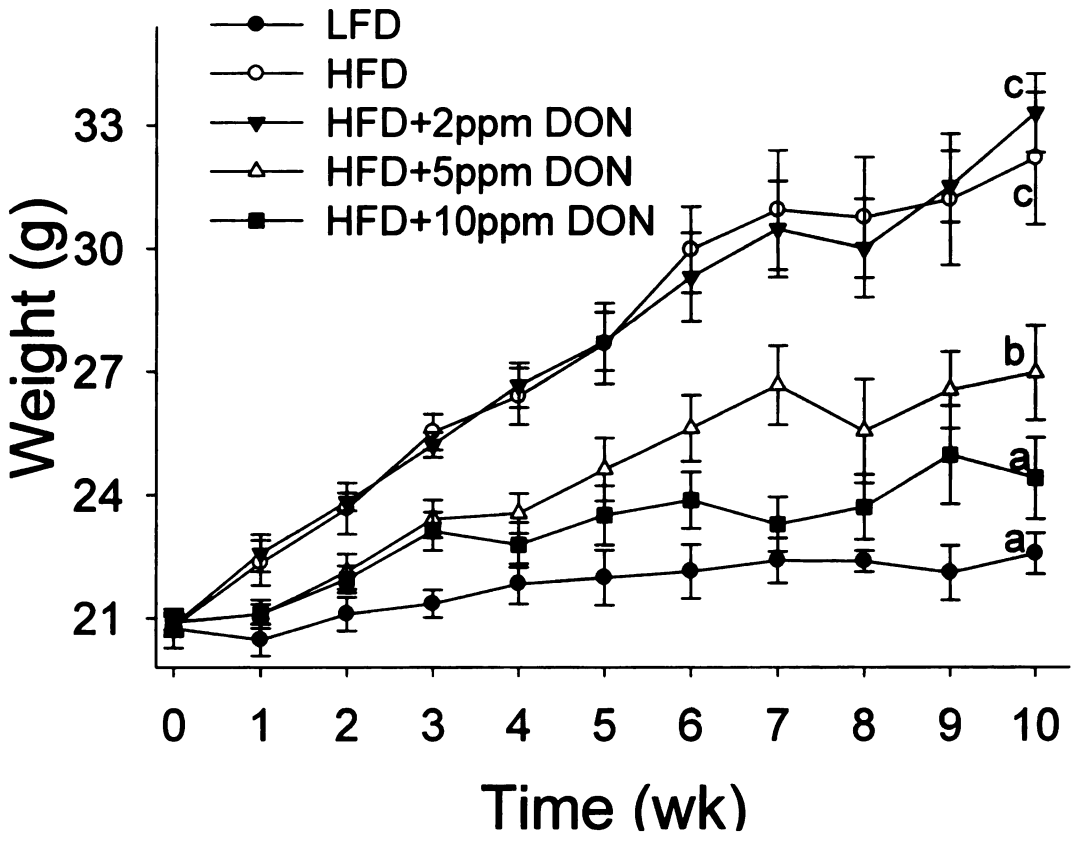

Figure 5.1 DON consumption suppresses weight gain in mice fed a high fat diet. Female B6C3F1 mice (10-11 wk old) were fed diets low fat diet (LFD), high fat diet (HFD), and HFD + increasing concentrations of DON (2, 5, 10 ppm) for 10 wk. Mice in different treatment groups were weighed weekly on a weighing scale. Data are mean ± SEM ($n \geq 6$). Means with different letters differ at 10 wk ($p < 0.05$).

on HFD alone. In summary, weight data indicated that DON dose-dependently prevented weight gain in mice on HFD, and the highest dose of DON nearly obliterated diet-related weight gain differences.

Adiposity correlates negatively with plasma DON concentrations

To confirm that plasma DON concentrations increases with increased DON consumption, an ultrasensitive ELISA was used to discern plasma DON concentrations. Mice on LFD and HFD alone had no measurable plasma DON (Figure 5.2A). However, increasing the dietary DON concentration resulted in corresponding increases in plasma DON. Mice fed 2, 5 and 10 ppm DON diets exhibited plasma DON concentrations of 3, 11 and 19 ng/ml respectively.

To relate DON exposure to adiposity effects, periuterine fat was excised at necropsy, weighed and related to body weight. Periuterine fat in mice fed HFD, or HFD + 2 ppm DON represented 6% of their body weight (Figure 5.2B). However, increasing dietary DON concentration to 5 and 10 ppm reduced periuterine fat to 3 and 1 % of body, respectively. By comparison, the periuterine fat in age-matched controls fed LFD was 1%. Overall, increasing concentrations of dietary DON resulted in increase plasma DON concentrations with a concomitant reduction in adiposity. Furthermore, plasma DON concentration negatively correlated with adiposity, with a Pearson correlation coefficient of -0.647 (Figure 5.3).

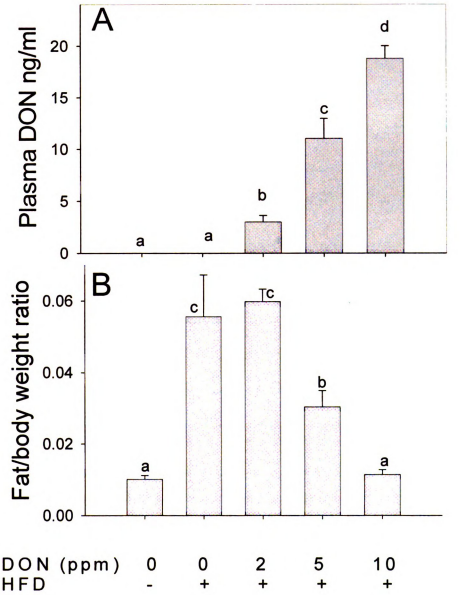

Figure 5.2 DON consumption increases plasma DON and reduces adiposity. Groups of mice in fed as in *Figure 5.1* above were sacrificed at the end of experiment (10 wk), organs and plasma were collected. Plasma was analyzed for DON by ELISA (A) and periuterine fat was excised, weighed and reported as a ratio to body weight (B). Data are mean ± SEM. (n =6). Means with different letters differ ($p < 0.05$).

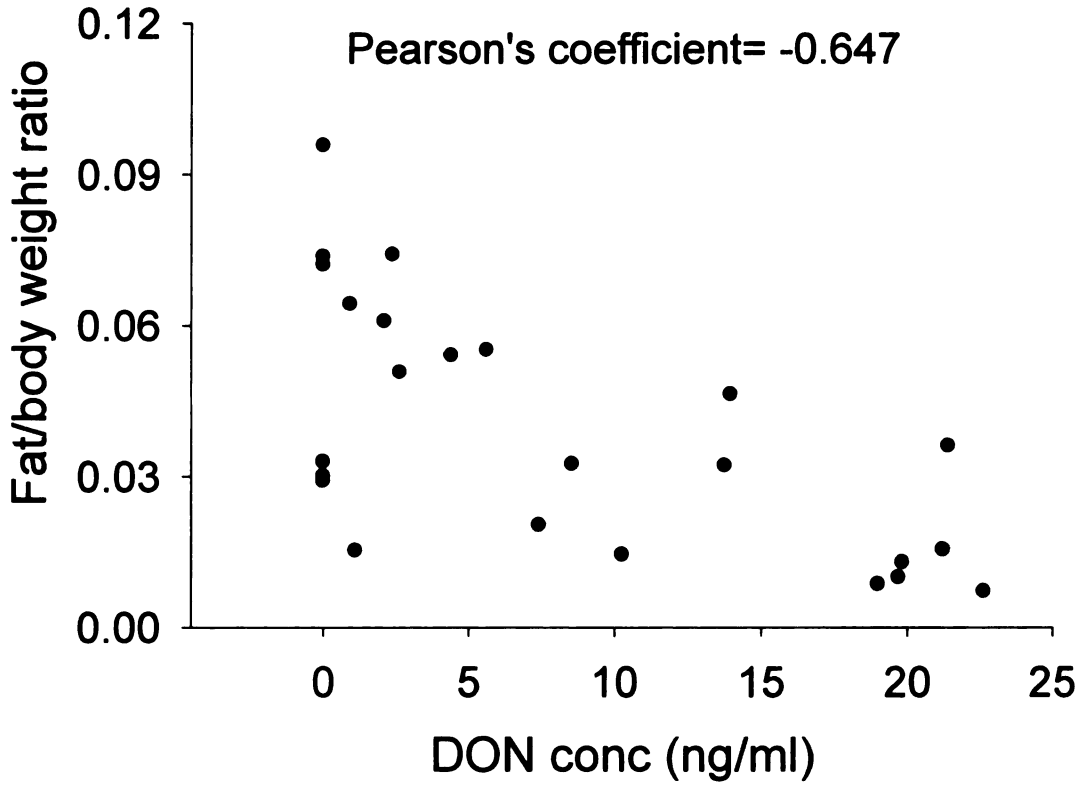

Figure 5.3 Plasma DON correlates negatively with adiposity in mice fed HFD. Pearson's correlation was used to relate plasma DON concentrations and adiposity data from *Figure 5.2*. Data are values from individual mice on HFD. There was a significant correlation (p=0.0006).

DON consumption attenuates diet-related IGFALS increase

Increases in IGF1 and its binding partners have been previously demonstrated in other models of diet-induced obesity (Fenton *et al.* 2009; Yakar *et al.* 2006) . Since we have demonstrated dietary DON-induced IGF1 and IGFALS reduction in lean mice (chapter 4), we assessed the levels of these markers in obese mice. HFD elevated the level of circulating IGF1 (490 ng/ml) above that of mice on LFD (372 ng/ml). DON consumption did not appear to affect IGF1 in mice on HFD (Figure5.4A). Circulating IGFALS, like IGF1, was significantly elevated in mice on HFD alone (11 μg/ml) over that of mice on LFD (7.5 μg/ml). This diet-related increase in IGFALS was dose-dependently reduced by dietary DON. Mice fed HFD + 10 ppm DON exhibited circulating IGFALS (7.6 μg/ml) that was remarkably similar to that of mice on LFD (Figure5.4B). In summary, the IGFALS data suggests that circulating IGFALS is dose-dependently impairment by dietary DON. Thus, the pattern of IGFALS impairment is similar to adiposity impairment in mice on high fat diet.

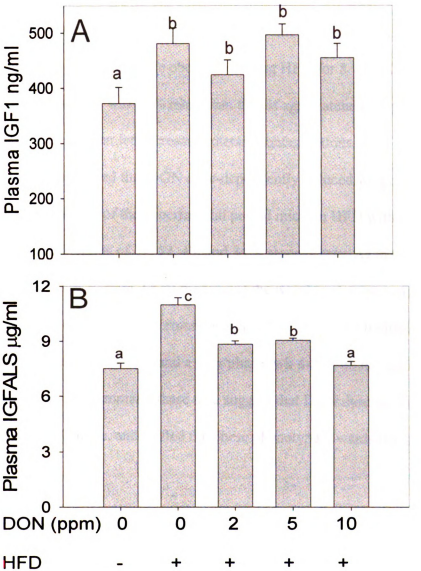

Figure 5.4 DON consumption prevents diet-related IGFALS increase in mice. Groups of mice were fed and organs collected as in *Figure 5.1,2* above . Plasma was analyzed for IGF1 (A) and IGFALS (B) by ELISA . Data are mean ± SEM. (n =6). Means with different letters differ ($p < 0.05$).

DON consumption causes weight loss in obese mice

DON's capacity to reduce weight in obese (DIO) mice was assessed. In this therapeutic model, mice were first made obese by feeding HFD for 8 wk (Figure5.5A), at which point their mean weight was 35% more than that of age-matched controls on LFD. Groups of obese mice were then fed increasing dietary concentrations of DON for another 8 wk. The data indicated that DON dose-dependently reduced weight in DIO mice (Figure5.5B). At the end of the experimental period mice on HFD with 0, 2, 5, and 10 ppm DON had mean weights of 51, 52, 43, and 34 g, respectively. As in the preventive model, at HFD+ 10 ppm, DON modulated the weights of mice on HFD to that in age-matched controls on LFD. Furthermore, weight plots revealed a biphasic pattern comprising initial weight loss (wk 0-4) and a later phase (wk 4-8) with no significant weight change at 10 ppm. In summary, these data suggest that DON dose-dependently caused weight loss in DIO mice, and shifted the obese phenotype towards that of lean mice.

DON consumption reduces adiposity in obese mice

DON feeding significantly reduced periuterine fat in DIO mice. In mice fed HFD+10 ppm DON, periuterine fat weight (6 %) was near that of age-matched controls on LFD (5.4 %). However, periuterine fat in mice on HFD + 2 and 5 ppm DON, 11 and 10 %, respectively were comparable to the 10% seen in mice on HFD alone (Figure 5.6).

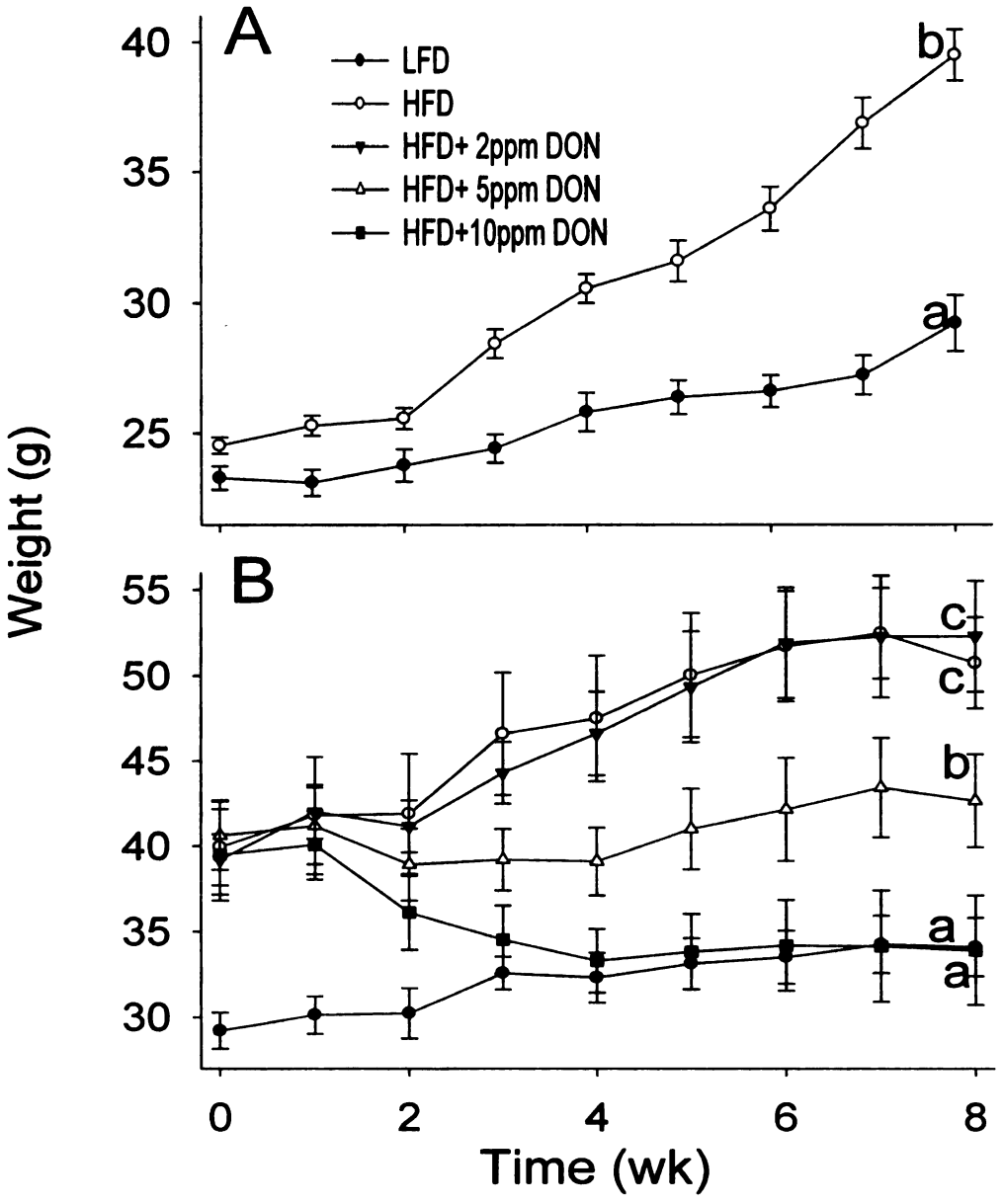

Figure 5.5 DON consumption induces weight loss in obese mice. Female B6C3F1 mice (12 wk old) Mice were made obese or kept lean by feeding HFD and LFD, respectively as in *Figure 5.1* above for 8 wk (A) . After obesity induction, obese mice were regrouped and were fed HFD + increasing concentrations of DON (0,2, 5, 10 ppm); while lean mice were maintained on LFD for another 8 wk (B). Mice in different treatment groups in experiments A and B were weighed weekly on a weighing scale. Data are mean ± SEM. ($n \leq 6$). Means with different letters differ at 8 wk within a figure ($p < 0.05$).

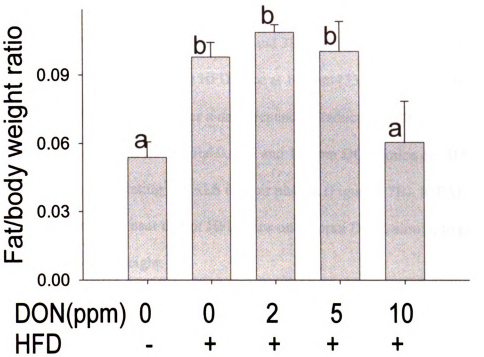

Figure 5.6 DON consumption reduces adiposity in obese mice. Groups of mice in fed as in *Figure 5.4* above were sacrificed at the end of experiment (8 wk), organs and plasma were collected. Periuterine fat was excised, weighed and reported as a ratio to body weight . Data are mean ± SEM. (n = 6). Means with different letters differ ($p \leq 0.05$).

DON consumption suppresses IGFALS in obese mice

DIO mice on HFD exhibited increases in circulating IGF1 and IGFALS. There was a reduction of IGF1 in DIO mice at all the DON doses (Figure 5.7A). Mice fed HFD+ 0, 2, and 5 ppm DON exhibited 490, 366 and 386 ng/ml of circulating IGF1, respectively. Circulating IGF1 levels in HFD mice at 10 ppm (332 ng/ml) was similar to that in LFD mice (355 ng/nl). There was a dose-dependent reduction of IGFALS occurred in DON-exposed mice. Mice fed 0, 2, 5 and 10 ppm DON, mice on HFD had 22, 19, 17 and 12 μg/ml of circulating IGFALS in their plasma (Figure 5.7B). IGFALS value in LFD mice (14 μg/ml) was near that of HFD mice on 10 ppm DON, similar to patterns observed for adiposity and weight.

Comparative analysis of the regulatory regions of human and rodent IGFALS gene

Since dose-dependent reduction of circulating IGFALS was observed in acute and chronic models of DON exposure (Chapters 4 and 5). It was necessary to compare the sequences of IGFALS gene in humans and rodents. Sequence similarities in the mouse, rat and human IGFALS gene was determined using rVISTA. The first 200 bases of IGFALS genes in all three species had greater than 70% sequence similarity. Furthermore, an rVISTA querry of all eukaryotic transcription factors in the rVISTA database predicted 25 conserved transcription factor binding sites on IGFALS gene in all three species. Notably, binding sites for cytokine signaling related transcription factors such as STATs were conserved in all three species. Transcription factors related to gluconeogenesis (HNF4) and lipogenesis (PPAR) were also predicted (Figure 5.7)

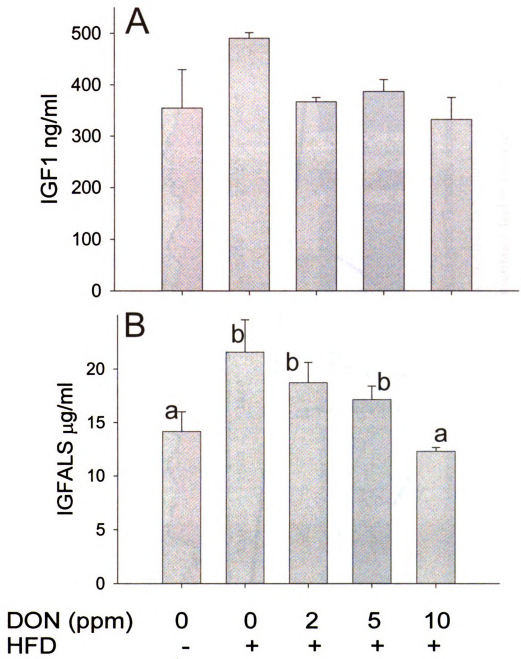

Figure 5.7 DON consumption reduces obesity-related IGFALS increase. Groups of mice were fed and organs collected as in *Figure 5.5-6* above . Plasma was analyzed for IGF1 (A) and IGFALS (B) by ELISA . Data are mean ± SEM. ($n \leq 6$). Means with different letters differ ($p < 0.05$).

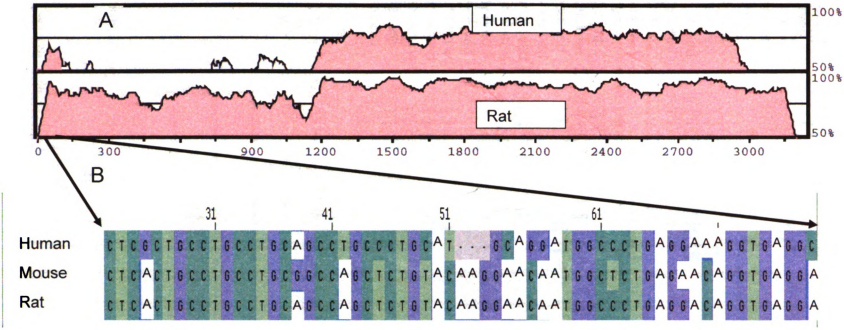

Figure 5.8 Comparative analysis of the regulatory regions of human and rodent IGFALS gene. Genomic sequences of mouse, rat, and human IGFALS gene were obtained from Pubmed, human and rat IGFALS were compared to a mouse background on rVISTA for sequence similarity (A). Conserved nucleotides (nt 23-79) (B) and predicted transcription factor binding sites (next page) (C) in the conserved regulatory region are shown. Modified Images were obtained from *rVISTA* (http://genome.lbl.gov/vista/rvista/submit.shtml).

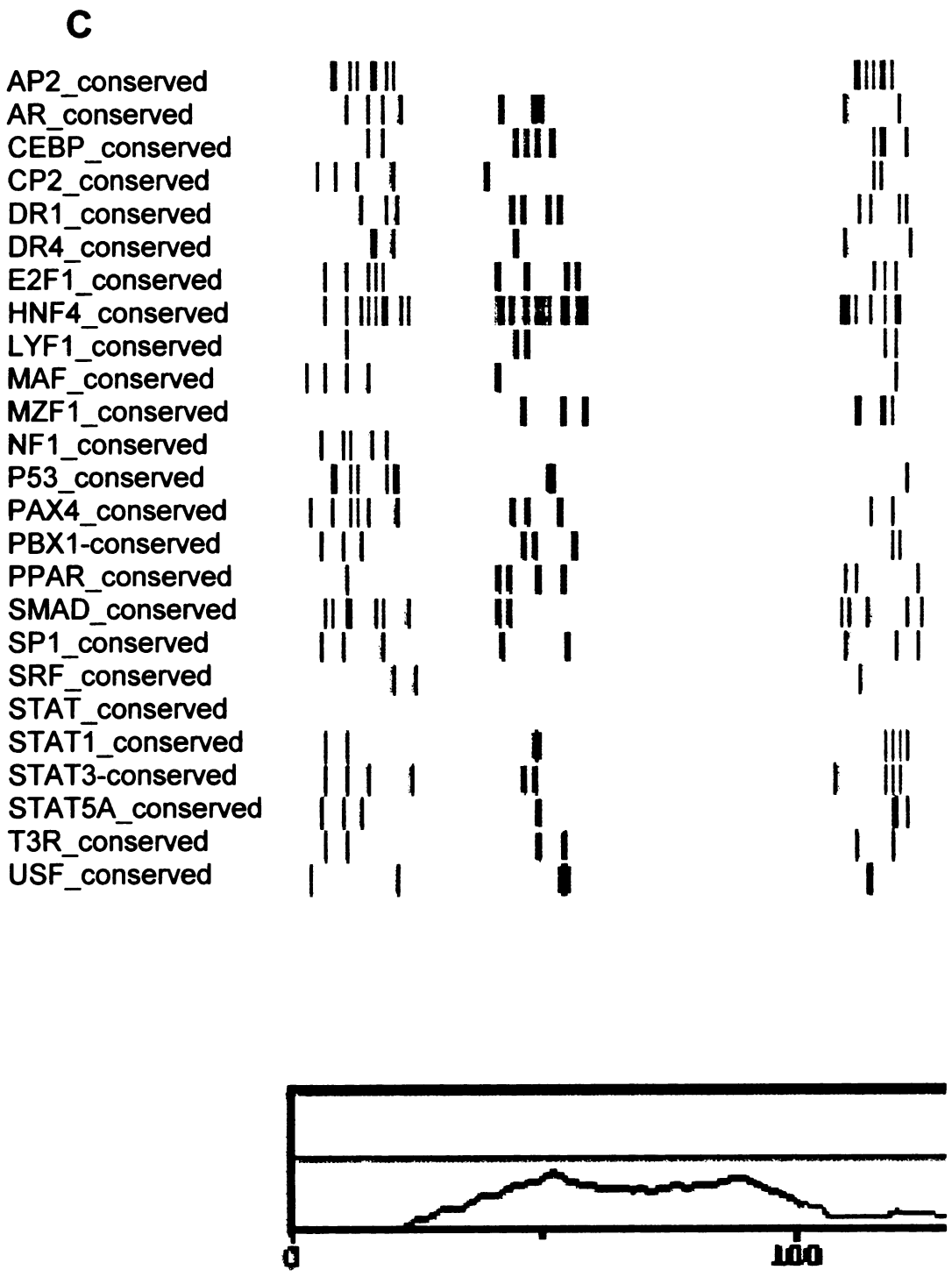

Figure 5.8 continued

Discussion

The results presented here show for the first time that DON dose-dependently prevents and ameliorates diet-induced obesity in mice. In our preventive model, DON feeding progressively reduced weight gain rates as DON concentration increased. The dose-dependent nature of weight gain prevention and its correlation with plasma DON suggests that DON intake might mediate the prevention. One interesting observation is the pattern of weight loss in the therapeutic model. Mice rapidly lost weight and appeared to stop when their weights reached the level of age-matched lean controls. The weight curve for mice on HFD and 10 ppm DON suggests a biphasic effect, comprising: (a) an initial rapid weight loss and (b) a later phase with no apparent weight change. Notably, the latter phase is similar to the weight curve in HFD+10 ppm DON for the preventive model, suggesting that the same mechanism(s) may have modulated these weight effects in the two models.

DON also reduced adiposity in both the preventive and therapeutic models. However, dose-dependent adiposity reduction was much clearer in the preventive model than the therapeutic model. A combination of the weight and adiposity measures in both experimental models offers some suggestions on mechanisms of DON's effect on adiposity. First, since mice fed HFD+10 ppm in the preventive model showed a static weight effect; DON could have prevented the attainment of adiposity (adipostatic effect). Second, mice on the therapeutic model showed an initial rapid weight loss (possible adipolytic effect), but maintained a static weight (adipostatic effect) once they reached the level of age-matched lean control. In the second scenario, DON might have induced a loss of adipose tissue and homeostatic adjustments could have prevented further loss once

mice reached a certain level. These observations raise the possibility that there could be a combination of DON-related adipolytic and adipostatic effects, depending on DON dose, duration of exposure and physiological state of mice. Future experiments will need to clarify these possibilities, perhaps by using imaging techniques like dual X-ray absorptiometry to determine dynamics of body fat composition during DON exposure. In the interim, DON-induced adiposity prevention and reduction holds promise for understanding mechanisms and targets for obesity prevention and control.

Plasma DON concentrations were negatively correlated with adiposity in mice. A single bolus of DON given by oral gavage is cleared within hours (Amuzie *et al.* 2008); but dietary DON results in a serum steady DON concentration within 2 wk of exposure (chapter 4). In the present study, increasing plasma DON was associated with increasing dietary DON through 10 weeks of exposure. The proportionate relationship between dietary DON and plasma DON suggest that the latter might be influenced by the former. This is notable because of the potential for feed refusal in DON-exposed animals. Although DON causes feed refusal at high doses (Pestka and Smolinski 2005), independent observations by our laboratory and Canadian researchers indicate no significant feed refusal at low doses ($\leq$ 10 ppm) (Forsell *et al.* 1986; Iverson *et al.* 1995). If mice in our study significantly refused feed at any dose, residual plasma DON may have been cleared within hours after feed refusal. Under a feed refusal scenario, correlation of plasma and dietary DON might have been obscured by an anorexic effect (especially at the higher doses). The strong correlation suggests that the observed weight changes might have resulted, in part, from pharmacological effects of plasma DON.

The role of central appetite control in DON-induced effects had been investigated (Prelusky 1993). Researchers could neither demonstrate a peripheral influence of anorexic proteins such as serotonin (Prelusky 1994), nor inhibit DON's weight effects with an appetite stimulant (cyproheptadine). They concluded that effects of dietary DON on weight gain appears to be influenced by factors more than reduced feed intake and may be due to secondary pharmacological actions (Prelusky *et al.* 1997). We agree with this conclusion and suggest an alternative hypothesis based on this study and previous data from our laboratory. DON-induced weight effects might be related to DON's ability to induce suppressors of signaling and impair IGFALS production.

We recently observed that DON induces SOCS proteins (chapter 3), which are negative regulators of cytokine and growth factor signaling pathways. Three mechanisms have been proposed to act either independently, or in concert to achieve the SOCS-induced negative regulation of signaling. These include (1) direct inhibition of signaling kinases, (2) competition with other SH2 domain-containing signaling proteins for binding sites on receptors, and (3) proteasomal degradation of receptors by SOCS box-elongin interactions (O'Sullivan *et al.* 2007). A potential consequence of SOCS upregulation in DON exposed mice could be attenuation of growth factor pathways which leads to cellular energy wasting. Interestingly, IL-6, a proinflammatory cytokine induced by DON, which also upregulates SOCS causes energy wasting (Wallenius *et al.* 2002). The possibility that DON exposure may activate energy wasting pathways in DIO mice needs to be investigated. Another consequence of SOCS upregulation is an impairment of GH signaling, and this has been demonstrated in DON-exposed mice.

An indication of impaired GH signaling is a reduction in IGF1 binding partner (IGFALS). We have shown a rapid, robust and sustained suppression of circulating IGFALS in lean mice (Chapter 4). This study confirms that DON suppresses IGFALS and extends the knowledge to different physiological states (lean and obese) and different dietary conditions (high and low fat). IGFALS reduction was also associated with weight reduction and adiposity reduction in both models of DON exposure. Since IGFALS reduction occurs rapidly (2 h) in DON-exposed mice (Chapter 4), it might be that IGFALS precedes adiposity reduction in DON-exposed mice. Future studies will need to determine the exact consequence of IGFALS reduction in DON-exposed mice, but the temporal relationships observed from our studies suggest that DON-induced IGFALS reduction is an early event, preceding IGF1, adiposity, and weight reductions; and that these later events may be related to IGFALS reduction. Taken together, our data indicate that dietary DON is taken up in plasma, prevents weight gain in lean mice, and induces weight loss in obese mice, despite HFD. The mechanism(s) that control these weight effects when clearly understood may hold promise for obesity prevention and therapy.

One unexpected finding is the inconsistent effect of DON on circulating IGF1. IGF1 circulates in plasma bound to IGFALS, and a third partner (IGFBP3). IGFALS extends the half-life of IGF1 from 15 min to 15 h (Guler *et al.* 1989). Such regulatory nature of IGFALS predicts a positive correlation with IGF1. However, in our study, reduction of circulating IGFALS did not always correlate with a reduction in circulating IGF1. Inconsistencies in circulating IGF1 have been reported in human models of obesity, where IGF1 is increased, reduced or remains unchanged (Maccario *et al.* 2000). However, IGF1 increase has also been associated with diet-induced obesity in pigs (Sebert *et al.*

2005), dogs (Gayet *et al.* 2004), mice (Ogus *et al.* 2003) and people (Rolland-Cachera *et al.* 1999). There are many reasons why IGF1 inconsistencies may exist. First, IGF1 is also produced by estradiol (Venken *et al.* 2005), in addition to GH. Such redundancy suggests that compensatory pathway(s) of IGF1 induction may modulate some effects of reduced IGFALS. In support of this compensatory hypothesis, we have shown a DON-related increased in IGF1 mRNA, despite IGFALS impairment in mice (chapter 4). Second, a feedback from increased/reduced IGF1 may dysregulate GH-induced IGF1 to achieve an opposite effect (Sebert *et al.* 2005). Third, IGF axis proteins fluctuate with age (De Benedetti *et al.* 1997) and our models involve two age groups (21 vs 29 wk), differences in IGF1 concentrations across model in our study might be related, in part, to the age differences. These inconsistencies notwithstanding, a combination of IGFALS and IGF1 data indicates a DON-related suppression of the GH/IGF axis in obese mice.

There are two potential ways that DON's impairment of IGF system may lead to reduction of adiposity and obesity. First, a direct reduction of IGF1 may reduce weight because of its anabolic and anti-lipolytic functions (Yakar *et al.* 2005). IGF1 drives maturation of preadipocytes to adipocytes (Rolland-Cachera *et al.* 1999) and induces glucose and lipid uptake in adipocytes (Sebert *et al.* 2005). The aforementioned studies support the possibility that IGF1 reduction will reduce adiposity. The second possibility is that IGFALS and IGF1 reduction may represent a broader dysregulation of metabolic pathways in DON-exposed animals. Such dysregulation could be mediated by transcription factors and kinases that regulate a broader metabolic network. In support of the second possibility, we have reported an increase in hepatic suppressors of cytokine signaling (chapter 3) and related such to an impairment of IGFALS transcription.

Since DON-induced IGFALS suppression occurs in mice of different physiological and dietary states, one obvious question is whether this effect will occur in other species and humans. In the future, demonstration of DON-induced IGFALS suppression in other species will be significant for DON risk assessment, and potentially in obesity prevention and therapy. In the interim, *r*VISTA was used to determine similarities in the regulatory regions of IGFALS gene in mice, human and rats. The first 200 bases in all 3 genes had greater than 70% sequence similarity. Furthermore, an *r*VISTA querry of all eukaryotic transcription factors using TRANSFAC predicted 25 conserved transcription factor binding sides. Notably, cytokine signaling related transcription factors such as STATs were conserved among all species. Transcription factors related to gluconeogenesis (HNF4) and lipogensis (PPAR) were also predicted (Figure5.8). The predicted conservation of STAT binding site on IGFALS promoter in multiple species is notable since STATs are central transcription factors in cytokine signaling. It might be speculated that DON sequentially induces cytokine and SOCS, and impairs STAT and IGFALS. Future studies need to identify which STATs or transcription factors are impaired on the IGFALS promoter and relate such to a broader metabolic context.

Finally, we have shown that DON prevents and ameliorates diet-induced obesity in mice. This study is the first, to our knowledge, that demonstrated a reduction in circulating IGFALS by an anti-obesity compound in mice. Overall, DON's control of obesity and its association with a conserved protein has the potential to help uncover novel targets of obesity prevention and control.

CONCLUSIONS

The work presented in this dissertation attempted to answer some questions for DON risk assessment. For airbone DON exposure, our oral and intranasal exposure route comparison suggests that similar doses of DON will induce greater proinflammatory cytokine mRNA via the nasal route than the oral route. For foodborne DON exposure, we have demonstrated, for the first time, that dietary DON exposure impairs GH/IGF axis, which might be responsible for reduced weight gain in DON-exposed mice. Perhaps the most significant finding is the identification of DON-induced circulating IGFALS suppression in both lean and obese mice. Thus, IGFALS is a biomarker candidate that might be translated to human DON exposure scenarios. The two most likely outcomes of human IGFALS will be critical for human DON risk assessment. First, if IGFALS suppression could be demonstrated in humans with higher DON intake, IGFALS might be used as biomarker of effect in human epidemiological surveillance and will aid risk assessment. Alternatively, if human IGFALS is not suppressed at any level of human DON exposure, it could mean that DON operates through different mechanism in humans or that human dietary DON exposures are not associated with significant biological effects. In either scenario, IGFALS measurements could lead to a significant reduction in the present uncertainties in risks associated with human DON exposures.

Regardless of the most likely outcome in humans, DON-induced IGFALS suppression also extends our knowledge of DON mechanisms (Figure 6). The pathway proposed is based on this work and those of other researchers, and is not intended to be exhaustive. Within the constraints of our experiment, the proposed pathway offers a framework that integrates DON-induced perturbations in the context of cytokine

signaling. Furthermore, the association of IGFALS suppression with both prevention and amelioration of obesity in DON-exposed mice is remarkable from a public health perspective. This research could potentially lead to a novel compound (DON) or a novel target (IGFALS) for obesity control.

This work is unique because it integrated known acute and chronic events of a foodborne toxin in a manner that identifies additional target tissue (liver), additional signaling molecules (SOCS) and pathway (GH/IGF), potential mechanism of action and potential usefulness for obese patients. In the future, additional studies will be necessary to identify the transcriptional/post-transcriptional trigger(s) that regulate IGFALS suppression and understand their role(s) in lean and obese states. Furthermore, scientists might use DON in body fluids as a biomarker of exposure to validate circulating IGFALS as a biomarker of effect in other mammalian species. IGFALS suppression could be used as a screening assay for other environmental chemicals that suppress weight gain with the hope of identifying a central mechanism. Overall, the identification of DON-induced IGFALS suppression has the potential to significantly impact risk assessment process and control human illnesses.

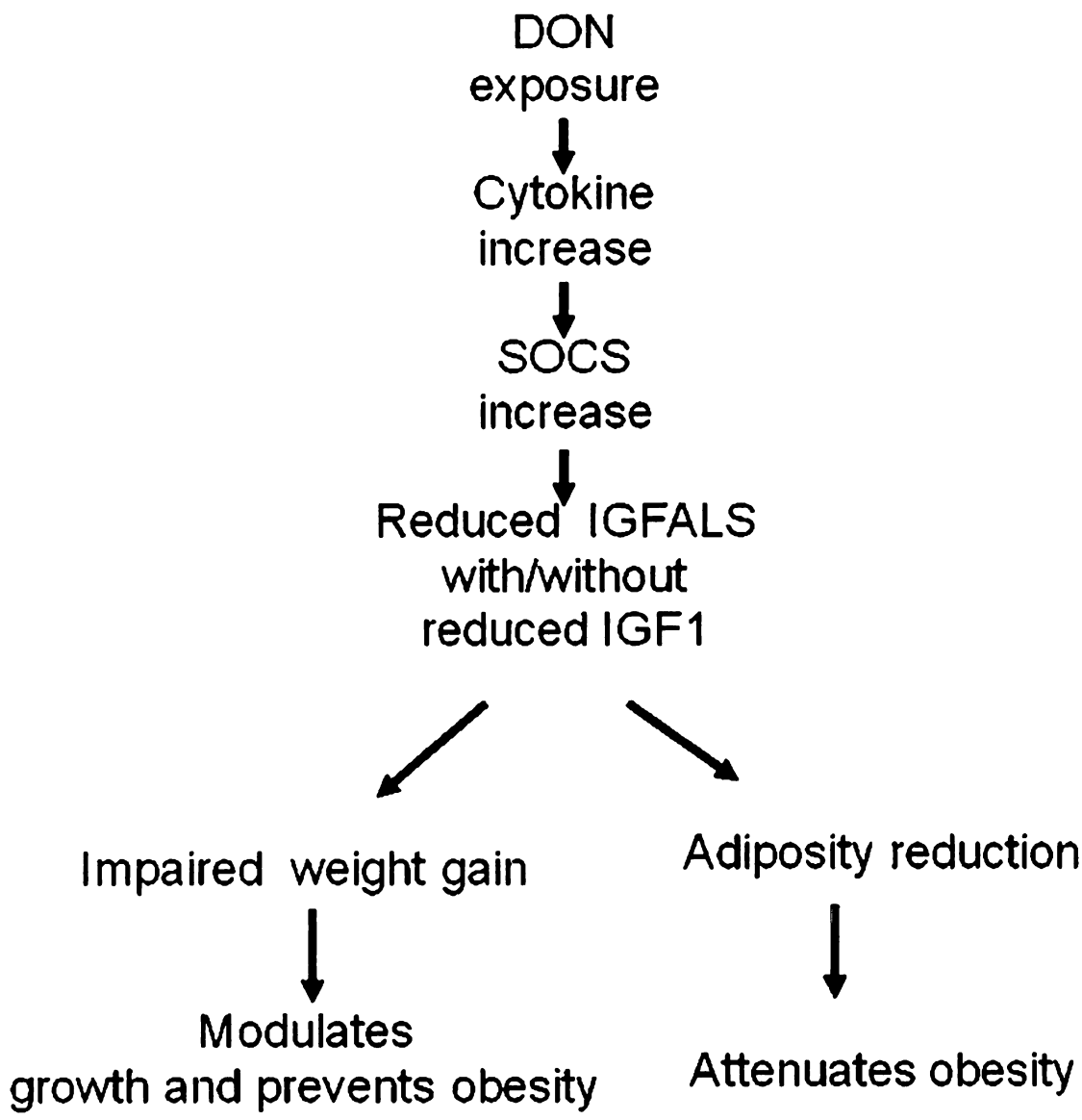

Figure 6: Proposed mechanism for modulation of murine growth and obesity by the trichothecene deoxynivalenol. Suppression of IGALS is a biomarker of murine DON effect in different physiological and dietary states.

REFERENCES

1. Ahmed, T. A., Buzzelli, M. D., Lang, C. H., Capen, J. B., Shumate, M. L., Navaratnarajah, M., Nagarajan, M., and Cooney, R. N. (2007). Interleukin-6 (IL-6) Inhibits Growth Hormone (GH) Mediated Gene Expression in Hepatocytes. *Am. J. Physiol Gastrointest. Liver Physiol.*

2. Alexander, W. S. (2002). Suppressors of cytokine signalling (SOCS) in the immune system. *Nat. Rev. Immunol.* **2**(6), 410-416.

3. Amuzie, C. J., Harkema, J. R., and Pestka, J. J. (2008). Tissue distribution and proinflammatory cytokine induction by the trichothecene deoxynivalenol in the mouse: comparison of nasal vs. oral exposure. *Toxicology* **248**(1), 39-44.

4. Arnold, D. L., Karpinski, K. F., McGuire, P. F., Nera, E. A., Zawidzka, Z. Z., Lok, E., Campbell, J. S., Tryphonas, L., and Scott, P. M. (1986a). A short-term feeding study with deoxynivalenol (vomitoxin) using rats. *Fundam. Appl. Toxicol.* **6**(4), 691-696.

5. Arnold, D. L., McGuire, P. F., Nera, E. A., Karpinski, K. F., Bickis, M. G., Zawidzka, Z. Z., Fernie, S., and Vesonder, R. F. (1986b). The toxicity of orally administered deoxynivalenol (vomitoxin) in rats and mice. *Food Chem. Toxicol.* **24**(9), 935-941.

6. Atkinson, T. J. (2008). Central and peripheral neuroendocrine peptides and signalling in appetite regulation: considerations for obesity pharmacotherapy. *Obes. Rev.* **9**(2), 108-120.

7. Atroshi, F., Rizzo, A. F., Veijalainen, P. L. L. A., Honkanen-Buzalski, T., Andersson, K., Hirvi, T., and Saloniemi, H. The effect of dietary exposure to DON and T-2 toxin on host resistance and serum immunoglobulins of normal and mastitic mice. J.Anim.Physiol. 71, 223-233. 1994.
Ref Type: Generic

8. Audige, A., Yu, Z. R., Frey, B. M., Uehlinger, D. E., Frey, F. J., and Vogt, B. (2003). Epithelial sodium channel (ENaC) subunit mRNA and protein expression in rats with puromycin aminonucleoside-induced nephrotic syndrome. *Clinical Science* **104**(4), 389-395.

9. Azcona-Olivera, J. I., Ouyang, Y., Murtha, J., Chu, F. S., and Pestka, J. J. (1995a). Induction of cytokine mRNAs in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): relationship to toxin distribution and protein synthesis inhibition. *Toxicol. Appl. Pharmacol.* **133**(1), 109-120.

10. Azcona-Olivera, J. I., Ouyang, Y. L., Warner, R. L., Linz, J. E., and Pestka, J. J. (1995b). Effects of vomitoxin (deoxynivalenol) and cycloheximide on IL-2, 4, 5 and 6 secretion and mRNA levels in murine CD4+ cells. *Food Chem. Toxicol.* **33**(6), 433-441.

11. Bae, H. K., and Pestka, J. J. (2008). Deoxynivalenol induces p38 interaction with the ribosome in monocytes and macrophages. *Toxicol. Sci.* **105**(1), 59-66.

12. Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993). Role of Insulin-Like Growth-Factors in Embryonic and Postnatal-Growth. *Cell* **75**(1), 73-82.

13. Bang, P., Brandt, J., Degerblad, M., Enberg, G., Kaijser, L., Thoren, M., and Hall, K. (1990). Exercise-induced changes in insulin-like growth factors and their low molecular weight binding protein in healthy subjects and patients with growth hormone deficiency. *Eur. J. Clin. Invest* **20**(3), 285-292.

14. Barnes, P. J. (2004). Mediators of chronic obstructive pulmonary disease. *Pharmacol. Rev.* **56**(4), 515-548.

15. Barreca, A., Ketelslegers, J. M., Arvigo, M., Minuto, F., and Thissen, J. P. (1998). Decreased acid-labile subunit (ALS) levels by endotoxin in vivo and by interleukin-1beta in vitro. *Growth Horm. IGF. Res.* **8**(3), 217-223.

16. Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Le, C. D., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., de, C. R., and Sinclair, D. A. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**(7117), 337-342.

17. Bazan, J. F. (1989). A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochem. Biophys. Res. Commun.* **164**(2), 788-795.

18. Bergad, P. L., Schwarzenberg, S. J., Humbert, J. T., Morrison, M., Amarasinghe, S., Towle, H. C., and Berry, S. A. (2000). Inhibition of growth hormone action in models of inflammation. *Am. J. Physiol Cell Physiol* **279**(6), C1906-C1917.

19. Bergsjo, B., Matre, T., and Nafstad, I. (1992). Effects of diets with graded levels of deoxynivalenol on performance in growing pigs. *Zentralbl. Veterinarmed. A* **39**(10), 752-758.

20. Boisclair, Y. R., Wang, J., Shi, J., Hurst, K. R., and Ooi, G. T. (2000). Role of the suppressor of cytokine signaling-3 in mediating the inhibitory effects of interleukin-1beta on the growth hormone-dependent transcription of the acid-labile subunit gene in liver cells. *J. Biol. Chem.* **275**(6), 3841-3847.

21. Borish, L. C., and Steinke, J. W. (2003). 2. Cytokines and chemokines. *J. Allergy Clin. Immunol.* **111**(2 Suppl), S460-S475.

22. Bray, G. A. (2008). Lifestyle and pharmacological approaches to weight loss: efficacy and safety. *J. Clin. Endocrinol. Metab* **93**(11 Suppl 1), S81-S88.

23. Buckley, D. B., and Klaassen, C. D. (2007). Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. *Drug Metab Dispos.* **35**(1), 121-127.

24. CAST. Task Force Report No. 139. 2003. Ames, IA, Council of Agricultural Science and Technology.
Ref Type: Generic

25. Chen, Y., Sun, D., Krishnamurthy, V. M., and Rabkin, R. (2007). Endotoxin attenuates growth hormone-induced hepatic insulin-like growth factor I expression by inhibiting JAK2/STAT5 signal transduction and STAT5b DNA binding. *Am. J. Physiol Endocrinol. Metab* **292**(6), E1856-E1862.

26. Chung, Y. J., Zhou, H. R., and Pestka, J. J. (2003). Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-alpha expression by deoxynivalenol (vomitoxin). *Toxicol. Appl. Pharmacol.* **193**(2), 188-201.

27. Clifford, L. J., Jia, Q., and Pestka, J. J. (2003). An improved method for the purification of the trichothecene deoxynivalenol (vomitoxin) from Fusarium graminearum culture. *J. Agric. Food Chem.* **51**(2), 521-523.

28. Creasia, D. A., Thurman, J. D., Jones, L. J., III, Nealley, M. L., York, C. G., Wannemacher, R. W., Jr., and Bunner, D. L. (1987). Acute inhalation toxicity of T-2 mycotoxin in mice. *Fundam. Appl. Toxicol.* **8**(2), 230-235.

29. Creasia, D. A., Thurman, J. D., Wannemacher, R. W., Jr., and Bunner, D. L. (1990). Acute inhalation toxicity of T-2 mycotoxin in the rat and guinea pig. *Fundam. Appl. Toxicol.* **14**(1), 54-59.

30. Creppy, E. E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol. Lett.* **127**(1-3), 19-28.

31. Croker, B. A., Kiu, H., and Nicholson, S. E. (2008). SOCS regulation of the JAK/STAT signalling pathway. *Semin. Cell Dev. Biol.* **19**(4), 414-422.

32. De Benedetti, F., Alonzi, T., Moretta, A., Lazzaro, D., Costa, P., Poli, V., Martini, A., Ciliberto, G., and Fattori, E. (1997). Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. *J. Clin. Invest* **99**(4), 643-650.

33. Denson, L. A., Held, M. A., Menon, R. K., Frank, S. J., Parlow, A. F., and Arnold, D. L. (2003). Interleukin-6 inhibits hepatic growth hormone signaling via upregulation of Cis and Socs-3. *Am. J. Physiol Gastrointest. Liver Physiol* **284**(4), G646-G654.

34. Domene, H. M., Bengolea, S. V., Martinez, A. S., Ropelato, M. G., Pennisi, P., Scaglia, P., Heinrich, J. J., and Jasper, H. G. (2004). Deficiency of the circulating insulin-like growth factor system associated with inactivation of the acid-labile subunit gene. *N. Engl. J. Med.* **350**(6), 570-577.

35. Dong, W., Azcona-Olivera, J. I., Brooks, K. H., Linz, J. E., and Pestka, J. J. (1994). Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. *Toxicol. Appl. Pharmacol.* **127**(2), 282-290.

36. EC. Opinion of the Scientific Committee on Food on Fusarium toxins. Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol. 2-26-2002. Brussels, Belgium, European Commission. Ref Type: Report

37. Ehrlich, K. C., and Daigle, K. W. (1987). Protein-Synthesis Inhibition by 8-Oxo-12,13-Epoxytrichothecenes. *Biochimica et Biophysica Acta* **923**(2), 206-213.

38. Eleswarapu, S., Gu, Z., and Jiang, H. (2008). Growth hormone regulation of insulin-like growth factor-I gene expression may be mediated by multiple distal signal transducer and activator of transcription 5 binding sites. *Endocrinology* **149**(5), 2230-2240.

39. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T., Kanakura, Y., Komiya, S., and Yoshimura, A. (1997). A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387**(6636), 921-924.

40. Fan, J., Char, D., Kolasa, A. J., Pan, W., Maitra, S. R., Patlak, C. S., Spolarics, Z., Gelato, M. C., and Lang, C. H. (1995). Alterations in hepatic production and peripheral clearance of IGF-I after endotoxin. *Am. J. Physiol* **269**(1 Pt 1), E33-E42.

41. FAO. Worldwide regulations for mycotoxins in food and feed in 2003. Food and Nutrition Paper No.**81** . 2003. Food and Agriculture Organization. Ref Type: Generic

42. Febbraio, M. A., and Pedersen, B. K. (2005). Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? *Exerc. Sport Sci. Rev.* **33**(3), 114-119.

43. Fenton, J. I., Nunez, N. P., Yakar, S., Perkins, S. N., Hord, N. G., and Hursting, S. D. (2009). Diet-induced adiposity alters the serum profile of inflammation in C57BL/6N mice as measured by antibody array. *Diabetes Obes. Metab* **11**(4), 343-354.

44. Fey, G., Hocke, G., Wilson, D., Ripperger, J., and Darlington, G. Cytokines and the acute phase response of the liver. Arias, I, Boyer, J, Fausto, N, Jacoby, W, Schachter, D, and Shafritz, D. The Liver: Biology and Pathobiology. 113-143. 1994. New York, Raven Pres Ltd.
Ref Type: Generic

45. Fitzpatrick, D. W., Boyd, K. E., and Watts, B. M. (1988). Comparison of the trichothecenes deoxynivalenol and T-2 toxin for their effects on brain biogenic monoamines in the rat. *Toxicol. Lett.* **40**(3), 241-245.

46. Forsell, J. H., Witt, M. F., Tai, J. H., Jensen, R., and Pestka, J. J. (1986). Effects of 8-week exposure of the B6C3F1 mouse to dietary deoxynivalenol (vomitoxin) and zearalenone. *Food Chem. Toxicol.* **24**(3), 213-219.

47. Garcia, M. C., Wernstedt, I., Berndtsson, A., Enge, M., Bell, M., Hultgren, O., Horn, M., Ahren, B., Enerback, S., Ohlsson, C., Wallenius, V., and Jansson, J. O. (2006). Mature-onset obesity in interleukin-1 receptor I knockout mice. *Diabetes* **55**(5), 1205-1213.

48. Gayet, C., Bailhache, E., Dumon, H., Martin, L., Siliart, B., and Nguyen, P. (2004). Insulin resistance and changes in plasma concentration of TNFalpha, IGF1, and NEFA in dogs during weight gain and obesity. *J. Anim Physiol Anim Nutr. (Berl)* **88**(3-4), 157-165.

49. Gesta, S., Tseng, Y. H., and Kahn, C. R. (2007). Developmental origin of fat: tracking obesity to its source. *Cell* **131**(2), 242-256.

50. Glund, S., and Krook, A. (2008). Role of interleukin-6 signalling in glucose and lipid metabolism. *Acta Physiol (Oxf)* **192**(1), 37-48.

51. Grove, J. F. (2007). The trichothecenes and their biosynthesis. *Fortschr. Chem. Org. Naturst.* **88**, 63-130.

52. Guler, H. P., Zapf, J., Schmid, C., and Froesch, E. R. (1989). Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol. (Copenh)* **121**(6), 753-758.

53. Halstensen, A. S., Nordby, K. C., Klemsdal, S. S., Elen, O., Clasen, P. E., and Eduard, W. (2006). Toxigenic Fusarium spp. as determinants of trichothecene mycotoxins in settled grain dust. *Journal of Occupational and Environmental Hygiene* **3**(12), 651-659.

54. Harkema, J. R., Carey, S. A., and Wagner, J. G. (2006). The nose revisited: A brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. *Toxicologic Pathology* **34**(3), 252-269.

55. He, P., Young, L. G., and Forsberg, C. (1992). Microbial transformation of deoxynivalenol (vomitoxin). *Appl. Environ. Microbiol.* **58**(12), 3857-3863.

56. Heath, K. E., Argente, J., Barrios, V., Pozo, J., az-Gonzalez, F., Martos-Moreno, G. A., Caimari, M., Gracia, R., and Campos-Barros, A. (2008). Primary acid-labile subunit deficiency due to recessive IGFALS mutations results in postnatal growth deficit associated with low circulating insulin growth factor (IGF)-I, IGF binding protein-3 levels, and hyperinsulinemia. *J. Clin. Endocrinol. Metab* **93**(5), 1616-1624.

57. Hintikka, E. L., and Nikulin, M. Airborne mycotoxins in agricultural and indoor environments. Indoor Air 4, 66-70. 1998.
Ref Type: Generic

58. Hirsch, J., and Batchelor, B. (1976). Adipose tissue cellularity in human obesity. *Clin. Endocrinol. Metab* **5**(2), 299-311.

59. Hopkins, S. J. (2007). Central nervous system recognition of peripheral inflammation: a neural, hormonal collaboration. *Acta Biomed.* **78 Suppl 1**, 231-247.

60. Hughes, D. M., Gahl, M. J., Graham, C. H., and Grieb, S. L. (1999). Overt signs of toxicity to dogs and cats of dietary deoxynivalenol. *J. Anim Sci.* **77**(3), 693-700.

61. Hursting, S. D., Nunez, N. P., Varticovski, L., and Vinson, C. (2007). The obesity-cancer link: lessons learned from a fatless mouse. *Cancer Res.* **67**(6), 2391-2393.

62. Hwang, D. L., Lee, P. D., and Cohen, P. (2008). Quantitative ontogeny of murine insulin-like growth factor (IGF)-I, IGF-binding protein-3 and the IGF-related acid-labile subunit. *Growth Horm. IGF. Res.* **18**(1), 65-74.

63. Idelevich, E., Kirch, W., and Schindler, C. (2009). Current pharmacotherapeutic concepts for the treatment of obesity in adults. *Ther. Adv. Cardiovasc. Dis.* **3**(1), 75-90.

64. Islam, Z., Gray, J. S., and Pestka, J. J. (2006). p38 Mitogen-activated protein kinase mediates IL-8 induction by the ribotoxin deoxynivalenol in human monocytes. *Toxicol. Appl. Pharmacol.* **213**(3), 235-244.

65. Islam, Z., Moon, Y. S., Zhou, H. R., King, L. E., Fraker, P. J., and Pestka, J. J. (2002). Endotoxin potentiation of trichothecene-induced lymphocyte apoptosis is mediated by up-regulation of glucocorticoids. *Toxicol. Appl. Pharmacol.* **180**(1), 43-55.

66. Islam, Z., and Pestka, J. J. (2003). Role of IL-1 beta in endotoxin potentiation of deoxynivalenol-induced corticosterone response and leukocyte apoptosis in mice. *Toxicological Sciences* **74**(1), 93-102.

67. Islam, Z., and Pestka, J. J. (2006). LPS priming potentiates and prolongs proinflammatory cytokine response to the trichothecene deoxynivalenol in the mouse. *Toxicol. Appl. Pharmacol.* **211**(1), 53-63.

68. Iverson, F., Armstrong, C., Nera, E., Truelove, J., Fernie, S., Scott, P., Stapley, R., Hayward, S., and Gunner, S. (1995). Chronic feeding study of deoxynivalenol in B6C3F1 male and female mice. *Teratog. Carcinog. Mutagen.* **15**(6), 283-306.

69. Joffe, A. Fusarium poae and F. sporotrichoides as principal causal agents of alimentary toxic aleukia. (Morehouse, T. W. Ed. Mycotoxic Fungi, Mycotoxins, Mycotoxicosis 3, 21. 1978. New York, Mercel Dekker.

70. Johnson, R. W. (1998). Immune and endocrine regulation of food intake in sick animals. *Domest. Anim Endocrinol.* **15**(5), 309-319.

71. Jungermann, K., and Kietzmann, T. (2000). Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology* **31**(2), 255-260.

72. Krysinska-Traczyk, E., Kiecana, I., Perkowski, J., and Dutkiewicz, J. (2001). Levels of fungi and mycotoxins in samples of grain and grain dust collected on farms in Eastern Poland. *Ann. Agric. Environ. Med.* **8**(2), 269-274.

73. Krysinska-Traczyk, E., Skorska, C., Prazmo, Z., Sitkowska, J., Dutkiewicz, J., and Cholewa, G. Bioaerosols as potential health risk factors for farmers at threshing grain. Med.Ogolna 5, 301-306. 1999.

74. Lang, C. H., Hong-Brown, L., and Frost, R. A. (2005). Cytokine inhibition of JAK-STAT signaling: a new mechanism of growth hormone resistance. *Pediatr. Nephrol.* **20**(3), 306-312.

75. Lappalainen, S., Nikulin, M., Berg, S., Parikka, P., Hintikka, E. L., and Pasanen, A. L. Fusarium toxins and fungi associated with handling of grain on eight Finnish farms. Atmos.Environ. 30, 3059-3065. 1996.
Ref Type: Generic

76. Laron, Z. (2004). Laron syndrome (primary growth hormone resistance or insensitivity): the personal experience 1958-2003. *J. Clin. Endocrinol. Metab* **89**(3), 1031-1044.

77. Leblanc, J. C., Tard, A., Volatier, J. L., and Verger, P. (2005). Estimated dietary exposure to principal food mycotoxins from the first French Total Diet Study. *Food Addit. Contam* **22**(7), 652-672.

78. Lee, S. A., Adhikari, A., Grinshpun, S. A., McKay, R., Shukla, R., and Reponen, T. (2006). Personal exposure to airborne dust and microorganisms in agricultural environments. *J. Occup. Environ. Hyg.* **3**(3), 118-130.

79. Legator, M. S. (1995). Application of Biomarkers: Getting our priorities straight. In *Biomarkers and Occupational Health: Progress and Perspectives* (M.L.Mendelsohn, J.P.Peeters, and M.J.Normandy, Eds.), pp. 61-68. Joseph Henry Press, Washington D.C.

80. Leroith, D., and Accili, D. (2008). Mechanisms of disease: using genetically altered mice to study concepts of type 2 diabetes. *Nat. Clin. Pract. Endocrinol. Metab* **4**(3), 164-172.

81. Leroith, D., and Yakar, S. (2007). Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. *Nat. Clin. Pract. Endocrinol. Metab* **3**(3), 302-310.

82. Li, S., Marquardt, R. R., Frohlich, A. A., Vitti, T. G., and Crow, G. (1997). Pharmacokinetics of ochratoxin A and its metabolites in rats. *Toxicol. Appl. Pharmacol.* **145**(1), 82-90.

83. Li, S., Ouyang, Y., Yang, G. H., and Pestka, J. J. (2000). Modulation of transcription factor AP-1 activity in murine EL-4 thymoma cells by vomitoxin (deoxynivalenol). *Toxicol. Appl. Pharmacol.* **163**(1), 17-25.

84. Lippmann, M., Yeates, D. B., and Albert, R. E. (1980). Deposition, retention, and clearance of inhaled particles. *Br. J. Ind. Med.* **37**(4), 337-362.

85. Liu, J. L., and Leroith, D. (1999). Insulin-like growth factor I is essential for postnatal growth in response to growth hormone. *Endocrinology* **140**(11), 5178-5184.

86. Lock, E. A., and Bonventre, J. V. (2008). Biomarkers in translation; past, present and future. *Toxicology* **245**(3), 163-166.

87. Lombaert, G. A., Pellaers, P., Roscoe, V., Mankotia, M., Neil, R., and Scott, P. M. (2003). Mycotoxins in infant cereal foods from the Canadian retail market. *Food Additives and Contaminants* **20**(5), 494-504.

88. Loots, G. G., Ovcharenko, I., Pachter, L., Dubchak, I., and Rubin, E. M. (2002). rVista for comparative sequence-based discovery of functional transcription factor binding sites. *Genome Res.* **12**(5), 832-839.

89. Maccario, M., Grottoli, S., Procopio, M., Oleandri, S. E., Rossetto, R., Gauna, C., Arvat, E., and Ghigo, E. (2000). The GH/IGF-I axis in obesity: influence of neuro-endocrine and metabolic factors. *Int. J. Obes. Relat Metab Disord.* **24 Suppl 2**, S96-S99.

90. Mao, Y., Ling, P. R., Fitzgibbons, T. P., McCowen, K. C., Frick, G. P., Bistrian, B. R., and Smith, R. J. (1999). Endotoxin-induced inhibition of growth hormone receptor signaling in rat liver in vivo. *Endocrinology* **140**(12), 5505-5515.

91. Matsumoto, A., Seki, Y., Kubo, M., Ohtsuka, S., Suzuki, A., Hayashi, I., Tsuji, K., Nakahata, T., Okabe, M., Yamada, S., and Yoshimura, A. (1999). Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. *Mol. Cell Biol.* **19**(9), 6396-6407.

92. McClellan, R. O. (1996). Reducing uncertainty in risk assessment by using specific knowledge to replace default options. *Drug Metab Rev.* **28**(1-2), 149-179.

93. Morrissey, R. E., and Vesonder, R. F. (1985). Effect of Deoxynivalenol (Vomitoxin) on Fertility, Pregnancy, and Postnatal-Development of Sprague-Dawley Rats. *Applied and Environmental Microbiology* **49**(5), 1062-1066.

94. Muoio, D. M., and Newgard, C. B. (2006). Obesity-related derangements in metabolic regulation. *Annu. Rev. Biochem.* **75**, 367-401.

95. Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., and Kishimoto, T. (1997). Structure and function of a new STAT-induced STAT inhibitor. *Nature* **387**(6636), 924-929.

96. Nordby, K. C., Andersen, A., and Kristensen, P. (2004a). Incidence of lip cancer in the male Norwegian agricultural population. *Cancer Causes Control* **15**(6), 619-626.

97. Nordby, K. C., Halstensen, A. S., Elen, O., Clasen, P. E., Langseth, W., Kristensen, P., and Eduard, W. (2004b). Trichothecene mycotoxins and their determinants in settled dust related to grain production. *Ann. Agric. Environ. Med.* **11**(1), 75-83.

98. O'Sullivan, L. A., Liongue, C., Lewis, R. S., Stephenson, S. E., and Ward, A. C. (2007). Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease. *Mol. Immunol.* **44**(10), 2497-2506.

99. Obol'skii, O. L., Kravchenko, L. V., Avren'eva, L. I., and Tutel'ian, V. A. (1998). [Effect of dietary selenium on the activity of UDP-glucuronosyltransferases and metabolism of mycotoxin deoxynivalenol in rats]. *Vopr. Pitan.*(4), 18-23.

100. Ogata, H., Chinen, T., Yoshida, T., Kinjyo, I., Takaesu, G., Shiraishi, H., Iida, M., Kobayashi, T., and Yoshimura, A. (2006). Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF-beta1 production. *Oncogene* **25**(17), 2520-2530.

101. Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J., and Flegal, K. M. (2006). Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA* **295**(13), 1549-1555.

102. Ogus, S., Ke, Y., Qiu, J., Wang, B., and Chehab, F. F. (2003). Hyperleptinemia precipitates diet-induced obesity in transgenic mice overexpressing leptin. *Endocrinology* **144**(7), 2865-2869.

103. Olden, K. (1995). The Role of the NIEHS in the Development of a National Program for Environmental Health Science Research. In *Biomarkers and Occupational Health: Progress and Perspectives* (M.L.Mendelsohn, J.P.Peeters, and M.J.Normandy, Eds.), pp. 25-34. Joseph Henry Press, Washington D.C.

104. Parker, G. A., and Picut, C. A. (2005). Liver immunobiology. *Toxicologic Pathology* **33**(1), 52-62.

105. Pedersen, B. K., and Febbraio, M. A. (2008). Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev.* **88**(4), 1379-1406.

106. Pestka, J. J. (2003). Deoxynivalenol-induced IgA production and IgA nephropathy-aberrant mucosal immune response with systemic repercussions. *Toxicol. Lett.* **140-141**, 287-295.

107. Pestka, J. J., Islam, Z., and Amuzie, C. J. (2008a). Immunochemical assessment of deoxynivalenol tissue distribution following oral exposure in the mouse. *Toxicol. Lett.* **178**(2), 83-87.

108. Pestka, J. J., Lin, W. S., and Miller, E. R. (1987a). Emetic activity of the trichothecene 15-acetyldeoxynivalenol in swine. *Food Chem. Toxicol.* **25**(11), 855-858.

109. Pestka, J. J., Moorman, M. A., and Warner, R. L. (1989). Dysregulation of IgA production and IgA nephropathy induced by the trichothecene vomitoxin. *Food Chem. Toxicol.* **27**(6), 361-368.

110. Pestka, J. J., and Smolinski, A. T. (2005). Deoxynivalenol: toxicology and potential effects on humans. *J. Toxicol. Environ. Health B Crit Rev.* **8**(1), 39-69.

111. Pestka, J. J., Tai, J. H., Witt, M. F., Dixon, D. E., and Forsell, J. H. (1987b). Suppression of immune response in the B6C3F1 mouse after dietary exposure to the Fusarium mycotoxins deoxynivalenol (vomitoxin) and zearalenone. *Food Chem. Toxicol.* **25**(4), 297-304.

112. Pestka, J. J., Yike, I., Dearborn, D. G., Ward, M. D., and Harkema, J. R. (2008b). Stachybotrys chartarum, trichothecene mycotoxins, and damp building-related illness: new insights into a public health enigma. *Toxicol. Sci.* **104**(1), 4-26.

113. Pestka, J. J., and Zhou, H. R. (2002). Effects of tumor necrosis factor type 1 and 2 receptor deficiencies on anorexia, growth and IgA dysregulation in mice exposed to the trichothecene vomitoxin. *Food Chem. Toxicol.* **40**(11), 1623-1631.

114. Pestka, J. J., Zhou, H. R., Moon, Y., and Chung, Y. J. (2004). Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. *Toxicol. Lett.* **153**(1), 61-73.

115. Pieters, M. N., Freijer, J., Baars, B. J., Fiolet, D. C., van, K. J., and Slob, W. (2002). Risk assessment of deoxynivalenol in food: concentration limits, exposure and effects. *Adv. Exp. Med. Biol.* **504**, 235-248.

116. Placinta, C. M., D'Mello, J. P. F., and Macdonald, A. M. C. (1999). A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Animal Feed Science and Technology* **78**(1-2), 21-37.

117. Pozo, J., Martos-Moreno, G. A., Barrios, V., and Argente, J. (2005). The IGF system in childhood: physiology and clinical implications. *J. Endocrinol. Invest* **28**(5 Suppl), 38-42.

118. Prelusky, D. B. (1993). The effect of low-level deoxynivalenol on neurotransmitter levels measured in pig cerebral spinal fluid. *J. Environ. Sci. Health B* **28**(6), 731-761.

119. Prelusky, D. B. (1994). The effect of deoxynivalenol on serotoninergic neurotransmitter levels in pig blood. *J. Environ. Sci. Health B* **29**(6), 1203-1218.

120. Prelusky, D. B. (1997). Effect of intraperitoneal infusion of deoxynivalenol on feed consumption and weight gain in the pig. *Nat. Toxins.* **5**(3), 121-125.

121. Prelusky, D. B., Hartin, K. E., Trenholm, H. L., and Miller, J. D. (1988). Pharmacokinetic fate of 14C-labeled deoxynivalenol in swine. *Fundam. Appl. Toxicol.* **10**(2), 276-286.

122. Prelusky, D. B., Rotter, B. A., Thompson, B. K., and Trenholm, H. L. (1997). Effect of the appetite stimulant cyproheptadine on deoxynivalenol-induced reductions in feed consumption and weight gain in the mouse. *J. Environ. Sci. Health B* **32**(3), 429-448.

123. Prelusky, D. B., and Trenholm, H. L. (1993). The efficacy of various classes of anti-emetics in preventing deoxynivalenol-induced vomiting in swine. *Nat. Toxins.* **1**(5), 296-302.

124. Probert, L., Akassoglou, K., Alexopoulou, L., Douni, E., Haralambous, S., Hill, S., Kassiotis, G., Kontoyiannis, D., Pasparakis, M., Plows, D., and Kollias, G. (1996). Dissection of the pathologies induced by transmembrane and wild-type tumor necrosis factor in transgenic mice. *J. Leukoc. Biol.* **59**(4), 518-525.

125. Radon, K., and Nowak, D. Respiratory diseases in European farmers. Part 1: Literary review. Pneumologie 57, 444-448. 2003.

126. Raghow, R., Yellaturu, C., Deng, X., Park, E. A., and Elam, M. B. (2008). SREBPs: the crossroads of physiological and pathological lipid homeostasis. *Trends Endocrinol. Metab* **19**(2), 65-73.

127. Ram, P. A., and Waxman, D. J. (1999). SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *J. Biol. Chem.* **274**(50), 35553-35561.

128. Renwick, A. G. (2000). The use of safety or uncertainty factors in the setting of acute reference doses. *Food Addit. Contam* **17**(7), 627-635.

129. Rico-Bautista, E., Flores-Morales, A., and Fernandez-Perez, L. (2006). Suppressor of cytokine signaling (SOCS) 2, a protein with multiple functions. *Cytokine Growth Factor Rev.* **17**(6), 431-439.

130. Rolland-Cachera, M. F., Deheeger, M., and Bellisle, F. (1999). Increasing prevalence of obesity among 18-year-old males in Sweden: evidence for early determinants. *Acta Paediatr.* **88**(4), 365-367.

131. Rotter, B. A., Prelusky, D. B., and Pestka, J. J. (1996). Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health* **48**(1), 1-34.

132. Rotter, B. A., Rotter, R. G., Thompson, B. K., and Trenholm, H. L. (1992). Investigations in the use of mice exposed to mycotoxins as a model for growing pigs. *J. Toxicol. Environ. Health* **37**(2), 329-339.

133. Rotter, B. A., Thompson, B. K., Lessard, M., Trenholm, H. L., and Tryphonas, H. (1994). Influence of low-level exposure to Fusarium mycotoxins on selected immunological and hematological parameters in young swine. *Fundam. Appl. Toxicol.* **23**(1), 117-124.

134. Salmon, W. D., Jr., and Daughaday, W. H. (1957). A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J. Lab Clin. Med.* **49**(6), 825-836.

135. Schaffler, A., Muller-Ladner, U., Scholmerich, J., and Buchler, C. (2006). Role of adipose tissue as an inflammatory organ in human diseases. *Endocr. Rev.* **27**(5), 449-467.

136. Schothorst, R. C., and van Egmond, H. P. (2004). Report from SCOOP task 3.2.10 "collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states". Subtask: trichothecenes. *Toxicol. Lett.* **153**(1), 133-143.

137. Sebert, S. P., Lecannu, G., Kozlowski, F., Siliart, B., Bard, J. M., Krempf, M., and Champ, M. M. (2005). Childhood obesity and insulin resistance in a Yucatan mini-piglet model: putative roles of IGF-1 and muscle PPARs in adipose tissue activity and development. *Int. J. Obes. (Lond)* **29**(3), 324-333.

138. Shargel, L., Wu-Pong, S., and Yu, A. B. C. Applied Biopharmaceutics and Pharmacokinetics. 2004. New York, McGraw-Hill Medical.
Ref Type: Serial (Book,Monograph)

139. Shumate, M. L., Yumet, G., Ahmed, T. A., and Cooney, R. N. (2005). Interleukin-1 inhibits the induction of insulin-like growth factor-I by growth hormone in CWSV-1 hepatocytes. *Am. J. Physiol Gastrointest. Liver Physiol* **289**(2), G227-G239.

140. Smolinski, A. T., and Pestka, J. J. (2005). Comparative effects of the herbal constituent parthenolide (Feverfew) on lipopolysaccharide-induced inflammatory gene expression in murine spleen and liver. *J. Inflamm. (Lond)* **2**, 6.

141. Starr, R., Willson, T. A., Viney, E. M., Murray, L. J. L., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997). A family of cytokine-inducible inhibitors of signalling. *Nature* **387**(6636), 917-921.

142. Swanson, S. P., Helaszek, C., Buck, W. B., Rood, H. D., Jr., and Haschek, W. M. (1988). The role of intestinal microflora in the metabolism of trichothecene mycotoxins. *Food Chem. Toxicol.* **26**(10), 823-829.

143. Tan, J. C., and Rabkin, R. (2005). Suppressors of cytokine signaling in health and disease. *Pediatr. Nephrol.* **20**(5), 567-575.

144. Thissen, J. P., Davenport, M. L., Pucilowska, J. B., Miles, M. V., and Underwood, L. E. (1992). Increased serum clearance and degradation of 125I-labeled IGF-I in protein-restricted rats. *Am. J. Physiol* **262**(4 Pt 1), E406-E411.

145. Thissen, J. P., and Verniers, J. (1997). Inhibition by interleukin-1 beta and tumor necrosis factor-alpha of the insulin-like growth factor I messenger ribonucleic acid response to growth hormone in rat hepatocyte primary culture. *Endocrinology* **138**(3), 1078-1084.

146. Thurman, J. D., Creasia, D. A., and Trotter, R. W. (1988). Mycotoxicosis caused by aerosolized T-2 toxin administered to female mice. *Am. J. Vet. Res.* **49**(11), 1928-1931.

147. Tritscher, A. M., and Page, S. W. (2004). The risk assessment paradigm and its application for trichothecenes. *Toxicology Letters* **153**(1), 155-163.

148. Trucksess, M. W., Thomas, F., Young, K., Stack, M. E., Fulgueras, W. J., and Page, S. W. (1995). Survey of Deoxynivalenol in Us 1993 Wheat and Barley

Crops by Enzyme-Linked-Immunosorbent-Assay. *Journal of Aoac International* **78**(3), 631-636.

149. Turner, P. C., Burley, V. J., Rothwell, J. A., White, K. L., Cade, J. E., and Wild, C. P. (2008a). Deoxynivalenol: rationale for development and application of a urinary biomarker. *Food Addit. Contam* **25**(7), 864-871.

150. Turner, P. C., Rothwell, J. A., White, K. L., Gong, Y., Cade, J. E., and Wild, C. P. (2008b). Urinary deoxynivalenol is correlated with cereal intake in individuals from the United kingdom. *Environ. Health Perspect.* **116**(1), 21-25.

151. Ueki, I., Giesy, S. L., Harvatine, K. J., Kim, J. W., and Boisclair, Y. R. (2009). The acid-labile subunit is required for full effects of exogenous growth hormone on growth and carbohydrate metabolism. *Endocrinology* **doi:10.1210/en.2008-1740**.

152. Ueno Y. Trichothecene mycotoxins mycology, chemistry and toxicology. In Draper, H. H. (ed.) Advances in Nutritional Research. 301-353. 1980. New York, Plenum Press.
Ref Type: Generic

153. Venken, K., Schuit, F., Van, L. L., Tsukamoto, K., Kopchick, J. J., Coschigano, K., Ohlsson, C., Moverare, S., Boonen, S., Bouillon, R., and Vanderschueren, D. (2005). Growth without growth hormone receptor: estradiol is a major growth hormone-independent regulator of hepatic IGF-I synthesis. *J. Bone Miner. Res.* **20**(12), 2138-2149.

154. Wallenius, V., Wallenius, K., Ahren, B., Rudling, M., Carlsten, H., Dickson, S. L., Ohlsson, C., and Jansson, J. O. (2002). Interleukin-6-deficient mice develop mature-onset obesity. *Nat. Med.* **8**(1), 75-79.

155. Wang, Y., Beydoun, M. A., Liang, L., Caballero, B., and Kumanyika, S. K. (2008). Will All Americans Become Overweight or Obese? Estimating the Progression and Cost of the US Obesity Epidemic. *Obesity (Silver. Spring)*.

156. Waxman, D. J., and O'Connor, C. (2006). Growth hormone regulation of sex-dependent liver gene expression. *Mol. Endocrinol.* **20**(11), 2613-2629.

157. Wolf, M., Bohm, S., Brand, M., and Kreymann, G. (1996). Proinflammatory cytokines interleukin 1 beta and tumor necrosis factor alpha inhibit growth hormone stimulation of insulin-like growth factor I synthesis and growth hormone receptor mRNA levels in cultured rat liver cells. *Eur. J. Endocrinol.* **135**(6), 729-737.

158. Wolf-Hall, C. E., and Schwarz, P. B. (2002). Mycotoxins and fermentation--beer production. *Adv. Exp. Med. Biol.* **504**, 217-226.

159. Wong, S., Schwartz, R. C., and Pestka, J. J. (2001). Superinduction of TNF-alpha and IL-6 in macrophages by vomitoxin (deoxynivalenol) modulated by mRNA stabilization. *Toxicology* **161**(1-2), 139-149.

160. Wong, S. S., Zhou, H. R., Marin-Martinez, M. L., Brooks, K., and Pestka, J. J. (1998). Modulation of IL-1beta, IL-6 and TNF-alpha secretion and mRNA expression by the trichothecene vomitoxin in the RAW 264.7 murine macrophage cell line. *Food Chem. Toxicol.* **36**(5), 409-419.

161. Wong, S. S., Zhou, H. R., and Pestka, J. J. (2002). Effects of vomitoxin (deoxynivalenol) on the binding of transcription factors AP-1, NF-kappaB, and NF-IL6 in raw 264.7 macrophage cells. *J. Toxicol. Environ. Health A* **65**(16), 1161-1180.

162. Wormald, S., Zhang, J. G., Krebs, D. L., Mielke, L. A., Silver, J., Alexander, W. S., Speed, T. P., Nicola, N. A., and Hilton, D. J. (2006). The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling. *J. Biol. Chem.* **281**(16), 11135-11143.

163. Worrell, N. R., Mallett, A. K., Cook, W. M., Baldwin, N. C., and Shepherd, M. J. (1989). The role of gut micro-organisms in the metabolism of deoxynivalenol administered to rats. *Xenobiotica* **19**(1), 25-32.

164. Wu, X., Murphy, P., Cunnick, J., and Hendrich, S. (2007). Synthesis and characterization of deoxynivalenol glucuronide: Its comparative immunotoxicity with deoxynivalenol. *Food Chem. Toxicol.* **45**(10), 1846-1855.

165. Yakar, S., Nunez, N. P., Pennisi, P., Brodt, P., Sun, H., Fallavollita, L., Zhao, H., Scavo, L., Novosyadlyy, R., Kurshan, N., Stannard, B., East-Palmer, J., Smith, N. C., Perkins, S. N., Fuchs-Young, R., Barrett, J. C., Hursting, S. D., and Leroith, D. (2006). Increased tumor growth in mice with diet-induced obesity: impact of ovarian hormones. *Endocrinology* **147**(12), 5826-5834.

166. Yakar, S., Sun, H., Zhao, H., Pennisi, P., Toyoshima, Y., Setser, J., Stannard, B., Scavo, L., and Leroith, D. (2005). Metabolic effects of IGF-I deficiency: lessons from mouse models. *Pediatr. Endocrinol. Rev.* **3**(1), 11-19.

167. Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., and Miyajima, A. (1995). A Novel Cytokine-Inducible Gene Cis Encodes An Sh2-Containing Protein That Binds to Tyrosine-Phosphorylated Interleukin-3 and Erythropoietin Receptors. *Embo Journal* **14**(12), 2816-2826.

168. Yumet, G., Shumate, M. L., Bryant, P., Lang, C. H., and Cooney, R. N. (2006). Hepatic growth hormone resistance during sepsis is associated with increased suppressors of cytokine signaling expression and impaired growth hormone signaling. *Critical Care Medicine* **34**(5), 1420-1427.

169. Yumet, G., Shumate, M. L., Bryant, P., Lin, C. M., Lang, C. H., and Cooney, R. N. (2002). Tumor necrosis factor mediates hepatic growth hormone resistance during sepsis. *Am. J. Physiol Endocrinol. Metab* **283**(3), E472-E481.

170. Zhou, H. R., Harkema, J. R., Hotchkiss, J. A., Yan, D., Roth, R. A., and Pestka, J. J. (2000). Lipopolysaccharide and the trichothecene vomitoxin (deoxynivalenol) synergistically induce apoptosis in murine lymphoid organs. *Toxicol. Sci.* **53**(2), 253-263.

171. Zhou, H. R., Islam, Z., and Pestka, J. J. (2003a). Rapid, sequential activation of mitogen-activated protein kinases and transcription factors precedes proinflammatory cytokine mRNA expression in spleens of mice exposed to the trichothecene vomitoxin. *Toxicol. Sci.* **72**(1), 130-142.

172. Zhou, H. R., Jia, Q., and Pestka, J. J. (2005). Ribotoxic stress response to the trichothecene deoxynivalenol in the macrophage involves the SRC family kinase Hck. *Toxicol. Sci.* **85**(2), 916-926.

173. Zhou, H. R., Lau, A. S., and Pestka, J. J. (2003b). Role of double-stranded RNA-activated protein kinase R (PKR) in deoxynivalenol-induced ribotoxic stress response. *Toxicol. Sci.* **74**(2), 335-344.

174. Zhou, H. R., Yan, D., and Pestka, J. J. (1998). Induction of cytokine gene expression in mice after repeated and subchronic oral exposure to vomitoxin (Deoxynivalenol): differential toxin-induced hyporesponsiveness and recovery. *Toxicol. Appl. Pharmacol.* **151**(2), 347-358.

175. Zhou, H. R., Yan, D., and Pestka, J. J. (1997). Differential cytokine mRNA expression in mice after oral exposure to the trichothecene vomitoxin (deoxynivalenol): dose response and time course. *Toxicol. Appl. Pharmacol.* **144**(2), 294-305.

176. Zhu, T., Goh, E. L., Graichen, R., Ling, L., and Lobie, P. E. (2001). Signal transduction via the growth hormone receptor. *Cell Signal* **13**(9), 599-616.