ASSESSING HEPATIC GENE EXPRESSION IN RESPONSE TO XENOBIOTIC EXPOSURE IN MICE

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Department of Animal and Poultry Sciences

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April 12, 2007

Blacksburg, Virginia

Keywords: Hepatic phase I and II enzymes, Gene expression, Sulforaphane, Ergotamine

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by

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(ABSTRACT)

Xenobiotics are plant derived compounds metabolized by phase I and II liver enzymes. Phase I enzymes increase, and phase II enzymes decrease, xenobiotic toxicity. Xenobiotics considered were ergotamine, associated with fescue toxicosis, and sulforaphane, a phase II inducer. Hypothesized responses in liver gene expression and enzyme activity due to exposure to these xenobiotics were tested. Polymorphic mice were gavaged with sulforaphane, ergotamine or control over four daily dosing periods (2, 5, 8 and 11 d), with at least 5 mice per treatment. Mice were killed and livers collected 24 h after last dosing. With ergotamine, expression of phase II genes catechol–O–amine methyltransferase 1 ($P = 0.009$) on d 8, and glutathione–S–transferase (**Gst**) mu1 (***Gstm1***; $P = 0.049$) on d 11 was increased, and sulfotransferase 5a1 on d 11 decreased ($P = 0.02$). Sulforaphane increased expression of cytochrome P450 1a2 on d 5 ($P = 0.02$) and flavin containing monooxygenases 1 on d 11 ($P = 0.002$), both phase I genes. It also increased expression of a phase II gene transcription factor ($P = 0.03$) and quinone reductase 02 ($P = 0.007$) on d 5, and *Gstm1* on d 8 ($P = 0.04$) and d 11 ($P = 0.01$). Moreover, sulforaphane treated mice had higher ($P < 0.05$) *Gstm1* expression across days. Among enzymes, only sufloraphane treated mice had higher ($P < 0.05$) Gst activity. The increase in both *Gstm1* expression and Gst activity indicate a consistent benefit of sufloraphane on phase II enzyme activity.

DEDICATION

To all the victims of Virginia Tech campus shooting on April 16, 2007

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to my advisor, Dr. Ron Lewis, for all of his support, guidance, and understanding. Thank you for your help, patience, expertise, and friendship that has assisted me in my career and allowed me to develop my goals. I am grateful for the opportunity to observe and work with such a talented and intelligent professor.

To my committee member Dr. Dennis Blodgett, I would like to express my deepest thanks for your help in writing this manuscript as well as the laboratory training. Thanks for teaching me the technique of scientific writing and honing my grammar skills.

I also wish to thank the other members of my committee. To Dr. Honglin Jiang and Dr. Eric Wong, thank you for your support, suggestions, teaching, and conversations and for your willingness to serve on my graduate committee. Your suggestions have been invaluable to the completion of my research.

I would like to express my sincere thanks to the faculty at Virginia Tech who have mentored me through their invaluable knowledge in quantitative genetics: Dr. Paul Siegel, Dr. David Notter, Dr. Ron Pearson, and Dr. Mc.Gilliard. I have learned a lot from the outstanding knowledge and instructions that you provide. I would also like to thank Dr. Kenneth Webb, Dr. Eric Wong, Dr. Dennis Blodgett and especially, Dr. Corl for allowing me to use their lab space and equipment.

To my fellow grad students, Larry, Bindu, Phoenix, Randy, Kathryn, Mike, Scott, and Joe, thank you for your help, support and friendship. I would also like to thank Elizabeth, Satyam and Bisi for their guidance in lab techniques. Thanks to Pat and Lee for being approachable and ever helping, and Sarah as well. Many thanks to the undergraduates involved with this project, especially Megan, Patrick, Josh, Sarah and Erica.

I am grateful to all my friends here at Blacksburg for helping me and keeping me motivated during my graduate career. Thanks to dear friend, Vidhi Mehta for taking care of me and making Blacksburg a place similar to home. Very special thanks to Dr. Esti Sheinberg for her friendship which is very dear and means a lot to me.

Finally, I would like to thank my parents, sister and jiju for encouraging me to come to graduate school and for always offering their support in all the choices I make.

ATTRIBUTIONS

The main manuscript in this thesis was written in a style that facilitates publication in scientific journals related to animal sciences. The manuscript has received contributions from multiple authors and I would like to specifically acknowledge the other listed authors.

Dr. Ron Lewis, my major professor, was actively involved in all aspects of this thesis. He contributed to the manuscript (Chapter 3) by having provided specific insights and expertise of subject matter, whenever appropriate, into statistical analysis, results, and general writing techniques. He offered guidance through discussion and suggestions, which improved the final outcome of the chapter.

Dr. Dennis Blodgett, my committee member, was intimately involved with this thesis. His contributions to the manuscript include having developed protocols for enzyme assays and assistance with general writing techniques.

Megan Carlidge, an undergraduate student, was a junior when I started work on my thesis and has recently graduated. Megan contributed to the manuscript by having assisted in the molecular laboratory work for the real-time PCR.

Sarah Blevins, an undergraduate student, was also a junior when I started work on my thesis and has recently graduated. Sarah's contribution to the manuscript is through having facilitated the mice gavage in both studies and having assisted in enzyme analysis for the preliminary study.

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INTRODUCTION

Xenobiotics are biologically active foreign chemicals that can be ingested or inhaled, and include both beneficial agents, like isothiocyanates, and potentially harmful agents, like heterocyclic amines. Two examples of xenobiotics are sulforaphane and ergotamine. Sulforaphane (**SFN**) is an isothiocyanate derived from broccoli. Ergotamine (**EGT**) is a member of the ergot alkaloid family and is derived from the fungal endophyte *Neotyphodium coenophialum*, which commonly infects tall fescue swards. Neither EGT nor SFN are found naturally in the animal's (including human's) body, but gain entry through ingested food.

Xenobiotics are modified in the body by metabolism, which occurs mainly in the liver. A large group of hepatic enzymes, collectively referred to as xenobiotic metabolizing enzymes, are involved in the process of metabolism. Generally, phase I hepatic enzymes activate lipophilic xenobiotics into reactive intermediate forms. Phase II enzymes conjugate endogenous compounds with these reactive intermediates making them water soluble, thereby facilitating their excretion through urine or bile. Thus phase I and II enzymes determine the fate of xenobiotic metabolites. Activity levels of these phase I and II enzymes varies between individuals due to polymorphisms in hepatic genes (Gonzalez and Yu, 2006).

Sulforaphane has received considerable attention in the past decade for its ability to induce phase II conjugating enzymes, especially glutathione-S-transferase (**Gst**) and quinone reductase (**Nq**). Hence, SFN is considered to be a mono-functional inducer of phase II enzymes (Zhang et al., 1994). In contrast to its monofunctional effect, SFN was reported to inhibit an isoenzyme of the cytochrome (**Cyp**) P450 enzymes, Cyp2E1. The inhibition of Cyp2E1 activity by SFN contributed to chemo protection against carcinogens (Barcelo et al., 1996). The unknown effects of SFN on phase I enzyme

systems, which are involved in activating a variety of carcinogens and other toxins, may also be important.

Ergotamine, a representative of ergot alkaloids, is considered therapeutically useful in early migraine attack because of its ability to produce vasoconstriction thereby acting as an analgesic. The phase I enzyme Cyp P450 3a 4 (**Cyp3a4**) is involved in the metabolism of EGT (Moubarak and Rosenkrans, 2000). Ergotamine toxicosis is associated with a deficiency of phase I enzymes necessary for the metabolism of EGT and EGT substrates (Schiff, 2006). Studies conducted in mice that were genetically resistant to ergot alkaloids had higher activity levels of phase I (Arthur et al., 2003) and phase II enzymes, namely Gst and UDP glucuronosyl transferase (**Ugt**) (Hohenboken and Blodgett, 1997; Wagner et al., 2000), when compared to susceptible mice.

Using mice as a model, the objectives of this study were: (i) to identify individual phase I and II genes that may be responsible for variation in response to oral administration of SFN and EGT; and, (ii) to elucidate any changes over time in the expression of these genes and associated enzyme activity given prolonged daily administration of these compounds. Real time - PCR and enzyme activity assays were used to quantify the expression of selected phase I and II genes and enzymes, respectively.

This thesis describes the role of hepatic metabolism, specifically phase I and II genes and enzymes, in dealing with xenobiotics, particularly SFN and EGT. In this first chapter, literature on the effects of these compounds on biotransformation systems in animal, human and *in vitro* studies is reviewed. The methods followed to identify hepatic genes responsive to SFN and EGT are explained in chapter 2. The design, conduct and results of two experiments, a preliminary and prolonged dosing (longitudinal) study, are described in chapter 3. Lastly, chapter 4 presents general conclusions and the implications of this research.

METABOLISM

Metabolism is a chemical or structural alteration of endogenous and exogenous (including xenobiotics) compounds by living cells. The structural modification of

xenobiotics may increase water solubility and hasten the process of their elimination from the body. Alternatively, it may create reactive intermediates that escape excretion and instead initiate mutagenesis, carcinogenesis and/or cell death by reacting with cellular constituents.

Several families of enzymes play pivotal roles in metabolism, elimination and/or detoxification of xenobiotics introduced into the body. Various tissues and organs in the body are well equipped with phase I and II metabolizing enzymes, and phase III transporters. These enzymes and transporters are present at basal levels and may be inducible after xenobiotic exposure (Rushmore and Kong, 2002). Hepatic phase I and II genes and enzymes involved in xenobiotic metabolism are discussed in this review. Depending on the similarity of their amino acid sequence, enzymes are classified into families (greater than 40% amino acid homology) and subfamilies (greater than 70% amino acid homology)(Plant, 2003).

Phase I metabolism

Phase I metabolism is the process of revealing or adding chemically reactive groups to the parent compounds to produce targets for phase II metabolism. This may result either in an inactive metabolite, which can be excreted directly, or in an active compound, which if not conjugated with phase II enzymes may be harmful. Two important phase I enzyme families are Cyp P450 and flavin containing monooxygenases (**Fmo**). Members of both families share the characteristic of diverse substrate specificity, multiple isozyme subtypes, and varied sensitivity to induction by different types of chemical inducers (Cashman et al., 1995). The mixed function oxidases involving Cyp P450's are common oxidation reactions of phase I metabolism, in addition to reduction, hydrolysis, hydration and isomerization reactions.

Cytochrome P450 family. The Cyp monooxygenase enzyme system in liver plays a central role in the oxidation of a wide variety of exogenous (pharmaceutical agents, chemical carcinogens and other lipophilic xenobiotics) and endogenous (steroids, fatty acids, prostaglandins and vitamin D_3) compounds (Gonzalez, 1990; Ryan and Levin,

1990). The structure of Cyp in archaebacteria (Poulos et al., 1987) and rabbits (Cosme and Johnson, 2000) are similar. However, polymorphisms in the coding and regulatory regions of Cyp enzymes explain variation in their coping with chemical exposures. The effect of these polymorphisms may be silent or non-silent, the latter resulting in enhanced or decreased enzyme production. The Cyp are divided into four subfamilies, Cyp1 through Cyp4, where the Cyp1 family regulates carcinogen or toxicant metabolism. Both Cyp2 and Cyp3 are mostly involved in metabolism of drugs and other compounds and ultimately result in phase II metabolism to form hydrophilic end products; however, a few exceptions exist.

Metabolism of polycyclic aromatic hydrocarbons is mainly by Cyp1a1 and Cyp1b1, whereas Cyp1a2 substrates are mostly N-heterocyclic amine and arylamines. Studies using knockout mice indicate that hepatic and intestinal Cyp1a1 and Cyp1a2 are more important in detoxification, whereas spleen and bone marrow Cyp1b1 are responsible for initiating toxicity of several aromatic hydrocarbons. Unlike *Cyp1a2*, expression of *Cyp1a1* gene in the liver of mice is only detected when induced; that is, under normal physiological conditions, expression of *Cyp1a1* gene is not detectable (Zacharova et al., 2003). In humans, Cyp1a2 enzyme activity, measured by caffeine metabolic ratios, is increased by intake of cruciferous vegetables (Vistisen et al., 1992), including broccoli (Kall et al., 1996).

Another subfamily of Cyp's, Cyp3a, is important because of its role in activation of a wide range of toxicological agents, particularly carcinogens. In the mouse, six Cyp3a genes are known: *Cyp3a11*, *Cyp3a13*, *Cyp3a16*, *Cyp3a25*, *Cyp3a41* and *Cyp3a44*. The gene *Cyp3a11* is expressed abundantly in liver (Yanagimoto et al., 1992), whereas *Cyp3a13* predominates in extrahepatic tissues (Yanagimoto et al., 1994). The genes *Cyp3a25* and *Cyp3a41* are also found in adult liver (Sakuma et al., 2000), whereas *Cyp3a16* is expressed mostly in fetal liver. In mice, a developmental change of major Cyp3a enzyme, from Cyp3a16 in fetal livers to Cyp3a11 in adult mouse livers, occurs (Itoh et al., 1994). High nucleotide sequence homology in coding regions of different isoforms of the Cyp3a family is typical, with 92.1% similarity between *Cyp3a16* and *Cyp3a41*, 92.4% between *Cyp3a16* and *Cyp3a44* and 95.3% between *Cyp3a41* and *Cyp3a44* (Sakuma et al., 2002).

Flavin containing monooxygenases. Flavin containing monooxygenases are microsomal enzymes that catalyze flavin adenine dinucleotide, nictotinamide adenine dinucleotide, and oxygen dependent oxidation of heteroatoms (nitrogen, sulfur, selenium, and phosphorous) present in many xenobiotic compounds (Ziegler, 1993). In mice, nine Fmo genes – *Fmo1-6* and *9*, *12* and *13* – are found (Hernandez et al., 2004). Although *Fmo9*, *12* and *13* are not significantly expressed in adult mice, expression of Fmo's *1- 5* is reported in mouse liver (Janmohamed et al., 2004).

Species, gender and tissue dependent expression of Fmo is well documented. In mice, *Fmo1* and *5* are expressed in fetal and adult liver, whereas *Fmo3* is only expressed in the adult liver. Gender dependent expression of *Fmo1* (2-3 times greater in the female than the male), gender specific expression of *Fmo3* (expressed only in females) and gender independent expression of *Fmo5* is well documented in mice (Hines et al., 1994; Falls et al., 1995). Important members of the Fmo family concerned with xenobiotic metabolism are *Fmo1* and *3* (Ziegler 1993).

Bioactivation is a major role of the Cyp and Fmo enzymes. Oxidation reactions by Cyp and Fmo result in a reactive center for phase II metabolism, thus resulting in bioactivation. The general stochiometry of oxidation reactions is:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O,$$

where R indicates the substrates chemical structure. The entire catalytic phase is divided into seven phases for Cyp (Figure 1.1; Segall et al., 1997) and four for Fmo (Figure 1.2; Zhang and Robertus, 2002).

Phase II metabolism

Phase II metabolism enhances the rate of excretion of chemically reactive compounds or phase I metabolites by adding larger polar molecules. This generally results in elimination of compounds either in urine or feces. Compounds excreted through feces are passed into bile and then to the intestines for excretion. If phase II conjugation

does not occur, then reactive compounds may cause cellular damage by forming DNA or protein adducts. Phase II conjugation may rarely result in xenobiotic metabolites becoming more lipophilic, which cannot be excreted easily and pose a danger to the body.

Phase II conjugating enzymes consist of the following superfamilies: Gst, Nq, epoxide hydrolase (**Ephx**), Ugt, sulfotransferases (**Sult**) and N-acetyltransferases (**Nat**).

Glutathione-S-transferases. Expression of Gst is highly inducible by certain foods and is usually considered protective against cancer. In mice, more than a hundred xenobiotics are reported to be capable of inducing Gst (Hayes and Pulford, 1995). Large and diverse Gst consist of eight families: alpha (**Gsta**), mu (**Gstm**), pi (**Gstp**), sigma (**Gsts**), theta (**Gstt**), zeta (**Gstz**), omega and kappa.

Polymorphisms in Gst may predispose individuals to cancer due to reduced detoxification. In humans, functional polymorphisms are reported in three Gst genes: *Gstm1*, *Gstt1* and *Gstp1*. In *Gstm1* polymorphisms, the variant allele is a deletion of the gene, and individuals homozygous for the deleted allele do not produce the enzyme and are said to possess a "null" genotype. Reduced Gstm1 enzyme activity in individuals with *Gstm1* null alleles reduces the metabolism of carcinogens, thus increasing the risk of cancers (Gawronska-Szklarz et al., 1999).

Conjugation of glutathione (**GSH**) with diverse electrophiles, including carcinogens, is chiefly catalyzed by Gst. Briefly, a thioether bond is formed between the sulfur atom of GSH and the reactive substrate, catalyzed by Gst. The general reaction is:

$$\text{R- epoxide + GSH} \rightarrow \text{R-GS},$$

where R indicates the substrate's base chemical structure. In GSH conjugation, the tripeptide GSH (glutamine-cysteine-glycine), a chemically reactive conjugate, usually targets only chemically reactive substrates. As a result of GSH conjugation with a reactive electrophile, a stable water soluble compound is usually formed; thus cellular components, like DNA, are protected from damage. Total Gst activity can be easily

measured *in vitro* by estimation of 1-chloro-2, 4, dinitrobenzene (CDNB) conjugation with GSH (Habig et al., 1974).

In contrast to the role of detoxification, in rare cases Gst conjugation reactions increases toxicity of synthetic compounds. For instance, in mice dichloromethane (a chemical solvent used in industry) is usually activated by Cyp2E1 enzyme. Instead, if dichloromethane directly conjugates with GSH, a reaction catalyzed by Gstt1, it produces a highly reactive episulfonium ion, which forms DNA or protein adducts (Sherratt et al., 2002).

Quinone reductase. Quinone reductase 01 (**Nq01**) and quinone reductase 02 (**Nq02**) are cytosolic flavoporoteins belonging to a phase II enzyme family. They are transcriptionally induced in response to various agents, including xenobiotics (Talalay et al., 1995). Quinone reductase is responsible for a two electron reduction of quinones to form hydroquinones, which are further metabolized to form water soluble conjugates that can be excreted in urine. If there is insufficient reduction of quinones by Nq, then quinones undergo one electron reduction catalyzed by NADPH-Cyp P450 reductase, a phase I enzyme, to hydroquinones. However, in the absence of conjugating reactions, the hydroquinones undergo oxidation to generate reactive oxygen species causing cell damage (Ross and Siegel, 2004; Iskander and Jaiswal, 2005).

Epoxide hydrolase. Microsomal Ephx catalyzes hydrolysis of a large number of epoxide intermediates. Hydration of arene epoxides that are toxic, carcinogenic and mutagenic to less reactive trans-dihydrodiols intermediates by Ephx is an important step in detoxification (Oesch and Daly, 1971). However, Ephx may activate certain polycyclic aromatic hydrocarbons that are further metabolized by the Cyp yielding highly reactive toxic compounds (Wood et al., 1976). Thus, Ephx plays a central role in both detoxification and activation of polycyclic aromatic hydrocarbons.

UDP-glucuronosyltransferases. Based on amino acid sequence, Ugt are divided into two families: Ugt1a and Ugt2a. All the members of Ugt1 family share greater than

50% identity among each other (Ritter et al., 1992). Polymorphisms in the conserved exon sequences result in certain clinically important syndromes in humans.

Glucose-1-phosphate, essential for cell glycolysis, is an extensively available conjugate for glucuronidation. A co-substrate, uridine diphosphate glucuronic acid (**UDPGA**), is required for the glucuronidation reaction, which is catalyzed by Ugt. The general stoichiometry of this reaction is:

$$R\text{-}OH + UDPGA \rightarrow R\text{-}OGA + UDP$$

where R indicates the substrate's chemical structure and UDPGA indicates the conjugate, UDP-glucuronic acid. However, the half life of xenobiotics metabolized through this pathway may be increased by a chemical inversion of the glucuronidation reaction, which enables enterohepatic circulation. β-glucuronidase in the intestinal microflora cleaves the conjugate from the compound and the parent compound is reabsorbed into the body. Formation of chemically reactive acyl-glucuronides under alkaline conditions may lead to irreversible binding as protein adducts (Plant, 2003).

Sulfotransferases. Sulfotransferases have been classified into five families. In mice, Sult isozymes present in liver are Sult1a1, 1c1, 1c2, 1d1, 2a1/2, 2b1, 3a1, 4a1, and 5a1. Single nucleotide polymorphisms associated with these genes are either silent or exhibit decreased enzyme activity. Sulfotransferases catalyze the transfer of sulfonate group (SO_3^-) from 3′- phosphoadenosine 5′-phosphosulfate (**PAPS**), an activated form of sulfate, to target xenobiotics. The general stoichiometry of their activity is:

$$R\text{-}OH + PAPS \rightarrow R\text{-}SO_4 + PAP$$

where R indicates the substrate's chemical structure and PAPS indicates the conjugate, 3'- phosphadenosine-5'phosphosulfate. Rats exposed to 2-nitropropane by oral or inhalation routes develop liver cancer due to electrophilic break down products of sulfate conjugates (Sodum et al., 1994).

N-acetyl transferases. Arylamine Nat catalyze an activation step, O- acetylation of N-hydroxylamine, and a detoxification step, N-acetylation of the arylamine. Isoenzymes of Nat in mice are coded by three genes: *Nat1*, *Nat2* and *Nat3*. The Nat2 enzyme, which is under genetic control of the *Nat2* gene, is not readily inducible with normal dietary consumption of vegetables (Vistisen et al., 1992).

LIVER

Anatomy

Liver is the largest organ and gland (as it secretes bile) in the body. Liver is a triangular macroscopic structure located on the upper right quadrant of the abdominal cavity below the diaphragm and above the stomach, intestines and kidneys. It is divided into a few lobes and numerous lobules that are made up of hepatic cells. Both the hepatic artery (30%) and portal vein (70%) supply blood to the liver and drain into the central hepatic vein. The hepatic artery carries oxygenated blood from the heart. The portal vein carries nutrient rich blood containing ingested xenobiotics from the stomach and intestines into the liver. Thus, liver is a potential target organ exposed to ingested xenobiotics and toxicants.

The functional unit of liver is called the acinus, which is supplied by the portal triad formed by the hepatic artery, portal vein and bile duct. The acinus is divided into three zones depending on the blood supply and their distance from the portal triad. The zone of permanent function (zone one), which is near the center of the acinus, has higher oxygen blood supply and a higher concentration of metabolites than the zone of intermediate function (zone two) and the zone of permanent repose (zone three). Zone three has the least supply of blood, oxygen and metabolites, as it is further away towards the peripheral border near the hepatic vein (Figure 1.3; Cunningham and Van horn, 2003).

Functions

More than 500 functions of the liver have been identified and can be broadly classified as: (i) *homeostasis*, regulation of levels of various chemicals (xenobiotic or endogenous) and amino acids in the blood, and the synthesis of glucose, proteins (e.g.albumin) and cholesterol; and, (ii) *storage*, the accumulation of glucose in the form of glycogen, fat soluble vitamins, etc.

An important homeostatic function of the liver is detoxification, which involves absorption, metabolism and excretion. The liver receives chemicals from the blood and at times prevents their entry into systemic circulation. A proportion of these absorbed chemicals are excreted directly into the bile without entry into the general circulation. The process is called the first pass effect or pre-systemic elimination.

The liver also plays an important role in metabolism of these absorbed substrates as it contains an abundance of enzymes involved in the metabolic pathways (phase I and II). It also has immediate access to these xenobiotic compounds after they are absorbed from the intestines. Immunohistochemistry studies in liver tissue for hepatotoxins show high Cyp P450 proteins and GSH levels at zone 3 (port of exit from the liver, perivenous), compared to zone 1 (the site of entry of blood, periportal. High expression and induction of Cyp (*Cyp1a1* and *Cyp1a2*) in the perivenous region makes the region vulnerable to damage (Oinonen et al., 1995). However, the damage is counteracted by expression of phase II enzymes, mainly Gst and Ugt, in the same region (Bengtsson et al., 1987).

Expression of Fmo in the liver lobule has a differential pattern, with *Fmo1* distributed in the perivenous region and *Fmo3* localized in the periportal region. Compounds like thiourea, phenylthiourea, and alpha naphthyl thiourea are toxic to mouse (C3H/10T1/2) cells expressing human *Fmo3* but not to those expressing human *Fmo1*. (Smith and Crespi, 2002).

For xenobiotics and other compounds to be excreted, there must be adequate formation of bile, a yellow-green fluid. Xenobiotics are absorbed from the blood by transporters on the sinusoidal membranes and enter the canalicular lumen with the help of exporters in the canalicular membrane. Hepatocytes secrete conjugates like GSH and

glucuronide along with bile salts into the lumen of bile canaliculi. Biliary epithelial cells expressing phase I and II enzymes may also help in biotransformation of xenobiotics entering into the bile canaliculi. Secretion of toxicants into bile ducts generally results in their excretion in feces, except in cases of enterohepatic cycling (Klaassen and Watkins, 2003).

Liver response to toxicant challenges

The response of liver to toxicity depends on the amount of the chemicals ingested, whether the exposure is acute or chronic, and the population of hepatic cells affected. It also depends on the amount of phase I and II enzyme production. Depending on the attributes of the toxicant and the enzymatic activity levels of the liver, the response to the toxic challenge may result in the successful removal of the chemical or the elimination of damaged cells.

Immediate response. Surprisingly, metabolism that enables safe and efficient removal of chemicals from the body may bioactivate chemicals. Bioactivation in cells is usually by formation of small reactive oxygen species (**ROS**) and large reactive groups. Oxidation stress occurs in cells due to decreased reduction-oxidation (redox) potential when ROS are produced. These ROS bind to substrates and form water, which is excreted in urine. However, in the absence of sufficient amounts of substrate, ROS attack the nucleus of electron deficient chemical groups (DNA, protein or lipid). The ROS form covalent bonds with chemical groups, called adducts, which disrupt cellular functions. Larger chemical reactive groups conjugate with GSH and are removed from the cell. Since the clean up process of chemicals within cells is mediated by Gst, they are called "biological hoovers" of the cell. However, limited levels of Gst and uneven levels of enzyme induction by chemicals (high levels of phase I and poor induction of Gst) may lead to toxicity (Plant, 2003).

Coordination of the response to reactive chemicals. The chemicals (ROS) produced as a result of immediate response of liver to toxicity alters the cellular

environment by affecting gene expression directly or indirectly. Surprisingly, the ROS produced tend to increase the expression of particular genes that are responsible for preventing adduct formations caused by ROS themselves. Direct activation of cysteine residues in Kelch-like ECH associating protein 1(**Keap1**) by ROS results in release of the transcription factor, nuclear factor E2 related factor 2 protein (**Nrf2**). After release, *Nrf2* translocates from the cytoplasm to the nucleus and binds to the antioxidant response element (**ARE**) and electrophile responsive element (**EpRE**) present in the promoter regions of most of the phase II enzymes. Thus, release of *Nrf2* coordinates a change in gene expression of phase II enzymes (Itoh et al., 1997). Additionally, initiation of signal transduction pathways, such as mitogen-activated protein kinase (**MAPK**) cascades, by low concentrations of ROS plays a key role in regulation of genes.

Repair of cellular damage. The DNA and protein damage in cells resulting from toxicants can be repaired. Repair of chemically-mediated damage to DNA is generally by base–excision repair and nucleotide–excision repair, where the correct nucleotides are replaced after removing damaged nucleotides. Exceptions are seen when chemicals (e.g., nickel, cobalt) damage repair systems by acting as co-mutagens (Hartwig and Schwerdtle, 2002). Proteins destroyed by adduct conjugation are degraded by ubiquination enzymes (Donohue, 2002) and removed from cells. Proteins with similar functions replace damaged proteins in cells.

Apoptosis and necrosis. As repair is not always possible, the body resorts to apoptosis or necrosis or both (Pierce et al., 2002). If injured cells are not removed from the body, they may pose a threat to the integrity of the organism. Programmed cell death of injured cells is called apoptosis, which removes injured cells from the body (Kerr et al., 1972). Necrosis is the death of tissue, generally surrounded by healthy tissue. Both apoptosis and necrosis have a similar result (cell death), but differ in their biological consequences and mode of functioning. Apoptosis involves the activation of signal transduction pathways (Hodgson et al., 1998) unlike necrosis, which is mediated by many enzymes.

Liver enzyme induction

Induction of phase I enzymes alone. In liver cells that are engineered to over-express Cyp2E1, a phase I enzyme, there is excessive generation of ROS, which lead to increased toxicity due to ferric-nitriloacetate (Sakurai and Cederbaum, 1998). In umu tester strains of *Salmonella typhimurium*, activation of phase I enzymes by heterocyclic aromatic amines results in genotoxicity. Activation of aflatoxin to aflatoxin B- 8, 9-epoxide by *Cyp3a4* and *Cyp1a2* also leads to genotoxicity of the tester strains (Oda et al., 2001). Transgenic *Cyp2E1* (-/-) knockout mice show more resistance to hepatic necrosis than wild type mice after high doses of paracetamol. This is because the over production of Cyp2E1 is inhibited in knockout mice, thus inhibiting toxic effects induced by *Cyp2E1* activation of paracetamol (Zaher et al., 1998). Human cells expressing high levels of *Fmo3* are susceptible to thio-urea induced toxicity (Smith and Crespi, 2002).

Induction of Phase II enzymes alone. Generally, induction of phase II enzymes by consumption of fruits and vegetables is suggested as a mechanism for preventing cancer (Smith and Yang, 1994). Such mono-functional induction of phase II enzymes usually does not have adverse effects. Exceptions are seen in very rare cases of reversible or irreversible binding of proteins in alkaline conditions by C2-C4 acyl glucuronides in Ugt-mediated toxicity (Spahn-Langguth and Benet, 1992) and liver cancer in rats exposed to 2-nitropropane due to sult-mediated toxicity (Sodum et al., 1994).

Balanced enzyme induction. Oltipraz, known for weak phase I enzyme induction but potent phase II enzyme induction, induces Cyp that activate aflatoxin B to aflatoxin B 8,9 epoxide. Further conjugation of aflatoxin B 8, 9 epoxide by Gst, not only results in resistance to aflatoxin but indicates that balanced enzyme production is required for maintaining homeostasis in the body. Many fruits and vegetables are multi-functional inducers. For example, ellagic acid in red grape skin induces several phase II enzymes as well as decreases phase I enzyme induction and thus has an anti-carcinogenic effect (Manson et al., 1997; Barch et al., 1995).

SULFORAPHANE

Introduction

Epidemiological studies suggest that fruits and vegetables play a vital role in preventing cancers (Steinmetz and Potter, 1991; Block et al., 1992). Cruciferous vegetables, particularly broccoli, provide protection against prostate (Jain et al., 1999), bladder (Michaud et al., 1999) and breast cancers (Terry et al., 2001). Cancer preventive attributes of broccoli are due to SFN, a breakdown product of glucosinolates (Verhoeven et al., 1997). Sulforaphane is also present in leaves of hoary cress and radish seeds (Schmid and Karrer, 1948). The molecular formula of SFN is $C_6H_{11}NOS_2$ and it is also known as *R*-1- isothiocyanato- 4 – methylsulfinyl-butane (Zhang et al., 1992).

Sulforaphane is released from broccoli by disruption of plant cells. In intact cells of broccoli, glucosinolates and myrosinase (a hydrolyzing enzyme) co-exist but are physically segregated. Cell disruption, generally due to mastication, results in hydrolysis of glucosinolates by myrosinase leading to formation of SFN (Figure 1.4; Fenwick et al., 1983). Isothiocyanates are absorbed into the digestive system and metabolized in the liver. In the liver, isothiocyanates conjugate with cellular GSH, mediated by Gst, to form dithiocarbamates, the major urinary metabolites of isothiocyanates (Seow et al., 1998).

Metabolism

Effect on Phase II enzyme activity. Sulforaphane, like many other monofunctional inducers, induces a large number of phase II enzymes. Unlike bi-functional inducers, which stimulate both phase I and II enzymes, mono-functional inducers, like SFN (Zhang et al., 1992), specifically induce phase II enzymes. Usually, phase II enzyme activity is increased by activation of the *ARE* in the 5` flanking region of these genes. Activation of *ARE* requires release of *Nrf2*, a transcription factor, usually tethered to the Keap1 protein in cytoplasm. Upon its release, *Nrf2* translocates into the

nucleus where, along with Maf proteins, it binds with the *ARE* and promotes transcription of phase II genes (Itoh et al., 1997).

Thimmulappa et al. (2002) identified several phase II genes that are up-regulated in the small intestine of mice having a functional *Nrf2* gene (*Nrf2* $^{+/+}$), in comparison with knockout mice (*Nrf2* $^{-/-}$) lacking *Nrf2*. Additionally, mice treated with SFN express *Nrf2* more than mice treated with a vehicle, corn oil. This study clearly showed that SFN increases the expression of *Nrf2,* which in turn induces several other phase II genes (Thimmulappa et al., 2002).

Treatment with SFN increases the activity of several members of phase II enzyme family, in in vitro, in vivo, time and dose dependent studies. Sulforaphane is a potent inducer of Gst and Nq in murine hepatoma cells (Zhang et al., 1992). Oral administration of SFN in rats for 10 days increases the activity of hepatic Nq at both normal (3 mg/kg body wt) and high (12 mg/kg body wt) dietary dose levels. However, Gst, Ephx and Ugt activities in the liver are not affected at either of the dose levels (Yoxall et al., 2005). Induction of phase II enzymes, like Gst or Nq, is considered a major mechanism of protection against initiation of cancer (Talalay et al., 1995). These phase II enzymes may in turn offer protection against certain carcinogens and other toxic electrophiles.

Effect on Phase I enzyme activity. Although considered a mono-functional inducer of phase II enzymes, SFN also affects a few phase I enzymes, both in vitro and in vivo. Sulforaphane inhibits Cyp2E1 activity (Barcelo et al., 1996) and decreases the activity of Cyp2b and Cyp3a enzymes (Yoxall et al., 2005). In contrast to decrease in phase I activity by SFN, Bacon et al. (2003) reported an increase in Cyp1a2 levels in human HepG2 cells (liver cells capable of expressing *Cyp1a2* genes) after 6 h of incubation with SFN. Administration of SFN in drinking water to rats for 10 days at doses equivalent to 3 and 12 mg/kg body wt elevates *Cyp1a2* expression levels in liver. Although gene expression levels of *Cyp1a2* are high when treated with SFN, Cyp1a2 activity does not increase (Yoxall et al., 2005). This may be due to: (i) binding of SFN metabolite to the Cyp and compromising its metabolic activity (Yoxall et al., 2005); or, (ii) deactivation of Cyp1a2 activity by the increase in *Gstm1* levels (Probst -Hensch et al., 1998).

Bioavailability

Bioavailability of SFN is influenced by age of the broccoli floret and cooking procedures. Sulforaphane availability is higher in younger broccoli florets, which contain 10 to 100 times more glucosinolates than mature florets (Fahey et al., 1997). Boiling or steaming vegetables destroys myrosinase present in the plant. Glucosinolate conversion then depends entirely on the limited amounts of myrosinase naturally present in intestinal bacteria, thus limiting conversion of glucosinolates into SFN. Furthermore, boiling vegetables also results in leaching of glucosinolates, with additional losses in SFN availability. Hence, uncooked broccoli consumption may be more beneficial than consuming cooked broccoli as it has three times more SFN (Conaway et al., 2000).

ERGOTAMINE

Introduction

Tall fescue (*Festuca arundinacea*) is a major forage crop of the southeastern United States. It is a hardy grass that has a long growing season, which starts early in the spring and lasts until late into the fall. It survives drought conditions that wither other grasses, and it is resistant to insects, disease and weed competition. These positive attributes of fescue are partially due to a symbiotic relationship with the fungal endophyte *Neotyphodium coenophialum* that infects most tall fescue fields. However, animals grazing on endophyte-infected tall fescue have reduced body weight, milk yield, and pregnancy rates (Porter and Thompson, 1992). Losses incurred in the beef cattle industry due to fescue toxicosis are approximately US $800 million each year (Ensley and Larsen, 2001). Current measures of controlling fescue toxicosis in animals, such as improving livestock management and treatment of affected animals, have very limited success (Wagner, 2000).

Endophytic fungi live between plant cells and produce ergot alkaloid toxins. Major classes of ergot alkaloids in endophyte-infected tall fescue are ergopeptides and

loline alkaloids. Ergovaline is the major ergot alkaloid present in tall fescue (Porter, 1995), but it is not commercially available for experimental purposes. Ergotamine is another ergopeptide that is commercially available. Ergotamine is very similar to ergovaline and differs in only one of the three amino acids (Lyons et al., 1986; Powell and Petroski, 1992; Porter, 1995). The chemical formula of EGT is $C_{33}H_{35}N_5O_5$ (Figure 1.5; Schiff, 2006).

Metabolism

Ergot alkaloids affect the Cyp P450 system, especially isoenzyme Cyp3a4, by binding to the isoenzyme as a substrate. Metabolic profiles of EGT are similar when incubated separately with rat livers treated with dexamethasone and beef liver microsomes isolated from steers grazing on endophyte-infected tall fescue. Dexamethasone is known for inducing Cyp3a4, an isoenzyme that results in ergotamine metabolism. It was inferred that beef liver microsomes contained high levels of Cyp P450 that are induced as a result of grazing on infected fescue (Moubarak and Rosenkrans, 2000). Ergotamine administration, along with compounds like ritonavir that inhibit Cyp3a4 activity, result in ergotism (Liaudet et al., 1999). Thus, Cyp3a4 plays a major role in EGT metabolism.

Mouse lines with measurable genetic difference in resistance to endophyte-infected fescue exhibit higher activity of phase I (Arthur et al., 2003) and phase II enzymes (Hohenboken and Blodgett, 1997; Wagner et al., 2000) compared to susceptible mice. The selection for resistance to endophyte-infected fescue seed also led to resistance to sporidesmin, another fungal toxin (Hohenboken et al., 2000).

If specific genes associated with variation in enzyme activity could be identified, animals possessing resistant genes (alleles) could be selected. Selection based on markers is not only a quick process, but also it may provide a long term and permanent solution to ameliorate fescue toxicosis in livestock animals.

TOXICOGENOMICS

Toxicogenomics combines toxicology (study of toxicants) with genomics (all genes within chromosomes). Upon exposure to toxicants, living cells respond by altering their gene expression pattern. Gene expression is transcribed into messenger RNA (mRNA) and translated into proteins. Production of proteins that have varied functions in response to toxicant exposure, may increase, decrease or remain unchanged depending on type of exposure and the cell's requirements.

Genes and their expression patterns

Genes are either constitutive or facultative (inducible) and their expression is basal or inducible. Genes that are expressed at all times and generally not influenced by any treatment are constitutive genes. Housekeeping genes, like glyceraldehyde-3-phosphate dehydrogensae (***Gapdh***) and beta-actin, are constitutive genes. Facultative genes are transcribed when needed by the cell, and are expressed in response to environmental factors like xenobiotics. Genes have a coding region and a regulating region, with the regulating region controlling expression of the coding region via promoters and enhancers. Minimum control is required for producing some level of gene expression and is referred to as basal gene expression. Inducible gene expression is seen as a response to specific stimuli, like xenobiotics. The stimulus changes the cell's environment and the result is an increase (switch on) or decrease (turn off) in the expression of the specific genes in the cell. Unlike basal genes, which depend only on their promoter regions for their expression, the inducible genes also depend on the enhancers (Plant, 2003).

Response coordination to toxic exposure

Exposure to xenobiotics results in activation of several genes and this coordinated response is due to: (i) overlap of ligands that can bind and activate the ligand-activated

receptors present on the cell surface; and/or, (ii) sharing of same response element by different genes. A certain extent of overlap exists among the ligand-activated receptors that bind to ligands and extracellular substances like xenobiotics. This overlap induces more than one gene and leads to transcription of gene expression, which is usually beneficial with the exception of some xenobiotic ligands. For instance, glucocorticoid binds to two ligand-activated receptors, glucocorticoid receptor and pregnane X receptor, both of which induces *Cyp3a4* and thereby increases its expression. Increased Cy3a4 may further lead to activation of xenobiotics and thus lead to detrimental results (El-Sankary et al., 2000). The *Nrf2* transcription factor binds with the *ARE* present in promoter regions of several phase II genes and activates them. Thus, sharing of *ARE* causes coordinated response in several phase II genes (Thimmulappa et al., 2002).

Variation in toxic response among and within species

Mutations are responsible for variations between individuals. Mutations in coding regions produce altered protein activity (generally decreased) and mutations in regulatory regions affect the amount of protein product formed. When mutations reach a level of greater than 1% in a population, they are called polymorphisms (Twyman, 2003). Variation in DNA sequences common in a population is called polymorphisms. Polymorphisms in genes associated with phase I and II enzymes have been shown to affect activity of enzymes. As mentioned earlier, decreased Gstm1 enzyme activity in individuals with *Gstm1* null alleles decreases metabolism of carcinogens. Thus, individuals with *Gstm1* null gene have 2.5 higher risk of cancer compared to individuals with functional *Gstm1* gene (Gawronska-Szklarz et al., 1999). Therefore, variation in hepatic enzyme activity among populations, due to polymorphisms in genes, affects the rate of metabolism, thus affecting the response to toxicants.

Variation in resistance to toxins in livestock populations is evident in the following cases. Sporidesmin released from fungal swards of *Pithomyces chartarum* on *Lolium perenne* (perennial ryegrass) and *Trifolium repens* (white clover) causes liver damage mainly by occluding bile ducts. Facial eczema, a secondary clinical sign of the toxin, is decreased in flocks selected for resistance to sporidesmin (Morris et al., 1995).

Among cattle breeds, varied response to fescue toxin was reported in British and Brahman crossbred animals with the former being more susceptible (Morrison et al., 1988). Hence, selection of animals resistant to sporidesmin and fescue toxicosis may help to alleviate the problem in sheep and cattle, respectively.

Studies of gene expression in response to toxins in higher mammals, including humans, is seldom possible because of ethical concerns. Hence, extrapolation of toxicological studies in lab animals to other mammalian species has been the norm. Lab animal studies assume that different mammalian species exhibit similar responses when exposed to specific toxins. The assumption has been partly justified by recent knowledge from gene sequencing, which revealed extensive similarities in the genome structures among mammals. For instance, humans and mice have greater than 31,000 genes in common, which translates to approximately 80% similarity. Apart from these reasons, lab animals are generally used for toxicological studies because they are economical, easy to handle and have shorter generation intervals.

Genetic difference between lab mice that are resistant and susceptible to fescue toxicosis serves as a good model for studying the variation in response to toxins. Eight generations of bi-directional selection in mice, based on average daily gain in weight when fed an endophyte-infected diet, successfully produced resistant and susceptible lines (Hohenboken and Blodgett, 1997). When fed with the diet containing the toxin, the susceptible line had lower reproductive fitness than the resistant line. Activities of phase I (Arthur et al., 2003) and phase II (Gst and Ugt) liver enzymes were higher in the resistant compared to the susceptible line (Hohenboken and Blodgett, 1997; Wagner et al., 2000). Hence, laboratory mice can serve as a model for studying variation in hepatic gene expression and enzyme activity in response to toxins or xenobiotics.

Identifying the specific genes responsible for variation in toxin response in populations is possible through toxicogenomic studies. These studies enable us to understand the mechanism of resistance, and may help in designing selection strategies to increase resistance to toxicants in populations.

Techniques used for studying gene expression

Advanced techniques in molecular biology enable study of the response of organisms challenged with a toxicant. Using these tools, an animal's overall genomic response (microarrays), or the expression of select genes (quantitative real time (**RT**) – PCR), to a toxicant challenge may be explored. Such information can be complemented by enzyme activity assays that aid in identifying the actual product level changes, i.e., the change in enzyme activity associated with a change in gene expression.

Microarray. Studying the response of the entire genome to a particular challenge may help in identifying novel genes regulated by the compound. Such would not have been possible without the development of microarray technology. Microarray technology allows "spotting" of several thousand gene fragments onto a single membrane or chip, and thereby enables study of a large portion of a genome at once. In toxicogenomic experiments, chips are hybridized with the cDNA of animals that have or have not (the control) been exposed to a toxicant or family of toxicants. Data mining is then used to distinguish genes that are differently expressed in treated vs. control animals (Plant, 2003).

Real time PCR. Once identifying genes with altered expression in response to a toxicant challenge using microarrays, experiments can then be directed towards quantification of these differently expressed genes. Assessing individual gene expression is necessary for validating the results of microarrays. Quantification of gene expression of individual genes is possible through RT-PCR.

Reverse transcription followed by RT-PCR is the most sensitive method to measure transcript abundance. Real time PCR depends on the relationship between amount of template added and number of cycles of amplification needed to produce a detectable product. An easy way to detect the product formed without interrupting the amplification process is to use a dye, SYBR green, which fluoresces when it binds to double-stranded DNA. Florescence of the dye increases with an increase in product accumulation. However, a florescence signal can be produced even when an incorrect

product is made. Hence, the specificity of the product formed should be confirmed. Greater specificity can be achieved by using probes that bind only to specific products (Taqman) or by a disassociation analysis, which shows a single peak for a single product formation. Taqman and SYBR green differ in their ability to detect low copy numbers and in their specificity. SYBR is less specific and only able to detect higher copy numbers (greater than 10 copies) whereas Taqman can detect low copy numbers (less than 10 copies; Applied Biosystems, 2004).

The amplification cycle in RT-PCR has four stages. Initially, in stage one, amplification occurs at below detection levels and the product formed cannot be detected. In the second exponential stage the concentration of the template and the product formed are still low, yet are increasing at an exponential rate. During this stage, the reagents are in excess and there is no competition between the renaturation of product and the primer binding. In the linear phase of amplification – the third stage – the renaturation of product competes with primer binding; hence product formation is extremely variable during this stage, even among replicate samples. Amplification rate ceases in the plateau stage and drops to zero, with little product formation. Real time quantification analysis is based on identifying the cycle number where amplification crosses the threshold, called the threshold cycle number (**C_T**). The C_T value coincides with a midway point in the exponential phase of the amplification reaction (Applied Biosystems, 2004).

Housekeeping genes, such as beta-actin and *Gapdh*, have high basal expression in cells and are assumed to be constitutively and uniformly expressed with no effect of treatment. Therefore, they are used as internal standards in RT-PCR reactions. Errors may occur at several steps in the RT-PCR procedure, such as during purification of RNA, and reverse transcription of the extracted RNA to cDNA. Expression of these housekeeping genes is thus used to standardize or normalize expression of target genes across samples.

Quantitative RT-PCR is used for absolute and relative quantification. Absolute quantification of genes gives the absolute amount of mRNA in an experimental sample. With relative quantification, the expression of a target gene is relative to that of the housekeeping gene. Analysis of RT-PCR results is done in three ways: (i) absolute standard curve; (ii) relative standard curve; or, (iii) comparative C_T method. An external

standard or control, which is identical to the target mRNA except for a small deletion or addition to the sequence, is used in the absolute standard curve method. Since the absolute amount of external control is known, the amount of external control that gives the same amount of amplification product as the unknown sample is taken as the amount of mRNA in the sample. An alternative to absolute quantification of gene expression is its relative quantification. The relative standard curve method requires a serial dilution of template cDNA standard, which is included on each plate. Concentration values of the standard cDNA template are used for calculating relative mRNA levels in unknown samples. The comparative C_T method assesses relative changes in mRNA levels between two samples. Although comparative C_T method does not need a template dilution of the standard, it requires amplification efficiencies of the standard and the target genes to be the same (Livak and Schmittgen, 2001).

Enzyme activity assays

Enzyme activity assays are used to determine proteins or enzyme levels in tissue samples as a result of xenobiotic exposure. A change in gene expression may not always bring about a change in protein formation as many cross-reactions in a biochemical process are responsible for enzyme production. Assessing enzyme activity involves separating cell contents into cytosolic and microsomal fractions. The cytosol contains enzymes such as Gst or Nq, while microsomes contains enzymes such as Ugt. Based on absorbance of a substrate or metabolite measured in a spectrophotometer, enzyme activity present in the sample is determined. A protein dye-binding method determines the total protein present in cytosolic and microsomal components with bovine serum albumin as the standard (Bradford, 1976). Activity of enzyme per milligram of total protein is commonly expressed in $\mu mol\ min^{-1}\ mg^{-1}$.

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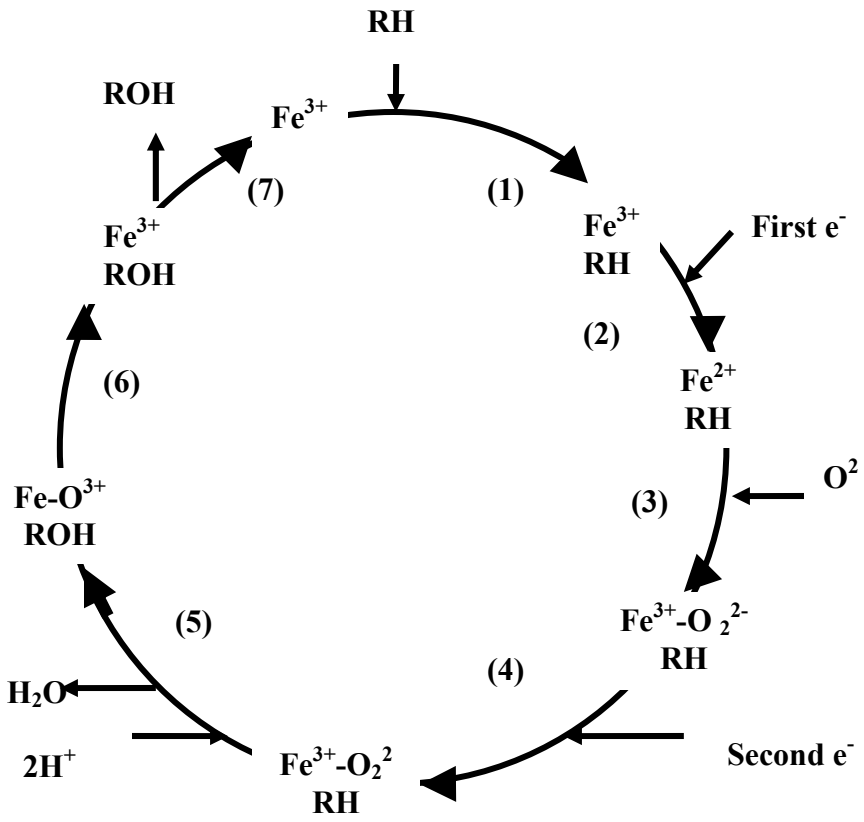

Figure 1. 1 Catalytic cycle of cytochrome P450

The intermediate states that are hypothetical are enclosed in a box. In step 1, substrate binds to the ferric heme. In step2, reduction of substrate bound ferric heme to ferrous heme by NADPH. In step 3, incorporation of molecular oxygen into the binary ferrous-Cyp-substrate complex. In step 4, reduction by addition of an electron to the complex. In step 5, addition of an oxygen atom in to the substrate. In step 6, the product is formed. In step 7, from the active site of the enzyme the product is released which returns to its initial state.

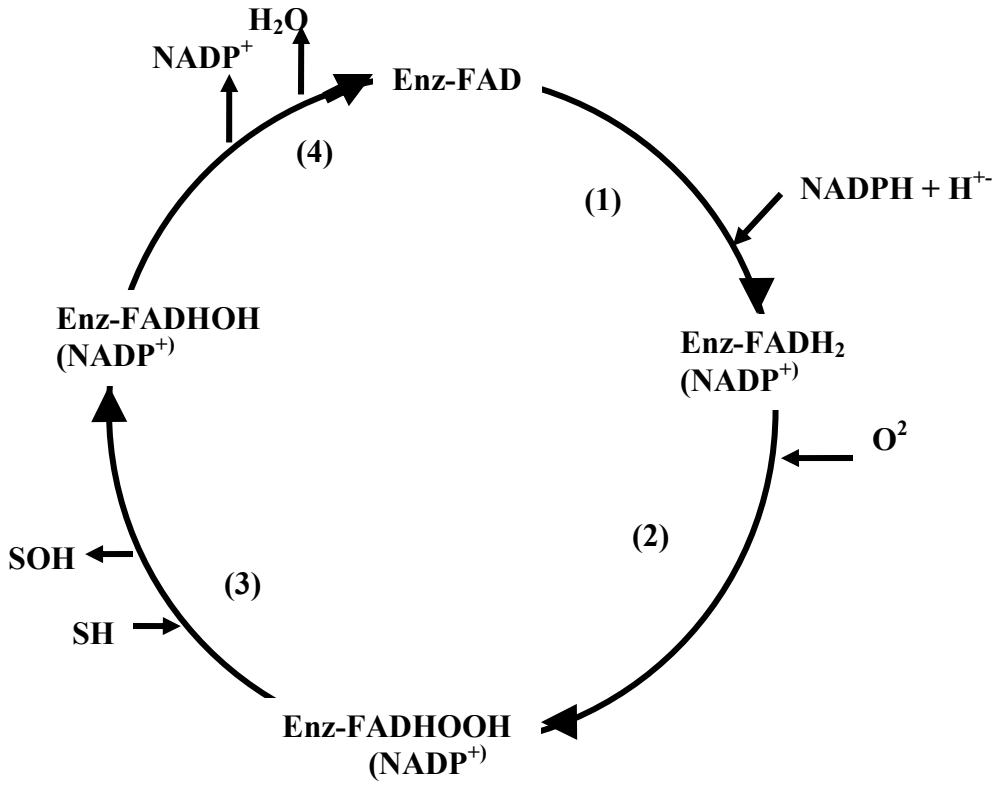

Figure 1. 2 Catalytic cycle of flavin monooxygenease.

Substrate independent NADPH oxidase indicated by dashed lines. In step 1, flavoprotein is reduced using NADPH. In step 2, addition of oxygen produces hydroperoxyflavin intermediate. In step 3, hydroperoxyflavin intermediate is open to nucleophilic attack by substrates at the terminal oxygen atom and oxygenated metabolite can be released. In step 4, water is released and fully oxidized flavoproteins can be reformed.

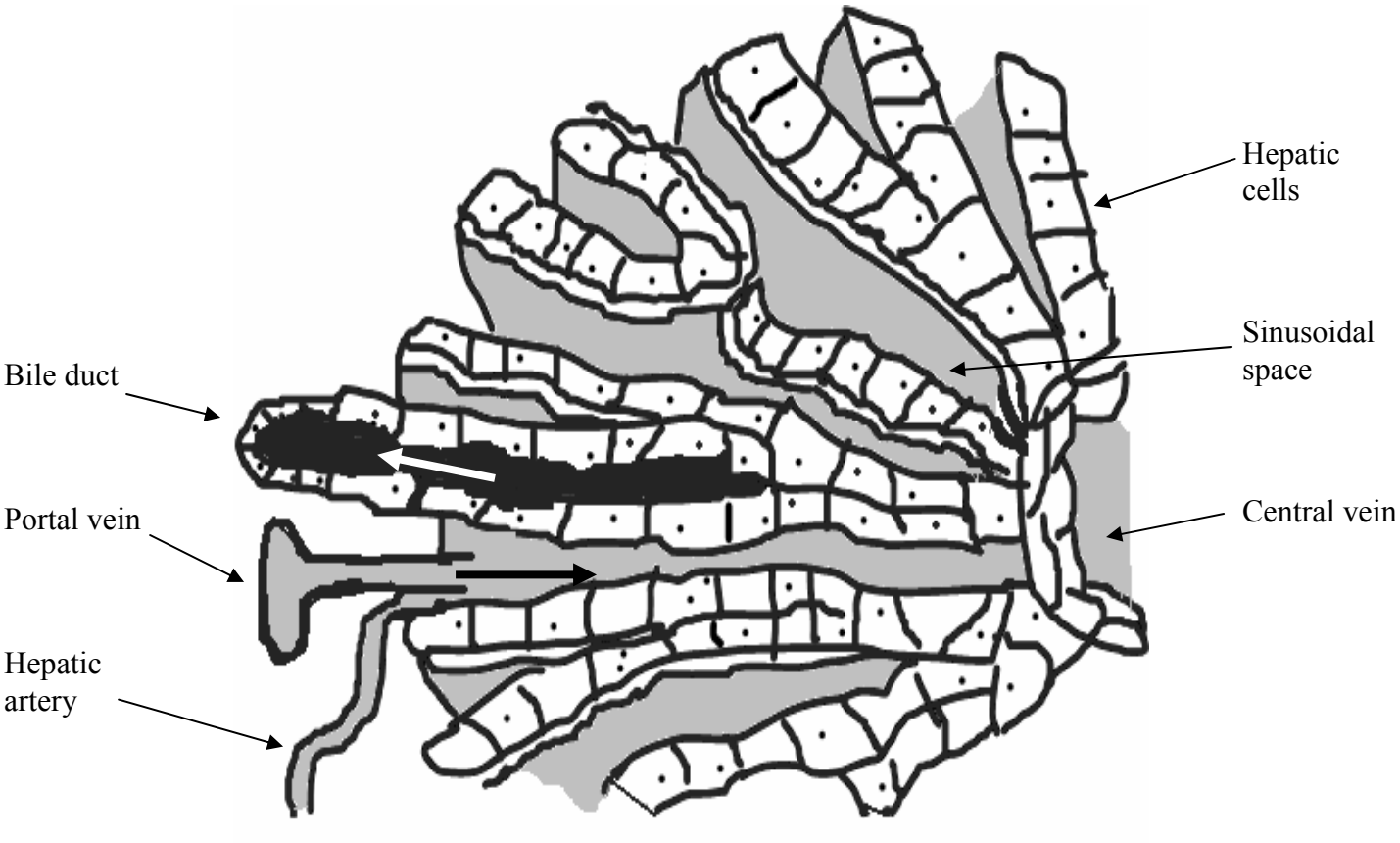

Figure 1. 3 Cross section of liver.

Blood flows from the portal vein and hepatic artery in the sinusoidal space between the hepatic cells, and drains into the central vein (black arrow). Bile flows in the direction opposite to the blood (white arrow) and collects in the bile duct. Bile duct, portal vein and hepatic artery form the portal triad and the region adjacent to these vessels is called periportal region. The region nearest the central vein is the perivenous zone.

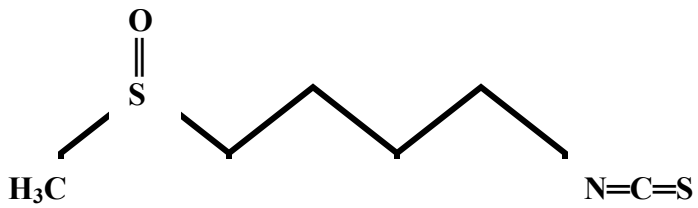

Figure 1. 4 Molecular structure of sulforaphane.

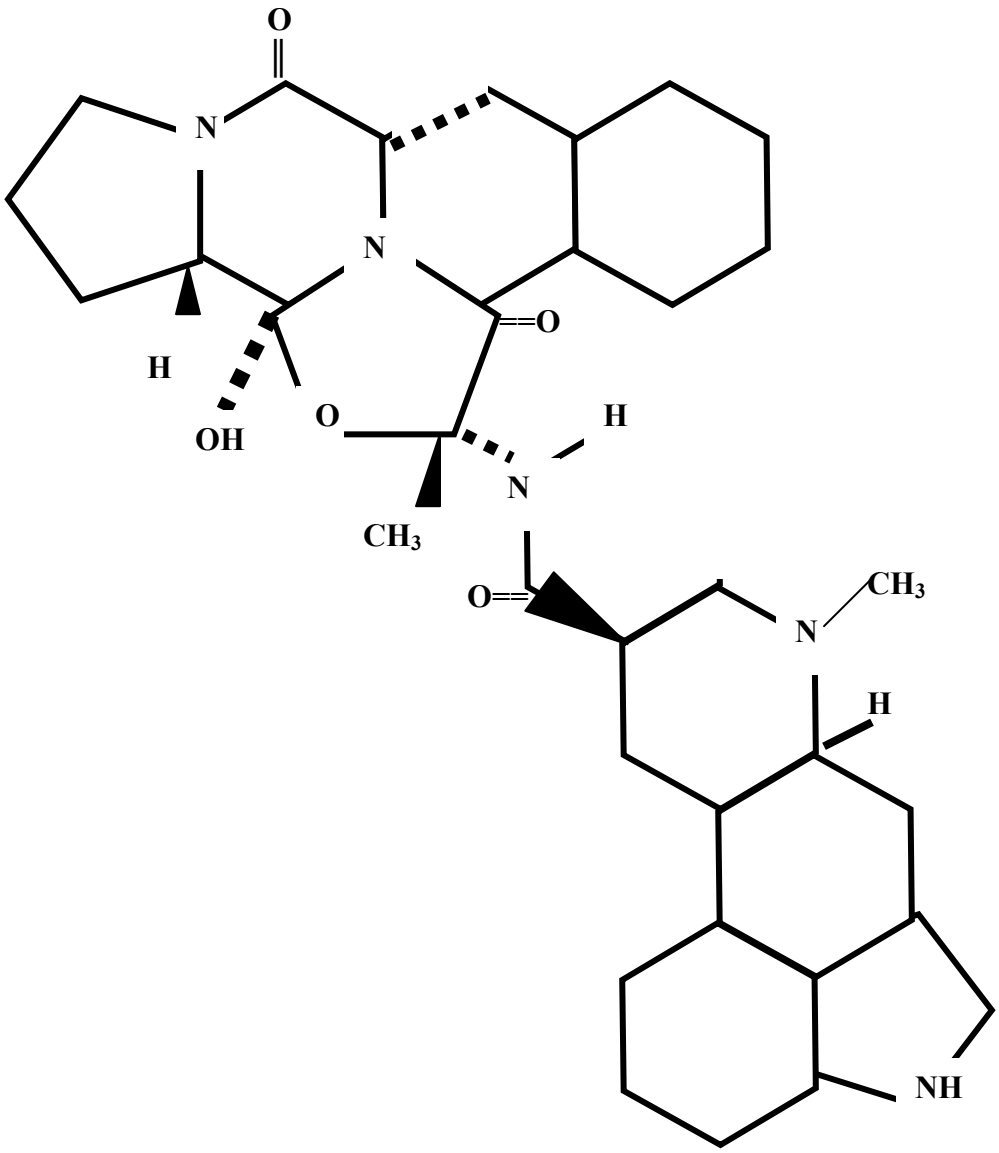

Figure 1. 5 Molecular structure of ergotamine.

CHAPTER 2
Genes of interest

INTRODUCTION

Several phase I hepatic enzymes may activate xenobiotic compounds into reactive metabolites. Metabolites are deactivated through conjugation reactions that involve phase II hepatic enzymes and result in increased polarity. These polar compounds are easily eliminated from the body in urine or feces. The effect of xenobiotics on activity of phase I and II liver enzymes have been studied extensively. However, research identifying specific genes responsible for biotransformation is limited.

In this study, we tried to identify specific genes responsible for variation in enzyme activity in animals challenged with xenobiotics. A preliminary study was conducted in which two xenobiotics, sulforaphane (**SFN**) and ergotamine (**EGT**) were orally administered (by gavage) to four female mice for 4 d. The mice were from a commercial polymorphic strain (ICR, Sprague Dawley). Mice were killed and liver samples were collected 24 h after last dosage. These liver samples were used for three types of laboratory analysis, two of which analyzed specific genes involved in biotransformation.

Two techniques for measuring gene expression, microarray and real time (**RT**)-PCR analysis, were used to identify specific genes involved in biotransformation of EGT and SFN. This chapter describes microarray analysis, and pre-requisite laboratory work involved in the RT-PCR analysis. The objectives of this chapter are: (i) to describe steps taken to identify genes associated with metabolism of these two compounds; and, (ii) to describe the approach used for quantifying expression of those genes.

IDENTIFYING GENES OF INTEREST

Introduction

To identify candidate genes associated with biotransformation, a microarray experiment was performed. Only 8 out of 40,000 genes on the chip were differentially expressed. None of these 8 genes appeared to be clearly associated with metabolism.

Therefore, based on literature and our knowledge of enzyme families known to be important in liver function, we identified 24 candidate genes of potential interest. In our list of chosen genes, we also included 3 genes differentially expressed in our microarray study to validate through RT-PCR analysis.

Microarray Analysis

Introduction. A DNA microarray or chip is formed by a collection of DNA spots on a small glass, plastic or silicon chip. This allows monitoring the expression profiles of thousands of genes simultaneously, and is particularly useful for efficiently comparing gene expression among treatments.

Materials and methods. Mice were dosed with one of the two treatments or a control (12 mice in total). Treatments were: (i) SFN (LKT Laboratories) at 2.5 $mg \cdot mouse^{-1} \cdot d^{-1}$; and, (ii) EGT (Sigma-Aldrich, Inc.) at 0.06 $mg \cdot mouse^{-1} \cdot d^{-1}$. Each treatment was diluted to deliver the desired mg amount in a volume of 0.25 ml. The SFN and EGT were diluted with a 50:50 mixture of dimethyl sulfoxide (**DMSO**) and distilled water. The control vehicle was a 50:50 mixture of DMSO and distilled water.

Microarray analyses were conducted using the Affymetrix GeneChip Mouse Genome 430 2.0 Array. Its 45,000 probe sets analyze the expression of over 39,000 transcripts and variants from over 34,000 well-characterized mouse genes. Total RNA was extracted from mice liver samples using a Qiagen RNeasy kit (Qiagen Inc., Valencia, CA). Quality of RNA was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies Gmbh., Berlin, Germany). Extracted RNA was analyzed separately for each sample (12 chips). Chips were analyzed in the Virginia Bioinformatics Institute (**VBI**) (Blacksburg, VA) core lab using the GeneChip Scanner 3000 (Santa Clara, CA) operated with GeneChip Operating Software.

Microarray data were analyzed by Z. Fei (VBI computational biology unit, Blacksburg, Virginia, personal communication). The analytical approach adopted entailed three steps: (i) checking the quality of array hybridizations (Eisen et al., 1998; Yeung and Ruzzo, 2001); (ii) normalizing, transforming and filtering data (Irizarry et al.,

2003; Wu et al., 2003); and, (iii) identifying differentially expressed genes due to treatments using SAM (Tusher et al., 2001) and LIMMA (Smyth, 2004) software. Gene expression was summarized by comparing mice treated with SFN and EGT as a ratio of the control treatment. A cutoff value of at least a two-fold change with an adjusted *P*-value < 0.05 was used to delineate differential expression.

Results. Of approximately 40,000 genes present on the chip, only 8 genes were differentially expressed by at least two-fold compared to the control ($P < 0.05$), with change consistent across all samples for only 4 genes (Table 2.1). None of the genes deemed to be associated with phase I or II biotransformation showed a consistent change in expression based on microarray analysis.

Three of the genes identified as differentially expressed for EGT or SFN treatment (on average, greater than 2-fold) were chosen for validation using RT-PCR (Table 2.1). However, as described subsequently, primer pairs chosen for amplification of one of these genes, hydroxysteroid dehydrogenase like 2 (***Hsdl2***), proved inadequate and thus the gene was excluded from the study. The abhydrolase domain containing 6 gene (***Abhd6***), showed no significant differential expression for EGT in RT-PCR analyses of the same samples (average of 0.62 ± 0.2; $P = 0.055$). The RT- PCR analyses indicated an increased expression (average of 0.82 ± 0.27; $P = 0.05$) of the glycerol phosphate dehydrogenase 2 (***Gpd2***) for the SFN treatment, however, the increased expression associated with EGT was not validated by RT-PCR (average of 0.66 ± 0.22; P = 0.06).

Discussion. Microarray analysis surprisingly did not identify differential expression in any genes considered to be associated with xenobiotic metabolism in the liver. Although the intent of this approach was to identify candidate genes for further study, our results proved uninformative. Instead, candidate genes were chosen either based on published literature or because they were considered pivotal in metabolism. The approach for testing these genes is described in the subsequent section.

Identifying candidate genes

Ergotamine is responsible for increasing the activity of the cytochrome P450 family, which are phase I enzymes (Arthur et al., 2003). Mice fed ground fescue seeds had increased activity of UDP glucuronosyl transferases and glutathione–S–transferases, which are phase II enzymes (Hohenboken and Blodgett, 1997).

Sulforaphane is a mono-functional inducer and thus anticipated to affect genes associated with phase II enzyme activity. Thimmulappa et al. (2002) found that the phase II enzyme families involved in metabolism of SFN are the glutathione–S–transferases, quinone reductases and epoxide hydrolases. These phase II enzymes are hypothesized to be induced by transcription factors like nuclear factor E2 p 45-related factors (*Nrf1* and *Nrf2*).

We were interested in gene expression profiles of the above enzyme families and hence chose 15 genes representative of each family. In addition, we selected 9 genes belonging to other important phase I (flavin monooxygenases) and II families (like sulfotransferases, methyl transferases, N-acetyl transferases, malic enzyme and multiple drug resistance protein). The three differentially expressed microarray genes and two housekeeping genes were included in the set. The genes considered in the study are listed in Table 2.2.

PRIMER SELECTION AND VALIDATION

Introduction

A primer is a nucleic acid strand that serves as a starting point for DNA replication. Primers are required because enzymes that catalyze DNA replication, DNA polymerases, cannot synthesize new DNA from scratch, but can only add nucleotides to an existing strand. Molecular biology techniques, like RT-PCR, require chemically synthesized short primers, which are about 20-25 base pairs in length.

In RT-PCR, primers are designed such that they correspond to the DNA sequence specific to the region of gene of interest. Primer pairs for the 29 genes were designed

using the Primer Express Software version 2 and are shown in Table 2.3. Primers had an optimum melting temperature and length of 60ºC and 20-30 nucleotide base pairs, respectively. However, some primers were ultimately excluded because no amplification product was formed with RT-PCR, or because they failed a validation test. The objective of this section is to describe and justify reasons for excluding specific primers.

Agarose Gel Electrophoresis

Introduction. Linear DNA fragments migrate in agarose gel with a speed inversely proportional to their molecular weight or number of base pairs. By using gels with different concentrations of agarose, different sizes of DNA fragments can be resolved. Generally, high (1-1.5%) agarose concentrations facilitate separation of small DNA fragments, whereas low concentrations (0.5-0.7%) allow resolution of larger fragments. In order to confirm the length of the DNA product formed after an RT-PCR run, we performed agarose gel electrophoresis of the amplified product.

Materials and methods. Total RNA from the liver of an 8-week old female mouse of ICR strain was used to validate the length of the amplification products for each set of primers for each gene. As the first step, cDNA was obtained by reverse transcribing the total RNA using the Applied Biosystems High Capacity cDNA archive kit (Foster City, CA). Pairs of primers for genes of interest were then used to amplify the cDNA template. The forward and reverse primers for each gene, at a concentration of 5 µM, were added to the SYBR green reagent, a fluorescent marker that binds with double stranded DNA. Diethyl pyrocarbonate (**DEPC**) treated water, was used to obtain a uniform master mix of sufficient volume, 23 µl per well for each gene. Using a single cDNA sample (2 µl of 3.33 ng/µl concentrations) and adding master mix specific for a gene to each well, RT-PCR was performed. After the run, an additional dissociation step was completed to verify that the amplification product formed was a single product. The product was loaded on to a 1% DNA agarose gel and electrophoresis was performed to confirm the RT-PCR product size.

The 1% DNA agarose gel was prepared by adding 1.2 g of agar to 120 ml of Tris-borate- EDTA buffer (4.84 g/ml), while simultaneously heating and stirring. Ethidium bromide (10 µg/µl), a staining dye used for nucleic acids, was added to the gel to enable its visualization under UV light. The amplification product formed in the RT-PCR well was mixed with 10 X loading buffer and then loaded in to the wells of the gel. Loading buffer contained 40% glycerol, which is dense and hence allowed the amplified DNA sample derived from the RT-PCR to settle into the wells of the gel. Loading buffer also contained two tracking dyes, 0.5 % bromo phenol blue and 0.5 % xylenol, which migrated in the gel and allow visual monitoring of the progress of electrophoretic process. As a standard indicator of the length of the amplification product formed, a DNA ladder of 50-5,000 base pair fragments was included. The gel was run for about 14 h at 20 V. After 14 h when adequate migration of DNA fragments had occurred, the gel was stained in ethidium bromide for about 20 min. Bands were visualized under UV light and a gel picture was taken.

Results. For most genes, the gel picture (Figure 2.1) had a single band formed between 100 and 200 base pairs, which validated our assumption of formation of a single amplification product. However, some genes had no amplified products, as indicated by the absence of a band (Figure 2.1). These genes were cytochrome P450 1a1 (***Cyp1a1***), *Cyp3a16*, multiple drug resistance protein (***Mrp1***), N-acetyl transferases 1 (***Nat1***), tryptase methyl transferases 1 (***Tmt1***) and malic enzyme 1 (***Mod1***). All these genes, with the exception of *Cyp3a16*, did not show any peak in the dissociation analysis. Also, *Cyp3a16* had a clear primer dimer formation in the dissociation analysis.

Discussion. Expression of *Cyp1a1*, a phase I gene, is very low in liver tissue under normal physiological conditions. Low levels of gene transcript, including *Cyp1a1*, are not detected by RT-PCR analysis (Zacharova et al., 2003). The apparent absence of a band is thus not surprising (Figure 2.1). However, expression of *Cyp1a1* can be induced by xenobiotics, like polycyclic aromatic hydrocarbons present in cigarette smoke. We therefore were interested if SFN or EGT might induce expression of *Cyp1a1*. For this reason, *Cyp1a1* was retained in the study.

In male mice, expression of the *Cyp3a16* gene decreases gradually after birth and is completely absent at 5 wk of age. Although, female mice continue to express the *Cyp3a16* gene, the expression is very low in liver after 5 wk of age (Sakuma et al., 2000). Mice used in the study were approximately 8 wk of age. Hence, expression of this gene was low; as a likely consequence, no amplification product could be detected by gel electrophoresis. In addition, a primer dimer was noticed in the dissociation step. Due to absence of a band on the gel, and mainly because of primer dimer formation, *Cyp3a16* was excluded from the study.

Multiple drug resistance protein is represented by three important genes that are expressed in liver: *Mrp1*, *Mrp2* and *Mrp3*. Although *Mrp2* and *Mrp3* show basal expression in liver, *Mrp1* alone appears to be inducible by xenobiotics (Ishikawa et al., 1996). When not induced, *Mrp1* is not detectable in mouse (Maher et al., 2005) and rat (Ogawa et al., 2000) Livers. Thus, unsurprisingly, it was not detected in the gel. However, as we were interested in any treatment effect on this protein, *Mrp1* was included as a gene in the study.

N-acetyl transferase 1 is found primarily in the liver. Boukovula et al. (2002) confirmed expression of *Nat1* in mouse liver using RT-PCR. However, no amplification product was found in our results for *Nat1*. Possible reasons are that the concentration of the primer pair chosen was not optimal for liver tissue or that the cDNA concentration was too low. An increase in input cDNA did not result in any expression of the gene (data not shown). Thus, apparently, the primer concentration was not optimized and hence *Nat1* was not included in the remainder of study.

Tryptase methyltransferase is abundant in secretory granules of mast cells and transfers metabolizing enzymes to the site of xenobiotics. Expression of *Tmt1* varies between different tissues in a mouse and between different strains. Intestinal tissues from two inbred strains of mice, BALB/c and C57BL/6, expressed higher levels of *Tmt1* compared to other tissues like liver, kidney, heart and brain. Transcript levels of mouse *Tmt1* were below detection in RNA blot analysis of liver samples from two inbred strains of mice (Wong et al., 1999). However, no data was available on the expression of *Tmt1* gene in polymorphic mice (ICR strain). In this study, only low transcript levels of *Tmt1*

in liver samples of ICR strain mice were observed. Hence, this gene was not considered further.

Malic enzyme is a NADPH regenerating enzyme induced by phase II transcription factor Nrf2, which is expressed in the liver (Thimulappa et al., 2002). Although literature on the enzyme is plentiful, information regarding the *Mod1* gene is scarce. Because no cDNA product was formed in the gel, different concentrations of cDNA (a range from 3.3 to 10 ng/µl) were used to test the optimal concentration required for *Mod1* expression. Even at higher concentrations of cDNA (10 ng/µl as opposed to the usual concentration of 3.3 ng/µl), there was no detectable expression of the *Mod1* gene. Hence, this gene was also disregarded from the study.

The gel picture showed that there was no amplification product formed when *Cyp1a1*, *Cyp3a16*, *Mrp1*, *Nat1*, *Tmt1* and *Mod1* genes were amplified. All the genes that showed no amplification product in the gel were targeted for exclusion from the study. However, our original interest in *Cyp1a1* and *Mrp1* was because these genes are known to be induced by certain compounds. Thus, these two genes were retained despite no detectable amplification in this preliminary work.

Amplification efficiency

Introduction. The rate at which an amplicon, a PCR product derived from a DNA or cDNA template, is generated is called the PCR amplification efficiency. Amplification efficiency is generally expressed in percentage. The comparative C_T ($\mathbf{\Delta\Delta C_T}$) method (Livak, 1997) of RT-PCR data analysis assumes: (i) the efficiency of amplification is constant and close to 100% in the exponential phase of the PCR, a phase in which PCR products accumulate at a steady rate; and, (ii) the amplification efficiencies of the target and the reference (housekeeping) genes are approximately equal (Livak, 1997). Usually, a validation test is done to test these assumptions.

If the amplification efficiency is 100%, then the fold change in gene expression can be expressed by relative quantification (RQ) as the value $2^{(-\Delta\Delta C_T)}$. Note that the 2 in the formula indicates that the RT-PCR product doubles in quantity after each cycle. Also, if the amplification efficiencies of target and reference gene are approximately equal,

then the calculation for RQ of the target gene can be based on the $\Delta\Delta C_T$ method without a standard curve on each plate (Livak and Schmittgen, 2001).

If primer pairs for a particular gene of interest do not meet the assumptions (i.e., fail the validation test), then the RQ method is not appropriate for analysis. As an alternative, absolute quantification could be used where a standard curve is fitted for each gene on the RT-PCR plate. Additionally, a different set of primers could be designed and the validation test repeated; this testing process would continue until suitable forward and reverse primers are identified.

Materials and methods. Four liver samples from mice belonging to either treatment or control were used for the validation test. Extraction of RNA from the liver samples was done using the RNeasy mini kit (Qiagen Inc., Valencia, CA). Concentrations of RNA were measured on a nanodrop (Wilmington, DE). Reverse transcription of RNA (initial concentration of 10 ng/µl) was done to produce cDNA using the High Capacity cDNA archive kit. (Foster City, CA). Serial dilutions of cDNA (of approximately 2-log range) were made by addition of DEPC–treated water. Seven 2-fold serial dilutions of the stock cDNA were made, resulting in concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 ng/µl. These cDNA concentrations were assumed to reflect the input RNA concentrations (Applied Biosystems, 2004).

For a target gene, each of the seven dilutions of cDNA (in triplicate) was run along with the reference gene, *Gapdh*, on the same plate and C_T values were obtained. In samples where the SE of triplicates was greater than 0.15 and one of the triplicates was clearly an outlier, it was removed. If the SE remained above 0.15 after exclusion of the outlier, or if no replicate was clearly extreme, then the gene along with the reference gene were reanalyzed.

The C_T values for each gene and dilution were averaged. The regression of average C_T value on the log input nucleic acid was fitted using PROC REG (SAS Institute Inc., Cary, NC, USA). Amplification efficiency (**E**), was calculated using the equation $E = [10^{(-1/\beta)} - 1] * 100$ (Applied Biosystems, 2004), where β is the slope of the regression line.

The differences between the average C_T values of target and reference gene were also calculated (ΔC_T). The regression of ΔC_T on the log input nucleic acid was fitted and the null hypothesis $\beta = 0$ tested. Failure to reject the null hypothesis implies the efficiency of target and reference genes are the same. It was suggested that if β is less than ± 0.1 (Livak, 1997), or if the ratio of amplification efficiency between target and reference gene was above 95% (Applied Biosystems, 2007), then their efficiency values were approximately the same. These benchmarks were used as additional criteria for assessing efficiencies.

Results. Slopes of the standard curves for individual efficiencies of genes ranged from -3.3 to -4.3. A slope of -3.33 coincides with 100% efficiency. For most of the genes, efficiency was less than 100%. Model R^2 statistics were high (greater than 98%) indicating good fit of efficiency curve (Table 2.4). The slope and SE from the regression of ΔC_T on log input nucleic acid, and the significance level of t-test for null hypothesis of $\beta = 0$, are shown in Table 2.5. Ratio of efficiency of each target gene to the reference gene (*Gapdh*) is also provided.

Discussion. Out of 22 primer pairs tested, slope of the regression of ΔC_T on log input nucleic acid did not differ from zero ($P < 0.05$) and/or was less than ± 0.1 for 10 primers (delineated with letter index A in Table 2.5). Amplification efficiency for these primers was also similar to their respective reference gene (within 5%).

Applied Biosystems (2007) suggested using dilutions covering a scale of 5 logs for an amplification study. When a narrower range of cDNA dilutions (2-3 logs) was used, greater variability in efficiencies was observed (82-112%). Due to limited amount of cDNA, we used dilutions covering approximately 2 logs. This may have contributed to the broad range of efficiencies observed (Table 2.5). For example, the range in efficiency values of *Gapdh* across plates was 74.9-88.5%.

Ratios of efficiency values of target to the reference gene were calculated (Table 2.5) and were within an acceptable range of 82 -112%. Using this criterion, an additional 8 primer pairs (designated with letter index B in Table 2.5) were retained in the study.

The amplification efficiencies of four primer pairs (indicated with letter index C in Table 2.5) clearly differed from their respective reference gene. These genes were *Ugt2a1*, *Nrf1*, *Nq01* and *Hsdl2,* and were excluded from further consideration.

CONCLUSIONS

Those genes that did not show an amplification product on the agarose gel (*Cyp3a16*, *Mrp1*, *Tmt1*, *Nat1* and *Mod1*), genes identified as differentially expressed exclusively in the microarray analyses (*Gpd2*, *Abhd6* and *Hsdl2*), and genes for which primer pairs failed the validation test (*Ugt2a1*, *Nrf1*, *Nq01*and *Hsdl2*) were excluded from the study and thus are not discussed hereafter. Although *Cyp1a1* and *Mrp1* did not show any amplification product in the gel, we were interested to see if they would be induced by the SFN and EGT treatments; hence, they were retained.

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Table 2. 1 Microarray data showing genes that were differentially regulated for the preliminary study [1]

Probe Set ID	Gene Bank Number	Gene description	EGT vs. C	SFN vs. C
1426856_at	BM200015	hydroxysteroid dehydrogenase like 2[2]	**2.26**[3]	2.12
1417434_at	NM_010274	glycerol phosphate dehydrogenase 2[2]	**3.55**[3]	2.89
1419103_a_at	NM_025341	abhydrolase domain containing 6[2]	**2.92**	2.46
1448944_at	AK011144	neuropilin 1	**2.27**	1.13
1429771_at	AK014252	RIKEN cDNA 3110073H01 gene	**1.53**	0.83
1453345_at	AK014427	RIKEN cDNA 3830408G10 gene	**2.27**	-0.60
1447502_at	AW112184	No description provided	-0.41	**-2.49**[3]
1448724_at	NM_009895	cytokine inducible SH2-containing protein	-1.81	**-8.80**

[1] Designations are C = Control, EGT = Ergotamine and SFN = Sulforaphane.

[2] Genes used for RT-PCR validation.

[3] Consistent greater than two-fold change in expression across all samples.

Table 2. 2 General description of mouse hepatic genes and their accession numbers

Gene symbol	General description	Accession number[1]
Phase I genes		
Cyp1a1	Cytochrome P450 1a1	NM_009992
Cyp1a2	Cytochrome P450 1a2	NM_009993
Cyp3a16	Cytochrome P450 3a16	NM_007820
Cyp3a41	Cytochrome P450 3a41	NM_017396
Cyp3a44	Cytochrome P450 3a44	NM_177380
Fmo1	Flavin monooxygenase 1	NM_010231
Phase II genes		
Gsta2	Glutathione-S-transferase a2	NM_008182
Gsta3	Glutathione-S-transferase a3	NM_010356
Gstm1	Glutathione-S-transferase mu1	NM_010358
Ugt1a1	UDP glucoronosyl transferase 1a1	NM_201645
Ugt2a1	UDP glucoronosyl transferase 2a1	NM_053184
Sult2a2	Sulfotransferase 2a2	NM_017465
Sult5a1	Sulfotransferase 5a1	NM_020564
Ephx1	Epoxide hydrolase 1	NM_010145
Nq01	Quinone reductase 01	NM_008706
Nq02	Quinone reductase 02	NM_020282
Mrp1	Multiple drug resistance protein 1	NM_008576
Comt1	Catechol amine methyl transferases 1	NM_007744
Tmt1	Tryptase methyl transferases 1	NM_012034
Nat1	N-acetyl transferases 1	NM_008317
Nat2	N-acetyl transferases 2	NM_010874
Mod1	Malic enzyme	NM_008615
Nrf1	Nuclear factor E2 p45-related factor 1	NM_010938
Nrf2	Nuclear factor E2 p45-related factor 2	U20532
Microarray genes		
Hsdl2	hydroxysteroid dehydrogenase like 2	BM200015
Gpd2	glycerol phosphate dehydrogenase 2	NM_010274
Abhd6	abhydrolase domain containing 6	NM_025341
Endogenous genes		
Gapdh[2]	Glyceraldehyde -3- phosphate dehydrogenase	BC083149
Actb6	Beta-actin	NM_007393

[1] Gene bank accession number used by National Center for Biotechnology Information to track the gene sequences.

[2] Gene used as the endogenous gene in the study.

Table 2. 3 Primer pairs for genes of interest

Gene symbol[1]	Primer pairs[2]		AL[3]
	Forward	Reverse	
Phase I genes			
Cyp1a1	AGCCTCATTGAGCATTGTCAGG	CCAGCTCCAAAGAGGTCCAAA	103
Cyp1a2	TTGGTGCCATGTGCTTTGG	TCCCTGAGGTGACATTCTCCAC	101
Cyp3a16	TCTGAAATCGAGATCACAGCCC	TGAGTGGCCAAGGAATACACG	100
Cyp3a41	TGCCATTTTTAGGCACTGTGC	GCATTTGACCATCAAACAACCC	107
Cyp3a44	TTGGTCCTGCTGGCAATCA	GCACAGTGCCTAAAAATGGCA	117
Fmo1	CCCTTCCTCGATGAATCCGTA	AGGCCAATCACAGCCAGAGTT	106
Phase II genes			
Gsta2	CCCAGACCAAAGAGAAGCCAA	GCCCACAAGGTAGTCTTGTCCA	101
Gsta3	AAGCCTTGCCAAGATCAAGGA	GCCTGTTGCCAACGAGATAATC	100
Gstm1	CACACAAGATCACCCAGAGCAA	CACAATGTCTGCACGGATCCT	100
Ugt1a1	ATTGCCATGCAGCCTGGAT	TCGCTGTAGGAAGTTCATGCG	105
Ugt2a1	TTGGCTAATGCGAACCTATTGG	CCTTAGGTAAAGGCTTGGCAGG	106
Sult2a2	AGGCCAAGGCGATCTATCTCA	TCCGAGTGACCCTGGATTCTT	103
Sult5a1	AGCGCATGAACACCACTGAAA	TGTGTCCTGGAACTGGAAGGAG	103
Ephx1	CAAAGCCATCAGCCAAAGAAG	TGCCCGGAACCTATCTATCCT	103
Nq01	AGAGTGGCATCCTGCGTTTCT	TTCCATCCTTCCAGGATCTGC	108
Nq02	GCTCTCCTTTCCTTAACCACGG	AGTGTACCATGCTGAAGTGGCC	103
Mrp1	CGCATGAACTTGGACCCTTTC	TGGTTCAGCTTGTCAGGCAAA	109
Comt	CATGTGCAGCAACACGCAA	TTGCGTCACCCACGTTCAT	102
Tmt1	GCCAGGCTTACAATAGTCCCA	ACTAGTGGCCCTCCAGAGTCAT	102
Nat1	GGCTTTGAAACCACAATGTTGG	TCCTGTCACTGATGGTCACCTG	105
Nat2	CCAGGAGCAAACTGGACTTGAA	CATGGATTCCCCACAATGGA	100
Mod1	TCTTCCTCACCACTCGTGAGGT	CGAAACGCCTCGAATGGTATT	100
Nrf1	CAGCACCTTTGGAGAATGTGGT	AATTAACCTCCTGTGGCGCAG	101
Nrf2	TACAGCCTCTGTCACCAGCTCA	TTTGATGACCAGGACTCACGG	101
Microarray genes			
Hsdl2	CGCAAAGGATGGAGCCAATAT	CCCTCCAGCTGCTTCAATTTC	108
Gpd2	GGAGAAGATGACAATTGCTGGC	CCGCACTTCATTCAGGATGAA	102
Abhd6	CCTGGCATTTGTTGCGTCTT	ATGGTGTGCGTAGCGAACTTG	105
Endogenous genes			
Gapdh[4]	GCAAAGTGGAGATTGTTGCCAT	CCTTGACTGTGCCGTTGAATTT	107
Actb6	TCCTGAGCGCAAGTACTCTGTG	CGGACTCATCGTACTCCTGCTT	100

[1]See Table 2.2 for gene description.

[2]All primer pairs were chosen using the primer express software version 2.

[3] AL = amplicon length.

[4] Gene used as the endogenous gene in the study.

Table 2. 4 Results of validation test showing the intercept, slope (± SE) and R^2 value from the fit of the regression of C_T values on log input amount of nucleic acid, and the associated amplification efficiency value of each gene

Gene symbol[1]	Intercept	Slope ± SE	R-square value	Efficiency (%)[2]
Phase I genes				
Cyp1a2	23.3	-3.41 ± 0.03	0.99	96.3
Cyp3a41	21.4	-3.71 ± 0.08	0.99	86.2
Cyp3a44	26.8	-4.31 ± 0.10	0.99	70.7
Fmo1	25.1	-3.81 ± 0.05	0.99	82.9
Phase II genes				
Gsta2	32.1	-3.89 ± 0.08	0.99	80.8
Gsta3	24.7	-4.07 ± 0.05	0.99	76.2
Gstm1	22.9	-3.67 ± 0.03	0.99	87.3
Ugt1a1	24.2	-3.72 ± 0.05	0.99	85.6
Ugt1a2	31.3	-3.5 ± 0.02	0.98	93.1
Sult2a2	26.3	-3.79 ± 0.09	0.98	83.5
Sult5a1	32.8	-3.76 ± 0.03	0.98	84.5
Ephx1	27.2	-3.78 ± 0.07	0.99	83.8
Nq01	30.6	-3.35 ± 0.03	0.99	98.8
Nq02	26.4	-3.81 ± 0.04	0.99	82.9
Comt1	25.5	-4.03 ± 0.08	0.99	77.2
Nat2	32.6	-4.11 ± 0.14	0.98	75.1
Gpd2	29.7	-3.78 ± 0.12	0.98	84.0
Abhd6	30.4	-3.72 ± 0.06	0.99	85.8
Hsdl2	31.7	-4.26 ± 0.08	0.99	71.6
Nrf1	30.7	-3.50 ± 0.07	0.99	93.1
Nrf2	28.9	-3.93 ± 0.07	0.99	79.6
Actb6	22.8	-3.56 ± 0.02	0.99	96.3

[1] See Table 2.2 for gene description.

[2] Amplification efficiency = $[10 \wedge (-1/\beta)] - 1$, where β is the slope (Applied Biosystems, 2004).

Table 2. 5 Results of validation test showing the slope (± SE) of the regression of normalized C_T values (ΔC_T) on log input amount of nucleic acid, the ratio between target and reference gene efficiency values in percentage (D.E.), and categories of primer pairs grouped by letter indices

Gene symbol[1]	ΔC_T slope ± SE	D.E.	Letter index[2]		
Cyp1a2	0.22* ± 0.02	109		B	
Cyp3a41	0.16* ± 0.06	106		B	
Cyp3a44	-0.41* ± 0.06	88		B	
Fmo1	-0.19* ± 0.03	93		B	
Gsta2	-0.22* ± 0.07	93		B	
Gsta3	-0.43* ± 0.04	86		B	
Gstm1	-0.04 ± 0.01	99	A		
Ugt1a1	-0.05 ± 0.04	98	A		
Ugt2a1	-0.52* ± 0.12	114			C
Sult2a2	0.08 ± 0.08	103	A		
Sult5a1	0.04 ± 0.06	101	A		
Ephx1	-0.10 ± 0.06	97	A		
Nq01	0.28* ± 0.02	112			C
Nq02	0.08* ± 0.03	103	A		
Comt1	-0.46* ± 0.16	96		B	
Nat2	0.10 ± 0.11	97	A		
Gpd2	-0.03 ± 0.09	101	A		
Abhd6	0.01 ± 0.05	100	A		
Hsdl2	-0.52* ± 0.04	114			C
Nrf1	0.61* ± 0.03	124			C
Nrf2	0.19* ± 0.04	95		B	
Actb6	0.07* ± 0.02	103	A		

An asterisk (*) indicates slope differs from zero ($P > 0.05$).

[1] See Table 2.2 for gene description.

[2] A = target genes with absolute value of the slope of the regression of ΔC_T on log input nucleic acid not differing from zero ($P < 0.05$) and/or was less than ± 0.; B = target genes having slope greater than ± 0.1, but do not differ substantially in their amplification efficiency from the reference gene (D.E. within range 82-112%); C = target genes differing substantially in amplification efficiency from the reference gene (D.E. outside range 82-112%) and hence eliminated from the study.

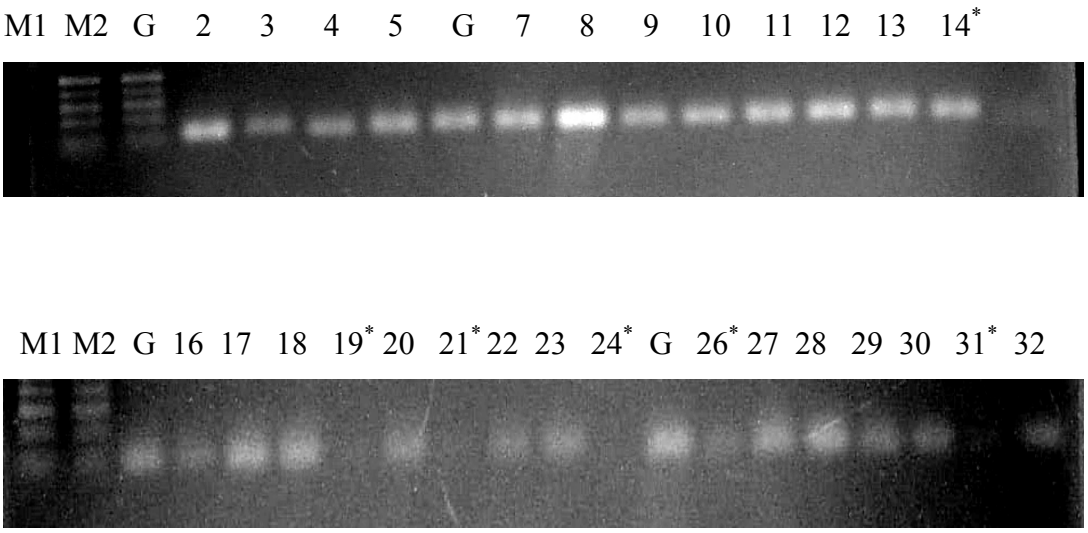

Figure 2. 1 Agarose gel electrophoresis showing the length of the amplification products being formed after real time PCR.

Designations are: M1, M2 = DNA size markers, G = house keeping gene, *Gapdh*; 2 = *Cyp3a44*, 3 = *Nat2*, 4 = *Actb6*, 5 = *Abhd6*, 7 = *Cyp3a41*, 8 = *Nrf2*, 9 = *Cyp1a2*, 10 = *Nrf1*, 11 = *Nq02*, 12 = *Comt1*, 13 = *Ephx1*, 14 = *Cyp3a16*, 16 = *Gsta2*, 17 = *Ugt1a1*, 18 = *Sult2a2*, 19 = *Nat1*, 20 = *Ugt2a1*, 21 = *Mrp1*, 22 = *Sult5a1*, 23 = *Hsdl2*, 24 = *Tmt1*, 26 = *Mod1*, 27 = *Nq01*, 28 = *Fmo1*, 29 = *Gsta3*, 30 = *Gpd2*, 31 = *Cyp1a1*, 32 = *Gstm1*. An asterisk (*) represent wells in the gel where product is not formed. All lanes in which products were formed showed a single band with a length of 100-200 base pairs.

CHAPTER 3

Assessing hepatic gene expression in response to xenobiotic exposure in mice.[1]

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ABSTRACT: Xenobiotics derived from plants are metabolized in the liver when ingested. Phase I liver enzymes change non-polar xenobiotics into reactive metabolites, often increasing toxicity. Phase II enzymes inactivate these metabolites with addition of water-soluble groups enabling excretion of metabolites in urine or bile. Xenobiotics considered in this study were ergotamine (**EGT**), which is associated with fescue toxicosis, and sulforaphane (**SFN**), which is considered a phase II enzyme inducer. The effect of SFN on expression of phase I genes is unclear. However, phase I enzymes are known to metabolize EGT. Our objective was to test whether predicted responses in liver enzyme activity and gene expression occurred when exposed to these xenobiotics. In a preliminary study, polymorphic mice were orally dosed by gavage for 4 d with SFN (2.5 $mg \cdot mouse^{-1} \cdot d^{-1}$), EGT (0.06 $mg \cdot mouse^{-1} \cdot d^{-1}$) or a control vehicle, with 4 mice per treatment. Control mice were dosed with a 50:50 mixture of dimethyl sulfoxide and water, the solution used to dissolve SFN and EGT. Results of the preliminary study were equivocal. Hence, a longitudinal study was conducted with four dosing periods (2, 5, 8 or 11 d), with at least 5 mice assigned to each dosing period and treatment. Mice were killed 24 h after last dosing and their livers collected for analysis of gene expression using real time-PCR and enzyme activity. Ergotamine treatment marginally increased expression of

[1] We thank D. Gemmell, his outstanding staff at the Lab Animal Research facility, and J. Boothe for their technical support, and E. Wong and K. Webb for use of lab space.

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the phase II genes catechol–O–amine methyltransferase 1 ($P = 0.009$) on d 8, and glutathione–S–transferase mu1 (***Gstm1***; $P = 0.049$) on d 11. However, EGT substantially decreased ($P = 0.02$) sulfotransferase 5a1 expression on d 11, which may impede hepatic detoxification. Sulforaphane increased the expression of two phase I genes: cytochrome P450 1a2 (***Cyp1a2)*** on d 5 ($P = 0.02$) and flavin containing monooxygenases 1 (***Fmo1)*** on d 11 ($P = 0.002$). It also increased the expression of phase II gene transcription factor, nuclear factor E2 p45-related factor 2 ($P = 0.03$) and quinone reductase 02 ($P = 0.007$) on d 5, and *Gstm1* on d 8 ($P = 0.04$) and d 11 ($P = 0.01$). Moreover, SFN treated mice had higher ($P < 0.05$) *Gstm1* expression levels across days compared to control mice. The downstream metabolism of *Fmo1* is not well known. However, as observed in this study, the deactivation of *Cyp1a2* in the presence of *Gstm1* has been documented previously. Both within and across days, no significant difference in activity level due to treatment was observed for the phase II enzyme quinone reductase 01. However, SFN treated mice had higher ($P < 0.05$) glutathione–S–transferase (**Gst**) enzyme activity vs. control. The increase in both *Gstm1* expression and Gst activity indicate a consistent beneficial impact of SFN on phase II enzyme activity.

INTRODUCTION

Xenobiotics are natural or synthetic foreign chemicals that often enter the body by ingestion. In vivo metabolism of xenobiotics occurs primarily in the liver. Lipophilic, fat soluble xenobiotic compounds are initially processed into hydrophilic, water-soluble metabolites by phase I hepatic enzymes. This phase of biotransformation often activates xenobiotics into reactive metabolites. Through addition of other water-soluble groups, phase II enzymes deactivate the reactive metabolites, at times carcinogens, excreting them in bile or urine. Thus, phase II enzymes protect cells against xenobiotic metabolites and toxicant damage.

Ergot alkaloids, which are lipophilic xenobiotics, are found in endophyte-infected (*Neotyphoidium coenophialum*) tall fescue. Ingestion of these ergot alkaloids by grazing animals causes fescue toxicosis, which negatively affects their average daily weight gain and reproductive efficiency. Economic losses due to fescue toxicosis in the beef industry

are approximately $800 million each year (Ensley and Larsen, 2001). Phase I enzymes, such as cytochrome P450 (**Cyp**) play a significant role in the metabolism of ergot alkaloids ((Moubarak and Rosenkrans, 2000; Arthur et al., 2003). However, phase II enzymes, specifically glutathione-S-transferase (**Gst**) and uridine diphosphate glucuronosyl transferase (**Ugt**), also had increased activities in a mouse line selected for resistance to fescue toxicosis (Hohenboken and Blodgett, 1997; Wagner et al., 2000). Ergovaline is considered the most toxic and principal ergot alkaloid (greater than 90%) present in endophyte-infected tall fescue (Lyons et al., 1986). Ergotamine, an alternative ergopeptine structurally similar to ergovaline (differs in only one of the three amino acids), is often used in animal studies as ergovaline is commercially unavailable.

Sulforaphane, also a lipophilic xenobiotic, is an isothiocyanate derived from cruciferous vegetables such as broccoli. Sulforaphane has received considerable attention in the past decade because of its ability to increase activity of phase II enzymes like Gst and quinone reductase (**Nq**) 01. Sulforaphane induces expression of nuclear factor E2 p45-related factor 2 (***Nrf2***), a transcription factor of phase II genes (Thimmulappa et al., 2002). Unlike bi-functional inducers, which induce both phase I and II enzymes, sulforaphane is considered to be a mono-functional inducer of phase II enzymes. Nonetheless, sulforaphane inhibits the phase I enzyme Cyp2E1, which activates several carcinogens, thereby providing protection against cancers (Barcelo et al., 1996). Despite its role as a mono-functional inducer of phase II enzymes, the effects of sulforaphane on phase I enzyme activity may also be important.

The mouse is commonly used as a model organism to understand the function of mammalian genes, and the role of specific proteins in health and disease. In mice fed diets including either endophyte-free or endophyte-infected ground fescue seed, Cyp concentrations were higher in females as compared to males (Duringer et al., 2005). This was not surprising because many compounds undergo differential metabolism between genders (Löfgren et al., 2004; Cotreau et al., 2005). Choosing one sex in the design of an experiment may be useful to circumvent gender effects.

The first objective of this study was to evaluate response of phase I and II genes and enzymes in female mice when orally dosed with sulforaphane and ergotamine. A second objective was to determine whether persistent trends in the expression patterns of

the phase I and II genes and enzymes occurred when mice were exposed to these xenobiotics over time. Ergotamine is a toxicant that may affect biotransformation by impeding detoxification. In contrast, sulforaphane is a beneficial nutrient that may facilitate the metabolism of harmful compounds. In this experiment, they were chosen to reflect compounds with complementary affects on liver activity.

MATERIALS AND METHODS

Animals

The following procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Tech. Two experiments that differed in their duration were conducted using female mice. A polymorphic strain (ICR) was used with mice purchased from several colonies of a commercial lab (Harlan Sprague Dawley). Mice were approximately eight wk of age at the start of each experiment. After one wk quarantine, three mice from the same colony, blocked by their average weight over 3 d, were housed in a standard sized cage (29 x 14 x 13 cm). All mice had free access to pelleted rodent food (Standard Harlan Teklad, chow 2018) and were provided ad libitum water throughout the quarantine and experimental periods.

The first experiment, a preliminary study, was conducted using 12 mice from three ICR colonies. Four mice were orally dosed by gavage for 4 d with one of three assigned treatments and killed on d 5. The second experiment, a longitudinal study, used 69 mice and involved three treatments for four different time periods of dosing: 2, 5, 8 and 11 d; mice were killed on the subsequent day. At least five cages were randomly assigned to a dosing period.

Treatments

Each mouse in a cage was randomly assigned to one of the following treatments: (i) DL-sulforaphane (**SFN**) (LKT Laboratories) at 2.5 $mg \cdot mouse^{-1} \cdot d^{-1}$; (ii) ergotamine (**EGT**) (Sigma-Aldrich, Inc.) at 0.06 $mg \cdot mouse^{-1} \cdot d^{-1}$; and, (iii) a control vehicle. Each

treatment was diluted to deliver the desired mg amount of the treatment in a volume of 0.25 ml. The SFN and EGT were diluted with a 50:50 mixture of dimethyl sulfoxide (**DMSO**) and distilled water. Therefore, a 50:50 mixture of DMSO and distilled water was used as the control.

The SFN was initially diluted to 20 mg/ml with DMSO and aliquoted to the volume needed for the individual dosing days. These aliquots were stored at -20°C. On each day of dosing, the SFN in DMSO aliquot was diluted with an equal volume of distilled water and vortexed. The final concentration of SFN was 10 mg/ml. The target amount of EGT was weighed into separate vials, a sufficient amount for a given day, and stored at -20°C until the day of dosing. On each day of dosing, EGT was dissolved in a 50:50 DMSO and distilled water mixture by vortexing. The final concentration of ergotamine was 0.24 mg/ml. Mice were gavaged at the same time each day with 0.25 ml of their assigned treatment for the duration of the experiment with the exception of the day of kill. Oral dosing was done using a 22-gauge animal feeding needle attached to a Teflon coated 250 µl syringe.

Sample Collection

Mice were euthanized by cervical dislocation 24 h after the last dose. Liver samples were collected, chopped into small pieces, and separated into three homogenous samples immediately. Each sample was wrapped in pre-labeled aluminum foil, and snap frozen in liquid nitrogen. Time from kill to freezing of the sample was generally less than 1 min. Collected samples were stored at -80^{o}C.

Laboratory Analyses

Total RNA Isolation. Liver samples were ground thoroughly using mortar and pestle that were cooled with liquid nitrogen. Total RNA from powdered liver samples was isolated using RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Concentrations of RNA were determined by measuring absorbance at 260/280 nm on a spectrophotometer (Hitachi Instrument Inc, Japan, Model

U-2000). Quality and integrity of RNA was verified by electrophoresis on formaldehyde-agarose gels. The RNA samples were stored at -80°C until further use.

cDNA Preparation. Reverse transcription was performed using Applied Biosystems High capacity cDNA Archive Kit (Foster City, CA). Each reverse transcription reaction contained 10 µl of RNA (200 ng/µl concentration) and an equal volume of master mix. The components of reverse transcription master mix for each reaction were 2 µl of 10X reverse transcription buffer, 0.8 µl of 25X dNTPs, 2 µl of 10X random primers, 1 µl of MultiScribe Reverse Transcriptase (50U/µl), made up to 10 µl by adding nuclease-free water. Reverse transcription was performed using a thermal cycling profile of 10 min incubation at 25° C with 120 min reverse transcription at 37° C. Following reverse transcription, cDNA was diluted 1:30 with diethyl pyrocarbonate (**DEPC**)-treated water, and the 1:30 dilutions were used for RT-PCR reactions. The cDNA samples were stored at -20°C.

Primer Design. Primers for 5 genes associated with phase I biotransformation, 11 genes associated with phase II biotransformation, a transcription factor for phase II genes, and 2 reference genes, glyceraldehyde-3-phosphate dehydrogenase (***Gapdh***) and beta actin (***Actb6***), were designed based on published GenBank mouse gene sequences, using the Primer Express (Applied Biosystems, Foster City, CA) software. Genes considered in the study are shown in Table 3.1 and their primer pairs are shown in Table 3.2.

The *Gapdh* and *Actb6* genes were each evaluated as reference gene by comparing expression across all treatments and, for the longitudinal study, across days. As expression of both genes was not significantly different from each other (data not shown), *Gapdh* was chosen as endogenous control. All primer pairs used in the study were validated for primer amplification efficiency. An efficiency ratio between target genes and the *Gapdh* within a range of 82 -112% was considered acceptable as per Applied Biosystems (2004) guidelines (see Table 2.5 from chapter 2).

Quantitative Real-Time PCR. Quantitative RT- PCR was performed on an Applied Biosystems 7300 machine (Foster City, CA) using the relative quantification method. Liver cDNA samples from three mice in a cage were assayed on the same plate for RT-PCR analysis. Samples were run in triplicate for 10 genes including *Gapdh* on each plate. For each 25 µl PCR reaction, 2 µl of 1:30 diluted cDNA, 12.5 µl of SYBR green master mix (Applied Biosystems, Foster City, CA), 0.5 µl of forward and reverse gene-specific primers at 5 µM concentration, and 9.5 µl of DEPC-treated water were added. Amplification was performed in 96-well MicroAmp optical reaction plates (Applied Biosystems, Foster City, CA).

Incubation parameters used for RT-PCR were incubation 2 min at 50°C, initial denaturation for 10 min at 95°C, followed by 40 amplification cycles of 15 s each at 95°C and then 1 min at 60°C. After the final cycle, dissociation analysis was performed to ensure gene specific amplification. Parameters for the dissociation step were 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C. Single dissociation peak for a gene ensured amplification of desired PCR product without primer-dimer formation.

The cycle threshold ($\mathbf{C_T}$) for each reaction well was obtained with Applied Biosystems 7300 software using manual analysis. In order to remove extraneous variation among triplicates for a sample, outliers were removed using a more conservative method than provided by the software (Grubb's statistic). When the SE was greater than 0.15, one of the triplicates that differed most in C_T value from its counterparts was removed as an outlier. Approximately 4 % of the C_T values were outliers. If outlier removal did not decrease the SE below 0.15, or no outlier could be clearly delineated as different among triplicates, RT-PCR was repeated for all three samples from the cage for the target gene along with *Gapdh*. Average of the triplicates, or in some cases duplicates, was calculated.

The average C_T values of a sample for a target gene were normalized using *Gapdh* to obtain ΔC_T values. The ΔC_T values of a treatment (SFN and EGT, separately) and the control were used for statistical analysis.

Enzyme activity assays. Liver samples were weighed and a 25% homogenate was prepared in 0.1 M phosphate buffer (pH 7.4 with 1.15% KCl) using a Polytron blender (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged for 10 min at

15,000 X g at 5°C. Supernatant was re-centrifuged for 60 min at 50,000 X g at 5°C. The supernatant, cytosol, was transferred and stored at -80°C until assayed.

Enzyme activity and protein assays were performed in separate 96- well micro titer plates. Activity of quinone reductase 01 (**Nq01**) and Gst were measured in liver cytosol fractions according to established procedures. Briefly, in the Nq01 assay the following stock solution was prepared for each set of assays: 7.5 ml of 0.5 M Tris-Cl (pH 7.4), 100 mg of bovine serum albumin, 1 ml of 1.5% Tween-20, 0.1 ml of 7.5 mM flavin adenine dinucleotide (FAD), 1 ml of 150 mM glucose 6-phosphate dehydrogenase, 90 µl of 50 mM nicotinamide adenine dinucleotide phosphate, 300 U of Yeast glucose-6-phosphate dehydrogenase, 45 mg of thiazolyl blue tetrazolium and distilled water to a final volume of 150 ml. Menadione (1 µl of 50 mM menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the mixture was dispensed into the microtiter plates. Each well in the plate contained 50 µl of 1: 8 diluted cytosol and 200 µl of the stock solution and the reaction was run for 5 min. To stop the reaction, 50 µl of dicoumarol (0.3 mM dicoumarol in 1.5% DMSO in 5 mM potassium phosphate buffer at pH 7.4) was added to each well in the plate, which was read at an absorbance of 610 nm. The extinction coefficient value used for substrate was 11,300 $M^{-1}\cdot cm^{-1}$ and path length of cuvette was 0.57 cm. The concentration of enzyme was expressed in $nmoles\cdot min^{-1}\cdot mg\ protein^{-1}$ (Prochaska and Santamaria, 1988).

The Gst assay was done using the following stock solution prepared for each set of assays: 5 ml of 20 mM glutathione in 0.1 M of sodium phosphate buffer (pH 6.5), 5 ml of 20 mM 1-chloro-2, 4-dinitrobenzene in 95% ethanol. Each well in the plate contained 25 µl of 1: 8 diluted cytosol and 245 µl of the assay buffer, 15 µl of 20 mM glutathione in buffer (final concentration of 1 mM in well (15 µl in 300 µl)) and 15 µl of 20 mM 1-chloro-2,4-dinitrobenzene in 95% ethanol. The reaction was run for 5 min at 30 C with readings at 15 s increments. The plate was read at an absorbance of 340 nm. The extinction coefficient value used for substrate was 9.6 $mM^{-1}\cdot cm^{-1}$ and path length of cuvette was 0.57 cm. The concentration of enzyme was expressed in $\mu moles\cdot min^{-1}\cdot mg\ protein^{-1}$ (Mannervik and Jemth, 1999; Habig et al., 1974; Kaplowitz et al., 1975).

Cytosolic protein was determined using the protein-dye binding method (Bradford, 1976) with bovine serum albumin as the standard. For an individual assay,

(Nq01, Gst or protein) samples from mice killed on a given day were analyzed on the same plate in triplicate.

Statistical Analyses

Initially, analyses of gene expression and enzyme activity were performed separately by day. A paired t-test was used in which a treatment, either EGT or SFN, was paired with its respective control within a cage. Since the two treatments were considered separately, this approach avoids heterogeneity of variance between treatments.

In addition, for the longitudinal study, response variables were analyzed using a statistical model that considered treatment and day as fixed effects. Each day (n = 4) had at least 5 cages. Therefore, cage was assumed nested within day and fitted as a random effect. The error term for testing day effects was cage. The statistical model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{(k)j} + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the observation, μ is the mean, α is the fixed effect of treatment i, β is the fixed effect due to day j, γ is the random effect of cage k nested within day j, $\alpha\beta$ is the interaction between treatment i and day j, and ε is the residual term. With four levels of day, three orthogonal contrasts were fitted to compare day means (d 2 vs. 5, 8 and 11; d 5 vs. 8 and 11; and d 8 vs. 11). Treatment means (control, EGT and SFN) were compared using 2 orthogonal contrasts: control vs. EGT and SFN, and EGT vs. SFN. However, comparison of EGT and SFN was not a focus of this study. Therefore, Fisher's least square difference was used to test for any differences among treatments and control.

The t-tests were performed using the analyst procedure of SAS and the ANOVA model was fitted using PROC GLM (SAS Institute Inc., Cary, NC, USA).

RESULTS

General effects of oral administration of SFN and EGT

Oral administration of SFN and EGT to mice in both the preliminary and longitudinal studies induced no mortality and no evidence of systemic toxicosis. Also, the treatments did not have any effect on the mean body weight gain of mice (data not shown). No treatment related gross lesions were identified in any animal at terminal necropsy.

Gene expression

Within day. Least squares means for differences in ΔC_T values for a treatment and its control are shown in Figure 3.1 for the preliminary study and in Figure 3.2 for the longitudinal study, for differentially expressed genes ($P < 0.05$). These values correspond with the log 2 of the ratio of expression for a treatment (either EGT or SFN) and control; in other words, they are the log 2 of the fold change in gene expression. Positive values indicate increase expression and vice versa. Although statistically significant, the changes in gene expression due to treatment were generally less than two-fold. The results for all genes analyzed in both studies are shown in the appendices (Table 5.1, 5.2 and 5.3).

In the preliminary study, EGT influenced phase I gene expression by increasing *Cyp3a44* ($P = 0.03$; Figure 3.1), and decreasing expression of flavin containing monooxygenases 1 (***Fmo1***; $P = 0.002$), relative to the control. The phase II gene catechol-O- amine methyl transferases 1 **(*Comt1***; $P = 0.03$) had decreased expression. No significant difference in gene expression was observed in SFN treated mice compared to the control in the preliminary study.

In the longitudinal study, after 2 d of dosing, there was no differential gene expression ($P > 0.05$) associated with EGT or SFN. However, expression levels of *Cyp1a2*, a phase I gene, increased in SFN treated mice with respect to the control ($P = 0.02$) by d 5 (Figure 3.2). Expression levels of *Nrf2* ($P = 0.03$) and *Nq02* ($P = 0.007$),

both phase II genes, were increased in SFN treated mice at d 5 compared to the control, but not in the EGT treated mice ($P > 0.05$). No differential expression was found for any other genes at d 5.

Expression levels of *Comt1* increased ($P = 0.009$; Figure 3.2) after 8 d of dosing with EGT compared to the control. After 8 days of dosing with SFN, *Gstm1* expression increased ($P = 0.04$). There was no significant difference in expression levels of any other gene after d 8.

After 11 d of dosing with SFN, the expression level of the phase I gene *Fmo1* was increased relative to the control ($P = 0.002$; Figure 3.2). For both treatments, *Gstm1* expression was increased, although more substantially for SFN ($P = 0.01$) than for EGT ($P = 0.049$). Expression of *Sult5a1,* another phase II gene, decreased ($P = 0.02$) in EGT treated mice compared to the control. There was no significant difference in the expression of any other genes on d 11.

Across days. In the longitudinal study, trends in gene expression over time were investigated using ΔC_T values. A negative ΔC_T value indicates increase in gene expression and vice versa. In Figures 4.3 to 4.6, the y-axis is minus ΔC_T to facilitate their interpretation.

Only for *Gstm1* in mice dosed with SFN was there a consistent increase in gene expression across days compared to control ($P < 0.05$; Figure 3.3). Expression of *Cyp3a44*, *Sult5a1* and *Nat2* genes differed ($P < 0.05$) across day (Figure 3.4) but not due to treatment ($P > 0.05$). Contrasts for d 8 vs. d 11 for *Cyp3a44* and *Nat2*, and for d 5 vs. d 8 and 11 for *Sult5a1,* were significant. Also, the interaction between day and treatment was significant for *Nat2*. No other gene identified as differentially expressed within a day had an overall treatment effect across days (Figures 3.5 and 3.6)

The importance of treatment, day and their interaction on gene expression in the longitudinal study, along with R-square values, are summarized for all genes in Table 3.3. Two genes, *Cyp1a1* and multiple drug resistance protein 1(***Mrp1***), failed to show any detectable expression due to treatment or in control groups and thus are excluded from Table 3.3.

Enzyme activity

Activity levels of Nq01 and Gst, both phase II enzymes, were assessed in the preliminary study and for all 4 time points in the longitudinal study. The complete results are shown in the appendix (Table 5.4).

When data from each day were analyzed separately, there was no significant effect of treatment on Gst activity in either study on any individual day ($P > 0.05$). There also was no overall treatment effect on Gst activity ($P > 0.1$). However, mice treated with SFN had higher Gst activity then the control ($P < 0.05$), with the difference tending to increase as dosing continued. Fit of the third order polynomial shows the pattern in Gst activity levels for the two treatments and the control across days (Figure 3.7). The increase in Gst enzyme activity for the SFN treatment was consistent with the increase in expression of the *Gstm1* gene.

Activity levels of Gst enzyme increased over days ($P < 0.05$) in SFN treated mice. The overall effect of day, and all orthogonal contrasts among selected days, defined variation in Gst activity ($P < 0.05$). The difference in mean activity level of d 5 vs. d 8 and 11 was most substantial ($P < 0.01$).

No overall treatment effect was found ($P > 0.1$) for Nq01 enzyme activity (Figure 3.8). However, similar to Gst, Nq01 activity differed significantly across days. The fitted contrasts identified differences ($P < 0.001$) in Nq01 activity for d 2 vs. other days, and for d 5 vs. 8 and 11. However, no difference in Nq01 activity was observed between d 8 and 11 ($P > 0.1$).

DISCUSSION

The dosage of xenobiotic compounds used for both the preliminary and longitudinal study was determined based on published literature. Sulforaphane induced activity of Nq and Gst in liver samples of mice dosed with 15 $\mu mol \cdot mouse^{-1} \cdot d^{-1}$ for 5 d (Zhang et al., 1992), which is similar to the dose used in this study (14.12 $\mu mol \cdot mouse^{-1} \cdot d^{-1}$). In a typical fescue experimental trial in mice, the diet contains 2 ppm ergovaline. Mice eating approximately 10% of their body weight would receive 0.2 mg

ergovaline·kg^{-1}·d^{-1} from the fescue diet. Physiological alterations were achieved with subcutaneous administration of ergotamine tartrate at 0.4, 2, 10 and 50 mg ·kg^{-1}·d^{-1} for 10 d in mice (Filipov et al., 1999). As no mortality was seen when mice were administered the above doses, approximately 2 mg·kg^{-1}·d^{-1} dose of EGT was used in our study.

Preliminary study

In the preliminary study, EGT increased expression of the phase I gene *Cyp3a44,* and decreased the expression of the phase II gene *Comt1;* this combination may accentuate toxicity. Boobis et al. (1995) hypothesized that excessive Cyp activity, without associated increase in phase II enzyme activity, may be a potential health risk. A decrease in *Fmo1* expression was also observed in the EGT treated mice. Although Fmo belong to the phase I enzyme family, they are involved in detoxification of xenobiotics, especially those derived from plants (Ziegler, 1990). Thus, a decrease in *Fmo1* expression in EGT treated mice may further impede detoxification.

There was no differential gene expression observed for any of the 17 genes in SFN treated mice in the preliminary study. Also, there was no significant difference in the enzyme activity levels of Gst and Nq01 for both treatments. Such was not our expectation, and suggested that either the duration (4 d) or dosage of challenge was insufficient. Consequently, a longitudinal study with 12 d of dosing was conducted. Since liver samples were collected at four equal intervals within the extended dosing period, patterns in the expression of genes over time were also investigated.

Longitudinal study

Genes responsive to Ergotamine. In the longitudinal study, EGT influenced phase II genes by decreasing the expression of *Sult5a1* on d 11, and increasing the expression of *Comt1* and *Gstm1* on d 8 and d 11, respectively.

Heifers fed with endophyte-infected fescue for 11 d have greater than 3.3-fold decrease in sulphotransferase expression when compared with heifers fed with endophyte-free fescue for 14 d (Jones et al., 2004). Thus, the decrease in expression of

Sult5a1, a phase II gene, after prolonged EGT administration in this study was to be anticipated. In contrast to the decrease in *Sult5a1* expression, there was an increase in expression of the phase II genes *Comt1* and *Gstm1* due to EGT treatment. Although the consequence of increased expression of *Gstm1* is unclear, the increase in *Comt1* expression may result in neuronal abnormalities (Filipov et al., 1999). Since *Comt1* is a phase II gene that harmful effect is not intuitive. However, mice administered with 0.4, 2, 10 or 50 mg/kg EGT subcutaneously for 10 d showed significant decrease in dopamine concentrations at all doses (Filipov et al., 1999). Neurotransmitters, like dopamine and catacholamine, are broken down by catalytic reactions mediated by Comt enzyme (Grossman et al., 1992). Thus, an increase in *Comt1* expression due to EGT treatment may increase the respective enzyme activity leading to depletion in dopamine levels and, potentially cause nervous disorders.

Genes responsive to Sulforaphane. Analyses on a day basis showed increase ($P < 0.05$) in expression of two phase I genes (*Cyp1a2* and *Fmo1*) and three phase II genes (*Nrf2*, *Nq02* and *Gstm1*) in SFN treated mice. When expression levels across days were considered, *Gstm1* alone had a significant increase in expression over time.

Expression of *Cyp1a2* was increased after 5 d of dosing with SFN in our study. Similarly, rats administered 30 and 120 mg/L concentrations of SFN (equivalent to daily doses of 3 and 12 mg/kg) in drinking water for 10 days exhibit increased levels of hepatic *Cyp1a2* as determined by immunological methods (Yoxall et al., 2005). Human HepG2 cells – cell lines expressing *Cyp1a2* – treated with 2 to 10 μM concentration of SFN for 24 h showed a 30 % increase in *Cyp1a2* expression after 6 hr, which returned to basal level after 24 h (Bacon et al., 2003). Similarly, in this study, expression of *Cyp1a2* increased after 5 d of dosing with SFN; however, expression levels were not significantly different from the control after d 8.

Consumption of broccoli caused an increase in Cyp1a2 activity in humans (Lampe et al., 2000). Probst-Hensch et al. (1998) hypothesized that induction of *Cyp1a2* was deactivated by *Gstm1*. In that study, human subjects who consumed broccoli as part of their normal diet were genotyped for *Gstm1* and tested for Cyp1a2 enzyme activity. Those subjects with non-functional *Gstm1* genes had a 21% increase in Cyp1a2 activity

compared to those with functional *Gstm1* genes (Probst-Hensch et al., 1998). They concluded that the deactivation of Cyp1a2 activity was due to the presence of functional *Gstm1* genes. The increased *Cyp1a2* expression in the current study after 5 d of dosing, but not thereafter, may be explained by the increase in *Gstm1* expression by d 8 suppressing *Cyp1a2*.

The expression of *Fmo1* was increased on d 11 in mice treated with SFN. The long term consequence of this increase on subsequent liver enzyme activity was not measured in this study, and appears not to be documented elsewhere in literature. However, activity of Fmo enzymes has been shown to facilitate detoxification of plant derived xenobiotics (Ziegler, 1990), and thus are not generally responsible for increasing toxicity. The increase in *Fmo1* expression may suggest a generalized increase in detoxification with SFN treatment.

Expression of *Nrf2*, a phase II genes transcription factor, was increased on d 5. Thimmulappa et al. (2002) administered SFN (9μmol·mouse^{-1}·d^{-1}) to *Nrf2* wild type ($^{+}/_{+}$) and knockout mice ($^{-}/_{-}$) for about 6 d, which increased the expression of *Nrf2* in the wild type mice. Microarray analyses documented that *Nrf2* was not only responsible for the basal expression of several phase II genes, including *Gstm1*, but also induced their expression when SFN was administered (Thimmulappa et al., 2002). In the current study, an increase in expression of the phase II gene *Nq02* was observed after 5 d of dosing with SFN. Wang and Jaiswal (2006) reported that over expression of *Nrf2* increased the expression of *Nq02*, while inhibition of *Nrf2* decreased the expression of *Nq02* in HepG2 cells. They also hypothesized that *Nrf2* along with *JunD*, a nuclear transcription factor, bind to the antioxidant response element present in the promoter region of *Nq02* and control its expression. This suggests *Nrf2* may have a role in the expression of *Nq02*. Thus, increased expression of *Nrf2* on d 5 may in part have been responsible for increased expression of *Nq02* (on d 5) and *Gstm1* (on d 8 and 11).

Enzyme activity assays

For analysis of individual days, there was no significant difference in activity of Gst or Nq01 enzymes for either treatment. In the longitudinal study, both enzymes

changed in activity level across days (Figure 3.7 and 3.8; $P < 0.001$), with the activity of Gst enzyme increasing over time (Figure 3.7). Additionally, SFN treated mice had higher ($P < 0.05$) Gst activity compared to the control, with the difference tending to increase as dosing continued. A gradual increase in Gst activity was also reported when rats were orally administered with allyl isothiocyanate (a compound similar to sulforaphane) at 40 $\mu mol \cdot kg^{-1} \cdot d^{-1}$ over a period of 21 d (Munday and Munday, 2004).

CONCLUSION

It is evident that SFN and EGT differ in their ability to induce phase I and II genes and detoxifying enzymes in the liver. Ergotamine significantly decreased the expression of the phase II gene *Sult5a1*, although its effect on phase II enzymes was minimal. With a higher dose of EGT, a larger response in gene expression and enzyme activity in the liver may have been observed.

Sulforaphane significantly increased expression of *Gstm1*, along with other phase II genes. Equally, Gst enzyme activity was higher. This coupling of gene expression and enzyme activity indicates a consistent and beneficial impact of SFN on phase II enzyme activity. As a next step, if polymorphisms in *Gstm1* could be identified that were associated with heightened sensitivity to induction, this gene may serve as a useful genetic marker in selection programs to enhance animals' capability to combat challenges from toxicants.

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Table 3. 1 Description of mouse hepatic genes used in the study

Gene symbol	General description	Gene Bank accession number[1]
Phase I genes		
Cyp1a1	Cytochrome P450 1a1	NM_009992
Cyp1a2	Cytochrome P450 1a2	NM_009993
Cyp3a41	Cytochrome P450 3a41	NM_017396
Cyp3a44	Cytochrome P450 3a44	NM_177380
Fmo1	Flavin mono-oxygenase 1	NM_010231
Phase II genes		
Gsta2	Glutathione-S-transferase a2	NM_008182
Gsta3	Glutathione-S-transferase a3	NM_010356
Gstm1	Glutathione-S-transferase mu1	NM_010358
Ugt1a1	UDP glucoronosyl-transferase 1a1	NM_201645
Sult2a2	Sulfotransferase 2a2	NM_017465
Sult5a1	Sulfotransferase 5a1	NM_020564
Ephx1	Epoxide hydrolase 1	NM_010145
Nq02	Quinone reductase 02	NM_020282
Comt1	Catechol-O-amine methyl transferases	NM_007744
Mrp1	Multiple drug resistance protein 1	NM_008576
Nat2	N-acetyl transferases 2	NM_010874
Nrf2	Nuclear factor E2 p45-related factor 2	U20532
Endogenous genes		
Gapdh[2]	Glyceraldehyde -3- phosphate dehydrogenase	BC083149
Actb6	Beta-actin	NM_007393

[1]Accession number used by National Center for Biotechnology Information to track the gene sequences.

[2]Used as the endogenous control gene in the study.

Table 3. 2 Primer pairs of mouse hepatic genes used in the study

Gene[1]	Primer pairs[2]	
	Forward	Reverse
Phase I genes		
Cyp1a1	AGCCTCATTGAGCATTGTCAGG	CCAGCTCCAAAGAGGTCCAAA
Cyp1a2	TTGGTGCCATGTGCTTTGG	TCCCTGAGGTGACATTCTCCAC
Cyp3a41	TGCCATTTTTAGGCACTGTGC	GCATTTGACCATCAAACAACCC
Cyp3a44	TTGGTCCTGCTGGCAATCA	GCACAGTGCCTAAAAATGGCA
Fmo1	CCCTTCCTCGATGAATCCGTA	AGGCCAATCACAGCCAGAGTT
Phase II genes		
Gsta2	CCCAGACCAAAGAGAAGCCAA	GCCCACAAGGTAGTCTTGTCCA
Gsta3	AAGCCTTGCCAAGATCAAGGA	GCCTGTTGCCAACGAGATAATC
Gstm1	CACACAAGATCACCCAGAGCAA	CACAATGTCTGCACGGATCCT
Ugt1a1	ATTGCCATGCAGCCTGGAT	TCGCTGTAGGAAGTTCATGCG
Sult2a2	AGGCCAAGGCGATCTATCTCA	TCCGAGTGACCCTGGATTCTT
Sult5a1	AGCGCATGAACACCACTGAAA	TGTGTCCTGGAACTGGAAGGAG
Ephx1	CAAAGCCATCAGCCAAAGAAG	TGCCCGGAACCTATCTATCCT
Nq02	GCTCTCCTTTCCTTAACCACGG	AGTGTACCATGCTGAAGTGGCC
Comt1	CATGTGCAGCAACACGCAA	TTGCGTCACCCACGTTCAT
Mrp1	CGCATGAACTTGGACCCTTTC	TGGTTCAGCTTGTCAGGCAAA
Nat2	CCAGGAGCAAACTGGACTTGAA	CATGGATTCCCCACAATGGA
Nrf2	TACAGCCTCTGTCACCAGCTCA	TTTGATGACCAGGACTCACGG
Endogenous genes		
Gapdh[3]	GCAAAGTGGAGATTGTTGCCAT	CCTTGACTGTGCCGTTGAATTT
Actb6	TCCTGAGCGCAAGTACTCTGTG	CGGACTCATCGTACTCCTGCTT

[1]See Table 3.1 for gene description.

[2]All primer pairs were chosen using the primer express software version 2.

[3]Used as the endogenous control gene in the study.

Table 3. 3 P-values for treatment, day and their interaction (Treatment*Day) effects along with R-square values for the longitudinal study [1]

Gene symbol[2]	Treatment	Day	Treatment*Day	R-square
Cyp1a2	0.06	0.42	0.60	0.82
Cyp3a41	0.87	0.93	0.57	0.94
Cyp3a44	0.29	**0.05**	0.87	0.51
Fmo1	0.20	0.15	0.65	0.95
Gsta2	0.08	0.40	0.21	0.80
Gsta3	1.00	0.36	0.39	0.97
Gstm1	**<0.0001**	0.85	0.80	0.85
Ugt1a1	0.41	0.14	0.11	0.77
Sult2a2	0.45	0.38	0.84	0.50
Sult5a1	0.94	**0.02**	0.08	0.79
Ephx1	0.41	0.16	0.59	0.91
Nq02	0.18	0.57	0.85	0.86
Comt1	0.96	0.28	0.43	0.86
Nat2	0.43	**0.02**	**0.04**	0.95
Nrf2	0.81	0.37	0.14	0.95

[1]Analyses were based on ΔC_T values of the control, sulforaphane and ergotamine treatments. Effects deemed statistically significant are highlighted in bold. Expression of cytochrome P450 1a1 (*Cyp1a1*) and multiple drug resistance protein 1 (*Mrp1*) were undetectable and hence are not included in this table.

[2]See Table 3.1 for gene descriptions.

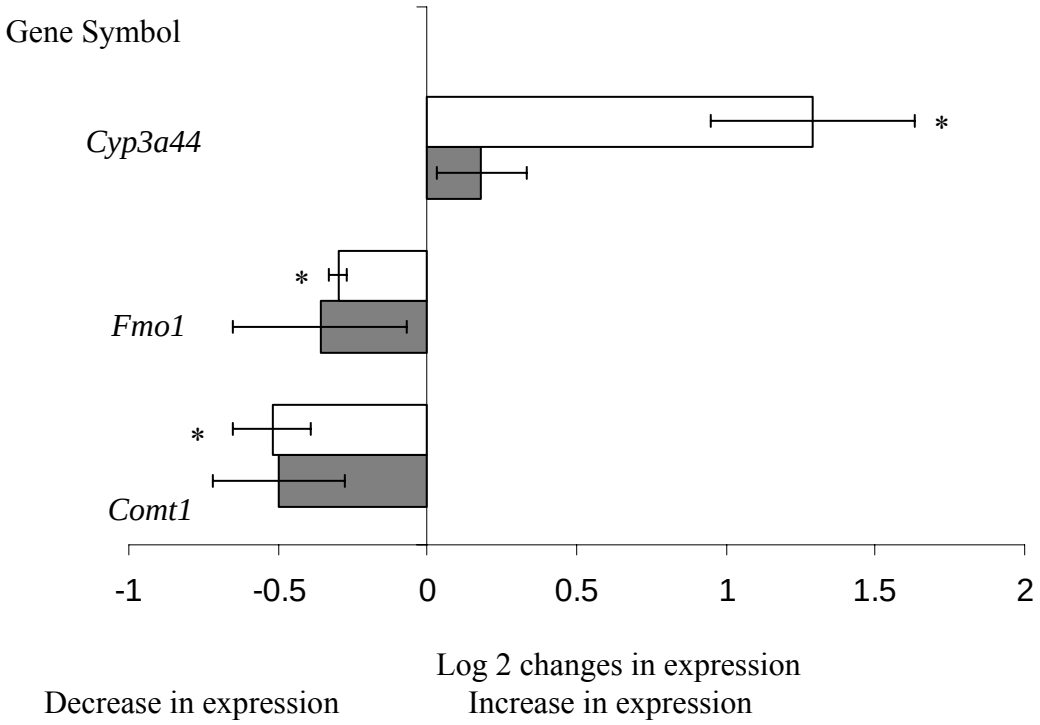


Figure 3. 1 Differentially expressed hepatic genes in preliminary study.
The figure shows the log 2 of the ratio of the expression of each gene in mice dosed with either ergotamine (open bar) or sulforaphane (solid bar) in comparison with control. An asterisk (*) indicates that expression due to treatment differs from the control ($P < 0.05$) for a gene. Gene symbols are defined in Table 3.1.

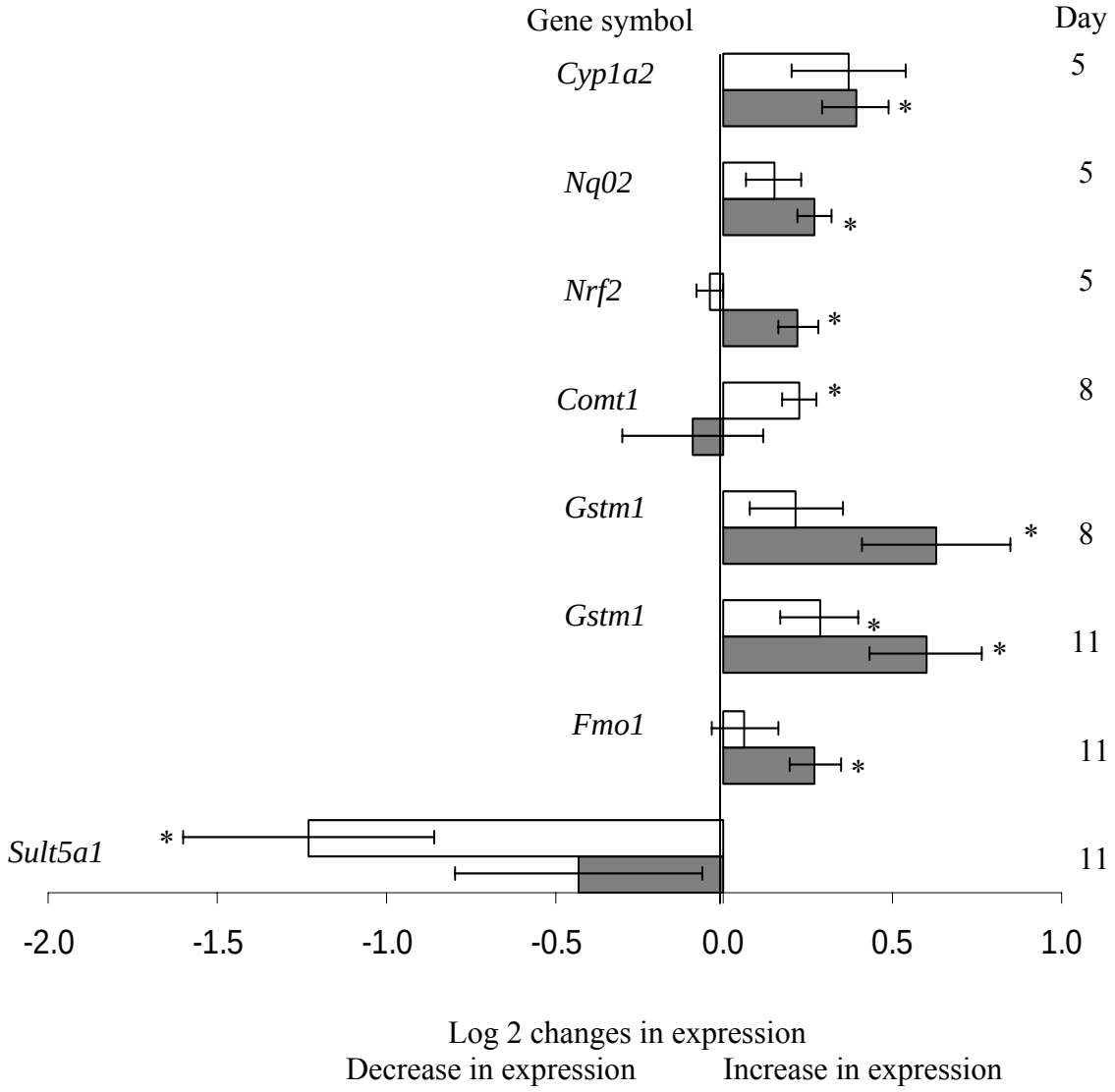


Figure 3. 2 Differentially expressed hepatic genes in longitudinal study.

The figure shows the log 2 of the ratio of the expression of each gene in mice dosed with either ergotamine (open bar) or sulforaphane (solid bar) in comparison with control. An asterisk (*) indicates that expression due to treatment differs from the control ($P < 0.05$) for a gene for the specified days of dosing. Gene symbols are defined in Table 3.1.

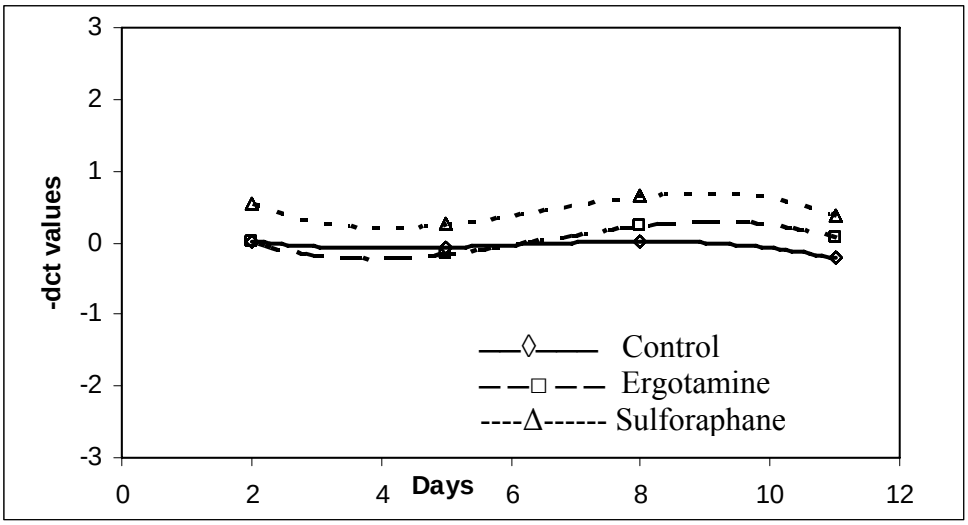
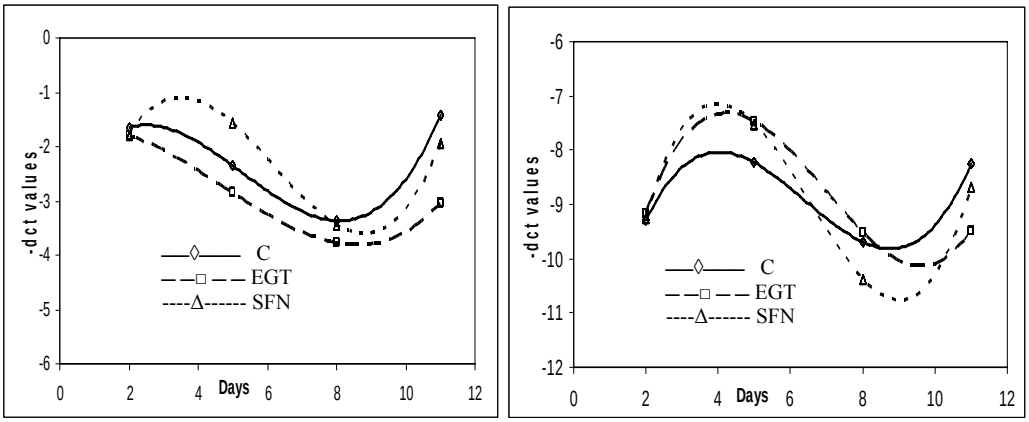


Figure 3. 3 Glutathione-S-transferase mu 1 gene expression in longitudinal study.

Sulforaphane had increased gene expression compared to control across days ($P < 0.05$). The y-axis is minus ΔC_T values and thus higher values correspond with increased expression and vice versa.

Cyp3a44 *Sult5a1*



Nat2[2]

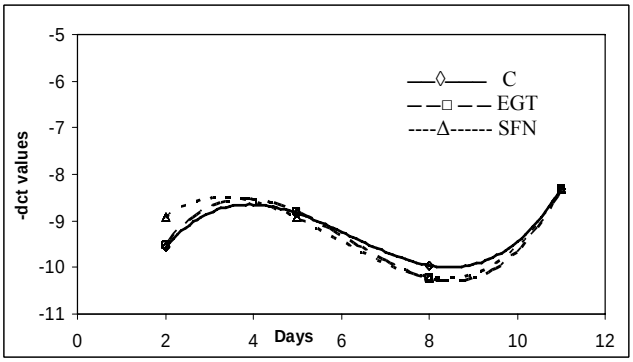


Figure 3. 4 Cytochrome P450 3a44 (Cyp3a44), sulfotransferase 5a1 (Sult5a1) and N-acetyl transferase 2 (Nat2) gene expression in the longitudinal study.
Day effects were significant for all genes ($P < 0.05$). An interaction between treatment and day was detected for *Nat2* ($P < 0.05$). The y-axis is minus ΔC_T values and thus higher values correspond with increased expression and vice versa. Symbol designations are C for control, EGT for ergotamine and SFN for sulforaphane.

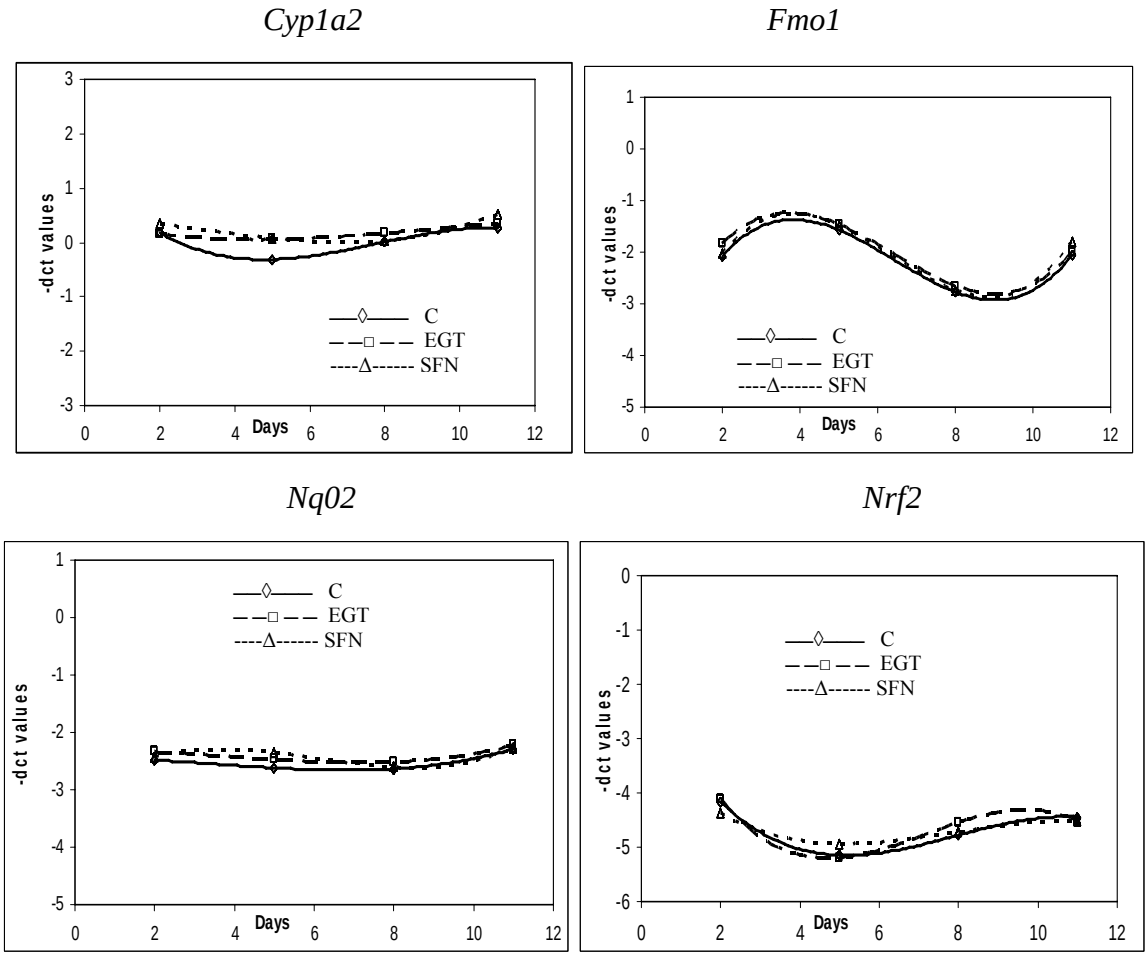


Figure 3. 5 Expression of cytochrome P450 1a2 (Cyp1a2), flavin monooxygenase 1 (Fmo1), quinone reductase 02 (Nq02), and nuclear factor E2 p45-related factor 2 (Nrf2) in the longitudinal study.

Expression of these genes was affected by sulforaphane on specific days ($P < 0.05$). However, no treatment or day effects were identified for any gene ($P > 0.05$) across days. The y-axis is minus ΔC_T values and thus higher values correspond with increased expression and vice versa. Symbol designations are C for control, EGT for ergotamine and SFN for sulforaphane.

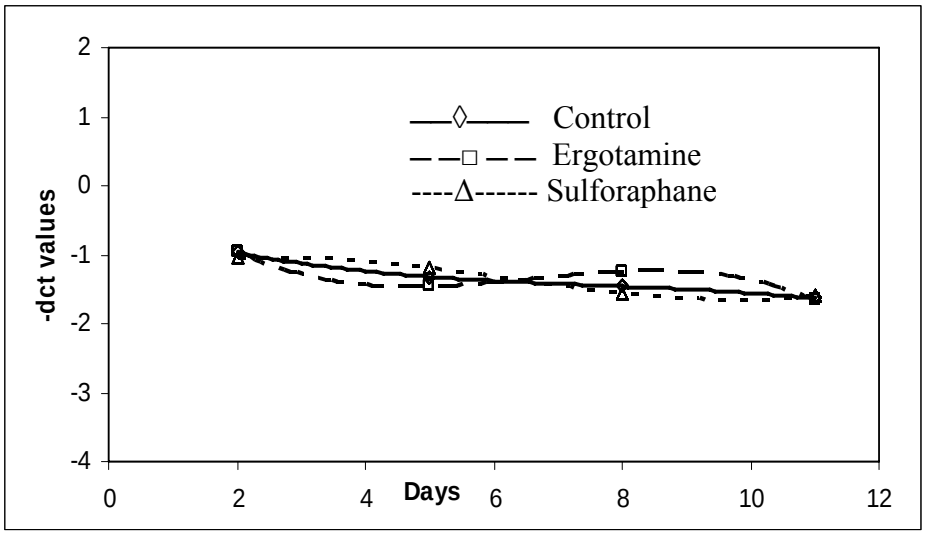


Figure 3. 6 Expression of catechol-O-amine methyl transferase 1 (Comt1) in the longitudinal study.

This gene was differentially expressed on d 8 due to ergotamine treatment ($P = 0.009$). However, no treatment or day effects were identified ($P > 0.05$) across days. The y-axis is minus ΔC_T values and thus higher values correspond with increased expression and vice versa.

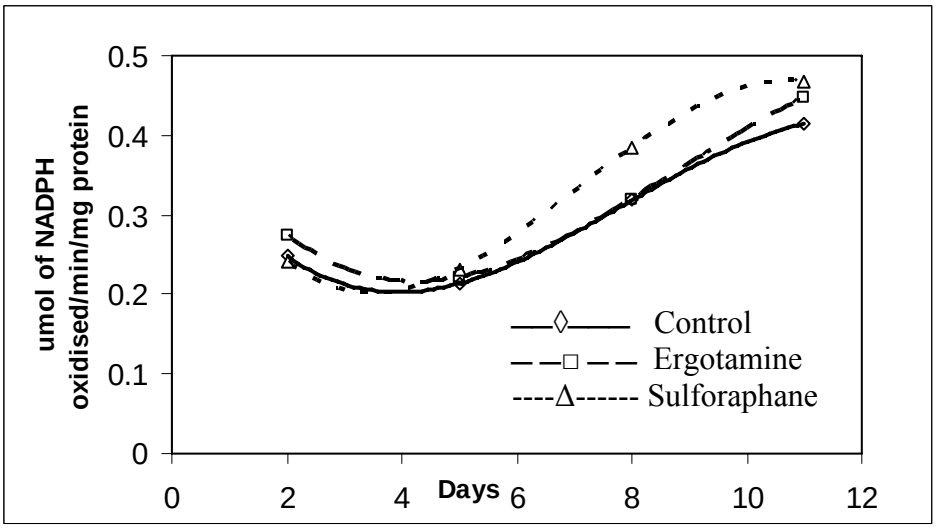


Figure 3. 7 Glutathione-S-transferase enzyme activity in longitudinal study. The fit of a third order polynomial is shown.

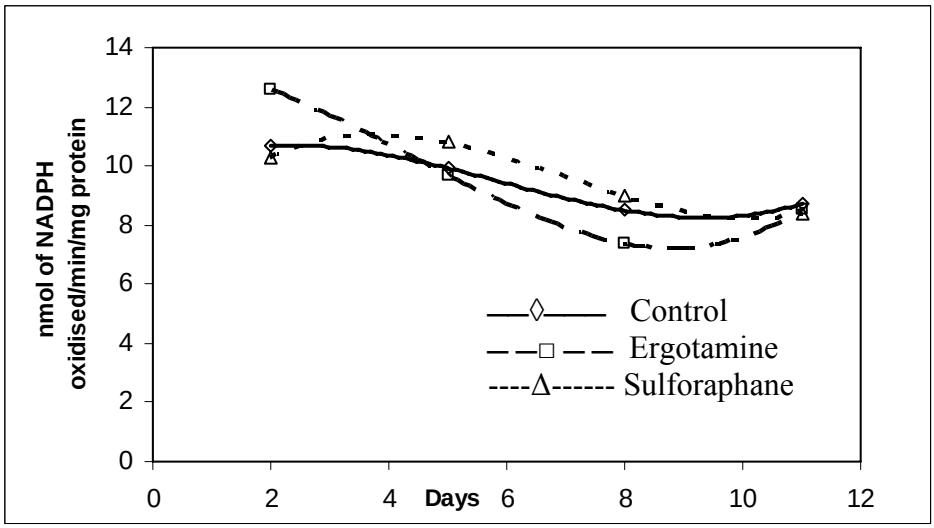


Figure 3. 8 Quinone reductase 01 enzyme activity in longitudinal study. The fit of a third order polynomial is shown.

CHAPTER 4
General conclusions and implications

Gene expression and enzyme activities involved in liver metabolic pathways were tested for two xenobiotics: ergotamine (**EGT**) and sulforaphane (**SFN**). Ergotamine is related to a fungal toxin produced in tall fescue, a common forage crop in southeastern United States. Unlike EGT, which is particularly relevant to the livestock industries, SFN is more closely aligned with biomedical research since it is considered an anticarcinogenic compound. Although some of the anticipated responses in gene expression and enzyme activity were observed in liver due to SFN treatment, EGT elicited relatively little response. This chapter deals with the implications of the results from the two experiments conducted involving these compounds, and suggestions for future studies.

Historically, mice were fed with endophyte-infected fescue to quantify differences in phase I and II enzyme activities. However, endophyte-infected fescue seed has a combination of ergot alkaloids like ergopeptines and loline alkaloids. Ergovaline is the major toxic ergot alkaloid present in tall fescue. Unfortunately, ergovaline is commercially unavailable. Hence, EGT, a commercially available compound that is very similar to ergovaline, was used. With few exceptions, EGT had almost no effect on either phase I or II genes, or on phase II enzyme activity. This may be because: (i) EGT dose was insufficient; (ii) tissue sampled is less responsive; and, (iii) EGT is not an appropriate representative of ergot alkaloids. As the dose used in the current study did not produce a measurable response in gene expression, effect of an increased dosage might be usefully tested. Apart from analyzing liver tissue, kidney or brain tissue could have been sampled as renal failure and neuronal symptoms are associated with ergotamine toxicity. If an increase in dosage and/or sampling of other tissue did not result in any change in gene expression or enzyme activity, then future experiments should consider using a different ergot alkaloid or an entirely different toxicant.

Genes coding for phase I and II detoxification enzymes had increased expression in liver of mice treated with SFN. An increase in expression of transcription factor nuclear factor E2 p 45 related factor 2 (***Nrf2)*** due to SFN is an exciting result since it

induces a plethora of phase II genes involved in detoxification. Expression of both *Cyp1a1* and *Fmo1* was increased with SFN treatment. No phase I enzymes were measured and thus, changes in their activity are unknown. Several phase II genes (*Nrf2*, *Nq02* and *Gstm1*), and an associated enzyme (Gst), showed increased activity. Therefore, with ingestion of SFN, an increase in phase II enzyme activity may occur to cope with an increase in reactive oxygen species generated by phase I detoxification pathways.

As a next step, individual animals could be genotyped to detect polymorphisms in phase II genes, like *Gstm1* or *Nq02*. If polymorphisms are in the coding region, they may alter the amino acid sequence affecting the specific protein formation. If polymorphisms are in the regulatory regions, then individuals may differ in the quantity of encoded protein. For example, a polymorphism detected in phase II gene, *Gstm1,* might code for increased *Gstm1* expression, and thereby increased Gst activity. Enhanced Gst activity would increase the animal's ability to cope with toxins.

Whole-flock genotyping identifies and allows for selection of animals carrying beneficial genetic variants, but genotyping large numbers of animals may be costly. Instead, by identifying phenotypes associated with beneficial polymorphisms, a more economical approach for selection may be possible. For instance, if hepatic enzyme levels in urine could be used as indicators of associated gene's expression, a selection program based on phenotypes for enzyme activity level may lead to increased genetic tolerance to harmful xenobiotics.

CHAPTER 5
Appendix

Table 5. 1 Least square (LS) means (± SE) of log 2 fold change values for gene expression in the preliminary study [1]

	Ergotamine		Sulforaphane	
Gene symbol[2]	LS means	SE	LS means	SE
Phase I genes				
Cyp1a2	-0.14	0.77	0.48	0.47
Cyp3a41	-0.04	0.09	-0.11	0.42
Cyp3a44	**1.29***	0.35	0.18	1.13
Fmo1	**-0.30***	0.03	-0.36	0.30
Phase II genes				
Gsta2	-0.14	0.71	0.80	1.03
Gsta3	-0.13	0.28	0.12	0.41
Gstm1	-0.57	0.97	0.78	3.36
Ugt1a1	0.05	0.74	0.66	0.65
Sult2a2	-0.04	0.83	0.69	0.92
Sult5a1	-1.33	0.91	-1.03	0.45
Ephx1	-0.49	0.40	0.00	0.00
Nq02	-0.21	0.13	-0.18	0.19
Comt1	**-0.52***	0.13	-0.55	0.22
Nat2	-0.48	0.29	-0.41	0.16
Nrf2	-0.23	0.19	0.00	0.00

[1]Values reflect log 2 fold differences in expression relative to control. Positive values infer up-regulation and negative values infer down regulation of a gene. An asterisk (*) indicates effect was highly significant ($P < 0.05$). Expression of Cytochrome P450 1a1 (Cyp1a1) and Multiple drug resistance protein 1 (Mrp1) were undetectable and hence not reported. [2]See Table 3.1 for gene description.

Table 5. 2 Least square (LS) means (± SE) of log 2 fold change values for gene expression in the longitudinal study on d 2 and 5 [1]

Gene symbol[2]	Day 2				Day 5			
	Ergotamine		Sulforaphane		Ergotamine		Sulforaphane	
	LS means	SE	LS means	SE	LS means	SE	LS means	SE
Phase I genes								
Cyp1a2	-0.02	0.34	0.17	0.24	0.37	0.17	**0.39***	0.10
Cyp3a41	0.14	0.14	0.11	0.24	-0.10	0.07	-0.01	0.16
Cyp3a44	-0.15	1.24	-0.16	0.97	-0.50	0.35	0.78	0.44
Fmo1	0.25	0.28	0.05	0.30	0.11	0.07	0.10	0.16
Phase II genes								
Gsta2	-0.57	0.48	-0.46	0.41	-0.15	0.22	-0.11	0.14
Gsta3	-0.18	0.09	-0.07	0.22	-0.05	0.09	-0.10	0.22
Gstm1	-0.02	0.18	0.52	-0.37	-0.09	0.16	0.32	0.20
Ugt1a1	-0.27	0.21	-0.17	0.19	0.03	0.23	0.37	0.22
Sult2a2	0.29	0.64	0.52	0.43	-0.38	0.76	-0.59	0.89
Sult5a1	0.12	0.24	0.04	0.42	0.76	0.57	0.70	0.88
Ephx1	0.29	0.29	0.56	0.41	-0.09	0.19	-0.11	0.15
Nq02	0.15	0.12	0.06	0.14	0.15	0.08	**0.27***	0.05
Comt1	0.02	0.21	-0.08	0.16	-0.13	0.10	0.13	0.16
Nat2	0.04	0.09	0.64	0.38	0.03	0.15	-0.09	0.21
Nrf2	0.04	0.19	-0.21	0.21	-0.04	0.04	**0.22***	0.06

[1]Differential expression of genes is in comparison with the control group having a value of zero. An asterisk (*) indicates effect was significant ($P < 0.05$). Expression of Cytochrome P450 1a1 (*Cyp1a1*) and Multiple drug resistance protein 1 (*Mrp1*) were undetectable and hence not reported.

[2]See Table 3.1 for gene description.

Table 5. 3 Least square (LS) means (± SE) of log 2 fold change values for genes that were not differentially expressed in the longitudinal study on d 8 and 11 [1]

	Day 8				Day 11			
	Ergotamine		Sulforaphane		Ergotamine		Sulforaphane	
Gene symbol[2]	LS means	SE	LS means	SE	LS means	SE	LS means	SE
Phase I gene								
Cyp1a2	0.17	0.23	0.03	0.09	0.08	0.10	0.25	0.13
Cyp3a41	0.15	0.13	0.12	0.16	-0.23	0.14	-0.10	0.09
Cyp3a44	-0.41	0.75	-0.08	1.31	-1.64	1.27	-0.53	0.63
Fmo1	0.13	0.20	0.04	0.21	0.06	0.10	0.27	0.08
Phase II gene								
Gsta2	-0.12	0.53	0.88	0.39	-0.03	0.32	0.56	0.33
Gsta3	0.13	0.11	-0.04	0.13	0.10	0.07	0.21	0.15
Gstm1	0.21	0.13	**0.63***	0.22	0.28	0.11	**0.60***	0.17
Ugt1a1	0.28	0.18	0.03	0.11	-0.10	0.08	0.13	0.15
Sult2a2	-0.92	1.59	0.84	1.32	-0.60	1.12	0.11	0.57
Sult5a1	0.17	0.38	-0.68	0.28	**-1.23***	0.37	-0.43	0.37
Ephx1	0.06	0.24	0.19	0.32	-0.35	0.22	-0.05	0.20
Nq02	0.12	0.17	0.03	0.08	0.06	0.11	0.02	0.09
Comt1	**0.22***	0.05	-0.09	0.20	-0.02	0.11	0.05	0.19
Nat2	-0.28	0.18	-0.25	0.18	-0.01	0.09	-0.02	0.19
Nrf2	0.23	0.13	0.06	0.10	-0.08	0.16	-0.08	0.14

[1]Differential expression of genes is in comparison with the control group having a value of zero. An asterisk (*) indicates effect was highly significant ($P < 0.05$). Expression of Cytochrome P450 1a1 (*Cyp1a1*) and Multiple drug resistance protein 1 (*Mrp1*) were undetectable and hence not reported.

[2]See Table 3.1 for gene description.

Table 5. 4 Quinone reductase 01 and glutathione-S-transferase activities of control and treatments (± SE) for preliminary and longitudinal studies [1]

	Quinone reductase 01			Glutathione –S- transferase		
Study	C ± SE	EGT ± SE	SFN ± SE	C ± SE	EGT ± SE	SFN ± SE
Preliminary						
	18.51 ± 1.46	20.08 ± 1.48	25.40 ± 2.95	0.24 ± 0.03	0.31 ± 0.1	0.30 ± 0.06
Longitudinal						
Day 2	10.67 ± 0.83	12.56 ± 0.28	10.31 ± 1.38	0.25 ± 0.02	0.27 ± 0.01	0.24 ± 0.02
Day 5	9.93 ± 1.02	9.69 ± 0.61	10.79 ± 0.81	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.02
Day 8	8.49 ± 0.43	7.38 ± 0.4	8.96 ± 0.44	0.32 ± 0.03	0.32 ± 0.03	0.38 ± 0.04
Day 11	8.72 ± 0.41	8.54 ± 0.47	8.41 ± 0.59	0.41 ± 0.05	0.45 ± 0.02	0.47 ± 0.04

[1] Designations are C = Control, EGT = Ergotamine, SFN = Sulforaphane.

Vita

Smitha Boorgula was born and brought up in Hyderabad, India. She was selected for pursuing Bachelor of Veterinary Sciences and Animal Husbandry in College of Veterinary Sciences, Rajendra Nagar, Hyderabad, in 1998. She pursued her degree and worked as a Veterinary Assistant Surgeon in Medak District for three months in 2004. She then moved to Virginia Tech in spring of 2005 to pursue her Masters. After her graduation she is interested to expand her knowledge in the field of statistical analysis, experimental design and molecular genetics.