Title
Xenobiotic regulation of Phase I and Phase II metabolism enzymes: beyond the Ah receptor paradigm

Permalink
https://escholarship.org/uc/item/5nz70459

Author
Bonzo, Jessica A.

Publication Date
2007

Peer reviewed|Thesis/dissertation
Xenobiotic Regulation of Phase I and Phase II Metabolism Enzymes:
Beyond the Ah Receptor Paradigm

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences

by

Jessica A. Bonzo

Committee in charge:
Professor Robert H. Tukey, Chair
Professor Daniel J. Donoghue
Professor Ronald M. Evans
Professor Michael Karin
Professor Julian I. Schroeder

2007
Copyright

Jessica A. Bonzo, 2007

All rights reserved.
The Dissertation of Jessica A. Bonzo is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007
Dedication

To my parents, Roy T. and Flora E. A. Bonzo.
# Table of Contents

Signature Page ................................................................. iii

Dedication .......................................................................... iv

Table of Contents .............................................................. v

List of Abbreviations .......................................................... vii

List of Figures ................................................................. xi

List of Tables ....................................................................... xiv

Acknowledgements ........................................................... xv

Vita .................................................................................... xix

Abstract of the Dissertation ............................................... xxiii

## Chapter I

**Introduction**

Historical Perspective on Phase I and Phase II Enzymes ........ 1
Xenobiotics and CYP and UGT Induction ............................ 11
The Ah Receptor Regulates Cytochrome P450 1A1.............. 16
Structure and Signal Transduction Pathway of the Ah Receptor... 18
Regulation of Ah Receptor Function ..................................... 22
Objectives of the Dissertation ............................................. 25

## Chapter II

**Materials and Methods** .................................................. 29

## Chapter III

**Results**

Effects of As$^{3+}$ on Apoptosis and Cell Cycle ..................... 48
Inhibition of CYP1A1 Induction During Cell Cycle Arrest ........ 52
As$^{3+}$ Treatment Blocks TCDD Induction of CYP1A1 .......... 54
The Actions of As$^{3+}$ on TCDD-Induced Transcriptional control of CYP1A1 .......................................................... 58
Gene and Ligand Dependence of As$^{3+}$ Inhibition of Ah Receptor Responsive Genes ........................................ 64
Chrysin Regulates the UGT1A1 Gene Through a XRE ........... 68
Effects of Chrysin on TCDD Binding to the Ah Receptor ....... 71
The Role of the Ah Receptor in Chrysin Induction of UGT1A1 ..... 75
Chrysin Activation of the UGT1A1 Promoter Occurs Through the MAP kinase Pathway ................................................................. 85
Actions of Oral Chrysin Treatment in Transgenic UGT1 (Tg-
UGT1) Mice .................................................................................. 87
Regulation of UGT1A6 by the Xenobiotic-Sensing Nuclear
Receptors PXR and CAR ................................................................. 91
Regulation of UGT1A6 by PPARα .................................................. 97
UGT1As and the High Fat Diet Model of Type 2 Diabetes ............. 101
High Fat Diet Influences on UGT1A Isoform Expression ............. 108

Chapter IV
Discussion
Summary ....................................................................................... 112
As³⁺ and CYP1A1 ........................................................................... 113
Chrysin and UGT1A1 ................................................................. 120
Nuclear Receptor Control of UGT1A6 Expression .................... 125
Implications for UGT1A Enzymes In Metabolic Syndrome ........ 132
Conclusions and Future Work ...................................................... 142

References .......................................................................................... 147
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ah</td>
<td>aryl hydrocarbon</td>
</tr>
<tr>
<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
</tr>
<tr>
<td>Arnt</td>
<td>Aryl hydrocarbon nuclear translocator</td>
</tr>
<tr>
<td>As&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>arsenite, inorganic trivalent arsenic</td>
</tr>
<tr>
<td>B[a]P</td>
<td>benzo[a]pyrene, 3,4-benzpyrene</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CN-I, -II</td>
<td>Crigler-Najjar Syndrome Type I or Type II</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRE</td>
<td>dioxin response element</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1 or 2, p44/42</td>
</tr>
<tr>
<td>EROD</td>
<td>ethoxyresorufin-O-deethylase</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehydes-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GI tract</td>
<td>gastrointestinal tract</td>
</tr>
<tr>
<td>GPR40</td>
<td>G-coupled protein receptor 40</td>
</tr>
<tr>
<td>GRDBD</td>
<td>glucocorticoid receptor DNA binding domain</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>GTT</td>
<td>glucose tolerance test</td>
</tr>
<tr>
<td>HAH</td>
<td>halogenated aromatic hydrocarbon</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HF</td>
<td>high-fat diet</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatic nuclear factor</td>
</tr>
<tr>
<td>hnRNA</td>
<td>heteronuclear RNA</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
</tr>
<tr>
<td>JNK1/2</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LXRα</td>
<td>liver x receptor α</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK ERK kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium</td>
</tr>
<tr>
<td>NEFA</td>
<td>non-esterified free fatty acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H:quinone oxidoreductase 1</td>
</tr>
<tr>
<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drug</td>
</tr>
</tbody>
</table>
PAH  polycyclic aromatic hydrocarbon
PARP  poly(ADP-ribose)polymerase
PAS  Per-Arnt-Sim
PCR  polymerase chain reaction
PD98059  2'-amino-3'-methoxyflavone
PKC  protein kinase C
polII  RNA polymerase II
PMSF  phenylmethylsulfonyl fluoride
PPARα, γ, δ peroxisome proliferator-activated receptor α, γ, δ
PPRE  PPAR response element
PXR  pregnane x receptor
RT-PCR  reverse transcriptase-polymerase chain reaction
SB203580  4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole
SNP  single nucleotide polymorphism
SP600125  1,9-pyrazoloanthrone
2,4,5-T  2,4,5-trichlorophenoxyacetic acid
TCDD  2,3,7,8-tetrachlorodibenzo-p-dioxin
TCDF  2,3,7,8-tetrachlorodibenzo-furan
TNFα  tumor necrosis factor α
UDP-GA  UDP-glucuronic acid
UGT/UDPGT  UDP-glucuronosyltransferase
UO126  1,2-diamino-2,3-dicyano-1,4-bis[2-aminophenylthiol] butadiene
WT  wild type
WY-14643  4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid
XAP2  hepatitis B virus X-associated protein 2
XRE  xenobiotic response element
List of Figures

Chapter I

Figure 1: Organization of the human UGT1 locus .................. 7
Figure 2: Ah receptor signal transduction pathway ................. 19

Chapter III

Figure 3: Cell viability, apoptosis, and cell cycle arrest after As$^{3+}$ exposure ........................................ 51
Figure 4: G2/M arrest inhibits CYP1A1 mRNA expression but not transcriptional activation .................... 53
Figure 5: CYP1A1 EROD activity, protein, and mRNA expression as a function of As$^{3+}$ treatment ............ 56
Figure 6: As$^{3+}$ inhibition of CYP1A1 induction in transgenic CYP1A1N$^{+/−}$ primary hepatocytes ............... 58
Figure 7: Activation of Ah receptor is unaffected by the presence of As$^{3+}$ ................................................. 59
Figure 8: Ah receptor nuclear translocation and DNA binding activity is unchanged by As$^{3+}$ treatment .... 60
Figure 9: Transcriptional control of CYP1A1 ......................... 63
Figure 10: Identification of As$^{3+}$ inhibition region on CYP1A1 promoter .................................................... 64
Figure 11: Inhibition of chrysin-mediated CYP1A1 induction by As$^{3+}$ ............................................................. 65
Figure 12: Induction of NQO1 by TCDD and As$^{3+}$ ................. 66
Figure 13: Differential regulation of UGT1A1 in response to TCDD and chrysin mixtures with As$^{3+}$ .......... 68
Figure 14: Chrysin induction of TCDD responsive genes .......... 70
Figure 15: Identification of the chrysin responsive region within the UGT1A1 promoter ............................... 71
Figure 16: Chrysin alters TCDD binding affinity to the Ah receptor. 73
Figure 17: Differential effects of TCDD and chrysin mixtures……… 75
Figure 18: Confirmation of Ah receptor/Arnt-binding complex on CYP1A1 and UGT1A1 XREs……………………………………… 76
Figure 19: Effects of chrysin on Ah receptor activation………………. 80
Figure 20: Transactivation of the Ah receptor by chrysin and TCDD 82
Figure 21: siRNA knockdown of Ah receptor………………………… 84
Figure 22: U0126 activates the Ah receptor………………………… 85
Figure 23: Involvement of MAP kinase pathway in chrysin induction of UGT1A1……………………………………….. 87
Figure 24: The actions of oral chrysin treatment…………………… 90
Figure 25: Induction of UGT1A6 in Tg-UGT1 primary hepatocytes. 93
Figure 26: UGT1A6 promoter constructs and previously characterized response elements…………………………………. 94
Figure 27: Preliminary identification of PXR and CAR responsive regions on the UGT1A6 promoter…………………………… 95
Figure 28: Identification of the PXR responsive region in the UGT1A6 promoter……………………………………………… 97
Figure 29: PPARα is functional in the presence of UGT1A6 DR1 sequence…………………………………………………… 100
Figure 30: Increased weight in response to a high fat diet……………. 104
Figure 31: Increased adiposity in high fat diet mice………………….. 105
Figure 32: High fat diet Tg-UGT1 male mice respond normally to insulin…………………………………………………….. 106
Figure 33: Tg-UGT1 male mice are resistant to high fat diet-induced hyperinsulinemia……………………………………… 107
Figure 34:  High fat diet decreases UGT1A protein expression……. 109
Figure 35:  Down-regulation of UGT1A mRNA in response to high fat diet…………………………………………………………. 110

Chapter IV

Figure 36:  Predicted nuclear receptor binding sites within the 5’ regulatory DNA of human $UGT1A6$…………………. 126
Figure 37:  Proposed model of UGT1A modulation of high fat diet-induced hyperinsulinemia……………………………… 142
List of Tables

Chapter II

Table 1: Oligonucleotides used for RT-PCR analysis…………… 37

Chapter III

Table 2: Regulation of human UGT1A6 through various transcription factors…………………………………… 91
Acknowledgements

Foremost, I thank my graduate advisor Dr. Robert H. Tukey for providing the guidance and support that has made me the scientist I am today. His enthusiasm for each new piece of data motivated me and his creativity opened my eyes when I could not see the story to be told. As well as being a professional mentor, he opened his home life to me which eased the loneliness of being away from my family and closest friends. I am also grateful for the opportunities afforded to me by Dr. Tukey to attend many professional meetings within the United States and abroad. Interacting with other scientists in our field has made me even more appreciative of the resources and experience available within the Tukey laboratory and UCSD community. I am also honored by the mentorship of Dr. Alain Bélanger, Professor at Laval University. His uncompromising belief in my scientific skills reaffirms that these long, hard years were worth it. He has also reminded me to appreciate the small pleasures; you may not always be able to walk on the beach on a warm, sunny day in February. I also thank my thesis committee members (Dr. Daniel J. Donoghue, Dr. Ronald M. Evans, Dr. Michael Karin, and Dr. Julian I. Schroeder) for giving their time to provide helpful suggestions.

The Tukey laboratory has been my second family for the past six years. As well as being terrific scientific colleagues, they have all offered friendship. Foremost, I thank Dr. Alema Galijatovic-Idrizbegovic for taking me under her wing and being an excellent role model. I might still be looking for my first project if it was not for her. Dr. Shujuan Chen and Dr. Mei-Fei Yueh have both provided me with a wealth of
experimental techniques and scientific discussion. They have also taught me an
appreciation for fine Asian cuisine. Aphone (Kathy) Seneko-Effenberger performed
the in vivo analysis of UGT1A regulation by PPARα as part of her Master thesis. I
could not have conducted the high fat diet study without Kathy’s assistance and she
should be credited for her participation should the study be published. On a personal
note, I thank Kathy and her husband Andrew for encouraging me to push my limits.
Erin Brace-Sinnokrak, Daniel Machemer, and Theresa Operaña shared the joys and
pains of the BMS graduate program with me. I look forward to reminiscing with them
about the “good” old times while sitting on the balcony of one of our many vacation
homes. Thank you to Deirdre Beaton LaPlaca for teaching me the skill of mouse
husbandry. And thank you to Nghia Nguyen who can do everything for you if you ask
politely.

I am indebted to my parents for providing me with the education of my
choosing. I also thank them for supporting my move to California to pursue my
academic ambitions. It was equally hard letting go. Traits inherited from both of my
parents made my scientific career possible: dad’s tinkering and mom’s logic. Thanks
Bryce for being there when I need you. Thank you to my extended William and Mary
family: Lisa, Jenn, Sam, and the Nicholson crew. You hung in there and provided me
with much needed support during the rough times and fun during the easy times.

Publications arising from this work are as follows:

Portions of Chapters II, III, and IV on the interaction of As^{3+} and TCDD
include material as published in Molecular Pharmacology 2005, Bonzo JA, Chen S,
Galijatovic A, Tukey RH. Arsenite inhibition of CYP1A1 induction by 2,3,7,8-
tetrachlorodibenzo-\textit{p}-dioxin is independent of cell cycle arrest. 67(4): 1247-1256. I was the primary investigator and author of this article.

Portions of Chapters II, III, and IV on the mechanism of chrysin induction of \textit{UGT1A1} include material as published in \textit{Hepatology} 2007, Bonzo JA, Belanger A, Tukey RH. The role of chrysin and the Ah receptor in induction of the human \textit{UGT1A1} gene in vitro and in transgenic \textit{UGTI} mice. 45 (2): 349-60. I was the primary investigator and author of this article.

The identification of the PPAR\(\alpha\) responsive element on the \textit{UGT1A6} promoter (Chapter III: Fig. 29) is reprinted from \textit{Drug Metabolism and Disposition} 2007, Senekoe-Effenberger K, Chen S, Yueh MF, Brace-Sinnokrak E, \textbf{Bonzo JA}, Argikar U, Kaeding J, Trottier J, Remmel RP, Ritter JK, Barbier O, Tukey RH. Expression of the human \textit{UGTI} locus in transgenic mice by Wy-14643 and implications on drug metabolism through PPAR\(\alpha\) activation. 35 (3): 419-27. I identified the PPRE sequences, constructed the PPRE2 luciferase vector, performed the luciferase experiments, and assisted with many of the in vivo experiments. The PPRE electrophoretic mobility shift assays (Fig. 29 B and C) were performed by Jenny Kaeding and Jocelyn Trottier in the laboratory of Dr. Olivier Barbier (CHUL Research Center, Laval University, Quebec, Canada).

Figure 18 (Chapter II I) is reprinted from \textit{Methods in Enzymology} 2005, Yueh MF, \textbf{Bonzo JA}, Tukey RH. The role of Ah receptor in induction of human UDP-
glucuronosyltransferase 1A1. 400: 75-91. I conducted this experiment and authored the accompanying text for this figure.
2005, Chen S, Operana T, **Bonzo J**, Nguyen N, Tukey RH. Erk kinase inhibition leads to induction of cellular Ah receptor levels. Implications for transcriptional activation and protein degradation. **280** (6): 4350-4359. I performed the electromobility shift experiment and participated in discussions in support of this article.
Vita

EDUCATION:

2001-2007 Ph.D. – Doctor of Philosophy (Biomedical Sciences Graduate Program), University of California San Diego, La Jolla, CA

1997-2001 B.S. – Bachelor of Science (Biology), College of William and Mary, Williamsburg, VA

FUNDING:

09/01/06-09/01/07 UCSD Chancellor’s Collaborations Graduate Student Fellowship. *Integrated Cost-Benefit Assessment of Biodiesel.*

07/01/02-09/01/07 Superfund Basic Research Program-Graduate Student Training Core

RESEARCH EXPERIENCE:

Sep 2001-Jun 2007 **PhD Student:** “Regulation of drug metabolizing enzymes: Effects of xenobiotics on expression of CYP1A1 and UGT1A proteins” supervised by Robert Tukey, PhD, Professor, Departments of Pharmacology and Chemistry/Biochemistry, University of California San Diego, La Jolla, CA.


Jan 2000-May 2001 **Undergraduate Biology Student,** College of William and Mary, Williamsburg, VA. Thesis Project: “Individual Variation in Photoperiodic Reproduction in *Peromyscus leucopus*” supervised by Paul Heideman, PhD, Professor, College of William and Mary, Williamsburg, VA.


**TEACHING EXPERIENCE:**

March 2003-June 2004 **Teaching Assistant** (Part Time) in undergraduate pharmacology and toxicology class. Department of Chemistry & Biochemistry, University of California, San Diego, La Jolla, CA.

**HONORS AND AWARDS:**

2006 ASPET Graduate Student travel award to the IUPHAR2006 meeting in Beijing, China
2006 ASPET Annual Meeting-Division of Drug Metabolism Best Graduate Student Poster Award: First Place
2006 ASPET Annual Meeting Graduate Student Travel Award
2005 ASPET Annual Meeting-Division of Molecular Pharmacology Best Graduate Student Poster Award: First Place
2004 Society of Toxicology Graduate Student Travel Award
2003 Outstanding Graduate Student Poster Award, Southern California Chapter of The Society of Toxicology: First place

**PUBLICATIONS:**


**INVITED PRESENTATIONS:**


**ABSTRACTS:**

1. Bonzo JA, Seneko-Effenberger, K, Tukey RH. Transgenic UDP-Glucuronosyltransferase 1 Mice are Protected from Obesity-Induced Type II Diabetes. 15th World Congress of Pharmacology in Beijing, China, July 2006.


ABSTRACT OF THE DISSERTATION

Xenobiotic Regulation of Phase I and Phase II Metabolism Enzymes:
Beyond the Ah Receptor Paradigm

by

Jessica A. Bonzo

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2007

Professor Robert H. Tukey, Chair

Phase I and Phase II metabolizing enzymes assist in the removal of endogenous and exogenous chemicals by increasing their hydrophilicity and thereby facilitating excretion from the body. One of the primary responses to exposure to the prevalent environmental carcinogens polycyclic and halogenated aromatic...
hydrocarbons (PAH/HAH) is activation of the aryl hydrocarbon (Ah) receptor to increase expression of the Phase I enzyme cytochrome P450 1A1 (CYP1A1) and the Phase II enzyme UDP-glucuronosyltransferase 1A1 (UGT1A1). In addition to these carcinogens, humans are exposed to many other substances which when combined, present a more complicated mechanism of carcinogenesis that may not be readily identified using the classically understood Ah receptor pathway. Using two different chemical induction models, we demonstrate further levels of complexity of Ah receptor-mediated transcription. First, I sought to elucidate the mechanism of As$^{3+}$ inhibition of TCDD-mediated CYP1A1 induction. As$^{3+}$ interferes with TCDD/Ah receptor-mediated recruitment of basal transcription factors to the CYP1A1 promoter but does not impair the basic functionality of the Ah receptor. The second model examined was induction of UGT1A1 by a new class of Ah receptor activators, the flavonoids. The flavonoid chrysin is shown to be a more effective inducer of the UGT1A1 gene than the most potent Ah receptor ligand, TCDD. We demonstrate that chrysin, as an Ah receptor ligand, induces CYP1A1 expression. In contrast, the Ah receptor is minimally involved in chrysin induction of UGT1A1 expression. This suggests that the role of the Ah receptor differs in accordance with the type of ligand and target gene.

In addition to Ah receptor regulation, nuclear receptors play an important role in UGT1A inducibility. As many of these receptors play key roles in disease, the effect of high fat diet-induced type 2 diabetes on UGT1A expression was examined in Tg-UGT1 mice. Unexpectedly, Tg-UGT1 while increasing adiposity do not develop the expected phenotype of hyperinsulinemia and glucose and insulin intolerance.
suggesting that overexpression of UGT1A proteins is protective against the early phases of type 2 diabetes. Paradoxically, human UGT1A expression was downregulated in high fat diet mice indicating that adiposity negatively impacts on UGT1A expression.
Chapter I

INTRODUCTION

Historical Perspective on Phase I and Phase II Enzymes

The human body is exposed to a constant flux of endogenously and exogenously synthesized chemical compounds. Many of these compounds are lipophilic to facilitate diffusion through the cell membrane to their effector sites. Processes that render compounds more hydrophilic evolved to facilitate the removal of these compounds from cells and the organism as a whole. In mammals, these processes have been classified into a three-tiered system of metabolism and transport. Phase I metabolism which is characterized by small chemical modifications such as hydroxylation, oxidation, and peroxidation to form hydrophilic reactive groups that lend the compound to excretion in the urine or as a substrate for further modification by Phase II metabolism. Phase II metabolism is characterized by conjugation of large, bulky groups such as glucuronic acid, glutathione, or sulfate to reactive moieties thereby significantly increasing the chemical’s molecular weight and water solubility. Phase III is the collection of transport proteins such as the P-glycoprotein, multidrug resistance-associated proteins, and organic anion transporters that help with absorption, distribution, and elimination of compounds. The development of these systems of chemical maintenance have been critical for the survival of cellular organisms. The Phase I enzyme family of cytochrome P450s (CYPs) have been identified in all eukaryotes studied as well as some prokaryotes, suggesting primitive metabolism enzymes have been present for 1.5 billion years (1;2).
The family of cytochrome P450 monooxygenases participates in the formation of hydroxylated intermediates during steroidogenesis and metabolizes approximately 55% of all pharmaceutical drugs (3;4). CYPs were originally identified as a yellow pigment present in liver microsomes that presented a spectral peak at 450 nM when bound to atmospheric oxygen (5;6). In 1962, Omura and Sato (7) coined the term cytochrome P450 to distinguish this new enzyme from cytochromes b5 and later proved that it is a heme-containing enzyme (8). CYPs are distinct from cytochromes a, c, and b5 in that they complex with molecular oxygen and insert this single atom of oxygen into an organic substrate. This process requires electron donation from NADPH cytochrome P450 reductase. The originally identified function of CYPs was the oxidation of steroids (9-11). It was well known before this time that drugs were oxidized by liver microsomes to more water-soluble metabolites (12). The concept that CYPs may be important in oxidation of pharmaceutical drugs was put forth by Kato in 1966 (13). To date, there are over 6700 identified CYPs spanning the prokaryotic to animal kingdoms (html://drnelson.utmem.edu/CytochromeP450.html). There are 57 human CYPs divided among 17 families which metabolize a wide range of substrates from exogenous chemicals also called xenobiotics to endogenous sterols, fatty acids, eicosanoids, and vitamins (reviewed in (14)). The original association of CYPs with steroidogenesis is somewhat overshadowed today by the role of CYPs in xenobiotic metabolism and pharmaceutical drug clearance in particular. CYPs are abundantly expressed in the organs of detoxification (liver and kidney) as well as organs of primary exposure including the lung and small intestine (14). Significant
protein expression and activity is not detectable until 1 month after birth (14). The major CYPs involved in endogenous chemical synthesis include 4A11 (fatty acids), 1B1, the 11Bs, 21A2 and other higher numbered CYPs (sterols), the 4Fs (eicosanoids), and the 26A and Bs (vitamins). The majority of pharmaceutical drug metabolites are formed from the actions of CYP3A4 followed by 2C9, 2D6, 2C19, and 2E1. Located on human chromosome 15 and sharing its 5′ regulatory region with CYP1A2, CYP1A1 is the only P450 not basally expressed in normal, unstimulated adult human tissues (reviewed in (15;16)). Reports have identified CYP1A1 mRNA and protein in stimulated peripheral blood cells and tumor tissues as well as tissues exposed to cigarette smoke such as the lung (17-23). CYP1A1 is highly inducible in all tissues studied, particularly the lung and liver, by the environmental contaminants associated with burning of fossil fuels and cigarettes (15). These xenobiotics are also metabolized by CYP1A1 to their ultimate carcinogenic forms. Today, in toxicological screens, a compound that activates CYP1A1 transcription is considered to have indirect carcinogenic potential due to increased CYP1A1 activity towards procarcinogens that may be present in the whole organism (24).

Coupled to the generation of these potentially harmful hydroxylated metabolites is inactivation by conjugation with glucuronic acid to form easily excretable glucuronides. The first isolation of a glucuronide, the dye Indian yellow, is attributed to Schmidt in 1855 who fed mango leaves to cows and isolated compounds from their urine (25;26). The major site of production of glucuronides was isolated to the liver by Lipschitz and Bueding (27). The active factor necessary for the formation of these glucuronides was discovered by Dutton and Storey in 1953 to be UDP-
glucuronic acid (UDP-GA) (28;29). The glycosyltransferase responsible for the transfer of glucuronic acid to an aglycone, UDP-glucuronosyltransferase (UGT or UDPGT), was localized to the microsomal fraction of the cell (30;31). UGTs were postulated to face the luminal side of the endoplasmic reticulum (32), a finding later confirmed by Shepherd et al in 1989 (33). During this time, glucuronides formed by UGT activity towards many endogenous hormones, steroids, and thyroxins, as well as the xenobiotics morphine, paracetamol, chloramphenicol, phenobarbital, and polycyclic aromatic hydrocarbons (PAHs) were isolated from blood and urine of laboratory animals and humans (reviewed in (25)). Perhaps the most important role of UGT activity in humans was discovered in 1956 with the identification of bilirubin glucuronide as the sole excretable metabolite of bilirubin (34-36). Bilirubin is a toxic breakdown product of hemoglobin which, when left unbound, causes unconjugated hyperbilirubinemia that can lead to neural toxicity in extreme cases.

Studies by Axelrod et al demonstrated the common feature of the UGT substrates to be a nucleophilic functional group (37). They and others postulated that a single UGT enzyme with a promiscuous active site was responsible for the transfer of the UDP moiety to the substrate (37;38). While, to other researchers, the functional heterogeneity of the UGT enzyme suggested that there were many transferases, each with its own substrate specificity (reviewed in (25)). It was not until Gorski and Kasper developed the technique of UGT purification by affinity chromatography with UDP-hexanolamine Sepharose that the question of heterogeneity could be definitively addressed (39). They succeeded in purifying to homogeneity a phenobarbital-inducible UGT that conjugated p-nitrophenol whereas others had been unable to purify stable,
functional UGTs. In the same year, Burchell also published the purification of UGTs to homogeneity using the method developed by Gorski (40). Furthermore, Gorski and Kasper (41) as well as Tukey et al (42) established the dependence of UGT activity on the presence of membrane phospholipids which confirmed earlier reports of UGTs integrated into the endoplasmic reticulum membrane. With a method to purify homogeneous, active UGTs established, inducible UGTs such as the 3-methylcholanthrene (3-MC) inducible UGT (43) were purified as well as the substrate-specific UGTs for oestrone (44) and testosterone (45). The purified UGTs displayed different substrate specificities strengthening the proposal that the functional heterogeneity results from a heterogeneous population of UGTs. In line with this general reasoning, Bock et al (46) and Burchell (47) independently proposed UGT nomenclature based on substrate and inducibility. Some of the more commonly referred to isoenzymes included bilirubin UDPGT, 4-nitrophenol UDPGT, phenol UDPGT, oestrone UDPGT and androsterone UDPGT and were classified as phenobarbital or 3-MC inducible (reviewed in (48)).

The generation of purified UGTs allowed for resolution of the question of UGT heterogeneity. An antibody raised against the purified UGTs was used to immunoprecipitate nascent proteins still translating from polysomes, resulting in the isolation of the first mouse UGT mRNAs (49). This technique was then independently used by two laboratories to isolate three rat liver UGT mRNAs (50;51). The mRNAs were divergent enough to suggest that there were multiple UGT isoforms within at least two distinct families and finally provided direct evidence for the heterogeneity of UGTs. In 1986, the first full-length cDNAs of rat UGTs were sequenced for the
phenobarbital-inducible isoforms today known as rUGT2B1 (52) and rUGT2B4 (53), androsterone UDPGT (rUGT2B2), the 17β-hydroxysteroid UDPGTs rUGT2B3 and rUGT2B5 (53) and the 3-MC inducible 4-nitrophenol UDPGT (rUGT1A6)(54). In 1987, Jackson et al cloned the first human UGT, UGT2B4 (55). A plethora of rat, mouse, and human UGT cDNAs soon followed and in 1991 the first in a series of nomenclature guidelines was issued (56).

With the isolation of many of the UGT isoforms, the high sequence homology (>80-95% in some cases) clearly delineated two gene families: the UGT1 and UGT2 families. The human UGT2 family resides in a gene cluster on chromosome 4q13 that encodes 7 functional UGT2B proteins as well as 3 UGT2A genes that have not been functionally characterized (57). Each gene consists of a promoter and six exons. The UGT2B gene cluster is similar to that seen with many of the CYP gene families (58). The activities of the UGT2B enzymes can be broadly assigned to metabolism of endogenous hormones, steroids, and fatty acids. The UGT1 gene family on human chromosome 2q37 evolved a unique organization distinct from the UGT2 gene cluster. In 1992, Ritter et al, after analyzing the cDNAs of several UGTs, concluded that the UGT1A family was encoded by a novel locus resulting from multiple gene duplication events (59). This locus contains six unique exons 1 with their accompanying 5’ regulatory region that are spliced to commonly shared exons 2-5 (59). This model was later refined to expand the locus to a 220 kb region with 13 unique exons 1, 4 of which are pseudogenes (60) (Fig. 1). This genomic organization produces UGT1A proteins which are composed of a variable amino region (exon 1) and an identical carboxyl region (exons 2–5) (59;61). Due to the conserved nature of the carboxy terminus, it is
believed to function as the UDP-glucuronic acid binding domain while the amino terminus confers substrate specificity (62). More recently, Levesque et al reported the identification of an alternative splice variant for UGT1A1 encoded by a second exon 5 (5b) that is downstream of the originally identified exon 5 (5a) (63). This encodes a UGT1A1 protein that is truncated in the C-terminus and lacks transferase activity. The in vivo role of this new variant has yet to be established.

Figure 1. Organization of the human UGTL locus. The locus spans 220 kb on chromosome 2q37 and contains 13 cassette exons 1 and a single complement of exons 2-5. There are 9 functional UGT1A transcripts and 4 pseudogenes (p). Each functional exon 1 is under the control of its own TATA box and unique 5′ regulatory DNA which allows for the independent initiation of RNA polymerase activity. The 3′ splice site of a cassette exon 1 is directly spliced to the 5′ splice site of exon 2, treating the intervening exons 1 as intronic sequence due to the absence of splice sites on their 5′ end. This method of transcription is termed exon sharing. Adapted from Gong et al (60).

Human UGT1A expression is regulated in a strict tissue-specific manner with each tissue having a unique complement of UGT1A isoforms. The liver expresses UGT1A1, 1A3, 1A4, 1A6, and 1A9 and is notably lacking UGT1A7, 1A8 and 1A10 (64). These isoforms are unique in that they are only expressed extrahepatically.
UGT1A8 and 1A10 are expressed throughout the gastrointestinal (GI) tract, particularly in the small intestine and colon (64-66) while UGT1A7 expression appears limited to the upper GI tract (65; 67-69) and pancreas (70). The colon has the greatest number of UGT1A isoforms with 1A7 the only lacking isoform (66). Interestingly, UGT1A6 is the only UGT1A isoform identified in the brain (71). Serotonin is a specific substrate probe for UGT1A6 activity, suggesting a possible role for glucuronidation in neurotransmission (72). UGT1A5 transcripts were only recently identified through the use of transgenic mice and thus no information is available regarding its human tissue distribution (73; 74). With the exception of UGT1A1, the enzymatic activities of the UGT1A isoforms are usually associated with the glucuronidation of xenobiotics such as pharmaceutical drugs, environmental toxicants, plant-derived compounds (i.e. flavonoids), and animal-derived fats but do participate in the glucuronidation of endogenous steroids, hormones, and fatty acids (75-77).

Along with glucuronidation of various xenobiotics, UGT1A1 is the sole enzyme responsible for the detoxification of bilirubin from the body. A genetic model of unconjugated hyperbilirubinemia in the rat was discovered in 1938 and named for its identifier, Gunn (78). It was determined that these rats lacked the enzyme necessary for bilirubin glucuronide synthesis (79). This finding was confirmed in 1989 after the Gunn rat UGT1A1 cDNA sequence was shown to contain a single base pair deletion which resulted in truncation of 115 amino acids from the C-terminus (80). This mutation is located in the common exon region and thus disrupts production of all function UGT1A proteins. In humans, defects in bilirubin conjugation are borne-out as the clinical diseases of unconjugated hyperbilirubinemia: neonatal jaundice, Gilbert’s,
and Crigler-Najjar I/II (CN-I, -II). There is no significant expression of the UGT1A enzymes in the human fetus (25;81;82). Glucuronidation activity does not reach near-adult levels until 20 weeks post-natal thus putting newborns at risk for drug toxicity as well as unconjugated hyperbilirubinemia (83-86). Gilbert’s syndrome is characterized by mild, chronic unconjugated hyperbilirubinemia and considered harmless in adults but with the possibility of some adverse drug reactions. This syndrome is due to autosomal recessive inheritance of an extra TA insertion in the TATAA element of the $UGT1A1$ promoter ($A(TA)_nTAA$ is normal) that reduces $UGT1A1$ transcription up to 70% (87). The CN-I and CN-II syndromes are characterized by more severe unconjugated hyperbilirubinemia and in the case of CN-I, patients are at risk for lethal neurotoxicity. Both CN-I and CN-II result from mutations in the coding regions of all exons as well as a TA insertion in the promoter (88-91). Interestingly, it was known for many years in the clinic that administration of phenobarbital lowers unconjugated bilirubin levels in neonates as well as Gilbert’s and CN patients (92). The discovery that the nuclear receptor CAR is the transcription factor responsible for phenobarbital induction of CYP2Bs (93;94) lead to the identification of a CAR response element in the $UGT1A1$ promoter (95). Therapeutic regiments targeting UGT1A1 for treatment of the diseases of hyperbilirubinemia has made UGT1A1 perhaps the most well-characterized UGT in regard to transcriptional regulation by xenobiotics.

In addition to regulation by xenobiotics, the surge in pharmacogenetic data in the recent years has provided evidence that the UGT1A enzymes are important in cancer and drug toxicity susceptibility. There are several reviews that catalogue the known human UGT1A polymorphisms and their relation to disease and toxicity (96-
Given the location of UGTs at the interfaces for xenobiotic entry into the body (upper and lower GI tract, skin, lung), it has been proposed that UGTs in these tissues may protect against carcinogenic xenobiotics. UGT1A7, which is highly expressed in these contact tissues, is known to glucuronidate, and therefore inactivate, carcinogenic compounds from cigarette smoke (69). One study found that individuals with low-activity UGT1A7 alleles were at an 8-10 fold higher risk of orolaryngeal cancer from smoking (99). Low-activity UGT1A7 alleles are also linked to an increased risk of heptocellular carcinoma and colon cancer (86;100) as well as dietary heterocyclic amine-linked colon cancer (101). UGT1A6 is primarily involved in the metabolism of small phenols and planar arylamines and conjugates many of the non-steroidal anti-inflammatory drugs (NSAIDs). While a low-activity allele of UGT1A6 has been identified, the wild-type genotype is associated with decreased protective effects of NSAIDs (due to increased glucuronidation and thus inactivation) against colon adenomas while the low-activity allele is associated with decreased recurrence of colorectal adenoma (102;103). The UGT1A1 Gilbert’s low expressing allele (A(AT)TAA) was positively associated with breast cancer in African-American women and suggests that UGT1A1 glucuronidation of estrogens may be protective (104). Perhaps the most heavily studied case of UGT1A1 polymorphisms and drug toxicity is with the camptothecin anticancer drug irinotecan used in the treatment of colon cancer. The active metabolite of irinotecan, SN-38, causes severe toxic reactions of diarrhea and neutropenia and is cleared through glucuronidation. Several UGT1A isoforms are responsible for the production of the SN-38 glucuronide but UGT1A1 is considered the major isoenzyme involved in SN-38 conjugation (98). Several studies
found that SN-38 associated toxicities are greater in patients who are also Gilbert’s due to decreased drug inactivation (105-108). Thus, it will be useful to design therapies that could increase expression and/or activity of the UGT1A enzymes to alleviate drug toxicities and perhaps reduce the risk of cancers from environmental exposures.

**Xenobiotics and CYP and UGT induction**

The role of metabolism in protection of an organism is well defined in the case of a class of carcinogenic compounds that were originally identified as aryl hydrocarbon hydroxylase inducers. The most intensely studied of these compounds are the PAHs and halogenated aromatic hydrocarbons (HAHs). PAHs are by-products of incomplete combustion that commonly occur during coal processing as well as pyrolysis of organic material such as tobacco. The more intensely studied compound of this class is benzo[a]pyrene (B[a]P). HAHs, typified by the compound 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD), are generated as by-products of chemical synthesis of chlorophenoxyl herbicides (most widely known is Agent Orange), incineration, metal-processing, and bleaching of paper pulp.

Association of combustion processes and cancer became evident by the turn of the 20\(^{th}\) century when it was noted that chimney sweeps and workers at gasworks and tar distilleries were more likely to be afflicted with cancer (109-111). In 1915, Yamagiwa and Ichikawa initiated multiple carcinomas in the ears of rabbits by administration of coal tar (reviewed in (111)). In 1933, Cook et al isolated the carcinogenic components of coal tar and identified them as PAHs, specifically
benzpyrene (today known as B[a]P) (112). By the late 1930s, research showed that aromatic hydrocarbons produced cancer in animals and a theory proposed that these compounds could be converted into more harmful metabolites and/or be detoxified by an enzyme system (113). This concept was demonstrated by PAH induction of an unknown enzyme that decreased the carcinogenicity of 3-methyl-4-monomethylaminoazobenzene, a chemical hepatocarcinogen still used today (114;115). The same group later demonstrated that the PAH 3,4-benzpyrene (also known as B[a]P) induced synthesis of its own hydroxylase, termed benzpyrene hydroxylase (116). Nebert and Gelboin renamed this PAH-induced microsomal activity “aryl hydroxylase” to reflect the broad array of polycyclic hydrocarbons that are metabolized by the system and formally assigned this activity to a cytochrome P450 (117;118). A series of papers in 1969-1970 from Mannering’s laboratory further characterized this aryl hydroxylase as being specific to a subset of P450s termed P1-450 (known today as CYP1A1) (119;120). It was known for many years that the aryl hydroxylase generated multiple hydroxylated metabolites of B[a]P but it was not until 1972 that the carcinogenicity of these metabolites was known. Conney and Jerina initiated an ambitious project to synthesize all possible combinations of B[a]P metabolites. They determined the carcinogenic potential of each metabolite and identified the P450-generated (+)-B[a]P 7,8-dihydrodiol-9,10-epoxide-2 (BPDE-2) as the ultimate carcinogen (reviewed in (121)). Thus, PAHs induce their own metabolism and carcinogenic potential by increasing P1-450 activity. In a negative feedback loop, as the PAHs are cleared through P1-450 metabolism, P1-450 activity decreases and the generation of carcinogenic metabolites is slowed. In the absence of this feedback loop,
the potential for excessive generation of reactive and carcinogenic metabolites exists, as is seen in the case of the HAH TCDD which is not metabolized by P450s.

TCDD was first identified in the mid-1950s to 1960s through several outbreaks of chloracne in chemical plants generating the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, Agent Orange). 2,4,5-T was found to be contaminated with several dioxins and furans of which, TCDD was the most potent inducer of chloracne ((122), reviewed in (123)). TCDD is sequestered in the fat and liver and has no known metabolic pathway for elimination leading to its estimated 7 ½ years half-life in the human body (124). Acute, high dose exposures result in chloracne, wasting syndrome, immunosuppression, and in rodent models, cancer (125). As well as linking TCDD-induced hepatic porphyria to increases in δ-aminolevulinic acid synthetase (ALA) activity (126), Poland and Glover demonstrated that TCDD is 1,000-30,000 times more potent (depending on species) than 3-MC, another highly active PAH, in inducing aryl hydroxylase activity (127;128). They also introduced the concept of an “induction receptor” that recognizes PAHs and HAHs to initiate events leading to aryl hydroxylase activity.

The genetic association of aryl hydroxylase activity came from the study of various mouse strains. In 1969, Nebert and Gelboin published the aryl hyroxyxylase activity after 3-MC treatment in six mouse strains (129). They found that two strains, DBA and AKR/N, lacked induction of aryl hydroxylase activity after 3-MC treatment while C57NK, Swiss, and C3H/HEJ showed the most robust aryl hydroxylase activity induction. Through breeding experiments, Gielen et al demonstrated the autosomal dominant Mendelian inheritance of aryl hydroxylase activity and termed the genetic
region responsible for this activity, the *Ah* locus (130). Through these studies, it was determined that different mouse strains were responsive (C57BL/6N, C57BL/6J, C3H/HeN, BALB/cAnN, and CBA/HN) or non-responsive (DBA/2N, DBA/2J, AKR/N, NZW/BLN, and NZW/BLN) to PAH induction of cytochrome P450 aryl hydroxylase activity (130;131). Concurrently, Thomas *et al* reached the same conclusion of an autosomal dominant gene responsible for aryl hydroxylase activity and named it *Ahh* (132). In crosses between a responsive and a non-responsive mouse strain, they also noticed an additive effect in the hybrid animals suggesting that different alleles may exist at the *Ahh* locus (133). Robinson *et al* also concluded that multiple alleles existed and further concluded that they are at two nonlinked genetic loci (134). Thomas *et al* identified that the locus associated with the inflammatory response that leads to papillomas initiated by PAH administration to the skin, *In*, was the same as or very closely linked to the *Ahh* locus (135). Thus, the *Ah* gene product determined the carcinogenic outcome of PAH exposure. Interestingly, administration of the more potent HAH, TCDD, lead to similar induction of cytochrome P1-450 in responsive and non-responsive mouse strains (131). Since TCDD is more potent than PAHs, the authors proposed that the allele in the nonresponsive mice encodes a mutation that interferes with recognition of the less potent PAHs. In 1975, a theoretical model of cytochrome P1-450 aryl hydroxylase induction was proposed that suggested the *Ah* gene product encodes a receptor which enhances the mRNA and protein levels of cytochrome P1-450 (136).

Concurrent to the studies on PAH and HAH induction of cytochrome P1-450 aryl hydroxylase activity, the induction of other microsomal enzyme activities was
noted, including UDP-glucuronosyltransferase activity. In 1957, Conney et al noted an increase in a polar conjugate of B[a]P after administration to rats (116). Inscoe and Axelrod followed with the report of induction of hepatic glucuronosyltransferase activity in B[a]P treated rats (137). Dutton and Stevenson reported increases in transferase activity in the skin after topical B[a]P administration (138). A decade later, these experiments were revisited by Howland and Burkhalter who observed increased glucuronidation after 3-MC administration to rats (139). Induction of rat hepatic glucuronidation was demonstrated with the final classic aryl hydroxylase inducer, TCDD, by Lucier et al (140;141). In 1973, Bock et al demonstrated differential substrate specificity between the 3-MC and phenobarbital-inducible UGTs (142;143). As well as providing a method for enrichment of UGTs for kinetic analysis, this was also the start of the delineation of two UGT activity populations (discussed in the previous section). The same responsive and nonresponsive mouse strains used to identify aryl hydroxylase induction also linked induction of UGT activity by B[a]P to the Ah locus gene product (144-146). In conjunction with the finding of induction of other membrane-bound monooxygenases activities not discussed here (147), the concept of an Ah gene battery was put forth. This gene battery coupled phase I and phase II enzymes in a coordinated cellular response to facilitate the metabolic detoxification of exogenous signals. The signaling mechanism in this response was hypothesized to be a receptor encoded by the Ah locus.
The Ah Receptor Regulates Cytochrome P450 1A1

Evidence for the existence of this Ah receptor was first obtained by Poland et al in 1976 when they identified high-affinity binding of [\textsuperscript{3}H]TCDD to mouse hepatic cytosols (148). Several reports followed also confirming the existence and purification of a cytosolic factor that binds TCDD and PAHs in rodent cell culture and whole animal models (149-152). Interestingly, PAHs bind to the Ah receptor cytosols with 1/2 to 1/25 of the affinity of TCDD but are dramatically less potent in activation of aryl hydroxylase activity suggesting deactivation of PAHs contributes significantly to the reduced potency (148). Regardless of inducer, the inducer-receptor complex translocates to the nucleus (153;154) and interacts with DNA (155). These basic properties of Ah receptor action were then used to clone cytochrome P\textsubscript{1}-450.

Using [\textsuperscript{32}P]cDNA probes prepared from 3-MC responsive and nonresponsive mice (156), a cDNA library generated from cytochrome P\textsubscript{1}-450 immunoprecipitated polysomes was screened, resulting in the first isolation of specific cytochrome P\textsubscript{1}-450 mRNA (157). The human P\textsubscript{1}-450 was cloned several years later by Jaiswal et al (158). The full-length mouse cDNAs and genes for both cytochrome P\textsubscript{1}-450 and P\textsubscript{3}-450 (a second highly inducible P450 later given the name Cyp1a2) were cloned in 1984 (159-161). The identification of the specific P\textsubscript{1}-450 transcript now allowed for investigation as to the mechanism by which Ah receptor binding to PAHs or TCDD leads to P\textsubscript{1}-450 aryl hydroxylase activity.

It was determined that the appearance of the Ah receptor in the nucleus correlated to large increases in cytochrome P\textsubscript{1}-450 mRNA and aryl hydroxylase activity, directing specific gene transcription (162-164). Analysis by chloramphenicol
acetyltransferase (CAT) activity, identified regions in the 5′ regulatory DNA of the $P_1$-$450$ gene that were ascribed to have positive and negative transcriptional regulatory roles in response to TCDD (165;166). For simplicity, only the course of research dealing with the identification of Ah receptor control of mouse $P_1$-$450/Cyp1a1$ will be discussed but it should be noted that other researchers were simultaneously characterizing TCDD responses for other genes and species. These regions of positive regulation by TCDD were determined to act as transcriptional enhancers regardless of orientation or distance to the promoter and were termed dioxin responsive elements (DREs, later revised to xenobiotic response element (XRE)) (167;168). During this time, three DREs in the mouse $Cyp1a1$ promoter were identified and the core binding sequence 5′-TNGCGTG-3′ was established as the minimum sequence necessary for Ah receptor binding but other flanking nucleotides were necessary to confer enhancer activity (169-171). This core binding sequence was later revised to incorporate functional XREs identified in other promoters and also reflect those nucleotides which confer transcriptional functionality: 5′-GCGTGNN(A/T)NNN(C/G)-3′ (172;173).

Surprisingly, the transcriptional regulation of mouse $Cyp1a1$ was fairly well characterized before the cloning of the transcription factor responsible for the inductions. The mouse Ah receptor was cloned in 1992 and the human form in 1993, both in the laboratory of Bradfield (174;175). Cloning of the murine and human Ah receptor genes allowed for more detailed investigation into the structure and function of the receptor.
Structure and Signal Transduction Pathway of the Ah Receptor

The 95-106 kDa (species varying) Ah receptor transcription factor is bound in the cytosol in the unliganded state and after ligand activation, translocates to the nucleus where in a heterodimeric complex, binds to specific DNA regulatory elements to initiate transcription (Fig. 2). The basic-helix-loop-helix (bHLH) motif and PAS domain in the N-terminus classifies the Ah receptor as a member of the Per-Arnt-Sim family of regulatory proteins. Ligand binding occurs in the PAS B domain, dimerization involves both the PAS and bHLH domains, and DNA binding occurs through the basic domain (174;176). The nuclear export signal (NES) and nuclear localization signal (NLS) are also located in the N-terminus (177). The carboxyl terminus is the transcription activation domain (178). Original protein isolation studies identified the 90 kDa heat shock protein (Hsp90) in the cytosolic Ah receptor complex and by similarity to glucocorticoid receptor regulation, was presumed to mask the DNA binding domain of the Ah receptor to maintain an inactive form (179). Through analysis of mouse hepatoma cells lines that were responsive or nonresponsive to PAHs, it was determined that a second protein was necessary for the activity of the Ah receptor inside the nucleus (180;181). In 1991, this protein, the Ah receptor nuclear translocator (Arnt), was cloned and identified as a bHLH protein with homology to the Per and Sim proteins of *Drosophila* (182). Arnt was also found to be a component of the nuclear Ah receptor DNA binding complex while Hsp90 was only bound to the Ah receptor in the cytosol (183). These initial findings provided the basis for the more detailed analysis of the signaling events and cofactors necessary for Ah receptor initiated transcription in the model mouse hepatoma cell line Hepa-1.
In the cytosol, the Ah receptor is bound to dimeric Hsp90, p23, and XAP2. Hsp90 maintains the receptor in a confirmation necessary for ligand binding and in the nucleus blocks Arnt from binding to the unliganded receptor thereby preventing unregulated Ah receptor transcription-initiation (184-186). When complexed to the liganded Ah receptor, Hsp90 is shuttled to the nucleus with the Ah receptor complex but is removed prior to Arnt dimerization and DNA binding (187). The immunophilin-like hepatitis B virus associated protein 2 (XAP2; In original Ah receptor pathway papers this protein was referred to as AIP or ARA9 but is now known to be the previously cloned XAP2.) binds to both Hsp90 and the Ah receptor through its C-
terminal half and is required for Ah receptor sequestration in the cytosol. XAP2 prevents importin-β binding to the Ah receptor complex as well as ubiquitin-mediated degradation by the proteosome (reviewed in (188-190). XAP2 also regulates the association of the Hsp90-associated co-chaperone protein p23 with the cytosolic Ah receptor complex (191). The role of p23 is still not very well understood but studies suggest that it facilitates binding of the complex to the nuclear import protein pendulin (192) and its absence enhances Arnt binding to the unliganded Ah receptor indicating it is removed from the complex prior to DNA binding (193). The current model of nuclear translocation proposes that XAP2 disassociates from the Ah receptor complex prior to nuclear entry and then Hsp90 and p23 are removed just prior to dimerization with Arnt. The heterodimeric Ah receptor-Arnt complex then binds to specific DNA sequences known as dioxin/xenobiotic response elements (DRE/XRE).

Given that the Ah receptor and Arnt are members of the bHLH family of proteins it is not surprising that the minimal XRE (5′-TNGCGTG-3′) is similar to the E-box enhancer sequence (5′-CANNTG-3′) bound by most bHLH dimers. However, the XRE is unique in that it is not palindromic. Arnt recognizes the 3′ half-site GTG while the Ah receptor binds with highest affinity to the more degenerate 5′ half-site TNGC (194). As discussed in the previous section, the first XREs were identified on the mouse Cyp1a1 promoter followed soon after by the discovery of XREs in the promoters of human CYP1A1 and CYP1A2 (195;196), human CYP1B1 (197), rat glutathione S-transferase Ya subunit (198), rat NAD(P)H:quinone reductase (NQO1) (199), human and rat UGT1A1 (200;201), and human and rat UGT1A6 (202-204).
These genes comprise the Ah receptor gene battery for the coordinated response of a cell to eliminate xenobiotics through metabolism.

Studies of the transcription initiation complex recruited by the Ah receptor-Arnt heterodimer have primarily focused on the XREs present in the mouse Cyp1a1 promoter. It was determined in Hepa-1 cells using micrococcal nuclease digestion that the Cyp1a1 promoter is occupied by two nucleosomes under non-induced conditions (205;206). These two sites become accessible after TCDD-mediated recruitment of the Ah receptor-Arnt complex to the enhancer. The presence of nucleosomes in the promoter will prevent RNA polymerase II initiation of transcription (207). The polymerase may still be capable of accumulating in the promoter or cis-enhancer regions awaiting a critical signal as was demonstrated on the hsp70 gene in Drosophila melanogaster (208) and dihydrofolate reductase (Dhfr) gene in mouse (209). Detailed analysis of the mammalian α globin gene locus presents a different model of poised gene expression in that factors recruited first to far cis elements direct hyperacetylation towards the promoter at which point, when most of the locus is acetylated, the polymerase is recruited and transcription is rapidly initiated (210;211).

It was shown by DNase I footprinting that Ah receptor binding led to recruitment of proteins at the TATA sequence and a CCAAT box (212) which were later confirmed to be the TATA binding protein (TBP) (213) and nuclear factor-1 (NF-1), respectively (214). The transactivation domain of the Ah receptor is necessary for nucleosome displacement at these promoter elements as well as binding to the TFIIB basal transcription factor in mice or TFIIF in humans (213). In order to facilitate the binding of the basic transcription machinery, it is necessary to remodel the surrounding
histones to allow transcription factor access to the DNA. An assortment of histone acetyltransferases (HATs) including NcoA2 (GRIP-1 and TIF-2), SRC-1, p/CIP, CBP, and p300 are known to interact with the Ah receptor-Arnt complex and are recruited to the Cyp1a1 promoter in Ah receptor-Arnt dependent manner (215-218). The ATP-dependent histone modifier Brg-1 is recruited in an Arnt-dependent manner (219). Finally, it was shown that Med130, Med220, and CDK8, all components of the Mediator complex, are recruited via the Ah receptor to the XRE (220). In addition, the release of histone deacetylase 1 (HDAC1) from the Cyp1a1 promoter was noted after B[a]P treatment (221). In contrast to these factors that assistant in activating transcription, the Ah receptor has been shown to interact with the nuclear receptor corepressor SMRT in vitro and thus may recruit HDACs to the XRE which would suppress transcription (222). The aforementioned studies also indicate that Ah receptor and coactivator binding to cis enhancer regions precedes polymerase recruitment to the promoter suggesting a model of poised gene expression more similar to that of the α globin gene.

**Regulation of Ah Receptor Function**

Due to the involvement of a multitude of other proteins, the Ah receptor activation pathway can be regulated through various mechanisms. A key mechanism of Ah receptor modulation is degradation through the ubiquitin-proteasome pathway. Early reports indicated that the binding of radioligands to the Ah receptor in cytosolic density gradients decreased rapidly after addition of the ligand (223;224). After the generation of specific Ah receptor antibodies, it was clearly established that Ah
receptor protein levels decrease after ligand stimulation. This down-regulation was not due to reduction in Ah receptor mRNA (225) but was blocked by 26S proteasome inhibitors and not other proteases (226). Ubiquitin-bound Ah receptor was also detected in Hepa-1 cells by Ma and Baldwin (227). The location of Ah receptor degradation is still uncertain but evidence suggests that degradation of liganded Ah receptor occurs after nuclear translocation (reviewed in (228). Very recently, Morales and Perdew demonstrated that the E3 ubiquitin ligase CHIP can promote the ubiquitination of both Hsp90 and the Ah receptor in vitro and that XAP2 interferes with CHIP targeting (229).

In 1999, Mimura et al while screening a mouse genomic library with an Ah receptor cDNA probe, identified a gene that had high sequence homology to the Ah receptor (230). This gene and its product named the Ah receptor repressor (AHRR) contained a similar bHLH and PAS-A domain but due to the absence of the PAS-B domain important (ligand binding) was absent and was unable to initiate transcription. AHRR competes with the Ah receptor for dimerization with Arnt and binding to the XRE. The Ahrr gene promoter contains an XRE and is inducible by Ah receptor ligands. Thus, the Ah receptor can increase the expression of its own inhibitor to attenuate Ah receptor-mediated gene transcription.

Modulation of Ah receptor functionality by ubiquitination and the AHRR are clearly understood mechanisms of Ah receptor regulation compared to the overwhelming data on the effects of signaling pathways on Ah receptor activation and Cyp1a1 transcription. Phosphorylation is important for the in vitro binding of the Ah receptor-Arnt complex to an XRE as was demonstrated by use of global phosphatases
during an electromobility shift assay (231). It has also been determined that the Ah receptor can be directly phosphorylated in vitro by PKC (232). Treatment of cells with phorbol esters which stimulate PKC activity also increased TCDD-mediated induction of CYP1A1 (233). Use of various PKC inhibitors inhibits TCDD-mediated induction of a CYP1A1-promoter luciferase construct but failed to show any direct modulation of Ah receptor abundance or nuclear translocation. This suggests that PKC modulation of CYP1A1 expression occurs through an indirect signaling mechanism such as the mitogen-activated protein kinases (MAPKs) (234).

The Ah receptor ligands TCDD and B[α]P activate the MAPKs extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase 1/2 (JNK1/2) (235-237). Chemical inhibitors of MAPKs inhibit TCDD-mediated CYP1A1 induction linking activation of MAPKs to Ah receptor functionality (235;238;239). However, several of the inhibitors used were later shown to be competitive ligands for the Ah receptor (240). During the course of my dissertation work, I participated in the identification of ERK1/2 as a modulator of Ah receptor transactivation (241). Inhibition of ERK1/2 by UO126, which is not a ligand for the Ah receptor, inhibits TCDD-mediated induction of Cyp1a1. ERK1/2 physically associates with the receptor, facilitating ligand-induced transactivation and targeting the receptor for degradation.

As well as inducing CYP1A1 expression, exposure to TCDD is known to have multiple detrimental effects including activation of inflammatory pathways. TCDD enhances release of tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) in mouse models and multiple cell lines (242-245) but is also highly immunosuppressive
These inflammatory cytokines also activate nuclear factor κB (NF-κB) mediated transcription of genes involved in innate immunity, cell survival, and even more inflammatory cytokines (247). The association of TCDD with inflammation has lead to many studies on possible interactions of the Ah receptor and NF-κB. Physical interaction of the p65 subunit of NF-κB and the Ah receptor has been demonstrated and this association was linked to reduced activity of both NF-κB and the Ah receptor (248). There are many studies that indicate both positive and negative effects of NF-κB on Ah receptor mediated induction of CYP1A1 with no clear understanding of the interaction of these two pathways. Even though the exact mechanisms of the interaction of master transcription regulators like the MAPKs, PKC, and NF-κB with the Ah receptor-Arnt transcription complex are not definitively known, it does suggest that a complex network exists to modify the actions of the Ah receptor and the accompanying transcription coactivators in a stimulus and cell-specific context.

**Objectives of the Dissertation**

At the start of my dissertation research, the key mechanisms involved in simple agonist-initiated Ah receptor regulation of xenobiotic metabolizing enzymes were understood. As typical Ah receptor ligands like the PAHs and TCDD are often co-localized with other environmental contaminants, it became evident that cellular and animal studies with a single contaminant may not accurately reflect the true nature of responses to environmental exposures. Indeed, rodents given large, acute doses of TCDD readily develop cancer while it has been difficult to prove a direct link in humans between TCDD exposure and carcinogenesis (124). In contrast,
epidemiological studies of the ubiquitous environmental heavy metal contaminant inorganic arsenic (As\(^{3+}\)) have linked As\(^{3+}\) exposure to skin, lung, liver, bladder, prostate and kidney cancers (249;250). However, exposure of laboratory animals to As\(^{3+}\) has failed to produce organ specific cancers without the addition of other known mutagens. This would indicate that cancers in humans linked to As\(^{3+}\) exposure may be associated with additional contaminants that work in concert to predispose humans to a carcinogenic episode.

Towards the goal of understanding the mechanism of carcinogenesis from environmental exposures, I chose to initially focus my research on the interaction of the contaminants TCDD and As\(^{3+}\) and the effect on CYP1A1 expression. At that time, reports were published on the inhibition of PAH-induced CYP1A1 activity in cells co-exposed to As\(^{3+}\) (251-254). Since the Ah receptor is activated in response to PAHs leading to transcriptional activation of \(CYP1A1\), I hypothesized that As\(^{3+}\) mediates the down-regulation of CYP1A1 through modulation of Ah receptor functionality. To this end, I initiated a series of experiments to identify the point of CYP1A1 disregulation. My results indicate that Ah receptor functionality is intact and that the inhibition is directed towards recruitment of the basic transcription machinery (255).

In the course of these studies, I also became interested in a new class of Ah receptor ligands, the flavonoids. While the flavonoid chrysin is a relatively poor CYP1A1 inducer, I discovered that it is a more robust inducer of another Ah receptor regulated gene, \(UGT1A1\). I hypothesized that this induction was due to enhanced Ah receptor functionality at the \(UGT1A1\) promoter. To my surprise, the data indicates that the Ah receptor plays a minimal role in chrysin induction of UGT1A1 and that a
previously unknown mechanism of UGT1A1 regulation exists through the MAPK pathway (256).

Concurrent with these projects, a transgenic UGT1 mouse (Tg-UGT1) was generated in our laboratory (73). With this mouse, many of the previously characterized UGT1A transcriptional regulation pathways were confirmed in vivo including regulation by the Ah receptor and the xenobiotic-sensing nuclear receptors PXR and CAR. Projects were also initiated to examine the potential for other nuclear receptors to regulate UGT1A expression in vivo. This lead to my participation in a collaboration with the laboratory of Olivier Barbier (Laval University, Quebec, Canada) identifying human UGT1A3 as a target for LXRα (257). I also assisted in the identification of the PPARα response element in the UGT1A6 promoter (258) which has lead to my further characterization of regulatory elements in this promoter. While gaining experience with the mouse model and agonist-receptor responses, I became interested in the potential to examine human UGT1A regulation during disease states.

The studies on fibrate induction of UGT1A expression through PPARα were conducted in healthy mice, and I reasoned that this class of drugs would not normally be given to a healthy person but rather, someone with dyslipidemia or metabolic syndrome. Thus, I initiated a simple model of metabolic syndrome in the Tg-UGT1 mice through a high-fat diet. The goal was to obtain mice with various metabolic alterations such as dyslipidemia, hyperinsulinemia, hyperglycemia, and possibly type 2 diabetes then examine UGT1A expression with or without PPARα agonist treatment. This would determine if UGT1A expression is regulated by PPARα agonists in a more realistic clinical situation and if this regulation would lead to
unexpected alterations in drug clearance due to increased glucuronidation. The Tg-
*UGT1* mice increased adiposity but to our surprise, did not develop hyperinsulinemia. Somewhat paradoxically, human UGT1A expression was down-regulated in the high-fat diet mice while the mouse *Ugt1a* expression was relatively unchanged. These results have now changed the focus of this pilot study from a simple drug interaction study to implicating UGT1A functionality in the progression of metabolic disorders.
Chapter II
MATERIALS AND METHODS

Chemicals and Reagents

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Wellington Laboratories (Guelph, ON, Canada). Pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid), commonly known as WY-14643, benzo[a]pyrene (B[a]P), chrysin (5,7-Dihydroxyflavone), dimethyl sulfoxide (DMSO), α-naphthoflavone, sodium arsenite and corn oil were all purchased from Sigma-Aldrich (St. Louis, MO). [³H]-TCDD (specific activity, 27.5 Ci/mmol) was purchased from EaglePicher Pharmaceutical (Lenexa, KS) and 2,3,7,8-tetrachlorodibenzo-furan (TCDF) was from Cambridge Isotope Laboratories (Andover, MA). UO126 (1,2-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) and PD98059 (2'-amino-3'-methoxyflavone) were purchased from Cell Signaling (Beverly, MA). SP600125 (1,9-pyrazoloanthrone) and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were from Calbiochem (San Diego, CA). All chemicals were dissolved in DMSO and final DMSO concentration in cell culture did not exceed 0.1%. Insulin (NovolinR, NDC 0169-1833-11) was from Novo Nordisk Pharmaceuticals (Princeton, NJ). Insulin and glucose were diluted in sterile saline to proper concentrations for injection. The transfection reagent GenePorter2 was from Genlantis (San Diego, CA) and Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). A mouse anti-human PARP-1 antibody was purchased from BD Pharmentechn (San Diego, CA) and anti-Pol II (sc-5943) was purchased from Santa Cruz
Biotechnology, Inc (Santa Cruz, CA). The anti-β-actin antibody was purchased from Sigma-Aldrich. The p-p44/42 and p44/42 antibodies were purchased from Cell Signaling. Rabbit anti-human CYP1A1 was a generous gift from Dr. Fred Guengerich (Vanderbilt University, Nashville, TN). The rabbit anti-mouse Ah receptor and anti-Arnt antibodies were gifts of Dr. Christopher Bradfield (University of Wisconsin, Madison, WI). The anti-human UGT antibody was a generous gift of Dr. Wilbert H. Peters (Radboud University Medical Center, Nijmegen, The Netherlands). The mouse anti-human UGT1A1 and UGT1A4 antibodies were gifts of Dr. Joseph K. Ritter (Virginia Commonwealth University, Medical College of Virginia, Richmond, VA), and the rabbit anti-human UGT1A common region antibody was a gift of Dr. Alain Bélanger (CHUQ Research Center, Laval University, Quebec, Canada). The horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling. All other chemicals and reagents were obtained through standard suppliers.

**Cell Culture**

The human hepatocarcinoma cell line HepG2 (American Type Tissue Culture), wild-type mouse hepalc1c7 and Arnt-defective BPRc1 cells (gifts from Dr. James Whitlock; Stanford University, Stanford, CA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with penicillin/streptomycin (10,000 units/ml). TV101L cells were derived from HepG2 cells that stably express a CYP1A1-luciferase reporter gene (259). MH1A1L cells were derived from HepG2 cells that stably express a human UGT1A1-promoter luciferase reporter gene (200). hPPARα-HepG2 cells derived from HepG2 cells that stably
express the human pRSV-hPPARα cDNA were a kind gift of Dr. Eric F. Johnson (The Scripps Research Institute, La Jolla, CA). TV101L, MH1A1L, and hPPARα-HepG2 cells were cultured under the same conditions as above, except Geneticin (G418; Gibco Brl, Gaithersburg, MD) was added to 0.8 mg/mL. Cells were incubated in a humidified incubator under 5% CO₂ at 37 °C.

Primary hepatocytes were isolated from 8-12 week old CYP1A1N⁺/⁻ mice (260) and Tg-UGT1⁺/⁻ mice. Mice were anesthetized by isoflurane inhalation. The portal vein was cannulated while the anterior vena cava was sectioned to allow flow-through from the liver. Perfusion of the liver was started with Hank’s balanced salt solution (no Ca²⁺ or Mg²⁺) containing 0.5 mM EGTA and 10 mM Hepes (pH 7.4) at a rate of 4 mL/min and continued for 4 minutes. The perfusate was then changed to Hank’s balanced salt solution (with Ca²⁺ and Mg²⁺) containing 10 mM Hepes (pH 7.4) and 0.2 mg/mL collagenase. The liver was gently teased apart while in a solution of DMEM medium containing 10% FBS supplemented with penicillin/streptomycin. The cells were filtered through a 70 μm cell strainer and washed twice by centrifugation at 50 × g for 5 minutes. The hepatocytes were cultured on 6-well collagen-coated tissue culture plates (Biocoat Collagen I, Becton Dickinson Labware, Two Oak Park, MA). Four hours after plating, the medium was replaced with treatment medium and cells collected at appropriate times thereafter.

**Animals and Treatments**

All animal experiments were carried out following University of California San Diego Institutional Animal Care and Use guidelines. A mouse line heterozygous
for the human \textit{UGT1} locus was generated as previously described (73). The Tg-\textit{UGT1} strains \textit{I}e and \textit{I}e showed strong induction of UGT1A1 by TCDD in small intestine, large intestine, and liver. Animals were housed 2 to 3 in plastic cages with hardwood chips for bedding in a 12-hour light, 12-hour dark cycle with water and food (#7912, Harlan-Teklad, Indianapolis, IN) \textit{ad libitum}. For plasma chrysin analysis, nine wild-type (WT) and eight Tg-\textit{UGT1e} mice were given a single oral dose of 100 \(\mu\)L (50 mg/kg) chrysin in vehicle (60\% corn oil, 40\% DMSO). Blood was collected 60 minutes after the dose and plasma separated. For analysis of UGT1A1 induction, 3 animals per group were orally gavaged with 100 \(\mu\)L vehicle for 7 days, 100 \(\mu\)L 50 mg/kg chrysin for 7 days, or 100 \(\mu\)L 100 mg/kg benzo[a]pyrene for 3 days. Mice were anesthetized by isoflurane inhalation, and the liver was perfused with ice-cold 1.15\% KCl. Small intestine, large intestine, and stomach were dissected lengthwise and rinsed in cold 1.15\% KCl. Organs were immediately frozen on dry ice after collection and stored at -80\°C until microsomes were prepared.

To investigate the effects of high fat diet-induced type 2 diabetes on human UGT1A proteins, 10 week old male and female WT and Tg-\textit{UGT1e} littermates were maintained on a 35\% fat diet (HF) to initiate markers of type 2 diabetes. Weight gain was monitored weekly for both normal (5\% fat) and HF diet fed mice for 16 weeks. At the end of the 16 weeks, insulin tolerance tests (ITTs) and glucose tolerance tests (GTTs) were performed. For the ITT and GTT, mice were fasted for 5 hours and then injected i.p. with 0.85 U/kg (100 \(\mu\)L final volume) insulin or 1 g/kg (100 \(\mu\)L final volume) glucose, respectively. Blood glucose levels were measured from a small cut at the tip of the mouse tail using the OneTouch Ultra glucose meter (LifeScan,
Milpitas, CA) every 15 minutes for 2 hours. Mice were allowed to recover for 24 hours and then anesthetized for cardiac puncture to obtain blood for measurement of serum insulin levels (#EZRMI-13K; Linco Research, St. Charles, MO) and serum free fatty acid content (NEFA-C #994-75409; Wako Chemicals USA, Richmond, VA). Organs were collected individually and immediately frozen on dry ice. RNA and microsomes were prepared from organs to measure UGT1A expression levels.

**Cell Viability Assay and Cell Cycle Analysis**

Cell viability was measured by MTT as described previously (261). After treatment with various chemicals for 18 hours, culture medium was replaced with serum-free medium containing 0.5 mg/mL MTT, and cultures were incubated for an additional 3 hours. Assay medium was removed, and 1 mL of isopropanol with 0.04% HCl was added. Absorbance values were determined at 570 and 630 nm. Results are displayed as percentage of viable cells compared to TCDD-treated cells.

Approximately $1 \times 10^6$ HepG2 cells were exposed to TCDD and As$^{3+}$ for 18 hours, and the cells were collected by trypsinization and pelleted at 1000 x g for 5 minutes. Cells were washed twice with 1x DPBS and resuspended in 50 µL of 1x DPBS. Cells were fixed by the slow addition of 70% ethanol with constant vortexing to a volume of 5 mL. The cells were pelleted and resuspended in 800 µL of 1x DPBS containing 3% fetal bovine serum. Then, 100 µL of PI solution (final concentration 50 µg/mL PI) and 100 µL of boiled RNase A (final concentration 1 mg/mL) were added and incubated at 37°C for 30 minutes. Approximately $1 \times 10^4$ cells were acquired on a FACSCalibur flow cytometer and analyzed using the CELLQuest software.
**CYPI1A1, UGT1A1 and UGT1A6 Promoter Cloning**

The primary plasmid used for induction studies on the CYPI1A1 promoter, $CYP1A1$-luciferase was previously cloned (259). Additional fragments of the CYPI1A1 promoter (5' -1704 to 3' +2366 and 5' -2629 to 3' +385) were amplified by PCR using the GC-Rich PCR system (Roche Applied Sciences, Indianapolis, IN) from a pBluescript-CYP1A1 plasmid (260) and cloned into the pGL3-basic vector (Promega, Madison, WI). Nucleotide positions refer from the transcription start site.

For isolation of the chrysin-responsive region of the UGT1A1 gene, luciferase constructs containing sections of the human UGT1A1 promoter (PR-3712, ER, and ER-XRE mut) were previously described (200). Additional constructs (PR-3377, PR-3289, and PR-2584) were cloned using the PR-3712-pGL3-basic vector as the PCR template. PCR products were then cloned into the pGL3-basic vector. A concatenated UGT1A1 XRE was generated by cloning of an oligonucleotide that contains three copies of the UGT1A1 XRE (5'-ggtaagCAGCAatgaac-3'; capital letters: core sequence) flanked by XHO restriction enzyme half-sites into the pGL3-promoter vector (Promega) which contains the SV40 viral promoter upstream of the luciferase gene.

Portions of the UGT1A6 10.0 kb promoter region were cloned from a BAC of the UGT1 locus with sequences published under GenBank accession number AF297093 (200). UGT1A6 enhancer region constructs ER-1 and ER-2 were cloned into the pGL3-promoter vector. The promoter constructs PR-3135, PR-2189, PR-1192, and PR-664 were cloned into the pGL3-basic vector. For the concatenated
UGT1A6 PPRE, oligonucleotides containing three copies of the putative DR1 site at -2692/-2680 bp (5′-ttatGGGTCGTAAGGTCAtac-3′; capital letters: core sequence) flanked by XHO restriction enzyme half-sites were synthesized and cloned into the pGL3-promoter vector. Note that nucleotide positions for the UGT1A1 and UGT1A6 genes refer from the initiation codon, ATG, and not the start of transcription. The fidelity of all PCR products cloned into pGL3 vectors was confirmed by DNA sequence analysis and comparison with previously published GenBank sequence data.

**Transient Transfections and Luciferase Assays**

Luciferase assays in the stably transfected cells MH1A1L and TV101L were performed as described previously (233). Cells were treated and lysed on plated in lysis buffer (1% Triton, 25 mM tricine pH 7.8, 15 mM MgSO4, 4 mM EDTA, and 1 mM DTT). Cell lysates were collected by centrifugation at 10,000 x g for 10 minutes at 4°C. Supernatant (20 μL) was mixed with 100 μL reaction buffer (25 mM tricine, 15 mM MgSO4, 4 mM EDTA, 15 mM KPO4 pH 7.8, 1 mM DTT, and 2 mM ATP). Reaction was started by addition of 50 μL luciferin (0.3 mg/mL), and light output measured for 2 seconds using a LMax II luminometer (Molecular Devices, Sunnyvale, CA). Results were normalized by protein concentration as determined by the Bradford protein assay and reported as fold increase of control treatment. All experiments were done in triplicate.

Dual luciferase assays were conducted following the manufacturer’s instructions (Promega). Briefly, HepG2 cells were transfected in 12-well plated for 24 hours with 500 ng of firefly luciferase plasmid and 20 ng phRL-SV40 Renilla
luciferase plasmid using GenePorter 2 or Lipofectamine 2000 protocols. Where indicated, cells were also co-transfected with 1 μg of plasmid that over-expressed a protein. Plasmids pCMV/GRBD/mAhR83-805 and p(GRE)$_2$T105Luc were kindly provided by Dr. Lawrence Poellinger (Karolinska Institute, Stockholm, Sweden). The plasmid pCMV/GRBD/mAhR83-805 contains an N-terminal zinc finger DNA-binding domain of the glucocorticoid receptor linked to the C-terminal amino acids 83-805 of the mouse Ah receptor. This construct can be activated by ligand as measured by glucocorticoid-binding enhancer sequence (GRE)-driven luciferase activity when cotransfected with the p(GRE)$_2$T105Luc plasmid (262). Plasmids pMEV-MEK1-WT (P1030a) and pMEV-MEK1-DN (P1030b) were purchased from Biomyx (San Diego, CA). The constitutively active forms of the nuclear receptors hPXR and CAR, VP-hPXR and VP-CAR respectively, were provided by Dr. Ronald M. Evans (The Salk Institute, La Jolla, CA). After transfection, cells were treated for indicated time, lysed, and 20 μL of lysate was used for dual-luciferase analysis using the LMax$^{384}$ luminometer. Firefly luciferase values were normalized to Renilla luciferase and protein concentration and reported as fold increase over control treatment. All experiments were conducted in triplicate.

**Measurement of Gene Expression by Real-Time PCR**

Total RNA was extracted from cells using TRIzol (Invitrogen) according to manufacturer's protocol and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Each preparation of RNA was treated with DNase using a DNA-free kit (Ambion, Austin, TX). cDNA was synthesized using oligo(dt)-primed reverse
transcription using 2 μg of total RNA with the Omniscript RT kit (Qiagen, Valencia, CA) followed by quantitative real-time PCR. Real-time PCR amplification was performed in 50-μL PCR reactions that contained 25 μL of QuantiTect SYBR Green PCR Master Mix (Qiagen), 100 nM each of forward and reverse primers, and 2 μL of cDNA. The initial activation proceeded at 95°C for 10 minutes followed by 40 cycles of amplification: 95°C for 30 seconds, specific annealing temperature for 1 minute (See Table 1), and 72°C for 45 seconds. Amplification was followed by DNA melt at 95°C for 1 minute and a 41-cycle dissociation curve starting at 55°C and ramping 1°C every 30 seconds. The MX4000 Multiplex QPCR (Stratagene, La Jolla, CA) was programmed to take three fluorescence data points at the endpoint of each annealing plateau. All PCR reactions were performed in triplicate. C(t) values were normalized to human GAPDH or mouse β-actin C(t) [ΔC(t)]. cDNA was expressed as fold induction of vehicle-treated cells using the equation ratio = 2-\(^{\Delta Ct\text{Sample}-\Delta Ct\text{Vehicle}}\) (263).

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotides used for RT-PCR analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>hCYP1A1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hCYP1A1 hnRNA(^{(215)})</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hCYP1A1 ChIP</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hUGT1A1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hUGT1A6</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>mUgt1a1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hNQO1(^{(264)})</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>hGAPDH</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>m-β-Actin</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Protein Preparations

Total cellular protein was obtained by lysing cells directly on the tissue culture plates in 25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM α-glycerophosphate, 0.5 mM DTT, 1 mM sodium orthovanadate, 0.1 µM okadaic acid, and 1 mM PMSF. The solubilized cell lysate was collected and centrifuged at 10,000 x g, and the supernatant was collected.

Cytosolic protein fractions were prepared as described previously (233). In brief, cells washed in HEPES were collected by scraping in HED buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, and 1 mM DTT). The cells were homogenized with a Dounce homogenizer and then diluted 1:1 with HED2G (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, and 20% glycerol). The homogenate was centrifuged at 105,000 x g for 1 hour, and the supernatant was collected.

Nuclear protein was prepared as described previously (233), and all procedures were performed at 4°C. After treatment, the tissue culture plates were washed twice with ice-cold 10 mM HEPES. Cells were collected by scraping into MDH buffer (3 mM MgCl₂, 25 mM HEPES, 1 mM DTT, 0.2 mM PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 2500 x g for 5 minutes, and the resulting nuclear pellet was resuspended and washed three times with MDHK buffer (3 mM MgCl₂, 25 mM HEPES, 0.1 M KCl, 1 mM DTT, 0.2 mM PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin). The nuclear fraction was resuspended in 100 µL of HKD buffer (25 mM HEPES, 0.4 M KCl, 1 mM DTT, 0.2 mM PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin), incubated for 30 minutes on ice, and centrifuged at 16,000 x g for 15
minutes. The resulting supernatant was transferred to ultracentrifuge tubes, adjusted to a final concentration of 10% glycerol, and centrifuged at 105,000 x g for 1 hour. Each nuclear protein aliquot was stored at -70°C.

Microsomal protein from HepG2 cells was obtained by scraping cells from the tissue culture plates in a suspension of 10 mM KH\textsubscript{2}PO\textsubscript{4}, 0.15 M KCl, 2 mM PMSF, 2 mg/mL aprotinin, 0.2 mg/mL benzamidine, 0.5 mg/mL leupeptin, and 1 μg/mL pepstatin. The cells were disrupted on ice by ultrasonic disruption using five repetitive 5-second bursts, followed by centrifugation at 10,000 x g. Supernatants were collected and centrifuged at 105,000 x g for 1 hour in a Beckman TL100 tabletop ultracentrifuge. Microsomal pellet was resuspended in 100 µl of the phosphate buffer and stored at -70°C.

For microsomal protein from mice, organs were collected from Tg-UGT1 and wild type mice. For the small and large intestine, the tissue was dissected lengthwise and the luminal surface gently rinsed in 1.15% KCl before freezing on dry ice. Tissue samples were then pulverized in liquid nitrogen with a porcelain mortar and pestle. A sample of the pulverized tissue was added to 5 volumes of 1.15% ice-cold KCl, and the tissue was homogenized using a motorized glass Teflon homogenizer. The tissue homogenate was first centrifuged at 2,000 x g for 10 minutes at 4 °C, and the supernatant was collected. The supernatant was then centrifuged at 9,000 x g for 10 minutes at 4 °C, and this resulting supernatant was centrifuged at 100,000 x g for 1 hour at 4 °C. The pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl\textsubscript{2}, 1 mM phenylmethylsulfonyl fluoride). All protein concentrations were determined by the Bradford method.
**Western Blot Procedure**

All Western blots were performed using NuPAGE gel electrophoresis units as outlined by the manufacturer (Invitrogen). Protein aliquots were heated under reducing conditions at 70°C in loading buffer and resolved on 10% or 4%-12% Bis-Tris gels under reducing conditions, and protein was transferred to a nitrocellulose membrane using a semi-dry transfer system (Novex, Invitrogen). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline for 1 hour at room temperature, followed by incubation with primary antibodies in Tris-buffered saline overnight. Membranes were then washed and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody at room temperature. The conjugated horseradish peroxidase was detected using ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ) and blots were exposed to X-ray film.

**Detection of Ethoxyresorufin O-deethylase Activity (EROD Assay)**

EROD measurement was performed as described previously (265). Approximately 2.5 x 10^5 cells/well were plated in six-well plates. Cells were exposed to TCDD and As^{3+} for 18 hours, and the medium was removed and replaced with DMEM containing 10% fetal bovine serum, 1.5 mM salicylamide, and 2.5 µM 7-ethoxyresorufin. After incubation for 30 minutes at 37°C, the medium was removed, and fluorescence was measured with 530-nm excitation and 590-nm emission on a FluoroMax-2 (HORIBA Jobin Yvon SPEX Instruments, Inc., Edison, NJ). Resorufin
standard curves were used to convert fluorescence to picomoles of resorufin formed. Results were normalized to reaction time and cellular protein.

**Electrophoretic Mobility Shift Assay (EMSA)**

As described previously (200), nuclear or cytosolic extracts were incubated on ice for 15 minutes with 2.2 µg of poly(dI-dC) and 1 µg of salmon sperm DNA in HEDG buffer (25 mM HEPES, pH 7.4, 1.5 mM EDTA, 10% glycerol, and 1 mM DTT). Oligonucleotide probes were 5′-end labeled using Klenow and [α-32P]-dCTP. The 32P-labeled oligonucleotide probe (5 x 10^5 cpm) was added, and the reaction was incubated at room temperature for 15 minutes. The probes used in these studies are as follows (core XRE sequence underlined): CYP1A1-XRE (5′-GATCCGGCTTTCTGACGCACTCCGAGCTCA-3′), UGT1A1-XRE (5′-GCTAGGCCACTTGGTAAGCGCAATCGACATGCA-3′), and the mutated UGT1A1-XRE (mXRE; 5′-GCTAGGCCACTTGGTAAGCGCAATCGACATGCA-3′). For competition experiments, 200-fold excess unlabeled probe was added to the binding reaction before addition of the labeled probe. For antibody competitions, 3 µL of either anti-Ah receptor or anti-Arnt antibody was added to the binding reaction. Loading dye was added, and the proteins were resolved on a 6% nondenaturing polyacrylamide gel. Radioactively bound proteins were visualized by exposure to a PhosphorImager plate and scanning with a Molecular Dynamics Storm 840 scanner (Amersham Biosciences). Relative band intensities were determined using ImageQuant 5.2 (Molecular Dynamics).
EMSAs examining the functionality of putative PPARα binding sites on the UGT1 locus were carried out in the laboratory of Dr. Olivier Barbier (Laval University, Quebec City, Quebec, Canada). This author contributed by finding putative PPARα binding sites on the UGT1A6 promoter through computer database searching and then providing the sequences for oligonucleotides to be used in binding experiments. These experiments were carried out as described previously (266) by examining the ability of PPARα and RXRα to associate with putative DR1 binding sequences on the human UGT1A6 gene. The human UGT1A6 promoter oligonucleotides –2680 (5′-ttatGGGTCGTAGGTCAtac-3′), and the UGT1A9 PPRE (5′-gacaTCACCTCTGACCTcaaggag-3′) were end-labeled with [γ-32P]ATP using T4-polynucleotide kinase, followed by incubation with PPARα and/or RXRα synthesized in vitro using the TnT Quick-Coupled Transcription/Translation System (Promega). For competition experiments showing specificity, various concentrations of cold UGT1A9 DR1 sequence were included in the binding assays. The protein-DNA complexes were resolved by 4% nondenaturing polyacrylamide gel electrophoresis in Tris-borate-EDTA buffer.

**Chromatin Immunoprecipitation Assay**

Method was based on those published previously (215;221). HepG2 cells were grown to confluence on 150-cm plates and then treated for 1 hour with DMSO, TCDD, 0.5 µM As3+ + TCDD, or 5 µM As3+ + TCDD. Cells were cross-linked by addition of formaldehyde to 1% directly to culture media for 10 minutes. Cross-linking was stopped by the addition of 125 mM glycine, and cells were incubated at room
temperature for 10 minutes with gentle rocking. Plates were washed with phosphate-buffered saline, and then cells were collected by scraping in ice-cold phosphate-buffered saline. Cellular pellet was lysed (1% SDS, 5 mM EDTA, 50 mM Tris, pH 8, and protease inhibitors) for 10 minutes on ice and then sonicated 3 x 15 seconds at 20 W in 1-minute intervals. The sample was cleared of cellular debris by centrifugation at 16,000 x g for 10 minutes at 4°C. One hundred-microliter aliquots were diluted to 1 mL in dilution buffer (1% Triton, 2 mM EDTA, 20 mM Tris, pH 8, 150 mM NaCl, and protease inhibitors) and precleared for 1 hour at 4°C with 50 µL of protein agarose A/G (Santa Cruz Biotechnology, Inc.) with 1 µg/µL salmon sperm DNA. Aliquots were removed at this time for use as input control and processed along with pull-down DNA at reversal of cross-linking step. Precleared supernatants were then incubated overnight at 4°C on a rotating platform with 1 µg of anti-Pol II. Fifty microliters of protein agarose A/G plus salmon sperm DNA was added and incubated on rocking platform for 1 hour at 4°C. Beads were pelleted and washed for 10 minutes each in the following buffers (buffer 1, 0.1% SDS, 2 mM EDTA, 20 mM Tris, and 150 mM NaCl; buffer 2, 0.1% SDS, 2 mM EDTA, 20 mM Tris, and 500 mM NaCl; and buffer 3, 1% LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris). Pellets were washed twice in Tris/EDTA buffer and then eluted in 100 µL of 1% SDS, 0.1 M sodium bicarbonate, and 0.2 M NaCl at 65°C overnight. Eluates were digested with proteinase K at 45°C for 1 hour and then purified using Qiagen spin columns. DNA was quantitated using a spectrophotometer. Equal amounts of pulled-down DNA as well as input controls were used for each quantitative real-time PCR reaction.
Ligand Binding Assay

Analysis of ligand binding to the Ah receptor was carried out by examining the ability of various chemicals to interfere with $[^3]$H]TCDD binding to the cytosolic receptor (267). In this assay, 500 µL of hepa1c1c7 cytosol (5 mg/mL) was incubated with 2 nM $[^3]$H]TCDD (original specific activity of 27.5 Ci/mmol). For each assay, to monitor competitive binding, a control binding assay was conducted that included 1 µM TCDF. Competition for binding was analyzed using different concentrations of chrysin and PD98059, as a control for competition. All reagents were incubated at 4 °C for 2 hours. To remove free $[^3]$H]TCDD, dextran-coated charcoal (5 mg of charcoal/5 mg of protein) was added to each binding reaction, the mixture was incubated for 10 minutes on ice, and the dextran-charcoal was removed by centrifugation. Three hundred µL of cytosol was layered onto linear 10–30% sucrose density gradients prepared in HEDG buffer (HED buffer supplement with 10% glycerol). Gradients were centrifuged at 4 °C for 16 hours at 235,000 × g in a Beckman Coulter SW60 Ti rotor. The gradients were collected by puncturing the bottom of the tube and collecting 150 µL per fraction, and the radioactivity in each fraction was determined by liquid scintillation counting.

siRNA Knockdown of the Ah Receptor

A SMARTpool siRNA directed against the human Ah receptor (si-AhR, cat #M-004990) was purchased from Dharmacon RNA Technologies (Boulder, CO). To measure knockdown of Ah receptor protein, HepG2 cells were plated in 6-well plates and transfected for 48 hours with 50 nM negative control siRNA (Dharmacon, cat #D-
001210-01) or 50 nM si-AhR following the Lipofectamine (Invitrogen) protocol for siRNA transfection. Whole-cell extracts were collected as described and 20 μg protein used for Western blot. Ah receptor band intensities were quantified using a GS-800 calibrated densitometer (Bio-Rad) and normalized to actin loading. Results shown are representative of 6 independent transfections.

For luciferase assays, the \(^{1612} CYP1A1\)-promoter or the \( UGT1A1 \) PR3.7 luciferase construct was co-transfected with 50 nM negative control siRNA or 50 nM si-AhR as well as phRL-SV40 for 36 hours following the Lipofectamine protocol. Cells were subsequently treated with DMSO, 1 nM TCDD, or 20 μM chrysin for 24 hours. Firefly luciferase values were normalized to \( Renilla \) luciferase and protein concentration and reported as fold increase over control treatment. All experiments were conducted in triplicate.

**Quantification of Plasma Chrysin Levels**

Chrysin was purchased from Sigma. Chrysin glucuronide was obtained from enzymatic assays using liver microsomes. Briefly, media from enzymatic assays were diluted with 0.1% formic acid and loaded on Strata X cartridges (60 mg; Phenomenex, Torrance, CA) preconditioned with methanol followed by 0.1% formic acid. After loading the sample, the cartridge was washed with ultrapure water and ethyl acetate to remove the unreacted chrysin. The chrysin glucuronide was eluted with a mixture of acetonitrile:water (90:10) and evaporated under nitrogen, diluted in methanol, and the purity of the compound was confirmed by HPLC-MS. An aliquot was treated with \( \beta \)-glucuronidase and the residue was quantified with a calibration curve of chrysin. The
concentration of chrysin obtained for the aliquot digested by β-glucuronidase was then converted to a concentration of chrysin glucuronide.

Plasma samples (10-50 μL) were diluted with 1 mL water and applied to Strata X 60-mg solid-phase extraction cartridges that had been preconditioned with methanol and water. The loaded cartridges were washed sequentially with water and 10% methanol. The washed cartridges were placed under full vacuum. The analytes were eluted with acetonitrile:water (90:10), and the solvent was evaporated under a stream of nitrogen (25°C). The residue was dissolved in mobile phase and then transferred into conical vial for injection into the mass spectrometer. Determinations of chrysin and chrysin glucuronide were performed using a standard curve containing chrysin (1-50 ng/mL) and chrysin glucuronide (1-50 ng/mL) extracted in the same conditions as sample.

The HPLC-45 system consisted of a mass spectrometer (model API 3000, Perkin Elmer Sciex, Thornhill, Canada). It was operated in multiple reactions monitoring mode and equipped with an electrospray ionization interface in positive ion mode and a HPLC pump plus autosampler Model 2690 (Waters, Milford, MA). Chromatographic separation was achieved with a C6 phenyl column 3 μm packing material, 100 × 4.6 mm (Phenomenex). Isocratic condition with 50% methanol:40% acetonitrile:10% water:0.1% acetic acid with a flow rate of 0.9 mL/min were used to elute chrysin and chrysin glucuronide.
Statistical Analysis

All experiments were performed in triplicate. Statistical analysis was performed where indicated using two-tailed student’s t-test assuming unequal variances. Differences were determined to be significant if $p \leq 0.05$. Significant differences are indicated on figures where appropriate with the following key: *, † $p \leq 0.05$; **, ‡ $p \leq 0.005$; ***, $p \leq 0.0005$.

Portions of this chapter are reprinted in:


CHAPTER III
RESULTS

Effect of As$^{3+}$ on Apoptosis and Cell Cycle

As$^{3+}$ has been shown to alter cell cycle control, causing $G_1$ and/or $G_2/M$ phase arrest with subsequent programmed cell death (268-270). Evidence suggests that the phase arrest is induced by DNA damage. Telomere shortening and chromosome end-to-end fusions (271), oxidative DNA base modifications (272), and DNA strand breaks (269) indicate that small amounts of direct DNA damage can be caused by low concentrations of As$^{3+}$. If DNA damage is not repaired, p53 induces cell arrest which eventually leads to apoptosis. It has been shown that p53 expression and ATM-dependent activation associated with $G_1$ and $G_2/M$ arrest and apoptosis is up regulated upon As$^{3+}$ treatment (269;270;273). Thus, modulations in cell cycle control by As$^{3+}$ exposure may impact on the expression of other cellular components.

Given the known effects of As$^{3+}$ on cell cycle control and apoptosis, the role of As$^{3+}$-induced $G_2/M$ arrest on TCDD-initiated induction of CYP1A1 in HepG2 cells was investigated. As$^{3+}$ has been shown to interrupt normal cell function by interfering with cell cycle control and by initiating apoptosis. To examine the impact of As$^{3+}$ on HepG2 cells, we treated cells with a range of As$^{3+}$ concentrations and measured cell viability by MTT analysis (Fig. 3A) and apoptosis by detecting caspases-activated PARP-1 cleavage (Fig. 3B). Comparisons were made over a range of As$^{3+}$ concentrations that also included cotreatment with 10 nM TCDD. At concentrations of As$^{3+}$ ranging from 0.5 to 25 µM, no changes in cell viability or the initiation of
apoptosis were noted. TCDD alone or in combination with As\(^{3+}\) at these concentrations did not affect cell function. However, cell viability was reduced 30% in HepG2 cells treated with 50 µM As\(^{3+}\), which correlated with a mild increase in caspases activated PARP-1 cleavage. When cell cycle status was evaluated, As\(^{3+}\) and TCDD treated HepG2 cells revealed an increase in G\(_2/M\) cell cycle arrest at concentrations that exceeded 12.5 µM (Fig. 3C). Analysis of HepG2 cells treated with As\(^{3+}\) alone (data not shown) was consistent with the results shown in Fig. 3C, indicating that the increase in G\(_2/M\) arrest was attributed solely to the actions of As\(^{3+}\).
Figure 3. **Cell viability, apoptosis, and cell cycle arrest after As$^{3+}$ exposure.** HepG2 and/or TV101L cells were treated with 10 nM TCDD and indicated concentrations of As$^{3+}$ for 18 hours. (A) Cell viability was measured using the MTT assay and expressed as percentage of viability compared with TCDD-treated cells. Significant decrease from viability of TCDD-treated cells is indicated (*, $p \leq 0.05$). (B) Twenty micrograms of whole cell extract was used for Western blot analysis of PARP-1 cleavage. Intact PARP-1 (116 kDa) and cleaved PARP-1 (85 kDa) are indicated. Extracts from benzo[a]pyrene-7, 8-dihydrodiol-9,10-epoxide treated cells were used as a positive control for cleavage. Blots were rewashed and exposed to anti-β-actin for loading control. (C) Ten thousand PI-stained cells were acquired on a FACSCalibur flow cytometer, and the percentage of cells in G2/M as a result of As$^{3+}$ treatment is shown.
A. % Viability

<table>
<thead>
<tr>
<th>TCDD (10nM)</th>
<th>Arsenite (μM)</th>
<th>+</th>
<th>–</th>
<th>+</th>
<th>–</th>
<th>+</th>
<th>–</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>+</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. PARP-1

<table>
<thead>
<tr>
<th>TCDD (10nM)</th>
<th>Arsenite (μM)</th>
<th>–</th>
<th>+</th>
<th>–</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>5</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. % of Cells in G2/M

<table>
<thead>
<tr>
<th>Arsenite (μM) + 10 nM TCDD</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 nM TCDD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCDD + 5 μM As³⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCDD + 25 μM As³⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Inhibition of CYP1A1 Induction During Cell Cycle Arrest

Cell cycle control has been shown to influence CYP1A1 expression through mechanisms involving the Ah receptor and other independent pathways. When murine hepa1c1c7 cells were treated with microtubule disrupters known to cause G2/M phase arrest, induction patterns of Cyp1a1 following exposure to TCDD were dramatically reduced (274). In addition, this arrest appeared to not disrupt Ah receptor functionality. Analysis of Ah receptor and Arnt proteins revealed no decrease in protein levels, nor was nuclear translocation of the activated Ah receptor impaired in G2/M arrested cells. Further implication that cell cycle control has an effect on CYP1A1 expression comes from data demonstrating that pRb binds to the Ah receptor and is necessary for maximal CYP1A1 induction by TCDD in G1 phase (275).

To determine if cell cycle control can modulate the induction of CYP1A1 by TCDD, I examined the induction of CYP1A1 in HepG2 cells that were arrested in G2/M. Vinblastine is an anti-cancer chemotherapeutic agent that is widely used to arrest cells in the G2/M cell cycle phase through a process of microtubule disruption (276). In hepa1c1c7 cells, it has been demonstrated that vinblastine treatment will chemically arrest cells in the G2/M phase of the cell cycle while inhibiting TCDD induction of Cyp1a1 RNA (274). HepG2 cells treated with varying concentrations of vinblastine elicited a dose-dependent G2/M cellular arrest (Fig. 4A, open bars). When 10 nM TCDD was added and the levels of CYP1A1 mRNA quantitated by real time RT-PCR, the increasing concentrations of vinblastine were followed by a dose-dependent decrease in TCDD-induction of CYP1A1 mRNA (Fig. 4A, black bars).
To compare the levels of CYP1A1 mRNA with that of transcriptional activation of CYP1A1, induction of TCDD initiated CYP1A1-luciferase reporter gene activity in TV101L cells was measured as a function of vinblastine concentration. As shown in Figure 4B, concentrations of vinblastine that arrested cells in G2/M and inhibited CYP1A1 mRNA induction by TCDD did not inhibit the Ah receptor dependent activation of the CYP1A1-luciferase reporter gene activity in TV101L cells. Instead, we observed a mild increase when cells were cotreated with vinblastine and TCDD. The inability of vinblastine to inhibit TCDD initiated induction of CYP1A1-
luciferase reporter gene activity indicates that G₂/M arrest does not interrupt the functional properties surrounding activation of the Ah receptor or its ability to initiate CYP1A1 transcription. This finding and the observation that G₂/M arrest leads to a concentration dependent reduction in CYP1A1 mRNA suggests that G₂/M arrest must be compromising late CYP1A1 transcriptional or posttranscriptional events. Since As³⁺ and vinblastine initiate G₂/M arrest, experiments were conducted to examine the concentration dependent impact of As³⁺ on the inducibility of CYP1A1 by TCDD.

**As³⁺ Treatment Blocks TCDD Induction of CYP1A1**

The cotreatment of HepG2 cells with As³⁺ and TCDD elicited significant inhibition of TCDD-dependent induction of EROD activity over a concentration range from 5 to 50 µM As³⁺ (Fig. 5). An excellent correlation was observed when EROD activity was measured both in whole cells (Fig. 5A) as well as in HepG2 cell microsomal preparations (Fig. 5B). The pattern of EROD activity correlated with a concentration-dependent reduction in CYP1A1 protein (Fig. 5C). This indicated that As³⁺ interferes with CYP1A1 expression at or above the level of translation.

Quantitation of TCDD inducible CYP1A1 mRNA by real-time RT-PCR demonstrated that the levels of mRNA after TCDD and As³⁺ treatment were concordant with the reductions in CYP1A1 protein. At 0.5 µM As³⁺, a concentration that has no detectable effect on cell cycle control, CYP1A1 mRNA induction decreased by 61% of the levels observed with TCDD treatment alone (Fig. 5D). At 50 µM As³⁺ cotreatment, a 90% decrease in TCDD induction of CYP1A1 mRNA was observed.
Figure 5. CYP1A1 EROD activity, protein, and mRNA expression as a function of As$_{3}^{+}$ treatment. HepG2 cells were treated with 10 nM TCDD and increasing concentrations of As$_{3}^{+}$ for 18 hours. (A) Cellular EROD activity was measured as outlined in Materials and Methods. (B) Microsomes were collected and microsomal EROD activity was determined using 20 µg protein in reaction mixture. (C) Ten µg of microsomes were used for western blot analysis using an anti-CYP1A1 antibody. (D) CYP1A1 mRNA expression was measured by quantitative RT-PCR. Results were normalized to GAPDH and expressed as % induction of TCDD treated cells. Significant decreases from TCDD treatment alone are indicated (*, p≤0.05; **, p≤0.005; ***, p≤0.0005).
The levels of As$^{3+}$ needed to block induction of CYP1A1 were 10-fold lower than those shown to stimulate G2/M arrest, indicating that those events associated with cell cycle control have limited impact on induction of CYP1A1. However, we cannot exclude the possibility that analysis of cell cycle control in the presence of lower concentrations of As$^{3+}$ may lie outside the detection limits of flow cytometry. To compensate for this possibility, an experiment was conducted using cultured mouse liver hepatocytes isolated from transgenic $CYP1A1N^{+/-}$ mice (260).

When primary hepatocytes are placed in culture, they become quiescent thereby limiting the impact of cell cycle control on gene expression patterns. Because $CYP1A1N^{+/-}$ mice express the full-length human $CYP1A1$ gene, induction of mouse Cyp1a1 and human CYP1A1 can be evaluated simultaneously. As shown in Fig. 6, treatment of hepatocytes with TCDD results in a marked induction of mouse and human CYP1A1. As$^{3+}$ cotreatment inhibited in a dose-dependent manner TCDD induction of both mouse and human CYP1A1. The inability of lower concentrations of As$^{3+}$ to inhibit cell cycle control in HepG2 cells combined with the observations that As$^{3+}$ can inhibit induction of CYP1A1 in $CYP1A1N^{+/-}$-derived primary hepatocytes indicates that the cellular mechanisms initiated by As$^{3+}$ on cell cycle control do not influence those events that lead to inhibition of CYP1A1 induction by Ah receptor ligands.
Figure 6. 

**As$^{3+}$ inhibition of CYP1A1 induction in transgenic CYP1AIN$^{+/\text{-}}$ primary hepatocytes.** Primary hepatocytes were isolated from CYP1AIN$^{+/\text{-}}$ transgenic mice and exposed in culture to TCDD and various concentrations of As$^{3+}$. Whole cell lysates were collected and 20µg of protein used for Western blot detection using the human CYP1A1 antibody that is cross-reactive with mouse Cyp1a1. Whole cell extracts from TCDD treated hepa1c1c7 cells were used as a positive control for mouse Cyp1a1 and TCDD treated HepG2 whole cell extracts were used as a human CYP1A1 positive control.

**The Actions of As$^{3+}$ on TCDD-Induced Transcriptional Control of CYP1A1**

The reduction in TCDD enhancement of CYP1A1 by As$^{3+}$ might indicate that cellular control of the Ah receptor is a potential target for the actions of As$^{3+}$. When cytosolic preparations from hepa1c1c7 cells are incubated with TCDD, the Ah receptor forms a complex with Arnt, generating a transcriptional complex capable of binding to XRE sequences. In Fig. 7, the addition of TCDD to hepa1c1c7 cytosol leads to the identification of an Ah receptor/Arnt/XRE complex (lane 5). The binding of activated Ah receptor was shown to be specific by the reduction in labeled protein/DNA interactions upon incubation with excess of unlabeled XRE oligonucleotide (Fig. 7, lane 6). Incubation with a competitive inhibitor (α-naphthoflavone) towards TCDD binding to the Ah receptor also demonstrates a specific reduction in binding (Fig. 7, lane 7). However, when increasing concentrations
of As$^{3+}$ were included in the binding reaction, no inhibition of Ah receptor binding to the XRE sequence was noted.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD (20 nM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arsenite (μM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>5</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

![Image of gel shift experiment](image)

**Figure 7. Activation of Ah receptor is unaffected by the presence of As$^{3+}$.** Cytosolic extracts (50 μg) from untreated Hepa1c1c7 cells were incubated with 20 nM TCDD and indicated As$^{3+}$ concentrations for 20 hours at 4°C. Gel shift was performed as described. Control reactions are as follows: free probe (lane 1), water (lane 2), DMSO (lane 3), 5 μM As$^{3+}$ (lane 4), 20 nM TCDD (lane 5), 20 nM TCDD and 200X unlabeled XRE (lane 6), and 20 nM TCDD + 1μM α-naphthoflavone (lane 7). Co-incubation of TCDD and As$^{3+}$ are indicated (lanes 8-12). Activated Ah receptor (AhR)/Arnt heterodimer is indicated. Gel shift is representative of three independent experiments.

To examine the impact of As$^{3+}$ on TCDD-induced nuclear accumulation of the Ah receptor, HepG2 cells were treated with TCDD or cotreated with TCDD and As$^{3+}$ for 18 hours. Nuclear protein preparations were then analyzed by EMSA. The treatment of HepG2 cells with 10 nM TCDD leads to the accumulation of nuclear Ah receptor (Fig. 8A, lane 4). This complex can be disrupted by incubation of the binding reaction with antibodies to the Ah receptor and Arnt protein (Fig. 8B). When HepG2 cells are cotreated with TCDD and varying concentrations of As$^{3+}$ for 18 hours, no disruption in the accumulation of nuclear Ah receptor complex is observed (Fig. 8A,
lanes 6-10). Further confirmation that As$_{3}^{+}$ does not interfere with TCDD activation of the Ah receptor was obtained by radioligand binding assay. This assay measures the association of [H$^{3}$]TCDD with cytosolic Ah receptor. Inclusion of 50 μM As$_{3}^{+}$ did not alter the ability of [H$^{3}$]TCDD to associate with the Ah receptor (Fig. 8C).

Figure 8. Ah receptor nuclear translocation and DNA binding activity is unchanged by As$_{3}^{+}$ treatment. (A) Nuclear protein was prepared from HepG2 cells treated for 18 hours with 10 nM TCDD and increasing concentrations of As$_{3}^{+}$. Ten μg of nuclear protein was incubated with $^{32}$P-XRE (5x10$^{5}$ cpm). Controls are as indicated: free probe (lane 1), DMSO treatment (lane 2), 5 μM As$_{3}^{+}$ treatment (lane 3), 10 nM TCDD treatment (lane 4), and TCDD treatment incubated with 200X unlabelled XRE as a specific competitor control (lane 5). Shifts of TCDD and As$_{3}^{+}$ cotreatments are indicated (lanes 6-10). (B) Supershift analysis was performed using 10 nM TCDD and 10 nM TCDD with 25 μM As$_{3}^{+}$ nuclear protein preparations. Activated AhR/Arnt binding complex was competed with 200X unlabelled XRE (lanes 2 and 7). Specificity of binding was determined by incubation with 200 ng anti-Ah receptor (lanes 4 and 9) and 100 ng anti-Arnt (lanes 5 and 10) antibodies. Nonspecific antibody binding was determined using excess anti-UGT (lanes 6 and 11). (C) Hepa1c1c7 cytosols (2.5 mg) were incubated with 2 nM [$^{3}$H]-TCDD and 50 μM As$_{3}^{+}$ for 2 hours then separated on 10-30% sucrose gradients. Fractions were collected and total cpm per fraction counted indicating [$^{3}$H]-TCDD/Ah receptor complexes.

To examine directly the impact of As$_{3}^{+}$ on TCDD-initiated CYP1A1 transcription, TV101L cells that express the human CYP1A1 promoter upstream of the
firefly luciferase reporter gene were used. Treatment with TCDD induced luciferase activity 50-fold over that of untreated cells (Fig. 9A). When TV101L cells were cotreated with 10 nM TCDD and varying concentrations of As$^{3+}$ for 18 h, As$^{3+}$ had negligible effects on TCDD-initiated induction of CYP1A1-luciferase activity.

Because activation of the Ah receptor and the initiation of transcription are not influenced by As$^{3+}$ exposure, we elected to determine whether the rate of CYP1A1 transcription was altered by measuring the levels of CYP1A1 hnRNA. Quantization of hnRNA is a measurement of the abundance of nuclear transcripts and reflects the rate of RNA synthesis at any steady-state level. In this experiment, cells were treated for 1 hour with either TCDD or cotreated with TCDD and As$^{3+}$. The nuclear CYP1A1 RNA was quantitated by real-time RT-PCR using primers that amplify the exon-intron boundary of exon 1 (215). Treatment of HepG2 cells with TCDD induced CYP1A1 hnRNA after a 1 hour treatment (Fig. 9B). When cells were cotreated with TCDD at either 0.1 or 0.5 µM As$^{3+}$, reduction in the abundance of the hnRNA transcript was noted. The low concentrations of As$^{3+}$ were comparable with those that inhibited the induction of mRNA and protein. Thus, As$^{3+}$ seems to interfere with the transcriptional processes that promote induction of CYP1A1.

Confirmation of the inhibition of transcription on the CYP1A1 promoter was obtained by the chromatin immunoprecipitation assay. HepG2 cells were treated for 1 hour with TCDD or TCDD with either 0.5 µM As$^{3+}$ or 5 µM As$^{3+}$. The pull down was performed with an antibody specific to RNA polymerase II (Pol II), and real-time PCR was performed with primers specific to the proximal promoter of CYP1A1. Because of the exponential nature of PCR, the C(t) value difference of 2 between DMSO- and
TCDD-treated cells indicates an approximately 4-fold enrichment of promoter sequences detected in TCDD-treated cells (Fig. 9C). Cotreatment with 5 µM As$^{3+}$ raised the C(t) value to almost basal levels, indicating a reduction in the amount of Pol II recruited to the promoter. Additional cloning of the CYP1A1 promoter to include sequences more 3’ from the start of transcription did result in reduction of luciferase activity (Fig. 10). These results suggest in conjunction with the hnRNA assay that As$^{3+}$ inhibits the recruitment of the basic transcription machinery necessary for TCDD induction of CYP1A1.
Figure 9. Transcriptional control of CYP1A1. (A) TV101L cells were co-treated for 18 hours with 10 nM TCDD and increasing concentrations of As$^{3+}$. Results are displayed as RLU per µg cellular protein. Significant increase from 10 nM TCDD treatment is indicated (*, p ≤ 0.05). (B) HepG2 cells were treated for 1 hour with TCDD or TCDD and As$^{3+}$. Quantitative RT-PCR results using primers specific for CYP1A1 hnRNA were normalized to GAPDH and expressed as % induction of TCDD treated cells. (C) ChIP assay was performed with HepG2 cells treated for 1 hour with TCDD or TCDD and As$^{3+}$. Cells were crosslinked and incubated with antibody specific for Pol II. Recovered DNA was subjected to PCR with primers specific to the proximal promoter of CYP1A1. Results are shown as raw C(t) value and are representative of three independent pull-down experiments.
Gene and Ligand Dependence of As\textsuperscript{3+} Inhibition of Ah Receptor Responsive Genes

The inhibition of the basic transcription machinery recruited to the \textit{CYP1A1} promoter suggests that any mechanism of Ah receptor-mediated CYP1A1 induction should be inhibited by the addition of As\textsuperscript{3+}. The flavonoid chrysin has been shown to induce the expression of \textit{CYP1A1} luciferase reporters as well as CYP1A1 mRNA and protein activity (277-280). While the mechanism of induction is unknown, the induction of CYP1A1 is almost exclusively controlled by Ah receptor activation and thus, chrysin has been presumed in the literature to be an Ah receptor ligand. Treatment of TV101L cells with chrysin and As\textsuperscript{3+} resulted in a similar luciferase
pattern to TCDD treatment: no inhibition and even a slight increase over chrysin treatment alone (Fig. 11A). Evaluation of CYP1A1 mRNA mirrored As$^{3+}$-inhibition of TCDD induction (Fig. 11B). This would suggest that while As$^{3+}$ does not impact the Ah receptor directly, the inhibition is associated with Ah receptor-mediated CYP1A1 inducible expression.

**Figure 11.** **Inhibition of chrysin-mediated CYP1A1 induction by As$^{3+}$.** (A) TV101L cells were treated with 20 μM chrysin and/or indicated concentrations of As$^{3+}$ for 48 hours. Luciferase assays were performed as described and results shown as fold over DMSO treatment alone. (B) HepG2 cells were treated with 20 μM chrysin and/or indicated concentrations of As$^{3+}$ for 48 hours. RNA was isolated and quantitative RT-PCR performed. Results are displayed as fold over DMSO treatment alone. Significant increase over DMSO treatment alone is indicated (***, p≤0.0005) and significant decrease from chrysin treatment is indicated (‡, p≤0.005).

To assess whether this interference with a TCDD-inducible gene is universal, the induction of two other Ah receptor responsive genes, *NQO1* and *UGT1A1*, was tested in the presence of As$^{3+}$. NQO1 is mildly induced (2-3 fold) by the Ah receptor ligand TCDD (281). Analysis of NQO1 transcript by RT-PCR shows an increase in transcript after TCDD treatment. The combination treatment of TCDD and 5 or 10 μM As$^{3+}$ resulted in a significant upregulation above TCDD treatment alone (Fig. 12). The increase in NQO1 with TCDD and As$^{3+}$ is also supported by a recent publication on
the interaction of metals and Ah receptor inducible genes (282). This increase may be
due to activation of the Nrf2 oxidative stress signaling pathway. As$^{3+}$ has previously
been shown to activate Nrf2 (283). NQO1 expression can be increased by Nrf2
activation by tert-butylhydroquinone (tBHQ) (284).

![Figure 12. Induction of NQO1 by TCDD and As$^{3+}$. HepG2 cells were treated for 48
hours with TCDD and/or As$^{3+}$. RNA was isolated and RT-PCR (22 cycles) performed
with primers specific for NQO1. Amplification of GAPDH was used as a loading
control.](image)

$UGT1A1$ regulation by the Ah receptor was previously characterized in our
laboratory (200). Interestingly, TCDD induction of $UGT1A1$-luciferase was slightly
inhibited (24% reduction) by As$^{3+}$ in MH1A1L cells, a HepG2 cell line that stably
expresses the $^{\text{3712}}UGT1A1$ promoter driving luciferase (Fig. 13A). However, TCDD is
a relatively weak inducer of this promoter (2 fold induction compared to 50-fold on
$CYP1A1$ promoter). Chrysin has also been shown to induce $UGT1A1$ protein
expression (285). Chrysin is a stronger inducer of $UGT1A1$-luciferase with an almost
10-fold increase over DMSO compared to 2-fold with TCDD treatment (Fig. 13A).
As$^{3+}$ dramatically inhibited (65% decrease) the chrysin-mediated induction of
$UGT1A1$-luciferase activity.

To determine if $UGT1A1$-luciferase results reflect a functional outcome,
$UGT1A$ mRNA was analyzed by quantitative RT-PCR. In contrast to the luciferase
results, TCDD-induced UGT1A1 expression was synergistically increased by the addition of As\textsuperscript{3+} (Fig. 13B). As\textsuperscript{3+} treatment alone increased UGT1A1 mRNA expression suggesting As\textsuperscript{3+} activates another transcription factor mediating UGT1A1 expression. Our laboratory recently published Nrf2-mediated control of UGT1A1 by tBHQ (286). Activation of Nrf2 by As\textsuperscript{3+} was confirmed in HepG2 cells (data not shown). This suggests that the Ah receptor and Nrf2 can simultaneously induce UGT1A1 expression. In contrast to the TCDD and As\textsuperscript{3+} synergistic effect, As\textsuperscript{3+} dramatically inhibited chrysin induction of UGT1A1 mRNA, consistent with the luciferase data (Fig. 13B). Given the differences in response to As\textsuperscript{3+} treatment between induction of \textit{CYP1A1}, \textit{NQO1}, and \textit{UGT1A1}, the inhibition of induction appears to be gene and inducer specific. This may be linked to the differences between basally expressed genes (\textit{NQO1} and \textit{UGT1A1}) and one that is transcriptionally poised (\textit{CYP1A1}).

The difference between As\textsuperscript{3+} effects on chrysin induction of \textit{UGT1A1} in contrast to the classic Ah receptor ligand TCDD raises questions about the role of the Ah receptor in chrysin-mediated UGT1A1 induction. It is possible that chrysin is activating the Ah receptor in a novel manner sensitive to As\textsuperscript{3+} exposure or that the Ah receptor is not involved in UGT1A1 induction by chrysin. When these experiments were conducted, there were no studies proving that chrysin is a ligand for the Ah receptor and activates Ah receptor-mediated transcription. To elucidate the mechanism of chrysin induction of UGT1A1, the following series of experiments were conducted measuring Ah receptor functionality and \textit{UGT1A1} inducibility in response to chrysin treatment.
Humans are exposed to a wide variety of natural polyphenolic plant compounds called flavonoids through food as well as the growing dietary supplement industry. The flavone chrysin (5,7-dihydroxyflavone) is found in *Passiflora coerulea* (tropical passion fruit flower) (287;288), *Pelargonium* (geranium-like plants) (289;290), and *Pinaceae* (pine trees), as well as in the honey and propolis from bees that collect popular and conifer tree pollen (288). Chrysin inhibits the aromatization of testosterone to estrogen by cytochrome P450 19 in cell culture models (291-294). These findings have led to the use of chrysin supplements by male athletes and
bodybuilders. Perhaps of greater importance to general human health are reports that chrysin induces detoxifying phase II xenobiotic metabolizing enzymes such as UGT1A1.

Induction of UGT1A1 catalytic activity by chrysin has been reported in primary human hepatocytes (277), the human hepatoma cell line HepG2 (277-280) and the human intestinal cell line Caco-2 (285). The exact mechanism of chrysin induction of UGT1A1 is unknown but several findings suggest a role for the Ah receptor. As well as inducing UGT1A1, chrysin weakly induces CYP1A1 which is almost exclusively controlled through activation of the Ah receptor (295-297). Chrysin induces luciferase constructs that contain XREs (295;296) and one report demonstrated the necessity for a functional XRE in the UGT1A1 promoter (278).

To confirm whether the increase in protein expression is attributable to regulation of the promoter, MH1A1L cells were treated with increasing concentrations of chrysin. The UGT1A1 promoter responded in a dose-dependent manner (Fig. 14A). The UGT1A1 promoter was previously identified as being TCDD responsive (200), and the XRE core sequence (CACGCA) was located between -3309 and -3304 bp. Chrysin induced the 3.7 kb UGT1A1 luciferase construct 6- to 9-fold compared with 2- to 4-fold by TCDD treatment (Fig. 14B). Conversely, treatment of TV101L cells resulted in minimal induction by chrysin compared to TCDD treatment (Fig. 14C). Activation of the human CYP1A1 and UGT1A1 promoters implicates activation of the Ah receptor by chrysin.
To determine whether the Ah receptor is directly linked to induction of the 
*UGT1A1* promoter by chrysin, further deletions in the *UGT1A1* promoter were 
examined in transient transfection experiments (Fig.15). Within a 380-bp enhancer 
fragment are located the binding sites for the pregnane X-receptor (PXR) DR3 (298), 
the constitutive androstane receptor (CAR; DR4) (95), the Ah receptor (XRE) (200), 
and peroxisome proliferator-activated receptor alpha (PPARα; DR1) (258). A region 
between -3377 and -3289 that contains the DR4 and XRE was shown to be essential 
for chrysin induction (Fig. 15A). Mutation of the XRE core sequence in the 380-bp 
enhancer region resulted in complete loss of chrysin induction (Fig. 15B). These data
support previous findings (278) that the XRE element in the enhancer region of the 
UGT1A1 gene is responsible for induction by chrysin.

**Effects of Chrysin on TCDD Binding to the Ah Receptor**

Chrysin activation of the UGT1A1 gene through a functional XRE suggests that chrysin is a ligand for the Ah receptor. To examine this possibility, a series of radioligand binding assays were conducted to establish the ability of chrysin to compete with [³H]TCDD binding to the Ah receptor. Using cytosols from hepahc7 cells (241), 2 nM [³H]-TCDD was allowed to equilibrate in solution and the nonspecifically bound label removed by extraction with dextran charcoal. Specifically
bound [³H]TCDD was identified as a single peak by sedimentation through a linear sucrose gradient (Fig. 16). The specificity of [³H]TCDD binding was confirmed by complete competition for binding when 1 μM TCDF, a competitive antagonist with higher Ah receptor binding affinity than TCDD, was included in the cytosol incubation. Chrysin inhibited [³H]TCDD binding in a concentration-dependent manner, demonstrating that chrysin was capable of displacing TCDD from the active site of the receptor with an IC₅₀ of 24.43 μM (Fig. 16A). Interestingly, however, at concentrations of chrysin ≤1 μM, an increase in [³H]-TCDD binding to the Ah receptor was observed (Fig. 16B). The MEK1/2 inhibitor PD98059 has previously been established as a competitive antagonist toward TCDD binding (241) and was used as a control for partial inhibition of [³H]-TCDD binding. The increase in TCDD bindin to the Ah receptor after incubation with low chrysin concentrations suggests that chrysin associates with the Ah receptor at a site different from the TCDD binding site. Alternatively, chrysin may be a poor ligand (Kᵢ = 4.88 μM compared to TCDD Kᵦ = 0.5 nM (299)) at the TCDD binding site but at low concentrations initiates a signaling pathway that enhances TCDD binding to the Ah receptor. These results indicate that the affinity of chrysin for the receptor is markedly lower than that for TCDD. Chrysin is capable of interfering with the binding of TCDD and thus may serve as a ligand for the Ah receptor.
Figure 16. **Chrysin alters TCDD binding affinity to the Ah receptor.** Hepa1c1c7 cytosols (2.5 mg) were incubated with 2 nM [3H]-TCDD and high (A) or low (B) chrysin (Ch) concentrations for 2 hr then separated on 10-30% sucrose gradients. TCDF (1 mM) was used as a competitive antagonist to determine non-specific binding and 20 μM PD98095 (PD) was used as a partial competitive agonist. Fractions were collected and total cpm per fraction counted indicating [3H]-TCDD/Ah receptor complexes.

To examine the possibility that chrysin mixtures can alter Ah receptor functionality, transcriptional induction of the *UGT1A1* promoter was examined. MH1A1L cells were treated for 24 hours with 0.1 nM TCDD (submaximal Ah receptor activation conditions) and increasing concentrations of chrysin (Fig. 17A). A statistically significant increase in luciferase activity over chrysin treatment alone was observed at 20 μM chrysin and 0.1 nM TCDD cotreatment. Enhancement of TCDD induction was also observed when increasing concentrations of TCDD were co-treated with 20 μM chrysin (Fig. 17B). The increase in transcriptional activity in either case is additive in nature. In contrast, cotreatment of the *CYP1A1* reporter line (TV101L cells) with increasing concentrations of TCDD and 20 μM chrysin resulted in suppression of the TCDD response to chrysin-alone levels (Fig. 17C). Finally, increasing concentrations of chrysin cotreated with 0.1 nM TCDD resulted in enhancement of luciferase activity at low chrysin concentrations and no effect on high
chrysin concentrations (Fig. 17D). The CYP1A1-luciferase data indicates that chrysin is a competitive antagonist towards the Ah receptor ligand TCDD in agreement with the high concentration chrysin ligand binding assay. The contrasting UGT1A1-luciferase data supports the low concentration ligand binding assay data in which TCDD association with the Ah receptor was enhanced by chrysin. While chrysin enhancement of TCDD binding is a possibility, low concentration chrysin did not enhance TCDD activation of CYP1A1-luciferase. These data suggest divergence of the chrysin mechanism of action at these two promoters. Thus, at the CYP1A1 promoter chrysin recruits the Ah receptor and is a competitive antagonist towards TCDD. At the UGT1A1 promoter, chrysin recruits a different complex that can function jointly with the Ah receptor to enhance UGT1A1 promoter transcription.
The findings that an XRE element was required for maximal induction of UGT1A1 reporter gene activity coupled with the observation that chrysin effectively displaced TCDD from the receptor led us to analyze the potential of chrysin to activate the Ah receptor. For these studies, EMSAs were performed using the XRE sequences from both the UGT1A1 and CYP1A1 promoters. Previously, I optimized EMSA conditions to compare Ah receptor/Arnt binding to XRE probes from both the
**CYP1A1** and **UGT1A1** promoters (Fig. 18; (300)). Recruitment of the specific Ah receptor/Arnt complex is generally weaker at the **UGT1A1** XRE (Fig. 18). This is consistent with luciferase results that show weaker inducibility by TCDD at the **UGT1A1** promoter compared to the **CYP1A1** promoter (Figs. 14B and 14C).

When HepG2 cells were treated with chrysin, weak binding of the nuclear Ah receptor occurred to both the **UGT1A1** and **CYP1A1** XREs (Fig. 19A). In contrast, significantly greater binding of the Ah receptor to the **UGT1A1** and **CYP1A1** XRE occurred after TCDD treatment. This pattern of XRE binding was not consistent with expression of **UGT1A1** promoter activity (Fig. 14B). Chrysin exhibited far greater transcriptional activation of the **UGT1A1** promoter than TCDD. Supershift analysis
with an anti-Ah receptor antibody confirmed only partial recruitment of the Ah receptor to the *UGT1A1* XRE as shown by the 60% reduction in banding intensity (Fig. 19B). Furthermore, unlabeled mutated XRE probe competed with the chrysin-induced band, whereas it did not compete with TCDD-induced Ah receptor recruitment to the *UGT1A1* XRE. This indicates that other factors may be associated at or near the *UGT1A1* XRE after chrysin treatment. EMSA with the labeled *UGT1A1* mutated XRE probe resulted in recruitment of a complex after chrysin treatment whereas TCDD treatment did not (Fig. 19C). While this binding may be the result of the in vitro nature of the assay, it does raise the possibility that chrysin initiates recruitment of a complex that is not dependent on the XRE core sequence for binding but is dependent on an intact XRE sequence for maximal transcriptional activation (Fig. 15B).

The relatively weak binding of nuclear Ah receptor to the XRE sequences after chrysin treatment was consistent with the concentration of nuclear Ah receptor protein identified by Western blot analysis (Fig. 19D). With TCDD treatment, cytosolic levels of the Ah receptor decreased dramatically while nuclear Ah receptor levels increased. When HepG2 cells were treated with chrysin, a reduction in the cytosolic concentration of the receptor was not apparent, yet a detectable but low accumulation of the Ah receptor could be seen in the nucleus. TCDD treatment also results in a decrease in total cellular Ah receptor. Agonists of the Ah receptor initiate ubiquitin-mediated degradation of the Ah receptor (241;301). However, chrysin treatment of HepG2 cells had no impact on the steady-state levels of the Ah receptor. These results
indicate that chrysin is unable to activate the Ah receptor in a TCDD-dependent manner.
Figure 19. Effects of chrysin on Ah receptor activation. (A) Nuclear protein was collected from HepG2 cells treated for 48 hours with DMSO, 10 nM TCDD, or 20 mM chrysin. Binding reactions were carried out in the presence of 10 mg nuclear protein and either the UGT1A1 or CYP1A1 32P-XRE probe. Competition reactions were performed in the presence of 200 fold-excess of unlabeled probe. (B) Competition experiments were performed in the presence of 200-fold excess of either wild type XRE (+ XRE) or mutated XRE (+ mXRE) unlabeled probe. Antibody supershift experiments were performed in the presence of 100 ng antibody directed against the AhR (+ AhR), Arnt (+ Arnt), or nonspecific rabbit IgG (+ IgG). Phosphorimager quantification (arbitrary units) of AhR/ARNT/XRE binding complexes is given below each lane. (C) Binding reactions were carried out using 32P-labeled mXRE. (D) HepG2 cells were treated for 48 hours with DMSO, 10 nM TCDD, or 20 μM chrysin, and cytosolic, nuclear, and whole cell extracts were collected. Ten micrograms of protein was used for Western blot analysis of Ah receptor protein using a rabbit anti-mouse Ah receptor antibody that is cross-reactive to the human Ah receptor.
### A

<table>
<thead>
<tr>
<th></th>
<th>UGT1A1 XRE</th>
<th>CYPIA1 XRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Probe</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>TCDD</td>
<td>17</td>
<td>62</td>
</tr>
<tr>
<td>TCDD + XRE</td>
<td>0.6</td>
<td>2</td>
</tr>
<tr>
<td>Chrysin</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Chrysin + XRE</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Chrysin + mXRE</td>
<td>0.6</td>
<td>2</td>
</tr>
</tbody>
</table>

**Intensities:**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR/Arnt</td>
<td>0</td>
<td>1</td>
<td>0.9</td>
<td>3</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th></th>
<th>TCDD</th>
<th>Chrysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>+ XRE</td>
<td>0.8</td>
<td>2</td>
</tr>
<tr>
<td>+ mXRE</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>+ AhR</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>+ Arnt</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>+ IgG</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

**Intensities:**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chrysin</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### C

<table>
<thead>
<tr>
<th></th>
<th>UGT1A1 mXRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Probe</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
</tr>
<tr>
<td>TCDD</td>
<td>0.9</td>
</tr>
<tr>
<td>Chrysin</td>
<td>3</td>
</tr>
</tbody>
</table>

**Intensities:**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### D

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>TCDD</th>
<th>Chrysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Intensities:**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
An alternative explanation for the high efficiency of \textit{UGT1A1}-driven luciferase activity by chrysin (Fig. 14B) may be the result of its ability to promote Ah receptor-dependent transactivation of gene transcription. To examine this possibility, an Ah receptor construct lacking the N-terminal basic helix-loop-helix domain fused to a functional glucocorticoid receptor DNA-binding domain (GRDBD) was used (Fig. 20). When co-transfected with a luciferase reporter construct under control of the glucocorticoid-binding enhancer sequence (GRE), expressed GRDBD will associate with the GRE, and the Ah receptor transactivation domain will promote transcriptional activation (262). Co-transfection of HepG2 cells with the GRDBD/\textit{mAhR83-805} and \textit{p(GRE)$_2$T105Luc} plasmids followed by treatment with TCDD led to a 25-fold induction of luciferase activity, demonstrating that TCDD promotes transactivation of the Ah receptor. However, when transfected cells were treated with a concentration of chrysin that promoted \textit{UGT1A1}-luciferase activity (20 μM), only a 1.6-fold induction in Ah receptor-dependent transactivation was measured. In addition, co-treatment of transfected cells with TCDD and chrysin led to a nearly 50% reduction in the ability of TCDD to promote transactivation. This result indicates that although chrysin is capable of inducing \textit{UGT1A1} transcription in an XRE-dependent fashion, its ability to activate the Ah receptor and promote transactivation is occurring in a manner that is independent from the processes underlying TCDD initiated activation of the receptor.
To conclusively determine the role of the Ah receptor in chrysin induction of \textit{UGT1A1}, siRNA directed against the Ah receptor (si-AhR) was employed. Transfection of HepG2 cells with 50 nM si-AhR results in an average 50% knockdown of Ah receptor protein (Fig. 21A). The effectiveness of Ah receptor protein knockdown was demonstrated by the complete inhibition of TCDD induction of the $^{-1612}CYP1A1$ promoter luciferase (Fig. 21B). Chrysin induction of \textit{CYP1A1}-promoter luciferase was also inhibited, indicating that the Ah receptor is the primary pathway of chrysin induction of CYP1A1. In contrast, chrysin induction of $^{3712}UGT1A1$ luciferase was only reduced by 30% after si-AhR transfection (Fig. 21B).

Lastly, a construct was made that contains three repeats of the 18 bp \textit{UGT1A1} XRE
driving the pGL3-promoter vector (3X-XRE-luc) (Fig. 21C). TCDD induction of the 3X-XRE-luc was completely abolished by si-AhR transfection while chrysin induction was unchanged. Interestingly, when the XRE is taken out of the context of the full \textit{UGT1A1} promoter, TCDD is the predominant inducer while chrysin only induces 3-fold, a result more similar to \textit{CYP1A1} promoter induction. This may indicate that other factors on the \textit{UGT1A1} promoter are tempering the gene’s response to Ah receptor ligands. Therefore, we conclude that the mechanisms of induction of \textit{CYP1A1} and \textit{UGT1A1} by chrysin are divergent. Chrysin induces \textit{CYP1A1} through weak activation of the Ah receptor, whereas induction of the \textit{UGT1A1} gene is dependent not only on the Ah receptor but also additional cellular factors.
Figure 21. **siRNA knockdown of Ah receptor.** (A) HepG2 cells were transfected for 48 hours with either 50 nM negative control siRNA (Mock) or 50 nM si-AhR. Whole-cell extract (20 mg) was used for Western blot analysis with the anti-Ah receptor antibody or β-actin. Ah receptor band intensities were quantified, normalized to β-actin, and represented as percent of mock transfection (100%). (B) HepG2 cells were transiently transfected with the 1.6 kb *CYP1A1*-luc or *UGT1A1* PR-3712 luciferase, phRL-SV40, and 50 nM negative control siRNA (mock; black bars) or 50 nM si-AhR (gray bars). Thirty-six hours after transfection, cells were treated with DMSO, 1 nM TCDD, or 20 mM chrysin for 24 hours. (C) HepG2 cells were transfected as described for (B) except with the UGT-3X-XRE luciferase construct. Firefly luciferase readings were normalized to *Renilla* luciferase and protein concentration and displayed as fold increase over the respective DMSO treatment. Significant decreases from mock transfection are indicated (*,p ≤ 0.05; **,p ≤ 0.005; ***,p ≤ 0.0005).
Chrysin Activation of the *UGT1A1* Promoter Occurs Through the MAP Kinase Pathway

ERK1/2 has been shown to be involved in Ah receptor stabilization and transactivation potential (241). Inhibition of ERK1/2 by U0126 leads to the inhibition of TCDD-initiated activation of Cyp1a1. ERK1/2 inhibition alone also activates Ah receptor translocation and binding to the *CYP1A1* XRE indicating a role for ERK1/2 in Ah receptor cytosolic sequestration (Fig. 22).

![Figure 22. U0126 activates the Ah receptor.](image)

(A) Hepa1c1c7 and Arnt deficient BPRc1 cells were treated with DMSO, 10 nM TCDD, or 10 µM U0126 for 18 hours. For EMSA, 10 µg of nuclear protein from each extract was incubated with 32P-labeled *CYP1A1*-XRE probe and subjected to 6% non-denaturing acrylamide gel electrophoresis. Competition was performed in the presence of a 200-fold excess of unlabeled *CYP1A1*-XRE.

To investigate whether similar MAP kinase pathways are involved in chrysin induction of *UGT1A1* through the Ah receptor, MH1A1 cells were treated with a panel of MAP kinase inhibitors (Fig. 23A). ERK1/2 was inhibited using the MEK1 inhibitors U0126 and PD98059, JNK1/2/3 was inhibited using SP600125, and p38
was inhibited by SB203580. All MAP kinase inhibitors suppressed chrysin induction of UGT1A1 luciferase. PD98059 and SP600125, as well as being MAP kinase inhibitors, are also competitive ligands for the TCDD binding site on the Ah receptor (240). The slight increase in UGT1A1 luciferase activity with PD98059 or SP600125 treatment alone may be the result of this Ah receptor activation. Thus, these MAP kinase inhibitors may be blocking access of chrysin to the receptor, resulting in only partial reduction of luciferase activity similar to the partial reduction observed with si-AhR. However, U0126 does not compete with TCDD for Ah receptor binding and has been used to demonstrate a direct link between ERK1/2 phosphorylation and Ah receptor activation (241). Additionally, the link between MEK1 activity and chrysin induction of UGT1A1 is shown by overexpression of a dominant negative MEK1 (MEK1-DN). When compared with wild-type MEK1 (MEK1-WT) expression, the MEK1-DN inhibits basal as well as chrysin-induced UGT1A1 luciferase activity in a manner similar to treatment with UO126 (Fig. 23B). ERK1/2 involvement in chrysin induction of UGT1A1 is further implicated by the observation that chrysin treatment of HepG2 cells leads to active, phosphorylated ERK1/2 at levels similar to TCDD (Fig. 23C). We conclude that, in conjunction with the Ah receptor, chrysin activates the MAP kinase signaling pathways to achieve maximal UGT1A1 induction.
Figure 23. **Involvement of MAP kinase pathway in chrysin induction of UGT1A1.** (A) MH1A1 cells were treated for 48 hours with a series of MAP kinase inhibitors alone or in combination with 20 μM chrysin: UO126 (UO, 20 μM), PD98059 (PD, 20 μM), SB203580 (SB, 20 μM), or SP600125 (SP, 20 μM). Luciferase values were normalized to protein concentration and displayed as fold increase over DMSO treatment. Significant increases over DMSO are indicated (*, p≤0.05; **p≤0.005; ***p≤0.0005). Significant decreases are indicated (†, p≤0.005; ††, p≤0.0005). (B) HepG2 cells were transiently transfected with a wild-type MEK1 (WT) or dominant negative MEK1 (DN) construct with the *UGT1A1* PR-3712 and phRL-SV40 luciferase constructs for 24 hours followed by 24 hours of treatment with DMSO or 20 μM chrysin. Firefly luciferase readings were normalized to Renilla luciferase and protein concentration and displayed as fold increase over MEK1-WT DMSO treatment. Significant decreases from WT-MEK1 transfection are indicated (*, p≤0.05). (C) Phosphorylated Erk1/2 (p-P44/42) and total Erk1/2 (P44/42) were measured in whole-cell extracts from HepG2 cells treated for 48 hours with 10 nM TCDD (T), 20 μM chrysin (C), and 10 μM UO126 (UO).

**Actions of Oral Chrysin Treatment in Transgenic UGT1 (Tg-UGT1) Mice**

One of the goals in defining the mechanisms underlying induction of the UGTs by chrysin and other flavonoids is to apply this knowledge to *in vivo* models. To examine the contribution of chrysin on human UGT1A1 expression *in vivo*, we have taken advantage of a recently developed transgenic mouse model (Tg-UGT1) that expresses the entire human *UGT1* locus (73). The *UGT1* locus in Tg-UGT1 mice has been shown to be regulated in a tissue-specific fashion that is comparable to gene
expression patterns observed in humans. In addition, the locus is regulated in vivo by activation of the Ah receptor and PXR (73), as well as the LXR (257).

To confirm that chrysin is adequately absorbed in wild-type (WT) and Tg-UGT1 mice, plasma samples were analyzed for chrysin and chrysin-glucuronide 60 minutes after a single oral dose of 50 mg/kg chrysin. The average concentration of chrysin in plasma from WT and Tg-UGT1 mice is 6.9 ng/mL and 2.7 ng/mL, respectively (Fig. 24A). Metabolism of chrysin to the glucuronide was observed in WT mice (average concentration, 581 ng/mL); however, the conversion to the glucuronide was nearly doubled in Tg-UGT1 mice (average concentration, 1124 ng/mL). The metabolic ratio of drug to metabolite in WT and Tg-UGT1 mice after a single dose of chrysin was 0.012 and 0.002, respectively. The very low metabolic ratio both in WT and Tg-UGT1 mice indicates that chrysin is rapidly metabolized after oral administration and possesses very poor bioavailability.

To determine whether oral chrysin administration can induce human UGT1A1 expression in the gastrointestinal tract and liver, Tg-UGT1 mice were administered chrysin (50 mg/kg) every day for 7 days and the expression of human UGT1A1 was evaluated in small intestine and liver microsomes by Western blot analysis. As previously described, Tg-UGT1 mice express low basal levels of UGT1A1 in liver and significantly higher basal levels in the small intestine (Fig. 24B). Oral chrysin treatment had no effect on facilitating induction of UGT1A1 in these tissues. However, when Tg-UGT1 mice were treated orally with B[a]P (100 mg/kg) for 3 days, UGT1A1 was induced in both small intestine and liver, demonstrating that agents capable of activating the Ah receptor in vivo will ultimately stimulate UGT1A1
induction (Fig. 24C). Chrysin did not induce expression of murine Cyp1a1 (data not shown). Overall, if the molecular mechanisms defined in this report are extrapolated to humans, the findings indicate that the high levels of glucuronidation in the gastrointestinal tract serve to limit the bioavailability of consumed flavonoids. Thus, the ability of these agents to regulate UGT1A1 and CYP1A1 through activation of the Ah receptor or other as yet unidentified pathways is limited.
**Figure 24. The actions of oral chrysin treatment.** (A) Wild-type (WT) and Tg-UGT1 (Tg) mice were given a single oral dose of chrysin (50 mg/kg). Plasma was collected 60 minutes after the dose and analyzed by HPLC for the presence of chrysin (circles) and the chrysin-glucuronide (squares). (B) Tg-UGT1 mice were orally gavaged for 8 days with vehicle (V, 40% DMSO/60% corn oil) or 50 mg/kg chrysin (C) in vehicle. Microsomes were prepared from liver and small intestine for Western blot analysis. Supersomes expressing UGT1A1 were used as a standard (S). Blots were incubated with the human-specific anti-UGT1A1 antibody. (C) Tg-UGT1 mice were gavaged for 3 days with vehicle or 100 mg/kg B[a]P and microsomes prepared from liver and small intestine for Western blot analysis of UGT1A1.
Regulation of \textit{UGT1A6} by Xenobiotic–Sensing Nuclear Receptors PXR and CAR

In addition to transcriptional control by the Ah receptor, UGT1A1 is regulated by the xenobiotic-sensing nuclear receptors PXR (human homologue SXR) and CAR (95;298). In Tg-\textit{UGT1} mice, all UGT1A isoforms are inducible in vivo by Ah receptor and PXR ligands in a tissue-specific manner (73). Other than UGT1A1, UGT1A6 is perhaps the most well-characterized isoform in terms of transcriptional control. It is the most widely expressed isoform, appearing in the human liver, small intestine, large intestine, larynx, stomach, kidney, and brain and is responsible for the glucuronidation of many small phenolic compounds that include the NSAIDs (305). Human UGT1A6 has been shown to be responsive to Ah receptor, PXR, and Nrf2 activation although only the Ah receptor and Nrf2 response elements have been mapped (Table 2). Additionally, a study in 1° human hepatocytes reported increased naphthol

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ah receptor</td>
<td>β-naphthoflavone</td>
<td>Caco-2 cells</td>
<td>(204)</td>
</tr>
<tr>
<td></td>
<td>PAHs</td>
<td>Caco-2 cells</td>
<td>(302)</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>Caco-2 cells</td>
<td>(202;303)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1° Tg-\textit{UGT1} hepatocytes</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1° human hepatocytes</td>
<td>(303)</td>
</tr>
<tr>
<td>Nrf2</td>
<td>tBHq</td>
<td>Tg-\textit{UGT1} whole liver</td>
<td>(73)</td>
</tr>
<tr>
<td>PXR/SXR</td>
<td>PCN</td>
<td>Tg-\textit{UGT1} whole liver, small intestine, large intestine</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td>Rifampicin</td>
<td>HepG2</td>
<td>(304)</td>
</tr>
</tbody>
</table>
glucuronidation (a somewhat specific UGT1A6 substrate) after carbamazepine (SXR ligand) or phenobarbital (CAR ligand) treatment (306). This provides indirect evidence for regulation of human UGT1A6 expression by SXR and CAR. As an aside, the report of a functional PXR/CAR DR3 response element in the rat Ugt1a6 gene has now been identified as being located in exon 6 of the rat Ugt2b1 gene (BLAST search for short, nearly identical matches using the sequence published in (298;307)). In addition, control by HNF1α in the rat has been characterized (308).

To establish which nuclear receptors may regulate UGT1A6 expression, primary hepatocytes from Tg-UGT1 mice were treated in culture for 48 hours with various inducers. As expected, UGT1A6 was induced by TCDD (Fig. 25). Surprisingly, the gene did not respond to PCN, the prototypical ligand for PXR and only weakly to TCPOBOP, a ligand for CAR. However, transfection with the constitutively active VP-PXR and VP-CAR did result in dramatic induction indicating that human UGT1A6 is indeed regulated by PXR and CAR. UGT1A6 was also induced by WY-14643, a ligand for PPARα. TO-901317, a LXR ligand, did not significantly induce UGT1A6. However, other preliminary experiments indicated mild induction of UGT1A6 (data not shown). Although human UGT1A6 was shown to be responsive to PXR activation at the level of protein and RNA induction, the regulatory region has yet to be identified. The next series of experiments were initiated to identify the PXR-responsive element on the human UGT1A6 promoter.
The UGT1A6 promoter spans a region of approximately 10 kb extending to the 3′ end of exon 1A7. The transcription start site was previously identified at -93 bp from the translation initiation ATG codon with two putative TATAA boxes located at -147 bp and -280 bp, relative to the ATG (Fig. 26, (202)). Additionally, an XRE element was identified between -1503 bp and -1498 bp, and three ARE sequences were identified in the proximal promoter region (202;204). A PXR binding site was previously predicted at -9216 bp through in silico modeling but was later determined to not be functional in a luciferase activity assay (309).
To isolate the region responsible for induction by PXR, the 5’ regulatory DNA was initially cloned in three parts, two enhancers (ER1 and ER2) and one promoter (PR-3135), into luciferase reporter vectors. HepG2 cells were transiently transfected with the indicated luciferase reporter plasmid and then either treated with TCDD (18 hours) or co-transfected with the VP-PXR or VP-CAR constructs for 48 hours (HepG2 cells do not express substantial levels of SXR or CAR, (310)). As shown in Fig. 27, the promoter region and ER1 were responsive to all three treatments while the intervening ER2 region is only responsive to PXR activation.
To isolate the PXR responsive region within the promoter, further promoter truncations were used for transfection with VP-PXR (Fig. 28). After an 18 hour transfection, the full-length promoter construct was induced 2-fold by VP-PXR. A significant increase in PXR responsiveness was noted in the PR-2189 construct which was lost after further truncation to PR-1192. This may indicate that the primary region of PXR responsiveness is located between -1192 bp and -2189 bp. The induction of luciferase activity was also noted for the PR-664 construct. Also, a poly (dC-dA) repeat that is located between -2200 bp and -2250 bp is suggested to be a region associated with negative DNA regulation (202). This may explain the slight but
statistically significant increase in PXR responsiveness between the PR-3135 and PR-2189 constructs. When the *UGT1A6* promoter sequence is applied to the transcription factor binding site prediction software, MatInspector7.4.5 (Genomatix), multiple binding sites are predicted for various nuclear hormone receptors. Interestingly, only one putative PXR/CAR site (-1759 bp) containing an inverted half site (solid underline; 5′-CTAGATGAACCTCAAAGCAAGATG-3′) was identified. A second more divergent inverted half site (dashed underline) is located four nucleotide spaces in the 3′ direction from this half site and together these half sites may comprise a DR4 response element. No PXR binding sites were predicted in the initial 660 bp of the promoter indicating that PXR may be utilizing other binding sites under artificial conditions. Further promoter deletion studies as well as electromobility shift assays will be necessary to clearly identify the PXR as well as CAR binding sites within the *UGT1A6* proximal promoter.
Regulation of *UGT1A6* by PPARα

Treatment of primary Tg-*UGT1* hepatocytes with the PPARα ligand, WY-14643, led to an average 3-fold induction of UGT1A6 transcript levels (Fig. 25). Concomitantly, the role of PPARα in in-vivo induction of the UGT1A locus was being investigated in Tg-*UGT1* mice. Oral administration of WY-14643 led to induction of UGT1A1, UGT1A3, UGT1A4, and UGT1A6 mRNA and protein in the liver, small intestine, large intestine, and kidney (258)). The most dramatic upregulation of UGT1A6 was observed in the liver, large intestine, and kidney. In
support of the in-vivo experiments, I initiated the characterization of the PPARα response element on the *UGT1A6* promoter.

In contrast to the PXR-induction studies, an in-silico approach was initially taken to identify PPARα response elements (PPREs) also known as DR1 elements. Using a consensus PPRE sequence (5′-a(a/t)ct(A/G)GGNCANAG(G/T)TCAn(g/t)n-3′; conserved core sequence in capital) compiled from a list of known functional PPREs, the 10 kb promoter region of *UGT1A6* was scanned for highly similar sequences (311). Three putative PPREs were identified within the first 6 kb of the promoter with two (PPRE1 and PPRE2) being near perfect matches (Fig. 29A). In collaboration with the laboratory of Dr. Olivier Barbier who previously identified the PPRE in the *UGT1A9* promoter (266), the three putative UGT1A6 PPREs were analyzed by EMSA. Interestingly, only PPRE2 (-2680) was able to recruit binding of the PPARα/RXRα heterodimer (Fig. 28B). Specificity of PPRE2 was further confirmed by competition assays with increasing concentrations of unlabeled 1A6-PPRE2, 1A9-PPREwt, or 1A9-PPREmut (Fig. 28C). As expected, PPARα/RXRα binding was competed away by the unlabeled 1A6-PPRE2 and 1A9-PPREwt but not by the 1A9-PPRE mutant probe.
Figure 29. **PPARα is functional in the presence of UGT1A6 DR1 sequence.** (A) Putative DR1-PPREs were identified in the *UGT1A6* promoter by searching with the consensus sequence compiled from multiple confirmed PPREs (291). (B) EMSAs were conducted with end-labeled PPRE probes in the presence of unprogrammed reticulocyte lysate, PPARα, or both PPARα and RXRα. After binding, the labeled proteins were resolved on a 4% nondenaturing polyacrylamide gel. The arrow indicates the retarded band with PPARα and RXRα added to the binding reactions. (C) Competition EMSAs with the radiolabeled DR1 sequences were performed by adding 1-, 10-, or 50-fold molar excesses of the indicated cold consensus DR1 sequences (D) Oligonucleotides encoding three copies of the *UGT1A6* PPRE2-DR1 element were cloned in both the sense and antisense directions into the pGL3-SV40 luciferase construct. After transfection into either HepG2 cells or PPARα-HepG2 cells, the cells were treated with WY-14643 (W, 100 μM) for 48 hours, and luciferase activity was measured. The induced levels are expressed as fold induction over DMSO-treated cells. Significant increases over DMSO treatment are indicated (*, p≤0.05; **, p≤0.005).
A

Consensus: aact AGGNCAGGGTCA ngn
t  G     T  t

PPRE1: caga AGGGCAAGGGTCA gag
PPRE2: t tat GGTCGTAAGGTCATac
PPRE3: cttt AGGAGGGGTTCA atg

B

1A9 UGT1A6 PPREs

PPARα + + + + + + + +
RXRα — + — — + — — + —

Non-specific

C

PPARα/RXRα

D

Fold Over Respective DMSO Control

PPRE2 sense
PPRE2 antisense

HepG2 PPARα-HepG2
To confirm the functionality of 1A6-PPRE2, an oligomer containing three direct repeats of the 1A6-PPRE2 was cloned into the pGL3-promoter vector in both the sense and antisense directions. This construct was then transfected into HepG2 cells that stably over-express human PPARα (312). When PPARα-HepG2 cells are treated with WY-14643, a significant increase in luciferase activity is observed for both the sense and antisense 1A6-PPRE2 plasmids (Fig. 29D). A minimal induction of is also observed in the control HepG2 cells which is attributable to the low endogenous levels of PPARα in this cell line (312). Preliminarily, the responsiveness of UGT1A6 to PPARα ligands can be attributed to regulation through the PPRE located at -2680 to -2692 bp.

**UGT1As and the High Fat Diet Model of Type 2 Diabetes**

The significant upregulation of the UGT1 locus after treatment with the fibrate drug WY-14643 raises important clinical questions. Fibrate drugs are known inducers of UGT1A expression through PPARα activation (258;266). Fibrates are also substrates for glucuronidation (313-315). Fibrates are widely prescribed for the treatment of hypertriglyceridemia and hypercholesterolemia (316), conditions associated with the generalized metabolic syndrome (317). Patients are often prescribed multiple drugs to reverse the development of metabolic syndrome. Thus, those taking fibrates may be at a higher risk of adverse drug reactions or ineffective therapy due to accelerated metabolism of both the fibrate and coadministered drugs. It has been shown clinically that the fibrate gimpibrozil interferes with glucuronidation of statins, resulting in increased myopathy (318;319). Traditionally, drug interactions
have been predicted through in vitro kinetic studies or using rodent models that may not accurately reflect the human compliment of UGTs. With the generation of the Tg-UGT1 mouse it is now possible to study the major Phase II metabolism enzymes in the context of a complex disease such as metabolic syndrome or type 2 diabetes (non-insulin dependent diabetes mellitus).

Studies of glucuronidation and diabetes are sparse and inconsistent. Glucuronidation was first linked to type 1 diabetes (insulin dependent diabetes mellitus) in studies of streptozocin treated Sprague-Dawley rats. It was found in several studies using the chemically-induced type 1 diabetes model that glucuronidation was increased (320-324). Conversely, decreases in glucuronidation have also been reported in streptozotocin- (325-328) and alloxan-treated rats (322;329). Braun L et al identified the induction of rUGT1A1 by Northern blot and Western blot in BB/Wor rats, a genetic model of type 1 diabetes (330). Study of the genetically obese Zucker rat, a model more closely resembling type 2 diabetes, showed decreases in glucuronidation of PPAR agonists attributed to decreases in rUGT1A1 and rUGT1A6 (331). Another study of obese Zucker rats demonstrated higher glucuronidation capacity (332). The majority of these studies were conducted prior to the identification of individual rat UGT1A isoforms and the development of specific RT-PCR and Western blot techniques. To date, there are no studies on the effects of high fat diet-induced type 2 diabetes on glucuronidation in rodent models or humans. The following study was initiated to evaluate the effect that high fat diet-induced type 2 diabetes may have on expression of human UGT1A proteins.
To simulate the initiation of metabolic syndrome/type 2 diabetes, 10-week old male and female wild type (WT) and Tg-UGT1 mice were fed a normal (5% fat, N) or high-fat diet (35% fat, HF) for 16 weeks. Weight gain was monitored weekly and at the end of the 16 week study, insulin tolerance tests (ITTs) and glucose tolerance tests (GTTs) were performed. Cardiac punctures were also performed for collection of serum to measure insulin and free fatty acid levels. Organs were harvested to examine the expression of UGT1A isoforms.

All mice fed a HF diet gained a significant amount of weight compared to the control mice on normal food (Fig. 30). There was no significant difference in the rate of weight gain between WT and Tg-UGT1 mice on a normal diet nor between the WT and Tg-UGT1 mice on HF diet (Fig. 30A). Male WT HF mice gained 90% of their original body mass (avg final mass 43 g) compared to male WT normal mice that gained 39% (avg final mass 31 g). Similarly, Tg-UGT1 male mice on a HF diet gained 100% of their original body mass (avg final mass 40 g) compared to normal diet which gained 42% (avg final mass 32 g). Female mice produced similar results. On a normal diet, WT and Tg-UGT1 female mice gained 48% (avg final mass 24.6 g) and 56% (avg final mass 24.1 g), respectively. On a HF diet, WT and Tg-UGT1 female mice gained 131% (avg final mass 44.5 g) and 115% (avg final mass 36.7 g) of their original mass, respectively. There did not appear to be any obvious changes in fat deposition between the HF WT and HF Tg-UGT1 mice although detailed analysis of fat deposition was not conducted (Fig. 31).
Figure 30. **Increased weight in response to a high fat diet.** WT and Tg-UGT1 mice were fed a 35% fat diet for 16 weeks and mass was measured weekly. (A) Weekly mass gain expressed as the percentage of the original mass. (B) Final mass at the end of 16 weeks of high fat feeding expressed as percentage of original mass.
Interestingly, differences were observed between WT and Tg-UGT1 mice when diabetic status was assessed. As expected, WT HF male mice displayed high blood glucose levels when challenged with insulin during the ITT (Fig. 32A). Surprisingly, Tg-UGT1 HF male mice displayed a normal blood glucose response during the ITT. While female mice did gain a significant amount of weight on the HF diet, neither WT nor Tg-UGT1 female mice had elevated glucose levels after insulin challenge (Fig. 32B). Due to the lack of response in the ITT, female mice were not subjected to the GTT. When challenged with a bolus of glucose for the GTT, WT HF male mice were unable to rapidly clear the excess glucose while Tg-UGT1 male mice cleared glucose at a similar rate as normal diet mice (Fig. 33A).
Serum insulin levels were elevated in WT HF male mice while Tg-UGT1 male mice serum insulin was comparable to all normal diet male mice (Fig. 33B). HF diet fed WT females did show a slightly elevated serum insulin level compared to normal diet female mice. This would indicate that the female mice develop hyperinsulinemia at a slower rate than males. Circulating free fatty acids were measured in the serum using an ELISA for the detection of total non-esterified free fatty acids (FFAs). No pattern can be identified due to the lack of sufficient serum samples and serum volume (Fig. 33C).
Figure 33. Tg-UGT1 male mice are resistant to high fat diet-induced hyperinsulinemia. (A) Male mice were fasted for 5 hours prior to i.p. administration of 1 g/kg glucose for the glucose tolerance test. Every 15 minutes, small blood samples from the tail were used to monitor blood glucose levels with a glucose meter. (B) Blood was collected by cardiac puncture after mice were fasted for 5 hours. Serum was separated from the whole blood and used for ELISA measurement of insulin as well as NEFA (C).

Another significant component involved in the progression towards type 2 diabetes is the activation JNK. It has been shown in obese mice that JNK activity is elevated and that JNK1 phosphorylates an inhibitory serine of the insulin receptor.
substrate-1 (IRS-1) (333-335). This phosphorylation then leads to the development of FFA-induced insulin resistance. Initial Western blot experiments to determine the level of phospho-Jnk1/2 in the liver were not successful (data not shown). This is likely due to activated phosphatases during repeated handling of tissue samples for UGT1A mRNA and protein analysis. The surprising lack of hyperinsulinemia and normal insulin and glucose tolerance tests in HF Tg-UGT1 mice indicate that this strain may be resistant to high fat diet-induced metabolic syndrome and type 2 diabetes.

High Fat Diet Influences on UGT1A Isoform Expression

To determine if UGT1A protein levels were affected by a high fat diet, microsomes from various tissues of the male mice were isolated for Western blot analysis. Female organs were not used for analysis due to the lack of response to the high fat diet in the WT as well as Tg-UGT1 mice. Blots using an antibody against the common region of human UGT1A proteins showed an overall decrease in human UGT1A proteins in the small intestine and liver of HF diet Tg-UGT1 mice (Fig. 34). Further analysis using antibodies specific for human UGT1A1 and UGT1A4 in the small intestine confirmed the repression. The liver of Tg-UGT1 mice does not express sufficient basal levels of UGT1A1 and UGT1A4 to measure a down-regulation with the isoform-specific antibodies. The pancreas was also analyzed for UGT1A expression. It was determined that the pancreas does not express human UGT1A proteins, which is consistent with the lack of reports characterizing UGT1A protein expression in the pancreas. The finding of down-regulation of UGT1A proteins is
consistent with earlier reports of decreased glucuronidation activity in streptozotocin-treated rats as well as Zucker rats.

![Table and Figure](image)

<table>
<thead>
<tr>
<th>A Small Intestine</th>
<th>B Liver</th>
<th>C Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td><strong>Tg-UGT1</strong></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>HF</td>
<td>N</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α-UGT1A1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α-UGT1A4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α-UGT1A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α-UGT1A</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 34.** **High fat diet decreases UGT1A protein expression.** Microsomes were prepared from the small intestine (A), liver (B), and pancreas (C) of male mice. Western blot analysis was performed with 20 μg of protein and blots exposed to a general anti-UGT1A antibody or the isoform specific anti-UGT1A1 and anti-UGT1A4. Due to decreased sensitivity of the anti-UGT1A antibody and/or the concentration of human UGT1A proteins in the mouse liver and pancreas, liver and pancreas microsome samples were pooled from individual tissues.

To establish if the down-regulation of UGT1A protein expression is a result of lower abundance of mRNA transcripts, RT-PCR was performed on RNA from the small intestine. All human UGT1A isoforms expressed in the small intestine (1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, 1A10) were down-regulated in HF mice indicating transcriptional regulation of the human UGT1 locus in response to obesity (data not shown). Additionally, quantitative RT-PCR analysis was performed to measure hUGT1A1 and mUgt1a1. Analysis of small intestine mRNA confirmed the down-regulation of the human transcripts in HF diet Tg-UGT1 mice (Fig. 35A).
mUgt1a1 in HF diet WT mice was similarly reduced. Interestingly, mUgt1a1 was not down-regulated in Tg-UGT1 HF diet mice. In contrast, mUgt1a1 levels in the liver of Tg-UGT1 HF diet mice were suppressed while human UGT1A1 transcripts were not significantly lowered (Fig. 35B). Due to the large variation in human UGT1A1 transcript detected, a larger sample population may reveal a statistically significant decrease. The overall decrease in human UGT1A protein in the HF diet male mice is intriguing given the apparent resistance to hyperinsulinemia. This suggests that increased glucuronidation activity is protective in preventing the onset of diet-induced type 2 diabetes. The disassociation of mouse and human gene regulation may indicate that the human genes are more sensitive to overall metabolic changes and their functionality can impact on regulation of the mouse locus.

![Graph A: Small Intestine](image1)

![Graph B: Liver](image2)

Figure 35. **Down-regulation of UGT1A mRNA in response to high fat diet.** RNA isolated from small intestine (A) and liver (B) tissues of male mice was analyzed by quantitative RT-PCR with primers specific to mouse Ugt1a1 or human UGT1A1. Values were normalized to mouse β-actin and expressed as fold change from the normal diet mouse of the same genotype.
Portions of this chapter are reprinted in:


Chapter IV
DISCUSSION

Summary

My dissertation research initially focused on transcriptional regulation of the xenobiotic metabolizing enzymes \textit{CYP1A1} and \textit{UGT1A1} through examination of the classical Ah receptor signaling pathway. The first model studied was the interaction of environmental contaminant mixtures on the expression of CYP1A1. I hypothesized that the heavy metal As$^{3+}$ interferes with TCDD activation of the Ah receptor signaling pathway resulting in down-regulation of TCDD-induced CYP1A1 expression. To this end, I examined CYP1A1 induction at the levels of protein activity, protein expression, mRNA expression, and transcription. The resulting data indicates that As$^{3+}$ does not directly affect the Ah receptor but rather interferes with the recruitment of RNA polymerase II. To further characterize the effects of As$^{3+}$ treatment, I expanded my studies to include another Ah receptor mediated gene, \textit{UGT1A1}, as well as another putative class of Ah receptor agonists, the flavonoids. It was evident that the flavonoid chrysin did not initiate the same responses as the classical Ah receptor ligand TCDD at the \textit{UGT1A1} promoter. This led to my characterization of chrysin induction of UGT1A1. I expanded my repertoire of techniques to examine Ah receptor functionality to include in-depth gel shift analysis, radioligand binding assays, and siRNA. I demonstrated the dual roles of chrysin as an Ah receptor ligand directing \textit{CYP1A1} transcription as well as initiating the MAP
kinase signaling pathway to induce UGT1A1 expression. In addition to the cell culture models studied, I expanded my work to include in vivo models of UGT1A regulation. Experiments conducted in Tg-UGT1 mice on flavonoid regulation of UGT1A1 did not validate the cell culture model. Thus, in-vitro screens may not be reflective of actual, in-vivo UGT1A1 regulation. The Tg-UGT1 mouse also allowed for the discovery of functional, in vivo transcriptional regulation by the nuclear receptors PXR, CAR, and PPARα that could then be characterized through traditional cell culture molecular biology techniques. Perhaps the most promising use of the Tg-UGT1 mouse is modeling of complex diseases such as metabolic syndrome to understand the effects disease has on xenobiotic clearance. Pilot experiments in which the early phase of type 2 diabetes was initiated by a high fat diet show dramatic regulation of the UGT1 locus. This may be a result of various physiological changes and signals that would not have been easily identified in cultured cells. And to our great surprise, UGT1A proteins may have an influence on the progression towards type 2 diabetes. The initial experiments revealed normal responses to glucose and insulin in the high fat diet Tg-UGT1 mice. Taken together, a more complex picture of the regulation of xenobiotic metabolizing enzymes is evolving that shows the sensitivity of these genes to various environmental chemical signals, transcription factors, and the overall health of the organism.

As$^{3+}$ and CYP1A1

Our results indicate that As$^{3+}$-initiated cell cycle arrest and the inhibition of CYP1A1 induction by TCDD are not associated with a common regulatory event. The
disparity in these events can be observed by the large differences in As$^{3+}$ concentrations needed to induce G$_2$/M arrest and the inhibition of TCDD-initiated CYP1A1 induction. Very clear reductions in the induction of CYP1A1 mRNA and protein are observed at 0.5 µM As$^{3+}$, whereas the early events leading to G$_2$/M arrest are not detectable until 12.5 µM As$^{3+}$ exposure. One may speculate that As$^{3+}$ is capable of initiating cell cycle arrest at very low concentrations, but the sensitivity of detecting G$_2$/M arrest exceeds the limits of our techniques. Yet, when we analyzed the potential for cell arrest by monitoring changes in cell cycle regulatory proteins such as the cyclins B1 and D1 (268;336), p21, cdc25A, and cdk1 (270), no observable changes in these proteins were detected with low concentrations of As$^{3+}$ (data not shown). Furthermore, studies conducted in primary hepatocytes would question the role of cell cycle arrest in As$^{3+}$ inhibition of TCDD-mediated CYP1A1 induction. Hepatocytes are quiescent cells in the intact liver but are capable of one to two progressions through the cell cycle during liver injury. In culture, primary hepatocytes progress through G$_1$ independent of stimulation but arrest in mid-G$_1$ after 42 hours in culture (337;338). The hepatocytes in our study were cultured for 48 hours before treatment, allowing for cessation of cycling. Inhibition of TCDD-mediated CYP1A1 induction by As$^{3+}$ was still observed and closely resembled the dose-response established in HepG2 cells. Thus, CYP1A1 induction was still dramatically inhibited in a quiescent cell model. These results demonstrate that the actions of As$^{3+}$ on blocking CYP1A1 induction by TCDD are initiated through alterations in CYP1A1 transcription and are independent of the regulatory mechanisms initiated by As$^{3+}$-induced cell arrest.
The reduction in TCDD-induced CYP1A1-specific EROD activity by As$^{3+}$ has been clearly established. Similar results have been observed when cells were cotreated with PAHs and As$^{3+}$ (252-254;339). It has been postulated that this inhibition is the result of interference by As$^{3+}$ with the catalytic potential of CYP1A1, either through reduction in cellular heme pools or by direct binding of As$^{3+}$ to CYP1A1. However, changes in cellular heme pools would not lead to the observed changes in CYP1A1 transcription and the resulting reduction in CYP1A1 mRNA and protein.

The unique ability of As$^{3+}$ to bind thiol groups is well established in vitro (340), whereas relevant cellular models of binding are more difficult to determine. As$^{3+}$ has been shown to inhibit steroid binding to the glucocorticoid receptor (341;342) and to inactivate the catalytic loop in the inhibitor of κB kinase β subunit (IκB-β), (343). Members of the cytochromes P450 superfamily contain a conserved cysteine residue that serves as the axial ligand for heme iron (344). P450s contain very few cysteine residues and in the crystal structure of CYP2C5, no other cysteines are found in proximity to the heme binding cysteine. Because coupling to adjacent cysteine residues is a prerequisite for inhibition of protein function by As$^{3+}$ and given the evidence presented here on down-regulation of CYP1A1 by a transcriptional mechanism, the inhibition of EROD activity is not the result of As$^{3+}$ directly associating with CYP1A1.

Recent work has demonstrated a similar pattern of PAH-induced CYP1A1 inhibition by chromium (221). This work suggests that heavy metals may be acting in a similar manner at the promoter to inhibit induction of CYP1A1. However, chromium and As$^{3+}$ show distinct patterns of gene expression alteration. As$^{3+}$ alters the
expression of a unique set of genes, most notably up-regulation of heme oxygenase 1, metallothionein, and \textit{NQO1}, whereas chromium inhibits the expression of metallothionein and NQO1 (345-348). Chromium's ability to cause DNA-protein cross-links has been suggested as a potential mechanism for HDAC1 sequestration on chromatin within the \textit{Cyp1a1} promoter (221). However, \textit{As}$_3^+$-induced cross-links and DNA damage have been repeatedly found using primarily very high cytotoxic concentrations (269;349;350). Thus, it is doubtful that the low concentrations of \textit{As}$_3^+$ used in our studies would impact on HDAC1 sequestration.

Our results suggest that the actions of \textit{As}$_3^+$ leading to inhibition of CYP1A1 induction in HepG2 cells occur after TCDD-initiated activation of the Ah receptor and binding of the receptor to DNA. Because the nuclear accumulation of the Ah receptor and the binding potential of the Ah receptor/Arnt complex to DNA is not compromised, the reductions in CYP1A1 hnRNA indicate that the rate of transcription initiated by TCDD may be slowed. The low concentrations of \textit{As}$_3^+$ needed to inhibit TCDD induction of CYP1A1 leads to the prediction that \textit{As}$_3^+$ is modifying essential regulatory proteins involved in the maintenance of polymerase-initiated transcription. In support of this, chromatin immunoprecipitation assays revealed a reduction in Pol II recruitment to the \textit{CYP1A1} proximal promoter. Given the specificity of \textit{As}$_3^+$ inhibition to TCDD-mediated upregulation of CYP1A1 compared to UGT1A1 and NQO1, this suggests blockage of a signaling event(s) involved in the recruitment and maintenance of Pol II specifically to \textit{CYP1A1} and not a general inhibition of inducible gene expression. Indeed, microarray experiments using mouse embryonic fibroblasts (MEFs) treated with B[\textit{a}]P, \textit{As}$_3^+$, or both chemicals revealed many genes that were
either increased or decreased with the combination treatment confirming that transcription is not globally repressed by As$^{3+}$ treatment (282). Additionally, no genes induced by B[a]P were inhibited when MEFs were co-cultured with B[a]P and As$^{3+}$. Cyp1a1 expression was not analyzed in this cell model but another Ah receptor-inducible cytochrome P450, Cyp1b1, was measured. As$^{3+}$ did not impact on Cyp1b1 expression. To date, *CYP1A1* is the only Ah receptor-inducible gene that is repressed after co-treatment with a classic Ah receptor ligand and As$^{3+}$. Furthermore, the distinguishing feature between *CYP1A1* and *UGT1A1*, *NQO1*, and *CYP1B1* is that *CYP1A1* is not basally expressed. Thus, the parameters necessary for As$^{3+}$ inhibition of Ah receptor-dependent gene activation may be further narrowed to genes that are not actively transcribed in the basal state.

In the majority of normal, non-stimulated, adult human tissues, CYP1A1 mRNA and protein is non-detectable or present at very low, highly variable levels but is dramatically inducible through Ah receptor activation (reviewed in (15)). Additionally, the model cell lines used to study *CYP1A1* regulation, HepG2 and the mouse hepa1c1c7 cells, do not basally express CYP1A1. This classifies *CYP1A1* as a gene “poised” for expression in comparison to a silent/non-inducible gene or an actively transcribed gene. ChIP and co-immunoprecipitation studies have revealed an ever-growing collection of coactivators that are recruited with the Ah receptor to the *Cyp1a1* promoter including the p160 HAT coactivators p300, SRC-1, NCoA-2, and p/CIP, the ATP-utilizing histone modification protein Brg-1, as well as the mediator/TRAP/DRIP/ARC complex (see Chapter I: Structure and Signal Transduction Pathway of the Ah Receptor). With the identification of the
transcriptional machinery recruited by the Ah receptor, it is possible to hypothesize the most likely points of As\textsuperscript{3+} disruption of Ah receptor initiated transcription.

As\textsuperscript{3+} may interfere with the removal of nucleosomes thereby preventing recruitment of general transcription factors. This mechanism would be distinct from chromium inhibition of TCDD induction of Cyp1a1 through crosslinking of DNA to histones (221). Indeed, microarray experiments measuring gene expression profiling in MEFs (282) treated with 5 μM B[a]P and/or 2 μM As\textsuperscript{3+} detected upregulation of the methylation imprinting and HDAC1 recruiting gene Dnmt3l (DNA (cytosine-5)-methyltransferase 3-like) (351;352) as well as histone H1e (Hist1h1e, human homologue H1\textsuperscript{S.4}) which is associated with regions of inactive chromatin (353-355). The microarray study was performed after several hours incubation of the cells with As\textsuperscript{3+} while I demonstrated inhibition of pol II recruitment 1 hour after treatment (Fig. 9). It is unlikely that the synthesis and incorporation of new H1e histones into DNA is the major component of As\textsuperscript{3+} inhibition of CYP1A1 transcription. Alteration of the histone methylation and acetylation patterns can occur rapidly and thus is a plausible mechanism of As\textsuperscript{3+} inhibition of CYP1A1 induction. In studies of acute promyelocytic leukemia, it was found that As\textsubscript{2}O\textsubscript{3} enhanced histone H3 phosphoacetylation of the CASPASE-10 gene within 12 hours (356). Additionally, investigation of the carcinogenic potential of sodium As\textsuperscript{3+} lead to the finding that the H3 histones of the proto-oncogenes c-fos and c-jun were phosphoacetylated within 1 hour of treatment (357). In contrast to the activation of chromatin, in Drosophila cells, sodium As\textsuperscript{3+} promotes generalized hypermethylation of histone H2B within 3 hours of treatment (358). These histone modifications are likely due to alteration of key signaling
pathways as was demonstrated for the c-fos and c-jun genes whose phosphoacetylation was ERK dependent (357).

As$^{3+}$ has been shown to affect multiple phosphorylation signaling cascades in studies of both its carcinogenic as well as therapeutic potential. The first studies of arsenic influences on phosphorylation identified the process of oxidative phosphorylation in the mitochondria. This studies suggested that (1) As$^{5+}$ disrupts phosphorylation reactions by substitution for phosphate (359) and (2) As$^{3+}$ reacts with a dithiol group to uncouple phosphorylation from oxidation in a manner that does not inhibit phosphate transfer reactions (360). More recently, As$^{3+}$ was shown to affect many of the major cellular signaling cascades including all three of the MAP kinase pathways (ERK, JNK, and p38). The majority of reports concluded that As$^{3+}$ activates ERK, JNK, and p38 (361-364). However, in PHA-stimulated mouse T lymphocytes, As$^{3+}$ prevented PHA-induced ERK phosphorylation (365). This indicates that the effects of As$^{3+}$ on MAP kinase signaling may be dependent on the presence of other chemical signals. As reviewed in Chapter I, Ah receptor signaling is regulated by ERK and thus, MAP kinases may be the signaling event through which As$^{3+}$ inhibits TCDD-initiated CYP1A1 expression. Inhibition of Ah receptor signaling occurs with inhibition of MAP kinase activation (241). The mode of action of As$^{3+}$ might be similar to the model proposed in stimulated T lymphocytes rather than the numerous reports of As$^{3+}$ activation of MAP kinases.

It may be the case that a different complement of coactivators is recruited along with the Ah receptor to the transcriptionally poised CYP1A1 promoter in contrast to the actively transcribed UGT1A1 and NQO1 promoters. ChIP and co-
immunoprecipitation experiments identified several coactivators that associate with
the mouse Ah receptor on the Cyp1a1 promoter (reviewed in Chapter I: Structure and
Signal Transduction Pathway of the Ah Receptor). To date, ChIP analysis has not been
performed using promoters from constitutively expressed genes that also contain
XREs such as UGT1A1, NQO1, CYP1B1, or CYP1A2. Thus, it is not known if the same
complement of coactivators is recruited with the Ah receptor to these transcriptionally
active promoters. As$^{3+}$ may be interfering with a factor that is specifically recruited to
the CYP1A1 promoter. Nrf2 is activated by As$^{3+}$ and UGT1A1 and NQO1 are both
regulated by Nrf2. Thus, the activation of Nrf2 may alter those factors which are
recruited by the Ah receptor to the UGT1A1 and NQO1 promoters to further enhance
transcription.

**Chrysin and UGT1A1**

In this study, we describe the mechanism of human UGT1A1 gene induction by
the dietary supplement chrysin. As demonstrated by electrophoretic mobility shift
assay and expression of UGT1A1 promoter and enhancer reporter gene constructs,
UGT1A1 gene induction by chrysin occurs after activation and XRE binding of the Ah
receptor. Mutation of the XRE binding sequence eliminates chrysin induction. Our
findings indicate that chrysin is capable of competing for TCDD binding to the Ah
receptor and thus serves as a ligand for the receptor. Like TCDD activation of the Ah
receptor and induction of CYP1A1, chrysin activation and induction of UGT1A1 is
dependent on activation of MAP kinase pathways. This suggests that TCDD and
chrysin may promote induction of UGT1A1 through conserved signaling pathways. In
addition, treatment of HepG2 cells with either chrysin or TCDD promotes the nuclear translocation of the Ah receptor in a manner that promotes binding of the receptor to DNA. Combined, this evidence suggests that chrysin induction of \textit{UGT1A1} is elicited through activation of the Ah receptor.

However, in HepG2 cells, dramatic differences have been observed in the molecular actions between TCDD and chrysin. For example, chrysin is a more effective inducer of \textit{UGT1A1} than TCDD in tissue culture. This finding is a surprise because TCDD activation of the Ah receptor predominates when compared with the ability of chrysin to induce \textit{CYPIA1} reporter activity. One might even speculate that a mechanism is in place on the \textit{UGT1A1} promoter to suppress responsiveness to the Ah receptor. Combined with the minimal impact of Ah receptor knockdown on chrysin induction of the \textit{UGT1A1} promoter, it is clear that although the Ah receptor may be recruited to the promoter, the Ah receptor is not the primary mechanism of chrysin induction of \textit{UGT1A1}. Conversely, an intact XRE sequence typically only associated with the Ah receptor is critical for chrysin induction of \textit{UGT1A1}, indicating other factors are recruited to this site in a MEK1-dependent manner.

The differential characteristics of chrysin induction of \textit{CYPIA1} and \textit{UGT1A1} are interesting. Further evidence that TCDD and chrysin differentially regulate induction in an Ah receptor-dependent fashion was demonstrated by examining the functional properties of the receptor. After treatment of HepG2 cells with TCDD, ligand binding to the Ah receptor stimulated both transactivation and proteolysis. These two activation processes are thought to be characteristic properties of ligands that activate the Ah receptor. In contrast, chrysin treatment of HepG2 cells does not
promote transactivation of the Ah receptor in a TCDD-dependent fashion and has no
effect on initiating proteolysis of the receptor. The near complete block in chrysin
induction of \textit{CYP1A1}-luciferase by si-AhR is strong evidence that chrysin is able to
activate transcription through the Ah receptor on the \textit{CYP1A1} promoter. This
activation may be the result of the weak Ah receptor transactivation potential of
chrysin or be dependent on the recruitment of coactivators.

Flavonoids have been described as health-promoting, disease-preventing
dietary supplements and have been widely proclaimed to possess biological activity as
cancer preventive agents (366). Part of the rationale for predicting the effectiveness of
flavonoids in disease prevention stems from reports that agents such as chrysin are
capable of inducing those enzymes that are involved in drug-elicited and dietary-
elicited detoxification, such as the UGTs (367). Yet, limited reports are available on
the effectiveness of flavonoids in regulation of the \textit{UGT} genes \textit{in vivo}. To directly
address the potential for chrysin to induce human UGT1A1 expression, transgenic
mice that express the human \textit{UGT1} locus (73) were treated orally with chrysin for 7
days. Oral treatment of chrysin had no effect on protein induction in the small
intestine. A similar result was noted when Tg-\textit{UGT1} liver microsomes were analyzed.
Because the oral treatment of B[a]P led to induction of UGT1A1 in both liver and
small intestine, the Ah receptor signaling pathway is intact in these mice. The lack of
in vivo induction by chrysin may be reflective of the bioavailability of chrysin. This
appears to be the case, as demonstrated by the rapid reduction of plasma chrysin and
the accumulation of chrysin glucuronide. Clearly, chrysin's ability to induce
detoxifying enzymes such as UGT1A1 is tempered by the low bioavailability of the
compound, which has been suggested to occur in humans (368). Although chrysin has been convincingly demonstrated to induce UGT1A1 expression in tissue culture models (285;369), these data indicate that oral chrysin consumption has little effect on regulating UGT1A1 expression in vivo.

Although other flavonoids have not been investigated, the utility of consuming these compounds for dietary or clinical use may be questionable. An example of the usefulness of flavonoid therapy is presented in individuals who are taking the prodrug irinotecan for solid tumor chemotherapy. Irinotecan is metabolized to the active metabolite SN-38, which is ultimately eliminated through glucuronidation (370-372). However, a heightened sensitivity to irinotecan-induced bone marrow toxicity as well as ileocolitis toxicity (373) is observed in patients with reduced UGT1A1 activity because adequate elimination of SN-38 is impaired (374). With available information that flavonoids can induce UGT1A1 in both human hepatoma and intestinal cells, a diet rich in flavonoids may help to counter the toxicities elicited by SN-38 treatment (370). Yet, the experiments that we conducted in Tg-UGT1 mice would indicate that oral flavonoid therapy may have limited potential as an inducer of human UGT1A1 in vivo. Our findings are consistent with a previous clinical study demonstrating that colorectal cancer patients on irinotecan therapy in combination with chrysin treatment did not significantly improve SN-38-induced gastrointestinal tract toxicity (375).

The current study reinforces the need for additional studies to be conducted in transgenic and humanized animal models as an endpoint to verify the importance of regulatory events that have been identified in other model systems such as tissue culture studies. Studies with newly identified exogenous and endogenous Ah receptor
ligands should include target genes other than CYPIA1 as these compounds may preferentially activate a different set of genes as was the case with chrysin. Chrysin utilizes the Ah receptor, ERK signaling pathway, and additional unidentified factors to induce UGT1A1 expression to a greater extent than TCDD in vitro. Evaluation of the signaling mechanisms of these endogenously active genes may provide insight into the Ah receptor’s role outside of xenobiotic sensing.

Identification of endogenous ligands for the Ah receptor has been sparse and dependent mainly on in vitro binding assays and competition experiments with TCDD. Candidate endogenous ligands include 7-ketocholesterol, bilirubin, lipoxin A₄, low-density lipoprotein, and multiple tryptophan derivatives (376-380). AhR⁻/⁻ mouse studies identified the necessity for the Ah receptor in development of the liver, immune system, heart, vasculature, and normal reproduction (381;382). One of the aforementioned endogenous chemicals may activate Ah receptor signaling during development. TCDD is classified as a teratogen which may be explained by the Ah receptor’s role in development. However, not all Ah receptor ligands are teratogens. Artificially high doses of the tryptophan derivative ITE were found to activate the Ah receptor in vivo (383). In contrast to TCDD, this method of Ah receptor activation was not teratogenic indicating that the adverse effects of Ah receptor activation are highly dependent on the stimulus. It remains to be seen if physiologically relevant levels of these endogenous Ah receptor ligands do indeed activate the Ah receptor in vivo and direct physiological responses. Additionally, understanding of the complexity of Ah receptor signaling and target genes may allow for more accurate refinement and assessment of cellular and whole animal responses to xenobiotic exposures.
Nuclear Receptor Control of UGT1A6 Expression

The family of UGT1A proteins metabolizes a diverse group of endogenous and exogenous substrates. As well as being substrates for glucuronidation, many xenobiotics are also regulators of UGT1A expression. The first fully characterized transcriptional control of a human UGT1A gene by a xenobiotic was regulation of UGT1A6 by the Ah receptor (202;203). Our laboratory later characterized Ah receptor control of human UGT1A1 (200). Soon after, the xenobiotic-sensing adopted orphan nuclear receptors PXR and CAR were shown to regulate human UGT1A1 (95;298). Later research expanded the repertoire of adopted nuclear receptor regulation to include the lipid-sensing PPARα and γ regulation of UGT1A9 and LXRxα regulation of UGT1A3. Thus, it was not surprising to observe upregulation of UGT1A6 in vivo after treatment with PXR, CAR (73), and PPARα ligands (258). The promoter scan software program MatInspector 7.4.5 (Genomatix) predicts a multitude of putative nuclear receptor binding sites within the 10 kb of the UGT1A6 5’ regulatory DNA. This includes response elements for the adopted orphan receptors LXR, PPARα, farnesyl x receptor (FXR), PXR, and CAR as well as the endocrine receptors GR, estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), retinoid A receptor (RAR), thyroid receptor (TR), and vitamin D receptor (VDR) (Fig. 36, (384)).
The adopted orphan nuclear receptors as well as the endocrine receptors RAR, TR, and VDR bind to a core response element sequence, (G/A)GGT(C/G)A, arranged as a direct repeat (DR), inverted/palindromic repeat (IR), or everted repeat (ER)/inverted palindrome (IP) with a varying number of spacer nucleotides (i.e. DR4: AGGTCAnnnnAGGTCA) (reviewed in (385;386)). The heterodimeric nuclear receptors tend to follow the 3-4-5 rule of half site direct repeat spacing (387). The VDR prefers a DR3, the TR, PXR, LXR, and CAR all prefer DR4, and the RAR prefers the DR5 motif (310;387;388). Additionally, PPARα preferentially binds DR1 motifs (311;389) and FXR prefers an IR1 motif (390). The aforementioned DNA response elements are representative of the more commonly characterized nuclear
receptor binding sites. As more genes are found to be regulated by the nuclear receptors, a larger catalogu of binding site preferences is sure to arise. The promiscuousness of nuclear receptors is exemplified by VDR affinity for an IP9 motif over its classically defined DR3 in the human calbindin D9k promoter (391). The similarity in motif sequences allows for the sharing of response elements as illustrated by PXR, CAR, and VDR shared affinity for DR3 elements (95;387). Even though nuclear receptor binding sequences are categorized by the number of spacer nucleotides, this does not mean that all nuclear receptors capable of binding a particular class of motif will. LXR which binds to a DR4 motif with a specific DNA sequence termed the LXRE will not bind other DR4 motifs that the TR has been shown to bind and vice versa (388).

With the complexity of nuclear receptor binding, the in silico prediction of nuclear receptor binding sites on the *UGT1A6* promoter is only a general guide to what factors may be regulating the gene *in vivo*. Indeed, Vyhlidal *et al.* identified a DR4 PXR binding site at -9240/-9224 bp (from ATG) which bound PXR/RXR in electromobility shift assays but did not activate a luciferase construct composed of tandem repeats of this DR4 (309). Luciferase assay using the 3.6 kb enhancer fragment ER1 that contains this putative PXR binding site as well as many other predicted nuclear receptor binding sites is highly responsive to PXR and CAR (Fig. 27). Thus, the in silico method of nuclear receptor binding site identification should only be used as a guide. More traditional promoter/enhancer truncation studies in combination with ChIP and electromobility shift assay will be necessary to clearly define nuclear receptor response sites.
This more traditional approach is being used to identify the PXR (and eventually CAR) responsive sites within the initial 3 kb promoter region of \textit{UGT1A6}. Promoter deletion analysis has narrowed the PXR response element to between -2189 kb and -1192 kb. Conveniently, promoter scan analysis does indicate the presence of a putative DR4 element at -1762/-1746 bp. Further truncations of the promoter as well as mutation of critical residues will be used to establish this site as the PXR response region along with electromobility shift assay to confirm PXR/RXR binding. Eventually, the PXR, CAR, and Ah receptor responsive elements in the entire 10 kb of 5’ regulatory DNA will be identified to more accurately predict xenobiotic regulation of \textit{UGT1A6} expression.

In addition to regulation by xenobiotic sensors, \textit{UGT1A6} expression may be regulated by dietary lipids though PPAR$\alpha$. In vivo treatment of Tg-\textit{UGT1} mice with the fibrate WY-14643 results in significant upregulation of UGT1A1, 1A3, 1A4, and 1A6 expression (258). Based on the consensus PPAR$\alpha$ binding site published by Johnson \textit{et al}. (311), in silico promoter screening of the \textit{UGT1A6} promoter region identified 3 putative PPAR$\alpha$ binding sites within the first 6 kb of the 5’ regulatory DNA (Fig. 29). Electrophoretic mobility shift assay using probes of the 3 putative PPREs identified only PPRE2 as capable of PPAR$\alpha$/RXR binding. Transactivation of the complex was confirmed using a luciferase reporter consisting of three tandem repeats of PPRE2. It is interesting to note the similarities in transcription factor binding site prediction methods. The MatInspector software identified 6 putative PPREs within the first 3.5 kb of the \textit{UGT1A6} promoter, only one of which overlapped with the manual consensus binding site search (PPRE2). This is indicative that this
PPRE2 site is responsible for PPARα regulation of *UGT1A6*. Confirmation of promoter activity will be obtained using the *UGT1A6* promoter truncation constructs described for the identification of the PXR responsive region. Regulation of UGT1A6 expression by PPARα also allows for the possibility of regulation by PPARγ and PPARδ due to the recognition of similar DR1 elements. Control by all PPARs may result in differential regulation patterns during times of metabolic disruptions such as fasting or obesity.

The identification of regulatory elements controlling inducibility may also explain basal expression patterns of UGT1A6. Human UGT1A6 presents a highly polymorphic expression pattern. Within the liver, UGT1A6 mRNA is consistently detected in all samples analyzed whereas it is detected in only 36% of gastric epithelium tissue samples (65). Analysis of the duodenum, jejenum, and ileum of the small intestine revealed consistent, but not 100%, expression of UGT1A6 transcripts in the duodenum and ileum while in the jejunum UGT1A6 transcripts were detected in only 1 of 5 samples (392). A disconnect therefore exists in the regulation of UGT1A6 expression between the liver and gastrointestinal tract.

The consistent basal expression in the liver may be due to regulation by liver specific transcription factors while the variation in the gastrointestinal tract could be due to xenobiotic regulation from the diet. Indeed, hepatic expression of the *UGT1A9* gene was shown to be controlled by the orphan nuclear receptor hepatic nuclear factor 4α (HNF4α) through a DR1 site (393). The exclusively extrahepatic UGT1A7, 1A8, and 1A10 isoforms were not regulated by HNF4α. Several HNF4α binding sites are predicted for the *UGT1A6* promoter as well as the putative PPREs which are also DR1
sites. Thus, consistent hepatic expression of the UGT1A isoforms may be obtained through HNF4α.

Variation in UGT1A6 expression within the gastrointestinal tract may arise from exposure to drugs, environmental toxicants, or dietary factors. PXR is often referred to as a promiscuous receptor that recognizes a variety of compounds most likely stemming from its large, flexible ligand binding pocket (394;395). PXR/SXR is activated by a wide variety of chemicals including endogenous sterols (396;397) and bile acids (398), hyperforin from St. John’s wort (399;400), and pharmaceuticals including SR12813, rifampicin (307;401), and clotrimazole (402). In studies with Tg-UGT1 mice bred into a PXR-null background, the basal levels of UGT1A6 as well as the other UGT1As was reduced in the small intestine (unpublished data; Chen S and Tukey RH). Thus, activation of PXR by endogenous and dietary factors may contribute to the basal maintenance of UGT1A6 expression. Ligands for human CAR tend to be xenobiotics such as phenobarbital (403), chlorinated pesticides, and polychlorinated biphenyls (404). As well as being a mediator for fatty acid signaling (405-407), PPARα has also been shown to be activated by the environmental contaminant trichloroethylene (408). Although the primary location of PXR and CAR is in the liver and small intestine, PXR transcripts have also been detected in human stomach (409;410). Endogenous compounds or oral exposure to anthropogenic compounds may regulate UGT1A6 gastrointestinal expression through PXR in the stomach as well as CAR and PXR in the small intestine. Nuclear receptor control is one possible mechanism explaining interindividual variation in UGT1A6 expression in the gastrointestinal tract.
Additionally, the recent identification of single nucleotide polymorphisms (SNPs) in the 5’ regulatory DNA of the human \textit{UGT1A6} gene may explain the interindividual variation. Recently, Krishnaswamy et al. (411) identified 8 SNPs within the first 2 kb of the \textit{UGT1A6} promoter. Using the MatInspector homology analysis program, the authors concluded that the SNPs did not occur in any major response element sequences. Comparison of the putatively identified PXR and PPAR\(\alpha\) response elements from this dissertation also did not reveal possible disruption of binding sites by these SNPs. However, the authors did not extend their analysis beyond 2 kb and the putative PPAR\(\alpha\) response element is located at -2692/-2680 bp. This SNP analysis was performed using 54 liver tissue samples and thus it is possible that additional SNPs in the regulatory DNA will be identified with a larger sample population. A significant precedence for SNP influence on glucuronidation was set with the discovery of a SNP in the promoter region of \textit{UGT1A1} which leads to Gilbert’s disease (87;412). The association of other known \textit{UGT1A} polymorphisms with disease and drug therapies is reviewed in greater detail in Chapter I: Historical Perspective on Phase I and Phase II Enzymes. With the increasing field of pharmacogenomics, there is great potential for the identification of SNPs in all of the \textit{UGT1A} genes. These SNPs may lead to interindividual variation in expression as well as toxic responses to pharmaceutical drugs and linkage to disease susceptibility.

The \textit{UGT1A6} and \textit{UGT1A1} promoters, so far, are responsive to the same set of transcription factors (Ah receptor, PXR, CAR, PPAR\(\alpha\), Nrf2), and possibly more as the locus is screened for nuclear receptor ligand responses. While the characterization of the PXR, CAR, and PPAR\(\alpha\) responsive elements is on-going, the demonstrated
regulation of UGT1A6 as well as in vivo regulation of most of the UGT1A isoforms by these nuclear receptors (73;258) highlights the role of UGTs in homeostasis. It is probable that the \textit{UGT1A} promoter regions evolved numerous elements that are responsive to a wide range of endogenous as well as dietary chemical exposures to better facilitate their removal and avoid a toxic episode.

\textbf{Implications for UGT1A enzymes In Metabolic Syndrome}

While the majority of studies focus on evaluating the risk of drug-drug interactions initiated by nuclear receptor regulation of detoxifying enzymes, the role of Phase II enzymes in maintaining metabolic homeostasis has largely been overlooked (the main exception being the diseases of unconjugated hyperbilirubinemia). If nuclear receptors control the UGT1A response to exogenous compounds, it is likely that the same signaling mechanisms would be utilized to clear endogenous compounds when concentrations rise due to disease.

The identification of PPAR regulation of the \textit{UGT1} locus highlights the potential involvement of UGT1A proteins in lipid homeostasis. The endogenous role of the three PPAR nuclear receptors (\(\alpha\), \(\gamma\), \(\delta\)) is to regulate the metabolism of dietary fatty acids (and their metabolites) and the storage and release of lipids (413). PPAR\(\alpha\) directs the upregulation of many enzymes involved in fatty acid oxidation including the fatty acyl-CoA oxidase, acyl-CoA synthetase, HMG-CoA, very long chain, long chain, and medium chain acyl-CoA dehydrogenases, cytochrome P450 4As, and liver fatty acid binding proteins (reviewed in (413)). The activation of PPAR\(\alpha\) results in lowered circulating triglycerides and reduced adiposity (414-416). Ppar\(\alpha\)-null mice
fed a high-fat diet increase adiposity but maintain a normal insulin response compared to hyperinsulinemia in WT (417). This paradoxically associates PPARα to development of insulin resistance in the context of a high fat diet. PPARγ’s effects are concentrated on adipose tissue, activating genes necessary for adipocyte differentiation as well as lipid storage and synthesis such as aP2, adipsin, lipoprotein lipase, GLUT4, FATP, CD36, and even PPARγ itself (418-420). Similar to Pparα-null mice, Pparγ heterozygote knockout mice have increased sensitivity to insulin during both normal and high fat diet, suggesting normal activation is also linked to development of insulin resistance (421;422). A liver-specific Pparγ knockout mouse develops hyperlipidemia and increased adiposity, possibly caused by reduced triglyceride uptake (423). Despite the ubiquitous expression of PPARδ, relatively little is known about specific target genes. Recent studies with Pparδ-null mice revealed the role of PPARδ in liver glycolysis and fat-burning in muscle (424;425). Clearly, the roles of PPARs in metabolic homeostasis are complex and provide for a diverse response to metabolic disruptions. The in vivo upregulation of UGT1A proteins with a PPARα agonist (258), identification of functional PPARα response elements in the promoters of individual UGT1A genes (1A1, 1A3, 1A6, 1A9), and the transcriptional control of UGT1A9 through PPARγ (266) implicates UGT1A proteins in the control of fatty acid metabolism and/or adipocyte differentiation and lipid synthesis. Furthermore, the potential exists for alterations in UGT1A expression through aberrant PPAR signaling during type 2 diabetes.

The United States has experienced a significant rise in abdominal obesity over the past 20 years (426;427). As well as increasing the risk for myocardial infarction
abdominal obesity is a risk factor for development of the components of metabolic syndrome as well as type 2 diabetes (429-432). Metabolic syndrome is a term used to describe a cluster of clinical disorders that elevate a patient’s risk for type 2 diabetes and cardiovascular disease (reviewed in (433)). The criteria for diagnosis include hyperglycemia, insulin resistance, abdominal obesity, hypertriglyceridemia, low HDL cholesterol, and high blood pressure (434). Many of the components of metabolic syndrome are also characteristic of the early phase of type 2 diabetes (435-437). In the pre-diabetic state, fasting blood glucose levels remain normal but insulin-stimulated glucose uptake is impaired. This mild insulin resistance is overcome by increased insulin secretion (i.e. hyperinsulinemia). Eventually pancreatic β-cells become desensitized to FFA-stimulation. The pancreas stops secreting insulin and circulating insulin levels return to normal levels. This leads to the development of hyperglycemia and full type 2 diabetes.

To examine the role of UGT1A proteins in a metabolic disorder, Tg-UGT1 mice were fed a normal or high fat diet for 16 weeks to stimulate the early phases of type 2 diabetes. Tg-UGT1 mice displayed similar weight gain as that of their WT littermates suggesting the early stages of type 2 diabetes should be occurring in the Tg-UGT1 mice. However, clear differences were observed when the mice were challenged with glucose or insulin. HFdiet Tg-UGT1 male mice were refractive to glucose and, even more clearly, to insulin while HF diet WT male mice were glucose and insulin intolerant. The apparent insulin sensitivity in Tg-UGT1 mice was further confirmed by measurement of serum insulin levels. HF diet WT male mice were hyperinsulinemic while Tg-UGT1 male mice maintained normal serum insulin levels.
Both HF diet strains of mice were normoglycemic indicating the mice were in the earliest stages of type 2 diabetes and resembling the metabolic syndrome. Studies conducted with female Tg-UGT1 and WT littermates did not reveal any differences between the two genotypes, and furthermore, the high-fat diet did not initiate insulin insensitivity or hyperinsulinemia in WT females. This sexual dimorphism in initiation of type 2 diabetes has been reported for several rodent models and may be linked to the estrogen receptor (438-442). Due to the lack hyperinsulinemia in the WT female mice, only male mice were used for further analysis. The apparent resistance to hyperinsulinemia in the male Tg-UGT1 mice suggests that over-expression of UGT1A proteins uncouples insulin resistance from obesity during the early stages of type 2 diabetes.

The mechanism by which UGT1A proteins may sensitize high-fat fed mice to insulin is pure speculation at this point but may involve metabolism of endogenous signaling compounds that are integral in insulin resistance: free fatty acids (FFAs). Clinically, elevated FFAs are associated with obesity, insulin resistance, hyperinsulinemia, and hyperglycemia (reviewed in (433;443)). Recently, through the use of knockout and transgenic mice it has been shown that the G protein-coupled receptor GPR40, located on the cell surface of pancreatic β-cells, is activated by medium and long chain fatty acids and regulates insulin secretion (444-446). During the early stages of type 2 diabetes, stimulation by elevated circulating FFAs leads to hyperinsulinemia (435). As the disease progresses, chronic GPR40 stimulation leads to impaired β-cell function and eventually lower insulin levels as is seen in overt type
2 diabetes (447). Since Tg-UGT1 mice do not display hyperinsulinemia on a high-fat diet, it is possible that UGT1A proteins affect the level of circulating FFAs.

Fatty acids and their derivatives are known to be substrates for glucuronidation in rodents and humans. In the 1950s, there was a surge in the identification of steroid glucuronides in human urine and plasma (reviewed in (25)). Some 80-95% of steroid metabolites found in urine are glucuronides including derivatives of estrogen, testosterone, androsterone, aldosterone, pregnenolone, and hydrocortisone. More recently, glucuronidation of estrogens by the UGT1A enzymes was confirmed while androgen glucuronidation is more likely due to the activities of the UGT2B family of enzymes (reviewed in (77)). Ingestion of diets enriched in medium-chain triglycerides (C8-10,3:1) as well as patients diagnosed with medium-chain acyl-CoA dehydrogenase deficiency results in large quantities of octanoylglucuronides excreted in the urine (448-450). Additionally, glucuronidation of arachidonic acid and linoleic acid derivatives (LTB4, PGB1, 12-HETE, 15-HETE, 20-HETE, 13-HODE), phytanic acid, docosahexaenoic acid, and farnesol by several members of the UGT1A family has been demonstrated using expressed human UGTs (451-454). 20-HETE glucuronides have been detected in human urine samples (455). As well as endogenous fatty acids, glucuronidation is a major route of metabolism for the pharmaceutical derivatives of fatty acids such as valproic acid (456), the non-steroidal anti-inflammatory drugs acetaminophen, oxaprozin, benoxaprofen (457-460), the peroxisome proliferator 2-ethylhexanoic acid (461)(HamdouneM1995ToxAppPhar), and the fibrate class of PPAR agonists (462;463). Although many of the medium and long chain fatty acids known to activate GPR40 (444) as well as biomarker FAs for
type 2 diabetes (464) have not been identified as substrates for glucuronidation, the existent of such a vast array of fatty acid derivative glucuronides is highly indicative of the potential for the family of UGT1A proteins to modulate those FFAs associated with insulin resistance in type 2 diabetes.

There is little study of the effects of the diabetic state on human glucuronidation capacity. An early report on the total concentration of glucuronides found in human serum concluded that there was no difference between normal and diabetic patients, although it is not known whether the patients were type 1 or type 2 diabetic (465). As was reviewed in Chapter III: UGT1As and the High Fat Diet Model of Type 2 Diabetes, there are few reports of the effects of the disease state on glucuronidation in rat or mouse models. Additionally, there are no studies analyzing the effects of high fat diet-induced type 2 diabetes on glucuronidation or UGT1A expression in rodent models or humans. In our Tg-UGTI mouse model, each UGT1A gene is under the control of its native promoter, not a constitutively active or tissue-specific directed promoter. Thus, while the mice initially express a full complement of mouse and human UGT1A isoforms, both are susceptible to transcriptional regulation during the onset of type 2 diabetes. Analysis of total UGT1A protein in Tg-UGTI mice revealed a surprising decrease in UGT1A levels in small intestine and liver of the high fat fed mice (Fig. 34). The common UGT1A antibody is only cross-reactive with the mouse isoforms in liver and a similar decrease in UGT1A levels was observed in WT high fat mice. Quantitative real-time PCR analysis of human UGT1A1 mRNA in small intestine was consistent with the down-regulation of protein suggesting a transcriptional mechanism of repression (Fig. 35). Curiously, the mouse Ugt1a1
mRNA was down-regulated in the small intestine of WT high fat mice but not in Tg-

UGT1 high fat mice. Conversely, liver mouse Ug1a1 transcripts were unaffected in WT mice but down-regulated in high fat Tg-UGT1 mice. Clearly, there is a disconnect in regulation between human and mouse UGTA transcriptional regulation. In addition, the human locus is impacting in some way on regulation of the mouse locus.

The mechanism of the down-regulation of human UGT1A mRNA transcripts is unknown but could involve the interplay between various circulating factors. The main similarity between HF diet Tg-UGT1 and WT mice is increased adiposity. Thus, it is likely that the repression mechanism of UGT1A repression is initiated through the adipose and not hyperinsulinemia as Tg-UGT1 mice are normoinsulinemic. Adipose tissue releases many circulating factors including leptin, TNFα, IL-6, resistin, and adiponectin (reviewed in (466)). Adiponectin is negatively correlated to adipose mass while leptin, TNFα, and resistin are positively correlated to adipose mass. Adiponectin stimulates FFA uptake from the blood and FA oxidation, functions also associated with PPARα activities (467). Increased adiposity lowers adiponectin synthesis while increasing leptin and inflammatory cytokine secretion. Leptin stimulates release of the anorexigen T4 while also inhibiting liver-specific glucocorticoid activation (and some reports of overall lower circulating glucocorticoids) (468). The glucocorticoid cortisone is inactive and converted to active cortisol by 11β-HSD1 and the reverse reaction is mediated by 11β-HSD2. In obese humans, hepatic 11β-HSD1 activity is impaired while adipose 11β-HSD1 activity is increased (reviewed in (469)). This mimicks and is often clinically confused with the effects of hypercorticolism seen in Cushing’s syndrome. In primary Tg-UGT1 hepatocyte cultures, the addition of very
low concentrations of the glucocorticoid dexamethasone increases basal UGT1A mRNA levels as well as enhances transcriptional responses to PXR and Ah receptor ligands (73). Additionally, leptin can activate the JAK/STAT5B signaling pathway which has been shown to inhibit basal levels of PPARα mediated genes involved in FA β-oxidation and PPARα-mediated fibrate induction of genes (470-473). Release of inflammatory cytokines such as TNFα induces NF-κB and JNK1/2 signaling pathways that lead to insulin resistance (333;474-476). Two studies provide evidence that LPS-induced inflammation in mice or inflammatory liver disease in humans generally down-regulates expression of UGT1A isoforms (477;478). Additionally, treatment of Tg-UGT1 primary hepatocytes with TNFα suppresses basal and inducible UGT1A expression (Chen, S. and Tukey, R.H., unpublished data). Thus, it is possible that circulating inflammatory cytokines and leptin released from the increasing adipose stores in Tg-UGT1 mice work to suppress basal UGT1A expression directly and through inhibition of glucocorticoid activation, respectively. Despite the general decrease in UGT1A expression in HF diet Tg-UGT1 mice, Tg-UGT1 mice on both the normal and high fat diet possess higher UGT1A protein levels and thus increased glucuronidation capacity than WT mice (Fig. 34). This laboratory previously demonstrated increased glucuronidation of various substrates in small intestine microsomes (73) as well as lowered unconjugated serum bilirubin levels in uninduced Tg-UGT1 mice (unpublished data). This elevated glucuronidation potential may be the underlying reason for the resistance to hyperinsulinemia in Tg-UGT1 mice.

Increased glucuronidation, and therefore increased elimination of FFAs presents a theoretical model of UGT1A mediated resistance to hyperinsulinemia (Fig.
When a diet high in triglycerides is consumed, the triglycerides are broken down to the glycerol and fatty acid components in the lumen of the small intestine by pancreatic lipase (triglyceride digestion and absorption reviewed in [479]). The fatty acids in conjunction with bile salts form micelles that are absorbed by the enterocytes of the small intestine. Within the endoplasmic reticulum, FAs are reassembled into triglycerides but would also encounter UGT1A enzymes which conjugate the FAs. The FA-glucuronide is now water soluble and enters the portal vein to the liver. The FA-glucuronide enters the systemic circulation for elimination in the kidneys via urine. Although, FA-glucuronides excreted in the bile could be unconjugated by glucuronidases present in the intestinal flora and re-enter the portal circulation [480].

Within the hepatocytes, FAs that were not initially subjected to glucuronidation in the small intestine (or that re-entered the portal circulation through enterohepatic recirculation) would now be subject to glucuronidation. As triglycerides are broken down to FAs and then subsequently subjected to β-oxidation for generation of acetyl CoA, many of the hydroxylated intermediates would be ideal substrates for glucuronidation. Thus, glucuronidation may interfere with gluconeogenesis in the liver and possible development of hyperglycemia. Additionally, FFAs released from the adipose tissue into the systemic circulation [481] would be targets for glucuronidation during filtration within the liver and kidneys. Although the pancreas is the site of FFA initiated insulin resistance [446], no human UGT1A proteins were detected in normal or HF diet Tg-UGT1 mice. Thus, FFA inactivation within the pancreas is unlikely to be the mechanism of resistance to hyperinsulinemia in Tg-UGT1 mice. However, UGT1A7 (and to a lesser extent UGT1A4 and UGT1A3) mRNA transcripts have been
detected in human pancreatic tissue allowing for possible pancreatic FFA metabolism in humans (70). The potential systemic reduction in FFAs due to increased glucuronidation may prevent (or retard) development of insulin resistance due to excess FFA stimulation of GPR40 in the pancreas (446). While glucuronidation slows the accumulation of FFAs, the high fat diet ensures a continuous supply of dietary FFAs and a certain percentage of FFAs will escape elimination. The FFAs will be taken up by the growing adipose (466). The adipose secretes leptin which inhibits formation of the active glucocorticoid cortisol in the liver (468). This may reduce the maintenance of basal UGT1A expression (73). Additionally, leptin suppression of PPARα may reduce basal UGT1A transcription. Inflammatory cytokines such as TNFα released from the ever increasing adipose tissue may also initiate signaling pathways that suppress UGT1A expression. The combination of these inhibitory signals may lead to significant reductions in UGT1A protein expression. Theoretically, if the Tg-UGT1 mice remain on the high fat diet for a long enough period of time, the protective effect will regress due to loss of the human UGT1A glucuronidation of FFAs and hyperinsulinemia will develop.
Figure 37. Proposed model of UGT1A modulation of high fat diet-induced hyperinsulinemia. Dietary triglycerides are broken down into fatty acid micelles in the small intestine where they are absorbed by the enterocytes. Within the enterocytes, UGT1A enzymes glucuronidate the fatty acids or the FFAs are re-packaged as triglycerides, both of which enter the portal vein for transport to the liver. In the liver, triglycerides are again broken down to glycerol and FFAs to be used for gluconeogenesis in the mitochondria. FFAs will also be subject to glucuronidation by the UGT1A enzymes in the ER. The FFAs and FA-glucuronides re-enter the systemic circulation. FFAs stimulate the pancreas to secrete insulin via GPR40 signaling. FFAs and FA-glucuronides will eventually be filtered through the kidneys for elimination in the urine. Excessive dietary fats will be more efficiently eliminated in mice that over-express UGT1A enzymes, thereby reducing FFA stimulation of GPR40-mediated insulin secretion and delaying onset of hyperinsulinemia due to desensitization of the receptor. However, fat tissue will accumulate that secretes large amounts of leptin and TNF-α which will inhibit UGT1A expression and/or activity leading eventually to a reversal of UGT1A-mediated protection from hyperinsulinemia and type 2 diabetes.

Conclusions and Future Work

The aim of this dissertation research is to unravel the complex nature of xenobiotic metabolism regulation. The induction of *CYP1A1* through activation of the Ah receptor by carcinogens been extensively studied as the classical pathway of xenobiotic-mediated carcinogenicity. The studies presented here expand the basic model of Ah receptor control of xenobiotic metabolism. The differential effects of
As$^{3+}$ on TCDD induction of \textit{CYP1A1} and \textit{UGT1A1} indicates differences in Ah receptor mediated recruitment of the transcription initiation complex. The signaling events involved in Ah receptor activation and transcription initiation have almost solely been conducted on the \textit{CYP1A1} gene. As \textit{CYP1A1} is a transcriptionally poised gene, the transcription initiation events associated with the Ah receptor do not represent those events occurring at genes that are functionally active. Thus, while it is important to study the possible carcinogenic mechanism through Ah receptor activation of \textit{CYP1A1}, more focused research on signaling pathways involved in induction of transcriptionally active genes such as the \textit{UGT1A} isoforms or \textit{NQO1} may shed light on the protective and endogenous roles of the Ah receptor. Presumably, throughout the course of evolution, organisms were not exposed to the same level of high affinity Ah receptor ligands that have been chemically synthesized in the modern era (i.e. TCDD). Rather, organisms encountered an array of plant and animal components that could be efficiently eliminated through minor alterations in xenobiotic metabolizing enzyme expression. I demonstrate that a natural plant extract, chrysin, while able to activate the Ah receptor to induce CYP1A1 expression, activates both the Ah receptor and an unidentified MAP kinase-dependent pathway in the induction of the Ah-receptor responsive gene, \textit{UGT1A1}. Moreover, this representative of natural Ah receptor ligands only weakly induces CYP1A1 expression compared to TCDD. Carcinogenic episodes associated with Ah receptor activation of CYP1A1 expression may be a recent anthropogenic phenomenon and not indicative of the helpful detoxification role that the Ah receptor and CYP1A1 were probably intended for throughout evolution. Additionally, several \textit{CYP1A1} and XRE luciferase
based assays have been marketed as toxicological screens to predict carcinogenicity arising from Ah receptor activation (295;482-484). Using these assays, natural plant flavonoids such as chrysin would be labeled as potentially carcinogenic. One of the goals of the U.S. EPA is to translate basic research technologies like the luciferase assay to field sites for better assessment of potentially hazardous sites that may contain hundreds of different chemicals. Assays that utilize XRE repeats or CYP1A1 promoter fragments are only valid in the context of activation of a single signaling pathway. The functional outcome of mixtures of environmental contaminants such as TCDD or PAHs and heavy metals like As$^{3+}$ would be misrepresented in a transcriptional CYP1A1-luciferase assay. As demonstrated in this body of work, CYP1A1-luciferase is not reflective of the inhibition of CYP1A1 expression by As$^{3+}$. Thus, many screens would overestimate the carcinogenic potential of environmental samples. Using a single gene toxicity screen, the induction of compensatory Ah receptor-mediated detoxification enzymes like UGT1A1 and NQO1 induction would also be overlooked.

The chrysin studies also introduce a potentially new pathway of UGT1A1 regulation through MAP kinase activation. The demonstrated necessity of functional ERK1/2 and MEK1/2 in flavonoid induction of the UGT1A1 gene suggests that activation of upstream receptors and effectors could regulate UGT1A1 expression. These may include the prominent signaling pathways elicited by the receptor tyrosine kinases EGF and IR, TGFβ/SMADs, and IL-6 receptor/JAK/STATs. By methodical examination of each upstream regulatory component through genetic and pharmacologic techniques, a larger picture of endogenous regulation of UGT1A1 will develop. Along with the characterization of xenobiotic- and lipid-sensing nuclear
receptor control of the UGT1A locus, this detailed understanding of regulatory pathways will assist in understanding the fate of the entire UGT1A family during whole organism activation of signaling pathways as occurs in type 2 diabetes.

While the finding of resistance to hyperinsulinemia in the Tg-UGT1 mice is perhaps the most tantalizing of the findings presented in this dissertation, much work is needed to confirm and understand this finding. Due to the unexpected lack of type 2 diabetes in female mice, the study population was drastically reduced. In addition, there are reports of strain variations in susceptibility to type 2 diabetes (442;485-488). Our studies were performed in second generation mice on a mixed C57BL/6J and 129SvJ background. Although the mice were littermates, it is conceivable that some mice displayed phenotypes more characteristic of one strain over another. The Tg-UGT1 mice may have received a genetic background less susceptible to diet-induced diabetes. To address this issue for future studies, the Tg-UGT1 strain has been bred through five generations into the pure C57BL/6J background. Follow-up studies will be conducted using this “pure” C57BL/6J line and conducted in male mice. Due to the inhibition of UGT1A expression after 16 weeks of a high fat diet, diabetic status will be assessed weekly for a longer period of time to detect delay of onset of hyperinsulinemia. As FFA metabolism is the current hypothesis for the observed hyperinsulinemia, larger blood samples will be collected and accurate serum chemistry analysis performed. In addition to studies in the Tg-UGT1 mouse, an Ugt1a knockout mouse has recently been generated in this laboratory. It will be interesting to determine if the loss of all functional Ugt1a enzymes will confer susceptibility to metabolic disorders such as type 2 diabetes. If the Tg-UGT1 and Ugt1a−/− mice provide
direct links to high fat diet induced type 2 diabetes, the potential for clinical studies does exist with Gilbert’s patients that are genetically deficient in UGT1A1 activity. With the observed regulation of UGT1A isoforms by numerous nuclear receptors, it is conceivable that prescription drugs given to treat specific components of metabolic syndrome such as fibrates for dyslipidemia are also increasing UGT1A protein expression. This may be an additional, hereto unknown therapeutic benefit as well as potential target for pharmacological treatment of metabolic syndrome.

Portions of this chapter are reprinted in:


REFERENCES


4. Guengerich FP. Cytochrome P450s and Other Enzymes in Drug Metabolism and Toxicity. The AAPS Journal 2006; 8(1):E101-E111.


UDPglucuronosyltransferase genes are encoded at the human UGT1 gene complex locus. Pharmacogenetics 2001; 11:357-368.


82. Coughtrie MWH, Burchell B, Leakey JEA, Hume R. The inadequacy of perinatal glucuronidation: Immunoblot analysis of the developmental


145. Nemoto N, Takayama S. Genetic differences between C57BL/6 and DBA/2 mice in the inductions of UDP-glucuronyl transferases for 3-hydroxybenzo(s)pyrene, p-nitrophenol, and bilirubin by 3-methylcholanthrene. Toxicol Lett 1980; 5:45-50.


147. Nebert DW, Considine N, Owens IS. Genetic expression of aryl hydrocarbon hydroxylase induction. VI Control of other aromatic hydrocarbon-inducible mono- oxygenase activities at or near the same genetic locus. Arch Biochem Biophys 1973; 157:148-159.


161


185. Coumailléau P, Poellinger L, Gustafsson JÅ, Whitelaw ML. Definition of a minimal domain of the dioxin receptor that is associated with hsp90 and


203. Munzel PA, Schmohl S, Heel H, Kalberer K, Bock-Hennig BS, Bock KW. Induction of human UDP glucuronosyltransferases (UGT1A6, UGT1A9, and UGT2B7) by t-butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-p-dioxin in Caco-2 cells. Drug Metab Dispos 1999; 27(5):569-573.


213. Rowlands JC, McEwan IJ, Gustafsson J-Å. Trans-activation by the human aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator proteins; direct interactions with basal transcription factors. Mol Pharmacol 1996; 50:538-548.


226. Davarinos NA, Pollenz RS. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytoplasmic proteasome following nuclear export. J Biol Chem 1999; 274(40):28708-28715.


229. Morales JL, Perdew GH. Carboxyl terminus of hsc70-interacting protein (CHIP) can remodel mature aryl hydrocarbon receptor (AhR) complexes and


232.  Minsavage GD, Park SK, Gasiewicz TA. The aryl hydrocarbon receptor (AhR) tyrosine 9, a residue that is essential for AhR DNA binding activity, is not a phosphoresidue but augments AhR phosphorylation. J Biol Chem 2004; 279(20):20582-20593.


244. Moos AB, Baecher-Steppan L, Kerkvliet NI. Acute inflammatory response to sheep red blood cells in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin: the role of proinflammatory cytokines, IL-1 and TNF. Toxicol Appl Pharmacol 1994; 127(2):331-335.


284. He X, Chen MG, Lin GX, Ma Q. Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2 x Keap1 x Cul3 complex and recruiting Nrf2 x Maf to the antioxidant response element enhancer. J Biol Chem 2006; 281(33):23620-23631.


298. Xie W, Yueh MF, Radominska-Pandya A, Saini SPS, Negishi Y, Bottroff BS, Cabrera GY, Tukey RH, Evans RM. Control of steroid, heme, and carcinogen
metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proceedings of the National Academy of Sciences of the United States of America 2003; 100(7):4150-4155.


409. Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S, Schuetz EG. PXR (NR1I2): splice variants in human tissues, including brain, and


