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UNIVERSITY OF CALIFORNIA SAN DIEGO

NRF2-Independent Regulation of Drug Metabolism by CAR and Prooxidants

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

in

Chemistry

by

Miles Paszek

Committee in charge:

Professor Robert Tukey, Chair
Professor Neal Devaraj, Co-Chair
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2020

The Dissertation of Miles Paszek is approved, and it is acceptable in quality and form
for publication on microfilm and electronically:

Co-chair

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University of California San Diego

2020

DEDICATION

For my parents

Without their foresight, love, and support I would be nothing

EPIGRAPH

“Research is what I’m doing when I don’t know what I’m doing.” – Wernher von Braun

“But I am very poorly today and very stupid and hate everybody and everything. One lives to only make blunders.” – Charles Darwin

“Life, uh, finds a way.” – Malcolm, Jurassic Park

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LIST OF ABBREVIATIONS

ARE:	antioxidant response element
CAR:	constitutive androstane receptor
CCRP:	cytoplasmic CAR retention protein
CITCO:	6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime
CYP450:	cytochrome P450
ERK:	extracellular signal-related kinase
GI:	gastrointestinal tract
GSH:	glutathione
GST:	glutathione S-transferase
HSP:	heat shock protein
IEC:	intestinal epithelial cell
ITC:	isothiocyanates
KEAP1:	kelch-like ECH associated protein 1
MAPK:	mitogen-activated protein kinase
NAC:	N-acetyl-L-cysteine
NCoR1:	nuclear receptor corepressor 1
NR:	nuclear receptor
NRF2:	nuclear factor erythroid 2 related factor 2
UGT:	UDP-glucuronosyltransferase
P38:	P38 MAPK
PB:	phenobarbital

PBREM:	phenobarbital-responsive enhancer module
PEITC:	phenylethyl isothiocyanate
PPAR α :	peroxisome proliferator-activated receptor alpha
PXR:	pregnane x receptor
ROS:	reactive oxygen species
RXR:	retinoid x receptor
SI:	small intestine
TCPOBOP:	1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene
TSB:	total serum bilirubin
Veh:	vehicle
XR:	xenobiotic receptor

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ACKNOWLEDGEMENTS

I would like to acknowledge Professor Robert Tukey for his support as my research advisor. His patience, feedback on my work, and depth of knowledge have been instrumental to my continuing development into a scientist.

I would also like to acknowledge my mentors: Shujuan Chen, Mei-Fei Yueh, and Emiko Yoda for their willingness to share their expertise have contributed immensely to my knowledge and skills.

I would like to thank my parents Michael and Lynette Paszek for their love and support, which was and still is essential to my development as a person and a scientist. I would also like to thank my friend Andrea Sitton for her help in editing chapters 1, 4, and 5.

Chapters 2 and 3, in part, are partial reprints of the material as they appear in the following, respectively:

Yoda E, Paszek M, Konopnicki C, Fujiwara R, Chen S, and Tukey RH (2017) Isothiocyanates induce UGT1A1 in humanized UGT1 mice in a CAR dependent fashion that is highly dependent upon oxidative stress. *Scientific Reports* vol 7. The dissertation author was the second investigator and author of this paper.

Paszek M and Tukey RH (2020) NRF2-independent regulation of intestinal constitutive androstane receptor by the pro-oxidants cadmium and isothiocyanate in hUGT1 mice. *Drug Metabolism and Disposition* vol 48. The dissertation author was the primary investigator and author of this paper.

Changes to the materials presented were made to maintain formatting consistency.

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- Paszek, M and Tukey, R. H. NRF2-Independent Regulation of Intestinal Constitutive Androstane Receptor by the Pro-Oxidants Cadmium and Isothiocyanate in hUGT1 Mice. *Drug Metab. Dispos.* 2020, 48, 25-30
- Yoda, E.; Paszek, M.; Konopnicki, C.; Fujiwara, R.; Chen, S.; Tukey, R. H. Isothiocyanates Induce UGT1A1 in Humanized UGT1 Mice in a CAR Dependent Fashion That Is Highly Dependent upon Oxidative Stress. *Sci. Rep.* 2017, 7.
- Lu, W.; Rettenmeier, E.; Paszek, M.; Yueh, M.-F.; Tukey, R. H.; Trottier, J.; Barbier, O.; Chen, S. Crypt Organoid Culture as an in Vitro Model in Drug Metabolism and Cytotoxicity Studies. *Drug Metab. Dispos.* 2017, 45, 748–754.
- Chen, S.; Lu, W.; Yueh, M.-F.; Rettenmeier, E.; Liu, M.; Paszek, M.; Auwerx, J.; Yu, R.T.; Evans, R. M.; Wang, K.; Karin, M.; Tukey, R. H. Intestinal NCoR1, a regulator of epithelial cell maturation, controls neonatal hyperbilirubinemia. *Proc. Natl. Acad. Sci.* 2017, 114, 1432-1440.

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ABSTRACT OF THE DISSERTATION

NRF2-Independent Regulation of Drug Metabolism by CAR and Prooxidants.

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Doctor of Philosophy in Chemistry

University of California San Diego, 2020

Professor Robert Tukey, Chair
Professor Neal Devaraj, Co-Chair

Exposure to toxicants that create oxidative stress, such as isothiocyanates (ITCs) and heavy metals, initiate inductions of antioxidant response genes and detoxification genes such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) by the NRF2-KEAP1 pathway. UGTs act on a wide range of hydrophobic compounds, including the heme-degradation product, bilirubin,

which can only be conjugated by UGT1A1. Newborn humans commonly exhibit an accumulation of bilirubin in the serum known as hyperbilirubinemia due to a developmental delay in the transcription of UGT1A1. This effect is replicated in neonatal mice that lack a functional mouse *Ugt1* locus but contain the human *UGT1* locus. These *hUGT1* mice experience neonatal hyperbilirubinemia that can be ameliorated by the induction of *UGT1A1* by active nuclear/xenobiotic receptors. The crossing of *hUGT1* mice with nuclear/xenobiotic receptor knockout mice creates a mouse model that links a readily observed phenotype, hyperbilirubinemia, with nuclear/xenobiotic receptor function following exposure to a desired compound. Phenylethyl isothiocyanate (PEITC) treated neonates experienced a reduction of total serum bilirubin levels, and an induction of hepatic and intestinal UGT1A1 and the CAR target gene, CYP2B10. Utilizing *hUGT1/Car^{-/-}*, it was determined that PEITC induced hepatic UGT1A1 in a CAR-dependent manner. It was also shown that some CAR-independent induction of intestinal UGT1A1 occurred. Inductions of CYP2B10 and UGT1A1 could be blocked by pretreatment with the antioxidant, N-acetyl cysteine (NAC), which indicates oxidative stress as a component to CAR activation. To examine the role of NRF2 on intestinal induction of UGT1A1 by PEITC, *hUGT1/Nrf2^{-/-}* mice were generated. Intestinal UGT1A1 induction by PEITC was determined to be partially dependent upon NRF2, while *Cyp2b10* and hepatic *UGT1A1* induction was retained in *hUGT1/Nrf2^{-/-}* mice. This indicated that CAR activity following PEITC exposure does not require NRF2. Similar results were obtained with the heavy metals arsenic and cadmium in the small intestine. Western blot analysis of intestinal fractions of *hUGT1/Nrf2^{-/-}* mice treated with CdCl₂

demonstrated increases in nuclear CAR accumulation, nuclear P38 content, and the rise and fall of ERK1/2 phosphorylation. Mitogen activated protein kinase activity may serve as the regulatory mechanism for CAR activation by oxidative stress.

CHAPTER 1: INTRODUCTION

Section: 1.1 Oxidative Stress

Under some conditions oxidative modifications to proteins, lipids, and small molecules serve necessary signaling functions¹. However, excessive modifications of proteins, lipids, and nucleic acids affect electrostatic interactions resulting in altered structures and in some cases a loss of function. Excessive loss of function puts the cell in a state of distress, which is known as oxidative stress. The redox state of any compartment within the cell is variable and is regulated by enzymatic and non-enzymatic redox reactions that alter the flux of reactive oxygen, nitrogen, carbon, and sulfur species.

Typically, the major contributors to the redox state of a cell are enzymatic/protein-related processes. For example, mitochondria are the principal source of reactive oxygen species (ROS), which are generated by NADPH oxidases, dehydrogenases, and respiratory complexes I-III^{2,3}. Most enzymatic methods reduce oxidative damage by either directly targeting ROS or the secondary oxidized products formed by ROS, such as lipid peroxides and glutathione disulfide (GSSG). Some of the most notable enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione-disulfide reductase (GSR).

Glutathione (GSH) is one of the most important intracellular antioxidants, and the ratio of reduced glutathione to oxidized glutathione is used as a loose measure of the oxidative state of a cell. The variability of glutathione concentrations and ratios are dependent upon the cell type and compartment, making certain organelles, cells, and tissues are more susceptible to oxidative damage. Those cells that experience

high rates of ROS generation or those compartments that have low levels of GSH available for an antioxidant response are more susceptible to oxidative stress. Indirectly, stress can be generated by depletion of cellular antioxidants and by less reactive small molecules that dysregulate the antioxidant response; therefore, these compounds must be detected and removed.

Experimentally, oxidative stress can be detected through RT-PCR of gene transcripts encoding antioxidant response proteins, spectrophotometric measurement of glutathione conjugates, and through immunohistochemical targeting of residues modified by reactive species.

Section: 1.2 Drug Metabolism

Drug metabolism (which herein refers to both xeno- and endobiotic metabolism) is described by 4 phases: absorption/uptake (phase 0), oxidation/reduction and hydrolysis (phase I), conjugation (phase II), and transport and excretion (phase III). Drug absorption is dependent upon several properties, such as route of exposure, hydrophobicity, and mechanism of uptake (e.g., passive diffusion, active transporters, etc.).

The major routes of exposure for most xenobiotics are inhalation via the lungs and orally via the gastrointestinal tract due to the relatively large surface areas that are essential for satisfactory gas exchange and nutrient uptake, respectively. Certain xenobiotics are absorbed only after their formation from pro-forms by the microbiota. An example are isothiocyanates (ITCs), such as sulforaphane and phenylethyl isothiocyanate, which exist as glucosinolates glucoraphanin and gluconasturtiin, respectively. These pungent compounds are natural pesticides found in cruciferous

vegetables that undergo cleavage by myrosinase upon damage to the plant by mastication, or by gut microbiota⁴. These small hydrophobic ITCs are readily and rapidly absorbed giving them a high bioavailability⁵.

In contrast to the unassisted diffusion that enables small hydrophobic compounds to be absorbed, certain toxicants like heavy metals As^{3+} and Cd^{2+} are primarily taken up by facilitated diffusion or active transport processes, which can limit their accumulation depending on protein levels and competition. For example, cadmium uptake is dependent upon DMT1⁶ and ZIP8⁷, which limits GI absorption to ~3%⁸. These transporters are also utilized for iron and zinc uptake, and competition with these transporters and other ion gates can disrupt regulatory processes⁶. Certain conditions, like anemia, facilitate Cd^{2+} uptake due to increased transporter expression, which can increase absorption up to 15-20%⁹. The complexity of toxicant absorption can be further increased if multiple routes of exposure are considered. For example, Cd^{2+} can also be taken up by the lungs from cigarette smoke, and the absorption is ~25% via inhalation¹⁰. Multiple exposure routes increase an individual's risks and impact which organs will experience toxic effects, particularly when those organs are poorly suited to perform phases I, II, and III of drug metabolism.

During phase I of drug metabolism, small molecules are typically oxidized by class of monooxygenases known as cytochrome P450s (CYP450s). Other reactions such as reductions and cleavages occur, but the most prominent phase I modification is oxidation. The oxidation of small hydrophobic molecules serves to increase the molecule's solubility, provide a functional group for further modifications, and sometimes inactivate the molecule. However, it should be noted that CYP450 action

can sometimes bioactivate molecules leading to an increase in their action, and in some cases, their toxicity. These CYP450s are localized to the cytoplasmic face of the endoplasmic reticulum and certain CYP450s, such as CYP3A4, have a dramatic range of pharmaceutical substrates. Competition for CYP450s and changes in their expression are major contributors to drug-drug interactions and the variability of drug efficacy and safety.

In phase II of drug metabolism, small hydrophobic molecules are made more water soluble by conjugation reactions. There are multiple reactions that solubilize or inactivate compounds, such as glucuronidation, sulfation, and glutathione conjugation among others. Glucuronidation represents a significant source of xenobiotic metabolism that requires consideration when evaluating drug therapeutics. Glucuronidation is performed by UDP-glucuronosyltransferases (UGTs), which are localized to the endoplasmic reticulum and utilize uridine diphosphate glucuronic acid as a substrate for the conjugation of small lipophilic compounds. There are 19 characterized in humans which are split into the UGT1, family containing 9 transferases, and the UGT2 family, containing 10 transferases¹¹. A variety of endogenous and exogenous compounds are metabolized by UGTs including the heme metabolite, bilirubin.

In addition to the antioxidant function previously introduced, glutathione also serves as a nucleophile for a variety of addition and substitution reactions that occur both enzymatically and non-enzymatically. The conjugation by glutathione serves to both inactivate electrophilic and reactive centers such as epoxides or metals while increasing solubility of the substrate. The enzymatic catalyzed conjugation reactions

of glutathione are performed by glutathione S-transferases (GSTs) which can be found in the cytosol, the endoplasmic reticulum, and the mitochondria.

Phase III of drug metabolism consists of transport of metabolites out of the liver or kidneys to be excreted from the body in the bile or urine. There are two superfamilies of drug transporters (also known as efflux pumps); they are the ATP-binding cassette (ABC) transporters and the solute carrier (SLC) transporters and are driven by ATP hydrolysis and electrochemical gradients, respectively. Examples of these include the multidrug resistance-associated proteins (MRPs) and glucose transporters (GLUTs). Many efflux pumps, such as P-glycoprotein (P-gp/MDR1), are capable of binding to variety of substrates. The regulation and coordination of the different phases of drug metabolism is mediated by multiple sensors known as nuclear (NR) and xenobiotic receptors (XR).

Nuclear and xenobiotic receptors are generally composed of a few domains, they contain a ligand independent activating function (AF1), a DNA binding domain (DBD), a short linker that enables regulation and conformation changes, a second activating function (AF2), and the ligand binding domain (LBD). Nuclear and xenobiotic receptors are capable of promiscuous binding to both endo- and xenobiotics, which induce conformational changes that enable specific protein-protein interactions and subsequent post-translational modifications. These signals both regulate the trafficking of the receptor and its transactivation activity through transcription factor and enhancer site recognition. Two nuclear/xenobiotic receptors that have been well characterized include CAR and NRF2.

Section: 1.3 CAR

The constitutive androstane receptor (CAR) also known as the constitutively active receptor was observed in HepG2 cells as a nuclear receptor that has high, basal levels of transcriptional activity and spontaneously localizes to the nucleus¹². CAR's activity is inhibited by cytoplasmic localization and can be activated by a direct ligand binding (direct activation) and or by signaling cascades (indirect activation). The ligand binding pocket of CAR prefers small hydrophobic compounds¹³ with some species-dependent ligand specificity; for example, mouse CAR (mCAR) is activated by TCPOBOP and human CAR (hCAR) is activated by CITCO¹⁴. Both the direct and indirect mechanisms make use of a single post translation modification to control CAR localization, which is the phosphorylation of the conserved threonine residue (38 in humans and 48 in mice)¹⁵.

Threonine 38 is between the two zinc finger motifs of the DBD, which are destabilized when Thr38 is phosphorylated¹⁵. This phosphorylation is sufficient to completely inhibit CAR DNA binding and prevent nuclear localization¹⁵. The importance of this residue to CAR has been studied extensively with phosphomimetic mutants (Thr38Asp) and “dephosphorylated” mutants (Thr38Ala). Until recently, the kinase responsible for the phosphorylation of Thr38 was unknown but was eventually determined to be the P38 mitogen-activated protein kinase (MAPK)¹⁶, which we propose is a critical component in the mechanism by which oxidative stress affects drug metabolism. Through *in vitro* work involving the ectopic expression of CAR and P38, the use of chemical activators, and phosphomimetic mutants, a Co-IP study demonstrated that P38, and more effectively p-P38, can physically interact with CAR

to increase its transcriptional activity and phosphorylate Thr38-CAR to induce cytoplasmic localization¹⁷.

The dephosphorylation of p-Thr38-CAR is performed by protein phosphatase 2A¹⁸, which is composed of the scaffolding subunit A and the catalytic subunit C, and it is currently unknown if a substrate adaptor, subunit B, is required for the dephosphorylation of CAR. It is known that the receptor for activated C kinase 1 (RACK1) is required for PP2A to dephosphorylate p-Thr38-CAR¹⁸. The regulation of PP2A interaction with CAR is regulated in two parts: the first is through Tyr52-RACK1 phosphorylation and the second is through accessibility to the PP2A:RACK1 binding interface of CAR¹⁹. The first method represents the canonical pathway by which CAR is indirectly activated by the suppression of receptor tyrosine kinases (RTKs) such as EGFR²⁰, HGFR²¹, and IGF1R²². Activation of RTKs leads to increases in phosphorylation of extracellular signal-regulated kinases (ERK1/2) and phosphorylation of Tyr52-RACK1, which prevents RACK1 from interacting with CAR and prevents PP2A from dephosphorylating p-Thr38-CAR¹⁸. RACK1 is phosphorylated by c-SRC, which is a proto-oncogene tyrosine-protein kinase Src¹⁸, and it should not be confused with steroid receptor coactivator 1 (SRC1), which increases CAR activity in the nucleus²³. c-SRC is activated in response to EGFR activation, and the inhibition of EGFR by phenobarbital is the classical indirect mechanism of CAR activation¹⁸.

Following the phosphorylation of Thr38, CAR is sequestered in the cytoplasm as a homodimer in a complex with the cytoplasmic CAR retention protein (CCRP/DNAJC7/HSP40), heat shock proteins 70 or 90 (HSP70 and HSP90), and

phosphorylated ERK1/2^{24,25}. Not much is known about the exchange of one heat shock protein for another in the context of CAR regulation; however, increases in heat stress cause an accumulation of HSP70, which reduces the proteasomal degradation of CCRP, which subsequently accumulates and sequesters CAR in the cytoplasm²⁴. Additionally, HSP70 directly interacts with CAR; however when CAR is directly activated by a ligand, an increase in CCRP ubiquitination occurs along with CAR translocation²⁴.

The role of ERK1/2 phosphorylation in CAR repression has been characterized as well. Phosphorylated ERK1/2 are only capable of binding p-Thr38-CAR at the xenobiotic response site (XRS) near the C-terminus of the LBD²⁶. The binding of ERK1/2 causes CAR to form a homodimer, which buries the interface for the binding of RACK1:PP2A, thereby preventing p-Thr38 from being dephosphorylated. The MEK1/2 inhibitor, U0126, is sufficient to both reduce the phosphorylation of ERK1/2, as expected, and increase nuclear localization of CAR²¹. It should be noted that only the basal transcriptional activity of CAR is increased without augmenting ligand induction. Less is known about how direct ligand binding causes CAR activation and what proteins are essential for the translocation processes. An overview of the regulation of CAR can be found in **Figure 1**.

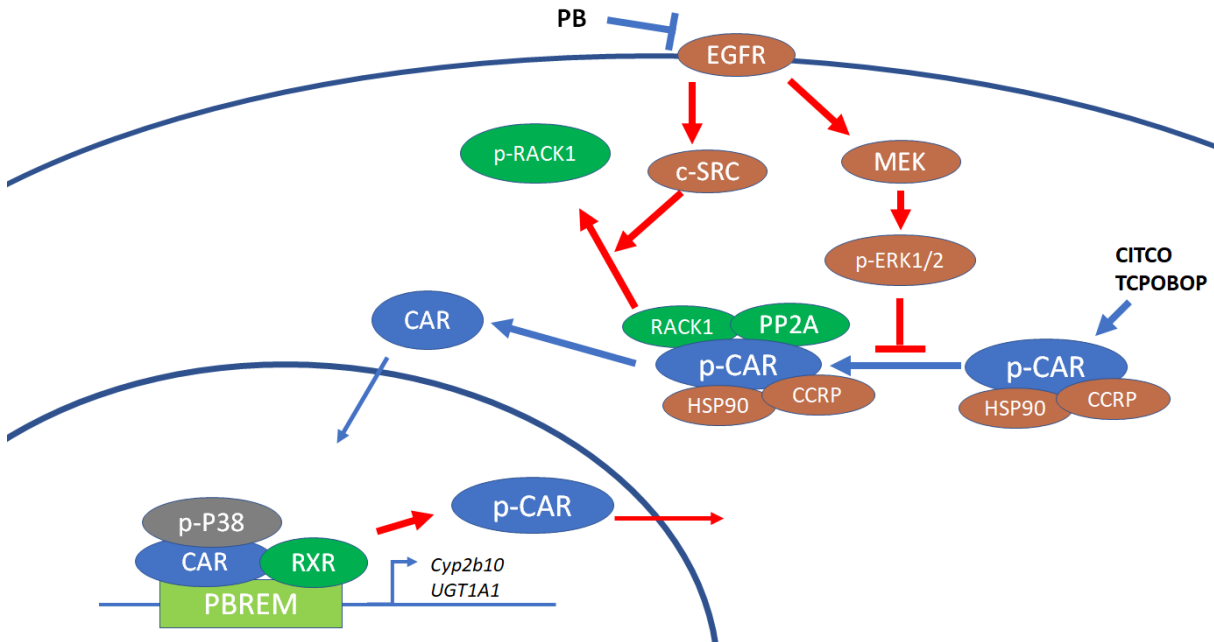


Figure 1. Regulation of the constitutive androstane receptor (CAR). CAR is regulated by direct ligand binding and indirect phosphorylation cascades. CITCO and TCPOBOP are human and mouse CAR direct activators and phenobarbital (PB) is an indirect activator that inhibits EGF binding to activate CAR. Both mechanisms require PP2A activity. PP2A binding is inhibited by CAR homodimerization, which is controlled by pERK1/2, while unphosphorylated RACK1 mediates the PP2A-CAR interaction. Following dephosphorylation of p-Thr38-CAR, CAR translocates to the nucleus where it dimerizes with RXR at the phenobarbital responsive enhancer module (PBREM) to activate transcription of target genes such as *Cyp2b10* and *UGT1A1*. Following transcription, p-P38 phosphorylates CAR initiating cytoplasmic translocation. Blue lines are CAR activating processes while red lines are CAR repressive processes.

Section: 1.4 NRF2

The Nuclear factor erythroid 2 like 2 (NRF2/NFE2L2) is a transcription factor that is sensitive to the redox state of the cell and regulates genes that mediate the antioxidant response. NRF2 is constitutively expressed and is regulated at the post-translation level by the kelch-like ECH associated protein 1 (KEAP1), which targets NRF2 for proteasomal degradation²⁷. The interaction between NRF2 and KEAP1 is mediated by the Neh2 domain of NRF2, which contains high (ETGE) and low affinity

(DLG) motifs that bind two separate molecules of KEAP1²⁸. Under basal conditions, KEAP1 orients the E3 ubiquitin ligase, CUL3, to enable proteasomal degradation of NRF2. Upon electrophilic modifications of KEAP1 cysteine residues, conformational changes occur that prevent ubiquitination of NRF2, however the precise changes that are responsible for the decrease in ubiquitination have not been completely characterized. Without degradation, NRF2 prevents KEAP1 from binding and degrading other NRF2 molecules, causing an accumulation of NRF2 and subsequently an increase in NRF2 activity²⁹. Other mechanisms exist where NRF2 is activated by sequestration of KEAP1 by P62³⁰. NRF2 and small Maf proteins (sMaf) bind to the antioxidant response element (ARE) to induce transcription of a variety of genes. These genes include the antioxidant proteins previously mentioned (section 1.1), as well as heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), GSTs, MRPs, and UGTs³¹.

Section: 1.5 The Humanized UGT1 Mouse Model

Bilirubin is a metabolite of heme, and its accumulation in tissues gives them a yellow color, which is a sign of liver dysfunction known as jaundice. During neonatal human development, increased red blood cell turnover causes the accumulation of bilirubin in the blood and other tissues. The liver glucuronidates bilirubin via UGT1A1; however, there is a developmental delay in the transcriptional regulation of UGT1A1, which enables the accumulation of unconjugated bilirubin. This is because there are no other enzymes that can conjugate bilirubin to increase its hydrophilicity to enable elimination via bile. Excessive accumulation of bilirubin in the serum is known as

hyperbilirubinemia and bilirubin accumulation in the brain causes damage known as kernicterus³². While kernicterus can be lethal, mild forms of hyperbilirubinemia, such as Gilbert's syndrome, have been associated with positive effects such as reduced rates of cardiovascular disease³³ these effects are putatively due to bilirubin's antioxidant capabilities³⁴. One of the tools created that could aid the study of these correlations is the humanized *UGT1* mouse model.

The generation of the humanized *UGT1* mouse model began with transgenic incorporation of the human *UGT1* locus (**Fig. 2**)³⁵. A separate model was created in which a null mutation for mouse *Ugt1* was generated by insertion of a neomycin resistance gene (neo) in place of the shared exon 4³⁶.

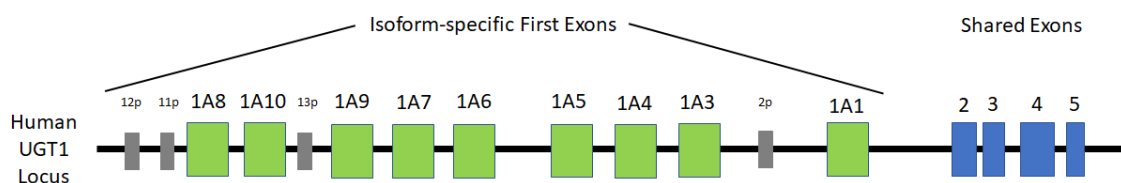


Figure 2. The human *UGT1* gene locus. The human *UGT1* locus is comprised of 9 functional isoforms that are differentiated by their first exon (green) and share the last 4 exons (blue). The shared exon structure is also present in mice, enabling a complete mouse *Ugt1*-null model to be generated by insertion of a *Neo* gene marker in the shared exon 4.

Crossing of *Ugt1*^{+/-} mice resulted in the death of the majority of *Ugt1*^{-/-} neonates by day seven. These deaths resulted from high levels of bilirubin causing kernicterus. However, by breeding the Tg-UGT1 mice with *Ugt1*^{+/-} mice, it was determined that neonatal *Ugt1*^{-/-} mice could be rescued by the human *UGT1* gene locus³⁷. Mice bearing the Tg-UGT1 in an *Ugt1*-null background will be referred to as *hUGT1* mice.

The UGT1 gene locus is regulated by many NRs and XRs³⁷ and changes in UGT1 transcript levels are assumed to result from changes in NR and XR activity when neonatal mice are treated with some compound as compared to controls. Changes in UGT1A1 expression can be observed in multiple ways, like the transcript and protein levels via RT-qPCR and Western blot analysis, respectively. Furthermore, those observations extend to metabolite concentrations, meaning TSB levels of mice can be leveraged as a phenotype indicative of NR and XR activity. The accumulation of bilirubin in serum, which is a spectroscopically observable phenotype is a direct measure of the global UGT1A1 enzyme activity; low UGT1A1 activity is correlated with high TSB levels. When a xenobiotic activates a NR or XR resulting in an increase in transcription of the UGT1A1 promotor, TSB levels drop. These assumptions and observations hold true in neonatal mice with hyperbilirubinemia that were acutely exposed to certain xenobiotics³⁸.

By crossing *hUGT1* mice with those containing NR or XR-null alleles, an *in vivo* model was created that enabled examination of the contribution of the individual NR or XR in the regulation of UGT1 genes following xenobiotic exposure. In order to determine which model needs to be utilized, target genes associated with specific NR and XRs were examined for changes in expression. These targets genes are regulated by promoters containing binding elements specific to a given NR or XR. For example, Cyp2b10 is regulated by CAR while NQO1 is regulated by NRF2. By examining loss of induction of target genes and TSB levels, assertions can be made about the relative role of NRs or XRs in responding to toxicants. Utilizing *hUGT1/CARKO* and *hUGT1/NRF2KO* mice the induction of drug metabolizing

enzymes by prooxidant toxicants was investigated and found to activate the constitutive androstane receptor in an NRF2-independent fashion. This establishes a new role for CAR and a new mechanism through which toxicants and oxidative stress can affect drug metabolism. This work may provide a new model for examining the effects of environmental exposure to toxicants on an individual's health and response to pharmaceutical therapies.

CHAPTER 2: THE ACTIVATION OF CAR BY OXIDATIVE STRESS

Taken from: Yoda E, Paszek M, Konopnicki C, Fujiwara R, Chen S, and Tukey RH (2017). Isothiocyanates induce UGT1A1 in humanized UGT1 mice in a CAR dependent fashion that is highly dependent upon oxidative stress. Scientific Reports Vol 7.

ABSTRACT

Isothiocyanates, such as phenethyl isothiocyanate (PEITC), are formed following the consumption of cruciferous vegetables and generates reactive oxygen species (ROS) that leads to the induction of cytoprotective genes such as the UDP-glucuronosyltransferases (UGTs). The induction of ROS activates the Nrf2-Keap 1 pathway leading to the induction of genes through antioxidant response elements (AREs). UGT1A1, the sole enzyme responsible for the metabolism of bilirubin, can be induced following activation of Nrf2. When neonatal humanized *UGT1* (*hUGT1*) mice, which exhibit severe levels of total serum bilirubin (TSB) because of a developmental delay in expression of the *UGT1A1* gene, were treated with PEITC, TSB levels were reduced. Liver and intestinal UGT1A1 were induced, along with murine CYP2B10, a consensus CAR target gene. In both neonatal and adult *hUGT1/Car^{-/-}* mice, PEITC was unable to induce CYP2B10. A similar result was observed following analysis of UGT1A1 expression in liver. However, TSB levels were still reduced in *hUGT1/Car^{-/-}* neonatal mice because of ROS induction of intestinal UGT1A1. When oxidative stress was blocked by exposing mice to N-acetylcysteine, induction of liver UGT1A1 and CYP2B10 by PEITC was prevented. Thus, new findings in this report link an important role in CAR activation that is dependent upon oxidative stress.

INTRODUCTION

The consumption of cruciferous vegetables such as broccoli, watercress, and cauliflower have been correlated through epidemiological studies with a lower incidence of cancer development^{39–41}. These vegetables are abundant in glucosinolates, which are hydrolyzed by plant myrosinase and by microflora of the gastrointestinal tract to form isothiocyanates (ITCs), commonly regarded as conveying active anticarcinogenic activity^{42,43}. The anticarcinogenic activity of ITCs has been linked to the activation of the nuclear factor erythroid 2 related factor 2 (Nrf2)-Keap1 signaling pathway⁴⁴, which is followed by binding of the transcriptional factor Nrf2 to antioxidant response elements (AREs)^{45,46}. Prominent in this induction process are the cluster of genes that encode proteins involved in limiting both endogenous and exogenous chemicals from initiating deleterious biological effects by directing their metabolism, an event that biologically inactivates the agents and in many cases, facilitates their excretion from the body. This cumulative defense system, carried out by proteins that have been classified to participate in Phase II drug metabolism, prevents chemical induced toxic/genetic mutations and limits induction of epigenetic events that might lead to cellular proliferation and tumor development⁴⁷.

The UDP-glucuronosyltransferase (UGT) family of proteins are part of the Phase II cluster and participate in the detoxification and genoprotective process by converting target substrates to water soluble products by glucuronidation^{48,49}. Once the glucuronides are formed, the soluble metabolites are transported out the cell, in many instances through high affinity transporters^{50–52}, and become sequestered in

the water compartments of the tissue that eventually lead to elimination. Thus, the UGTs play a key role in detoxification of many endogenous agents, such as steroids and bile acid, hundreds of therapeutically consumed drugs, and environmental toxicants that are known mutagens and carcinogens⁴⁹. The substrate specificity attributed to UGTs occurs in part because of a large and diversified gene family of proteins. In human, 19 UGTs have been identified, with each being classified into the UGT1 (UGT1A1, -1A3, -1A4, -1A5, -1A6, -1A7, -1A8, -1A9 and -1A10) or UGT2 family (-A1, A2, -A3, -B4, -B7, -B10, -B11, -B15, -B17 and -B28)⁵³. Each of the *UGT* genes is dynamically regulated, as evident from unique expression patterns observed in human tissues⁴⁹. When human tissues were examined to document gene expression patterns, each tissue displayed a unique complement of the UGTs, providing evidence that tight tissue specific regulation leads to diversified glucuronidation activity^{54–57}.

Cellular and molecular events that underlie the regulation of the UGTs, especially those encoded by the human *UGT1* gene family, have been studied in humanized transgenic mice that express the human *UGT1* locus in a *Ugt1*-null background (*humanized UGT1* mice)⁵⁸. Developmental and tissue specific regulation of *UGT1A* genes has been shown to reflect similar patterns of expression as characterized in human tissues³⁵. For example, the human *UGT1A1* gene, which is developmentally delayed in newborns⁵⁹, is also developmentally repressed in *hUGT1* neonatal mice⁶⁰. Since UGT1A1 is the sole enzyme responsible for the metabolism of serum bilirubin⁶¹, this delay in UGT1A1 expression leads to severe neonatal hyperbilirubinemia in *hUGT1* mice. The *UGT1A1* gene can be transcriptionally

induced by a host of xenobiotic nuclear receptors, including the pregnane X receptor (PXR)⁶², the constitutive androstane receptor (CAR)⁶³, the peroxisome proliferator-activated receptor alpha (PPAR α)³⁸, the liver X receptor (LXR), and the environmental sensors, Ah receptor^{64,65} and Nrf2⁶⁶. The oral or intraperitoneal administration of ligands capable of activating these receptors in neonatal *hUGT1* mice leads to the induction of liver or intestinal UGT1A1 followed by dramatic reductions in total serum bilirubin (TSB) levels. Induction of UGT1A1 following activation of the Nrf2-Keap1 signaling pathway by oxidants, such as tert-butylhydroquinone, implicates a potential role for the AREs responding to oxidative stress in control of the *UGT1A1* gene⁶⁶.

It is generally believed that ITCs, such as phenethyl isothiocyanate (PEITC), induce UGT1A1 through activation of the Nrf2-Keap1 pathway, since the *UGT1A1* gene is a known target of Nrf2⁶⁶. We have extended these findings by treating neonatal and adult *hUGT1* mice with PEITC and have evaluated the impact of ITCs in vivo for its capability to reduce serum bilirubin in neonatal mice while inducing a genome wide array of potential target genes. We will demonstrate that along with Nrf2 response genes, such as *Nqo1* being significantly induced in adult hepatic tissue, CAR dependent target genes, such as *Cyp2b10*, are also upregulated. In addition, evidence will be presented linking the generation of reactive oxygen species (ROS) by PEITC in neonatal intestinal tissue to activation of intestinal epithelial cell maturation, a process that was recently shown to be linked to derepression of the nuclear corepressor protein NCoR1 and induction of intestinal UGT1A1⁶⁷. These observations led us to examine in greater detail the potential cross-talk between the

PEITC generated antioxidant response and CAR activation, and the implications of reactive oxygen species (ROS) on activation and induction of UGT1A1.

MATERIALS AND METHODS

Chemical reagents

Phenethyl isothiocyanate (PEITC) and N-acetyl-L-cysteine (NAC) were obtained from SigmaAldrich (St. Louis, MO). Rabbit anti-UGT1A1 monoclonal antibody was purchased from abcam (Cambridge, UK). Mouse anti-GAPDH monoclonal antibody and rabbit anti-CYP2B9/10 antibody were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-p-p38, anti-p38, anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody and anti-rabbit IgG HRP conjugated antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

Animals

Transgenic mice expressing the human *UGT1* locus (*UGT1A1*1*) in a *Ugt1*^{-/-} background (*hUGT1*)⁶⁸ were developed previously⁵⁸. *hUGT1/Car*^{-/-} mice were constructed previously⁶⁰. All animals were housed at the University of California San Diego (UCSD) Animal Care Facility and received food and water ad libitum. All animal use protocols, mouse handling, and experimental procedures were approved by the UCSD Animal Care and Use Committee (IACUC), and these protocols were conducted in accordance with federal regulations.

For neonatal studies, 10-day-old *hUGT1*, *hUGT1/Car*^{+/-} or *hUGT1/Car*^{-/-} mice were treated by oral gavage with corn oil (vehicle) or 50 to 200 mg/kg PEITC dissolved in vehicle. Blood and tissues were collected as indicated in the figures. For

adult studies, vehicle or PEITC (200 mg/kg) was orally administrated to 6-week-old male mice every day for 4 days and tissues were collected 5 days after PEITC administration.

Administration of PEITC, arsenic and antioxidant NAC

Adult *hUGT1* male mice (3 weeks old) were divided into 4 groups (n = 3-4 per group): control + vehicle group was given drinking water for 3 weeks and then administrated vehicle (corn oil) orally for 4 days; control + PEITC group was given drinking water for 3 weeks and then PEITC (200 mg/kg) was administrated orally for 4 days; N-acetyl cysteine (NAC) + vehicle group was given 40 mM NAC in drinking water for 3 weeks and then vehicle (corn oil) was administrated orally for 4 days; NAC + PEITC group was given 40 mM NAC in drinking water for 3 weeks and PEITC (200 mg/kg) was administrated once a day for 4 days by oral gavage. Tissues were isolated five days after vehicle or PEITC administration.

Neonatal *hUGT1* mice (10 days old) were given an oral dose of NAC (75 mg/kg) 1 hour prior to the oral administration of arsenic (As^{3+}) at a dose of 10 mg/kg. Forty-eight hours later TSB levels were taken and tissue was collected. For PEITC treatment, neonatal mice were given an oral dose of 200 mg/kg and TSB levels measured after 48 hours and tissues collected.

Bilirubin measurements

A few drops of blood were obtained from the submandibular vein and centrifuged at 2,000 x g for 5 min. Serum samples were immediately measured for total serum bilirubin (TSB) levels using a Unistat Bilirubinometer (Reichert, Inc.).

Reverse Transcription Quantitative-PCR

Tissue samples were homogenized in 0.5 mL TRIzol (Invitrogen, Waltham, MA) and total RNA from whole tissues was isolated using TRIzol reagents per the manufacturer's instructions. Using iScript Reverse Transcriptase (Bio-Rad Laboratories, Hercules, CA), 1 µg of total RNA was used for the generation of cDNA in a total volume of 20 µL as outlined by the manufacturer. Following cDNA synthesis, quantitative PCR was carried out on a CFX96 qPCR system (BioRad) by using SsoAdvanced SYBR Green Supermix (BioRad). Primers were designed through the mouse primer depot (<https://mouseprimerdepot.ncimci.nih.gov/>). The sequences of the primers used are listed in **Table 1**.

Western blot analysis

Tissues (0.1 mg) were homogenized in 0.5 mL 1 x RIPA lysis buffer (EMD Millipore, Billerica, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Western blots were performed by using NuPAGE 4-12 % BisTris-polyacrylamide gels (Invitrogen) with the protocols described by the manufacturer. Protein (20 µg) was electrophoresed at 200 V for 50 min and transferred at 30 V for 2 hours to PVDF membranes (EMD Millipore). Membranes were blocked with either 5% skim milk or 5% BSA (fraction V) at room temperature for 1 hour and incubated with primary antibodies (rabbit antihuman UGT1A1 (ab 194697), mouse anti-GAPDH (sc-47724), rabbit anti-CYP2B9/10 antibody, rabbit anti-p-p38 (cs-9211) or rabbit anti-p38 (cs-9212)) at 4°C overnight. Membranes were washed and exposed to HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG) for 1 hour at room

temperature. Protein was detected by the ECL Plus Western blotting detection system (PerkinElmer) and was visualized by the BioRad gel documentation system. All Western blots are cropped from the full length blots that have been included in the Supplemental Material.

Statistical analysis

Statistical significances were determined by analysis of variance by using Student's t test. *P* values <0.05 were considered statistically significant, and statistically significant differences are indicated with *, *P*<0.05; **, *P*<0.01; ****P*<0.001.

Table 1. Primer sequences used for Reverse Transcription Quantitative PCR (RT-qPCR)

Gene	Forward (5'→3')	Reverse (5'→3')
<i>Akp3</i>	CTGGAGCCCTACACCGACT	AGGCTTCTGGCGCTGTTAT
<i>Cph</i>	CAGACGCCACTGTCGCTT	TGTCTTTGGAACCTTGTCTGC
<i>Cyp2b10</i>	AAAGTCCCGTGGCAACTTCC	CATCCCAAAGTCTCTCATGG
<i>Glb1</i>	CACTGCTGCAACTGCTGG	ATGTATCGGAATGGCTGTCC
<i>GstA1</i>	AGCCCGTGCTTCACTACTTC	TCTTCAAACCTCCACCCCTGC
<i>GstA2</i>	GAATCAGCAGCCTCCCAAT	TCCATCAATGCAGCCACACT
<i>hUGT1A1</i>	CCTTGCCTCATCAGAATTCCTTC	ATTGATCCCAAAGAGAAAACCAC
<i>Krt20</i>	CCTGCGAATTGACAATGCTA	CCTTGGAGATCAGCTTCCAC
<i>Lrp2</i>	CCAGGATTCTGGTGATGAGG	CGGGAACCTCATCACAACT
<i>Nox4</i>	TCTGGAAACCTTCCTGCTG	CCGGCACATAGGTAAAAGGA
<i>Nqo1</i>	TTTAGGGTCGTCTTGGCAAC	GTCTTCTCTGAATGGGCCAG
<i>Sis</i>	ACCCCTAGTCCTGGAAGGTG	CACATTTTGCCTTTGTTGGATGC

RESULTS

Impact of PEITC in neonatal *hUGT1* mice.

We have shown previously that xenobiotic induction of either gastrointestinal (GI) tract or liver UGT1A1 will promote the reduction of total serum bilirubin (TSB) in neonatal *hUGT1* mice^{58,69}. The impact of oral PEITC treatment to neonatal mice would be expected to induce ARE target genes both in the GI tract as absorption is occurring as well as in the liver prior to movement of PEITC into the systemic circulation. After a single oral dose of 200 mg/kg to 10-day-old *hUGT1* mice, TSB levels were reduced to normal levels after 48 hours (**Fig. 3A**), indicating that UGT1A1 was induced. Analysis of gene expression and protein in small intestine (SI) and liver shows induction of UGT1A1 (**Fig. 3B and 3C**). Target genes of the antioxidant response, *Nqo1*, *Gsta1* and *Gsta2* were not induced dramatically in either tissue (**Fig. S1**), indicating that an alternative mechanism other than an antioxidant response was underlying the induction of UGT1A1. Analysis of additional potential gene expression differences that might exist between liver and SI following PEITC administration confirmed induction of the *Cyp2b10* gene and protein expression in both tissues (**Fig. 4**), an observation that implicates a role for CAR in the induction process.

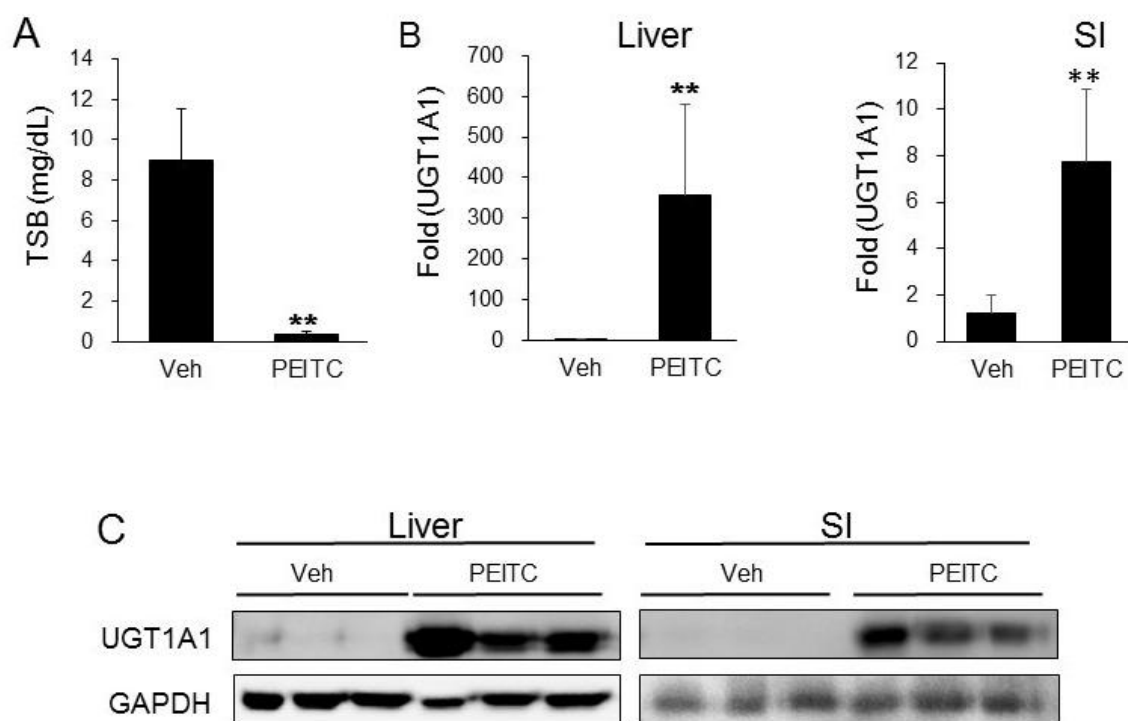


Figure 3. Induction of UGT1A1 by PEITC in neonatal *hUGT1* mice. Ten day old neonatal *hUGT1* mice were treated with 200 mg/kg PEITC or vehicle by p.o. administration. **(A)** On the second day, total serum bilirubin (TSB) levels were taken. **(B)** Liver and small intestine (SI) were pulverized under liquid nitrogen and used for the preparation of total RNA or whole tissue extracts. From RNA preparations, *UGT1A1* gene expression was determined by real time PCR and expressed as fold induction. **(C)** Using whole tissue extracts, Western blots were performed from liver and SI tissue to examine UGT1A1 expression and imaged on a BioRad ChemiDoc Touch Imaging System. The bands have been cropped from the full-length blots but not enhanced in anyway (**Fig. S4**). Values are the means of $\pm$ SD ($n > 3$). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: **, $P < 0.01$).

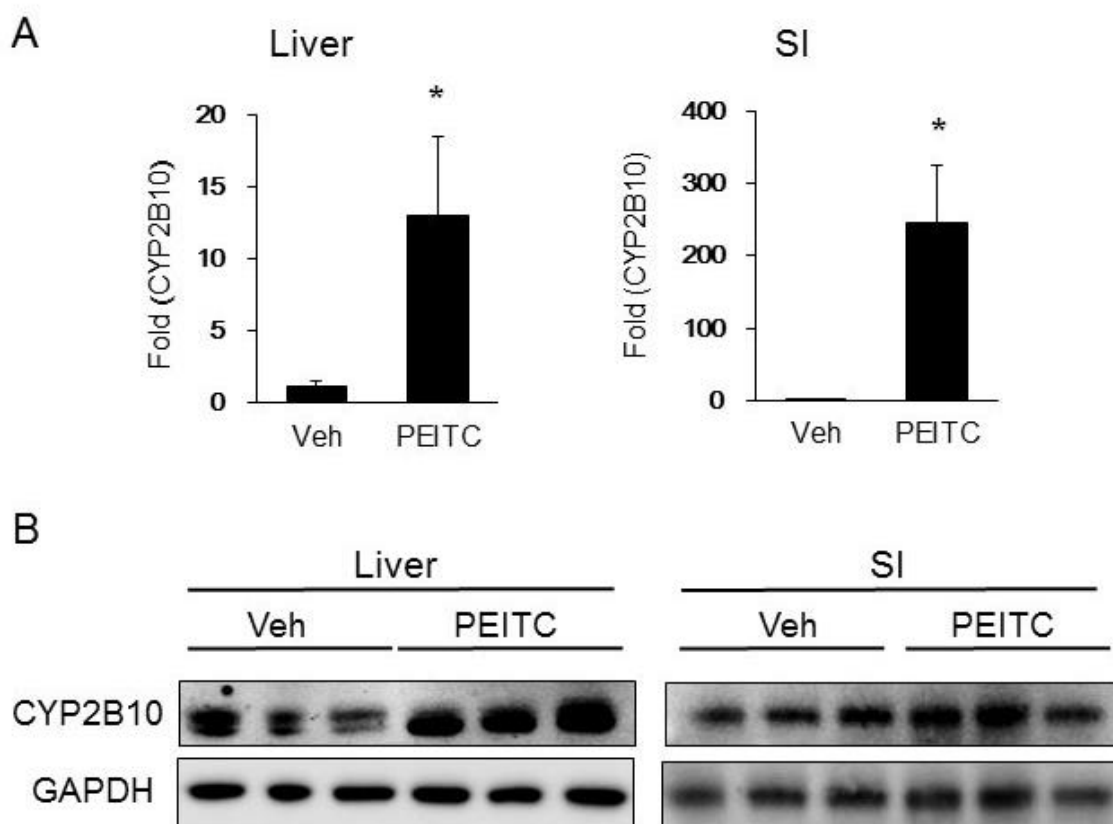


Figure 4. PEITC induction of CYP2B10 in neonatal *hUGT1* mice. Following the treatment of neonatal *hUGT1* mice with 200 mg/kg PEITC for three days (see Figure 1), *Cyp2b10* gene and CYP2B10 protein expression were analyzed. **(A)** Using RNA isolated from liver and small intestines (SI), mRNA induction was quantitated by real time PCR. **(B)** Total tissue extracts prepared from the same tissues were used for Western blot analysis to examine CYP2B10 expression. The bands have been cropped from the full-length blots but not enhanced in anyway (**Fig. S5**). Values are the means of $\pm$ SD ($n > 3$). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, $P < 0.05$).

To examine the possibility that CAR is playing a role following exposure to PEITC, 10-day-old *hUGT1/Car^{-/-}* mice were treated orally with PEITC and analysis of both TSB levels, UGT1A1 and CYP2B10 expression were monitored in both liver and SI (**Fig. 5**). TSB levels were completely reduced in *hUGT1/Car^{+/-}* mice and significantly reduced in *hUGT1/Car^{-/-}* litter mates but were not as low as those observed in *hUGT1/Car^{+/-}* mice (**Fig. 5A**). Deletion of CAR led to the complete lack of PEITC initiated induction of CYP2B10 and UGT1A1 expression in liver (**Fig. 5B**), confirming that PEITC exposure activates liver CAR. When we examined SI, CYP2B10 expression was absent in *hUGT1/Car^{-/-}* mice, but induction of UGT1A1 was observed (**Fig. 5B**). When gene expression patterns were examined in liver, induction of *Cyp2b10* and *UGT1A1* gene expression was absent in *hUGT1/Car^{-/-}* mice, confirming the role of CAR in activation of these genes in hepatic tissue (**Fig. 5C**). Intestinal tissue showed a different induction pattern. The *Cyp2b10* gene was regulated in concordance with PEITC activation of CAR, showing no activation of the gene in *hUGT1/Car^{-/-}* mice. In contrast, PEITC initiated induction of *UGT1A1* gene expression equally in the SI of both *hUGT1/Car^{+/-}* and *hUGT1/Car^{-/-}* mice (**Fig. 5D**), a finding that would suggest the induction of UGT1A1 in intestinal tissue is being controlled by an alternate mechanism that is not linked to CAR activation.

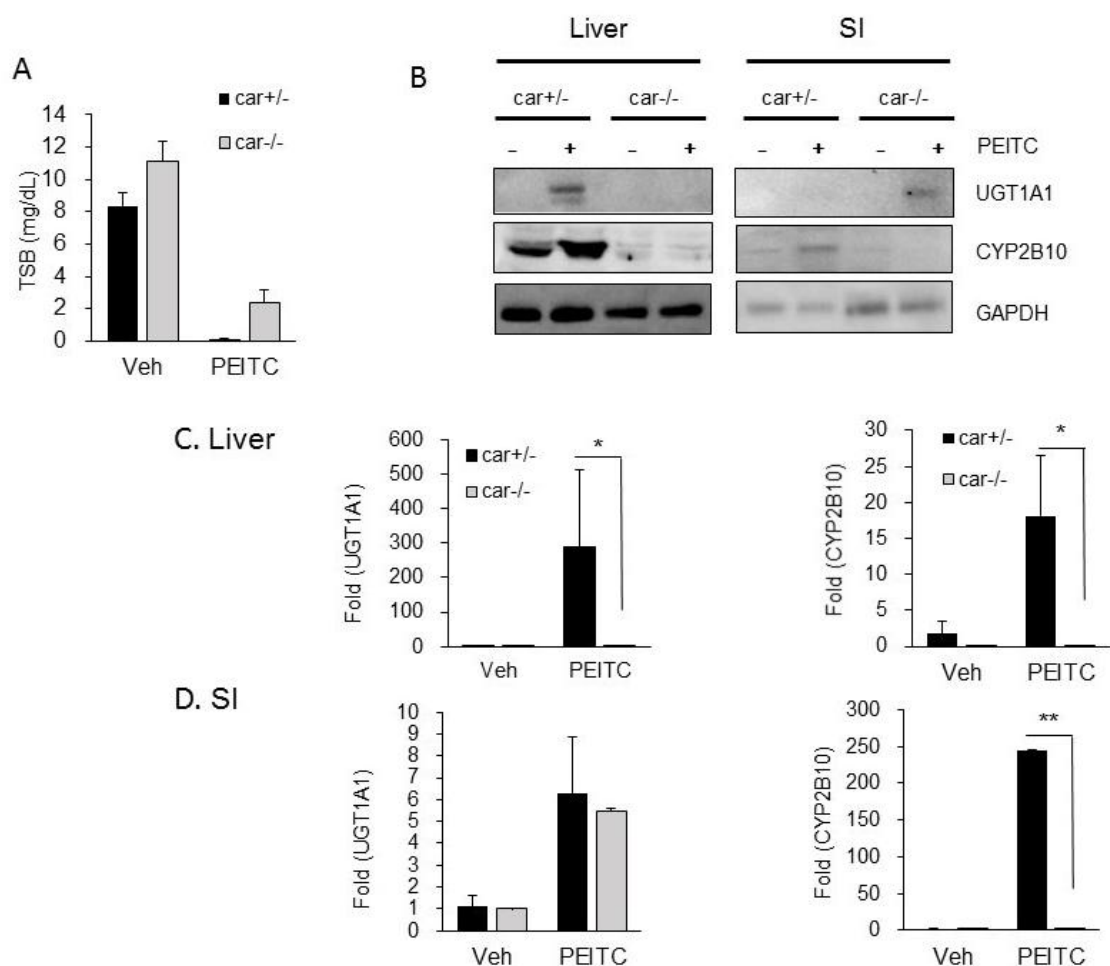


Figure 5. PEITC induction in *hUGT1/Car*-null mice. Litters were bred to produce *hUGT1/Car^{-/-}* and *hUGT1/Car^{+/-}* mice and 10-day old neonatal mice treated with 200 mg/kg on day 10, followed by TSB analysis and tissue preparation on day 12. **(A)** After treatment, TSB levels were quantitated in *hUGT/Car^{+/-}* (*car^{+/-}*) and *hUGT1/Car^{-/-}* (*car^{-/-}*) mice. **(B)** Using total cell extracts from liver and small intestines (SI), Western blots were performed using antibodies toward human UGT1A1 and mouse CYP2B10. The bands have been cropped from the full-length blots but not enhanced in anyway (**Fig. S6**). **(C)** Total RNA was prepared from liver and small intestines and mRNA expression determined by real time PCR. Values are the means of $\pm$ SD ($n > 3$). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, $P < 0.05$; **, $P < 0.01$).

To examine this possibility in greater detail, we undertook a series of experiments to determine if an oxidative stress response could be induced in the

intestines. We have shown previously that oral arsenic (As^{3+}) exposure to neonatal *hUGT1* mice induce *Cyp2b10* and *UGT1A1* gene expression⁶⁹. If we treat neonatal *hUGT1* mice with As^{3+} and measure phosphorylated p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) activation, both markers of oxidative stress⁷⁰, induction is observed as early as 2 hours after treatment (**Fig. 6A**). N-acetylcysteine (NAC), which serves as a precursor to elevate intracellular GSH⁷¹, blocks the effects of oxidative stress. We treated 10-day old *hUGT1* mice with oral NAC and then challenged the mice with oral As^{3+} , measuring gene expression after 24 hours. Arsenic treatment lead to a dramatic reduction in TSB levels that was blocked when the mice were pretreated with NAC. Arsenic induced *UGT1A1*, *Cyp2b10* and *Gsta1* gene expression, all of which were inhibited by NAC treatment (**Fig. 6B**). When we measured phosphorylated p38 MAPK after PEITC treatment as an index of oxidative stress, there was a rapid and sustained activation of p38 MAPK even through 24 hours (**Fig. 6C**), demonstrating that PEITC elicited a strong oxidative stress response in intestinal tissue. This was also verified by an increase in GSSG in intestinal tissue (**Fig. S2**). However, this response was not sufficient to activate Nrf2 target genes (**Fig. S1**).

It has recently been demonstrated that nuclear receptor corepressor 1 (NCoR1) in intestinal epithelial tissue controls neonatal intestinal maturation by repressing gene expression⁶⁷. When NCoR1 is deleted by targeted gene interruption in intestinal tissue, epithelial cell maturation is accelerated in neonatal *hUGT1* mice resulting in derepression of *UGT1A1* gene expression with the near complete reduction in TSB levels. Intestinal epithelial cell (IEC) maturation can be analyzed by

measurement of IEC specific maturation markers, such as the upregulated *Sis*, *Akp3*, and *Krt20* genes, and the downregulated *Glb1*, *Nox4* and *Lrp2* genes. Intestinal maturation can be induced by activated cellular kinases, which are believed to target phosphorylation of NCoR1 leading to derepression of gene expression⁷². Since PEITC is a potent inducer of intestinal p38 MAPK, we examined these maturation marker genes (**Fig. 6D**). Regulation of these genes followed closely the patterns of expression that were documented during neonatal intestinal maturation by expressed IKK β and following targeted deletion of NCoR1⁶⁷. The possibility exists that the sharp increase in oxidative stress by PEITC promotes intestinal maturation, leading to derepression of the *UGT1A1* gene and the reductions we have observed in TSB levels.

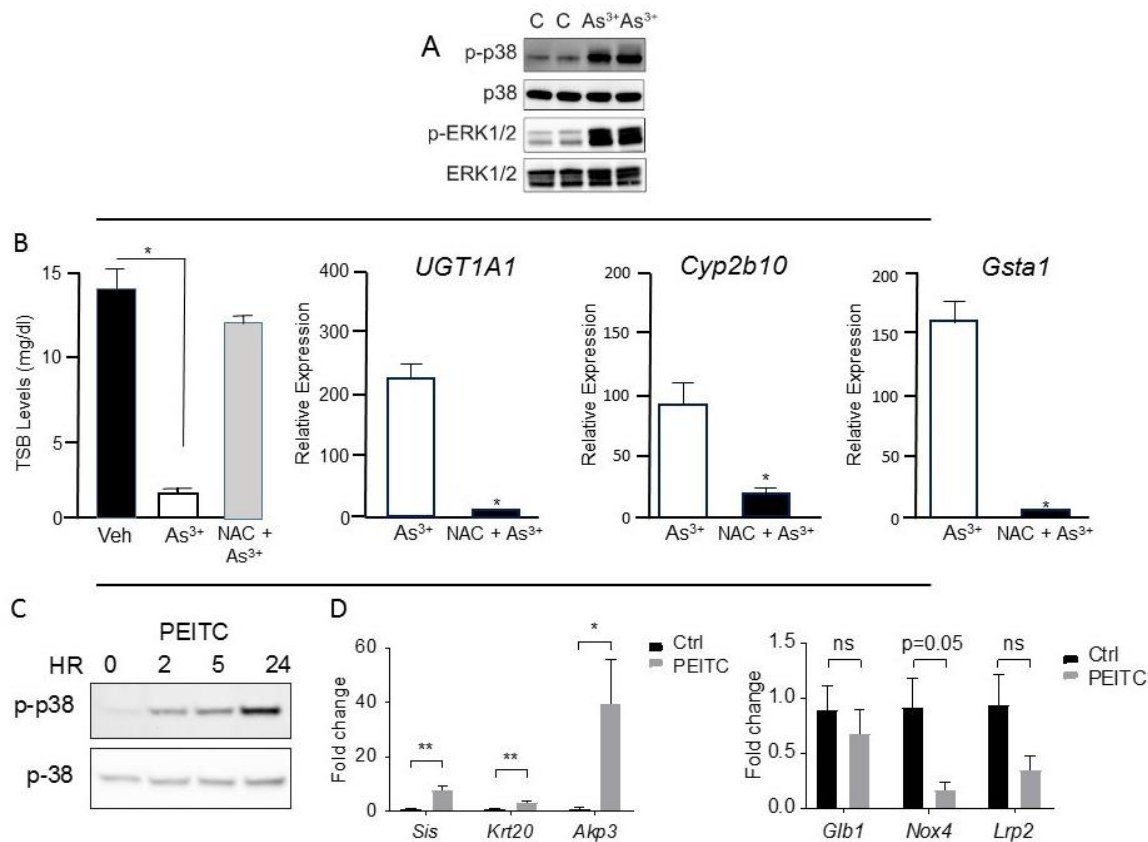


Figure 6. Induction of oxidative stress induces UGT1A1 in intestinal tissue. Neonatal *hUGT1* mice were given a dose of either arsenic (As³⁺) or PEITC and markers of oxidative stress determined. **(A)** Ten day old mice were given a single oral dose of As³⁺ (10 mg/kg) and tissue extracts from the small intestine prepared after 4 hours. Western blots were prepared and phosphorylated p38 MAPK and ERK determined. The bands have been cropped from the full-length blots but not enhanced in anyway. **(B)** Ten day old mice were pre-treated with 75 mg/kg N-acetylcysteine (NAC) for one hour prior to As³⁺ exposure. Forty-eight hours after oral As³⁺ exposure, RNA was isolated from small intestine and RTqPCR was conducted for *UGT1A1*, *Cyp2b10* and *Gsta1* expression. Data is shown as fold induction of the value observed in mice treated with As³⁺ and water. **(C)** Ten-day-old mice were given an oral dose of PEITC (200 mg/kg) and intestinal tissue isolated at 0, 2, 5, and 24 hours after treatment. Western blots were performed to examine activation of phosphorylated p38 MAPK (**Fig. S7**). **(D)** Twenty-four hours after PEITC treatment, RNA was prepared from small intestines and RT-qPCR performed to examine intestinal maturation marker gene expression. Genes *Sis*, *Akp3* and *Krt20* are upregulated during maturation while genes *Glb1*, *Nox4* and *Lrp2* are downregulated.

Impact of PEITC in adult *hUGT1* mice

To examine the association of CAR activation and the anti-oxidative response in adult mice, we treated *hUGT1/Car^{+/-}* and *hUGT1/Car^{-/-}* mice orally with PEITC and evaluated gene and protein expression in liver and SI (**Fig. 7**). As observed in neonatal *hUGT1* mice, PEITC led to induction of *UGT1A1* and *Cyp2b10* gene expression in liver as well as SI in *hUGT1/Car^{+/-}* mice. Protein expression paralleled the findings of gene expression, with Western blots confirming induction of UGT1A1 and CYP2B10 in liver and SI. The induction of CYP2B10 in liver is completely dependent upon CAR, since no induction was noted in *hUGT1/Car^{-/-}* mice (**Fig. 7A**). There was also a dramatic reduction in the induction of UGT1A1 in *hUGT1/Car^{-/-}* mice, implicating an important role for CAR in PEITC activation. However, we did detect both at the mRNA and protein level minor induction of UGT1A1 in *hUGT1/Car^{-/-}* mice, indicating that an alternative mechanism exists. Analysis of Nrf2 target genes confirmed induction was taking place by an oxidative stress response (**Fig. S3**). In the SI, PEITC treatment led to induction of the *UGT1A1* gene and protein expression in both *hUGT1/Car^{+/-}* and *hUGT1/Car^{-/-}* mice (**Fig. 7B**). We have confirmed that PEITC does activate CAR in the SI, as shown by CAR dependent activation of the *Cyp2b10* gene. While *UGT1A1* is also a target following CAR activation by PEITC, the ability to induce UGT1A1 in *hUGT1/Car^{-/-}* mice indicates that in this tissue the actions of PEITC are activating *UGT1A1* gene expression and protein induction through an alternative signaling pathway. Since ARE genes are induced in the SI following treatment with PEITC (**Fig. S3**), it is reasonable to assume that an antioxidant response by PEITC is initiating induction of UGT1A1 in the SI.

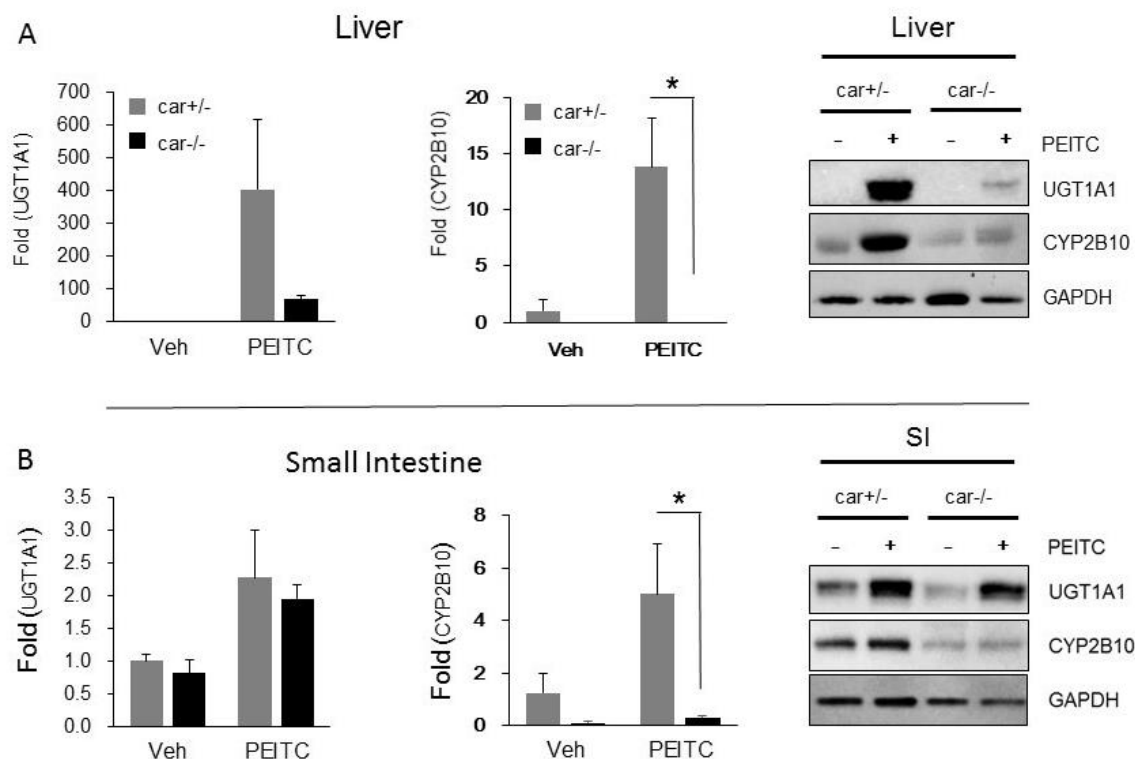


Figure 7. Induction by PEITC in adult *hUGT1/Car*^{-/-} mice. Adult (8 week) *hUGT1/Car*^{-/-} and *hUGT1/Car*^{+/-} mice were treated by p.o administration with 200 mg/kg/day for three days. After treatment, RNA and total cell extracts were prepared. (A) From liver tissue, *UGT1A1* and *Cyp2b10* gene expression was analyzed by real time PCR and expressed as Fold induction. From the same tissues, total cell extracts were combined and used for Western blot analysis using anti-human UGT1A1 and anti-mouse CYP2B10 antibodies. The bands have been cropped from the full-length blots but not enhanced in anyway (Fig. S8). (B) From small intestine, a similar series of experiments were performed. Values are the means of $\pm$ SD (n>3). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, *P*<0.05).

The role of oxidative stress and control of CAR.

To separate an anti-oxidative response initiated by PIETC from activation of CAR and induction of UGT1A1, we first administered adult *hUGT1* mice N-acetylcysteine (NAC) in their drinking water for 2 weeks. NAC prevents oxidative damage in tissues and cells by altering the redox-state and provides a reducing environment by increasing intracellular glutathione concentrations⁷¹. After two weeks of NAC exposure, *hUGT1* mice were administered PIETC. Examining liver, anti-oxidant-generated gene expression patterns for *Nqo1*, *Gsta1* and *Gsta2*, which were induced by PIETC, were blocked following NAC exposure (**Fig. 8**). Similarly, induction of gene expression for both liver *UGT1A1* and *Cyp2b10* by PEITC was inhibited because of NAC treatment (**Fig. 8B**). For UGT1A1 expression, NAC exposure completely blocked PEITC induction of UGT1A1. These results indicate that by replenishing intracellular antioxidant glutathione levels, NAC blocked PIETC-mediated antioxidant responses, and more surprisingly, abolished induction of both *Cyp2b10* and *UGT1A1* gene expression. These findings strongly suggesting that the Nrf2-ARE signaling pathway is actively involved in regulating these genes. Since we have demonstrated in liver that induction of the *Cyp2b10* and *UGT1A1* genes is driven by activation of CAR following PIETC exposure, this finding indicates that there is an important association between the production of ROS by PIETC and activation of CAR.

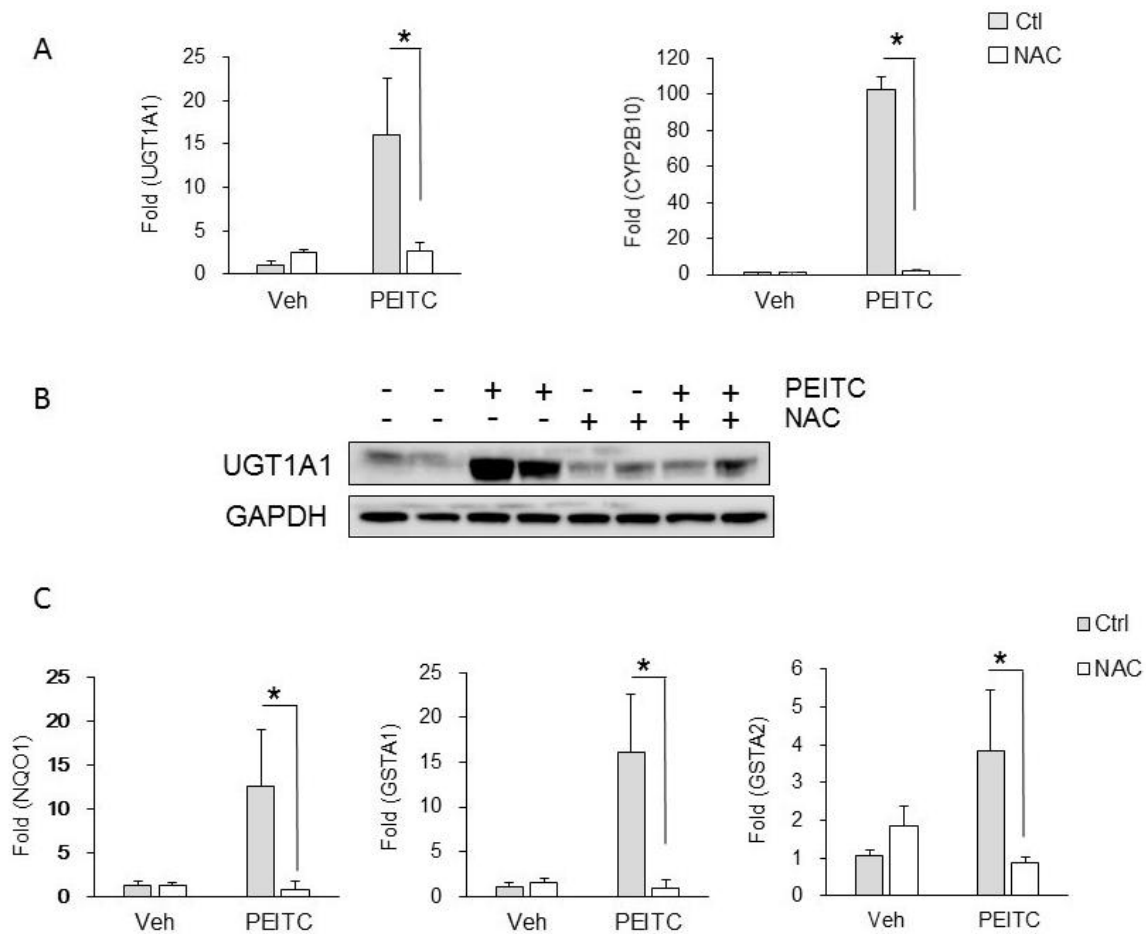


Figure 8. Impact of N-acetylcysteine on PEITC induction of UGT1A1. N-acetylcysteine (NAC) was added to the drinking water at a concentration of 40 mM for 3 weeks. After NAC exposure, adult *hUGT1* mice were treated by p.o. administration with 200 mg/kg PEITC once each day for three days. Liver was isolated after PEITC treatment. **(A)** From total RNA, human *UGT1A1* and mouse *Cyp2b10* gene expression was determined by real time PCR. **(B)** Using total cellular lysates, UGT1A1 expression was determined by Western blot analysis. The bands have been cropped from the full-length blots but not enhanced in anyway (**Fig. S8**). **(C)** *Nqo1*, *Gsta1* and *Gsta2* gene expression by real time PCR. Values are means $\pm$ SD (n=3). Statistically significant differences between Vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, *P* < 0.05)

DISCUSSION

The antioxidant initiated induction patterns following exposure to PEITC have been shown to result from activation of the transcription factor Nrf2 that leads to its binding to ARE elements followed by transcriptional activation^{73–75}. Activation of the Nrf2-Keap1 pathway in response to chemical and oxidative stress leads to the induction of a host of cytoprotective enzymes that provide an active defense mechanism against cellular damage. This unique signaling pathway was first discovered when ARE sequences were identified on two genes that encode enzymes involved in detoxification processes, NQO1⁷⁶ and GSTA2⁷⁷. The *Nqo1* and *Gsta2* genes are induced through the ARE elements following exposure to H₂O₂ and other agents that undergo redox cycling, or by compounds that are electrophilic. Other agents, such as the isothiocyanate PEITC, react with sulfhydryl groups to elevate the levels of ROS and reduce the antioxidant capacity, which in turn trigger the transcriptional response mediated by the AREs. Other genoprotective genes associated with cellular detoxification processes, such as UGTs, have long been implicated as important target genes that are induced following chemical and oxidative stress. A cluster of ARE elements in the phenobarbital response enhancer module (PBREM) in the *UGT1A1* gene has been identified and promotes Nrf2 dependent transcriptional activation⁶⁶. Based upon these findings, it would be an obvious assumption that induction of the human *UGT1A1* gene following PEITC exposure would result from the production of an antioxidant response.

The previous development of *hUGT1* mice offered us the opportunity to examine activation of the *UGT1A1* gene since chemicals or agents administered to

neonatal *hUGT1* mice that result in the reduction of TSB levels are directly inducing UGT1A1 expression^{58,60,69,78,79}. When PEITC was administered orally to neonatal *hUGT1* mice, within 24 hours the TSB levels were reduced to those measured in wild type mice. Induction of *UGT1A1* gene and protein expression is significant in both SI and liver, indicating that a prominent antioxidant response was initiated following PEITC dosing. However, there was no significant induction of other key antioxidant target genes related to the Nrf2 signaling pathway, such as *Nqo1*, *Gsta1* or *Gsta2*, leaving us to speculate that induction of UGT1A1 was occurring independently from a Nrf2-mediated antioxidant response in the neonatal mice, although we would not rule out the possibility that Nrf2 is not fully activated at our experimental dose. Examination of additional gene expression patterns confirmed that PEITC dramatically induced *Cyp2b10* gene and protein expression in both SI and liver, an important target gene that is regulated following activation of CAR. When we extended these experiments to examine the impact of oral PEITC on TSB levels and UGT1A1 expression in neonatal *hUGT1/Car^{-/-}* mice, TSB levels were reduced. However, there was no induction of UGT1A1 or CYP2B10 in liver tissue, confirming that PEITC induction of the *UGT1A1* and *Cyp2b10* genes in neonatal *hUGT1* mice are CAR dependent. While intestinal *Cyp2b10* gene expression is absent in neonatal *hUGT1/Car^{-/-}* mice, we still observed induction of *UGT1A1* gene expression, leaving us to conclude that the actions of PEITC and induction of UGT1A1 in SI were occurring independently of CAR.

To examine this possibility, we first looked at the possibility of As³⁺, a known activator of oxidative stress and potent inducer of neonatal UGT1A1 and CYP2B10⁶⁹,

to activate stress linked proteins such as p38 MAPK. Oral As³⁺ rapidly activated phosphorylated p38 MAPK, demonstrating that oxidative stress was induced. When this process was blocked by pre-treatment with NAC, induction of *UGT1A1* gene expression and the reduction in TSB values were blocked. This experiment indicated that activation of cellular kinases by oxidative stress may contribute to induction of intestinal UGT1A1. When we administered PEITC to neonatal *hUGT1* mice, intestinal phosphorylated p-38 MAPK was dramatically activated at least through 24 hours. Recent experiments conducted in our laboratory have confirmed that derepression of NCoR1 in neonatal intestinal tissue results in activation of intestinal maturation and the induction of UGT1A1⁶⁷. This process may be linked to the actions of PEITC since the mechanism that leads to NCoR1 derepression requires direct phosphorylation⁸⁰, an event that may be linked to PEITC activated p38 MAPK. This possibility is supported by analysis of maturation gene expression profiles, an event that we have shown leads to induction of intestinal UGT1A1 and a reduction in TSB levels⁶⁷.

In adult mice, PEITC administration led to prominent induction of UGT1A1 and CYP2B10 in liver and SI tissue, but there was a clear dominance of the induction pattern in liver. In contrast to induction patterns characterized in neonatal *hUGT1* mice, PEITC treatment to adult mice led to significant induction of an antioxidant response as confirmed by induction of target genes *Nqo1*, *Gst1a* and *Gsta2*. However, deletion of CAR in adult *hUGT1/Car^{-/-}* mice resulted in the elimination of both *UGT1A1* and *Cyp2b10* gene induction by PEITC, confirming previous findings that induction of these genes is dependent upon CAR. Collectively, these results indicate that PEITC has dual effects on activating both CAR and Nrf2, conveying its

antioxidant activity by inducing cellular detoxification processes – including the phase I *Cyp2b10* and phase II, *UGT1A1*, *Nqo1*, *Gsta1* and *Gsta2* genes. These genes are regulated in an age- and tissue-specific fashion by PEITC with different modes of actions: Following PEITC treatment, CAR appears to be the dominant regulator in CYP2B10 induction, whereas activation of both CAR and Nrf2 is associated with UGT1A1 induction. In line with these results is the evidence that the PBREM but not the ARE region has been identified in the *Cyp2b10* promoter¹², although it has recently been suggested that Nrf2 is involved in phorone induction of *Cyp2b10*⁸¹. In contrast, both response elements have been proven to be functional and located adjacent to each other within *UGT1A1* promoter sequences⁶⁶. In addition, in neonates CAR has a preferential role in regulating UGT1A1, and the Nrf2-antioxidant pathway becomes more responsive to PEITC as mice reach maturity. Although liver and SI tissues generally exhibit similar induction patterns responding to PEITC exposure, our results suggest that induction of hepatic UGT1A1 is predominantly regulated by CAR, and its expression in SI may be attributed to both CAR and Nrf2.

To isolate the antioxidant response from CAR dependency in PEITC induction of UGT1A1 and CYP2B10, we exposed adult *hUGT1* mice to NAC to block PEITC induced ROS production. Addition of the free radical scavenger NAC led to not only the complete inhibition of PEITC-induced expression of antioxidant genes *Nqo1*, *Gsta1* and *Gsta2* but also the blockage of CAR-initiated induction of the *UGT1A1* and *Cyp2b10* genes. This finding is significant because it indicates PEITC is the source of ROS enhanced expression of the *UGT1A1* and *Cyp2b10* genes through a ROS-dependent mechanism, further supporting the involvement of redox-sensitive

transcription factor Nrf2 in controlling expression of UGT1A1 and CYP2B10. In combination with our findings that CAR is essential for PEITC initiated induction of UGT1A1, NAC blocks the PEITC induced antioxidant response, impacting induction of *UGT1A1* and *Cyp2b10* gene expression, suggesting an interaction between PEITC-produced oxidative stress and CAR activation, however the mechanism underlying the crosstalk between CAR and Nrf2 still needs to be determined. An outline of the proposed mechanism of PEITC initiated induction of the *Cyp2b10* and *UGT1A1* genes in liver and small intestine is outlined in **Fig. 9**.

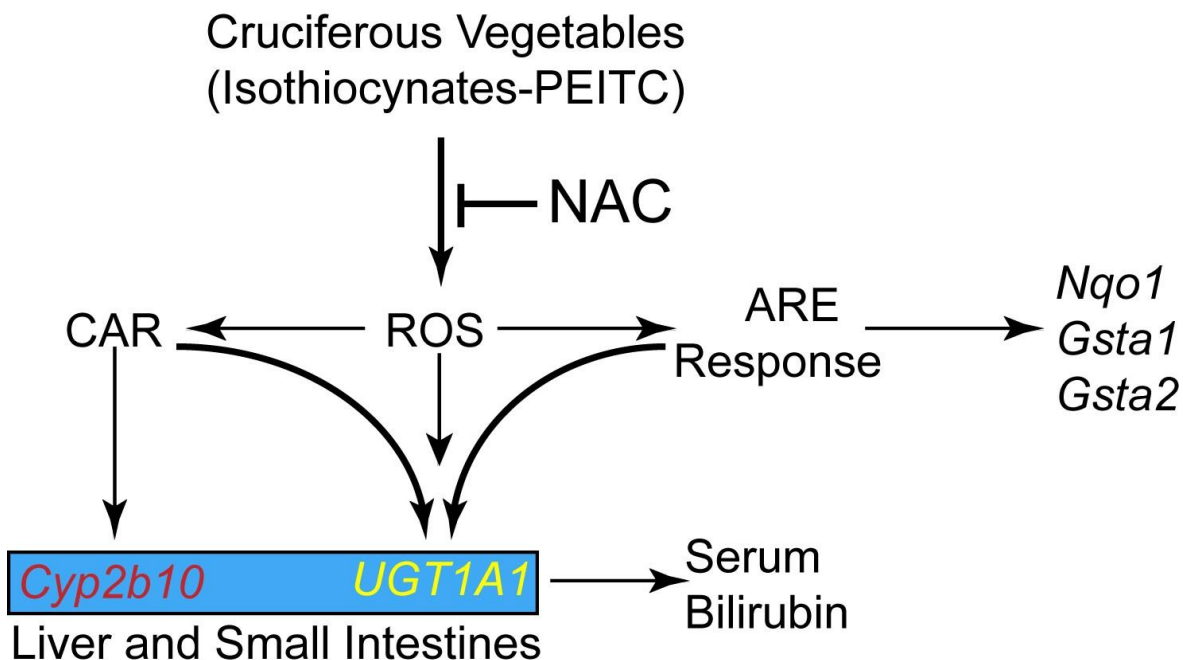


Figure 9. The actions of PEITC and regulation of UGT1A1 gene expression. Consumption of cruciferous vegetables leads to the generation of reactive oxygen species (ROS). The ROS generates signals that leads to the activation Nrf2, facilitating an ARE response on susceptible target genes. In addition, ROS are linked to the activation of CAR in both liver and small intestines, which leads to the induction of *Cyp2b10* and *UGT1A1*. In neonatal mice, it is also predicted that ROS has the potential to induce intestinal tissue maturation, an event that has been shown to induce the *UGT1A1* gene. The induction of *UGT1A1* in either the liver or small intestine will promote the metabolism and elimination of serum bilirubin. The addition of N-acetylcysteine (NAC) blocks the ROS response, nullifying both CAR and Nrf2 activation.

In adult *hUGT1* mice, PEITC exposure produces an antioxidant response in both liver and SI, but the same response is not achieved in neonatal *hUGT1* mice. Bilirubin is a natural antioxidant when bound to albumin³⁴ and slightly elevated levels in individuals that inherit Gilbert's syndrome have been documented to have reduced rates of cardiovascular disease in addition to certain cancers³³. Hence, the elevated concentrations of TSB in neonatal *hUGT1* mice may be sufficient to prevent PEITC induced ROS production, which is necessary to initiate an antioxidant response. When maternal mice were administered a daily dose of PEITC, nursing pups demonstrated a reduction in TSB levels (data not shown). The reduction in TSB levels correlated with induction of intestinal UGT1A1 expression. These findings indicate that neonatal hyperbilirubinemia can possibly be controlled through a maternal diet.

Our studies indicate that certain dietary ingredients present in cruciferous vegetables have a beneficial effect by providing a reductive capacity in tissues while also controlling important detoxification pathways such as glucuronidation, which are controlled at the transcriptional level through multiple signaling pathways. In addition to the important antioxidant response that leads to activation of the Nrf2-Keap1 pathway, we show here that ITCs regulate significant gene expression through a CAR dependent mechanism. However, the CAR dependent induction process, which we show exists for both the human *UGT1A1* gene and the murine *Cyp2b10* gene, appears to be linked mechanistically with a controlled antioxidant response. This relationship between CAR and Nrf2, which are both activated by PEITC, may serve

as a double-edged-sword with regards to liver disease. Under these circumstances, agents that activate CAR stimulate the proliferative process and serve as tumor promoters⁸². Recently, it has been demonstrated that the accumulation of p62, a protein that can activate multiple signaling pathways, is induced in liver disease such as liver fibrosis and HCC. Induction of p62 leads to the activation of Nrf2, and sustained Nrf2 activation has been shown to be oncogenic⁸³. Thus, in situations of liver disease, a diet rich in cruciferous vegetables would promote tumorigenesis through the activation of CAR while stimulating additional oncogenesis by activation of Nrf2. While a diet that includes cruciferous vegetables is believed to promote health by activating cellular defense systems, their consumption following liver disease may be detrimental.

ACKNOWLEDGEMENTS:

This study was funded in part by U.S. Public Health Service Grants ES010337, GM100481, GM086713 (R.H.T.) and R21-ES024818 (S.C). We thank Mr. Ryo Mitsugi (Department of Pharmaceutics, School of Pharmacy, Kitasato University) for his technical assistance.

AUTHOR CONTRIBUTIONS: Conceptualization: E.Y, R.F. and R.H.T.; Investigation. E.Y., M.P., C.K., and S.C.; Formal analysis. E.Y. and R.H.T. Writing-Reviewing and editing, all authors. Funding acquisition; R.H.T. and S.C.

Chapter 2, in part, is an inexact reprint of the material as it appears in the following: Yoda E, Paszek M, Konopnicki C, Fujiwara R, Chen S, and Tukey RH (2017) Isothiocyanates induce UGT1A1 in humanized UGT1 mice in a CAR dependent fashion that is highly dependent upon oxidative stress. Scientific Reports Vol 7. The dissertation author was the second investigator and author of this paper. Changes to the material presented were made to maintain format consistency. Supplementary materials can be found in the appendix.

CHAPTER 3: NRF2-INDEPENDENT REGULATION OF CAR BY OXIDATIVE STRESS

Taken from: Paszek M and Tukey RH. (2020) NRF2-independent regulation of intestinal constitutive androstane receptor by the pro-oxidants cadmium and isothiocyanate in *hUGT1* mice. Drug Metab. Dispos. Vol. 48, Issue 1. 25-30.

Abstract

Environmental toxicants such as heavy metals from contaminated water or soil and isothiocyanates (ITC) from dietary sources act as pro-oxidants by directly generating reactive oxygen species (ROS) or through depleting cellular antioxidants such as glutathione. Toxicants can alter drug metabolism and it was reported that CYP2B10 and UGT1A1 are induced by phenethyl isothiocyanate (PEITC) through the constitutive androstane receptor (CAR). The possibility that NRF2, the master regulator of the antioxidant response, could co-activate CAR was investigated in neonatal *hUGT1/Nrf2^{-/-}* mice. Neonatal mice were treated with PEITC or cadmium (Cd^{2+}) by oral gavage for two days. Both PEITC and Cd^{2+} induced UGT1A1 RNA and protein in intestinal tissues in both *hUGT1/Nrf2^{+/-}* and *hUGT1/Nrf2^{-/-}* neonates, indicating NRF2-independent regulation of UGT1A1. Increases in CYP2B10 RNA in intestinal tissues were observed following PEITC or Cd^{2+} exposure. Activation of intestinal CAR by Cd^{2+} exposure was directly assessed by nuclear fractionation and Western blot analyses at 0.5, 1, 2, and 4 hours after treatment in *hUGT1* neonates and after 48 hours in *hUGT1/Nrf2^{+/-}* and *hUGT1/Nrf2^{-/-}* neonates. CAR localized to the nucleus independent of NRF2 48 hours after exposure. Substantial CAR localization to the nucleus occurred at the 2- and 4-hour time points, coinciding with a

decrease in cytoplasmic ERK1/2 phosphorylation and a nuclear increase in P38/p-P38 content. This suggests a novel oxidative stress-MAPK-CAR axis exists with phenotypic consequences.

Significance Statement

Pro-oxidant toxicants can alter drug metabolism through activation of CAR, independent of the NRF2-KEAP1 signaling pathway. The changes in proteins associated with drug metabolism are likely mediated through an oxidative stress-MAPK-CAR axis that have been linked to increases in intestinal maturation.

Introduction

Exposure to certain environmental toxicants is capable of altering drug metabolism through the activation of selective transcription factors, such as xenobiotic and nuclear receptors (XRs and NRs). The activation of XRs and NRs typically occurs through direct ligand binding to the receptor or through post translation modifications. In vitro models enable medium- to high- throughput screening of compounds abilities to activate nuclear receptors while in vivo models provide more complex details such as absorption, elimination, and tissue specific metabolism.

An in vivo model that lends itself well to reverse genetic approaches is the humanized *UDP-glucuronosyltransferase 1 (hUGT1)* mouse model due to the readily quantifiable phenotype that changes following XR or NR activation^{37,58,60}. The phenotype is presented as neonatal hyperbilirubinemia, a condition unique to humans in which increased turnover of red blood cells causes heme degradation to bilirubin which, accumulates systemically⁸⁴. The accumulation of bilirubin occurs

because hepatic expression of UGT1A1, the only enzyme capable of conjugating bilirubin for excretion ⁶¹, is developmentally delayed at the transcriptional level ⁸⁵. However, we have demonstrated that control of intestinal UGT1A1 during development plays a key role in the clearance of serum bilirubin during the neonatal period ^{69,86}. Induction of the *UGT1A1* gene in *hUGT1* mice can be mediated by multiple XRs and NRs, such as the pregnane X-receptor (PXR) ⁷⁹, constitutive androstane receptor (CAR) ⁸⁷, peroxisome proliferator-activated receptor α (PPAR α) ³⁸, as well as environmental toxicant sensors such as the aryl hydrocarbon receptor (AhR) ^{65,88}, and the nuclear factor erythroid 2-related factor 2 (NRF2) ⁶⁶. Following exposure, activation of one or more of these XRs and NRs induces *UGT1A1* gene expression resulting in a reduction of total serum bilirubin (TSB) levels ^{37,89}. Known transcriptional targets can be examined to determine which XR or NR is inducing *UGT1A1*, for example CAR induces the *Cyp2b10* ⁹⁰ gene while activated NRF2 can induce genes such as *Gsta1/2* ⁹¹, which are sensitive to oxidative stress. The receptor mediated induction of *UGT1A1* can be confirmed in neonatal *hUGT1* mice that express a null allele for the XRs or NRs, where elevated TSB levels following exposure can be directly linked to a dependency on the deleted receptor ^{69,92,93}.

Recently, it was reported that the dietary pro-oxidants isothiocyanates (ITCs) induce hepatic UGT1A1 in a CAR-dependent fashion ⁹³. This is significant because the mechanisms of direct and indirect activation of CAR are well characterized ^{24–26,94} but have never directly implicated oxidative stress as a CAR activator. Additionally, other environmentally relevant toxicants, such as cadmium (Cd²⁺) and arsenic (As³⁺) that increase reactive oxygen species (ROS) have been reported to induce intestinal

Cyp2b10 and *UGT1A1* gene expression in *hUGT1* mice ^{60,93}. However, *UGT1A1* induction by pro-oxidants has been linked to NRF2 ⁶⁶, the master regulator of the antioxidant response ⁹⁵. The induction mechanism of intestinal *UGT1A1* in *hUGT1* mice by agents such as Cd²⁺, ITCs, and As³⁺ are examined with respect to their link towards activation of NRF2 and CAR.

Materials and Methods

Animal Studies.

All animals were housed at the University of California San Diego Animal Care Facility with access to food and water ad libitum. All animal use including experimental procedures, handling, and protocols were approved by the UCSD Animal Care and Use Committee (IACUC) and adhere to the National Institute of Health Guide for the Care and Use of Laboratory Animals. UCSD's Animal Care Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Humanized *UGT1* (*hUGT1*) mice were previously generated as described. *Nrf2*^{-/-} mice were purchased from Jackson Laboratory (Stock No: 017009), and *hUGT1/Nrf2*^{+/-} mice were generated and crossed for comparison to *hUGT1/Nrf2*^{+/+} mice, utilizing both males and females. Additional studies involved breeding *hUGT1/Nrf2*^{+/-} with *hUGT1/Nrf2*^{-/-} to generate male and female mice used for neonatal studies. Neonates were treated on day 10 and 11 with CdCl₂·2 ½ H₂O (Cd²⁺) or NaAsO₂ (As³⁺) (Sigma-Aldrich) at 10mg/kg or PEITC (Sigma Aldrich) at 200 mg/kg by oral gavage or with water or corn oil (5µL/g bw) for vehicle control. The dosages for CdCl₂ and PEITC treatments were selected from dose-response curves where both the greatest reduction in total serum bilirubin levels and no observable

behavioral or gross anatomical phenotypes of toxicity were obtained. Animals were anesthetized with isoflurane and euthanized by cervical dislocation.

Real-time PCR.

Intestines were collected and briefly washed with ice cold PBS 3 times before snap freezing in liquid nitrogen. Livers were collected and washed once with ice cold PBS before snap freezing in liquid nitrogen. RNA was extracted with TRIzol™ Reagent (ThermoFisher Scientific) as described by the manufacturer's protocol. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed using a CFX96™ Real-Time PCR Detection system (Bio-Rad) and SSoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) as described by the manufacturer's protocol. All values represent independent biological replicates and technical singlets as analyzed by the $\Delta\Delta CT$ method. Primers utilized for real time PCR analysis of selected genes can be found in Table 2.

Table 2. Primers utilized for real-time PCR analysis of selected genes

Gene	Forward (5'→3')	Reverse (5'→3')
<i>Cph</i>	CAGACGCCACTGTCGCTT	TGTCTTTGGAACCTTGTCTGC
<i>Cyp1a1</i>	TGCCCTTCATTGGTCACATG	CACGTCCCCATACTGCTGACT
<i>Cyp2b10</i>	AAGGAGAAGTCCAACCAGCA	CTCTGCAACATGGGGGTACT
<i>Cyp3a11</i>	ACAAACAAGCAGGGATGGAC	CCCATATCGGTAGAGGAGCA
<i>Cyp4a10</i>	CACACCCTGATCACCAACAG	TCCTTGATGCACATTGTGGT
<i>Glb1</i>	CACTGCTGCAACTGCTGG	ATGTATCGGAATGGCTGTCC
<i>Gsta1</i>	AGCCCGTGCTTCACTACTTC	TCTTCAAACCTCCACCCCTGC
<i>Gsta2</i>	AATCAGCAGCCTCCCCAAT	TCCATCAATGCAGCCACACT
<i>Gstm3</i>	AAACCTGAGGGACTTCCTGG	AACACAGGTCTTGGGAGGAA
<i>Ho-1</i>	ACAGGGTGACAGAAGAGGCTAAGAC	ATTTTCCTCGGGGCGTCTCT
<i>hUGT1A1</i>	CCATCATGCCCAATATGGTT	CCACAATTCCATGTTCTCCA
<i>Il-1b</i>	GCAACTGTTCTGAACTCAAC	ATCTTTTGGGGTCCGTCAACT
<i>Il-6</i>	ACCAGAGGAAATTTTCAATAGGC	TGATGCACTTGCAGAAAACA
<i>Krt20</i>	CCTGCGAATTGACAATGCTA	CCTTGGAGATCAGCTTCCAC
<i>Lrp2</i>	CCAGGATTCTGGTGATGAGG	CGGGAACTCCATCACAACT
<i>Nqo1</i>	TTTAGGGTCGTCTTGGCAAC	GTCTTCTCTGAATGGGCCAG
<i>Sis</i>	ACCCCTAGTCCTGGAAGGTG	CACATTTTGCCTTTGTTGGATGC
<i>Tnfa</i>	GATCGGTCCCCAAAGGGATG	GGCTACAGGCTTGTCACTCG

Western Blot Analysis.

Whole lysate was obtained from homogenization of pulverized intestinal tissues in RIPA buffer (Millipore Sigma Cat. No. 20-188) containing protease (ThermoFisher Scientific™ Cat. No. 78429) and phosphatase (ThermoFisher Scientific™ Cat. No. 78420) inhibitors. Pulverized tissues in lysis buffer (1:4 wt/vol) were blade homogenized and incubated on ice for 15 min before centrifugation (10,000xg, 4°C, 20 min) and supernatant storage at -80°C.

Nuclear fractionation used a two-buffer extraction. Buffer A contained 10mM HEPES, 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, and 0.05% Igepal at pH 7.9 with protease and phosphatase inhibitors. Buffer B contained 5mM HEPES, 1.5mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, and 26% glycerol (v/v) at pH 7.9 with protease and phosphatase inhibitors. Pulverized tissues were homogenized in buffer A (1:4 wt:vol) using a Potter-Elvehjem homogenizer for 10 passes. Homogenates were incubated on ice for 10 minutes prior to centrifugation (1500xg, 4°C, 10 min). The supernatant containing the cytosol was stored at -80°C. The pellet containing the nuclear contents was washed with 200uL of buffer A before resuspension in 400uL of Buffer B. After resuspension, NaCl was added to a final concentration of 300mM and the solution was sonicated for 5 sec and this repeated three times. Samples were incubated on ice for 30 minutes before centrifugation (21,000xg, 4°C, 20 min). The supernatant containing the nuclear fraction was stored at -80°C. Protein concentration was determined *via* Bradford assay (Bio-Rad), and BSA (New England BioLabs) protein standards were created for simple linear regressions.

Samples were run on NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) before transferring to PVDF membranes. Membranes were blocked with 5% nonfat milk or BSA for phospho-antibodies in TBST and incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at RT. Imaging was performed on a ChemiDoc™ Touch imaging system (Bio-Rad) using Clarity™ Western ECL substrate (Bio-Rad). All antibodies used for protein expression and localization analyses can be found in Table 3. All samples represent biological replicates and technical singlets.

Table 3. Antibodies utilized for Western blot analysis

Antibody	Supplier	Product #
Anti-mouse IgG, HRP-linked	Cell Signaling Technology	#7076
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	#7074
CAR	ABclonal	A1970
ERK1/2	Cell Signaling Technology	#9102
Phospho-ERK1/2	Cell Signaling Technology	#9101
GAPDH	Santacruz Biotechnology	sc-47724
HDAC-1	Santacruz Biotechnology	sc-6298
Histone 3	ABclonal	A2352
P38	Cell Signaling Technology	#9212
Phospho-P38	Cell Signaling Technology	#9211
Tubulin	Sigma-Aldrich	T9026
UGT1A1	Abcam	Ab170858

Bilirubin measurement.

Approximately 50 μ L of blood was obtained from the submandibular vein. Blood was centrifuged for 2 minutes at 16,000xg and the supernatant was measured using a Unistat Bilirubinometer (Reichert Inc.) to determine total serum bilirubin (TSB) levels.

Statistical analysis

All statistical analyses were performed using GraphPad Prism's (V 6.07) two-way ANOVA with multiple comparisons unless indicated otherwise. Averages $\pm$ SEM are shown. Differences were considered statistically significant when $p < 0.05$. Statistical significance is represented by the following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Cadmium treatment induces oxidative stress markers in an NRF2-dependent manner

Intestinal tissues were collected from *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonates treated orally with 10mg/kg Cd²⁺ and gene targets known to be induced by NRF2 in response to oxidative stress examined by RT-qPCR (**Fig. 10**).

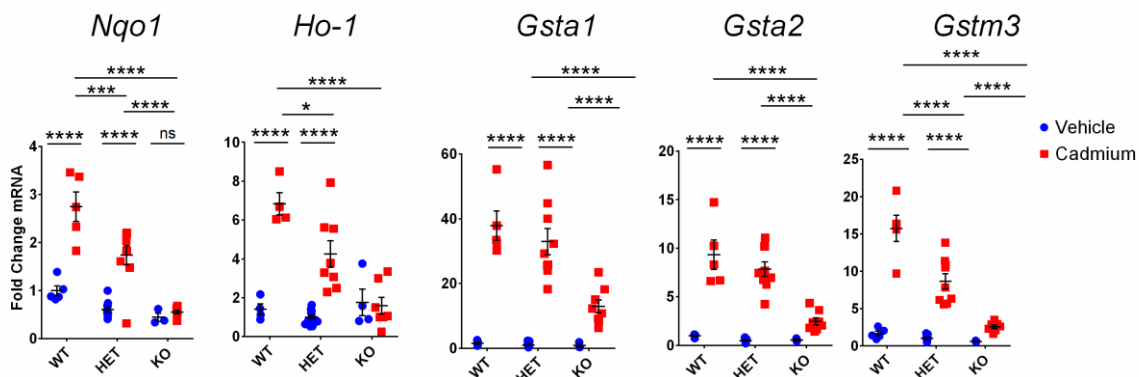


Figure 10. NRF2-dependency of antioxidant response genes in neonatal *hUGT1* mice. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10mg/kg od. po.) for two days before collecting intestinal tissue for RT-qPCR analysis of oxidative stress markers. The genes examined include *Nqo1*, *Ho-1*, *Gsta1*, *Gsta2*, and *Gstm3*. (Mean $\pm$ SEM, Two-way ANOVA; * P<0.05, *** P<0.001, **** P<0.0001)

Statistically significant inductions were detected in Cd²⁺ exposed *hUGT1/Nrf2*^{+/+} and *hUGT1/Nrf2*^{+/-} neonates for *Nqo1* (2.7- and 1.7-fold), *Ho-1* (6.8- and 4.3-fold), *Gsta1* (37.8- and 33.0-fold), *Gsta2* (9.3- and 7.9-fold), and *Gstm3* (15.8- and 8.7-fold) genes. A gene dosage effect comparing responses between *hUGT1* and *hUGT1/Nrf2*^{+/-} neonatal mice was observed for the induction of the *Nqo1*, *Ho-1*, and *Gstm3* genes. This indicates haploinsufficiency related to NRF2's induction of antioxidant response genes. When we examined the induction of these genes in *hUGT1/Nrf2*^{-/-} mice following Cd²⁺ treatment, there was no statistically significant

difference from the vehicle treatment of *hUGT1* mice, confirming that the induction of these genes in the gastrointestinal tract originates from the generation of ROS in the intestines. With oral Cd^{2+} treatment, there was no induction of these genes in hepatic tissue.

Cadmium induces intestinal UGT1A1 and lowers TSB levels in an NRF2 independent fashion.

It was previously determined that oral Cd^{2+} treatment to neonatal *hUGT1* mice led to the induction of intestinal UGT1A1⁶⁹. To determine if induction of the intestinal *UGT1A1* gene by Cd^{2+} was dependent upon the generation of ROS and activation of the NRF2-KEAP1 pathway, *hUGT1/Nrf2*^{+/-} mice were mated and 10-day old newborns treated with 10mg/kg Cd^{2+} or water by oral gavage once per day for two days before collecting blood and intestinal tissue. With *hUGT1*, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} mice, Cd^{2+} treatment eliminated circulating TSB levels within two days, indicating that a mechanism had been activated to induce intestinal UGT1A1 (**Fig. 11A**).

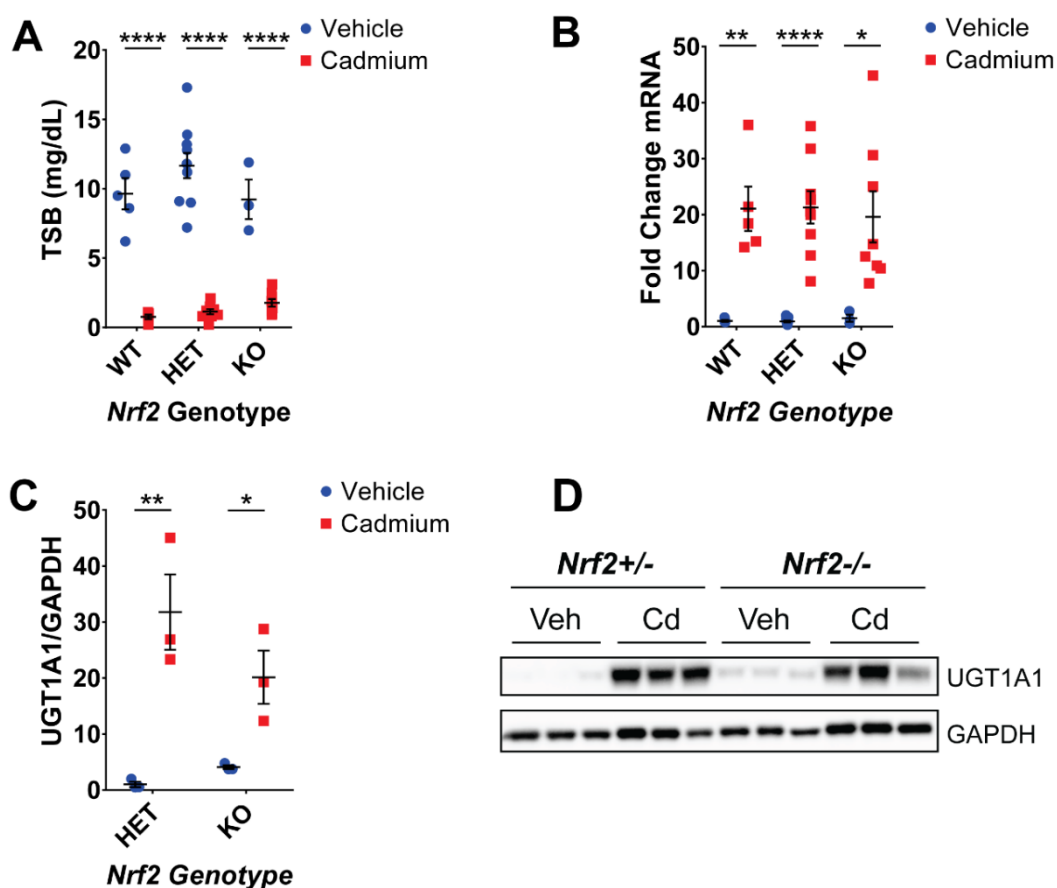


Figure 11. Cadmium reduces TSB levels in *hUGT1* neonatal mice in an NRF2 independent fashion. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10mg/kg od. po.) (red squares) for two days before collecting blood and intestinal tissues. (A) Changes in TSB levels (mg/dL) from mice treated with Cd²⁺. (B) Changes in the expression of the *UGT1A1* gene. (C) Changes in the expression of the UGT1A1 protein as visualized by Western blot analysis and (D) quantified by normalizing to GAPDH expression and set relative to vehicle treated *hUGT1/Nrf2*^{+/+} mice. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, **** P<0.0001)

Similar reductions were observed for PEITC and As³⁺ treated neonatal mice (Fig. S10). Analysis of UGT1A1 expression both at the gene and protein level firmly establish that Cd²⁺ exposure induces intestinal UGT1A1 (Fig. 2B and C). Unlike the Cd²⁺ initiated induction of the *Nqo1*, *Ho-1*, *Gsta1*, *Gsta2*, and *Gstm3* genes (Fig. 10), which are dependent upon NRF2, induction of the intestinal *UGT1A1* gene by Cd²⁺ is

not linked to the NRF2-KEAP1 pathway. It should be noted that an antioxidant-response element (ARE) has previously been identified flanking the *UGT1A1* gene in the phenobarbital-response enhancer module region (PBREM), and has been demonstrated to bind the antioxidant *tert*-butylhydroquinone (tBHQ) inducible NRF2 in treated HepG2 cells ⁶⁶. In addition, the intraperitoneal administration of tBHQ to adult TgUGT1 mice led to induction of intestinal UGT1A1, implicating the NRF2-KEAP1 pathway as a regulator of the *UGT1A1* gene ⁶⁶. However, the oral administration of Cd²⁺ and induction of intestinal UGT1A1 in *hUGT1* neonatal mice does not require NRF2-KEAP1 signaling.

Intestinal *Cyp2b10* is positively regulated by cadmium exposure and induces nuclear accumulation of CAR

The nuclear receptors that induce *UGT1A1* were examined for activation by RT-qPCR analysis of their known target genes (**Fig. S11**). Only *Cyp2b10* was substantially induced by Cd²⁺ in *hUGT1/Nrf2^{+/+}* (8.4-fold), *hUGT1/Nrf2^{+/-}* (13.5-fold), and *hUGT1/Nrf2^{-/-}* (9.8-fold) neonates, however only heterozygotes reached statistical significance (**Fig. 12A**). Furthermore, Western blot analysis of CYP2B10 was inconclusive with no apparent detection in either vehicle or Cd²⁺ treated mice. Given that basal expression of CYP2B10 in neonatal intestinal tissue is low, CAR activation was assayed more directly via nuclear localization.

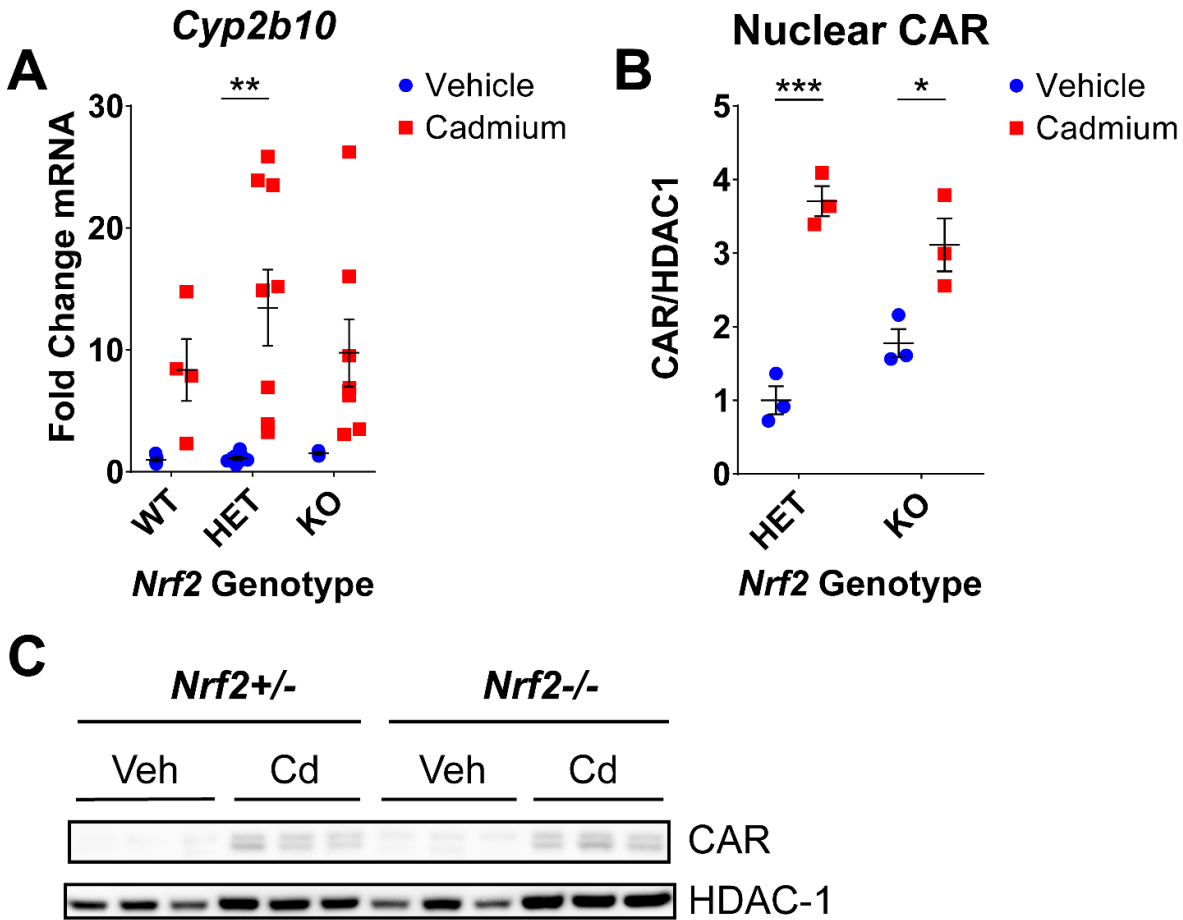


Figure 12. NRF2-independent changes of intestinal *Cyp2b10* gene expression and CAR localization. Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonatal mice were treated with vehicle (blue circles) or Cd²⁺ (10mg/kg od. po.) (red squares) for two days before collecting intestinal tissues. (A) Changes in the expression of the *Cyp2b10* gene were observed by RT-qPCR. (B) Nuclear localization of CAR was determined by Western blot analysis of fractionated intestinal protein extracts. (C) Expression of CAR was quantified by normalizing to HDAC-1 and set relative to vehicle treated *hUGT1/Nrf2*^{+/+} mice. (Mean $\pm$ SEM, Two-way ANOVA; * P<0.05, ** P<0.01, *** P<0.001)

Intestinal tissues taken from *hUGT1/Nrf2*^{+/+} and *hUGT1/Nrf2*^{-/-} neonates exposed to Cd²⁺ were fractionated and examined for nuclear CAR accumulation by Western blot analysis (**Fig. 12B**). There was a statistically significant increase in the nuclear accumulation of CAR in *hUGT1/Nrf2*^{+/+} (3.7-fold) and in *hUGT1/Nrf2*^{-/-} mice (1.8-fold) following exposure to Cd²⁺. This localization can occur independently of

NRF2 but is 2-fold less in *hUGT1/Nrf2*^{-/-} compared to *hUGT1/Nrf2*^{+/-} neonatal mice. This indicates that NRF2 is not responsible for the activation of CAR by Cd²⁺.

In vivo Cd²⁺ exposure induces mitogen-activated protein kinase (MAPK) phosphorylation and CAR localization

The regulation of CAR localization makes use of ERK1/2 and P38 phosphorylation, which was examined by Western blot analysis. Neonatal *hUGT1* mice were treated orally with a single dose of Cd²⁺ (10mg/kg) or water and intestinal tissues were collected 30 minutes, 60 minutes, 120 minutes, and 240 minutes after exposure. Following treatment, the neonatal mice experienced dramatic changes in MAPK phosphorylation, where a large increase in cytoplasmic phosphorylated ERK1/2 occurs in intestinal tissue between 0 and 30 minutes after oral treatment before decaying (**Fig. 13B**). Additionally, there is an accumulation of P38 and phosphorylated-P38 in the nucleus (**Fig. 13A**). Similar phosphorylation patterns were observed in adult mice (data not shown). Consistent increases in CAR accumulation in the nucleus were observed in neonatal mice after 2 hours (**Fig. 13C**) coinciding with the dephosphorylation of ERK1/2 and the nuclear accumulation of P38.

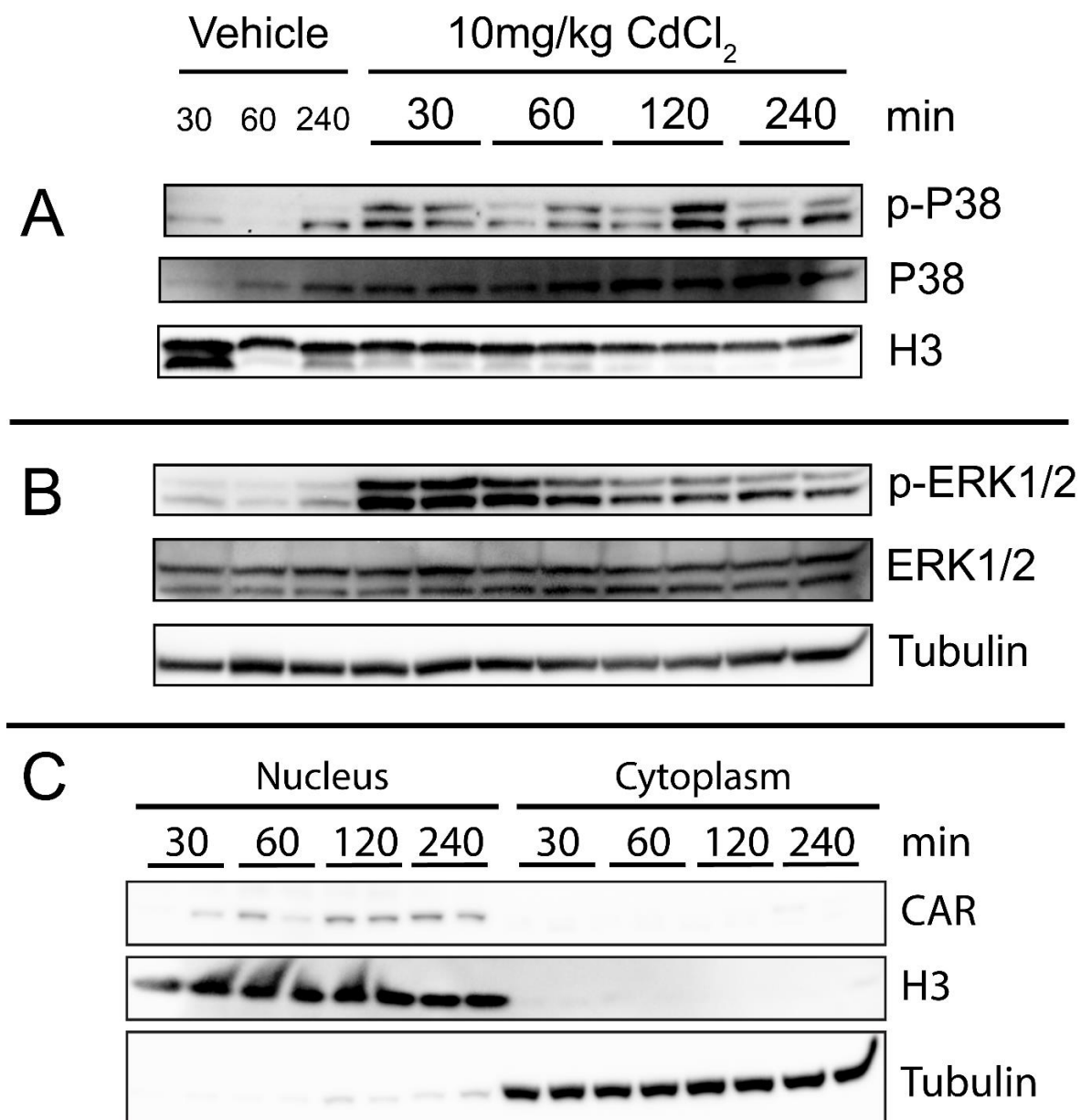


Figure 13. Changes in intestinal MAPK phosphorylation and CAR localization following Cd²⁺ treatment in *hUGT1* neonates. Twelve-day old neonatal *hUGT1* mice were treated with vehicle or Cd²⁺ (10mg/kg po.) and intestinal tissues were collected 30, 60, 120, and 240 minutes after exposure. Intestinal protein extracts were fractionated into nuclear and cytosolic components, and changes in (A) nuclear P38 content and phosphorylation status, (B) cytosolic ERK1/2 content and phosphorylation status, and (C) CAR localization were visualized by Western blot analysis with H3 and tubulin for nuclear and cytoplasmic loading controls. Quantifications and statistical analyses for A, B, and C can be found in supplementary figures 13, 14, and 15, respectively.

The observed changes in MAPK activation are consistent with known regulatory mechanisms for CAR by ERK ²¹ and P38 ¹⁷, supporting the observation that cadmium exposure activates intestinal CAR. The increased oxidative state following Cd²⁺ treatment, the activation of MAPK pathways, and the commensurate nuclear accumulation of CAR underlies the induction of intestinal UGT1A1.

PEITC induction of UGT1A1 retains CAR-dependency pattern in *hUGT1/Nrf2*^{-/-} mice.

The observation of CAR-dependent regulation of hepatic UGT1A1 by pro-oxidants was observed using neonatal *hUGT1/Car*^{-/-} mice treated with PEITC ⁹³. The tissue-specific induction patterns of hepatic and intestinal *UGT1A1* and *Cyp2b10* genes were observed in the *hUGT1/Nrf2*^{-/-} neonatal mice. The increased clearance of bilirubin in neonates treated with PEITC occurred concomitantly with increases in liver and intestinal expression of *UGT1A1* and *Cyp2b10* (**Fig. 14**). The inducibility of intestinal *UGT1A1* by PEITC was reduced in *hUGT1/Nrf2*^{-/-} (16.2-fold) mice compared to *hUGT1/Nrf2*^{+/-} mice (30.3-fold) (**Fig. 14**). No difference was observed in liver *UGT1A1* induction between *hUGT1/Nrf2*^{+/-} and *hUGT1/Nrf2*^{-/-} neonates and the induction of *Cyp2b10* was greatest in the liver (**Fig. 14**). These observations are consistent with the previous report on CAR-dependency of hepatic *UGT1A1* induction and CAR-independent intestinal *UGT1A1* induction by PEITC ⁹³.

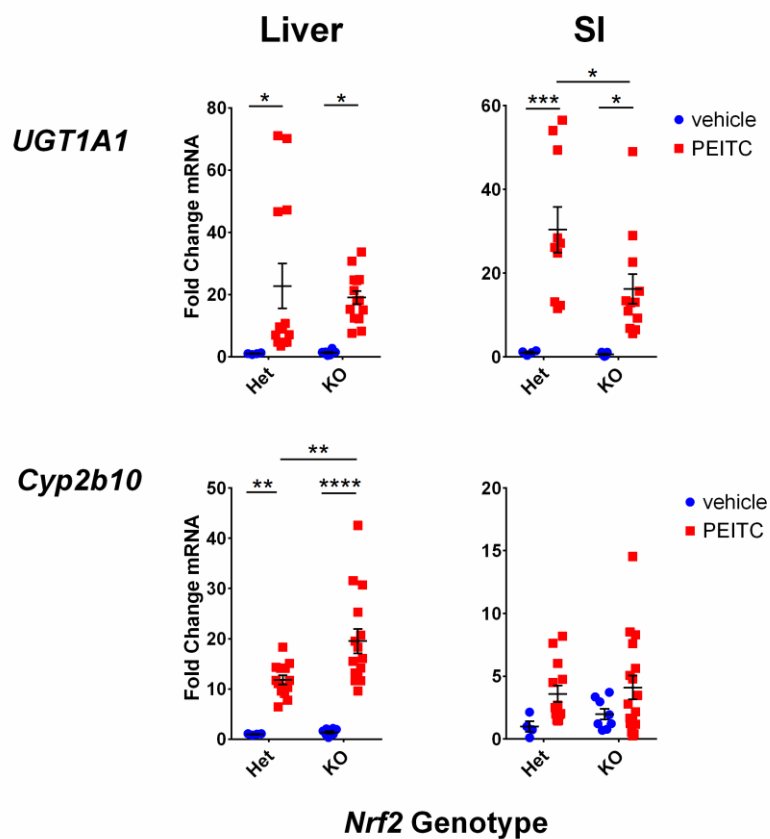


Figure 14. Hepatic and intestinal induction of *UGT1A1* and *Cyp2b10* in *hUGT1/Nrf2* neonatal mice. Ten-day old *hUGT1/Nrf2*^{+/+} and *hUGT1/Nrf2*^{-/-} neonates were treated with vehicle (blue circles) or PEITC (200mg/kg od. po.) (red squares) for two days before collecting liver and small intestine. Gene expression for *UGT1A1* and *Cyp2b10* in each tissue were examined by RT-qPCR. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001)

Discussion

It has been previously demonstrated that ITCs are capable of CAR-dependent induction of liver UGT1A1 ⁹³. It is well-established that NRF2 is the master regulator of the antioxidant response and can induce UGT1A1 ⁶⁶. Nuclear receptors are susceptible to high levels of mutual regulation ⁹⁶ from shared protein interaction partners or through binding interactions at regulatory elements, therefore it was hypothesized that oxidative stress could be co-activating CAR through NRF2. The role of oxidative stress in the regulation of NRF2 and CAR target genes in *hUGT1/Nrf2*^{-/-} mice was investigated. As anticipated, NRF2-deficient mice do not generate the antioxidant response when challenged with Cd²⁺. However, the *hUGT1/Nrf2*^{+/-} mice exhibited a diminished antioxidant response in comparison to WT mice, suggesting haploinsufficiency.

By breeding *Nrf2*^{-/-} mice with *hUGT1* mice, pro-oxidants could be screened for their ability to reduce TSB levels and examine the impact of this action in an NRF2-dependent fashion. The NRF2-KEAP1 pathway is not required for Cd²⁺ and ITCs to induce intestinal UGT1A1 in *hUGT1* mice to reduce TSB levels. This supports the hypothesis that CAR can be activated by oxidative stress ⁹³. In analysis of NR activity, we observed suppression of target genes for PXR and PPAR α and slight induction of the AhR target gene, *Cyp1a1*, in WT mice (**Fig. S11**). The reason for the suppression is unclear, but the latter induction could have resulted from co-regulation of *Cyp1a1* by NRF2 and the AhR ⁹⁷. The loss of this induction in the heterozygous mice can be linked to haploinsufficiency of NRF2, and there was little change in the *Nrf2*^{-/-} mice. From the examined NRs, there was evidence for CAR's activation from

induction of the *Cyp2b10* gene. Attempts were made to confirm protein induction of CYP2B10, but the expression levels were too low for detection. Subsequently, CAR's activation was assayed more directly. In the absence of a readily available antibody for phosphorylated CAR, fractionated intestinal samples from neonates treated with Cd^{2+} were analyzed for CAR nuclear localization. After an acute cadmium exposure, CAR localized to the nucleus in an NRF2-independent manner, which indicates that co-activation of CAR by NRF2 is not responsible for the induction of intestinal UGT1A1 and the reduction of TSB levels.

The increase in ERK1/2 phosphorylation is a confounding factor in the mechanism of CAR's activation, given that p-ERK1/2 inhibits the activation of p-Thr38-CAR, keeping it sequestered in the cytoplasm ²⁶. However, in cardiac ventricular myocytes, it has been reported that ERK1/2 can be dephosphorylated by PP2A activity that is dependent upon the activation of P38 ⁹⁸. Furthermore, the phosphorylation status of ERK1/2 and P38 can be increased by H_2O_2 and As^{3+} respectively, both of which are linked to oxidative stress. The observation of a consistent nuclear accumulation of CAR during the decay of the p-ERK signal at the 2- and 4-hour time points suggests the reduction in ERK1/2 activity is sufficient to permit CAR activation, which occurs concurrently with the nuclear accumulation of P38/p-P38. In order for CAR to translocate to the nucleus, it must first be dephosphorylated by PP2A ²⁵ in order to interact with P38 to increase CAR's transactivation activity ¹⁷. Activated P38 phosphorylates CAR at the conserved Thr38 residue to export CAR from the nucleus. These changes in the phosphorylation status of P38 and ERK1/2 were also observed in whole-cell lysate from a *hUGT1*

adult time course (data not shown), supporting our hypothesis of the oxidative stress-MAPK-CAR axis.

It has been reported that intestinal differentiation and crypt formation involves ROS and the activation of P38^{99,100}. Intestinal maturation is essential to both nutrient uptake and metabolic waste/toxicant removal. From studies in *hUGT1* mice, early intestinal expression of UGT1A1 is important to metabolize bilirubin while hepatic UGT1A1 expression is developmentally delayed⁵⁸. Maturation of intestinal epithelial cells can be estimated by the decreases in genes related to cell stemness and the increase of functional genes related to the differentiated cell types. For example, the brush border glucosidase known as sucrose isomaltase, *SIS*, is only expressed in maturing and mature duodenum and jejunum enterocytes¹⁰¹. Alternatively, the *Lrp2* gene encodes for a protein known as megalin, which mediates nutrient uptake by endocytosis, and its expression in the jejunum and ileum is down regulated as those tissues develop¹⁰². These and other genes, like *Glb1* and *Krt20* are markers for intestinal maturation when down- and up-regulated, respectively⁶⁷. Intestinal maturation markers were examined and it was determined that low doses of Cd²⁺ may induce intestinal maturation as inferred by inhibition of the *Glb1* and *Lrp2* genes and the induction of the *Sis* and *Krt20* genes (**Fig. S12**). Recently, it was reported that the nuclear receptor corepressor 1 (NCoR1), which regulates CAR activity at the PBREM²³, is also capable of regulating intestinal maturation⁶⁷. Intestinal epithelial cell specific knockout of NCoR1 leads to changes in maturation markers and induction of UGT1A1 similar to what was observed herein⁶⁷. Thus, it may be

possible that Cd²⁺ cadmium inhibits NCoR1, which increases maturation and de-represses CAR's transcriptional activity.

The most obvious implication of the oxidative stress-MAPK-CAR signaling axis is that those individuals with higher intestinal and hepatic CAR activity from environmental pro-oxidants, such as heavy metals from smoking or contaminated drinking water and soil, or from indirect antioxidants found in certain diets and commercial supplements will also have increased metabolism of UGT1A1 and CYP2B6 substrates. For example, bupropion, an anti-depressant and smoking cessation aid, is hydroxylated by CYP2B6 in the liver to hydroxybupropion, which is both pharmacologically active and the major metabolite ¹⁰³. Alternatively, the chemotherapeutic agent cyclophosphamide is bio-activated in the liver by CYP2B6, which would increase both toxicity and therapeutic effectiveness ¹⁰⁴. Regarding UGT1A1, irinotecan is a chemotherapeutic agent for colorectal cancer and the activated metabolite SN-38 is detoxified by UGT1A1, increased intestinal and colonic activity of UGT1A1 would reduce the treatment side effects ⁹². Additionally, raloxifene is used to treat osteoporosis and the major metabolites are glucuronides where increases in UGT1A1 would decrease availability and treatment effectiveness ¹⁰⁵. It is possible that future studies making use of RNA-seq, metabolomics, and exposure data may reveal a correlation between pro-oxidant, toxicant exposure, and an individual variability in xenobiotic metabolism stemming from CAR activity.

Acknowledgments

This work was supported by U.S. Public Health Service Grants ES010337 and GM126074 and the UCSD Graduate Training Program in Cellular and Molecular Pharmacology, GM007752.

Author Contributions

Participated in research design: Paszek, Tukey

Conducted experiments: Paszek

Performed data analysis: Paszek

Wrote or contributed to the writing of the manuscript: Paszek, Tukey

Chapter 3, in part, is a reprint of Paszek M and Tukey RH. (2020) NRF2-independent regulation of intestinal constitutive androstane receptor by the pro-oxidants cadmium and isothiocyanate in *hUGT1* mice. Drug Metabolism and Disposition. Vol. 48, Issue 1. 25-30. The dissertation author was the primary investigator and author of this paper. Changes to the material presented were made to maintain format consistency. Supplementary materials can be found in the appendix.

CHAPTER 4: MECHANISTIC CONSIDERATIONS OF CAR ACTIVATION BY OXIDATIVE STRESS

Section 4.1 CAR and NRF2 Crosstalk

Arsenic and cadmium are environmentally relevant toxicants that are ranked number 1 and 7, respectively, on the agency for toxic substances and disease registry's (ATSDR) substance priority list. The dietary prooxidant PEITC and its antitumoral properties¹⁰⁶ has been the subject of many investigations, including that of an interventional clinical trial (NCT03700983). These toxicants can exert their effects on drug metabolism through the depletion of cellular glutathione to induce oxidative stress and activate the NRF2-KEAP1 pathway. The activation of CAR by PEITC and heavy metals, as examples of prooxidant toxicants in general, was initially thought to be a form of crosstalk between the master regulator of the antioxidant response, NRF2, and CAR. An alternative pathway for the regulation of drug metabolism by oxidative stress could be inferred by the observation that P38, could increase CAR transcriptional activity and initiation cytoplasmic localization¹⁶. This is of note because it was previously understood that ERK1/2, another MAPK, had a repressive effect on CAR. Furthermore, NF-kB activates following substantial amounts of oxidative stress, which leads to the repression of many nuclear receptors¹⁰⁷, making CAR a potential target. At first glance the action of MAPK and NF-kB would suggest that CAR is inhibited by oxidative stress. However, CAR is not directly repressed by NF-kB like other nuclear receptors, and there are multiple reports that indicate a crosstalk between CAR and NRF2, suggesting that NRF2 activity would be the source of CAR activation. These investigations included the

activation of CAR by phorone⁸¹, oltripaz¹⁰⁸, allyl sulfide¹⁰⁹, trans-stilbene oxide¹¹⁰, and the induction of NRF2 targets by CAR activators¹¹¹. The latter of these is of interest in the context of nuclear receptor crosstalk because CAR activity precedes the activation of NRF2 but does not appear to be dependent upon CYP450-induced oxidative stress, which is a reported mechanism for ROS production^{112,113}. All of the above chemicals can deplete cellular glutathione, but only trans-stilbene oxide has been examined for direct CAR binding and was determined to be a ligand based upon concentration dependent recovery of CAR target gene induction from androstenol inverse agonism¹¹⁰. It may prove useful to establish if other xenobiotics activate CAR independently of NRF2 *in vivo* by using the *hUGT1/NRF2* mouse model.

In contrast to the crosstalk others have observed, NRF2-independent induction of UGT1A1 by prooxidants suggests another redox sensitive pathway is activating CAR. While there are no independent reports of Cd²⁺ activating CAR, there are reports of PEITC inducing cyp2b15¹¹⁴ and sulforaphane increasing bilirubin glucuronidation¹¹⁵ and CYP2B activity¹¹⁶. It has been reported *in vitro* that cancer cells experience reduced EGFR activation when treated with PEITC¹¹⁷. This raises the possibility that indirect activation, not oxidative stress, could be the cause of CAR activation; however, these effects were modest and occurred in prostate cancer cells. Additionally, we were unable to activate CAR with PEITC *in vitro* using mouse primary hepatocytes, which are responsive to indirect mechanisms of activation. A direct ligand binding assay showed no activation by PEITC (**Fig. S16**). It appears PEITC is not capable of activating mouse CAR from a phenobarbital-like indirect

mechanism. One reason that PEITC may not be able to induce UGT1A1 in primary hepatocyte culture but is capable *in vivo* could be either by gut microflora or first pass metabolism through the intestine. However, when neonatal mice were intraperitoneally treated with 50mg/kg PEITC, treated mice experienced reductions in TSB values in an NRF2-independent manner (**Fig. S17**). This I.P. administration bypasses potential bioactivation from microbial or intestinal metabolism. Additionally, supplementing mice with N-acetylcysteine in drinking water prevented hepatic induction of oxidative stress genes and CAR target genes *Cyp2b10* and *UGT1A1*, which suggests oxidative stress rather than EGFR inhibition.

Section 4.2 CAR and MAPKs

The activation of the MAPK's is of interest because they control a variety of cellular responses to different types of stress. Following its activation by oxidative stress, P38 both activates and inhibits CAR. Negishi and colleagues proposed that p-P38 increases the activity of transcription factors that increase CAR activity. These factors include PGC1 α ¹¹⁸, C/EBP α ¹¹⁹, HFN4 α ¹²⁰, and SRC1¹²¹. All but the SRC1 study have directly implicated P38 phosphorylation of the transcription factor as an activating step. This would either require multiple p-P38 proteins as CAR is also phosphorylated by p-P38 and/or an exchange of the phosphate group from one of the transcription cofactors to CAR, thereby decreasing the activity of the cofactor and excluding CAR from the nucleus. In this way, both the activating function of P38 and the phosphorylation of Thr38 could be explained. However, under this hypothesis, the activation of CAR by prooxidants does not explain the initial increase in nuclear CAR accumulation. The increase in CAR activity by the examined toxicants could be

due to derepression instead of or in addition to the proposed mechanism above as they are not mutually exclusive.

In this derepression hypothesis, the combination of three separate phosphorylation changes prompted by p-P38 could explain both the activation of CAR by oxidative stress, as we have observed and repression following transcription as it has been described by Negishi and colleagues. The activation of P38 leads to increases in PP2A activity, which has two separate activating steps; the dephosphorylation of ERK1/2 and the dephosphorylation of Thr38-CAR. The basis for oxidative stress activation of PP2A by P38 has been examined *in vitro* using several cell types. The first study details the increase in P38 phosphorylation following sodium arsenite exposure in cardiac ventricular myocytes, which subsequently dephosphorylates H₂O₂-activated ERK1/2 in an PP2A-dependent manner⁹⁸. The second study utilized endothelial somatic hybrid cells to demonstrate that P38 mediates a PP2A-dependent inhibition of the MEK-ERK pathway¹²². The third study was performed in human skin fibroblasts and demonstrated that arsenite activated P38, leading to PP2A dephosphorylation of the phorbol ester-activated MEK-ERK pathway¹²³.

ERK1/2 acts as a major repressor of CAR nuclear localization as it has been demonstrated that a reduction in ERK1/2 phosphorylation by MEK inhibitor U0126 is sufficient to induce CAR translocation to the nucleus²¹. First, the dephosphorylation of ERK1/2 allows the CAR homodimer complex to dissociate and reveal the RACK1:PP2A binding interface¹⁹. Second, the RACK1:PP2A complex dephosphorylates Thr38, which is the essential regulatory step for cytoplasmic

localization¹⁸. The last phosphorylation event is performed by p-P38 acting on Thr38-CAR to initiate cytoplasmic localization¹⁷. Between the second and third steps, changes in phosphorylation drive p-P38 to activate cofactors and increase CAR transcriptional activity. Alternatively, p-P38 may lead to increased activity of PP2A and decreased ERK1/2 phosphorylation.

The phosphorylation of ERK1/2 is a rapid event that peaks within 30 minutes and then decays, potentially derepressing CAR. It is unclear if the activation of ERK1/2 causes additional repression because CAR is already principally localized in the cytoplasm under basal conditions. Decreases in CAR phosphorylation and dissociation of the CAR homodimer complex can be expected following the drop in ERK1/2 phosphorylation; however, only one phospho-specific antibody for CAR has ever been created and could not be obtained for use. The nuclear accumulation of intestinal CAR following cadmium exposure occurs concomitantly with the drop in ERK1/2 phosphorylation, which suggests a derepression mechanism. Additional time course experiments with other indirect NRF2/CAR activators such as oltipraz or arsenic may show similar patterns of localization and phosphorylation.

Section 4.3 CAR and HIF1a

Other redox sensitive proteins were investigated as potential sources of CAR activation, such as HIF1a and FOXO1. There have been reports that ROS can stabilize HIF1a¹²⁴. A report of crosstalk between HIF1a and CAR implicated HIF1a as a potential coactivator of CAR¹²⁵. In HepG2 cells and the livers of mice, CoCl₂, a stabilizer of HIF1a, led to the increase of CAR transcriptional activity and increased association of both CAR and HIF1a at the PBREM¹²⁵. However, these effects were

not investigated in small intestine. To examine the effect of HIF1a on CAR activation in the intestines, neonatal *hUGT1* mice were treated with CoCl₂ or water on day 10 and CdCl₂ on day 11. TSB values were collected on days 11 and 12 to determine if CoCl₂ treatment was sufficient to induce UGT1A1. As shown in **Figure S18**, CoCl₂ does not induce UGT1A1 by itself but pretreatment with CoCl₂ followed by CdCl₂ had a greater effect in lowering TSB values than water pretreatment. The effect of HIF1a in this experiment is likely intestinal as CdCl₂ does not accumulate in the liver to a significant degree using this protocol. A caveat is that this experiment was performed in *hUGT1* mice, and so the contribution of NRF2 to the lowering of TSB values and the possible interaction of HIF1a and NRF2 prevents confirming the crosstalk between CAR and HIF1a. Performing this experiment in *Nrf2*-null and *Car*-null mice would be a better approach; however, it is known that CdCl₂ destabilizes HIF1a and so two treatments of CdCl₂ may reduce HIF1a to levels where ROS activation has a negligible effect. This calls into question the relevance of HIF1a to ROS activation of CAR, as a single dose of 10mg/kg CdCl₂ does not typically lower TSB values to 0-1, but these values can be obtained with two doses, by which point HIF1a levels might be too low to have an effect. The effect observed by increasing HIF1a content is likely due to another mechanism by which CAR activity is increased rather than what is observed with PEITC and Cd²⁺.

The validity of overexpressing components to determine their role in CAR activation is a recurring issue for *in vitro* mechanism studies as they diverge from the complexity associated with a full biological system but are necessary to obtain positive data. For example, in immortalized cell lines CAR is typically overexpressed

by transient or stable transfection, and the transformed CAR is constitutively active in hepatomas, which are useful cell lines given the *in vivo* expression of CAR is highest in the liver; however, primary hepatocyte cultures do not exhibit constitutive nuclear accumulation of CAR. There are phenotypic consequences to studying CAR in different models as phenobarbital is incapable of inducing CAR target gene expression in hepatomas but does induce expression in primary hepatocytes. The reason for this is unknown but may have something to do with low levels of P38 activity in HepG2 cells, which may prevent the initiation of cytoplasmic localization following transcription activation.

Section 4.4 CAR and FOXO1

The transcription factor FOXO1 increases CAR transcriptional activity at its expense due to sequestration from its target binding elements upon interacting with CAR¹²⁶. This is the basis for some of CAR's ability to regulate energy homeostasis through mechanisms like inhibiting expression of the rate limiting enzyme for gluconeogenesis, PEPCK1 (*Pck1*). The redox regulation of FOXO1 is mediated through its phosphorylation by AKT that signals for nuclear exclusion and through increasing mRNA stability by RNA-binding proteins containing reactive cysteine residues¹²⁷. Following PEITC exposure in *hUGT1* neonates, hepatic *Pck1* mRNA expression was inconsistently down regulated between independent experiments while *Foxo1* mRNA was unaffected. Similar inconsistencies were observed in neonatal intestinal samples of *hUGT1/Nrf2* mice treated with CdCl₂. In *hUGT1* neonatal mice, FOXO1 protein could not be detected, therefore adults were utilized. A time course was performed in adults treated with PEITC and their livers were

fractionated for nuclear content. Following PEITC administration FOXO1 is excluded from the nucleus (**Fig. S19**), presumably due to increased AKT activity, and thus is not responsible for the increase in CAR activity.

Much of the work presented herein has been in examination of redox sensitive factors that could be responsible for increases of CAR target gene expression following prooxidant exposure. While other mechanisms may exist that act in an *in vivo* context to coactivate CAR, it seems likely that MAPK regulation is responsible for the increases of CAR nuclear accumulation and activity.

CHAPTER 5: CLOSING COMMENTS

The activation of CAR by the prooxidants arsenic, cadmium, and PEITC represents a novel mechanism for the regulation of drug metabolism in an *in vivo* context with phenotypic consequences. Initial observations of ITCs inducing phase II drug metabolizing enzymes led to the study of PEITC in the humanized *UGT1* mouse model. The use of neonatal hyperbilirubinemia enables faster determinations of nuclear receptor dependency of regulation of drug metabolism by xenobiotics. In the course of these examinations, it was determined that CAR was responsible for the induction of UGT1A1 and CYP2B10 in neonatal and adult mice. The CAR dependency, however, was a tissue-specific phenomenon. While hepatic control of UGT1A1 was CAR-dependent, intestinal induction of UGT1A1 was, in part, dependent upon NRF2. This was concluded through gene expression analysis and the generation of a new humanized *UGT1* mouse model in an *Nrf2*-null background. The generation of the *hUGT1/Nrf2* mouse model and the potential for a *Car/Nrf2* double knockout mouse model comes at an opportune time given the recent reports detailing CAR-NRF2 crosstalk. However, such crosstalk was not responsible for the activity of CAR following exposure to prooxidants, which begs the question what redox sensitive mechanism is responsible for regulating CAR.

These preliminary investigations suggest that HIF1a and FOXO1 are not likely to be responsible for CAR's increased nuclear accumulation, which is an underlying feature of CAR activity. It is likely that the increase in CAR activity by oxidative stress is a multifactorial response given the numerous redox sensitive pathways that either directly or indirectly affect CAR. Some clues to the activation of CAR may come from

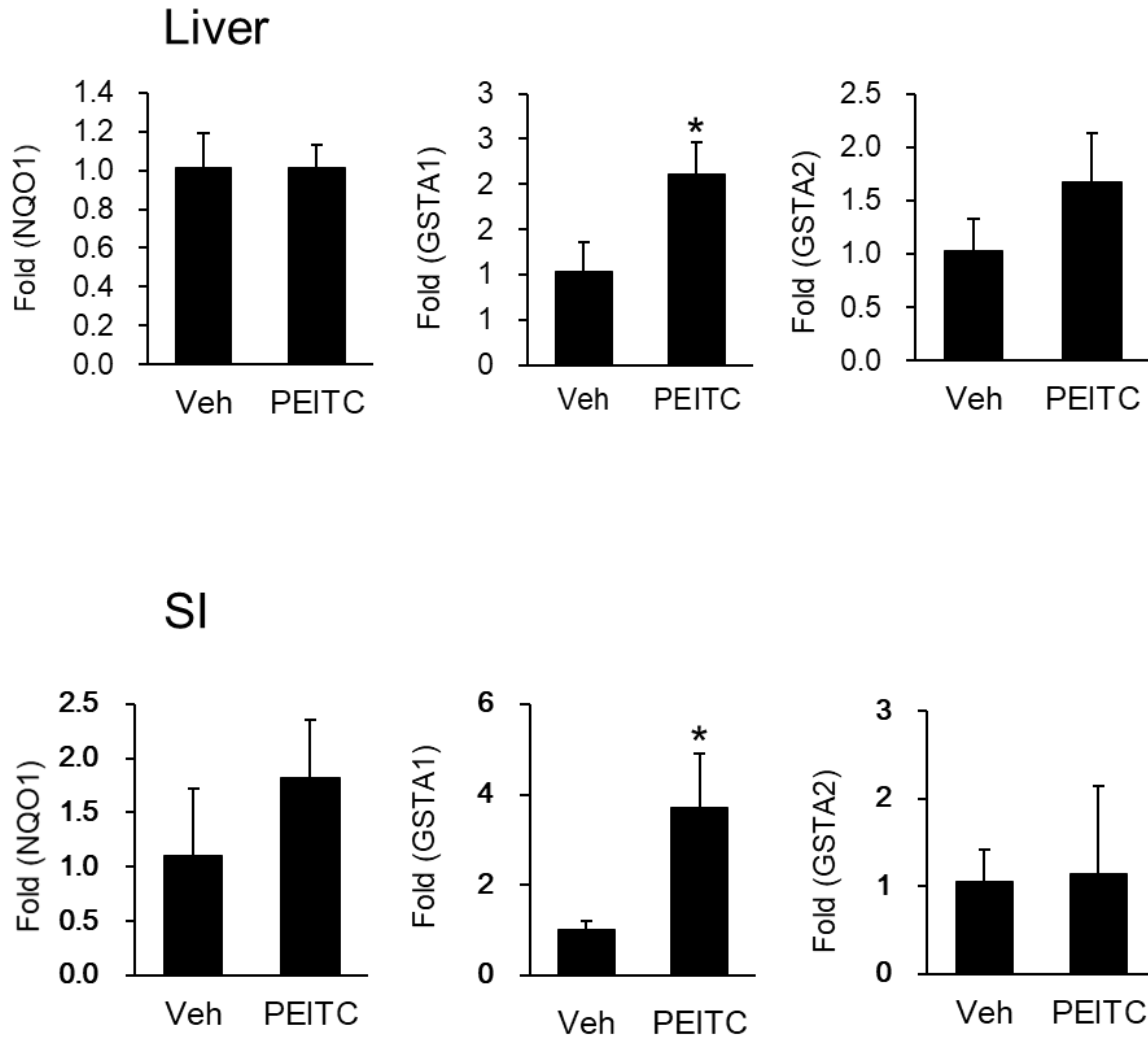
tissue specific dependency of UGT1A1 induction by PEITC; however, PEITC alone is insufficient to implicate oxidative stress as the essential regulatory component given that a variety of mechanisms of action have been reported for its antitumor properties. By expanding the investigation of prooxidants to the environmentally relevant toxicants arsenic and cadmium, the role of oxidative stress is reinforced and the implications of this work are potentially extended to the study of environmental factors that may explain individual variability in pharmaceutical treatments. It is worth noting that all of the experiments presented herein were acute exposures, and studies into the regulation of CAR by constant oxidative stress may yield different results.

An additional avenue of research is the effect of oxidative toxicants on the development of the intestines. Are the changes in maturation markers following PEITC and cadmium exposure reflected in proliferative cell populations? If so, is there a link to the increase in UGT1A1 expression as it relates to CAR and NRF2 dependency? Some answers may reside in other regulatory factors such as NCoR1, but other poorly understood avenues remain, such as the role of AMPK signaling or microRNA regulation. What is clear in any event is that the regulation of CAR localization is dependent upon proper recognition and activity of PP2A. The mechanism by which nuclear accumulation of CAR by Thr38 dephosphorylation is unknown and may present additional explanations of CAR activity following prooxidant exposure. Herein, a definitive link between the activation of CAR by diverse toxicants that induce oxidative stress has been shown. This activation of CAR

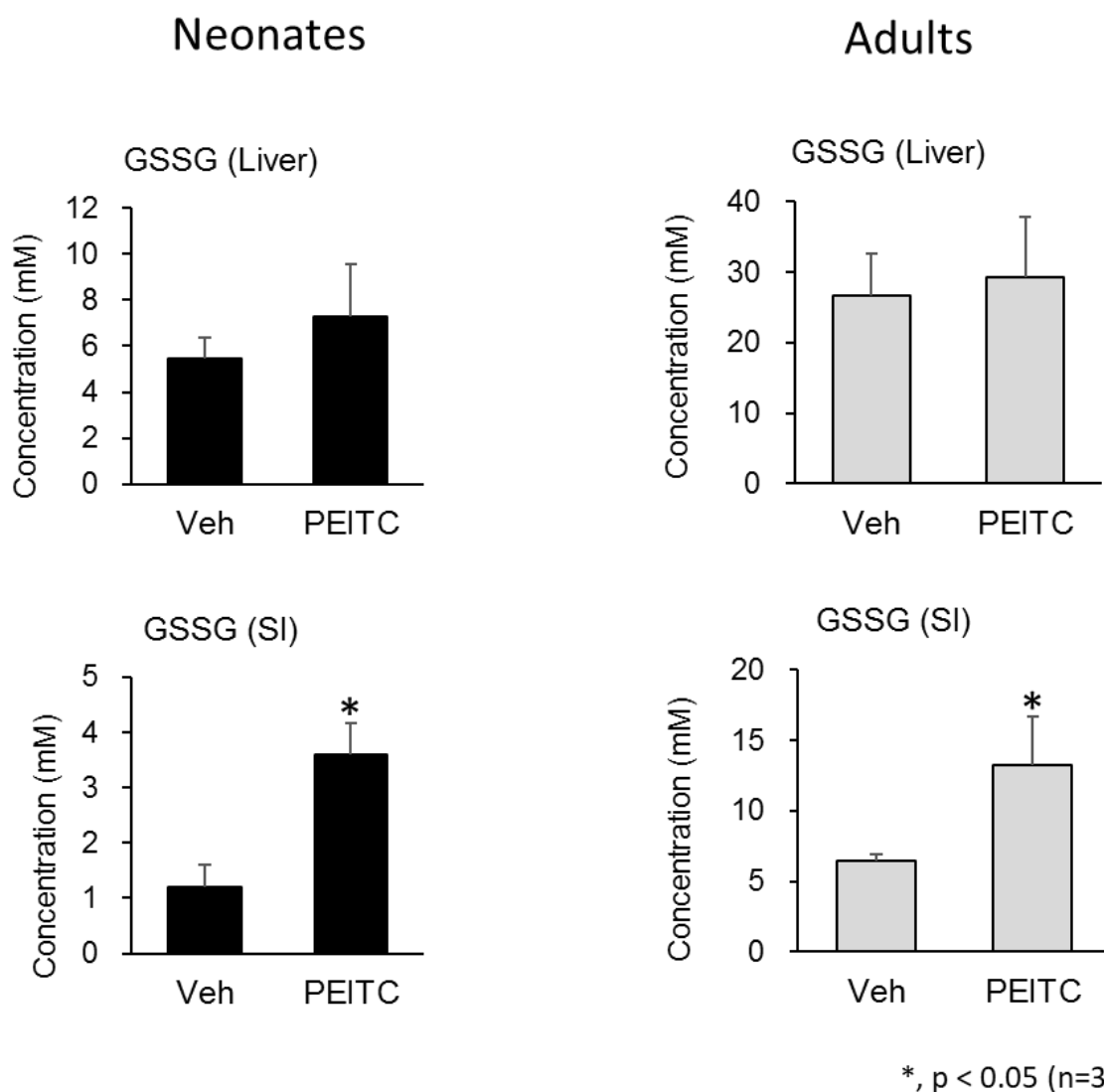
regulates drug metabolism with phenotypic consequences and is not a result of crosstalk with the master regulator of the antioxidant response, NRF2.

APPENDIX (Supplemental Materials)

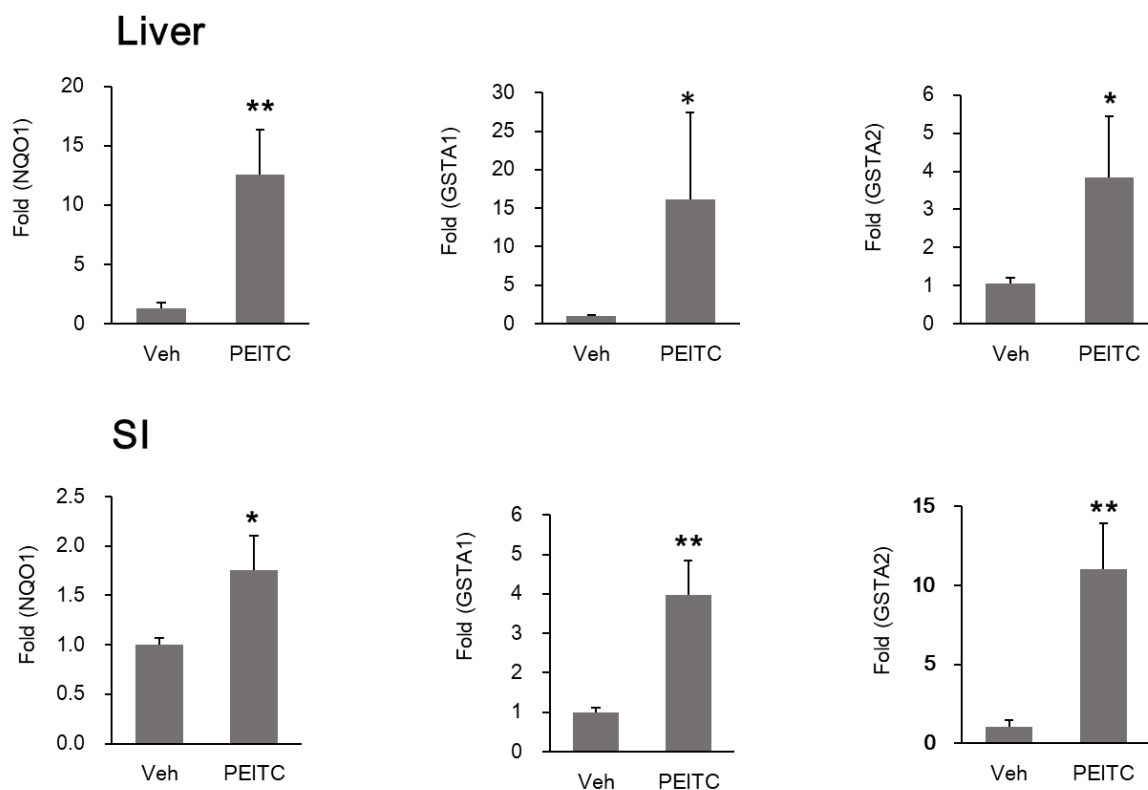
Supplemental Figures



Supplemental Figure 1. Analysis of *Nrf2* target genes. Using RNA isolated in Figure 1 (neonatal *hUGT1* mice), gene expression patterns for *Nqo1*, *Gsta1* and *Gsta2* were quantitated by RT-qPCR expressed as Fold induction. Values are the means of $\pm$ SD ($n=3$). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, $P<0.05$).



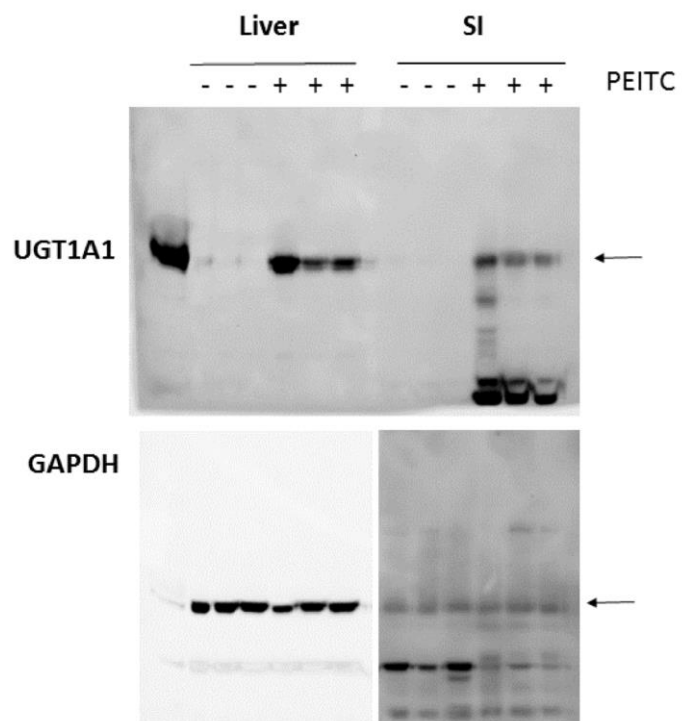
Supplemental Figure 2. Oxidative stress following PEITC treatment. **A.** Neonatal *hUGT1* mice were given an oral dose of PEITC (200 mg/kg) and GSSG levels measured in liver and small intestine (SI) after 24 hours. **B.** The same experiment was conducted on adult (6 week old) *hUGT1* mice. Values are the means of $\pm$ SD (n=3). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, $P < 0.05$).



Supplemental Figure 3. Impact of PEITC on Nrf2 target genes in adult mice.

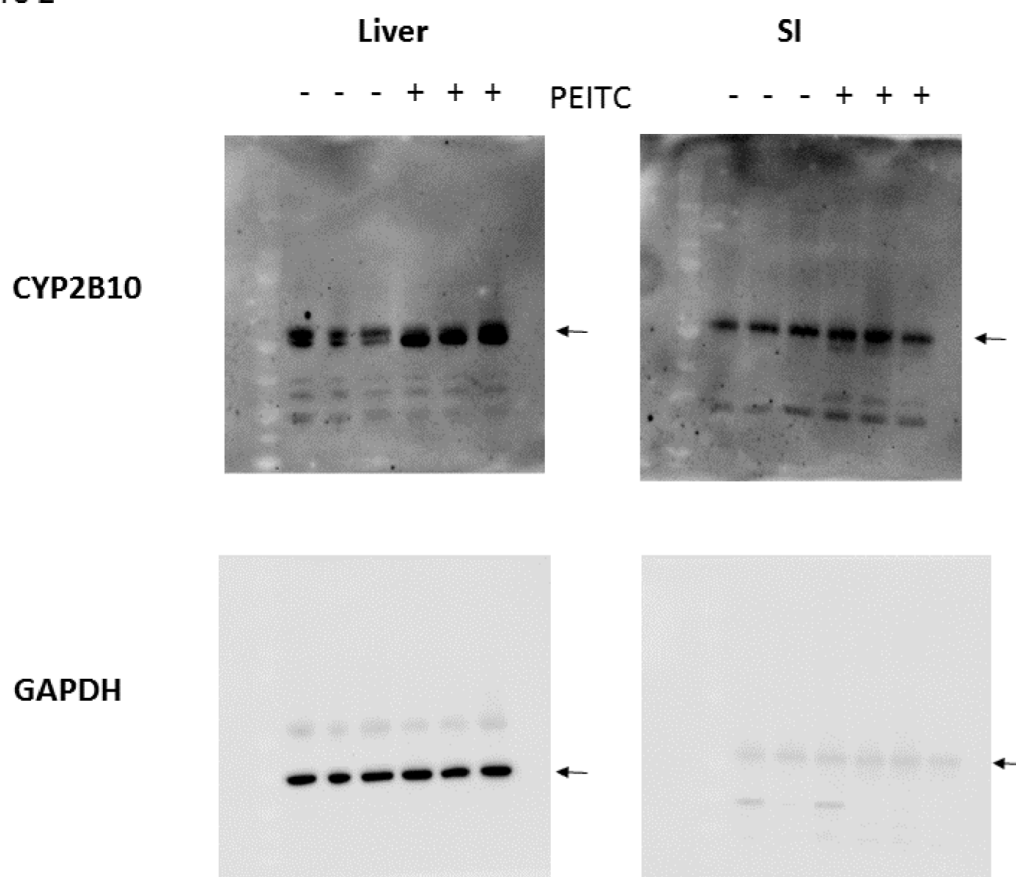
RNA prepared from PEITC treated *hUGT1/Car^{+/-}* and *hUGT1/Car^{-/-}* mice from liver and small intestine (Figure 4) was used to quantitate *Nqo1*, *Gsta1* and *Gsta2* gene expression by real time PCR. Values are the means of $\pm$ SD ($n > 3$). Statistically significant differences between vehicle (Veh) and PEITC are indicated by asterisks (Student *t* test: *, $P < 0.05$; **, $P < 0.01$).

Figure 1

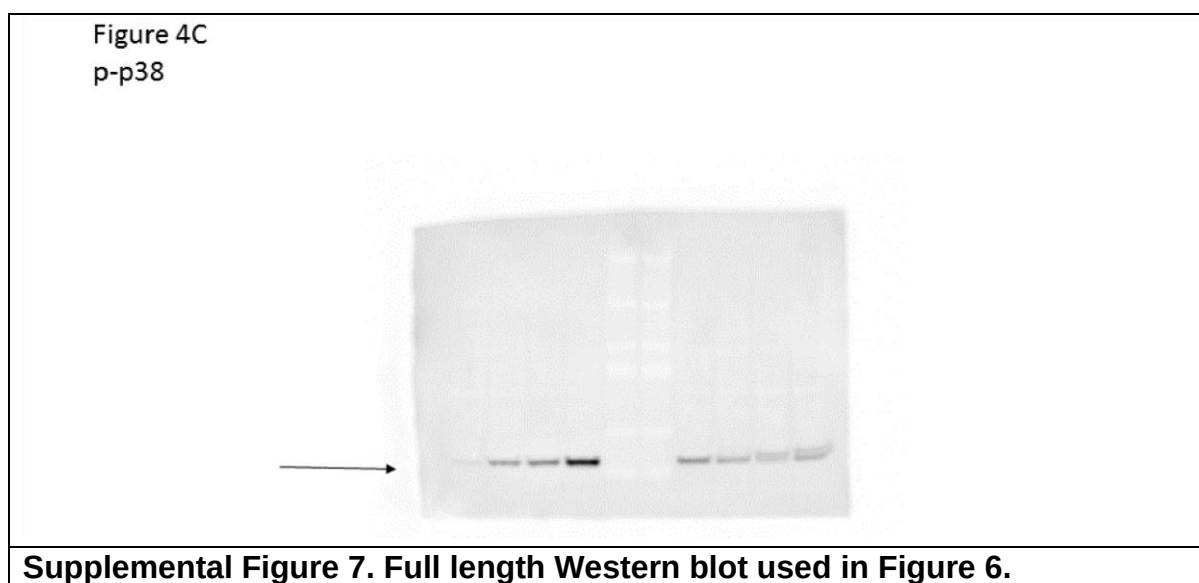
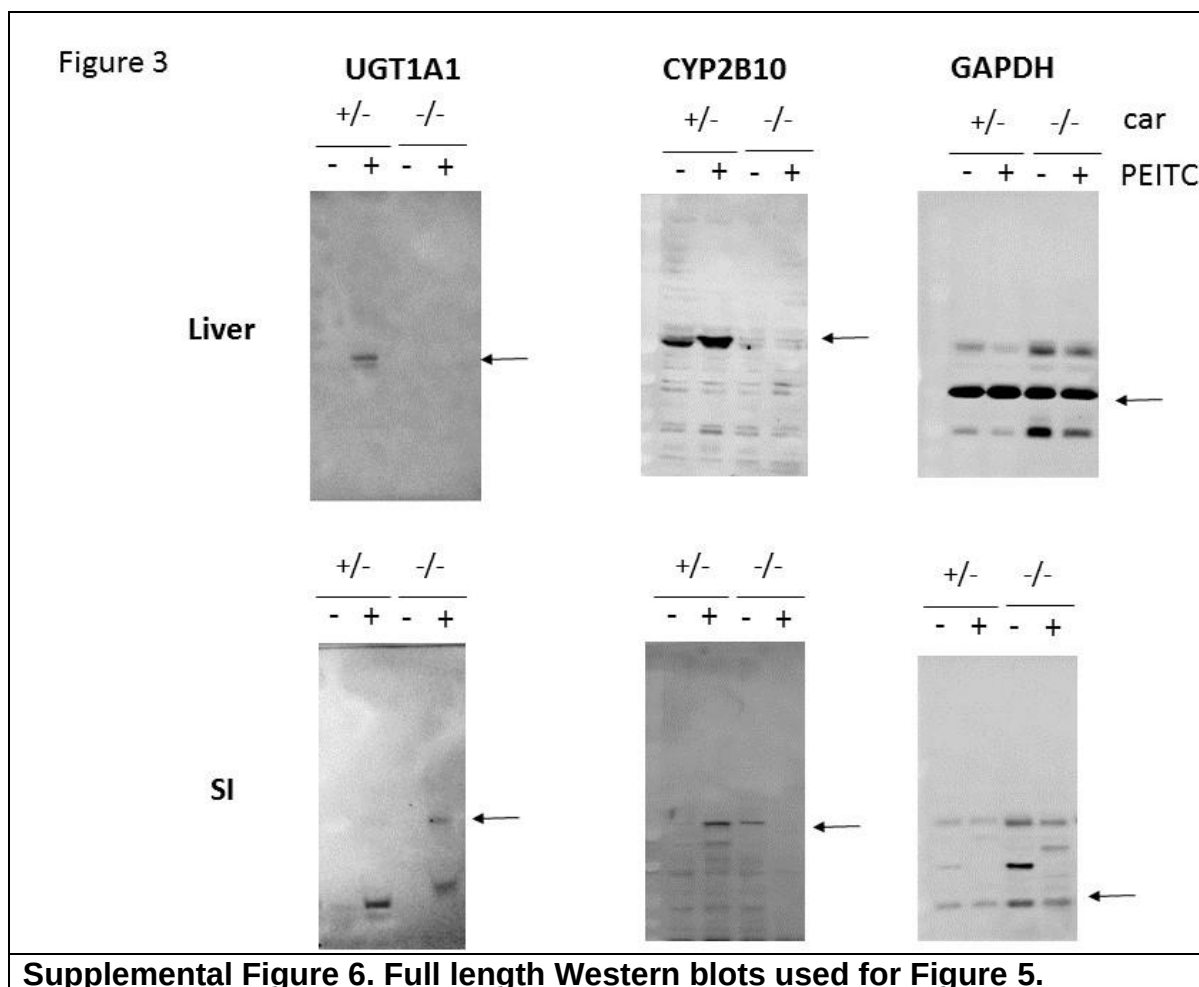


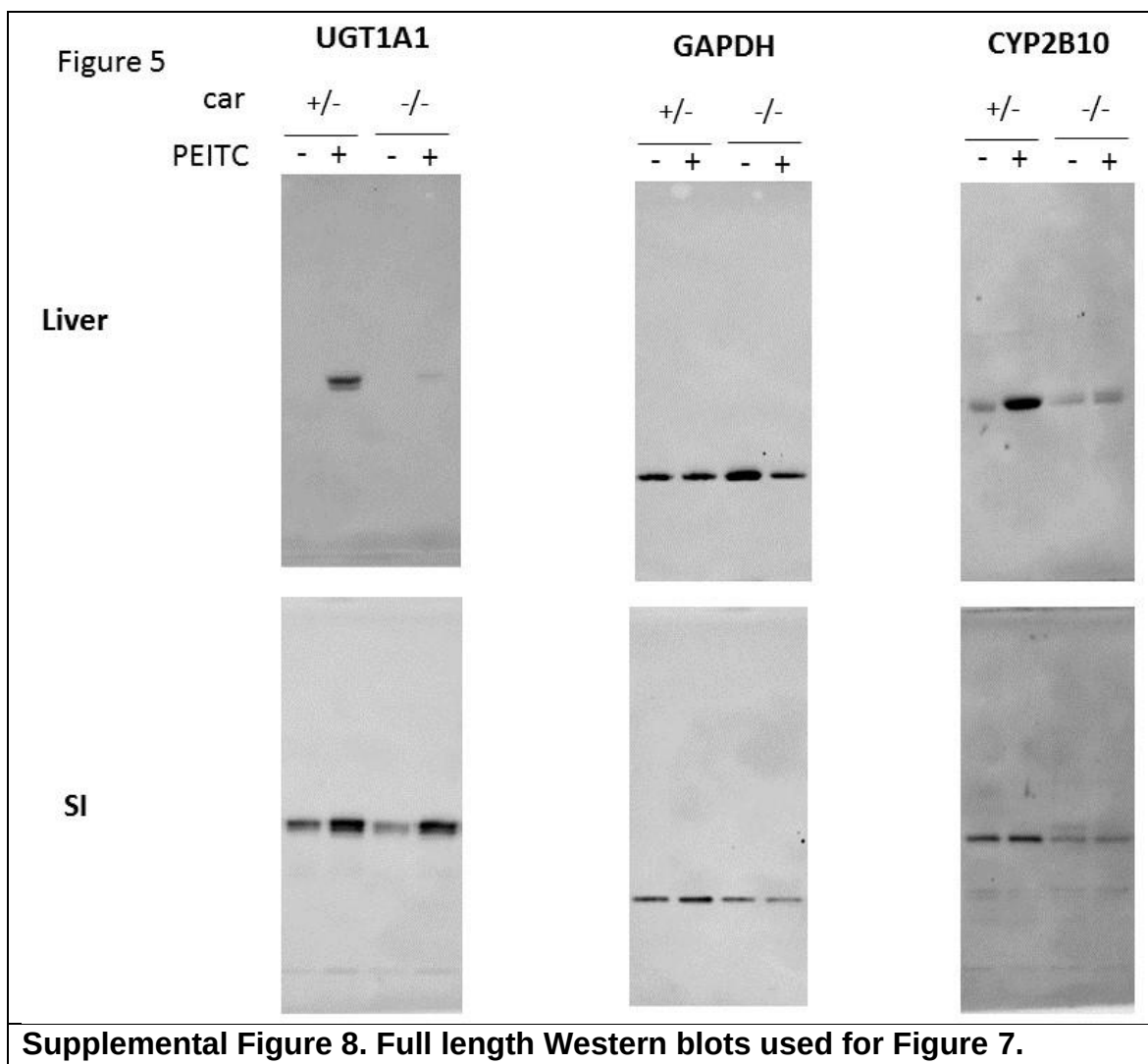
Supplemental Figure 4. Full length Western blot used for Figure 3.

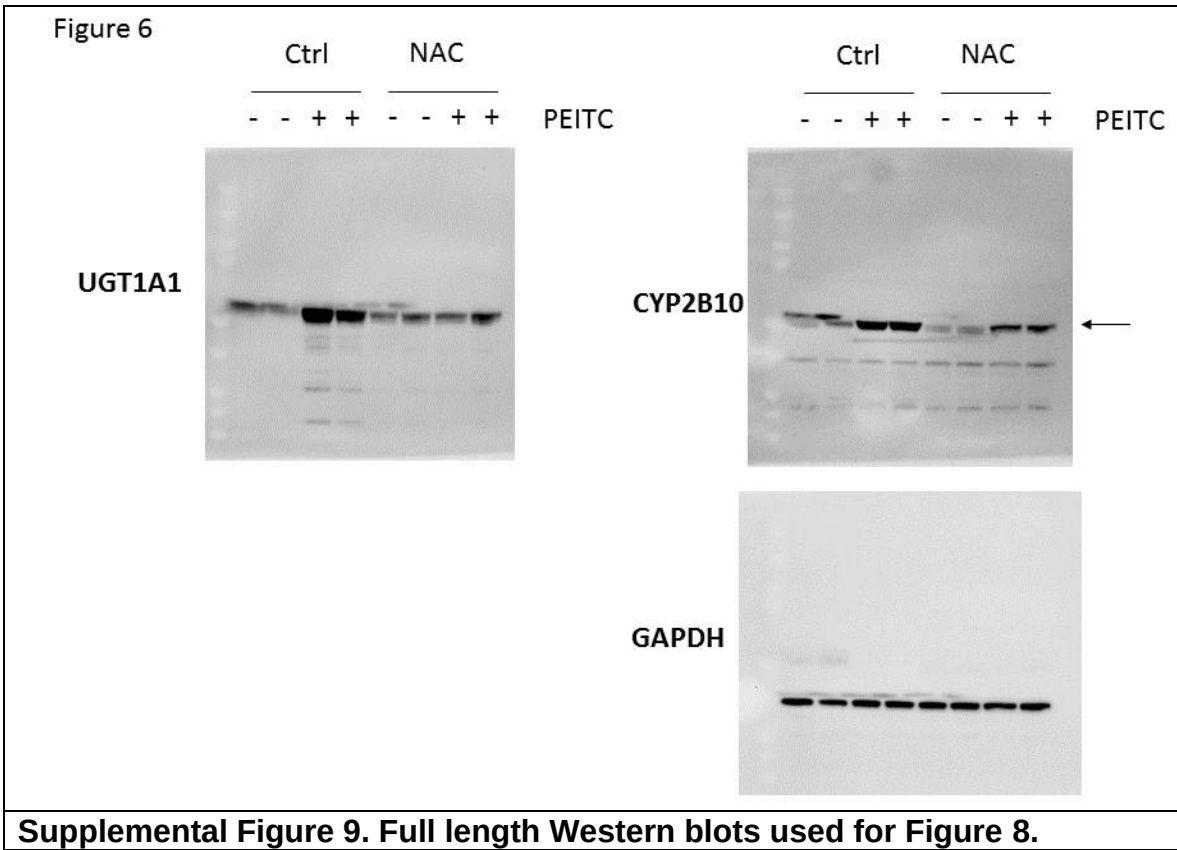
Figure 2

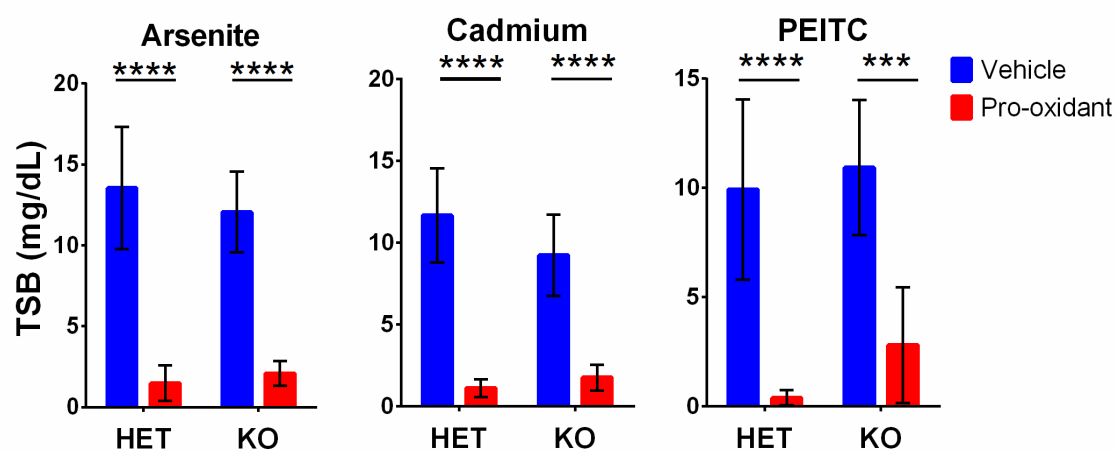


Supplemental Figure 5. Full length Western blots used for Figure 4.



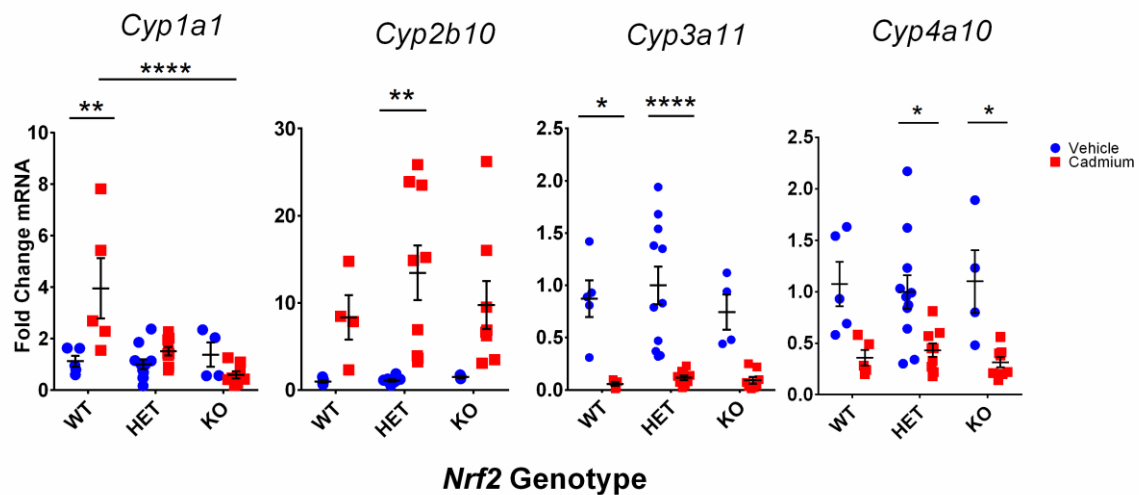




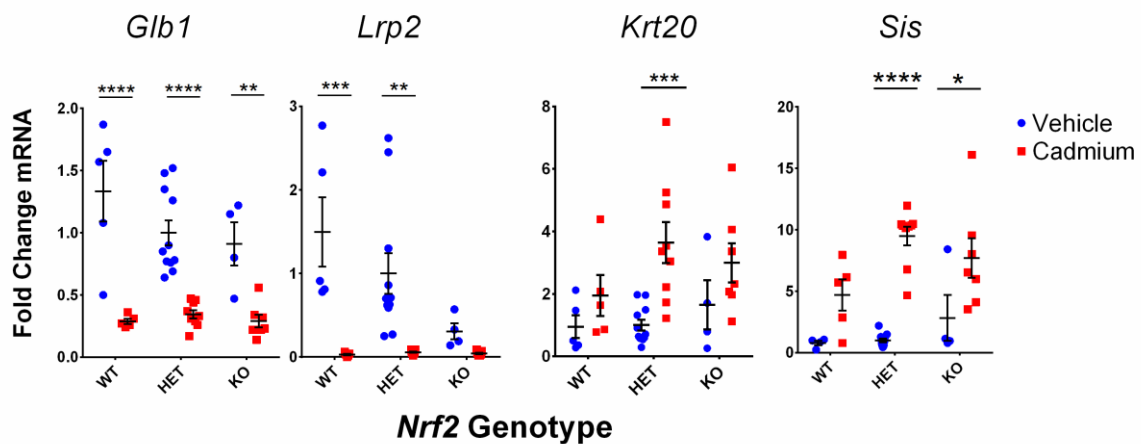


Nrf2 Genotype

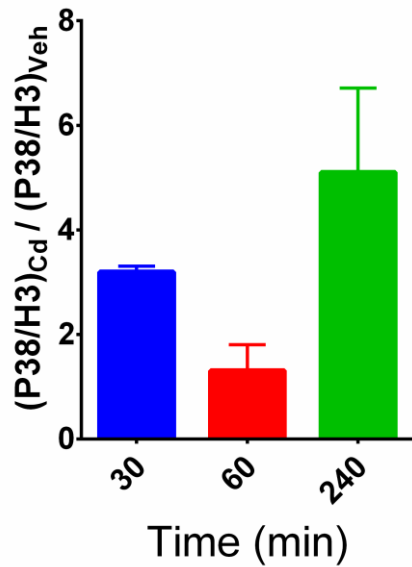
Supplemental Figure 10. Changes in TSB levels of *hUGT1/Nrf2* neonates treated with As^{3+} , Cd^{2+} , or PEITC. Ten-day old *hUGT1/Nrf2*^{+/+} and *hUGT1/Nrf2*^{-/-} neonates were treated with vehicle (water or corn oil) or As^{3+} (10 mg/kg od. po.) or Cd^{2+} or PEITC (200 mg/kg od. po.) for two days before collecting blood for TSB measurements (mg/dL). (Mean $\pm$ SEM, Two-way ANOVA; *** $P < 0.001$, **** $P < 0.0001$)



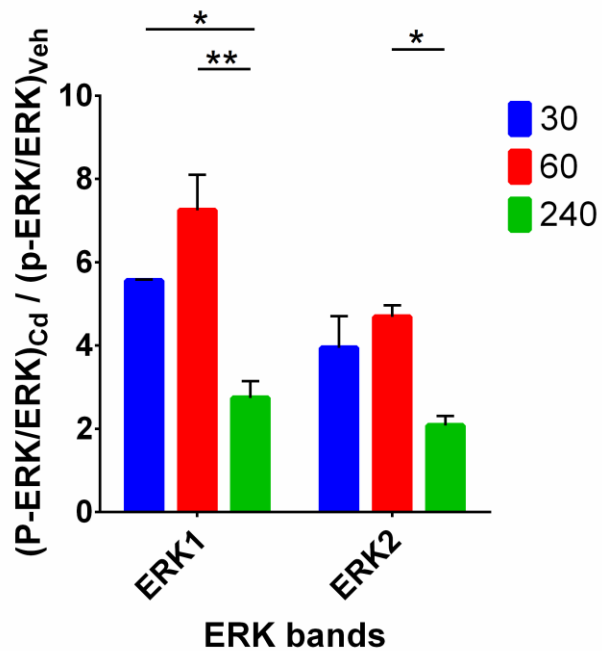
Supplemental Figure 11. Changes of intestinal *Cyp* gene expression in *hUGT1/Nrf2* neonates exposed to Cd^{2+} . Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonates were treated with vehicle (blue circles) or Cd^{2+} (10 mg/kg od. po.) (red squares) for two days before collecting intestinal tissues. Changes in the expression of *Cyp1a1*, *Cyp2b10*, *Cyp3a11*, and *Cyp4a10* were examined by RT-qPCR. (Mean +/- SEM, Two-way ANOVA; * P<0.05, ** P<0.01, **** P<0.0001)



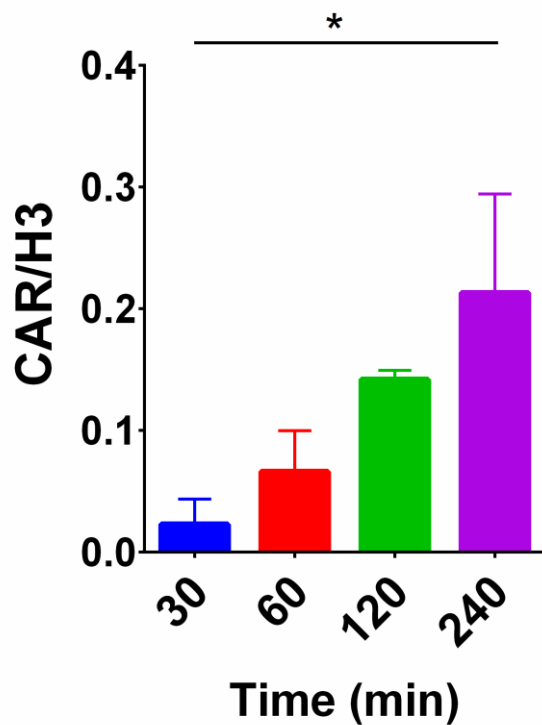
Supplemental Figure 12. Changes of intestinal maturation markers in *hUGT1/Nrf2* neonates treated with Cd^{2+} . Ten-day old *hUGT1/Nrf2*^{+/+}, *hUGT1/Nrf2*^{+/-}, and *hUGT1/Nrf2*^{-/-} neonates were treated with vehicle (blue circles) or Cd^{2+} (10 mg/kg od. po.) (red squares) for two days before collecting intestinal tissues. Exposure to Cd^{2+} altered intestinal gene expression of *Glb1*, *Lrp2*, *Krt20*, and *Sis* as examined by RT-qPCR. (Mean $\pm$ SEM, Two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)



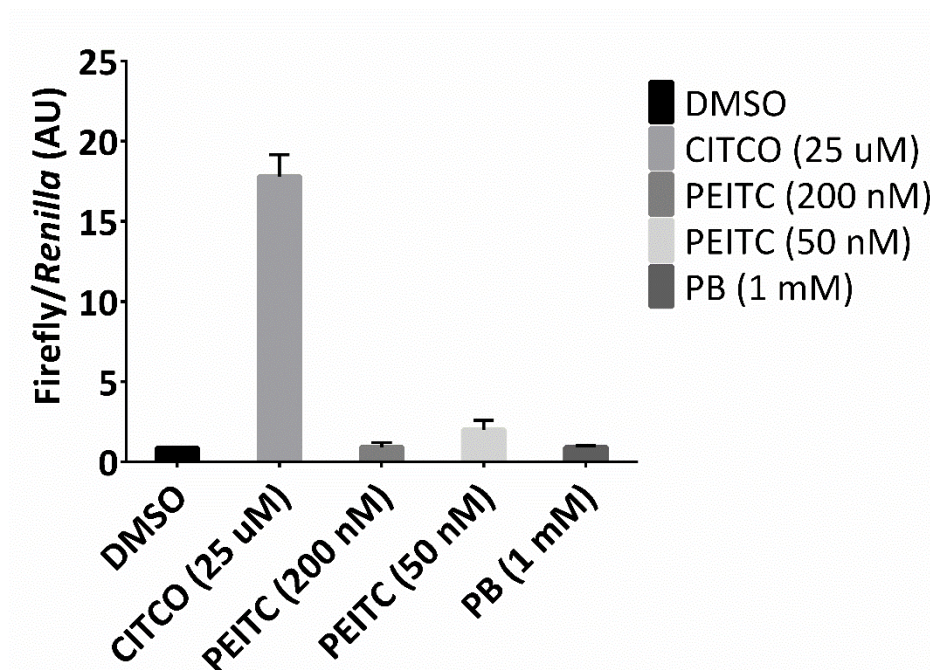
Supplemental Figure 13. Ratio of intestinal, nuclear P38 at various time points from Cd²⁺ exposed *hUGT1* neonates relative to vehicle treated littermates. Day-12 neonatal *hUGT1* mice treated with vehicle or cadmium (10mg/kg po.) and sacrificed at 30, 60, 120, and 240 minutes following exposure. Intestinal samples were collected, and nuclear and cytoplasmic protein fractions were isolated. Western blot analysis was performed on the nuclear fractions for P38, P-P38, and H3. Bands were quantified and ratios of relative nuclear P38 content (P38/H3) from cadmium exposed *hUGT1* neonates were set relative to vehicle treated littermates. (Mean +/- SEM, One-way ANOVA)



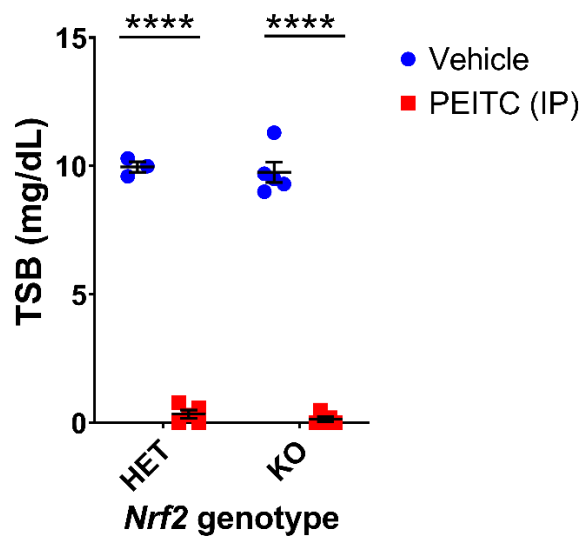
Supplemental Figure 14. Ratios of phosphorylated to unphosphorylated ERK1/2 in Cd²⁺ exposed *hUGT1* neonates set relative to vehicle treated littermates at various time points. Day-12 neonatal *hUGT1* mice treated with vehicle or cadmium (10mg/kg po.) and sacrificed at 30, 60, 120, and 240 minutes following exposure. Intestinal samples were collected and nuclear and cytoplasmic protein fractions were isolated. Western blot analysis was performed on the cytosolic fractions for ERK1/2, P-ERK1/2, and Tubulin. Bands were quantified and a comparison of the ratios of P-ERK1/2 / ERK1/2 for cadmium exposed mice relative to vehicle treated littermates was created. (Mean +/- SEM, One-way ANOVA, * P<0.05, ** P<0.01)



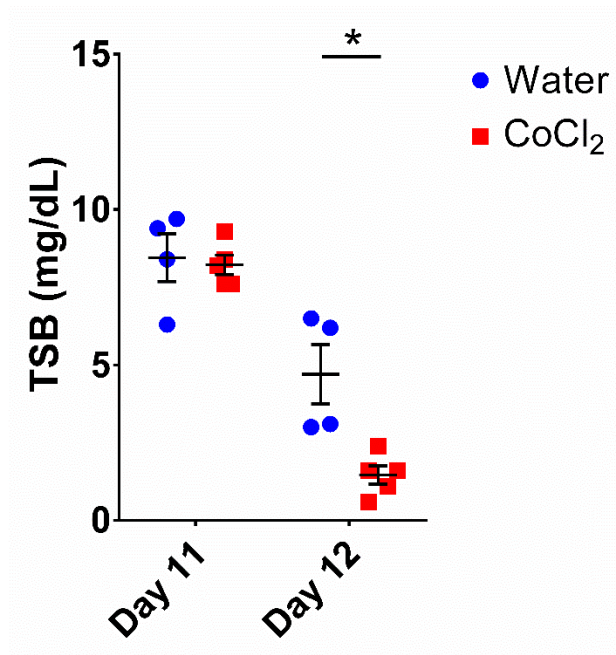
Supplemental Figure 15. Quantification of nuclear CAR localization at various time points following Cd²⁺ exposure in *hUGT1* neonates. Day-12 neonatal *hUGT1* mice treated with vehicle or cadmium (10mg/kg po.) and sacrificed at 30, 60, 120, and 240 minutes following exposure. Intestinal samples were collected, and nuclear and cytoplasmic protein fractions were isolated. Western blot analysis was performed on the nuclear fractions for CAR and H3. Bands were quantified and ratios of nuclear CAR relative to total protein (H3) were established. (Mean $\pm$ SEM, One-way ANOVA, * P<0.05)



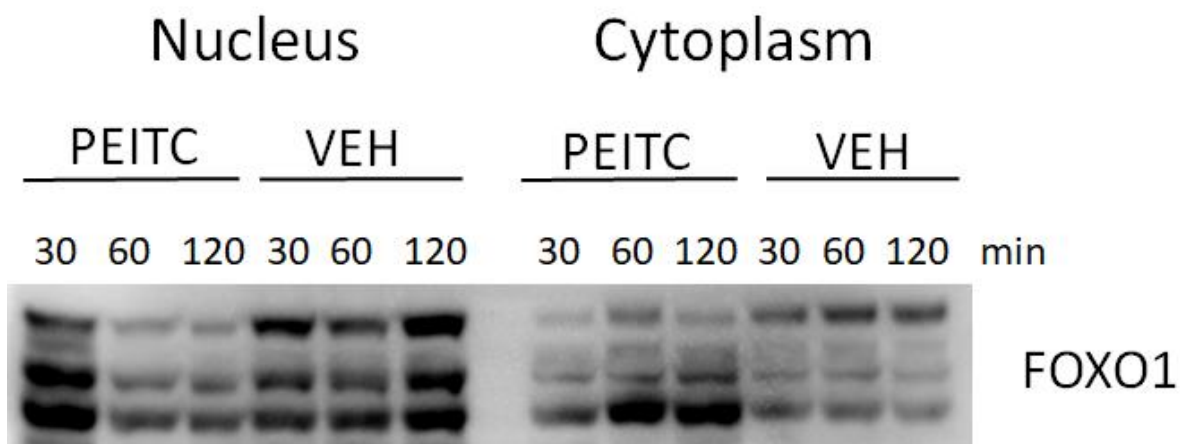
Supplemental Figure 16. PEITC Transactivation of GAL4 DBD-hCAR LBD. CV-1 cells were transfected with plasmids encoding for: (1) the GAL4 DNA binding domain fused to the hCAR ligand binding domain, (2) the firefly luciferase gene driven by the GAL4 promoter, and (3) a control *Renilla* luciferase. The assay was performed using the Dual-Glo® Luciferase Assay System by Promega. Cells were treated with vehicle DMSO, direct ligand CITCO, nontoxic concentrations of PEITC, and indirect activator phenobarbital (PB). Firefly luciferase activity was measured in accordance with the manufacturer's protocol following a 24hr incubation period with the respective compounds. Values are representative of two independent experiments with five biological replicates per group.



Supplemental Figure 17. Total serum bilirubin levels of *hUGT1/Nrf2* neonatal mice following IP administration of PEITC. Day-10 neonatal *hUGT1/Nrf2*^{+/−} and *hUGT1/Nrf2*^{−/−} mice were treated with vehicle (blue circles) or 50mg/kg PEITC (red squares) by I.P. for two days before collecting blood for TSB (mg/dL) measurements. (Mean ± SEM, Two-way ANOVA; **** P<0.0001)



Supplemental Figure 18. Total serum bilirubin levels of *hUGT1* neonatal mice following oral pretreatment with CoCl₂ and challenge with CdCl₂. Day-10 neonatal *hUGT1* mice were treated with vehicle (blue circles) or 70mg/kg CoCl₂ (red squares) by oral gavage. On day 11, blood was collected and all mice were treated orally with 10mg/kg CdCl₂. Blood was collected on day 12 and TSB values were measured (mg/dL). (Mean ± SEM, student's t-test; * P<0.05)



Supplemental Figure 19. Hepatic FOXO1 localization following oral PEITC treatment of *hUGT1* adults. Adult mice (12 weeks old) were treated orally with PEITC 200mg/kg and livers were collected at various time points. Livers were fractionated for nuclear and cytoplasmic content and a Western blot analysis was performed for FOXO1.

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