The Mechanistic Involvement of Vanin-1 in Protection against Acetaminophen Hepatotoxicity

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The Mechanistic Involvement of Vanin-1 in Protection against Acetaminophen Hepatotoxicity

Daniel W. Ferreira, Ph.D.

University of Connecticut, 2013

Pretreatment with peroxisome proliferators (PPs) is protective in rodent models of acetaminophen (APAP)-induced hepatotoxicity. Protection is associated with an induction of vascular non-inflammatory molecule 1 (Vanin-1; Vnn1) gene expression in liver. This thesis investigates whether Vnn1 expression is protective against APAP-induced toxicity and whether Vnn1 upregulation is involved in the PP-mediated mechanism of protection.

Mice lacking Vnn1 are more susceptible to APAP hepatotoxicity despite no apparent differences in APAP bioactivation or detoxification pathways. Enhanced susceptibility to APAP in Vnn1 knockout mice is associated with a deficiency in compensatory immune cell infiltration and hepatocellular repair in and around areas of hepatic centriflobular necrosis 48 hours after APAP treatment. Gene induction of cytokines involved with pro-inflammatory M1 stimulation is also deficient in the livers of Vnn1 knockout mice at the same time point.

A Vnn1 overexpression construct was stably transfected into the human hepatocyte cell line HC04. Exposure of HC04-VN1 cells to APAP
treatment resulted in modest cytoprotection compared to HC04-EV cells as determined by the leakage of lactate dehydrogenase into media. Together, the data indicate that Vnn1 expression is protective against APAP-induced toxicity both \textit{in vitro} and \textit{in vivo}.

An \textit{in vitro} model for investigating the mechanism of PP-mediated protection against APAP is also described. HC04 cells exhibit time- and dose-dependent increases in cytotoxicity following APAP exposure. Treatment with Wy-14,643 results in the induction of peroxisome proliferator activated receptor alpha (PPARα)-responsive genes acyl CoA oxidase 1 (Acox1), adipose differentiation related protein (Adrp) and Vnn1. Pretreatment with Wy-14,643 is partially protective in HC04 cells following APAP-induced cytotoxicity, though neither Vnn1 protein nor enzymatic activity are enhanced in whole cell lysates. Together, the studies suggest that enhanced Vnn1 activity is not involved with the observed partial protection by Wy-14,643 in HC04 monocultures.

Together, these investigations provide evidence that Vnn1 expression modulates APAP toxicity both \textit{in vivo} and \textit{in vitro}. Furthermore, the HC04-VN1 and PP-treated HC04 cell models described here represent two potential systems in investigating protective mechanisms against APAP-induced toxicity.
The Mechanistic Involvement of Vanin-1 in Protection against Acetaminophen Hepatotoxicity

Daniel W. Ferreira

B.S., Northeastern University, 2007

A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut

2013
Doctor of Philosophy Dissertation

The Mechanistic Involvement of Vanin-1 in Protection against Acetaminophen Hepatotoxicity

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University of Connecticut
2013
DEDICATION

To Mom and Dad, Grandma and Grandpa and Aunt Arlene:

Thank you for your unconditional love, support and faith.

I could not have done this without you.
ACKNOWLEDGEMENTS

I owe a debt of gratitude to many people who have guided me during my graduate career. Dr. José Manautou provided trust, patience and a constructive learning environment during my time in his laboratory. I am grateful for all of his guidance that has contributed to my professional development. I also owe thanks to Dr. Angela Slitt, Dr. Mike Lawton and Dr. Ted Rasmussen for serving as associate advisors. I am deeply appreciative of their scientific input as well as the moral (and material) support and kindness that they have always extended to me. I truly look up to all four of my committee members and consider myself very fortunate to have had their assistance throughout this process. In large part, this research would not have been possible without the collaboration of Dr. Philippe Naquet and his laboratory. His expertise has been central to the work, along with the knockout mice that he has made available to this cause!

I would also like to thank the past and present members of our laboratory, especially Dr. Xinsheng Gu, for their support, technical assistance and friendship. Thank you to Dr. Igor Gurevich who has always been available to bounce ideas and problems off of since my first day at UConn. Finally, on a personal note, I would also like to thank Nastassja, who has supported me in every possible way outside of the laboratory. Her influence is throughout this text, though she did not pick up a pipette. I owe her everything.
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Chapter 1

Review of Literature

1.1 Liver Physiology and Role in Pharmacology

Liver Structure and Function

The liver contains many basic structural units referred to as lobules. Each lobule consists of a central vein which is surrounded by several portal triads. The portal triad is made up of a hepatic arteriole, portal vein and bile duct. The majority of blood entering the liver is from the intestine, which enters through the portal vein and passes through the sinusoidal space between radially-aligned hepatocytes. Oxygenated blood from the heart also enters the portal triads in this way via the hepatic arterioles. In both cases, the blood filters through the hepatocytes and exits the liver by way of the central veins. At the same time, bile is synthesized within the hepatocytes and flows away from the central vein through canaliculi. These passageways are formed by the contact of apical surfaces of adjacent hepatocytes and create a canicular lumen between them. Once synthesized, bile enters this luminal space following transport across the apical membrane, filtering into the bile ducts and eventually reaching the gall bladder for storage.

The functional unit of the liver is described as the acinus and is divided into three zones. The first zone, or periportal region, represents the hepatocytes closest to the portal triad. The second zone is located mid-way between the portal triad and central vein. The hepatocytes in this zone are commonly referred to as midzonal hepatocytes. Finally, the hepatocytes
surrounding the central vein represent zone three, or the centrilobular region. These three zones vary in localization as well as functionality. In general, zone one hepatocytes reside in an oxygen and bile salt-rich environment because of their proximity to the portal triad. By contrast, zone three hepatocytes exist in an oxygen-poor environment since they are the farthest from the portal vein. Zone three hepatocytes have high expression of cytochrome P450 (CYP450) enzymes, important contributors to phase I drug metabolism, while zone one hepatocytes have high concentrations of glutathione (GSH), an important detoxification molecule. These features contribute to the zonal patterns of liver damage commonly seen following exposure to hepatotoxicants in which one zone is preferentially damaged (i.e. centrilobular necrosis following carbon tetrachloride or acetaminophen poisoning).

There are several resident cell types within the liver. The parenchymal cell is the hepatocyte, which makes up the bulk of the liver mass. These cells are polarized epithelial cells and have both a basolateral and apical surface. Whereas the apical surface is in contact with the canalicular lumen, the basolateral membrane is adjacent to the sinusoidal blood flow. Within the sinusoids are several cell types that make up the nonparenchymal cell population including Kupffer cells, stellate cells and sinusoidal endothelial cells. The Kupffer cells make up roughly 15% of all hepatic cells and are the resident macrophages of the liver. Their functions include the phagocytosis of foreign and cellular debris as well as cell signaling through cytokine release.
These cells will be discussed in more depth in the next section. Hepatic stellate cells, also known as Ito cells, make up approximately 5% of the cells within the liver. They reside in the Space of Disse between the sinusoids and hepatocytes. These cells are responsible for storing fat and also depositing collagen in response to hepatic injury. Lastly, sinusoidal endothelial cells line approximately 6-8% of the total sinusoidal length (Braet and Wisse 2002). These specialized endothelial cells have fenestrations that allow the exchange of nutrients between the sinusoid and hepatocytes. The centrilobular region has a higher density of sinusoidal endothelial cells and is therefore more porous than the periportal region (Braet and Wisse 2002).

Kupffer Cells

The Kupffer cells make up the largest population of tissue macrophages in the body. Circulating monocytes differentiate into mature Kupffer cells upon stimulation by granulocyte macrophage colony-stimulating factor (GM-CSF) as well as other growth factors. Upon release of growth factors and chemotactic proteins (for example the CC and CXC families of chemokines) from either Kupffer or hepatic stellate cells, circulating monocytes enter the liver sinusoids via extravasation from the vasculature and differentiate into their mature form. Mature Kupffer cells express scavenger receptors on their surface that assist in their phagocytic activity. Kupffer cells also assist in both lipid metabolism and the production of bilirubin (Naito, Hasegawa et al. 2004). Although these cells are found
throughout the liver lobule, they are roughly twice as abundant in zone one compared to zone three. In general, zone one Kupffer cells are typically larger in size and exhibit enhanced phagocytic and lysosomal activities (Naito, Hasegawa et al. 2004).

Kupffer cells are known for their ability to secrete numerous cytokines upon their activation. These cytokines can either stimulate other immune cells or even hepatocytes. For example, tumor necrosis factor (Tnfα) can stimulate an immune response through the initiation of macrophage and neutrophil infiltration. In hepatocytes, Tnfα signaling stimulates DNA synthesis and liver regeneration and is thought to prime the parenchymal cells to respond to growth factors (Ramadori and Armbrust 2001). Tnfα signaling can also result in activation of either pro- or anti-apoptotic signaling pathways. Tnfα directly stimulates the production of pro-inflammatory cytokines which can initiate apoptosis through the activation of caspases, or can activate nuclear factor kappa-light chain enhancer of activated B cells (NF-κB), a transcription factor involved in the synthesis of both pro- and anti-apoptotic gene products. Multiple factors such as cell type and context of activation likely influence the final outcome of NF-κB stimulation (Barkett and Gilmore 1999; Ramadori and Armbrust 2001).

**Hepatic Metabolizing Systems**

The liver is an organ with many important metabolic functions. The liver receives the majority of its blood flow from the intestine, meaning that it
receives absorbed material soon after oral consumption. The liver is exposed to high concentrations of absorbed compounds due to factors such as high blood flow, the inherent lipophilicity of drugs and pollutants and the presence of hepatic fenestrated sinusoids. The goal of hepatic metabolism is to modify ingested chemicals to decrease their inherent toxicity and increase water solubility to quicken elimination. The liver is properly equipped to accept and alter countless numbers of compounds as evidenced by the complexity of its inherent metabolizing and detoxifying systems.

The liver expresses a myriad of enzymes to metabolize various compounds. These enzymes fall into one of three categories or phases. Phase I metabolism involves enzymes that catalyze hydrolysis, reduction or oxidation reactions. Arguably, the most important family of these is the microsomal cytochrome P450 (CYP450) superfamily, which to date includes fifty seven identified enzymes in humans. These oxidize a wide variety of substrates from xenobiotics to endogenous molecules such as bile acids, steroids, fatty acids and vitamins. Other phase I enzymes include carboxylesterases and epoxide hydrolases (which catalyze hydrolysis reactions) as well as flavin-containing monooxygenases (catalysts of oxidation).

Phase II enzymes catalyze conjugation reactions which attach a large hydrophilic moiety to the compound. These reactions differ from phase I reactions, which typically introduce a small water-soluble functional group (such as –OH, COOH, etc.). Phase two enzymes include members of the
UDP-glucuronosyltransferase (microsomal), sulfotransferase (cytosolic and microsomal) and GSH transferase (cytosolic, microsomal and mitochondrial) phase II enzyme families.

Phase III metabolism is performed by transporters that regulate cellular uptake of chemicals from the blood and their efflux to either the blood or bile following phase I and/or phase II reactions. For the purposes of this thesis only three members of the ABCC transport family will be discussed: multidrug resistance associated protein (Mrp) 2, 3 and 4. Within hepatocytes, Mrp2 resides within the apical membrane and is responsible for the efflux of chemicals and metabolites into bile. Substrates for Mrp2 include glucuronide and GSH conjugates. Mrps 3 and 4, on the other hand, are basolateral efflux transporters that release metabolites back into the blood. Transporters commonly exhibit broad and overlapping substrate specificities.

Typically the result of a xenobiotic undergoing phase I, II or III metabolism is a reduction in both toxicity and lipophilicity. However, metabolism can also result in an enhancement of toxicity in certain cases. For example, phase I reactions can increase the toxicity of xenobiotics through the formation of reactive metabolites. Some of these metabolites readily bind covalently with cellular targets (namely proteins, DNA and lipids) and can result in dysfunction of macromolecules and oxidative stress. Common examples of xenobiotic activation by CYP450 enzymes include acetaminophen (APAP), carbon tetrachloride, benzene and benzo[a]pyrene.
1.2 Hepatic Metabolism and Toxicity of Acetaminophen

Metabolism of Acetaminophen

APAP undergoes all three phases of hepatic metabolism as depicted in Figure 1.1. Following a therapeutic dose, it enters the hepatocytes by passive transport. More than 90% of the parent compound undergoes phase II metabolism by glucuronidation and sulfation (Jollow, Thorgeirsson et al. 1974) and is effluxed to the blood and bile. A small percentage of APAP is metabolized by several members of the CYP450 superfamily including Cyp2E1, 1A2 and 3A4. This reaction results in the formation of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) which is typically detoxified by GSH conjugation and is effluxed from the hepatocyte. Although APAP conjugates are relatively inactive, the GSH conjugate can undergo cleavage to form a cysteine conjugate (APAP-CYS) and subsequent acetylation to the mercapturic acid conjugate (APAP-NAC).

In terms of phase III metabolism, Mrp2 is able to efflux the sulfate (APAP-SULF), glucuronide (APAP-GLUC) and GSH (APAP-GSH) conjugates into bile. Mrp3 is the primary transporter involved with efflux of APAP-GLUC, for which it has a higher affinity compared to Mrp2 (Manautou, de Waart et al. 2005). Both Mrp3 and 4 are able to efflux APAP-SULF and Mrp4 upregulation has been hypothesized to increase resistance to APAP-induced hepatotoxicity (Aleksunes, Campion et al. 2008).

Although APAP is safe at recommended doses even after long term treatment (Temple, Benson et al. 2006), overdose is associated with almost
Figure 1.1
Figure 1.1. Hepatic Metabolism of Acetaminophen

APAP is metabolized by several families of enzymes located in both the cytoplasm and ER of hepatocytes. Upon conjugation, APAP metabolites are effluxed into either blood (major metabolites: APAP-GLUC, -CYS, -SULF) by Mrp3 and 4, or bile (major metabolites: APAP-GSH) by Mrp2. Small amounts of APAP-NAC are found in both compartments. Less than 5% of total APAP is recovered unchanged, most of which is effluxed basolaterally.
50% of all acute liver failure cases in the United States (Lee 2010). This percentage accounts for both accidental and intentional overdose cases. Upon a supratherapeutic dose of APAP, the glucuronidation and sulfation pathways become saturated, permitting more parent compound to be bioactivated to NAPQI by CYP450s. This in turn causes depletion of intracellular GSH stores and allows free NAPQI to bind to cellular macromolecules (Mitchell, Jollow et al. 1973). These events are associated with cell death of hepatocytes in zone three, resulting in centrlobular necrosis which can progress to acute liver failure.

_Treatment of Acetaminophen Overdose_

Current strategies to limit APAP hepatotoxicity involve enhancing GSH synthesis, although GSH itself is not a sufficient protector against APAP toxicity because of poor cellular uptake. The current antidotal treatment for APAP poisoning involves the administration of N-acetyl cysteine (NAC), a cysteine analog. Cysteine provides the reactive sulfhydryl group responsible for the proton donation of the GSH tripeptide. NAC increases the availability of intracellular cysteine and in turn enhances the rate of formation of the gamma-glutamylcysteine dipeptide from available glutamate and cysteine amino acids. The formation of this dipeptide is considered the rate limiting step of GSH synthesis and is catalyzed by gamma-glutamylcysteine synthetase (γGCS). GSH synthesis is then completed by addition of glycine to
the dipeptide by GSH synthetase. Thus, NAC is protective against APAP through the supplementation of cellular GSH.

There are limitations to successful antidotal NAC treatment, however. NAC is most effective when administered 8-12 hours following APAP ingestion, although symptoms frequently do not occur until later (Larson, Polson et al. 2005; Kanter 2006). This is a significant limitation in patients who overdose unintentionally and do not report to a doctor until they feel discomfort. Adverse reactions to NAC are not uncommon (though rarely life threatening) and is also difficult to administer orally to children due to its unpleasant odor. For these reasons, this thesis will discuss the Vanin-1 gene in the interest of developing additional effective treatments against the toxicity of APAP-induced hepatotoxicity.

1.3 Contributions of the Immune System in Modulating Acetaminophen Hepatotoxicity

Influence of Immune Cells

Upon initiation of hepatocellular damage the immune cells remove cellular debris by phagocytosis and stimulate hepatocyte regeneration by the release of cytokines and growth factors. Following the initiation of APAP injury by covalent binding of NAPQI and the production of oxidative stress, the contents of necrotic hepatocytes are released into the extracellular matrix. Adjacent macrophages become activated and phagocytose the debris. The activated cells produce cytokines and chemokines that recruit and activate
other immune cell types involved in innate immunity, including neutrophils and circulating monocytes.

The overall impact of immune cell activation following APAP intoxication remains controversial. Studies using gadolinium chloride (GdCl$_3$) to deplete macrophages have shown protection against toxic APAP exposure. Originally it was thought that GdCl$_3$ was protective by depleting the liver of Kupffer cells, which were believed to exhibit a pro-inflammatory phenotype and elicit tissue destructive effects. Protection from GdCl$_3$ treatment was found to be associated with reduced nitric oxide (NO) and peroxynitrite formation in the liver following APAP treatment. NO is produced by macrophages following activation of inducible nitric oxide synthase (iNos) by pro-inflammatory cytokines such as Tnf$\alpha$ and interferon gamma (Ifn$\gamma$), as well as lipopolysaccharide (LPS), a bacterial cell wall product (Figure 1.2.). It should be noted that hepatocytes and activated neutrophils can also express iNos and produce NO following stimulation by pro-inflammatory mediators, although it is unknown to what degree these cells contribute to the total amount of NO produced in the liver upon APAP intoxication.

Once produced, NO is able to react with superoxide (likely from multiple sources, also produced by macrophages expressing NADPH oxidase) to form peroxynitrite. While NO, superoxide and peroxynitrite are produced primarily as a defensive mechanism against bacterial infection, these reactive molecules can also produce oxidative stress within the surrounding parenchyma. In the case of peroxynitrite, it is able to covalently
Figure 1.2. Regulation of iNos Expression and Nitrotyrosine Formation as a result of Pro-Inflammatory Signaling.

Ifγ regulates the expression of iNos through the activation of the Jak/STAT pathway. STAT1α is phosphorylated as a result of Jak stimulation and forms a homodimer. Transcription of Irf1 is increased by binding of p-STAT1α and in turn initiates transcription of iNos mRNA. Binding to TnfR and TLR4 by Tnfa and LPS, respectively, results in the release of NF-κB from IκB upon its phosphorylation by Iκk. NF-κB enters the nucleus and binds to the iNos promoter. Interaction of Irf1 and NF-κB results in a synergistic effect on iNos transcription. Translation of iNos and homodimer formation are required for the oxidation of L-arginine to citrulline and the release of NO. Reaction of NO with O$_2^\cdot$ causes the production of peroxynitrite, a reactive nitrogen species that preferentially binds exposed Tyr residues of proteins.
bind tyrosine residues and result in the formation of nitrotyrosine adducts within cellular proteins. iNos is further implicated in APAP-induced injury by the use of iNos-deficient mice, which are resistant to APAP hepatotoxicity and exhibit a reduction in hepatic nitrotyrosine staining compared to wild-type mice (Gardner, Laskin et al. 2002). Mice treated with either the Nos inhibitor NG-Nitro-L-arginine methyl ester (L-NAME) or NO scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) eight hours after APAP challenge also results in significant protection against increases in ALT and aspartate aminotransferase (AST) (Ishida, Kondo et al. 2002).

Other studies suggest that Kupffer cells are protective against APAP hepatotoxicity. Treatment of mice with clodronate liposomes results in the depletion of resident Kupffer cells (as determined immunohistochemically by a reduction in F4/80 positive cells). Subsequent treatment of these mice with APAP results in pronounced enhancement of hepatotoxicity despite no observed differences in hepatic GSH content or the livers’ ability to bioactivate the parent compound (Campion, Johnson et al. 2008). These data are inconsistent with the findings of studies using GdCl₃ and suggest that the presence of F4/80 positive macrophages is important in limiting APAP injury.

In recent years, a hypothesis has been formed in an attempt to explain the seemingly contradictory effects of Kupffer cell depletion models of APAP intoxication. Kupffer cells, although they are all resident macrophages, are not a homogeneous population of cells (Kono, Fujii et al. 2002). That is, macrophages, including mature Kupffer cells, exist as subpopulations that
range from pro-inflammatory to anti-inflammatory, depending on their environment and stimuli. Upon activation by Tnfα, IL-1β, LPS or Ifnγ, macrophages polarize towards an M1 pro-inflammatory or classically activated phenotype. These cells are highly phagocytic, express high levels of iNos and produce large amounts of NO. By contrast, activation by IL-4 or IL-10 causes macrophages to polarize towards an M2 anti-inflammatory, or alternatively activated phenotype. These cells primarily promote angiogenesis and tissue remodeling.

Both the GdCl3 and clodonate treatment experiments assume that these chemicals deplete all macrophages in the liver. More likely, each agent depletes a specific subset of Kupffer cells or alters their phenotype as a result of phagocytosis. Support for this hypothesis comes from the finding that GdCl3 treatment does not deplete rats of hepatic CD68-positive (a pan-macrophage marker) macrophages although CD163-positive (a marker of anti-inflammatory macrophages) macrophages were depleted by over 95% (Lee, Yeoh et al. 2004; Eligini, Crisci et al. 2012). Furthermore, macrophages are known to shed CD163 under certain circumstances (Tippett, Cheng et al. 2011; Moreno, Ortega-Gomez et al. 2012) and typically gain or lose expression of specific surface receptors depending on their phenotype. Consequently, GdCl3 may be targeted and phagocytosed by only a specific subpopulation of macrophages, or it may cause a shift in macrophage polarization following its uptake. Based on these observations it is likely that macrophage polarization affects the extent of injury following APAP overdose.
Although one might expect that an M1 phenotype might promote and enhance APAP-induced inflammation, this does not appear to be the case. Mice that lack Tnfr1, a receptor for Tnfα, are more susceptible to APAP-induced liver injury (Gardner, Laskin et al. 2003), suggesting that although Tnfα is generally referred to as “pro-inflammatory” and partially regulates iNos expression, its signaling may be important in limiting hepatotoxicity by APAP in mice. Tnfα signaling is involved with multiple cellular pathways, including activation steps for transcription factors NF-κB and AP-1, as well as Caspase 3, a pro-apoptotic protein. In this way, Tnfα is able to influence inflammation, as well as survival and apoptotic signaling pathways. In hepatocytes, the release of Tnfα by activated macrophages and subsequent hepatocellular NF-κB activation can lead to either DNA synthesis and repair or apoptosis upon binding to Tnfr1. The final outcome of NF-κB activation is likely influenced on additional cell signaling pathways (Barkett and Gilmore 1999; Ramadori and Armbrust 2001).

Influence of Cytokines

Although immune cells are the effectors of the immune response, the exact response is dictated by cytokine release. Cytokines influence not only the exact response of immune cells (for example M1 versus M2 polarization), but also their accumulation to sites of injury by the release of chemokines. Because of their functions as cellular sensors, individual cytokines and chemokines have been investigated to determine their influence during APAP
hepatotoxicity. To this end, knockout mouse models have been developed for many of these proteins and treated with APAP to determine how alterations in cytokine signaling affect susceptibility to APAP injury.

Hepatocytes and macrophages alike express monocyte chemoattractant protein (Mcp-1), which is released upon acute injury to stimulate the migration of monocytes and macrophages to damaged areas of the liver. Its receptor, C-C chemokine receptor 2 (Ccr2), is expressed by leukocytes of the monocyte/macrophage lineage but not by hepatocytes. Ccr2 knockout mice have been used to determine what effect monocyte/macrophage recruitment has on the extent of APAP injury. Both Ccr2 knockout and wild-type mice exhibit similar upregulation of hepatic Mcp-1 mRNA following APAP administration, although recruitment of pro-inflammatory macrosidealin-positive macrophages is limited in Ccr2 knockouts (Dambach, Watson et al. 2002). Interestingly, there is no enhancement of APAP toxicity in the null mice, suggesting that accumulation of the pro-inflammatory Ccr2-dependent macrophage subpopulation itself does not alter the development or extent of injury.

Neutrophils are also recruited during APAP injury by members of the CXC chemokine ligands. In particular, macrophage inflammatory protein 2 (Cxcl2) is a potent chemoattractant for neutrophils (as well as monocytes). Experiments performed in Cxcl2 knockout mice show attenuation of APAP hepatotoxicity associated with a decrease in both neutrophil and macrophage accumulation in liver (Ishida, Kondo et al. 2006). These findings correlated
with reductions in iNos mRNA expression and nitrotyrosine staining compared to wild-type mice treated with APAP.

The involvement of neutrophils in the progression of APAP injury is controversial. In the same experiments described above, the authors induced neutropenia in wild-type mice by neutralizing antibody treatment. These mice have reduced neutrophil, but not macrophage, accumulation in liver (in contrast to the Cxcl2 knockouts) following toxic APAP treatment and show greater protection than Cxcl2 knockout mice. Although the data might suggest that protection was due to neutrophil rather than macrophage depletion, the data should be interpreted with caution. Neutralizing antibodies used to deplete neutrophils have been shown to upregulate metallothionein and other genes that are protective against APAP (Jaeschke and Liu 2007). Mice that are treated with this antibody after APAP treatment but prior to neutrophil accumulation are not protected, suggesting that the protective effect seen in these mice is unrelated to neutrophilic accumulation and activation (Cover, Liu et al. 2006).

Aside from chemokines, individual pro- and anti-inflammatory cytokines are also able to modulate susceptibility to APAP hepatotoxicity in vivo. Ifnγ, a pro-inflammatory cytokine, is elevated upon APAP treatment. Ifnγ is a potent activator of macrophages secreted by activated cytotoxic lymphocytes as well as macrophages. Although hepatocytes can also express Ifnγ, they are not significant contributors to its production (Horras, Lamb et al. 2011). Ifnγ knockout mice are resistant to APAP hepatotoxicity as
well as hepatic mRNA induction of several inflammatory mediators and chemokines Mcp-1, Cxcl2 and Cxcl1 (Ishida, Kondo et al. 2002). In these studies, protection correlated with decreases in hepatic neutrophils, macrophages and T cells both 10 and 24 hours after APAP. ALT reductions in Ifnγ knockout mice occurred during the onset (6-24 hours) but not recovery phase (48 hours) of injury. Additionally, Ifnγ-neutralizing antibody treatment in wild-type mice results in a reduction of plasma ALT when administered two, but not eight hours following APAP treatment. Together, the data suggest that Ifnγ signaling contributes to the severity of APAP-induced hepatotoxicity by affecting the initiation and/or progression phases of injury.

A lack of Ifnγ signaling during APAP toxicity could be protective by multiple mechanisms. As shown by Ishida et al., the recruitment of multiple cell types involved in the innate immune system is deficient in the absence of Ifnγ. It stands to reason that Ifnγ may be involved in the recruitment or polarization of cells detrimental to the resolution of injury since it is involved in programming immune cells towards a pro-inflammatory M1 phenotype.

Another possible explanation is through Ifnγ-dependent regulation of iNos (Figure 1.2). As mentioned previously in this chapter, iNos has been implicated as a possible contributor to APAP injury in several models because of its pro-oxidative role. Binding of Ifnγ to its receptors (IfnR1 and 2) results in the phosphorylation of STAT1α. Phospho-STAT1α stimulates the production of the transcription factor interferon response factor-1 (Irf1), which in turn promotes the transcription of iNos. Interaction of NF-κB (which regulates iNos
transcription following activation by Tnfα) and Irf1 synergistically enhances iNos transcription (Lorenzi, Forloni et al. 2012). Therefore, the protective effect observed in Ifnγ knockout mice may be the result of the prevention of iNos synergistic upregulation mediated by Irf1-Nf-κB interaction.

Several anti-inflammatory cytokines have also been investigated for their role in APAP-induced liver injury. IL-10 is recognized as one of the primary cytokines involved in M2 polarization. Mice lacking IL-10 are more susceptible to APAP hepatotoxicity and induction of pro-inflammatory mRNAs for IL-1α and Tnfα in liver despite no differences in APAP covalent binding (Bourdi, Masubuchi et al. 2002). Induction of hepatic iNos mRNA and elevated serum concentrations of nitrite and nitrates are seen in IL-10 knockouts and correlate with the extent of injury. APAP injury is not exacerbated in IL-10/iNos double knockouts, indicating that the enhanced susceptibility of IL-10-null mice may be related to a shift towards a pro-inflammatory phenotype following APAP treatment (Bourdi, Masubuchi et al. 2002). Additional in vitro studies have suggested that IL-10 prevents NO production by inhibiting the intracellular transport of arginine by the CAT-2 transporter and/or by regulating iNos synthesis in macrophages at the transcriptional (Cunha, Moncada et al. 1992; Becherel, Le Goff et al. 1995) and/or post-translational level (Huang, Stevens et al. 2002).

IL-4 is another cytokine involved in stimulating cells towards an M2 phenotype. IL-4 knockout mice are also more susceptible to APAP toxicity, although this effect is most likely due to a reduction in GSH synthesis as a
result of decreased expression of gamma-glutamylcysteine ligase (γGCL) at the transcriptional level (Ryan, Bourdi et al. 2012). Both endpoints are rescued in knockout mice by treatment with recombinant IL-4. Double knockout of IL-4 and IL-10 in mice also results in a reduction of GSH, although the IL-4/10/6 triple knockout has no such phenotype (Bourdi, Eiras et al. 2007).

In summary, the data indicate that pro- and anti-inflammatory cytokines, as well as chemokines, are able to modulate APAP toxicity not only by priming immune cells but also by influencing the defense capabilities of hepatocytes against oxidative stress. As of yet there are no clear-cut conclusions as to which immune cells or cytokines are protective in response to APAP toxicity because of the multiple cell types and mechanistic pathways affected by each cytokine. Overall, the data support a pathogenic role of iNos and the production of peroxynitrite in response to APAP injury and suggest that pro- and anti-inflammatory programming of immune cells has the ability to affect the extent of damage and repair.

1.4 Peroxisome Proliferators and Protection from Acetaminophen Hepatotoxicity

*Biological Significance of Peroxisome Proliferators*

Peroxisome proliferators represent a hypolipidemic class of compounds that activate the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). PPARα controls the regulation of genes
involved in β-oxidation of fatty acids as well as cellular and peroxisomal proliferation (Holden and Tugwood 1999). Peroxisome proliferators are diverse in chemical structure and origin. Endogenous fatty acids, industrial chemicals (plasticizers) and the fibrate class of drugs are all activators of PPARα. Upon PPARα activation, a heterodimer is formed with the retinoid x receptor (RXR), which then binds to peroxisome proliferator response elements (PPREs) within the promoter regions of target genes.

Upregulation of the fatty acid β-oxidation occurs in both mitochondria and peroxisomes in response to PPARα activation. The majority of short and medium chain fatty acids derived from the diet are oxidized by mitochondria. Long and very long chain fatty acids that cannot enter the mitochondrial matrix are instead shortened by peroxisomal β-oxidation, though they can also be metabolized by CYP4A enzymes. CYP4A-mediated ω-oxidation results in the formation of dicarboxylic acids that serve as substrates for β-oxidation.

Prior to undergoing the four catalytic steps of β-oxidation, fatty acids are targeted for degradation by coenzyme A (CoA), forming a fatty acyl-CoA thioester upon binding by the reactive thiol group of CoA. In the first step of β-oxidation, the acyl-CoA chain undergoes an initial oxidation step catalyzed by acyl-CoA dehydrogenase and in-so-doing reduces FAD. This process also produces hydrogen peroxide through redox cycling of reduced FADH₂ with molecular oxygen. In the second step of β-oxidation, hydroacetyl-CoA is formed through the addition of water by enoyl-CoA hydratase. Oxidation of
the hydroxyl group at the β position is catalyzed by L-Hydroxyacyl-CoA dehydrogenase and NAD+, forming β-ketoacyl-CoA and NADH. Finally, thiolase cleaves β-ketoacyl-CoA to form acetyl-CoA and a shortened acyl(n-2)-CoA. The shortened acyl-CoA will either re-enter the cycle or migrate to the mitochondria for further oxidation. Generated acetyl-CoA is further metabolized in the tricarboxylic (TCA) cycle or is used as a substrate in amino acid biosynthesis.

Species Differences in PPARα Activation

Although peroxisome proliferators activate PPARα in both rodents and humans there are differences in their downstream effects. For example, in mice and rats peroxisome proliferator treatment results in the enhancement of the size and number of peroxisomes in hepatocytes—a process that does not occur in humans. Hepatic hyperplasia, hypertrophy, enhanced DNA synthesis, hepatomegaly and hepatocellular carcinomas are common in rodents following short and long-term treatment with peroxisome proliferators, although there is no such evidence of hepatocarcinogenicity in man (Frick, Elo et al. 1987; Holden and Tugwood 1999). Other non-rodent mammals such as guinea pigs, dogs and non-human primates are also resistant to hepatocellular changes associated with carcinogenicity.

Studies in PPARα knockout mice indicate that hypertrophy and hyperplasia resulting from peroxisome proliferator treatment are dependent on the expression of PPARα (Lee, Pineau et al. 1995; Gonzalez 1997).
Although mice and rats are more sensitive to PPARα activation, humans do elicit some peroxisome proliferator-mediated effects. Most notably, long-term treatment with fibrates in humans has proven to be clinically effective in lowering serum triglyceride and cholesterol levels as well as reducing the incidence of coronary heart disease (Frick, Elo et al. 1987).

The data indicate that human liver elicits the hypolipidemic but not the proliferative effects of peroxisome proliferators—a concept that has been thoroughly researched. *In vitro* studies echoed animal data by finding that the expression of PPARα target genes are much more inducible in primary rat hepatocytes compared to human (Ammerschlaeger, Beigel et al. 2004). PPARα content is roughly ten times lower in human compared to rodent and was thought to be a contributing factor to the lack of responsiveness. This hypothesis was disproven by transfection studies performed in human HepG2 cells that were transfected with either human or rat PPARα overexpression constructs. Neither construct was able to enhance PPARα activity, suggesting that PPARα activity was being regulated by additional mechanisms (Ammerschlaeger, Beigel et al. 2004).

The identification of a human transcript variant of PPARα that lacks exon 6 was identified (Gervois, Torra et al. 1999). The deletion leads to the introduction of a premature stop codon and results in the formation of truncated protein. Although initially it was thought that this could contribute to a lack of PPARα activity in humans, further study indicated that truncated PPARα exists in similar proportion in rats (Hanselman, Vartanian et al. 2001).
It now appears that differences in PPAR co-regulators or miRNAs could contribute to the differential response between species. Studies using mice with humanized livers exhibit PPARα responsiveness similar to humans. That is, lipid metabolism pathways are upregulated in response to peroxisome proliferator treatment, though proliferative pathways are not (Yang, Nagano et al. 2008). Several genes are dysregulated in the humanized mice including the gene encoding for the oncogenic let-7c miRNA. Let-7c has since been shown to destabilize mRNA for c-myc, a transcription factor involved in proliferation. Whereas wild-type mice respond to peroxisome proliferator treatment by downregulating let-7c, humanized mice retain let-7c regulatory control of c-myc mRNA (Gonzalez and Shah 2008). This mechanism is currently thought to contribute to the rodent-specific hepatocarcinogenic properties of peroxisome proliferators.

_Peroxisome Proliferator-Mediated Protection from Acetaminophen_

The protective effects of peroxisome proliferators against hepatotoxicants have been known for several decades. Rats were first treated with various hypolipidemic compounds to examine their effectiveness in reducing the toxicity of cerium chloride, the salt of a rare earth metal that increases hepatic triglycerides and leads to hepatic steatosis and necrosis. It was found that pretreatment with nafenopin, a peroxisome proliferator, yielded the greatest protection against hepatotoxicity and elevations in liver triglycerides (Tuchweber and Salas 1978). Years later the peroxisome
proliferator Di(2-ethylhexyl)phthalate (DEHP) was found to reduce weight loss and hepatic steatosis associated with tetrachlorodibenzo-p-dioxin (TCDD) treatment in rats (Tomaszewski, Montgomery et al. 1988). Subsequently, peroxisome proliferators have been shown to protect against other model hepatotoxicants such as carbon tetrachloride, chloroform, bromobenzene and APAP (Nicholls-Grzemski, Calder et al. 1992; Manautou, Silva et al. 1998).

During APAP-induced hepatotoxicity NAPQI preferentially binds to specific cellular proteins in both mouse and human (Bartolone, Beierschmitt et al. 1989; Birge, Bartolone et al. 1990). Partial sequencing of one particular hepatic 58kD APAP-binding protein (ABP) suggested that it was a selenoprotein (Bartolone, Birge et al. 1992). Around the same time, DEHP had been shown to stimulate the incorporation of selenium into a 58kD hepatocellular protein (Garberg and Hogberg 1991).

To determine whether peroxisome proliferators could modulate APAP toxicity by increasing the expression of the 58kD protein, mice were pretreated with the peroxisome proliferator clofibrate (CFB) for ten days prior to APAP intoxication. Surprisingly, the covalent binding of several major ABPs was decreased in both cytosolic and microsomal hepatic fractions in pretreated animals (Manautou, Hoivik et al. 1994). Reduced covalent binding was associated with the prevention of APAP-mediated increases in plasma sorbitol dehydrogenase activity and hepatic centrilobular necrosis. Depletion of hepatic non-protein sulfhydryls (NPSH) was also prevented by CFB.
treatment, suggesting that protection could be a result of reduced APAP bioactivation.

Follow-up experiments determined that mice were protected from APAP toxicity following a single dose of CFB (Manautou, Emeigh Hart et al. 1996). Protection occurred despite no alterations in hepatic covalent binding or NPSH content, indicating that the protective mechanism is independent of APAP bioactivation or detoxification. Additionally, PPARα activation is a requirement for protection, as PPARα knockout mice are not protected by CFB (Chen, Hennig et al. 2000).

In liver, PPARα is activated by endogenous fatty acids as well as peroxisome proliferators and is involved in lipid metabolism and energy production pathways by inducing the β-oxidation of fatty acids. PPARα activation also results in upregulation of the antioxidant genes catalase and NAD(P)H:quinone oxidoreductase 1 (Nqo1) since lipid catabolism is a significant source of intracellular reactive oxygen species (ROS). Although these genes are upregulated following CFB treatment, they do not contribute to the mechanism of protection (Chen, Hennig et al. 2002; Moffit, Aleksunes et al. 2007). A gene array analysis identified a positive correlation between Vanin-1 expression and protection against APAP in CFB treated mice. This correlation is strengthened by the observation that the basal expression and induction of Vanin-1 by CFB are dependent on the presence of PPARα (Moffit, Koza-Taylor et al. 2007). Further investigation of the protective effects
of Vanin-1 in APAP-mediated toxicity will be discussed in the following chapters.

1.5 Biochemistry of Vanin-1, Pantetheinase and Cysteamine

Introduction

Cysteamine is a molecule that has been studied for decades due to its cytoprotective properties. It is an effective protectant in models of neurodegeneration, lipid peroxidation, radiation, inflammation and oxidative stress. In comparison, relatively little is known about pantetheinase (EC 3.5.1.92), the ectoenzyme responsible for endogenous cysteamine production through the hydrolysis of pantetheine. Even less is known about Vascular non-inflammatory molecule 1 (Vanin 1; Vnn1), the gene that encodes for pantetheinase.

Pantetheinase catalyzes an important reaction during CoA synthesis, hydrolyzing pantetheine to pantothenic acid, a CoA precursor. CoA is a critical part of multiple pathways involved in cellular respiration through the formation of acetyl CoA. During the β-oxidation pathway of lipid metabolism, acyl CoA synthetases attach the thiol group of CoA to fatty acids of various lengths to form acyl CoA chains. These multi-carbon chains are further processed to acetyl CoA for use in either cholesterol synthesis or NADH production via the tricarboxylic (TCA) cycle. Alternatively, acetyl CoA can also be formed from CoA and pyruvate in a reaction catalyzed by pyruvate dehydrogenase. In either case, acetyl CoA is required for initiation of the TCA
cycle, which produces NADH for the electron transport chain and drives mitochondrial ATP production.

Vanin-1 is believed to be the primary transcript responsible for pantetheinase activity, although one and two other isoforms of the gene have been identified in mouse (Vnn2) and human (Vnn2 and 3), respectively. Vnn1 is inducible by PPAR activators and inducers of oxidative stress. Vnn1-mediated catalysis is also believed to be the primary source of the endogenous antioxidant cysteamine, since Vnn1 knockout mice do not have detectable levels of the thiol. Thus, Vnn1 and pantetheinase likely have important roles in regulating both metabolism and oxidative stress. The known and hypothesized hepatic functions of Vnn1, pantetheinase and cysteamine will be discussed in the remainder of this chapter.

**GPI Biosynthesis and Membrane Targeting**

Pantetheinase is an ectoenzyme that is attached to the outer plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. In general, GPI anchors consist of three domains: a phosphoethanolamine linker that attaches to the C terminal end of the target protein, a conserved glycan core and a phospholipid tail for anchoring to the lipid membrane (Paulick and Bertozzi 2008). Successful attachment of GPI following its synthesis in the ER requires two signal sequences within the protein. The first is an N terminal signal peptide (amino acids 1-21 of human pantetheinase) that retains the target protein within the ER lumen. The second necessary sequence is a C-
terminal 22 amino acid propeptide downstream of the Gly491 residue, termed the ω site, which is removed and replaced by the GPI anchor. This process is mediated by a GPI-transamidase complex composed mainly of members of the phosphatidylinositol glycan (PIG) family, including PIG-K, the catalytic subunit (Maeda and Kinoshita 2011). Once attached, GPI-anchored proteins (GPI-APs) exit the ER and are transported to the cell surface by way of the Golgi. There, GPI-APs are inserted within the outer leaflet of the plasma membrane by the phospholipid tail portion of the GPI-linker.

In general, GPI-APs are recruited to the apical surface of polarized hepatocytes by an indirect transcytotic pathway that differs from the transport of other proteins. In the direct pathway, proteins leave the trans-Golgi Network (TGN) and are targeted to either the apical or basolateral membrane by a signal peptide. By contrast, GPI-APs leave the TGN and first arrive at the basolateral surface prior to being selectively internalized and transcytosed to the apical surface. The mechanism of internalization used by GPI-APs is not completely understood although it is dependent on dynamin and flotillin-2 and independent of clathrin (Ait-Slimane, Galmes et al. 2009). The basolateral versus apical distribution of Vanin-1 on hepatocytes has not been investigated.

Involvement of Vanin-1 and Pantetheinase in Liver Homeostasis

Although no thorough investigation of pantetheinase expression has been performed in human tissues, the Vnn1 gene is widely expressed.
Expression is most prevalent in highly metabolic tissues: liver, kidney, small intestine, heart and lung (Su, Wiltshire et al. 2004; Jansen, Kamsteeg et al. 2009). Functionally, it is not surprising that Vanin-1 is highly expressed in liver given its involvement in at least two important hepatic processes: lipid metabolism and taurine biosynthesis.

Vnn1 is upregulated by both fatty acids (Motomura, Yoshizaki et al. 2012) and PPAR agonists (Wong and Gill 2002; Yamazaki, Kuromitsu et al. 2002; Moffit, Koza-Taylor et al. 2007) and is likely an important regulator of CoA metabolism by regulating the availability of pantothenic acid. Pantetheinase hydrolyzes pantetheine to pantothenic acid (Vitamin B5) in a reaction that cleaves cysteamine from the pantetheine molecule (Figure 1.3.). During CoA synthesis, pantothenic acid is first phosphorylated to 4-phosphopantothenate by pantothenate kinases in a reaction that is considered the rate limiting step of CoA synthesis. Phosphopantothenate-cysteine ligase adds cysteine to form 4-phosphopantetheine in a step that will provide the CoA molecule with its reactive thiol group necessary to bind fatty acids. 4-phosphopantetheine is then converted to 3-dephospho-CoA by pantetheine-phosphate adenyllyltransferase (PPAT) and finally to CoA by dephospho-CoA kinase (DPCK) by consecutive phosphorylation steps (Daugherty, Polanuyer et al. 2002).

PPARα agonists are well known for their ability to upregulate genes involved in lipid metabolism, including the β-oxidation pathway. Mice protected against APAP by CFB pretreatment exhibit a seven-fold
Figure 1.3. Pantetheine Hydrolysis and Downstream Pathways.

Pantetheinase activity regulates the availability of pantothenic acid and cysteamine which are involved in several critical pathways of cell homeostasis including lipid catabolism, synthesis of cholesterol, taurine and NADH as well as maintenance of cellular redox balance.
enhancement in hepatic Vnn1 gene expression, as well as increased concentrations of pantothenic acid and cystamine (the oxidized form of cysteamine) in liver (Moffit, Koza-Taylor et al. 2007). This suggests that PPARα-mediated upregulation of Vnn1 translates to enhanced pantetheinase activity in vivo. Furthermore, it is likely that pantetheinase activity can directly influence lipid metabolism, as rats treated with a novel pantetheinase inhibitor in their drinking water have increased free fatty acids and decreased cholesterol in plasma (Jansen, van Diepen et al. 2013). Presumably, these effects are due to decreased availability of pantothenic acid, resulting in suppression of CoA activity. In this way, pantetheinase likely acts as a regulator of CoA-dependent metabolism by controlling the availability of pantothenic acid in liver.

The second major hepatic pathway involving pantetheinase is taurine biosynthesis. In vivo, taurine can be synthesized from either cysteine or cysteamine. pantetheinase-mediated catalysis is the main source of endogenous cysteamine in mice (Pitari, Malergue et al. 2000). During taurine synthesis, cysteamine is converted to hypotaurine by the addition of molecular oxygen catalyzed by cysteamine dioxygenase (ADO) (Cavallini, De Marco et al. 1966; Dominy, Simmons et al. 2007). Hypotaurine is then oxidized to taurine enzymatically in a reaction using NAD+ and H2O as cofactors (Sumizu 1962; Oja and Kontro 1981).

It is unclear to what extent cysteamine contributes to the total pool of hepatic taurine. It is currently believed that the majority of hepatic taurine is
synthesized from cysteine by cysteine dioxygenase (CDO) due to higher concentration of the enzyme compared to ADO in this organ (Dominy, Simmons et al. 2007). However, no comparative analysis of ADO and CDO kinetics has been reported in the literature. Also, estimates of free hepatic cysteine (20-100μM) (Stipanuk, Dominy et al. 2006) and cysteamine (20-50μM) (Duffel, Logan et al. 1987; Moffit, Koza-Taylor et al. 2007) are similar.

The importance of cysteamine-mediated taurine synthesis may be increased in tissues that have either low cysteine dioxygenase activity (i.e. the brain) (Dominy, Simmons et al. 2007) or are depleted of cysteine (i.e. the liver during APAP hepatotoxicity). Taurine and hypotaurine treatments are protective in rodents during APAP overdose (Acharya and Lau-Cam 2010) and other acute and chronic models of hepatotoxicity (Miyazaki and Matsuzaki 2012). Likewise, normal liver concentrations of taurine (4-11mM) are depleted during disease (van Stijn, Vermeulen et al. 2012). Given that pantetheinase-mediated hydrolysis is the primary mechanism of cysteamine formation, it is possible that pantetheinase activity is protective by augmenting the availability of taurine, although the significance of this contribution remains unknown.

1.6 Transcriptional Regulation of Vanin-1

*Vanin-1 Expression in Normal and Diseased Liver*

As described earlier in this chapter, Vnn1 is upregulated in mice following activation of PPARα. Mice lacking PPARα are refractory to hepatic...
Vnn1 upregulation by the PPARα agonist CFB (Moffit, Koza-Taylor et al. 2007). These mice also have reduced expression of Vnn1 compared to wild-type, suggesting that PPARα may be involved in Vnn1 basal expression as well as its inducibility in mouse liver. *In vitro*, Vnn1 gene expression is also induced in the human hepatocyte cell line HepG2 following the activation of PPAR alpha, delta and gamma (Tachibana, Kobayashi et al. 2005), indicating that this is not an isoform or species-specific effect of PPAR activation. Although direct binding of Vnn1 by PPARs has not been confirmed, it is supported by enhanced luciferase activity following transfection of HepG2 cells with a predicted PPRE within the Vnn1 sequence (Rakhshandehroo, Knoch et al. 2010). Analysis of the human promoter using MatInspector software predicts an additional PPAR and RXR binding site roughly 570 bases upstream of the transcription start site (unpublished observation), although the functionality of this potential PPRE has not yet been investigated.

Vnn1 is also inducible by promoters of oxidative stress. Berruyer et al. demonstrated that the mouse Vnn1 promoter contains at least two functional antioxidant response elements (AREs) (Berruyer, Martin et al. 2004). The first of these sites, located 67 bases upstream of the transcription starting site, is stimulated by tert-butylhydroquinone (tBHQ) treatment as determined by an increase in luciferase activity in transfected MTE-4-14 cells. This site also binds JunB and c-Fos, subunits of activator protein 1 (AP-1) in thymic endothelial cells (Berruyer, Martin et al. 2004).
Within the liver, gene expression of JunB and c-Fos are increased in toxic models of bile acid accumulation both *in vitro* and *in vivo* (Bernt, Vennegeerts et al. 2006; Denk, Cai et al. 2006). Despite this, mice that have undergone bile duct ligation (BDL) for seven days have reduced expression of Vnn1 mRNA (Denk, Cai et al. 2006). Consequently, AP-1 does not appear to be an effective inducer of hepatic Vnn1 transcription during chronic obstructive cholestatic injury. It should be noted that BDL surgery also results in a significant reduction in the expression of PPARα and several downstream PPAR-regulated genes (Denk, Cai et al. 2006), arguably strengthening the association between PPARα and Vnn1 expression.

Patients with obstructive cholestasis have increased Specificity Protein 1 (SP-1) protein (Chai, He et al. 2012), which represents another potential mechanism of Vnn1 downregulation during this disease state. SP-1 has been demonstrated to bind the second functional ARE elucidated by Berruyer et al., located 2016 bases upstream of the mouse Vnn1 transcription start site (Berruyer, Martin et al. 2004). Luciferase reporter experiments revealed that binding of SP-1 to this ARE resulted in a repression of promoter activity following the treatment of thymic endothelial cells with tBHQ. Therefore, reduced PPARα or enhanced SP-1 signaling represent two potential mechanisms of Vnn1 downregulation during chronic obstructive cholestatic disease.

By contrast, Vnn1 mRNA expression is upregulated in several mouse models of non-alcoholic fatty liver disease (NAFLD) (Yu, Viswakarma et al. 2012).
2004; Sato, Horie et al. 2006; Guillen, Navarro et al. 2009). Additional studies indicate that Vnn1 upregulation occurs in both mice and human hepatoma Huh-7 cells prior to hepatocyte lipid accumulation (Motomura, Yoshizaki et al. 2012), suggesting that Vnn1 mRNA induction occurs shortly after an increased availability of lipids rather than as a result of disease.

1.7 Cysteamine

Antioxidant Properties of Cysteamine

Cysteamine supplementation provides protection in models of neurodegeneration, irradiation and cancer as well as acute and chronic models of liver disease. Endogenous cysteamine concentrations in the liver have been estimated to be between 20-50μM in mice (Duffel, Logan et al. 1987; Moffit, Koza-Taylor et al. 2007), appreciably lower than that of GSH, which accounts for the bulk of hepatic non-protein sulfhydryls. Consequently, it is unlikely that the endogenous pool of cysteamine has a high capacity to detoxify reactive species, although the subcellular localization of cysteamine is likely important to its protection. For example, low doses of cysteamine (0.75ug, delivered intratesticularly) to mice are known to be radioprotective to sperm following exposure to $^{125}$I and $^{210}$Po (Rao, Narra et al. 1991), radionuclides that preferentially bind to DNA. It has since been shown that $^{35}$S)cysteamine given at a similar concentration and route of administration results in roughly 1% of the total dose of cysteamine being associated with the DNA fraction, suggesting that although the total amount of cysteamine is
low, its proximity to DNA appears sufficient to yield radioprotection (Harapanhalli, Narra et al. 1993).

Other experiments further suggest that cysteamine could be a “low capacity, high affinity” scavenger of reactive species. Hydrogen peroxide decay experiments indicate that cysteamine is able to scavenge H₂O₂ more efficiently than GSH, an effect that is likely due to the lower pKₐ of cysteamine’s active thiol group (Winterbourn and Metodiewa 1999). Skrede and Christopherson demonstrated that cysteamine protected isolated rat liver mitochondria from ascorbate-induced lipid peroxidation in vitro (Skrede and Christophersen 1966). These experiments revealed that malondialdehyde formation was inhibited similarly by cysteamine and GSH in fresh mitochondria, but only the former was inhibitory in boiled mitochondria, suggesting that inhibition by cysteamine does not depend on heat-sensitive coenzymes (Christophersen 1968).

Protection against Hepatotoxicants by Cysteamine/Cystamine

Cysteamine and cystamine, the oxidized disulfide form of cysteamine, have been proven to be protective against several model hepatotoxicants in both rodents and man. Early experiments involving rats treated with carbon tetrachloride, an inducer of lipid peroxidation, showed that pretreatment with cystamine was protective (Castro, De Ferreyra et al. 1973). Livers from rats receiving cystamine displayed reductions in lipid peroxidation and hepatocellular necrosis within the liver. Cystamine also reduced CCl₄ covalent
binding to microsomal lipids and suggested that the protective effects may have been due to decreased bioactivation of CCl₄ to the reactive trichloromethyl radical. A subsequent study was performed in which rats were treated with cystamine 3, 6 or 12 hours after CCl₄ and sacrifice at 24 hours. Additional tested compounds that inhibited CCl₄ bioactivation did not provide protection when given three hours after CCl₄, suggesting that the early events of bioactivation and the initiation of lipid peroxidation had already occurred (Ferreyra, de Fenos et al. 1977). Cystamine was protective at all three time points, and surprisingly, showed a trend of increased protection when dosed 12 hours post CCl₄ rather than 3 hours as determined by histopathology and liver enzyme activity in plasma. Cysteine was also protective in these experiments, although the authors noted that it provided less protection against necrosis compared to cystamine (Ferreyra, de Fenos et al. 1977). Five day repeated treatment of pantethine (the oxidized disulfide form of pantetheine), pantothenic acid, or cystamine show similar protective effects in rats when dosed prior to CCl₄, suggesting that at least a portion of the inherent protective effects of cystamine may be shared by these related compounds (Nagiel-Ostaszewski and Lau-Cam 1990).

In contrast to CCl₄, galactosamine induces acute liver hepatotoxicity without requiring bioactivation to a reactive intermediate. Instead, it depletes hepatic uridine by forming uridine-5’-diphosphate hexosamines and is associated with hepatic necrosis and causes elevations in the activity of liver enzymes in plasma. Toxicity can be prevented by uridine treatment 2 hours,
but not 6 hours, following galactosamine intoxication. By contrast, cystamine treatment was protective at time points examined from 30 minutes preceding to 12 hours following galactosamine treatment (MacDonald, Gandolfi et al. 1984), further indicating that cystamine is hepatoprotective without interfering with bioactivation processes and is more likely protective by altering later biochemical events. Furthermore, isolated hepatocytes from galactosamine treated rats are protected from toxicity when treated with 3-30\(\mu\)M cystamine \textit{in vitro}, demonstrating that protection is likely an effect exerted (at least partially) on hepatocytes directly, rather than immune cells or extra-hepatic stimuli (MacDonald, Thayer et al. 1987). The non-protein sulfhydryl content of hepatocytes was also unchanged by cystamine treatment.

Cysteamine has been used clinically as an antidote for APAP overdose (Prescott, Newton et al. 1974). Cysteine and other APAP antidotes that enhance GSH synthesis lose their protective effect in rodents when GSH synthesis is inhibited by buthionine sulfoximine (BSO) treatment. Cysteamine remains protective in these conditions (Miners, Drew et al. 1984), suggesting that the mechanism of protection is independent of GSH synthesis. Although administration of cysteamine has been shown by some to partially inhibit CYP450 activity (Peterson, Peterson et al. 1989), this is unlikely to be the cause of antidotal protection because the proportion of a dose of APAP that is bioactivated to the reactive metabolite is unchanged following cysteamine treatment (Miller and Jollow 1986; Peterson and Brown 1992).
It is unclear how cysteamine and cystamine protect against these model hepatotoxicants. The aforementioned studies suggest that protection occurs without any significant direct binding to reactive metabolites or augmenting the availability of GSH. The observation that cysteamine and cystamine supplementation are both hepatoprotective raises the question as to whether one species is predominantly responsible. Although the reduced form is an effective scavenger of ROS, cysteamine can auto-oxidize at neutral to slightly alkaline pH due to the reactivity of its sulfhydryl group. Furthermore, an increase in fatty acid oxidation could enhance the oxidation of cysteamine due to an increase in H$_2$O$_2$ generation. Support for this hypothesis is seen in mice treated with CFB which have an enhanced capacity for fatty acid metabolism. These mice exhibit increased hepatic concentrations of oxidized cystamine (and an increase in Vnn1 gene expression), while concentrations of reduced cysteamine are unchanged (Moffit, Koza-Taylor et al. 2007). Interestingly, these mice are also protected against subsequent APAP intoxication.

Flavin-containing monooxygenase (FMO) enzymes can also oxidize cysteamine to the disulfide. The $K_m$ of FMO-mediated oxidation of cysteamine has been estimated to be 105 and 48μM in mouse and pig liver microsomal preparations, respectively (Tynes and Hodgson 1985). These values do not vary greatly from observed hepatic cysteamine concentrations of 20-50μM in mice. Furthermore, yeast FMO has shown to contain redox-sensitive Cys residues and can be inhibited by GSSG accumulation (Suh, Poulsen et al.
Together, these data suggest that FMO provides an additional regulatory mechanism for the maintenance of reduced:oxidized cysteamine.

The fact that both the reduced and disulfide forms of the molecule appear to be protective speaks to the reactivity of its thiol group. In vivo, the balance of reduced to disulfide cysteamine:cystamine ratio is almost certainly regulated by many factors including subcellular localization, pH, reducing capacity, FMO status and metabolic rate. It is uncertain whether one species is responsible for protection, although the protective mechanism likely involves regulation of the redox state of the cell by maintenance of disulfide bonds. The following subsections will discuss the possible mechanisms of cysteamine-mediated protection in further depth.

**Protein Disulfide Exchange (Cysteaminylation)**

One possible explanation for the hepatoprotective effects of cysteamine/cystamine is that they are important sensors of oxidative stress. Maintenance of the thiol:disulfide balance has been well studied in relation to cell homeostasis and is known to affect proliferative and apoptotic pathways (Townsend 2007) and may also influence inflammation (Iyer, Accardi et al. 2009). Oxidized glutathione (GSSG), cystine and cystamine are all thought to regulate these pathways by protein disulfide exchange, which can result in the inactivation of enzymes through the modification of critical cysteine sites.

In comparison to cystine and GSSG, cystamine appears to be particularly efficient at catalyzing disulfide exchange reactions. Among 16
tested peptide sequences, each containing a Cys residue with varying pK\textsubscript{a}s, cystamine exhibited the highest observed rates of thiol-disulfide exchange almost uniformly (Bulaj, Kortemme et al. 1998). These data suggest that the ability of cystamine to undergo disulfide exchange could be of importance even when its concentration is less than that of other thiol disulfide species.

Specifically, in vitro assays have shown that cystamine is able to inhibit several key enzymes involved in metabolism, proliferation and apoptosis including glycogen phosphorylase b, aldose reductase and caspase 3 (Miller, Sies et al. 1990; Cappiello, Voltarelli et al. 1996; Mallis, Buss et al. 2001; Lesort, Lee et al. 2003; O'Brian and Chu 2005). It has been hypothesized that cystamine can modulate the immune system by a similar inhibitory mechanism (Hsu, Chiang et al. 2007; Ozaki, Kaibori et al. 2007). Under normal conditions these inhibitory effects are most likely very limited due to the high intracellular reducing environment, but could have expanded roles at times when cellular redox status is altered or in cell compartments such as the ER, where protein disulfide exchange commonly occurs during protein folding. It is possible then that oxidized cystamine is an intracellular sensor with the ability to limit the production of ROS within the cell through the inhibition of thiol-sensitive cellular processes that can produce oxidative stress (i.e. glucose metabolism).

Cystamine's ability to assist in protein disulfide exchange also likely has another important role: the folding of disulfide-containing proteins. Addition of cystamine to a solution of a 28-amino acid peptide containing two
reduced Cys residues resulted in the formation of mixed disulfides at a rate of 8 times faster than that achieved by adding GSSG as the oxidizing agent (Darby, Freedman et al. 1994). A subsequent peptide bond between the two Cys residues can then catalyzed by protein disulfide isomerase (PDI) to complete folding. Similar experiments showed that the addition of 200μM cystamine could restore roughly 70% of enzyme activity to denatured lysozyme, whereas GSSG treatment was ineffective (Wang, Dong et al. 2011). These experiments suggest that cystamine has the ability to restore, as well as regulate, the activity of enzymes through maintenance of their disulfide bonds. Many proteins that require disulfide bonds for their biological activity are involved in immune cell adhesion and programming, including the IfnγR (Stuber, Friedlein et al. 1993) and IL-4 (Curbo, Gaudin et al. 2009), proteins important in M1 and M2 immune cell signaling, respectively. High mobility group box 1 (HMGB1), a regulator of inflammation, requires a Cys23-Cys45 disulfide bond in order to induce nuclear NF-kB translocation (Yang, Lundback et al. 2012). On the other hand, the activity of Integrin αIIbβ3, which mediates platelet adhesion and aggregation, is enhanced following the reduction of its disulfide bonds (Yan and Smith 2001). Thus, cystamine may exert its hepatoprotective effects through post-translational modification of proteins that influence important cell processes such as cell survival and polarization of immune phenotypes (M1 versus M2). The aforementioned experiments indicate that cystamine has the ability to influence redox signaling in vitro. In vivo data suggesting that cystamine
regulates these processes at endogenous concentrations under physiological conditions are largely limited to studies performed in Vnn1 knockout mice. These mice lack measurable amounts of cysteamine (Pitari, Malergue et al. 2000) and exhibit alterations in immune cell and inflammatory responses upon exposure to sources of oxidative stress (Table 1.1). In general, these studies show that a lack of Vnn1 results in a protective effect that is accompanied by reductions in inflammatory endpoints, suggesting that Vnn1 can control inflammation following exposure to oxidative stress. Furthermore, cyst(e)amine treatment restores the wild-type phenotype in many of these models (Berruyer, Martin et al. 2004; Martin, Penet et al. 2004; Berruyer, Pouyet et al. 2006; Roisin-Bouffay, Castellano et al. 2008; Pouyet, Roisin-Bouffay et al. 2010), indicating that at least some of the effects mediated by a loss in Vnn1 are due to a lack of its catalytic byproducts and that pantetheinase activity is physiologically relevant in normal tissue homeostasis in mice. These studies need to be interpreted with caution, however, because Vnn1 knockout mice bred on a BALB/c background have an approximate 50% increase in γGCS activity and GSH stores in both liver and intestine compared to wild-type (Martin, Penet et al. 2004). This effect is also reversed by cystamine treatment and is consistent with the finding that γGCS can be inhibited by cystamine-mediated protein disulfide exchange (Lebo and Kredich 1978). It should also be noted that Vnn1 knockout mice bred on a C57Bl/6 background have no such modification in basal GSH content in
### Table 1.1. Models of Oxidative Stress and Inflammation Tested in the Vnn1 Knockout Mouse

<table>
<thead>
<tr>
<th>Model</th>
<th>Phenotype of KO</th>
<th>Endpoints</th>
<th>Reversed by CEA?</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Inflammation (indomethacin)</td>
<td>↓ Inflammation</td>
<td>↓ iNOS and Cox2 mRNA</td>
<td>Yes</td>
<td>(Martin, Penet et al. 2004)</td>
</tr>
<tr>
<td>Chronic Inflammation (schistosoma)</td>
<td>↓ Inflammation</td>
<td>↑ Survival</td>
<td>NR</td>
<td>(Martin, Penet et al. 2004)</td>
</tr>
<tr>
<td>Diabetes (streptozotocin)</td>
<td>↑ Incidence</td>
<td>↑ Islet cell nuclei fragmentation ↑ Cleaved Caspase 3</td>
<td>Yes</td>
<td>(Roisin-Bouffay, Castellano et al. 2008)</td>
</tr>
<tr>
<td>Diabetes (NOD mice)</td>
<td>↑ Incidence</td>
<td>↓ Mac-1+ cells ↑ CD4+ cells</td>
<td>NR</td>
<td>(Roisin-Bouffay, Castellano et al. 2008)</td>
</tr>
<tr>
<td>Bacterial Infection (C. burnetti)</td>
<td>↓ Granuloma Formation</td>
<td>↓ iNos, Mcp-1 mRNA ↑ Il-10, arginase mRNA ↓ Macrophage migration</td>
<td>NR</td>
<td>(Meghari, Berruyer et al. 2007)</td>
</tr>
<tr>
<td>Colitis (TNBS-induced)</td>
<td>↑ Survival</td>
<td>↓ Colonic lesions</td>
<td>Yes</td>
<td>(Bernt, Vennegeerts et al. 2006)</td>
</tr>
<tr>
<td>Colitis-associated colon cancer</td>
<td>↑ Survival</td>
<td>↓ Tumor incidence</td>
<td>Yes</td>
<td>(Pouyet, Roisin-Bouffay et al. 2010)</td>
</tr>
<tr>
<td>Gamma irradiation</td>
<td>↓ Inflammation</td>
<td>↑ Survival ↓ Apoptosis</td>
<td>Yes</td>
<td>(Berruyer, Martin et al. 2004)</td>
</tr>
</tbody>
</table>
liver (Chapter 2), suggesting that the phenotype of suppressed GSH content in BALB/c mice may be background specific.

While increased GSH concentration likely contributes to the protection of BALB/c Vnn1 knockout mice in these studies, the immune effects of Vnn1 likely extend beyond this influence. For example, wild-type mice treated with an antibody raised against Vnn1 have a decrease in the migration and adhesion of immature T cells in the thymus (Aurrand-Lions, Galland et al. 1996). Furthermore, APAP treatment of C57Bl/6 Vnn1 knockout mice results in enhanced centrilobular necrosis and a deficiency in immune cell migration to sites of injury without any alterations in hepatic GSH content or utilization (Chapter 2). Together, the experiments performed in Vnn1 knockout mice further support the hypothesis that the presence of pantetheinase and/or cystamine affects cell homeostasis and inflammation at endogenous concentrations in vivo.

1.8 Roles of Vanin-1 in Oxidative Stress and Disease

Vnn1: Protector of or Contributor to Toxicity?

Inflammation is often a double-edged sword. The redox and seemingly “pro-inflammatory” roles of Vnn1 and its catalytic products have piqued the interest of investigators in recent years. Two separately identified single nucleotide polymorphisms within the hVnn1 sequence have been pursued in the field of hypertension. A N131S missense mutation has been associated with increased hypertension in both African and Mexican, but not European,
Americans (Zhu and Cooper 2007). A second identified SNP, T26I, lies within a splicing regulation site within the protein sequence although further investigation has not shown any association with increases in hypertension or cardiovascular effects (Fava, Montagnana et al. 2013). Both SNPs occur within the CN Hydrolase domain of hVnn1, although it is not clear how either affects the functionality of the enzyme.

The redox function of cysteamine can have positive or negative effects. Oral administration of cysteamine causes duodenal ulceration in rats. This toxicity appears to occur following its auto-oxidation to cystamine and the generation of H$_2$O$_2$ in the presence of transition metals. The produced H$_2$O$_2$ can in turn form hydroxyl radicals by the Fenton reaction, thereby promoting oxidative stress. Toxicity resulting from cysteamine auto-oxidation can be prevented both in vitro and in vivo by catalase but not superoxide dismutase treatment, indicating that H$_2$O$_2$ generation is the initiating event responsible for toxicity (Issels, Biaglow et al. 1984; Khomenko, Deng et al. 2003).

The high reactivity of cysteamine can also be beneficial. Cysteamine bitartrate is used clinically as a treatment for cystinosis, a condition characterized by a mutation in the gene encoding for the lysosomal cysteine transporter. Cystinosis patients are unable to efflux lysosomal cysteine disulfides, leading to cystine accumulation and eventually to nephropathic disease. Delivered cysteamine initiates disulfide exchange with cystine to form a cysteamine-cysteine mixed disulfide, which exits the lysosome and reduces cystine burden.
The models of oxidative stress investigated in Vnn1 knockout mice associate a loss of Vnn1 with a phenotype of reduced inflammation as shown in Table 1.1. As discussed earlier, the direct effect of knocking out Vnn1 is difficult to discern from the studies using BALB/c mice due to a loss of cysteamine-mediated inhibition of γGCS and a resultant elevation in hepatic GSH. While the majority of published studies utilize Vnn1 knockout mice on the BALB/c background, Meghari et al. utilized C57Bl/6 Vnn1 knockout mice which do not have elevated hepatic GSH. The authors found that C57Bl/6 Vnn1 knockouts had fewer infiltrating macrophages in liver and were resistant to granuloma formation (a macrophage-driven process) relative to wild-type mice following *Coxiella burnetti* infection (Meghari, Berruyer et al. 2007).

Cultured bone marrow-derived macrophages from C57Bl/6 Vnn1 knockouts exhibited decreased mRNA expression of Mcp-1 and iNos, in addition to increases in arginase and IL-10 expression, indicating a shift from a pro-inflammatory M1 phenotype towards an anti-inflammatory M2 phenotype. Similar results were obtained by exposing the cultures to LPS, suggesting that macrophages from Vnn1 knockouts did not undergo M1 activation. An M1 phenotype is characterized by high phagocytic activity and respiratory burst, whereas M2 macrophages favor tissue repair and angiogenesis. The mannose receptor, a scavenger receptor associated with anti-inflammatory macrophages has been shown ability to bind mannose residues within GPI anchors and in-so-doing drives macrophage polarization towards an M2 phenotype (Dangaj, Abbott et al. 2011). While the mannose
receptor has not yet been shown to bind the GPI anchor of pantetheinase directly, this or a similar mechanism represents an additional possible role for Vnn1 during tissue repair. Together, these data support a role for Vnn1 in immune cell programming and suggest that Vnn1 may influence the immune system in addition to cellular redox status.

Summary

Cysteamine is a reactive thiol that likely is at least partially responsible for the redox and immune effects of Vnn1 and pantetheinase. Though it is an effective antioxidant in its own right, it exists endogenously at low concentrations in liver and in all probability represents a "low-capacity, high-affinity" scavenger of reactive oxygen species. Its high reactivity also allows it to auto-oxidize to the cystamine disulfide. Cysteamine is protective in animal models when administered up to 12 hours after various hepatotoxicants, suggesting that protection is the result of effects that occur downstream of bioactivation and covalent binding of the hepatotoxicants to target cellular macromolecules.

Cystamine is able to undergo mixed disulfide exchange and has been shown to inhibit several thiol-sensitive enzymes by this mechanism in vitro. Under normal conditions, this mechanism would be most effective where the reducing capacity is low (i.e., extracellularly or within the endoplasmic reticulum), but may become enhanced when the cell’s redox balance is altered to a pro-oxidative (i.e., highly metabolic or during times of oxidative
stress) state. Many inflammatory mediators also contain active Cys sites or depend on maintenance of their disulfide bonds for their activation.

Pantetheinase appears to be the primary source of endogenous cysteamine in mice. The full influence of this enzyme remains poorly understood, but appears to be involved in multiple metabolic and immune pathways. Metabolically, Vnn1 regulates the availability of pantothenic acid, a major precursor of CoA and is thus involved with lipid metabolism and energy production by the β-oxidation pathway and TCA cycle, respectively. Vnn1 may also regulate important cell processes by producing cysteamine/cystamine which has been shown to inactivate multiple proteins involved in glucose metabolism in vitro. Immunologically, Vnn1 may influence cell signaling indirectly through maintenance of disulfide bonds. This thesis describes work that has been done to better understand the protective effects of Vanin-1 in both murine in vivo and human in vitro models of APAP injury.
Chapter 2

Enhanced Susceptibility of Vanin-1 Knockout Mice to Acetaminophen Hepatotoxicity is Associated with Altered Compensatory Proliferative and Immune Responses

2.1 Abstract

Vanin-1 (Vnn1) knockout mice are more susceptible to APAP hepatotoxicity (400mg/kg, i.p.) despite no differences in hepatic glutathione (GSH) content or gene expression of APAP metabolizing enzymes or transporters. In vitro, livers from both genotypes show similar capabilities to bioactivate APAP to its reactive metabolite (~1.8 nmol APAP-NAC/min/mg protein) as well as detoxify the parent compound by glucuronidation (~1.7 nmol APAP-Gluc/min/mg protein) and sulfation (~15.6 pmol APAP-Sulf/min/mg protein). Together, these data strongly suggest that the enhanced susceptibility of Vnn1 knockout mice to APAP toxicity is not due to differences in APAP metabolism. Immunohistochemistry of formalin-fixed liver sections following APAP treatment revealed a lack of PCNA-positive hepatocytes and F4/80-positive macrophages in and around the areas of centrilobular necrosis in Vnn1 knockouts at 48 hours. qRT-PCR from total RNA isolated from whole liver indicated that inducible nitric oxide synthase (iNos) and interleukin-4 were reduced by 2.9 and 4.3 fold, respectively, in control treated Vnn1 knockout mice relative to wild-types. Following APAP treatment, Vnn1 knockout mice had reductions in hepatic expression of the
pro-inflammatory genes interferon γ, iNos, tumor necrosis factor and chemokine ligand 2 by 2.7, 2.8, 5.1 and 38 fold at 48 hours. Myeloperoxidase also exhibited a trend of decreased expression in Vnn1 knockouts at 48 hours but was not statistically significant. Together, these results indicate that mice lacking Vnn1 have alterations in compensatory repair and immune responses following toxic APAP exposure and that these mechanisms may contribute to the enhanced hepatotoxicity seen.

**Keywords:** acetaminophen, pantetheine hydrolase, cysteamine, vanin
2.2 Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is present in many prescription and non-prescription medications. Although safe at therapeutic doses, roughly half of all acute liver failure cases in the United States are caused by APAP overdose (Lee 2010). Upon therapeutic administration, the majority of APAP is metabolized in the liver by glucuronidation and sulfation reactions and the resulting conjugates are safely eliminated via multidrug resistance-associated proteins (Mrps) into both blood and bile (Manautou, de Waart et al. 2005; Zamek-Gliszczynski, Hoffmaster et al. 2005; Zamek-Gliszczynski, Nezasa et al. 2006). At therapeutic doses also, a small fraction of APAP also undergoes bioactivation by cytochrome P450 to generate N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite that can be detoxified by hepatic glutathione (GSH) and excreted into bile by Mrp2 (Chen, Hennig et al. 2003). APAP overdose causes the saturation of detoxification pathways, resulting in a greater amount of APAP undergoing cytochrome P450 metabolism. While the exact mechanism of APAP toxicity is unknown, excessive NAPQI generation causes GSH depletion. This permits free NAPQI to bind to cellular macromolecules and promotes oxidative stress, resulting in hepatic centrilobular necrosis (Hinson, Roberts et al. 2010).

Peroxisome proliferators (PPs), in particular the fibrate class of hypolipidemic drugs, have been shown to protect rodents from APAP hepatotoxicity (Nicholls-Grzemski, Calder et al. 1992; Manautou, Hoivik et al. 1994; Manautou, Silva et al. 1998). Peroxisome proliferators are a large
group of endogenous and exogenous chemicals that activate the peroxisome proliferator-activated receptor alpha (PPARα) nuclear receptor and increase the capacity of the liver to metabolize fatty acids by β oxidation. In mice, protection by clofibrate (CFB), a PP, does not appear to be due to alterations in APAP metabolism (Manautou, Tveit et al. 1996; Chen, Hennig et al. 2000) or by increased detoxification by catalase (Chen, Hennig et al. 2002) or NAD(P)H quinone oxidoreductase 1 (Nqo1) (Moffit, Aleksunes et al. 2007). Protection also occurs independently of changes in APAP-protein adduct formation and GSH depletion (Manautou, Emeigh Hart et al. 1996). The mechanism of this protection remains poorly understood, although our laboratory has shown that it is dependent on PPARα activation (Chen, Hennig et al. 2000).

Gene array studies revealed that mice receiving CFB before toxic APAP dosing had a 7-fold induction in Vanin-1 (Vnn1) gene expression (Moffit, Koza-Taylor et al. 2007). Similar to the mechanism of PP-mediated protection, Vnn1 induction is also PPARα dependent, as no induction is observed in PPARα knockout mice (Moffit, Koza-Taylor et al. 2007). The Vnn1 gene encodes for pantethine hydrolase, a glycosylphosphatidylinositol (GPI)-anchored ectoenzyme that catalyzes the hydrolysis of pantethine to pantothenic acid (Vitamin B5). Pantothenic acid is a precursor of Coenzyme A, an important cofactor in fatty acid metabolism as well as the tricarboxylic acid (TCA) cycle. Pantethine hydrolysis also results in the production of cysteamine (2-aminoethanethiol), a thiol with antioxidant properties (Pitari,
Malergue et al. 2000). Similar to other thiols, cysteamine exists endogenously in both reduced and oxidized disulfide (cystamine) forms and has shown to be protective against lipid peroxidation (Skrede and Christophersen 1966; Christophersen 1968; Winterbourn and Metodiewa 1999) and aldehyde overload (Wood, Khan et al. 2007). In addition to cysteamine production, Vnn1 also modulates immune function by contributing to the extravasation of inflammatory cells to sites of injury (Meghari, Berruyer et al. 2007). Vnn1 also controls the homing of immature T lymphocytes to the thymus during their development (Aurrand-Lions, Galland et al. 1996).

Since CFB-mediated protection against APAP is associated with enhanced Vnn1 gene expression (Moffit, Koza-Taylor et al. 2007) we set out to investigate the role of Vnn1 in APAP hepatotoxicity by studying the susceptibility of Vnn1 knockout mice to APAP liver injury. Here we show for the first time that mice lacking Vnn1 have an increased susceptibility to APAP hepatotoxicity. We determined that this increased susceptibility to APAP toxicity is not due to compensatory differences in APAP bioactivation or detoxification in Vnn1 null mice. Finally, we show that enhanced toxicity in Vnn1 knockout mice is associated with a decrease in immune cell infiltration and compensatory hepatic repair in response to APAP hepatotoxicity. The identification of specific protective mechanisms mediated by Vnn1 may be useful in developing more effective treatments for patients with APAP overdose by modulating these compensatory pathways following APAP injury.
2.3 Materials and Methods

Animal Care and Treatment

Vnn1 knockout mice backcrossed to a C57BL/6 background were kept in a pathogen-free mouse facility. Experiments were performed according to an experimental protocol as approved by the Institutional Animal Care and Use Committee of the Université de la Méditerranée in Marseille, France. Vnn1 knockout and wild-type mice receiving drug treatment were fasted the night before receiving intraperitoneal injection with either APAP (400mg/kg in 50% propylene glycol) or vehicle. Food was returned at 8 hours following treatment. Mice were sacrificed at 24 and 48 hours after treatment. Blood was collected by orbital bleeds and plasma was separated following centrifugation. Livers were also collected, half of which was snap frozen in liquid nitrogen, while the remaining half was fixed in formalin.

ALT Activity Assay

Hepatotoxicity was determined by measuring ALT activity in plasma samples. ALT activity was measured using the Infinity GPT Reagent (Thermo Scientific) as recommended by the manufacturer and read on a 96-well plate reader (BioTek PowerwaveX, BioTek, Winooski, VT).

Histopathology and Immunohistochemistry

Paraffin sections were prepared from formalin-fixed livers. Hepatotoxicity was also assessed following hematoxylin and eosin staining.
Sections of liver samples were examined and scored on a 0-4 scale (0.5 intervals) according to the severity of centrilobular necrosis. Immunohistochemistry for the presence of proliferating cell nuclear antigen (PCNA) and F4/80 were performed on paraffin-embedded liver sections following heat-induced antigen retrieval and blocking steps for endogenous peroxidase, avidin and biotin. PCNA was stained using a kit (#931143, Invitrogen, Carlsbad, CA) and by incubating sections with biotinylated anti-PCNA primary antibody for 30 minutes at room temperature. F4/80 detection was achieved by incubating rat monoclonal anti-F4/80 antibody (#Ab6640, Abcam, Cambridge, MA) for 30 minutes at room temperature at a 1:200 dilution. Appropriate Vectastain ABC (Vector Laboratories, Burlingame, CA) biotinylated secondary antibody kits were used before developing with 3, 3’ diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories). Negative controls were generated by omitting the corresponding primary antibodies.

**qRT-PCR**

Total RNA was isolated from frozen liver following homogenization in TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and random primers. qRT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using SYBR green and species-specific primer pairs for each gene (Table 2.1). Gene expression was quantified by the ΔΔCT method and normalized to the expression of β-actin.
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<th>Reverse Sequence</th>
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<td>CCC AAG AAG GAA GGC TGG A</td>
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</table>
**GSH Assay**

Total hepatic GSH concentrations were quantified by the recycling method as previously described (Rahman, Kode et al. 2006). Briefly, 15-25mg of liver tissue was added to 500µl of 5mM EDTA disodium salt in 0.1M potassium phosphate buffer containing protease inhibitors. Tissues were kept on ice and homogenized by hand in a dounce homogenizer for 5-6 strokes. Equal volumes of 0.67mg/ml 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) and 3.33 units/ml glutathione reductase (Sigma Aldrich, St. Louis, MO) in 5mM EDTA disodium salt in 0.1M potassium phosphate buffer were mixed and 120µl was added to 20µl of each liver homogenate in a 96 well plate. 60µl of 0.67mg/ml β-NADPH (Sigma) in 5mM EDTA disodium salt in 0.1M potassium phosphate buffer was then added to each well. Absorbance was read immediately at 412nm, taking measurements every 30 seconds for 2 minutes. The assay was performed in duplicate for each sample and compared to a standard curve made from twofold serial dilutions (211.2nmol/ml to 1.65nmol/ml) of GSH standard (Sigma).

**In Vitro Metabolism of APAP**

Hepatic microsomal and cytosolic fractions were prepared from untreated Vnn1 knockout and wild-type mice. 0.3-0.5g of frozen liver was homogenized in 9 volumes of 0.1M potassium phosphate buffer (pH = 7.4) containing 0.15M potassium chloride, 1.0mM EDTA and protease inhibitors. Liver homogenates were prepared using cold Teflon dounce homogenizers.
and kept on ice. Homogenates were centrifuged at 9000 x g for 20 minutes at 4°C and supernatants were centrifuged again at 100,000 x g for 60 minutes at 4°C. The cytosolic supernatant was frozen until analysis. Microsomal pellets were resuspended in 5ml of 100mM sodium pyrophosphate buffer with 0.1mM EDTA (pH = 7.4) and centrifuged at 100,000 x g for an additional 60 minutes at 4°C. The supernatant was decanted and the microsomal pellet was resuspended in 3-500μl of 250mM sucrose depending on original liver weight. Protein concentrations of cytosolic and microsomal preparations were determined by the DC Lowry assay kit (BioRad, Hercules, CA).

*In vitro* biochemical analyses of acetaminophen (APAP) metabolism were performed as previously described (Manautou, Tveit et al. 1996; Moffit, Aleksunes et al. 2007; Reisman, Csanaky et al. 2009; Xu, Kulkarni et al. 2012) with some modifications. Glucuronidation was assessed by adding 50μl of 0.05% Brij58 to 250μg of microsomal protein into glass test tubes. Solutions of 25mM Uridine 5'-diphosphorogluconic acid (UDPGA) and 50mM MgCl2 in 0.1M sodium phosphate buffer (pH=7.8) were added to the samples and preincubated at room temperature for 10 minutes. Upon addition of APAP, tubes were incubated in a 37°C water bath for 60 minutes. The final 500μl reaction volume contained 0.5mg/ml microsomal protein, 10mM MgCl2, 5mM APAP and 4mM UDPGA in 0.1M sodium phosphate buffer. Incubations were stopped by adding 50μl of 6% perchloric acid. Samples were chilled on ice and centrifuged at 3000 x g for 10 minutes at 4°C. 50μl of collected supernatants were injected onto the HPLC for analysis. APAP glucuronide
(APAP-Gluc) formation was confirmed by conducting control incubations lacking APAP, microsomes, or UDPGA in which no APAP-Gluc metabolite was formed. Quantification of the APAP-Gluc peak was achieved by comparing peak areas of APAP-Gluc metabolite with an authentic APAP-Gluc standard (McNeil-PPC, Inc, Fort Washington, PA) and expressed as nmol APAP-Gluc/min/mg protein.

Sulfation of APAP was carried out by adding 125μl 3’-phosphoadenosine 5’-phosphosulfate (PAPS) and 50μl of dithiothreitol (DTT) containing 0.5% bovine serum albumin (BSA) in 0.1M sodium phosphate buffer (w/v, pH=7.8) into glass test tubes. 250μg of cytosolic protein was then added. This incubation mixture was diluted with 0.1M sodium phosphate buffer to a total volume of 350μl. Samples were placed in a 37°C water bath and 50μl of APAP solution was added and mixed gently. The final reaction mixture (400μl final volume) contained 0.625mg/ml cytosolic protein, 1mM APAP, 8mM DTT, 0.0625% BSA and 0.1mM PAPS. Incubations were stopped after 120 minutes by adding 400μl of ice-cold methanol. Samples were centrifuged at 3000 x g for 10 minutes at 4°C and 50μl of supernatant was analyzed by HPLC. Acetaminophen sulfate (APAP-Sulf) formation was confirmed by conducting control incubations lacking APAP, cytosol, or PAPS in which no APAP-Sulf metabolite was formed. Quantification of the APAP-Sulf peak was achieved by comparing peak areas of APAP-Sulf metabolite with an authentic APAP-Sulf standard (McNeil-PPC, Inc.) and expressed as pmol APAP-Sulf/min/mg protein.
Bioactivation of APAP was measured by incubating 250μg of microsomal protein with β-Nicotinamide adenine dinucleotide phosphate (NADP), N-acetyl-L-cysteine (NAC), MgCl2, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PD). Samples were pre-incubated at 37°C in an oscillating water bath for 10 minutes prior to the addition of APAP. The 300μl incubation volume consisted of 0.83mg/ml protein, 0.56mM NADP, 10mM MgCl2, 13.4mM G-6-P, 0.67mM NAC, 4IU G-6-PD and 20mM APAP in 0.1M sodium phosphate buffer. After a 20-minute incubation period, the reaction was stopped by adding 300μl of ice-cold methanol. Samples were centrifuged at 3000 x g for 30 minutes at 4°C. 50μl of the supernatant was then injected onto the HPLC for analysis. Acetaminophen mercapturate (APAP-NAC) formation was confirmed by conducting control incubations lacking APAP, microsomes, or NAC in which no APAP-NAC metabolite was formed. Quantification of the APAP-NAC peak was achieved by comparing peak areas of APAP-NAC metabolite with an authentic APAP-NAC standard (McNeil-PPC, Inc.) and expressed as nmol APAP-NAC/min/mg protein.

HPLC analysis of biochemical assays was performed using a Shimadzu LC-6AD pump with a SPD-20A UV-Vis detector (Shimadzu Scientific Instruments, Inc, Columbia, MD) at a wavelength of 254nm. Analyte separation was achieved using a 250 x 4.6mm Ultrasphere C18 column with 5μm particle size (Beckman, Brea, CA) and mobile phase containing 12.5% methanol and 1% acetic acid at a flow rate of 1.2ml/min.
Statistical Analysis

Results are expressed as means ± standard error. Statistical analysis of the data was compared using the student’s t test or ANOVA followed by post-hoc analysis. Differences were considered significant at $p<0.05$.

2.4 Results

*Vanin-1 null mice are sensitive to acetaminophen-induced hepatotoxicity*

Previous gene array studies in our laboratory established an association between Vnn1 gene upregulation and protection against APAP in mice receiving CFB pretreatment (Moffit, Koza-Taylor et al. 2007). To determine whether the absence of Vnn1 expression renders mice susceptible to APAP hepatotoxicity, male Vnn1-null mice were treated with APAP (400mg/kg, i.p.). Liver toxicity was assessed by plasma alanine aminotransferase (ALT) activity and histopathological analysis of liver sections at 24 and 48 hours after treatment. At 24 hours the mean plasma ALT activity was three-fold higher in the knockouts, 768 ± 161 U/L compared to 255 ± 75 U/L in wild-type mice (Figure 2.1A). By 48 hours, the mean plasma ALT activity in Vnn1 knockout mice was four times that of wild-type mice (936 ± 151 and 223 ± 55 U/L, respectively). The result of the histopathological analysis of liver samples is shown in Figure 2.1B and supports the ALT findings. The tissue scores (on a scale of 0 to 4) show that there was more severe hepatocellular damage in Vnn1 knockout mice than in wild-types at both 24 and 48 hours. Together, these results show that mice
Figure 2.1

A

Plasma ALT Activity (U/L)

Wildtype
Vnn1 KO

0hr 24hr 48hr

Time after acetaminophen treatment

B

Necrosis Score

Control WT Control KO 24hr WT 24hr KO 48hr WT 48hr KO
**Figure 2.1.** Plasma ALT Activity and Hepatic Necrosis Scoring after Acetaminophen Treatment. Plasma and livers were isolated from mice 24 and 48hr following 400mg/kg APAP or vehicle treatment. (A) Data presented as mean plasma ALT activity (U/L) ± SEM (n = 6-10 animals). (B) Hepatic necrosis score for each wild-type (closed shapes) and Vnn1 knockout (open shapes) mice (n = 4-5 animals). Horizontal lines represent the mean score for each group. Liver sections were stained with hematoxylin and eosin and scored on a 0-4 scale in 0.5 increments. Asterisks (*) represent a statistical difference (p < 0.05) between wild-type and knockout at same time point.
lacking Vnn1 are more susceptible to APAP hepatotoxicity, which is consistent with the hypothesis that Vnn1 expression is protective against APAP injury.

Susceptibility of Vanin-1 null mice to APAP is not due to differences in APAP metabolism

Enhanced APAP toxicity may be caused by increased bioactivation to the reactive metabolite NAPQI or by a decreased ability to detoxify APAP or its reactive intermediate. Therefore, the higher susceptibility of Vnn1 knockout mice to APAP toxicity could be the result of genotype-related differences in the expression of APAP metabolizing or detoxifying enzymes between Vnn1 nulls and wild-types. To examine this possibility, qRT-PCR was performed on livers collected from naïve, fasted Vnn1 knockout and wild-type mice. Figure 2.2A shows that no differences in hepatic mRNA expression of APAP bioactivating enzymes Cyp1a2, 3a11 or 2e1 are observed between genotypes. Additionally, no differences were evident in the gene expression of UDP-glucuronosyltransferase 1a6 (Ugt1a6), a major enzyme involved in APAP detoxification. Lastly, no alterations in the expression of genes involved in the disposition of APAP metabolites, such as Mrp 2, 3 and 4 were detected either (Figure 2.2B). Together, these data indicate that Vnn1 knockout mice do not have compensatory differences in the expression of hepatic genes important during APAP metabolism and disposition.
Figure 2.2
Figure 2.2. Hepatic Gene Expression of Drug Metabolizing Enzymes and Transporters Involved in Acetaminophen Metabolism and Disposition. qRT-PCR analysis was performed on naïve wild-type and Vnn1 knockout mice following overnight fast. mRNA was quantified following extraction of total RNA from liver as described in “Materials and Methods.” (A) Gene expression of acetaminophen bioactivating (Cyp1a2, 2e1, 3a11) and detoxifying (Ugt1a6) enzymes. (B) Gene expression of canalicular (Mrp2) and basolateral (Mrp3, 4) efflux transporters involved in the transport of acetaminophen and its metabolites. The data are presented as mean gene expression ± SEM (n = 4 animals).
To ensure that the enhanced susceptibility of Vnn1 knockout mice to APAP toxicity is not due to differences in the ability of the liver to bioactivate or detoxify APAP, hepatic metabolism of APAP was performed in vitro. Microsomal incubations were prepared from frozen livers of both genotypes and their ability to bioactivate APAP was assessed by measuring the formation of APAP-NAC metabolite following the addition of NAC, NADP and APAP. Livers from both Vnn1 knockout and wild-type mice formed the APAP-NAC metabolite at similar rates (Figure 2.3A), indicating that a comparable amount of NAPQI was being generated in liver microsomal preparations of both genotypes.

The capacity of the liver to detoxify APAP by glucuronidation and sulfation was also assessed in vitro. APAP-Gluc formation was determined by incubating microsomal fractions with APAP and UDPGA, while APAP sulfation was monitored by incubating cytosolic fractions with PAPS, DTT and APAP. In both instances, incubations from both Vnn1 knockout and wild-type livers generated the APAP-Gluc and APAP-Sulf metabolites at similar rates (Figure 2.3B and C, respectively). Collectively, gene expression for drug metabolizing enzymes and transporters as well as in vitro metabolism analysis strongly indicate that the increased susceptibility of Vnn1 knockout mice to APAP hepatotoxicity is unlikely due to differences in bioactivation, detoxification or disposition of APAP between genotypes.
Figure 2.3

A

nmol APAP-NAC/min/mg protein

Wildtype  Vnn1 KO

B

nmol APAP-Gluc/min/mg protein

Wildtype  Vnn1 KO

C

pmol APAP-Sulf/min/mg protein

Wildtype  Vnn1 KO
**Figure 2.3. In Vitro** Biochemical Analysis of Acetaminophen Bioactivation, Glucuronidation and Sulfation. Previously frozen livers were collected from untreated Vnn1 knockout and wild-type mice. Analyses were performed on microsomal (bioactivation, glucuronidation) and cytosolic (sulfation) fractions to determine the livers’ ability to form major APAP metabolites. Rates of formation of (A) APAP-NAC, (B) APAP-Gluc, and (C) APAP-Sulf metabolites. The data are presented as mean metabolite formation per minute per mg protein ± SEM (n = 4 animals).
Vanin-1 null mice have no deficiencies in APAP detoxification capacity by GSH

GSH status is an important factor in determining the extent of damage following APAP treatment, as covalent binding of APAP to cellular macromolecules is not detected until GSH is sufficiently depleted (Mitchell, Jollow et al. 1973). To investigate whether Vnn1 knockout mice have deficiencies in GSH synthesis and/or content, we first examined the hepatic mRNA expression of the catalytic subunit of gamma glutamylcysteine synthetase (Gclc). Vnn1 knockout mice exhibited 33% higher expression of Gclc when compared to wild-types, a slight but significant enhancement (Figure 2.4A). To determine whether an increase in Gclc gene expression translates into an increase in basal hepatic glutathione concentration in Vnn1 knockout mice, total GSH was quantified. Our analysis did not detect any variation in basal GSH concentration between genotypes, as livers contained a mean GSH content of 10.3 and 10.1 µmol/g wet liver tissue in Vnn1 knockout and wild-type mice, respectively (Figure 2.4B). It is therefore unlikely that the susceptibility of Vnn1 knockout mice to APAP hepatotoxicity is due to altered GSH content in liver.

A time-dependent GSH depletion study was performed to establish whether Vnn1 knockout mice utilize hepatic GSH differently than wild-types following APAP dosing. Differences between genotypes in GSH depletion or repletion following APAP administration would suggest that the livers of Vnn1-null mice have an altered capacity to regenerate GSH or to neutralize the reactive
Figure 2.4

A

Gclc mRNA (Fold Induction)

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B

total GSH (nmol/mg wet weight)

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* statistically significant difference
Figure 2.4. Glutathione Detoxification Pathway in Livers from Naïve Wild-type and Vnn1 Knockout Mice. (A) qRT-PCR was performed to quantify hepatic Gclc mRNA expression among both genotypes. (B) Total GSH content was examined in frozen livers from naïve Vnn1 knockout and wild-type mice as described in "Materials and Methods" and is expressed as nmol total GSH/mg wet liver weight. The data are presented as mean ± SEM (n = 4 animals). An asterisk (*) represents a significant difference (p < 0.05) between wild-type and Vnn1 knockout animals.
metabolite of APAP via GSH conjugation. Mice of both genotypes were treated with 400mg/kg APAP by i.p. injection and sacrificed immediately and at 2, 4 or 24 hours afterward. Total hepatic GSH concentration was then analyzed. APAP treatment resulted in >70% depletion of total hepatic GSH in both genotypes by 2 hours. GSH levels remained equally depleted at 4 hours in both Vnn1 null and wild-type mice (Figure 2.5A). Interestingly, GSH replenishment was more pronounced in Vnn1 knockout mice at 24 hours. Induction of hepatic Gclc gene expression was also greater at 2 and 4 hours after APAP treatment in the Vnn1 knockout mice relative to wild-types (Figure 2.5B). Together, these data show that hepatic basal GSH content and utilization after APAP treatment in Vnn1 knockout and wild-type mice are similar, thus suggesting that the enhanced APAP toxicity seen in knockout mice is not due to impaired detoxification by GSH.

Vanin-1 knockout mice exhibit deficiencies in compensatory hepatocellular proliferation and F4/80-positive macrophage infiltration following toxic acetaminophen insult

Activation of compensatory repair mechanisms is thought to be an important determinant of the extent of hepatotoxicity following toxic APAP insult (Jaeschke, Williams et al. 2011). This response involves the recruitment of immune cells to sites of injury and the proliferation of viable hepatocytes surrounding necrotic areas in centrilobular regions. It is possible then that disruption of these compensatory mechanisms could result in enhanced
Figure 2.5

A

![Graph showing total GSH (nmol/mg wet weight) over time (0 to 24 hours). The graph compares Wildtype and Vnn1 KO groups. The x-axis represents hours, and the y-axis represents total GSH (nmol/mg wet weight). The graph includes error bars indicating variability.]

B

![Bar graph showing hepatic mRNA expression (fold change) at different hours (0, 2, 4, 24). The graph compares Wildtype and Vnn1 KO groups. The x-axis represents hours, and the y-axis represents hepatic mRNA expression (fold change). The graph includes error bars indicating variability.]

Figure 2.5. Timecourse of Glutathione Homeostasis Following Depletion by Acetaminophen Treatment in Vnn1 Knockout and Wild-type Mice. Livers were collected 2, 4 and 24 hrs following 400mg/kg APAP treatment and analyzed as described in “Materials and Methods”. (A) Hepatic total GSH is depleted in both genotypes following APAP treatment. Data are expressed as nmol total GSH/mg protein. (B) Hepatic gene expression of Gclc is enhanced in Vnn1 knockout mice following APAP treatment. An asterisk (*) represents a statistical difference ($p < 0.05$) between genotypes at the same time point ($n = 3$-7 animals).
Figure 2.6
Figure 2.6. Histopathological Analysis of Livers from Vnn1 Wild-type and Null Mice Receiving Acetaminophen. 48 hours after treatment, less infiltration by immune cells (small blue nuclei) is evident within areas of centrilobular necrosis. Also, less compensatory proliferation of hepatocytes is seen surrounding the necrotic areas as illustrated by the presence of fewer binucleated hepatocytes (arrowheads). Magnification = 20x.
APAP toxicity. To investigate this, we analyzed H&E stained liver sections from Vnn1 knockout and wild-type mice sacrificed 48 hours after 400mg/kg APAP treatment. The Vnn1 knockout mice had noticeably less infiltration of immune cells within areas of centrilobular necrosis compared to wild-type mice (Figure 2.6) despite having more severe hepatocellular damage. This is interesting because the amount of infiltrating immune cells generally correlates with the severity of damage following APAP injury (Laskin, Pilaro et al. 1986; Laskin and Laskin 2001). The knockouts also displayed decreased numbers of bi-nucleated hepatocytes surrounding the areas of injury (Figure 2.6), which can be considered as an indirect indication of lower compensatory hepatocyte proliferation.

To more conclusively determine whether Vnn1 knockout mice have a deficiency in compensatory hepatocellular proliferation and recruitment of immune cells following APAP injury, paraffin embedded liver sections were stained for specific antigens by immunohistochemistry. Actively mitotic cells were stained with an antibody recognizing proliferating cell nuclear antigen (PCNA). PCNA-positive hepatocytes were evident in approximately equal amounts surrounding areas of centrilobular damage in both genotypes 24 hours after APAP (Figure 2.7B, E). However, positive staining of nuclei was less prevalent in the Vnn1 knockout compared to wild-type mice at 48 hours, indicating a delayed deficiency in cellular proliferation in response to APAP injury in these mice (Figure 2.7C, F).
Figure 2.7
Figure 2.7. Hepatic PCNA Protein Expression is Enhanced in Vnn1 Knockout and Wild-type Mice following Toxic Acetaminophen Exposure. Livers were removed either 24 (B, E) or 48 (C, F) hours following 400mg/kg APAP treatment and fixed in formalin. Immunohistochemistry was performed on paraffin embedded sections as described in “Materials and Methods.”
Figure 2.8
Figure 2.8. Hepatic F4/80 Protein Expression in Vnn1 Knockout and Wild-type Mice following Toxic Acetaminophen Exposure. Livers were removed either 24 (B, E) or 48 (C, F) hours following 400mg/kg APAP treatment and fixed in formalin. Immunohistochemistry was performed on paraffin embedded sections as described in “Materials and Methods.” Fewer F4/80-positive cells were evident within areas of centrilobular necrosis in Vnn1 knockout mice 48 hours (but not 24 hours) after APAP treatment. Rather, the majority of F4/80-positive cells instead resided in periportal regions.
Liver sections were also stained with anti-F4/80 antibody, a marker of resident macrophages. Once again, no differences in F4/80 staining were observed between genotypes at 24 hours (Figure 2.8B, E). In contrast, F4/80 positive cells were less likely to be found within areas of necrosis in Vnn1 knockouts compared to wild-type mice at 48 hours (Figure 2.8C, F). Instead, F4/80-positive cells in the knockout mice primarily resided in periportal regions despite the clear presence of centrilobular necrosis, suggesting a decrease in the infiltration of these cells 48 hours after injury.

Altered Expression of Genes involved in Macrophage Polarization in Vanin-1 Knockout Mice

The number of hepatic F4/80 positive cells has been shown to decrease in mice after APAP treatment, presumably as a result of macrophage activation (Dambach, Watson et al. 2002; Holt, Cheng et al. 2008). Thus, the observed decrease in infiltrating F4/80 positive cells within necrotic areas in Vnn1 knockout mice treated with APAP could be the result of a difference in macrophage polarization rather than a decrease in the number of mature macrophages present. To investigate whether macrophage polarization could be altered in Vnn1 knockout mice we selected several “M1” (pro-inflammatory) and “M2” (anti-inflammatory) genes for analysis by qRT-PCR using total RNA from whole liver obtained at 48 hours following APAP treatment. The M1 genes included in this analysis were tumor necrosis factor alpha (Tnfa), interferon gamma (Ifng), chemokine ligand 2 (Ccl2), inducible
nitric oxide synthase (iNos), myeloperoxidase (Mpo) and interleukins 1 beta (II-1β) and 6 (II-6). The M2 genes examined included transforming growth factor beta 1 (Tgfβ1), chemokine receptor 2 (Ccr2) and interleukins 4 (II-4) and 10 (II-10). Ifnγ is a primary cytokine involved in developing an M1 phenotype, while II-4 and II-10 are cytokines directly involved in M2 stimulation (Mantovani, Sozzani et al. 2002). In vehicle-treated mice, expression of iNos, an M1 gene, and IL-4, an M2 gene, were reduced in Vnn1 knockouts by 2.9 and 4.3 fold, respectively (Figure 2.9). No other genes examined varied in their expression between genotypes following vehicle treatment (control mouse livers). APAP treatment resulted in the differential expression of several M1 genes. Gene expression of Ifnγ, iNos, Tnfα, and Ccl2 were reduced by 2.7, 2.8, 5.1 and 38 fold in Vnn1 knockout mice, respectively (Figure 2.9A). In contrast, no differences in expression were seen in any of the M2 genes at the same time point (48 hours) (Figure 2.9B), suggesting a decrease in overall “pro-inflammatory” signaling in livers of Vnn1 knockout mice.

2.5 Discussion

In the present study, we investigated the susceptibility of Vnn1 knockout mice to APAP hepatotoxicity. 400mg/kg APAP treatment resulted in enhanced hepatotoxicity in the knockouts at both 24 and 48 hours as measured by plasma ALT activity and histopathological scoring of liver sections. This increase in toxicity occurs despite no observed differences in
Figure 2.9

![Graph A](image1)

![Graph B](image2)
Figure 2.9. Hepatic Gene Expression of Markers Involved in Macrophage Polarization. qRT-PCR analysis was performed on wild-type and Vnn1 knockout mice following control or 48hr APAP treatment (400mg/kg, i.p.). mRNA was quantified following extraction of total RNA from liver as described in "Materials and Methods." (A) Expression of pro-inflammatory genes. (B) Expression of anti-inflammatory genes. The data are presented as mean relative gene expression ± SEM (n = 4-6 animals). Asterisks (*) represent a statistical difference (p < 0.05) between wild-type and knockout at same time point.
gene expression of APAP metabolizing enzymes (Cyp1a2, 2e1, 3a11, Ugt1a6) or hepatic efflux transporters (Mrp2, 3 and 4). In vitro APAP metabolism studies confirmed that there are no significant alterations in the capacity of livers from Vnn1 knockout mice to bioactivate or detoxify APAP by CYP450 and glucuronidation or sulfation reactions, respectively. Interestingly, Vnn1 knockout mice bred on a BALB/c background (instead of the C57BL6/J used in the present studies) have significantly greater basal hepatic GSH concentrations and γ-glutamylcysteine synthetase (γ-GCS) activity in comparison to their wild-type counterparts (Martin, Penet et al. 2004). As a result, these mice exhibit resistance to toxicity and/or disease development in multiple models of oxidative stress (Berruyer, Martin et al. 2004; Martin, Penet et al. 2004). For reasons unknown, the phenotype of enhanced hepatic GSH content is not evident in Vnn1 knockout mice bred on a C57Bl/6 background as shown in Figure 2.4.

Gene expression of Gclc, the catalytic subunit of γ-GCS, is slightly but significantly elevated in the livers of naïve Vnn1 knockout mice, although this does not translate into increased hepatic total basal GSH content. Similarly, no differences in hepatic GSH utilization after APAP treatment were seen until the recovery phase (24hr) at which time GSH repletion was augmented in the Vnn1 knockout mice (Figure 2.5). These data are consistent with a possible inhibitory effect of GSH synthesis by cystamine in wild-type mice and also the enhanced gene expression of Gclc observed in the knockouts. Alternatively, elevated expression of Gclc and GSH repletion in the Vnn1 knockout mice
may be explained by an increased activation of compensatory repair pathways (i.e. Nrf2) as a result of enhanced toxicity. Overall, the current data suggest that the increased susceptibility of Vnn1 knockouts is not due to a decrease in the capacity of the liver to detoxify APAP via GSH.

Tissue repair is an important process in determining the outcome of acute liver injury (Mehendale 2005). One possible explanation for the heightened susceptibility of Vnn1 knockout mice to APAP is that Vnn1 may have a participatory role in compensatory repair mechanisms following tissue injury. This mechanistic concept is supported by the decrease in bi-nucleated hepatocytes we detected in areas surrounding foci of centrilobular necrosis (Figure 2.6). Additional support to the impaired compensatory hepatocellular proliferation in the Vnn1 knockout mice is provided by the lack of PCNA-positive hepatocytes (Figure 2.7) noticed after toxic APAP exposure.

Vnn1 knockout mice had less infiltration of immune cells 48 hours after APAP treatment despite having more necrosis (Figure 2.6), an unexpected result given that the amount of infiltrating immune cells generally correlates with the severity of APAP injury (Laskin, Pilaro et al. 1986; Laskin and Laskin 2001). This finding was confirmed by a prominent decrease in the number of F4/80-positive cells within areas of hepatic centrilobular necrosis at 48 hours after APAP administration (Figure 2.8), indicating that some of the cells that are normally present at this time point after APAP treatment appear to be mature macrophages. Moreover, hepatic gene expression of Ccl2, a monocyte chemokine associated with pro-inflammatory activity, was greatly
reduced in Vnn1 knockout mice at the same time point. In agreement with this, a deficiency in hepatic macrophage recruitment in Vnn1 knockout mice following bacterial infection has been previously documented (Meghari, Berruyer et al. 2007), further suggesting that migration of the monocyte/macrophage lineage is likely affected by a lack of Vnn1 expression. This lineage has been shown to be protective during APAP intoxication, as mice depleted of Kupffer cells (the resident macrophages of the liver) are more susceptible to subsequent APAP hepatotoxicity (Ju, Reilly et al. 2002; Campion, Johnson et al. 2008).

Overall, none of the four anti-inflammatory M2 genes probed was differentially regulated in Vnn1 knockout livers at 48 hours after APAP treatment (Figure 2.9). By contrast, the gene expression of four out of seven pro-inflammatory M1 targets was significantly reduced, suggesting a shift from a pro-inflammatory environment in livers of Vnn1 knockout mice. Phagocytosis is a function primarily attributed to the M1 phenotype of macrophages and is important in the clearance of necrotic debris. A decrease in debris clearance could therefore contribute to the enhanced APAP-induced toxicity in Vnn1 knockout mice. In addition to macrophages, Vnn1 likely contributes to the migration of other populations of immune cells following APAP treatment that we have not yet investigated. For example, anti-Vnn1 antibody treatment inhibits the entry of immature T lymphocytes into the thymus in mice (Aurrand-Lions, Galland et al. 1996). Together, these data are suggestive that Vnn1 contributes to the recruitment and possibly the
polarization of multiple populations of immune cells. In this way, a lack of Vnn1 may compromise repair processes following toxic APAP treatment by an immune-mediated mechanism.

Vnn2, another GPI-linked protein that shares roughly 64% sequence similarity with Vnn1, is present on human neutrophils and CD-14 positive monocytes and has been studied as a possible regulator of leukocyte migration and adhesion (Maras, Barra et al. 1999; Suzuki, Watanabe et al. 1999; Sendo, Takeda et al. 2003). It is hypothesized that GPI anchored proteins are involved in signal transduction, cell adherence and macrophage polarization (Sendo and Araki 1999; Huang, Takeda et al. 2004; Paulick and Bertozzi 2008; Dangaj, Abbott et al. 2011) and that these signals are propagated through the engagement of lipid rafts on the cell surface (Simons and Toomre 2000). Thus, as a GPI-anchored protein, Vnn1 may have important cell signaling functions beyond its recognized function as pantetheinase, although additional experiments are needed to confirm this hypothesis.

Cysteamine, the catalytic product of pantetheine hydrolysis, has been used clinically as an antidote for APAP overdose (Prescott, Newton et al. 1974); however, the mechanism by which cysteamine acts has not been elucidated. Cysteine and other APAP antidotes that enhance GSH synthesis lose their protective effect when GSH synthesis is inhibited by buthionine sulfoximine treatment. Cysteamine remains protective in these conditions (Miners, Drew et al. 1984), suggesting that the mechanism of protection is
independent of GSH synthesis. Although administration of cysteamine has been shown to partially inhibit CYP450 activity (Peterson, Peterson et al. 1989), this is unlikely to be the cause of antidotal protection because the proportion of a dose of APAP that is bioactivated to the reactive metabolite is unchanged following cysteamine treatment (Miller and Jollow 1986; Peterson and Brown 1992).

Given the protective properties of cysteamine, Vnn1’s pantetheinase activity provides another potential mechanism of protection against APAP hepatotoxicity. It is unknown whether cysteamine is protective against APAP hepatotoxicity at endogenous concentrations. The 20-50µM cysteamine concentration in mouse liver is appreciably lower than other non-protein thiols such as GSH (Duffel, Logan et al. 1987; Moffit, Koza-Taylor et al. 2007), suggesting that any direct binding of NAPQI to cysteamine is minimal. Also, mice that are protected from APAP by CFB pretreatment have increased hepatic concentrations of oxidized cystamine (and an increase in Vnn1 gene expression), while concentrations of reduced cysteamine are unchanged (Moffit, Koza-Taylor et al. 2007).

Maintenance of thiol:disulfide balance has been well studied in relation to cell homeostasis and is known to affect proliferative and apoptotic pathways (Townsend 2007) and may also influence inflammation (Iyer, Accardi et al. 2009). Oxidized glutathione, cystine and cystamine are all thought to regulate these pathways by protein disulfide exchange, which results in the inactivation of enzymes through the modification of critical
cysteine sites. Specifically, *in vitro* assays have shown that cystamine can inhibit several key enzymes involved in metabolism, proliferation and apoptosis including glycogen phosphorylase b, aldose reductase and caspase 3 (Miller, Sies et al. 1990; Cappiello, Voltarelli et al. 1996; Mallis, Buss et al. 2001; Lesort, Lee et al. 2003; O'Brian and Chu 2005). It has been suggested that cystamine can modulate the immune system by a similar inhibitory mechanism (Hsu, Chiang et al. 2007; Ozaki, Kaibori et al. 2007). One can anticipate that under oxidative stress and acute GSH depletion, cystamine dominates over cysteamine and elicits its inhibitory effect on these pathways. It is likely that oxidized cystamine acts as a sensor when the cellular redox status is altered, inhibiting thiol-sensitive cellular processes that can produce oxidative stress (i.e. glucose metabolism) in an attempt to reduce the production of ROS within the cell. Thus, a lack of cysteamine/cystamine signaling could represent the loss of a feedback loop during times of oxidative stress (for example, following APAP treatment) and may result in enhanced ROS production and augmented hepatotoxicity.

The studies presented here indicate that mice lacking Vnn1 are more susceptible to APAP hepatotoxicity without any differences in bioactivation or detoxification of the parent compound. The mechanism of this susceptibility remains unknown, but our experiments demonstrate that compensatory immune and repair responses are deficient in Vnn1 knockout mice and may contribute to the enhanced toxicity seen. Additional experiments are needed to further characterize the recruitment of specific populations of immune cells
following APAP toxicity and to determine whether a deficiency in redox signaling may also contribute to the heightened hepatotoxicity seen in mice lacking Vnn1.
Chapter 3

Vanin-1 Overexpression is Protective against Acetaminophen Cytotoxicity in vitro although it Unlikely Contributes to Peroxisome Proliferator-mediated Protection Observed in HC04 cells

3.1 Abstract

Mice pretreated with peroxisome proliferators are protected against subsequent toxic APAP exposure. These mice exhibit elevations in hepatic mRNA and catalytic products of Vanin-1 (Vnn1). To determine whether Vnn1 contributes to the mechanism of protection, the human cell line HC04 was employed. HC04 responsiveness to APAP (0.2-15mM APAP) and the peroxisome proliferators clofibrate (CFB; 0.5-1.0mM) and Wy-14,643 (50-100μM) was examined. 15mM APAP treatment for 24hrs resulted in moderate cytotoxicity as determined by 49% lactate dehydrogenase (LDH) leakage. 48hr 100μM Wy-14,643 produced peak upregulation of peroxisome proliferator activated receptor (PPAR)-responsive genes acyl CoA oxidase 1 (Acox1), adipocyte differentiation related protein (Adrp) and Vnn1 by 2.1, 3.1 and 6.3 fold, respectively. This treatment regimen also protected HC04 cells from subsequent cytotoxicity from 15mM APAP as determined by a 25% reduction in LDH leakage compared to vehicle treatment. To determine whether Vnn1 might be a contributor to the mechanism of protection, Vnn1 protein and pantetheinase activity were measured in HC04 cells following Wy-14,643 treatment. Vnn1 protein was unchanged and activity was reduced to
10% of the basal rate, suggesting that Vnn1 is not involved with the protective effect. The reduction in pantetheinase activity was due to serum starvation in the treatment medium, although its addition failed to increase activity beyond the basal rate. Furthermore, the addition of serum to the media did not increase the protective effect of Wy-14,643. To determine whether Vnn1 overexpression was protective against APAP cytotoxicity, Vnn1 was stably transfected into HC04 cells. When treated with APAP, HC04-VN1 cells exhibited a mild but reproducible resistance as determined by a 10% reduction in LDH leakage. HC04-VN1 and PP-treated HC04 cells provide two new mechanistic tools to explore protection against APAP toxicity.

**Keywords:** peroxisome proliferators, acetaminophen, Vanin-1
3.2 Introduction

Acetaminophen (APAP) accounts for nearly half of all acute liver failure cases in the United States (Lee 2010). The majority of a therapeutic dose of APAP is metabolized safely via glucuronidation and sulfation conjugation pathways. As a minor route of metabolism, bioactivation occurs via oxidation by cytochrome P450s 1A2, 2E1 and 3A4 to form the reactive metabolite n-acetyl-p-benzoquinone imine (NAPQI) which is typically detoxified by intracellular glutathione (GSH). Following APAP overdose, glucuronidation and sulfation pathways become saturated and GSH stores become depleted from increased generation of NAPQI. Free NAPQI is then available to bind to cellular macromolecules within the liver in the absence of GSH, eventually leading to centrilobular necrosis (Jollow, Mitchell et al. 1973).

Peroxisome proliferators (PPs) are a large collection of endogenous and exogenous chemicals that activate the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα). Activated PPARα forms a heterodimer with the retinoid x receptor (RXR) and binds to peroxisome proliferator response elements (PPREs) in the promoter region of target genes, stimulating their transcription. Many PPARα target genes are involved in the β-oxidation of fatty acids and thus increase the capacity of the liver to metabolize lipids. In hepatocytes, β-oxidation occurs within both peroxisomes and mitochondria in a process that is initiated by the binding of Coenzyme A (CoA) and free fatty acids.
The hypolipidemic fibrate drugs are a well-known class of PPs and are protective against APAP hepatotoxicity in rodents both \textit{in} and \textit{ex vivo} (Nicholls-Grzemski, Calder et al. 1992; Manautou, Hoivik et al. 1994; Manautou, Silva et al. 1998). Protection against APAP by clofibrate (CFB), a PP, does not appear to be due to alterations in APAP metabolism (Manautou, Tveit et al. 1996; Chen, Hennig et al. 2000) or by PPAR-mediated inductions in catalase (Chen, Hennig et al. 2002) or NAD(P)H quinine oxidoreductase 1 (Nqo1) (Moffit, Aleksunes et al. 2007). Additionally, protection is dependent on PPAR\(\alpha\) activation (Chen, Hennig et al. 2000) but is independent of changes in APAP-protein adduct formation and GSH depletion (Manautou, Emeigh Hart et al. 1996).

PPAR\(\alpha\) activation also results in the upregulation of Vascular non-inflammatory molecule 1 (Vanin-1; Vnn1). The Vnn1 gene encodes for pantetheinase, a glycosylphosphatidylinositol (GPI)-anchored ectoenzyme that hydrolyzes pantetheine to pantothenic acid and cysteamine (2-aminoethanethiol). Pantothenic acid is a major precursor of CoA, suggesting that pantetheinase may have a regulatory role in CoA synthesis. In support of this hypothesis, rats treated with a novel pantetheinase inhibitor have increased hepatic triglyceride and decreased cholesterol content (Jansen, van Diepen et al. 2013), presumably due to a decrease in available CoA.

Cysteamine, the other product of pantetheine hydrolysis, exists in both a reduced and oxidized disulfide form (cystamine). Vnn1 knockout mice lack endogenous cysteamine, suggesting that pantetheinase is the primary source
of the thiol in vivo (Pitari, Malergue et al. 2000). Cysteamine is a relatively reactive small molecule—the pKa of its thiol group is similar to that of cysteine and lower than glutathione (GSH), dithiothreitol (DTT) and N-acetylcysteine (NAC) (Winterbourn and Metodiewa 1999). The reactivity of cysteamine has been demonstrated in several in vitro models that illustrate its ability to undergo thiol disulfide exchange with redox sensitive cysteine groups of numerous proteins including glycogen phosphorylase b, aldose reductase, h-ras, caspase 3 and gamma-glutamylcysteine synthetase (γGCS) (Griffith, Larsson et al. 1977; Lebo and Kredich 1978; Miller, Sies et al. 1990; Cappiello, Voltarelli et al. 1996; Mallis, Buss et al. 2001; Lesort, Lee et al. 2003). Furthermore, cysteamine protects rodents against several model hepatotoxicants, including APAP (Castro, De Ferreyra et al. 1973; MacDonald, Gandolfi et al. 1984; Miners, Drew et al. 1984).

Vnn1 mRNA is upregulated in mice following PP treatment and has been identified as a potential target gene for contributing to the mechanism of protection against APAP (Moffit, Koza-Taylor et al. 2007). Hepatic pantothenic acid and cystamine content are also increased in CFB pretreated animals, suggesting that Vnn1 upregulation results in a physiologically-relevant increase in pantetheinase catalytic activity. Furthermore, our laboratory has shown that mice lacking Vnn1 are susceptible to APAP toxicity (see Chapter 2). This enhanced toxicity occurs despite there being no differences in APAP bioactivation or detoxification and is correlated with deficiencies in compensatory immune response and hepatocellular repair.
The current experiments have been carried out to investigate whether induction of Vnn1 contributes to the PP-mediated protection against APAP in vitro. Here we show that the PP-mediated mechanism of protection against APAP seen in rodents is relevant to human HC04 cells in vitro. Furthermore, protection is observed in a hepatocyte monoculture, indicating that at least a portion of the protective mechanism occurs without contributions of the immune system. Finally, additional experiments reveal that the protection mediated by Wy-14,643 in HC04 cells is unlikely due to Vnn1, although Vnn1 overexpression results in partial protection against APAP cytotoxicity.

3.3 Materials and Methods

Cell Culture and Treatment

All materials were purchased from Sigma Aldrich (St. Louis, MO) unless stated otherwise. HC04 cells were obtained as a gift from Dr. Urs Boelsterli and housed in a humidified chamber with 5% CO₂ at 37°C. Cells were maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 10mM glucose. 2x10⁵ cells were seeded onto sterile 24-well plates in 1.0ml of complete DMEM for 24hrs prior to treatment. APAP was dissolved in DMEM directly, while Wy-14,643 and CFB were dissolved in DMSO prior to addition to DMEM. Final concentration of DMSO in media was 0.1%. For protection studies, PP treatment lasted for 48hrs following the initial 24hr plating period. The cells were then rinsed with PBS before addition of DMEM containing
APAP for an additional 24hrs (96hr total culture period) before determining LDH leakage.

Cytotoxicity Assay

Lactate Dehydrogenase (LDH) leakage was assessed with the Tox-7 kit according to the manufacturer's instructions. Briefly, media was collected from culture dishes following treatment and diluted in Triton-X100 (1% final volume) and set aside. Remaining media was removed from cultures and replaced with fresh media containing 1% Triton-X100. The cells were incubated for 30-45 minutes at 37°C. After confirming cell lysis, lysates were collected and centrifuged for one minute at 12,000 x g. LDH content in media and lysate fractions were determined in a 96 well plate following incubation with cofactor, substrate and dye as supplied by the manufacturer. Leakage is expressed as a percentage in media compared to total (media + lysate).

Preparation of cDNA and qRT-PCR

Total RNA was prepared from HC04 cell lysates using the RNeasy Mini kit (Qiagen). cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) and random primers. qRT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY) using SYBR green and species-specific primer pairs for each gene—β Actin forward CCA TCG AGC ACG GCA TC and reverse ATT GTA GAA GTG GTG GTG CA GA; Vnn1 forward TTC TTG TAC TGG GGT CTG GC and reverse GCT GGA
ACT TCA ACA GGG AC; Acox1 forward ATG CCC AAG TGA AGA TCC AG 
and reverse GAA GAT GAG GGA GTT TGG CA; Adrp forward AGG GGC 
TAG ACA GGA TTG AGG A and reverse TTT TCT ACG CCA CTG CTC 
ACG. Gene expression is quantified by the ΔΔCT method and normalized to 
β Actin.

*Western Blotting*

Protein was quantified from whole cell lysates prepared from HC04 
cells by standard procedures following the assay of total protein by the DC 
Protein Assay (Bio-Rad, Hercules, CA). Whole cell lysates were boiled at 
95°C for 5 minutes in 2x sample buffer prior to gel loading. PVDF membranes 
were blocked with 5% nonfat dry (NFD) milk in tris buffered saline with 0.2% 
Tween (TBS-T) overnight at 4°C. Membranes were incubated with 
appropriate primary antibodies for Vnn1 (#Ab96171; Abcam, Cambridge, MA) 
or βActin (Ab8227; Abcam) for 2hrs at room temperature in 2% NFD milk in 
TBS-T and then incubated with peroxidase conjugated anti-rabbit IgG 
secondary at room temperature for an additional 2hrs. Finally, probed 
membranes were incubated in ECL western blot detection reagent (GE 
Healthcare, Buckinghamshire, UK) according to the manufacturer’s 
instructions prior to exposure to X-ray film.
**Vnn1 Construct Assembly and Overexpression**

Vnn1 cDNA obtained from HC04 cells was amplified by PCR before purification with the QIAquick PCR purification kit (Qiagen, Germantown, MD). After ligation to the pcDNA 3.1 plasmid vector (Invitrogen, Carlsbad, CA) (3:1 vector to insert ratio) and transformation of DH5α, colonies were selected from agar plates containing 100μg/ml ampicillin. Plasmid DNA from collected colonies was isolated using a QIAprep Miniprep kit (Qiagen). Following sequencing, the DNA insert was compared to the published coding sequence of hVnn1. After confirming the sequence of the inserted DNA, the construct was amplified in DH5α and purified by GenElute Plasmid Maxiprep kit.

Prior to transfection, HC04 cells were seeded in a 6-well plate for 24hrs. Transfection of cells with TransIT 2020 reagent and either empty vector (EV) or Vnn1 construct (VN) occurred over a 48hr period to allow for integration with DNA according to the manufacturer’s instructions (Mirus Bio, Madison, WI). Following transfection, cells were selected with complete DMEM containing 2.0mg/ml G418 (Invitrogen) for two weeks, followed by 1.0mg/ml G418 treatment for an additional 3 weeks, changing media twice per week.

**Pantetheinase Activity Assay**

Pantetheinase activity was measured as previously described by Ruan, et al. (Ruan, Cole et al. 2010) with several modifications. Whole cell lysates were prepared in RIPA buffer and diluted 1:10 in PBS prior to the
addition of 50μl to each well of a black 96 well plate (Fisher Scientific, Waltham, MA). A standard curve was also prepared by diluting human recombinant VNN1 (Sino Biological, Beijing, China) in PBS. Pantothenate-AMC (P-AMC), a gift from Pfizer (Cambridge, MA), was added so that each well contained 100μl of Vnn1 standard or cell lysate (1:20 v/v), 20μM P-AMC, and 5% RIPA in PBS. Fluorescence (ex: 350nm, em: 460nm) was measured every 2 minutes over a 40 minute period. Pantetheinase activity was calculated by measuring the slope of the reaction (RFU/sec) and normalizing to total protein content by the DC Protein Assay (described above).

Statistical Analysis

Results are expressed as means ± standard error. Statistical analysis of the data was compared using the student’s t test or ANOVA followed by post-hoc analysis. Differences were considered significant at p<0.05.

3.4 Results

Response of HC04 Cells to Acetaminophen Treatment

To determine whether HC04 cells would serve as a suitable model for examining the PP-mediated protection against APAP, we examined their responsiveness to both APAP and peroxisome proliferator treatment. HC04 cells were plated in complete media before being treated with APAP in serum-free media 24hrs later. Following a 24hr exposure to APAP, the cells exhibit a time and dose-dependent increase in cytotoxicity (Figure 3.1A).
Figure 3.1

A  HC04

B  Mouse Primary Hepatocytes
Figure 3.1. Dose-Dependent Increases in Acetaminophen Cytotoxicity *in vitro*. Toxicity assessed by leakage of Lactate Dehydrogenase into media. (A) Human HC04 cells were exposed to 0.2-15mM APAP for either 24 or 48hrs. (B) Primary hepatocytes were exposed to 0.25-25mM APAP for 24hrs following their isolation from naïve mice. Data presented as percent leakage (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between the indicated treatment group relative to the appropriate control at the same time point.
At this time point, 15mM APAP was required to achieve 49% LDH leakage. For comparison, primary murine hepatocytes were isolated and seeded onto culture plates as described in Materials and Methods. A similar dose response was performed following 24hr exposure to APAP, at which time 40% LDH leakage was observed in cells treated with 5mM (Figure 3.1B), suggesting that HC04 cells were similarly sensitive to APAP cytotoxicity.

Response of HC04 Cells to Peroxisome Proliferator Treatment

HC04 cells were treated with 0.5 or 1.0mM CFB for 48 hours to examine their response to PP treatment. PPARα-responsive genes acyl CoA oxidase (Acox1) and Vnn1 were upregulated by 2.6 and 18.8 fold, respectively, at 1.0mM but not 0.5mM (Figure 3.2A). LDH leakage was also elevated from 9.3 to 60.6% at this dose compared to control, indicating that upregulation of Vnn1 could be due to cytotoxicity rather than the direct result of PPAR activation (Figure 3.2B).

To investigate whether HC04 cells are protected against APAP cytotoxicity by CFB at the lower 0.5mM dose, cells were pretreated with 0.5mM CFB for 48hrs prior to 15mM APAP treatment for an additional 24hrs. Assessment of LDH leakage revealed that 43% of total LDH leaked into the media in cells treated with APAP with or without CFB pretreatment, indicating that CFB exposure was not protective (Figure 3.3). Together the data demonstrate that CFB treatment only induced PPARα-responsive genes at
Figure 3.2

A

[CFB] (mM)

mRNA Fold Change

ACOX1

VNN1

*

B

[CFB] (mM)

LDH Leakage (%)

0
20
40
60
80
*

*
**Figure 3.2.** HC04 Cells are Refractory to Upregulation of PPAR Responsive Genes upon Subtoxic Exposure to Clofibrate. Cells were treated with the indicated concentrations of CFB in media containing 0.1% DMSO for 48hr. (A) Treatment with 1.0mM, but not 0.5mM, CFB resulted in the upregulation of mRNAs for both Acox1 and Vnn1 (n=4 per group). (B) Cytotoxicity occurred in HC04 cells following CFB treatment at both intermediate (0.75mM) and high (1.0mM) concentrations (n=3 per group). Data presented as mean ± SEM for all groups. Asterisks (*) represent a statistical difference (p < 0.05) between the indicated treatment group relative to the appropriate control.
Figure 3.3

LDH Leakage (%)

Control 0.5mM CFB 15mM APAP CFB + APAP

* * *

0 10 20 30 40 50
Figure 3.3. Clofibrate Pretreatment does not Protect HC04 Cells against Subsequent Acetaminophen-induced Cytotoxicity. Where indicated, cells were pretreated with 0.5mM CFB for 48hrs prior to APAP exposure for an additional 24hrs. Data presented as mean ± SEM (n=4 per group). Asterisks (*) represent a statistical difference ($p < 0.05$) between the indicated treatment group relative to control.
the high 1.0mM cytotoxic dose, and that the 0.5mM dose that did not result in 
gene induction was insufficient in protecting HC04 cells against APAP.

HC04 cells were then treated with Wy-14,643 to determine whether 
insensitivity to PPARα activation by CFB was compound-specific. Following 
48hr treatment, Vnn1 mRNA was induced in a dose and time-dependent 
manner with a maximum induction of four-fold over control (Figure 3.4). The 
most pronounced induction of Vnn1 was achieved by treatment with 100μM 
Wy-14,643 for 48hrs, therefore this treatment regimen was chosen for 
subsequent experiments. Furthermore, gene induction occurred in the 
absence of any elevation in LDH leakage, suggesting that induction of Vnn1 
mRNA was the result of PPAR activation rather than cytotoxicity.

To ensure that Wy-14,643 treatment resulted in activation of PPARα, a 
time course was performed to examine the induction of PPAR target genes by 
qRT-PCR. mRNA for both Acox1 and adipocyte differentiation related protein 
(Adrp) were upregulated by 100μM Wy-14,643 treatment (Figure 3.5A, B). 
Acox1 mRNA was slightly but significantly upregulated in Wy-14,643-treated 
HC04 cells by 1.4 and 2.1 fold at 24 and 48hrs, respectively, compared to 
vehicle-treated cells. Wy-14,643-mediated upregulation of Adp was seen as 
early as 6hrs and continued through 48hrs of treatment at which time a 
maximum induction of 3.1 fold over vehicle control was observed (Figure 
3.5C). Similar to Acox1, Vnn1 upregulation did not occur until 24hrs after 
treatment—inductions of 4.2 and 6.3 fold were observed at 24 and 48hr, 
respectively.
Figure 3.4
Figure 3.4. Wy-14,643 Induces Vnn1 Gene Expression in HC04 Cells at Subtoxic Concentrations. Induction of Vnn1 mRNA by Wy-14,643 in HC04 cells is both time and dose dependent. Data presented as mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) in Vnn1 gene expression between the indicated treatment group relative to the appropriate control.
Figure 3.5

A

ACOX1

0.1% DMSO
100uM Wy-14,643

mRNA Fold Change

Hours

B

ADRP

0.1% DMSO
100uM Wy-14,643

mRNA Fold Change

Hours

C

VNN1

0.1% DMSO
100uM Wy-14,643

mRNA Fold Change

Hours
Figure 3.5. Time Course of PPAR Responsive Gene Upregulation in HC04 Cells following Wy-14,643 Exposure. Cells were exposed to 100uM Wy-14,643 for 1-48hrs prior to isolation of total RNA. (A) Acyl CoA Oxidase (ACOX1) and (C) VNN1 mRNAs were upregulated in Wy-treated cells relative to 0.1% DMSO vehicle control at both 24 and 48hrs. (B) Adipose Differentiation Related Protein (ADRP) mRNA was upregulated as early as 6hrs following Wy-14,643 treatment. Data are normalized to vehicle control at 1hr and presented as mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between the indicated treatment group relative to vehicle control at the same time point.
To investigate whether Wy-14,643 treatment protects HC04 cells against APAP cytotoxicity, cells were pretreated with 100μM Wy-14,643 for 48hrs prior to treatment with 15mM APAP for an additional 24hrs. Moderate cytotoxicity was achieved in cells receiving APAP as determined by an increase in LDH leakage from 9 to 38% compared to control (Figure 3.6). By contrast, LDH leakage was only 28.5% in cells receiving Wy-14,643 prior to APAP, indicating a 25% protection against APAP-induced cytotoxicity in these cells.

Following the observation that Wy-14,643 treatment increases the gene expression of Vnn1 in HC04 cells (Figure 3.5C), we wanted to determine whether PP-mediated protection in HC04 cells could be due to enhanced Vnn1 protein expression and/or catalytic activity. No induction of Vnn1 protein was observed following the same Wy-14,643 treatment (Figure 3.7A). Furthermore, pantetheinase activity quantified by a fluorescent signal following pantothenate-AMC hydrolysis revealed that hydrolytic activity was reduced by 10 fold in whole-cell lysates from both vehicle- and Wy-14,643-treated cells.

In an additional experiment, we demonstrated that the decrease in pantetheinase activity in both the 0.1% DMSO and 100μM Wy-14,643 treatment groups was due to a lack of FBS in the media during the 48hr treatment period (Figure 3.8A). When the cells were instead treated with either vehicle or Wy-14,643 in complete media, pantetheinase activity in
Figure 3.6

LDH Leakage (%)
Figure 3.6. HC04 Cells are Partially Protected against Acetaminophen Cytotoxicity by Wy-14,643 Pretreatment. Where indicated, cells were treated with 100uM Wy-14,643 for 48hr prior to 24hr APAP exposure. Data presented as mean ± SEM (n=4 per group). Asterisks (*) represent a statistical difference ($p < 0.05$) between the indicated treatment group relative to control. # indicates statistical significance ($p < 0.05$) among APAP treated groups.
Figure 3.7

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vnn1</th>
<th>βActin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive HC04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100μM Wy-14-643</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinant hVnn1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Pantetheinase Activity (RFU/s/mg protein)

- Untreated
- 0.1% DMSO
- 100μM Wy

* Significant difference
Figure 3.7. Vnn1 is an Unlikely Contributor to PPAR-mediated Protection against Acetaminophen in HC04 Cells. Cells were exposed to 100uM Wy-14,643 for 48hrs prior to collection of whole lysates as described in the Materials and Methods. (A) No changes in Vnn1 protein were observed following vehicle control or Wy-14,643 treatment. (B) Pantetheinase activity is reduced following vehicle or Wy-14,643 treatment relative to untreated HC04 cells. Pantetheinase activity is expressed as the mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) between the indicated treatment group relative to untreated cells.
Figure 3.8

**A**
Pantetheinase Activity (RFU/s/mg protein)

Untreated
Untreated (SF)
0.1% DMSO
100μM Wy

**B**

Vnn1

βActin

**C**

LDH Leakage (%)

Control
100μM Wy
15mM APAP
Wy + APAP

* Significance compared to control.
# Significance compared to 100μM Wy.
Figure 3.8. Serum Starvation of HC04 Cells Results in Inhibition of Pantetheinase Activity without Affecting Vnn1 Protein Content or Protection against Acetaminophen. HC04 cells were exposed to either complete or serum-free (SF) media during the 48hr treatment period. (A) Cells exposed to SF media for 48hrs without any other treatment resulted in a drastic reduction of Pantetheinase activity. (B) No alterations in Vnn1 protein concentration were observed in any of the treatment groups. (C) The degree of protection seen in HC04 cells against APAP by Wy-14,643 pretreatment is unchanged by treating in complete media. Pantetheinase activity is expressed as the mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference (p < 0.05) compared to all other groups.
whole cell lysates was maintained at a basal rate, though neither treatment exceeded this. Additionally, Wy-14,643 treatment in complete media did not result in an increase of Vnn1 protein (Figure 3.8B) or additional protection from APAP (Figure 3.8C) in HC04 cells. Interestingly, the decrease in pantetheinase activity seen in serum-starved cells was not accompanied by a decrease in Vnn1 protein, suggesting that inhibition of Vnn1 catalytic activity may be due to posttranslational modification. Overall, the data suggest that the mechanism of protection afforded by Wy-14,643 is not due to enhanced Vnn1 protein expression or catalytic activity.

**Cells Stably Overexpressing Vnn1 are Partially Protected from Acetaminophen Cytotoxicity**

Cells were transfected with a construct containing the human Vnn1 coding sequence as described in the Materials and Methods. Following selection of G418-resistant cells over the course of four weeks, cells were grown in 10cm dishes to confluence. Gene and protein expression of Vnn1 were measured to confirm overexpression. Cells transfected with the Vnn1 construct (VN) exhibited a 250-fold upregulation of Vnn1 gene expression compared to both wild-type (WT) and cells transfected with empty vector (EV) (Figure 3.9A). Protein expression and enzyme activity were increased in whole cell lysates collected from VN cultures (Figure 3.9B, C). Pantetheinase activity was 17.7-fold higher compared to EV cultures, suggesting that translated Vnn1 was being processed into the functional enzyme.
Figure 3.9

A

VNN1

mRNA Fold Change

Wildtype EV VN

0 100 200 300 *

B

Pantetheinase Activity (RFU/s/mg protein)

EV VN

0 10 20 30 40 50 400 500 600 700 800 *

C

Pantetheinase Activity (RFU/s/mg protein)

EV VN

0 10 20 30 40 50 60 70 80 90 100
Figure 3.9. Characterization of HC04 Cells Stably Overexpressing Vnn1. Stable overexpression was achieved as described in Materials and Methods. (A) Gene and (B) protein expression of Vnn1 following transfection of either Empty Vector (EV) or Vnn1 construct (VN). (C) Pantetheinase activity in HC04 whole cell lysates following transfection with either EV or VN. Data are expressed as mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) in the VN group relative to EV cells.
To determine whether HC04 cells overexpressing Vnn1 are protected against APAP hepatotoxicity, EV and VN cells were treated with 15mM APAP in complete media for 24hrs. APAP treatment of VN cells resulted in a slight but statistically significant decrease in LDH leakage of 28.7% compared to 32.5% in EV cells, a 12% reduction (Figure 3.10). No protection was observed when the cells were treated with APAP in serum free media (data not shown), suggesting that Vnn1 catalytic activity is important to the modest protective effect seen.

3.5 Discussion

The present experiments describe the investigation of whether the mechanism of PP-mediated protection from APAP-induced toxicity involves Vnn1. Human hepatocyte-derived HC04 cells were chosen for our model based on the observation that these cells retain some expression of APAP-metabolizing CYP450s in culture (Lim, Tan et al. 2007). As a result, HC04 cells exhibit a cytotoxic response when exposed to APAP that is similar to primary human hepatocytes while other cell lines such as HepG2 are refractory (Lim, Tan et al. 2007). In our hands, APAP treatment results in cytotoxicity of HC04 cell cultures at a dose similar to that required in primary murine hepatocytes. Although HC04 cells did not upregulate PPAR-responsive genes at subtoxic doses of CFB, 100μM Wy-14,643 treatment resulted in both time and dose-dependent inductions of Acox1, Adrp and Vnn1. As expected based on these results, CFB pretreatment did not protect
Figure 3.10
**Figure 3.10.** HC04 Cells Overexpressing Vnn1 Exhibit Modest Resistance against Acetaminophen-induced Cytotoxicity. EV and VN transfected cells were treated with 15mM APAP for 24hrs in complete media. Data are represented as the mean (n=3 per group) ± SEM. Asterisks (*) represent a statistical difference ($p < 0.05$) between the indicated treatment group relative to control. # indicates statistical significance ($p < 0.05$) among APAP treated groups.
against toxic APAP exposure, whereas Wy-14,643 pretreatment reduced APAP-induced LDH leakage by 25%.

While 48hr pretreatment of HC04 cells with Wy-14,643 is partially protective against APAP in our model in vitro, mice pretreated with CFB repeatedly in vivo are completely protected against APAP-induced changes in liver histopathology and elevations in plasma sorbitol dehydrogenase (SDH) activity (Manautou, Hoivik et al. 1994). The reason for this discrepancy in the degree of protection between model systems is unclear. Mice that are pretreated with just a single dose of CFB prior to APAP intoxication are partially, but not completely protected against increases in plasma SDH activity (Manautou, Emleigh Hart et al. 1996). Furthermore, Nicholls-Grzemski and colleagues demonstrated that hepatocytes are protected against paraquat in vitro following their isolation from CFB treated mice (Nicholls-Grzemski, Burcham et al. 1996), though the protected cells exhibit mild cytotoxicity at higher doses of paraquat. Therefore, it is possible that in our model either the protective effect is simply being overcome at higher doses of APAP, or that a mechanism contributing to protection is lost when going from an in vivo to an in vitro system (i.e. loss of any immune-mediated effects). The current monoculture experiments indicate that although extra-hepatocellular mechanisms may contribute to the protective effect stimulated by PPs, protection is at least partially elicited directly on the hepatocyte.

Similar to PP exposure, expression of Vnn1 is also protective against APAP toxicity in vivo. Mice lacking Vnn1 are more susceptible to APAP
hepatotoxicity and have deficiencies in compensatory hepatic immune response and repair pathways (Chapter 2). Since PP treatment in rodents results in protection against APAP and is associated with an upregulation in Vnn1 gene expression and catalytic products in liver (Moffit, Koza-Taylor et al. 2007), we investigated whether Vnn1 upregulation contributes to the PP-mediated mechanism of protection against APAP injury.

As described above, pretreatment of HC04 cells with Wy-14,643 in serum-free media resulted in partial protection against APAP cytotoxicity. Protection by Wy-14,643 was associated with an induction of Vnn1 mRNA. Further investigation revealed that Vnn1 protein concentration was unchanged and catalytic activity was reduced to 10% of the basal rate, suggesting that Vnn1 protein was unlikely contributing to the mechanism of protection in these cells. An additional experiment performed in media containing 10% FBS indicated that the decrease in pantetheinase activity was due to serum starvation.

The reduction in pantetheinase activity without a loss of Vnn1 protein in serum-starved cells indicates that the enzyme may be posttranslationally modified following 48hr incubation in serum-free media. Although Vnn1 protein contains several sites for N-glycosylation, the molecular weight did not appear to change as determined by western blotting, providing evidence that glycosylation of the mature protein was unaltered by serum-free treatment. Pantetheinase catalytic activity is inhibited by disulfides such as pantethine and cystamine (Pitari, Maurizi et al. 1994), suggesting that changes in the
The redox environment of serum-starved HC04 cells *in vitro* may impair Vnn1-dependent pantetheinase activity.

Similar experiments were performed to determine whether Vnn1 activity would be enhanced by Wy-14,643 treatment in media containing 10% FBS. After 48hrs, neither Vnn1 protein nor activity increased above control levels. Additionally, the inclusion of serum in Wy-14,643 treatment medium had no effect on the magnitude of protection against APAP. Together, the above experiments indicate that Wy-14,643 does not enhance Vnn1 protein expression or catalytic activity and further indicate that Vnn1 is unlikely involved in the mechanism of PP protection in HC04 cells.

An upregulation in Vnn1 mRNA without an increase in protein has previously been observed in mice fed a high-fat lard diet (Kirpich, Gobejishvili et al. 2011). Similarly, Wy-14,643 treatment of HC04 cells resulted in upregulation of Vnn1 mRNA without increasing protein in whole cell lysates after the 48hr treatment period. Although the data are suggestive that there are post-transcriptional regulatory mechanisms that control Vnn1 protein expression, we cannot rule out the possibility that a soluble form of Vnn1 (lacking the GPI anchor) is produced and released into the media upon Wy-14,643 treatment. A soluble form of human Vnn2 has been shown to be released from neutrophils upon stimulation by Tnfα (Nitto, Araki et al. 2002); a similar transcriptional mechanism represents a possible explanation as to why Vnn1 protein did not increase in whole cell lysates despite the induction of mRNA following PPAR activation.
Following the Wy-14,643 treatment studies we wanted to investigate whether overexpression of Vnn1 protein could protect against APAP *in vitro*. HC04 cells were stably transfected with either a Vnn1 overexpression construct or empty vector. Successful selection of HC04-VN1 cells was confirmed by enhanced mRNA and protein expression of hVnn1, as well as catalytic activity compared to empty vector transfected cells (Figure 3.9A-C). Upon exposure to APAP in complete media, HC04-VN1 cells exhibited a slight but significant and reproducible resistance to cytotoxicity (Figure 3.10). This resistance was not evident when the cells are exposed to APAP in serum-free media (data not shown), suggesting that components within FBS are critical to the protective effect mediated by Vnn1 overexpression.

There are multiple factors that likely limit the effectiveness of Vnn1 overexpression in protecting against APAP in our *in vitro* model. For example, pantetheine, the endogenous substrate of pantetheinase, cannot be synthesized by mammalian cells and is instead acquired from the diet. DMEM is not supplemented with pantetheine and our suppliers do not routinely measure the pantetheine content in FBS. The observation that Vnn1 overexpression is partially cytoprotective in complete but not serum-free media indicates that components within FBS are necessary for the protective effect in Vnn1 overexpressing cells. These findings are consistent with experiments showing a reduction in pantetheinase activity in cell lysates following the incubation of HC04 cells in serum-free media. Together, the data suggest that Vnn1 catalytic activity is required for the protective effect
seen in APAP treated HC04-VN1 cells. Presumably, protection by this mechanism stems from the production of cysteamine that occurs from Vnn1-mediated pantetheine hydrolysis, though we have not yet confirmed this hypothesis.

Our cell culture system also neglects the immune effects of Vnn1. Vnn1 has been shown to contribute to the accumulation of distinct populations of immune cells by multiple loss of function models through the use of Vnn1 knockout mice and neutralizing antibodies (Aurrand-Lions, Galland et al. 1996; Meghari, Berruyer et al. 2007). Macrophages are among the cells whose migration appears to be affected by a loss of Vnn1 (Meghari, Berruyer et al. 2007) and are also believed to play a protective role in the immune response following APAP hepatotoxicity in vivo (Campion, Johnson et al. 2008). The use of HC04-VN1 cells in a co-culture model may then add additional insight into the immune effects of Vnn1 as well as increase the window of protection afforded to HC04-VN1 cells following toxic APAP exposure.

As mentioned throughout this text, previous work has demonstrated that rodents are protected against APAP toxicity following either single or repeated PP pretreatment (Manautou, Hoivik et al. 1994; Manautou, Emeigh Hart et al. 1996). The current experiments provide evidence that the protective mechanism is not a species-specific effect of PPs and is likely relevant to human health. Our studies in human HC04 cells are in agreement with studies performed in humanized mice treated with fenofibrate showing
that the PP-mediated protective mechanism is extended to human PPARα (Patterson, Shah et al. 2012).

Patterson et al. have recently investigated the involvement of mitochondria in the PP-mediated mechanism of protection following the observation that prolonged Jun N-terminal kinase (JNK) activation mediates hepatocellular destruction following toxic APAP exposure through the collapse of the mitochondrial permeability transition (Han, Shinohara et al. 2010; Win, Than et al. 2011). They have shown that Wy-14,643 treatment prevented APAP-induced phosphorylation of JNK, a transcriptional regulator of genes involved in survival/apoptosis, proliferation and inflammatory pathways. Furthermore, pretreatment of mice with Wy-14,643 prevented mitochondrial, but not whole liver, GSH depletion by APAP. APAP-induced oxidative stress was also reduced as measured by mitochondrial H$_2$O$_2$ concentration, together suggesting a reduction in mitochondrial stress in the protected mice (Patterson, Shah et al. 2012).

Mice lacking uncoupling protein 2 (UCP2) are not protected against APAP by Wy-14,643 pretreatment, suggesting that UCP2 is an important contributor to the protective effects of PPs against APAP hepatotoxicity (Patterson, Shah et al. 2012). UCP2 is a mitochondrial anion carrier within the inner mitochondrial membrane (IMM) that acts as a mild uncoupler of the electron transport chain (ETC). Activation of UCP2 occurs by mechanisms that are not yet fully understood (Brand and Esteves 2005). Upregulation of UCP2 following PP treatment suggests that it is a negative regulator of the
ETC during times of enhanced β-oxidation and may be protective by limiting the production of reactive oxygen species (ROS) during cellular respiration. Activation of redox sensitive UCP2 may occur directly by increased mitochondrial ROS during times of enhanced fatty acid catabolism. Opening of UCP2 decreases the proton gradient between the intermembrane space and the mitochondrial matrix—lowering the mitochondrial membrane potential and reducing the production of both ATP and ROS. This “mild” uncoupling of the mitochondrial ETC differs from the classical concept of mitochondrial uncoupling in which the proton gradient is irreversibly compromised.

The current experiments describe the development of a human in vitro model to investigate the protective mechanism against APAP by PPs. Here we demonstrate that the mechanism of protection is not species-specific and can be at least partially reproduced in a monoculture developed from a human hepatocyte cell line. Although the data indicate that Vnn1 upregulation is not involved in the protective effect observed in HC04 cells, stable overexpression of Vnn1 provides these cells with a modest resistance from APAP cytotoxicity. Additional experiments are needed to characterize this resistance, which may be further optimized by the addition of pantetheine or the implementation of a co-culture system.

3.6 Acknowledgements
The authors would like to thank Dr. Urs Boelsterli for the gift of the HC04 cells. We would also like to thank Dr. Margaret Fleming and Pfizer for the gift
of the P-AMC Vnn1 substrate used in the pantetheinase activity assay. Finally, we thank Dr. Xinsheng Gu for his technical advice regarding the development of the HC04-VN1 cells.
Chapter 4
Conclusions

Vnn1 is a gene involved with both redox maintenance and immune cell recruitment processes. Catalysis of pantetheine by pantetheinase, the enzyme encoded by Vnn1, is the main source of the endogenous thiol cysteamine in mice in vivo (Pitari, Malergue et al. 2000). Administration of cysteamine is protective in multiple murine models of hepatotoxicity including APAP, galactosamine and CCl₄ (Castro, De Ferreyra et al. 1973; Ferreyra, de Fenos et al. 1977; MacDonald, Gandolfi et al. 1984; Miners, Drew et al. 1984; MacDonald, Thayer et al. 1987) and has been used clinically as an antidote to APAP poisoning (Prescott, Newton et al. 1974; Prescott, Sutherland et al. 1976). The precise mechanism of protection is not well understood but does not appear to involve alterations in toxicant bioactivation or detoxification by direct binding or enhancing GSH synthesis.

Vnn1 is also a contributor to the extravasation and targeting of multiple populations of immune cells, including macrophages (Aurrand-Lions, Galland et al. 1996; Meghari, Berruyer et al. 2007). Studies that deplete Kupffer Cells by clodronate liposome treatment indicate that these macrophages limit APAP-induced hepatotoxicity (Campion, Johnson et al. 2008). Furthermore, cultured bone marrow-derived macrophages isolated from mice lacking Vnn1 have deficiencies in pro-inflammatory M1 polarization following stimulation by LPS (Meghari, Berruyer et al. 2007), suggesting that Vnn1 may influence the polarization of immune cells in addition to their recruitment.
This thesis evaluates the contribution of Vnn1 as a protective gene during APAP-induced hepatotoxicity both in vivo and in vitro. In Chapter 2 we examined the impact of a loss of Vnn1 in the susceptibility of mice to APAP injury. We hypothesized that a loss of Vnn1 would render mice more susceptible to APAP, based on previous data indicating that increases in Vnn1 mRNA and catalytic byproducts in liver correlated with protection against APAP by peroxisome proliferators (Moffit, Koza-Taylor et al. 2007).

The present studies confirm that Vnn1 knockout mice are more susceptible to hepatotoxicity by APAP despite no alterations in the capacity for hepatic bioactivation of the parent compound or detoxification by glucuronidation, sulfation or GSH pathways. Results indicate that compensatory proliferation of hepatocytes surrounding the areas of centrilobular necrosis is impaired in Vnn1 knockout mice relative to wild-types 48hr following APAP injury. At the same time point, F4/80-positive cells are deficient within necrotic centrilobular foci of Vnn1 knockouts, suggesting that the recruitment and/or polarization of macrophages are altered in response to APAP treatment. qRT-PCR analysis of markers for M1 and M2 polarization revealed that four out of seven genes associated with M1 polarization were deficient in Vnn1 knockout livers compared to wild-type mice 48hrs after APAP. By contrast, none of the four M2 polarization genes was differentially expressed. Together, our studies indicate that the expression of Vnn1 is protective against APAP-induced hepatotoxicity in vivo and that
compensatory pathways involved in hepatocellular proliferation and immune cell recruitment or polarization are deficient in mice lacking Vnn1.

Future studies to continue research in this area should address the hypothesis that differences in recruited immune cells are responsible for the differential susceptibility to APAP hepatotoxicity. A full characterization of immune cells whose recruitment may be deficient in Vnn1 knockout mice should be explored by either FACS or immunofluorescent techniques. Because we see changes in some, but not all cytokines, it is unlikely that a loss of Vnn1 results in a global deficiency in immune cell recruitment. It is far more likely that certain cell types, or even subpopulations of the same cell type (i.e. M1 vs M2 macrophage subpopulations) are selectively affected.

It is also possible that genotype-specific differences in cell death programming could contribute to differential APAP susceptibility. To address this possibility, laser capture microdissection of perinecrotic hepatocytes could be performed to investigate differential expression patterns of genes involved in apoptotic, repair and proliferative pathways in Vnn1 wild-type and knockout mice following APAP treatment. Additionally, a genotype- and time-dependent comparison among gene expression signatures of cytokines and chemokines in necrotic and perinecrotic cells could identify hepatocellular differences responsible for the attraction of different cell populations.

In Chapter 3 the development of an in vitro model to examine the contribution of Vnn1 upregulation during peroxisome proliferator-mediated protection against APAP toxicity is discussed. Based on in vivo data from
studies performed in mice we hypothesized that peroxisome proliferator treatment of HC04 cells would result in elevated Vnn1 protein and in turn contribute to protection against APAP cytotoxicity. HC04 cells were selected for the model based on the observation that they maintain some expression of CYP450 enzymes responsible for bioactivation of APAP (Lim, Tan et al. 2007). APAP cytotoxicity in HC04 cells occurred in a dose and time dependent manner that was similar to freshly isolated murine hepatocytes.

While cells were refractory to PPAR activation by subtoxic concentrations of CFB, treatment with Wy-14,643 was successful in inducing the PPAR regulated genes Adrp, Acox1 and Vnn1 without any observed cytotoxicity. 48hr treatment with 100μM Wy-14,643 treatment resulted in resistance to APAP-induced cytotoxicity in HC04 cells. Although Vnn1 mRNA was upregulated in these cells, no increase in Vnn1 protein was detected and basal pantetheinase activity in whole cell lysates was dramatically reduced. Additional experiments indicated that the reduction in pantetheinase activity was due to treatment in serum-free media. Addition of serum to the Wy-14,643 treatment media did not increase Vnn1 protein or pantetheinase activity beyond basal values, nor did it increase the window of protection against APAP cytotoxicity. Together, our studies involving Wy-14,643 treatment indicate that although Vnn1 mRNA is upregulated, Vnn1 is unlikely to be a contributor to the peroxisome proliferator-mediated protection against APAP observed in HC04 cells.
Although the current studies suggest that Vnn1 protein is not increased in HC04 cells by Wy-14,643 treatment, the possibility remains that protein was produced and not detected by western blotting of whole cell lysates. For example, we cannot rule out the possibility that a soluble form of Vnn1 is synthesized and released into the culture medium upon PPAR activation. A soluble form of Vnn2 has been shown to be secreted by neutrophils following stimulation by Tnfα (Nitto, Araki et al. 2002), indicating that at least one member of the Vnn family of proteins does not necessarily need to be bound by a GPI anchor. In our current model, any soluble Vnn1 protein produced would be removed from the cultures along with the Wy-14,643 treatment media prior to the addition of fresh media containing APAP. In a future experiment it would be interesting to perform an ELISA assay for human Vnn1 with media collected following either Wy-14,643 or vehicle treatment to determine whether this is a possibility.

Furthermore, our monoculture system cannot exactly replicate the responses of the liver in vivo following peroxisome proliferator treatment. Though our model resulted in partial protection against APAP by Wy-14,643 pretreatment, CFB pretreatment results in complete protection in vivo (Manautou, Hoivik et al. 1994). We were not able to replicate complete protection in our model, suggesting that the protective mechanism(s) may not entirely reflect the in vivo situation. Despite this caveat, the present results indicate that the mechanism of protection is not a rodent-specific effect and occurs at least in part in a hepatocyte monoculture without the involvement of
other cell types. Additional experiments are needed in order to conclusively
determine whether Vnn1 is involved with peroxisome proliferator-mediated
protection against APAP \textit{in vivo}. To do this, Vnn1 wild-type and knockout
mice should be treated with CFB prior to toxic APAP exposure. A loss of the
protective effect in the knockout mice would indicate the involvement of Vnn1
\textit{in vivo}.

In the final section of Chapter 3, we investigated whether enhanced
Vnn1 protein expression in HC04 cells is protective against APAP. Cells
transfected with Vnn1 were selected and confirmed by elevated mRNA and
protein expression, as well as pantetheinase expression compared to empty
vector transfected cells. Exposure of HC04-VN1 cells to APAP in complete
media resulted in a modest, but significant and highly reproducible resistance
to LDH leakage compared to HC04-EV cells. By contrast, exposure of the
cells to APAP in serum-free media did not result in protection.

This model has the potential for further optimization to increase the
protection against APAP observed in HC04-VN1 cells in future studies. The
observation that protection occurs only in the presence of serum in the
treatment media suggests that components in FBS are necessary for the
Vnn1-mediated protective effect. For example, pantetheine, the substrate of
Vnn1 catalysis cannot be synthesized by mammalian cells. It is not a
component of the DMEM media, nor is it measured by our suppliers of FBS. It
is possible that pantetheine is needed for the protective effect and that its
addition could increase the resistance to APAP cytotoxicity observed in HC04-VN1 cells.

It is also a distinct possibility that proper redox balance is not maintained in the extracellular environment of our *in vitro* model. Vnn1 is an ectoenzyme that is anchored to the external layer of the plasma membrane and is sensitive to the external redox environment. Pantethine and cystamine disulfides are known to inhibit Vnn1 activity; thus, the accumulation of disulfides in the media following APAP treatment may elicit an inhibitory effect on the enzyme anchored to HC04-VN1 cells. For the above reasons, it would be an interesting experiment to add reduced pantetheine to the APAP treatment media prior to exposure of HC04-VN1 and HC04-EV cells. This would not only provide the cells with Vnn1 substrate but also increase the availability of reduced thiol in the media.

Our hepatocyte monoculture model ignores the contribution of other cell types that may provide additional protection against APAP. Since Vnn1 has distinct immune effects involving the recruitment and polarization of immune cells, co-culture studies using HC04-VN1 cells along with non-parenchymal cells such as macrophages and neutrophils could be employed in the future. These studies could provide evidence as to whether Vnn1 is protective through the involvement of specific populations (or polarizations) of immune cells. Co-cultures treated with APAP could subsequently be probed for differences in gene expression profiles related to cytokine signaling and oxidative stress.
Together, the present studies indicate that Vnn1 expression is protective during APAP-mediated toxicity *in vivo* and *in vitro*. Though our *in vitro* model has not implicated Vnn1 in the protective effect elicited by Wy-14,643 treatment of HC04 cells, this mechanism of protection appears to be relevant to humans and occurs at least partially without the influence of extra-hepatocellular stimuli.

Our *in vivo* studies indicate that a lack of Vnn1 reduces both compensatory repair and immune recruitment mechanisms that occur following APAP insult. Additional experiments are needed to determine whether these deficits are central to the susceptibility of Vnn1 knockout mice to APAP or whether their occurrence is secondary to changes in hepatocyte programming. It is clearly established in the literature that the immune system has the capability to either alleviate or aggravate APAP hepatotoxicity—our studies in Vnn1 knockout mice underline this potential. Vnn1 is a gene that establishes a clear link between oxidative stress and the immune system, making it a valid candidate for further mechanistic study involving APAP injury. Further optimization of the *in vitro* models described in this thesis may give additional insight to downstream effects of Vnn1 expression following APAP exposure and the involvement of both redox and immune signaling. Together with the use of Vnn1 knockout mice, these *in vivo* and *in vitro* approaches represent valuable tools in investigating the full influence of Vnn1 during APAP-induced injury.
References


