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Role of Nrf2 in the Regulation of Ppar Expression in Mice

Fabio Carvalho
fabio.carvalho@uconn.edu

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Role of Nrf2 in the Regulation of Ppar Expression in Mice

Fábio V. Carvalho

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Role of Nrf2 in the Regulation of Ppar Expression in Mice

Presented by

Fábio V. Carvalho, B.S.

Major Advisor _____

José E. Manautou, Ph.D.

Associate Advisor _____

Brian Aneskievich, Ph.D.

Associate Advisor _____

Yvonne Will, Ph.D.

University of Connecticut

2017

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LIST OF ABBREVIATIONS

58kD APAP-binding protein	58-ABP
Acetaminophen	APAP
Activator protein 1	Ap-1
Acyl-CoA oxidase	Aox
Acyl-CoA oxidase 1	Acox1
Aldo-keto reductase 1c1	Akr1c1
Antioxidant response element	ARE
Basic leucine zipper	bZIP
Breast cancer resistance protein	Bcrp
Catalase	Cat
Chromatin immunoprecipitation	ChIP
Clofibrate	CFB
Coenzyme A	CoA
Cytochrome P450	Cyp
Double glycine repeat	DGR
Drug induced liver injury	DILI
Erythroid Krüppel-like factor	EKLF
Fatty acid binding protein 4	Fabp4
Fatty acid transport protein	Fatp
Fatty acids	FA
Free iron	Fe(II)
GATA binding protein 1	GATA-1
Glucose transporter type 4	Glut4
Glutamate-cysteine ligase	Gcl
Glutamate-cysteine ligase catalytic subunit	Gclc
Glutamate-cysteine ligase modifying subunit	Gclm
Glutathione	GSH
Glutathione S-transferase	GST
Glycosylphosphatidyl inositol	GPI
Heme oxygenase 1	Hmox1
Hydrogen peroxide	H ₂ O ₂
Hypersensitive sites	HS
Interleukin	IL
Kelch-like ECH-associated protein 1	Keap1
Lipoprotein lipase	Lpl
Locus control region	LCR
Messenger ribonucleic acid	mRNA
Multidrug resistance-associated protein	Mrp
N-acetyl-para-benzo-quinone imine	NAPQI
N-Acetylcysteine	NAC
NAD(P)H:quinone oxidoreductase 1	Nqo1
Notch intracellular domain	NICD
Nrf2-ECH homology domain	NEH
Nuclear factor erythroid 2	NF-E2
Nuclear factor erythroid 2-related factor 2	Nrf2

Nuclear factor- κ B	NF- κ B
Peroxisome proliferator activated receptor	Ppar
Peroxisome proliferator response element	PPRE
Ppar γ coactivator	PGC
Pyruvate dehydrogenase	PDH
Pyruvate dehydrogenase kinase 4	Pdk4
Reactive oxygen species	ROS
Real time polymerase chain reaction	RT-PCR
Recombining binding protein suppressor of hairless	Rbpjk
Retinoic acid receptors	Rars
Retinoic acid response element	RARE
Retinoid X receptor	Rxr
Retinol binding protein 1	Rbp1
Superoxide dismutase	Sod
Thiazolidinediones	TZDs
Solute carrier	Slc
Sulfotransferase	Sult
Thyroid hormone receptors	Tr
Triacylglycerol	TAG
UDP-glucuronosyl transferase	Ugt
Vanin 1	Vnn1
Very low-density lipoproteins	VLDL
Vitamin D receptors	Vdr

Chapter 1

Literature Review

1.1 Liver Function and Structure:

The anatomy and physiology of the liver have been extensively studied and are well understood. The following overview is adapted from *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Jaeschke, 2012) and *Comprehensive Toxicology: Hepatic Toxicology* (McQueen, 2010).

Liver Physiology:

The liver's positioning between the intestines and the rest of the body allows it to effectively maintain metabolic homeostasis by metabolizing, detoxifying and excreting ingested nutrients, xenobiotics and environmental toxicants. These foreign compounds are brought to the liver, via the portal vein, following absorption in the stomach or intestine. Along with its major role in regulating metabolic homeostasis, the liver is also responsible for the synthesis of plasma proteins, such as albumin, the synthesis and secretion of bile and cholesterol, and the metabolism of fats.

Liver Anatomy:

In humans, the liver is a four lobed structure covered by a thin layer of connective tissue, known as Glisson's capsule. The liver is composed of various cell types, which consist of

hepatocytes and the cells of the sinusoid. Hepatocytes are the parenchymal cells of the liver and make up 60% of the cell population, as well as 80% of the volume of the liver. They are large cells with large amounts of mitochondria and endoplasmic reticulum and round nuclei containing peripherally dispersed chromatin. Hepatocellular nuclei vary in size and can often be binucleated. The hepatocytes are organized in a three-dimensional lattice where the spaces between cords of hepatocytes consist of the sinusoids, which act as the hepatocyte blood supply. Sinusoids consist mainly of endothelial cells, but also contain stellate cells (act in storage of vitamin A and synthesis of extracellular matrix proteins), Kupffer cells (resident macrophages of the liver), lymphocytes, and natural killer cells.

There are two ways in which the liver can be structurally organized, these being the lobule and the acinus. The lobular classification organizes the liver into hexagonal lobules around a central vein. At each of the six corners of the hexagon are portal triads, consisting of a portal vein, hepatic arteriole and bile duct. Blood entering the liver through both the portal vein and hepatic arteriole mix together before entering the sinusoids making its way to the central vein, where it exits the liver. The lobular structure consists of three zones (centrilobular, midzonal and periportal), which are often used in the description of pathological parenchymal lesions.

The acinar classification better depicts a functional hepatic unit. The acinus is diamond shaped and is centered on the terminal branches of the portal vein and hepatic artery. There are three zones of decreasing blood oxygen concentrations moving away from the portal blood vessels towards the central vein. Zone 1 is closest to the blood supply and contains blood with the highest oxygen content, zone 3 is closest to the central vein and blood is most depleted of

oxygen and zone 2 is located between the two. Due to the higher level of oxygen, hepatocytes in zone 1 are mitochondria-rich and are the most involved in fatty acid oxidation and gluconeogenesis. There is also a gradient of proteins and enzymes involved in xenobiotic metabolism and detoxification. Hepatocytes in zone 1 have higher concentrations of the antioxidant glutathione, whereas hepatocytes in zone 3 contain the bulk of the metabolic cytochrome P450 enzymes.

1.2 Nrf2:

Discovery:

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that acts in the activation and regulation of the cellular response to oxidative stress. It does this by inducing the transcription of a series of antioxidant-related genes, which allows it to regulate cell survival and defense (Jaramillo and Zhang, 2013). Nrf2 was first isolated during a screen for proteins that bind the locus control region (LCR) located at the 5' end of the β -globin gene cluster (Moi et al., 1994). The β -globin gene cluster on chromosome 11 consists of five genes, which encode for components of the globulin family of proteins, such as hemoglobin (Levings and Bungert, 2002). The LCR is a regulatory element in the β -globin locus containing four sites that are hypersensitive to DNase I. These four hypersensitive sites (HS) serve as binding sites for transcription factors, such as GATA binding protein 1 (GATA-1; regulator of blood cell differentiation in erythroid cells), Erythroid Krüppel-like factor (EKLF; erythroid transcription factor needed for human β -globin gene expression) and NF-E2 (nuclear factor erythroid 2) (Levings and Bungert, 2002).

From the LCR binding assay it was found that the protein now known as Nrf2 binds to the same hypersensitive site (HS2) as NF-E2. Nrf2 and NF-E2 were found to be a part of the same family of cap 'n' collar basic leucine zipper (bZIP) transcription factors and both act as strong activators of RNA polymerase II (Moi et al., 1994). There is, however, a difference in the expression levels of the two genes. NF-E2 is mainly expressed in erythroid cells, whereas Nrf2 is ubiquitously expressed at low levels. The highest levels of Nrf2 can be found in the liver, kidney and lung (Chan et al., 1996).

Regulation:

In normal cellular conditions Nrf2 is sequestered in the cytoplasm by the E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (Keap1). E3 ubiquitin ligases act to catalyze the isopeptide binding of ubiquitin to target proteins, thus targeting these proteins for proteasomal degradation (Kobayashi et al., 2004). Sequestration of Nrf2 occurs through an association between the Nrf2-ECH homology (NEH) 2 domain and the Keap 1 double glycine repeat (DGR) region, located at the C-terminal domain of the protein (Itoh et al., 1999). Interactions between the intervening region (IVR) domain of Keap1 and the Cul3-type E3 ligase complex then act in the ubiquitination of Nrf2 and promote its subsequent degradation.

In the presence of electrophiles, the repression of Nrf2 by Keap1 is inhibited and newly synthesized Nrf2 is able to translocate to the nucleus, where it can bind to the antioxidant response element (ARE) of target genes and mediate transcription of these genes (Watai et al., 2007). The exact mechanism of how electrophiles disrupt the repression is still unclear, however, it is known that the electrophile acts to prevent Keap1-mediated ubiquitination, and

subsequent degradation of Nrf2. Five models have been proposed to explain the mechanism behind the loss of Nrf2 repression. A Hinge and Latch model suggests that cysteine residues on Keap1 are modified by electrophiles, causing a disruption of the Keap1-Cul3 complex and preventing Nrf2 ubiquitination (Tong et al., 2006). Another postulate suggests that a Cul1 interference factor, cullin associated and neddylation dissociated 1 (CAND1), disrupts the Keap1-Cul3 complex. A third model suggests that oxidative stress promotes Keap1 self-ubiquitination and degradation. The other two mechanisms suggest disruption of a nuclear export signal, either on Keap1 or Nrf2, that senses oxidative stress and promotes nuclear accumulation of either the Keap1-Nrf2 complex or just Nrf2, respectively (Watai et al., 2007).

Antioxidant Response:

Nrf2 acts in the cell's response to oxidative stress by binding the ARE and regulating the transcription of genes involved in various antioxidant pathways.

Nrf2 controls the production of the antioxidant cofactor glutathione (GSH), which acts to scavenge free radicals in the cell. Nrf2 regulates the transcription of the two subunits of glutamate-cysteine ligase (Gcl), mainly Gclm (modifying subunit) and Gclc (catalytic subunit). Gcl catalyzes the rate-limiting step of GSH synthesis (Gorrini et al., 2013). Nrf2 also regulates the expression of NAD(P)H:quinone oxidoreductase 1 (Nqo1), which is involved in the reduction of quinones to hydroquinones. This prevents the formation of semiquinone radical intermediates and reduces cellular oxidative stress (Atia et al., 2014).

Oxygen is required by aerobic organisms to generate energy in the form of ATP, but oxygen can also be toxic. During normal metabolic function oxygen is converted into various

oxygen free radicals and oxygen species that cause cellular oxidative damage. It has been hypothesized that over time, oxidative stress builds up and causes a gradual reduction in cellular function. In this oxidative stress theory of aging it is believed that the gradual reduction in cellular function caused by oxidative stress is one explanation for the phenotype known as aging (Harman, 1956). Nrf2 regulates a series of genes, such as superoxide dismutase (Sod) and catalase (Cat), which function in the detoxification of damaging oxygen species that are key contributors to aging (Zhang et al., 2015). Sod is a family of enzymes responsible for catalyzing the dismutation reaction that converts superoxide anion radicals into oxygen and hydrogen peroxide. Cat is then able to catalyze the detoxification of hydrogen peroxide to oxygen and water (Zhang et al., 2015).

Nrf2 is also involved in the regulation of pathways that indirectly causes decreased oxidative stress. For example, Nrf2 regulates the transcription of heme oxygenase 1 (Hmox1). Hmox1 catabolizes heme molecules to produce free iron (Fe(II)). Fe(II) is a cofactor in the conversion of hydrogen peroxide to a very reactive hydroxyl radical and build up of Fe(II) in the cell results in increased levels of oxidative stress through the production of this radical. The increased formation of reactive oxygen species (ROS) is avoided by the detoxification and sequestration of excess Fe(II) through the actions of the ferritin complex, whose subunits are also regulated by Nrf2 (Gorrini et al., 2013).

Another function of Nrf2 is in the transcriptional regulation of transporters. These include multidrug resistance-associated protein 2 (Mrp2; Abcc2), multidrug resistance-associated protein 3 (Mrp3; Abcc3), multidrug resistance-associated protein 4 (Mrp4; Abcc4) and breast cancer resistance protein (Bcrp/Abcg2). The Mrps are members of the ATP-binding

cassette (ABC) family of ATP dependent efflux transporters. Mrp2 is mainly expressed in the hepatocyte canalicular membrane and functions in the export of glucuronate, sulfate and GSH conjugates into the bile for excretion (Jedlitschky et al., 2006). Mrp3 is a basolateral efflux transporter and acts as an alternate route for the export of bile acids and glucuronide conjugates into portal circulation (Belinsky, 2005). Mrp4 is another basolateral efflux transporter that acts to export bile acids and GSH conjugates. It also has a role in cell signaling through regulation of intracellular levels of cyclic nucleotides, such as cAMP (Russel et al., 2008). Bcrp is a canalicular efflux transporter and mediates the export of endogenous compounds, such as urate and folate, as well as many xenobiotics into the bile (Eldasher et al., 2013). The regulation of efflux transporters by Nrf2 serves as a protective mechanism by increasing the excretion of toxic compounds and decreasing cellular exposure to xenobiotics with the potential to produce oxidative damage.

1.3 Ppar:

The peroxisome proliferator activated receptors (Ppars) consist of three transcription factors (α , β/δ , and γ) that are part of the nuclear receptor superfamily. They heterodimerize with retinoid X receptor (Rxr) and function in energy metabolism via the regulation of fatty acids (FA) (Tyagi et al., 2011). Nuclear receptors share a well-conserved structure that consists of a DNA-binding domain and ligand-binding domain. The DNA-binding domain of Ppars recognize a six nucleotide direct repeat region in the promoter of target genes known as the peroxisome proliferator response element (PPRE). The binding of a Ppar-specific ligand allows dissociation of the heterodimer from co-repressor proteins and association with co-activator

proteins, such as Pparγ coactivator-1α (PGC1α), that are necessary for the association of a transcription-initiation complex (Kersten, 2014). The formation of the complex is followed by association of the heterodimer complex with the PPRE of target genes and modulation of the transcription of these genes.

1.3.1 Pparα:

Pparα is mainly expressed in the liver, however it is also expressed at lower levels in skeletal muscle, heart and bone. It functions in the regulation of hepatic FA oxidation (Tyagi et al., 2011) and also has protective functions in the antioxidant response (Manautou et al., 1994). Activation of Pparα is also involved in repression of tissue inflammation, (i.e. vasculature, spleen, liver). One mechanism explaining this effect is that Pparα activation antagonizes the nuclear factor-κB (NF-κB) signaling pathway, which is involved in the immune response to infection (Delerive, 2001). Pparα is typically activated by FAs during periods of fasting, but can also be activated by exogenous ligands known as peroxisome proliferators (Contreras et al., 2013).

Discovery:

Cloning of Pparα was first reported in 1990 following a screen of two murine cDNA libraries using two isolated clones. From this screening, a 468 amino acid protein, now referred to as Pparα, was identified (Issemann and Green, 1990). The screen was conducted following a study suggesting the presence of a peroxisome proliferator binding protein and another study that showed the ability of peroxisome proliferators to rapidly alter the transcription of specific

genes. Peroxisome proliferators are chemicals, such as the fibrate-type class of drugs, which cause hepatic proliferation of peroxisomes and hepatic hyperplasia when administered to rodents. Effects of administration of this class of compounds includes increased transcription of genes involved in peroxisomal FA β -oxidation (catabolism of long chain FAs) and increased transcription of the cytochrome P450 IV family genes (Isseman and Green, 1990).

Activation of Ppara:

The actions of Ppara activation occur through the modulation of Ppara-responsive genes. These hundreds of genes, which contain a PPRE in their promoter, encode proteins involved in functions such as microsomal, peroxisomal and mitochondrial FA oxidation, gluconeogenesis, triglyceride turnover, bile synthesis, bile secretion, and retinoid metabolism (Kersten, 2014). The genes targeted by Ppara activation are numerous and encode proteins such as Vanin 1 (Vnn1), Acyl-CoA oxidase 1 (Acox1), pyruvate dehydrogenase kinase 4 (Pdk4) and Cyp4a10.

Vanin 1: Vnn1 is an enzyme, specifically a pantetheinase, which is anchored to the cell surface by a glycosylphosphatidylinositol (GPI) moiety. Vnn1 is mostly expressed in the liver, kidney and gastrointestinal tract and it functions in the hydrolysis of pantetheine to vitamin B5 (pantotheic acid) and cysteamine (Chen et al., 2014). Vitamin B5 is a required component of Coenzyme A (CoA) synthesis. CoA is a coenzyme that plays a role in the synthesis and degradation of FA by transporting intermediates between enzyme active sites (Leonardi and Jackowski, 2007). Cysteamine is an aminothiols, which has

been shown to possess some antioxidant activity. Cysteamine appears to decrease ROS mainly through conversion to the amino acid taurine and increasing levels of the antioxidant enzyme superoxide dismutase. Cysteamine also has functions as an iron chelator, which reduces the production of reactive hydroxyl radicals in the cell (Petrov et al., 2012).

Acyl-CoA oxidase 1: Acox1 was the first Ppara-responsive gene to be identified and encodes the enzyme involved in the first (and rate-limiting) step of peroxisomal long-chain FA oxidation (Kersten, 2014).

Pyruvate dehydrogenase kinase 4: Pdk4 is an enzyme that is normally activated during periods of fasting. It inactivates pyruvate dehydrogenase (PDH) through phosphorylation. Inactivation of PDH results in a switch in fuel sources from oxidation of carbohydrates to oxidation of fat (Wang and Sahlin, 2012).

Cyp4a10: Cyp4a10 is a member of the Cyp4a subfamily of phase I metabolic enzymes known as cytochrome P450s. The Cyp4a subfamily is expressed in the rough endoplasmic reticulum of hepatocytes and functions in the ω hydroxylation of medium and long chain FAs. They oxidize the terminal C-H bond of FAs, which initiates the degradation and conversion of the FA to dicarboxylic acids. The dicarboxylic acids are then converted to fuels by β -oxidation in the mitochondria or peroxisome (Nyagode et al., 2014).

Species Differences:

Peroxisome proliferators are classified as non-genotoxic hepatocarcinogens, meaning that they have been demonstrated to produce liver tumors when chronically administered in rodents (Gonzalez and Shah, 2008). The peroxisome proliferators are classified as non-genotoxic because they cause tumor formation without direct interaction with DNA. Non-genotoxic carcinogens typically act by increasing cellular proliferation, disrupting cellular structures or creating an environment that increases the risk of mutations being formed (Lee et al., 2013). In rodents, the administration of peroxisome proliferators causes increased liver size (hepatomegaly), hepatocyte proliferation, peroxisome proliferation and oxidative stress. The oxidative stress is mostly due to the increased production of the acyl-CoA oxidase (Aox) enzyme. During the AOX-catalyzed step in fatty acid oxidation, hydrogen peroxide (H_2O_2) is produced as a byproduct (Gonzalez and Shah, 2008). Increased levels of H_2O_2 yields increased oxidative stress, which could lead to the formation of DNA adducts and subsequent gene mutations. Also, due to the ability of H_2O_2 to diffuse out of peroxisomes, damage to other cellular structures can occur. H_2O_2 has also been shown to interfere with the cell survival and apoptosis pathways through activation of NF- κ B, protein kinase C (a cell cycle regulator) and activator protein 1 (Ap-1; a cell growth and apoptosis regulator) (Reuter et al., 2010). The increased oxidative stress, damage to cellular structures, increased cell survivability and decreased apoptosis serve as possible mechanisms for hepatocarcinogenesis in rodents treated chronically with peroxisome proliferators.

Peroxisome proliferation and liver tumor formation, however, have only been observed in rodents. A one year feed study in rats and mice with the peroxisome proliferator WY-14,643 resulted in a 100% incidence of liver tumors, whereas no incidences have been observed in epidemiological studies for human patients receiving similar doses of fibrate drug treatments (Gonzalez et al., 1998). The species difference could be partially due to the tenfold decrease in Ppar α messenger RNA (mRNA) expression in humans compared to mice. This difference suggests that mice are much more susceptible to activation of Ppar α signaling (Palmer et al., 1998). Another difference lies in the lack of a functional PPRE in the promoter of human AOX. In rodents, peroxisome proliferators increase the transcription of the Aox gene in a Ppar α -dependent manner, however the human AOX gene is not responsive to peroxisome proliferators (Lambe et al., 1999).

Despite the apparent decrease in Ppar α function and the resistance to the proliferative effects of Ppar α activation, the hypolipidemic effects of peroxisome proliferators are still present in humans and other non-rodent mammals (Yang et al., 2008). Studies performed in Ppar α -humanized mice determined that there are differences in the regulation of the cell-cycle control gene cMyc in wild type and the humanized mice. In wild type mice let-7c, a microRNA regulating c-myc RNA, is suppressed by Ppar α activation, resulting in increased levels of c-myc RNA and increased proliferation. In the Ppar α -humanized mice, however, Ppar α activation did not lead to suppression of let-7c. As a result, there is no increase in c-myc RNA and proliferation is not altered (Gonzalez and Shah, 2008).

1.3.2 Ppar β/δ :

Ppar β/δ was cloned in both humans and *Xenopus* in 1992 (Dreyer et al., 1992; Schmidt et al., 1992). Ppar β/δ is ubiquitously expressed, but is expressed at the highest levels in the brain, adipose tissue and skin. The functions and targets of Ppar β/δ are not very well known, however it is known to have some function in FA metabolism and in the inhibition of macrophage derived inflammation (Tyagi et al., 2011).

1.3.3 Ppar γ :

Ppar γ was first cloned in *Xenopus* in 1992, which was followed by its cloning in humans in 1995 (Greene et al., 1995; Schmidt et al., 1992). Ppar γ has two protein isoforms in humans that are differentially expressed throughout the body. $\gamma 1$ is ubiquitously expressed and $\gamma 2$ is found in adipose tissue (Zieleniak et al., 2008).

Ppar γ is activated by a wide variety of ligands, including FA derivatives, prostaglandin derivatives and thiazolidinediones (TZDs). TZDs are a drug class with have high affinity for Ppar γ and act to increase insulin responsiveness in type II diabetes mellitus patients (Zieleniak et al., 2008). Ppar γ activation, through ligand binding, is followed by association with the PPRE of target genes, which leads to the modulation of their transcription. These target genes encode proteins involved in the regulation of FA storage, glucose metabolism and adipocyte differentiation (Tyagi et al., 2011). Activation of Ppar γ has also been associated with anti-inflammatory effects, due to inhibition of inflammatory cytokines (Delerive, 2001).

Activation of Ppar γ :

Ppar γ , just like Ppar α , enacts its effects through the regulation of target gene transcription. These PPRE-containing genes encode proteins involved in adipocyte differentiation, lipid and glucose storage and in decreasing inflammation. Ppar γ targets numerous genes, such as solute carrier family 27 member 1 (Slc27a1; fatty acid transporter 1 (Fatp1)), lipoprotein lipase (Lpl), glucose transporter type 4 (Glut4; solute carrier family 2 member 4 (Slc2a4)), and fatty acid binding protein 4 (Fabp4).

Slc27a1: Slc27a1, or Fatp1, is a member of the fatty acid transport protein (Fatp) family of transporters involved in the uptake of long chain FAs. It is mainly expressed in the heart, adipose and skeletal muscle, but also has low levels of expression in the liver, kidney, brain and lung (Guitart et al., 2014).

Lipoprotein lipase: Lpl is the enzyme that catalyzes the rate-limiting step of triacylglycerol (TAG) hydrolysis. Lpl acts to metabolize TAGs, located in chylomicrons, and very low-density lipoproteins (VLDL) into free FAs, which can then be stored as TAGs in adipocytes or be oxidized and used for energy. It is widely distributed throughout the body, including in adipose tissue, liver, kidney, heart and skeletal muscle. Due to the role of Lpl in lipid metabolism it is important to note that the enzymatic activity of Lpl is tissue-specific and depends on whether the system is in a fed or fasted state. For example, during a period of fasting, Lpl expression is decreased in adipose tissue and increased in the heart, resulting in free FAs being used for energy instead of being stored (Braun and Severson, 1992).

Glucose transporter type 4: Glut4 is a member of a family of sugar transporters, which either import or export sugar molecules through facilitated diffusion. Glut4 is the isoform involved in the uptake of glucose from systemic circulation and thus plays a large role in maintaining glucose homeostasis. It is expressed at high levels in skeletal muscle and adipose tissue, but can also be found at low levels in other tissues, such as the liver. Alterations to the expression of Glut4 transporters have serious consequences. For example, depletion of Glut4 leads to insulin resistance and the development of diabetes (Huang and Czech, 2007).

Fatty acid binding protein 4: Fabp4 is a member of the fatty acid binding protein family of cytoplasmic proteins. Fabp4 is mainly expressed in adipose tissue and macrophages, but also has some expression in the liver. It acts as a chaperone for FAs in various signaling pathways by transporting intracellular lipids to biological targets (Garin-Shkolnik et al., 2014).

1.4 Rxr:

The retinoid X receptors (Rxrs) are a family of nuclear receptors that bind retinoic acid metabolites, such as 9-cis retinoic acid, and have the ability to form stable heterodimers with many receptors, including retinoic acid receptors (Rars), Ppars, vitamin D receptors (Vdr) and thyroid hormone receptors (Tr) (Mangelsdorf et al., 1992). The various Rxr heterodimers act in the transcriptional regulation of energy balance, through appetite and fuel usage. Heterodimers

formed with Rxr can either be permissive or non-permissive. The Rxr-Ppar heterodimer is permissive because only binding of a Ppar-selective ligand is required to activate the complex. Rxr-Rar, Rxr-Vdr and Rxr-Tr heterodimers are non-permissive, meaning activation can only be achieved through separate binding of an Rxr-selective and a partner-selective ligand.

There are three differentially expressed subtypes of Rxr, mainly Rxr α , Rxr β , and Rxr γ . Rxr α is mainly expressed in the liver, kidney, intestine and muscle. Rxr β is expressed throughout the body. Rxr γ is expressed in the brain, cardiac muscle, and skeletal muscle (Pérez et al., 2012). Studies in knockout mice have demonstrated that loss of Rxr α produces more severe effects than knockout of either Rxr β or Rxr γ . In fact, absence of Rxr β , Rxr γ , or both can be compensated for in most tissues by the action of Rxr α (Ahuja et al., 2003).

There is not much evidence suggesting the three Rxr subtypes have different heterodimer partners or functions, however, it has been demonstrated that Rxr α is the most prominent form involved in development. Rxr α knockout in mice is embryonic lethal due to cardiac ventricle hypoplasia and errors in the division of the ventricles. The lethal effects arise from the inability of Rxr-Rar heterodimer formation, which prevents the activation of downstream Rar-targets. In a Ppar-dependent manner, Rxr α is also involved in the differentiation of adipocytes, the regulation of lipolysis and in regulating hepatocyte lifespan and regeneration (Ahuja et al., 2003). Rxr β and Rxr γ play roles in the dopamine-signaling pathway and double knockout in mice causes defects in locomotion as a result of decreased dopamine signaling. Knockout of Rxr β causes embryonic lethality in about 50% of mutant mice due to defects in placenta formation (Ahuja et al., 2003). Knockout of Rxr γ , however, produces mice that mostly appear unaffected. Some observed effects include increased metabolic rate,

reduced food consumption and increased activity of lipoprotein lipase in skeletal muscle (Pérez et al., 2012).

Activation of Rxr α :

Rxr α , either as a homodimer or heterodimer, acts to regulate the activity of other nuclear receptors. In the absence of a ligand, Rxr α binds a complex of co-repressors that prevent its activity. Binding of a ligand displaces this complex of co-repressors and recruits a complex of co-activators that allow for Rxr α to bind DNA and regulate transcription, depending on the heterodimer partner. Permissive heterodimers are activated by ligand binding to Rxr α , however, non-permissive heterodimers require both the Rxr α -specific ligand and the partner-specific ligand to be bound. In both cases, activation of the Rxr α heterodimer targets numerous genes involved in many different regulatory pathways. Some of the proteins encoded by these genes are the retinoic acid receptor α (Rar α), deiodinase I (Dio1), Cyp2r1, and retinol binding protein 1 (Rbp1).

Retinoic acid receptor α : Rar α is a subtype of the Rar family of nuclear receptors. Rar forms a heterodimer with Rxr, which then binds to the retinoic acid response element (RARE) in the promoters of target genes. The Rar-Rxr heterodimer regulates the transcription of genes involved in cell-cycle arrest, cell differentiation and cell death (Kalitin and Karamysheva, 2016).

Deiodinase 1: Dio1 is a hepatic enzyme that converts the pro-hormone thyroxine (T_4) to the active thyroid hormone triiodothyronine (T_3) (Yu and Koenig, 2006). Dio1 is a downstream target of thyroid hormone receptor activity and is regulated by the Rxr-thyroid hormone receptor heterodimer.

Cyp2r1: Cyp2r1 is a member of the cytochrome P450 family of phase I metabolic enzymes. Cyp2r1 catalyzes the second step in the synthesis of active vitamin D. This second step occurs in the liver and hydroxylates carbon 25 of vitamin D to form 25-hydroxyvitamin D_3 . The final step occurs via cytochrome P450 metabolism in the kidney, which produces the Vdr ligand $1\alpha,25$ -dihydroxyvitamin D_3 (Cheng et al., 2004). Cyp2r1 is a downstream target of vitamin D receptor activity and is regulated by the Rxr-Vdr heterodimer.

Retinol binding protein 1: Rbp1 is a $Rar\alpha$ target gene that functions as a chaperone protein for retinol. Retinol bound to Rbp1 is the preferred substrate for retinol dehydrogenase, which converts retinol to all-*trans* retinoic acid. All-*trans* retinoic acid is required for proper signaling and differentiation in several tissue types (Pierzchalski et al., 2014).

1.5 Acetaminophen:

Acetaminophen (APAP; Paracetamol) is a commonly used analgesic and antipyretic compound that is found in many over the counter and prescription pain relief medications.

Administration of therapeutic doses (roughly four grams over a 24 hour period) does not typically cause any adverse effects, however, the common consumption of much larger doses has made APAP one of the leading causes of drug induced liver injury (DILI). APAP overdose in the United States alone is responsible for approximately 30,000 hospitalizations and 300 deaths annually (Yoon et al., 2016).

Mechanism of Hepatotoxicity:

Acetaminophen is a weak acid that is rapidly absorbed into the systemic circulation from the duodenum after oral administration, but the rate of absorption can be delayed by the concurrent consumption of food. The metabolism of APAP occurs mostly by microsomal enzymes, with only about two percent of APAP being excreted in the urine unchanged.

At therapeutic levels, APAP phase II conjugation enzymes, mainly UDP-glucuronosyl transferase (Ugt) and sulfotransferase (Sult), metabolize about 90 percent of APAP. Ugt and Sult catalyze the addition of polar groups to APAP, resulting in the formation of polar glucuronidated and sulfated metabolites that are more easily excreted in the urine. The remaining APAP enters a phase I oxidative pathway catalyzed mainly by the cytochrome P450 enzyme Cyp2e1, with contributions also by Cyp1a2 and Cyp3a4. The APAP entering this pathway is metabolized into the highly reactive N-acetyl-para-benzo-quinone imine (NAPQI). Normally, NAPQI is scavenged and detoxified by intracellular GSH stores. The metabolites of APAP are then exported from the hepatocytes by phase III transporters, either into the bile or into blood for subsequent urinary excretion (Yoon et al., 2016).

At supratherapeutic doses of APAP the Ugt and Sult pathways become saturated and a greater portion of APAP is metabolized to NAPQI by Cyp2e1. The increased production of NAPQI depletes GSH stores, allowing NAPQI to form protein adducts. The formation of protein adducts in the mitochondria causes mitochondrial oxidative stress, opening of the mitochondrial permeability transition pore and eventually mitochondrial lysis. This leads to cellular DNA damage, ATP depletion, and eventually centrilobular hepatocellular necrosis (McGill et al., 2012).

Treatment of Acetaminophen-Induced Hepatotoxicity:

The only available treatment for APAP-induced hepatotoxicity is to replenish GSH stores using a sulfhydryl donor, such as N-Acetylcysteine (NAC). NAC is preferred to other sulfhydryl donors, such as methionine, cysteine and cysteamine, due to its low toxicity and the ease of administration. Administration of NAC, either orally or intravenously, within 12 to 24 hours following APAP overdose, serves as an effective antidote to APAP hepatotoxicity. NAC acts to increase the synthesis of GSH, providing the hepatocytes with an increased ability to scavenge and detoxify NAPQI and also to counteract the oxidative stress produced by this reactive metabolite (Lauterburg et al., 1983). NAC serves as an effective treatment for APAP-induced hepatotoxicity, as long as symptoms can be identified early. The lack of other treatments highlights the need for a better understanding of the mechanism of APAP hepatotoxicity and the identification of other factors that can be useful in treatment or prevention.

1.5.1 Role of Nrf2 in Protection from Acetaminophen-Induced Hepatotoxicity:

In the presence of oxidative stress Nrf2 is able to translocate and accumulate in the nucleus, where it upregulates the transcription of a series of antioxidant response genes. Studies have shown that there is a linear relationship between increasing APAP dose (from nontoxic to toxic doses) and increased nuclear accumulation of Nrf2 in mouse liver. The increased nuclear accumulation is also accompanied by an increase in Nrf2 activity, as shown by elevated mRNA expression of the Nrf2-dependent genes Nqo1, Hmox1 and Gclc (Aleksunes, 2006; Goldring et al., 2004). This is supported by studies analyzing the effects of hepatocyte-specific knockouts of Keap1 in mice. These mice have elevated levels of Nrf2 in the liver, as well as increased accumulation of Nrf2 in the nucleus and increased mRNA expression of the Nrf2 target genes Nqo1 and Gclc. Also, the Keap1 mutant mice display very few signs of liver injury, compared to wild type mice, following administration of normally toxic doses of APAP (Okawa et al., 2006).

Studies using Nrf2 knockout mice have illustrated that lack of Nrf2 function causes an increased susceptibility to APAP-induced liver insults. The knockout mice had high mortality rates at doses that are nonlethal in wild type mice. This is in part due to significantly lower intracellular stores of GSH that accompany knockout of Nrf2 (Chan et al., 2001). Nrf2 knockout mice also exhibit decreased expression of mRNA and protein for Nqo1, Gclc and other antioxidant genes, supporting the decreased ability to tolerate oxidative stress and the decreased production of GSH (Aleksunes et al., 2008).

1.5.2 Role of Ppara in Protection from Acetaminophen-Induced Hepatotoxicity:

Administration of peroxisome proliferators prior to APAP dosing protects against the hepatotoxic effects of high APAP doses. Ten day repeated dosing of mice using the peroxisome proliferator clofibrate (CFB) followed by an APAP challenge was sufficient to protect against the hepatotoxic effects of APAP (Manautou et al., 1994; Nicholls-Grzemeski et al., 1992). The CFB pretreated mice displayed larger livers, increased intracellular GSH stores, lower levels of GSH depletion and decreased protein adduct formation in comparison to APAP-treated mice not receiving CFB pretreatment (Manautou et al., 1994). In the development of APAP-induced hepatotoxicity, NAPQI covalently binds to selective cellular proteins to form adducts. One of these targets is the cytosolic 58kD APAP-binding protein (58-ABP) (Hoivik et al., 1996). The ten-day pretreatment with CFB did not change the amount of cytosolic 58-ABP, however it did decrease the covalent binding of NAPQI to 58-ABP. Repeated dosing of peroxisome proliferators appears to confer protection against APAP hepatotoxicity, possibly due to an increase in the production of GSH (Manautou et al., 1994).

Unlike in the ten-day dosing model, a single dose of CFB does not alter hepatic GSH levels, however, a single dose of CFB, prior to APAP dosing, is partially protective against APAP hepatotoxicity, without significantly altering protein adduct formation or initial GSH depletion. Compared to APAP-treated control mice, APAP-treated mice receiving CFB pretreatment display faster restoration of GSH to control levels (Manautou et al., 1996). This supports the protective role of CFB against APAP toxicity because GSH restoration occurs more rapidly when APAP dosing does not culminate in hepatic toxicity. Furthermore, a single pretreatment of CFB protects against APAP hepatotoxicity without affecting hepatic covalent binding. Hepatic covalent binding is a key component of APAP-induced hepatotoxicity, but it is not sufficient by

itself for the onset of toxicity. Oxidative events, occurring after initial covalent binding, are believed to play a significant role in the production of cellular damage. It is possible that the partial protection against APAP toxicity observed following a single pretreatment dose of CFB may be mechanistically linked to disruption of oxidative events that occur subsequent to covalent binding (Manautou et al., 1996).

The protective effect of CFB is dependent on the ability of Ppar α to be activated. A study using the ten-day CFB pretreatment model in Ppar α knockout mice found that, unlike wild type mice, the mutant mice were not protected by CFB from the hepatotoxic effects of APAP. Also, these mutant mice are resistant to typical peroxisome proliferator effects, such as hepatomegaly, peroxisome proliferation and enzyme induction (Chen et al., 2000). The protective effects of peroxisome proliferator pretreatment against APAP hepatotoxicity are occurring in a manner dependent on Ppar α activation.

Another possible mechanism for the protective role of Ppar α activation against chemically induced oxidative stress could be through the regulation of protein folding genes and genes involved in proteasomal degradation. Anderson et al. (2004) performed a transcript profiling study using wild type and Ppar α -null mouse livers after seven days of dosing with the Ppar α activator WY-14,643. This study revealed that Ppar α activation alters the expression of genes encoding heat-shock proteins, chaperone proteins, and proteasome subunits. The possible role of Ppar α activation in the induction of genes involved in the protein folding pathway and genes involved in proteasomal degradation of damaged proteins may serve as a protective mechanism against chemically induced oxidative stress (Anderson et al., 2004).

1.6 Signaling Pathway Interactions

The activation of a receptor or transcription factor leads to the transduction of a signal along a cellular pathway, with the end goal of altering intracellular physiology. Signaling pathways, however, are often complex and can involve the integration, or crosstalk, between multiple pathways. Crosstalk between various transcription factors highlights the existence of regulatory and compensatory mechanisms needed to maintain proper cellular function. Additionally, different transcription factors can regulate genes with similar functions or even the same genes. This redundancy exists to ensure proper cellular function in case one signaling pathway becomes inhibited or is otherwise malfunctioning.

1.6.1 Nrf2 Signaling Interactions

Evidence suggests that Nrf2, the transcription factor involved in cellular response to oxidative stress, has interactions with other signaling pathways, including Notch and NF- κ B.

The Notch receptors are important in early cell signaling, cellular differentiation, organogenesis and organ repair. Notch1 appears to be the receptor subtype with the most activity in the liver (Wakabayashi et al., 2015). Ligand binding to Notch1 is followed by two proteolytic cleavage events that allow the Notch intracellular domain (NICD) to translocate into the nucleus. The NICD associates with the transcription factor recombining binding protein suppressor of hairless (Rbpjk) and binds DNA, leading to transcription of target genes. A recognition sequence for the Notch1 modulator protein Rbpjk has been identified in the human Nrf2 promoter (Wakabayashi et al., 2015). Also, a functional ARE was identified in the promoter region of the Notch1 gene, suggesting the two pathways interact. Partial hepatectomy studies

in Nrf2-null mice demonstrated that the loss of Nrf2 function causes a significant reduction in liver regeneration, compared to wild type mice. NICD rescue allowed for normal liver regeneration in Nrf2-null mice, suggesting that the decreased regenerative capacity of Nrf2-null mouse livers is due to an interplay between Nrf2 and Notch1 (Wakabayashi et al., 2010).

The NF- κ B family of transcription factors are activated by pro-inflammatory cytokines and are involved in the immune response against bacterial and viral pathogens, inflammation, cell proliferation and protection against ultraviolet radiation (Wardyn et al., 2015). Studies done in primary mouse astrocytes, isolated from both wild type and Nrf2-null mice, analyzed NF- κ B activity following “scratch” injury. Scratching a monolayer of wild type astrocytes with a 26G needle causes increased DNA binding by NF- κ B and increased expression of pro-inflammatory cytokines. Applying a scratch to an astrocyte monolayer isolated from Nrf2-null mice produced higher NF- κ B activity compared to wild type (Pan et al., 2012). This study suggests that Nrf2 has a regulatory role in NF- κ B signaling. Studies in the human liver cancer cell line HepG2 and human embryonic kidney cell line HEK293 determined that NF- κ B activity acts to antagonize Nrf2 signaling. This inhibition occurs through interactions between the NF- κ B subunit P65 and Keap1, the Nrf2 regulator. The P65-Keap1 interaction results in translocation of Keap1 to the nucleus, where it sequesters Nrf2 and prevents Nrf2 binding to the ARE of target genes (Yu et al., 2011). These studies depict a regulatory interplay between the Nrf2 and NF- κ B signaling pathways, suggesting a connection between the cellular response to oxidative stress and inflammation.

1.6.2 Ppar Signaling Interactions

The Ppars are a family of transcription factors that act to regulate FA metabolism. Ppar γ activation plays an important role in adipogenesis, and activation of all three Ppar subtypes has been linked to anti-inflammatory effects, suggesting a potential interaction with Notch and NF- κ B respectively.

Activation of Ppar γ , by either endogenous ligands or TZDs, is all that is needed for adipogenesis. Deficiencies in the Notch1 signaling pathway, however, prevents adipocyte differentiation in 3T3-L1 mouse embryonic fibroblasts. The effect appears to be due to Ppar γ downregulation following Notch1 abrogation (Garces et al., 1997). In another study, treatment of mouse adipose-derived mesenchymal stem cells with the Notch1 ligand Jagged1 resulted in increased adipocyte formation. It is believed that this effect is due to Ppar γ induction that was observed following Notch1 signaling activation (Ba et al., 2012). These studies provide evidence for a possible interaction between the Notch and Ppar γ signaling pathways in at least the process of adipogenesis.

Activation of Ppars, such as fibrate activation of Ppar α and TZD activation of Ppar γ , demonstrates anti-inflammatory actions. This effect appears to occur through antagonism of inflammatory pathways, such as NF- κ B and AP-1, and inhibition of inflammatory cytokine production. Studies performed in human aortic smooth muscle cells used interleukin 1 β (IL-1 β) to induce mRNA expression of IL-6, a cytokine and known marker of vascular inflammation. The increased expression of IL-6 was reduced in cells co-treated with IL-1 β and the Ppar α agonist WY-14,643. The decreased expression of the IL-6 cytokine appears to be due to Ppar α -mediated repression of IL-6 promoter activation through interactions with p65 and c-Jun (a subunit of AP-1). The exact mechanisms of these interactions are not well-known, however it is

known that Ppar α can directly bind the DNA binding domain of p65 and N-terminal domain of c-Jun (Delerive et al., 1999). The authors also performed transfection assays in COS-1 cells (a monkey fibroblast-like kidney cell line) in which the activity of a PPRE-driven promoter was analyzed in the presence of Ppar α and WY-14,643. In these assays the reporter activity of the PPRE-containing gene was analyzed in the presence of increasing concentrations of the NF- κ B subunit p65 and the AP-1 subunit c-Jun. p65 and c-Jun inhibited reporter activity in a dose-dependent manner, suggesting that the negative regulation between Ppar α and NF- κ B or AP-1 is bidirectional (Delerive et al., 1999). Altogether, the studies performed by Delerive et al. suggest the presence of a connection between the pathways regulating cytokine production and FA metabolism.

1.6.3 Interaction Between Nrf2 and Rxr α

Rxr α , the heterodimer partner for many nuclear receptors including the Ppars, is crucial for Ppar signaling. Interaction of Rxr α with other signaling molecules could have effects on the signaling of its many heterodimer partners. Experimental evidence indicates that Rxr α does take part in interactions with other signaling pathways, one of which is Nrf2.

A genome-wide binding study (ChIP-sequencing) for human Nrf2 was performed in cultured human lymphoblastoid cells, following Nrf2 activation by the dietary antioxidant sulforaphane. Analysis of high confidence regions of Nrf2-associated DNA sequences, referred to as Nrf2-bound peaks, identified Rxr α and the Ppar co-activator Ppar γ coactivator 1-beta (Pgc1 β) as ARE-dependent targets of Nrf2 binding (Chorley et al., 2012). Quantitative real time PCR (qRT-PCR) analysis of Rxr α revealed that mRNA expression of Rxr α increased following Nrf2

activation, either chemically using sulforaphane or through shRNA knockdown of Keap1 in 3T3-L1 mouse embryonic fibroblast cells. Accordingly, mRNA expression of Rxr α decreased following shRNA knockdown of Nrf2 (Chorley et al., 2012).

Experimental evidence also indicates a role for Rxr α in the inhibition of Nrf2 transcriptional activity. Knockdown of Rxr α using siRNA produced a doubling of basal mRNA expression for the Nrf2-dependent genes aldo-keto reductase 1c1 (Akr1c1) and Hmox1 in Caco2 human colon cancer cells. Conversely, overexpression of Rxr α , by transfection of an Rxr α expression vector into Caco2 cells, led to reduced basal expression of the same genes. This study denotes that Rxr α plays a role in the regulation of Nrf2-dependent genes (Wang et al., 2013).

In order to determine the extent of the Nrf2-Rxr α interaction, a glutathione S-transferase (GST) pull-down assay was performed to examine protein-protein interactions. Two separate assays were performed that revealed strong interactions between Nrf2 and Rxr α . A series of plasmids encoding GST-tagged Nrf2 or Rxr α were constructed and expressed in *Escherichia coli* (*E. coli*). Protein was purified using GSH-Sepharose beads and resolved on a 10%SDS-PAGE gel. The first assay used isolated GST-tagged Nrf2 incubated with histidine-tagged Rxr α protein. A GST-tagged control bound Rxr α non-specifically, whereas GST-tagged Nrf2 binds strongly to his-tagged Rxr α . A second assay, using isolated GST-tagged Rxr α protein incubated with histidine-tagged Nrf2 protein, was performed to confirm the specificity of binding between Nrf2 and Rxr α . Nonspecific binding by the GST-tagged control and strong binding between Rxr α and Nrf2 confirmed that the interaction between the two proteins is specific. The authors also performed GST pull-down assays using truncated GST-tagged

constructs of Nrf2 and Rxr α protein to determine what regions are involved in binding. The strongest interactions were observed between the Neh7 domain of Nrf2 and the DNA-binding domain of Rxr α (Wang et al., 2013).

ChIP assays performed in MCF7 human breast cancer cells, following Nrf2 activation using the synthetic antioxidant tert-butylhydroquinone, revealed that both Nrf2 and Rxr α were able to bind ARE sites in the promoters of Nrf2-dependent genes. The binding of Rxr α to AREs was much weaker than the binding exhibited by Nrf2 and depletion of Nrf2 reduced the ability of Rxr α to bind AREs. The authors concluded that Rxr α possesses an inhibitory effect on Nrf2 signaling, most likely through the formation of an inactive Nrf2-Rxr α complex bound to the ARE in target genes (Wang et al., 2013).

1.6.4 Interaction Between Nrf2 and Ppar γ

Transcription factor signaling is comprised of a complex series of signaling and regulatory events that often involve interactions with other transcription factors. The presence of some overlapping actions between Nrf2 and the Ppars suggest that these transcription factors may have interacting signaling pathways. There is some evidence supporting the existence of this interaction.

Protein analysis of Ppar γ in wild type and Nrf2-null mouse lungs revealed that Nrf2-null mice possess a decreased basal level of Ppar γ protein expression. Also, Ppar γ protein expression in wild type mice increased significantly following 48-hour exposure to hyperoxic conditions, whereas exposing Nrf2-null mice to the same treatment produced no changes in Ppar γ protein levels. These results imply that Nrf2 plays some undefined role in the regulation

of Ppar γ (Cho et al., 2010). The authors proceeded to perform bioinformatics analysis of the Ppar γ promoter and identified a potential ARE. They then cloned a region of the Ppar γ promoter containing a wild type version of the sequence and another containing a mutated sequence into luciferase reporter vectors. Analysis of luciferase activity, following transfection of the vectors into Nrf2-overexpressing airway epithelial cells revealed that in both normal and hyperoxic conditions, cells containing the mutated sequence had lower luciferase activity than cells containing the wild type form. Through this study the authors determined that Nrf2 binding to the ARE sequence in the Ppar γ promoter is required for the induction of Ppar γ expression following exposure to hyperoxia (Cho et al., 2010).

Another group of researchers studying adipose tissue found that the Nrf2-mediated regulation of Ppar γ extends to adipogenesis. Protein and mRNA expression of Ppar γ is decreased in adipose tissue isolated from Nrf2-null mice and following shRNA knockdown of Nrf2 in 3T3-L1 mouse embryonic fibroblasts. The decreased expression of Ppar γ leads to impaired adipogenesis in the knockout mice and cell line. Conversely, activating Nrf2, through knockdown of Keap1, results in increased adipocyte differentiation in 3T3-L1 cells. This effect is due to increased Nrf2 binding in the Ppar γ promoter (Pi et al., 2010).

1.6.5 Interaction Between Nrf2 and Ppar α

The positive regulation of Rxr α by Nrf2 activation and the negative regulation of Nrf2 by Rxr α suggest the presence of a regulatory feedback loop between Nrf2 and Rxr α signaling. Nrf2 has also been revealed to play a direct role in the regulation of Ppar γ expression in models of acute lung injury and adipogenesis. Nrf2 has known interactions with the heterodimer partner

of Ppar α and with a member of the Ppar family of nuclear receptors. The extent to which these interactions are involved in other tissues are not known, however the presence of these interactions hint at a possible interaction, either direct or indirect, between Nrf2 and Ppar α signaling.

As a subset to a study on the role of Ppar α in the regulation of proteome maintenance genes, Anderson et al. (2004) analyzed mRNA expression of stress modifier genes in the livers of wild type and Nrf2-null mice treated with the Ppar α activator WY-14,643 for seven days. The goal of this experiment was to determine whether or not Ppar α was regulating stress modifier genes independently of Nrf2. The authors also included several Ppar α target genes in their mRNA analysis, most likely to confirm that WY-14,643-mediated activation of Ppar α was occurring. The mRNA expression of Ppar α and the Ppar α -dependent genes (Acox1, Cyp4a10, Cyp4a14, Fabp4) is elevated by WY-14,643 treatment in wild type mice. The authors also observed significantly higher mRNA expression of the Ppar α target genes in WY-14,643-treated Nrf2-null mice, compared to all other treatment groups (Anderson et al., 2004). The up-regulation of Ppar α target genes in the absence of Nrf2 suggests a possible role for Nrf2 in the regulation of Ppar α signaling.

Another group measured the mRNA expression of the Ppar gamma coactivator 1-alpha (Pgc1 α) in the livers of wild type, Nrf2-null and Nrf2-overexpressing mice. They were interested in Nrf2-mediated protection of mitochondria in response to oxidative stress and found that Nrf2-null mice had lower hepatic mitochondrial content compared to wild type. Their analysis revealed that hepatic Pgc1 α mRNA expression was elevated in Nrf2-null mice after 24 hours of fasting, compared to wild type and Nrf2-overexpressing mice (Zhang et al., 2013). Pgc1 α also

serves as a co-activator for all three members of the Ppar family. In regards to Ppar α , periods of fasting cause Ppar α induction and co-activation by Pgc1 α , leading to the induction of genes involved in hepatic fatty acid oxidation. This serves to switch fuel usage from glucose to fatty acids in order to preserve glucose for use by the central nervous system (Liang and Ward, 2006).

Evidence suggests that there is some level of interaction between the Nrf2 and Ppar α signaling pathways. Both Nrf2 and Ppar α are protective against chemically induced oxidative stress and individual activation of either Nrf2 or Ppar α has been demonstrated to confer tolerance to drug-induced hepatotoxicity. Nrf2 is already known to be involved in the regulation of several signaling pathways, so a permissive role for Nrf2 in the regulation of Ppar α activity would not be surprising. This proposed interaction, however, is most likely not direct, since there is no indication of direct binding. The studies presented in this thesis have been performed to identify a possible mechanism for the interaction between Nrf2, a regulator of the antioxidant response and Ppar α , a regulator of lipid homeostasis. It is believed that a crosstalk between the Nrf2 and Ppar α signaling pathways contributes to changes in hepatic gene expression by treatment with Ppar α activators. The discovery of a mechanism for the interaction between Nrf2 and Ppar α would provide a new insight into how the body responds to chemically induced oxidative stress and may provide new targets for the treatment or prevention of drug induced liver injury.

Chapter 2

Role of Nrf2 in the regulation of Ppar expression in mice

2.1 Abstract:

The peroxisome-proliferator activated receptors (Ppars) are a family of ligand-dependent nuclear receptors, which promote transcriptional activation of target genes. Ppar α regulates genes involved in hepatic fatty acid transport, uptake and metabolism. Several of these target genes are upregulated by hypolipidemic fibrate-type drugs. Clofibrate (CFB) is a prototype for this class, and a well-known Ppar α activator. CFB treatment also prevents liver injury by acetaminophen (APAP) in a Ppar α -dependent manner. Similarly, nuclear factor erythroid 2-related factor 2 (Nrf2) is an important genetic determinant of susceptibility to APAP hepatotoxicity. The purpose of this study was to analyze the potential interaction between Ppar α and Nrf2 signaling. Analysis of mRNA expression of Ppar α -related genes in wild type and Nrf2 knockout (Nrf2-null) mice treated for five days with 250mg/kg CFB suggests that Nrf2 regulates Ppar α function. Gene expression of Ppar α was not altered basally between wild type and Nrf2-null mice; however, expression was elevated significantly in CFB-treated Nrf2-null mice, compared to wild types. For Ppar α -dependent genes analyzed, mRNA levels were significantly increased with CFB treatment in both genotypes. Elevation was significantly higher in CFB-treated Nrf2-null compared to wild types also receiving CFB. This suggests that the ability of Ppar α target genes to be induced is heightened in the absence of Nrf2. Also, mRNA expression of Ppar gamma coactivator 1-alpha (Pgc1 α) was elevated in Nrf2-null mouse liver,

independent of CFB treatment. Altogether, these results are indicative of a potential regulatory link between Nrf2 and Ppara signaling.

2.2 Introduction:

Acetaminophen (APAP; Paracetamol) is a commonly used analgesic and antipyretic compound that is found in many over the counter and prescription pain relief medications. APAP is safe when consumed at therapeutic doses. However, consumption of supra-therapeutic doses has made APAP one of the leading causes of drug-induced liver injury (DILI). In the United States alone, APAP overdose is responsible for approximately 30,000 hospitalizations and 300 deaths annually (Yoon et al., 2016). A therapeutic dose of APAP is mostly metabolized in the liver via phase II glucuronidation and sulfation reactions, which produce conjugates that are easily excreted into the blood and bile. The fraction of the APAP dose that remains unconjugated can be bioactivated into the highly reactive metabolite N-acetyl-para-benzo-quinone imine (NAPQI) by cytochrome P450 enzymes. The NAPQI that is formed is then scavenged and detoxified by intracellular stores of glutathione (GSH) before being excreted (Yoon et al., 2016). In cases of APAP overdose, the phase II conjugation reactions become saturated and cytochrome P450 enzymes metabolize a larger portion of the parent APAP. The excess NAPQI that is produced depletes GSH stores, allowing free NAPQI to form protein adducts, which causes cellular oxidative stress, formation of reactive oxygen species (ROS) and eventually necrosis of centrilobular hepatocytes (McGill et al., 2012).

Oxidative stress, such as through the formation of ROS, is a common occurrence in the liver due to the large number of mitochondria present in hepatocytes, as well as a function of

the liver's oxidative metabolism. Large levels of oxidative stress have the potential to disrupt cellular homeostasis, resulting in cellular necrosis if ROS are not neutralized (Jaeschke and Ramachandran, 2011). For this reason, it is crucial for the liver to possess mechanisms that counteract the metabolic generation of reactive intermediates.

In the liver, heightened levels of oxidative stress elicit an antioxidant response, which serves to prevent cell death through reduction of cellular oxidative stress. A major regulator of the antioxidant response is the transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2). A positive linear relationship exists between APAP dosing and the accumulation of Nrf2 in the nucleus of mouse hepatocytes. Nuclear accumulation of Nrf2 is accompanied by an increase in Nrf2 activity, as indicated by the increased mRNA expression of Nrf2-dependent genes (Aleksunes, 2006; Goldring et al., 2004). Nrf2 acts in the activation and regulation of the cellular response to oxidative stress. It does this by regulating the transcription of a series of antioxidant-response genes, which help regulate cell survival (Jaramillo and Zhang, 2013). Nrf2 is a member of the cap "n" collar basic leucine zipper family and is regulated by the E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (Keap1). In normal conditions, or absence of a pro-oxidant state, Nrf2 is sequestered and ubiquitinated in the cytosol through the actions of Keap1. Ubiquitination marks Nrf2 for rapid proteasomal degradation. In the presence of reactive oxygen species (ROS), however, Keap1 is prevented from sequestering Nrf2, allowing Nrf2 to accumulate in the cytosol before its translocation to the nucleus. Once in the nucleus, Nrf2 is able to bind to antioxidant response elements (ARE) in the promoter of target genes, enabling transcriptional regulation of a series of genes involved in the antioxidant response (Watai et al., 2007).

The protective role of Nrf2 against APAP-induced hepatotoxicity has been well demonstrated in models of increased Nrf2 activation using Keap1-null mice and in Nrf2 loss-of-function models using Nrf2-null mice. Hepatocyte-specific knockout of Keap1 results in elevated levels of Nrf2 in the liver and increased nuclear accumulation. There is also increased activity of Nrf2 as illustrated by increased mRNA expression of the Nrf2-dependent genes NAD(P)H:quinone oxidoreductase 1 (Nqo1) and glutamate-cysteine ligase catalytic subunit (Gclc) among others (Okawa et al., 2006). Nqo1 acts to decrease oxidative stress by reducing quinones to hydroquinones, preventing the formation of semiquinone radical intermediates (Atia et al., 2014). Gclc is one of two subunits that make up glutamate-cysteine ligase (Gcl), which catalyzes the rate-limiting step of GSH synthesis. The formation of GSH helps decrease oxidative stress through the scavenging of free radicals and other reactive intermediates in the cell (Gorrini et al., 2013). The elevated levels of Nqo1, Gclc and other Nrf2 target genes in the conditional Keap1-null mice allow these mice to better tolerate doses of APAP that are highly toxic to wild type mice. In fact, at the doses of APAP tested, Keap1-null mice exhibited minimal evidence of liver injury (Okawa et al., 2006). At the other end of the spectrum, whole body knockout of murine Nrf2 results in heightened susceptibility to APAP hepatotoxicity. In fact, doses that are typically nonlethal to wild type mice, cause high mortality rates when administered to Nrf2-null mice. This could be due to the significantly lower intracellular stores of GSH and lower basal expression of multiple antioxidant genes observed in these mice (Aleksunes et al., 2008; Chan et al., 2001).

Activation of Ppar α in mice with peroxisome proliferator treatment prior to APAP dosing also provides protection against APAP hepatotoxicity (Chen et al., 2000; Manautou et al., 1994,

1996). The peroxisome-proliferator activated receptors (Ppars) are a family of ligand-dependent nuclear receptors, which bind to a peroxisome proliferator response elements (PPREs) in the promoter of target genes and regulate the expression of these genes. In order to bind the PPREs, Ppars form a heterodimer complex in the nucleus with the retinoid X receptor (Rxr). In the absence of ligand the heterodimer forms a complex with co-repressor proteins that prevent DNA binding. The binding of a Ppar-specific ligand allows the heterodimer to dissociate from these co-repressor proteins and enables association with co-activator proteins, such as Ppar γ coactivator-1 α (Pgc1 α). Co-activator proteins are necessary for the association of a transcription-initiation complex with the heterodimer. This heterodimer complex is able to bind DNA and induce transcription of target genes (Kersten, 2014).

There are three different members of the Ppar family (α , β/δ and γ). Ppar α regulates proteins that act to release, uptake or transport fatty acids (FAs) and also regulates proteins involved in FA β -oxidation. Activation of Ppar α , in response to factors such as fasting or fibrate drug dosing, results in increased cellular uptake of FAs and a decreased inflammatory response (Delerive, 2001). Ppar γ is involved in FA metabolism, glucose storage and adipocyte differentiation. Activation of Ppar γ limits cellular proliferation and migration, as a way to conserve cellular energy (Tyagi et al., 2011). Ppar β/δ also acts in the regulation of genes involved in FA catabolism, but is not as well studied as the other Ppars (Tyagi et al., 2011).

The hepatoprotective role of Ppar α has been addressed using models of Ppar α activation through peroxisome proliferator dosing and in mice genetically deficient in Ppar α . A commonly used method for activating Ppar α in rodents is through dosing with a class of chemicals known as peroxisome proliferators. The fibrates are a group of hypolipidemic drugs,

originally used in the treatment of hypercholesterolemia, that were discovered to cause peroxisome proliferation in rodents. Clinically, fibrate-type drugs act to decrease FA and triglyceride levels through activation of peroxisomal β -oxidation (Pahan, 2006). Clofibrate (CFB), the first of the fibrate-type drugs, dosed daily in CD-1 mice for ten days affords near complete protection against APAP hepatotoxicity. This could be due to the increased intracellular GSH stores, lower levels of GSH depletion and decreased APAP protein adduct formation observed in CFB pretreated mice, compared to vehicle pretreated controls also receiving APAP (Manautou et al., 1994). In order to determine if the protective effect of CFB was Ppar α -dependent, Chen et al. (2000) examined the susceptibility of Ppar α -null mice to APAP hepatotoxicity following CFB pretreatment. Unlike wild type mice, Ppar α -null mice pretreated with CFB were not protected from APAP hepatotoxicity, demonstrating that the mechanism of CFB-mediated protection is dependent on Ppar α presence and activation (Chen et al., 2000).

Pretreatment with just a single dose of CFB was demonstrated to partially protect against APAP toxicity. The mode of protection, however, appears to differ from that observed in the ten-day pretreatment model. Unlike repeated CFB dosing, a single CFB dose does not alter the initial GSH depletion or level of protein adducts formed following APAP challenge. Covalent binding is necessary, but not sufficient, for APAP-induced hepatotoxicity. Since the single dose model of CFB pretreatment does not affect hepatic covalent binding of APAP, it is believed that partial protection is mediated through disruption of later oxidative events (Manautou et al., 1996).

Activation of Nrf2 and Ppar α in mice act to protect against toxic APAP insult. However, it is unknown whether these two pathways interact with one another in mediating

hepatoprotection. Direct binding of Nrf2 to the promoter of *Rxra* and *Ppar γ* at ARE consensus sites has been described (Cho et al., 2010; Chorley et al., 2012). The interaction between Nrf2 and *Rxra* appears to occur through a feedback loop, with increased Nrf2 activation promoting *Rxra* expression and increased *Rxra* expression inhibiting Nrf2-mediated transcription (Chorley et al., 2012; Wang et al., 2013). As for *Ppar γ* , the upregulation of *Ppar γ* in response to hyperoxia in the lungs and during adipogenesis appears to be dependent on Nrf2 expression and its binding to the *Ppar γ* promoter (Cho et al., 2010; Pi et al., 2010). Evidence suggests that Nrf2 may interact with *Ppar α* , however, a mechanism is yet to be determined.

As a subset to a study by Anderson et al. (2004) on the role of *Ppar α* in the regulation of proteome maintenance genes, wild type and Nrf2-null mice were treated with the *Ppar α* -activator WY-14,643 for seven days. The authors analyzed the mRNA expression of a series of stress modifier genes to determine whether Nrf2 was involved in their regulation. *Ppar α* and several *Ppar α* -target genes were also included in the mRNA analysis, most likely to show *Ppar α* was being activated by WY-14,643. Increased mRNA expression of *Ppar α* and *Ppar α* -dependent genes was observed in both genotypes. The increase in mRNA expression was higher in WY-14,643 treated Nrf2-null mice compared to all other treatment groups (Anderson et al., 2004). In another study, wild type and Nrf2-null mice were fasted as a means of inducing oxidative stress in mouse livers. Following a 24-hour fast, mRNA expression for the *Ppar* co-activator protein *Pgc1 α* was elevated in both genotypes, with expression being higher in Nrf2-null mice (Zhang et al., 2013). *Ppar α* activation in mouse liver by FAs has been reported following fasting (Bouwens et al., 2007). Analysis of *Pgc1 α* expression following chemically induced *Ppar α* activation, however, has not been well studied in wild type and Nrf2-null mouse liver.

Both Nrf2 and Ppar α regulate genes involved in the anti-inflammatory response and in the response to chemically induced oxidative stress. An interaction between the two would most likely not be direct since there is no indication of direct binding between the two transcription factors. The discovery of a potential interaction between Nrf2 and Ppar α would provide new insights into how these two transcription factors coordinately regulate gene expression in response to xenobiotics that induce oxidative stress and on adaptive response that confer tolerance to toxicants. Furthermore, it may provide leads on novel therapeutic targets for the treatment or prevention of drug induced liver injury. In order to determine if a loss of Nrf2 function alters the ability of fibrate-type drugs to activate Ppar α , wild type and Nrf2 knockout mice were treated for five days with 250mg/kg of CFB. This study also determines whether or not Nrf2 regulates expression of Ppar α and Rxr α in mouse liver. Liver samples from CFB-treated wild type and Nrf2-null mice were used to analyze mRNA expression of Ppar α , Ppar γ , Rxr α , Nrf2, Pgc1 α and their target genes. Results of mRNA expression analysis indicate that Nrf2-null mice are more susceptible to Ppar α activation by the peroxisome proliferator CFB, possibly through increased expression of Pgc1 α .

A follow up study was performed in order to determine if CFB pretreatment is able to protect Nrf2-null mice against a toxic dose of APAP. Nrf2-null mice were pretreated for five days with 250mg/kg of the fibrate drug CFB and challenged on day six with 300mg/kg APAP. Analysis of serum alanine aminotransferase (ALT) activity and hepatic non-protein sulfhydryl (NPSH) content suggests that Ppar α activation also has the potential to partially protect against oxidative stress in Nrf2-null mice, as we have previously shown in wild type mice.

2.3 Materials and Methods:

Chemicals. Acetaminophen (APAP), Clofibrate (CFB) and corn oil (CO) were purchased from Sigma Aldrich (St. Louis, MO). All of the other reagents used were commercially available and of reagent grade or better.

Treatment of Animals. Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were acclimated for one week upon arrival. Male Nrf2-null mice on a mixed C57BL/6 and AKR background were bred at the University of Connecticut. Mice were housed in an environment with a 12-hour light/dark cycle, controlled temperature and controlled humidity. Mice were fed laboratory rodent diet (Harlan Teklad 2018, Madison, WI) and water *ad libitum*. A modified version of a ten day, 500mg/kg CFB dosing model was adapted from Nicholls-Grzemeski et al. (1992) due to the inability of Nrf2-null mice to tolerate a 500mg/kg dose of CFB for more than a few days. Groups of mice (n = 4-5; 8-10 weeks) received daily doses of either vehicle (corn oil) or CFB (250mg/kg) via intraperitoneal (i.p.) injection for five days. 24 hours after the final day of CFB dosing, blood was collected in 1.5mL microcentrifuge tubes for ALT analysis. Livers were removed and portions were either snap frozen in liquid nitrogen and stored at -80°C or fixed in formalin.

An APAP challenge study was also conducted. Groups of Nrf2-null mice (n=3-5; 8-12 weeks) were dosed i.p. for five days with either CFB or vehicle. At the end of day five mice were fasted overnight. On day six, they were given a challenge dose of either vehicle (50% propylene glycol; 5mL/kg i.p.) or APAP (300mg/kg). Typically a dose of at least 400mg/kg APAP is used in wild type mice, however, Nrf2-null mice have increased susceptibility to APAP toxicity,

therefore the dose was lowered to 300mg/kg APAP to reduce the possibility of lethality (Aleksunes et al., 2008). Food was returned 8 hours after dosing. 24 hours after APAP dosing, blood was collected in 1.5mL microcentrifuge tubes for ALT analysis. Livers were removed and portions were either snap frozen in liquid nitrogen and stored at -80°C, fixed in formalin or placed in trichloroacetic acid (TCA) buffer (5% TCA in 10⁻³M EDTA) for non-protein sulfhydryl (NPSH) analysis. All animal studies were conducted in accordance with the standards of the National Institutes of Health and the *Guide for the Care and Use of Laboratory Animals*. Studies were approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC Protocol no. A15-041).

Alanine Aminotransferase (ALT) Activity Assay. Hepatotoxicity was determined by measuring ALT activity in serum samples using an Infinity ALT (GPT) Reagent from Thermo Scientific (Waltham, MA) using the recommendations of the manufacturer. Whole blood was allowed to clot for 30-minutes and serum was isolated following a 15-minute spin at 2000 x g in a tabletop centrifuge. Samples were read on a BioTek PowerwaveX 96-well plate reader (Winooski, VT).

Histology. Liver samples were fixed in 10% zinc formalin before paraffin embedding. 5µm sections from the paraffin embedded samples were stained with hematoxylin and eosin and observed using light microscopy for the presence of hepatomegaly, hepatocellular necrosis and other alterations to liver structure.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from mouse liver samples using TRIzol reagent (Life Technologies, Carlsbad, CA)

according to the manufacturer's recommendations. Total RNA was then reverse-transcribed into cDNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA). qRT-PCR was run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR green master mix (BioRad, Hercules, CA) and species-specific primers for each gene (Table 2.1). mRNA expression was quantified using the $\Delta\Delta C_T$ method and was normalized to β -actin.

Protein Isolation and Western Blot Analysis. Portions of livers, snap frozen in liquid nitrogen, were homogenized in sucrose-Tris (ST) buffer (150mM sucrose, 10mM Tris-HCl, pH 7.5) containing 1% Halt protease inhibitor cocktail (100X; Thermo Scientific, Waltham, MA). Protein concentrations of whole liver and nuclear homogenates were assayed using BioRad DC protein assay reagents (BioRad, Hercules, CA), and using dilutions of bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) to develop a standard curve. Whole liver and nuclear homogenates were diluted 1:1 with 2x Kaman buffer (2.3% sodium dodecyl sulfate (SDS), 10% Glycerol, 5% 2-Mercaptoethanol, 62.5mM Tris-HCl) and boiled for 5 minutes at 95°C before gel loading. Protein samples were resolved with gel electrophoresis using polyacrylamide gels (10% resolving, 4% stacking; run for 120min at 120v; transferred for 90min at 105v) and trans-blotted onto Polyvinylidene Fluoride (PVDF) membranes (Micron Separations, Westboro, MA). Membranes were blocked overnight in 5% non-fat dry milk dissolved in Tris-buffered saline containing .1% Tween-20 (TBS-T, pH 7.4). Membranes were incubated overnight with primary antibody diluted in blocking buffer, followed by a wash with TBS-T. The appropriate primary antibody was used for Vnn1 (Ab96171; Abcam, Cambridge, MA), β -actin (Ab8227; Abcam), and Nqo1 (ab2346; Abcam). Membranes were then incubated for 2 hours with species-specific

Gene ID	Forward	Reverse
mAbcc2	TCCAGGACCAAGAGATTTGC	TCTGTGAGTGCAAGAGACAGGT
mAbcc3	AGTGGCTGTGATAGTCTTGCTGATA	CCG TTCAGGATCTCGCTCAT
mAbcc4	ACCTCTGCTCGCGCGTGTCT	CCAGTACCGTTGAAGCTCCTCTCC
mAcox1	CCTGATTCAGCAAGGTAGGG	TCGCAGACCCTGAAGAAATC
mBcrp	TCGGAACTCAGTTTATCCGTGGCA	GCATTGCTGTGCTTGAGTCCAAA
mCatalase	TGGCACACTTTGACAGAGAGC	CCTTTGCCTTGGAGTATCTGG
mCyp2r1	AAACTACAACCAATGTGCTCCG	ATTCCCAAGAAGGTCTCCTGT
mCyp4a10	TTCCCTGATGGACGCTCTTTA	GCAAACCTGGAAGGGTCAAAC
mDio1	GCTGAAGCGGCTTGTGATATT	GTTGTCAGGGGCGAATCGG
mFabp4	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTTCATAACACATTCC
mGclc	CTGCACATCTACCACGCAGT	TTCATGATCGAAGGACACCA
mGlut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
mHmox1	GAGCCTGAATCGAGCAGAAC	CCTTCAAGGCCTCAGACAAA
mLpl	GGGAGTTTGGCTCCAGAGTTT	TGTGTGTTCAAGGGGTCCTTAG
mNqo1	AGGATGGGAGGTACTCGAATC	TGCTAGAGATGACTCGGAAGG
mNrf2	TCTTGGAGTAAGTCGAGAAGTGT	GTTGAAACTGAGCGAAAAAGGC
mPck1	CTGCATAACGGTCTGGACTTC	CAGCAACTGCCCGTACTCC
mPdk4	AGGGAGGTCGAGCTGTTCTC	GGAGTGTTCACTAAGCGGTCA
mPgc1 α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
mPpara α	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
mPpar γ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
mRar α	ATGTACGAGAGTGTGGAAGTCG	ACAGGCCCGGTTCTGGTTA
mRbp1	AACGTGGCCTTACGCAAAATC	GCGGTCGTCTATGCCTGTC
mRxr α	ATGGACACCAAACATTTCTGCG	CCAGTGGAGAGCCGATTCC
mSlc27a1	CGCTTTCTGCGTATCGTCTG	GATGCACGGGATCGTGTCT
mVanin1	ACCACAGGTGCGTAAATTGG	TTATGCCTTTGGAGCCTTTG
m β actin	GCAACGAGCGGTTCCG	CCCAAGAAGGAAGGCTGGA

Table 2.1. Primer Sequences for Genes Examined by RT-PCR

peroxidase-labeled secondary antibodies (Sigma-Aldrich, St. Louis, MO) diluted in blocking buffer. After another wash period in TBS-T, blots were coated in Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) for 1 minute. This was followed by exposure to X-ray film. Protein expression was quantified, using ImageJ image processing software, and normalized to β -actin (whole liver).

Non-Protein Sulfhydryl Assay. The total concentration of GSH in the liver was quantified from the supernatant of a 20% liver homogenates in 5% TCA in 3mM EDTA. The colorimetric assay of Ellman was used for quantifying NPSH, as adapted by Manautou et al. (1994) (Ellman, 1959; Manautou et al., 1994). Samples were read on a BioTek PowerwaveX 96-well plate reader (Winooski, VT) at 490nm. The levels of NPSH in liver were determined through comparison to a GSH standard curve.

Statistical Analysis. The statistical significance between groups was determined using the Student's t-test or one-way ANOVA with the Newman-Keuls post hoc test. The Student's t-test was used to compare means of two different treatment groups and ANOVA was used to compare the means of more than two treatment groups normally distributed with a common variance. Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA). Data are presented as the mean \pm standard error of the mean (SEM), with $p < 0.05$ considered statistically significant.

2.4 Results:

Clofibrate increases liver to bodyweight ratio in Nrf2 but not wild type mice

In a ten day model of 500mg/kg CFB dosing, wild type mice receiving CFB had greater liver to bodyweight ratio than mice receiving only vehicle (Manautou et al., 1994). With the reduced dose (250mg/kg CFB) and reduced duration of dosing (five days) employed in the current study, there were no significant changes in liver to bodyweight ratios in wild type mice treated with CFB (Figure 2.1a). This suggests that this alternative CFB dosing regimen is not sufficient to produce the marked hepatomegaly previously reported in rodents. Histological analysis of vehicle and CFB-treated wild type mouse livers did not reveal any obvious morphological differences in hepatocytes between the two treatment groups (data not shown). However, the basal liver to bodyweight ratio in Nrf2-null mice was reduced compared to wild types. This finding has been previously reported and could possibly be due to decreased rates of hepatocellular proliferation in Nrf2-null mice (Beyer et al., 2008). Although the liver to bodyweight ratio was unchanged in wild type mice following CFB treatment, a significant increase was observed in CFB-treated Nrf2-null mice. The drug-induced increase in liver to bodyweight ratio in null mice does not seem to be accompanied by hepatocellular injury or inflammation, since no ALT elevations were observed (Figure 2.1b). Furthermore, histological analysis of vehicle and CFB-treated Nrf2-null mouse livers did not reveal the presence of injury or inflammation in either treatment group (data not shown). It is possible that the increased liver to bodyweight ratio observed in the CFB-treated Nrf2-null mice may be due to hepatocellular hyperplasia or enlargement of hepatocytes. Treatment of mice for five days with 250mg/kg CFB does not seem to be sufficient to cause the hepatomegaly observed in wild type mice in our previously used ten-day model.

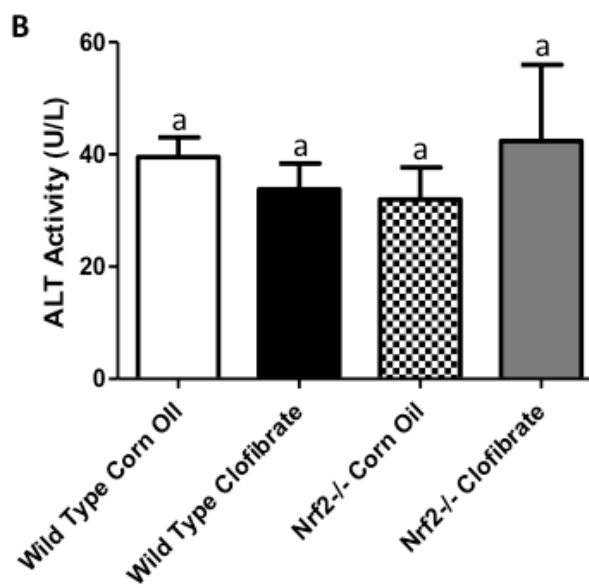
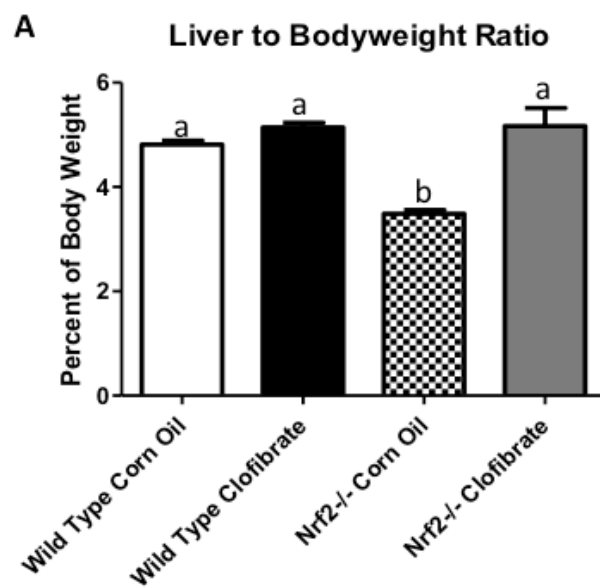


Figure 2.1: *Liver to Bodyweight Ratio and ALT Activity Analysis Following Clofibrate Treatment.* Livers and serum were isolated from mice 24 hours after the final dose of CFB. (A) Liver to bodyweight ratio is presented as percent of total body weight \pm SEM (n=4-5 animals). (B) Data presented as mean serum ALT activity (U/L) \pm SEM. Values with different superscripts are significantly different from one another ($p < .05$).

Nrf2-null mice have increased sensitivity to Ppara activation

Nrf2 and Ppara have both been linked to protection against APAP hepatotoxicity. However, it is not known if the two transcription factors engage in crosstalk to mediate this protection. Peroxisome proliferators exert their effects (i.e. hepatomegaly, hepatic peroxisome proliferation) through activation of the Ppara receptor in mice. The ability of peroxisome proliferators, such as CFB, to activate Ppara in the absence of Nrf2 has not been extensively studied and may provide some insight into whether or not the Nrf2 and Ppara pathways are interconnected and operating in a coordinated manner to protect the liver.

A gene array study on CFB-treated wild type and Ppara-null mouse livers identified the Ppara target gene Vanin1 (Vnn1) as being heavily induced by CFB treatment in wild type mice, compared to vehicle. Furthermore, analysis of mRNA expression revealed that the basal expression of Vnn1 is significantly reduced in Ppara-null mice, compared to wild type (Moffit et al., 2007). Vnn1 is a pantetheinase that functions in the hydrolysis of pantetheine into the CoA precursor vitamin B5 (pantoic acid) and the antioxidant cysteamine (Chen et al., 2014). Studies in Vnn1-null mice determined that Vnn1 is involved in Ppara-mediated protection against APAP hepatotoxicity, as evidenced by the greater susceptibility of Vnn1-null mice to APAP hepatotoxicity (Ferreira et al., 2016). Due to its induction by CFB and its involvement in Ppara-mediated protection against APAP hepatotoxicity (Ferreira et al., 2016; Moffit et al., 2007), Vnn1 expression should be a good indicator of potential Ppara activation in the Nrf2-null mice.

In order to investigate the effect of Nrf2 knockout on Ppar α expression and activation, qRT-PCR was performed on cDNA isolated from vehicle and CFB-treated wild type and Nrf2-null livers. mRNA expression was determined for Ppar α , Vnn1 (Vnn1) and other known Ppar α target genes (i.e. Acyl-CoA oxidase 1 (Acox1), Pyruvate dehydrogenase kinase 4 (Pdk4), Cyp4a10). Acox1 was the first Ppar α target gene to be identified, which encodes the enzyme involved in the first step of peroxisomal long-chain FA oxidation (Kersten, 2014). Pdk4 is an enzyme normally activated during periods of fasting. It is involved in switching fuel sources from oxidation of carbohydrates to oxidation of fat (Wang and Sahlin, 2012). Cyp4a10 is a member of the Cyp4a subfamily of cytochrome P450s. The Cyp4a subfamily is expressed in the rough endoplasmic reticulum in the liver and functions in the hydroxylation of medium and long chain FAs. Long chain FAs are then β -oxidized in the mitochondria or peroxisomes for subsequent use as fuel (Nyagode et al., 2014).

Our results indicate that there are no changes in basal Ppar α hepatic mRNA expression between wild type and Nrf2-null mice, or between vehicle and CFB-treated wild type mice. CFB treatment in Nrf2-null mice did lead to a significant increase in Ppar α mRNA expression (Figure 2.2a). There were no changes in the basal mRNA expression of Vnn1, Acox1, Pdk4 and Cyp4a10 between vehicle-treated wild type and Nrf2-null mice. Compared to vehicle-treated controls, a significant increase in mRNA expression was observed for all target genes following CFB treatment in both genotypes (Figures 2.2b-e). Compared to all other treatment groups, mRNA expression for Vnn1, Pdk4 and Cyp4a10 was significantly higher in CFB-treated Nrf2-null mice. A similar trend was observed in Acox1 expression, but it was not significant. These data indicate that CFB-mediated Ppar α activation is greater in Nrf2-null mice than in wild type mice.

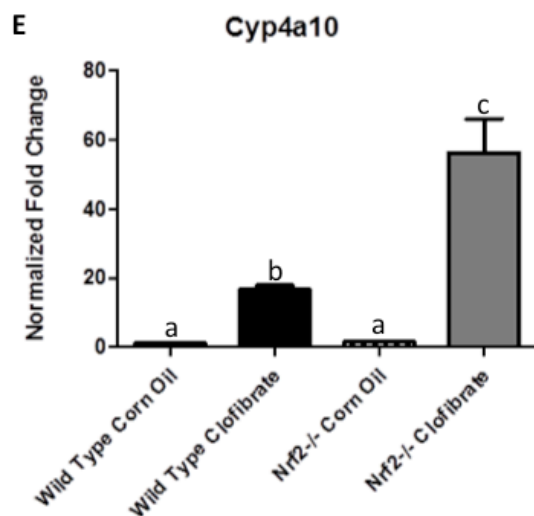
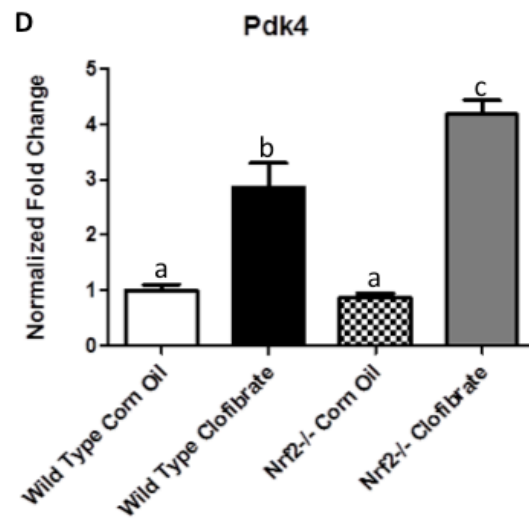
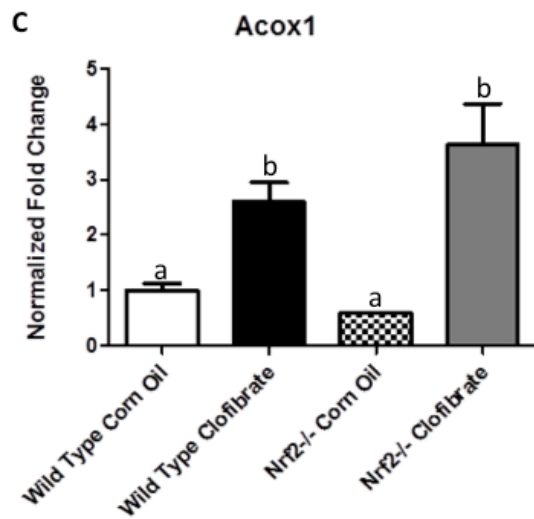
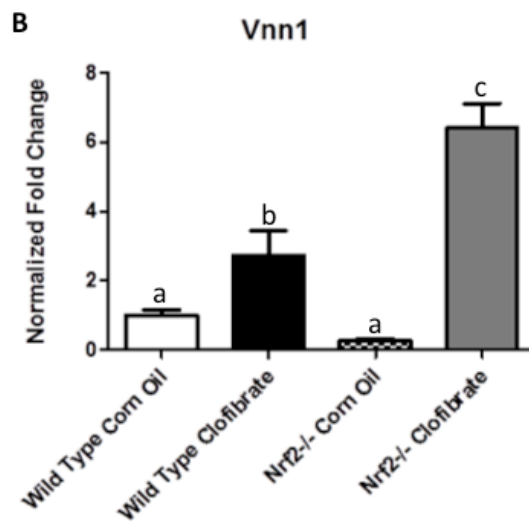
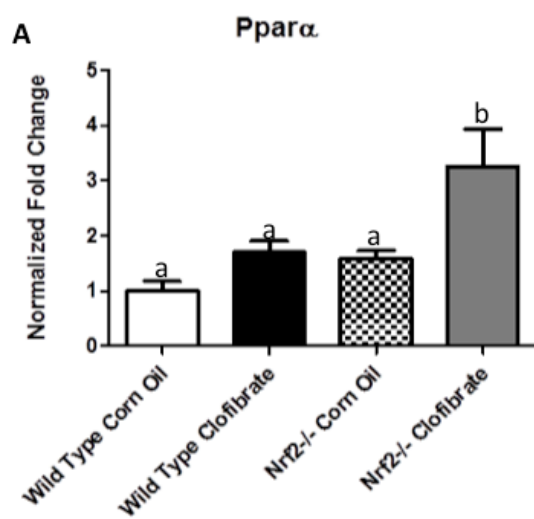


Figure 2.2: *Hepatic Gene Expression of Ppara and Ppara-Dependent Genes.* qRT-PCR was performed using cDNA isolated from wild-type and Nrf2-null mouse livers 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). (A) Gene expression of Ppara. (B-E) Gene expression of Ppara-dependent genes involved in the cellular response to oxidative stress (Vnn1) and in fatty acid oxidation (Vnn1, Acox1, Pdk4 and Cyp4a10). Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

Nrf2-null mice have decreased basal hepatic mRNA expression of Ppar γ

A literature search to determine if a possible connection between Nrf2 and Ppar signaling pathways has already been established revealed the existence of such a connection. Ppar γ is part of the Ppar family of nuclear receptors and acts in FA metabolism, glucose storage and adipocyte differentiation. It is highly expressed in adipose tissue, but is also ubiquitously expressed in other tissue types, such as the liver. CFB-mediated activation of Ppar γ has been reported in zebrafish hepatocytes (Ibabe et al., 2005). Studies of acute lung injury in Nrf2-null mice identified an ARE binding site for Nrf2 in the Ppar γ promoter and determined that Nrf2 has a role in the regulation of Ppar γ (Cho et al., 2010). Another group of investigators discovered that Nrf2 is important for regulating Ppar γ expression during adipogenesis. Nrf2-null mice exhibited impaired adipogenesis due to decreased mRNA and protein expression of Ppar γ (Pi et al., 2010). It is clear that Nrf2 is involved in the regulation of Ppar γ , however, it is unknown whether or not this regulation extends to the liver.

In order to determine if Nrf2 affects Ppar γ expression in the liver and if CFB treatment influences Ppar γ activation, qRT-PCR was performed on Ppar γ and Ppar γ target genes, such as fatty acid transport protein 1 (Fatp1; Slc27a1), lipoprotein lipase (Lpl), glucose transporter type 4 (Glut4) and fatty acid binding protein 4 (Fabp4). These genes were selected due to their high levels of expression in adipose tissue and their roles in the transport or storage of FAs or glucose. Slc27a1 is a transport protein involved in the uptake of long chain FAs (Guitart et al., 2014). Lpl catalyzes the hydrolysis of triacylglycerol and very low-density lipoproteins into free FAs. These free FAs can then be stored in adipose as triacylglycerol or oxidized and used for

energy (Braun and Severson, 1992). Glut4 is a transport protein involved in the uptake of glucose from the systemic circulation into mainly muscle and adipose tissue (Huang and Czech, 2007). Fabp4 is a chaperone protein for FAs and acts to transport intracellular lipids to biological targets (Garin-Shkolnik et al., 2014).

Basal mRNA expression of Ppar γ was significantly lower in Nrf2-null compared to wild type mice. There was no significant alteration in Ppar γ mRNA expression in wild type mice following CFB treatment; while the CFB-treated Nrf2-null mice exhibited similarly repressed levels of Ppar γ mRNA as vehicle-treated null mice (Figure 2.3a). Furthermore, there was no change in the basal mRNA expression of Slc27a1, Lpl, Glut4 or Fabp4 between genotypes (Figure 2.3b-e). There were also no significant changes in mRNA expression of these genes in wild type mice treated with CFB. However, in Nrf2-null mice receiving CFB the mRNA expression of Slc27a1, Lpl, Glut4, and Fabp4 was significantly increased. This suggests that the expression of the Ppar γ -dependent genes is being regulated by Ppar α activation. Due to the major role of the Ppars in FA metabolism, it is not surprising that there is a degree of overlap between genes that are targeted by Ppar γ and genes that are targeted by Ppar α . Slc27a1, Lpl and Fabp4 have all been shown to be upregulated by Ppar α activation in liver macrophages (Kupffer cells) (Rakhshandehroo et al., 2010). Despite this, it is clear that the lack of Nrf2 is causing a decrease in the basal expression of Ppar γ mRNA, which suggests that Ppar γ expression is Nrf2-dependent in the mouse liver.

Clofibrate does not affect the expression of Rxra and Rxra target genes

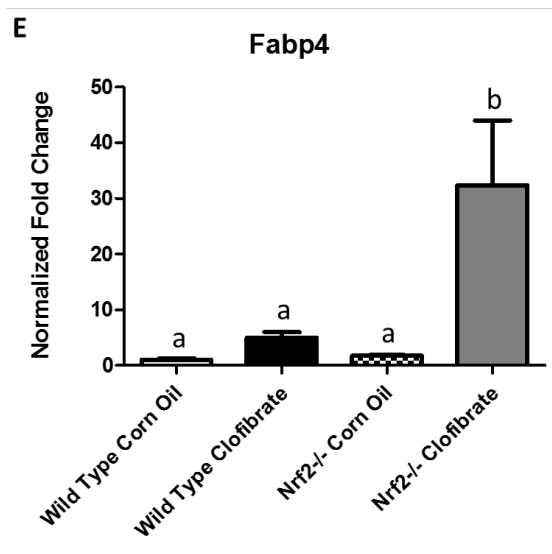
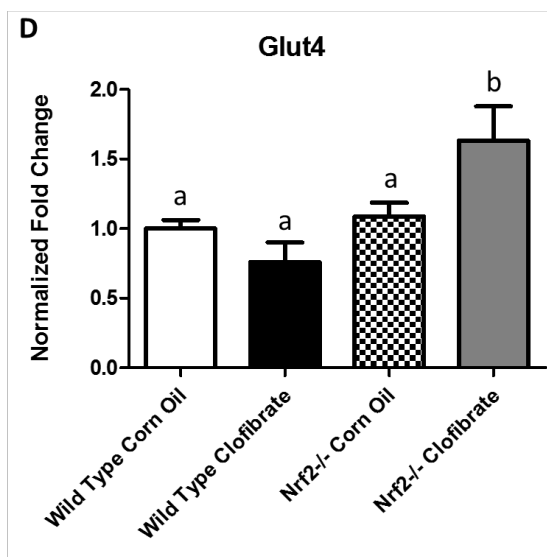
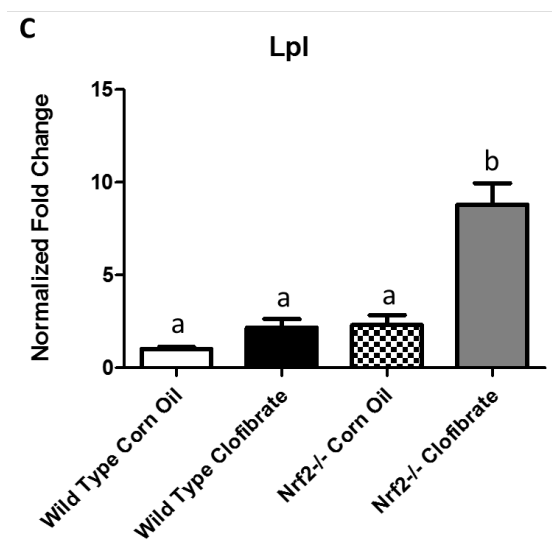
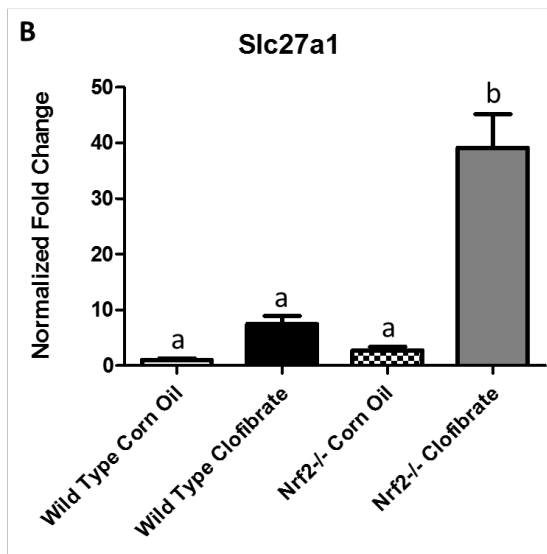
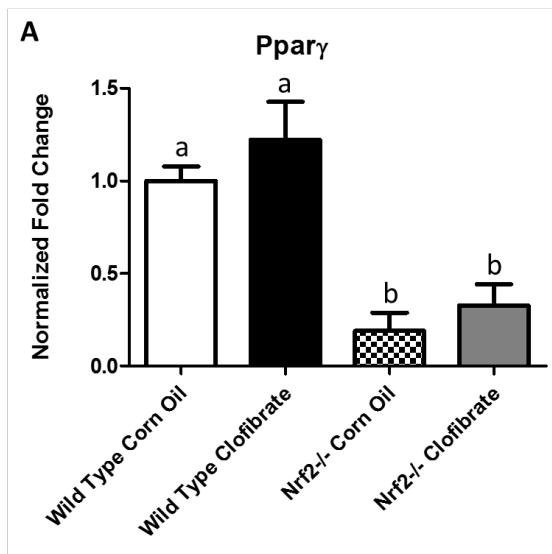


Figure 2.3: *Hepatic Gene Expression of Ppar γ and Ppar γ Target Genes.* qRT-PCR was performed using cDNA isolated from wild-type and Nrf2-null mouse livers 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). (A) Gene expression of Ppar γ . (B-E) Gene expression of Ppar γ target genes involved in fatty acid transport (Slc27a1, Fabp4), lipid metabolism (Lpl) and glucose transport (Glut4). Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

Rxra is a member of the retinoid X receptor (Rxr) family of nuclear receptors, along with Rxrb, and Rxry. The Rxr family acts as a heterodimer partner for many nuclear receptors, including retinoic acid receptors (Rars), the Ppars, vitamin D receptors (Vdr) and thyroid hormone receptors (Tr) (Mangelsdorf et al., 1992). The Rxra subtype was chosen for analysis in mouse liver due to its high expression in the liver, kidney, intestine and muscle (Pérez et al., 2012).

Rxra is a crucial component of Ppar signaling, and expression and regulation of Rxra could have direct effects on Ppar signaling. There is experimental evidence supporting the regulation of Rxra by Nrf2. A genome-wide binding study for human Nrf2 revealed that Rxra is an ARE-dependent target of Nrf2 binding. Also, mRNA expression of Rxra is increased by Nrf2 activation and decreased by Nrf2 knockdown in mouse embryonic fibroblast cells (Chorley et al., 2012). It appears that Rxra may also play a role in the regulation of Nrf2 activity. Overexpression of Rxra in human colon cancer cells results in reduced basal mRNA expression of Nrf2-dependent genes and Rxra knockdown results in increased basal mRNA expression for these genes (Wang et al., 2013). Altogether these results suggest that there is a regulatory feedback loop present between Nrf2 and Rxra.

Analysis of mRNA expression for Rxra and Rxra target genes, such as Retinoic acid receptor α (Rar α), Deiodinase 1 (Dio1), Cyp2r1, and Retinol binding protein 1 (Rbp1), was performed to determine if CFB treatment or loss of Nrf2 is having any effect on Rxra expression or activity. Rar α is a heterodimer partner for Rxra. The Rar-Rxr heterodimer acts in the transcriptional regulation of genes involved in cell-cycle arrest, cell differentiation and cell death (Kalitin and Karamysheva, 2016). Dio1 is the enzyme, present in the liver, responsible for

the conversion of the thyroid pro-hormone thyroxine to its active form triiodothyronine. Dio1 is a target gene of the thyroid hormone receptor, which forms a heterodimer with Rxr α (Yu and Koenig, 2006). Cyp2r1 catalyzes a step in the synthesis of the active form of vitamin D. Cyp2r1 is regulated by the vitamin D receptor, which forms a heterodimer with Rxr α (Cheng et al., 2004). Rbp1 is a target gene of Rar α that acts as a chaperone protein for retinol (Pierzchalski et al., 2014).

Analysis of Rxr α mRNA expression shows no changes between Nrf2-null and wild type mice or between vehicle and CFB treatment groups (Figure 2.4a). There was also no change in mRNA expression of Rar α or Cyp2r1 between genotypes or treatment groups (Figure 2.4b and c). Basal mRNA expression for Dio1 was significantly lower in Nrf2-null mouse liver compared to wild types. However, no significant changes were observed in response to CFB treatment in either genotype (Figure 2.4d). Basal mRNA expression for Rbp1 was significantly increased in Nrf2-null compared to wild type. For all genes tested, CFB treatment in both genotypes did not produce any significant changes in mRNA expression (Figure 2.4e). Loss of Nrf2 appears to have an effect on the expression of some Rxr α target genes. These effects do not appear to be due to Nrf2-mediated changes in Rxr α expression. Also, CFB treatment does not appear to have any effect on the mRNA expression of Rxr α or its target genes.

Clofibrate increases the mRNA expression of Nrf2 in wild type mice

Nrf2 acts to regulate the transcription of genes involved in protecting against oxidative stress. In the absence of Nrf2 cells become more susceptible to oxidative damage. CFB treatment, in a Ppar α -dependent manner, has been demonstrated to induce the mRNA and

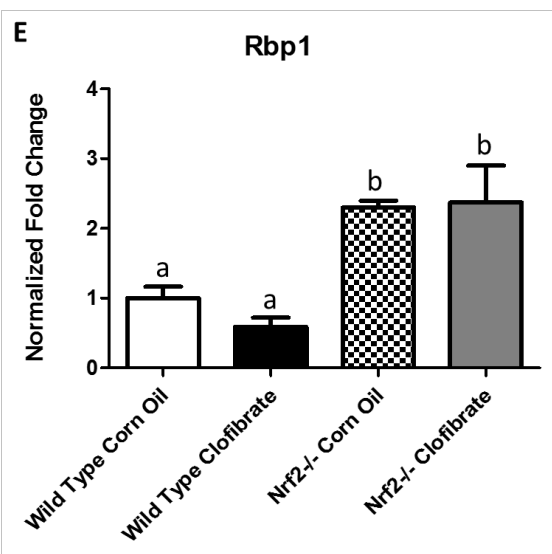
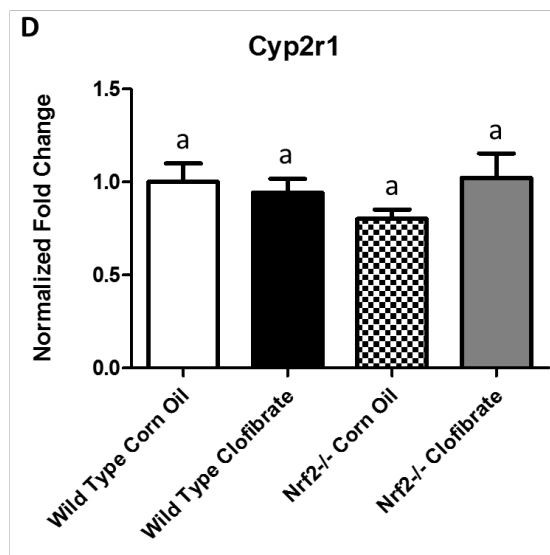
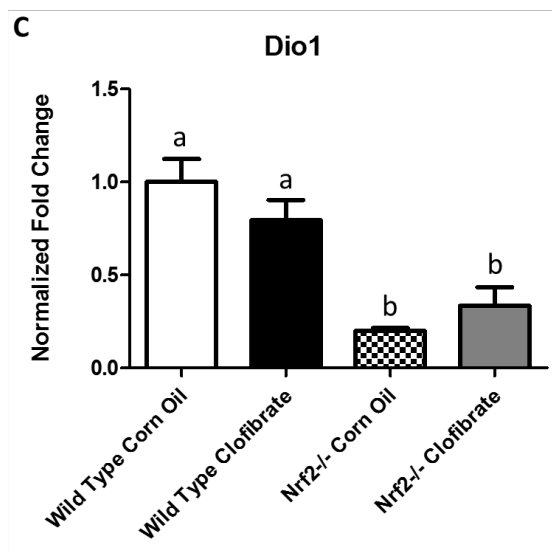
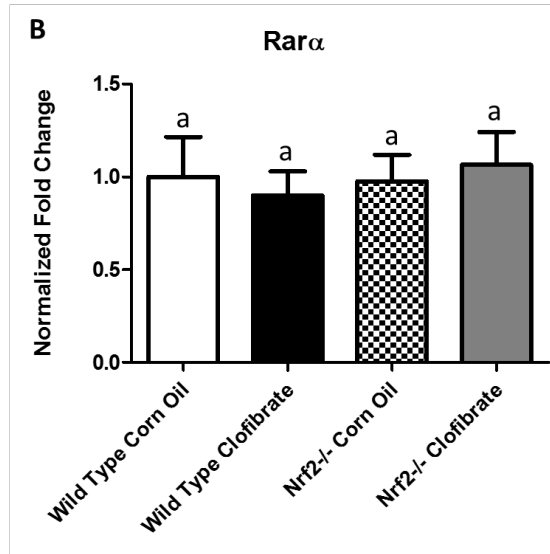
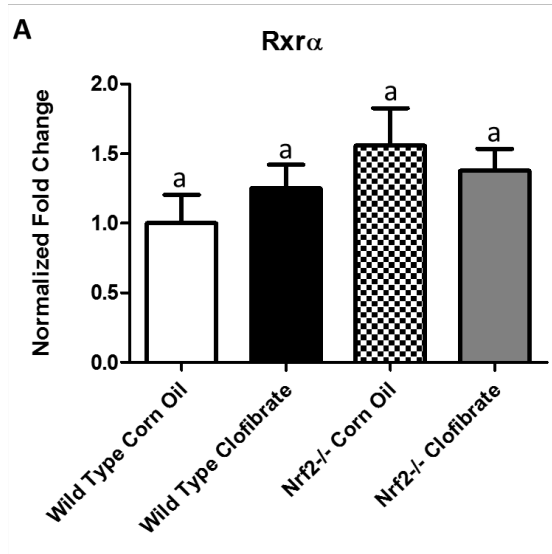


Figure 2.4: *Hepatic Gene Expression of Rxra and Rxra–Dependent Genes.* qRT-PCR was performed using cDNA isolated from wild-type and Nrf2-null mouse livers 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). (A) Gene expression of Rxra. (B) Gene expression of the Rxra heterodimer partner Rara. (C-E) Gene expression of non-Ppar, Rxra heterodimer target genes involved in thyroid hormone activation (Dio1), vitamin D metabolism (Cyp2r1) and retinol transport (Rbp1). Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

protein expression of several efflux transporters that are targets of Nrf2 (Moffit, 2006). In order to determine the effect of CFB treatment on Nrf2 in the five day CFB dosing mouse model, qRT-PCR analysis was performed to analyze the mRNA expression of Nrf2 and Nrf2 target genes, such as glutamate-cysteine ligase catalytic subunit (Gclc), NAD(P)H:quinone oxidoreductase 1 (Nqo1), catalase (Cat), heme oxygenase 1 (Hmox1), multi-drug resistance protein 2 (Mrp2; Abcc2), multi-drug resistance protein 3 (Mrp3; Abcc3), multi-drug resistance protein 4 (Mrp4; Abcc4) and breast cancer resistance protein (Bcrp/Abcg2).

Gclc is the catalytic subunit for Gcl, which functions to catalyze the rate-limiting step of GSH synthesis (Gorrini et al., 2013). Nqo1 acts in the reduction of quinones to prevent the formation of reactive semiquinone radicals (Atia et al., 2014). Cat is the enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen. It acts as an antioxidant by detoxifying hydrogen peroxide and preventing oxidative cellular damage (Reuter et al., 2010). Hmox1 is involved in the breakdown of heme into free iron, which is later sequestered and detoxified by the ferritin complex (Gorrini et al., 2013). Mrp2 is mainly expressed in the hepatocyte canalicular membrane and functions in the export of glucuronate, sulfate and GSH conjugates into the bile (Jedlitschky et al., 2006). Mrp3 is a basolateral efflux transporter and acts in the export of bile acids and glucuronide conjugates into portal circulation (Belinsky, 2005). Mrp4 is a basolateral efflux transporter that exports bile acids and GSH conjugates into portal circulation. It also has a role in intracellular signaling through regulation of intracellular levels of cyclic nucleotides, such as cAMP (Russel et al., 2008). Bcrp is a canalicular efflux transporter that exports endogenous compounds, such as urate and folate, and many xenobiotics into the bile (Eldasher et al., 2013).

Expression of Nrf2 mRNA was significantly increased following CFB treatment in wild type mouse liver (Figure 2.5a). This suggests that Ppara activation is having an effect on the expression of Nrf2. Gclc mRNA expression displays significantly decreased basal expression in Nrf2-null mice, compared to wild type. Expression was also significantly increased following CFB treatment in wild type mice (Figure 2.5b). Basal mRNA expression for Nqo1 was significantly lower in Nrf2-null mice than in wild type. Expression was significantly increased following CFB treatment in wild type, but not in Nrf2-null mice (Figure 2.5c). Basal mRNA expression for Cat was significantly decreased in Nrf2-null mice, compared to wild type. CFB treatment had no effect on Cat expression in wild type or Nrf2-null mice (Figure 2.5d). Furthermore, there were no changes in Hmox1 mRNA expression between genotypes or treatment groups (Figure 2.5e). Basal expression of Mrp2 mRNA was unchanged between Nrf2-null and wild type mice. There was a trend for increased mRNA expression following CFB treatment in wild type mice, with a significant increase detected in Nrf2-null mice (Figure 2.5f).

In the ten day model of CFB dosing (500mg/kg i.p.) Mrp3, Mrp4 and Bcrp mRNA and protein expression have been shown to increase in wild type mouse liver (Moffit, 2006). In the five-day model, Mrp3 displayed reduced basal expression in Nrf2-null mice and increased expression following CFB treatment in wild type (Figure 2.5g). There was no significant change in mRNA expression for Mrp4, however there does appear to be a trend for decreased basal expression in Nrf2-null mice (Figure 2.5h). Basal expression of Bcrp was reduced in Nrf2-null mice, which was increased to wild type basal levels of expression following CFB treatment in Nrf2-null mice (Figure 2.5i).

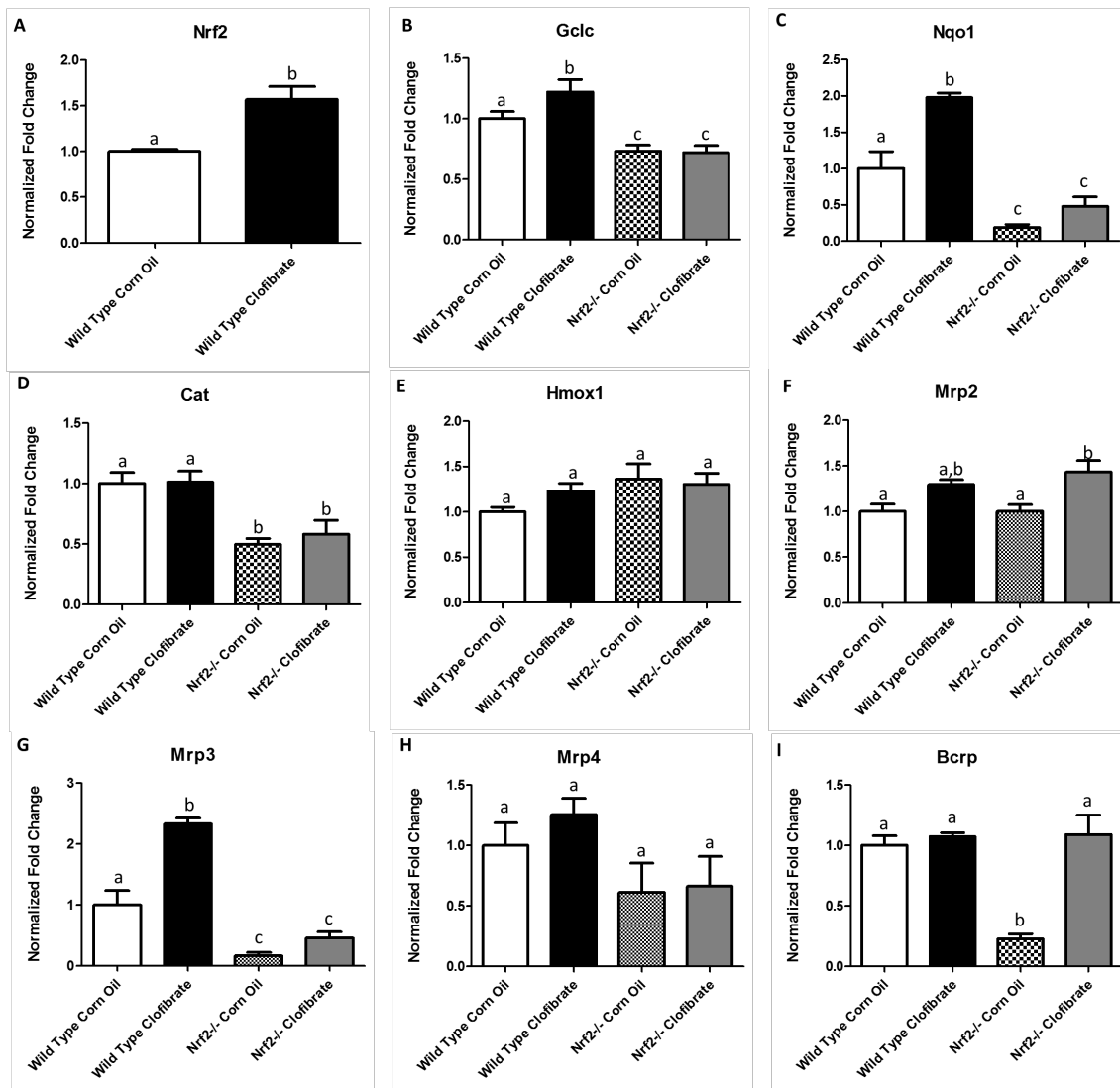


Figure 2.5: *Hepatic Gene Expression of Nrf2 and Nrf2–Dependent Genes.* qRT-PCR was performed using cDNA isolated from wild-type and Nrf2-null mouse livers 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). (A) Gene expression of Nrf2. (B-H) Gene expression of Nrf2-dependent genes involved in combating oxidative stress (Gclc, Nqo1, Cat, and Hmox1), canalicular efflux transport (Mrp2, Bcrp) and basolateral efflux transport (Mrp3, Mrp4). Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

Altogether these results suggest Ppar α may be involved in the regulation of transcription for Nrf2 and some of its target genes. Activation of Ppar α by CFB treatment in wild type mice increases the mRNA expression of Nrf2, and, as already demonstrated by Moffit et al. (2006, 2007), several Nrf2 target genes. In the absence of Nrf2, the majority of Nrf2 target genes tested did not display any CFB-mediated alterations to expression. Following CFB treatment, however, Mrp2 and Bcrp displayed significant increases in mRNA expression in Nrf2-null, compared to vehicle-treated null mice. These data suggest that Ppar α activation may regulate Nrf2 expression and may play a role in the transcriptional regulation of several Nrf2 target genes in the absence of Nrf2.

Loss of Nrf2 increases mRNA expression of Pgc1 α in mouse liver

Ppar gamma co-activator 1-alpha (Pgc1 α) is a member of the Pgc1 family of inducible transcriptional co-activators. Pgc1 is part of the co-activator complex that directly enhances transcription initiation in a series of transcription factors, including both nuclear and non-nuclear receptors, involved in cellular metabolism (Finck, 2006). Unlike in muscle and adipose, Pgc1 α and Pgc1 β are lowly expressed in mouse liver. During a period of fasting, however, hepatic expression of Pgc1 α and Pgc1 β increases. During fasting, FA oxidation is increased in the liver in order to supply the body with energy. This is mediated through Pgc1 α and Pgc1 β , which function as co-activators for Ppar α and act to increase transcription of Ppar α target genes involved in FA oxidation (Lin et al., 2003; Yoon et al., 2001). In hepatocytes isolated from fasted Pgc1 α -null mice, FA oxidation is diminished and steatosis is evident (Leone et al., 2005).

This suggests that Pgc1 α is important in the regulation of hepatic FA oxidation through co-activation of Ppar α .

Hepatic mRNA expression of Pgc1 α has been shown to be induced following fasting in both Nrf2-null and C57BL/6 wild type mice. Compared to all other treatment groups, fasted Nrf2-null mice displayed the highest levels of Pgc1 α expression (Zhang et al., 2013). In another study, Sanderson et al. (2009) performed ChIP analysis on fasted wild type (129S1/SvImJ) and Ppar α -null mice. In concordance with Zhang et al. (2013) they observed increased mRNA expression of Pgc1 α in following fasting in both genotypes. ChIP analysis displayed enhanced recruitment of Pgc1 α to the transcription start site of the representative Ppar α target genes fatty aldehyde dehydrogenase (Aldh3a2) and carnitine palmitoyltransferase 2 (Cpt2) in wild type mice. This recruitment, however, was not present in Ppar α -null mice, suggesting that Pgc1 α binding to these genes is Ppar α dependent (Sanderson et al., 2009). Analysis of Pgc1 α expression, following chemical activation of Ppar α , could provide insight into the interaction between Nrf2 and Ppar α . This is because increased expression of a known Ppar α co-activator, in the absence of Nrf2 expression, provides a possible mechanism for the increased sensitivity of Nrf2-null mice to CFB-mediated Ppar α activation.

Analysis of Pgc1 α mRNA expression was conducted to establish whether or not CFB has any effect on Pgc1 α expression. Basal mRNA expression of Pgc1 α was significantly increased in Nrf2-null mice compared to wild type. Expression was not altered by CFB treatment in either genotype (Figure 2.6). CFB acts as a synthetic ligand for Ppar α . When a ligand binds to nuclear receptors, it causes disassociation of the co-repressor protein complex. This enables association of co-activator proteins, which then act to enhance transcriptional initiation at the promoter of

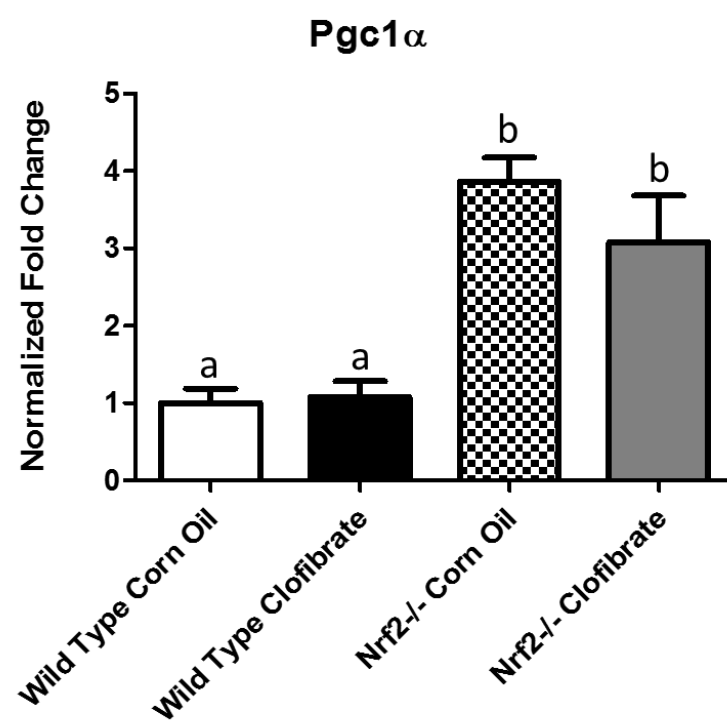


Figure 2.6: *Hepatic Gene Expression of Pgc1 α* . qRT-PCR was performed using cDNA isolated from wild-type and Nrf2-null mouse livers 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). Gene expression of Pgc1 α . Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another (p<.05).

target genes. Increased expression of the Ppar α co-activator protein Pgc1 α could allow for increased association with ligand bound Ppar α and increased transcription of Ppar α target genes.

Clofibrate treatment increases protein expression of Vanin-1 in wild type and Nrf2-null mouse liver

Western blot analysis of Vnn1 was conducted to determine if the absence of Nrf2 has any effect on the CFB-mediated induction of protein expression for Ppar α target genes. Expression of Vnn1 was significantly increased following CFB treatment, with no differences in the magnitude of induction by genotype. Protein expression, however, was not significantly different between CFB-treated wild type and Nrf2-null mice (Figure 2.7a). Further protein analysis of Ppar α and other Ppar α target genes are necessary to determine the effect of CFB on Ppar α activation in Nrf2-null mouse liver beyond our gene expression analysis.

Analysis of protein expression for the prototypical and representative Nrf2-dependent gene Nqo1 was also conducted. Protein expression for Nqo1 closely resembles mRNA expression. Expression was significantly increased in CFB-treated wild type mice, compared to vehicle-treated wild type mice (Figure 2.7b). Nrf2-null mice demonstrated significantly decreased basal protein expression of Nqo1, which is expected since Nqo1 is an Nrf2-dependent gene. CFB-treated Nrf2-null mice displayed no alterations to protein expression, compared to vehicle-treated null mice.

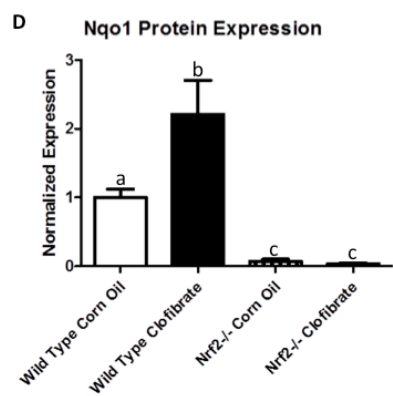
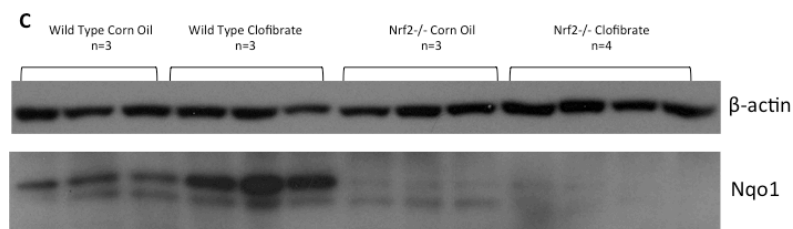
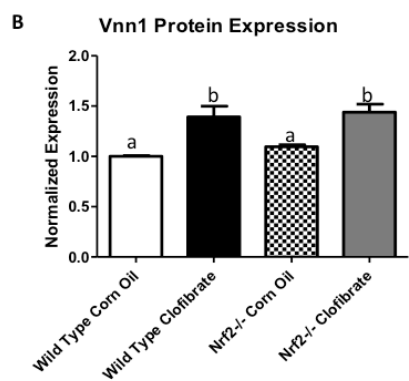
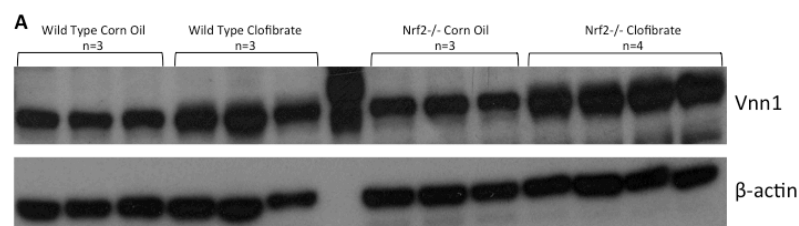


Figure 2.7: *Hepatic protein expression of Vnn1.* Western Blots were performed using whole-liver homogenates isolated from wild-type and Nrf2-null mice 24 hours after the last of five daily doses of CFB (250mg/kg; i.p.). (A) Protein expression of Vnn1. (B) Quantitation of Vnn1 protein expression, normalized to β -actin. (C) Protein expression of Nqo1. (D) Quantitation of Nqo1 protein expression normalized to β -actin. Data is presented as mean gene expression \pm SEM (n=4-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

Clofibrate pretreatment may partially protect Nrf2-null mice against Acetaminophen hepatotoxicity

Pretreatment of wild type mice with the fibrate-type drug CFB is known to provide protection against a challenge dose of APAP, however, the protective role of CFB has not been investigated in Nrf2-null mice. In order to determine if CFB pretreatment, in the absence of Nrf2, is able to protect against a toxic insult of APAP, Nrf2-null mice were pretreated for five days with 250mg/kg of CFB and challenged with 300mg/kg of APAP on day six. There was no significant change in the liver to bodyweight ratios between treatment groups 24 hours after APAP challenge (Figure 2.8a). This indicates that this alternative CFB treatment regimen of reduced dose and length did not result in hepatomegaly. Compared to vehicle-treated control mice, there was a significant increase in ALT activity in vehicle-treated mice challenged with APAP. In CFB pretreated Nrf2-null mice receiving APAP, ALT activity was also increased slightly, however, these values were not significantly different from either vehicle treated controls or vehicle-pretreated mice challenged with APAP (Figure 2.8b). In this study, ALT values at the dose of APAP used (300mg/kg) are much lower than our historical values. This indicates that the APAP dose in the current study did not produce sufficient toxicity to distinguish any potential differences in response by treatment. This is supported by the lack of any histological indications of hepatotoxicity (i.e. centrilobular necrosis) in control mice receiving APAP challenge (data not shown). A significant decrease in liver non-protein sulfhydryl (NPSH) content was observed in vehicle-pretreated mice challenged with APAP, compared to the mice receiving CFB only. Compared to all other groups, there was a trend for decreased NPSH content in vehicle-pretreated mice receiving APAP. Compared to all other groups there was a

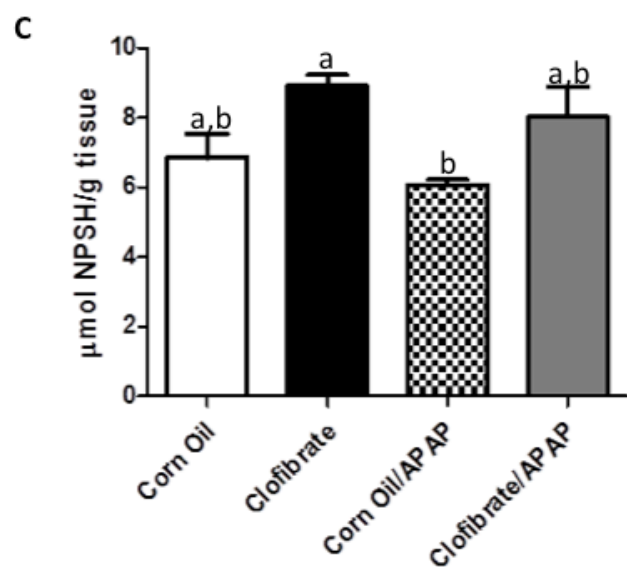
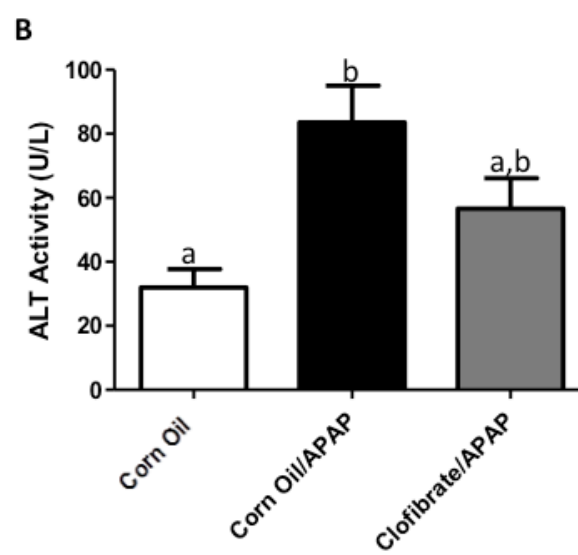
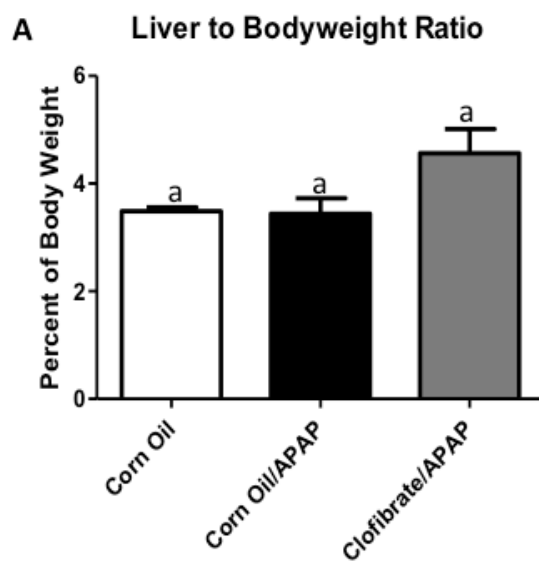


Figure 2.8: *Liver to Bodyweight Ratio, ALT Activity and Hepatic Non-Protein Sulfhydryl Content Following APAP challenge.* Liver and serum was isolated from Nrf2-null mouse livers 24 hours after APAP challenge (300mg/kg; 50% propylene glycol, i.p.). (A) Liver to bodyweight ratio presented as percent of total bodyweight. (B) ALT activity (U/L). (C) Hepatic NPSH content presented as μmol of NPSH per gram of liver. Data is presented as mean gene expression \pm SEM (n=3-5 mice). Values with different superscripts are significantly different from one another ($p < .05$).

trend for increased NPSH content in mice receiving only CFB (Figure 2.8c). Similarly, NPSH content was higher in CFB pretreated mice than in vehicle control or vehicle-pretreated mice. However, this elevation in NPSH was not significantly different from any other treatment groups. This suggests that CFB pretreated Nrf2-null mice may be less susceptible to GSH depletion following a toxic insult of APAP.

2.5 Discussion:

The Nrf2 and Ppar α transcription factors have both been demonstrated to play a role in protection against APAP-induced hepatotoxicity (Goldring et al., 2004; Nicholls-Grzemeski et al., 1992). The dependence of Ppar γ action on Nrf2 signaling has been demonstrated in lung and adipose tissue, and appears to act through binding of Nrf2 to an ARE in the Ppar γ promoter. The importance of this regulation was demonstrated in Nrf2-null mice, which display decreased basal expression of Ppar γ , increased susceptibility to acute lung injury, and impaired adipogenesis (Cho et al., 2010; Pi et al., 2010). Positive regulation of the Ppar heterodimer partner, Rxr α , through Nrf2 binding to an ARE in the promoter has also been shown *in vitro* (Chorley et al., 2012). Despite some experimental evidence supporting an interaction, a regulatory link between Nrf2 and Ppar α is yet to be established.

In the present study, the increased susceptibility of Nrf2-null mice to Ppar α activation, by means of CFB treatment, was demonstrated. Vehicle-treated Nrf2-null mice had a decreased liver to bodyweight ratio compared to vehicle-treated wild type mice. This decrease did not occur in CFB-treated Nrf2-null mice. There was also no change in the liver to bodyweight ratio of CFB-treated wild type mice. In the literature, 500mg/kg CFB treatment for ten days has been

reported to cause hepatomegaly in wild type mice (Manautou et al., 1994). Due to this treatment regimen being toxic in Nrf2-null mice, a new regimen of CFB dosing at 250mg/kg for five days was implemented. In the present study, the new treatment model was not sufficient to produce hepatomegaly in wild type or Nrf2-null mice; however, it was sufficient to cause an increase in the liver to bodyweight ratio of CFB-treated Nrf2-null mice, compared to vehicle-treated Nrf2-null mice. No evidence for hepatotoxicity was noted and animal viability was not compromised.

CFB is a member of the fibrate-type class of hypolipidemic drugs that act through activation of the Ppar α signaling pathway (Chen et al., 2000). In the present study, mRNA expression of Ppar α was significantly elevated in Nrf2-null mice following CFB treatment. Analysis of mRNA expression for Ppar α -dependent genes revealed a pattern of increased mRNA expression for both wild type and Nrf2-null mice treated with CFB. A greater fold change was observed in Nrf2-null mice. These findings suggest that Nrf2-null mice exhibit a greater increase in Ppar α activation by CFB than wild type mice. Western blot analysis of Vnn1 demonstrated significantly increased protein expression following CFB treatment in both wild type and Nrf2-null mice. Protein expression, however, did not significantly differ between the two genotypes following CFB treatment. This indicates that induction of Vnn1 protein by CFB is not influenced by Nrf2 expression or function, at least not with this regimen of CFB treatment. Further protein analysis of Ppar α and other target genes, such as Acox1, Pdk4 and Cyp4a10, are needed to determine if the data obtained from mRNA analysis is corroborated by data obtained from protein analysis. This would provide a better understanding as to the effect of CFB treatment on Ppar α activation in Nrf2-null mouse liver.

The Ppar family of transcription factors consists of three members (Ppar α , Ppar γ , and Ppar β/δ), which are differentially expressed throughout the body. Ppar γ is mainly expressed in adipose tissue (Zieleniak et al., 2008). Interactions between Nrf2 and Ppar γ are present in mouse adipose and lung tissue, but this has not been studied in the mouse liver. Our results show that Ppar γ mRNA expression in the mouse liver was not affected by CFB treatment, but was significantly decreased in vehicle-treated Nrf2-null compared to wild type mice. This is consistent with previous reports examining mouse lung and adipose tissue (Cho et al., 2010; Pi et al., 2010). Analysis of Ppar γ protein expression is still required, but Ppar γ mRNA expression suggests that Nrf2 may be a positive regulator of Ppar γ in the mouse liver. Analysis of mRNA expression for some Ppar γ target genes displayed no significant change following CFB treatment in wild type mice. Expression of these genes in Nrf2-null mice, however, was significantly increased by CFB treatment. It appears that in the mouse liver Ppar α predominantly regulates these selected Ppar γ target genes. This is not surprising because Ppar γ and Ppar α have both been demonstrated to regulate a set of overlapping genes involved in lipid metabolism (i.e. Lpl, Fabp4) (Nakachi et al., 2008; Rakhshandehroo et al., 2010). Also, Ppar γ acts mainly in adipose tissue as a regulator of lipid and glucose metabolism, and is only expressed at low levels in the liver. Ppar α , on the other hand, is highly expressed in the liver and acts as a main regulator of hepatic lipid metabolism (Tyagi et al., 2011). These findings suggest that, when analyzed in the liver, these selected Ppar γ target genes (i.e. Slc27a1, Lpl, Fabp4, Glut4) serve as better indicators of Ppar α activity than as indicators of Ppar γ activity.

Rxr α is a member of the Rxr family of transcription factors with high levels of expression in the liver (Pérez et al., 2012). It acts as a heterodimer partner for many transcription factors

and is required for Ppar α action. Our data show that Rxr α mRNA expression is not altered between mouse genotypes or treatment group, indicating that Rxr α expression is not affected by Nrf2 expression or function. This finding is contradictory to previous reports that mRNA expression of Rxr α is decreased by knocking down Nrf2 function (Chorley et al., 2012). This apparent discrepancy may be due to differences in experimental models. Our study analyzed mRNA expression of Rxr α *in vivo*, using wild type and Nrf2-null mice, whereas Chorley et al. (2012) analyzed mRNA expression *in vitro*, using shRNA knockdown of Nrf2 in mouse embryonic fibroblasts.

Analysis of mRNA expression for all of the Rxr α target genes tested revealed that CFB treatment had no effect on expression. Two of the analyzed target genes, mainly Dio1 and Rbp1, displayed altered basal mRNA expression in Nrf2-null compared to wild type mouse liver. Basal expression of Dio1 was significantly decreased in Nrf2-null compared to wild type mice and the basal expression of Rbp1 was significantly increased in Nrf2-null compared to wild type mice. Other target genes (i.e Rar α , Cyp2r1), however, displayed no change in basal expression between genotypes. The cause of the Nrf2-dependent changes in basal expression of Dio1 and Rbp1 are not clear, but it seems that the changes are not mediated by Rxr α signaling.

CFB has been previously reported to increase protein and mRNA expression of several Nrf2 target genes, including Mrp3, Mrp4 and Bcrp (Moffit, 2006). Interestingly, mRNA expression of Nrf2 was increased in wild type mice following CFB treatment. Protein expression is required to confirm this finding. As previously demonstrated by Moffit et al. (2006, 2007) CFB treatment caused increases in mRNA expression for the Nrf2 target genes Gclc, Nqo1 and Mrp3 in wild type mouse liver. Protein expression for Nqo1 was also increased in wild type following

CFB treatment. With the exception of Mrp2 and Bcrp, the majority of Nrf2 target genes tested, did not display any CFB-mediated alterations to expression in the absence of Nrf2. Mrp2 and Bcrp, however, displayed significant increases in mRNA expression in CFB-treated Nrf2-null, compared to vehicle-treated null mice. Furthermore, mRNA expression for Nrf2 was elevated in CFB-treated wild type mice compared to vehicle-treated controls. Peroxisome proliferators are known to cause oxidative stress in rodents thorough the indirect production of hydrogen peroxide (Gonzalez and Shah, 2008). The observed increases in Nrf2 mRNA expression may thus be a direct result of CFB dosing. Further studies of Nrf2 expression in the absence of Ppar α activity are required to confirm this. Altogether, these data suggest Ppar α activation may play a role in the regulation of Nrf2 expression and in the transcriptional regulation of some Nrf2 target genes in an Nrf2-independent manner.

Once the Ppar α -Rxr α heterodimer is ligand bound it is able to associate with co-activator proteins. Co-activator proteins, such as Pgc1 α , increase transcription of target genes by interacting with histone acetyltransferases, which act to remodel chromatin so that the transcriptional machinery has improved access to the target gene (Finck, 2006). Pgc1 α mRNA and protein expression have been demonstrated to increase in livers from 24 hour fasted rats and mice (Yoon et al., 2001; Zhang et al., 2013). Fasting is a natural way of indirectly activating Ppar α . Fasting causes increased production of FAs, which act as ligands for activation of Ppar α (Bouwens et al., 2007). CFB, on the other hand, is a synthetic ligand for the Ppar α nuclear receptor and acts to directly activate Ppar α , thus inducing transcription of Ppar α -dependent genes involved in FA oxidation. Analysis of Pgc1 α mRNA expression following five-day CFB treatment (250mg/kg) revealed that its basal expression is elevated in Nrf2-null mice,

compared to wild type mice. Increased expression could explain the observed sensitivity of Nrf2-null mice to Ppar α activation. Since Pgc1 α is also a known co-activator of Ppar α (Barberá et al., 2001), increased expression of Pgc1 α in Nrf2-null mice suggests that these mice may have increased ligand-mediated activation of Ppar α . Recruitment of Pgc1 α to the Ppar α heterodimer, following ligand binding, would induce transcription of Ppar α target genes, as depicted in Figure 2.9. Protein expression of Pgc1 α would provide further insight into the effect of loss of Nrf2 function on Pgc1 α expression and could provide a possible mechanism for the apparent interaction between Nrf2 and Ppar α .

A follow-up study was performed that involved challenging CFB-pretreated Nrf2-null mice with a toxic dose of APAP. This was performed to determine if the protective effect of Ppar α activation against APAP hepatotoxicity is still present in the absence of Nrf2. A challenge dose of 300mg/kg (50% propylene glycol; i.p.) was selected due to the increased sensitivity of Nrf2-null mice to APAP. Analysis of serum ALT levels revealed that, compared to vehicle-treated control mice, there was a significant increase in ALT activity in vehicle-treated mice challenged with APAP. ALT activity was also increased slightly in CFB pretreated mice, however, these values were not significantly different from either vehicle treated control or vehicle-pretreated mice. The observed ALT levels at the dose employed, however, do not match our historical data from previous APAP toxicity studies. This indicates that the APAP dose of 300mg/kg used in the present study was not sufficient to produce enough hepatotoxicity in Nrf2-null mice to distinguish potential differences in response by treatment. Although this dose should have produced noticeable toxicity in Nrf2-null mice our historical experience with APAP toxicity studies has demonstrated that seasonal variations (i.e. time of year) in responsiveness to APAP

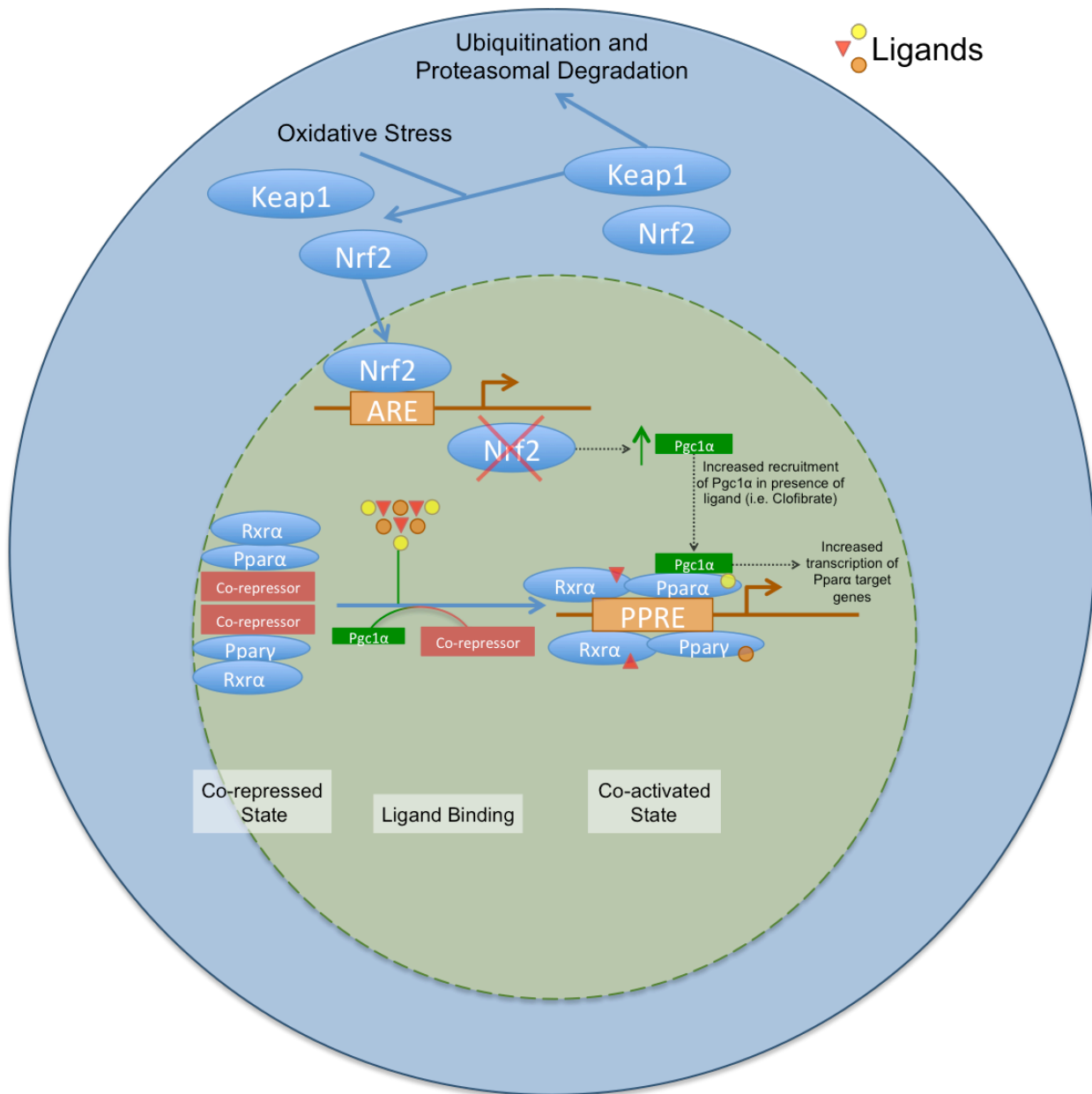


Figure 2.9: *Potential mechanism for increased activation of Ppara in Nrf2-null mice.* Depicts activation and nuclear translocation of Nrf2 in the presence of oxidative stress, as well as activation of Ppara through ligand binding, co-repressor dissociation and co-activator association. Increased expression of Pgc1 α observed in the absence of Nrf2 may result in increased ligand-dependent recruitment of Pgc1 α to the Ppara heterodimer complex. This may then allow for increased transcriptional regulation of Ppara target genes.

toxication can be prominent. Analysis of hepatic NPSH content revealed a trend for increased basal levels of GSH in CFB-treated, compared to vehicle-treated Nrf2-null mice. This is consistent with studies of repeated CFB dosing in wild type mice (Manautou et al., 1994). There was also a trend for decreased GSH restoration in vehicle-pretreated, compared to CFB-pretreated, APAP challenge groups. This indirectly suggests that higher GSH content following CFB pretreatment, even in the absence of Nrf2, may have the potential to partially protect against oxidative stress, such as that produced by APAP's reactive intermediate.

2.6 Conclusion:

The present study indicates that Nrf2 and Ppar α interact in a way that most likely does not require direct binding between the two transcription factors. Further studies are required to determine the exact mechanism of this interaction, however, it appears that the mechanism may involve regulation of the co-activator protein Pgc1 α . ChIP analysis could be utilized to determine the level of Pgc1 α being recruited to the transcription start site of Ppar α target genes in vehicle- and CFB-treated wild type and Nrf2-null mouse livers. This would provide insight as to whether or not Pgc1 α plays a role for the increased sensitivity of Nrf2-null mice to Ppar α activation.

Further studies on the induction of Nrf2 expression following CFB treatment could provide additional insight into the interaction between Nrf2 and Ppar α . These studies could include protein analysis of Nrf2 following CFB pretreatment in wild type mice. Also, analysis of Nrf2 mRNA and protein expression could be conducted in vehicle and CFB-treated Ppar α -null mice to determine if the observed increase in Nrf2 mRNA expression following CFB treatment is

Ppar α dependent. This study may also benefit from extending CFB treatment (250mg/kg) from five days to ten days. It has been established that Nrf2-null mice can tolerate 250mg/kg of CFB for five days. Extending treatment to ten days may further induce the observed CFB-mediated activation of Ppar α in both wild type and Nrf2-null mice. It is also possible that extending CFB treatment to ten days may be sufficient to produce hepatomegaly, which was observed in the ten day, 500mg/kg model of CFB dosing. It is also possible that protein expression of Vnn1 may better reflect the observed mRNA expression if CFB treatment was extended.

Developing a mechanism for the interaction between Nrf2 and Ppar α would provide a better understanding of APAP-induced liver injury and may result in improved understanding of how Nrf2 and Ppar α protect against this liver injury.

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