ABSTRACT
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Regulation of the Cholesterol-Biosynthesis Pathway Influences Effects of Peroxisome Proliferators in Mouse Liver
Under the direction of Ivan Rusyn

Phthalates and a variety of other environmental compounds belong to a class of chemicals known as peroxisome proliferators (PPs). PPs are a widely studied class of compounds that cause non-genotoxic liver carcinogenesis in rodents. The mechanism by which PPs cause cancer and a number of other pleotropic effects, including induction of peroxisomes, hepatocellular hypertrophy and oxidative stress involve activation of the nuclear receptor peroxisome proliferator activated receptor (PPAR) alpha. While it is known that PPARα is required for these effects, the exact mechanism of action is unknown. In order to determine how human health is affected by these compounds it is necessary to elucidate the mechanism of action. A number of studies have suggested that PPARα plays a role in the cholesterol biosynthesis (mevalonate) pathway. We hypothesize that by affecting the key regulators of this pathway, we can modulate the effects of peroxisome proliferators in the mouse liver. Here, we explored the consequences of blocking the mevalonate pathway with lovastatin or increasing the availability of isoprenoids by treating with farnesol on PP induced liver effects. While farnesol had no effect on PP-induced ACO activity (i.e., peroxisome proliferation), it blocked the increase in cell proliferation.

Collectively, these results indicated that disregulation of the mevalonate pathway affects the promotional ability of PPs in liver, but not their ability to cause induction of peroxisomes. Thus, the perturbations of lipid metabolism in liver that are caused by PPs may be linked to the ability of the agents to induce cell proliferation.

To my Mom and Dad, Melissa, my family, friends and PSU friends thank you for your love and support. To my friends that I have made at Carolina, I will always cherish your friendship and guidance.

I would like to express my gratitude to the Rusyn Lab. I have learned so much from working with you. I will never be able to express how much I appreciate the encouragement and guidance I have received from this group. I would also I like to acknowledge the professors in the department and my committee members for their guidance and for pushing me to do my best.

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Chapter 1

Literature Review

Phthalates are a family of industrial compounds that have been used for a variety of purposes since the 1930s and belong to the class of chemicals known as peroxisome proliferators. Phthalates can be found in personal care products, paints, industrial plastics, certain medical devices and pharmaceuticals. Globally, more than 18 billion pounds of phthalates are used each year (Latini 2005). Di-(2-ethylhexyl) phthalate (DEHP) is used as an industrial plasticizer, and it is the most abundant phthalate in the environment with well above 2 million tons of DEHP produced annually worldwide (Latini 2005). DEHP is also the most studied of the phthalate esters and is on the EPA's list of priority pollutants (http://www.ecy.wa/gov/programs/eap/pbt/rule/docs/summary). These plasticizers give polyvinyl chloride (PVC) its flexibility. They have been shown to leach from plastic products at a constant rate in to the environment. These materials also enter the environment through incineration, landfills and effluent of numerous industrial plants (http://epa.gov/safewater/dwh/c-soc/phthalat.html). DEHP is persistent in the environment due to the large amounts produced and disposed of on a daily basis. In 1998, 669 pounds of DEHP were released into surface waters and 207,795 pounds were released into the air on a national level. North Carolina is one of the top ten states for DEHP discharge, where 23,082 pounds were released into the

environment in 2005 (National Safety Council, 2005). Phthalates have also been shown to be present in household wastewater. A housing area in Sweden which used a water system that had separate flows for grey-water and black-water was the basis for an interesting study. Grey-water is defined as household wastewater without input from toilets, meaning this is wastewater from bathing, showering, hand washing, laundry and the kitchen sink. Grey-water has been estimated to account for about 70-75 vol. % of domestic wastewater (Palmquist and Hanaeus 2005). Phthalates were identified in the grey-water, and they are believed to accumulate as a result of activities such as dishwashing, cleaning, etc. (Palmquist and Hanaeus 2005). Wastewater and industrial effluent account for the point sources that lead to water contamination and serve as a source for human exposure.

Humans can be exposed to these compounds through ingestion, inhalation and dermal exposure over the course of a life time. The greatest absorption of DEHP occurs from oral exposure in the GI tract (Rusyn *et al.* 2006; Schmid and Schlatter 1985). The margin of exposure (MOE) has been recommend at 20-25mg/kg/day the level at which is there is no observable effect level (NOEL) in mice (Doull *et al.* 1999). On a daily basis, human exposure is estimated to be in the range of 4-30μg of DEHP/kg/day, which is well below the MOE (Latini 2005; Moore *et al.* 2001). The relevance of this value is under debate because humans are also exposed to many other types of phthalates and peroxisome proliferators. The effects of these compounds maybe additive and this would lead to significantly greater exposure levels (Rusyn *et al.* 2006). Exposure from medical devices and occupational exposure can be much greater, in the range of 475 μg/kg/day - 700 μg/kg/day

mainly via inhalation or intravenous routes respectively and they are still approximately 2 orders of magnitude below the MOE for rodents (Doull *et al.* 1999).

Concentration levels of phthalates in drinking water are regulated by the US Environmental Protection Agency (EPA) because short-term effects and potential for long-term effects from exposure to these agents have been reported. Short-term exposures to phthalates in the drinking water can result in mild gastrointestinal disturbances, nausea and vertigo (http://epa.gov/safewater/dwh/c-soc/phthalat.html). Long-term exposures have the potential to cause liver and testicular damage, reproductive effects (i.e.-acting as endocrine disrupting agents) and cancer (http://epa.gov/safewater/dwh/c-soc/phthalat.html). Phthalates have been shown to be animal carcinogens especially in rodents, and can cause fetal death, malformations, testicular injury, liver and kidney injury, anti-androgenic activity, teratogenicity, peroxisome proliferation and marked reproductive toxicity and reduced fertility in laboratory animals (Latini 2005; ECY 2005). The persistence and concentrations at which phthalates are found in the environment make this class of chemicals an important environmental agent to monitor and regulate.

DEHP and other phthalates are of interest to researchers and regulators because they have been shown to be reproductive toxicants by acting as an endocrine disrupting compound. This is an exciting and important area of research; however, this review will focus on phthalates as peroxisome proliferators and non-genotoxic rodent carcinogens. In addition, while DEHP is an environmental agent of interest, we will be using a more potent peroxisome proliferator Wyeth 14,643 (Wy) in the majority of our studies. In using Wy as a model peroxisome proliferator, we

will be able to see the effects of peroxisome proliferation and increased cell proliferation more clearly and at an earlier time point. It is our goal to identify the critical steps in the molecular and cellular pathways of non-genotoxic liver carcinogenesis by peroxisome proliferators.

Because DEHP is ubiquitous in the environment and there is a potential for adverse health effects, many regulatory agents have justified the need to monitor these agents and set daily limits for the public and for occupational exposures. The Safe Drinking Water Act requires the EPA to determine safe levels of chemicals in drinking water which may or may not cause health problems. The Maximum Contaminant Level Goal (MCLG) for DEHP has been set at zero, and the Maximum Contaminant Level (MCL) has been set at 6 parts per billion (ppb). The MCL is the enforceable standard that is set by the EPA (http://epa.gov/safewater/dwh/c-soc/phthalat.html). Several states have examined phthalates as a class of chemicals of concern and regulate them accordingly. For example, California has its own MCL values and they are set at 4 ppb which is below EPA's standards (Wang 1994). Washington State has included phthalate esters on their proposed list of persistent bioaccumulative toxins (PBT). The criteria for including them on this list are their persistence in the environment, the ability to bioaccumulate in the environment, and toxicity (ECY 2005). Other organizations have published reasonable limits of exposure for DEHP (ECY 2005).

ATSDR (2002) - MRL (0.1mg/kg/day); chronic exposure (0.06mg/kg/day)
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National Toxicology Program (2004)-reasonably anticipated to be a human carcinogen
International Agency for Research on Cancer IARC (2004) - downgraded to not classifiable as to carcinogenicity in humans.
Environment Canada- set a NOEL at 44mg/ [kg (BW)*d] (Canada, 1994)
U.S. Occupational Safety and Health Administration (OSHA)- Permissible Exposure Limit: 5mg/m3
American Conference of Governmental Industrial Hygienists (ACGIH) - Threshold Limit Value: 5mg/m3
National Institute for Occupational Safety and Health (NIOSH) - Recommended Exposure Limit: 5mg/m3

Most of the above reports rely on animal studies since there is little epidemiological data available on human exposure to phthalates. It is essential that more research be done on phthalates and other peroxisome proliferators to determine the mechanisms of action both as non-genotoxic carcinogens and endocrine disrupting compounds in order to establish guidelines of acceptable dose and associated risk to humans. It is necessary to elucidate the differences between rodents and humans to explain why humans appear to be less sensitive to the effects of these compounds. Molecular biology and toxicological research may help us achieve these goals.

Peroxisome Proliferators are a class of chemicals that are ubiquitous in the environment. Environmentally relevant compounds within this class include phthalates, industrial solvents, such as trichloroethylene (TCE), perfluorooctanoic acids (PFOA), herbicides, and pesticides. Hypolipidemic drugs such as fibrates, endogenous fatty acids, and leukotriene antagonists are also known to cause peroxisome proliferation (Eacho *et al.* 1991; Holden and Tugwood 1999; Marsman *et al.* 1988; Reddy and Lalwani 1983). A phenomenon referred to as peroxisome proliferation was first described in 1960; it was observed that a hypolipidemic drug was able to increase the number and size of peroxisomes in rodents (Hess *et al.* 1965; Peters *et al.* 2005). Compounds classified as peroxisome proliferators stimulate hepatic peroxisomes, which are hydrogen peroxide producing organelles within the liver (Reddy and Rao 1989). Peroxisomes play a role in cholesterol and lipid regulation within the liver and the metabolism of fatty acids (Lai 2004). Liver hypertrophy, hyperplasia and tumor formation have been shown to occur in rodent studies (Rusyn *et al.* 2006). A "tumor triad" composed of pancreatic, liver, and Leydig cell tumors has been hypothesized to be induced by peroxisome proliferators in rodents (Lai 2004). Because epidemiological data is weak, it is still difficult to draw a conclusion as to the carcinogenicity of peroxisome proliferators in humans (Lai 2004). However, the possibility of adverse health effects ultimately leading to cancer as a result of chronic exposure to peroxisome proliferators is still possible. (http://epa.gov/safewater/dwh/c-soc/phthalat.html).

The mechanism of action for peroxisome proliferators leading to non-genotoxic rodent carcinogenesis is not well known. However, the postulated modes of action

and key events have been studied and are reviewed in numerous articles (Klaunig *et al.* 2003; Peraza *et al.* 2006; Peters *et al.* 2005). The relevance to humans is a controversial issue in this field and more research must be done to elucidate the mechanism of action leading to hepatocarcinogenesis (Cattley *et al.* 1998).

Peroxisome proliferators are able to interact with the Peroxisome Proliferator Activated Receptor α (PPARα), a nuclear receptor that is expressed in the cells of the liver, heart, and kidney (Klaunig *et al.* 2003). Upon ligand activation, PPARα heterodimerizes with Retinoid X Receptor alpha (RXRα), and translocates to the nucleus where it acts as a transcription factor for a number of target genes (Klaunig *et al.* 2003; Peters *et al.* 2005). These target genes and other genes responsive to PPs are involved in a number of physiological responses including fatty acid catabolism, cell proliferation, apoptosis (programmed cell death), inflammation and oxidative stress (Peters *et al.* 2005). With PPARa activation, cell proliferation increases via an increase in DNA synthesis and mitosis while apoptosis is decreased (ATSDR 2002). By using a PPARα null mouse model, it was shown that PPARα ligand-induced rodent liver cancer is dependent on PPARα (Hays *et al.* 2005; Peraza *et al.* 2006; Peters *et al.* 1997). Continued research will allow us to elucidate the various pathways in which phthalates acts as a liver toxicant.

As previously mentioned, phthalates are classified by the EPA as probable human carcinogens and IARC has downgraded their classification to "not classifiable as to carcinogenicity in humans." These classifications are mostly based on animal data and the mode of action for rodent liver carcinogenesis. The key events for this mode of action are summarized in a review by Klaunig et al. Events with high

specificity and strong weight of evidence for the development of rodent liver carcinogenesis are 1) the activation of PPARα, 2) expression of peroxisomal genes and 3) peroxisomal proliferation (Klaunig *et al.* 2003). These events can be measured by protein or mRNA expression of PPARα, acyl Co A oxidase (ACO) and cytochrome P450 4A (Cyp4A). Other events such as non-peroxisomal lipid gene expression, disregulation of cell proliferation and apoptosis, and Kupffer cell-mediated events have strong weight of evidence with low specificity. Low specificity indicates that these events are not specific to a PPARα mode of action (MOA).

The International Life Science Institute (ILSI) workgroup has also weighed in on the topic of human relevance. They have concluded that "despite the presence of similar pathways in humans, it is unlikely that the proposed MOA for rodent tumors is plausible in humans" (Lai 2004). However, regardless of this conclusion it is still unknown how humans are affected by these compounds at ambient exposure levels over the course of a lifetime. It was recommended by a panel of scientists that risk assessments for these compounds should be conducted on a case-by-case basis (Cattley *et al.* 1998).

Human relevance of peroxisome proliferator induced hepatocarcinogenesis is an important question for risk assessors. Species differences in key mechanisms of action in hepatocarcinogenesis or in expression, structure and/or function of important mediators of PP-induced events may be critical in understanding the extent of the effect of PPs on humans. It has been shown that rodents such as mice and rats are the most sensitive species to peroxisome proliferators (Klaunig *et al.* 2003). Humans have a functional PPARα that can modulate gene expression;

however there is no conclusive epidemiological data that suggest an increased rate of liver cancer in patients treated with hypolipidemic drugs (Peters *et al.* 1997). One hypothesis for the reduced susceptibility of humans to peroxisome proliferators is the fact that humans have approximately 1/10 the protein expression and binding activity of PPARα compared to rodents. However, this may not be the only reason that humans respond differently (Klaunig *et al.* 2003; Palmer *et al.* 1998). The functionality of the human receptor itself is an important aspect of the relevance to humans that is currently being studied. Previous in vitro studies demonstrated that the human PPARα is functional (Sher *et al.* 1993). Recently, the humanized PPARα mouse (hPPARalpha), which expresses the human form of PPARα has been an interesting new tool for determining the relevance of the human PPARα in the development of liver tumors and for evaluating risk (Morimura *et al.* 2006). Results from a long term Wy feeding studies indicate that the hPPARα mouse is more resistant to the development of liver tumors compared to its wild-type, mPPARalpha, counterpart (Morimura *et al.* 2006). Interestingly it is important to note that there have been studies that showed an increase in peroxisome proliferation with a therapeutic treatment of Clofibrate (a peroxisome proliferator used to treat high cholesterol) in a human study (Hanefeld *et al.* 1983).

There are other species differences that should be noted. Guinea pigs and non-human primates also appear to be unaffected by PPs (Foxworthy *et al.* 1990; Holden and Tugwood 1999; Makowska *et al.* 1992). Hamsters respond to peroxisome proliferators, however this response is less robust compared to rodents (Klaunig *et al.* 2003). Using mouse models such as the hPPARα mouse and inbred

mouse strains of varying PPARα expression studies may be useful for identifying the role of PPARα in peroxisome proliferator-induced carcinogenesis.

PPARα plays an essential role in lipid homeostasis (Peters *et al.* 2005). Fibrates are hypolipidemic drugs that reduce serum LDL cholesterol and triglycerides in humans. Stimulation of peroxisomes by these drugs leads to induction of enzymes that are involved in metabolism of fatty acids (Klaunig *et al.* 2003). Triglyceride levels are reduced in the liver by increasing the expression of lipoprotein lipase and inhibiting apolipoprotein C-III (Kota *et al.* 2005). This results in the increased clearance of triglycerides (Jossic-Corcos *et al.* 2004). Lipid metabolism and cholesterol biosynthesis play key roles in many biological processes responsible for liver function. Thus we hypothesize that by affecting key regulators of the cholesterol biosynthesis (mevalonate) pathway, we can modulate the effects of peroxisome proliferators in mouse liver. The focus of this report is to examine the role of PPARα activation on the lipid mevalonate pathway and its impact on cell proliferation. The remainder of this review will provide a brief overview of the main components of the cholesterol synthesis (mevalonate) pathway. Scheme 1 represents our hypothesis of how PPARa activation is associated with the mevalonate pathway.

Our hypothesis begins with the activation of PPARα. Activation leads to an induction of a peroxisomal enzyme Acyl CoA oxidase which produces hydrogen peroxide and is metabolized to HMGCoA. This induction of hydrogen peroxide is hypothesized to be causally associated to the hepatocarcinogenic effects observed with PPARα activation (Peters *et al.* 2005). HMGCoA is converted to HMGCoA

reductase and mevalonate. HMGCoA reductase has been an important target for a new line of cholesterol lowering drugs known as statins. Statins such as lovastatin are cholesterol lowering drugs that block HMGCoA reductase which inhibits mevalonate the precursor to cholesterol and isoprenoids. This pathway as a whole is known to regulate cell growth and includes products such as isoprenoids, small GTPases and cholesterol (Johnson and Ledwith 2001). Statins have a negative impact on downstream intermediates such as isoprenoids, and small GTPases such as Ras and Rho (Liao 2002). It was shown that by inhibiting mevalonate, farnesylation of Rho and Ras which is required for their activation could be prevented (Ambrosi *et al.* 2003). More recent data has suggested that the inhibition of mevalonate may result in an upregulation of small GTPases (Holstein *et al.* 2003).

Isoprenoids, such as farnesol and farnesyl pyrophosphate (f-pp) are key intermediates in the mevalonate pathway. Isoprenoids are needed to produce cholesterol and to activate small GTPases. These chemicals are produced within the body and can be found in a number of fruits and vegetables (Rao *et al.* 2002). Within the peroxisome, mevalonate is converted to farnesol phosphate (FMP), farnesyl diphosphate (FDP) synthase and other enzymes needed to form cholesterol (Liliom *et al.* 2006). Research now supports the conversion between farnesol (FOH) and farnesyl pyrophosphate (FPP) (Holstein and Hohl 2004). It is still unclear what role isoprenoids play in cell proliferation. It has been suggested that farnesol can inhibit cell proliferation by inhibiting HMGCoA, and that farnesyl pyrophosphate can prevent the up regulation of GTPases such as Ras, suggesting that farnesol may be chemo-preventive (Holstein *et al.* 2003; Ong *et al.* 2006). Conversely, others have

shown that isoprenoids such as farnesol are required for the activation of Ras via prenylation (Bifulco *et al.* 1999). They have demonstrated *in vitro* that the effect of inhibiting cell growth with statins can be reversed with isoprenoids such as all trans-farnesol (Corsini *et al.* 1999). There are chemo-preventive drugs currently being developed known as farnesyltransferase inhibitors (FTIs). These drugs are designed to inhibit farnesyl transferase the enzyme required for activating the Ras oncogene via prenylation (Cascinu *et al.* 2006).

Small GTPases such as Ras and Rho play important roles in regulating cell survival, proliferation, differentiation and cytoskeletal organization (Holstein *et al.* 2003). These proteins become active when the lipid tail of an isoprenoid covalently binds to the GTPase and the protein translocates to the membrane where it binds to GTP and becomes active. This action known as prenylation and is important in regulating signal transduction, cytoskeletal regulation, cell proliferation, and apoptosis (programmed cell death) (Bifulco *et al.* 1999). This entire process is regulated by a number of feedback mechanisms to keep the mevalonate pathway in check. Exogenous and endogenous compounds can impact this pathway in various ways depending on the cholesterol state within the body.

The mevalonate pathway is an important pathway to study with relation to PPARα. PPARα plays an important role in lipid homeostasis and humans take therapeutic drugs to reduced serum triglycerides and LDL cholesterol. It has been shown that PPARα activation leads to an increase in HMGCoA synthase and F-pp synthase, two key regulators in the mevalonate pathway (Wheeler *et al.* 2003) Furthermore, it has also been shown that there is a connection between changes in

the mevalonate pathway, the prenylation of Ras and cell proliferation (Mo and Elson 2004). The mechanism of action leading to peroxisome proliferator induced cell proliferation is unknown. However, we have a good reason to hypothesize that the mevalonate pathway is involved due to previous research that has demonstrated that PPARα can have an effect on regulatory enzymes of this pathway. We currently do not know how PPARα activation or exposure to peroxisome proliferators results in cell proliferation. A lack of understanding in this area inhibits our ability to understand the risk associated with human exposure to peroxisome proliferators. In this work, we attempt to determine whether modulators of cholesterol regulation can also modulate peroxisome proliferator induced cell proliferation in mouse liver.

Chapter 2

Introduction

Peroxisome Proliferator Induced Cell Proliferation and the Mevalonate Pathway
How do these pathways interact?

Phthalates are a family of industrial compounds that have been used for a variety of purposes since the 1930s and belong to the class of chemicals known as peroxisome proliferators PPs. Phthalates can be found in personal care products, paints, industrial plastics, certain medical devices and pharmaceuticals. Globally, more than 18 billion pounds of phthalates are used each year (Latini 2005); ASTDR 2002). Peroxisome proliferators are a widely studied class of compounds that cause non-genotoxic liver carcinogenesis in rodents. Many other structurally unrelated compounds are grouped as peroxisome proliferators, based on their unique ability to cause and increase in peroxisome proliferation (Lai 2004). Phthalates are model peroxisome proliferators due to their persistence in the environment and the body of research for these compounds is fairly large. While phthalates are peroxisome proliferators that humans are exposed to on a daily basis, Wyeth 14,643 (Wy) is a model peroxisome proliferator that is used in many studies due to the pronounced peroxisome proliferation that occurs with exposure. The mechanism by which PPs cause cancer and a number of other pleotropic effects, including induction of peroxisomes, hepatocellular hypertrophy and oxidative stress involve activation of

the nuclear receptor peroxisome proliferator activated receptor alpha (PPARα). In fact, presence of active PPARα is required for the effects of PPs on liver carcinogenesis. In addition, a number of studies have suggested that PPARα plays a role in the cholesterol biosynthesis (mevalonate) pathway. It has been shown that 2 regulatory enzymes of this pathway can be affected by PPARα activation (Wheeler *et al.* 2003). Lipid metabolism and cholesterol synthesis play a key role in many biological processes important to normal liver function. Thus, we hypothesized that by affecting key regulators of the lipid metabolism/mevalonate pathway we can modulate the effects of peroxisome proliferators in mouse liver. The purpose of this study is to determine *in vivo* if PPARα activation is required for an increase in the prenylation of small GTPases which is associated with increased cell proliferation. Specifically, we hypothesize that PPARα activation perturbs the lipid metabolism and cholesterol synthesis (mevalonate) pathways leading to the activation of Ras and induction of cell proliferation. Un-published *in vitro* data from collaborators show that TNFα is required for Wy-induced increases in DNA synthesis in pure hepatocytes. Interestingly, it was shown that by blocking the mevalonate pathway the effect of Wy-induced DNA synthesis was reduced and restoring this pathway with isoprenoids (F-pp), increased DNA synthesis (Parzefall *et. al.* 2001). It is our goal to recreate a similar model *in vivo* to understand the relationship between the mevalonate pathway and PPARα activation. Cholesterol bio-synthesis is a critical part of our hypothesis because we propose that PPARα activation can increase the pool of isoprenoids which are involved in sterol synthesis and required for Ras activation which may drive cell proliferation. Alternatively, it has been recently suggested that

farnesol can inhibit cell proliferation by degrading HMGCoA reductase, and that farnesyl pyrophosphate can prevent the up regulation of GTPases such as Ras, suggesting that farnesol may be chemo-preventive (Holstein and Hohl 2004; Ong *et al.* 2006). This work seeks to examine the effects of perturbing the mevalonate pathway through PPARα activation on cell proliferation.

We attempted to model the effects of PPARα activation on the mevalonate pathway observed *in vitro* in an *in vivo* system. To accomplish this, wild-type and PPARα (-/-) mice on a C57Bl6J background were fed a diet containing either Wy (0.1%) or control NIH-07 diet for 72 hours. Mice were dosed (i.g.), with an isoprenoid alcohol, farnesol alone (250 mg/kg of body weight/day) or with Wy containing diet. Lovastatin, a specific HMGCoA reductase inhibitor, was administered alone (20mg/kg of body weight/day) or with Wy containing diet. Lastly, a treatment group was administered all three, Wy diet, farnesol and lovastatin.

In this work, the effect of PPARα of the mevalonate pathway will be evaluated by analysis of endpoints such as cell proliferation, ACO activity, protein expression of PPARα and ACO, liver to body weight and clinical chemistry. Results from this study will contribute to the understanding of the mechanism of action for peroxisome proliferator induced mouse liver carcinogenesis. The link between PPARα activation and cell proliferation is currently unknown. This study provides further evidence for the interaction between PPARα activation and the mevalonate pathway, a pathway that is currently targeted for cholesterol lowering drugs and is involved in cell cycle regulation and cell proliferation.

Chapter 3

Methods and Materials

Animals and Diets

Adult aged (6-8 weeks) male wild-type C57BL6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). We chose this strain because it is the background strain for the PPARα knockout mouse that was used in this study (at lease F5 generation); they have a low rate of spontaneous tumor formation but are sensitive to peroxisome proliferators (Cattley R.C., personal communication). Animals were maintained on NIH-07 chow. Treated animals were given the same rodent chow blended with WY-14,643 at a target concentration of 0.1% (1000ppm). Wild type and knockout mice were gavaged with either Farnesol (250mg/kg/day, Alfa Aesar, Ward Hill, MA), Lovastatin (20mg/kg/day, Tocris-Ellisville, MS), or vehicle (0.5% methyl cellulose). Treatment groups consisted of (FOH) and Wy or control diet, Lovastatin (Lov) and Wy or control diet, or Farnesol, Lovastatin and Wy diet. Mice were maintained in a temperature- and light-controlled facility at UNC-Chapel Hill, and permitted *ad libitum* consumption of food and water. At the end of the study, liver and body weight measurements were collected. A portion of liver tissue was preserved in formalin for histological sections and the remaining tissue was snapped frozen in liquid nitrogen and stored at –80°C for later analyses. All animals were given humane care in compliance with NIH and institutional guidelines and studies

were performed according to protocols approved by the appropriate institutional review board.

Genotyping

Animals were genotyped to ensure homozygosity. DNA was isolated using the DNeasy tissue kit (Qiagen, Valencia CA). Following manufactures protocol.

PCR

Expected results: (+/+) ~400bp, Null allele: ~650 base pairs

Forward primer (aF1) 5'-3', GAGAAGTTGCAGGAGGGGATTGTG

Reverse primer (aR1) 5'-3', CCCATTTCGGTAGCAGGTAGTCTT

NEO primer (NEOR1) 5'-3', GCAATCCATCTTGTTCAATGGC

Program:

1) T=94°C 5min
2) T= 94°C 1min
3) T=60°C 1min
4) T=72°C 1min
5) Go to 2 repeat 29 x's

Clinical Chemistry

A standard kinetic assay for measuring the disappearance of NADH was used to measure serum ALT. Thermo Trace cat # TR 71021; normal control cat # 1902-050; abnormal control # 1902-050 (Thermo Scientific Waltham, MA).

ALT buffer is allowed to come to room temperature, and then warmed to 37 °C. Plate reader is warmed to 37 °C and wavelength is set to 340nm. 20 μL of sample

or control is added to each well of a 95 well plate. Plate is placed in the plate reader and allowed to warm to 37 °C for 5 minutes. 200 μL of Thermo trace is applied into each well of the plate and incubate for 30 sec at 37 °C. Results are read in the kinetic mode. Changes in absorbance are read in 60 second intervals for 2-3 minutes.

For cholesterol and triglyceride measurements serum samples were sent to the Animal Clinical Laboratory Core Facility at UNC. Clinical chemistries were measured by using an automated Clinical Chemical Analyzer, Vitro 250 (Ortho-Clinical Diagnostic Inc, Rochester NY).

Immunohistochemical Detection of 5-bromo-2'-deoxyuridine (BrdU)

Formalin-fixed, paraffin-embedded liver sections (6 μm) were mounted on glass slides. Sections were deparaffinized in xylene, rehydrated in a series of graded alcohol concentrations, and placed in PBS with 1% Tween 20. Immunostaining was performed using DAKO EnVision System HRP (Dako Cytomation, Carpinteria, CA) with primary Monoclonal antibody BrdU (DAKO, clone Bu20a) diluted in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. Slides were counterstained with hematoxylin. In order to ensure the quantitative measurement of each immunoreaction, all sections from each animal and group to be compared were processed in parallel. Changes in liver morphology were evaluated from H&E stained slides by Dr. John Troutman a veterinary pathologist. Quantitative analysis of immunostained liver sections was performed using BIOQUANT software (BIOQUANT Image Analysis, Nashville, TN) by averaging percent positively stained nuclei to total nuclei within 10 random fields at 200×.

cDNA was purified using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA). cDNA was diluted to an appropriate concentration. Stratagene (La Jolla, CA) full velocity Master Mix was prepared according to protocol and with the appropriate primers of interest. Farnesyl diphosphate farnesyl transferase Mm00815354_s11; PPARα Mm00440939_m1; GAPDH as a house keeping gene Mm99999915_m; Applied Biosystems, Foster City, CA). 19 μL of master mix was added to 1μL of diluted cDNA to each well of a 96 well plate. Real time PCR was run on the Stratagene MX 3000 real-time PCR system.

Preparation of Cytosolic and Nuclear Extracts

All of the following steps took place at 4°C. 50mg of liver tissue was homogenized in 400ul of Buffer A (10mM HEPES, ph 7.9; 10mM KCL; 0.1mM EGTA; 0.1mM EDTA; 1mM DTT; 0.5mM PMSF). 10% NP-40 (sigma) was added to the homogenate, vortexed and centrifuged at 15,000 RPM for 30 seconds to obtain the cytosolic fraction. Cells were re-suspended in buffer A with 10% NP-40. Cells were centrifuged once again at 15,000 RPM for 30 seconds and the supernatant was removed. The pellet was re-suspended in approximately 200ul of Buffer C (20mM HEPES; 0.4 M NaCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 1mM PMSF; 1mM Na3VO4. Cells were then centrifuged for 5 minutes at 14,000 RPM, and the supernatant nuclear extract was transferred to a new tube. Protein concentration was determined with the Pierce BCA Protein assay kit (Rockford, IL). Aliquots of cytosol and nuclear extract were then stored at -80c.

Preparation of Cytosolic and Membrane Extracts

Livers obtained from mice were homogenized in a buffer containing 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 0.25 M sucrose and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was then centrifuged at 100,000 g for 1 h at 4°C to prepare cleared cytosol. The resulting pellet was solubilized with 2% (vol/vol) Triton X-100 for 1 h at 4°C and then centrifuged at 100,000 g for 1 h at 4°C and was used as the membrane extract. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Wheeler *et al.* 2003).

Western Blot analysis

Protein extracts were separated by 4-12% Invitrogen (Carlsbad, CA) Novex Bis Tris gels and transferred to Immobilon-P, Millipore PVDF membranes (Billerica, MA). Membranes are blocked for 1 hour with 5% milk in TBST solution. Washed 3 times in TBST and incubated over night with the primary antibodies PPARa (ab24509, abcam), Beta Actin loading control (ab8227, abcam), and Ras (Cell biolabs) followed by a 1 hour incubation with either peroxidase labeled anti-mouse, or anti-rabbit (Amersham Pharmacia Biotech). Immunoblots were visualized by enhanced chemiluminescence autoradiography (ECL, Amersham).

Chapter 4

Results

Effects of Farnesol, Lovastatin and Wy on general liver function and morphology

To determine the effects that sub acute treatment with farnesol (Foh), lovastatin (Lov) and Wy had on general liver function and morphology several endpoints were measured, including liver weight and serum clinical chemistry. In addition, histopathological changes were scored by a veterinary pathologist. Liver to body weight ratios are useful in establishing gross changes in liver weight due to treatment with a drug or chemical. An increase in liver weight with a PPARα agonist is indicative of a decrease in apoptosis (Klaunig *et al.* 2003). It was observed that Wy treatment resulted in an increase in liver to body weight ratios in wild-type mice. As seen in Figure 1A, wild-type mice treated with Wy+Foh+Lov had a significantly higher liver to body weight ratio compared to Wy alone. This suggests that farnesol and lovastatin are having a synergistic effect on increasing liver weight. No change in liver to body weight ratios were observed in PPARα null mice (Figure 1B).

Normal serum ALT levels indicate that no liver damage occurs at the chosen doses and time point. Values are not significantly different than control values and are all well below 100 U/L (Figure 2A&B). Liver pathology assessed by H&E staining also revealed that there was no significant liver damage (Figure 2C).

Wy and lovastatin are therapeutic drugs used to lower LDL cholesterol and triglycerides. Figure 3A indicates that Wy and Wy+Lov did significantly lower the serum cholesterol in wild-type mice as expected. PPARα-null mice are known to have higher cholesterol levels compared to wild-type mice. This observation is seen across all treatment groups (Figure 3B). The effect of lovastatin on PPARα null mice is unknown since this was not collected and there is no historical information. Wy 14,643 is also known to reduce serum triglyceride levels in wild-type mice. We see that Wy treatment does result in a lowering of triglyceride levels in wild-type mice (Figure 4). While statistics could not be preformed for the PPARα null treatment groups it appears that farnesol alone has a triglyceride lowering affect in these animals.

Farnesol, Lovastatin and Wy Have a Synergistic Effect on PPARα Activation

To examine the combined effects of peroxisome proliferators and modulators of cholesterol pathway on lipid metabolism, it is important to determine whether these compounds cause significant changes in PPARα expression and activity.

PPARα western blots demonstrate the protein expression of PPARα in all treatment groups is increased with Wy. In wild-type mice, all groups treated with Wy show an increase in PPARα that appears to correlate with liver weight data. Where there is a noticeable increase in PPARα expression in the Wy+Foh+Lov groups (figure not shown). PPARα null treatment groups revealed little to no PPARα expression as predicted.

Levels of H_2O_2 as a measure of acyl-CoA oxidase (ACO) activity is one of the most reliable markers for peroxisome proliferation (Klaunig *et al.* 2003). Results in

Figure 5A again parallel the results from our PPARα western blot and liver to body weight ratios. These results further demonstrate that there is an affect on PPARα induction that is not explained by Wy treatment alone. Treatment with Wy+Foh+Lov caused an increase in ACO activity that is significantly greater than Wy treatment alone. Western blots for ACO protein shown in Figure 5B demonstrate a similar trend where Wy and Wy+Foh+Lov result in a significant increase compared to control.

Wy-14,643 Causes a Significant Increase in Farnesyl-Transferase mRNA Expression

Our objective was to use farnesol, an isoprenoid that is metabolized to farnesyl pyrophosphate, to mimic the effects of Wy on cell proliferation. It was important to ensure that Wy and farnesol have the same impact on down stream enzymes of the lipid metabolism pathway. To determine this, RT-PCR was performed to establish mRNA expression of farnesyl transferase. Farnesyl transferase is an important enzyme in the mevalonate pathway. This enzyme is required for cholesterol synthesis and for the membrane localization of Ras (Liliom *et al.* 2006).

Figure 6 demonstrates an approximately 1.5 fold increase in farnesyl transferase mRNA expression with Wy alone and farnesol alone indicating that we are in fact causing an increase in a down stream enzyme of the mevalonate pathway with Wy treatment in wild-type mice. Unexpectedly there was an approximate 4 fold increase in expression in the Wy+Lov group.

Co-Treatment with Farnesol and Wy Does Not Lead to an Increase in Cell Proliferation

Immunohistochemical detection of 5-bromo-2'-deoxyuridine (BRDU), a measure of cell proliferation, was performed using formalin fixed paraffin embedded sections of liver tissue. Cell proliferation in all treatment groups in PPARα knockout mice was consistently at wild-type control levels. Wild-type treatment groups responded with a significant increase in cell proliferation in the Wy-only treated group. Interestingly, as demonstrated in Figure7, the Wy+Foh group did not demonstrate a significant increase in cell proliferation over control levels, while Wy+Lov caused an increase in cell proliferation which was consistent with Wy treatment alone. Farnesol appears to attenuate the Wy induced cell proliferation at 72 hours. These results do no correlate with liver to body weight ratios observed. The liver is increased in size, yet there is no increase in cell proliferation. In addition, we observed no increase in BRDU labeling in the PPARα null (-/-) mice (Figure 7B).

Western blot analysis was performed to compare the level of Ras protein expression in the membrane fraction of the protein compared to the cytosolic fraction. Ras protein is located in the membrane fraction when it is in its active form. A trend while not statistically significant, in this data shows a consistent increase in Ras protein expression in the membrane fraction of all treatment groups compared to the cytosol fraction.

Chapter 5

Discussion

The mechanism of action for peroxisome proliferators leading to non-genotoxic rodent carcinogenesis is not well understood. Identifying the critical steps in the molecular and cellular pathways of non-genotoxic liver carcinogenesis by peroxisome proliferators is needed to understand how humans may be affected by these compounds. Based on previous *in vitro* and *in vivo* studies, we hypothesized that by affecting key regulators of cholesterol biosynthesis/mevalonate pathway we can modulate the effects of peroxisome proliferators in mouse liver. It was our intent to examine the effects of perturbing the mevalonate pathway through PPARα activation on cell proliferation. Previous *in vitro* studies have shown that blocking the mevalonate pathway can have a negative effect on Ras activation and ultimately cell proliferation. Understanding how this pathway is impacted by PPARα activation may lead to a better understanding of the mechanism of action and potential targets for therapeutic agents.

There were a number of endpoints collected in this study and most results have advanced our knowledge of the interaction between PPARα and the mevalonate pathway. This study sheds more light on the complexity of the mevalonate pathway and the importance of targeting this pathway for a variety of therapeutic interventions.

Increased farnesyl transferase (fdft) mRNA expression in the Wy alone and farnesol alone treatment groups demonstrate that both compounds are acting similarly on fdft mRNA expression. We had hypothesized that both compounds would have a similar effect on down stream enzymes of the mevalonate pathway resulting in an increase in cell proliferation by increasing the pool of available isoprenoids for Ras activation. The RTPCR results for the Wy+Lov group were unexpected yet others have shown that there is a considerable increase in fdft concentration when this pathway is inhibited (Bergstrom *et al.* 1993; Liliom *et al.* 2006). In this case cholesterol levels are significantly lowered due to co-treatment with Wy and Lovastatin, both cholesterol lowering drugs. This inhibition might have resulted in a non-functional compensatory response. In order to examine why Wy and not farnesol treatment resulted in cell proliferation and why Wy+Lov was significantly up-regulated, a measure of fdft activity is required.

Using BrdU as a marker of cell proliferation, we observed no increase in cell proliferation in the Wy+Foh treated mice. Lovastatin treatment alone caused an increase in cell proliferation comparable to that caused by Wy alone. Interestingly, mice treated with Wy+Foh+Lov exhibited lower levels of hepatocellular cell proliferation than Wy+Lov, which suggest an inhibitory effect by farnesol treatment. Despite a statistically significant increase in liver weight, the wild-type mice treated with Wy and farnesol had levels of cell proliferation similar to control groups. This observation demonstrates that Wy is able to cause peroxisomal induction as seen in the increased liver weight, ACO activity and ACO protein expression. At the same time, farnesol is able to attenuate the effects of cell proliferation. Excess farnesol

has been associated with the reduction of cell proliferation and has the potential to be used as a chemotherapeutic agent (Rao *et al.* 2002). It is hypothesized that farnesol may degrade HMG CoA reductase (Rao *et al.* 2002). To further understand the role farnesol may be playing one would have to examine HMG CoA protein expression. If farnesol is degrading HMG CoA, then one is essentially blocking the mevalonate pathway with farnesol instead of lovastatin resulting in an outcome that we had originally hypothesized.

We can conclude that the disregulation of the mevalonate pathway affects the promotional ability of peroxisome proliferators in liver, but has no effect on the induction of peroxisomes. Additionally, the perturbations of lipid metabolism in liver that are caused by peroxisome proliferators may be linked to the ability of these agents to induce cell proliferation. Research surrounding the mechanism of action of peroxisome proliferator induced cell proliferation is an important venture in risk assessment and public health since there are many unanswered questions regarding the safety of these compounds. This study provides new evidence for the interaction between PPARα activation and the mevalonate pathway, a pathway that is currently the targeted for new cancer treatment drugs.

Chapter 6

Future Directions

It is important to address areas of this study where more research is needed. While we used farnesol and lovastatin for the specific purpose of reinstating and blocking the mevalonate pathway respectively, it is important to note that both drugs have non-target effects, and it is possible that they may have effects on PPARα activation itself. These effects may be due to a deprivation in the mevalonate pathway via feed-back loops. It has been shown that inhibition of HMGCoA reductase by statins may lead to an induction of PPARα activity (Martin *et al.* 2001). In addition, exogenous farnesol may be converted to farnesoic acid and other intermediates which may serve as activators of PPARα and the farnesoid X receptor (FXR) another nuclear hormone receptor (O'Brien *et al.* 2005). Because of this we can not be certain that farnesol was only acting on PPARα. The sub acute doses and time point were chosen from the literature to prevent toxicity. While we did prevent toxicity, the dose of lovastatin given may not have been potent enough to off set the large dose of Wy given in the diet. Alternatively, 72 hours may not have been an adequate length of time to see an effect of reduced cell proliferation. Lovastatin will not be used in future studies since farnesol has provided the desired effect of attenuating cell proliferation. On a similar note, since farnesol has off target effects on PPARα activation, another compound should be used in its place. A

farnesyl transferase inhibitor, FTI-276 is a compound that targets and inhibits the prenylation of Ras. A pilot study has demonstrated *in vivo* that FTI-276 can attenuate Wy induced cell proliferation in mice.

While there were changes in farnesyl transferase mRNA expression, mRNA expression does not necessarily translate into protein expression or activity. The current results appear contradictory since similar levels are observed between the two treatment groups, yet Wy resulted in a significant increase in cell proliferation and farnesol did not. It is important to determine the corresponding activity of farnesyl transferase in the Wy alone and farnesol alone groups.

In order to determine what is driving the cell proliferation western blots can be done to assess protein expression of various cell cycle proteins and small GTPases. Cdc2p34 kinase is one protein that may prove useful to examine, it is required for the G2 to M transition in vertebrate cells and is known to be up-regulated with Wy treatment (Peters *et al.* 1998). A western blot for Ras localized in the membrane compared to the cytosol would again help us determine the downstream effects of our treatment groups.

Humans are exposed to phthalates and other peroxisome proliferators in the environment at low levels through out their life. They may also be exposed to these compounds by taking fibrates which are cholesterol lowering drugs. It is yet to be fully understood how PPARα activation leads to an increase in cell proliferation. This knowledge is necessary to accurately assess the human risk associated with exposure. Ultimately we want to determine if humans and rodents have similar or different mechanisms of action. Some compounds have proven to be only relevant

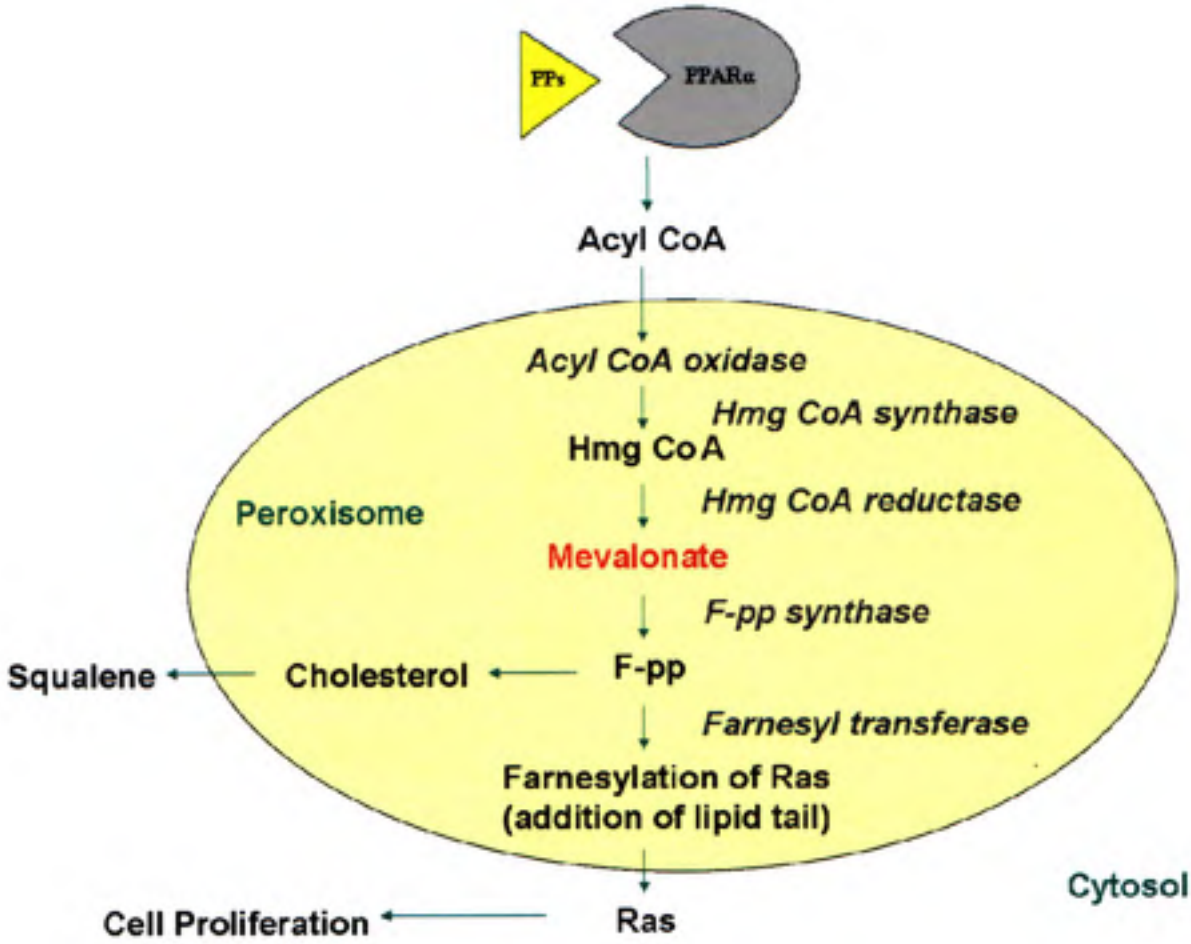
in certain species due to differences in metabolism and expression of certain receptors or enzymes. Furthermore, certain populations may be more or less susceptible based on the premise that levels of metabolizing enzymes vary across individuals. These are important issues that need to be addressed in the regulation of phthalates and other peroxisome proliferators as potential non-genotoxic human carcinogens.

This study has highlighted a number of questions that need to be addressed. Understanding how peroxisome proliferators are involved in the mevalonate pathway will further science on two fronts. This pathway is targeted for a number of new and useful therapeutic agents. A better understanding of this pathway will help develop new drugs that aid in reducing cholesterol such as statins and serve as chemo preventive agents. This research has demonstrated that farnesol may be a useful agent in preventing peroxisome proliferator induced cell proliferation. Statins and fibrates are often used together as a therapy for reducing LDL cholesterol levels. A better understanding how these compounds interact will also be useful in determining the risk of using fibrates for long term treatment.

In conclusion, a better understanding of the mechanism of action of peroxisome proliferator-induced rodent hepatocarcinogenesis will help risk assessors determine the significance of exposure to phthalates and other known peroxisome proliferators to human to health. This work has shown that peroxisome proliferator induced cell proliferation in rodents can be affected by changes in the mevalonate pathway. This indicates that a pathway similar in rodents and man may potentially be involved in PP induced cell proliferation. This study does not provide

us with conclusive information to make new recommendations in the way that phthalates or peroxisome proliferators are regulated; however this work allowed us to take a closer look at the interaction between persistent environmental agents and an important regulatory pathway in the human body that may play a key role in the mechanism of action of peroxisome proliferator induced hepatocarcinogenesis.

Appendix



Scheme 1:
Displayed are key regulators of the cholesterol biosynthesis/mevalonate pathway. Our hypothesis begins with PPARα activation and the induction of peroxisomal enzymes (ACO). Mevalonate and F-pp are the main regulators of this pathway. Where as prenylation of Ras and Cholesterol synthesis are the endpoints of this pathway.

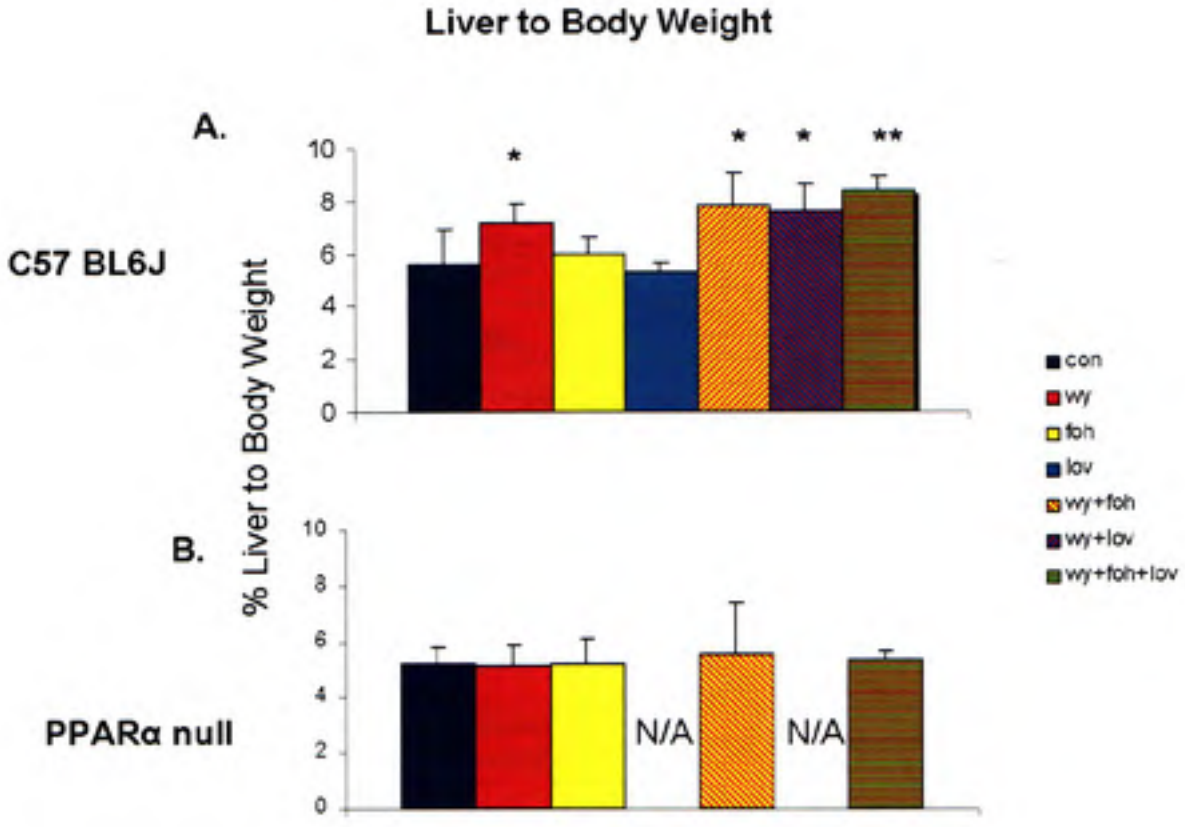
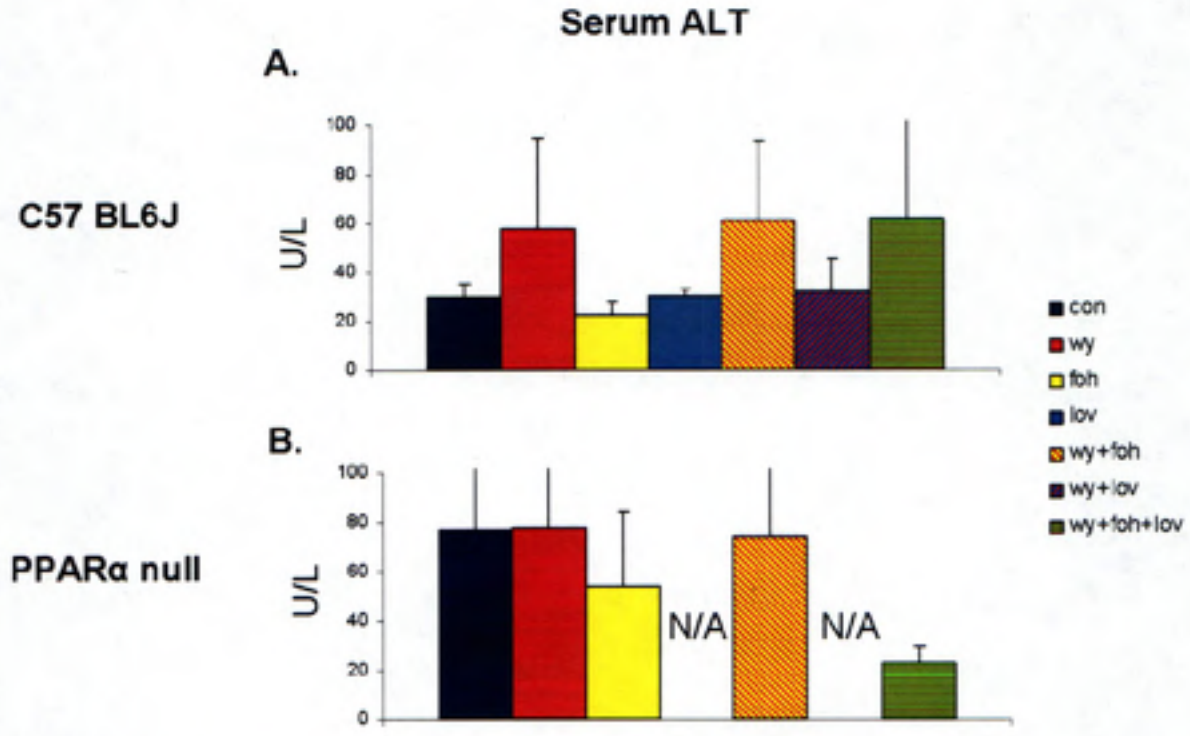


Figure 1. Effects of Wy, Farnesol and Lovastatin on Liver Weight
Figure 1A. Wy treatment resulted in an increase in the liver to body weight ratio across all groups in wild-type mice. Interestingly, the Wy+Lov+Foh group had a significantly higher ratio compared to Wy alone. Figure 1B. An increase in liver to body weight ration was not observed in PPARα null mice. Data is shown as mean ± S.D. *, ** $P<0.05$, as compared to control, and Wy alone respectively; ttest.


Serum ALT
A.
C57 BL6J
U/L
100
80
60
40
20
0
con
wy
foh
lov
wy+foh
wy+lov
wy+foh+lov
B.
PPARα null
U/L
100
80
60
40
20
0
N/A
N/A

C.

Histology Score

	(+/+)						(-/-)		
	mc	mc	foh	foh	wf	wf	con	foh	foh
Slide 1/20/2006 AB#:	12	13	14	15	16	17	18	20	21
LIVER	N	(+)	N	(+)	N	N	(+)	(+)	N
Focus (i) inflammatory cell infiltration +/- necrosis	.	2	.	1	.	.	1	1	.
Single cell necrosis +/- inflammatory cell infiltrate	.	.	.	.	.	.	.	.	.
Hepatocyte hypertrophy centrilobular	.	.	.	.	.	.	.	.	.
Hepatocyte vacuolation	.	.	.	.	.	.	.	.	.
Calcification	.	.	.	.	.	.	.	.	.

Key	Morphology Severity
. NOT RECORDED	1 minimal
N N.A.D.	2 slight
O MISSING	3 moderate
(+) MORPHOLOGY PRESENT	4 marked
X NO SEVERITY	

Figure 2. Effects of Wy, Farnesol and Lovastatin on Serum ALT Levels and liver injury

Figure 2A, 2B. Serum ALT levels indicate that at the chosen doses and time point there is no significant liver damage in any of the treatment groups. Data is shown as mean ± S.D. * $P<0.05$ as compared to control, ttest. Figure 2C. Histology scores were compiled by Dr. John Troutman. No significant liver injury was observed.

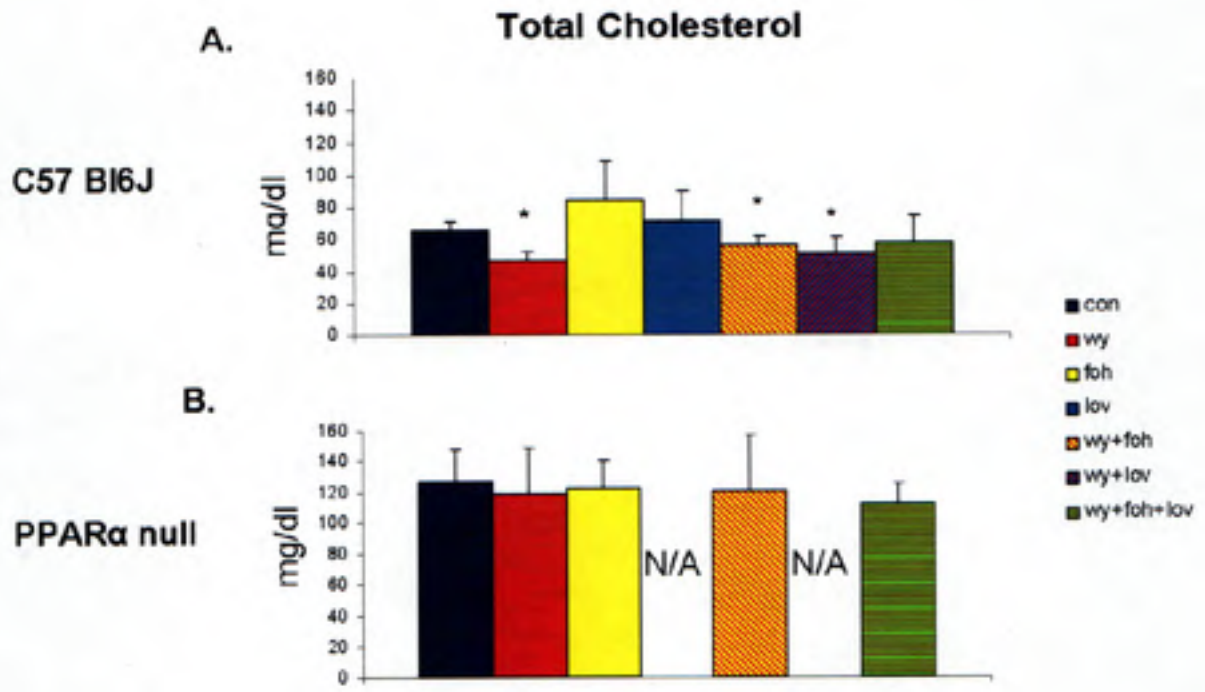


Figure 3. Effects of Wy, Farnesol and Lovastatin on Total Serum Cholesterol Levels

Figure 3A. Total serum cholesterol was significantly reduced in Wy treated wild-type mice. Figure 3B. PPARα null mice naturally have high cholesterol levels compared to wild-type mice. Cholesterol levels were not reduced in these mice. Data is shown as mean ± S.D. * $P<0.05$ as compared to control, ttest.

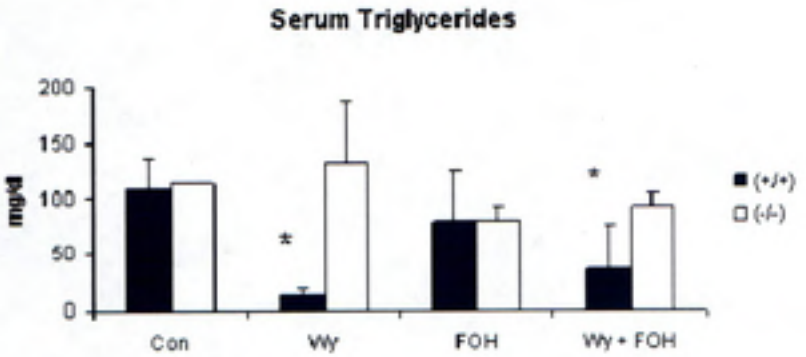


Figure 4. Effects of Wy, Farnesol and Lovastatin on Serum Triglyceride Levels Serum Triglyceride levels were significantly reduced in Wy and Wy+Foh treated wild-type mice. Data is shown as mean ± S.D. * $P<0.05$ as compared to control, ttest.

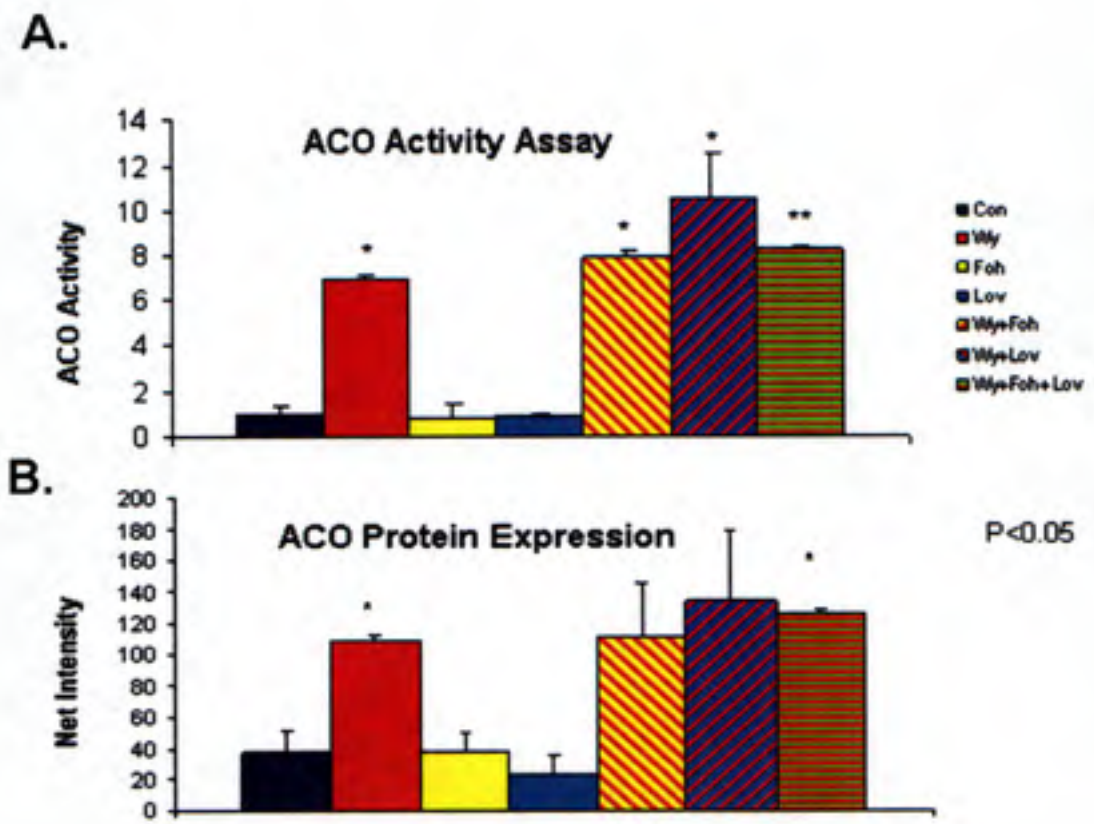


Figure 5. Activity and Protein Expression of the Peroxisomal Enzyme Acyl-CoA Oxidase in Wild-Type Mice

Figure 5A. These results parallel our liver to body weight ratio data where Wy+Foh+Lov treated mice have a significant increase in ACO activity compared to Wy alone. Figure 5B. ACO protein expression was measured by western blot analysis. There was a significant increase in ACO protein expression in Wy and Wy+Foh+Lov treatment groups. Data is shown as mean ± S.D. *,** $P<0.05$ as compared to control or Wy, respectively, ttest.

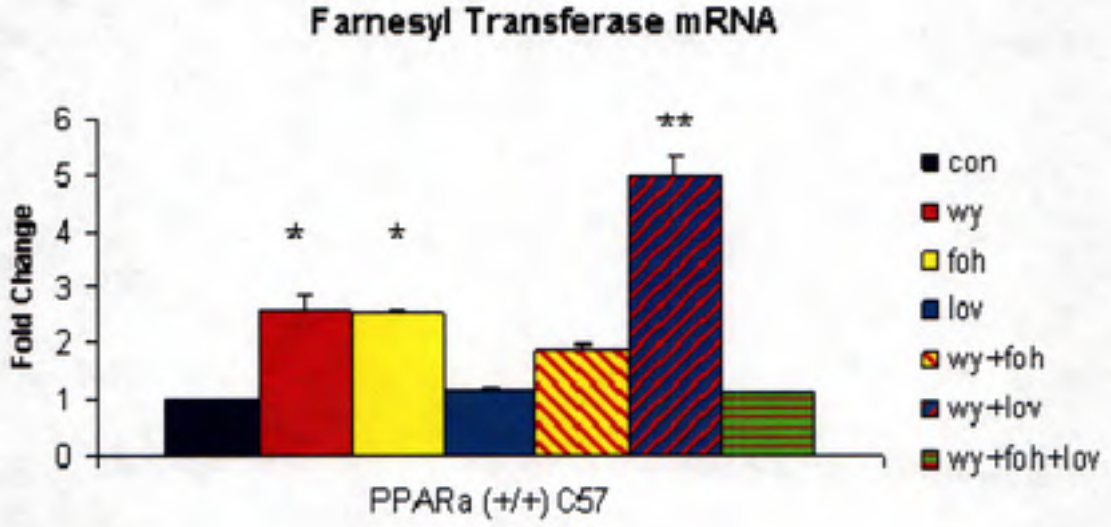


Figure 6. RTPCR- Farnesyl Transferase mRNA Expression.
These results demonstrate a 1.6 fold increase in farnesyl transferase mRNA expression with Wy alone and farnesol alone indicating that there is an increase in the down stream enzymes of the cholesterol synthesis pathway with Wy treatment in wild-type mice. Data is shown as mean ± S.D. *,** $P<0.05$ as compared to control or Wy, respectively, ttest.

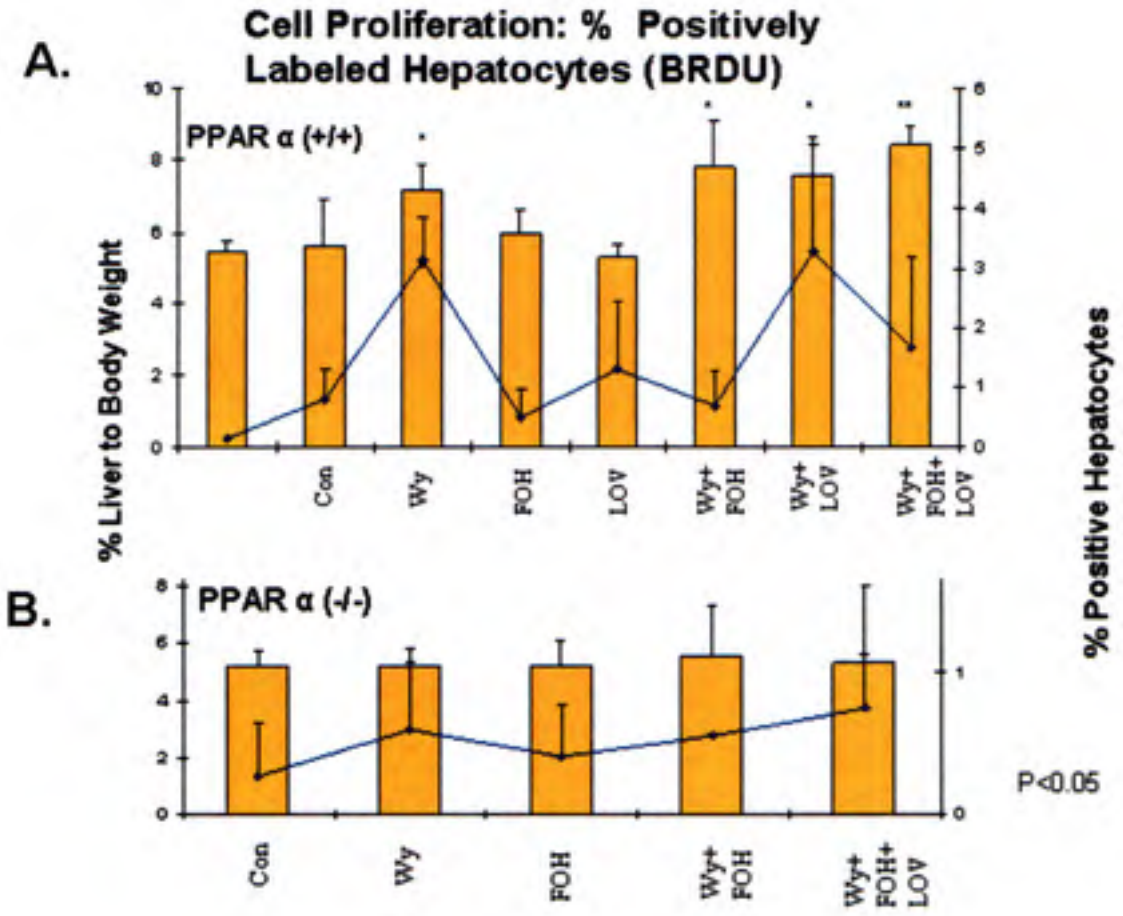


Figure 7. Effect of Wy, Farnesol and Lovastatin on Liver Weight (Bars) and Cell Proliferation (Line) in PPARα Wild-Type and Null Mice

Figure 7A. In wild-type mice a significant increase in cell proliferation in response to Wy was observed. Wy treated groups with farnesol resulted in control level cell proliferation, while Wy + lovastatin had an increase in cell proliferation consistent with Wy treatment. Figure 7B. There was no increase in cell proliferation in PPARα null mice. Data is shown as mean ± S.D. *P<0.05 as compared to control.

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